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(54) **Title:** COMPOSITIONS AND METHODS FOR THE DELIVERY OF NITRIC OXIDE

(57) **Abstract:** H-NOX proteins are mutated to exhibit improved or optimal kinetic and thermodynamic properties for blood gas NO delivery. The engineered H-NOX proteins comprise mutations that impart altered NO or O_2 ligand-binding relative to the corresponding wild-type H-NOX domain, and are operative as physiologically compatible mammalian blood NO gas carriers. The invention also provides pharmaceutical compositions, kits, and methods that use wild-type or mutant H-NOX proteins for the treatment of any condition for which delivery of NO is beneficial.

WO 2007/139767 A2

COMPOSITIONS AND METHODS FOR THE DELIVERY OF NITRIC OXIDE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. provisional application serial number _____, filed May 22, 2006 by Michael A. Marletta, Stephen P.L. Cary, Elizabeth M. Boon, and Jonathan A. Winger, entitled "Engineering H-NOX Proteins for Therapeutic Nitric Oxide and Oxygen Delivery" (UC Case No. B06-084). This U.S. provisional application was converted from U.S. utility application serial number 11/440,588, filed May 22, 2006, to a provisional application on May 1, 2007, the disclosures of which are each hereby incorporated by reference in their entireties.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This work was supported by Grant No. DE-AC03-76SF. The U.S. government may have rights in any patent issuing on this application.

TECHNICAL FIELD

[0003] This application pertains to H-NOX proteins and methods of using them to deliver nitric oxide (NO). H-NOX proteins provide a new therapeutic tool for delivering NO to humans and, for veterinary purposes, to animals.

BACKGROUND OF THE INVENTION

[0004] NO acts as a chemical messenger in the control of many important processes *in vivo*, including vasodilation, neurotransmission, inflammation, platelet aggregation, and regulation of gastrointestinal and vascular smooth muscle tone. Since the discovery in 1867 by Drs. Lauder Brunton and William Murrell that nitroglycerin (GTN) is capable of treating heart disease conditions such as angina pectoris, organic nitrates have been widely used to treat acute cases of vasoconstriction. Within the last several decades, the mechanism of vasodilation has been elucidated. NO, which is synthesized in endothelial cells, diffuses to smooth muscle cells and activates soluble guanylate cyclase (sGC) to produce cyclic GMP, and thereby induce vasodilation.

The clinical mechanism of action of organic nitrates, then, is presumed to require their biotransformation to NO and subsequent activation of sGC. However, organic nitrates cease to be effective in patients after 24-48 hours, due to a phenomenon called tolerance. Thus, for treatment of chronic cases of hypertension, compounds such as β-blockers and ACE inhibitors are used, although they too have limitations and side effects. Thus, nitrovasodilators are most useful in treating acute situations where rapid vasodilation is required to alleviate symptoms such as angina and myocardial infarction. Prolonged administration of organic nitrates results in reduced efficacy, and the vasculature becomes non-responsive; this tolerance prevents their further use both in chronic and acute cases. Thus, for acute treatment, non-continuous nitrovasodilator use is employed with limited effect. For chronic cases of vasoconstriction, other avenues of treatment are employed, typically using a mixed regimen of organic nitrates and NO-independent blood pressure medications, with mixed success.

[0005] Two major competing theories on the mechanism for tolerance run parallel to the search for the mechanism of biotransformation of nitrates that leads to release of NO. Because NO is believed to be the mediator of the vasodilatory effects of organic nitrates, the mechanism of release of NO from organic nitrates may become inhibited, resulting in tolerance. But how organic nitrates metabolically release NO in tissues is not understood. Furthermore, the mechanism-based theory for tolerance is problematic because tolerance also reduces the efficacy of endogenous NO and exogenous NO gas in mediating vasodilation. Thus, the mechanism of biotransformation of organic nitrates appears to be separate from the reason for tolerance. A competing theory posits that the response to NO from organic nitrates becomes dampened in the target tissue, perhaps because the generation of NO and the by-products of the reaction eventually inhibit the response to NO, or because acute activation of the NO pathway has a feedback mechanism that desensitizes it to further stimulation. This theory is known as end-organ tolerance. Recently, a unifying theory has been proposed that includes aspects of the biotransformation of organic nitrates as well as end-organ desensitization to NO. Essentially, biotransformation of organic nitrates appears to result in higher levels of superoxide ($O_2^{\cdot}$) in tissues. Superoxide reacts at the rate of diffusion with NO to produce peroxynitrite (OONO). This reaction essentially traps and destroys basal NO, preventing it from activating sGC. Reduced NO levels leads to vasoconstriction, and $OONO^{-}$ is a powerful oxidant that damages tissues. Prolonged treatment with organic nitrates such as GTN can result in

hypertension and tissue damage in patients, and this can be moderated with co-administration of antioxidants such as ascorbate. Thus, improved therapeutics for delivering NO to organs and tissues to alleviate vasoconstriction is a major therapeutic goal.

[0006] Some research has been conducted on the use of hemoglobin-based carriers to deliver NO. However, hemoglobin-based carriers are limited due to their reactivity with NO in the presence of O_2, which leads to the inactivation of hemoglobin-based carriers. NO reacts directly with O_2 that is bound to hemoglobin to form methemoglobin and nitrate. Both the heme iron and NO become oxidized by the bound oxygen atoms, and the reaction occurs so rapidly that no replacement of O_2 by NO is observed (see, *e.g.*, U.S. Pat. No. 6,455,676).

[0007] Since NO is produced and consumed on a continuous basis, there is a natural turnover of NO *in vivo*. When cell-free hemoglobin is administered, the balance between NO production and consumption is altered by reactions with cell-free hemoglobin. The oxidative reaction between NO and O_2 bound to hemoglobin is irreversible, resulting in the destruction of NO, O_2, and hemoglobin. NO binding to hemoglobin without O_2 bound is effectively irreversible on physiologic timescales since the half-life for dissociation of nitrosylhemoglobin is 5-6 hours, thereby effectively inactivating hemoglobin as a cell-free NO carrier. Once an NO molecule reacts with hemoglobin, it is eliminated from the pool of signal molecules, thereby causing certain adverse conditions. For example, the binding of NO to hemoglobin (with or without O_2 bound) can prevent vascular relaxation and potentially lead to hypertension, which is sometimes observed after the administration of certain extracellular hemoglobin solutions.

[0008] NO is also needed to mediate certain inflammatory responses. For example, NO produced by the endothelium inhibits platelet aggregation. Consequently, as NO is bound by cell-free hemoglobin (with or without O_2 bound), platelet aggregation may increase. As platelets aggregate, they release potent vasoconstrictor compounds such as thromboxane A_2 and serotonin. These compounds may act synergistically with the reduced NO levels caused by hemoglobin scavenging to produce significant vasoconstriction. In addition to inhibiting platelet aggregation, NO also inhibits neutrophil attachment to cell walls, which in turn can lead to cell wall damage. Endothelial cell wall damage has been observed with the infusion of certain hemoglobin solutions. Hemoglobin-based NO carriers are also hindered by the rapid clearance of cell-free hemoglobin from plasma due the presence of receptors for hemoglobin that remove cell-free hemoglobin from

plasma. Cell-free hemoglobin may also cause kidney toxicity, possibly due to NO depletion in glomeruli, causing constriction and subsequent dysfunction.

[0009] Due to the limitations of current nitrovasodilator therapies, there remains a significant interest in and need for additional or alternative therapies for delivering NO. In particular, NO carriers that produce less tolerance are needed. Additionally, NO carriers with a low rate of inactivation by NO in the presence of O_2 are desired, such as NO carriers that have a low NO reactivity and/or a low affinity for O_2. NO carriers with NO dissociation constants or NO dissociation rates that are appropriate for particular clinical or industrial applications are also needed.

BRIEF SUMMARY OF THE INVENTION

[0010] The present invention is based in part on the surprising discovery that wild-type and mutant H-NOX proteins have a much lower NO reactivity than hemoglobin and thus are desirable NO carriers. If desired, mutations can be introduced into H-NOX proteins to alter their binding of NO and O_2 ligands to further optimize the use of H-NOX proteins as NO carriers. In some embodiments, use of an H-NOX protein as an NO carrier produces less tolerance than the use of current vasodilators, such as organic nitrates.

[0011] In one aspect, the invention features mutant H-NOX proteins. Accordingly, in some embodiments, the invention provides an isolated H-NOX protein comprising at least one mutation that alters the NO dissociation constant or NO reactivity compared to that of a corresponding wild-type H-NOX protein. In some embodiments, the NO dissociation constant of the mutant H-NOX protein is within 2 orders of magnitude of that of hemoglobin, and the NO reactivity of the mutant H-NOX protein is at least 10-fold lower than that of hemoglobin. In some embodiments, the NO reactivity of the mutant H-NOX protein is at least 100-fold lower than that of hemoglobin, such as at least 1,000-fold lower than that of hemoglobin. In some embodiments, the k_{off}, k_1, or k_2 for NO of the mutant H-NOX protein is between about 1 x $10^{-4}s^{-1}$ to about 10 s^{-1} at 37 °C, such as about 1 x $10^{-4}s^{-1}$ to about 0.012 s^{-1} or about 1 x $10^{-4}s^{-1}$ to about 1 x $10^{-3}s^{-1}$ at 37 °C. In some embodiments, the O_2 dissociation constant of the mutant H-NOX protein is at least about 1 μM at 37 °C, such as at least about 10 μM or at least about 50 μM at 37 °C.

[0012] In some embodiments, the invention provides an isolated H-NOX protein comprising at least one mutation that alters the k_{off}, k_1, or k_2 for NO or alters the O_2 dissociation constant compared to that of a corresponding wild-type H-NOX protein. In some embodiments, the k_{off}, k_1, or k_2 for NO of the mutant H-NOX protein is between about 1 x $10^{-4}s^{-1}$ to about 10 s^{-1} at 37 °C, and the O_2 dissociation constant of the mutant H-NOX protein is at least about 1 μM at 37 °C. In some embodiments, the k_{off}, k_1, or k_2 for NO of the mutant H-NOX protein is between about 1 x $10^{-4}s^{-1}$ to about 0.012 s^{-1} or about 1 x $10^{-4}s^{-1}$ to about 1 x $10^{-3}s^{-1}$ at 37 °C. In some embodiments, the O_2 dissociation constant of the mutant H-NOX protein is at least about 10 μM, such as at least about 50 μM at 37 °C. In some embodiments, the NO reactivity of the mutant H-NOX protein is at least 10-fold lower than that of hemoglobin, such as at least 100-fold lower than that of hemoglobin or at least 1,000-fold lower than that of hemoglobin.

[0013] In some embodiments, the invention provides an isolated H-NOX protein selected from the group consisting of *T. tengcongensis* H-NOX I5A, *T. tengcongensis* H-NOX I5L, *T. tengcongensis* H-NOX I5L-P115A, *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX W9F-Y140L, *T. tengcongensis* H-NOX W9F-Y140H*T. tengcongensis* H-NOX W9F-N74A, *T. tengcongensis* H-NOX W9Y, *T. tengcongensis* H-NOX W9N, *T. tengcongensis* H-NOX W9H, *T. tengcongensis* H-NOX N74E, *T. tengcongensis* H-NOX N74A, *T. tengcongensis* H-NOX N74H, *T. tengcongensis* H-NOX N74A-Y140H, *T. tengcongensis* H-NOX F78Y-Y140F, *T. tengcongensis* H-NOX P115A, *T. tengcongensis* H-NOX R135Q, *T. tengcongensis* H-NOX Y140F, *T. tengcongensis* H-NOX Y140H, *T. tengcongensis* H-NOX Y140A, *T. tengcongensis* I75F-His6, *T. tengcongensis* I75F, *T. tengcongensis* L144F-His6, *T. tengcongensis* L144F, *L2* F9W-F142Y, *D. desulfuricans* H-NOX(728-899), *D. desulfuricans* H-NOX Y139L, β1(1-385), β1(1-385) I145Y, β1(1-385) I145H, β1(1-194), β1(1-194) I145Y, β1(1-194) L9W-I145Y, β2(1-217), β2(1-217) I142Y, *C. botulinum* H-NOX(1-175), *C. botulinum* H-NOX(1-186), *C. acetobutylicum* H-NOX(1-197), *C. acetobutylicum* H-NOX(1-183), and *C. elegans* H-NOX GCY-35(1-252). In some embodiments, the β1 or β2 protein is derived from a *R. norvegicus* or *H. sapiens* β1 or β2 protein.

[0014] In some embodiments, the invention provides an isolated H-NOX protein selected from the group consisting of *T. tengcongensis* H-NOX I5A, *T. tengcongensis* H-NOX I5L, *T. tengcongensis* H-NOX I5L-P115A, *T. tengcongensis* H-NOX W9F-Y140L, *T. tengcongensis* H-NOX W9F-Y140H, *T. tengcongensis* H-NOX W9F-N74A, *T. tengcongensis* H-NOX W9Y, *T.*

tengcongensis H-NOX W9N, *T. tengcongensis* H-NOX W9H, *T. tengcongensis* H-NOX N74E, *T. tengcongensis* H-NOX N74A, *T. tengcongensis* H-NOX N74H, *T. tengcongensis* H-NOX N74A-Y140H, *T. tengcongensis* H-NOX F78Y-Y140F, *T. tengcongensis* H-NOX P115A, *T. tengcongensis* H-NOX R135Q, *T. tengcongensis* H-NOX Y140H, *T. tengcongensis* H-NOX Y140A, *T. tengcongensis* I75F-His6, *T. tengcongensis* I75F, *T. tengcongensis* L144F-His6, *T. tengcongensis* L144F, *L. pneumophilia 2* F9W-F142Y, *D. desulfuricans* H-NOX(728-899), *D. desulfuricans* H-NOX Y139L, β1(1-385) I145H, β1(1-194), β1(1-194) I145Y, β1(1-194) L9W-I145Y, β2(1-217), β2(1-217) I142Y, *C. botulinum* H-NOX(1-175), *C. botulinum* H-NOX(1-186), *C. acetobutylicum* H-NOX(1-197), *C. acetobutylicum* H-NOX(1-183), and *C. elegans* H-NOX GCY-35(1-252). In some embodiments, the β1 or β2 protein is derived from a *R. norvegicus* or *H. sapiens* β1 or β2 protein.

[0015] In some embodiments of the isolated H-NOX proteins, the NO dissociation constant of the H-NOX protein is between 0.1 to 10-fold of that of hemoglobin, such as between 0.5 to 2-fold of that of hemoglobin. In some embodiments of the isolated H-NOX proteins, the NO dissociation constant of the H-NOX protein is within 2 orders of magnitude of that of *Homo sapiens* hemoglobin alpha, such as an NO dissociation constant between 0.1 to 10-fold or between 0.5 to 2-fold of that of *Homo sapiens* hemoglobin alpha. In some embodiments of the isolated H-NOX proteins, the NO reactivity of the H-NOX protein is at least 10-fold lower than that of *Homo sapiens* hemoglobin alpha, such as at least 100-fold or 1,000-fold lower than that of *Homo sapiens* hemoglobin alpha. In some embodiments of the isolated H-NOX proteins, the NO reactivity of the H-NOX protein is less than about 700 s^{-1} at 20 °C, such as less than about 600 s^{-1}, 500 s^{-1}, 400 s^{-1}, 300 s^{-1}, 200 s^{-1}, 100 s^{-1}, 75 s^{-1}, 50 s^{-1}, 25 s^{-1}, 20 s^{-1}, 10 s^{-1}, 50 s^{-1}, 3 s^{-1}, 2 s^{-1}, 1.8 s^{-1}, 1.5 s^{-1}, 1.2 s^{-1}, 1.0 s^{-1}, 0.8 s^{-1}, 0.7 s^{-1}, or 0.6 s^{-1} at 20 °C. In some embodiments of the isolated H-NOX proteins, the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, such as at least about 10 μM or at least about 50 μM at 37 °C. In some embodiments of the isolated H-NOX proteins, the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ to about 10 s^{-1} at 37 °C, and the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C. In some embodiments of the isolated H-NOX proteins, the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ to about 10 s^{-1} at 37 °C, and the NO reactivity of the H-NOX protein is less than about 700 s^{-1} at 20 °C (*e.g.*, less than about 600 s^{-1}, 500 s^{-1}, 100 s^{-1}, 20 s^{-1}, or 1.8 s^{-1}at 20 °C). In some embodiments of

the isolated H-NOX proteins, the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and the NO reactivity of the H-NOX protein is less than about 700 s^{-1} at 20 °C (*e.g.*, less than about 600 s^{-1}, 500 s^{-1}, 100 s^{-1}, 20 s^{-1}, or 1.8 s^{-1}at 20 °C). In some embodiments of the isolated H-NOX proteins, the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C. In some embodiments of the isolated H-NOX proteins, the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ to about 10 s^{-1} at 37 °C, and the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C. In some embodiments of the isolated H-NOX proteins, the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C. In some embodiments of the isolated H-NOX proteins, the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C, and the NO reactivity of the H-NOX protein is less than about 700 s^{-1} at 20 °C (*e.g.*, less than about 600 s^{-1}, 500 s^{-1}, 100 s^{-1}, 20 s^{-1}, or 1.8 s^{-1}at 20 °C).

[0016] In some embodiments of the isolated H-NOX proteins, the H-NOX protein contains one or more mutations (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mutations) compared to the H-NOX protein from which it was derived. In various embodiments, the H-NOX protein contains less than 20, 15, 12, 10, 9, 8, 7, 6, 5, 4, 3, or 2 mutations compared to the H-NOX protein from which it was derived. In some embodiments, the H-NOX protein has at least one distal pocket mutation. In some embodiments, the H-NOX protein has at least one mutation that is not in the distal pocket. In some embodiments, the H-NOX protein has at least one mutation in which a residue that corresponds to Tyr140 of *T. tengcongensis* H-NOX or Phe142 of *L. pneumophila 2* is replaced by any other amino acid. In some embodiments, the H-NOX protein has at least two mutations, wherein at least one mutation is the replacement of a residue that corresponds to Tyr140 of *T. tengcongensis* H-NOX or Phe142 of *L. pneumophila 2* by any other amino acid. In some embodiments, the mutation in the H-NOX protein corresponds to a Y140F mutation or a Y140L mutation of *T. tengcongensis* or a F142Y mutation of *L. pneumophila 2*. In some embodiments of the isolated H-NOX proteins, at least one C-terminal amino acid (such as at least about 50 contiguous C-terminal amino acids or between about 25 to about 200 contiguous C-terminal amino acids) in the H-NOX protein has been removed compared to the corresponding wild-type protein. In some embodiments, the H-NOX protein is a deletion that contains the first 194, 217, or 385 amino acids of an H-NOX protein such as *R. norvegicus* or *H. sapiens* β1 or β2 protein.

[0017] In some embodiments of the isolated H-NOX proteins, the H-NOX protein is derived from a mammalian protein (*e.g.*, a human protein such as β1). In various embodiments of the isolated H-NOX proteins, the H-NOX protein derived from a bacterial protein (*e.g.*, a *T. tengcongensis* protein). In some embodiments of the isolated H-NOX proteins, the H-NOX protein is covalently bound to another molecule or moiety, such as polyethylene glycol. Heme may or may not be bound to the H-NOX protein. In some embodiments of the isolated H-NOX proteins, NO is bound to the H-NOX protein. In some embodiments of the isolated H-NOX proteins, the H-NOX protein is a fusion protein that includes an H-NOX domain and part or all of another protein, such as albumin (*e.g.*, human serum albumin).

[0018] In some embodiments of the isolated H-NOX proteins, the H-NOX protein is not *T. tengcongensis* H-NOX Y40L, wild-type *T. tengcongensis* H-NOX, wild-type *R. norvegicus* sGC, or *L. pneumophilia* 2 H-NOX F142Y. In some embodiments of the isolated H-NOX proteins, the H-NOX protein is not *T. tengcongensis* H-NOX F78Y/Y140L. In some embodiments of the isolated H-NOX proteins, the H-NOX protein is not wild-type *L. pneumophilia* 2 H-NOX, wild-type *H. sapiens* β1 H-NOX, *R. norvegicus* sGC β1 H-NOX (1-385), wild-type *R. norvegicus* β1 H-NOX, wild-type *D. melangaster* β1 H-NOX, wild-type *D. melangaster* CG14885-PA H-NOX, wild-type *C. elegans* GCY-35 H-NOX, wild-type *N. punctiforme* H-NOX, wild-type *C. crescentus* H-NOX, wild-type *S. oneidensis* H-NOX, or wild-type *C. acetobutylicum* H-NOX. In some embodiments of the isolated H-NOX proteins, the H-NOX protein is not *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX Y140F, *R. norvegicus* sGC β1 H-NOX H105G, *R. norvegicus* sGC β1 H-NOX H105F, *R. norvegicus* sGC β1 H-NOX I145Y, *R. norvegicus* sGC β1 H-NOX C78S, or *R. norvegicus* sGC β1 H-NOX C78E. In some embodiments of the isolated H-NOX proteins, the H-NOX protein is not *R. norvegicus* β2(1-217), *R. norvegicus* β1(1-194), *R. norvegicus* β1(1-385), or *R. norvegicus* β1(1-385) I145Y. In some embodiments of the isolated H-NOX proteins, the H-NOX protein is not *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX Y140F, or *H. sapiens* β1 H-NOX (1-385) I145Y. In some embodiments of the isolated H-NOX proteins, the H-NOX protein is not *T. tengcongensis* H-NOX Y140H, *H. sapiens* β1 I140Y, or *H. sapiens* β1 I145Y. In some embodiments of the isolated H-NOX proteins, the H-NOX protein is not *T. tengcongensis* H-NOX Y40L, *T. tengcongensis* H-NOX F78Y/Y140L, *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX Y140F, wild-type *T. tengcongensis* H-NOX, *L. pneumophilia* 2 H-NOX F142Y, wild-type *L.*

pneumophilia 2 H-NOX, *H. sapiens* β1 H-NOX I140Y, *H. sapiens* B1 I145Y, wild-type *H. sapiens* β1 H-NOX, *R. norvegicus* sGC β1 H-NOX (1-385), *R. norvegicus* sGC β1 H-NOX (1-385) I145Y, *R. norvegicus* sGC β1 H-NOX H105G, *R. norvegicus* sGC β1 H-NOX H105F, *R. norvegicus* sGC β1 H-NOX I145Y, wild-type *R. norvegicus* β1 H-NOX, wild-type *D. melangaster* β1 H-NOX, wild-type *D. melangaster* CG14885-PA H-NOX, wild-type *C. elegans* GCY-35 H-NOX, wild-type *N. punctiforme* H-NOX, wild-type *C. crescentus* H-NOX, wild-type *S. oneidensis* H-NOX, or wild-type *C. acetobutylicum* H-NOX. In some embodiments of the isolated H-NOX proteins, the H-NOX protein is not any of the following H-NOX proteins that are listed by their gene name, followed by their species abbreviation and Genbank Identifiers (such as the following protein sequences available as of May 21, 2006; May 22, 2006; May 21, 2007; or May 22, 2007): Npun5905_Npu_23129606, alr2278_Ana_17229770, SO2144_Sone_24373702, Mdeg1343_Mde_23027521, VCA0720_Vch_15601476, CC2992_Ccr_16127222, Rsph2043_Rhsp_22958463 (gi:46192757), Mmc10739_Mcsp_22999020, Tar4_Tte_20807169, Ddes2822_Dde_23475919, CAC3243_Cac_15896488 , gcy-31_Ce_17568389, CG14885_Dm_24647455, GUCY1B3_Hs_4504215, HpGCS-beta1_Hpul_14245738, Gycbeta100B_Dm_24651577, CG4154_Dm_24646993 (gi:NP_650424.2, gi:62484298), gcy-32_Ce_13539160,gcy-36_Ce_17568391 (gi:32566352, gi:86564713), gcy-35_Ce-17507861 (gi:71990146), gcy-37_Ce_17540904 (gi:71985505), GCY1α3_Hs_20535603, GCY1α2-Hs_899477, or GYCα-99B_Dm_729270 (gi:68067738) (Lakshminarayan *et al.* (2003). "Ancient conserved domains shared by animal soluble guanylyl cyclases and bacterial signaling proteins," *BMG Genomics* 4:5-13). The species abbreviations used in these names include Ana – *Anabaena Sp*; Ccr – *Caulobacter crescentus*; Cac – *Clostridium acetobutylicum*; Dde – *Desulfovibrio desulfuricans*; Mcsp – *Magnetococcus sp.*; Mde – *Microbulbifer degradans*; Npu – *Nostoc punctiforme*; Rhsp – *Rhodobacter sphaeroides*; Sone – *Shewanella oneidensis*; Tte – *Thermoanaerobacter tengcongensis*; Vch – *Vibrio cholerae*; Ce – *Caenorhabditis elegans*; Dm – *Drosophila melanogaster*; Hpul – *Hemicentrotus pulcherrimus*; Hs – *Homo sapiens*. In some embodiments of the isolated H-NOX proteins, the H-NOX protein is not any of the following H-NOX proteins that are listed by their organism name and Pfam database accession number (such as the following protein sequences available as of May 21, 2006; May 22, 2006; May 17, 2007; May 21, 2007; or May 22, 2007): *Caenorhabditis briggsae* Q622M5_CAEBR, *Caenorhabditis briggsae*

Q61P44_CAEBR, *Caenorhabditis briggsae* Q61R54_CAEBR, *Caenorhabditis briggsae* Q61V90_CAEBR, *Caenorhabditis briggsae* Q61A94_CAEBR, *Caenorhabditis briggsae* Q60TP4_CAEBR, *Caenorhabditis briggsae* Q60M10_CAEBR, *Caenorhabditis elegans* GCY37_CAEEL, *Caenorhabditis elegans* GCY31_CAEEL, *Caenorhabditis elegans* GCY36_CAEEL, *Caenorhabditis elegans* GCY32_CAEEL, *Caenorhabditis elegans* GCY35_CAEEL, *Caenorhabditis elegans* GCY34_CAEEL, *Caenorhabditis elegans* GCY33_CAEEL, *Oryzias curvinotus* Q7T040_ORYCU, *Oryzias curvinotus* Q75WF0_ORYCU, *Oryzias latipes* P79998_ORYLA, *Oryzias latipes* Q7ZSZ5_ORYLA, *Tetraodon nigroviridis* Q4SW38_TETNG, *Tetraodon nigroviridis* Q4RZ94_TETNG, *Tetraodon nigroviridis* Q4S6K5_TETNG, *Fugu rubripes* Q90VY5_FUGRU, *Xenopus laevis* Q6INK9_XENLA, *Homo sapiens* Q5T8J7_HUMAN, *Homo sapiens* GCYA2_HUMAN, *Homo sapiens* GCYB2_HUMAN, *Homo sapiens* GCYB1_HUMAN, *Gorilla gorilla* Q9N193_9PRIM, *Pongo pygmaeus* Q5RAN8_PONPY, *Pan troglodytes* Q9N192_PANTR, *Macaca mulatta* Q9N194_MACMU, *Hylobates lar* Q9N191_HYLLA, *Mus musculus* Q8BXH3_MOUSE, *Mus musculus* GCYB1_MOUSE, *Mus musculus* Q3UTI4_MOUSE, *Mus musculus* Q3UH83_MOUSE, *Mus musculus* Q6XE41_MOUSE, *Mus musculus* Q80YP4_MOUSE, *Rattus norvegicus* Q80WX7_RAT, *Rattus norvegicus* Q80WX8_RAT, *Rattus norvegicus* Q920Q1_RAT, *Rattus norvegicus* Q54A43_RAT, *Rattus norvegicus* Q80WY0_RAT, *Rattus norvegicus* Q80WY4_RAT, *Rattus norvegicus* Q8CH85_RAT, *Rattus norvegicus* Q80WY5_RAT, *Rattus norvegicus* GCYB1_RAT, *Rattus norvegicus* Q8CH90_RAT, *Rattus norvegicus* Q91XJ7_RAT, *Rattus norvegicus* Q80WX9_RAT, *Rattus norvegicus* GCYB2_RAT, *Rattus norvegicus* GCYA2_RAT, *Canis familiaris* Q4ZHR9_CANFA, *Bos taurus* GCYB1_BOVIN, *Sus scrofa* Q4ZHR7_PIG, *Gryllus bimaculatus* Q59HN5_GRYBI, *Manduca sexta* O77106_MANSE, *Manduca sexta* O76340_MANSE, *Apis mellifera* Q5UAF0_APIME, *Apis mellifera* Q5FAN0_APIME, *Apis mellifera* Q6L5L6_APIME, *Anopheles gambiae* str PEST Q7PYK9_ANOGA, *Anopheles gambiae* str PEST Q7Q9W6_ANOGA, *Anopheles gambiae* str PEST Q7QF31_ANOGA, *Anopheles gambiae* str PEST Q7PS01_ANOGA, *Anopheles gambiae* str PEST Q7PFY2_ANOGA, *Anopheles gambiae* Q7KQ93_ANOGA, *Drosophila melanogaster* Q24086_DROME, *Drosophila melanogaster* GCYH_DROME, *Drosophila melanogaster* GCY8E_DROME, *Drosophila melanogaster* GCYDA_DROME, *Drosophila melanogaster* GCYDB_DROME, *Drosophila melanogaster*

Q9VA09_DROME, *Drosophila pseudoobscura* Q29CE1_DROPS, *Drosophila pseudoobscura* Q296C7_DROPS, *Drosophila pseudoobscura* Q296C8_DROPS, *Drosophila pseudoobscura* Q29BU7_DROPS, *Aplysia californica* Q7YWK7_APLCA, *Hemicentrotus pulcherrimus* Q95NK5_HEMPU, *Chlamydomonas reinhardtii,* Q5YLC2_CHLRE, *Anabaena sp* Q8YUQ7_ANASP, *Flavobacteria bacterium BBFL7* Q26GR8_9BACT, *Psychroflexus torquis* ATCC *700755* Q1VQE5_9FLAO, marine gamma proteobacterium HTCC2207 Q1YPJ5_9GAMM, marine gamma proteobacterium HTCC2207 Q1YTK4_9GAMM, *Caulobacter crescentus* Q9A451_CAUCR, *Acidiphilium cryptum* JF-5 Q2DG60_ACICY, *Rhodobacter sphaeroides* Q3J0U9_RHOS4, *Silicibacter pomeroyi* Q5LPV1_SILPO, *Paracoccus denitrificans* PD1222, Q3PC67_PARDE, *Silicibacter sp TM1040* Q3QNY2_9RHOB, *Jannaschia sp* Q28ML8_JANSC, *Magnetococcus sp* MC-1 Q3XT27_9PROT, *Legionella pneumophila* Q5WXP0_LEGPL, *Legionella pneumophila* Q5WTZ5_LEGPL, *Legionella pneumophila* Q5X268_LEGPA, *Legionella pneumophila* Q5X2R2_LEGPA, *Legionella pneumophila subsp pneumophila* Q5ZWM9_LEGPH, *Legionella pneumophila subsp pneumophila* Q5ZSQ8_LEGPH, *Colwellia psychrerythraea* Q47Y43_COLP3, *Pseudoalteromonas atlantica* T6c Q3CSZ5_ALTAT, *Shewanella oneidensis* Q8EF49_SHEON, *Saccharophagus degradans* Q21E20_SACD2, *Saccharophagus degradans* Q21ER7_SACD2, *Vibrio angustum* S14 Q1ZWE5_9VIBR, *Vibrio vulnificus* Q8DAE2_VIBVU, *Vibrio alginolyticus* 12G01 Q1VCP6_VIBAL, *Vibrio sp* DAT722 Q2FA22_9VIBR, *Vibrio parahaemolyticus* Q87NJ1_VIBPA, *Vibrio fischeri* Q5E1F5_VIBF1, *Vibrio vulnificus* Q7MJS8_VIBVY, *Photobacterium sp SKA34* Q2C6Z5_9GAMM, *Hahella chejuensis* Q2SFY7_HAHCH, *Oceanospirillum sp* MED92 Q2BKV0_9GAMM, *Oceanobacter sp* RED65 Q1N035_9GAMM, *Desulfovibrio desulfuricans* Q310U7_DESDG, *Halothermothrix orenii* H 168 Q2AIW5_9FIRM, *Thermoanaerobacter tengcongensis* Q8RBX6_THETN, *Caldicellulosiruptor saccharolyticus* DSM 8903 Q2ZH17_CALSA, *Clostridium acetobutylicum* Q97E73_CLOAB, *Alkaliphilus metalliredigenes* QYMF Q3C763_9CLOT, *Clostridium tetani* Q899J9_CLOTE, and *Clostridium beijerincki NCIMB 8052* Q2WVN0_CLOBE. In some embodiments of the isolated H-NOX proteins, the H-NOX protein does not have a mutation in the Y-S-R motif, which includes Tyr135, Ser137, and Arg139 of human H-NOX.

[0019] In one aspect, the invention features a recombinant nucleic acid encoding any one or more of the mutant H-NOX proteins described herein. In particular embodiments, the nucleic acid

includes a segment of or the entire nucleic acid sequence of any of the nucleic acids shown in FIGS. 2-4D or 8A-8DD. In some embodiments, the nucleic acid encodes a fusion protein that includes an H-NOX domain and part or all of another protein, such as albumin (*e.g.*, human serum albumin). In some embodiments, the nucleic acid includes at least about any of 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, or more contiguous nucleotides from an H-NOX nucleic acid and contains one or more mutations (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mutations) compared to the H-NOX nucleic acid from which it was derived. In various embodiments, a mutant H-NOX nucleic acid contains less than about any of 20, 15, 12, 10, 9, 8, 7, 6, 5, 4, 3, or 2 mutations compared to the H-NOX nucleic acid from which it was derived. The invention also features degenerate variants of any nucleic acid encoding a mutant H-NOX protein.

[0020] In yet another aspect, the invention provides a vector that includes any one or more of the mutant H-NOX nucleic acids described herein. In another aspect, the invention features a cell that includes any one or more of the mutant H-NOX nucleic acids described herein. In one aspect, the invention features a cell that includes any vector described herein.

[0021] In one aspect, the invention features a method of producing an H-NOX protein. This method involves culturing a cell having a nucleic acid encoding any one or more of the mutant H-NOX proteins described herein under conditions suitable for production of the mutant H-NOX protein. In some embodiments, the invention further includes the step of purifying the mutant H-NOX protein.

[0022] In one aspect, the invention features pharmaceutical compositions that include one or more H-NOX proteins, such as any of the wild-type or mutant H-NOX proteins described herein. In some embodiments, the pharmaceutical composition includes a pharmaceutically acceptable amount of an H-NOX protein described herein and a pharmaceutically acceptable carrier. In some embodiments, the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about $1 \times 10^{-4} s^{-1}$ to about $10\ s^{-1}$ at 37 °C, and the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C. In some embodiments, the NO dissociation constant of the H-NOX protein is within 2 orders of magnitude of that of hemoglobin, and the NO reactivity of the H-NOX protein is at least 10-fold lower than that of hemoglobin.

[0023] In some embodiments of the pharmaceutical compositions, the NO dissociation constant of the H-NOX protein is within 2 orders of magnitude of that of *Homo sapiens* hemoglobin

alpha, such as an NO dissociation constant between 0.1 to 10-fold or between 0.5 to 2-fold of that of *Homo sapiens* hemoglobin alpha. In some embodiments of the pharmaceutical compositions, the NO reactivity of the H-NOX protein is at least 10-fold lower than that of *Homo sapiens* hemoglobin alpha, such as at least 100-fold or 1,000-fold lower than that of *Homo sapiens* hemoglobin alpha. In some embodiments of the pharmaceutical compositions, the H-NOX protein is a wild-type protein. In some embodiments of the pharmaceutical compositions, the H-NOX protein is a mutant protein as described herein. In various embodiments of the pharmaceutical compositions, the H-NOX protein has at least one mutation that alters the NO dissociation constant, the k_{off} for NO, the k_1 for NO, the k_2 for NO, the O_2 dissociation constant, the NO stability, the NO reactivity the rate of heme autoxidation, or any combination of two or more of the foregoing compared to that of a corresponding wild-type protein. In some embodiments of the pharmaceutical compositions, the H-NOX protein is a selected from the group consisting of wild-type *T. tengcongensis* H-NOX, *T. tengcongensis* H-NOX I5A, *T. tengcongensis* H-NOX I5L, *T. tengcongensis* H-NOX I5L-P115A, *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX W9F-Y140L, *T. tengcongensis* H-NOX W9F-Y140H*T. tengcongensis* H-NOX W9F-N74A, *T. tengcongensis* H-NOX W9Y, *T. tengcongensis* H-NOX W9N, *T. tengcongensis* H-NOX W9H, *T. tengcongensis* H-NOX N74E, *T. tengcongensis* H-NOX N74A, *T. tengcongensis* H-NOX N74H, *T. tengcongensis* H-NOX N74A-Y140H, *T. tengcongensis* H-NOX F78Y-Y140F, *T. tengcongensis* H-NOX F78Y/Y140L, *T. tengcongensis* H-NOX P115A, *T. tengcongensis* H-NOX R135Q, *T. tengcongensis* H-NOX Y140F, *T. tengcongensis* H-NOX Y40L, *T. tengcongensis* H-NOX Y140H, *T. tengcongensis* H-NOX Y140A, *T. tengcongensis* I75F-His6, *T. tengcongensis* I75F, *T. tengcongensis* L144F-His6, *T. tengcongensis* L144F, *L. pneumophilia* 2 H-NOX F142Y, wild-type *L. pneumophilia* 1 H-NOX, wild-type *L. pneumophilia* 2 H-NOX, *L. pneumophilia 2* F9W-F142Y, wild-type *D. desulfuricans* H-NOX, *D. desulfuricans* H-NOX(728-899), *D. desulfuricans* H-NOX Y139L, wild-type *H. sapiens* β1 H-NOX, *H. sapiens* β1 I140Y, *H. sapiens* β1 I145Y, *H. sapiens* β1(1-385), *H. sapiens* β1(1-385) I145Y, *H. sapiens* β1(1-385) I145H, *H. sapiens* β1(1-194), *H. sapiens* β1(1-194) I145Y, *H. sapiens* β1(1-194) L9W-I145Y, *H. sapiens* β2(1-217), *H. sapiens* β2(1-217) I142Y, *H. sapiens* β1 H-NOX H105G, *H. sapiens* β1 H-NOX H105F, *H. sapiens* β1 H-NOX C78S, *H. sapiens* β1 H-NOX C78E, wild-type *R. norvegicus* β1 H-NOX, *R. norvegicus* β1(1-385), *R. norvegicus* β1(1-385) I145Y, *R. norvegicus* β1(1-385) I145H, *R. norvegicus* β1(1-194), *R. norvegicus* β1(1-194)

I145Y, *R. norvegicus* β1(1-194) L9W-I145Y, *R. norvegicus* β2(1-217), *R. norvegicus* β2(1-217) I142Y, *R. norvegicus* β1 H-NOX H105G, *R. norvegicus* β1 H-NOX H105F, *R. norvegicus* sGC β1 H-NOX C78S, *R. norvegicus* sGC β1 H-NOX C78E, *C. botulinum* H-NOX(1-175), *C. botulinum* H-NOX(1-186), wild-type *C. acetobutylicum* H-NOX, *C. acetobutylicum* H-NOX(1-197), *C. acetobutylicum* H-NOX(1-183), wild-type *C. elegans* GCY-35 H-NOX, *C. elegans* H-NOX GCY-35(1-252), wild-type *D. melangaster* β1 H-NOX, wild-type *D. melangaster* CG14885-PA, wild-type *D. melangaster* CG14886, wild-type *D. melangaster* CG4154; wild-type *N. punctiforme* H-NOX, wild-type *C. crescentus* H-NOX, wild-type *S. oneidensis* H-NOX, wild-type *M. musculus* H-NOX , wild-type *C. familiaris* H-NOX, wild-type *B. Taurus* H-NOX, wild-type *R. norvegicus;* wild-type *X. laevis* H-NOX, wild-type *O. latipes* H-NOX, wild-type *O. curivatus* H-NOX, wild-type *F. rubripes* H-NOX, wild-type *A. gambiae* H-NOX, wild-type *M. sexta* H-NOX; wild-type *C. elegans* gcy-31, *C. elegans* gcy-32, wild-type *C. elegans* gcy-33, wild-type *C. elegans* gcy-34, wild-type *C. elegans* gcy-35, wild-type *C. elegans* gcy-36, wild-type *C. elegans* gcy-37; wild-type *V. cholera* H-NOX, wild-type *V. fischerii* H-NOX, and wild-type *N. punctiforme* H-NOX. In particular embodiments of the pharmaceutical compositions, the H-NOX protein is a selected from the group consisting of wild-type *R. norvegicus* sGC, wild-type *R. norvegicus* β1(1-385), *R. norvegicus* β1(1-217), *R. norvegicus* β1(1-194), wild-type *T. tengcongensis* H-NOX, *T. tengcongensis* H-NOX Y140L, *T. tengcongensis* H-NOX Y140F, wild-type *L. pneumophilia* 1 H-NOX, wild-type *L. pneumophilia* 2 H-NOX, and *L. pneumophilia* 2 H-NOX F142Y. In some embodiments of the pharmaceutical compositions, the pharmaceutical composition includes one or more liposomes or nanoparticles that include or encapsulate the H-NOX protein.

[0024] In some embodiments of the pharmaceutical compositions, the H-NOX protein is not *T. tengcongensis* H-NOX Y40L, wild-type *T. tengcongensis* H-NOX, wild-type *R. norvegicus* sGC, or *L. pneumophilia* 2 H-NOX F142Y. In some embodiments of the pharmaceutical compositions, the H-NOX protein is not *T. tengcongensis* H-NOX F78Y/Y140L. In some embodiments of the pharmaceutical compositions, the H-NOX protein is not wild-type *L. pneumophilia* 2 H-NOX, wild-type *H. sapiens* β1 H-NOX, *R. norvegicus* sGC β1 H-NOX (1-385), wild-type *R. norvegicus* β1 H-NOX, wild-type *D. melangaster* β1 H-NOX, wild-type *D. melangaster* CG14885-PA H-NOX, wild-type *C. elegans* GCY-35 H-NOX, wild-type *N. punctiforme* H-NOX, wild-type *C. crescentus* H-NOX, wild-type *S. oneidensis* H-NOX, or wild-type *C. acetobutylicum* H-NOX. In some

embodiments of the pharmaceutical compositions, the H-NOX protein is not *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX Y140F, *R. norvegicus* sGC β1 H-NOX H105G, *R. norvegicus* sGC β1 H-NOX H105F, *R. norvegicus* sGC β1 H-NOX I145Y, *R. norvegicus* sGC β1 H-NOX C78S, or *R. norvegicus* sGC β1 H-NOX C78E. In some embodiments of the pharmaceutical compositions, the H-NOX protein is not *R. norvegicus* β2(1-217), *R. norvegicus* β1(1-194), *R. norvegicus* β1(1-385), or *R. norvegicus* β1(1-385) I145Y. In some embodiments of the pharmaceutical compositions, the H-NOX protein is not *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX Y140F, or *H. sapiens* β1 H-NOX (1-385) I145Y. In some embodiments of the pharmaceutical compositions, the H-NOX protein is not *T. tengcongensis* H-NOX Y140H, *H. sapiens* β1 I140Y, or *H. sapiens* β1 I145Y. In some embodiments of the pharmaceutical compositions, the H-NOX protein is not *T. tengcongensis* H-NOX Y40L, *T. tengcongensis* H-NOX F78Y/Y140L, *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX Y140F, wild-type *T. tengcongensis* H-NOX, *L. pneumophilia* 2 H-NOX F142Y, wild-type *L. pneumophilia* 2 H-NOX, *H. sapiens* β1 H-NOX I140Y, *H. sapiens* B1 I145Y, wild-type *H. sapiens* β1 H-NOX, *R. norvegicus* sGC β1 H-NOX (1-385), *R. norvegicus* sGC β1 H-NOX (1-385) I145Y, *R. norvegicus* sGC β1 H-NOX H105G, *R. norvegicus* sGC β1 H-NOX H105F, *R. norvegicus* sGC β1 H-NOX I145Y, wild-type *R. norvegicus* β1 H-NOX, wild-type *D. melangaster* β1 H-NOX, wild-type *D. melangaster* CG14885-PA H-NOX, wild-type *C. elegans* GCY-35 H-NOX, wild-type *N. punctiforme* H-NOX, wild-type *C. crescentus* H-NOX, wild-type *S. oneidensis* H-NOX, or wild-type *C. acetobutylicum* H-NOX. In some embodiments of the pharmaceutical compositions, the H-NOX protein is not any of the following H-NOX proteins that are listed by their gene name, followed by their species abbreviation and Genbank Identifiers (such as the following protein sequences available as of May 21, 2006; May 22, 2006; May 21, 2007; or May 22, 2007): Npun5905_Npu_23129606, alr2278_Ana_17229770, SO2144_Sone_24373702, Mdeg1343_Mde_23027521, VCA0720_Vch_15601476, CC2992_Ccr_16127222, Rsph2043_Rhsp_22958463 (gi:46192757), Mmc10739_Mcsp_22999020, Tar4_Tte_20807169, Ddes2822_Dde_23475919, CAC3243_Cac_15896488 , gcy-31_Ce_17568389, CG14885_Dm_24647455, GUCY1B3_Hs_4504215, HpGCS-beta1_Hpul_14245738, Gycbeta100B_Dm_24651577, CG4154_Dm_24646993 (gi:NP_650424.2, gi:62484298), gcy-32_Ce_13539160,gcy-36_Ce_17568391 (gi:32566352, gi:86564713), gcy-35_Ce-17507861 (gi:71990146), gcy-

37_Ce_17540904 (gi:71985505), GCY1α3_Hs_20535603, GCY1α2-Hs_899477, or GYCα-99B_Dm_729270 (gi:68067738) (Lakshminarayan *et al.* (2003). "Ancient conserved domains shared by animal soluble guanylyl cyclases and bacterial signaling proteins," *BMG Genomics* 4:5-13). The species abbreviations used in these names include Ana – *Anabaena Sp*; Ccr – *Caulobacter crescentus*; Cac – *Clostridium acetobutylicum*; Dde – *Desulfovibrio desulfuricans*; Mcsp – *Magnetococcus sp.*; Mde – *Microbulbifer degradans*; Npu – *Nostoc punctiforme*; Rhsp – *Rhodobacter sphaeroides*; Sone – *Shewanella oneidensis*; Tte – *Thermoanaerobacter tengcongensis*; Vch – *Vibrio cholerae*; Ce – *Caenorhabditis elegans*; Dm – *Drosophila melanogaster*; Hpul – *Hemicentrotus pulcherrimus*; Hs – *Homo sapiens*. In some embodiments of the pharmaceutical compositions, the H-NOX protein is not any of the following H-NOX proteins that are listed by their organism name and Pfam database accession number (such as the following protein sequences available as of May 21, 2006; May 22, 2006; May 17, 2007; May 21, 2007; or May 22, 2007): *Caenorhabditis briggsae* Q622M5_CAEBR, *Caenorhabditis briggsae* Q61P44_CAEBR, *Caenorhabditis briggsae* Q61R54_CAEBR, *Caenorhabditis briggsae* Q61V90_CAEBR, *Caenorhabditis briggsae* Q61A94_CAEBR, *Caenorhabditis briggsae* Q60TP4_CAEBR, *Caenorhabditis briggsae* Q60M10_CAEBR, *Caenorhabditis elegans* GCY37_CAEEL, *Caenorhabditis elegans* GCY31_CAEEL, *Caenorhabditis elegans* GCY36_CAEEL, *Caenorhabditis elegans* GCY32_CAEEL, *Caenorhabditis elegans* GCY35_CAEEL, *Caenorhabditis elegans* GCY34_CAEEL, *Caenorhabditis elegans* GCY33_CAEEL, *Oryzias curvinotus* Q7T040_ORYCU, *Oryzias curvinotus* Q75WF0_ORYCU, *Oryzias latipes* P79998_ORYLA, *Oryzias latipes* Q7ZSZ5_ORYLA, *Tetraodon nigroviridis* Q4SW38_TETNG, *Tetraodon nigroviridis* Q4RZ94_TETNG, *Tetraodon nigroviridis* Q4S6K5_TETNG, *Fugu rubripes* Q90VY5_FUGRU, *Xenopus laevis* Q6INK9_XENLA, *Homo sapiens* Q5T8J7_HUMAN, *Homo sapiens* GCYA2_HUMAN, *Homo sapiens* GCYB2_HUMAN, *Homo sapiens* GCYB1_HUMAN, *Gorilla gorilla* Q9N193_9PRIM, *Pongo pygmaeus* Q5RAN8_PONPY, *Pan troglodytes* Q9N192_PANTR, *Macaca mulatta* Q9N194_MACMU, *Hylobates lar* Q9N191_HYLLA, *Mus musculus* Q8BXH3_MOUSE, *Mus musculus* GCYB1_MOUSE, *Mus musculus* Q3UTI4_MOUSE, *Mus musculus* Q3UH83_MOUSE, *Mus musculus* Q6XE41_MOUSE, *Mus musculus* Q80YP4_MOUSE, *Rattus norvegicus* Q80WX7_RAT, *Rattus norvegicus* Q80WX8_RAT, *Rattus norvegicus* Q920Q1_RAT, *Rattus norvegicus*

Q54A43_RAT, *Rattus norvegicus* Q80WY0_RAT, *Rattus norvegicus* Q80WY4_RAT, *Rattus norvegicus* Q8CH85_RAT, *Rattus norvegicus* Q80WY5_RAT, *Rattus norvegicus* GCYB1_RAT, *Rattus norvegicus* Q8CH90_RAT, *Rattus norvegicus* Q91XJ7_RAT, *Rattus norvegicus* Q80WX9_RAT, *Rattus norvegicus* GCYB2_RAT, *Rattus norvegicus* GCYA2_RAT, *Canis familiaris* Q4ZHR9_CANFA, *Bos taurus* GCYB1_BOVIN, *Sus scrofa* Q4ZHR7_PIG, *Gryllus bimaculatus* Q59HN5_GRYBI, *Manduca sexta* O77106_MANSE, *Manduca sexta* O76340_MANSE, *Apis mellifera* Q5UAF0_APIME, *Apis mellifera* Q5FAN0_APIME, *Apis mellifera* Q6L5L6_APIME, *Anopheles gambiae* str PEST Q7PYK9_ANOGA, *Anopheles gambiae* str PEST Q7Q9W6_ANOGA, *Anopheles gambiae* str PEST Q7QF31_ANOGA, *Anopheles gambiae* str PEST Q7PS01_ANOGA, *Anopheles gambiae* str PEST Q7PFY2_ANOGA, *Anopheles gambiae* Q7KQ93_ANOGA, *Drosophila melanogaster* Q24086_DROME, *Drosophila melanogaster* GCYH_DROME, *Drosophila melanogaster* GCY8E_DROME, *Drosophila melanogaster* GCYDA_DROME, *Drosophila melanogaster* GCYDB_DROME, *Drosophila melanogaster* Q9VA09_DROME, *Drosophila pseudoobscura* Q29CE1_DROPS, *Drosophila pseudoobscura* Q296C7_DROPS, *Drosophila pseudoobscura* Q296C8_DROPS, *Drosophila pseudoobscura* Q29BU7_DROPS, *Aplysia californica* Q7YWK7_APLCA, *Hemicentrotus pulcherrimus* Q95NK5_HEMPU, *Chlamydomonas reinhardtii,* Q5YLC2_CHLRE, *Anabaena sp* Q8YUQ7_ANASP, *Flavobacteria bacterium BBFL7* Q26GR8_9BACT, *Psychroflexus torquis* ATCC *700755* Q1VQE5_9FLAO, marine gamma proteobacterium HTCC2207 Q1YPJ5_9GAMM, marine gamma proteobacterium HTCC2207 Q1YTK4_9GAMM, *Caulobacter crescentus* Q9A451_CAUCR, *Acidiphilium cryptum* JF-5 Q2DG60_ACICY, *Rhodobacter sphaeroides* Q3J0U9_RHOS4, *Silicibacter pomeroyi* Q5LPV1_SILPO, *Paracoccus denitrificans* PD1222, Q3PC67_PARDE, *Silicibacter sp TM1040* Q3QNY2_9RHOB, *Jannaschia sp* Q28ML8_JANSC, *Magnetococcus sp* MC-1 Q3XT27_9PROT, *Legionella pneumophila* Q5WXP0_LEGPL, *Legionella pneumophila* Q5WTZ5_LEGPL, *Legionella pneumophila* Q5X268_LEGPA, *Legionella pneumophila* Q5X2R2_LEGPA, *Legionella pneumophila subsp pneumophila* Q5ZWM9_LEGPH, *Legionella pneumophila subsp pneumophila* Q5ZSQ8_LEGPH, *Colwellia psychrerythraea* Q47Y43_COLP3, *Pseudoalteromonas atlantica* T6c Q3CSZ5_ALTAT, *Shewanella oneidensis* Q8EF49_SHEON, *Saccharophagus degradans* Q21E20_SACD2, *Saccharophagus degradans* Q21ER7_SACD2, *Vibrio angustum* S14 Q1ZWE5_9VIBR, *Vibrio vulnificus* Q8DAE2_VIBVU,

Vibrio alginolyticus 12G01 Q1VCP6_VIBAL, *Vibrio sp* DAT722 Q2FA22_9VIBR, *Vibrio parahaemolyticus* Q87NJ1_VIBPA, *Vibrio fischeri* Q5E1F5_VIBF1, *Vibrio vulnificus* Q7MJS8_VIBVY, *Photobacterium sp SKA34* Q2C6Z5_9GAMM, *Hahella chejuensis* Q2SFY7_HAHCH, *Oceanospirillum sp* MED92 Q2BKV0_9GAMM, *Oceanobacter sp* RED65 Q1N035_9GAMM, *Desulfovibrio desulfuricans* Q310U7_DESDG, *Halothermothrix orenii* H 168 Q2AIW5_9FIRM, *Thermoanaerobacter tengcongensis* Q8RBX6_THETN, *Caldicellulosiruptor saccharolyticus* DSM 8903 Q2ZH17_CALSA, *Clostridium acetobutylicum* Q97E73_CLOAB, *Alkaliphilus metalliredigenes* QYMF Q3C763_9CLOT, *Clostridium tetani* Q899J9_CLOTE, and *Clostridium beijerincki NCIMB 8052* Q2WVN0_CLOBE. In some embodiments of the pharmaceutical compositions, the H-NOX protein does not have a mutation in the Y-S-R motif, which includes Tyr135, Ser137, and Arg139 of human H-NOX.

[0025] Unless otherwise explicitly noted or dictated by context, all wild-type and mutant H-NOX proteins described herein may be used in any of the pharmaceutical compositions described herein. The H-NOX protein may or may not have heme and/or NO bound and may or may not be covalently bound to another molecule or moiety, such as polyethylene glycol. In some embodiments, the H-NOX protein is a fusion protein that includes an H-NOX domain and part or all of another protein, such as albumin (*e.g.*, human serum albumin).

[0026] In one aspect, the invention provides methods of delivering NO to an individual (*e.g.*, a mammal, such as a primate (*e.g.*, a human, a monkey, a gorilla, an ape, a lemur, *etc*), a bovine, an equine, a porcine, a canine, or a feline) using an H-NOX protein. In some embodiments, the individual is suffering from or at risk for a cardiovascular condition, hypertension, a condition exacerbated by hypertension, a vasoconstrictive condition, stroke, or a functional NO deficiency. In particular embodiments, the condition exacerbated by hypertension is heart failure, renal failure, or a stroke.

[0027] Accordingly, in some embodiments, the invention provides a method of delivering NO to an individual (*e.g.*, a human) by administering to an individual in need thereof an H-NOX protein in an amount sufficient to deliver an effective amount of NO to the individual. In some embodiments, the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x 10^{-4} s^{-1} to about 10 s^{-1} at 37 °C, and the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C. In some embodiments, the NO dissociation constant of the H-NOX protein is within 2 orders of

magnitude of that of hemoglobin, and the NO reactivity of the H-NOX protein is at least 10-fold lower than that of hemoglobin.

