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(54) Title: NEXT-GENERATION ELECTROCHEMICAL BIOSENSORS

(57) Abstract: The present invention relates to enzymes, oxidoreductase enzymes, polypeptides and their use in biosensors. More particularly, the present invention relates to improved biosensors optimised for target detection under physiological conditions, having enhanced dynamic ranges and improved signal-to-noise ratio.



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NEXT-GENERATION ELECTROCHEMICAL BIOSENSORS

TECHNICAL FIELD

5 The present invention relates to improved biosensors. In particular, the present invention relates to improved electrochemical biosensors that are suitable for detection of one or more target molecules in a sample. The biosensors of the present invention may also relate to the field of synthetic biology such as for constructing artificial cellular or extracellular signalling networks.

10

BACKGROUND

Detection of target molecules or analytes in biological samples is central to diagnostic monitoring of health and disease (1-3). Key requirements of analyte detection are
15 specificity and sensitivity, particularly when the target molecule or analyte is in a limiting amount or concentration in a biological sample. Previously described biosensors, such as electrochemical biosensors, have addressed a need to develop quantitative, relatively inexpensive and easily produced molecular biosensors capable of readily detecting the presence or the activity of target molecules (e.g. analytes) on short time scales that are
20 compatible with treatment regimens (4-7) (see also e.g., WO 2016/191812). These biosensors typically have specificity for a target molecule and produce an electrical response to signal detection of the target molecule.

Previous biosensors of the prior art may comprise an oxidoreductase enzyme or a variant
25 thereof to provide the catalytic output. Some previously described biosensors of the prior art comprise a calmodulin protein to regulate to the catalytic activity of the oxidoreductase enzyme. A previously developed “two-component” biosensor architecture is generally applicable as it is composed of two interchangeable modules: the binders responsible for the capture of the analyte and the allosteric reporter that converts the binding event into a
30 biochemical activity.

There remains a need in the art for further improved biosensors that are optimised for detection in physiological conditions, have improved signal-to-noise ratio and which provide increased sensitivity of detection. The present inventors have identified biosensor improvements that provide an enhanced dynamic range, optimise the biosensor for
5 functioning in physiological conditions and have lower levels of background activation in the absence of a target. The present invention will be described below in further detail.

SUMMARY

10 The present invention relates to improved oxidoreductase enzymes and enzymes, which may be used in biosensors. In particular, the improved oxidoreductase enzymes and enzymes have one or more features specifically adapted for detection of target molecules in physiological conditions. These features give rise to corresponding improvements in the biosensors, resulting in biosensors that are also specifically adapted for detection of target
15 molecules in physiological conditions. Physiological samples, including biological samples such as blood and saliva, typically comprise high concentrations of calcium, such as between 500 μ M and 5 mM. The present inventors have developed an improved oxidoreductase enzyme comprising a calmodulin protein insert acting to regulate said enzyme that is insensitive to changes in calcium concentration within the physiological
20 range. Biosensors comprising this enzyme are specifically adapted for detection in physiological conditions. The biosensors of the invention have been compared to established clinical diagnostic platforms and the results show excellent correlation. The results demonstrate that the sensitivity of the biosensors of the invention is higher than required for the detection of target molecules at clinically relevant concentrations. This
25 demonstrates that the biosensors of the invention are able to specifically and sensitively detect and quantify target molecules in clinical samples.

The present inventors have also developed an enzyme comprising a further improvement that results in biosensors with enhanced sensitivity. Enzymes comprising a heterologous,
30 sensor amino acid insert, used in biosensors, such as an oxidoreductase enzyme comprising a calmodulin protein insert, where binding of a target molecule to the insert regulates the catalytic activity of the enzyme, can be improved by engineering particular forms of

linkers between the insert and the enzyme. The present inventors have developed linkers which interact with each other when they are in proximity. Binding of the target molecule to the heterologous amino acid sequence brings the linkers into proximity and activates the catalytic activity of the enzyme. The interaction between the linkers fixes the enzyme in this activated state and thereby enhances the activation of the enzyme. Biosensors comprising such linkers have improved sensitivity and enhanced dynamic ranges making them specifically adapted for detection of analytes that are typically at low concentrations in physiological conditions.

10 The present inventors have also developed a polypeptide that may be used in a biosensor to reduce the background activation of the biosensor in the absence of the target molecule of the biosensor. The present inventors have developed a modified calmodulin protein with a reduced affinity for calmodulin binding peptides (CaM-BPs) that can be used to assemble a caged calmodulin binding peptide (CaM-BP). The caged CaM-BP will only activate an
15 oxidoreductase enzyme comprising a calmodulin protein insert when the two modules are in close proximity. Two component biosensors comprising the oxidoreductases described herein and this caged CaM-BP module shows reduced background, which enable detection of target molecules that are at low concentrations in physiological conditions. The modified calmodulin protein with a reduced affinity for calmodulin binding peptides
20 (CaM-BPs) can also be used with two component biosensors, such as those described herein, to reduce background and enable detection of target molecules that are at varying concentrations in physiological conditions. For example, the modified calmodulin protein can bind to the component of the biosensor comprising the calmodulin binding peptide, thereby preventing activation of the component of the biosensor comprising the enzyme
25 until the two components of the biosensor are localised by the presence of the target molecule. Such biosensors have a reduced dependence on the concentration of the biosensor components and therefore are adapted for detection of analytes that are present at different concentrations, such as high concentrations where a high concentration of the biosensor may be used.

30 Similarly, the present inventors have developed variant CaM-BPs that bind to calmodulin and block binding of an activating CaM-BP, optionally a wild-type CaM-BP. The variant

CaM-BPs bind to a calmodulin protein insert in an oxidoreductase enzyme but will not result in activation of the enzyme. These variant CaM-BPs can be used to block activation of an enzyme in a biosensor until two components of the biosensor are localised by the presence of the target molecule. Such biosensors also display reduced background, which
5 enables detection of target molecules that are at low concentrations in physiological conditions. The present inventors have also developed variant CaM-BPs with a reduced affinity for calmodulin, *i.e.*, a lower affinity for calmodulin than a wild-type CaM-BP.

The present inventors have also developed circularly permuted oxidoreductase enzymes
10 comprising first and second binding moieties (whose interaction may depend on the presence of a target molecule), and in addition a heterologous amino acid sequence which releasably maintains the enzyme in a catalytically inactive state by allosteric regulation and is responsive to a molecule to release said regulation. The allosteric control and interaction
15 between binding moieties provides for reversible regulation of catalytic activity of the enzyme in a single component system, advantageously reducing noise associated with multiple sensor components and providing for high sensitivity and avidity. A further advantage is that system is not prone to “antigen poisoning” –that may occur in two component systems based on non-cooperative binders. This is a consequence of increased proportion of biosensor components forming binary complexes with the excess of antigen
20 and not an active ternary complex.

The present inventors have also engineered oxidoreductase enzymes comprising a heterologous amino acid sequence and which are regulated by a peptide (whose binding to the heterologous amino acid sequence reversibly regulates catalytic activity of the enzyme)
25 and a further polypeptide, providing three component sensors with highly specific and sensitive response to a target molecule. The oxidoreductase enzyme comprises a first interaction domain which is capable of interacting with a second interaction domain on the further polypeptide, for example in the presence of an interaction ligand. The further polypeptide also comprises a second binding moiety capable of interacting with a first
30 binding moiety on the peptide. Binding between the first and second binding moieties, and the first and second interaction domains colocalises the enzyme and peptide and thus provides for regulation of catalytic activity. This architecture enables individual expression

of components which is helpful when different domains have different optimal expression regimes.

The improvements described herein enable the sensitivity of the biosensor to be tuned
5 without significantly affecting its dynamic range. Micromolar and higher concentrations
of the biosensor components are required for many applications, including in screen
printed electrodes used in point of care diagnostics. However, the background activation
of the biosensor is increased when such high concentrations are used, which increases
noise and limits the dynamic range of the biosensor. Thus the dynamic range of the two-
10 component system is influenced by the relative concentration of the sensor components.
The present invention provides general strategies for tuning the performance of the
biosensor without modification of the design. For example, addition of a modified
calmodulin protein, with a reduced affinity for calmodulin binding peptides, complexes
with the calmodulin binding peptide of the biosensor, preventing its spontaneous
15 association with the calmodulin insert in the oxidoreductase enzyme of the biosensor.
Thus, the added calmodulin acts as an additional thermodynamic barrier to activation of
the biosensor. Similarly, using variant CaM-BPs with a reduced binding affinity for the
calmodulin insert in the oxidoreductase enzyme of the biosensor and caged calmodulin
binding peptides as described herein can similarly reduce spontaneous association of the
20 peptides with the calmodulin insert. Thereby, the sensitivity of the biosensor can be tuned
without affecting its dynamic range.