[0028] In some embodiments of the methods, NO is bound to the H-NOX protein prior to the administration of the H-NOX protein to the individual. In some embodiments of the methods, NO is not bound to the H-NOX protein prior to the administration of the H-NOX protein to the individual, and the H-NOX protein transports NO from one location in the individual to another location in the individual. In some embodiments of the methods, the H-NOX protein is administered orally, rectally, or to the blood of the individual. In particular embodiments of the methods, the H-NOX protein is administered to the blood of the individual. In some embodiments of the methods, the H-NOX protein is administered to the individual at least twice.

[0029] In some embodiments of the methods, the NO dissociation constant of the H-NOX protein is within 2 orders of magnitude of that of *Homo sapiens* hemoglobin alpha, such as an NO dissociation constant between 0.1 to 10-fold or between 0.5 to 2-fold of that of *Homo sapiens* hemoglobin alpha. In some embodiments of the methods, the NO reactivity of the H-NOX protein is at least 10-fold lower than that of *Homo sapiens* hemoglobin alpha, such as at least 100-fold or 1,000-fold lower than that of *Homo sapiens* hemoglobin alpha. In some embodiments of the methods, the H-NOX protein is a wild-type protein. In some embodiments of the methods, the H-NOX protein is a mutant protein as described herein. In various embodiments of the methods, the H-NOX protein has at least one mutation that alters the NO dissociation constant, the k_{off} for NO, the k_1 for NO, the k_2 for NO, the O_2 dissociation constant, the NO stability, the NO reactivity the rate of heme autoxidation, or any combination of two or more of the foregoing compared to that of a corresponding wild-type protein. In some embodiments of the methods, the H-NOX protein is a selected from the group consisting of wild-type *T. tengcongensis* H-NOX, *T. tengcongensis* H-NOX I5A, *T. tengcongensis* H-NOX I5L, *T. tengcongensis* H-NOX I5L-P115A, *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX W9F-Y140L, *T. tengcongensis* H-NOX W9F-Y140H*T. tengcongensis* H-NOX W9F-N74A, *T. tengcongensis* H-NOX W9Y, *T. tengcongensis* H-NOX W9N, *T. tengcongensis* H-NOX W9H, *T. tengcongensis* H-NOX N74E, *T. tengcongensis* H-NOX N74A, *T. tengcongensis* H-NOX N74H, *T. tengcongensis* H-NOX N74A-Y140H, *T. tengcongensis* H-NOX F78Y-Y140F, *T. tengcongensis* H-NOX F78Y/Y140L, *T. tengcongensis* H-NOX P115A, *T. tengcongensis* H-NOX R135Q, *T. tengcongensis* H-NOX Y140F, *T. tengcongensis* H-NOX Y40L,

T. tengcongensis H-NOX Y140H, *T. tengcongensis* H-NOX Y140A, *T. tengcongensis* I75F-His6, *T. tengcongensis* I75F, *T. tengcongensis* L144F-His6, *T. tengcongensis* L144F, *L. pneumophilia* 2 H-NOX F142Y, wild-type *L. pneumophilia* 1 H-NOX, wild-type *L. pneumophilia* 2 H-NOX, *L. pneumophilia 2* F9W-F142Y, wild-type *D. desulfuricans* H-NOX, *D. desulfuricans* H-NOX(728-899), *D. desulfuricans* H-NOX Y139L, wild-type *H. sapiens* β1 H-NOX, *H. sapiens* β1 I140Y, *H. sapiens* β1 I145Y, *H. sapiens* β1(1-385), *H. sapiens* β1(1-385) I145Y, *H. sapiens* β1(1-385) I145H, *H. sapiens* β1(1-194), *H. sapiens* β1(1-194) I145Y, *H. sapiens* β1(1-194) L9W-I145Y, *H. sapiens* β2(1-217), *H. sapiens* β2(1-217) I142Y, *H. sapiens* β1 H-NOX H105G, *H. sapiens* β1 H-NOX H105F, *H. sapiens* β1 H-NOX C78S, *H. sapiens* β1 H-NOX C78E, wild-type *R. norvegicus* β1 H-NOX, *R. norvegicus* β1(1-385), *R. norvegicus* β1(1-385) I145Y, *R. norvegicus* β1(1-385) I145H, *R. norvegicus* β1(1-194), *R. norvegicus* β1(1-194) I145Y, *R. norvegicus* β1(1-194) L9W-I145Y, *R. norvegicus* β2(1-217), *R. norvegicus* β2(1-217) I142Y, *R. norvegicus* β1 H-NOX H105G, *R. norvegicus* β1 H-NOX H105F, *R. norvegicus* sGC β1 H-NOX C78S, *R. norvegicus* sGC β1 H-NOX C78E, *C. botulinum* H-NOX(1-175), *C. botulinum* H-NOX(1-186), wild-type *C. acetobutylicum* H-NOX, *C. acetobutylicum* H-NOX(1-197), *C. acetobutylicum* H-NOX(1-183), wild-type *C. elegans* GCY-35 H-NOX, *C. elegans* H-NOX GCY-35(1-252), wild-type *D. melangaster* β1 H-NOX, wild-type *D. melangaster* CG14885-PA, wild-type *D. melangaster* CG14886, wild-type *D. melangaster* CG4154; wild-type *N. punctiforme* H-NOX, wild-type *C. crescentus* H-NOX, wild-type *S. oneidensis* H-NOX, wild-type *M. musculus* H-NOX , wild-type *C. familiaris* H-NOX, wild-type *B. Taurus* H-NOX, wild-type *R. norvegicus;* wild-type *X. laevis* H-NOX, wild-type *O. latipes* H-NOX, wild-type *O. curivatus* H-NOX, wild-type *F. rubripes* H-NOX, wild-type *A. gambiae* H-NOX, wild-type *M. sexta* H-NOX; wild-type *C. elegans* gcy-31, *C. elegans* gcy-32, wild-type *C. elegans* gcy-33, wild-type *C. elegans* gcy-34, wild-type *C. elegans* gcy-35, wild-type *C. elegans* gcy-36, wild-type *C. elegans* gcy-37; wild-type *V. cholera* H-NOX, wild-type *V. fischerii* H-NOX, and wild-type *N. punctiforme* H-NOX. In some embodiments of the methods, the H-NOX protein is a selected from the group consisting of wild-type *R. norvegicus* sGC, wild-type *R. norvegicus* β1(1-385), *R. norvegicus* β1(1-217), *R. norvegicus* β1(1-194), wild-type *T. tengcongensis* H-NOX, *T. tengcongensis* H-NOX Y140L, *T. tengcongensis* H-NOX Y140F, wild-type *L. pneumophilia* 1 H-NOX, wild-type *L. pneumophilia* 2 H-NOX, and *L. pneumophilia* 2 H-NOX F142Y. In some

embodiments of the methods, one or more liposomes or nanoparticles that include or encapsulate the H-NOX protein.

[0030] In some embodiments of the methods, the H-NOX protein is not *T. tengcongensis* H-NOX Y40L, wild-type *T. tengcongensis* H-NOX, wild-type *R. norvegicus* sGC, or *L. pneumophilia* 2 H-NOX F142Y. In some embodiments of the methods, the H-NOX protein is not *T. tengcongensis* H-NOX F78Y/Y140L. In some embodiments of the methods, the H-NOX protein is not wild-type *L. pneumophilia* 2 H-NOX, wild-type *H. sapiens* β1 H-NOX, *R. norvegicus* sGC β1 H-NOX (1-385), wild-type *R. norvegicus* β1 H-NOX, wild-type *D. melangaster* β1 H-NOX, wild-type *D. melangaster* CG14885-PA H-NOX, wild-type *C. elegans* GCY-35 H-NOX, wild-type *N. punctiforme* H-NOX, wild-type *C. crescentus* H-NOX, wild-type *S. oneidensis* H-NOX, or wild-type *C. acetobutylicum* H-NOX. In some embodiments of the methods, the H-NOX protein is not *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX Y140F, *R. norvegicus* sGC β1 H-NOX H105G, *R. norvegicus* sGC β1 H-NOX H105F, *R. norvegicus* sGC β1 H-NOX I145Y, *R. norvegicus* sGC β1 H-NOX C78S, or *R. norvegicus* sGC β1 H-NOX C78E. In some embodiments of the methods, the H-NOX protein is not *R. norvegicus* β2(1-217), *R. norvegicus* β1(1-194), *R. norvegicus* β1(1-385), or *R. norvegicus* β1(1-385) I145Y. In some embodiments of the methods, the H-NOX protein is not *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX Y140F, or *H. sapiens* β1 H-NOX (1-385) I145Y. In some embodiments of the methods, the H-NOX protein is not *T. tengcongensis* H-NOX Y140H, *H. sapiens* β1 I140Y, or *H. sapiens* β1 I145Y. In some embodiments of the methods, the H-NOX protein is not *T. tengcongensis* H-NOX Y40L, *T. tengcongensis* H-NOX F78Y/Y140L, *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX Y140F, wild-type *T. tengcongensis* H-NOX, *L. pneumophilia* 2 H-NOX F142Y, wild-type *L. pneumophilia* 2 H-NOX, *H. sapiens* β1 H-NOX I140Y, *H. sapiens* B1 I145Y, wild-type *H. sapiens* β1 H-NOX, *R. norvegicus* sGC β1 H-NOX (1-385), *R. norvegicus* sGC β1 H-NOX (1-385) I145Y, *R. norvegicus* sGC β1 H-NOX H105G, *R. norvegicus* sGC β1 H-NOX H105F, *R. norvegicus* sGC β1 H-NOX I145Y, wild-type *R. norvegicus* β1 H-NOX, wild-type *D. melangaster* β1 H-NOX, wild-type *D. melangaster* CG14885-PA H-NOX, wild-type *C. elegans* GCY-35 H-NOX, wild-type *N. punctiforme* H-NOX, wild-type *C. crescentus* H-NOX, wild-type *S. oneidensis* H-NOX, or wild-type *C. acetobutylicum* H-NOX. In some embodiments of the methods, the H-NOX protein is not any of the following H-NOX proteins that are listed by their gene name, followed by their species

abbreviation and Genbank Identifiers (such as the following protein sequences available as of May 21, 2006; May 22, 2006; May 21, 2007; or May 22, 2007): Npun5905_Npu_23129606, alr2278_Ana_17229770, SO2144_Sone_24373702, Mdeg1343_Mde_23027521, VCA0720_Vch_15601476, CC2992_Ccr_16127222, Rsph2043_Rhsp_22958463 (gi:46192757), Mmc10739_Mcsp_22999020, Tar4_Tte_20807169, Ddes2822_Dde_23475919, CAC3243_Cac_15896488 , gcy-31_Ce_17568389, CG14885_Dm_24647455, GUCY1B3_Hs_4504215, HpGCS-beta1_Hpul_14245738, Gycbeta100B_Dm_24651577, CG4154_Dm_24646993 (gi:NP_650424.2, gi:62484298), gcy-32_Ce_13539160,gcy-36_Ce_17568391 (gi:32566352, gi:86564713), gcy-35_Ce-17507861 (gi:71990146), gcy-37_Ce_17540904 (gi:71985505), GCY1α3_Hs_20535603, GCY1α2-Hs_899477, or GYCα-99B_Dm_729270 (gi:68067738) (Lakshminarayan *et al.* (2003). "Ancient conserved domains shared by animal soluble guanylyl cyclases and bacterial signaling proteins," *BMG Genomics* 4:5-13). The species abbreviations used in these names include Ana – *Anabaena Sp*; Ccr – *Caulobacter crescentus*; Cac – *Clostridium acetobutylicum*; Dde – *Desulfovibrio desulfuricans*; Mcsp – *Magnetococcus sp.*; Mde – *Microbulbifer degradans*; Npu – *Nostoc punctiforme*; Rhsp – *Rhodobacter sphaeroides*; Sone – *Shewanella oneidensis*; Tte – *Thermoanaerobacter tengcongensis*; Vch – *Vibrio cholerae*; Ce – *Caenorhabditis elegans*; Dm – *Drosophila melanogaster*; Hpul – *Hemicentrotus pulcherrimus*; Hs – *Homo sapiens*. In some embodiments of the methods, the H-NOX protein is not any of the following H-NOX proteins that are listed by their organism name and Pfam database accession number (such as the following protein sequences available as of May 21, 2006; May 22, 2006; May 17, 2007; May 21, 2007; or May 22, 2007): *Caenorhabditis briggsae* Q622M5_CAEBR, *Caenorhabditis briggsae* Q61P44_CAEBR, *Caenorhabditis briggsae* Q61R54_CAEBR, *Caenorhabditis briggsae* Q61V90_CAEBR, *Caenorhabditis briggsae* Q61A94_CAEBR, *Caenorhabditis briggsae* Q60TP4_CAEBR, *Caenorhabditis briggsae* Q60M10_CAEBR, *Caenorhabditis elegans* GCY37_CAEEL, *Caenorhabditis elegans* GCY31_CAEEL, *Caenorhabditis elegans* GCY36_CAEEL, *Caenorhabditis elegans* GCY32_CAEEL, *Caenorhabditis elegans* GCY35_CAEEL, *Caenorhabditis elegans* GCY34_CAEEL, *Caenorhabditis elegans* GCY33_CAEEL, *Oryzias curvinotus* Q7T040_ORYCU, *Oryzias curvinotus* Q75WF0_ORYCU, *Oryzias latipes* P79998_ORYLA, *Oryzias latipes* Q7ZSZ5_ORYLA, *Tetraodon nigroviridis* Q4SW38_TETNG,

Tetraodon nigroviridis Q4RZ94_TETNG, *Tetraodon nigroviridis* Q4S6K5_TETNG, *Fugu rubripes* Q90VY5_FUGRU, *Xenopus laevis* Q6INK9_XENLA, *Homo sapiens* Q5T8J7_HUMAN, *Homo sapiens* GCYA2_HUMAN, *Homo sapiens* GCYB2_HUMAN, *Homo sapiens* GCYB1_HUMAN, *Gorilla gorilla* Q9N193_9PRIM, *Pongo pygmaeus* Q5RAN8_PONPY, *Pan troglodytes* Q9N192_PANTR, *Macaca mulatta* Q9N194_MACMU, *Hylobates lar* Q9N191_HYLLA, *Mus musculus* Q8BXH3_MOUSE, *Mus musculus* GCYB1_MOUSE, *Mus musculus* Q3UTI4_MOUSE, *Mus musculus* Q3UH83_MOUSE, *Mus musculus* Q6XE41_MOUSE, *Mus musculus* Q80YP4_MOUSE, *Rattus norvegicus* Q80WX7_RAT, *Rattus norvegicus* Q80WX8_RAT, *Rattus norvegicus* Q920Q1_RAT, *Rattus norvegicus* Q54A43_RAT, *Rattus norvegicus* Q80WY0_RAT, *Rattus norvegicus* Q80WY4_RAT, *Rattus norvegicus* Q8CH85_RAT, *Rattus norvegicus* Q80WY5_RAT, *Rattus norvegicus* GCYB1_RAT, *Rattus norvegicus* Q8CH90_RAT, *Rattus norvegicus* Q91XJ7_RAT, *Rattus norvegicus* Q80WX9_RAT, *Rattus norvegicus* GCYB2_RAT, *Rattus norvegicus* GCYA2_RAT, *Canis familiaris* Q4ZHR9_CANFA, *Bos taurus* GCYB1_BOVIN, *Sus scrofa* Q4ZHR7_PIG, *Gryllus bimaculatus* Q59HN5_GRYBI, *Manduca sexta* O77106_MANSE, *Manduca sexta* O76340_MANSE, *Apis mellifera* Q5UAF0_APIME, *Apis mellifera* Q5FAN0_APIME, *Apis mellifera* Q6L5L6_APIME, *Anopheles gambiae* str PEST Q7PYK9_ANOGA, *Anopheles gambiae* str PEST Q7Q9W6_ANOGA, *Anopheles gambiae* str PEST Q7QF31_ANOGA, *Anopheles gambiae* str PEST Q7PS01_ANOGA, *Anopheles gambiae* str PEST Q7PFY2_ANOGA, *Anopheles gambiae* Q7KQ93_ANOGA, *Drosophila melanogaster* Q24086_DROME, *Drosophila melanogaster* GCYH_DROME, *Drosophila melanogaster* GCY8E_DROME, *Drosophila melanogaster* GCYDA_DROME, *Drosophila melanogaster* GCYDB_DROME, *Drosophila melanogaster* Q9VA09_DROME, *Drosophila pseudoobscura* Q29CE1_DROPS, *Drosophila pseudoobscura* Q296C7_DROPS, *Drosophila pseudoobscura* Q296C8_DROPS, *Drosophila pseudoobscura* Q29BU7_DROPS, *Aplysia californica* Q7YWK7_APLCA, *Hemicentrotus pulcherrimus* Q95NK5_HEMPU, *Chlamydomonas reinhardtii,* Q5YLC2_CHLRE, *Anabaena sp* Q8YUQ7_ANASP, *Flavobacteria bacterium BBFL7* Q26GR8_9BACT, *Psychroflexus torquis* ATCC *700755* Q1VQE5_9FLAO, marine gamma proteobacterium HTCC2207 Q1YPJ5_9GAMM, marine gamma proteobacterium HTCC2207 Q1YTK4_9GAMM, *Caulobacter crescentus* Q9A451_CAUCR, *Acidiphilium cryptum* JF-5 Q2DG60_ACICY, *Rhodobacter sphaeroides* Q3J0U9_RHOS4, *Silicibacter pomeroyi*

Q5LPV1_SILPO, *Paracoccus denitrificans* PD1222, Q3PC67_PARDE, *Silicibacter sp TM1040* Q3QNY2_9RHOB, *Jannaschia sp* Q28ML8_JANSC, *Magnetococcus sp* MC-1 Q3XT27_9PROT, *Legionella pneumophila* Q5WXP0_LEGPL, *Legionella pneumophila* Q5WTZ5_LEGPL, *Legionella pneumophila* Q5X268_LEGPA, *Legionella pneumophila* Q5X2R2_LEGPA, *Legionella pneumophila subsp pneumophila* Q5ZWM9_LEGPH, *Legionella pneumophila subsp pneumophila* Q5ZSQ8_LEGPH, *Colwellia psychrerythraea* Q47Y43_COLP3, *Pseudoalteromonas atlantica* T6c Q3CSZ5_ALTAT, *Shewanella oneidensis* Q8EF49_SHEON, *Saccharophagus degradans* Q21E20_SACD2, *Saccharophagus degradans* Q21ER7_SACD2, *Vibrio angustum* S14 Q1ZWE5_9VIBR, *Vibrio vulnificus* Q8DAE2_VIBVU, *Vibrio alginolyticus* 12G01 Q1VCP6_VIBAL, *Vibrio sp* DAT722 Q2FA22_9VIBR, *Vibrio parahaemolyticus* Q87NJ1_VIBPA, *Vibrio fischeri* Q5E1F5_VIBF1, *Vibrio vulnificus* Q7MJS8_VIBVY, *Photobacterium sp SKA34* Q2C6Z5_9GAMM, *Hahella chejuensis* Q2SFY7_HAHCH, *Oceanospirillum sp* MED92 Q2BKV0_9GAMM, *Oceanobacter sp* RED65 Q1N035_9GAMM, *Desulfovibrio desulfuricans* Q310U7_DESDG, *Halothermothrix orenii* H 168 Q2AIW5_9FIRM, *Thermoanaerobacter tengcongensis* Q8RBX6_THETN, *Caldicellulosiruptor saccharolyticus* DSM 8903 Q2ZH17_CALSA, *Clostridium acetobutylicum* Q97E73_CLOAB, *Alkaliphilus metalliredigenes* QYMF Q3C763_9CLOT, *Clostridium tetani* Q899J9_CLOTE, and *Clostridium beijerincki NCIMB 8052* Q2WVN0_CLOBE. In some embodiments of the methods, the H-NOX protein does not have a mutation in the Y-S-R motif, which includes Tyr135, Ser137, and Arg139 of human H-NOX.

[0031] Unless otherwise explicitly noted or dictated by context, all wild-type and mutant proteins and all pharmaceutical compositions described herein may be used in any of the methods of delivering NO described herein. The H-NOX protein may or may not have heme and/or NO bound and may or may not be covalently bound to another molecule or moiety, such as polyethylene glycol. In some embodiments, the H-NOX protein is a fusion protein that includes an H-NOX domain and part or all of another protein, such as albumin (*e.g.*, human serum albumin).

[0032] In one aspect, the invention features kits that include one or more H-NOX proteins. In some embodiments, the invention provides a kit that includes an H-NOX protein and instructions for using the kit to deliver NO to an individual. In some embodiments, the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ to about 10 s^{-1} at 37 °C, and the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C. In some embodiments, the NO

dissociation constant of the H-NOX protein is within 2 orders of magnitude of that of hemoglobin, and the NO reactivity of the H-NOX protein is at least 10-fold lower than that of hemoglobin. Unless otherwise explicitly noted or dictated by context, all wild-type and mutant proteins and all pharmaceutical compositions described herein may be used in any of the kits described herein. The H-NOX protein may or may not have heme and/or NO bound and may or may not be covalently bound to another molecule or moiety, such as polyethylene glycol. In some embodiments, the H-NOX protein is a fusion protein that includes an H-NOX domain and part or all of another protein, such as albumin (*e.g.*, human serum albumin).

[0033] In one aspect, the invention features an H-NOX protein (such as any of the wild-type or mutant proteins described herein) for use as a medicament. In some embodiments, the invention features an H-NOX protein for use in a method of delivering NO to an individual. In some embodiments, the H-NOX protein is used to treat any condition for which delivery of NO is beneficial, such as a cardiovascular condition, hypertension, a condition exacerbated by hypertension (*e.g.*, heart failure, renal failure, or a stroke) , a vasoconstrictive condition, stroke, or a functional NO deficiency.

[0034] In some embodiments, the invention features the use of an H-NOX protein (such as any of the wild-type or mutant proteins described herein) for the manufacture of a medicament, such as a medicament for delivering NO to an individual. In some embodiments, the invention features the use of an H-NOX protein for delivering NO to an individual. In some embodiments, the H-NOX protein is used to treat any condition for which delivery of NO is beneficial, such as a cardiovascular condition, hypertension, a condition exacerbated by hypertension (*e.g.*, heart failure, renal failure, or a stroke) , a vasoconstrictive condition, stroke, or a functional NO deficiency.

BRIEF DESCRIPTION OF THE DRAWINGS

[0035] FIG. 1A is a picture of the three dimensional structure of distal pocket residues of NO-binding and O_2-binding H-NOX proteins (above heme). Heme coordination residues of NO-binding and O_2-binding H-NOX proteins are also shown (below heme). FIG. 1A is based on the three-dimensional structure of *T. tengcongensis* H-NOX reported by Pellicena, P. *et al.* (August 31, 2004). "Crystal Structure of An Oxygen-Binding Heme Domain Related to Soluble Guanylate Cyclases," *Proc Natl. Acad Sci USA* 101(35):12854-12859.

[0036] FIG. 1B is a stereo side view of the three dimensional structure of *T. tengcongensis* HNOX illustrating structural features of the H-NOX domain. The protein fold is represented by ribbon diagrams. The heme, dioxygen ligand, and proximal histidine are shown as ball-and-stick models. α-helices are labeled A-G according to the nomenclature shown in FIG. 5B. β-strands are labeled 1–4. FIG. 1B is from Pellicena, P. *et al.* (August 31, 2004). "Crystal Structure of An Oxygen-Binding Heme Domain Related to Soluble Guanylate Cyclases," *Proc Natl. Acad Sci USA* 101(35):12854-12859.

[0037] FIGS. 1C-1H are pictures of the three dimensional structure of *T. tengcongensis* HNOX illustrating exemplary distal pocket residues in *T. tengcongensis* HNOX. The following residues depicted in FIGS. 1C-1H are the main residues comprising the H-NOX distal pocket: Thr4, Ile5, Thr8, Trp9, Trp67, Asn74, Ile75, Phe78, Phe82, Tyr140, and Leu144, which are contained within helices A, D, E, and G. FIGS. 1C-1H were created using PYMOL (DeLano Scientific, LLP).

[0038] FIG. 2 is a sequence alignment of the following H-NOX proteins that bind or are predicted to bind O_2 and NO: Majority (SEQ ID NO:1); Ce. gcy-31 (SEQ ID NO:2); Ce. gcy-33 (SEQ ID NO:3); Ce. gcy-35 (SEQ ID NO:4); Dm. CG14885 HNOX (SEQ ID NO:5); Dm. CG4154 HNOX (SEQ ID NO:6); Ms. Beta3 HNOX (SEQ ID NO:7); *Tt* HNOX (SEQ ID NO:8); and Ca HNOX (SEQ ID NO:9). These H-NOX proteins are predicted to bind O_2 as well as NO because they have a tyrosine at the position corresponding to Y140 of *T. tengcongensis* H-NOX. The amino acid numbering used in FIG. 2 starts with the first amino acid in the H-NOX domain or full-length protein as residue number 1. The alignment was generated using the default parameters in the program MegAlign. The abbreviations used in FIG. 2 are described below with respect to FIGS. 4A-4D.

[0039] FIG. 3A-3D are a sequence alignment of the following H-NOX proteins that bind or are predicted to bind NO but not O_2: Majority (SEQ ID NO:10); Dm. sGC beta1 protein (SEQ ID NO:11); sGC beta1 protein (SEQ ID NO:12); hs. sGC beta1 protein (SEQ ID NO:13); hs. beta2 protein (SEQ ID NO:14); Ms. sGC beta1 protein (SEQ ID NO:15); Mm. sGCbeta1 protein (SEQ ID NO:16); Np. beta1HD-like (SEQ ID NO:17); Tr. sGC beta1 protein (SEQ ID NO:18); Anopheles_gambiae|XP_310919 (SEQ ID NO:19); Apis_mellifera|NP_001011632 (SEQ ID NO:20); Bt. sGC beta1 protein (SEQ ID NO:21); Chlamydomonas_reinhardtii|AAR02 (SEQ ID NO:22); Oryzias_curvinotus|BAC98396 (SEQ ID NO:23); Oryzias_latipes|BAA76691 (SEQ ID

NO:24); Strongylocentrotus_purpuratus|X (SEQ ID NO:25); and Sus scrofa beta1|NP_001018042+ (SEQ ID NO:26). The alignment was generated using the default parameters in the program MegAlign. The abbreviations used in FIGS. 3A-3D are described below with respect to FIG. 4.

[0040] FIGS. 4A-4D are a sequence alignment of H-NOX proteins from FIGS. 2 and 3A-3D: Majority (SEQ ID NO:27); Dm. sGC beta1 protein (SEQ ID NO:11); sGC beta1 protein (SEQ ID NO:12); hs. sGC beta1 protein (SEQ ID NO:13); hs. beta2 protein (SEQ ID NO:14); Mm. sGC beta1 protein (SEQ ID NO:16); Np. beta1HD-like (SEQ ID NO:17); Tr. sGC beta1 protein (SEQ ID NO:18); Chlamydomonas_reinhardtii|AAR02 (SEQ ID NO:22); Oryzias_curvinotus|BAC98396 (SEQ ID NO:23); Strongylocentrotus_purpuratus|X (SEQ ID NO:25); Sus scrofa beta1|NP_001018042 (SEQ ID NO:26); gcy-31a (SEQ ID NO:2); gcy-33 (SEQ ID NO:3); Ca. HNOX (SEQ ID NO:9); T. beta1HD-like (SEQ ID NO:8); Ms. sGc beta 3 protein (SEQ ID NO:7); CG14885 (SEQ ID NO:5); and Dm. sGC short variant (SEQ ID NO:6). The alignment was generated using the default parameters in the program MegAlign. For FIGS. 2-4D, "Dm. sGC beta1 protein" denotes *Drosophila melanogaster* β1 H-NOX; "sGC beta1 protein" denotes *Rattus norvegicus* β1 H-NOX; "hs. sGC beta1 protein" denotes *Homo sapiens* β1 H-NOX; "hs. beta2 protein" denotes *Homo sapiens* β2 H-NOX; "Mm. sGC beta1 protein" denotes *Mus musculus* β1 H-NOX; "Np. beta1HD-like" denotes *Nostoc punctiforme* H-NOX; "Tr. sGC beta1 protein" denotes *Takifugu rubripes* β1 H-NOX; "Anopheles_gambiae|XP_310919" denotes *Anopheles gambiae* β1 H-NOX; "Apis_mellifera|NP_001011632" denotes *Apis mellifera* β1 H-NOX; "Bt. sGC beta1 protein" denotes *Bos taurus* β1 H-NOX; "Chlamydomonas_reinhardtii|AAR02" denotes *Chlamydomonas reinhardtii* β1 H-NOX; "Oryzias_curvinotus|BAC98396 denotes *Oryzias curvinotus* β1 H-NOX; "Oryzias_latipes|BAA76691" denotes *Oryzias latipes* β1 H-NOX; "Strongylocentrotus_purpuratus|X" denotes *Strongylocentrotus purpuratus* β1 H-NOX; "Sus scrofa beta1|NP_001018042+" denotes *Sus scrofa* β1 H-NOX; "gcy-31a" denotes *Caenorhabditis elegans* Gcy-31a H-NOX; "gcy-33" denotes *Caenorhabditis elegans* Gcy-33 H-NOX; "gcy-35" denotes *Caenorhabditis elegans* Gcy-35 H-NOX; "Ca. HNOX" denotes *Clostridium acetobutiylicum* H-NOX; "T. beta1HD-like" denotes *Thermoanaerobacter tengcongensis* H-NOX; "Ms. sGc beta 3 protein" denotes *Manduca sexta* β3 H-NOX; "CG14885" denotes *Drosophila melanogaster* CG14885 H-NOX; "Dm. sGC short variant" denotes *Drosophila melanogaster* Gcy-88-E-S H-NOX, and "Dm. CG4154 HNOX" denotes *Drosophila melanogaster* CG4154 H-NOX.

[0041] FIG. 5A is a sequence alignment of members of the H-NOX family. The sequence numbering is that of *T. tengcongensis* H-NOX. Invariant residues are indicated by a "V", very highly conserved residues are indicated by "s". Y140 of *T. tengcongensis* H-NOX is indicated by a "H." Predicted distal pocket tyrosine residues that may stabilize an Fe^{II}-O_2 complex in other H-NOX proteins are: position 70 for *Caenorhabditis elegans* GCY-35; position 140 in *Drosophila melanogaster* CG14885-PA; position 138 of *Caenorhabditis elegans* GCY-35; position 140 of *Clostridium acetobutylicum*; numbered according to *Thermoanaerobacter tengcongensis*. Accession numbers are: *Homo sapiens* β1 [gi:2746083] (SEQ ID NO:28), *Rattus norvegicus* β1 [gi:27127318] (SEQ ID NO:29), *Drosophila melangaster* β1 [gi:861203] (SEQ ID NO:30), *Drosophila melangaster* CG14885-PA [gi:23171476] (SEQ ID NO:31), *Caenorhabditis elegans* GCY-35 [gi:52782806] (SEQ ID NO:32), *Nostoc punctiforme* [gi:23129606] (SEQ ID NO:33), *Caulobacter crescentus* [gi:16127222] (SEQ ID NO:34), *Shewanella oneidensis* [gi:24373702] (SEQ ID NO:35), *Legionella pneumophila* (ORF 2) [CUCGC_272624] (SEQ ID NO:36), *Clostridium acetobutylicum* [gi:15896488] (SEQ ID NO:37), and *Thermoanaerobacter tengcongensis* [gi:20807169] (SEQ ID NO:38). Alignments were generated using the program MegAlign, Lasergene, DNA Star, (see, the world-wide web at "dnastar.com/products/megalign.php"). Clustal-W default parameters were used.

[0042] FIG. 5B is a sequence alignment of exemplary H-NOX domains. The secondary structure annotations and the numbering on top of the alignment correspond to the H-NOX domain from *T. tengcongensis*. α-helices are represented by spirals, and β-strands by arrows. The distal pocket is defined by α-helices αA, αD, αE, and αG. Pubmed/NCBI accession numbers are as follows: Ther_tengcongensis gi | 20807169 | (SEQ ID NO:39), Clos_acetobutylicum gi | 15896488 | (SEQ ID NO:40), Clos_tetani GI:75543266 (SEQ ID NO:41), Desu_desulfuricans gi | 23475919 | (SEQ ID NO:42), Vibr_vulnificus gi | 27361734 | (SEQ ID NO:43), Caul_crescentus gi | 16127222 | (SEQ ID NO:44), Micr_degradans gi | 23027521 | (SEQ ID NO:45), Vibr_cholerae gi | 15601476 | (SEQ ID NO:46), Shew_oneidensis gi | 24373702 | (SEQ ID NO:47), Rat_beta1_sGC gi | 27127318 | (SEQ ID NO:48), Rat_beta2_sGC gi | 21956635 | (SEQ ID NO:49), Nost_punctiforme gi | 23129606 | (SEQ ID NO:50), and Nost_sp. gi | 17229770 | (SEQ ID NO:51). The consensus sequence is shown a the bottom of FIG. 5 B (SEQ

ID NO:52). The alignments were generated using the program MULTALIN (Corpet, F. (1988) *Nucleic Acids Res.* 16:10881–10890), and FIG. 5B was prepared using the program ESPRIPT (Gouet, P. *et al.* (1999) *Bioinformatics* 15: 305–308.).

[0043] FIGS. 6A and 6B are pictures of the three dimensional structure of the heme environment of the *T. tengcongensis* H-NOX domain. FIG.S. 6A and 6B are from Pellicena, P. *et al.* (August 31, 2004). "Crystal Structure of An Oxygen-Binding Heme Domain Related to Soluble Guanylate Cyclases," *Proc Natl. Acad Sci USA* 101(35):12854-12859.

[0044] FIGS. 7A-7F are graphs of the UV-visible spectroscopy of H-NOX proteins after anaerobic reduction (Fe^{II} unligated complexes; top line in each graph) before and after being exposed to air (Fe^{II}-O_2 complexes; bottom line in each graph) for *Tt* H-NOX (FIG. 7A), *Tt* Y140L (FIG. 7B), *Tt* W9F-Y140L (FIG. 7C), *Tt* F78Y-Y140L (FIG. 7D), *L2* H-NOX and *L2* F142Y (FIG. 7E), and β1(1-385) and β1(1-385) I145Y (FIG. 7F). In addition to the Fe^{II} and Fe^{II}-O_2 complexes of *L2* F142Y and β1(1-385) I145Y, the spectrum of wild-type *L2* H-NOX and β1-(1-385) H-NOX after reduction and exposure to air are shown in the middle line in FIG. 7E and 7F, respectively, to demonstrate that these proteins do not bind O_2 before the addition of a distal pocket tyrosine. The two or three numbers written in the upper left corner of each panel represent the wavelength for the peak of the lines in the graph. The numbers are written vertically in the order in which the corresponding lines appear vertically in the graph. For example, the 430 nm value in FIG. 7A denotes the peak of the wavelength for the top line in the graph (which represents a Fe^{II} unligated complex), and the 416 nm value in FIG. 7A denotes the peak of the wavelength for the bottom line in the graph (which represents a Fe^{II}-O_2 complex). A shift in the wavelength in the presence of air indicates that the protein binds O_2. The formation of a double peak between 500 and 600 nm in the presence of air is also indicative of O_2 binding. FIGS. 7A-7F are from Boon, E. M. *et al.* (2005). "Molecular Basis For NO Selectivity in Soluble Guanylate Cyclase," *Nature Chem. Biol.* 1:53-59.

[0045] FIGS. 8A-8DD contain polynucleotide sequences of exemplary nucleic acids that encode H-NOX proteins and the amino acid sequences of the corresponding H-NOX proteins (SEQ ID NOS:53-162).

DETAILED DESCRIPTION OF THE INVENTION

[0046] The present invention is based in part on the surprising discovery that H-NOX proteins have a much lower NO reactivity than hemoglobin. This intrinsic low NO reactivity (and high NO stability) makes wild-type and mutant H-NOX proteins desirable NO carriers because of the lower probability of inactivation of H-NOX proteins by NO in the presence of O_2. Importantly, the presence of a distal pocket tyrosine in some H-NOX proteins (Pellicena, P. *et al.* (August 31, 2004). "Crystal Structure of An Oxygen-Binding Heme Domain Related to Soluble Guanylate Cyclases," *Proc Natl. Acad Sci USA* 101(35):12854-12859) is suggestive of undesirable, *high* NO reactivity, contraindicating use as an NO carrier. For example, by analogy, a *Mycobacterium tuberculosis* hemoglobin protein, with a structurally analogous distal pocket tyrosine, reacts extremely rapidly with NO, and is used by the Mycobacterium to effectively scavenge and avoid defensive NO produced by an infected host (Ouellet, H. *et al.* (April 30, 2002). "Truncated Hemoglobin HbN Protects Mycobacterium Bovis From Nitric Oxide," *Proc. Natl. Acad. Sci. U S A* 99(9):5902-5907). However, we surprisingly discovered that H-NOX proteins actually have a much lower NO reactivity than that of hemoglobin making their use as NO carriers possible.

[0047] Additionally, it was discovered that the usefulness of H-NOX proteins as NO carriers can be improved by modifying their affinities for NO or O_2 to maximize the amount of NO that is bound to the H-NOX protein and to reduce the amount of H-NOX protein that is oxidized by the reaction of NO with O_2 bound to the H-NOX protein. In particular, the affinity of H-NOX proteins for NO or O_2 and the ability of H-NOX proteins to discriminate between NO and O_2 ligands can be altered by the introduction of one or more amino acid mutations, allowing H-NOX proteins to be tailored to bind NO or O_2 with desired affinities. For example, the dissociation constant or dissociation rate for NO or O_2 binding by H-NOX proteins can be altered the introduction of a single amino acid mutation. Additional mutations can be introduced to further alter the affinity for NO and/or O_2. The H-NOX protein family can therefore be manipulated to exhibit improved or optimal kinetic and thermodynamic properties for NO delivery. For example, mutant H-NOX proteins have been generated with altered dissociation constants and/or dissociation rates for NO binding that improve the usefulness of H-NOX proteins for a variety of clinical and industrial applications. In some embodiments, an H-NOX protein with a low affinity for O_2 (such as an O_2 dissociation constant of at least about 1 μM at 37 °C) is used to minimize the amount of O_2 that

binds the H-NOX protein, thereby facilitating the binding of NO to the H-NOX protein and reducing the amount of H-NOX protein that is oxidized due to the reaction of NO with O_2 bound to the heme of the H-NOX protein. This reduction in the oxidation of H-NNOX proteins results in less destruction of NO and O_2 that can be used by the organs, tissues, and cells of the treated individual. The ability to tune H-NOX proteins to bind and deliver NO is a therapeutic avenue that addresses and overcomes the central shortcomings of current vasodilators. Accordingly, the present invention provides proteins, compositions, kits, and methods for the delivery of NO.

[0048] There are numerous benefits of using H-NOX proteins for NO delivery. Organic nitrates are effective for a limited length of time due to tolerance. Since H-NOX proteins delivery NO directly to individuals without requiring the bioconversion of nitrates to NO, the effectiveness of H-NOX proteins as NO carriers is not limited by inhibition of this bioconversion pathway. Major limitations of hemoglobin-based NO carriers are their high affinity for O_2 and their propensity to be inactivated by NO. As mentioned above, destruction of even low levels of NO by hemoglobin-based carriers can have serious effects on the tonic resting state of the vasculature and organs and leads to hypertension and gastrointestinal distress. Intra- and inter-molecular cross-linking have been used to minimize the toxicity of hemoglobin-based vehicles when used as oxygen carriers ("Blood Substitutes," R. Winslow ed. Academic Press, 2006). While these modifications overcame some of the severe toxicity issues related to extravasation of hemoglobin, the high NO reactivity remained. In contrast, H-NOX proteins have a much lower NO reactivity than hemoglobin. This lower reactivity leads to less destruction of NO, O_2, and H-NOX protein since less NO reacts with O_2 bound to the H-NOX protein. The ability to select H-NOX proteins with desired dissociation constants and dissociation rates for NO can also minimize side-effects by preventing too much NO from being released (causing hypotension) and prevent NO from being released at undesired sites (*e.g.*, sites that are not vasoconstricted). Engineering H-NOX proteins to bind and deliver NO with minimal NO reactivity provides a new blood gas NO carrier where the H-NOX proteins deliver NO without being inactivated by NO. These H-NOX proteins, compositions, kits, and methods are described further herein.

[0049] For delivery of NO, engineered H-NOX proteins represent an important alternative that overcomes the persistent problem of tolerance with current nitrovasodilators. The use of H-

NOX proteins as delivery vehicles for NO provides a new therapeutic venue for treating diseases exacerbated by chronic hypertension.

H-NOX Proteins

Overview of H-NOX Protein Family

[0050] Unless otherwise indicated, any wild-type or mutant H-NOX protein can be used in the compositions, kits, and methods as described herein. As used herein, an "H-NOX protein" means a protein that has an H-NOX domain (named for Heme-Nitric oxide and OXygen binding domain). An H-NOX protein may or may not contain one or more other domains in addition to the H-NOX domain. H-NOX proteins are members of a highly-conserved, well-characterized family of hemoproteins (Iyer, L. M. *et al.* (February 3, 2003). "Ancient Conserved Domains Shared by Animal Soluble Guanylyl Cyclases And Bacterial Signaling Proteins," *BMC Genomics* 4(1):5; Karow, D. S. *et al.* (August 10, 2004). "Spectroscopic Characterization of the Soluble Guanylate Cyclase-Like Heme Domains From Vibrio Cholerae And Thermoanaerobacter Tengcongensis," *Biochemistry* 43(31):10203-10211; Boon, E. M. *et al.* (2005). "Molecular Basis For NO Selectivity in Soluble Guanylate Cyclase," *Nature Chem. Biol.* 1:53-59; Boon, E. M. *et al.* (October 2005). "Ligand Discrimination in Soluble Guanylate Cyclase and the H-NOX Family of Heme Sensor Proteins," *Curr. Opin. Chem. Biol.* 9(5):441-446; Boon, E. M. *et al.* (2005). "Ligand Specificity of H-NOX Domains: From sGC to Bacterial NO Sensors," *J. Inorg. Biochem.* 99(4):892-902). H-NOX proteins are also referred to as Pfam 07700 proteins or HNOB proteins (Pfam - A database of protein domain family alignments and Hidden Markov Models, Copyright (C) 1996-2006 The Pfam Consortium; GNU LGPL Free Software Foundation, Inc., 59 Temple Place - Suite 330, Boston, MA 02111-1307, USA). In some embodiments, an H-NOX protein has, or is predicted to have, a secondary structure that includes six alpha-helices, followed by two beta-strands, followed by one alpha-helix, followed by two beta-strands. An H-NOX protein can be an apoprotein that is capable of binding heme or a holoprotein with heme bound. An H-NOX protein can covalently or non-covalently bind a heme group. Some H-NOX proteins bind NO but not O_2, and others bind both NO and O_2. H-NOX domains from facultative aerobes that have been isolated bind NO but not O_2. H-NOX proteins from obligate aerobic prokaryotes, *C. elegans*, and *D. melanogaster* bind NO and O_2. Mammals have two H-NOX proteins: β1 and β2. An alignment of mouse, rat, cow, and human

H-NOX sequences shows that these species share >99% identity. In some embodiments, the H-NOX domain of an H-NOX protein or the entire H-NOX protein is at least about any of 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 95, 97, 98, 99, or 99.5% identical to that of the corresponding region of a naturally-occurring *Thermoanaerobacter tengcongensis* H-NOX protein or a naturally-occurring sGC protein (*e.g.*, a naturally-occurring sGC β1 protein). As discussed further herein, an H-NOX protein may optionally contain one or more mutations relative to the corresponding naturally-occurring H-NOX protein. In some embodiments, the H-NOX protein includes one or more domains in addition to the H-NOX domain. In particular embodiments, the H-NOX protein includes one or more domains or the entire sequence from another protein. For example, the H-NOX protein may be a fusion protein that includes an H-NOX domain and part or all of another protein, such as albumin (*e.g.*, human serum albumin). In some embodiments, only the H-NOX domain is present.

[0051] A crystal structure of a prokaryotic O_2-binding H-NOX from *Thermoanaerobacter tengcongensis* (Nioche, P. *et al.* (November 26, 2004). "Femtomolar Sensitivity of a NO Sensor From Clostridium Botulinum," *Science* 306(5701):1550-1553; Pellicena, P. *et al.* (August 31, 2004). "Crystal Structure of An Oxygen-Binding Heme Domain Related to Soluble Guanylate Cyclases," *Proc Natl. Acad Sci USA* 101(35):12854-12859) shows that a tyrosine side chain hydroxyl group makes a critical H-bond to the Fe^{II}-O_2 moiety. This distal pocket hydrogen-bonding network, involving principally Y140, stabilizes an Fe^{II}-O_2 complex (FIG. 6B). This tyrosine is not present in H-NOX proteins that discriminate against O_2 and only bind NO. For example, this hydrogen-bonding network is predicted to be absent in the H-NOX proteins from sGCs and aerobic prokaryotes, suggesting this as a key molecular factor in the remarkable ligand selectivity against O_2 displayed by these heme proteins. FIGS. 7A-7G clearly demonstrate that the addition of a tyrosine in the distal pocket of a wild-type H-NOX protein that binds NO but not O_2 can enable the mutant H-NOX protein to bind O_2. Thus, a tyrosine in the distal heme pocket of the H-NOX heme fold acts like a switch to turn on or off O_2 binding.

[0052] As illustrated in FIGS. 6A and 6B, the structure of the porphyrin is highly distorted. As illustrated in FIG. 6A, the conserved Y-S-R motif makes hydrogen-bonding interactions with the propionic acid side chains of the heme group. FIG. 6B, the conserved H102 is the proximal ligand to the heme (FIG. 6B).

[0053] As used herein, a "protein" includes proteins and fragments of proteins whether isolated from natural sources, produced by recombinant techniques, or chemically synthesized. A protein may have one or more modifications, such as a post-translational modification (*e.g.*, glycosylation, *etc*) or any other modification (*e.g.*, PEGylation, *etc*). The protein may contain one or more non-naturally-occurring amino acids (*e.g.*, such as an amino acid with a side chain modification). In various embodiments, the H-NOX protein has at least about 50, 100, 150, 181, 200, 250, 300, 350, 400, or more amino acids. In some embodiments, the H-NOX proteins may include from about 50 to about 600 amino acids, such as about 100 to about 500 amino acids, about 150 to about 400 amino acids, about 150 to about 300 amino acids, or about 175 to about 200 amino acids.

Sources of H-NOX Proteins

[0054] H-NOX proteins from any genus or species can be used in the compositions, kits, and methods described herein. In various embodiments, the H-NOX protein is a protein from a mammal (*e.g.*, a primate (*e.g.*, human, monkey, gorilla, ape, lemur, *etc*), a bovine, an equine, a porcine, a canine, or a feline), an insect, a yeast, or a bacteria or is derived from such a protein. Exemplary mammalian H-NOX proteins include wild-type human and rat soluble guanylate cyclase (such as the β1 subunit). Examples of H-NOX proteins include wild-type mammalian H-NOX proteins, *e.g. H. sapiens, M. musculus, C. familiaris, B. taurus* and *R. norvegicus;* and wild-type non-mammalian vertebrate H-NOX proteins, *e.g,. X. laevis, O. latipes, O. curivatus,* and *F. rubripes.* Examples of non-mammalian wild-type NO-binding H-NOX proteins include wild-type H-NOX proteins of *D. melanogaster, A. gambiae,* and *M. sexta*; examples of non-mammalian wild-type O_2-binding H-NOX proteins include wild-type H-NOX proteins of *C. elegans* gcy-31, gcy-32, gcy-33, gcy-34, gcy-35, gcy-36, and gcy-37; *D. melanogaster* CG14885, CG14886, and CG4154; and *M. sexta* beta-3; examples of prokaryotic wild-type H-NOX proteins include *T. tengcongensis, V. cholera, V. fischerii, N. punctiforme, D. desulfuricans, L. pneumophila 1, L. pneumophila 2,* and *C. acetobutylicum.*

[0055] NCBI Accession numbers for exemplary H-NOX proteins include the following: *Homo sapiens* β1 [gi:2746083], *Rattus norvegicus* β1 [gi:27127318], *Drosophila melangaster* β1 [gi:861203], *Drosophila melangaster* CG14885-PA [gi:23171476], *Caenorhabditis elegans* GCY-

35 [gi:52782806], *Nostoc punctiforme* [gi:23129606], *Caulobacter crescentus* [gi:16127222], *Shewanella oneidensis* [gi:24373702], *Legionella pneumophila* (ORF 2) [CUCGC_272624], *Clostridium acetobutylicum* [gi:15896488], and *Thermoanaerobacter tengcongensis* [gi:20807169].

[0056] Exemplary H-NOX protein also include the following H-NOX proteins that are listed by their gene name, followed by their species abbreviation and Genbank Identifiers (such as the following protein sequences available as of May 21, 2006; May 22, 2006; May 21, 2007; or May 22, 2007, which are each hereby incorporated by reference in their entireties): Npun5905_Npu_23129606, alr2278_Ana_17229770, SO2144_Sone_24373702, Mdeg1343_Mde_23027521, VCA0720_Vch_15601476,CC2992_Ccr_16127222, Rsph2043_Rhsp_22958463 (gi:46192757), Mmc10739_Mcsp_22999020, Tar4_Tte_20807169,Ddes2822_Dde_23475919,CAC3243_Cac_15896488 ,gcy-31_Ce_17568389, CG14885_Dm_24647455,GUCY1B3_Hs_4504215, HpGCS-beta1_Hpul_14245738, Gycbeta100B_Dm_24651577, CG4154_Dm_24646993 (gi:NP_650424.2, gi:62484298), gcy-32_Ce_13539160,gcy-36_Ce_17568391 (gi:32566352, gi:86564713), gcy-35_Ce-17507861 (gi:71990146), gcy-37_Ce_17540904 (gi:71985505), GCY1a3_Hs_20535603, GCY1a2-Hs_899477, or GYCa-99B_Dm_729270 (gi:68067738) (Lakshminarayan *et al.* (2003). "Ancient conserved domains shared by animal soluble guanylyl cyclases and bacterial signaling proteins," *BMG Genomics* 4:5-13). The species abbreviations used in these names include Ana – *Anabaena Sp*; Ccr – *Caulobacter crescentus*; Cac – *Clostridium acetobutylicum*; Dde – *Desulfovibrio desulfuricans*; Mcsp – *Magnetococcus sp.*; Mde – *Microbulbifer degradans*; Npu – *Nostoc punctiforme*; Rhsp – *Rhodobacter sphaeroides*; Sone – *Shewanella oneidensis*; Tte – *Thermoanaerobacter tengcongensis*; Vch – *Vibrio cholerae*; Ce – *Caenorhabditis elegans*; Dm – *Drosophila melanogaster*; Hpul – *Hemicentrotus pulcherrimus*; Hs – *Homo sapiens.*

[0057] Other exemplary H-NOX proteins include the following H-NOX proteins that are listed by their organism name and Pfam database accession number (such as the following protein sequences available as of May 21, 2006; May 22, 2006; May 17, 2007; May 21, 2007; or May 22, 2007, which are each hereby incorporated by reference in their entireties): *Caenorhabditis briggsae* Q622M5_CAEBR, *Caenorhabditis briggsae* Q61P44_CAEBR, *Caenorhabditis briggsae* Q61R54_CAEBR, *Caenorhabditis briggsae* Q61V90_CAEBR, *Caenorhabditis briggsae* Q61A94_CAEBR, *Caenorhabditis briggsae* Q60TP4_CAEBR, *Caenorhabditis briggsae*

Q60M10_CAEBR, *Caenorhabditis elegans* GCY37_CAEEL, *Caenorhabditis elegans* GCY31_CAEEL, *Caenorhabditis elegans* GCY36_CAEEL, *Caenorhabditis elegans* GCY32_CAEEL, *Caenorhabditis elegans* GCY35_CAEEL, *Caenorhabditis elegans* GCY34_CAEEL, *Caenorhabditis elegans* GCY33_CAEEL, *Oryzias curvinotus* Q7T040_ORYCU, *Oryzias curvinotus* Q75WF0_ORYCU, *Oryzias latipes* P79998_ORYLA, *Oryzias latipes* Q7ZSZ5_ORYLA, *Tetraodon nigroviridis* Q4SW38_TETNG, *Tetraodon nigroviridis* Q4RZ94_TETNG, *Tetraodon nigroviridis* Q4S6K5_TETNG, *Fugu rubripes* Q90VY5_FUGRU, *Xenopus laevis* Q6INK9_XENLA, *Homo sapiens* Q5T8J7_HUMAN, *Homo sapiens* GCYA2_HUMAN, *Homo sapiens* GCYB2_HUMAN, *Homo sapiens* GCYB1_HUMAN, *Gorilla gorilla* Q9N193_9PRIM, *Pongo pygmaeus* Q5RAN8_PONPY, *Pan troglodytes* Q9N192_PANTR, *Macaca mulatta* Q9N194_MACMU, *Hylobates lar* Q9N191_HYLLA, *Mus musculus* Q8BXH3_MOUSE, *Mus musculus* GCYB1_MOUSE, *Mus musculus* Q3UTI4_MOUSE, *Mus musculus* Q3UH83_MOUSE, *Mus musculus* Q6XE41_MOUSE, *Mus musculus* Q80YP4_MOUSE, *Rattus norvegicus* Q80WX7_RAT, *Rattus norvegicus* Q80WX8_RAT, *Rattus norvegicus* Q920Q1_RAT, *Rattus norvegicus* Q54A43_RAT, *Rattus norvegicus* Q80WY0_RAT, *Rattus norvegicus* Q80WY4_RAT, *Rattus norvegicus* Q8CH85_RAT, *Rattus norvegicus* Q80WY5_RAT, *Rattus norvegicus* GCYB1_RAT, *Rattus norvegicus* Q8CH90_RAT, *Rattus norvegicus* Q91XJ7_RAT, *Rattus norvegicus* Q80WX9_RAT, *Rattus norvegicus* GCYB2_RAT, *Rattus norvegicus* GCYA2_RAT, *Canis familiaris* Q4ZHR9_CANFA, *Bos taurus* GCYB1_BOVIN, *Sus scrofa* Q4ZHR7_PIG, *Gryllus bimaculatus* Q59HN5_GRYBI, *Manduca sexta* O77106_MANSE, *Manduca sexta* O76340_MANSE, *Apis mellifera* Q5UAF0_APIME, *Apis mellifera* Q5FAN0_APIME, *Apis mellifera* Q6L5L6_APIME, *Anopheles gambiae* str PEST Q7PYK9_ANOGA, *Anopheles gambiae* str PEST Q7Q9W6_ANOGA, *Anopheles gambiae* str PEST Q7QF31_ANOGA, *Anopheles gambiae* str PEST Q7PS01_ANOGA, *Anopheles gambiae* str PEST Q7PFY2_ANOGA, *Anopheles gambiae* Q7KQ93_ANOGA, *Drosophila melanogaster* Q24086_DROME, *Drosophila melanogaster* GCYH_DROME, *Drosophila melanogaster* GCY8E_DROME, *Drosophila melanogaster* GCYDA_DROME, *Drosophila melanogaster* GCYDB_DROME, *Drosophila melanogaster* Q9VA09_DROME, *Drosophila pseudoobscura* Q29CE1_DROPS, *Drosophila pseudoobscura* Q296C7_DROPS, *Drosophila pseudoobscura* Q296C8_DROPS, *Drosophila pseudoobscura* Q29BU7_DROPS, *Aplysia californica*

Q7YWK7_APLCA, *Hemicentrotus pulcherrimus* Q95NK5_HEMPU, *Chlamydomonas reinhardtii,* Q5YLC2_CHLRE, *Anabaena sp* Q8YUQ7_ANASP, *Flavobacteria bacterium BBFL7* Q26GR8_9BACT, *Psychroflexus torquis* ATCC *700755* Q1VQE5_9FLAO, marine gamma proteobacterium HTCC2207 Q1YPJ5_9GAMM, marine gamma proteobacterium HTCC2207 Q1YTK4_9GAMM, *Caulobacter crescentus* Q9A451_CAUCR, *Acidiphilium cryptum* JF-5 Q2DG60_ACICY, *Rhodobacter sphaeroides* Q3J0U9_RHOS4, *Silicibacter pomeroyi* Q5LPV1_SILPO, *Paracoccus denitrificans* PD1222, Q3PC67_PARDE, *Silicibacter sp TM1040* Q3QNY2_9RHOB, *Jannaschia sp* Q28ML8_JANSC, *Magnetococcus sp* MC-1 Q3XT27_9PROT, *Legionella pneumophila* Q5WXP0_LEGPL, *Legionella pneumophila* Q5WTZ5_LEGPL, *Legionella pneumophila* Q5X268_LEGPA, *Legionella pneumophila* Q5X2R2_LEGPA, *Legionella pneumophila subsp pneumophila* Q5ZWM9_LEGPH, *Legionella pneumophila subsp pneumophila* Q5ZSQ8_LEGPH, *Colwellia psychrerythraea* Q47Y43_COLP3, *Pseudoalteromonas atlantica* T6c Q3CSZ5_ALTAT, *Shewanella oneidensis* Q8EF49_SHEON, *Saccharophagus degradans* Q21E20_SACD2, *Saccharophagus degradans* Q21ER7_SACD2, *Vibrio angustum* S14 Q1ZWE5_9VIBR, *Vibrio vulnificus* Q8DAE2_VIBVU, *Vibrio alginolyticus* 12G01 Q1VCP6_VIBAL, *Vibrio sp* DAT722 Q2FA22_9VIBR, *Vibrio parahaemolyticus* Q87NJ1_VIBPA, *Vibrio fischeri* Q5E1F5_VIBF1, *Vibrio vulnificus* Q7MJS8_VIBVY, *Photobacterium sp SKA34* Q2C6Z5_9GAMM, *Hahella chejuensis* Q2SFY7_HAHCH, *Oceanospirillum sp* MED92 Q2BKV0_9GAMM, *Oceanobacter sp* RED65 Q1N035_9GAMM, *Desulfovibrio desulfuricans* Q310U7_DESDG, *Halothermothrix orenii* H 168 Q2AIW5_9FIRM, *Thermoanaerobacter tengcongensis* Q8RBX6_THETN, *Caldicellulosiruptor saccharolyticus* DSM 8903 Q2ZH17_CALSA, *Clostridium acetobutylicum* Q97E73_CLOAB, *Alkaliphilus metalliredigenes* QYMF Q3C763_9CLOT, *Clostridium tetani* Q899J9_CLOTE, and *Clostridium beijerincki NCIMB 8052* Q2WVN0_CLOBE. These sequences are predicted to encode H-NOX proteins based on the identification of these proteins as belonging to the H-NOX protein family using the Pfam database as described herein.