Accordingly the invention provides:

25 An oxidoreductase enzyme comprising a calmodulin protein or a functional fragment
thereof, wherein binding of a peptide to the calmodulin protein or functional fragment
thereof reversibly regulates the catalytic activity of the enzyme, wherein binding of the
peptide is capable of reversibly regulating the catalytic activity of the enzyme in the
presence of physiological calcium concentrations.

30

An oxidoreductase enzyme comprising a heterologous amino acid sequence which is
responsive to a target molecule, wherein binding of the target molecule to the heterologous

amino acid sequence reversibly regulates catalytic activity of the enzyme, wherein the oxidoreductase enzyme comprises the heterologous amino acid sequence in a location corresponding to the loop connecting beta-sheets 5 and 6 of a PQQ-GDH.

- 5 An enzyme comprising a heterologous amino acid sequence which is responsive to a target molecule, wherein binding of the target molecule to the heterologous amino acid sequence reversibly regulates catalytic activity of the enzyme; wherein the heterologous amino acid sequence is provided as an insert within the amino acid sequence of the enzyme flanked on either side by linkers, wherein binding of the target molecule to the heterologous amino acid sequence brings the linkers into proximity with each other; and wherein interaction
10 between the linkers enhances activation of the catalytic activity of the enzyme, optionally wherein said interaction maintains the linkers in proximity with each other.

- A polypeptide comprising a calmodulin binding peptide and a calmodulin protein or
15 functional fragment thereof, wherein the calmodulin protein or functional fragment thereof comprises one or more modifications resulting in a reduced binding affinity for the calmodulin binding peptide as compared to a corresponding wild type calmodulin protein or functional fragment thereof.

- 20 A polypeptide comprising a calmodulin protein or functional fragment thereof, wherein the calmodulin protein or functional fragment thereof comprises one or more modifications resulting in a reduced binding affinity for a calmodulin binding peptide as compared to a corresponding wild type calmodulin protein or functional fragment thereof.

- 25 A variant calmodulin binding peptide (CaM-BP) which is capable of binding to a calmodulin protein or a functional fragment thereof provided as an insert within the amino acid sequence of an enzyme, wherein the calmodulin protein or functional fragment thereof activates the catalytic activity of the enzyme on binding of a corresponding activating CaM-BP, and wherein binding of the variant CaM-BP to said calmodulin protein or
30 functional fragment thereof inhibits binding of the corresponding activating CaM-BP, thereby preventing activation of said enzyme.

An enzyme comprising (i) a calmodulin protein or functional fragment thereof provided as an insert within the amino acid sequence of the enzyme, and (ii) a variant CaM-BP, wherein the calmodulin protein or functional fragment thereof activates the catalytic activity of the enzyme on binding of a corresponding activating CaM-BP, wherein binding
5 of the variant CaM-BP to the calmodulin protein or functional fragment thereof inhibits binding of said corresponding activating CaM-BP, and wherein binding of the variant CaM-BP to the calmodulin protein or functional fragment thereof prevents activation of the catalytic activity of the enzyme.

10 An oxidoreductase enzyme comprising a heterologous amino acid sequence that releasably maintains the enzyme in a catalytically inactive state by allosteric regulation and which is responsive to a molecule to release said regulation, a first binding moiety and a second binding moiety, wherein the amino acid sequence of the enzyme is circularly permuted, and wherein (i) release of the allosteric regulation by the heterologous amino acid
15 sequence and (ii) interaction between the binding moieties, reversibly regulates the catalytic activity of the enzyme.

An oxidoreductase enzyme comprising a heterologous amino acid sequence which is responsive to a peptide comprising a first binding moiety, and a first interaction domain
20 capable of interacting with a second interaction domain of a polypeptide, wherein the polypeptide further comprises a second binding moiety capable of interacting with said first binding moiety, and wherein binding between the first and second binding moieties and interaction of the first interaction domain of the enzyme with the second interaction domain of the polypeptide act to colocalise the enzyme and the peptide, wherein binding of
25 the peptide to the heterologous amino acid sequence reversibly regulates catalytic activity of the enzyme.

The present invention also provides biosensors, compositions and kits and detection devices comprising the enzymes, oxidoreductase enzymes, polypeptides and variant CaM-
30 BPs of the invention. The present invention also provides methods of detecting a target molecule, methods of diagnosis of a disease or condition in an organism, methods of assaying for protein-protein or protein-small molecule interactions that use the biosensors,

enzymes, oxidoreductase enzymes, polypeptides and variant CaM-BPs of the invention. The present invention also provides nucleic acids encoding the biosensors, enzymes, oxidoreductase enzymes, polypeptides and variant CaM-BPs of the invention.

5

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Effect of Ca^{2+} the performance of the CaM-GDH based biosensors and construction of an alternative $\beta 5/6$ -CaM-GDH switch module. (A) Absorbance traces of a titration of 20nM of CaM-GDH-FKBP (similar results were also obtained with 10nM CaM-GDH-FKBP) and 30nM of CalA/CalB-CaM-BP with increasing concentrations of tacrolimus (indicated on the right hand side of the plot) at 50 μM CaCl_2 . **(B)** as in (A) but in the presence of 2.5mM CaCl_2 . Only the lowest and the highest concentration of tacrolimus is shown. **(C)** Assessment of the activation range of 20 nM of CaM-GDH-FKBP with 100 nM of CaM-BP at different Ca^{2+} concentrations. The assays were performed as described above but only the zero and 20 min points were plotted. **(D)** Rate response of tacrolimus 10nM of CaM-GDH-FKBPFRB and 30nM of CalA/CalB-CaM-BP mix to the increasing concentrations of tacrolimus at different concentrations of Ca^{2+} . **(E)** The data from the experiment shown in D plotted as a fold of rate change. The fold change represents the increase in K_{obs} between the background activity and the maximal observed rate at saturating concentrations of tacrolimus. **(F)** Structure of PQQ-GDH in ribbon representation with labeled calmodulin insertion sites. PQQ cofactor is displayed in ball and stick representation colored in golden while glucose is colored in green. The coordinated Ca^{2+} in the active site is shown as a magenta ball. The β -sheets of the molecule are numbered 1 to 6. **(G)** A model of CaM-GDH chimera with CaM inserted in the loop connecting β -sheets 5 and 6. The molecules are displayed in ribbon representation. The Ca^{2+} ions are labeled as magenta balls. The β -sheets are labeled with numbers.

Figure 2: The functional mechanism and Ca^{2+} dependence of $\beta 5/6$ -CaM-GDH chimera
(A) Active site of PQQ-GDH showing the loop connecting β -sheets 5 and 6 that was used for CaM insertion. The Arg 406 and Arg 408 are shown in the ball and stick representation and the hydrogen bonds with carboxyl groups of PQQ are shown as strings of blue balls. **(B)** GDH activity of 10 nM GDH-CaM chimera in response to increasing concentration

CaM-BP in buffer containing 1 mM CaCl₂. (C) Fit of the data shown in B to a quadratic equation that gives a value of 21 nM for the K_d (D) Performance analysis of rapamycin biosensor based on the β5/6-CaM-GDH module. In the experiment 10 nM FKBP-CaM-GDH and 30 nM FRB -CaM-BP were incubated with different concentrations of rapamycin and CaCl₂ and the observed reaction rates were plotted against the concentration of rapamycin. The blue graph represents an experiment where 50% serum and 5 mM CaCl₂ were combined. (E) Performance analysis of rapamycin biosensor based on the β5/6-CaM-GDH chimera biosensor. In the experiment 10 nM β5/6-CaM-GDH was incubated with different concentrations of CaM-BP and CaCl₂ and the observed reaction rates were plotted against the concentration of CaM-BP. The blue graph represents an experiment where 50% serum and 5 mM CaCl₂ were combined.