[0058] Additional H-NOX proteins and nucleic acids, which may be suitable for use in the pharmaceutical compositions and methods described herein, can be identified using standard methods. For example, standard sequence alignment and/or structure prediction programs can be used to identify additional H-NOX proteins and nucleic acids based on the similarity of their

primary and/or predicted protein secondary structure with that of known H-NOX proteins and nucleic acids. For example, the Pfam database uses defined alignment algorithms and Hidden Markov Models (such as Pfam 21.0) to categorize proteins into families, such as the H-NOX protein family (Pfam - A database of protein domain family alignments and Hidden Markov Models, Copyright (C) 1996-2006 The Pfam Consortium; GNU LGPL Free Software Foundation, Inc., 59 Temple Place - Suite 330, Boston, MA 02111-1307, USA). Standard databases such as the swissprot-trembl database (world-wide web at "expasy.org", Swiss Institute of Bioinformatics Swiss-Prot group CMU - 1 rue Michel Servet CH-1211 Geneva 4 , Switzerland) can also be used to identify members of the H-NOX protein family. The secondary and/or tertiary structure of an H-NOX protein can be predicted using the default settings of standard structure prediction programs, such as PredictProtein (630 West, 168 Street, BB217, New York, N.Y. 10032, USA). Alternatively, the actual secondary and/or tertiary structure of an H-NOX protein can be determined using standard methods.

[0059] In some embodiments, the H-NOX protein has the same amino acid in the corresponding position as any of following distal pocket residues in *T. tengcongensis* H-NOX: Thr4, Ile5, Thr8, Trp9, Trp67, Asn74, Ile75, Phe78, Phe82, Tyr140, Leu144, or any combination of two or more of the foregoing. In some embodiments, the H-NOX protein has a proline or an arginine in a position corresponding to that of Pro115 orArg135 of *T. tengcongensis* H-NOX, respectively, based on sequence alignment of their amino acid sequences. In some embodiments, the H-NOX protein has a histidine that corresponds to His105 of *R. norvegicus* β1 H-NOX. In some embodiments, the H-NOX protein has or is predicted to have a secondary structure that includes six alpha-helices, followed by two beta-strands, followed by one alpha-helix, followed by two beta-strands. This secondary structure has been reported for H-NOX proteins.

[0060] If desired, a newly identified H-NOX protein can be tested to determine whether it binds heme using standard methods. The ability of an H-NOX protein to function as an NO carrier can be tested by determining whether the H-NOX protein binds NO using standard methods, such as those described herein. If desired, one or more of the mutations described herein can be introduced into the H-NOX protein to optimize its characteristics as an NO carrier. For example, one or more mutations can be introduced to alter its NO dissociation constant, k_{off} for NO, k_1 for NO, k_2 for NO, O_2 dissociation constant, NO stability, NO reactivity, rate of heme autoxidation, or any combination

of two or more of the foregoing. Standard techniques such as those described herein can be used to measure these parameters.

[0061] As discussed herein, mutant H-NOX proteins (*e.g.*, class I and class II mutants discussed below) may be derived by mutagenesis from these or other natural wild-type source sequences (*e.g.*, the sequences listed in FIG. 2-4D or 8A-8DD or any other sequence described herein). As used herein, "derived from" refers to the source of the protein into which one or more mutations is introduced. For example, a protein that is "derived from a mammalian protein" refers to protein of interest that results from introducing one or more mutations into the sequence of a wild-type (*i.e.*, a sequence occurring in nature) mammalian protein.

Mutant H-NOX Proteins

[0062] As discussed further herein, an H-NOX protein may contain one or more mutations, such as a mutation that alters the NO dissociation constant, the k_{off} for NO, the O_2 dissociation constant, the k_{off} for O_2, the rate of heme autoxidation, the NO reactivity, the NO stability, or any combination of two or more of the foregoing compared to that of the corresponding wild-type protein. Panels of engineered H-NOX proteins may be generated by random mutagenesis followed by empirical screening for requisite or desired dissociation constants, dissociation rates, NO-reactivity, stability, physio-compatibility, or any combination of two or more of the foregoing in view of the teaching provided herein using techniques as described herein and, additionally, as known by the skilled artisan. Alternatively, mutagenesis can be selectively targeted to particular regions or residues such as distal pocket residues apparent from the experimentally determined or predicted three-dimensional structure of an H-NOX protein (FIG. 1A herein; and see, for example, Boon, E. M. *et al.* (2005). "Molecular Basis For NO Selectivity in Soluble Guanylate Cyclase," *Nature Chemical Biology* 1:53-59, which is hereby incorporated by reference in its entirety, particularly with respect to the sequences of wild-type and mutant H-NOX proteins) or evolutionarily conserved residues identified from sequence alignments (FIGS. 2-4D herein; and see, for example, Boon E.M. *et al.* (2005). "Molecular Basis For NO Selectivity in Soluble Guanylate Cyclase," *Nature Chemical Biology* 1:53-59, which is hereby incorporated by reference in its entirety, particularly with respect to the sequences of wild-type and mutant H-NOX proteins).

[0063] As used herein, a "mutant protein" means a protein with one or more mutations compared to a protein occurring in nature. In one embodiment, the mutant protein has a sequence that differs from that of all proteins occurring in nature. In various embodiments, the amino acid sequence of the mutant protein is at least about any of 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 95, 97, 98, 99, or 99.5% identical to that of the corresponding region of a protein occurring in nature. In some embodiments, the mutant protein is a protein fragment that contains at least about any of 25, 50, 75, 100, 150, 200, 300, or 400 contiguous amino acids from a full-length protein. Sequence identity can be measured, for example, using sequence analysis software with the default parameters specified therein (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). This software program matches similar sequences by assigning degrees of homology to various amino acids replacements, deletions, and other modifications.

[0064] As used herein, a "mutation" means an alteration in a reference nucleic acid or amino acid sequence occurring in nature. Exemplary nucleic acid mutations include an insertion, deletion, frameshift mutation, silent mutation, nonsense mutation, or missense mutation. In some embodiments, the nucleic acid mutation is not a silent mutation. Exemplary protein mutations include the insertion of one or more amino acids (*e.g.*, the insertion of 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids), the deletion of one or more amino acids (*e.g.*, a deletion of N-terminal, C-terminal, and/or internal residues, such as the deletion of at least about any of 5, 10, 15, 25, 50, 75, 100, 150, 200, 300, or more amino acids or a deletion of about any of 5, 10, 15, 25, 50, 75, 100, 150, 200, 300, or 400 amino acids), the replacement of one or more amino acids (*e.g.*, the replacement of 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids), or combinations of two or more of the foregoing. An exemplary functional truncation of an H-NOX protein includes residues 1-385 of the β1 sequence. In some embodiments, a mutant protein has at least one amino acid alteration compared to a protein occurring in nature. In some embodiments, a mutant nucleic acid sequence encodes a protein that has at least one amino acid alteration compared to a protein occurring in nature. In some embodiments, the nucleic acid is not a degenerate version of a nucleic acid occurring in nature that encodes a protein with an amino acid sequence identical to a protein occurring in nature. The nomenclature used in referring to a particular amino acid mutation first identifies the wild-type

amino acid, followed by the residue number and finally the substitute amino acid. For example, Y140L means that tyrosine has been replaced by a leucine at residue number 140.

[0065] An "evolutionary conserved mutation" is the replacement of an amino acid in one protein by an amino acid in the corresponding position of another protein in the same protein family. Exemplary evolutionary conserved mutations (also denoted class I mutations) are listed in Table 1A. In Table 1A, mutations are numbered/annotated according to the sequence of human β1 H-NOX, but are analogous for all H-NOX sequences. Thus, the corresponding position in any other H-NOX protein can be mutated to the indicated residue. For example, Phe4 of human β1 H-NOX can be mutated to a tyrosine since other H-NOX proteins have a tyrosine in this position. The corresponding phenylalanine residue can be mutated to a tyrosine in any other H-NOX protein. In particular embodiments, the one or more mutations are confined to evolutionarily conserved residues. In some embodiments, the one or more mutations may include at least one evolutionarily conserved mutation and at least one non-evolutionarily conserved mutation. If desired, these mutant H-NOX proteins are subjected to empirical screening for NO/O_2 dissociation constants, NO-reactivity, stability, and physio-compatibility in view of the teaching provided herein.

Table 1A. Exemplary Class I H-NOX mutations targeting evolutionary conserved residues

F4Y	Q30G	I145Y
F4L	E33P	I145H
H7G	N61G	K151E
A8E	C78H	I157F
L9W	A109F	E183F

[0066] In some embodiments, the mutation is a distal pocket mutation, such as mutation of a residue in alpha-helix A, D, E, or G (Pellicena, P. *et al.* (August 31, 2004). "Crystal Structure of An Oxygen-Binding Heme Domain Related to Soluble Guanylate Cyclases," *Proc Natl. Acad Sci USA* 101(35):12854-12859). Exemplary distal pocket mutations (also denoted class II mutations) are listed in Table 1B. In Table 1B, mutations are numbered/annotated according to the sequence of human β1 H-NOX, but are analogous for all H-NOX sequences. Because several substitutions provide viable mutations at each recited residue, the residue at each indicated position can be

changed to any other naturally or non-naturally-occurring amino acid (denoted "X"). Such mutations can produce H-NOX proteins with a variety of desired affinity, stability, and reactivity characteristics.

Table 1B. Exemplary Class II H-NOX mutations targeting distal pocket residues

V8X	M73X	I145X
L9X	F77X	I149X
F70X	C78X	

[0067] In particular embodiments, the mutation is a heme distal pocket mutation. As described herein, a crucial molecular determinant that prevents O_2 binding in NO-binding members of the H-NOX family is the lack of a H-bond donor in the distal pocket of the heme. Accordingly, in some embodiments, the mutation alters H-bonding between the H-NOX domain and the ligand within the distal pocket. In some embodiments, the mutation disrupts an H-bond donor of the distal pocket and/or imparts reduced O_2 ligand-binding relative to the corresponding wild-type H-NOX domain. Exemplary distal pocket residues include Thr4, Ile5, Thr8, Trp9, Trp67, Asn74, Ile75, Phe78, Phe82, Tyr140, and Leu144of *T. tengcongensis* H-NOX and the corresponding residues in any other H-NOX protein.

[0068] Residues that are not in the distal pocket can also affect the three-dimensional structure of the heme group; this structure in turn affects the binding of O_2 and NO to iron in the heme group. Accordingly, in some embodiments, the H-NOX protein has one or more mutations outside of the distal pocket. Examples of residues that can be mutated but are not in the distal pocket include Pro115 and Arg135 of *T. tengcongensis* H-NOX. In some embodiments, the mutation is in the proximal pocket which includes His105 as a residue that ligates to the heme iron.

[0069] In some embodiments when two or more mutations are present; at least one mutation is in the distal pocket, and at least one mutation is outside of the distal pocket (*e.g.*, a mutation in the proximal pocket). In some embodiments, all the mutations are in the distal pocket.

[0070] In some embodiments, the amino acid sequence of the H-NOX protein is not identical to the sequence of a protein that is produced by an organism in nature. In some embodiments, the amino acid sequence of the H-NOX protein is not identical to a sequence found in

any database on May 21, 2006 or May 22, 2006 (such as all known sequences predicted or known to be an H-NOX nucleic acid or amino acid sequence). In some embodiments, the amino acid sequence of the H-NOX protein is not identical to a sequence found in any database on May 21, 2007 or May 22, 2007 (such as all known sequences predicted or known to be an H-NOX nucleic acid or amino acid sequence).

[0071] To reduce the immunogenicity of H-NOX proteins derived from sources other than humans, amino acids in an H-NOX protein can be mutated to the corresponding amino acids in a human H-NOX. For example, one or more amino acids on the surface of the tertiary structure of a non-human H-NOX protein can be mutated to the corresponding amino acid in a human H-NOX proteins. In some variations, mutation of one or more surface amino acids may be combined with mutation of two or more distal pocket residues, mutation of one or more residues outside of the distal pocket (*e.g.*, a mutation in the proximal pocket), or combinations of two or more of the foregoing.

[0072] Exemplary mutations are shown in Table 2. In addition, any of the residues listed in Table 2 can be mutated to any other amino acid. The invention also relates to any combination of mutation described herein, such as double, triple, or higher multiple mutations. For example, combinations of any of the mutations described herein can be made in the same H-NOX protein. Note that mutations in equivalent positions in other mammalian or non-mammalian H-NOX proteins are also encompassed by this invention. If desired, residues other than the ones mentioned in Table 2 can also be mutated. Exemplary mutant H-NOX proteins comprise one or more mutations that impart altered NO or O_2 ligand-binding relative to the corresponding wild-type H-NOX domain and are operative as a physiologically compatible mammalian NO blood gas carrier.

[0073] In Table 2 and all subsequent tables, the residue number for a mutation indicates the position in the sequence of the particular H-NOX protein being described. For example, *T. tengcongensis* I5A refers to the replacement of isoleucine by alanine at the fifth position in *T. tengcongensis* H-NOX. The same isoleucine to alanine mutation can be made in the corresponding residue in any other H-NOX protein (this residue may or may not be the fifth residue in the sequence of other H-NOX proteins). Since the amino acid sequences of mammalian β1 H-NOX domains differ by at most two amino acids, mutations that produce desirable mutant H-NOX proteins when introduced into wild-type rat β1 H-NOX proteins are also expected to produce

desirable mutant H-NOX proteins when introduced into wild-type β1 H-NOX proteins from other mammals, such as humans.

[0074] In some embodiments, the H-NOX protein has at least one mutation in which a residue that corresponds to Ile5, Trp9, Asn74, Pro115, Arg135, or Tyr140 of *T. tengcongensis* H-NOX, I145 of β1(1-385), or Phe142 of *L. pneumophila 2* is replaced by any other amino acid. In some embodiments, the H-NOX protein has at least two mutations, wherein at least one mutation is the replacement of a residue that corresponds to Ile5, Trp9, Asn74, Pro115, Arg135, or Tyr140 of *T. tengcongensis* H-NOX, I145 of β1(1-385), or Phe142 of *L. pneumophila 2* by any other amino acid. In some embodiments, the mutation in the H-NOX protein corresponds to a I5A mutation, a I5L mutation, a W9F mutation, a Y140F mutation, a Y140L mutation, a Y140H mutation, a W9F Y140H double mutation, or a F78Y Y140F double mutation of *T. tengcongensis* or a I145Y mutation of β1. In some embodiments, the mutation in the H-NOX protein corresponds to a W9Y mutation, a W9H mutation, a W9N mutation, a N74H mutation, a N74E mutation, a N74A mutation, a P115A mutation, a R135Q mutation, a I5L P115A double mutant, a N74A Y140H double mutant, or a W9F N74A double mutant of *T. tengcongensis*. In some embodiments, at least one C-terminal amino acid (such as at least about 50 contiguous C-terminal amino acids or between about 25 to about 200 contiguous C-terminal amino acids) in the H-NOX protein has been removed compared to the corresponding wild-type protein (such as *R. norvegicus* or *H. sapiens* β1).

Table 2. Exemplary H-NOX mutants from *T. tengcongensis* (*Tt*), *L. pneumophila* (*Lp*), *D. desulfuricans* (*Dd*), *V. cholera* (*Vc*), *N. punctiforme* (*Np*), *C. botulinium* (*Cb*), *C. acetobutylicum*, (*Ca*), rat, human, *C. elegans* (*Ce*).

Tt	*Lp*	*Dd*	Other Bacteria	Rat	Human	Worm
Tt H-NOX	*L2* H-NOX	*Dd* H-NOX(728-899)	*Vc* H-NOX	β1(1-385)	β1(1-385)	*Ce* GCY-35(1-252)
Tt H-NOX His6	*L2* F142Y	*Dd* Y139L	*Np* H-NOX	β1(1-385) I145Y	β1(1-385) I145Y	
Tt I5A	*L2* F9W-F142Y		*Cb* H-NOX(1-175)	β1(1-385) I145H	β1(1-385) I145H	
Tt I5L	*L1* H-NOX		*Cb* H-NOX(1-186)	β1(1-385) C78Y	β1(1-385) C78Y	
Tt I5L-P115A	*L1* F142Y		*Ca* H-NOX(1-197)	β1(1-194)	β1(1-194)	
Tt W9F			*Ca* H-NOX(1-183)	β1 H105F	β1 H105F	
Tt W9F-Y140L				β1 H105G	β1 H105G	
Tt W9F-Y140H				β1(1-194) I145Y	β1(1-194) I145Y	
Tt W9F-N74A				β1(1-194) L9W-I145Y	β1(1-194) L9W-I145Y	
Tt W9Y				β2(1-217)	β2(1-217)	
Tt W9N				β2(1-217) I142Y	β2(1-217) I142Y	
Tt W9H						
Tt N74E						
Tt N74A						

Tt	*Lp*	*Dd*	Other Bacteria	Rat	Human	Worm
Tt N74H						
Tt N74A-Y140H						
Tt I75F His6						
Tt F78Y-Y140L						
Tt F78Y-Y140F						
Tt P115A						
Tt R135Q His6						
Tt Y140F						
Tt Y140L						
Tt Y140H						
Tt Y140A						
Tt L144F His6						

Modifications to H-NOX Proteins

[0075] Any of the wild-type or mutant H-NOX proteins can be modified and/or formulated using standard methods to enhance therapeutic or industrial applications. For example, and particularly as applied to heterologous engineered H-NOX proteins, a variety of methods are known in the art for insulating such agents from immune surveillance, including crosslinking, PEGylation, carbohydrate decoration, *etc.* (*e.g.*, Rohlfs, R. J. *et al.* (May 15, 1998). "Arterial Blood Pressure Responses to Cell-Free Hemoglobin Solutions And The Reaction With Nitric Oxide," *J. Biol. Chem.* 273(20):12128-12134; Migita, R. *et al.* (June 1997). "Blood Volume And Cardiac Index in Rats After Exchange Transfusion With Hemoglobin-Based Oxygen Carriers," *J. Appl. Physiol.* 82(6):1995-2002; Vandegriff, K. D. *et al.* (August 15, 2004). "Kinetics of NO and O_2 Binding to a Maleimide Poly(ethylene glycol)-Conjugated Human Haemoglobin," *Biochem J.* 382(Pt 1):183-189, which are each hereby incorporated by reference in their entireties, particularly with respect to the modification of proteins) as well as other techniques known to the skilled artisan. Fusing an H-NOX protein with a human protein such as human serum albumin can increase the serum half-life, viscosity, and colloidal oncotic pressure. In some embodiments, an H-NOX protein is modified

during or after its synthesis to decrease its immunogenicity and/or to increase its plasma retention time. H-NOX proteins can also be encapsulated (such as encapsulation within liposomes or nanoparticles).

Characteristics of Wild-type and Mutant H-NOX Proteins

[0076] As described herein, a large number of diverse H-NOX mutant proteins providing ranges of NO dissociation constants, O_2 dissociation constants, NO k_{off}, O_2 k_{off}, NO reactivity, and stability have been generated. In some embodiments, an H-NOX protein has a similar or improved NO dissociation constant, O_2 dissociation constant, NO k_{off}, O_2 k_{off}, NO reactivity, autoxidation rate, plasma retention time, or any combination of two or more of the foregoing compared to any currently used compound for delivering NO, such as any organic nitrate for bioconversion into NO.

[0077] As discussed above, the intrinsic low NO reactivity (and high NO stability) makes wild-type and mutant H-NOX proteins desirable NO carriers because of the lower probability of inactivation of H-NOX proteins by NO in the presence of O_2. In some embodiments, an H-NOX protein has a low affinity for O_2 (such as an O_2 dissociation constant of at least about 1 μM at 37 °C) or no detectable affinity for O_2. Since little, if any, O_2 is bound to the H-NOX protein, there is minimal oxidation by NO of O_2 bound to the heme of the H-NOX protein. Thus, minimal NO, O_2, and H-NOX protein is inactivated by this NO oxidation. Thus, more NO can be delivered to desired sites in an individual and less O_2 that could be used by the tissues in the individual is destroyed.

[0078] As used herein, "hemoglobin" means a protein or a mutant thereof from the well-characterized family of hemoglobins, which are iron-containing O_2-transport metalloproteins in red blood cells. Purified, stroma-free, human hemoglobin has a kinetic K_D for O_2 of about 200-500 nM. This value is subunit dependent.

[0079] By "a 6-coordinate Fe^{II}-NO complex" is meant a 6-coordinate ferrous-nitrosyl that produces a UV-Vis Soret peak at approximately 416-422 nm, as described, *e.g.*, by Boon, E. M. *et al.*, (August 2006), "Nitric Oxide Binding to Prokaryotic Homologs of the Solube Guanylate Cyclase β1 H0NOX Domain," *J. Biol. Chem.* 281(31): 21892-21902, which is hereby incorporated by reference in its entirety, particularly with respect to the determination of the percentage of a H-NOX protein sample that contains a 6-coordinate Fe^{II}-NO complex and the percentage of a H-NOX protein sample that contains a 5-coordinate Fe^{II}-NO complex.

[0080] By "a 5-coordinate Fe^{II}-NO complex" is meant a 5-coordinate ferrous-nitrosyl that produces a UV-Vis Soret peak at approximately 397-400 nm, as described, *e.g.*, by Boon, E. M. *et al.*, (August 2006), "Nitric Oxide Binding to Prokaryotic Homologs of the Solube Guanylate Cyclase β1 H0NOX Domain," *J. Biol. Chem.* 281(31): 21892-21902, which is hereby incorporated by reference in its entirety, particularly with respect to the determination of the percentage of a H-NOX protein sample that contains a 6-coordinate Fe^{II}-NO complex and the percentage of a H-NOX protein sample that contains a 5-coordinate Fe^{II}-NO complex.

[0081] As used herein, a "k_{off}" means a dissociation rate, such as the rate of release of NO or O_2 from a protein. A lower numerical lower k_{off} indicates a slower rate of dissociation. For an H-NOX protein with a 6-coordinate Fe^{II}-NO complex, the k_{off} for NO is calculated as described by Boon, E. M. *et al.*, (August 2006), "Nitric Oxide Binding to Prokaryotic Homologs of the Solube Guanylate Cyclase β1 H0NOX Domain," *J. Biol. Chem.* 281(31): 21892-21902 and Boon, E.M. *et al.* (2005). "Molecular Basis For NO Selectivity in Soluble Guanylate Cyclase," *Nature Chemical Biology* 1:53-59, which are each hereby incorporated by reference in their entireties, particularly with respect to the calculation of NO k_{off} for H-NOX proteins. For an H-NOX protein with a 5-coordinate Fe^{II}-NO complex, the k_{off} for NO is described by the k_1 for NO and the k_2 for NO, as described by Winger, J. A. *et al.*, (January 2007) "Dissociation of Nitric Oxide from Soluble Guanylate Cyclase and Heme-Nitric Oxide/Oxygen Binding Domain Constructs" *J. Biol. Chem.* 282(2): 897-907, which is hereby incorporated by reference in its entirety, particularly with respect to the calculation of NO k_{off}, NO k_1, and NO k_2 for H-NOX proteins. For an H-NOX protein that contains a mixture of 5-coordinate and 6-coordinate Fe^{II}-NO complexes, the k_{off} for NO is described by the k_1 for NO and the k_2 for NO, as described by Winger, J. A. *et al.*, (January 2007) "Dissociation of Nitric Oxide from Soluble Guanylate Cyclase and Heme-Nitric Oxide/Oxygen Binding Domain Constructs" *J. Biol. Chem.* 282(2): 897-907, which is hereby incorporated by reference in its entirety, particularly with respect to the calculation of NO k_{off}, NO k_1, and NO k_2 for H-NOX proteins.

[0082] In some embodiments, the k_{off}, k_1, or k_2 for NO for the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ to about 10 s^{-1} at 37 °C, such as between about 1 x $10^{-4}s^{-1}$ to about 0.012 s^{-1}, about 1 x $10^{-4}s^{-1}$ to about 0.007 s^{-1}, about 0.005 s^{-1} to about 0.011 s^{-1}, or about 1 x $10^{-4}s^{-1}$ to about 1 x $10^{-3}s^{-1}$ at 37 °C. In various embodiments, the k_{off} for O_2 for an H-NOX protein is between about 1 to about

1,000 s^{-1} at 37 °C, such as about 1 to about 50 s^{-1}, about 50 to about 100 s^{-1}, about 100 to about 250 s^{-1}, about 250 to about 500 s^{-1}, about 500 to about 750 s^{-1}, or about 750 to about 1,000 s^{-1} at 37 °C.

[0083] By a "k_{on}" is meant an association rate, such as the rate of binding of NO or O_2 to a protein. A lower numerical lower k_{on} indicates a slower rate of association. In various embodiments, the k_{on} for O_2 for an H-NOX protein is between about 0.14 to about 60 $\mu M^{-1}s^{-1}$ at 20 °C, such as about 6 to about 60 $\mu M^{-1}s^{-1}$, about 6 to 12 $\mu M^{-1}s^{-1}$, about 15 to about 60 $\mu M^{-1}s^{-1}$, about 5 to about 18μM^{-} s^{-1}, or about 6 to about 15 $\mu M^{-1}s^{-1}$.

[0084] By "dissociation constant" is meant a "kinetic dissociation constant" or a "calculated dissociation constant." A "kinetic dissociation constant" or "K_D" means a ratio of kinetic off-rate (k_{off}) to kinetic on-rate (k_{on}), such as a K_D value determined as an absolute value using standard methods (*e.g.*, standard spectroscopic, stopped-flow, or flash-photolysis methods) including methods known to the skilled artisan and/or described herein. "Calculated dissociation constant" or "calculated K_D" refers to an approximation of the kinetic dissociation constant based on a measured k_{off}. For the calculated K_D for NO, the value for the k_{on} for NO for an H-NOX protein is assumed to be 710 $\mu M^{-1}s^{-1}$, which is a reported k_{on} for β1(1-385) that was measured at 4 °C and does not increase significantly at 37 °C (Zhao, *et. al.*, (1999). "A Molecular Basis for Nitric Oxide Sensing by Soluble Guanylate Cyclase," *PNAS.* 96:14753-14758, which is hereby incorporated by reference in its entirety, particularly with respect to the calculation of NO k_{on} for H-NOX proteins). For the calculated K_D for O_2, a value for the k_{on} is derived via the correlation between kinetic K_D and k_{off} as described herein.

[0085] In various embodiments, the kinetic or calculated K_D for NO binding by an H-NOX protein is within about 0.01 to about 100-fold of that of hemoglobin under the same conditions (such as at 20 °C), such as between about 0.1 to about 10-fold or between about 0.5 to about 2-fold of that of hemoglobin under the same conditions (such as at 20 °C). In some embodiments, the NO dissociation constant of the H-NOX protein is between about 0.1 to about 20 pM at 37 °C, such as about 0.5 to about 15, about 0.5 to about 12, about 0.7 to about 4, or about 0.7 to about 3 at 37 °C. In some embodiments, the NO dissociation constant of the H-NOX protein is at least about 0.1 pM at 37 °C, such as at least about any of 0.5, 1, 3, 5, 10, 12, 50, 100, 400, 500, 1000, 2000, 3000, or 4000 pM at 37 °C. In some embodiments, the NO dissociation constant of the H-NOX protein is

less than about 5000 pM at 37 °C, such as less than about any of 4000 pM, 3000 pM, 2000 pM, 1000 pM, 500 pM, 400 pM, 100 pM, 50 pM, 12 pM, 10 pM, 5 pM, 3 pM, or 1 pM at 37 °C.

[0086] In various embodiments, the kinetic or calculated K_D for O_2 binding by an H-NOX protein is within about 0.01 to about 100-fold of that of hemoglobin under the same conditions (such as at 20 °C), such as between about 0.1 to about 10-fold or between about 0.5 to about 2-fold of that of hemoglobin under the same conditions (such as at 20 °C). In some embodiments, the O_2 dissociation constant of the H-NOX protein it at least about 1 μM at 37 °C, such as at least about any of 5 μM, 10 μM, 20 μM, 30 μM, 40 μM, 50 μM, 60 μM, 70 μM, 80 μM, 90 μM, or 100 μM at 37 °C. In some embodiments, there is no detectable binding to O_2 at 37 °C, such as the lack of detectable O_2 binding using UV-visible spectroscopy as described herein (*e.g.*, a lack of an observable peak at ~418 nm in the presence of O_2, such as when the Soret peak remains at ~431 nm as seen in the absence of O_2 or when the Soret peak shifts to ~410 nm due to oxidized protein).

[0087] As used herein, "NO affinity" is a qualitative term that refers to the strength of NO binding to a protein (such as binding to a heme group or to an oxygen bound to a heme group associated with a protein). This affinity is affected by both the k_{off} and k_{on} for NO. A numerically lower NO K_D value means a higher affinity. "Oxygen affinity" is a qualitative term that refers to the strength of oxygen binding to the heme moiety of a protein. This affinity is affected by both the k_{off} and k_{on} for oxygen. A numerically lower oxygen K_D value means a higher affinity.

[0088] As used herein, "NO stability" refers to the stability or resistance of a protein to oxidation by NO in the presence of oxygen. For example, the ability of the protein to not be oxidized when bound to NO in the presence of oxygen is indicative of the protein's NO stability. In some embodiments, less than about any of 50, 40, 30, 10, or 5% of an H-NOX protein is oxidized after incubation for about any of 1, 2, 4, 6, 8, 10, 15, or 20 hours at 20 °C.

[0089] As used herein, "NO reactivity" refers to the rate at which iron in the heme of a heme-binding protein is oxidized by NO in the presence of oxygen at a concentration of 2 μM protein. A lower numerical value for NO reactivity in units of s^{-1} indicates a lower NO reactivity. In various embodiments, the NO reactivity of an H-NOX protein is less than about 700 s^{-1} at 20 °C, such as less than about 600 s^{-1}, 500 s^{-1}, 400 s^{-1}, 300 s^{-1}, 200 s^{-1}, 100 s^{-1}, 75 s^{-1}, 50 s^{-1}, 25 s^{-1}, 20 s^{-1}, 10 s^{-1}, 50 s^{-1}, 3 s^{-1}, 2 s^{-1}, 1.8 s^{-1}, 1.5 s^{-1}, 1.2 s^{-1}, 1.0 s^{-1}, 0.8 s^{-1}, 0.7 s^{-1}, or 0.6 s^{-1} at 20 °C. In various embodiments, the NO reactivity of an H-NOX protein is between about 0.1 to about 600 s^{-1} at 20

°C, such as between about 0.5 to about 400 s^{-1}, about 0.5 to about 100 s^{-1}, about 0.5 to about 50 s^{-1}, about 0.5 to about 10 s^{-1}, about 1 to about 5 s^{-1}, or about 0.5 to about 2.1 s^{-1} at 20 °C. In various embodiments, the reactivity of an H-NOX protein is at least about 10, 100, 1,000, or 10,000 fold lower than that of hemoglobin under the same conditions, such as at 20 °C.

[0090] As used herein, an "autoxidation rate" refers to the rate at which iron in the heme of a heme-binding protein is autoxidized. A lower numerical autoxidation rate in units of s^{-1} indicates a lower autoxidation rate. In various embodiments, the rate of heme autoxidation of an H-NOX protein is less than about 1.0 h^{-1}at 37 °C, such as less than about any of 0.9 h^{-1}, 0.8 h^{-1}, 0.7 h^{-1}, 0.6 h^{-1}, 0.5 h^{-1}, 0.4 h^{-1}, 0.3 h^{-1}, 0.2 h^{-1}, 0.1 h^{-1}, or 0.05 h^{-1} at 37 °C. In various embodiments, the rate of heme autoxidation of an H-NOX protein is between about 0.006 to about 5.0 h^{-1} at 37 °C, such as about 0.006 to about 1.0 h^{-1}, 0.006 to about 0.9 h^{-1}, or about 0.06 to about 0.5 h^{-1} at 37 °C.

[0091] In various embodiments, a mutant H-NOX protein has (a) an NO or O_2 dissociation constant, association rate (k_{on} for NO or O_2), or dissociation rate (k_{off} for NO or O_2) within 2 orders of magnitude of that of hemoglobin, (b) has an NO affinity weaker (*e.g.*, at least about 10-fold, 100-fold, or 1000-fold weaker) than that of sGC β1 (c) an NO reactivity with bound O_2 at least 1000-fold less than hemoglobin, (d) an *in vivo* plasma retention time at least 2, 10, 100, or 1000-fold higher than that of hemoglobin, or (e) any combination of two or more of the foregoing.

[0092] Exemplary suitable NO carriers provide dissociation constants within two orders of magnitude of that of hemoglobin, *i.e.* between about 0.01 and 100-fold, such as between about 0.1 and 10-fold, or between about 0.5 and 2-fold of that of hemoglobin. A variety of established techniques may be used to quantify dissociation constants, such as the techniques described herein (Boon, E. M. *et al.* (2005). "Molecular Basis For NO Selectivity in Soluble Guanylate Cyclase," *Nature Chem. Biol.* 1:53-59; Boon, E. M. *et al.* (October 2005). "Ligand Discrimination in Soluble Guanylate Cyclase and the H-NOX Family of Heme Sensor Proteins," *Curr. Opin. Chem. Biol.* 9(5):441-446; Boon, E. M. *et al.* (2005). "Ligand Specificity of H-NOX Domains: From sGC to Bacterial NO Sensors," *J. Inorg. Biochem.* 99(4):892-902), Vandegriff, K. D. *et al.* (August 15, 2004). "Kinetics of NO and O_2 Binding to a Maleimide Poly(ethylene glycol)-Conjugated Human Haemoglobin," *Biochem J.* 382(Pt 1):183-189, which are each hereby incorporated by reference in their entireties, particularly with respect to the measurement of dissociation constants), as well as those known to the skilled artisan. Exemplary NO carriers provide low or minimized NO reactivity

of the H-NOX protein with bound O_2, such as an NO reactivity lower than that of hemoglobin. In some embodiments, the NO reactivity is much lower, such as at least about 10, 100, 1,000, or 10,000-fold lower than that of hemoglobin. A variety of established techniques may be used to quantify NO reactivity (Boon, E. M. *et al.* (2005). "Molecular Basis For NO Selectivity in Soluble Guanylate Cyclase," *Nature Chem. Biol.* 1:53-59; Boon, E. M. *et al.* (October 2005). "Ligand Discrimination in Soluble Guanylate Cyclase and the H-NOX Family of Heme Sensor Proteins," *Curr. Opin. Chem. Biol.* 9(5):441-446; Boon, E. M. *et al.* (2005). "Ligand Specificity of H-NOX Domains: From sGC to Bacterial NO Sensors," *J. Inorg. Biochem.* 99(4):892-902), Vandegriff, K. D. *et al.* (August 15, 2004). "Kinetics of NO and O_2 Binding to a Maleimide Poly(ethylene glycol)-Conjugated Human Haemoglobin," *Biochem J.* 382(Pt 1):183-189, which are each hereby incorporated by reference in their entireties, particularly with respect to the measurement of NO reactivity) as well as those known to the skilled artisan. Because wild-type *T. tengcongensis* H-NOX has such a low NO reactivity, other wild-type H-NOX proteins and mutant H-NOX proteins may have a similar low NO reactivity. For example, *T. tengcongensis* H-NOX Y140H has an NO reactivity similar to that of wild-type *T. tengcongensis* H-NOX.

[0093] Exemplary mutants for NO delivery have an NO affinity weaker, preferably at least 10-fold, 100-fold, or 1000-fold weaker than that of sGC β1. For therapeutic NO delivery (*e.g.*, during/following a heart attack, open heart surgery, or stroke) a range of engineered H-NOX proteins with varying affinities are empirically tested for efficacy in particular disease states, with a range in some embodiments of NO affinities of 0.1 to 1000 nM.

[0094] In addition, suitable NO carriers provide high or maximized stability, particularly *in vivo* stability. A variety of stability metrics may be used, such as oxidative stability (*e.g.*, stability to autoxidation or oxidation by NO), temperature stability, and *in vivo* stability. A variety of established techniques may be used to quantify stability, such as the techniques described herein (Boon, E. M. *et al.* (2005). "Molecular Basis For NO Selectivity in Soluble Guanylate Cyclase," *Nature Chem. Biol.* 1:53-59; Boon, E. M. *et al.* (October 2005). "Ligand Discrimination in Soluble Guanylate Cyclase and the H-NOX Family of Heme Sensor Proteins," *Curr. Opin. Chem. Biol.* 9(5):441-446; Boon, E. M. *et al.* (2005). "Ligand Specificity of H-NOX Domains: From sGC to Bacterial NO Sensors," *J. Inorg. Biochem.* 99(4):892-902), as well as those known to the skilled artisan. For *in vivo* stability in plasma, blood, or tissue, exemplary metrics of stability include

retention time, rate of clearance, and half-life. H-NOX proteins from thermophilic organisms are expected to be stable at high temperatures. In various embodiments, the plasma retention times are at least about 2-, 10-, 100-, or 1000-fold greater than that of hemoglobin (*e.g.* Bobofchak, K. M. *et al.* (August 2003). "A Recombinant Polymeric Hemoglobin With Conformational, Functional, And Physiological characteristics of an in vivo O2 transporter," *Am. J. Physiol. Heart Circ. Physiol.* 285(2):H549-H561). As will be appreciated by the skilled artisan, hemoglobin-based carriers are limited by the rapid clearance of cell-free hemoglobin from plasma due the presence of receptors for hemoglobin that remove cell-free hemoglobin from plasma. Since there are no receptors for H-NOX proteins in plasma, wild-type and mutant H-NOX proteins are expected to have a longer plasma retention time than that of hemoglobin. If desired, the plasma retention time can be increased by PEGylating or crosslinking an H-NOX protein or fusing an H-NOX protein with another protein using standard methods (such as those described herein and those known to the skilled artisan).

[0095] In various embodiments, the NO dissociation constant of the H-NOX protein is within 2 orders of magnitude of that of hemoglobin, and the NO reactivity of the H-NOX protein is at least 10-fold lower than that of hemoglobin. In some embodiments, the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ to about 10 s^{-1} at 37 °C, and the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C. In some embodiments, the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ to about 10 s^{-1} at 37 °C, and the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C. In some embodiments, the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ to about 10 s^{-1} at 37 °C, and the NO reactivity of the H-NOX protein is less than about 700 s^{-1} at 20 °C (*e.g.*, less than about 600 s^{-1}, 500 s^{-1}, 100 s^{-1}, 20 s^{-1}, or 1.8 s^{-1}at 20 °C). In some embodiments, the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and the NO reactivity of the H-NOX protein is less than about 700 s^{-1} at 20 °C (*e.g.*, less than about 600 s^{-1}, 500 s^{-1}, 100 s^{-1}, 20 s^{-1}, or 1.8 s^{-1}at 20 °C). In some embodiments, the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ to about 10 s^{-1} at 37 °C, and the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C. In some embodiments, the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C. In some embodiments, the rate of heme autoxidation of the H-NOX protein is

less than about 1 h^{-1}at 37 °C, and the NO reactivity of the H-NOX protein is less than about 700 s^{-1} at 20 °C (*e.g.*, less than about 600 s^{-1}, 500 s^{-1}, 100 s^{-1}, 20 s^{-1}, or 1.8 s^{-1}at 20 °C). In some embodiments, the viscosity of the H-NOX protein solution is between 1 and 4 centipoise (cP). In some embodiments, the colloid oncotic pressure of the H-NOX protein solution is between 20 and 50 mm Hg.

[0096] Table 3 lists exemplary sizes, oxygen affinities, autoxidation stabilities, NO reactivity rates, and modifications for wild-type and mutant H-NOX proteins. In Table 3, the vehicle size refers to the molecular weight of a modified (*e.g.*, PEGylated) or unmodified H-NOX protein.

Table 3: Exemplary Embodiments for H-NOX proteins

Vehicle size	Oxygen Affinity	Stability (autoxidation)	NO affinity	NO reactivity (s^{-1})	Particle decoration
>1 MD	at least 1 μM	1 hour	1 pM	0.01 to 0.1	Cross-liking
0.5 kD to 1 MD	at least 10 μM	1 h to 12 h	500 pM	0.1 to 1	PEGylation
0.1 kD to 0.5 kD	at least 50 μM	12 h to 48 h	1 nM	1 to 10	Encapsulation
0.01 kD to 0.1 kD	at least 75 μM	48 h to 2 weeks	1 μM	10 to 100	

[0097] Exemplary data for particular mutants are reported in Tables 4-14. In Tables 4-14, β1 and B2 refer to proteins derived from rat H-NOX proteins. Since the amino acid sequences of mammalian β1 H-NOX domains differ by at most two amino acids, similar results are expected for the corresponding mutations in other mammalian β1 H-NOX proteins, such as human β1.

[0098] Table 4 demonstrates that the dissociation constant for NO binding can be significantly changed by mutating one or more residues in H-NOX proteins. Additionally, the ability of allosteric regulators to dramatically affect the dissociation constant and dissociation rate of NO for sGC supports the ability of mutations that alter the structure of sGC or other H-NOX proteins to alter the dissociation constant and dissociation rate of NO. If desired, the dissociation constant for NO binding can be further altered by combining any of the mutations listed in Table 4 or by introducing one or more additional mutations into an H-NOX protein, as described herein.

Table 4. K_D values for NO binding to H-NOX and other hemoproteins

Hemoprotein	k_{on} ($\mu M^{-1} s^{-1}$)	k_{off} (s^{-1})	K_D (pM) 37 °C [a]
sGC	> 140 [b]	0.001 to 0.66 [c, d]	0.71 to 4710
β1 (1-385)	710 [b]	0.0023 to 0.0087 [c, d]	3.24 to 12.3
Hb (T)	18 [e, f]	0.004 [e, f]	411 [g] (222)
Hb (R)	18 [e, f]	0.00005 [e, f]	5.14 [g] (2.28)
Mb	17 [e, h]	0.00012 [e, h]	13.1 [g] (7.06)
Tt H-NOX	I	0.00056 [j]	0.78
Tt H-NOX Y140L	I	0.00013 [j]	0.18
L1 H-NOX	I	0.00103 to 0.0087 [j]	1.45 to 12.3
L2 H-NOX	I	0.00036 to 0.00218 [j]	0.51 to 3.1
L2 H-NOX F142Y	I	0.00051 [j]	0.72

[a]calculated from the ratio k_{off}:k_{on}; [b] Zhao, *et. al.*, (1999). "A Molecular Basis for Nitric Oxide Sensing by Soluble Guanylate Cyclase,"*PNAS.* 96:14753-14758, measured at 4 °C, rates approach rate of diffusion and do not increase significantly at 37 °C; [c] Winger, J. A. *et al.*, (January 2007) "Dissociation of Nitric Oxide from Soluble Guanylate Cyclase and Heme-Nitric Oxide/Oxygen Binding Domain Constructs" *J. Biol. Chem.* 282(2):897-907; [d]k values bracket broadest ranges of fits from NO dissociation from 5-coordinate and 6-coordinate, each averaged from 2 – 4 dissociation experiments using saturating CO and 30 mM $Na_2S_2O_4$ as the NO trap and containing 0.88 – 2.2 μM protein. Data were best fit by a double exponential equation:

$\Delta A_t = \Delta A_1(1 - e^{-k_1 t}) + \Delta A_2(1 - e^{-k_2 t})$; [e]measured at 20 °C; [f] Morris, *et. al.*, 1980 J Biol. Chem. 255: 8050-8053; [g]K_D calculation, adjusted to 37 °C, assuming rate-doubling every 10 °C, value at 20 °C shown in brackets; [h]Moore, *et. al.*, (1976). "Cooperativity in the Dissociation of Nitric Oxide from Hemoglobin," *J Biol. Chem.* 251: 2788-2794; [i]assuming the same k_{on} as β1(1-385) (710 $\mu M^{-1}s^{-1}$); [j] Boon, E. M. *et al.*, (August 2006), "Nitric Oxide Binding to Prokaryotic Homologs of the Solube Guanylate Cyclase β1 H0NOX Domain," *J. Biol. Chem.* 281(31): 21892-21902.

[0099] Table 5 demonstrates that the dissociation rate (k_{off}) for NO binding can be significantly changed by mutating one or more residues in H-NOX proteins. The k_{off} for these exemplary mutant H-NOX proteins range from 0.00013 to 0.011 s^{-1} at 37 °C. For Table 5, NO dissociation rates from hemoproteins are derived using chemical traps as indicated in each cited reference. For comparison, NO dissociation rates from organic nitrates and NONOates are measured using a NO electrode and confirmed using an oxyhemoglobin trap. Where necessary, values are adjusted to 37 °C using the fact that rates double approximately for every 10 °C. If desired, the k_{off} for NO binding can be further altered by combining any of the single or double mutations listed in Table 5 or by introducing one or more additional mutations into an H-NOX protein, as described herein.

Table 5. Comparison of hemoprotein and nitrovasodilator NO dissociation rates at 37 °C.

Hemoprotein or Nitrovasodilator	k_{off} (s^{-1})
sGC	0.001 to 0.66 [a]
β1 (1-385)	0.0023 to 0.0087 [a]
β2 (1-217)	0.0069 to 0.011 [a]
β1 (1-194)	0.0009 to 0.0041 [a]
Tt H-NOX	0.00056 [b]
Tt H-NOX Y140L	0.00013 [b]
Tt H-NOX Y140F	$2.0 \pm 0.3 \times 10^{-4}$ [g]
L1 H-NOX	0.00103 to 0.0087 [b]
L2 H-NOX	0.00036 to 0.00218 [b]
L2 H-NOX F142Y	0.00051 [b]
Hb (T)	0.004 [c]
Hb (R)	0.00005 [c]
Mb	0.00012 [d]
Nitrophorin (pH 5.0)	0.02 to 21 [c]
Nitrophorin (pH 8.0)	0.6 to 15 [e]
DEA/NO	0.0083 [f]
nPRONO	0.00012 [f]
ClCH2CH2ONO	0.000022 [f]
tBuONO	0.000008 [f]

[a]Winger, J. A. *et al.*, (January 2007) "Dissociation of Nitric Oxide from Soluble Guanylate Cyclase and Heme-Nitric Oxide/Oxygen Binding Domain Constructs" *J. Biol. Chem.* 282(2): 897-907; [b]Boon, E. M. *et al.*, (August 2006), "Nitric Oxide Binding to Prokaryotic Homologs of the Solube Guanylate Cyclase β1 H0NOX Domain," *J. Biol. Chem.* 281(31): 21892-21902; [c]Morris, *et. al.*, (1980). "The role of diffusion in limiting the rate of ligand binding to hemoglobin" *J Biol. Chem.* 255:8050-8053; [d]Moore, *et. al.*, (1976). "Cooperativity in the dissociation of nitric oxide from hemoglobin," *J Biol. Chem.* 251: 2788-2794; [e]Maes, *et. al.*, (2004) "Role of Binding Site Loops in Controlling Nitric Oxide Release: Structure and Kinetics of Mutant Forms of Nitrophorin 4" *Biochemistry* 43(21):6679-90; [f]Artz, J.D. *et. al.*, (1998) " NO Release from NO Donors and Nitrovasodilators: Comparisons between Oxyhemoglobin and Potentiometric Assays," *Chem. Res. Toxicol.* 11(12):1393-1397; [g]Boon, E. M *et al.*, (2006) "Sensitive and Selective Detection of Nitric Oxide Using an H-NOX Domain," *JACS* 128:10022-10023.

[0100] As shown in Table 6, introducing one or more mutations into wild-type H-NOX proteins allows the autoxidation rate and O_2 dissociation rate to be altered. If desired, the autoxidation rate or O_2 dissociation rate can be further altered by combining any of the single or double mutations listed in Table 6 or by introducing one or more additional mutations into an H-NOX protein, as described herein.

Table 6. Stability to autoxidation, O_2-binding properties (such as rate of O_2 dissociation) and distal pocket H-bonding residues are listed for wild-type and class II mutant H-NOX proteins

Protein	Stability	O_2-binding activity[b]	Distal pocket residues
Tt H-NOX, a prokaryotic H-NOX and a strong O_2 binder			
Tt H-NOX	$k_{ox} \sim 0$[c]	$k_{off} = 1.22$	Trp9, Phe78, Tyr140
Tt Y140F	$k_{ox} = 0.05$	$k_{off} = 15.7$[d]	Trp9, Phe78, Phe140
Tt Y140L	$k_{ox} = 0.19$	$k_{off} = 20.$[d]	Trp9, Phe78, Leu140
Tt Y140H	$k_{ox} = 0.87$	$k_{off} = 5.03$	Trp9, Phe78, His140
Tt Y140A	Stable[a]	Partial complex[d,e]	Trp9, Phe78, Ala140
Tt W9F	$k_{ox} \sim 0$[c]	$k_{off} = 1.84$	Phe9, Phe78, Tyr140
Tt W9F-Y140L	$k_{ox} = 0.12$	No complex formed	Phe9, Phe78, Leu140
Tt W9F-Y140H	$k_{ox} = 0.11$	$k_{off} = 23.4$	Phe9, Phe78, His140
Tt F78Y-Y140L	$k_{ox} \sim 0$[c]	$k_{off} = 0.83$	Trp9, Tyr78, Leu140
Tt F78Y-Y140F	$k_{ox} \sim 0$[c]	$k_{off} = 1.48$	Trp9, Tyr78, Phe140
Prokaryotic H-NOX proteins for which the wild-type protein does not bind O_2			
L2 H-NOX	Stable[a]	No complex formed	Phe9, Phe78, Phe142
L2 F142Y	Stable[f]	$k_{off} = 3.68$	Phe9, Phe78, Tyr142
L2 F9W-F142Y	Stable[f]	Binds O_2[e]	Trp9, Phe78, Tyr142
L1 H-NOX	$k_{ox} = 0.31$	No complex formed	Leu9, Leu78, Phe142
L1 F142Y	$k_{ox} = 1.8$	$k_{off} = 1.73$[d]	Leu9, Leu78, Tyr142

Eukaryotic H-NOX for which the wild-type protein does not bind O_2			
β2(1-217)	k_{ox} = 0.18	No complex formed	Leu9, Cys76, Ile142
β2(1-217) I142Y		g	Leu9, Cys76, Tyr142
β1(1-194)	k_{ox} = 4.3	No complex formed	Leu9, Cys78, Ile145
β1(1-194) I145Y	k_{ox} = 2.8	g	Leu9, Cys78, Tyr145
β1(1-194) L9W-I145Y	k_{ox} ~ 10	g	Trp9, Cys78, Tyr145
β1(1-385)	Stable[e]	No complex found	Leu9, Cys78, Ile145
β1(1-385) I145Y	k_{ox} = 0.72	k_{off} = 2.69	Leu9, Cys78, Tyr145
β1(1-385) I145H			Leu9, Cys78, His145
β1(1-385) C78Y			Leu9, Tyr78, Ile145
Other H-NOX predicted to bind O_2 as the wild-type construct			
Dd H-NOX(728-899)	k_{ox} = 0.98	k_{off} = 5.80	Phe9, Phe75, Tyr139
Dd Y139L			Phe9, Phe75, Leu139
Cb H-NOX(1-175)	Not stable construct[h]	g	Trp9, Phe78, Tyr140
Cb H-NOX(1-186)	Slightly more stable[i]	g	Trp9, Phe78, Tyr140
Ca H-NOX(1-197)	Not stable construct[h]	g	Trp9, Phe78, Tyr140
Ca H-NOX(1-183)	Slightly more stable[i]	g	Trp9, Phe78, Tyr140
Ce GCY-35(1-252)	Stable	Binds O_2[e]	Phe9, Thr78, Tyr144

[a]The construct is stable to oxidation (evaluated by the rate of autoxidation, k_{ox} [h^{-1}] at 37 °C) and/or heme loss. [b]O_2-binding activity was evaluated by the rate of O_2 dissociation from the heme at 20 °C (s^{-1}). [c]After 24 hours at 37 °C, there is still no indication of autoxidation. [d]Only a small portion of the protein forms a complex with O_2, the rate reported represents the kinetics for this population. [e]The protein binds O_2 but the k_{off} was not determined. [f]Although relatively stable, this protein precipitated as it oxidized, making it difficult to measure k_{ox}. [g]Not applicable due to instability or rapid oxidation.

[h]"Not stable construct" means the protein oxidizes immediately under the conditions tested.
[i]"Slightly more stable" means the protein oxidizes over a period of minutes to hours, but does not remain stable beyond 24 hours under the conditions tested.

[0101] Table 7 illustrates the alteration of the O_2 association rate (k_{on}), O_2 dissociation rate (k_{off}), O_2 dissociation constant (K_D), and autoxidation rate (k_{ox}) in H-NOX proteins by the introduction of one or more mutations. In some embodiments, any of the single or double mutations listed in Table 7 are combined with another mutation (such as another mutation in Table 7 or any other mutation described herein) to further alter the O_2 association rate, O_2 dissociation rate, O_2 dissociation constant, autoxidation rate, or combinations of two or more of the foregoing.