Figure 3: Performance analysis of β5/6-CaM-GDH-based Tacrolimus biosensor at different tacrolimus and Ca²⁺ concentrations. (A) Ca²⁺ dependency of rapamycin biosensor based on β5/6-CaM-GDH. In the experiment 10 nM FKBP-CaM-GDH and 30 nM FRB -CaM-BP were incubated with different concentrations of rapamycin and CaCl₂ and the observed reaction rates were plotted against the concentration of rapamycin. (B) Activation of 10 nM β5/6-CaM-GDH FKBP and 30 nM Calceinurina/B-CaM-BP with increasing concentrations of the tacrolimus in the presence of 1mM CaCl₂. (C) Activity of the tacrolimus biosensor at different concentrations of Ca²⁺. The experiment shown in (B) was repeated at the indicated concentrations of Ca²⁺ and Tacrolimus.

Figure 4: Construction of GDH-based two component biosensor of α-amylase and its benchmarking against enzymatic test. (A) A model of α-amylase biosensor in complex with its analyte based on X-ray structure of α-amylase bound to VHH domains (model based on PDB entries 1KXQ and 1KXT). (B) A plot of the observed reaction rates of 20 nM of α-amylase biosensor (20 nM of VHH1-CaM-GDH and 100nM VHH2-CaM-BP) at different concentrations of α-amylase. (C) Detection of α-amylase in human saliva using Ca²⁺ tolerant α-amylase biosensor (X-axis) and its comparison with the traditionally used clinical assay based on the measurement of α-amylase enzymatic activity (Y-axis). The reactions were performed in 1ml volume cuvettes containing 20 nM solution of VHH1-CaM-GDH and 100 nM of VHH2-CaM-BP and 1 μl of undiluted human saliva. The

enzymatic assays of the same saliva samples were performed by a certified clinical laboratory (central laboratory of Queensland Health).

Figure 5: Testing and optimization of two component biosensors. (A) GDH activity of 10nM of “ratcheted” CaM-GDH chimera in response to increasing concentration CaM-BP in buffer containing 1mM CaCl₂. (B) Fit of the activity data of 10nM of cysteine “ratcheted” CaM-GDH-Protein G fusion and 100nM anti-HSA-VHH-CaM-BP in the presence of the indicated concentrations of HSA. The observed rates were fitted leading to a K_d of 5nM. (C) Quantification of HSA concentration in serum of human donors using HSA biosensor (X-axis) or clinical chemistry analyser (Y-axis). (D) Fit of the titration data of 5nM of “ratcheted” GDH-CaM-Cyclophilin and 30nM of CalA/CalB-CaM-BP with increasing concentration of cyclosporine in the presence of 25% human serum. The fit of the data led to a K_d value of 14nM. (E) Analysis of human serum samples spiked with different concentrations of cyclosporine using the cyclosporine biosensor from (D).

15

Figure 6: Cysteine “ratcheted” version of β 5/6-CaM-GDH. (A) GDH activity of 10 nM R β 5/6GDH-CaM chimera in response to increasing concentration CaM-BP in buffer containing 1 mM CaCl₂. (B) Fit of the data shown in (A) to a quadratic equation that gives a value of 20 nM for the K_d. (C) Titration of 10 nM of GDH-CaM-FKBP and 30 nM of FRB-CaM-BP with increasing concentrations of rapamycin in buffer containing 1 mM CaCl₂. (D) Fit of the data shown in (C) leading of an apparent dissociated constant of 7nM. (E) Titration of 10 nM of GDH-CaM-FKBP and 30 nM of CalA/CalB-CaM-BP with increasing concentrations of tacrolimus in buffer containing 1 mM CaCl₂. (F) Fit of the data shown in (E) leading of an apparent dissociated constant of 12 nM.

25

Figure 7: Mass-spectrometric analysis of “ratcheted” CaM-GDH chimera in the absence and presence of CaM-BP. A 4 μ M solution of “ratcheted” CaM-GDH was incubated for 10 minutes in the presence (B) or absence (A) of M13 CaM-BP and subjected to the mass spectroscopy on SCIEX Triplex TOF 5600 MALDI-TOF and the data was analyzed with Analyst® TF 1.6 Software. The calculated molecular weight of the reduced form of “ratcheted” CaM-GDH with intact acetylated N-terminal methionine is 68611 Da. The mass change is consistent with the formation of a disulfide bond.

30

Figure 8: Biosensor of human serum albumin (HSA) based on a “ratcheted” CaM-GDH switch module. (A) GDH activity of HSA biosensor at different concentrations of purified HSA. The reaction contained 10nM CaM-GDH -Protein G, and 100nM VHH-CaM-BP and 1mM CaCl₂. (B) Calibration plot of K_{obs} rates as function of HSA concentrations based on the data shown in (A). (C) Quantification of HSA in urine of healthy individuals and diabetic patients using GDH-based HSA biosensor and the clinical immunochemistry assay. These experiments correctly identifying individuals with normal HSA levels (HSA concentrations below 0.3mg/L) as well as individuals with microalbuminuria (HSA concentrations 0.3–0.3 mg/L) and gross albuminuria (HSA concentrations above 0.3 mg/L).

Figure 9: Biosensors of macrocyclic compounds based on a “ratcheted” CaM-GDH switch module. (A) GDH activity of solutions of 5nM GDH-CaM-Cyclophilin and 30nM of CalA/CalB-CaM-BP at indicated concentrations of the drug in buffer containing 1 mM CaCl₂. (B) Fit of the K_{obs} from A to a K_d of 8nM. (C) same as A but reactions performed in the presence of 25 % human serum (D) Fit of the K_{obs} from C to a K_d of 14nM.

Figure 10: Signal reduction in equilibrium associative biosensing systems operating at or below the K_d of the binding domains. (A) At high concentration of the analyte it drives the assembly of the biosensor resulting in the proportional increase in the output. (B) At the low concentration of the analyte the assembly of the functional complex is a less frequent and the resulting complex is transient resulting in low output. (C) A proposed non-equilibrium system where assembly of the receptor complex leads to a production of multiple signal mediators that in turs can activate the reporter switch.

Figure 11: A design principle for non-equilibrium biosensor system with high sensitivity. Activating ligand brings into proximity an autoinhibited protease and caged CaM-BP connected to its caging CaM via a linker with a protease cleavage site. Intermolecular swap results in cleavage of the linker and dissociation of the CaM-BP that subsequently activates CaM-GDH reporter. If the binder with caged CaM-BP is present in

excess than reaction can proceed in multiple turnover mode generating more and more CaM-BP and activating more CaM-GDH.

Figure 12: Caged CaM-BP as a messenger activating CaM-BP in proteolytic

5 **biosensors. (A)** Titration of 10 nM of β 5/6- CaM-GDH with increasing concentrations of CaM-CaM-BP fusion. The wt CaM-BP was used as the assay control (green line). **(B)** Activation of a two component proteolytic biosensor system composed of 50 nM solution FKBP-TVMV-AI and 50 nM FRB-CaM-CaM-BP incubated with different concentrations of rapamycin in presence of 20 nM of CaM-GDH (Q2CaM-GDH). **(C)** The absorbance at
10 data collected at 1000 sec. in the experiment shown in B was plotted against the concentration of rapamycin and fitted to a quadratic equation leading to a K_d of 11 nM.

Figure 13: Use of caged CaM-BP in a two-component system. Schematic showing the structure of the two-component biosensors comprising the caged CaM-BP.

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Figure 14: Independence of two component biosensor from caged CaM-BP

component concentrations. (A) Titration of the 10 nM of FKBP-GDH-CaM with increasing concentrations of FRB-mut CaM- CaM-BP in the absence of Rapamycin. The green trace represents the control reaction containing 10 nM FKBP-GDH-CaM and
20 200 nM wt CaM-BP. This reveals the possible dynamic range. **(B)** As in A but in the presence of saturating concentrations of Rapamycin.

Figure 15: Independence of two component biosensor from caged CaM-BP

component concentrations. (A) As in Fig. 12 but using stoichiometric concentrations of the biosensors components and equimolar amount of Rapamycin. Both biosensor and
25 components are present at 1 mM concentration. The reaction contained 20 mM Tris-HCl pH8.0, 20 mM NaCl, 2.5 mM CaCl_2 . The concentration of PQQ was kept at 0.1 nM. **(B)** As in A, but biosensor and components are present at 10 μM . **(C)** As in A, but biosensor and components are present at 20 μM .