Table 7. O_2-binding kinetic constants for histidyl-ligated Fe^{II} heme proteins

Protein	K_D[a]	k_{on}[b]	k_{off}[c]	k_{ox}[d]	Ref.
Tt H-NOX	89.7 ± 6.2	13.6 ± 1.0	1.22 ± 0.09	e	I
Tt P115A	21.2 ± 2.1	10.4 ± 1.1	0.22 ± 0.01	e	J
Tt I5A	~80		0.82 ± 0.03	0.7	J
Tt I5L	~1000		9.50 ± 0.64	0.6	J
Tt I5L-P115A	~30		0.28 ± 0.01	0.6	J
Tt W9F	305 ± 31	6.02 ± 0.62	1.84 ± 0.17	e	I
Tt Y140F	f	15.7 ± 1.4	15.7 ± 9.8	0.05	J
Tt Y140L	~2000	Geminal	20.1 ± 2.0	0.19	I
Tt Y140H	~500		5.03 ± 0.69	0.87	J
Tt W9F-Y140H	~2500		23.4 ± 3.7	0.11	J
Tt W9F-Y140L	No complex with O_2 observed			0.12	I
Tt F78Y-Y140F	~150		1.48 ± 0.33	e	J
Tt F78Y-Y140L	~80		0.83 ± 0.17	e	I
Tt W9F-N74A	Millimolar	very slow			J
Dd H-NOX	Millimolar	very slow	7.13 ± 0.45	0.14	J
Dd Y139L	No complex with O_2 observed				j
β1(1-385) I145Y	70,000,00	0.00004	2.69 ± 0.61	0.72	i
L2 F142Y	9200 ± 3000	0.40 ± 0.14	3.68 ± 0.71		i
Hs Hb beta	267	60	16		n
Hs Hb alpha	560	50	28		k
Sw Mb	880	17	15	0.006	k
Bj FixL	140,000	0.14	20	2.7	l

HemAT-B	720	32	23	0.06	m

[a]dissociation constant at 20 °C (nM); [b]rate of O_2 association to the heme at 20 °C ($\mu M^{-1}s^{-1}$); [c]rate of O_2 dissociation from the heme at 20 °C (s^{-1}); [d]rate of heme autoxidation (h^{-1}) at 37 °C; [e]after 24 hours at 37 °C, still no indication of autoxidation; [f]only a small portion of the protein forms a complex with O_2, although the kinetics for this population could be measured; [i]Boon, E.M. *et al.* (June 2005). "Molecular Basis For NO Selectivity in Soluble Guanylate Cyclase," *Nature Chemical Biology* 1(1):53-59, [j]unpublished data; [k]Springer, B. A. *et al.* (1994) "Family Physicians Key Partners in Preventing Suicide Among Youth," *Chem. Rev.* 94:699–714; [l]Gilles-Gonzalez *et al.* (1994) "Heme-Based Sensors, Exemplified by the Kinase FixL, are a New Class of Heme Protein with Distinctive Ligand Binding and Autoxidation," *Biochemistry* 33:8067-8073. [m]Aono, S. *et al.* (2002) "Resonance Raman and Ligand Binding Studies of the Oxygen-Sensing Signal Transducer Protein HemAT from Bacillus Subtilis,"*J. Biol. Chem.* 277:13528-13538. [n] Antonini, E. *et al.* (1971). "Hemoglobin and Myoglobin in Their Reactions with Ligands," North-Holland Publ., Amsterdam.

[0102] Table 8 illustrates that the O_2 association rate, O_2 dissociation rate, O_2, autoxidation rate, NO reactivity, and stability of Fe^{II}-O_2 complexes in H-NOX proteins may be altered by the introduction of one or more mutations. In some embodiments, any of the single or double mutations listed in Table 8 are combined with another mutation (such as another mutation in Table 8 or any other mutation described herein) to further alter the O_2 association rate, O_2 dissociation rate, O_2, autoxidation rate, NO reactivity, or stability of Fe^{II}-O_2 complexes in an H-NOX protein. As will be appreciated by the skilled artisan, introduction of one or more additional mutations, such as those described herein, may be used to further alter these values.

Table 8. O_2 association rate, O_2 dissociation rate, O_2, autoxidation rate, NO reactivity, and stability of Fe^{II}-O_2 complexes in H-NOX proteins.

Protein	k_{on}[a]	K_{off}[b]	k_{ox}[c]	NO reactivity[d]	stability of FeII-O_2 complex
Hs Hb	23	11	0.006	<0.001 s (~7,000 s^{-1})[e]	oxidizes o/n in air at RT, stable at 4 °C in air, stable anaerobic
Tt H-NOX	13.6	1.22	Very slow	0.54 ± 0.07 s^{-1}	always stable
Tt Y140H	~10	5.03	0.87	1.7 ± 0.4 s^{-1}	oxidizes o/n in air at RT, stable at 4 °C in air, stable anaerobic
β1(1-385) I145Y	~105	2.69	0.72	slow to Fe^{III}-NO	oxidizes o/n in air at RT, stable at 4 °C in air, stable anaerobic

[a]rate of O_2 association to the heme at 20 °C (μM-1s-1); [b]rate of O_2 dissociation from the heme at 20 °C (s-1); [c]rate of heme autoxidation (h-1) at 37 °C; [d]For determination of NO reactivities: purified proteins (*Tt* WT HNOX, *Tt* Y140H HNOX, *Homo sapiens* hemoglobin (Hs Hb)) were prepared at 2 μM in buffer A and nitric oxide (NO) was prepared at 200 μM in Buffer A (Buffer A: 50 mM Hepes, pH 7.5, 50 mM NaCl) at 20 °C. Using stopped flow spectroscopy, the protein was rapidly mixed with NO in a 1:1 ratio with an integration time of 0.00125 seconds. The wavelengths of maximum change were fit to a single exponential, essentially measuring the rate-limiting step of oxidation by NO. The end products of the reaction were ferric-NO for the HNOX proteins and ferric-aquo for Hs Hb. [e]For *Hs* Hb, the reaction of the protein with NO was so fast that the reaction was completed within the dead time of the experiment (0.001 seconds). The NO reactivity for hemoglobin is approximately 7,000 s^{-1} at 20 °C based on Eich, R. F. *et al.* (1996) "Mechanism of NO-Induced Oxidation of Myoglobin and Hemoglobin," *Biochemistry* 35:6976-6983.

[0103] Table 9 demonstrates that the dissociation constant for O_2 binding can be significantly changed by mutating one or more residues in H-NOX proteins. The kinetic K_D values for these exemplary H-NOX proteins range from 21.20 nM to 1000000.00 nM at 20 °C. If desired, the dissociation constant for O_2 binding can be further altered by combining any of the single or double mutations listed in Table 9 or by introducing one or more additional mutations into an H-NOX protein, as described herein.

Table 9. Wild-type and mutant H-NOX proteins and reference proteins arranged by the value of the dissociation constant for O_2 binding

Protein	Kinetic K_D (nM)	±	Calculated K_D (nM)
Tt P115A	21.2	2.1	
Tt N74H			27
Tt I5L-P115A			30
Tt N74A			32
Tt I5A			80
Tt F78Y-Y140L			80
Tt H-NOX His6			89
Tt H-NOX	89.7	6.2	
Tt wt			90
Tt F78Y-Y140F			150
Tt W9Y			218
Tt R135Q His6			252
Hs Hb beta			267
Tt W9F	305	31	
Tt W9H			456
Tt Y140H			500
Hs Hb alpha			560
Tt W9N			573
Tt I75F-His6			713-773
HemAT-B			720
Sw Mb			880
Tt I5L			1000
Tt L144F-His6			1092-1185
Tt Y140L			2000
Tt W9F-Y140H			2500
L2 F142Y	9200	3000	
Bj FixL			140000
Tt W9F-N74A			1000000
Dd H-NOX			1000000
β1(1-385) I145Y			1000000

[0104] Table 10 demonstrates that the dissociation rates for O_2 binding can be significantly changed by mutating one or more residues in H-NOX proteins. The dissociation rates for these exemplary H-NOX proteins range from 0.21 s^{-1} to 23.4 s^{-1} at 20 °C. If desired, the dissociation rate for O_2 binding can be further altered by combining any of the single or double mutations listed in Table 10 or by introducing one or more additional mutations into an H-NOX protein, as described herein.

Table 10. Wild-type and mutant H-NOX proteins and reference proteins arranged by the value of the dissociation rate for O_2 binding

Protein	$k_{off}(s^{-1})$	±
Tt N74A	0.21	0.004
Tt P115A	0.22	0.01
Tt I5L-P115A	0.28	0.03
Tt N74E	0.38	0.01
Tt N74H	0.44	0.01
Tt I5A	0.82	0.03
Tt F78Y-Y140L	0.83	0.17
Tt H-NOX His6	1.2	0.02
Tt H-NOX	1.22	0.09
Tt F78Y-Y140F	1.48	0.33
L1 F142Y	1.73	
Tt W9F	1.84	0.17
β1(1-385) I145Y	2.69	0.61
Tt W9Y	3.07	0.1
Tt R135Q His6	3.56	0.08
L2 F142Y	3.68	0.71
Tt Y140H	5.03	0.69
Tt W9H	6.42	0.11
Dd H-NOX	7.13	0.45
Tt W9N	8.09	0.14
Tt I5L	9.5	0.64
Tt I75F-His6	10.48	0.12
Sw Mb	15	
Tt Y140F	15.7	9.8
Hs Hb beta	16	
Tt L144F-His6	16.06	0.21
Bj FixL	20	
Tt Y140L	20.1	2
HemAT-B	23	
Tt W9F-Y140H	23.4	3.7
Hs Hb alpha	28	

[0105] Table 11 demonstrates that the association rates for O_2 binding can be significantly changed by mutating one or more residues in H-NOX proteins. The association rates for these exemplary H-NOX proteins range from 60 $\mu M^{-1}s^{-1}$ to 0.14 $\mu M^{-1}s^{-1}$ at 20 °C. If desired, the association rate for O_2 binding can be further altered by combining any of the single or double mutations listed in Table 11 or by introducing one or more additional mutations into an H-NOX protein, as described herein.

Table 11. Wild-type and mutant H-NOX proteins and reference proteins arranged by the value of the association rate for O_2 binding

Protein	k_{on} ($\mu M^{-1}s^{-1}$)	±
Hs Hb beta	60	
Hs Hb alpha	50	
HemAT-B	32	
Sw Mb	17	
Tt Y140F	15.7	1.4
Tt H-NOX	13.6	1
Tt P115A	10.4	1.1
Tt W9F	6.02	0.62
L2 F142Y	0.4	0.14
Bj FixL	0.14	
Tt W9F-N74A	very slow[a]	
Dd H-NOX	very slow[a]	
β1(1-385) I145Y	very slow[a]	
[a]By "very slow" is meant slower than hemoglobin, such as approximately one to two orders of magnitude slower than hemoglobin.		

[0106] Table 12 illustrates the effect of exemplary H-NOX mutations on NO and O_2-binding. Each number listed in Table 12 for the Fe-unligated form is for a single peak (which is listed in between the β and α columns). When NO or O_2 binds, this single peak splits into two peaks, β and α (which are listed below the β and α columns, respectively). If desired, NO or

O_2-binding can be further altered by combining any of the single or double mutations listed in Table 12 or by introducing one or more additional mutations into an H-NOX protein, as described herein.

Table 12: UV-visible peak positions[a] for some histidyl-ligated Fe^{II} heme protein complexes

Protein	Soret	β	α
Fe^{II} unligated complex			
sGC	431	555	
β1(1-385) I145Y	429	549	
Tt H-NOX	431	565	
Tt W9F-Y140L	430	560	
Vc H-NOX	429	568	
Np H-NOX	430	555	
L2 H-NOX	428	557	
L2 F142Y	428	557	
Tt I75F-His6	431	569	
Tt L144F-His6	433	564	
Hb	430	555	
Fe^{II}-NO complex			
sGC	398	537	572
β1(1-385) I145Y	399/416	542	574
Tt H-NOX	420	547	575
Tt W9F-Y140L	423	540	573
Vc H-NOX	398	540	573
Np H-NOX	416/400	543	576
L2 H-NOX	399/416	544	575
L2 F142Y	417	544	578
Tt I75F-His6	418	545	574
Tt L144F-His6	416	544	574

Hb	418	545	575
Fe^{II}-O_2 complex			
sGC	No complex observed		
β1(1-385) I145Y	416	541	575
Tt H-NOX	416	556	591
Tt W9F-Y140L	No complex observed		
Vc H-NOX	No complex observed		
Np H-NOX	No complex observed		
L2 H-NOX	No complex observed		
L2 F142Y	417	542	577
Tt I75F-His6	416	552	589
Tt L144F-His6	416	544	574
Hb	415	541	577
[a]nm (at 20 °C)			

[0107] Table 13 contains UV-visible peak positions for some Fe (II), Fe (III), Fe(II)-NO, and Fe(II)-O_2 complexes. When a hemoglobin or H-NOX protein is anaerobic, it has a Soret peak at ~431 nm, and it is in an unligated state. If the H-NOX protein does not bind NO, then the Soret peak will not change when NO is added. If the H-NOX protein binds NO and forms a 6-coordinate ferrous-nitrosyl complex, then its Soret peak will shift to between 420 nm and 424 nm when NO is added. If the H-NOX protein binds NO and forms a 5-coordinate ferrous-nitrosyl complex, the Soret peak will shift to ~399 nm. If the H-NOX protein does not bind O_2, then the Soret peak will not change when O_2 is added. If the H-NOX protein does bind O_2, then its Soret peak will shift to between 414 nm and 418 nm when O_2 is added, which is the same shift that occurs in hemoglobin, indicative of O_2 bound to the heme. Soret peaks for oxidized H-NOX (Fe(III)) may be relevant to the state of the H-NOX protein after storage or use. If desired, NO or O_2-binding can be further altered by combining any of the single or double mutations listed in Table 13 or by introducing one or more additional mutations into an H-NOX protein, as described herein.

Table 13. UV-visible peak positions for some Fe (II), Fe (III), Fe(II)-NO, and Fe(II)-O_2 complexes.

Complex	Protein	Soret	β	α
Fe (II)	*Tt* wt	430	563	
	Tt W9Y	430	569	
	Tt N74A	433	558	
	Tt N74H	431	561	
	Tt N74A-Y140H	430	567	
	Tt W9H	431	563	
	Tt N74E	433	559	
	Tt W9N	431	569	
	Tt wt His_6	430	565	
Complex	**Protein**	**Soret**	**β[a]**	**α**
Fe (III)	*Tt* wt	413	550	585
	Tt W9Y	409	N.A.	
	Tt N74A	416	554	586
	Tt N74H	408	N.A.	
	Tt N74A-Y140H	407	N.A.	
	Tt W9H	407	N.A.	
	Tt N74E	408	N.A.	
	Tt W9N	408	N.A.	
	Tt wt His_6	413	550	586

[a]"N.A." denotes nonassignable α and β bands due to low signal at longer wavelengths.

Complex	Protein	Soret	β	α
Fe(II) – NO	*Tt* wt	420	550	578
	Tt W9Y	420	552	576
	Tt N74A	421	572	
	Tt N74H	424	562	
	Tt N74A-Y140H	421	549	576
	Tt W9H	420	548	575
	Tt N74E	422	544	571
	Tt W9N	421	541	576

	Tt wt His_6	420	547	576
Complex	**Protein**	**Soret**	**β**	**α**
Fe(II)-O_2	*Tt* wt	416	556	591
	Tt W9Y	416	555	590
	Tt N74A	418	553	589
	Tt N74H	418	553	589
	Tt N74A-Y140H	414	555	584
	Tt W9H	418	556	589
	Tt N74E	417	555	587
	Tt W9N	416	588	553
	Tt wt His_6	416	556	591

[0108] Table 14 contains autoxidation rates for exemplary *T. tengcongensis* H-NOX proteins. If desired, the autoxidation rate can be further altered by combining any of the mutations listed in Table 14 or by introducing one or more additional mutations into an H-NOX protein, as described herein. The 2 nm and 3 nm values mean in Table 14 refer to a shift in the UV-Vis Soret peak by 2 to 3 nm over the time period of the observation; this extremely small change may be due to autoxidation.

Table 14. Autoxidation rates for *T. tengcongensis* (*Tt*) H-NOX proteins

Protein	Autoxidation Rate (25 °C, hr^{-1})[a]
Tt wt	Stable
Tt W9Y	Stable
Tt N74A	Stable
Tt N74H	stable at 4 °C, very slow at RT (2 nm)
Tt W9H	Stable
Tt N74E	very slow at 4 °C (2 nm), slow at RT
Tt W9N	stable at 4 °C, very slow at RT (3 nm)
Tt wt His_6	Stable
Tt I75F-His6	Stable
Tt L144F-His6	Stable

[a]"Stable" denotes lack of heme oxidation after at least 24 hours.

"RT" denotes room temperature.

H-NOX Nucleic Acids

[0109] The invention also features nucleic acids encoding any of the mutant H-NOX proteins described herein. As used herein, a "nucleic acid" refers to two or more deoxyribonucleotides and/or ribonucleotides in either single or double-stranded form, and unless otherwise limited, encompasses known analogs of naturally-occurring nucleotides that hybridize to nucleic acids in a manner similar to nucleotides occurring in nature. In some embodiments, the nucleic acid is a recombinant nucleic acid. By "recombinant nucleic acid" means a nucleic acid of interest that is free of one or more nucleic acids (*e.g.*, genes) which, in the genome occurring in nature of the organism from which the nucleic acid of interest is derived, flank the nucleic acid of interest. In some embodiments, an H-NOX nucleic acid is operably linked to another nucleic acid

encoding all or a portion of another protein such that the recombinant nucleic acid encodes a fusion protein that includes an H-NOX protein (*e.g.*, an H-NOX domain with or without another domain from an H-NOX protein) and all or part of another protein, such as human serum albumin. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (*e.g.*, a cDNA, a genomic DNA fragment, or a cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences.

[0110] The invention also features vectors with one more nucleic acids encoding any of the mutant H-NOX proteins that are described herein. As used herein, a "vector" means a construct that is capable of delivering, and optionally expressing one or more nucleic acids of interest in a host cell. Examples of vectors include, but are not limited to, plasmids, viral vectors, DNA or RNA expression vectors, cosmids, and phage vectors. In some embodiments, the vector contains a nucleic acid under the control of an expression control sequence. An "expression control sequence" means a nucleic acid sequence that directs transcription of a nucleic acid of interest. An expression control sequence can be a promoter, such as a constitutive or an inducible promoter, or an enhancer. The expression control sequence is operably linked to the nucleic acid segment to be transcribed.

[0111] In particular embodiments, the nucleic acid includes a segment of or the entire nucleic acid sequence of any of the nucleic acids shown in FIGS. 2-4D or 8A-8DD. In some embodiments, the nucleic acid includes at least about 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, or more contiguous nucleotides from a H-NOX nucleic acid and contains one or more mutations (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mutations) compared to the H-NOX nucleic acid from which it was derived. In various embodiments, a mutant H-NOX nucleic acid contains less than about 20, 15, 12, 10, 9, 8, 7, 6, 5, 4, 3, or 2 mutations compared to the H-NOX nucleic acid from which it was derived. The invention also features degenerate variants of any nucleic acid encoding a mutant H-NOX protein.

[0112] The invention also includes a cell or population of cells containing at least one nucleic acid encoding a mutant H-NOX protein described herein. Exemplary cells include insect,

plant, yeast, bacterial, and mammalian cells. These cells are useful for the production of mutant H-NOX proteins using standard methods, such as those described herein.

Formulations of H-NOX Proteins

[0113] Any wild-type or mutant H-NOX protein described herein may be used for the formulation of pharmaceutical or non-pharmaceutical compositions. As discussed further below, these formulations are useful in a variety of therapeutic and industrial applications.

[0114] In some embodiments, the pharmaceutical composition includes one or more wild-type or mutant H-NOX proteins (such as any of the H-NOX wild-type or mutant proteins described herein) and a pharmaceutically acceptable carrier. In various embodiments, the H-NOX protein is an isolated or purified protein. By "pharmaceutically acceptable carrier" is meant any material which, when combined with an active ingredient, allows the ingredient to retain biological activity and does not provoke an unacceptable immune response (*e.g.*, a severe allergy or anaphylactic shock) based on the knowledge of a skilled practitioner. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as phosphate buffered saline solutions, water, emulsions such as oil/water emulsion, and various types of wetting agents. Exemplary diluents for aerosol or parenteral administration are phosphate buffered saline or normal (0.9%) saline. Compositions comprising such carriers are formulated by well known conventional methods (see, for example, *Remington's Pharmaceutical Sciences*, 18th edition, A. Gennaro, ed., Mack Publishing Co., Easton, PA, 1990; and *Remington, The Science and Practice of Pharmacy* 20th Ed. Mack Publishing, 2000, which are each hereby incorporated by reference in their entireties, particularly with respect to formulations).

[0115] While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending on the mode of administration. Compositions can be formulated for any appropriate manner of administration, including, for example, intravenous, intra-arterial, intravesicular, inhalation, intraperitoneal, intrapulmonary, intramuscular, subcutaneous, intra-tracheal, transmucosal, intraocular, intrathecal, or transdermal administration. For parenteral administration,

such as subcutaneous injection, the carrier may include, *e.g.*, water, saline, alcohol, a fat, a wax, or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, or magnesium carbonate, may be employed. Biodegradable microspheres (*e.g.*, polylactate polyglycolate) may also be used as carriers.

[0116] In some embodiments, the pharmaceutical or non-pharmaceutical compositions include a buffer (*e.g.*, neutral buffered saline, phosphate buffered saline, *etc*), a carbohydrate (*e.g.*, glucose, mannose, sucrose, dextran, *etc*), an antioxidant, a chelating agent (*e.g.*, EDTA, glutathione, *etc.*), a preservative, another compound useful for binding and/or transporting NO, an inactive ingredient (*e.g.*, a stabilizer, filler, *etc*), or combinations of two or more of the foregoing. In some embodiments, the composition is formulated as a lyophilizate. H-NOX proteins may also be encapsulated within liposomes or nanoparticles using well known technology. Other exemplary formulations that can be used for H-NOX proteins are described by, *e.g.*, U.S. Pat. Nos. 6,974,795, and 6,432,918, which are each hereby incorporated by reference in their entireties, particularly with respect to formulations of proteins.

[0117] The compositions described herein may be administered as part of a sustained release formulation (*e.g.*, a formulation such as a capsule or sponge that produces a slow release of compound following administration). Such formulations may generally be prepared using well known technology and administered by, for example, oral, rectal or subcutaneous implantation, or by implantation at the desired target site. Sustained-release formulations may contain an H-NOX protein dispersed in a carrier matrix and/or contained within a reservoir surrounded by a rate controlling membrane. Carriers for use within such formulations are biocompatible, and may also be biodegradable. In some embodiments, the formulation provides a relatively constant level of H-NOX protein release. The amount of H-NOX protein contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release, and the nature of the condition to be treated or prevented.

[0118] In some embodiments, the pharmaceutical composition contains an effective amount of a wild-type or mutant H-NOX protein. The term "effective amount" intends such amount of one

or more proteins described herein which in combination with its parameters of efficacy and toxicity should be effective in a given therapeutic form based on the knowledge of the practicing specialist. As is understood in the art, an effective amount can be in one or more doses. As is understood in the clinical context, an effective dosage of a pharmaceutical composition may or may not be achieved in conjunction with another drug, compound, or pharmaceutical composition. Thus, an effective amount can be considered in the context of administering one or more therapeutic agents, and a single agent can be considered to be given in an effective amount if, in conjunction with one or more other agents, a desirable or beneficial result can be or is achieved.

[0119] An exemplary dose of hemoglobin as a blood substitute is from about 10 mg to about 5 grams or more of extracellular hemoglobin per kilogram of patient body weight. Similar doses of H-NOX proteins can be use for the delivery of NO. Thus, in some embodiments, an effective amount of an H-NOX protein for administration to a human is between a few grams to over about 350 grams. Other exemplary doses of an H-NOX protein include about any of 4.4., 5, 10, or 13 G/DL (where G/DL is the concentration of the H-NOX protein solution prior to infusion into the circulation) at an appropriate infusion rate, such as about 0.5 ml/min (see, for example, Winslow, R. Chapter 12 *In* Blood Substitutes). In some embodiments, a dose of less than 10 mg of H-NOX protein is used for temporary vasodilation. It will be appreciated that the unit content of active ingredients contained in an individual dose of each dosage form need not in itself constitute an effective amount since the necessary effective amount could be reached by the combined effect of a plurality of administrations. The selection of the amount of an H-NOX protein to include in a pharmaceutical composition depends upon the dosage form utilized, the condition being treated, and the particular purpose to be achieved according to the determination of the ordinarily skilled artisan in the field.

[0120] Exemplary compositions include genetically engineered, recombinant H-NOX proteins, which may be isolated or purified, comprising one or more mutations that collectively impart altered NO or O_2 ligand-binding relative to the corresponding wild-type H-NOX protein, and operative as a physiologically compatible mammalian blood gas NO carrier. For example, mutant H-NOX proteins as described herein.

[0121] The invention also provides NO carriers comprising or consisting essentially of one or more wild-type or mutant H-NOX proteins. Suitable buffers and other ingredients for formulating proteins (such as proteins delivered to the blood or gastrointestinal system) are known in the art.

[0122] To reduce or prevent an immune response in human subjects who are administered a pharmaceutical composition, human H-NOX proteins (either wild-type human proteins or human proteins into which one or more mutations have been introduced) or other non-antigenic H-NOX proteins (*e.g.*, mammalian H-NOX proteins) can be used. To reduce or eliminate the immunogenicity of H-NOX proteins derived from sources other than humans, amino acids in an H-NOX protein can be mutated to the corresponding amino acids in a human H-NOX. For example, one or more amino acids on the surface of the tertiary structure of a non-human H-NOX protein can be mutated to the corresponding amino acid in a human H-NOX protein.

Therapeutic Applications of H-NOX Proteins

[0123] Any of the wild-type or mutant H-NOX proteins (*e.g.*, isolated or purified H-NOX proteins) or pharmaceutical compositions described herein may be used in therapeutic applications.

Particular H-NOX proteins can be selected for such applications based on the desired

[0124] NO dissociation constant, O_2 dissociation constant, NO k_{off}, O_2 k_{off}, NO reactivity, NO stability, autoxidation rate, plasma retention time, half-life, or any combination of two or more of the foregoing for the particular indication being treated. H-NOX proteins can be used to treat any condition for which delivery of NO is beneficial. Exemplary target indications include diseases of functional NO deficiency, such as where a vasodilator or an NO carrier is indicated, including conditions exacerbated by chronic hypertension, such as heart failure, renal failure, and stroke. In various embodiments, the treated condition is a cardiovascular condition (*e.g.*, myocardial infarction or heart surgery), hypertension, a vasoconstrictive condition (*e.g.*, stroke), erectile dysfunction, constipation, or bowel obstruction. For the treatment of constipation or bowel obstruction, H-NOX proteins can be used to deliver NO to treat a sphincter control deficit, thereby relaxing the smooth muscle. For example, H-NOX proteins that function in the digestive system can relax the smooth

muscle of the ileum as the H-NOX proteins pass through the digestive system. The methods and compositions are applicable to both acute (providing rapid NO to tissues or a specific site, *e.g.*, acute myocardial infarction or stroke) and chronic situations (*e.g.*, chronic hypertension or post-acute recovery from cardiac infarction or stroke).

[0125] In various embodiments, the invention features a method of delivering NO to an individual (*e.g.*, a mammal, such as a primate (*e.g.*, a human, a monkey, a gorilla, an ape, a lemur, *etc*), a bovine, an equine, a porcine, a canine, or a feline) by administering to an individual in need thereof a wild-type or mutant H-NOX protein in an amount sufficient to deliver NO to the individual. In some embodiments, the invention provides methods of carrying or delivering blood gas to an individual such as a mammal, comprising the step of delivering to the blood of the individual (*e.g.*, a mammal) one or more of H-NOX compositions. Methods for delivering proteins to the blood, digestive system, or tissues (*e.g.*, mammalian blood or tissues) are known in the art. In various embodiments, the H-NOX protein is an apoprotein that is capable of binding heme or is a holoprotein with heme bound. The H-NOX protein may or may not have heme bound prior to the administration of the H-NOX protein to the individual. In some embodiments, NO is bound to the H-NOX protein before it is delivered to the individual. In other embodiments, NO is not bound to the H-NOX protein prior to the administration of the protein to the individual, and the H-NOX protein transports NO from one location in the individual to another location in the individual. For example, in particular embodiments, H-NOX proteins bind NO in the blood stream and only release it where NO concentrations are very low (such as sites of vasoconstriction). This targeted delivery of NO may produce fewer side-effects than conventional vasodilators that release NO independent of local NO concentration and thus function systemically, with side effects such as headaches and peripheral tingling.

[0126] The methods of the present invention can be used to treat any individual. For use herein, unless clearly indicated otherwise, "an individual" as used herein intends a mammal, including but not limited to, a primate (*e.g.*, a human, monkey, gorilla, ape, lemur, *etc.*), a bovine, an equine, a porcine, a canine, and a feline. Thus, the invention finds use in both human medicine and in the veterinary context, including use in agricultural animals and domestic pets. The individual may have been diagnosed with, is suspected of having, or is at risk of developing an

indication, such as a cardiovascular condition (*e.g.*, myocardial infarction or heart surgery), hypertension, a condition exacerbated by hypertension (*e.g.*, heart failure, renal failure, or stroke), a vasoconstrictive condition (*e.g.*, stroke), a functional NO deficiency, erectile dysfunction, constipation, or bowel obstruction. The individual may exhibit one or more symptoms associated with the indication. The individual can be genetically or otherwise predisposed to developing such a condition.

[0127] As used herein, "in need thereof" includes individuals who have a condition or disease (*e.g.*, as a cardiovascular condition such as myocardial infarction or heart surgery, hypertension, a condition exacerbated by hypertension such as heart failure, renal failure, or stroke, a vasoconstrictive condition such as stroke, a functional NO deficiency, erectile dysfunction, constipation, or bowel obstruction) or are "at risk" for the condition or disease. As used herein, an "at risk" individual is an individual who is at risk of development of a condition, such as a cardiovascular condition (*e.g.*, myocardial infarction or heart surgery), hypertension, a condition exacerbated by hypertension (*e.g.*, heart failure, renal failure, or stroke), a vasoconstrictive condition (*e.g.*, stroke), a functional NO deficiency, erectile dysfunction, constipation, or bowel obstruction. An individual "at risk" may or may not have a detectable disease or condition, and may or may not have displayed detectable disease prior to the treatment methods described herein. "At risk" denotes that an individual has one or more so-called risk factors, which are measurable parameters that correlate with development of a disease or condition and are known in the art. An individual having one or more of these risk factors has a higher probability of developing the disease or condition than an individual without these risk factor(s). These risk factors include, but are not limited to, age, sex, race, diet, history of previous disease, presence of precursor disease, genetic (*i.e.*, hereditary) considerations, and environmental exposure.

[0128] These methods can be used to treat or delay any condition for which delivery of NO is beneficial. By "treatment" or "treating" is meant an approach for obtaining a beneficial or desired result, including clinical results. For purposes of this invention, beneficial or desired results include, but are not limited to, alleviation of symptoms associated with a condition (such as, but not limited to, a cardiovascular condition such as myocardial infarction or heart surgery, hypertension, a condition exacerbated by hypertension such as heart failure, renal failure, or stroke, a

vasoconstrictive condition such as stroke, a functional NO deficiency, erectile dysfunction, constipation, or bowel obstruction) diminishment of the extent of the symptoms associated with a condition, or prevention of a worsening of the symptoms associated with a condition. In some embodiments, treatment with a one or more proteins disclosed herein is accompanied by no or fewer side effects than are associated with currently available therapies.

[0129] As used herein, "delaying" development of a disease or condition means to defer, hinder, slow, retard, stabilize and/or postpone development of the disease or condition, such as a cardiovascular condition (*e.g.*, myocardial infarction or heart surgery), hypertension, a condition exacerbated by hypertension (*e.g.*, heart failure, renal failure, or stroke), a vasoconstrictive condition (*e.g.*, stroke), a functional NO deficiency, erectile dysfunction, constipation, or bowel obstruction. This delay can be of varying lengths of time, depending on the history of the disease and/or individual being treated. As is evident to one skilled in the art, a sufficient or significant delay can, in effect, encompass prevention, in that the individual does not develop the disease or condition. For example, the method may reduce the probability of disease development in a given time frame and/or reduce the extent of the disease in a given time frame, when compared to not using the method. In some embodiments, such comparisons are based on clinical studies using a statistically significant number of subjects. Disease development can be detectable using standard clinical techniques. Development may also refer to disease progression that can be initially undetectable and includes occurrence, recurrence, and onset.

[0130] In some embodiments for the direct delivery of an H-NOX protein with bound NO to a particular site in the body (such as a tissue or organ), the k_{off} for NO is more important than the K_D because NO is already bound to the protein (making the k_{on} less important) and NO needs to be released at or near a particular site in the body (at a rate influenced by the k_{off}). In some embodiments for the treatment of acute conditions, the H-NOX protein has a relatively high k_{off}, k_1, or k_2 for NO (such as at least about any of 0.05 s^{-1}, 0.1 s^{-1}, or 1.0 s^{-1}) so that vasodilation occurs rapidly. In some embodiments for systemic administration, the H-NOX protein has a relatively low k_{off}, k_1, or k_2 for NO (such as at less than about any of 0.05 s^{-1}, 0.01 s^{-1}, or 0.001 s^{-1}) so that NO is not released until the H-NOX protein reaches a site of low NO concentration (*e.g.*, a vasoconstricted site).

[0131] H-NOX proteins can also be used for imaging. In particular, light imaging (*e.g.*, optical coherence tomography; *see*, for example, Villard, J. W. (2002). "Use of a Blood Substitute to Determine Instantaneous Murine Right Ventricular Thickening with Optical Coherence Tomography," *Circulation* 105:1843-1849, which is incorporated by reference in its entirety particularly with respect to optical coherence tomography) is obfuscated by erythrocytes. Perfusion with an H-NOX solution allows for clearer images of the circulation and vessel walls because the H-NOX protein is much smaller than erythrocytes.

[0132] H-NOX proteins and pharmaceutical compositions of the invention can be administered to an individual by any conventional means such as by oral, topical, intraocular, intrathecal, intrapulmonary, intra-tracheal, or aerosol administration; by transdermal or mucus membrane adsorption; or by injection (*e.g.*, subcutaneous, intravenous, intra-arterial, intravesicular, or intramuscular injection). H-NOX proteins may also be included in large volume parenteral solutions for administration to the blood. In exemplary embodiments, the H-NOX protein is administered to the blood (*e.g.*, administration to a blood vessel such as a vein, artery, or capillary) of the individual.

[0133] In some embodiments, a sustained continuous release formulation of the composition is used. Administration of an H-NOX protein can occur, *e.g.*, for a period of seconds to hours depending on the purpose of the administration. For acute conditions, an exemplary time course of administration is as rapid as possible. Other exemplary time courses include about 10, 20, 30, 40, 60, 90, or 120 minutes. Exemplary infusion rates for H-NOX solutions are from about 30 mL/hour to about 13,260 mL/hour, such as about 100 mL/hour to about 3,000 mL/hour. An exemplary total dose of H-NOX protein is about 900 mg/kg administered over 20 minutes at 13,260 mL/hour. An exemplary total dose of H-NOX protein for a swine is about 18.9 grams.

[0134] Exemplary dosing frequencies include, but are not limited to, at least 1, 2, 3, 4, 5, 6, or 7 times (*i.e.*, daily) a week. In some embodiments, an H-NOX protein is administered at least 2, 3, 4, or 6 times a day. The H-NOX protein can be administered, *e.g.*, over a period of a few days or weeks. In some embodiments, the H-NOX protein is administrated for a longer period, such as a

few months or years. The dosing frequency of the composition may be adjusted over the course of the treatment based on the judgment of the administering physician.

[0135] As noted above, the selection of dosage amounts for H-NOX proteins depends upon the dosage form utilized, the frequency and number of administrations, the condition being treated, and the particular purpose to be achieved according to the determination of the ordinarily skilled artisan in the field. In some embodiments, an effective amount of an H-NOX protein for administration to human is between a few grams to over 350 grams.

[0136] In some embodiments, two or more different H-NOX proteins are administered simultaneously, sequentially, or concurrently. In some embodiments, another compound or therapy useful for the delivery of NO is administered simultaneously, sequentially, or concurrently with the administration of one or more H-NOX proteins.

Industrial Applications of H-NOX Proteins

[0137] The H-NOX proteins and composition described herein can also be used for a number of *in vitro* or industrial applications (see, *e.g.*, U.S. Pat. No. 6,455,676, which is hereby incorporated by reference in its entirety, particularly with respect to *in vitro* or industrial applications). Particular H-NOX proteins can be selected for such applications based on the desired NO dissociation constant, O_2 dissociation constant, NO k_{off}, O_2 k_{off}, NO reactivity, NO stability, autoxidation rate, half-life, or any combination of two or more of the foregoing for the particular application. In various embodiments of industrial applications, the H-NOX protein is an apoprotein that is capable of binding heme or is a holoprotein with heme bound.

[0138] H-NOX proteins can be used to add NO to solutions for which NO is desirable. In embodiments that use bioreactors that require anaerobic fermentation, H-NOX proteins are used to deliver NO delivery to cells. For example, the delivery of NO to mitochondria may limit oxidative phosphorylation and enhance metabolism through the lactate pathway. The H-NOX protein in *Clostridium acetobutylicum,* which is cultured under anaerobic fermentation as a biofuel generator, may naturally serve this function. Moreover, the H-NOX proteins can be used to remove NO from solutions requiring the removal of NO. For example, H-NOX proteins may be used to absorb or

remove NO in bioreactors where NO is an inhibitor of cellular proliferation and/or mitochondrial function. Removing NO may improve mitochondrial function, limit apoptosis, increase per-cell productivity, or any combination of two or more of the foregoing.

Kits with H-NOX proteins

[0139] Also provided are articles of manufacture and kits that include any of the H-NOX proteins described herein and suitable packaging. In some embodiments, the invention includes a kit with (i) an H-NOX protein (such as a wild-type or mutant H-NOX protein described herein or formulations thereof as described herein) and (ii) instructions for using the kit to deliver NO to an individual. In various embodiments, the invention features a kit with (i) an H-NOX protein (such as a wild-type or mutant H-NOX protein described herein or formulations thereof as described herein) and (ii) instructions for using the kit for any of the industrial uses described herein (*e.g.*, addition of NO to a solution or removal of NO from a solution).

[0140] Suitable packaging for compositions described herein are known in the art, and include, for example, vials (*e.g.*, sealed vials), vessels, ampules, bottles, jars, flexible packaging (*e.g.*, sealed Mylar or plastic bags), and the like. These articles of manufacture may further be sterilized and/or sealed. Also provided are unit dosage forms comprising the compositions described herein. These unit dosage forms can be stored in a suitable packaging in single or multiple unit dosages and may also be further sterilized and sealed. Instructions supplied in the kits of the invention are typically written instructions on a label or package insert (*e.g.*, a paper sheet included in the kit), but machine-readable instructions (*e.g.*, instructions carried on a magnetic or optical storage disk) are also acceptable. The instructions relating to the use of H-NOX proteins generally include information as to dosage, dosing schedule, and route of administration for the intended treatment or industrial use. The kit may further comprise a description of selecting an individual suitable or treatment.

[0141] The containers may be unit doses, bulk packages (*e.g.*, multi-dose packages) or sub-unit doses. For example, kits may also be provided that contain sufficient dosages of H-NOX proteins disclosed herein to provide effective treatment for an individual for an extended period,

such as about any of a week, 2 weeks, 3 weeks, 4 weeks, 6 weeks, 8 weeks, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, or more. Kits may also include multiple unit doses of H-NOX proteins and instructions for use and packaged in quantities sufficient for storage and use in pharmacies, for example, hospital pharmacies and compounding pharmacies. In some embodiments, the kit includes a dry (*e.g.*, lyophilized) composition that can be reconstituted, resuspended, or rehydrated to form generally a stable aqueous suspension of H-NOX protein.

Exemplary Methods for Production of H-NOX Proteins

[0142] The present invention also provides methods for the production of any of the mutant H-NOX proteins described herein. In some embodiments, the method involves culturing a cell that has a nucleic acid encoding a mutant H-NOX protein under conditions suitable for production of the mutant H-NOX protein. In various embodiments, the mutant H-NOX is also purified (such as purification of the H-NOX protein from the cells or the culture medium).

[0143] As noted above, the sequences of several wild-type H-NOX proteins and nucleic acids are known and can be use to generate mutant H-NOX proteins and nucleic acids of the present invention. Techniques for the mutation, expression, and purification of recombinant H-NOX proteins have been described by, *e.g.*, Boon, E.M. *et al.* (2005). "Molecular Basis For NO Selectivity in Soluble Guanylate Cyclase," *Nature Chemical Biology* 1:53-59 and Karow, D. S. *et al.* (August 10, 2004). "Spectroscopic Characterization of The Soluble Guanylate Cyclase-Like Heme Domains From Vibrio Cholerae And Thermoanaerobacter Tengcongensis," *Biochemistry* 43(31):10203-10211, which is hereby incorporated by reference in its entirety, particularly with respect to the mutation, expression, and purification of recombinant H-NOX proteins. These techniques or other standard techniques can be used to generate any mutant H-NOX protein.

[0144] In particular, mutant H-NOX proteins described herein can be generated a number of methods that are known in the art. Mutation can occur at either the amino acid level by chemical modification of an amino acid or at the codon level by alteration of the nucleotide sequence that codes for a given amino acid. Substitution of an amino acid at any given position in a protein can be achieved by altering the codon that codes for that amino acid. This can be accomplished by site-

directed mutagenesis using, for example: (i) the Amersham technique (Amersham mutagenesis kit, Amersham, Inc., Cleveland, Ohio) based on the methods of Taylor, J.W. *et al.* (December 20, 1985). "The Use of Phosphorothioate-Modified DNA in Restriction Enzyme Reactions to Prepare Nicked DNA," *Nucleic Acids Res.* 13(24):8749-8764; Taylor, J.W. et al. (December 20, 1985). "The Rapid Generation of Oligonucleotide-Directed Mutations at High Frequency Using Phosphorothioate-Modified DNA," *Nucleic Acids Res.* 13(24):8765-8785; Nakamaye, K. L. *et al.* (December 22, 1986). "Inhibition of Restriction Endonuclease Nci I Cleavage by Phosphorothioate Groups and its Application to Oligonucleotide-Directed Mutagenesis," *Nucleic Acids Res.* 14(24):9679-9698; and Dente *et al.* (1985). in DNA Cloning, Glover, Ed., IRL Press, pages 791-802, (ii) the Promega kit (Promega Inc., Madison, Wis.), or (iii) the Biorad kit (Biorad Inc., Richmond, Calif.), based on the methods of Kunkel, T. A. (January 1985). "Rapid And Efficient Site-Specific Mutagenesis Without Phenotypic Selection," *Proc. Natl. Acad. Sci. USA* 82(2):488-492; Kunkel, T. A. (1987). "Rapid And Efficient Site-Specific Mutagenesis Without Phenotypic Selection," *Methods Enzymol.* 154:367-382; Kunkel, U.S. Pat. No. 4,873,192, which are each hereby incorporated by reference in their entireties, particularly with respect to the mutagenesis of proteins. Mutagenesis can also be accomplished by other commercially available or non-commercial means, such as those that utilize site-directed mutagenesis with mutant oligonucleotides.

[0145] Site-directed mutagenesis can also be accomplished using PCR-based mutagenesis such as that described in Zhengbin *et al.* (1992). pages 205-207 in PCR Methods and Applications, Cold Spring Harbor Laboratory Press, New York; Jones, D. H. *et al.* (February 1990). "A Rapid Method For Site-Specific Mutagenesis And Directional Subcloning by Using the Polymerase Chain Reaction to Generate Recombinant Circles," *Biotechniques* 8(2):178-183; Jones, D. H. *et al.* (January 1991). "A Rapid Method For Recombination And Site-Specific Mutagenesis by Placing Homologous Ends on DNA Using Polymerase Chain Reaction," *Biotechniques* 10(1):62-66, which are each hereby incorporated by reference in their entireties, particularly with respect to the mutagenesis of proteins. Site-directed mutagenesis can also be accomplished using cassette mutagenesis with techniques that are known to those of skill in the art.

[0146] A mutant H-NOX nucleic acid can be incorporated into a vector, such as an expression vector, using standard techniques. For example, restriction enzymes can be used to cleave the mutant H-NOX nucleic acid and the vector. Then, the compatible ends of the cleaved mutant H-NOX nucleic acid and the cleaved vector can be ligated. The resulting vector can be inserted into a cell (*e.g.*, an insect cell, a plant cell, a yeast cell, or a bacterial cell) using standard techniques (*e.g.*, electroporation) for expression of the encoded H-NOX protein.

[0147] In particular, heterologous proteins have been expressed in a number of biological expression systems, such as insect cells, plant cells, yeast cells, and bacterial cells. Thus, any suitable biological protein expression system can be utilized to produce large quantities of recombinant H-NOX protein. In some embodiments, the H-NOX protein (*e.g.*, a mutant or wild-type H-NOX protein) is an isolated protein. As used herein, an "isolated protein" means a protein separated from one or more components with which the protein is naturally associated in nature, including, for example, nucleic acids, lipids, and other proteins. An isolated protein also does not occur in a library of proteins, such as a library of 2, 5, 10, 20, 50 or more different proteins. An isolated protein can be obtained, for example, by expression of a recombinant nucleic acid encoding the protein or by chemical synthesis of the protein.

[0148] If desired, H-NOX proteins can be purified using standard techniques. As used herein, a "purified protein" means a protein (*e.g.*, a mutant or wild-type H-NOX protein) that has been separated from one or more components that are present when the protein is produced. In some embodiments, the protein is at least about 60%, by weight, free from other components that are present when the protein is produced. In various embodiments, the protein is at least about 75%, 90%, or 99%, by weight, pure. A purified protein can be obtained, for example, by purification (*e.g.*, extraction) from a natural source, a recombinant expression system, or a reaction mixture for chemical synthesis. Exemplary methods of purification include immunoprecipitation, column chromatography such as immunoaffinity chromatography, magnetic bead immunoaffinity purification, and panning with a plate-bound antibody, as well as other techniques known to the skilled artisan. Purity can be assayed by any appropriate method, *e.g.*, by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. In some embodiments, the purified protein is incorporated into a pharmaceutical composition of the invention or used in a method of the

invention. The pharmaceutical composition of the invention may have additives, carriers, or other components in addition to the purified protein.

EXAMPLES

[0149] The examples, which are intended to be purely exemplary of the invention and should therefore not be considered to limit the invention in any way, also describe and detail aspects and embodiments of the invention discussed above. The examples are not intended to represent that the experiments below are all or the only experiments performed. Unless indicated otherwise, temperature is in degrees Centigrade and pressure is at or near atmospheric.

Example 1: Production of Wild-type and Mutant H-NOX proteins

[0150] Wild-type and mutant H-NOX proteins were produced, expressed, and purified using standard methods, essentially as described by Boon, E.M. *et al.* (2005). "Molecular Basis For NO Selectivity in Soluble Guanylate Cyclase," *Nature Chemical Biology* 1:53-59 and Karow, D. S. *et al.* (August 10, 2004). "Spectroscopic Characterization of The Soluble Guanylate Cyclase-Like Heme Domains From Vibrio Cholerae And Thermoanaerobacter Tengcongensis," *Biochemistry* 43(31):10203-10211, which are both hereby incorporated by reference in their entireties, particularly with respect to the mutagenesis, expression, and purification of H-NOX proteins. Mutagenesis was performed using the QuickChange® protocol from Strategene (La Jolla, CA). Expression of the proteins in cell culture and subsequent purification of the proteins was performed as described by Karow, D. S. *et al.* (August 10, 2004). "Spectroscopic Characterization of The Soluble Guanylate Cyclase-Like Heme Domains From Vibrio Cholerae And Thermoanaerobacter Tengcongensis," *Biochemistry* 43(31):10203-10211.

Example 2: Characterization of Mutant H-NOX Proteins

Calculated K_D for NO: Ratio of k_{off} to k_{on}

[0151] To determine the calculated K_D for NO, the value for the k_{on} for NO for an H-NOX protein is assumed to be 710 $\mu M^{-1}s^{-1}$, and the dissociation rate for NO (k_{off} for an H-NOX protein with a 6-coordinate Fe^{II}-NO complex or k_1 or k_2 for an H-NOX protein with a 5-coordinate Fe^{II}-NO complex) is determined as described below.

k_{off}, k_1, and k_2 (NO dissociation rates)

k_{off} values for H-NOX proteins with a 6-coordinate Fe^{II}-NO complex

[0152] For an H-NOX protein with a 6-coordinate Fe^{II}-NO complex, the k_{off} for NO is calculated as described by Boon, E. M. *et al.*, (August 2006), "Nitric Oxide Binding to Prokaryotic Homologs of the Solube Guanylate Cyclase β1 H0NOX Domain," *J. Biol. Chem.* 281(31): 21892-21902 and Boon, E.M. *et al.* (2005). "Molecular Basis For NO Selectivity in Soluble Guanylate Cyclase," *Nature Chemical Biology* 1:53-59, which are each hereby incorporated by reference in their entireties, particularly with respect to the calculation of NO k_{off} for H-NOX proteins. Briefly, Fe^{II}-NO complexes of H-NOX protein (5 μM heme final concentration) diluted in anaerobic 50 mM triethanolamine, 50 mM NaCl, pH 7.5, buffer were rapidly mixed with a saturated carbon monoxide and 30 mM (final concentration) dithionite trap ($Na_2S_2O_4$) in the same buffer (anaerobic) (Kharitonov, V. G. *et al.* (1997). *Biochemistry* 36:6814- 6818 and Moore, E. G. *et al.* (1976). *J. Biol. Chem.* 251:2788-2794, which are each hereby incorporated by reference in their entireties, particularly with respect to the calculation of NO dissociation rates). It has been established previously that CO binding is not rate-limiting in these experiments (Kharitonov, V. G. *et al.* (1997) *Biochemistry* 36:6814 – 6818); this was confirmed in experiments using only 30 mM $Na_2S_2O_4$ without CO as a trap. Data were acquired by scanning periodically on a Cary 3E spectrophotometer equipped with a Neslab RTE-100 constant temperature bath set to varying temperatures (0–70 °C) using a quartz cuvette with a size of 100 μL to 1 mL and a path-length of 1-cm (Cary 3E, Varian, Inc., Palo Alto, CA). The dissociation of NO from the heme was monitored as the formation of the Fe^{II}-CO complex at 423 nm. Difference spectra were calculated by subtracting the first scan from

each subsequent scan. The NO dissociation rate was determined from the increase in absorbance at 423 nm versus time and fit with a single exponential of the form $f(x) = A \times (1 - e^{-kx})$ using Kaleidagraph 3.X (Synergy Software, Reading, PA). In particular, a single exponential increase in the concentration of heme-CO (due to CO binding from the NO trap) can be described by equation 1:

$$\Delta A_t = \Delta A_T (1 - e^{-k_1 t}) \qquad \text{(equation 1)}$$

[0153] where ΔA_t is the change in signal amplitude at time *t*; ΔA_T is the total change in signal amplitude, and k_1 is the observed reaction rate constant. Each experiment was performed a minimum of six times, and the resulting rates were averaged. The dissociation rates measured are independent of CO and dithionite concentration (3, 30, and 300 mM dithionite were tested).

k_1 and k_2 values for H-NOX proteins with a 5-coordinate Fe^{II}-NO complex

[0154] For an H-NOX protein with a 5-coordinate Fe^{II}-NO complex, the k_{off} for NO is described by the k_1 for NO and the k_2 for NO, as described by Winger, J. A. *et al.*, (January 2007) "Dissociation of Nitric Oxide from Soluble Guanylate Cyclase and Heme-Nitric Oxide/Oxygen Binding Domain Constructs" *J. Biol. Chem.* 282(2): 897-907, which is hereby incorporated by reference in its entirety, particularly with respect to the calculation of NO k_{off}, NO k_1, and NO k_2 for H-NOX proteins. Briefly, the dissociation of NO from the heme of H-NOX proteins with a 5-coordinate Fe^{II}-NO complex was measured at 37 and 10 °C using the CO/dithionite trapping method described previously (Cary, S. P. L., *et al.* (2005) *Proc. Natl. Acad. Sci. U. S. A.* 102: 13064 –13069 and Kharitonov, V. G. *et al.* (1997) *Biochemistry* 36:6814-6818, which are each hereby incorporated by reference in their entireties, particularly with respect to the calculation of NO dissociation rates). The trapping solution was prepared as follows: a solution of sodium dithionite ($Na_2S_2O_4$) in 50 mM HEPES, pH 7.4, 50 mM NaCl was prepared in a Teflon-sealed Reacti-Vial (Pierce) using an anaerobic chamber (Coy Laboratory Products). The solution was removed from the anaerobicchamber and saturated with CO by bubbling the gas through the solution for 10 minutes. H-NOX protein-NO complexes were formed by incubation with excess DEA/NO (in 10

mM NaOH) at 25 °C in 50 mM HEPES, pH 7.4, 50 mM NaCl for 10 min. Complete conversion to the nitrosyl species was verified by following the shift in the Soret maximum from 431 to 399 nm. H-NOX proteins were placed in a septum-sealed anaerobic cuvette (a quartz cuvette with a size of 100 μL to 1 mL and a path-length of 1-cm) and deoxygenated using an oxygen-scavenged gas train. A small amount of DEA/NO (~3 eq) was added just before deoxygenation to maintain the nitrosyl species (any remainder was subsequently destroyed by the large excess of $Na_2S_2O_4$ in the trapping solution). The head space of the anaerobic cuvette was replaced with CO, and the cuvette and trap solutions were equilibrated at assay temperature for 1 minute. The reaction was initiated by addition of CO/dithionite solution to the anaerobic cuvette with a Hamilton gas-tight syringe and mixing. The final concentration of $Na_2S_2O_4$ in the reaction mixture was 30 mM. Final protein concentrations were 1.9 to 2.5 μM for β1(1–194), β1(1–385), and β2(1–217), and 0.88 to 2.5 μM for sGC. Data collection was initiated 10 seconds after trap addition. The reaction was monitored by electronic absorption spectroscopy using a Cary 3E spectrophotometer equipped with a Neslab RTE-100 temperature controller (Cary 3E, Varian, Inc., Palo Alto, CA). Data were collected over the range of 380 - 450 nm at 909 nm/min with a 1.5-nm data point interval. Spectra were recorded every 18 seconds for 5 minutes, every 1 minute for 10 minutes, and every 2 minutes thereafter for a total of 3 hours, or until the reaction was complete. A buffer base line was subtracted from each spectrum, and spectra were corrected for base-line drift by normalization to an isosbestic point at 410 nm. Difference spectra were obtained by subtraction of the time 0 spectrum from all subsequent spectra. Values for the change in absorbance at 423 nm (ΔA_{423}; β1(1-194) and β1(1-385)) or 424 nm (ΔA_{424}; sGC and β2(1-217)) were extracted from the difference spectra and plotted versus time to obtain dissociation time courses for each experiment. Dissociation time courses were obtained in duplicate or triplicate, and each experiment was repeated 2–5 times over several days. Generally, because of the relative difficulty in obtaining large amounts of purified sGC, ΔA_{424} values for full-length sGC, which are proportional to the experimental protein concentrations, were smaller than for the heme domain constructs.

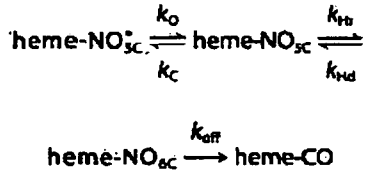
[0155] Curve fitting, data analysis, and figure generation were carried out using Kaleidagraph 3.X (Synergy Software Reading, PA). The data from each dissociation experiment were fit to a double exponentials as shown in Equation 2 below to obtain observed rate constants.

In particular, equation 2 describes a two-exponential increase in the concentration of heme-CO (due to CO binding from the NO trap):

$$\Delta A_t = \Delta A_1 (1 - e^{-k_1 t}) + \Delta A_2 (1 - e^{-k_2 t}) \qquad \text{(equation 2)}$$

where ΔA_t is the change in signal amplitude at time *t*; ΔA_1 and ΔA_2 are the contributions of each exponential process to the total change in signal amplitude, and k_1 and k_1 are the observed rate constants for each process.

[0156] The observed data are consistent with a model where dissociation proceeds from an initial equilibrium mixture of two 5-coordinate heme-NO complexes, as outlined in Scheme 1. Accordingly, k_1 corresponds to the dissociation of NO from the heme-NO_{5C} conformation, whereas k_2 represents the observed rate of reaction, corresponding to $k_O - k_C$, that is limited by the slower conversion from heme-NO NO^*_{5C} to heme-NO_{5C}.



Scheme 1

k_1 and k_2 values for H-NOX proteins with mixture of 5-coordinate and 6-coordinate Fe^{II}-NO complexes

[0157] For an H-NOX protein that contains a mixture of 5-coordinate and 6-coordinate Fe^{II}-NO complexes, the k_{off} for NO is described by the k_1 for NO and the k_2 for NO. The k_1 and the k_2 for NO are measured as described above for H-NOX proteins with a 5-coordinate Fe^{II}-NO complex, as described by Winger, J. A. *et al.*, (January 2007) "Dissociation of Nitric Oxide from Soluble Guanylate Cyclase and Heme-Nitric Oxide/Oxygen Binding Domain Constructs" *J. Biol. Chem.*

282(2): 897-907, which is hereby incorporated by reference in its entirety, particularly with respect to the calculation of NO k_{off}, NO k_1, and NO k_2 for H-NOX proteins.