30

Figure 16: Construction of auto-inhibited CaM-GDH module and its activation using calmodulin binding peptides. (A) Possible response of the CaM-GDH fusion to different

CaM-BPs. **(B)** Analysis of the effect of synthetic CaM-BPs derived from the high resolution structures of CaM:CaM-BP complexes on the activity of the CaM-GDH fusion. In the experiment the FKBP-CaM-GDH generation 4 was incubated with 200nM of the respective peptide and 600 nM 2-6-dichlorophenyl-Indophenol (DCPIP), 600 nM phenazine methosulfate (PSF), 60 nM PQQ and 20 mM glucose buffered with 1 mM CaCl₂ and 20 mM K₂HPO₄ at pH 7.0. The reaction is monitored by measuring the decrease in absorbance at 620 nm in the 96 well plate using plate reader. **(C)** Schematic representation of a protease sensor based on the autoinhibited CaM-GDH fused to an inhibitory peptide. **(D)** A two component system that in the presence of a ligand brings autoinhibited CaM-GDH into molecular proximity with autoinhibited protease resulting in the intermolecular swap of the inhibitor and substrate sequence and the cleavage of the latter. Leading to the activation of the GDH activity.

Figure 17: Generation 5 two component biosensor of Serum Human Albumin (HSA). **(A)** GDH activity of two component HSA biosensor and different concentrations of purified HSA. **(B)** Fit of the titration data between 0 and 25 nM of HSA to quadratic equation leading to K_d of 5nM. The reaction contained 10 nM GDH-CalM-GA fusion and 100nM VHH-CaM-BP and 1mM CaCl₂ in addition to the normal reaction buffer.

Figure 18: Analysis of the effect of different CaM-BPs on CaM-GDH chimera with the insertion at position 330 (open bars) and CaM-GDH chimera with the insertion at position 403 (filled bars). In the assay 5nM solution of CaM-GDH was incubated with 200nM of the respective peptide for 20 minutes and the reactions were triggered by the addition of glucose and changes in absorption were monitored for 10 minutes. The slope (k_{obs}) of the obtained curve was used as a measure of chimera's activity. The activity in the absence of CaM-GDH was considered as zero and activity of M13 peptide (peptide 1 here) was taken as 100% and used to scale the data.

Figure 19: Calmodulin-based artificial allosteric switch modules and thereon based two component biosensors. **(A)** Schematic representation of the conformation changes in calmodulin protein induced by the calmodulin binding peptide. The four dotted balls attached to calmodulin represent Ca²⁺ ions **(B)** An example of calmodulin-operated switch

- unit constructed on the basis of PQQ-glucose dehydrogenase. (C) A two component biosensor based on the allosteric switch module shown in (B). Ligand mediated scaffolding increases the local concentration of the calmodulin-binding peptide in the vicinity of the calmodulin and drives the conformation change of the reporter chimera. (D and E)
- 5 Activity analysis of two component Tacrolimus receptor performed at increasing concentrations of the biosensor components. 10nM of cysteine “ratcheted” CaM-GDH-FKBP and 30nM of CalA/CalB-CaM-BP were used for (D), and 1uM of cysteine “ratcheted” CaM-GDH-FKBP and 1.1uM of CalA/CalB-CaM-BP were used for (E). High concentrations of the biosensor components leads to an increase in background activation.
- 10 The concentration of the GDH cofactor PQQ was kept at 1nM to reduce the efficiency of the electron transfer. (F) Two component system where the dynamic range of the assay is tuned by the presence of the CaM mutant. (G) An example of biosensor response shown in (F) in the presence of 20μM of CaM mutant.
- 15 **Figure 20: Adoption of the two-component biosensor assay to higher concentration of the components and electrochemical readout.** (A) Schematic representation of the two component biosensor system with the “scavenger” low affinity calmodulin mutant. (B) as in Fig. 19E, but supplementing the assay with 20μM E83S, F92A, L105A and F141A calmodulin mutant. (C) Amperometric analysis of the reaction mixture containing 1μM
- 20 GDH-CaM-FKBP, 2μM CalA/CalB-CaM-BP, 20μM E83S, F92A, L105A and F141A calmodulin mutant and 1.5μM PQQ in the absence or presence of 10μM of tacrolimus. Activated sensor was incubated with 2mM mPMS mediator and 20mM glucose for 10 minutes, followed by polarization on a disposable electrode to yield a current related to the remaining oxidized mPMS after enzymatic reduction. Enzyme activity signal (μA)
- 25 proportional to mPMS reduced was calculated via subtraction of a blank value.

- Figure 21: Stability analysis of CaM-GDH based biosensors.** (A) Activity analysis of 10nM solution of CaM-GDH made from protein stock that either was sorted frozen or was freeze-dried and reconstituted after seven days storage at room temperature in the presence
- 30 or absence of 100nM of M13 peptide. (B) Activity analysis of solution of 20nM CaM-GDH-α-amylase-VHH1 and 100nM α-amylase VHH2-BP in the presence or absence of 25nM of purified salival α-amylase. Comparison of frozen and dried samples was

performed as in (A). (C) as in (B) but using 10nM CaM-GDH-FKBP and 30nM of CalA/CalB-CaM-BP in the presence or absence of 50nM of tacrolimus. (D) as in (C) but using 5nM CaM-GDH –Cyclophilin and 30nM CalA/CalB-CaM-BP in the presence or absence of 30nM of Cyclosporine A. (E) as in D but using 10nM CaM-GDH-GA and
 5 100nM of HSA-VHH-BP.

Figure 22: Allosteric peptide biosensors based on Affinity clamp-GDH chimera. (A) Schematic of GDH affinity clamp chimera and its interaction with affinity clamp binding peptide. (B) Activity of 10nM of affinity clamp-GDH chimera at different concentrations
 10 of binding peptide in the buffer containing 50uM CaCl₂.

Figure 23: Conversion of constitutively active protein reporters into peptide regulated allosteric modules by calmodulin domain insertion. (A) Activity of 10 nM calmodulin-dehydrofolate reductase (DHFR) chimera as a function of Cam-BP
 15 concentration. (B) Schematic representation of a single ligand two state allosteric switch. (C) Schematic representation of an allosteric switch based on operably coupled receptor and reporter domains (D) A two component system developed in this study where the system's specificity is "outsourced" to the ligand binding domains while a low affinity allosteric ligand activates a generic allosteric actuator.

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Figure 24: Three component biosensor system based on 5th generation version of GDH-CaM. (A) Schematic showing the design for a three-component biosensor, where Binders 1 and 2 bind to the Target. Constructs expressed in e coli periplasm to facilitate formation of disulfide bonds. (B) GDH activity of three component amylase biosensor
 25 (10nM GDH-CaM-SH3-FKBP, 20nM FRB-SH3L-VHH1, 100nM VHH2-CaMBP) at different concentrations of α -amylase in 1ml reaction comprising 0.6mM PMS, 20mM glucose, 1mM CaCl₂ and 250nM rapamycin, with 15 min pre-incubation time. (C) Fit of the titration data between 0 and 250 nM of α -amylase to quadratic equation leading to K_d of 13nM.

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Figure 25: Single component glucose dehydrogenase (GDH) sensors. (A) Novel biosensor architecture based on engineered GDH. A: Schematic representation of wild type

- GDH and its enzymatic reaction utilised by common glucose monitors. B: A schematic of the circular permuted GDH (cpGDH). C: Introduction of the conformational inhibitory calmodulin domain into the circular permuted GDH. D: The inactive Calmodulin-GDH chimera is flanked by binders to a target analyte of choice. E: Analyte and calmodulin
- 5 binding peptide-driven conformational change and activation of the GDH biosensor. **(B)** GDH activity of single component rapamycin biosensor (10nM of FKBP-cpGDH-CaM-FRB) at different concentrations of rapamycin in 1ml reaction comprising 0.6mM PMS, 20mM glucose, 2.5mM CaCl₂, 250nM M13 Calmodulin binding peptide and 1μM PQQ, with 15 min pre-incubation time. **(C)** GDH activity of single component FK506 biosensor
- 10 (2.5nM of calcineurin-cpGDH-CaM-FKBP) at different concentrations of FK506 in 1ml reaction comprising 0.6mM PMS, 20mM glucose, 2.5mM CaCl₂, 500nM M13 Calmodulin binding peptide and 1μM PQQ, with 30 min pre-incubation time. **(D)** Fit of the titration data between 0 and 10 nM of FK506 to quadratic equation leading to K_d of 1.4nM.
- 15 **Figure 26: Single component cyclosporine A sensor. (A)** GDH activity of single component cyclosporine A biosensor (2.5nM of calcineurin-cpGDH-CaM-cyclophilin) at different concentrations of cyclosporine A in 1ml reaction comprising 0.6mM PMS, 20mM glucose, 2.5mM CaCl₂, 500nM M13 Calmodulin binding peptide and 1μM PQQ, with 30 min pre-incubation time. **(B)** Fit of the titration data between 0 and 25 nM of cyclosporine
- 20 A to quadratic equation leading to K_d of 0.3nM. **(C)** Comparing the performance of single vs two component cyclosporine A GDH-based biosensors. Concentration of the drug cyclosporine A was measured in the same patient samples (1μl of whole blood per assay).

- Figure 27: Single component amylase sensor. (A)** GDH activity of single component amylase biosensor (5nM of VHH1-cpGDH-CaM-VHH2) at different concentrations of amylase in 1ml reaction comprising 0.6mM PMS, 20mM glucose, 2.5mM CaCl₂, 500nM M13 Calmodulin binding peptide and 1μM PQQ, with 30 min pre-incubation time. **(B)** Fit of the titration data between 0 and 100 nM of amylase to quadratic equation leading to K_d of 5.6nM. **(C)** Detection of amylase in saliva sample using single component amylase
- 30 GDH-based biosensor.

DETAILED DESCRIPTION

The present invention relates to improved oxidoreductase enzymes, enzymes and polypeptides which may be used in biosensors, including single, two-component and three-component biosensors, which are preferably capable of detecting the presence of a target molecule. The biosensors may comprise two or three components, wherein each component comprises a number of discrete functional domains, optionally linked by linkers. Each biosensor component, comprising the different domains, optionally linked by linkers, is typically a single contiguous protein amino acid sequence. Thus, the two or three components of the biosensors may comprise two or three separate protein sequences. Typically, co-localisation of the two or three components, for example, by binding a target molecule, activates the biosensor. The various aspects of the biosensor architecture described herein are generally applicable to different biosensor types that make use of the improved biosensor features of the invention. Accordingly, the following description describes general features of the biosensor architecture, particularly the different domains and components, followed by specific details of the various improvements embodied in the invention, which may be integrated into this architecture in various combinations.

The improved oxidoreductase enzymes, enzymes and polypeptides of the present invention may be used within biosensors of the present invention that are improved over biosensors described in the prior art in that they are optimised for the detection of target molecules in physiological conditions. Thus, the biosensors of the present invention are optimised to function in physiological conditions, such as at physiological calcium levels. Additionally, in physiological conditions target molecules are typically present at vastly different concentrations. Thus, the biosensors of the invention are adapted for detection of target molecules across a wide range of concentrations by improvements in the biosensors that provide reduced background and increased sensitivity giving an enhanced signal-to-noise ratio and an improved dynamic range. Different improvements in various aspects of the biosensor architecture provided by the present invention are summarised below:

In a first aspect the present invention provides an oxidoreductase enzyme comprising a calmodulin protein or a functional fragment thereof, wherein binding of a peptide to the calmodulin protein or functional fragment thereof reversibly regulates the catalytic activity

of the enzyme, wherein binding of the peptide is capable of reversibly regulating the catalytic activity of the enzyme in the presence of physiological calcium concentrations. Biosensors comprising such an oxidoreductase enzyme are insensitive to changing calcium concentrations within the physiological range of typically between 500 μ M and 5 mM calcium. Thus, biosensors comprising this oxidoreductase enzyme of the invention are adapted for use in physiological conditions and in biological samples, such as saliva and blood.

In a second aspect, the present invention provides an oxidoreductase enzyme comprising a heterologous amino acid sequence which is responsive to a target molecule, wherein binding of the target molecule to the heterologous amino acid sequence reversibly regulates catalytic activity of the enzyme, wherein the oxidoreductase enzyme comprises the heterologous amino acid sequence in a location corresponding to the loop connecting beta-sheets 5 and 6 of a PQQ-GDH. The present inventors have identified that specific insertion sites in oxidoreductase enzymes provide better calcium insensitivity. Thus, as described above, biosensors comprising oxidoreductases where the heterologous amino acid sequence is inserted in a location corresponding to the loop connecting beta-sheets 5 and 6 of a PQQ-GDH are adapted for use in physiological conditions and in biological samples.

In a third aspect, the present invention provides an enzyme comprising a heterologous amino acid sequence which is responsive to a target molecule, wherein binding of the target molecule to the heterologous amino acid sequence reversibly regulates catalytic activity of the enzyme; wherein the heterologous amino acid sequence is provided as an insert within the amino acid sequence of the enzyme flanked on either side by linkers, wherein binding of the target molecule to the heterologous amino acid sequence brings the linkers into proximity with each other; and wherein interaction between the linkers enhances activation of the catalytic activity of the enzyme, optionally wherein said interaction maintains the linkers in proximity with each other. Biosensors comprising such enzymes show enhanced sensitivity. The linkers between the heterologous amino acid insert and the enzyme are adapted so that when they are brought into proximity they interact so as to maintain their proximity, thereby prolonging and enhancing the activation

of the biosensor. This enhanced sensitivity improves the dynamic range of the biosensors comprising these enzymes allowing for detection of the target molecule of the biosensor across a wide range of concentrations.

- 5 In a fourth aspect, the present invention provides a polypeptide comprising a calmodulin binding peptide and a calmodulin protein or functional fragment thereof, wherein the calmodulin protein or functional fragment thereof comprises one or more modifications resulting in a reduced binding affinity for the calmodulin binding peptide as compared to a
- 10 polypeptides function to cage the calmodulin binding peptide so that it is not free in solution. These polypeptides can be incorporated into biosensors and cage the calmodulin binding peptide, thereby preventing activation of the enzyme of the biosensor, until the two components of the biosensor are brought into proximity, for example, by the presence of the target molecule of the biosensor. Such biosensors have reduced background and hence
- 15 are better adapted for detection of target molecules that may be at low concentrations in physiological samples and conditions.

- Similarly, as part of the fourth aspect, the present invention also provides a polypeptide comprising a calmodulin protein or functional fragment thereof, wherein the calmodulin
- 20 protein or functional fragment thereof comprises one or more modifications resulting in a reduced binding affinity for the calmodulin binding peptide as compared to a corresponding wild type calmodulin protein or functional fragment thereof. These polypeptides also function to cage the calmodulin binding peptide so that it is not free in solution, but may not be linked to the CaM-BP. These polypeptides can be incorporated
- 25 into the biosensors of the invention and cage the calmodulin binding peptide of the biosensor, thereby preventing activation of the enzyme of the biosensor, until the two components of the biosensor are brought into proximity, for example, by the presence of the target molecule of the biosensor. Such biosensors have reduced background, lower sensitivity to the component's concentrations and hence are better adapted for detection of
- 30 target molecules that may be at broadly varying concentrations in physiological samples and conditions.

In a fifth aspect, the present invention provides a variant calmodulin binding peptide (CaM-BP) which is capable of binding to a calmodulin protein or a functional fragment thereof provided as an insert within the amino acid sequence of an enzyme, wherein the calmodulin protein or functional fragment thereof activates the catalytic activity of the enzyme on binding of a corresponding activating CaM-BP, optionally a wild-type CaM-BP, and wherein binding of the variant CaM-BP to said calmodulin protein or functional fragment thereof inhibits binding of the corresponding activating CaM-BP, optionally a wild type CaM-BP, thereby preventing activation of said enzyme. These variant CaM-BPs may be used in biosensors to block activation of the enzyme of the biosensor until the two components of the biosensor have been brought into proximity, for example by the presence of the target molecule of the biosensor. Thus, as above, such biosensors have reduced background and hence are better adapted for detection of target molecules that may be at low concentrations in physiological samples and conditions.

In a sixth aspect, the present invention provides an oxidoreductase enzyme comprising a heterologous amino acid sequence that releasably maintains the enzyme in a catalytically inactive state by allosteric regulation and which is responsive to a molecule to release said regulation, a first binding moiety and a second binding moiety, wherein the amino acid sequence of the enzyme is circularly permuted, and wherein (i) release of the allosteric regulation by the heterologous amino acid sequence and (ii) interaction between the binding moieties, reversibly regulates the catalytic activity of the enzyme. This oxidoreductase enzyme may be used as a single component biosensor (alongside a molecule regulating the heterologous amino acid sequence), with binding interactions regulating enzyme activity occurring within a single component.