Calculated K_D for NO

[0158] For the calculated K_D for NO, the value for the k_{on} for NO for an H-NOX protein is assumed to be 710 $\mu M^{-1}s^{-1}$, which is a reported k_{on} for β1(1-385) that was measured at 4 °C and does not increase significantly at 37 °C (Zhao, *et. al.*, (1999). "A Molecular Basis for Nitric Oxide Sensing by Soluble Guanylate Cyclase," *PNAS.* 96:14753-14758, which is hereby incorporated by reference in its entirety, particularly with respect to the calculation of NO k_{on} for H-NOX proteins). Thus, the calculated K_D for NO is determined by calculating the ratio of either k_{off}, k_1, or k_2 (measured as described above) to k_{on} (assumed to be 710 $\mu M^{-1}s^{-1}$).

Kinetic K_D for O_2: Ratio of k_{off} to k_{on}

[0159] The kinetic K_D value for O_2 was determined for wild-type and mutant H-NOX proteins essentially as described by Boon, E.M. *et al.* (2005). "Molecular Basis For NO Selectivity in Soluble Guanylate Cyclase," *Nature Chemical Biology* 1:53-59, which is hereby incorporated by reference in its entirety, particularly with respect to the measurement of O_2 association rates, O_2 dissociation rates, dissociation constants for O_2 binding, autoxidation rates, and NO dissociation rates.

k_{on} (O_2 Association Rate)

[0160] O_2 association to the heme was measured using flash photolysis at 20 °C. It was not possible to flash off the Fe^{II}–O_2 complex as a result of the very fast geminate recombination kinetics; thus, the Fe^{II}–CO complex was subjected to flash photolysis with laser light at 560 nm (Hewlett-Packard, Palo Alto, CA), producing the 5-coordinate Fe^{II} intermediate, to which the binding of molecular O_2 was followed at various wavelengths. Protein samples were made by anaerobic reduction with 10 mM dithionite, followed by desalting on a PD-10 column (Millipore, Inc., Billerica, MA). The samples were then diluted to 20 μM heme in 50 mM TEA, 50 mM NaCl,

pH 7.5 buffer in a controlled-atmosphere quartz cuvette, with a size of100 µL to 1 mL and a path-length of 1-cm. CO gas was flowed over the headspace of this cuvette for 10 minutes to form the Fe^{II}–CO complex, the formation of which was verified by UV-visible spectroscopy (Soret maximum 423 nm). This sample was then either used to measure CO-rebinding kinetics after flash photolysis while still under 1 atmosphere of CO gas, or it was opened and stirred in air for 30 minutes to fully oxygenate the buffer before flash photolysis to watch O_2-rebinding events. O_2 association to the heme was monitored at multiple wavelengths versus time. These traces were fit with a single exponential using Igor Pro software (Wavemetrics, Inc., Oswego, OR; latest 2005 version). This rate was independent of observation wavelength but dependent on O_2 concentration. UV-visible spectroscopy was used throughout to confirm all the complexes and intermediates (Cary 3K, Varian, Inc. Palo Alto, CA). Transient absorption data were collected using instruments described in Dmochowski, I. J. *et al.* (August 31, 2000). "Enantiomeric Discrimination of Ru-Substrates by Cytochrome P450cam," *J Inorg Biochem.* *81*(3):221-228, which is hereby incorporated by reference in its entirety, particularly with respect to instrumentation. The instrument has a response time of 20 ns, and the data are digitized at 200 megasamples s^{-1}.

k_{off} (O_2 dissociation rate)

[0161] To measure the k_{off}, Fe^{II}–O_2 complexes of protein (5 µM heme), diluted in anaerobic 50 mM TEA, 50 mM NaCl, pH 7.5 buffer, were rapidly mixed with an equal volume of the same buffer (anaerobic) containing various concentrations of dithionite and/or saturating CO gas. Data were acquired on a HI-TECH Scientific SF-61 stopped-flow spectrophotometer equipped with a Neslab RTE-100 constant-temperature bath set to 20 °C (TGK Scientific LTD., Bradford On Avon, United Kingdom). The dissociation of O_2 from the heme was monitored as an increase in the absorbance at 437 nm, a maximum in the Fe^{II} – Fe^{II}-O_2 difference spectrum, or 425 nm, a maximum in the Fe^{II} – Fe^{II}-CO difference spectrum. The final traces were fit to a single exponential using the software that is part of the instrument. Each experiment was done a minimum of six times, and the resulting rates were averaged. The dissociation rates measured are independent of dithionite concentration (100, 50, 25, 10, 5 and 2.5 mM dithionite were tested) and independent of saturating CO as a trap for the reduced species, both with and without 10 mM dithionite present.

Kinetic K_D for O_2

[0162] The kinetic K_D for O_2 is determined by calculating the ratio of k_{off} to k_{on} using the measurements of k_{off} and k_{on} described above.

Calculated K_D

[0163] To measure the calculated K_D, the values for the k_{off} and kinetic K_D that were obtained as described above were graphed. A linear relationship between k_{off} and kinetic K_D was defined by the equation (y=mx+b). k_{off} values were then interpolated along the line to derive the calculated K_D using Excel: MAC 2004 (Microsoft, Redmond, WA). In the absence of a measured k_{on}, this interpolation provides a way to relate k_{off} to K_D.

Rate of Autoxidation

[0164] To measure the rate of autoxidation, the protein samples were anaerobically reduced, then diluted to 5 μM heme in aerobic 50 mM TEA, 50 mM NaCl, pH 7.5 buffer. These samples were then incubated in a Cary 3E spectrophotometer equipped with a Neslab RTE-100 constant-temperature bath set to 37 °C and scanned periodically (Cary 3E, Varian, Inc., Palo Alto, CA). The rate of autoxidation was determined from the difference between the maximum and minimum in the $Fe^{III} - Fe^{II}$ difference spectrum plotted versus time and fit with a single exponential using Excel: MAC 2004 (Microsoft, Redmond, WA).

Rate of reaction with NO

[0165] NO reactivity was measured using purified proteins (*Tt* WT HNOX, *Tt* Y140H HNOX, and *Homo sapiens* hemoglobin (Hs Hb)) prepared at 2 μM in buffer A and NO prepared at 200 μM in Buffer A (Buffer A: 50 mM Hepes, pH 7.5, 50 mM NaCl). Data were acquired on a HI-TECH Scientific SF-61 stopped-flow spectrophotometer equipped with a Neslab RTE-100 constant-temperature bath set to 20 °C (TGK Scientific LTD., Bradford On Avon, United Kingdom). The protein was rapidly mixed with NO in a 1:1 ratio with an integration time of 0.00125 sec. The

wavelengths of maximum change were fit to a single exponential using the software that is part of the spectrometer, essentially measuring the rate-limiting step of oxidation by NO. The end products of the reaction were ferric-NO for the HNOX proteins and ferric-aquo for Hs Hb.

p50 measurements

[0166] If desired, the p50 value for mutant or wild-type H-NOX proteins can be measured as described by Guarnone, R. *et al.* (September/October 1995). "Performance Characteristics of Hemox-Analyzer For Assessment of The Hemoglobin Dissociation Curve," *Haematologica* 80(5):426-430, which is hereby incorporated by reference in its entirety, particularly with respect to the measurement of p50 values. The p50 value is determined using a HemOx analyzer. The measurement chamber starts at 0% oxygen and slowly is raised, incrementally, towards 100% oxygen. An oxygen probe in the chamber measures the oxygen saturation %. A second probe (UV-Vis light) measures two wavelengths of absorption, tuned to the alpha and beta peaks of the hemoprotein's (*e.g.*, a protein such as H-NOX complexed with heme) UV-Vis spectra. These absorption peaks increase linearly as hemoprotein binds oxygen. The percent change from unbound to 100% bound is then plotted against the % oxygen values to generate a curve. The p50 is the point on the curve where 50% of the hemoprotein is bound to oxygen.

[0167] Specifically, the Hemox-Analyzer (TCS Scientific Corporation, New Hope, PA) determines the oxyhemoprotein dissociation curve (ODC) by exposing 50 μL of blood or hemoprotein to an increasing partial pressure of oxygen and deoxygenating it with nitrogen gas. A Clark oxygen electrode detects the change in oxygen tension, which is recorded on the x-axis of an x-y recorder. The resulting increase in oxyhemoprotein fraction is simultaneously monitored by dual-wavelength spectrophotometry at 560 nm and 576 nm and displayed on the y-axis. Blood samples are taken from the antemedial vein, anticoagulated with heparin, and kept at 4°C on wet ice until the assay. Fifty μL of whole blood are diluted in 5 μL of Hemox-solution, a manufacturer-provided buffer that keeps the pH of the solution at a value of 7.4±0.01. The sample-buffer is drawn into a cuvette that is part of the Hemox-Analyzer and the temperature of the mixture is equilibrated and brought to 37°C; the sample is then oxygenated to 100% with air. After adjustment of the pO_2 value the sample is deoxygenated with nitrogen; during the deoxygenation process the curve is

recorded on graph paper. The P50 value is extrapolated on the x-axis as the point at which O_2 saturation is 50% using the software that is part of the Hemox-Analyzer. The time required for a complete recording is approximately 30 minutes.

[0168] The p50 values for any of the H-NOX proteins can be compared to that of hemoglobin as an indication of the relative affinity of the H-NOX protein for O_2 compared to that of hemoglobin. Table 15 lists previously reported p50 values for hemoglobin.

Table 15. Hemoglobin variants and their reported oxygen affinities

Name	Modification	K_D (nM)	p50 (mmHg)	Reference/Manufacturer
Hemoglobin (stroma-free)		~400	14	
Hemoglobin (RBC's)			27	
Hemopure (HBOC-201)	Bovine polymerized		36	Biopure
Oxyglobin (HBOC-301	Bovine polymerized		54	Biopure
Hemospan (MP4)	Maleimide-PEG		5	Sangart
Polyheme	Pyridoxal		28-30	Northfield Labs
Hemolink	0-raffinose		40	Hemosol
Hemassist	Diaspirin		32	Baxter

Viscosity measurements

[0169] If desired, the viscosity of the H-NOX solutions can be measured using a cone/plate rheometer (model DV-III, Brookfield; Middleboro, MA) with the CPE-40 cone spindle at a shear rate of 200/s. Solutions with viscosities between 1 and 4 centipoise (cP) administered in hemodilution oxygen delivery experiments are reported as safe (Winslow, R. M. *et al.* (October 2004). "Comparison of PEG-Modified Albumin And Hemoglobin in Extreme Hemodilution in the Rat," *J Appl Physiol.* 97(4):1527-1534, U.S. Pat. Nos. 6,974,795, and 6,432,918, which are each hereby incorporated by reference in their entireties, particularly with respect to the measurement of viscosity). Accordingly, in some embodiments, the viscosity of the H-NOX protein solution is between 1 and 4 cP.

Colloid oncotic pressure measurements

[0170] If desired, the colloid oncotic pressure can be measured using a colloid osmometer according to the manufacturer's instructions (model 4420, Wescor; Logan, UT). Exemplary methods to measure colloid oncotic pressure are described in Vandegriff, K. D. *et al.* (November 1997). "Colloid Osmotic Properties of Modified Hemoglobins: Chemically Cross-Linked Versus Polyethylene Glycol Surface-Conjugated," *Biophys. Chem.* 69(1):23-30, in the world-wide web at "anaesthesiamcq.com/FluidBook/fl2_4.php;" U.S. Pat. Nos. 6,974,795, and 6,432,918, which are each hereby incorporated by reference in their entireties, particularly with respect to measuring colloid oncotic pressure. Solutions with colloid oncotic pressure between 20 and 50 mm Hg administered in hemodilution oxygen delivery experiments are reported as safe (Winslow, R. M. *et al.* (October 2004). "Comparison of PEG-Modified Albumin And Hemoglobin in Extreme Hemodilution in the Rat," *J. Appl. Physiol.* 97(4):1527-1534). Accordingly, in some embodiments, the colloid oncotic pressure of the H-NOX protein solution is between 20 and 50 mm Hg.

Example 3: Heart disease models for NO carrier H-NOX mutants

[0171] Two animal models can be used to compare the efficacy of H-NOX proteins as NO carriers to standard nitrate therapy. To compare the effects of H-NOX proteins with authentic NO delivery with isosorbide dinitrate (ISDN) in a rat model of chronic cardiovascular disease, an adaptation of an established protocol (Shimamura, S. *et al.* (2006). *J Vet Med Sci Vol.* 68(3):213-7, which is hereby incorporated by reference in its entirety, particularly with respect to models of cardiovascular disease) can be performed using male Wistar rats. To simulate cardiovascular hypertrophy, the abdominal aorta is constricted (abdominal aorta constriction or "AC" model) via ventral abdominal laparotomy and application of a constriction tie over an inserted 21-gauge needle, which is then removed to permit uniform vessel constriction. Sham-operated rats undergo similar surgery, but without creation of AC. After surgery, the rats are randomly divided into treatment groups of 14 animals per group (7 on drug and 7 on placebo), and allowed to recover for 7 days. Treatments per group (control groups are paired within each test case as placebo) are: (1) AC rats administered oral sr-ISDN or placebo; (2) AC rats administered IV one or more wild-type or mutant H-NOX proteins described herein or placebo (*e.g.*, an inactivated H-NOX protein); (3) and (4)

sham- operated rats treated as in (1) or (2), respectively. Treatments are once a day for 12 weeks, after which the animals are sacrificed, and the hearts are excised for standard histopathological analyses for the determination of cardiomyocyte morphology, fibrosis, collage deposition, ventricular diameter, aortic morphology, and other standard analyses for assessing disease progression or prevention.

[0172] To compare the efficacy of H-NOX proteins to that of ISDN in mediating long-term left ventricular remodeling following acute myocardial infarction, a canine model, in which ISDN has shown some efficacy via chronic administration, is performed using a standard protocol (Bodh I. Jugdutt, MBChB, MSc; Mohammad I. Khan, MBBS (1994). *Circulation* 89(S)). For each experiment, forty healthy mongrel dogs (weight, 16 to 29 kg) of either sex are given a left lateral thoracotomy under general anesthesia (sodium pentobarbital, 30 mg/kg IV). Polyethylene catheters are inserted in the external jugular vein, internal carotid artery, and left atrium, filled with heparinized saline, and their ends exteriorized behind the neck. A silk ligature is placed around the mid left anterior descending coronary artery, between the first and second diagonal branches, and tied. Metal beads are sutured on the anterior, lateral, and posterior epicardial surfaces in the short-axis plane at the mid left ventricular level for consistent echocardiographic orientation for serial topography. The pericardium and chest are then closed. Penicillin (1 million units) and streptomycin (1 g) are given intramuscularly, and the dogs are returned to their cages.

[0173] Two days after coronary artery ligation, the 70 healthy survivors are randomized to nitrate therapy (n=10), H-NOX protein therapy (*e.g.*, one or more wild-type or mutant H-NOX proteins that optionally have been characterized *in vitro* using any of the assays described herein and optionally have undergone optimization for toxicity and/or pharmacokinetics) (10), and matching control subgroups (no treatment, n=20): subgroup 1 (10 control, 10 nitrate), and subgroup 2 (10 control, 10 H-NOX protein). The dogs are allowed free access to fluids, and no attempt is made to treat heart failure by fluid restriction or pharmacotherapy. At six weeks, the surviving dogs are anesthetized, and the hearts are arrested in diastole with an overdose of intravenous potassium chloride, excised, washed in normal saline solution, and weighed. Blood samples are taken for monitoring blood gases, hemograms, and electrolytes. Using standard procedures, the measurements during healing are made (such as ECG's, hemodynamics, *etc.*), and post-mortem

analyses include those measures described above for chronic heart failure (*e.g.*, collagen accumulation, myocyte morphology, *etc.*). The H-NOX proteins that are as effective or more effective than ISDN (the standard of care for nitrate-based therapies for acute and chronic heart failure) in the myocardial infarction and/or the chronic AC model experiments are particularly useful for the treatment of myocardial infarction and/or chronic AC. Such H-NOX proteins are expected to also be useful to treat other indications for which delivery of NO is beneficial.

[0174] The foregoing examples and detailed description are offered by way of illustration and not by way of limitation. All publications, patent applications, and patents cited in this specification are herein incorporated by reference as if each individual publication, patent application, or patent were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

[0175] Unless defined otherwise, the meanings of all technical and scientific terms used herein are those commonly understood by one of skill in the art to which this invention belongs. One of skill in the art will also appreciate that any methods and materials similar or equivalent to those described herein can also be used to practice or test the invention.

[0176] For use herein, unless clearly indicated otherwise, use of the terms "a", "an," and the like refers to one or more.

[0177] Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se. For example, description referring to "about X" includes description of "X."

[0178] It is understood that aspect and embodiments of the invention described herein include "comprising," "consisting," and "consisting essentially of" aspects and embodiments.

CLAIMS

What is claimed as new and desired to be protected by Letters Patent of the United States is:

1. A pharmaceutical composition comprising (i) a pharmaceutically acceptable amount of an H-NOX protein, wherein the NO dissociation constant of the H-NOX protein is within 2 orders of magnitude of that of hemoglobin, and wherein the NO reactivity of the H-NOX protein is at least 10-fold lower than that of hemoglobin, and (ii) a pharmaceutically acceptable carrier.

2. The pharmaceutical composition of claim 1, wherein the NO dissociation constant of the H-NOX protein is between 0.1 to 10-fold of that of hemoglobin.

3. The pharmaceutical composition of claim 2, wherein the NO dissociation constant of the H-NOX protein is between 0.5 to 2-fold of that of hemoglobin.

4. The pharmaceutical composition of any one of claims 1-3, wherein the NO reactivity of the H-NOX protein is at least 100-fold lower than that of hemoglobin.

5. The pharmaceutical composition of claim 4, wherein the NO reactivity of the H-NOX protein is at least 1,000-fold lower than that of hemoglobin.

6. The pharmaceutical composition of any one of claims 1-5, wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

7. The pharmaceutical composition of any one of claims 1-6, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C.

8. The pharmaceutical composition of claim 7, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 0.012 s^{-1} at 37 °C.

9. The pharmaceutical composition of claim 8, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 1 x $10^{-3}s^{-1}$ at 37 °C.

10. The pharmaceutical composition of any one of claims 1-9, wherein the O_2 dissociation constant of the H-NOX protein it at least about 1 μM at 37 °C.

11. The pharmaceutical composition of claim 10, wherein the O_2 dissociation constant of the H-NOX protein it at least about 10 μM at 37 °C.

12. The pharmaceutical composition of claim 11, wherein the O_2 dissociation constant of the H-NOX protein it at least about 50 μM at 37 °C.

13. The pharmaceutical composition of any one of claims 1-12, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C.

14. The pharmaceutical composition of any one of claims 1-13, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

15. The pharmaceutical composition of any one of claims 1-14, wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

16. The pharmaceutical composition of any one of claims 1-15, wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

17. The pharmaceutical composition of any one of claims 1-16, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

18. The pharmaceutical composition of any one of claims 1-17, wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

19. The pharmaceutical composition of any one of claims 1-18, wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

20. The pharmaceutical composition of any one of claims 1-19, wherein heme is bound to the H-NOX protein.

21. The pharmaceutical composition of any one of claims 1-20, wherein the H-NOX protein is a mutant protein.

22. The pharmaceutical composition of claim 21, wherein the H-NOX protein comprises at least one distal pocket mutation.

23. The pharmaceutical composition of any one of claims 20 and 21, wherein the H-NOX protein comprises at least one mutation that is not in the distal pocket.

24. The pharmaceutical composition of any one of claims 1-23, wherein the H-NOX protein comprises at least one mutation that alters the NO dissociation constant, the k_{off} for NO, the k_1 for NO, the k_2 for NO, the O_2 dissociation constant, the NO stability, the rate of heme autoxidation, or any combination of two or more of the foregoing compared to that of a corresponding wild-type protein.

25. The pharmaceutical composition of any one of claims 1-24, wherein the H-NOX protein comprises at least one mutation that alters the NO reactivity compared to that of the corresponding wild-type protein.

26. The pharmaceutical composition of any one of claims 1-25, wherein the H-NOX protein comprises at least one mutation in which a residue that corresponds to Tyr140 of *T. tengcongensis* H-NOX is replaced by any other amino acid.

27. The pharmaceutical composition of any one of claims 1-26, wherein the H-NOX protein comprises at least one mutation in which a residue that corresponds to Phe148 of *L. pneumophilia* 8 H-NOX is replaced by any other amino acid.

28. The pharmaceutical composition of any one of claims 1-27, wherein at least one C-terminal amino acid in the mutant H-NOX protein has been removed compared to a corresponding wild-type protein.

29. The pharmaceutical composition of claim 28, wherein at least about 50 contiguous C-terminal amino acids in the mutant H-NOX protein have been removed compared to the corresponding wild-type protein.

30. The pharmaceutical composition of any one of claims 28 and 29, wherein between about 25 to about 200 contiguous C-terminal amino acids in the mutant H-NOX protein have been removed compared to the corresponding wild-type protein.

31. The pharmaceutical composition of any one of claims 1-30, wherein the H-NOX protein is a selected from the group consisting of wild-type *T. tengcongensis* H-NOX, *T. tengcongensis* H-NOX I5A, *T. tengcongensis* H-NOX I5L, *T. tengcongensis* H-NOX I5L-P115A, *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX W9F-Y140L, *T. tengcongensis* H-NOX W9F-Y140H*T. tengcongensis* H-NOX W9F-N74A, *T. tengcongensis* H-NOX W9Y, *T. tengcongensis* H-NOX W9N, *T. tengcongensis* H-NOX W9H, *T. tengcongensis* H-NOX N74E, *T. tengcongensis* H-NOX N74A, *T. tengcongensis* H-NOX N74H, *T. tengcongensis* H-NOX N74A-Y140H, *T. tengcongensis* H-NOX F78Y-Y140F, *T. tengcongensis* H-NOX F78Y/Y140L, *T. tengcongensis* H-NOX P115A, *T. tengcongensis* H-NOX R135Q, *T. tengcongensis* H-NOX Y140F, *T. tengcongensis* H-NOX Y40L, *T. tengcongensis* H-NOX Y140H, *T. tengcongensis* H-NOX Y140A, *T. tengcongensis* I75F-His6, *T. tengcongensis* I75F, *T. tengcongensis* L144F-His6, *T. tengcongensis* L144F, *L. pneumophilia* 2 H-NOX F142Y, wild-type *L. pneumophilia* 1 H-NOX, wild-type *L. pneumophilia* 2 H-NOX, *L. pneumophilia 2* F9W-F142Y, wild-type *D. desulfuricans* H-NOX, *D. desulfuricans* H-NOX(728-899), *D. desulfuricans* H-NOX Y139L, wild-type *H. sapiens* β1 H-NOX, *H. sapiens* β1 I140Y, *H. sapiens* β1 I145Y, *H. sapiens* β1(1-385), *H. sapiens* β1(1-385) I145Y, *H. sapiens* β1(1-385) I145H, *H. sapiens* β1(1-194), *H. sapiens* β1(1-194) I145Y, *H. sapiens* β1(1-194) L9W-I145Y, *H. sapiens* β2(1-217), *H. sapiens* β2(1-217) I142Y, *H. sapiens* β1 H-NOX H105G, *H. sapiens* β1 H-NOX H105F, *H. sapiens* β1 H-NOX C78S, *H. sapiens* β1 H-NOX C78E, wild-type *R. norvegicus* β1 H-NOX, *R. norvegicus* β1(1-385), *R. norvegicus* β1(1-385) I145Y, *R. norvegicus* β1(1-385) I145H, *R. norvegicus* β1(1-194), *R. norvegicus* β1(1-194) I145Y, *R. norvegicus* β1(1-194) L9W-I145Y, *R. norvegicus* β2(1-217), *R. norvegicus* β2(1-217) I142Y, *R. norvegicus* β1 H-NOX H105G, *R. norvegicus* β1 H-NOX H105F, *R. norvegicus* sGC β1 H-NOX C78S, *R. norvegicus* sGC β1 H-NOX C78E, *C. botulinum* H-NOX(1-175), *C. botulinum* H-NOX(1-186), wild-type *C. acetobutylicum* H-NOX, *C. acetobutylicum* H-NOX(1-197), *C. acetobutylicum* H-NOX(1-183), wild-type *C. elegans* GCY-35 H-NOX, *C. elegans* H-NOX GCY-35(1-252), wild-type *D. melangaster* β1 H-NOX, wild-type *D. melangaster* CG14885-PA, wild-type *D. melangaster* CG14886, wild-type *D. melangaster* CG4154; wild-type *N. punctiforme* H-NOX, wild-type *C. crescentus* H-NOX, wild-type *S.*

oneidensis H-NOX, wild-type *M. musculus* H-NOX , wild-type *C. familiaris* H-NOX, wild-type *B. Taurus* H-NOX, wild-type *R. norvegicus;* wild-type *X. laevis* H-NOX, wild-type *O. latipes* H-NOX, wild-type *O. curivatus* H-NOX, wild-type *F. rubripes* H-NOX, wild-type *A. gambiae* H-NOX, wild-type *M. sexta* H-NOX; wild-type *C. elegans* gcy-31, *C. elegans* gcy-32, wild-type *C. elegans* gcy-33, wild-type *C. elegans* gcy-34, wild-type *C. elegans* gcy-35, wild-type *C. elegans* gcy-36, wild-type *C. elegans* gcy-37; wild-type *V. cholera* H-NOX, wild-type *V. fischerii* H-NOX, and wild-type *N. punctiforme* H-NOX.

32. The pharmaceutical composition of claim 31, wherein the H-NOX protein is a selected from the group consisting of wild-type *R. norvegicus* sGC, wild-type *R. norvegicus* β1(1-385), *R. norvegicus* β1(1-217), *R. norvegicus* β1(1-194), wild-type *T. tengcongensis* H-NOX, *T. tengcongensis* H-NOX Y140L, *T. tengcongensis* H-NOX Y140F, wild-type *L. pneumophilia* 1 H-NOX, wild-type *L. pneumophilia* 2 H-NOX, and *L. pneumophilia* 2 H-NOX F142Y.

33. The pharmaceutical composition of any one of claims 1-32, wherein the H-NOX protein is a mammalian protein or is derived from a mammalian protein.

34. The pharmaceutical composition of claim 33, wherein the H-NOX protein is a human protein or is derived from a human protein.

35. The pharmaceutical composition of claim 34, wherein the human protein is β1 or is derived from β1.

36. The pharmaceutical composition of any one of claims 1-32, wherein the H-NOX protein is a bacterial protein or is derived from a bacterial protein.

37. The pharmaceutical composition of claim 36, wherein the H-NOX protein is a *T. tengcongensis* protein or is derived from a *T. tengcongensis* protein.

38. The pharmaceutical composition of any one of claims 1-37, wherein NO is bound to the H-NOX protein.

39. The pharmaceutical composition of any one of claims 1-38, comprising one or more liposomes or nanoparticles that comprise the H-NOX protein.

40. The pharmaceutical composition of any one of claims 1-38, wherein the H-NOX protein is covalently bound to another molecule or moiety.

41. The pharmaceutical composition of claim 40, wherein the H-NOX protein is covalently bound to polyethylene glycol.

42. The pharmaceutical composition of any one of claims 1-41, wherein the H-NOX protein is a mutant H-NOX protein of any one of claims 245-314.

43. A pharmaceutical composition comprising (i) a pharmaceutically acceptable amount of an H-NOX protein, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and (ii) a pharmaceutically acceptable carrier.

44. The pharmaceutical composition of any one of claims 43, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 0.012 s^{-1} at 37 °C.

45. The pharmaceutical composition of claim 44, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 1 x $10^{-3}s^{-1}$ at 37 °C.

46. The pharmaceutical composition of any one of claims 43-45, wherein the O_2 dissociation constant of the H-NOX protein it at least about 10 μM at 37 °C.

47. The pharmaceutical composition of claim 46, wherein the O_2 dissociation constant of the H-NOX protein it at least about 50 μM at 37 °C.

48. The pharmaceutical composition of any one of claims 43-47, wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

49. The pharmaceutical composition of any one of claims 43-48, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C.

50. The pharmaceutical composition of any one of claims 43-49, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

51. The pharmaceutical composition of any one of claims 43-50, wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

52. The pharmaceutical composition of any one of claims 43-51, wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

53. The pharmaceutical composition of any one of claims 43-52, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

54. The pharmaceutical composition of any one of claims 43-53, wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

55. The pharmaceutical composition of any one of claims 43-54, wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

56. The pharmaceutical composition of any one of claims 43-55, wherein heme is bound to the H-NOX protein.

57. The pharmaceutical composition of any one of claims 43-56, wherein the H-NOX protein is a mutant protein.

58. The pharmaceutical composition of claim 57, wherein the H-NOX protein comprises at least one distal pocket mutation.

59. The pharmaceutical composition of any one of claims 57 and 58, wherein the H-NOX protein comprises at least one mutation that is not in the distal pocket.

60. The pharmaceutical composition of any one of claims 43-59, wherein the H-NOX protein comprises at least one mutation that alters the NO dissociation constant, the k_{off} for NO, the k_1 for NO, the k_2 for NO, the O_2 dissociation constant, the NO stability, the rate of heme autoxidation, or any combination of two or more of the foregoing compared to that of a corresponding wild-type protein.

61. The pharmaceutical composition of any one of claims 43-60, wherein the H-NOX protein comprises at least one mutation that alters the NO reactivity compared to that of the corresponding wild-type protein.

62. The pharmaceutical composition of any one of claims 43-61, wherein the H-NOX protein comprises at least one mutation in which a residue that corresponds to Tyr140 of *T. tengcongensis* H-NOX is replaced by any other amino acid.

63. The pharmaceutical composition of any one of claims 43-62, wherein the H-NOX protein comprises at least one mutation in which a residue that corresponds to Phe148 of *L. pneumophilia* 8 H-NOX is replaced by any other amino acid.

64. The pharmaceutical composition of any one of claims 43-63, wherein at least one C-terminal amino acid in the mutant H-NOX protein has been removed compared to a corresponding wild-type protein.

65. The pharmaceutical composition of claim 64, wherein at least about 50 contiguous C-terminal amino acids in the mutant H-NOX protein have been removed compared to the corresponding wild-type protein.

66. The pharmaceutical composition of any one of claims 64 and 65, wherein between about 25 to about 200 contiguous C-terminal amino acids in the mutant H-NOX protein have been removed compared to the corresponding wild-type protein.

67. The pharmaceutical composition of any one of claims 43-66, wherein the H-NOX protein is a selected from the group consisting of wild-type *T. tengcongensis* H-NOX, *T. tengcongensis* H-NOX I5A, *T. tengcongensis* H-NOX I5L, *T. tengcongensis* H-NOX I5L-P115A, *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX W9F-Y140L, *T. tengcongensis* H-NOX W9F-Y140H*T. tengcongensis* H-NOX W9F-N74A, *T. tengcongensis* H-NOX W9Y, *T. tengcongensis* H-NOX W9N, *T. tengcongensis* H-NOX W9H, *T. tengcongensis* H-NOX N74E, *T. tengcongensis* H-NOX N74A, *T. tengcongensis* H-NOX N74H, *T. tengcongensis* H-NOX N74A-Y140H, *T. tengcongensis* H-NOX F78Y-Y140F, *T. tengcongensis* H-NOX F78Y/Y140L, *T. tengcongensis* H-NOX P115A, *T. tengcongensis* H-NOX R135Q, *T. tengcongensis* H-NOX Y140F, *T. tengcongensis* H-NOX Y40L, *T. tengcongensis* H-NOX Y140H, *T. tengcongensis* H-NOX

Y140A, *T. tengcongensis* I75F-His6, *T. tengcongensis* I75F, *T. tengcongensis* L144F-His6, *T. tengcongensis* L144F, *L. pneumophilia* 2 H-NOX F142Y, wild-type *L. pneumophilia* 1 H-NOX, wild-type *L. pneumophilia* 2 H-NOX, *L. pneumophilia 2* F9W-F142Y, wild-type *D. desulfuricans* H-NOX, *D. desulfuricans* H-NOX(728-899), *D. desulfuricans* H-NOX Y139L, wild-type *H. sapiens* β1 H-NOX, *H. sapiens* β1 I140Y, *H. sapiens* β1 I145Y, *H. sapiens* β1(1-385), *H. sapiens* β1(1-385) I145Y, *H. sapiens* β1(1-385) I145H, *H. sapiens* β1(1-194), *H. sapiens* β1(1-194) I145Y, *H. sapiens* β1(1-194) L9W-I145Y, *H. sapiens* β2(1-217), *H. sapiens* β2(1-217) I142Y, *H. sapiens* β1 H-NOX H105G, *H. sapiens* β1 H-NOX H105F, *H. sapiens* β1 H-NOX C78S, *H. sapiens* β1 H-NOX C78E, wild-type *R. norvegicus* β1 H-NOX, *R. norvegicus* β1(1-385), *R. norvegicus* β1(1-385) I145Y, *R. norvegicus* β1(1-385) I145H, *R. norvegicus* β1(1-194), *R. norvegicus* β1(1-194) I145Y, *R. norvegicus* β1(1-194) L9W-I145Y, *R. norvegicus* β2(1-217), *R. norvegicus* β2(1-217) I142Y, *R. norvegicus* β1 H-NOX H105G, *R. norvegicus* β1 H-NOX H105F, *R. norvegicus* sGC β1 H-NOX C78S, *R. norvegicus* sGC β1 H-NOX C78E, *C. botulinum* H-NOX(1-175), *C. botulinum* H-NOX(1-186), wild-type *C. acetobutylicum* H-NOX, *C. acetobutylicum* H-NOX(1-197), *C. acetobutylicum* H-NOX(1-183), wild-type *C. elegans* GCY-35 H-NOX, *C. elegans* H-NOX GCY-35(1-252), wild-type *D. melangaster* β1 H-NOX, wild-type *D. melangaster* CG14885-PA, wild-type *D. melangaster* CG14886, wild-type *D. melangaster* CG4154; wild-type *N. punctiforme* H-NOX, wild-type *C. crescentus* H-NOX, wild-type *S. oneidensis* H-NOX, wild-type *M. musculus* H-NOX , wild-type *C. familiaris* H-NOX, wild-type *B. Taurus* H-NOX, wild-type *R. norvegicus;* wild-type *X. laevis* H-NOX, wild-type *O. latipes* H-NOX, wild-type *O. curivatus* H-NOX, wild-type *F. rubripes* H-NOX, wild-type *A. gambiae* H-NOX, wild-type *M. sexta* H-NOX; wild-type *C. elegans* gcy-31, *C. elegans* gcy-32, wild-type *C. elegans* gcy-33, wild-type *C. elegans* gcy-34, wild-type *C. elegans* gcy-35, wild-type *C. elegans* gcy-36, wild-type *C. elegans* gcy-37; wild-type *V. cholera* H-NOX, wild-type *V. fischerii* H-NOX, and wild-type *N. punctiforme* H-NOX.

68. The pharmaceutical composition of claim 67, wherein the H-NOX protein is a selected from the group consisting of wild-type *R. norvegicus* sGC, wild-type *R. norvegicus* β1(1-385), *R. norvegicus* β1(1-217), *R. norvegicus* β1(1-194), wild-type *T. tengcongensis* H-NOX, *T. tengcongensis* H-NOX Y140L, *T. tengcongensis* H-NOX Y140F, wild-type *L. pneumophilia* 1 H-NOX, wild-type *L. pneumophilia* 2 H-NOX, and *L. pneumophilia* 2 H-NOX F142Y.

69. The pharmaceutical composition of any one of claims 43-68, wherein the H-NOX protein is a mammalian protein or is derived from a mammalian protein.

70. The pharmaceutical composition of claim 69, wherein the H-NOX protein is a human protein or is derived from a human protein.

71. The pharmaceutical composition of claim 70, wherein the human protein is β1 or is derived from β1.

72. The pharmaceutical composition of any one of claims 43-68, wherein the H-NOX protein is a bacterial protein or is derived from a bacterial protein.

73. The pharmaceutical composition of claim 72, wherein the H-NOX protein is a *T. tengcongensis* protein or is derived from a *T. tengcongensis* protein.

74. The pharmaceutical composition of any one of claims 43-73, wherein NO is bound to the H-NOX protein.

75. The pharmaceutical composition of any one of claims 43-74, comprising one or more liposomes or nanoparticles that comprise the H-NOX protein.

76. The pharmaceutical composition of any one of claims 43-75, wherein the H-NOX protein is covalently bound to another molecule or moiety.

77. The pharmaceutical composition of claim 76, wherein the H-NOX protein is covalently bound to polyethylene glycol.

78. The pharmaceutical composition of any one of claims 43-77, wherein the H-NOX protein is a mutant H-NOX protein of any one of claims 245-314.

79. A method of delivering NO to an individual comprising administering to an individual in need thereof an H-NOX protein in an amount sufficient to deliver an effective amount of NO to the individual, wherein the NO dissociation constant of the H-NOX protein is within 2 orders of magnitude of that of hemoglobin, and wherein the NO reactivity of the H-NOX protein is at least 10-fold lower than that of hemoglobin.

80. The method of claim 79, wherein the NO dissociation constant of the H-NOX protein is between 0.1 to 10-fold of that of hemoglobin.

81. The method of claim 80, wherein the NO dissociation constant of the H-NOX protein is between 0.5 to 2-fold of that of hemoglobin.

82. The method of any one of claims 79-81, wherein the NO reactivity of the H-NOX protein is at least 100-fold lower than that of hemoglobin.

83. The method of claim 82, wherein the NO reactivity of the H-NOX protein is at least 1,000-fold lower than that of hemoglobin.

84. The method of any one of claims 79-83, wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

85. The method of any one of claims 79-84, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C.

86. The method of claim 85, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 0.012 s^{-1} at 37 °C.

87. The method of claim 86, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 1 x $10^{-3}s^{-1}$ at 37 °C.

88. The method of any one of claims 79-87, wherein the O_2 dissociation constant of the H-NOX protein it at least about 1 μM at 37 °C.

89. The method of claim 88, wherein the O_2 dissociation constant of the H-NOX protein it at least about 10 μM at 37 °C.

90. The method of claim 89, wherein the O_2 dissociation constant of the H-NOX protein it at least about 50 μM at 37 °C.

91. The method of any one of claims 79-90, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C.

92. The method of any one of claims 79-91, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

93. The method of any one of claims 79-92, wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

94. The method of any one of claims 79-93, wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

95. The method of any one of claims 79-94, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

96. The method of any one of claims 79-95, wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

97. The method of any one of claims 79-96, wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

98. The method of any one of claims 79-97, wherein heme is bound to the H-NOX protein.

99. The method of any one of claims 79-98, wherein the H-NOX protein is a mutant protein.

100. The method of claim 99, wherein the H-NOX protein comprises at least one distal pocket mutation.

101. The method of any one of claims 99 and 100, wherein the H-NOX protein comprises at least one mutation that is not in the distal pocket.

102. The method of any one of claims 79-101, wherein the H-NOX protein comprises at least one mutation that alters the NO dissociation constant, the k_{off} for NO, the k_1 for NO, the k_2 for NO, the O_2 dissociation constant, the NO stability, the rate of heme autoxidation, or any combination of two or more of the foregoing compared to that of a corresponding wild-type protein.

103. The method of any one of claims 79-102, wherein the H-NOX protein comprises at least one mutation that alters the NO reactivity compared to that of the corresponding wild-type protein.

104. The method of any one of claims 79-103, wherein the H-NOX protein comprises at least one mutation in which a residue that corresponds to Tyr140 of *T. tengcongensis* H-NOX is replaced by any other amino acid.

105. The method of any one of claims 79-104, wherein the H-NOX protein comprises at least one mutation in which a residue that corresponds to Phe1410 of *L. pneumophilia* 10 H-NOX is replaced by any other amino acid.

106. The method of any one of claims 79-105, wherein at least one C-terminal amino acid in the mutant H-NOX protein has been removed compared to a corresponding wild-type protein.

107. The method of claim 106, wherein at least about 50 contiguous C-terminal amino acids in the mutant H-NOX protein have been removed compared to the corresponding wild-type protein.

108. The method of any one of claims 106 and 107, wherein between about 25 to about 200 C-terminal amino acids in the mutant H-NOX protein have been removed compared to the corresponding wild-type protein.

109. The method of any one of claims 79-108, wherein the H-NOX protein is a selected from the group consisting of wild-type *T. tengcongensis* H-NOX, *T. tengcongensis* H-NOX I5A, *T. tengcongensis* H-NOX I5L, *T. tengcongensis* H-NOX I5L-P115A, *T. tengcongensis* H-NOX W10F, *T. tengcongensis* H-NOX W10F-Y140L, *T. tengcongensis* H-NOX W10F-Y140H*T. tengcongensis* H-NOX W10F-N74A, *T. tengcongensis* H-NOX W10Y, *T. tengcongensis* H-NOX W10N, *T. tengcongensis* H-NOX W10H, *T. tengcongensis* H-NOX N74E, *T. tengcongensis* H-NOX N74A, *T. tengcongensis* H-NOX N74H, *T. tengcongensis* H-NOX N74A-Y140H, *T. tengcongensis* H-NOX F710Y-Y140F, *T. tengcongensis* H-NOX F710Y/Y140L, *T. tengcongensis* H-NOX P115A, *T. tengcongensis* H-NOX R135Q, *T. tengcongensis* H-NOX Y140F, *T. tengcongensis* H-NOX Y40L, *T. tengcongensis* H-NOX Y140H, *T. tengcongensis* H-NOX Y140A, *T. tengcongensis* I75F-His6, *T. tengcongensis* I75F, *T. tengcongensis* L144F-His6, *T. tengcongensis* L144F, *L. pneumophilia* 2 H-NOX F142Y, wild-type *L. pneumophilia* 1 H-NOX, wild-type *L. pneumophilia* 2 H-NOX, *L.*

pneumophilia 2 F10W-F142Y, wild-type *D. desulfuricans* H-NOX, *D. desulfuricans* H-NOX(7210-101010), *D. desulfuricans* H-NOX Y1310L, wild-type *H. sapiens* β1 H-NOX, *H. sapiens* β1 I140Y, *H. sapiens* β1 I145Y, *H. sapiens* β1(1-3105), *H. sapiens* β1(1-3105) I145Y, *H. sapiens* β1(1-3105) I145H, *H. sapiens* β1(1-1104), *H. sapiens* β1(1-1104) I145Y, *H. sapiens* β1(1-1104) L10W-I145Y, *H. sapiens* β2(1-217), *H. sapiens* β2(1-217) I142Y, *H. sapiens* β1 H-NOX H105G, *H. sapiens* β1 H-NOX H105F, *H. sapiens* β1 H-NOX C710S, *H. sapiens* β1 H-NOX C710E, wild-type *R. norvegicus* β1 H-NOX, *R. norvegicus* β1(1-3105), *R. norvegicus* β1(1-3105) I145Y, *R. norvegicus* β1(1-3105) I145H, *R. norvegicus* β1(1-1104), *R. norvegicus* β1(1-1104) I145Y, *R. norvegicus* β1(1-1104) L10W-I145Y, *R. norvegicus* β2(1-217), *R. norvegicus* β2(1-217) I142Y, *R. norvegicus* β1 H-NOX H105G, *R. norvegicus* β1 H-NOX H105F, *R. norvegicus* sGC β1 H-NOX C710S, *R. norvegicus* sGC β1 H-NOX C710E, *C. botulinum* H-NOX(1-175), *C. botulinum* H-NOX(1-1106), wild-type *C. acetobutylicum* H-NOX, *C. acetobutylicum* H-NOX(1-1107), *C. acetobutylicum* H-NOX(1-1103), wild-type *C. elegans* GCY-35 H-NOX, *C. elegans* H-NOX GCY-35(1-252), wild-type *D. melangaster* β1 H-NOX, wild-type *D. melangaster* CG1410105-PA, wild-type *D. melanogaster* CG1410105, wild-type *D. melangaster* CG1410106, wild-type *D. melangaster* CG4154; wild-type *N. punctiforme* H-NOX, wild-type *C. crescentus* H-NOX, wild-type *S. oneidensis* H-NOX, wild-type *M. musculus* H-NOX , wild-type *C. familiaris* H-NOX, wild-type *B. Taurus* H-NOX, wild-type *R. norvegicus;* wild-type *X. laevis* H-NOX, wild-type *O. latipes* H-NOX, wild-type *O. curivatus* H-NOX, wild-type *F. rubripes* H-NOX, wild-type *A. gambiae* H-NOX, wild-type *M. sexta* H-NOX; wild-type *C. elegans* gcy-31, *C. elegans* gcy-32, wild-type *C. elegans* gcy-33, wild-type *C. elegans* gcy-34, wild-type *C. elegans* gcy-35, wild-type *C. elegans* gcy-36, wild-type *C. elegans* gcy-37; wild-type *V. cholera* H-NOX, wild-type *V. fischerii* H-NOX, and wild-type *N. punctiforme* H-NOX.

110. The method of claim 109, wherein the H-NOX protein is a selected from the group consisting of wild-type *R. norvegicus* sGC, wild-type *R. norvegicus* β1(1-3105), *R. norvegicus* β1(1-217), *R. norvegicus* β1(1-1104), wild-type *T. tengcongensis* H-NOX, *T. tengcongensis* H-NOX Y140L, *T. tengcongensis* H-NOX Y140F, wild-type *L. pneumophilia* 1 H-NOX, wild-type *L. pneumophilia* 2 H-NOX, and *L. pneumophilia* 2 H-NOX F142Y.

111. The method of any one of claims 79-110, wherein the H-NOX protein is a mammalian protein or is derived from a mammalian protein.

112. The method of claim 111, wherein the H-NOX protein is a human protein or is derived from a human protein.

113. The method of claim 112, wherein the human protein is β1 or is derived from β1.

114. The method of any one of claims 79-110, wherein the H-NOX protein is a bacterial protein or is derived from a bacterial protein.

115. The method of claim 114, wherein the H-NOX protein is a *T. tengcongensis* protein or is derived from a *T. tengcongensis* protein.

116. The method of any one of claims 79-115, comprising one or more liposomes or nanoparticles that comprise the H-NOX protein.

117. The method of any one of claims 79-116, wherein the H-NOX protein is covalently bound to another molecule or moiety.

118. The method of claim 117, wherein the H-NOX protein is covalently bound to polyethylene glycol.

119. The method of any one of claims 79-118, wherein NO is bound to the H-NOX protein prior to the administration of the H-NOX protein to the individual.

120. The method of any one of claims 79-118, wherein NO is not bound to the H-NOX protein prior to the administration of the H-NOX protein to the individual, and wherein the H-NOX protein transports NO from one location in the individual to another location in the individual.

121. The method of any one of claims 79-120, wherein the H-NOX protein is administered orally, rectally, or to the blood of the individual.

122. The method of any one of claims 79-121, wherein the individual is a human.

123. The method of any one of claims 79-122, wherein the individual is suffering from or at risk for a cardiovascular condition, hypertension, a condition exacerbated by hypertension, or a functional NO deficiency.

124. The method of claim 123, wherein the condition exacerbated by hypertension is heart failure, renal failure, or a stroke.

125. The method of any one of claims 79-124, wherein the H-NOX protein is administered to the individual at least twice.

126. The method of any one of claims 79-125, wherein the H-NOX protein is a mutant H-NOX protein of any one of claims 245-314.

127. A method of delivering NO to an individual comprising administering to an individual in need thereof an H-NOX protein in an amount sufficient to deliver an effective amount of NO to the individual, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C.

128. The method of claim 127, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 0.012 s^{-1} at 37 °C.

129. The method of claim 128, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 1 x $10^{-3}s^{-1}$ at 37 °C.

130. The method of any one of claims 127-129, wherein the O_2 dissociation constant of the H-NOX protein it at least about 10 μM at 37 °C.

131. The method of claim 130, wherein the O_2 dissociation constant of the H-NOX protein it at least about 50 μM at 37 °C.

132. The method of any one of claims 127-131, wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

133. The method of any one of claims 127-132, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C.

134. The method of any one of claims 127-133, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

135. The method of any one of claims 127-134, wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

136. The method of any one of claims 127-135, wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

137. The method of any one of claims 127-136, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

138. The method of any one of claims 127-137, wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

139. The method of any one of claims 127-138, wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

140. The method of any one of claims 127-139, wherein heme is bound to the H-NOX protein.

141. The method of any one of claims 127-140, wherein the H-NOX protein is a mutant protein.

142. The method of claim 141, wherein the H-NOX protein comprises at least one distal pocket mutation.

143. The method of any one of claims 141 and 142, wherein the H-NOX protein comprises at least one mutation that is not in the distal pocket.

144. The method of any one of claims 127-143, wherein the H-NOX protein comprises at least one mutation that alters the NO dissociation constant, the k_{off} for NO, the k_1 for NO, the k_2 for NO, the O_2 dissociation constant, the NO stability, the rate of heme autoxidation, or any combination of two or more of the foregoing compared to that of a corresponding wild-type protein.

145. The method of any one of claims 127-144, wherein the H-NOX protein comprises at least one mutation that alters the NO reactivity compared to that of the corresponding wild-type protein.

146. The method of any one of claims 127-145, wherein the H-NOX protein comprises at least one mutation in which a residue that corresponds to Tyr140 of *T. tengcongensis* H-NOX is replaced by any other amino acid.

147. The method of any one of claims 127-146, wherein the H-NOX protein comprises at least one mutation in which a residue that corresponds to Phe1410 of *L. pneumophilia* 10 H-NOX is replaced by any other amino acid.

148. The method of any one of claims 127-147, wherein at least one C-terminal amino acid in the mutant H-NOX protein has been removed compared to a corresponding wild-type protein.

149. The method of claim 148, wherein at least about 50 contiguous C-terminal amino acids in the mutant H-NOX protein have been removed compared to the corresponding wild-type protein.

150. The method of any one of claims 148 and 149, wherein between about 25 to about 200 contiguous C-terminal amino acids in the mutant H-NOX protein have been removed compared to the corresponding wild-type protein.

151. The method of any one of claims 127-150, wherein the H-NOX protein is a selected from the group consisting of wild-type *T. tengcongensis* H-NOX, *T. tengcongensis* H-NOX I5A, *T. tengcongensis* H-NOX I5L, *T. tengcongensis* H-NOX I5L-P115A, *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX W9F-Y140L, *T. tengcongensis* H-NOX W9F-Y140H*T. tengcongensis* H-NOX W9F-N74A, *T. tengcongensis* H-NOX W9Y, *T. tengcongensis* H-NOX W9N, *T. tengcongensis* H-NOX W9H, *T. tengcongensis* H-NOX N74E, *T. tengcongensis* H-NOX N74A, *T. tengcongensis* H-NOX N74H, *T. tengcongensis* H-NOX N74A-Y140H, *T. tengcongensis* H-NOX F78Y-Y140F, *T. tengcongensis* H-NOX F78Y/Y140L, *T. tengcongensis* H-NOX P115A, *T. tengcongensis* H-NOX R135Q, *T. tengcongensis* H-NOX Y140F, *T. tengcongensis* H-NOX Y40L, *T. tengcongensis* H-NOX Y140H, *T. tengcongensis* H-NOX Y140A, *T. tengcongensis* I75F-His6, *T. tengcongensis* I75F, *T. tengcongensis* L144F-His6, *T. tengcongensis* L144F, *L. pneumophilia* 2 H-NOX F142Y, wild-type *L. pneumophilia* 1 H-NOX, wild-type *L. pneumophilia* 2 H-NOX, *L.*

pneumophilia 2 F9W-F142Y, wild-type *D. desulfuricans* H-NOX, *D. desulfuricans* H-NOX(728-899), *D. desulfuricans* H-NOX Y139L, wild-type *H. sapiens* β1 H-NOX, *H. sapiens* β1 I140Y, *H. sapiens* β1 I145Y, *H. sapiens* β1(1-385), *H. sapiens* β1(1-385) I145Y, *H. sapiens* β1(1-385) I145H, *H. sapiens* β1(1-194), *H. sapiens* β1(1-194) I145Y, *H. sapiens* β1(1-194) L9W-I145Y, *H. sapiens* β2(1-217), *H. sapiens* β2(1-217) I142Y, *H. sapiens* β1 H-NOX H105G, *H. sapiens* β1 H-NOX H105F, *H. sapiens* β1 H-NOX C78S, *H. sapiens* β1 H-NOX C78E, wild-type *R. norvegicus* β1 H-NOX, *R. norvegicus* β1(1-385), *R. norvegicus* β1(1-385) I145Y, *R. norvegicus* β1(1-385) I145H, *R. norvegicus* β1(1-194), *R. norvegicus* β1(1-194) I145Y, *R. norvegicus* β1(1-194) L9W-I145Y, *R. norvegicus* β2(1-217), *R. norvegicus* β2(1-217) I142Y, *R. norvegicus* β1 H-NOX H105G, *R. norvegicus* β1 H-NOX H105F, *R. norvegicus* sGC β1 H-NOX C78S, *R. norvegicus* sGC β1 H-NOX C78E, *C. botulinum* H-NOX(1-175), *C. botulinum* H-NOX(1-186), wild-type *C. acetobutylicum* H-NOX, *C. acetobutylicum* H-NOX(1-197), *C. acetobutylicum* H-NOX(1-183), wild-type *C. elegans* GCY-35 H-NOX, *C. elegans* H-NOX GCY-35(1-252), wild-type *D. melangaster* β1 H-NOX, wild-type *D. melangaster* CG14885-PA, wild-type *D. melangaster* CG14886, wild-type *D. melangaster* CG4154; wild-type *N. punctiforme* H-NOX, wild-type *C. crescentus* H-NOX, wild-type *S. oneidensis* H-NOX, wild-type *M. musculus* H-NOX , wild-type *C. familiaris* H-NOX, wild-type *B. Taurus* H-NOX, wild-type *R. norvegicus;* wild-type *X. laevis* H-NOX, wild-type *O. latipes* H-NOX, wild-type *O. curivatus* H-NOX, wild-type *F. rubripes* H-NOX, wild-type *A. gambiae* H-NOX, wild-type *M. sexta* H-NOX; wild-type *C. elegans* gcy-31, *C. elegans* gcy-32, wild-type *C. elegans* gcy-33, wild-type *C. elegans* gcy-34, wild-type *C. elegans* gcy-35, wild-type *C. elegans* gcy-36, wild-type *C. elegans* gcy-37; wild-type *V. cholera* H-NOX, wild-type *V. fischerii* H-NOX, and wild-type *N. punctiforme* H-NOX.

152. The method of claim 151, wherein the H-NOX protein is a selected from the group consisting of wild-type *R. norvegicus* sGC, wild-type *R. norvegicus* β1(1-3105), *R. norvegicus* β1(1-217), *R. norvegicus* β1(1-1104), wild-type *T. tengcongensis* H-NOX, *T. tengcongensis* H-NOX Y140L, *T. tengcongensis* H-NOX Y140F, wild-type *L. pneumophilia* 1 H-NOX, wild-type *L. pneumophilia* 2 H-NOX, and *L. pneumophilia* 2 H-NOX F142Y.

153. The method of any one of claims 127-152, wherein the H-NOX protein is a mammalian protein or is derived from a mammalian protein.

154. The method of claim 153, wherein the H-NOX protein is a human protein or is derived from a human protein.

155. The method of claim 154, wherein the human protein is β1 or is derived from β1.

156. The method of any one of claims 127-152, wherein the H-NOX protein is a bacterial protein or is derived from a bacterial protein.

157. The method of claim 156, wherein the H-NOX protein is a *T. tengcongensis* protein or is derived from a *T. tengcongensis* protein.

158. The method of any one of claims 127-157, wherein one or more liposomes or nanoparticles comprise the H-NOX protein.

159. The method of any one of claims 127-158, wherein the H-NOX protein is covalently bound to another molecule or moiety.

160. The method of claim 159, wherein the H-NOX protein is covalently bound to polyethylene glycol.

161. The method of any one of claims 127-160, wherein NO is bound to the H-NOX protein prior to the administration of the H-NOX protein to the individual.