In a seventh aspect, the present invention provides an oxidoreductase enzyme comprising a heterologous amino acid sequence which is responsive to a peptide comprising a first binding moiety, and a first interaction domain capable of interacting with a second interaction domain of a polypeptide, wherein the polypeptide further comprises a second binding moiety capable of interacting with said first binding moiety, and wherein binding between the first and second binding moieties and interaction of the first interaction domain of the enzyme with the second interaction domain of the polypeptide act to

colocalise the enzyme and the peptide, wherein binding of the peptide to the heterologous amino acid sequence reversibly regulates catalytic activity of the enzyme. This oxidoreductase enzyme may be used as part of a three-component system in combination with the polypeptide and peptide, with binding interactions between binding
5 moieties/interaction domains of the three components regulating catalytic activity of the enzyme.

These different improvements in various aspects of the biosensor architecture may be incorporated in different combinations into biosensors of the invention. For example, the
10 enzyme of the third aspect discussed above may correspond to the oxidoreductase enzyme of either the first or second aspect. Similarly, the modified calmodulin protein or functional fragment thereof of the fourth aspect discussed above may be included in a biosensor with the oxidoreductase of the first, second, fifth or sixth aspects described above, such that the CaM-BP that activates the oxidoreductase is caged until interaction
15 between the components (such as two components) of the biosensor. Thus, biosensors according to the present invention may be assembled that have low background, high sensitivity and are adapted for detection in physiological conditions. Further details of aspects of the architecture of the biosensors and the specific improvements provided in the present invention are described below.

20

Enzymes

The present invention relates to enzymes, particularly oxidoreductase enzymes, and biosensors comprising these enzymes. Typically, the enzyme, when catalytically active, is
25 capable of reacting with or acting upon a substrate molecule to thereby elicit a detectable signal. Non-limiting examples of suitable enzymes include β -lactamase, β -galactosidase, glucose oxidase, lysozyme, malate dehydrogenase, peroxidases (*e.g.*, HRP), phosphatases (*e.g.*, alkaline phosphatase), luciferase, transferases, ATPases, nucleases (*e.g.*, ribonucleases), kinases, synthases, oxidoreductases and dehydrogenases such as glucose
30 dehydrogenase, flavin adenine dinucleotide-dependent glucose dehydrogenase (FADGDH) and pyranose dehydrogenase (PDH). Non-limiting examples of suitable enzyme substrates include those that enable the generation of chromogenic, fluorescent, light (*e.g.*,

bioluminescent), electrical, radioactive and other detectable signals. In preferred instances, the enzyme is an oxidoreductase enzyme.

Typically, an oxidoreductase enzyme is a protein capable of displaying catalytic activity
5 towards a substrate molecule to thereby produce one or more electrons. The enzyme may be any enzyme capable of reacting with a substrate molecule to thereby produce one or more electrons. Preferably, the enzyme is an oxidoreductase, such as a glucose dehydrogenase (GDH), glucose oxidase, lactose dehydrogenase (LDH) or dihydrofolate reductase (DHFR). In some instances, the enzyme is an oxidoreductase and the activity is
10 oxidoreductase activity. The enzyme/oxidoreductase enzyme may be glucose oxidase and the substrate is glucose. The enzyme/oxidoreductase enzyme may be DHFR and the substrate molecule is dihydrofolic acid. The catalytic activity may thus be dihydrofolate reductase activity, which may be measured as described in the Materials and Methods. The DHFR preferably comprises the sequence of SEQ ID NO: 71, or a variant thereof.
15 The DHFR enzyme comprising a calmodulin protein as an insert may comprise, or consist of, the sequence of SEQ ID NO: 70. A DHFR may be encoded by a nucleic acid sequence encoding SEQ ID NO: 71. The enzyme/oxidoreductase enzyme may be LDH and the substrate molecule is lactate. Preferably the enzyme or oxidoreductase enzyme is GDH and the substrate molecule is glucose. The catalytic activity may thus be glucose
20 dehydrogenase activity, which may be measured as described in the Materials and Methods. The glucose dehydrogenase may be a pyrroloquinoline quinone-GDH (PQQ-GDH) or a flavin adenine dinucleotide-GDH (FAD-GDH). In preferred instances, the enzyme or oxidoreductase enzyme is a PQQ-GDH. A PQQ-GDH preferably comprises the sequence of SEQ ID NO: 3 or a variant thereof. A PQQ-GDH may be encoded by a
25 nucleic acid sequence encoding SEQ ID NO: 3.

The enzyme or oxidoreductase enzyme of the present invention comprises a heterologous amino acid sequence which is responsive to a target molecule, wherein binding of the target molecule to the heterologous amino acid sequence reversibly regulates catalytic
30 activity of the enzyme. The enzyme/oxidoreductase enzyme of the invention is thus engineered to be switchable from a state of reduced catalytic activity to a more catalytically active state based on whether the target molecule of the heterologous amino

acid sequence is bound. The enzyme/oxidoreductase enzyme is typically further engineered such that catalytic activity of the enzyme is further regulated by binding of a further target molecule, the target molecule of the biosensor, where this target molecule is typically an analyte to be detected. The binding of both target molecules may be necessary
5 for regulation of catalytic activity.

In some instances, binding of the target molecule to the heterologous amino acid sequence releasably maintains the enzyme in a state of reduced catalytic activity and loss of binding of the peptide may switch the enzyme from a state of reduced catalytic activity to a more
10 catalytically active state. However, in preferred instances, the heterologous amino acid sequence releasably maintains the enzyme in a state of reduced catalytic activity and is responsive to binding of the target molecule to switch the enzyme from a state of reduced catalytic activity to a more catalytically active state. Thus, binding of the target molecule to the heterologous amino acid sequence reversibly regulates catalytic activity of the
15 enzyme. Preferably, binding of the target molecule to the heterologous amino acid sequence activates the catalytic activity of the enzyme. The heterologous amino acid sequence may be displaced by binding of the target molecule or, preferably, may undergo a conformational change upon binding of the target molecule, optionally also in the presence of the further target molecule (the analyte) of the biosensor, to thereby catalytically
20 activate the enzyme. The heterologous amino acid sequence can thus allosterically regulate the catalytic activity of the enzyme.

Thus, the enzyme/oxidoreductase enzyme has a reduced or, preferably, enhanced state of catalytic activity when the target molecule is bound to the heterologous amino acid
25 sequence. In some instances, the oxidoreductase enzyme has a reduced or enhanced state of catalytic activity when the target molecule is bound to the heterologous amino acid sequence and a further target molecule (analyte) of the biosensor is also present. The reduction or preferably enhancement of catalytic activity may be of any magnitude. The reduction or preferably enhancement of catalytic activity is typically of a magnitude
30 sufficient to allow for correlation with the presence of the target molecule of the heterologous amino acid sequence or the presence of both the target molecule of the heterologous amino acid sequence and the target molecule of the biosensor. The skilled

person is able to determine whether binding of the target molecule regulates catalytic activity of the enzyme by comparing the activity of the enzyme with and without the target molecule.

- 5 The enzyme/oxidoreductase enzyme may be described as being catalytically active or in a catalytically active state when the target molecule is bound to the heterologous amino acid sequence or when the target molecule is bound to the heterologous amino acid sequence and a further target molecule (analyte) of the biosensor is also present. It should be understood that wild-type catalytic activity may not be conferred by binding of the target
- 10 molecule. Typically, an enzyme is catalytically active if it is capable of displaying specific enzyme activity towards a substrate molecule to produce a detectable signal, such as light, fluorescence, or a coloured product, under appropriate reaction conditions. Typically, an oxidoreductase enzyme is catalytically active if it is capable of displaying specific enzyme activity towards a substrate molecule to produce one or more electrons under appropriate
- 15 reaction conditions. As generally used herein catalytically inactive and catalytically inactive state may refer to an enzyme that is substantially incapable of displaying specific enzyme activity towards a substrate molecule under appropriate reaction conditions. Typically, the detectable signal (e.g., electrons) produced would be substantially less compared to that produced by a corresponding catalytically active enzyme or
- 20 oxidoreductase enzyme. Production of the detectable signal (e.g., electrons) may be entirely absent.