162. The method of any one of claims 127-160, wherein NO is not bound to the H-NOX protein prior to the administration of the H-NOX protein to the individual, and wherein the H-NOX protein transports NO from one location in the individual to another location in the individual.

163. The method of any one of claims 127-162, wherein the H-NOX protein is administered orally, rectally, or to the blood of the individual.

164. The method of any one of claims 127-163, wherein the individual is a human.

165. The method of any one of claims 127-164, wherein the individual is suffering from or at risk for a cardiovascular condition, hypertension, a condition exacerbated by hypertension, a vasoconstrictive condition, stroke, or a functional NO deficiency.

166. The method of claim 165, wherein the condition exacerbated by hypertension is heart failure, renal failure, or a stroke.

167. The method of any one of claims 127-166, wherein the H-NOX protein is administered to the individual at least twice.

168. The method of any one of claims 127-167, wherein the H-NOX protein is a mutant H-NOX protein of any one of claims 245-314.

169. A kit comprising (i) a pharmaceutically acceptable amount of an H-NOX protein, wherein the NO dissociation constant of the H-NOX protein is within 2 orders of magnitude of that of hemoglobin, and wherein the NO reactivity of the H-NOX protein is at least 10-fold lower than that of hemoglobin, and (ii) a pharmaceutically acceptable carrier.

170. The kit of claim 169, wherein the NO dissociation constant of the H-NOX protein is between 0.1 to 10-fold of that of hemoglobin.

171. The kit of claim 170, wherein the NO dissociation constant of the H-NOX protein is between 0.5 to 2-fold of that of hemoglobin.

172. The kit of any one of claims 169-171, wherein the NO reactivity of the H-NOX protein is at least 100-fold lower than that of hemoglobin.

173. The kit of claim 172, wherein the NO reactivity of the H-NOX protein is at least 1,000-fold lower than that of hemoglobin.

174. The kit of any one of claims 169-173, wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

175. The kit of any one of claims 169-174, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C.

176. The kit of claim 175, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 0.012 s^{-1} at 37 °C.

177. The kit of claim 176, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 1 x $10^{-3}s^{-1}$ at 37 °C.

178. The kit of any one of claims 169-177, wherein the O_2 dissociation constant of the H-NOX protein it at least about 1 μM at 37 °C.

179. The kit of claim 178, wherein the O_2 dissociation constant of the H-NOX protein it at least about 10 μM at 37 °C.

180. The kit of claim 179, wherein the O_2 dissociation constant of the H-NOX protein it at least about 50 μM at 37 °C.

181. The kit of any one of claims 169-180, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C.

182. The kit of any one of claims 169-181, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

183. The kit of any one of claims 169-182, wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

184. The kit of any one of claims 169-183, wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

185. The kit of any one of claims 169-184, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

186. The kit of any one of claims 169-185, wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

187. The kit of any one of claims 169-186, wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

188. The kit of any one of claims 169-187, wherein heme is bound to the H-NOX protein.

189. The kit of any one of claims 169-188, wherein the H-NOX protein is a mutant protein.

190. The kit of claim 189, wherein the H-NOX protein comprises at least one distal pocket mutation.

191. The kit of any one of claims 189 and 190, wherein the H-NOX protein comprises at least one mutation that is not in the distal pocket.

192. The kit of any one of claims 169-191, wherein the H-NOX protein comprises at least one mutation that alters the NO dissociation constant, the k_{off} for NO, the k_1 for NO, the k_2 for NO, the O_2 dissociation constant, the NO stability, the rate of heme autoxidation, or any combination of two or more of the foregoing compared to that of a corresponding wild-type protein.

193. The kit of any one of claims 169-192, wherein the H-NOX protein comprises at least one mutation that alters the NO reactivity compared to that of the corresponding wild-type protein.

194. The kit of any one of claims 169-193, wherein the H-NOX protein comprises at least one mutation in which a residue that corresponds to Tyr140 of *T. tengcongensis* H-NOX is replaced by any other amino acid.

195. The kit of any one of claims 169-194, wherein the H-NOX protein comprises at least one mutation in which a residue that corresponds to Phe149 of *L. pneumophilia* 9 H-NOX is replaced by any other amino acid.

196. The kit of any one of claims 169-195, wherein at least one C-terminal amino acid in the mutant H-NOX protein has been removed compared to a corresponding wild-type protein.

197. The kit of claim 196, wherein at least about 50 contiguous C-terminal amino acids or between about 25 to about 200 C-terminal amino acids in the mutant H-NOX protein have been removed compared to the corresponding wild-type protein.

198. The kit of any one of claims 169-197, wherein the H-NOX protein is a selected from the group consisting of wild-type *T. tengcongensis* H-NOX, *T. tengcongensis* H-NOX I5A, *T. tengcongensis* H-NOX I5L, *T. tengcongensis* H-NOX I5L-P115A, *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX W9F-Y140L, *T. tengcongensis* H-NOX W9F-Y140H*T. tengcongensis* H-NOX W9F-N74A, *T. tengcongensis* H-NOX W9Y, *T. tengcongensis* H-NOX W9N, *T. tengcongensis* H-NOX W9H, *T. tengcongensis* H-NOX N74E, *T. tengcongensis* H-NOX N74A, *T. tengcongensis* H-NOX N74H, *T. tengcongensis* H-NOX N74A-Y140H, *T. tengcongensis* H-NOX

F79Y-Y140F, *T. tengcongensis* H-NOX F79Y/Y140L, *T. tengcongensis* H-NOX P115A, *T. tengcongensis* H-NOX R135Q, *T. tengcongensis* H-NOX Y140F, *T. tengcongensis* H-NOX Y40L, *T. tengcongensis* H-NOX Y140H, *T. tengcongensis* H-NOX Y140A, *T. tengcongensis* I75F-His6, *T. tengcongensis* I75F, *T. tengcongensis* L144F-His6, *T. tengcongensis* L144F, *L. pneumophilia* 2 H-NOX F142Y, wild-type *L. pneumophilia* 1 H-NOX, wild-type *L. pneumophilia* 2 H-NOX, *L. pneumophilia 2* F9W-F142Y, wild-type *D. desulfuricans* H-NOX, *D. desulfuricans* H-NOX(729-999), *D. desulfuricans* H-NOX Y139L, wild-type *H. sapiens* β1 H-NOX, *H. sapiens* β1 I140Y, *H. sapiens* β1 I145Y, *H. sapiens* β1(1-395), *H. sapiens* β1(1-395) I145Y, *H. sapiens* β1(1-395) I145H, *H. sapiens* β1(1-194), *H. sapiens* β1(1-194) I145Y, *H. sapiens* β1(1-194) L9W-I145Y, *H. sapiens* β2(1-217), *H. sapiens* β2(1-217) I142Y, *H. sapiens* β1 H-NOX H105G, *H. sapiens* β1 H-NOX H105F, *H. sapiens* β1 H-NOX C79S, *H. sapiens* β1 H-NOX C79E, wild-type *R. norvegicus* β1 H-NOX, *R. norvegicus* β1(1-395), *R. norvegicus* β1(1-395) I145Y, *R. norvegicus* β1(1-395) I145H, *R. norvegicus* β1(1-194), *R. norvegicus* β1(1-194) I145Y, *R. norvegicus* β1(1-194) L9W-I145Y, *R. norvegicus* β2(1-217), *R. norvegicus* β2(1-217) I142Y, *R. norvegicus* β1 H-NOX H105G, *R. norvegicus* β1 H-NOX H105F, *R. norvegicus* sGC β1 H-NOX C79S, *R. norvegicus* sGC β1 H-NOX C79E, *C. botulinum* H-NOX(1-175), *C. botulinum* H-NOX(1-196), wild-type *C. acetobutylicum* H-NOX, *C. acetobutylicum* H-NOX(1-197), *C. acetobutylicum* H-NOX(1-193), wild-type *C. elegans* GCY-35 H-NOX, *C. elegans* H-NOX GCY-35(1-252), wild-type *D. melangaster* β1 H-NOX, wild-type *D. melangaster* CG14995-PA, wild-type *D. melanogaster* CG14995, wild-type *D. melangaster* CG14996, wild-type *D. melangaster* CG4154; wild-type *N. punctiforme* H-NOX, wild-type *C. crescentus* H-NOX, wild-type *S. oneidensis* H-NOX, wild-type *M. musculus* H-NOX , wild-type *C. familiaris* H-NOX, wild-type *B. Taurus* H-NOX, wild-type *R. norvegicus;* wild-type *X. laevis* H-NOX, wild-type *O. latipes* H-NOX, wild-type *O. curivatus* H-NOX, wild-type *F. rubripes* H-NOX, wild-type *A. gambiae* H-NOX, wild-type *M. sexta* H-NOX; wild-type *C. elegans* gcy-31, *C. elegans* gcy-32, wild-type *C. elegans* gcy-33, wild-type *C. elegans* gcy-34, wild-type *C. elegans* gcy-35, wild-type *C. elegans* gcy-36, wild-type *C. elegans* gcy-37; wild-type *V. cholera* H-NOX, wild-type *V. fischerii* H-NOX, and wild-type *N. punctiforme* H-NOX.

199. The kit of claim 198, wherein the H-NOX protein is a selected from the group consisting of wild-type *R. norvegicus* sGC, wild-type *R. norvegicus* β1(1-395), *R. norvegicus* β1(1-217), *R. norvegicus* β1(1-194), wild-type *T. tengcongensis* H-NOX, *T. tengcongensis* H-NOX

Y140L, *T. tengcongensis* H-NOX Y140F, wild-type *L. pneumophilia* 1 H-NOX, wild-type *L. pneumophilia* 2 H-NOX, and *L. pneumophilia* 2 H-NOX F142Y.

200. The kit of any one of claims 169-199, wherein the H-NOX protein is a mammalian protein or is derived from a mammalian protein.

201. The kit of claim 200, wherein the H-NOX protein is a human protein, is derived from a human protein, is human β1, or is derived from human β1.

202. The kit of any one of claims 169-199, wherein the H-NOX protein is a bacterial protein or is derived from a bacterial protein.

203. The kit of claim 202, wherein the H-NOX protein is a *T. tengcongensis* protein or is derived from a *T. tengcongensis* protein.

204. The kit of any one of claims 169-203, wherein NO is bound to the H-NOX protein.

205. The kit of any one of claims 169-204, comprising one or more liposomes or nanoparticles that comprise the H-NOX protein.

206. The kit of any one of claims 169-205, wherein the H-NOX protein is covalently bound to another molecule or moiety.

207. The kit of claim 206, wherein the H-NOX protein is covalently bound to polyethylene glycol.

208. The kit of any one of claims 169-207, wherein the H-NOX protein is a mutant H-NOX protein of any one of claims 245-314.

209. A kit comprising (i) an H-NOX protein, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and (ii) instructions for using the kit to deliver NO to an individual.

210. The kit of claim 209, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 0.012 s^{-1} at 37 °C.

211. The kit of claim 210, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 1 x $10^{-3}s^{-1}$ at 37 °C.

212. The kit of any one of claims 209-211, wherein the O_2 dissociation constant of the H-NOX protein it at least about 10 μM at 37 °C.

213. The kit of claim 212, wherein the O_2 dissociation constant of the H-NOX protein it at least about 50 μM at 37 °C.

214. The kit of any one of claims 209-213, wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

215. The kit of any one of claims 209-214, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C.

216. The kit of any one of claims 209-215, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

217. The kit of any one of claims 209-216, wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

218. The kit of any one of claims 209-217, wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

219. The kit of any one of claims 209-218, wherein the k_{off}, k_1, or k_2 for NO of the H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

220. The kit of any one of claims 209-219, wherein the O_2 dissociation constant of the H-NOX protein is at least about 1 μM at 37 °C, and wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C.

221. The kit of any one of claims 209-220, wherein the rate of heme autoxidation of the H-NOX protein is less than about 1 h^{-1}at 37 °C, and wherein the NO reactivity of the H-NOX protein is less than about 700 s^{-1}.

222. The kit of any one of claims 209-221, wherein heme is bound to the H-NOX protein.

223. The kit of any one of claims 209-222, wherein the H-NOX protein is a mutant protein.

224. The kit of claim 223, wherein the H-NOX protein comprises at least one distal pocket mutation.

225. The kit of any one of claims 223 and 224, wherein the H-NOX protein comprises at least one mutation that is not in the distal pocket.

226. The kit of any one of claims 209-225, wherein the H-NOX protein comprises at least one mutation that alters the NO dissociation constant, the k_{off} for NO, the k_1 for NO, or the k_2 for NO, the O_2 dissociation constant, the NO stability , the rate of heme autoxidation, or any combination of two or more of the foregoing compared to that of a corresponding wild-type protein.

227. The kit of any one of claims 209-226, wherein the H-NOX protein comprises at least one mutation that alters the NO reactivity compared to that of the corresponding wild-type protein.

228. The kit of any one of claims 209-227, wherein the H-NOX protein comprises at least one mutation in which a residue that corresponds to Tyr140 of *T. tengcongensis* H-NOX is replaced by any other amino acid.

229. The kit of any one of claims 209-228, wherein the H-NOX protein comprises at least one mutation in which a residue that corresponds to Phe149 of *L. pneumophilia* 9 H-NOX is replaced by any other amino acid.

230. The kit of any one of claims 209-229, wherein at least one C-terminal amino acid in the mutant H-NOX protein has been removed compared to a corresponding wild-type protein.

231. The kit of claim 230, wherein at least about 50 contiguous C-terminal amino acids in the mutant H-NOX protein have been removed compared to the corresponding wild-type protein.

232. The kit of any one of claims 230 and 231, wherein between about 25 to about 200 contiguous C-terminal amino acids in the mutant H-NOX protein have been removed compared to the corresponding wild-type protein.

233. The kit of any one of claims 209-232, wherein the H-NOX protein is a selected from the group consisting of wild-type *T. tengcongensis* H-NOX, *T. tengcongensis* H-NOX I5A, *T. tengcongensis* H-NOX I5L, *T. tengcongensis* H-NOX I5L-P115A, *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX W9F-Y140L, *T. tengcongensis* H-NOX W9F-Y140H*T. tengcongensis* H-NOX W9F-N74A, *T. tengcongensis* H-NOX W9Y, *T. tengcongensis* H-NOX W9N, *T. tengcongensis* H-NOX W9H, *T. tengcongensis* H-NOX N74E, *T. tengcongensis* H-NOX N74A, *T. tengcongensis* H-NOX N74H, *T. tengcongensis* H-NOX N74A-Y140H, *T. tengcongensis* H-NOX F78Y-Y140F, *T. tengcongensis* H-NOX F78Y/Y140L, *T. tengcongensis* H-NOX P115A, *T. tengcongensis* H-NOX R135Q, *T. tengcongensis* H-NOX Y140F, *T. tengcongensis* H-NOX Y40L, *T. tengcongensis* H-NOX Y140H, *T. tengcongensis* H-NOX Y140A, *T. tengcongensis* I75F-His6, *T. tengcongensis* I75F, *T. tengcongensis* L144F-His6, *T. tengcongensis* L144F, *L. pneumophilia* 2 H-NOX F142Y, wild-type *L. pneumophilia* 1 H-NOX, wild-type *L. pneumophilia* 2 H-NOX, *L. pneumophilia 2* F9W-F142Y, wild-type *D. desulfuricans* H-NOX, *D. desulfuricans* H-NOX(728-899), *D. desulfuricans* H-NOX Y139L, wild-type *H. sapiens* β1 H-NOX, *H. sapiens* β1 I140Y, *H. sapiens* β1 I145Y, *H. sapiens* β1(1-385), *H. sapiens* β1(1-385) I145Y, *H. sapiens* β1(1-385) I145H, *H. sapiens* β1(1-194), *H. sapiens* β1(1-194) I145Y, *H. sapiens* β1(1-194) L9W-I145Y, *H. sapiens* β2(1-217), *H. sapiens* β2(1-217) I142Y, *H. sapiens* β1 H-NOX H105G, *H. sapiens* β1 H-NOX H105F, *H. sapiens* β1 H-NOX C78S, *H. sapiens* β1 H-NOX C78E, wild-type *R. norvegicus* β1 H-NOX, *R. norvegicus* β1(1-385), *R. norvegicus* β1(1-385) I145Y, *R. norvegicus* β1(1-385) I145H, *R. norvegicus* β1(1-194), *R. norvegicus* β1(1-194) I145Y, *R. norvegicus* β1(1-194) L9W-I145Y, *R. norvegicus* β2(1-217), *R. norvegicus* β2(1-217) I142Y, *R. norvegicus* β1 H-NOX H105G, *R. norvegicus* β1 H-NOX H105F, *R. norvegicus* sGC β1 H-NOX C78S, *R. norvegicus* sGC β1 H-NOX C78E, *C. botulinum* H-NOX(1-175), *C. botulinum* H-NOX(1-186), wild-type *C. acetobutylicum* H-NOX, *C. acetobutylicum* H-NOX(1-197), *C. acetobutylicum* H-NOX(1-183), wild-type *C. elegans* GCY-35 H-NOX, *C. elegans* H-NOX GCY-35(1-252), wild-type *D. melangaster* β1 H-NOX, wild-type *D. melangaster* CG14885-PA, wild-type *D. melangaster* CG14886, wild-type *D. melangaster* CG4154; wild-type *N. punctiforme* H-NOX, wild-type *C. crescentus* H-NOX, wild-type *S.*

oneidensis H-NOX, wild-type *M. musculus* H-NOX , wild-type *C. familiaris* H-NOX, wild-type *B. Taurus* H-NOX, wild-type *R. norvegicus;* wild-type *X. laevis* H-NOX, wild-type *O. latipes* H-NOX, wild-type *O. curivatus* H-NOX, wild-type *F. rubripes* H-NOX, wild-type *A. gambiae* H-NOX, wild-type *M. sexta* H-NOX; wild-type *C. elegans* gcy-31, *C. elegans* gcy-32, wild-type *C. elegans* gcy-33, wild-type *C. elegans* gcy-34, wild-type *C. elegans* gcy-35, wild-type *C. elegans* gcy-36, wild-type *C. elegans* gcy-37; wild-type *V. cholera* H-NOX, wild-type *V. fischerii* H-NOX, and wild-type *N. punctiforme* H-NOX.

234. The kit of claim 233, wherein the H-NOX protein is a selected from the group consisting of wild-type *R. norvegicus* sGC, wild-type *R. norvegicus* β1(1-395), *R. norvegicus* β1(1-217), *R. norvegicus* β1(1-194), wild-type *T. tengcongensis* H-NOX, *T. tengcongensis* H-NOX Y140L, *T. tengcongensis* H-NOX Y140F, wild-type *L. pneumophilia* 1 H-NOX, wild-type *L. pneumophilia* 2 H-NOX, and *L. pneumophilia* 2 H-NOX F142Y.

235. The kit of any one of claims 209-234, wherein the H-NOX protein is a mammalian protein or is derived from a mammalian protein.

236. The kit of claim 235, wherein the H-NOX protein is a human protein or is derived from a human protein.

237. The kit of claim 236, wherein the human protein is β1 or is derived from β1.

238. The kit of any one of claims 209-234, wherein the H-NOX protein is a bacterial protein or is derived from a bacterial protein.

239. The kit of claim 238, wherein the H-NOX protein is a *T. tengcongensis* protein or is derived from a *T. tengcongensis* protein.

240. The kit of any one of claims 209-239, wherein NO is bound to the H-NOX protein.

241. The kit of any one of claims 209-240, comprising one or more liposomes or nanoparticles that comprise the H-NOX protein.

242. The kit of any one of claims 209-241, wherein the H-NOX protein is covalently bound to another molecule or moiety.

243. The kit of claim 242, wherein the H-NOX protein is covalently bound to polyethylene glycol.

244. The kit of any one of claims 209-243, wherein the H-NOX protein is a mutant H-NOX protein of any one of claims 245-314.

245. An isolated H-NOX protein comprising at least one mutation that alters the NO dissociation constant or NO reactivity compared to that of a corresponding wild-type H-NOX protein, wherein the NO dissociation constant of the mutant H-NOX protein is within 2 orders of magnitude of that of hemoglobin, wherein the NO reactivity of the mutant H-NOX protein is at least 10-fold lower than that of hemoglobin, and wherein the mutant H-NOX protein is not *T. tengcongensis* H-NOX Y40L, wild-type *T. tengcongensis* H-NOX, wild-type *R. norvegicus* sGC, or *L. pneumophilia* 2 H-NOX F142Y.

246. The isolated H-NOX protein of claim 245, wherein the mutant H-NOX protein is not *T. tengcongensis* H-NOX F78Y/Y140L.

247. The isolated H-NOX protein of any one of claims 245 and 246, wherein the mutant H-NOX protein is not wild-type *L. pneumophilia* 2 H-NOX, wild-type *H. sapiens* β1 H-NOX, *R. norvegicus* sGC β1 H-NOX (1-385), wild-type *R. norvegicus* β1 H-NOX, wild-type *D. melangaster* β1 H-NOX, wild-type *D. melangaster* CG14885-PA H-NOX, wild-type *C. elegans* GCY-35 H-NOX, wild-type *N. punctiforme* H-NOX, wild-type *C. crescentus* H-NOX, wild-type *S. oneidensis* H-NOX, or wild-type *C. acetobutylicum* H-NOX.

248. The isolated H-NOX protein of any one of claims 245-247, wherein the mutant H-NOX protein is not *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX Y140F, *R. norvegicus* sGC β1 H-NOX (1-385) I145Y, *R. norvegicus* sGC β1 H-NOX H105G, *R. norvegicus* sGC β1 H-NOX H105F, *R. norvegicus* sGC β1 H-NOX I145Y, *R. norvegicus* sGC β1 H-NOX C78S, or *R. norvegicus* sGC β1 H-NOX C78E.

249. The isolated H-NOX protein of any one of claims 245-248, wherein the mutant H-NOX protein is not *T. tengcongensis* H-NOX Y140H or *H. sapiens* β1 I140Y.

250. The isolated H-NOX protein of any one of claims 245-249, wherein the NO dissociation constant of the mutant H-NOX protein is between 0.1 to 10-fold of that of hemoglobin.

251. The isolated H-NOX protein of claim 250, wherein the NO dissociation constant of the mutant H-NOX protein is between 0.5 to 2-fold of that of hemoglobin.

252. The isolated H-NOX protein of any one of claims 245-251, wherein the NO reactivity of the mutant H-NOX protein is at least 100-fold lower than that of hemoglobin.

253. The isolated H-NOX protein of claim 252, wherein the NO reactivity of the mutant H-NOX protein is at least 1,000-fold lower than that of hemoglobin.

254. The isolated H-NOX protein of any one of claims 245-253, wherein the NO reactivity of the mutant H-NOX protein is less than about 700 s^{-1}.

255. The isolated H-NOX protein of any one of claims 245-254, wherein the k_{off}, k_1, or k_2 for NO of the mutant H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C.

256. The isolated H-NOX protein of any one of claims 245-255, wherein the k_{off}, k_1, or k_2 for NO of the mutant H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 0.012 s^{-1} at 37 °C.

257. The isolated H-NOX protein of any one of claims 245-256, wherein the k_{off}, k_1, or k_2 for NO of the mutant H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 1 x $10^{-3}s^{-1}$ at 37 °C.

258. The isolated H-NOX protein of any one of claims 245-257, wherein the O_2 dissociation constant of the mutant H-NOX protein it at least about 1 μM at 37 °C.

259. The isolated H-NOX protein of any one of claims 245-258, wherein the O_2 dissociation constant of the mutant H-NOX protein it at least about 10 μM at 37 °C.

260. The isolated H-NOX protein of claim 259, wherein the O_2 dissociation constant of the mutant H-NOX protein it at least about 50 μM at 37 °C.

261. The isolated H-NOX protein of any one of claims 245-260, wherein the k_{off}, k_1, or k_2 for NO of the mutant H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the O_2 dissociation constant of the mutant H-NOX protein is at least about 1 μM at 37 °C.

262. The isolated H-NOX protein of any one of claims 245-261, wherein the k_{off}, k_1, or k_2 for NO of the mutant H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the NO reactivity of the mutant H-NOX protein is less than about 700 s^{-1}.

263. The isolated H-NOX protein of any one of claims 245-262, wherein the O_2 dissociation constant of the mutant H-NOX protein is at least about 1 μM at 37 °C, and wherein the NO reactivity of the mutant H-NOX protein is less than about 700 s^{-1}.

264. The isolated H-NOX protein of any one of claims 245-263, wherein the rate of heme autoxidation of the mutant H-NOX protein is less than about 1 h^{-1}at 37 °C.

265. The isolated H-NOX protein of any one of claims 245-264, wherein the k_{off}, k_1, or k_2 for NO of the mutant H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the rate of heme autoxidation of the mutant H-NOX protein is less than about 1 h^{-1}at 37 °C.

266. The isolated H-NOX protein of any one of claims 245-265, wherein the O_2 dissociation constant of the mutant H-NOX protein is at least about 1 μM at 37 °C, and wherein the rate of heme autoxidation of the mutant H-NOX protein is less than about 1 h^{-1}at 37 °C.

267. The isolated H-NOX protein of any one of claims 245-266, wherein the rate of heme autoxidation of the mutant H-NOX protein is less than about 1 h^{-1}at 37 °C, and wherein the NO reactivity of the mutant H-NOX protein is less than about 700 s^{-1}.

268. The isolated H-NOX protein of any one of claims 245-267, comprising at least one distal pocket mutation.

269. The isolated H-NOX protein of any one of claims 245-268, comprising at least one mutation that is not in the distal pocket.

270. The isolated H-NOX protein of any one of claims 245-269, comprising at least one mutation in which a residue that corresponds to Tyr140 of *T. tengcongensis* H-NOX is replaced by any other amino acid.

271. The isolated H-NOX protein of any one of claims 245-270, comprising at least one mutation in which a residue that corresponds to Phe142 of *L. pneumophilia* 2 H-NOX is replaced by any other amino acid.

272. The isolated H-NOX protein of any one of claims 245-271, wherein at least one C-terminal amino acid has been removed compared to the corresponding wild-type protein.

273. The isolated H-NOX protein of claim 272, wherein at least about 50 contiguous C-terminal amino acids have been removed compared to the corresponding wild-type protein.

274. The isolated H-NOX protein of any one of claims 272 and 273, wherein between about 25 to about 200 contiguous C-terminal amino acids have been removed compared to the corresponding wild-type protein.

275. The isolated H-NOX protein of any one of claims 245-274, wherein the corresponding wild-type H-NOX protein is a mammalian protein.

276. The isolated H-NOX protein of claim 275, wherein the corresponding wild-type H-NOX protein is a human protein.

277. The isolated H-NOX protein of claim 276, wherein the corresponding wild-type H-NOX protein is β1.

278. The isolated H-NOX protein of any one of claims 245-274, wherein the corresponding wild-type H-NOX protein is a bacterial protein.

279. The isolated H-NOX protein of claim 278, wherein the corresponding wild-type H-NOX protein is a *T. tengcongensis* protein.

280. The isolated H-NOX protein of any one of claims 245-279, wherein the mutant H-NOX protein is covalently bound to another molecule or moiety.

281. The isolated H-NOX protein of claim 280, wherein the mutant H-NOX protein is covalently bound to polyethylene glycol.

282. An isolated H-NOX protein comprising at least one mutation that alters the k_{off}, k_1, or k_2 for NO or alters the O_2 dissociation constant compared to that of a corresponding wild-type H-NOX protein, wherein the k_{off}, k_1, or k_2 for NO of the mutant H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, wherein the O_2 dissociation constant of the mutant H-NOX protein is at least about 1 μM at 37 °C, and wherein the mutant H-NOX protein is not *T. tengcongensis* H-NOX Y40L, wild-type *T. tengcongensis* H-NOX, wild-type *R. norvegicus* sGC, or *L. pneumophilia* 2 H-NOX F142Y.

283. The isolated H-NOX protein of claim 282, wherein the mutant H-NOX protein is not *T. tengcongensis* H-NOX F78Y/Y140L.

284. The isolated H-NOX protein of any one of claims 282 and 283, wherein the mutant H-NOX protein is not wild-type *L. pneumophilia* 2 H-NOX, wild-type *H. sapiens* β1 H-NOX, *R. norvegicus* sGC β1 H-NOX (1-385), wild-type *R. norvegicus* β1 H-NOX, wild-type *D. melangaster* β1 H-NOX, wild-type *D. melangaster* CG14885-PA H-NOX, wild-type *C. elegans* GCY-35 H-NOX, wild-type *N. punctiforme* H-NOX, wild-type *C. crescentus* H-NOX, wild-type *S. oneidensis* H-NOX, or wild-type *C. acetobutylicum* H-NOX.

285. The isolated H-NOX protein of any one of claims 282-284, wherein the mutant H-NOX protein is not *T. tengcongensis* H-NOX W9F, *T. tengcongensis* H-NOX Y140F, *R. norvegicus* sGC β1 H-NOX (1-385) I145Y, *R. norvegicus* sGC β1 H-NOX H105G, *R. norvegicus* sGC β1 H-NOX H105F, *R. norvegicus* sGC β1 H-NOX I145Y, *R. norvegicus* sGC β1 H-NOX C78S, *R. norvegicus* sGC β1 H-NOX C78E, or *H. sapiens* β1 H-NOX (1-385) I145Y.

286. The isolated H-NOX protein of any one of claims 282-285, wherein the mutant H-NOX protein is not *T. tengcongensis* H-NOX Y140H or *H. sapiens* β1 I140Y.

287. The isolated H-NOX protein of any one of claims 282-286, wherein the k_{off}, k_1, or k_2 for NO of the mutant H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 0.012 s^{-1} at 37 °C.

288. The isolated H-NOX protein of claim 287, wherein the k_{off}, k_1, or k_2 for NO of the mutant H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 1 x $10^{-3}s^{-1}$ at 37 °C.

289. The isolated H-NOX protein of any one of claims 282-288, wherein the O_2 dissociation constant of the mutant H-NOX protein it at least about 10 μM at 37 °C.

290. The isolated H-NOX protein of claim 289, wherein the O_2 dissociation constant of the mutant H-NOX protein it at least about 50 μM at 37 °C.

291. The isolated H-NOX protein of any one of claims 282-290, wherein the NO reactivity of the mutant H-NOX protein is less than about 700 s^{-1}.

292. The isolated H-NOX protein of any one of claims 282-291, wherein the k_{off}, k_1, or k_2 for NO of the mutant H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the O_2 dissociation constant of the mutant H-NOX protein is at least about 1 μM at 37 °C.

293. The isolated H-NOX protein of any one of claims 282-292, wherein the k_{off}, k_1, or k_2 for NO of the mutant H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the NO reactivity of the mutant H-NOX protein is less than about 700 s^{-1}.

294. The isolated H-NOX protein of any one of claims 282-293, wherein the O_2 dissociation constant of the mutant H-NOX protein is at least about 1 μM at 37 °C, and wherein the NO reactivity of the mutant H-NOX protein is less than about 700 s^{-1}.

295. The isolated H-NOX protein of any one of claims 282-294, wherein the rate of heme autoxidation of the mutant H-NOX protein is less than about 1 h^{-1}at 37 °C.

296. The isolated H-NOX protein of any one of claims 282-295, wherein the k_{off}, k_1, or k_2 for NO of the mutant H-NOX protein is between about 1 x $10^{-4}s^{-1}$ and about 10 s^{-1} at 37 °C, and wherein the rate of heme autoxidation of the mutant H-NOX protein is less than about 1 h^{-1}at 37 °C.

297. The isolated H-NOX protein of any one of claims 282-296, wherein the O_2 dissociation constant of the mutant H-NOX protein is at least about 1 μM at 37 °C, and wherein the rate of heme autoxidation of the mutant H-NOX protein is less than about 1 h^{-1}at 37 °C.

298. The isolated H-NOX protein of any one of claims 282-297, wherein the rate of heme autoxidation of the mutant H-NOX protein is less than about 1 h^{-1}at 37 °C, and wherein the NO reactivity of the mutant H-NOX protein is less than about 700 s^{-1}.

299. The isolated H-NOX protein of any one of claims 282-298, comprising at least one distal pocket mutation.

300. The isolated H-NOX protein of any one of claims 282-299, comprising at least one mutation that is not in the distal pocket.

301. The isolated H-NOX protein of any one of claims 282-300, comprising at least one mutation in which a residue that corresponds to Tyr140 of *T. tengcongensis* H-NOX is replaced by any other amino acid.

302. The isolated H-NOX protein of any one of claims 282-301, comprising at least one mutation in which a residue that corresponds to Phe142 of *L. pneumophilia* 2 H-NOX is replaced by any other amino acid.

303. The isolated H-NOX protein of any one of claims 282-302, wherein at least one C-terminal amino acid has been removed compared to the corresponding wild-type protein.

304. The isolated H-NOX protein of claim 303, wherein at least about 50 contiguous C-terminal amino acids have been removed compared to the corresponding wild-type protein.

305. The isolated H-NOX protein of any one of claims 303 and 304, wherein between about 25 to about 200 contiguous C-terminal amino acids have been removed compared to the corresponding wild-type protein.

306. The isolated H-NOX protein of any one of claims 282-305, wherein the corresponding wild-type H-NOX protein is a mammalian protein.

307. The isolated H-NOX protein of claim 306, wherein the corresponding wild-type H-NOX protein is a human protein.

308. The isolated H-NOX protein of claim 307, wherein the corresponding wild-type H-NOX protein is β1.

309. The isolated H-NOX protein of any one of claims 282-305, wherein the corresponding wild-type H-NOX protein is a bacterial protein.

310. The isolated H-NOX protein of claim 309, wherein the corresponding wild-type H-NOX protein is a *T. tengcongensis* protein.

311. The isolated H-NOX protein of any one of claims 282-310, wherein the mutant H-NOX protein is covalently bound to another molecule or moiety.

312. The isolated H-NOX protein of claim 311, wherein the mutant H-NOX protein is covalently bound to polyethylene glycol.

313. The isolated H-NOX protein of any one of claims 279-395, wherein the NO dissociation constant of the H-NOX protein is within 2 orders of magnitude of that of *Homo sapiens* hemoglobin alpha.

314. The isolated H-NOX protein of any one of claims 279-396, wherein the NO reactivity of the H-NOX protein is at least 10-fold lower than that of *Homo sapiens* hemoglobin alpha.

315. A recombinant nucleic acid encoding an H-NOX protein of any one of claims 245-314.

316. A vector comprising a nucleic acid of claim 315.

317. A cell comprising a nucleic acid of claim 315.

318. A cell comprising a vector of claim 316.

319. A method of producing an H-NOX protein comprising culturing a cell comprising a nucleic acid encoding an H-NOX protein of any one of claims 245-314 under conditions suitable for production of the protein.

320. The method of claim 319, further comprising the step of purifying the H-NOX protein.

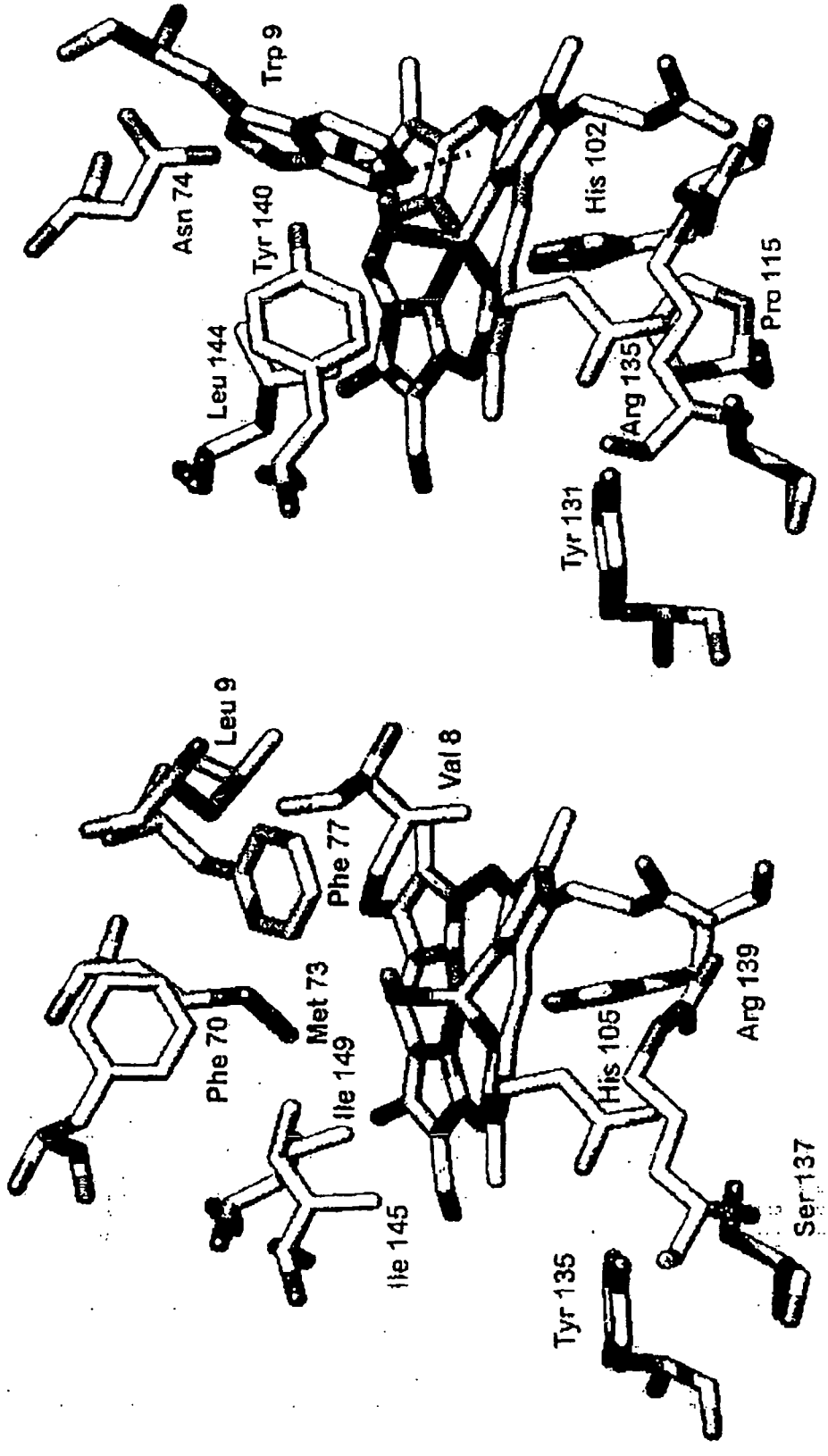

Leu 9
Phe 77
Val 8
Phe 70
Met 73
Ile 149
Ile 145
His 105
Arg 139
Tyr 135
Ser 137
Homo sapiens β1 homology model
Trp 9
Asn 74
Tyr 140
Leu 144
His 102
Pro 115
Arg 135
Tyr 131
Ser 133
T. tengcogensis H-NOX

Figure 1A

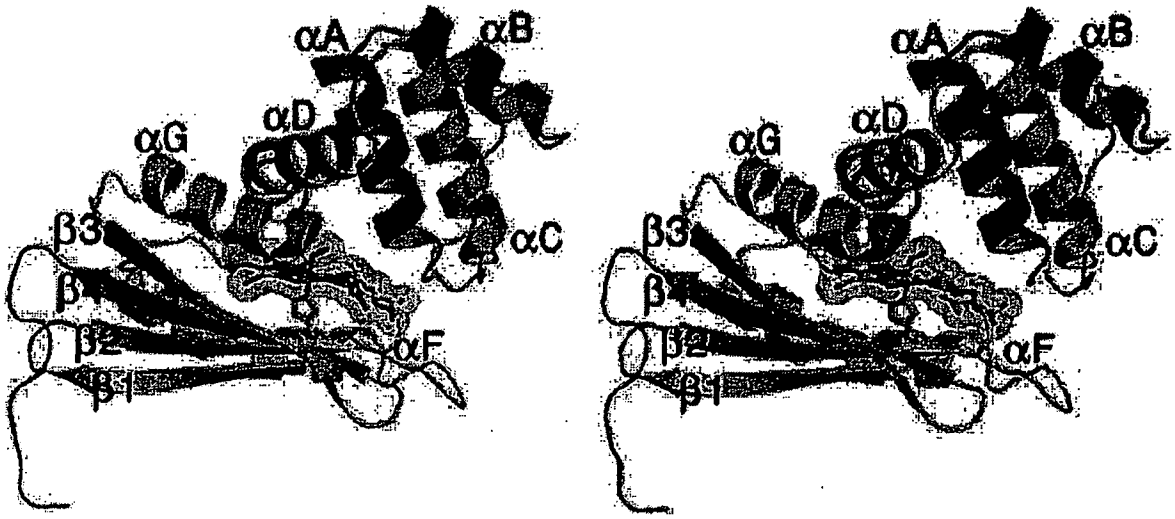
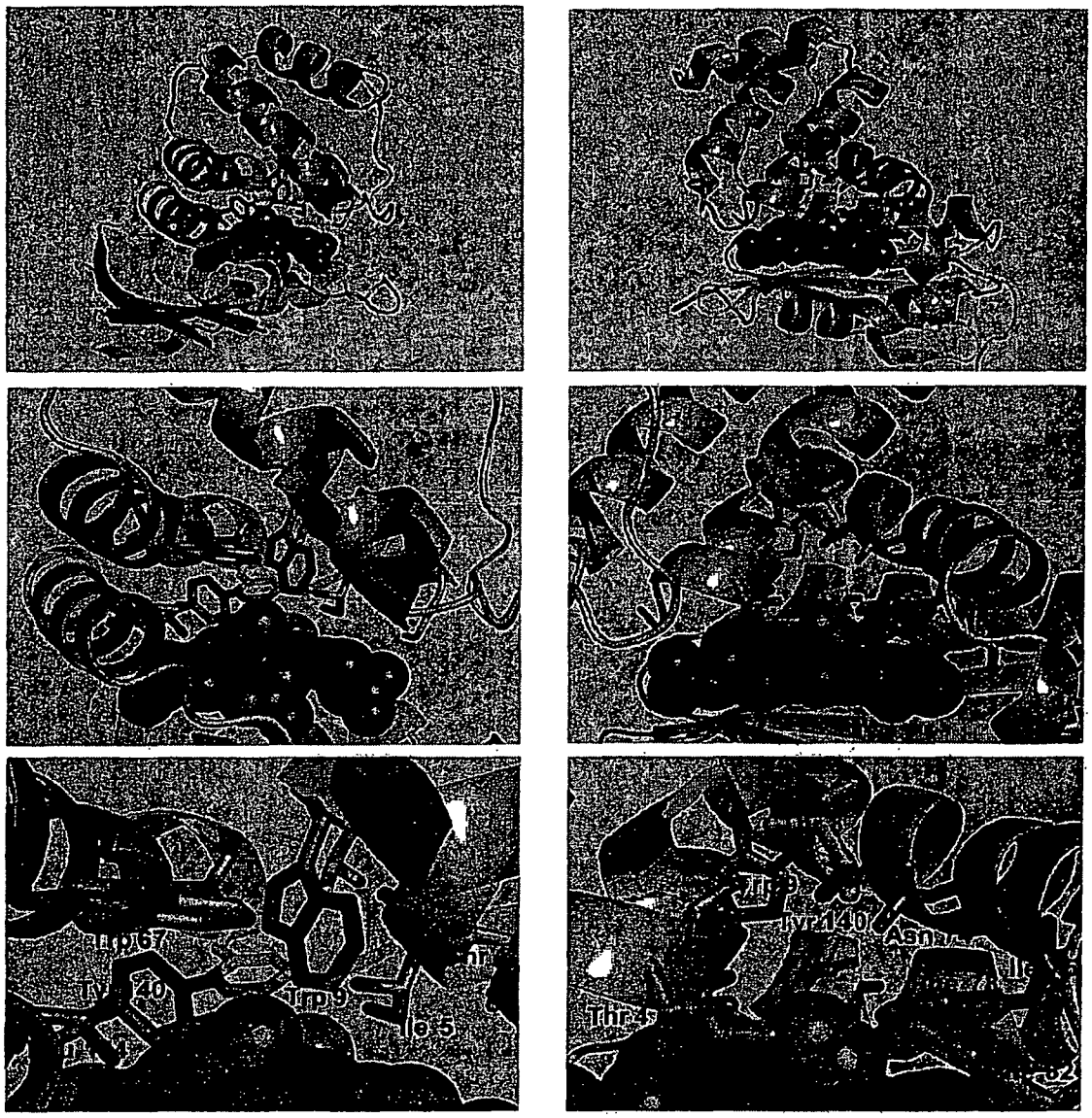


Figure 1B



Figures 1C-1H

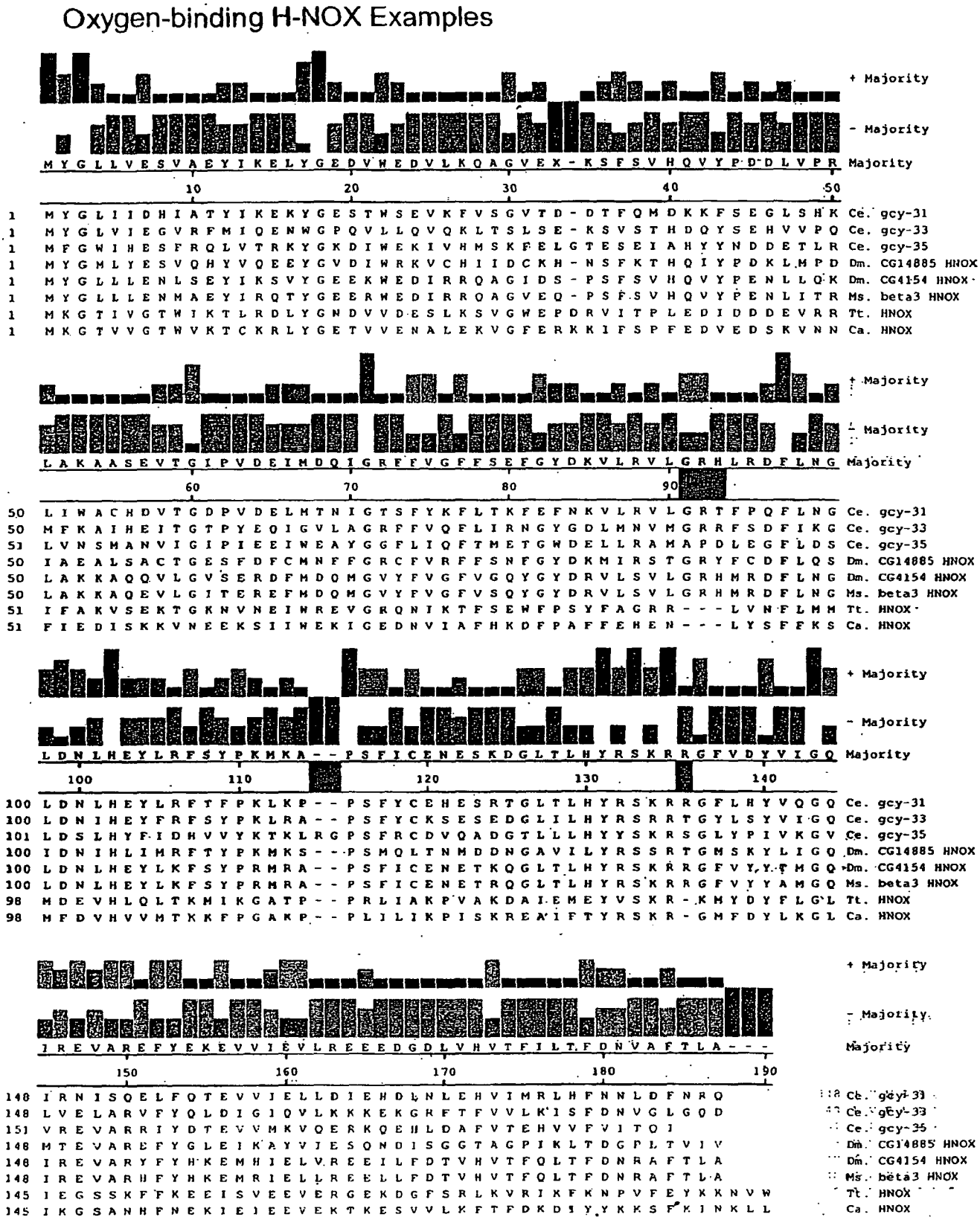

Oxygen-binding H-NOX Examples
+ Majority
- Majority
Majority
Ce. gcy-31
Ce. gcy-33
Ce. gcy-35
Dm. CG14885 HNOX
Dm. CG4154 HNOX
Ms. beta3 HNOX
Tt. HNOX
Ca. HNOX

Figure 2

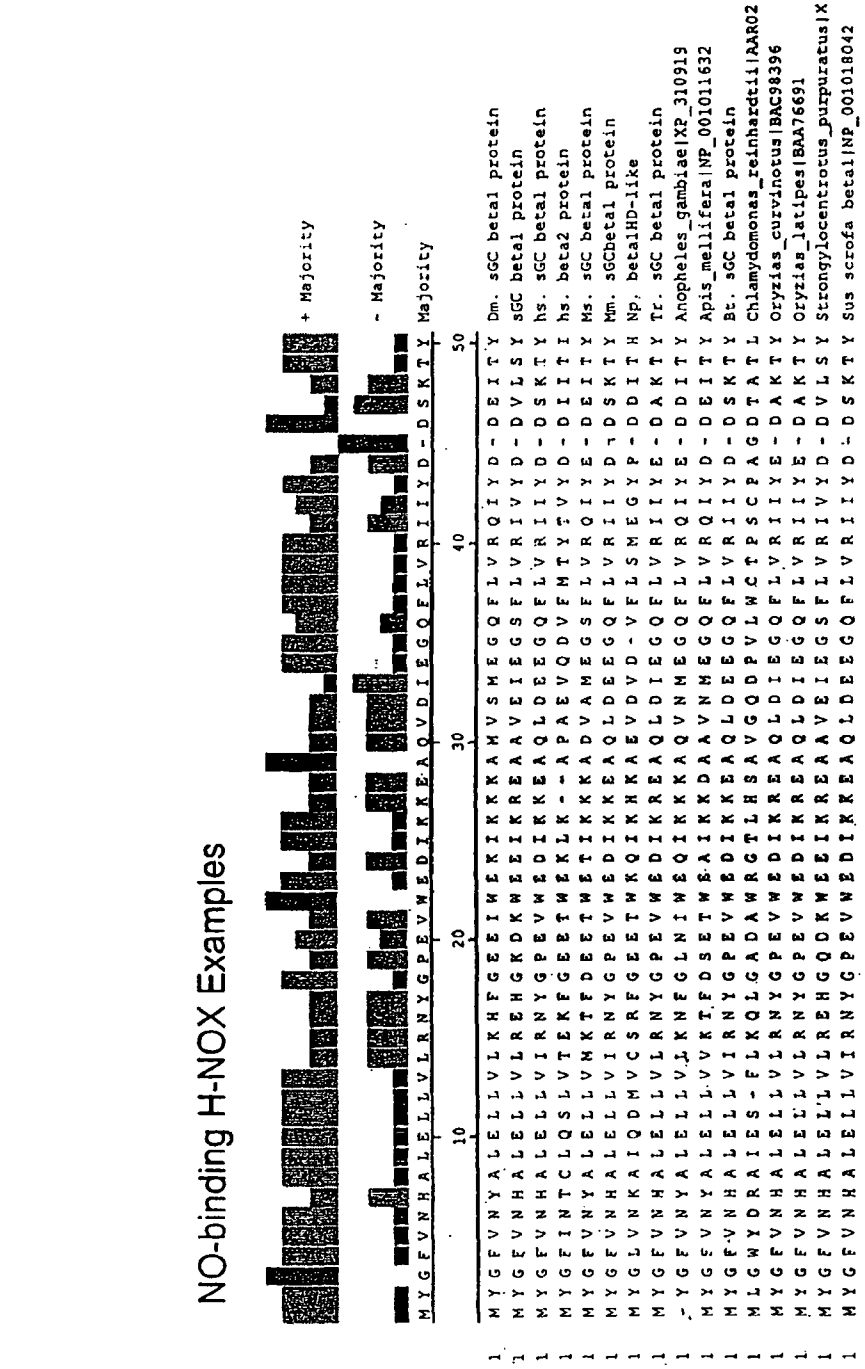

NO-binding H-NOX Examples
+ Majority
- Majority
Majority
Dm. sGC beta1 protein
sGC beta1 protein
hs. sGC beta1 protein
hs. beta2 protein
Ms. sGC beta1 protein
Mm. sGCbeta1 protein
Np. beta1HD-like
Tr. sGC beta1 protein
Anopheles_gambiae|XP_310919
Apis_mellifera|NP_001011632
Bt. sGC beta1 protein
Chlamydomonas_reinhardti1|AAR02
Oryzias_curvinotus|BAC98396
Oryzias_latipes|BAA76691
Strongylocentrotus_purpuratus|X
Sus scrofa beta1|NP_001018042

Figure 3A

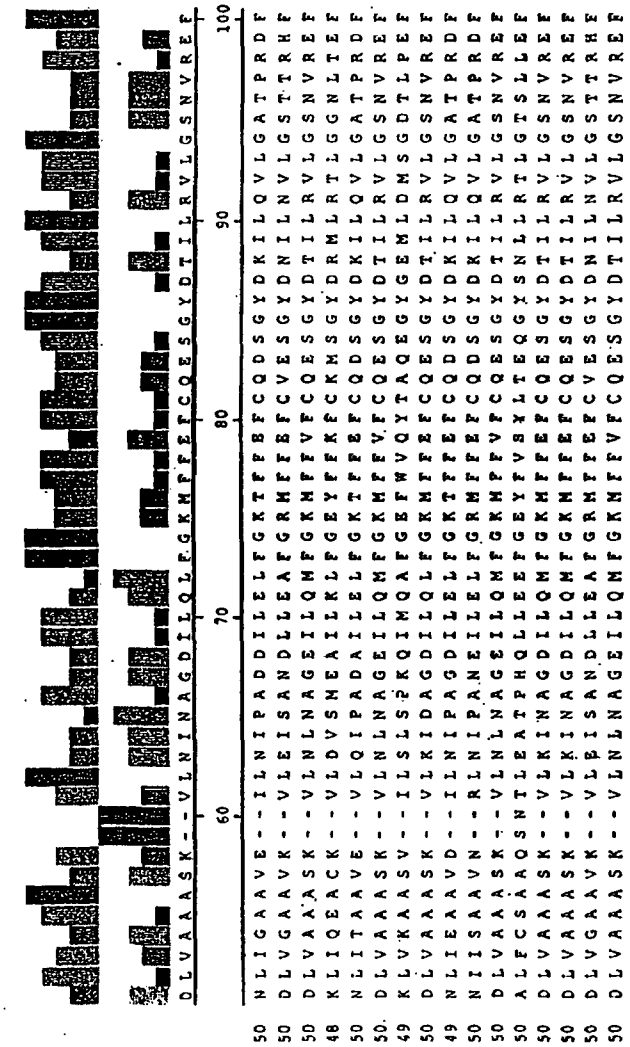

+ Majority
- Majority
Majority
Dm. sGC beta1 protein
hs. sGC beta1 protein
hs. sGC beta1 protein
hs. beta2 protein
Ms. sGC beta1 protein
Mm. sGCbeta1 protein
Np. beta1HD-like
Tr. sGC beta1 protein
Anopheles_gambiae|XP_310919
Apis_mellifera|NP_001011632
Bt. sGC beta1 protein
Chlamydomonas_reinhardtii|AAR02
Oryzias_curvinotus|BAC98396
Oryzias_latipes|BAA76691
Strongylocentrotus_purpuratus|X
Sus scrofa beta1|NP_001018042

Figure 3B

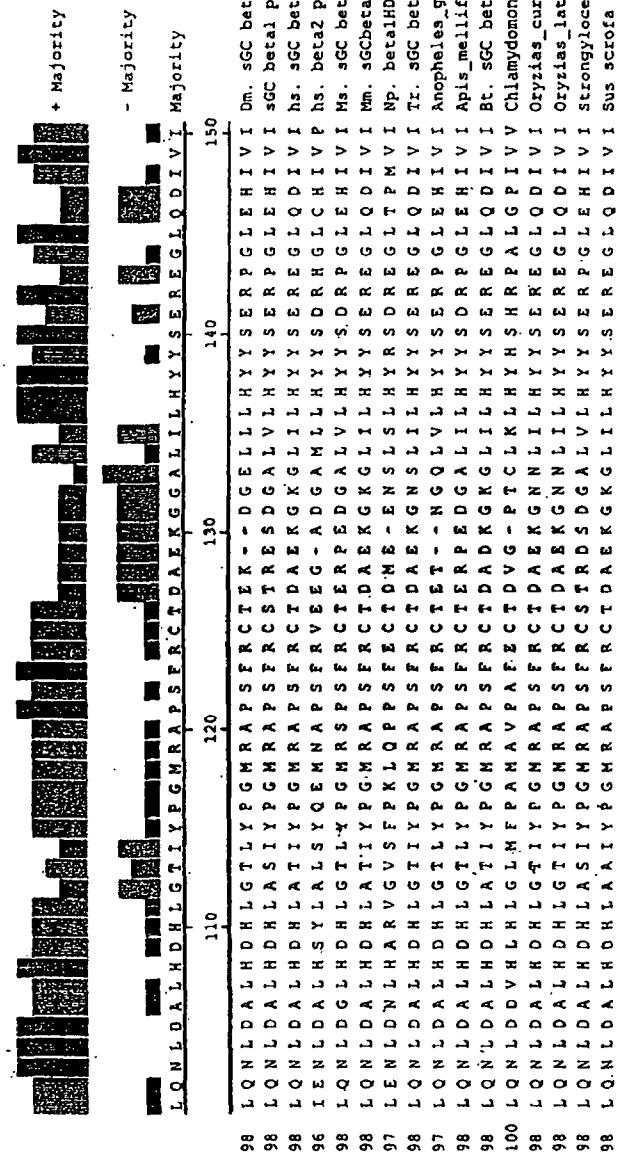

+ Majority
- Majority
Majority
110
120
130
140
150
98 LQNLDALHDHLGTLYPGMRAPSFRCTEK--DGELLHYYSERPGLEHIVI Dm. sGC beta1 protein
98 LQNLDALHDHLASIYPGMRAPSFRCSTRESDGALVLHYYSERPGLEHIVI sGC beta1 protein
98 LQNLDALHDHLATIYPGMRAPSFRCTDAEKGKGLILHYYSEREGLQDIVI hs. sGC beta1 protein
96 IENLDALHSYLALSYQEMNAPSFRVEEG-ADGAMLLHYYSDRHGLCHIVP hs. beta2 protein \
98 LQNLDGLHDHLGTLYPGMRSPSFRCTERPEDGALVLHYYS.DRPGLEHIVI Ms. sGC beta1 protein
98 LQNLDALHDHLATIYPGMRAPSFRCTDAEKGKGLILHYYSEREGLQDIVI Mm. sGCbeta1 protein
97 LENLDNLHARVGVSFPKLQPPSFECTDME-ENSLSLHYRSDREGLTPMVI Np. beta1HD-like
98 LQNLDALHDHLGTIYPGMRAPSFRCTDAEKGNSLILHYYSEREGLQDIVI Tr. sGC beta1 protein
97 LQNLDALHDHLGTLYPGMRAPSFRCTET--NGQLVLHYYSERPGLEHIVI Anopheles_gambiae|XP_310919
98 LQNLDALHDHLGTLYPGMRAPSFRCTERPEDGALILHYYSDRPGLEHIVI Apis_mellifera|NP_001011632
98 LQNLDALHDHLATIYPGMRAPSFRCTDADKGKGLILHYYSEREGLQDIVI Bt. sGC beta1 protein
100 LQNLDDVHLHLGLMFPAMAVPAFECTDVG-PTCLKLHYHSHRPALGPIVV Chlamydomonas_reinhardtii|AAR02
98 LQNLDALHDHLGTIYPGMRAPSFRCTDAEKGNNLILHYYSEREGLQDIVI Oryzias_curvinotus|BAC98396
98 LQNLDALHDHLGTIYPGMRAPSFRCTDAEKGNNLILHYYSEREGLQDIVI Oryzias_latipes|BAA76691
98 LQNLDALHDHLASIYPGMRAPSFRCSTRDSDGALVLHYYSERPGLEHIVI Strongylocentrotus_purpuratus|X
98 LQNLDALHDHLAAIYPGMRAPSFRCTDAEKGKGLILHYYSEREGLQDIVI Sus scrofa beta1|NP_001018042

Figure 3C

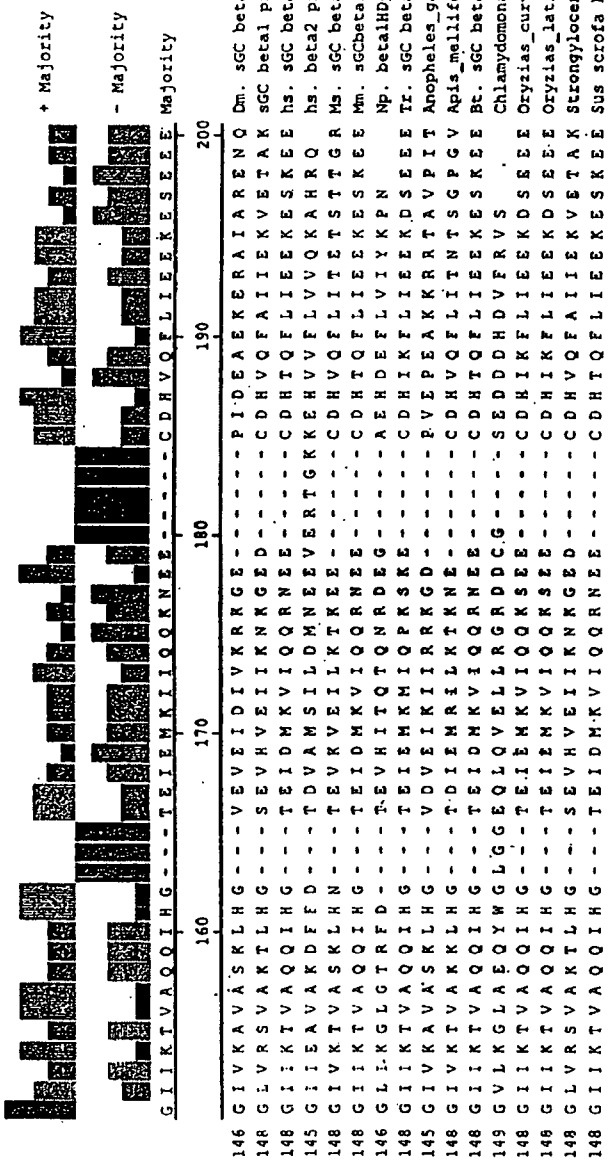
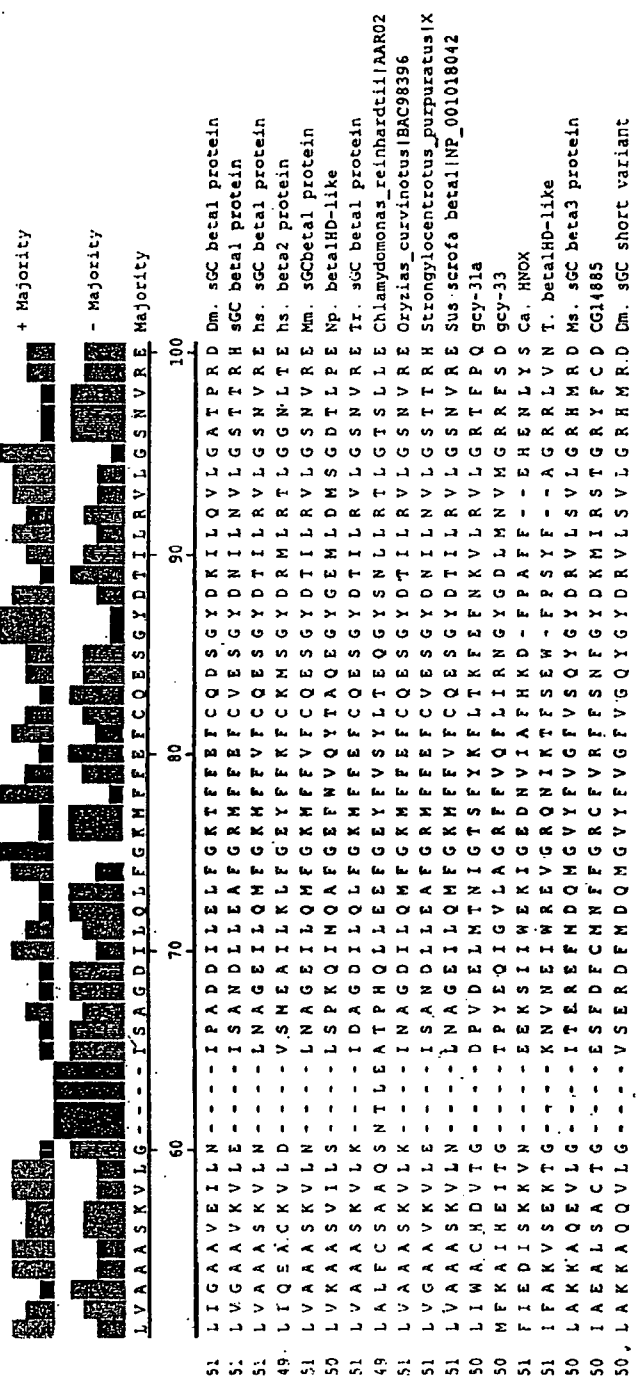

+ Majority
- Majority
Majority
160
170
180
190
200
Dm. sGC beta1 protein
sGC beta1 protein
hs. sGC beta1 protein
hs. beta2 protein
Ms. sGC beta1 protein
Mm. sGCbeta1 protein
Np. beta1HD-like
Tr. sGC beta1 protein
Anopheles_gambiae|XP_310919
Apis_mellifera|NP_001011632
Bt. sGC beta1 protein
Chlamydomonas_reinhardtii|AAR02
Oryzias_curvinotus|BAC98396
Oryzias_latipes|BAA76691
Strongylocentrotus_purpuratus|X
Sus scrofa beta1|NP_001018042

Figure 3D

Oxygen and NO-binding H-NOX Examples
+ Majority
- Majority
Majority
10
20
30
40
50
1 MYGFVNYALELLVLKHFGEEIWEKIKKKAMVSMEGQFLVRQIYDDEITYN Dm. sGC beta1 protein
1 MYGFVNHALELLVLREHGKDKWEEIKREAAVEIEGSFLVRIVYDDVLSYD sGC beta1 protein
1 MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYD hs. sGC beta1 protein
1 MYGFINTCLQSLVTEKFGEETWEKLK--APAEVQDVFMTYTVYDDIITIK hs. beta2 protein
1 MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYD Mm. sGCbeta1 protein
1 MYGLVNKAIQDMVCSRFGEETMKQIKHKAEVD-VDVFLSMEGYPDDITHK Np. beta1HD-like
1 MYGFVNHALELLVLRNYGPEVWEDIKREAQLDIEGQFLVRIIYEDAKTYD Tr. sGC beta1 protein
1 NLGWYDRAIESFLK-QLGADAWRGTLHSAVGQ-DPVLWCTPSCPAGDTAT Chlamydomonas_reinhardtii|AAR02
1 MYGFVNHALELLVLRNYGPEVWEDIKREAQLDIEGQFLVRIIYEDAKTYD Oryzias_curvinotus|BAC98396
1 MYGFVNHALELLVLREHGQDKWEEIKREAAVEIEGSFLVRIVYDDVLSYD Strongylocentrotus_purpuratus|X
1 MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYD Sus scrofa beta1|NP_001018042
1 MYGLIIDHIATYIKEKYGESTWSEVKFVSGVT-DDTFQMDKKFSEGLSHK gcy-31a
1 MYGLVIEGVRFMIQENWGPQVLLQVQKLTSLS-EKSVSTHDQYSEHVVPQ gcy-33
1 MKGTVVGTWVKTCKRLYGETVVENALEKVGFERKKIFSPFEDVEDSKVNN Ca. HNOX
1 MKGTIVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRR T. beta1HD-like
1 MYGLLLENMAEYIRQTYGEERWEDIRRQAGVE-QPSFSVHQVYPENLITR Ms. sGC beta3 protein
1 MYGMLYESVQKYVQEEYGVDIWRKVCHIIDCK-HNSFKTHQIYPDKLMPD CG14885
1 NYGLLLENLSEYIKSVYGEEKWEDIRRQAGID-SPSFSVHQVYPENLLQK Dm. sGC short variant

Figure 4A


+ Majority
- Majority
Majority
60
70
80
90
100
Dm. sGC beta1 protein
sGC beta1 protein
hs. sGC beta1 protein
hs. beta2 protein
Mm. sGCbeta1 protein
Np. beta1HD-like
Tr. sGC beta1 protein
Chlamydomonas_reinhardtii|AAR02
Oryzias_curvinotus|BAC98396
Strongylocentrotus_purpuratus|X
Sus scrofa beta1|NP_001018042
gcy-31a
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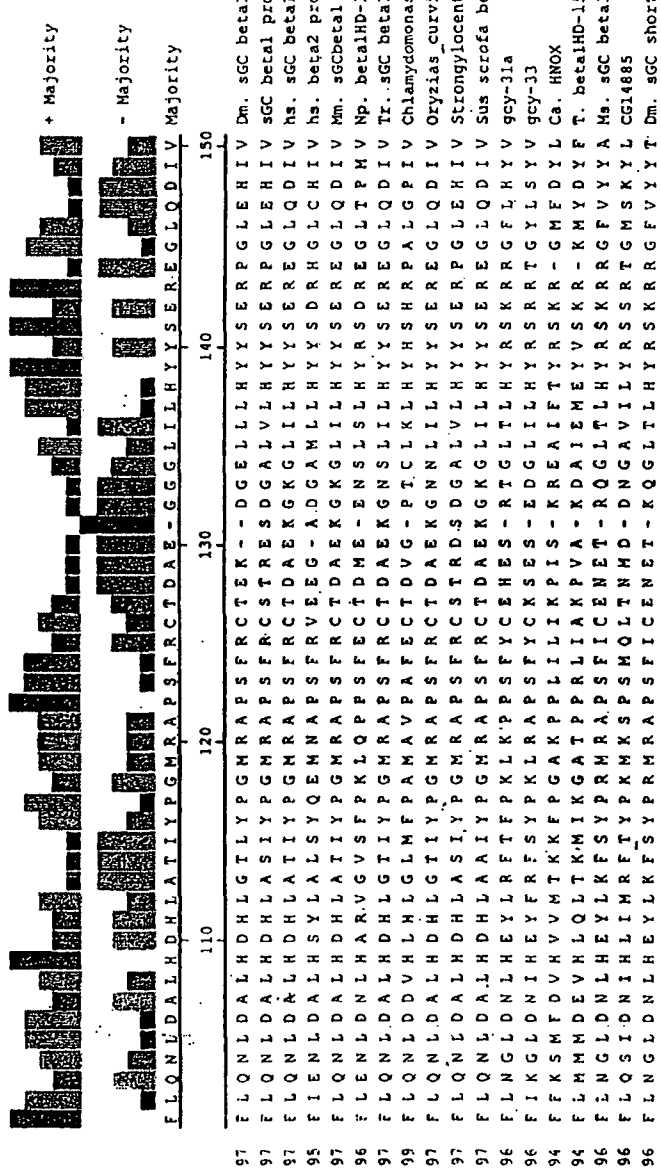
Ca. HNOX
T. beta1HD-like
Ms. sGC beta3 protein
CG14885
Dm. sGC short variant

Figure 4B


+ Majority
- Majority
Majority
110
120
130
140
150
Dm. sGC beta1 protein
sGC beta1 protein
hs. sGC beta1 protein
hs. beta2 protein
Mm. sGCbeta1 protein
Np. beta1HD-like
Tr. sGC beta1 protein
Chlamydomonas_reinhardtii|AAR02
Oryzias_curvinotus|BAC98396
Strongylocentrotus_purpuratus|X
Sus scrofa beta1|NP_001018042
gcy-31a
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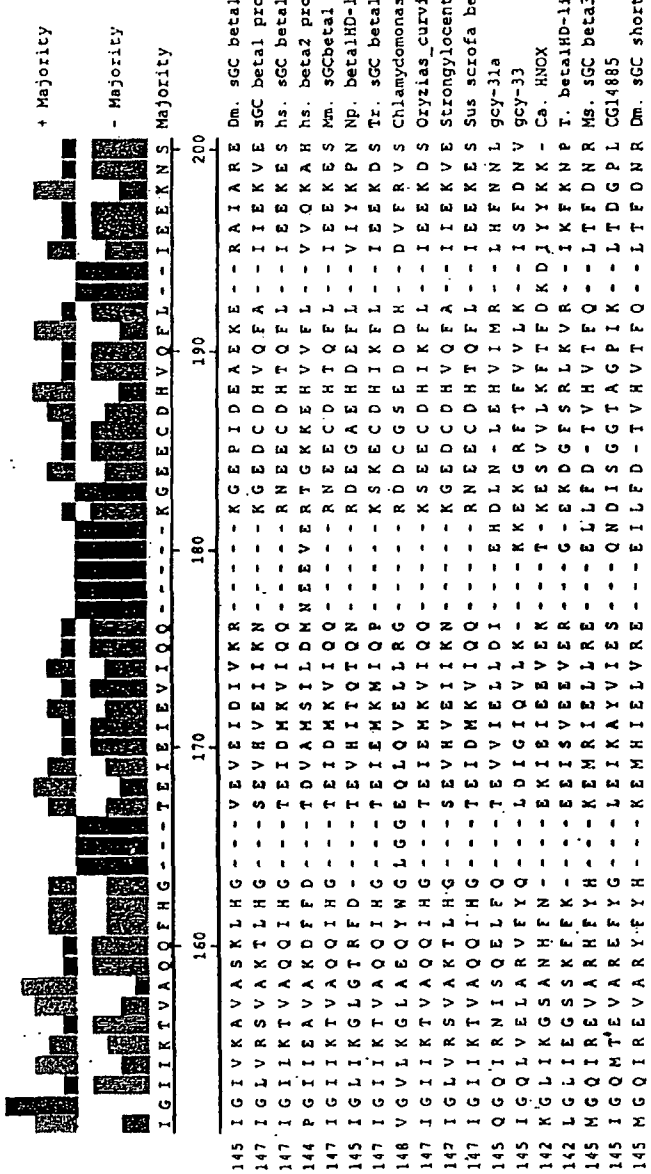
Ms. sGC beta3 protein
CG14685
Dm. sGC short variant

Figure 4C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- Majority
Majority
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180
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sGC beta1 protein
hs. sGC beta1 protein
hs. beta2 protein
Mm. sGCbeta1 protein
Np. beta1HD-like
Tr. sGC beta1 protein
Chlamydomonas_reinhardtii|AAR02
Oryzias_curvinotus|BAC98396
Strongylocentrotus_purpuratus|X
Sus scrofa beta1|NP_001018042
gcy-31a
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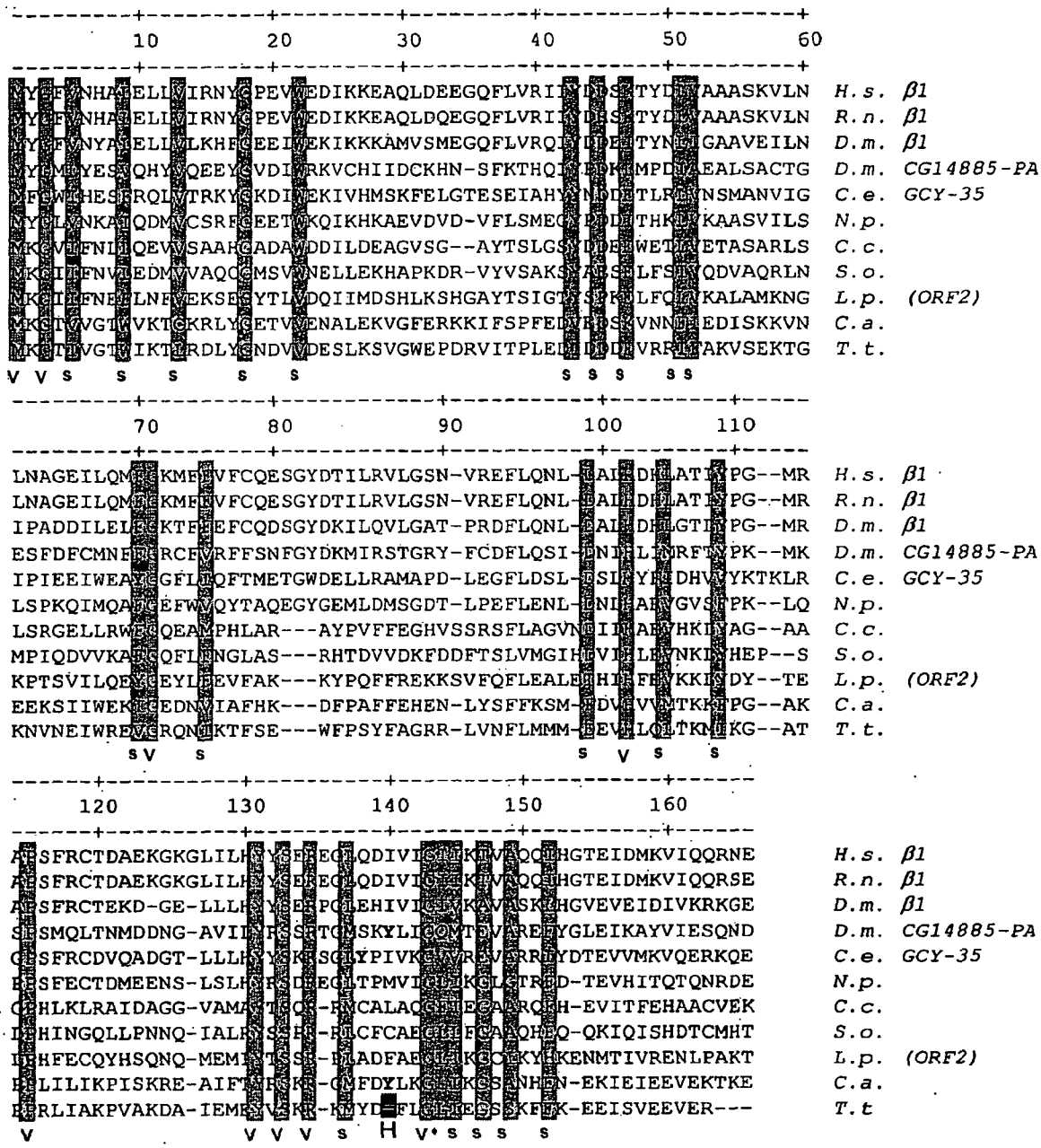
Ca. HNOX
T. beta1HD-like
Ms. sGC beta3 protein
CG14885
Dm. sGC short variant

Figure 4D

Figure 5A



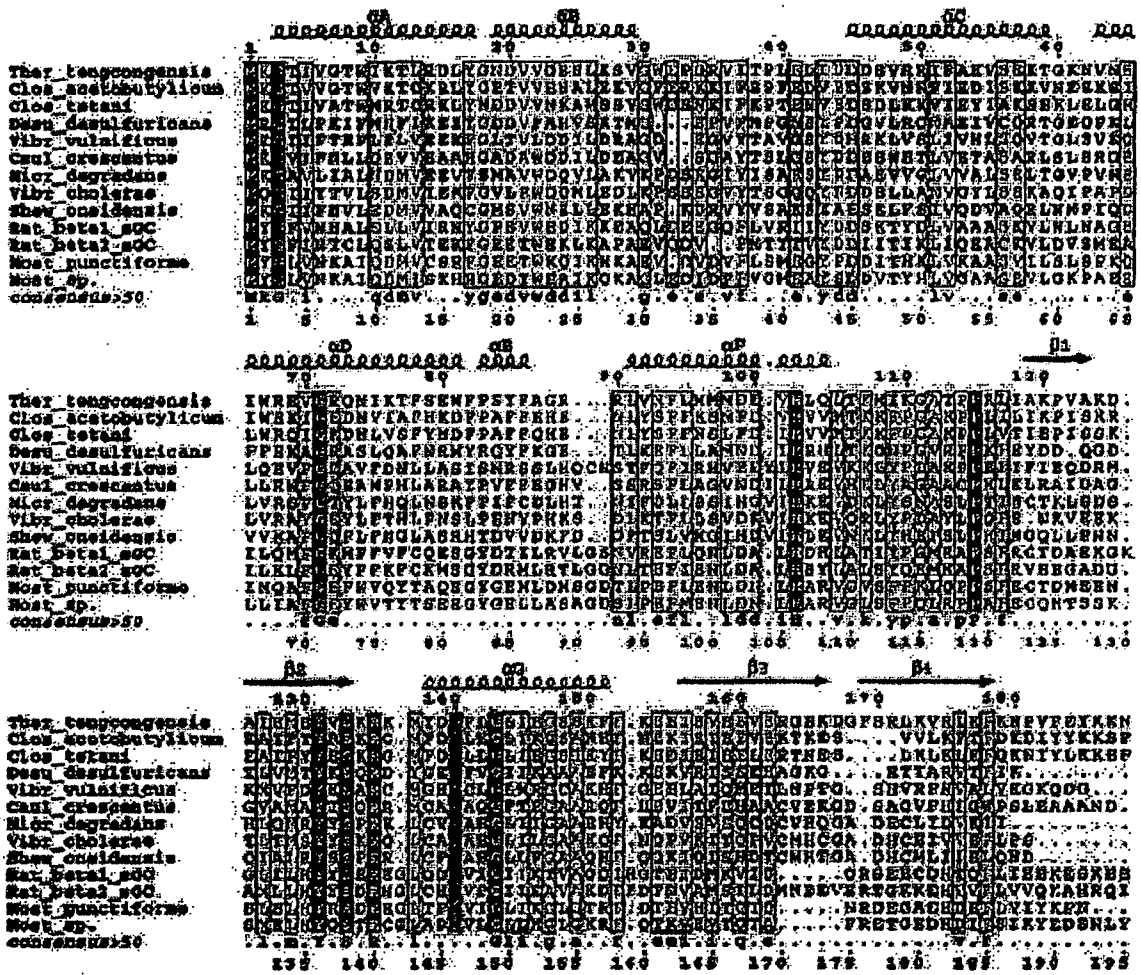
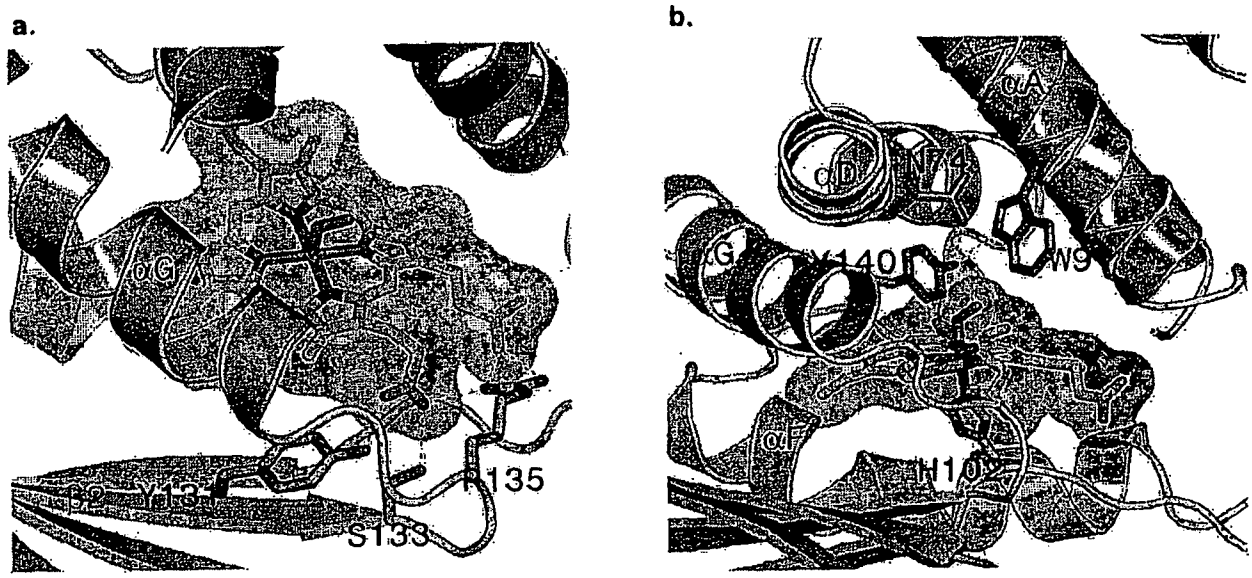
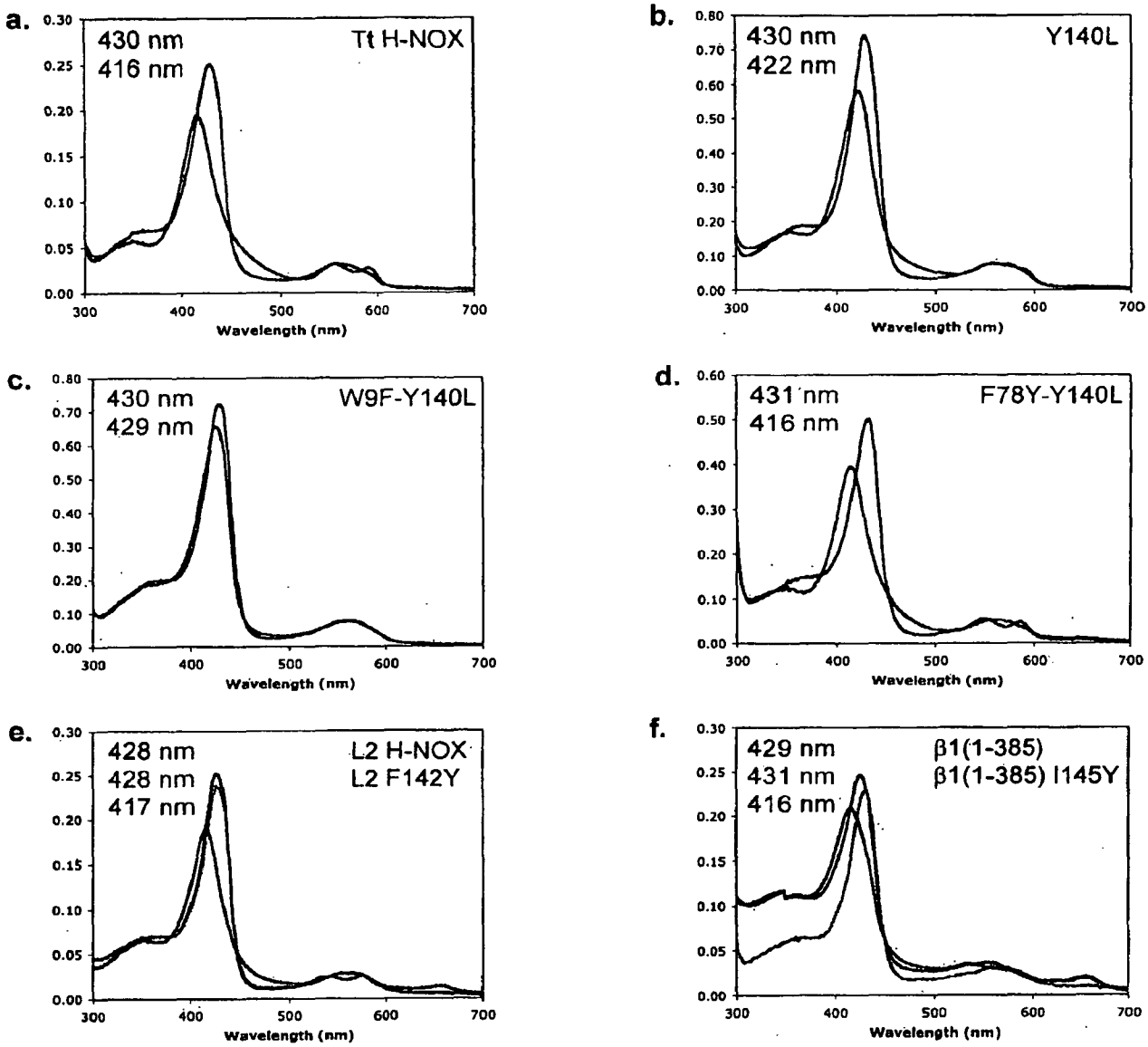


Figure 5B

Figures 6A and 6B



Figures 7A Thru 7F



Sequences of Mutant H-NOX and the Parent WT H-NOX

NUCLEOTIDES followed by AMINO ACIDS

Thermoanaerobacter tengcongensis H-NOX

***Tt.* WT**

(SEQ ID NO:53)
ATGAAGGGGACAATCGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTGA

(SEQ ID NO:54)
MKGTIVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFE

***Tt.* Y140F**

(SEQ ID NO:55)
ATGAAGGGGACAATCGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTTCTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTGA

(SEQ ID NO:56)
MKGTIVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDFFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFE

Figure 8A

***Tt.* Y140L**

(SEQ ID NO:57)
ATGAAGGGGACAATCGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATCTTTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTGA

(SEQ ID NO:58)
MKGTIVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDLFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFE

***Tt.* Y140H**

(SEQ ID NO:59)
ATGAAGGGGACAATCGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATCACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTGA

(SEQ ID NO:60)
MKGTIVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDHFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFE

***Tt.* Y140A**

(SEQ ID NO:61)
ATGAAGGGGACAATCGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATGCCTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTGA

(SEQ ID NO:62)
MKGTIVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDAFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFE

Figure 8B

***Tt.* W9F**

(SEQ ID NO:63)
ATGAAGGGGACAATCGTCGGGACATTTATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTGA

(SEQ ID NO:64)
MKGTIVGTFIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFE

***Tt.* W9F/Y140L**

(SEQ ID NO:65)
ATGAAGGGGACAATCGTCGGGACATTTATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTTTTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTGA

(SEQ ID NO:66)
MKGTIVGTFIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDFFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFE

***Tt.* W9F/Y140H**

(SEQ ID NO:67)
ATGAAGGGGACAATCGTCGGGACATTTATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATCACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTGA

(SEQ ID NO:68)
MKGTIVGTFIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDHFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFE

Figure 8C

Tt. W9F-N74A

(SEQ ID NO:69)
ATGAAGGGGACAATCGTCGGGACATTTATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGGCAATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTGA

(SEQ ID NO:70)
MKGTIVGTFIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQAIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFE

Tt. W9Y

(SEQ ID NO:71)
ATGAAGGGGACAATCGTCGGGACATACATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTGA

(SEQ ID NO:72)
MKGTIVGTYIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFE

Tt. W9N

(SEQ ID NO:73)
ATGAAGGGGACAATCGTCGGGACAAATATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTGA

(SEQ ID NO:74)
MKGTIVGTNIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFE

Figure 8D

***Tt.* W9H**

(SEQ ID NO:75)
ATGAAGGGGACAATCGTCGGGACACACATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTGA

(SEQ ID NO:76)
MKGTIVGTHIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFE

***Tt.* I5A**

(SEQ ID NO:77)
ATGAAGGGGACAGCAGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTGA

(SEQ ID NO:78)
MKGTAVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNE
IWREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFE

***Tt.* I5L**

(SEQ ID NO:79)
ATGAAGGGGACACTTGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTGA

(SEQ ID NO:80)
MKGTLVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNE
IWREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFE

Figure 8E

***Tt.* I5L-P115A**

(SEQ ID NO:81)
ATGAAGGGGACACTTGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTGCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTGA

(SEQ ID NO:82)
MKGTLVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNE
IWREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPARLIAKPVAKDAIEME
YVSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFE

***Tt.* P115A**

(SEQ ID NO:83)
ATGAAGGGGACAATCGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTGCCAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTGA

(SEQ ID NO:84)
MKGTIVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPARLIAKPVAKDAIEME
YVSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFE

***Tt.* N74E**

(SEQ ID NO:85)
ATGAAGGGGACAATCGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGGAAATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTATAAGAAA
AATTGA

(SEQ ID NO:86)
MKGTIVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQEIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEMEY
VSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFEYKKN

Figure 8F

***Tt.* N74A/Y140H**

(SEQ ID NO:87)
ATGAAGGGGACAATCGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGGCCATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATCACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTATAAGAAA
AATTGA

(SEQ ID NO:88)
MKGTIVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQAIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDHFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFEYKKN

***Tt.* R135Q-His6**

(SEQ ID NO:89)
ATGAAGGGGACAATCGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAACAGAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTATAAGAAA
AATCTCGAGCACCACCACCACCACCACTGA

(SEQ ID NO:90)
MKGTIVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKQKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFEYKKNLEHHHHHH

Figure 8G

Tt. N74A

(SEQ ID NO:91)
ATGAAGGGGACAATCGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGGCCATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTATAAGAAA
AATTGA

(SEQ ID NO:92)
MKGTIVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQAIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFEYKKN

Tt. N74A-His6

(SEQ ID NO:93)
ATGAAGGGGACAATCGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGGCCATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTATAAGAAA
AATCTCGAGCACCACCACCACCACCACTGA

(SEQ ID NO:94)
MKGTIVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQAIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFEYKKNLEHHHHHH

Figure 8H

Tt. W9N

(SEQ ID NO:95)
ATGAAGGGGACAATCGTCGGGACAAATATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTATAAGAAA
AATTGA

(SEQ ID NO:96)
MKGTIVGTNIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFEYKKN

Tt. W9H

(SEQ ID NO:97)
ATGAAGGGGACAATCGTCGGGACACATATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTATAAGAAA
AATTGA

(SEQ ID NO:98)
MKGTIVGTHIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFEYKKN

Figure 8I

***Tt.* N74H**

(SEQ ID NO:99)
ATGAAGGGGACAATCGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGCATATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTATAAGAAA
AATTGA

(SEQ ID NO:100)
MKGTIVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQHIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFEYKKN

***Tt.* I75F**

(SEQ ID NO:101)
ATGAAGGGGACAATCGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACTTCAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGCACCACCAC
CACCACCACTGA

(SEQ ID NO:102)
MKGTIVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNFKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFEHHHHHH

Figure 8J

Tt. L144F

(SEQ ID NO:103)
ATGAAGGGGACAATCGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGTTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGCACCACCAC
CACCACCACTGA

(SEQ ID NO:104)
MKGTIVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDYFLGFIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFEHHHHHH

Tt. WT-His6

(SEQ ID NO:105)
ATGAAGGGGACAATCGTCGGGACATGGATAAAGACCCTGAGGGACCTTTACGGGAATGATGT
GGTTGATGAATCTTTAAAAAGTGTGGGTTGGGAACCAGATAGGGTAATTACACCTCTGGAGG
ATATTGATGACGATGAGGTTAGGAGAATTTTTGCTAAGGTGAGTGAAAAAACTGGTAAAAAT
GTCAACGAAATATGGAGAGAGGTAGGAAGGCAGAACATAAAAACTTTCAGCGAATGGTTTCC
CTCCTATTTTGCAGGGAGAAGGCTAGTGAATTTTTTAATGATGATGGATGAGGTACACCTACA
GCTTACCAAGATGATAAAAGGAGCCACTCCTCCAAGGCTTATTGCAAAGCCTGTTGCAAAAG
ATGCCATTGAAATGGAGTACGTTTCTAAAAGAAAGATGTACGATTACTTTTTAGGGCTTATAG
AGGGTAGTTCTAAATTTTTCAAGGAAGAAATTTCAGTGGAAGAGGTCGAAAGAGGCGAAAAA
GATGGCTTTTCAAGGCTAAAAGTCAGGATAAAATTTAAAAACCCCGTTTTTGAGTATAAGAAA
AATCTCGAGCACCACCACCACCACCACTGA

(SEQ ID NO:106)
MKGTIVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRRIFAKVSEKTGKNVNEI
WREVGRQNIKTFSEWFPSYFAGRRLVNFLMMMDEVHLQLTKMIKGATPPRLIAKPVAKDAIEME
YVSKRKMYDYFLGLIEGSSKFFKEEISVEEVERGEKDGFSRLKVRIKFKNPVFEYKKNLEHHHHHH

Figure 8K

Legionella pneumophila ORF2

***L2* WT**

(SEQ ID NO:107)
ATGATGTCTATGAAAGGAATCATATTCAACGAATTTCTCAATTTTGTAGAAAAAAGTGAATCC
TACACCCTGGTAGATCAAATTATTATGGATAGTCATTTGAAGTCCCATGGTGCCTACACGTCT
ATCGGTACATACTCTCCCAAAGAATTATTTCAATTGGTTAAAGCGCTTGCTATGAAAAATGGC
AAACCAACATCAGTGATTTTACAAGAATATGGTGAGTATTTGTTTGAGGTTTTTGCAAAAAAA
TATCCTCAATTTTTCAGGGAAAAAAAGTCGGTGTTTCAATTTTTGGAAGCGCTTGAAACACAT
ATTCATTTCGAAGTGAAAAAATTGTATGACTATACTGAACTACCCCATTTTGAATGCCAATAT
CACAGTCAAAATCAAATGGAAATGATTTACACTTCTTCGCGTCCTTTGGCCGATTTTGCGGAA
GGTTTAATAAAAGGTTGTATTAAATATCATAAAGAAAACATGACTATTGTTCGTGAAAATCTG
CCTGCAAAAACAGGCTTTAAGGTAAGATTTGTATTAACAAAAGGCGATCCTGATGAGTGA

(SEQ ID NO:108)
MMSMKGIIFNEFLNFVEKSESYTLVDQIIMDSHLKSHGAYTSIGTYSPKELFQLVKALAMKNGKPT
SVILQEYGEYLFEVFAKKYPQFFREKKSVFQFLEALETHIHFEVKKLYDYTELPHFECQYHSQNQM
EMIYTSSRPLADFAEGLIKGCIKYHKENMTIVRENLPAKTGFKVRFVLTKGDPDE

***L2* F142Y**

(SEQ ID NO:109)
ATGATGTCTATGAAAGGAATCATATTCAACGAATTTCTCAATTTTGTAGAAAAAAGTGAATCC
TACACCCTGGTAGATCAAATTATTATGGATAGTCATTTGAAGTCCCATGGTGCCTACACGTCT
ATCGGTACATACTCTCCCAAAGAATTATTTCAATTGGTTAAAGCGCTTGCTATGAAAAATGGC
AAACCAACATCAGTGATTTTACAAGAATATGGTGAGTATTTGTTTGAGGTTTTTGCAAAAAAA
TATCCTCAATTTTTCAGGGAAAAAAAGTCGGTGTTTCAATTTTTGGAAGCGCTTGAAACACAT
ATTCATTTCGAAGTGAAAAAATTGTATGACTATACTGAACTACCCCATTTTGAATGCCAATAT
CACAGTCAAAATCAAATGGAAATGATTTACACTTCTTCGCGTCCTTTGGCCGATTATGCGGAA
GGTTTAATAAAAGGTTGTATTAAATATCATAAAGAAAACATGACTATTGTTCGTGAAAATCTG
CCTGCAAAAACAGGCTTTAAGGTAAGATTTGTATTAACAAAAGGCGATCCTGATGAGTGA

(SEQ ID NO:110)
MMSMKGIIFNEFLNFVEKSESYTLVDQIIMDSHLKSHGAYTSIGTYSPKELFQLVKALAMKNGKPT
SVILQEYGEYLFEVFAKKYPQFFREKKSVFQFLEALETHIHFEVKKLYDYTELPHFECQYHSQNQM
EMIYTSSRPLADYAEGLIKGCIKYHKENMTIVRENLPAKTGFKVRFVLTKGDPDE

Figure 8L

***L2* F9W-F142Y**

(SEQ ID NO:111)
ATGATGTCTATGAAAGGAATCATATGGAACGAATTTCTCAATTTTGTAGAAAAAAGTGAATCC
TACACCCTGGTAGATCAAATTATTATGGATAGTCATTTGAAGTCCCATGGTGCCTACACGTCT
ATCGGTACATACTCTCCCAAAGAATTATTTCAATTGGTTAAAGCGCTTGCTATGAAAAATGGC
AAACCAACATCAGTGATTTTACAAGAATATGGTGAGTATTTGTTTGAGGTTTTTGCAAAAAAA
TATCCTCAATTTTTCAGGGAAAAAAAGTCGGTGTTTCAATTTTTGGAAGCGCTTGAAACACAT
ATTCATTTCGAAGTGAAAAAATTGTATGACTATACTGAACTACCCCATTTTGAATGCCAATAT
CACAGTCAAAATCAAATGGAAATGATTTACACTTCTTCGCGTCCTTTGGCCGATTATGCGGAA
GGTTTAATAAAAGGTTGTATTAAATATCATAAAGAAAACATGACTATTGTTCGTGAAAATCTG
CCTGCAAAAACAGGCTTTAAGGTAAGATTTGTATTAACAAAAGGCGATCCTGATGAGTGA

(SEQ ID NO:112)
MMSMKGIIWNEFLNFVEKSESYTLVDQIIMDSHLKSHGAYTSIGTYSPKELFQLVKALAMKNGKP
TSVILQEYGEYLFEVFAKKYPQFFREKKSVFQFLEALETHIHFEVKKLYDYTELPHFECQYHSQNQ
MEMIYTSSRPLADYAEGLIKGCIKYHKENMTIVRENLPAKTGFKVRFVLTKGDPDE

Legionella pneumophila ORF1

***L1* WT**

(SEQ ID NO:113)
ATGAAAGGTATCGTTTTTACCTCCTTAAATGACATGATTATAGAACAATTTGGCATAGAAACC
TGGGACCAACTCGTATCCTCACTAGACCTTCCAAGTGGTGGAAGTTATACAGCAGGCGGCACT
TACTCGGATACAGAATTTCAGCAATTGATTAAGGCCATTGCGAAGAGGACCAATCAGCACGCT
TCTGTTTTTTTAGAGGCCTTTGGTGAATACATGTTTCCTATCTTATCGAGTAAGTGCGCAATTTT
TTTAAAAAAGGACATGACATTAAAAGAATTTTTAAAAAGCATTGATGGAACAATTCATGTGG
AAGTAGAAAAGTTATACCCAGATGAAACATTACCTACCATTAGCTATGAAGAGCCTGCTGCA
AACCAATTGGTTATGGTGTATCGATCGCATAGAAGACTCTGTCATTTTGCAATGGGGCTCATC
CAGGGAGCAGCGCAACATTTTAAAAAGAAAATTACCATTAAGCAGACTCACTGCATGTTAAA
AAAAGATGATCATTGTCGTTTGGAGATTACCTTTGAGTGA

(SEQ ID NO:114)
MKGIVFTSLNDMIIEQFGIETWDQLVSSLDLPSGGSYTAGGTYSDTEFQQLIKAIAKRTNQHASVFL
EAFGEYMFPILSSKCAIFLKKDMTLKEFLKSIDGTIHVEVEKLYPDETLPTISYEEPAANQLVMVYR
SHRRLCHFAMGLIQGAAQHFKKKITKQTHCMLKKDDHCRLEITFE

Figure 8M

***LI* F142Y**

(SEQ ID NO:115)
ATGAAAGGTATCGTTTTTACCTCCTTAAATGACATGATTATAGAACAATTTGGCATAGAAACC
TGGGACCAACTCGTATCCTCACTAGACCTTCCAAGTGGTGGAAGTTATACAGCAGGCGGCACT
TACTCGGATACAGAATTTCAGCAATTGATTAAGGCCATTGCGAAGAGGACCAATCAGCACGCT
TCTGTTTTTTTAGAGGCCTTTGGTGAATACATGTTTCCTATCTTATCGAGTAAGTGCGCAATTTT
TTTAAAAAAGGACATGACATTAAAAGAATTTTTAAAAAGCATTGATGGAACAATTCATGTGG
AAGTAGAAAAGTTATACCCAGATGAAACATTACCTACCATTAGCTATGAAGAGCCTGCTGCA
AACCAATTGGTTATGGTGTATCGATCGCATAGAAGACTCTGTCATTACGCAATGGGGCTCATC
CAGGGAGCAGCGCAACATTTTAAAAAGAAAATTACCATTAAGCAGACTCACTGCATGTTAAA
AAAAGATGATCATTGTCGTTTGGAGATTACCTTTGAGTGA

(SEQ ID NO:116)
MKGIVFTSLNDMIIEQFGIETWDQLVSSLDLPSGGSYTAGGTYSDTEFQQLIKAIAKRTNQHASVFL
EAFGEYMFPILSSKCAIFLKKDMTLKEFLKSIDGTIHVEVEKLYPDETLPTISYEEPAANQLVMVYR
SHRRLCHYAMGLIQGAAQHFKKKITIKQTHCMLKKDDHCRLEITFE

Desulfovibrio desulfuricans

***Dd* H-NOX(728-899)**

(SEQ ID NO:117)
ATGAAGATGCGCGGTATTTTGCCGAAAATATTTATGAATTTTATAAAAGAGATCTATGGGGAT
GACGTGTTTGCTCATGTTTCTAAAACCATGGGCGAGCCTGTCTTCATGCCGGGAAATTCCTACC
CTGATCAGGTGTTGCGCCAGATGGCTGAAATAGTATGCCAGCGCACGGGCGAACAGCCCAAG
TTGTTTTTTGAAAAAGCAGGGCGTGCAAGCCTGCAGGCTTTTAACAGAATGTACAGGCAGTAC
TTTAAAGGGGAAACCCTTAAAGAGTTTCTGCTGGCCATGAATGATATCCACAGGCACCTGACA
AAGGACAATCCCGGCGTACGCCCGCCTAAATTTGAGTATGACGATCAGGGCGATACGCTTGTT
ATGACATATAAGTCGCAGAGGGATTACGGAGAATACTTTGTGGGCATCATCAAGGCAGCTGC
GGAGTTTAAAAAGGAAAAAGTGCGTATCAGCTCGGAGCATGCCGGTAAGGGGCGAACAACG
GCAAGGGTTACATTTATTAAATGA

(SEQ ID NO:118)
MKMRGILPKIFMNFIKEIYGDDVFAHVSKTMGEPVFMPGNSYPDQVLRQMAEIVCQRTGEQPKLF
FEKAGRASLQAFNRMYRQYFKGETLKEFLLAMNDIHRHLTKDNPGVRPPKFEYDDQGDTLVMTY
KSQRDYGEYFVGIIKAAAEFKKEKVRISSEHAGKGRTTARVTFIK

Figure 8N

***Dd.* Y139L (728-899)**

(SEQ ID NO:119)
ATGAAGATGCGCGGTATTTTGCCGAAAATATTTATGAATTTTATAAAAGAGATCTATGGGGAT
GACGTGTTTGCTCATGTTTCTAAAACCATGGGCGAGCCTGTCTTCATGCCGGGAAATTCCTACC
CTGATCAGGTGTTGCGCCAGATGGCTGAAATAGTATGCCAGCGCACGGGCGAACAGCCCAAG
TTGTTTTTTGAAAAAGCAGGGCGTGCAAGCCTGCAGGCTTTTAACAGAATGTACAGGCAGTAC
TTTAAAGGGGAAACCCTTAAAGAGTTTCTGCTGGCCATGAATGATATCCACAGGCACCTGACA
AAGGACAATCCCGGCGTACGCCCGCCTAAATTTGAGTATGACGATCAGGGCGATACGCTTGTT
ATGACATATAAGTCGCAGAGGGATTACGGAGAACTTTTTGTGGGCATCATCAAGGCAGCTGC
GGAGTTTAAAAAGGAAAAAGTGCGTATCAGCTCGGAGCATGCCGGTAAGGGGCGAACAACG
GCAAGGGTTACATTTATTAAATGA

(SEQ ID NO:120)
MKMRGILPKIFMNFIKEIYGDDVFAHVSKTMGEPVFMPGNSYPDQVLRQMAEIVCQRTGEQPKLF
FEKAGRASLQAFNRMYRQYFKGETLKEFLLAMNDIHRHLTKDNPGVRPPKFEYDDQGDTLVMTY
KSQRDYGELFVGIIKAAAEFKKEKVRISSEHAGKGRTTARVTFIK

Homo sapiens β1(1-385)

***Hs.* WT (1-385)**

(SEQ ID NO:121)
ATGTACGGATTTGTGAATCACGCCCTGGAGTTGCTGGTGATCCGCAATTACGGCCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCACAGTTAGATGAAGAAGGACAGTTTCTTGTCAGAATAAT
ATATGATGACTCCAAAACTTATGATTTGGTTGCTGCTGCAAGCAAAGTCCTCAATCTCAATGC
TGGAGAAATCCTCCAAATGTTTGGGAAGATGTTTTTCGTCTTTTGCCAAGAATCTGGTTATGAT
ACAATCTTGCGTGTCCTGGGCTCTAATGTCAGAGAATTTCTACAGAACCTTGATGCTCTGCAC
GACCACCTTGCTACCATCTACCCAGGAATGCGTGCACCTTCCTTTAGGTGCACTGATGCAGAA
AAGGGCAAAGGACTCATTTTGCACTACTACTCAGAGAGAGAAGGACTTCAGGATATTGTCATT
GGAATCATCAAAACAGTGGCACAACAAATCCATGGCACTGAAATAGACATGAAGGTTATTCA
GCAAAGAAATGAAGAATGTGATCATACTCAATTTTTAATTGAAGAAAAAGAGTCAAAAGAAG
AGGATTTTTATGAAGATCTTGACAGATTTGAAGAAAATGGTACCCAGGAATCACGCATCAGCC
CATATACATTCTGCAAAGCTTTTCCTTTTCATATAATATTTGACCGGGACCTAGTGGTCACTCA
GTGTGGCAATGCTATATACAGAGTTCTCCCCCAGCTCCAGCCTGGGAATTGCAGCCTTCTGTCT
GTCTTCTCGCTGGTTCGTCCTCATATTGATATTAGTTTCCATGGGATCCTTTCTCACATCAATAC
TGTTTTTGTATTGAGAAGCAAGGAAGGATTGTTGGATGTGGAGAAATTAGAATGTGAGGATG
AACTGACTGGGACTGAGATCAGCTGCTTACGTCTCAAGGGTCAAATGATCTACTTACCTGAAG
CAGATAGCATACTTTTTCTATGTTCACCAAGTGTCATGAACCTGGACGATTTGACAAGGAGAG
GGCTGTATCTAAGTGACATCCCTCTGCATGATGCCACGCGCGATCTTGTTCTTTTGGGAGAAC
AATTTAGAGAGGAATACAAACTCACCCAAGAACTGGAAATCCTCACTGACAGGCTACAGCTC
ACGTTAAGAGCCCTGGAAGATTGA

(SEQ ID NO:122)
MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAGE
ILQMFGKMFFVFCQESGYDTILRVLGSNVREFLQNLDALHDHLATIYPGMRAPSFRCTDAEKGKG
LILHYYSEREGLQDIVIGIIKTVAQQIHGTEIDMKVIQQRNEECDHTQFLIEEKESKEEDFYEDLDRF
EENGTQESRISPYTFCKAFPFHIIFDRDLVVTQCGNAIYRVLPQLQPGNCSLLSVFSLVRPHIDISFHG
ILSHINTVFVLRSKEGLLDVEKLECEDELTGTEISCLRLKGQMIYLPEADSILFLCSPSVMNLDDLTR
RGLYLSDIPLHDATRDLVLLGEQFREEYKLTQELEILTDRLQLTLRALED