- The enzymes, oxidoreductase enzymes and biosensors described herein produce the detectable signal, preferably electrons, by reacting with substrate molecules in response to
- 25 binding, interacting with or otherwise detecting one or more target molecules. In this context react, reaction or reacting with a substrate molecule means enzymatically transforming the substrate molecule into one or more product molecules wherein the reaction produces a detectable signal or the product molecule may be directly or indirectly detected. For example, reacting with a substrate molecule may mean enzymatically
- 30 transforming the substrate molecule into one or more product molecules with a net or overall production of one or a plurality of electrons per substrate molecule. In preferred instances the biosensor acts as an electron donor, whereby the electrons produced by the

reaction may flow either directly or via an electron shuttle (*i.e.*, an electron mediator) such as, but not limited to, phenazine methosulfate or potassium ferrocyanide, to thereby act as an anode. The resulting change in potential between anode and cathode may be detected by an electronic detector.

5

In some embodiments the oxidoreductase enzymes and biosensors described herein may be attached to an electrode. The mode of attachment may permit direct electron transfer from the oxidoreductase enzyme or biosensor to the electrode. Typically, the biosensor or enzyme acts as an electron donor and electrons produced by the reaction may flow directly to the electrode to form the anode. The electrode may be composed of carbon nanotubes or graphene. The oxidoreductase enzyme or biosensor may be attached to the electrode surface using 1-pyrenebutanoic acid succinimidyl ester (PBSE) as a hetero-bifunctional linker, wherein the active ester groups of the PBSE linker may react with the amino groups of lysine residues in the oxidoreductase enzyme or biosensor. The electrode may be a screen printed electrode layered with a dry mixture comprising the oxidoreductase enzyme and/or biosensor of the invention and preferably further comprising an electron mediator. The enzyme may be immobilised on the electrode via a modified co-factor (such as PQQ for example). The modified co-factor may be functionalised with a linker that is attached to the surface of the electrode either covalently or non-covalently, for example through an attached group (for example through a pyrene- carbon nanotube interaction).

The enzymes, oxidoreductase enzymes and biosensors of the present invention may be lyophilised (*i.e.*, freeze-dried, cryodesiccated), for example by using a typical low temperature dehydration process that is well-known in the art. The enzymes, oxidoreductase enzymes and biosensors of the present invention may be lyophilised and re-hydrated (*i.e.*, reconstituted) and retain their activity, *i.e.*, do not show a significantly reduced activity after lyophilisation and re-hydration, as compared to their activity prior to lyophilisation. The enzymes, oxidoreductase enzymes and biosensors of the present invention may be lyophilised, re-hydrated and stored at room temperature for at least 7 days and retain their activity.

30

Heterologous Amino Acid Sequence

As described above, the present invention provides an enzyme or oxidoreductase enzyme comprising a heterologous amino acid sequence which is responsive to a molecule, target molecule, or peptide, wherein binding of the molecule, target molecule or peptide to the
5 heterologous amino acid sequence reversibly regulates catalytic activity of the enzyme. The heterologous amino acid sequence is preferably provided as an insert within the amino acid sequence of the enzyme or oxidoreductase enzyme. However, fusions of the heterologous amino acid sequence at the N- or C-terminus of the amino acid sequence of the enzyme or oxidoreductase enzyme are also possible. Thus, preferably, the enzyme/
10 oxidoreductase enzyme amino acid sequence and the heterologous amino acid sequence are present in, or form at least part of a single, contiguous amino acid sequence.

When provided as an insert, the heterologous amino acid sequence is contiguous with, respective portions, sub-sequences or fragments of the enzyme. The insertion is made at a
15 position in the amino acid sequence of the enzyme which tolerates said insertion without steric clashes preventing stable folding of the enzyme. In some instances, the heterologous amino acid sequence is provided as an insert within the amino acid sequence of the enzyme flanked on either side by linkers. The linkers may be added between the insert and the sequence of the enzyme to assist toleration of the insertion. The linkers may be an amino
20 acid sequence and may be between 1-20, 1-15, 1-10 or 1-5 amino acids in length, preferably between 1-10 amino acids in length. Typically, linkers comprise glycine and serine, preferably at least 50%, 60%, 70%, 80%, 90% glycine and serine.

The enzyme/oxidoreductase enzyme may comprise the heterologous amino acid sequence
25 at a loop or turn region in the structure of the enzyme, which functionally tolerates the heterologous amino acid sequence. The enzyme/oxidoreductase enzyme may comprise the heterologous amino acid sequence at a location in a region of the enzyme (such as a loop or turn region) which comprises one or more amino acid residues which influence substrate binding and/or catalytic activity of the enzyme. The heterologous amino acid sequence
30 insert may thus displace one or more residues which influence substrate binding and/or catalytic activity of the enzyme, such that catalytic activity of the enzyme is regulated by the heterologous amino acid sequence. The heterologous amino acid sequence insert may

displace one more residues which influence substrate binding and one or more residues which influence catalytic activity. The heterologous amino acid sequence insert may prevent or reduce substrate binding to the enzyme and/or may switch the enzyme to a state of reduced catalytic activity or a catalytically inactive state.

5

As described above, the binding of the target molecule to the heterologous amino acid sequence reversibly regulates catalytic activity of the enzyme. The binding of the target molecule to the heterologous amino acid sequence may thus reverse the displacement of one or more residues which influence substrate binding and/or catalytic activity of the enzyme. The catalytic activity of the enzyme may accordingly be regulated by the conformational status of the heterologous amino acid sequence, as affected by binding of the target molecule. The heterologous amino acid sequence provided as an insert may reversibly regulate catalytic activity through inducing a conformational change in the enzyme, typically at the substrate binding region and/or active site of the enzyme. The heterologous amino acid sequence typically undergoes a conformational change in the presence of the target molecule which acts to regulate catalytic activity of the enzyme. The heterologous amino acid sequence may thus allosterically regulate the catalytic activity of the enzyme in the presence of the target molecule.

In a preferred embodiment, the heterologous amino acid sequence is inserted in an oxidoreductase enzyme, preferably a GDH enzyme. The heterologous amino acid sequence may be inserted in the oxidoreductase enzyme in a location capable of regulating the position of the catalytic residues of the oxidoreductase enzyme. Preferably, the heterologous amino acid sequence may be inserted in the oxidoreductase enzyme in a location capable of regulating the position of the catalytic residues of the oxidoreductase enzyme corresponding to Arg406 and/or Arg408 of PQQ-GDH. The heterologous amino acid sequence may be inserted into a loop region of the oxidoreductase enzyme. Preferably, the heterologous amino acid sequence may be inserted in a location corresponding to the loop connecting beta-sheets 5 and 6 of a PQQ-GDH. Thus, the heterologous amino acid sequence may be inserted in a location corresponding to any position between positions 401-407 of PQQ-GDH of SEQ ID NO: 3. Preferably, the heterologous amino acid sequence is inserted in a location corresponding to positions 403-

405 (amino acid residues Ser403 to Asn405) of PQQ-GDH of SEQ ID NO: 3. This insertion may delete the amino acids at position 404 (Asn404) of PQQ-GDH of SEQ ID NO: 3 or at a corresponding position thereto. The skilled person is able to identify corresponding locations in other enzymes from structural analysis and sequence alignment.

- 5 A corresponding location is typically one which accommodates the inserted heterologous amino acid sequence such that it reversibly regulates catalytic activity of the enzyme as described above.

- 10 For example, the heterologous amino acid sequence, such as calmodulin binding protein or functional fragment thereof, may be inserted into an DHFR enzyme in a location capable of regulating the position of the catalytic residues of the DHFR enzyme. The heterologous amino acid sequence may be inserted in the DHFR enzyme in a location corresponding to Gly86 and/or Val88 of the DHFR enzyme (SEQ ID NO: 71). The heterologous amino acid sequence may be inserted in a location corresponding to any position between positions 15 80-90, preferably positions 85-88, of SEQ ID NO: 71.