Figure 8O

***Hs.* β1(1-385) I145Y**

(SEQ ID NO:123)
ATGTACGGATTTGTGAATCACGCCCTGGAGTTGCTGGTGATCCGCAATTACGGCCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCACAGTTAGATGAAGAAGGACAGTTTCTTGTCAGAATAAT
ATATGATGACTCCAAAACTTATGATTTGGTTGCTGCTGCAAGCAAAGTCCTCAATCTCAATGC
TGGAGAAATCCTCCAAATGTTTGGGAAGATGTTTTTCGTCTTTTGCCAAGAATCTGGTTATGAT
ACAATCTTGCGTGTCCTGGGCTCTAATGTCAGAGAATTTCTACAGAACCTTGATGCTCTGCAC
GACCACCTTGCTACCATCTACCCAGGAATGCGTGCACCTTCCTTTAGGTGCACTGATGCAGAA
AAGGGCAAAGGACTCATTTTGCACTACTACTCAGAGAGAGAAGGACTTCAGGATTATGTCATT
GGAATCATCAAAACAGTGGCACAACAAATCCATGGCACTGAAATAGACATGAAGGTTATTCA
GCAAAGAAATGAAGAATGTGATCATACTCAATTTTTAATTGAAGAAAAAGAGTCAAAAGAAG
AGGATTTTTATGAAGATCTTGACAGATTTGAAGAAAATGGTACCCAGGAATCACGCATCAGCC
CATATACATTCTGCAAAGCTTTTCCTTTTCATATAATATTTGACCGGGACCTAGTGGTCACTCA
GTGTGGCAATGCTATATACAGAGTTCTCCCCCAGCTCCAGCCTGGGAATTGCAGCCTTCTGTCT
GTCTTCTCGCTGGTTCGTCCTCATATTGATATTAGTTTCCATGGGATCCTTTCTCACATCAATAC
TGTTTTTGTATTGAGAAGCAAGGAAGGATTGTTGGATGTGGAGAAATTAGAATGTGAGGATG
AACTGACTGGGACTGAGATCAGCTGCTTACGTCTCAAGGGTCAAATGATCTACTTACCTGAAG
CAGATAGCATACTTTTTCTATGTTCACCAAGTGTCATGAACCTGGACGATTTGACAAGGAGAG
GGCTGTATCTAAGTGACATCCCTCTGCATGATGCCACGCGCGATCTTGTTCTTTTGGGAGAAC
AATTTAGAGAGGAATACAAACTCACCCAAGAACTGGAAATCCTCACTGACAGGCTACAGCTC
ACGTTAAGAGCCCTGGAAGATTGA

(SEQ ID NO:124)
MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAGE
ILQMFGKMFFVFCQESGYDTILRVLGSNVREFLQNLDALHDHLATIYPGMRAPSFRCTDAEKGKG
LILHYYSEREGLQDYVIGIIKTVAQQIHGTEIDMKVIQQRNEECDHTQFLIEEKESKEEDFYEDLDRF
EENGTQESRISPYTFCKAFPFHIIFDRDLVVTQCGNAIYRVLPQLQPGNCSLLSVFSLVRPHIDISFHG
ILSHINTVFVLRSKEGLLDVEKLECEDELTGTEISCLRLKGQMIYLPEADSILFLCSPSVMNLDDLTR
RGLYLSDIPLHDATRDLVLLGEQFREEYKLTQELEILTDRLQLTLRALED

Figure 8P

***Hs.* β1(1-385) I145H**

(SEQ ID NO:125)
ATGTACGGATTTGTGAATCACGCCCTGGAGTTGCTGGTGATCCGCAATTACGGCCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCACAGTTAGATGAAGAAGGACAGTTTCTTGTCAGAATAAT
ATATGATGACTCCAAAACTTATGATTTGGTTGCTGCTGCAAGCAAAGTCCTCAATCTCAATGC
TGGAGAAATCCTCCAAATGTTTGGGAAGATGTTTTTCGTCTTTTGCCAAGAATCTGGTTATGAT
ACAATCTTGCGTGTCCTGGGCTCTAATGTCAGAGAATTTCTACAGAACCTTGATGCTCTGCAC
GACCACCTTGCTACCATCTACCCAGGAATGCGTGCACCTTCCTTTAGGTGCACTGATGCAGAA
AAGGGCAAAGGACTCATTTTGCACTACTACTCAGAGAGAGAAGGACTTCAGGATCATGTCATT
GGAATCATCAAAACAGTGGCACAACAAATCCATGGCACTGAAATAGACATGAAGGTTATTCA
GCAAAGAAATGAAGAATGTGATCATACTCAATTTTTAATTGAAGAAAAAGAGTCAAAAGAAG
AGGATTTTTATGAAGATCTTGACAGATTTGAAGAAAATGGTACCCAGGAATCACGCATCAGCC
CATATACATTCTGCAAAGCTTTTCCTTTTCATATAATATTTGACCGGGACCTAGTGGTCACTCA
GTGTGGCAATGCTATATACAGAGTTCTCCCCCAGCTCCAGCCTGGGAATTGCAGCCTTCTGTCT
GTCTTCTCGCTGGTTCGTCCTCATATTGATATTAGTTTCCATGGGATCCTTTCTCACATCAATAC
TGTTTTTGTATTGAGAAGCAAGGAAGGATTGTTGGATGTGGAGAAATTAGAATGTGAGGATG
AACTGACTGGGACTGAGATCAGCTGCTTACGTCTCAAGGGTCAAATGATCTACTTACCTGAAG
CAGATAGCATACTTTTTCTATGTTCACCAAGTGTCATGAACCTGGACGATTTGACAAGGAGAG
GGCTGTATCTAAGTGACATCCCTCTGCATGATGCCACGCGCGATCTTGTTCTTTTGGGAGAAC
AATTTAGAGAGGAATACAAACTCACCCAAGAACTGGAAATCCTCACTGACAGGCTACAGCTC
ACGTTAAGAGCCCTGGAAGATTGA

(SEQ ID NO:126)
MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAGE
ILQMFGKMFFVFCQESGYDTILRVLGSNVREFLQNLDALHDHLATIYPGMRAPSFRCTDAEKGKG
LILHYYSEREGLQDHVIGIIKTVAQQIHGTEIDMKVIQQRNEECDHTQFLIEEKESKEEDFYEDLDRF
EENGTQESRISPYTFCKAFPFHIIFDRDLVVTQCGNAIYRVLPQLQPGNCSLLSVFSLVRPHIDISFHG
ILSHINTVFVLRSKEGLLDVEKLECEDELTGTEISCLRLKGQMIYLPEADSILFLCSPSVMNLDDLTR
RGLYLSDIPLHDATRDLVLLGEQFREEYKLTQELEILTDRLQLTLRALED

Figure 8Q

***Hs.* β1(1-385) C78Y**

(SEQ ID NO:127)
ATGTACGGATTTGTGAATCACGCCCTGGAGTTGCTGGTGATCCGCAATTACGGCCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCACAGTTAGATGAAGAAGGACAGTTTCTTGTCAGAATAAT
ATATGATGACTCCAAAACTTATGATTTGGTTGCTGCTGCAAGCAAAGTCCTCAATCTCAATGC
TGGAGAAATCCTCCAAATGTTTGGGAAGATGTTTTTCGTCTTTTACCAAGAATCTGGTTATGAT
ACAATCTTGCGTGTCCTGGGCTCTAATGTCAGAGAATTTCTACAGAACCTTGATGCTCTGCAC
GACCACCTTGCTACCATCTACCCAGGAATGCGTGCACCTTCCTTTAGGTGCACTGATGCAGAA
AAGGGCAAAGGACTCATTTTGCACTACTACTCAGAGAGAGAAGGACTTCAGGATATTGTCATT
GGAATCATCAAAACAGTGGCACAACAAATCCATGGCACTGAAATAGACATGAAGGTTATTCA
GCAAAGAAATGAAGAATGTGATCATACTCAATTTTTAATTGAAGAAAAAGAGTCAAAAGAAG
AGGATTTTTATGAAGATCTTGACAGATTTGAAGAAAATGGTACCCAGGAATCACGCATCAGCC
CATATACATTCTGCAAAGCTTTTCCTTTTCATATAATATTTGACCGGGACCTAGTGGTCACTCA
GTGTGGCAATGCTATATACAGAGTTCTCCCCCAGCTCCAGCCTGGGAATTGCAGCCTTCTGTCT
GTCTTCTCGCTGGTTCGTCCTCATATTGATATTAGTTTCCATGGGATCCTTTCTCACATCAATAC
TGTTTTTGTATTGAGAAGCAAGGAAGGATTGTTGGATGTGGAGAAATTAGAATGTGAGGATG
AACTGACTGGGACTGAGATCAGCTGCTTACGTCTCAAGGGTCAAATGATCTACTTACCTGAAG
CAGATAGCATACTTTTTCTATGTTCACCAAGTGTCATGAACCTGGACGATTTGACAAGGAGAG
GGCTGTATCTAAGTGACATCCCTCTGCATGATGCCACGCGCGATCTTGTTCTTTTGGGAGAAC
AATTTAGAGAGGAATACAAACTCACCCAAGAACTGGAAATCCTCACTGACAGGCTACAGCTC
ACGTTAAGAGCCCTGGAAGATTGA

(SEQ ID NO:128)
MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAGE
ILQMFGKMFFVFYQESGYDTILRVLGSNVREFLQNLDALHDHLATIYPGMRAPSFRCTDAEKGKG
LILHYYSEREGLQDIVIGIIKTVAQQIHGTEIDMKVIQQRNEECDHTQFLIEEKESKEEDFYEDLDRF
EENGTQESRISPYTFCKAFPFHIIFDRDLVVTQCGNAIYRVLPQLQPGNCSLLSVFSLVRPHIDISFHG
ILSHINTVFVLRSKEGLLDVEKLECEDELTGTEISCLRLKGQMIYLPEADSILFLCSPSVMNLDDLTR
RGLYLSDIPLHDATRDLVLLGEQFREEYKLTQELEILTDRLQLTLRALED

Figure 8R

***Hs.* β1 (1-385) H105F**

(SEQ ID NO:129)
ATGTACGGATTTGTGAATCACGCCCTGGAGTTGCTGGTGATCCGCAATTACGGCCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCACAGTTAGATGAAGAAGGACAGTTTCTTGTCAGAATAAT
ATATGATGACTCCAAAACTTATGATTTGGTTGCTGCTGCAAGCAAAGTCCTCAATCTCAATGC
TGGAGAAATCCTCCAAATGTTTGGGAAGATGTTTTTCGTCTTTTGCCAAGAATCTGGTTATGAT
ACAATCTTGCGTGTCCTGGGCTCTAATGTCAGAGAATTTCTACAGAACCTTGATGCTCTGTTCG
ACCACCTTGCTACCATCTACCCAGGAATGCGTGCACCTTCCTTTAGGTGCACTGATGCAGAAA
AGGGCAAAGGACTCATTTTGCACTACTACTCAGAGAGAGAAGGACTTCAGGATATTGTCATTG
GAATCATCAAAACAGTGGCACAACAAATCCATGGCACTGAAATAGACATGAAGGTTATTCAG
CAAAGAAATGAAGAATGTGATCATACTCAATTTTTAATTGAAGAAAAAGAGTCAAAAGAAGA
GGATTTTTATGAAGATCTTGACAGATTTGAAGAAAATGGTACCCAGGAATCACGCATCAGCCC
ATATACATTCTGCAAAGCTTTTCCTTTTCATATAATATTTGACCGGGACCTAGTGGTCACTCAG
TGTGGCAATGCTATATACAGAGTTCTCCCCCAGCTCCAGCCTGGGAATTGCAGCCTTCTGTCTG
TCTTCTCGCTGGTTCGTCCTCATATTGATATTAGTTTCCATGGGATCCTTTCTCACATCAATACT
GTTTTTGTATTGAGAAGCAAGGAAGGATTGTTGGATGTGGAGAAATTAGAATGTGAGGATGA
ACTGACTGGGACTGAGATCAGCTGCTTACGTCTCAAGGGTCAAATGATCTACTTACCTGAAGC
AGATAGCATACTTTTTCTATGTTCACCAAGTGTCATGAACCTGGACGATTTGACAAGGAGAGG
GCTGTATCTAAGTGACATCCCTCTGCATGATGCCACGCGCGATCTTGTTCTTTTGGGAGAACA
ATTTAGAGAGGAATACAAACTCACCCAAGAACTGGAAATCCTCACTGACAGGCTACAGCTCA
CGTTAAGAGCCCTGGAAGATTGA

(SEQ ID NO:130)
MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAGE
ILQMFGKMFFVFCQESGYDTILRVLGSNVREFLQNLDALFDHLATIYPGMRAPSFRCTDAEKGKGL
ILHYYSEREGLQDIVIGIIKTVAQQIHGTEIDMKVIQQRNEECDHTQFLIEEKESKEEDFYEDLDRFE
ENGTQESRISPYTFCKAFPFHIIFDRDLVVTQCGNAIYRVLPQLQPGNCSLLSVFSLVRPHIDISFHGI
LSHINTVFVLRSKEGLLDVEKLECEDELTGTEISCLRLKGQMIYLPEADSILFLCSPSVMNLDDLTRR
GLYLSDIPLHDATRDLVLLGEQFREEYKLTQELEILTDRLQLTLRALED

Figure 8S

***Hs.* β1 (1-385) H105G**

(SEQ ID NO:131)
ATGTACGGATTTGTGAATCACGCCCTGGAGTTGCTGGTGATCCGCAATTACGGCCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCACAGTTAGATGAAGAAGGACAGTTTCTTGTCAGAATAAT
ATATGATGACTCCAAAACTTATGATTTGGTTGCTGCTGCAAGCAAAGTCCTCAATCTCAATGC
TGGAGAAATCCTCCAAATGTTTGGGAAGATGTTTTTCGTCTTTTGCCAAGAATCTGGTTATGAT
ACAATCTTGCGTGTCCTGGGCTCTAATGTCAGAGAATTTCTACAGAACCTTGATGCTCTGGGT
GACCACCTTGCTACCATCTACCCAGGAATGCGTGCACCTTCCTTTAGGTGCACTGATGCAGAA
AAGGGCAAAGGACTCATTTTGCACTACTACTCAGAGAGAGAAGGACTTCAGGATATTGTCATT
GGAATCATCAAAACAGTGGCACAACAAATCCATGGCACTGAAATAGACATGAAGGTTATTCA
GCAAAGAAATGAAGAATGTGATCATACTCAATTTTTAATTGAAGAAAAAGAGTCAAAAGAAG
AGGATTTTTATGAAGATCTTGACAGATTTGAAGAAAATGGTACCCAGGAATCACGCATCAGCC
CATATACATTCTGCAAAGCTTTTCCTTTTCATATAATATTTGACCGGGACCTAGTGGTCACTCA
GTGTGGCAATGCTATATACAGAGTTCTCCCCCAGCTCCAGCCTGGGAATTGCAGCCTTCTGTCT
GTCTTCTCGCTGGTTCGTCCTCATATTGATATTAGTTTCCATGGGATCCTTTCTCACATCAATAC
TGTTTTTGTATTGAGAAGCAAGGAAGGATTGTTGGATGTGGAGAAATTAGAATGTGAGGATG
AACTGACTGGGACTGAGATCAGCTGCTTACGTCTCAAGGGTCAAATGATCTACTTACCTGAAG
CAGATAGCATACTTTTTCTATGTTCACCAAGTGTCATGAACCTGGACGATTTGACAAGGAGAG
GGCTGTATCTAAGTGACATCCCTCTGCATGATGCCACGCGCGATCTTGTTCTTTTGGGAGAAC
AATTTAGAGAGGAATACAAACTCACCCAAGAACTGGAAATCCTCACTGACAGGCTACAGCTC
ACGTTAAGAGCCCTGGAAGATTGA

(SEQ ID NO:132)
MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAGE
ILQMFGKMFFVFCQESGYDTILRVLGSNVREFLQNLDALGDHLATIYPGMRAPSFRCTDAEKGKG
LILHYYSEREGLQDIVIGIIKTVAQQIHGTEIDMKVIQQRNEECDHTQFLIEEKESKEEDFYEDLDRF
EENGTQESRISPYTFCKAFPFHIIFDRDLVVTQCGNAIYRVLPQLQPGNCSLLSVFSLVRPHIDISFHG
ILSHINTVFVLRSKEGLLDVEKLECEDELTGTEISCLRLKGQMIYLPEADSILFLCSPSVMNLDDLTR
RGLYLSDIPLHDATRDLVLLGEQFREEYKLTQELEILTDRLQLTLRALED

***Hs.* β1(1-194)**

(SEQ ID NO:133)
ATGTACGGATTTGTGAATCACGCCCTGGAGTTGCTGGTGATCCGCAATTACGGCCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCACAGTTAGATGAAGAAGGACAGTTTCTTGTCAGAATAAT
ATATGATGACTCCAAAACTTATGATTTGGTTGCTGCTGCAAGCAAAGTCCTCAATCTCAATGC
TGGAGAAATCCTCCAAATGTTTGGGAAGATGTTTTTCGTCTTTTGCCAAGAATCTGGTTATGAT
ACAATCTTGCGTGTCCTGGGCTCTAATGTCAGAGAATTTCTACAGAACCTTGATGCTCTGCAC
GACCACCTTGCTACCATCTACCCAGGAATGCGTGCACCTTCCTTTAGGTGCACTGATGCAGAA
AAGGGCAAAGGACTCATTTTGCACTACTACTCAGAGAGAGAAGGACTTCAGGATATTGTCATT
GGAATCATCAAAACAGTGGCACAACAAATCCATGGCACTGAAATAGACATGAAGGTTATTCA
GCAAAGAAATGAAGAATGTGATCATACTCAATTTTTAATTGAAGAAAAAGAGTCAAAAGAAG
AGGATTTTTATGAAGATTGA

(SEQ ID NO:134)
MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAGE
ILQMFGKMFFVFCQESGYDTILRVLGSNVREFLQNLDALHDHLATIYPGMRAPSFRCTDAEKGKG
LILHYYSEREGLQDIVIGIIKTVAQQIHGTEIDMKVIQQRNEECDHTQFLIEEKESKEEDFYED

Figure 8T

***Hs.* β1(1-194) I145Y**

(SEQ ID NO:135)
ATGTACGGATTTGTGAATCACGCCCTGGAGTTGCTGGTGATCCGCAATTACGGCCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCACAGTTAGATGAAGAAGGACAGTTTCTTGTCAGAATAAT
ATATGATGACTCCAAAACTTATGATTTGGTTGCTGCTGCAAGCAAAGTCCTCAATCTCAATGC
TGGAGAAATCCTCCAAATGTTTGGGAAGATGTTTTTCGTCTTTTGCCAAGAATCTGGTTATGAT
ACAATCTTGCGTGTCCTGGGCTCTAATGTCAGAGAATTTCTACAGAACCTTGATGCTCTGCAC
GACCACCTTGCTACCATCTACCCAGGAATGCGTGCACCTTCCTTTAGGTGCACTGATGCAGAA
AAGGGCAAAGGACTCATTTTGCACTACTACTCAGAGAGAGAAGGACTTCAGGATTATGTCATT
GGAATCATCAAAACAGTGGCACAACAAATCCATGGCACTGAAATAGACATGAAGGTTATTCA
GCAAAGAAATGAAGAATGTGATCATACTCAATTTTTAATTGAAGAAAAAGAGTCAAAAGAAG
AGGATTTTTATGAAGATTGA

(SEQ ID NO:136)
MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAGE
ILQMFGKMFFVFCQESGYDTILRVLGSNVREFLQNLDALHDHLATIYPGMRAPSFRCTDAEKGKG
LILHYYSEREGLQDYVIGIIKTVAQQIHGTEIDMKVIQQRNEECDHTQFLIEEKESKEEDFYED

***Hs.* β1(1-194) L9W-I145Y**

(SEQ ID NO:137)
ATGTACGGATTTGTGAATCACGCCTGGGAGTTGCTGGTGATCCGCAATTACGGCCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCACAGTTAGATGAAGAAGGACAGTTTCTTGTCAGAATAAT
ATATGATGACTCCAAAACTTATGATTTGGTTGCTGCTGCAAGCAAAGTCCTCAATCTCAATGC
TGGAGAAATCCTCCAAATGTTTGGGAAGATGTTTTTCGTCTTTTGCCAAGAATCTGGTTATGAT
ACAATCTTGCGTGTCCTGGGCTCTAATGTCAGAGAATTTCTACAGAACCTTGATGCTCTGCAC
GACCACCTTGCTACCATCTACCCAGGAATGCGTGCACCTTCCTTTAGGTGCACTGATGCAGAA
AAGGGCAAAGGACTCATTTTGCACTACTACTCAGAGAGAGAAGGACTTCAGGATTATGTCATT
GGAATCATCAAAACAGTGGCACAACAAATCCATGGCACTGAAATAGACATGAAGGTTATTCA
GCAAAGAAATGAAGAATGTGATCATACTCAATTTTTAATTGAAGAAAAAGAGTCAAAAGAAG
AGGATTTTTATGAAGATTGA

(SEQ ID NO:138)
MYGFVNHAWELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAG
EILQMFGKMFFVFCQESGYDTILRVLGSNVREFLQNLDALHDHLATIYPGMRAPSFRCTDAEKGK
GLILHYYSEREGLQDYVIGIIKTVAQQIHGTEIDMKVIQQRNEECDHTQFLIEEKESKEEDFYED

Figure 8U

Rattus norvegicus β1(1-385)

***Rn.* WT (1-385)**

(SEQ ID NO:139)
ATGTACGGTTTTGTGAACCATGCCCTGGAGCTGCTGGTGATCCGCAATTACGGTCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCGCAGCTGGATGAAGAAGGCCAGTTTCTTGTGAGAATAAT
CTACGATGATTCCAAAACCTATGACTTGGTGGCTGCTGCGAGCAAAGTCCTCAACCTCAATGC
TGGTGAAATCCTGCAGATGTTTGGGAAGATGTTTTTCGTCTTCTGTCAAGAGTCTGGCTATGAT
ACCATCTTGCGTGTCCTGGGATCTAATGTCAGGGAGTTTTTGCAGAACCTCGACGCCCTGCAC
GACCACCTCGCCACCATCTACCCAGGGATGCGCGCACCTTCCTTCCGGTGCACCGATGCAGAA
AAAGGCAAAGGGCTCATTCTGCACTACTACTCGGAAAGAGAGGGGCTTCAGGACATTGTGAT
CGGGATTATCAAGACTGTAGCTCAACAGATCCATGGCACTGAGATAGACATGAAGGTTATTCA
GCAAAGAAGTGAAGAATGTGATCATACCCAATTTTTAATTGAAGAAAAAGAATCAAAAGAAG
AGGATTTTTATGAAGATCTGGACAGGTTTGAAGAGAACGGTACCCAGGACTCCCGTATCAGCC
CGTACACCTTCTGCAAAGCGTTTCCTTTTCACATCATATTTGACCGGGACCTAGTAGTCACGCA
GTGTGGAAATGCTATCTACAGAGTGCTCCCCCAGCTCCAGCCTGGGAAGTGCAGCCTTCTGTC
TGTCTTCTCTCTGGTCCGCCCTCATATTGACATCAGTTTCCACGGGATTCTTTCACACATCAAT
ACCGTCTTTGTACTGAGAAGCAAGGAAGGGTTGCTGGATGTTGAGAAACTTGAATGTGAGGA
TGAACTGACTGGGGCAGAGATTAGCTGCCTCCGTCTCAAAGGCCAAATGATCTATTTACCGGA
AGCAGATAGCATCCTCTTCCTCTGTTCACCAAGTGTGATGAACTTGGATGACCTAACAAGAAG
AGGCCTGTACCTGAGTGACATCCCTCTCCATGATGCTACACGAGACCTGGTCCTTTTGGGAGA
ACAGTTCCGGGAGGAGTACAAACTGACACAAGAGCTGGAAATCCTCACAGACAGGCTGCAGC
TCACACTGAGGGCTTTGGAGGATTGA

(SEQ ID NO:140)
MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAGE
ILQMFGKMFFVFCQESGYDTILRVLGSNVREFLQNLDALHDHLATTYPGMRAPSFRCTDAEKGKG
LILHYYSEREGLQDIVIGIIKTVAQQIHGTEIDMKVIQQRSEECDHTQFLIEEKESKEEDFYEDLDRFE
ENGTQDSRISPYTFCKAFPFHIIFDRDLVVTQCGNAIYRVLPQLQPGKCSLLSVFSLVRPHIDISFHGI
LSHINTVFVLRSKEGLLDVEKLECEDELTGAEISCLRLKGQMIYLPEADSILFLCSPSVMNLDDLTR
RGLYLSDIPLHDATRDLVLLGEQFREEYKLTQELEILTDRLQLTLRALED

Figure 8V

***Rn.* β1(1-385) I145Y**

(SEQ ID NO:141)
ATGTACGGTTTTGTGAACCATGCCCTGGAGCTGCTGGTGATCCGCAATTACGGTCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCGCAGCTGGATGAAGAAGGCCAGTTTCTTGTGAGAATAAT
CTACGATGATTCCAAAACCTATGACTTGGTGGCTGCTGCGAGCAAAGTCCTCAACCTCAATGC
TGGTGAAATCCTGCAGATGTTTGGGAAGATGTTTTTCGTCTTCTGTCAAGAGTCTGGCTATGAT
ACCATCTTGCGTGTCCTGGGATCTAATGTCAGGGAGTTTTTGCAGAACCTCGACGCCCTGCAC
GACCACCTCGCCACCATCTACCCAGGGATGCGCGCACCTTCCTTCCGGTGCACCGATGCAGAA
AAAGGCAAAGGGCTCATTCTGCACTACTACTCGGAAAGAGAGGGGCTTCAGGACTACGTGAT
CGGGATTATCAAGACTGTAGCTCAACAGATCCATGGCACTGAGATAGACATGAAGGTTATTCA
GCAAAGAAGTGAAGAATGTGATCATACCCAATTTTTAATTGAAGAAAAAGAATCAAAAGAAG
AGGATTTTTATGAAGATCTGGACAGGTTTGAAGAGAACGGTACCCAGGACTCCCGTATCAGCC
CGTACACCTTCTGCAAAGCGTTTCCTTTTCACATCATATTTGACCGGGACCTAGTAGTCACGCA
GTGTGGAAATGCTATCTACAGAGTGCTCCCCCAGCTCCAGCCTGGGAAGTGCAGCCTTCTGTC
TGTCTTCTCTCTGGTCCGCCCTCATATTGACATCAGTTTCCACGGGATTCTTTCACACATCAAT
ACCGTCTTTGTACTGAGAAGCAAGGAAGGGTTGCTGGATGTTGAGAAACTTGAATGTGAGGA
TGAACTGACTGGGGCAGAGATTAGCTGCCTCCGTCTCAAAGGCCAAATGATCTATTTACCGGA
AGCAGATAGCATCCTCTTCCTCTGTTCACCAAGTGTGATGAACTTGGATGACCTAACAAGAAG
AGGCCTGTACCTGAGTGACATCCCTCTCCATGATGCTACACGAGACCTGGTCCTTTTGGGAGA
ACAGTTCCGGGAGGAGTACAAACTGACACAAGAGCTGGAAATCCTCACAGACAGGCTGCAGC
TCACACTGAGGGCTTTGGAGGATTGA

(SEQ ID NO:142)
MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAGE
ILQMFGKMFFVFCQESGYDTILRVLGSNVREFLQNLDALHDHLATIYPGMRAPSFRCTDAEKGKG
LILHYYSEREGLQDYVIGIIKTVAQQIHGTEIDMKVIQQRSEECDHTQFLIEEKESKEEDFYEDLDRF
EENGTQDSRISPYTFCKAFPFHIIFDRDLVVTQCGNAIYRVLPQLQPGKCSLLSVFSLVRPHIDISFHG
ILSHINTVFVLRSKEGLLDVEKLECEDELTGAEISCLRLKGQMIYLPEADSILFLCSPSVMNLDDLTR
RGLYLSDIPLHDATRDLVLLGEQFREEYKLTQELEILTDRLQLTLRALED

Figure 8W

***Rn.* β1(1-385) I145H**

(SEQ ID NO:143)
ATGTACGGTTTTGTGAACCATGCCCTGGAGCTGCTGGTGATCCGCAATTACGGTCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCGCAGCTGGATGAAGAAGGCCAGTTTCTTGTGAGAATAAT
CTACGATGATTCCAAAACCTATGACTTGGTGGCTGCTGCGAGCAAAGTCCTCAACCTCAATGC
TGGTGAAATCCTGCAGATGTTTGGGAAGATGTTTTTCGTCTTCTGTCAAGAGTCTGGCTATGAT
ACCATCTTGCGTGTCCTGGGATCTAATGTCAGGGAGTTTTTGCAGAACCTCGACGCCCTGCAC
GACCACCTCGCCACCATCTACCCAGGGATGCGCGCACCTTCCTTCCGGTGCACCGATGCAGAA
AAAGGCAAAGGGCTCATTCTGCACTACTACTCGGAAAGAGAGGGGCTTCAGGACCATGTGAT
CGGGATTATCAAGACTGTAGCTCAACAGATCCATGGCACTGAGATAGACATGAAGGTTATTCA
GCAAAGAAGTGAAGAATGTGATCATACCCAATTTTTAATTGAAGAAAAAGAATCAAAAGAAG
AGGATTTTTATGAAGATCTGGACAGGTTTGAAGAGAACGGTACCCAGGACTCCCGTATCAGCC
CGTACACCTTCTGCAAAGCGTTTCCTTTTCACATCATATTTGACCGGGACCTAGTAGTCACGCA
GTGTGGAAATGCTATCTACAGAGTGCTCCCCCAGCTCCAGCCTGGGAAGTGCAGCCTTCTGTC
TGTCTTCTCTCTGGTCCGCCCTCATATTGACATCAGTTTCCACGGGATTCTTTCACACATCAAT
ACCGTCTTTGTACTGAGAAGCAAGGAAGGGTTGCTGGATGTTGAGAAACTTGAATGTGAGGA
TGAACTGACTGGGGCAGAGATTAGCTGCCTCCGTCTCAAAGGCCAAATGATCTATTTACCGGA
AGCAGATAGCATCCTCTTCCTCTGTTCACCAAGTGTGATGAACTTGGATGACCTAACAAGAAG
AGGCCTGTACCTGAGTGACATCCCTCTCCATGATGCTACACGAGACCTGGTCCTTTTGGGAGA
ACAGTTCCGGGAGGAGTACAAACTGACACAAGAGCTGGAAATCCTCACAGACAGGCTGCAGC
TCACACTGAGGGCTTTGGAGGATTGA

(SEQ ID NO:144)
MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAGE
ILQMFGKMFFVFCQESGYDTILRVLGSNVREFLQNLDALHDHLATIYPGMRAPSFRCTDAEKGKG
LILHYYSEREGLQDHVIGIIKTVAQQIHGTEIDMKVIQQRSEECDHTQFLIEEKESKEEDFYEDLDRF
EENGTQDSRISPYTFCKAFPFHIIFDRDLVVTQCGNAIYRVLPQLQPGKCSLLSVFSLVRPHIDISFHG
ILSHINTVFVLRSKEGLLDVEKLECEDELTGAEISCLRLKGQMIYLPEADSILFLCSPSVMNLDDLTR
RGLYLSDIPLHDATRDLVLLGEQFREEYKLTQELEILTDRLQLTLRALED

Figure 8X

***Rn.* β1(1-385) C78Y**

(SEQ ID NO:145)
ATGTACGGTTTTGTGAACCATGCCCTGGAGCTGCTGGTGATCCGCAATTACGGTCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCGCAGCTGGATGAAGAAGGCCAGTTTCTTGTGAGAATAAT
CTACGATGATTCCAAAACCTATGACTTGGTGGCTGCTGCGAGCAAAGTCCTCAACCTCAATGC
TGGTGAAATCCTGCAGATGTTTGGGAAGATGTTTTTCGTCTTCTATCAAGAGTCTGGCTATGAT
ACCATCTTGCGTGTCCTGGGATCTAATGTCAGGGAGTTTTTGCAGAACCTCGACGCCCTGCAC
GACCACCTCGCCACCATCTACCCAGGGATGCGCGCACCTTCCTTCCGGTGCACCGATGCAGAA
AAAGGCAAAGGGCTCATTCTGCACTACTACTCGGAAAGAGAGGGGCTTCAGGACATTGTGAT
CGGGATTATCAAGACTGTAGCTCAACAGATCCATGGCACTGAGATAGACATGAAGGTTATTCA
GCAAAGAAGTGAAGAATGTGATCATACCCAATTTTTAATTGAAGAAAAAGAATCAAAAGAAG
AGGATTTTTATGAAGATCTGGACAGGTTTGAAGAGAACGGTACCCAGGACTCCCGTATCAGCC
CGTACACCTTCTGCAAAGCGTTTCCTTTTCACATCATATTTGACCGGGACCTAGTAGTCACGCA
GTGTGGAAATGCTATCTACAGAGTGCTCCCCCAGCTCCAGCCTGGGAAGTGCAGCCTTCTGTC
TGTCTTCTCTCTGGTCCGCCCTCATATTGACATCAGTTTCCACGGGATTCTTTCACACATCAAT
ACCGTCTTTGTACTGAGAAGCAAGGAAGGGTTGCTGGATGTTGAGAAACTTGAATGTGAGGA
TGAACTGACTGGGGCAGAGATTAGCTGCCTCCGTCTCAAAGGCCAAATGATCTATTTACCGGA
AGCAGATAGCATCCTCTTCCTCTGTTCACCAAGTGTGATGAACTTGGATGACCTAACAAGAAG
AGGCCTGTACCTGAGTGACATCCCTCTCCATGATGCTACACGAGACCTGGTCCTTTTGGGAGA
ACAGTTCCGGGAGGAGTACAAACTGACACAAGAGCTGGAAATCCTCACAGACAGGCTGCAGC
TCACACTGAGGGCTTTGGAGGATTGA

(SEQ ID NO:146)
MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAGE
ILQMFGKMFFVFYQESGYDTILRVLGSNVREFLQNLDALHDHLATIYPGMRAPSFRCTDAEKGKG
LILHYYSEREGLQDIVIGIIKTVAQQIHGTEIDMKVIQQRSEECDHTQFLIEEKESKEEDFYEDLDRFE
ENGTQDSRISPYTFCKAFPFHIIFDRDLVVTQCGNAIYRVLPQLQPGKCSLLSVFSLVRPHIDISFHGI
LSHINTVFVLRSKEGLLDVEKLECEDELTGAEISCLRLKGQMIYLPEADSILFLCSPSVMNLDDLTR
RGLYLSDIPLHDATRDLVLLGEQFREEYKLTQELEILTDRLQLTLRALED

Figure 8Y

***Rn.* β1 (1-385) H105F**

(SEQ ID NO:147)
ATGTACGGTTTTGTGAACCATGCCCTGGAGCTGCTGGTGATCCGCAATTACGGTCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCGCAGCTGGATGAAGAAGGCCAGTTTCTTGTGAGAATAAT
CTACGATGATTCCAAAACCTATGACTTGGTGGCTGCTGCGAGCAAAGTCCTCAACCTCAATGC
TGGTGAAATCCTGCAGATGTTTGGGAAGATGTTTTTCGTCTTCTGTCAAGAGTCTGGCTATGAT
ACCATCTTGCGTGTCCTGGGATCTAATGTCAGGGAGTTTTTGCAGAACCTCGACGCCCTGTTCG
ACCACCTCGCCACCATCTACCCAGGGATGCGCGCACCTTCCTTCCGGTGCACCGATGCAGAAA
AAGGCAAAGGGCTCATTCTGCACTACTACTCGGAAAGAGAGGGGCTTCAGGACATTGTGATC
GGGATTATCAAGACTGTAGCTCAACAGATCCATGGCACTGAGATAGACATGAAGGTTATTCA
GCAAAGAAGTGAAGAATGTGATCATACCCAATTTTTAATTGAAGAAAAAGAATCAAAAGAAG
AGGATTTTTATGAAGATCTGGACAGGTTTGAAGAGAACGGTACCCAGGACTCCCGTATCAGCC
CGTACACCTTCTGCAAAGCGTTTCCTTTTCACATCATATTTGACCGGGACCTAGTAGTCACGCA
GTGTGGAAATGCTATCTACAGAGTGCTCCCCCAGCTCCAGCCTGGGAAGTGCAGCCTTCTGTC
TGTCTTCTCTCTGGTCCGCCCTCATATTGACATCAGTTTCCACGGGATTCTTTCACACATCAAT
ACCGTCTTTGTACTGAGAAGCAAGGAAGGGTTGCTGGATGTTGAGAAACTTGAATGTGAGGA
TGAACTGACTGGGGCAGAGATTAGCTGCCTCCGTCTCAAAGGCCAAATGATCTATTTACCGGA
AGCAGATAGCATCCTCTTCCTCTGTTCACCAAGTGTGATGAACTTGGATGACCTAACAAGAAG
AGGCCTGTACCTGAGTGACATCCCTCTCCATGATGCTACACGAGACCTGGTCCTTTTGGGAGA
ACAGTTCCGGGAGGAGTACAAACTGACACAAGAGCTGGAAATCCTCACAGACAGGCTGCAGC
TCACACTGAGGGCTTTGGAGGATTGA

(SEQ ID NO:148)
MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAGE
ILQMFGKMFFVFCQESGYDTILRVLGSNVREFLQNLDALFDHLATIYPGMRAPSFRCTDAEKGKGL
ILHYYSEREGLQDIVIGIIKTVAQQIHGTEIDMKVIQQRSEECDHTQFLIEEKESKEEDFYEDLDRFEE
NGTQDSRISPYTFCKAFPFHIIFDRDLVVTQCGNAIYRVLPQLQPGKCSLLSVFSLVRPHIDISFHGIL
SHINTVFVLRSKEGLLDVEKLECEDELTGAEISCLRLKGQMIYLPEADSILFLCSPSVMNLDDLTRR
GLYLSDIPLHDATRDLVLLGEQFREEYKLTQELEILTDRLQLTLRALED

Figure 8Z

***Rn.* β1 (1-385) H105G**

(SEQ ID NO:149)
ATGTACGGTTTTGTGAACCATGCCCTGGAGCTGCTGGTGATCCGCAATTACGGTCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCGCAGCTGGATGAAGAAGGCCAGTTTCTTGTGAGAATAAT
CTACGATGATTCCAAAACCTATGACTTGGTGGCTGCTGCGAGCAAAGTCCTCAACCTCAATGC
TGGTGAAATCCTGCAGATGTTTGGGAAGATGTTTTTCGTCTTCTGTCAAGAGTCTGGCTATGAT
ACCATCTTGCGTGTCCTGGGATCTAATGTCAGGGAGTTTTTGCAGAACCTCGACGCCCTGGGG
GACCACCTCGCCACCATCTACCCAGGGATGCGCGCACCTTCCTTCCGGTGCACCGATGCAGAA
AAAGGCAAAGGGCTCATTCTGCACTACTACTCGGAAAGAGAGGGGCTTCAGGACATTGTGAT
CGGGATTATCAAGACTGTAGCTCAACAGATCCATGGCACTGAGATAGACATGAAGGTTATTCA
GCAAAGAAGTGAAGAATGTGATCATACCCAATTTTTAATTGAAGAAAAAGAATCAAAAGAAG
AGGATTTTTATGAAGATCTGGACAGGTTTGAAGAGAACGGTACCCAGGACTCCCGTATCAGCC
CGTACACCTTCTGCAAAGCGTTTCCTTTTCACATCATATTTGACCGGGACCTAGTAGTCACGCA
GTGTGGAAATGCTATCTACAGAGTGCTCCCCCAGCTCCAGCCTGGGAAGTGCAGCCTTCTGTC
TGTCTTCTCTCTGGTCCGCCCTCATATTGACATCAGTTTCCACGGGATTCTTTCACACATCAAT
ACCGTCTTTGTACTGAGAAGCAAGGAAGGGTTGCTGGATGTTGAGAAACTTGAATGTGAGGA
TGAACTGACTGGGGCAGAGATTAGCTGCCTCCGTCTCAAAGGCCAAATGATCTATTTACCGGA
AGCAGATAGCATCCTCTTCCTCTGTTCACCAAGTGTGATGAACTTGGATGACCTAACAAGAAG
AGGCCTGTACCTGAGTGACATCCCTCTCCATGATGCTACACGAGACCTGGTCCTTTTGGGAGA
ACAGTTCCGGGAGGAGTACAAACTGACACAAGAGCTGGAAATCCTCACAGACAGGCTGCAGC
TCACACTGAGGGCTTTGGAGGATTGA

(SEQ ID NO:150)
MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAGE
ILQMFGKMFFVFCQESGYDTILRVLGSNVREFLQNLDALGDHLATIYPGMRAPSFRCTDAEKGKG
LILHYYSEREGLQDIVIGIIKTVAQQIHGTEIDMKVIQQRSEECDHTQFLIEEKESKEEDFYEDLDRFE
ENGTQDSRISPYTFCKAFPFHIIFDRDLVVTQCGNAIYRVLPQLQPGKCSLLSVFSLVRPHIDISFHGI
LSHINTVFVLRSKEGLLDVEKLECEDELTGAEISCLRLKGQMIYLPEADSILFLCSPSVMNLDDLTR
RGLYLSDIPLHDATRDLVLLGEQFREEYKLTQELEILTDRLQLTLRALED

***Rn.* β1(1-194)**

(SEQ ID NO:151)
ATGTACGGTTTTGTGAACCATGCCCTGGAGCTGCTGGTGATCCGCAATTACGGTCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCGCAGCTGGATGAAGAAGGCCAGTTTCTTGTGAGAATAAT
CTACGATGATTCCAAAACCTATGACTTGGTGGCTGCTGCGAGCAAAGTCCTCAACCTCAATGC
TGGTGAAATCCTGCAGATGTTTGGGAAGATGTTTTTCGTCTTCTGTCAAGAGTCTGGCTATGAT
ACCATCTTGCGTGTCCTGGGATCTAATGTCAGGGAGTTTTTGCAGAACCTCGACGCCCTGCAC
GACCACCTCGCCACCATCTACCCAGGGATGCGCGCACCTTCCTTCCGGTGCACCGATGCAGAA
AAAGGCAAAGGGCTCATTCTGCACTACTACTCGGAAAGAGAGGGGCTTCAGGACATTGTGAT
CGGGATTATCAAGACTGTAGCTCAACAGATCCATGGCACTGAGATAGACATGAAGGTTATTCA
GCAAAGAAGTGAAGAATGTGATCATACCCAATTTTTAATTGAAGAAAAAGAATCAAAAGAAG
AGGATTTTTATGAAGATTGA

(SEQ ID NO:152)
MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAGE
ILQMFGKMFFVFCQESGYDTILRVLGSNVREFLQNLDALHDHLATIYPGMRAPSFRCTDAEKGKG
LILHYYSEREGLQDIVIGIIKTVAQQIHGTEIDMKVIQQRSEECDHTQFLIEEKESKEEDFYED

Figure 8AA

***Rn.* β1(1-194) I145Y**

(SEQ ID NO:153)
ATGTACGGTTTTGTGAACCATGCCCTGGAGCTGCTGGTGATCCGCAATTACGGTCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCGCAGCTGGATGAAGAAGGCCAGTTTCTTGTGAGAATAAT
CTACGATGATTCCAAAACCTATGACTTGGTGGCTGCTGCGAGCAAAGTCCTCAACCTCAATGC
TGGTGAAATCCTGCAGATGTTTGGGAAGATGTTTTTCGTCTTCTGTCAAGAGTCTGGCTATGAT
ACCATCTTGCGTGTCCTGGGATCTAATGTCAGGGAGTTTTTGCAGAACCTCGACGCCCTGCAC
GACCACCTCGCCACCATCTACCCAGGGATGCGCGCACCTTCCTTCCGGTGCACCGATGCAGAA
AAAGGCAAAGGGCTCATTCTGCACTACTACTCGGAAAGAGAGGGGCTTCAGGACTACGTGAT
CGGGATTATCAAGACTGTAGCTCAACAGATCCATGGCACTGAGATAGACATGAAGGTTATTCA
GCAAAGAAGTGAAGAATGTGATCATACCCAATTTTTAATTGAAGAAAAAGAATCAAAAGAAG
AGGATTTTTATGAAGATTGA

(SEQ ID NO:154)
MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAGE
ILQMFGKMFFVFCQESGYDTILRVLGSNVREFLQNLDALHDHLATIYPGMRAPSFRCTDAEKGKG
LILHYYSEREGLQDYVIGIIKTVAQQIHGTEIDMKVIQQRSEECDHTQFLIEEKESKEEDFYED

***Rn.* β1(1-194) L9W-I145Y**

(SEQ ID NO:155)
ATGTACGGTTTTGTGAACCATGCCTGGGAGCTGCTGGTGATCCGCAATTACGGTCCCGAGGTG
TGGGAAGACATCAAAAAAGAGGCGCAGCTGGATGAAGAAGGCCAGTTTCTTGTGAGAATAAT
CTACGATGATTCCAAAACCTATGACTTGGTGGCTGCTGCGAGCAAAGTCCTCAACCTCAATGC
TGGTGAAATCCTGCAGATGTTTGGGAAGATGTTTTTCGTCTTCTGTCAAGAGTCTGGCTATGAT
ACCATCTTGCGTGTCCTGGGATCTAATGTCAGGGAGTTTTTGCAGAACCTCGACGCCCTGCAC
GACCACCTCGCCACCATCTACCCAGGGATGCGCGCACCTTCCTTCCGGTGCACCGATGCAGAA
AAAGGCAAAGGGCTCATTCTGCACTACTACTCGGAAAGAGAGGGGCTTCAGGACTACGTGAT
CGGGATTATCAAGACTGTAGCTCAACAGATCCATGGCACTGAGATAGACATGAAGGTTATTCA
GCAAAGAAGTGAAGAATGTGATCATACCCAATTTTTAATTGAAGAAAAAGAATCAAAAGAAG
AGGATTTTTATGAAGATTGA

(SEQ ID NO:156)
MYGFVNHAWELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLNAG
EILQMFGKMFFVFCQESGYDTILRVLGSNVREFLQNLDALHDHLATIYPGMRAPSFRCTDAEKGK
GLILHYYSEREGLQDYVIGIIKTVAQQIHGTEIDMKVIQQRSEECDHTQFLIEEKESKEEDFYED

Figure 8BB

***Rattus norvegicus* β2**

(SEQ ID NO:157)
ATGTATGGATTCATCAACACCTGCCTGCAGTCTCTTGTGACAGAGAAATTTGGTGAGGAGACA
TGGGAGAAGCTGAAGGCTCCTGCAGAAGTGCAAGATGTCTTCATGACCTACACCGTGTATGAT
GACATCATCACCATTAAGCTCATCCAAGAAGCCTGCAAGGTTCTGGATGTGTCCATGGAAGCC
ATTCTGAAGCTCTTTGGCGAATACTTCTTTAAGTTCTGTAAGATGTCTGGCTATGACAGGATGC
TGCGGACACTTGGAGGAAATCTCACCGAGTTTATTGAAAACCTAGATGCACTCCACAGTTACC
TGGCACTGTCCTATCAGGAAATGAACGCACCATCCTTTCGAGTGGAGGAAGGAGCTGACGGG
GCGATGCTTCTCCACTACTACTCAGACAGACATGGTCTGTGTCACATTGTACCAGGTATCATTG
AAGCTGTGGCCAAGGACTTCTTTGACACTGATGTGGCCATGAGTATCCTGGATATGAACGAAG
AGGTGGAAAGGACAGGGAAGAAAGAACATGTTGTGTTTCTGGTCGTGCAGAAGGCTCACAGA
CAGATAAGAGGAGCAAAGGCAAGCCGGCCACAAGGCAGTGAGGACAGCCAGGCAGACCAGG
AGGCTCTCCAGGGAACACTCCTTCGGATGAAGGAGAGATATTTAAACATCCCTGTTTGCCCTG
GGGAGAAATCTCACTCAACTGCTGTGAGGGCATCGGTCCTTTTTGGAAAAGGGCCCCTCAGGG
ACACCTTCCAGCCCGTCTATCCTGAGAGACTATGGGTCGAAGAGGAGGTGTTCTGTGATGCTT
TTCCTTTCCACATTGTCTTTGATGAAGCACTAAGGGTCAAGCAAGCTGGAGTGAATATTCAGA
AGTATGTCCCTGGAATCTTAACCCAGAAGTTTGCACTAGATGAGTATTTTTCCATCATCCACCC
TCAAGTTACTTTCAACATCTCCAGCATCTGCAAGTTCATTAACAGTCAGTTTGTCTTGAAGACA
AGAAAAGAAATGATGCCCAAAGCAAGGAAGAGCCAGCCGATGCTCAAACTCCGGGGTCAGA
TGATCTGGATGGAGTCTCTGAGGTGCATGATCTTCATGTGTTCCCCAAACGTCCGCAGCCTGC
AAGAGCTGGAAGAGAGCAAGATGCATCTTTCTGATATCGCTCCGCACGACACGACCAGGGAT
CTCATCCTCCTCAACCAGCAGAGGCTGGCAGAGATGGAGCTGTCCTGCCAACTGGAAAAGAA
GAAGGAGGAGTTGCGTGTCCTTTCCAATCACCTGGCCATCGAGAAGAAGAAGACAGAGACCT
TGCTGTATGCCATGCTGCCTGAACATGTGGCCAACCAACTCAAGGAGGGCAGAAAGGTGGCT
GCAGGAGAATTTGAAACATGTACAATCCTTTTCAGCGATGTTGTGACATTTACCAACATCTGT
GCAGCCTGTGAACCTATCCAAATCGTGAACATGCTGAATTCAATGTACTCCAAGTTTGACAGG
TTAACCAGTGTCCATGATGTCTACAAAGTAGAAACAATAGGGGATGCTTACATGGTGGTGGGT
GGAGTACCAGTACCCGTTGAAAGCCATGCTCAAAGAGTCGCCAATTTTGCTCTGGGGATGAGA
ATTTCTGCAAAAGAAGTGATGAATCCTGTCACTGGGGAACCTATCCAGATCAGAGTGGGAATC
CACACTGGACCAGTCTTAGCAGGTGTTGTGGGAGACAAGATGCCTCGGTACTGCTTGTTTGGT
GACACTGTAAACACAGCCTCTAGGATGGAAAGTCACGGGCTTCCCAGCAAAGTGCATCTGAG
CCCCACAGCCCACAGAGCCCTGAAAAACAAAGGGTTTGAAATTGTCAGGAGAGGCGAGATCG
AAGTGAAGGGGAAAGGAAAGATGACCACATACTTTCTGATCCAGAACCTGAATGCCACCGAG
GATGAGATAATGGGGCGACCTTCAGCCCCCGCTGATGGGAAGGAAGTATGTACTCCCGGAAA
CCAAGTCAGGAAGTCCCCTGCTGTCCCGAGGAACACAGACCATCAGCAACAAGTCTACAAAG
GAGACCCAGCAGACGCTTCTAATGAAGTCACACTTGCTGGGAGCCCAGTGGCAGGGCGAAAC
TCCACAGATGCAGTCAATAACCAGCCATCACCAGATGAGACCAAGACAAGTGTCGTTGCTAG
TGGCCCTGTGCTGTCTGCTTTCTGTGTTGTGCTGTGA

(SEQ ID NO:158)
MYGFINTCLQSLVTEKFGEETWEKLKAPAEVQDVFMTYTVYDDIITIKLIQEACKVLDVSMEAILK
LFGEYFFKFCKMSGYDRMLRTLGGNLTEFIENLDALHSYLALSYQEMNAPSFRVEEGADGAMLLH
YYSDRHGLCHIVPGIIEAVAKDFFDTDVAMSILDMNEEVERTGKKEHVVFLVVQKAHRQIRGAKA
SRPQGSEDSQADQEALQGTLLRMKERYLNIPVCPGEKSHSTAVRASVLFGKGPLRDTFQPVYPERL
WVEEEVFCDAFPFHIVFDEALRVKQAGVNIQKYVPGILTQKFALDEYFSIIHPQVTFNISSICKFINSQ
FVLKTRKEMMPKARKSQPMLKLRGQMIWMESLRCMIFMCSPNVRSLQELEESKMHLSDIAPHDT
TRDLILLNQQRLAEMELSCQLEKKKEELRVLSNHLAIEKKKTETLLYAMLPEHVANQLKEGRKVA
AGEFETCTILFSDVVTFTNICAACEPIQIVNMLNSMYSKFDRLTSVHDVYKVETIGDAYMVVGGVP
VPVESHAQRVANFALGMRISAKEVMNPVTGEPIQIRVGIHTGPVLAGVVGDKMPRYCLFGDTVNT
ASRMESHGLPSKVHLSPTAHRALKNKGFEIVRRGEIEVKGKGKMTTYFLIQNLNATEDEIMGRPSA
PADGKEVCTPGNQVRKSPAVPRNTDHQQQVYKGDPADASNEVTLAGSPVAGRNSTDAVNNQPSP
DETKTSVVASGPVLSAFCVVL

Figure 8CC

***Homo sapiens* β2 (1-217)**

(SEQ ID NO:159)
ATGTATGGATTCATCAACACCTGCCTGCAGTCTCTTGTGACAGAGAAATTTGGTGAGGAGACA
TGGGAGAAGCTGAAGGCTCCTGCAGAAGTGCAAGATGTCTTCATGACCTACACCGTGTATGAT
GACATCATCACCATTAAGCTCATCCAAGAAGCCTGCAAGGTTCTGGATGTGTCCATGGAAGCC
ATTCTGAAGCTCTTTGGCGAATACTTCTTTAAGTTCTGTAAGATGTCTGGCTATGACAGGATGC
TGCGGACACTTGGAGGAAATCTCACCGAGTTTATTGAAAACCTAGATGCACTCCACAGTTACC
TGGCACTGTCCTATCAGGAAATGAACGCACCATCCTTTCGAGTGGAGGAAGGAGCTGACGGG
GCGATGCTTCTCCACTACTACTCAGACAGACATGGTCTGTGTCACATTGTACCAGGTATCATTG
AAGCTGTGGCCAAGGACTTCTTTGACACTGATGTGGCCATGAGTATCCTGGATATGAACGAAG
AGGTGGAAAGGACAGGGAAGAAAGAACATGTTGTGTTTCTGGTCGTGCAGAAGGCTCACAGA
CAGATAAGAGGAGCAAAGGCAAGCCGGCCACAAGGCAGTGAGGACAGCCAGGCAGACCAGG
AGGCTCTCCAGGGAACACTCCTT

(SEQ ID NO:160)
MYGFINTCLQSLVTEKFGEETWEKLKAPAEVQDVFMTYTVYDDIITIKLIQEACKVLDVSMEAILK
LFGEYFFKFCKMSGYDRMLRTLGGNLTEFIENLDALHSYLALSYQEMNAPSFRVEEGADGAMLLH
YYSDRHGLCHIVPGIIEAVAKDFFDTDVAMSILDMNEEVERTGKKEHVVFLVVQKAHRQIRGAKA
SRPQGSEDSQADQEALQGTLL

***Homo sapiens* β2 (1-217) I142Y**

(SEQ ID NO:161)
ATGTATGGATTCATCAACACCTGCCTGCAGTCTCTTGTGACAGAGAAATTTGGTGAGGAGACA
TGGGAGAAGCTGAAGGCTCCTGCAGAAGTGCAAGATGTCTTCATGACCTACACCGTGTATGAT
GACATCATCACCATTAAGCTCATCCAAGAAGCCTGCAAGGTTCTGGATGTGTCCATGGAAGCC
ATTCTGAAGCTCTTTGGCGAATACTTCTTTAAGTTCTGTAAGATGTCTGGCTATGACAGGATGC
TGCGGACACTTGGAGGAAATCTCACCGAGTTTATTGAAAACCTAGATGCACTCCACAGTTACC
TGGCACTGTCCTATCAGGAAATGAACGCACCATCCTTTCGAGTGGAGGAAGGAGCTGACGGG
GCGATGCTTCTCCACTACTACTCAGACAGACATGGTCTGTGTCACTATGTACCAGGTATCATTG
AAGCTGTGGCCAAGGACTTCTTTGACACTGATGTGGCCATGAGTATCCTGGATATGAACGAAG
AGGTGGAAAGGACAGGGAAGAAAGAACATGTTGTGTTTCTGGTCGTGCAGAAGGCTCACAGA
CAGATAAGAGGAGCAAAGGCAAGCCGGCCACAAGGCAGTGAGGACAGCCAGGCAGACCAGG
AGGCTCTCCAGGGAACACTCCTT

(SEQ ID NO:162)
MYGFINTCLQSLVTEKFGEETWEKLKAPAEVQDVFMTYTVYDDIITIKLIQEACKVLDVSMEAILK
LFGEYFFKFCKMSGYDRMLRTLGGNLTEFIENLDALHSYLALSYQEMNAPSFRVEEGADGAMLLH
YYSDRHGLCHYVPGIIEAVAKDFFDTDVAMSILDMNEEVERTGKKEHVVFLVVQKAHRQIRGAK
ASRPQGSEDSQADQEALQGTLL

Figure 8DD