- Thus, the present invention provides an oxidoreductase enzyme comprising a heterologous amino acid sequence which is responsive to a target molecule, wherein binding of the target molecule to the heterologous amino acid sequence reversibly regulates catalytic 20 activity of the enzyme, wherein the oxidoreductase enzyme comprises the heterologous amino acid sequence in a location corresponding to the loop connecting beta-sheets 5 and 6 of a PQQ-GDH. In preferred instances, the invention provides a GDH enzyme comprising a heterologous amino acid sequence inserted between positions 403 and 405 of SEQ ID NO: 3 or a variant thereof. Such an enzyme may comprise from N-terminus to C-terminus, 25 the sequences of SEQ ID NO: 38 (residues 1-403) or a variant thereof, the heterologous amino acid sequence, and SEQ ID NO: 39 (residues 405-455) or a variant thereof.

- The heterologous amino acid sequence provided as an insert within the amino acid sequence of the enzyme may be flanked on either side by linkers, i.e., the sequences of the 30 enzyme and the heterologous amino acid sequence may be separated by linkers. The linkers may be an amino acid sequence and may be between 1-20, 1-15, 1-10 or 1-5 amino acids in length, preferably between 1-10 amino acids in length. Typically, linkers

comprise glycine and serine, preferably at least 50%, 60%, 70%, 80%, 90% glycine and serine. The some instances, the linkers have the sequences GSGG and GGSGG.

The heterologous amino acid sequence may be any binding moiety for any target molecule
5 that undergoes a conformational changes upon binding of the target molecule. The heterologous amino acid sequence may comprise one more domains (such as one or two domains) which undergo structural rearrangement (*i.e.*, conformational change) upon binding of the target molecule. The heterologous amino acid sequence may comprise an unstructured or unfolded amino acid sequence which undergoes a structural rearrangement
10 or conformational change upon binding of the target molecule which optionally creates one or more folded protein domains. Preferably, the heterologous amino acid sequence undergoes a structural rearrangement or conformational change upon binding of the peptide that increases or decreases the distance in space between the N- and C- termini of the heterologous amino acid sequence. The heterologous amino acid sequence may be a binding
15 moiety as described below. The heterologous amino acid sequence may be an affinity clamp.

Calcium-binding proteins, typically calmodulin proteins

20

Preferably, the heterologous amino acid sequence is an amino acid sequence of a calcium-binding protein, or a functional fragment thereof. Where the heterologous amino acid sequence is a calcium-binding protein or functional fragment thereof, regulation of catalytic activity of the enzyme may requires the presence of calcium ions. In preferred instances, the
25 heterologous amino acid sequence is a calmodulin protein or a functional fragment thereof. The calmodulin protein or functional fragment thereof may be calcium-insensitive. A calmodulin protein as described herein may be any calmodulin protein or domain previously described and may be a derivative or variant of a calmodulin protein or domain. A calmodulin protein or functional fragment thereof is capable of reversibly regulating,
30 preferably activating, the catalytic activity of an enzyme upon binding of a peptide to the calmodulin protein or functional fragment thereof, where the calmodulin protein or a functional fragment thereof is provided as an insert within the amino acid sequence of the

enzyme, as described herein. The heterologous amino acid sequence may comprise a calmodulin protein comprising a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or at least 100% sequence identity to SEQ ID NO: 2, or a functional fragment thereof. The heterologous amino acid sequence may comprise or consist of the
5 sequence of SEQ ID NO: 2, or a variant or functional fragment thereof. Variants and functional fragments typically retain calmodulin activity. For example, variants and functional fragments typically retain calcium-binding activity and/or calmodulin-binding peptide binding activity, preferably both activities. Variants and functional fragments may not have calcium-binding activity and/or be calcium-insensitive; such variants and
10 functional fragments typically retain calmodulin-binding peptide binding activity. Where a calmodulin protein or functional fragment thereof is calcium-insensitive, it may bind calcium, but such calcium-binding may not significantly affect the conformation of the calmodulin protein or functional fragment thereof. In some instances, the heterologous amino acid sequence may comprise a calmodulin protein or functional fragment thereof
15 comprising one or more modifications resulting in a reduced binding affinity for a calmodulin-binding peptide as described further below.

The term variant as used herein may describe functional fragments of proteins or peptides of the invention, suitably retaining their relevant catalytic activity or binding activity as
20 applicable. Variants may include amino acid sequences comprising deletion or insertions as compared to any of the amino acid sequences disclosed herein. Such deletions or insertions may be 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids in length, or between 1-5, 1-10, 1-20, 1-30, 1-40 or 1-50 amino acids in length, preferably between 1-10 amino acids in length. Variants may preferably include amino acid sequences comprising mutations (*i.e.*, substitutions, point
25 mutations) relative to the corresponding wild type amino acid sequence or relative to the corresponding amino acid sequence disclosed herein. Variants may comprise up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90 or 100 mutations, or between 1-5, 1-10, 1-15, 1-20, 1-30, 1-40 or 1-50 mutations, preferably between 1-20 mutations relative to the corresponding wild type amino acid sequence or relative to the corresponding amino acid
30 sequence disclosed herein. For example, typically, conservative amino acid variations may be made without an appreciable or substantial change in function. For example, conservative amino acid substitutions may be tolerated where charge, hydrophilicity, hydrophobicity, side

chain “bulk”, secondary and/or tertiary structure (*e.g.* helicity), target molecule binding, protease activity and/or protease inhibitory activity are substantially unaltered or are altered to a degree that does not appreciably or substantially compromise the function of the biosensor.

5

Fragments are typically N- and/or C- terminal truncations. Protein fragments may comprise up to 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, preferably up to 80%, 85%, more preferably up to 90% or up to 95-99% of an amino acid sequence disclosed herein. In some embodiments, the protein fragment may comprise up to
10 5, 10, 20, 40, 50, 70, 80, 90, 100, 120, 150, 180 200, 220, 230, 250, 280, 300, 330, 350, 400 or 450 amino acids of an amino acid sequence disclosed herein. Variants may include amino acid sequences having at least 80%, at least 85%, preferably at least 90%, 91%, 92%, 93%, 94% 95%, 96%, 97%, 98% or 99% sequence identity with any of the amino acid sequences disclosed herein.

15

The target molecule of the heterologous amino acid sequence may be any ligand, analyte, small organic molecule, epitope, domain, fragment, subunit, moiety or combination thereof. The target molecule of the heterologous amino acid sequence may be a protein, including antibodies and antibody fragments, antigens, enzymes, phosphoproteins, glycoproteins,
20 lipoproteins and glycoproteins. The target molecule of the heterologous amino acid sequence may be lipid, phospholipids, carbohydrates (including simple sugars, disaccharides and polysaccharides), nucleic acids, nucleoprotein or any other molecule or analyte. The target molecule of the heterologous amino acid sequence may be a small molecule, such as a drug or other pharmaceuticals including antibiotics.

25

In preferred instances, the target molecule of the heterologous amino acid sequence is a peptide. The peptide may have any sequence, but is capable of binding to the heterologous amino acid sequence. The peptide may be a calmodulin-binding peptide (where the heterologous amino acid sequence is calmodulin), a peptide binding an affinity clamp (*e.g.*,
30 a PDZ domain binding peptide, where the heterologous amino acid sequence is an ePDZ domain), an SH3 domain binding peptide (where the heterologous amino acid sequence is an SH3 domain), an antibody binding peptide (where the heterologous amino acid sequence

is an antibody), or a leucine zipper peptide (where the heterologous amino acid sequence is a second leucine zipper peptide). The peptide may be between 1-10, 1-15, 1-20, 1-30, 1-40, 1-50, 1-100, 1-200 amino acids in length, preferably between 1-20 amino acids in length. The peptide may comprise or consist of a linear binding epitope that binds the heterologous amino acid sequence. In some instances, the target molecule of the heterologous amino acid sequence is a peptide that binds to calmodulin. In preferred instances, the target molecule of the heterologous amino acid sequence is a calmodulin-binding peptide (CaM-BP). A CaM-BP preferably comprises any amino acid sequence (preferably a linear peptide epitope of 1-20 amino acids) that is capable of specifically binding to calmodulin and inducing a structural rearrangement or conformational change in the calmodulin that brings the N- and C- termini of calmodulin into proximity. Preferably, a CaM-BP is capable of binding to a calmodulin protein or a functional fragment thereof that is provided as an insert within the amino acid sequence of an enzyme and thereby reversibly regulating, preferably activating, the catalytic activity of the enzyme, as described herein. A CaM-BP may be any previously described CaM-BP or a derivative or variant of any CaM-BP. The CaM-BP may comprise a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or at least 100% sequence identity to, preferably at least 80% sequence identity to, any one of SEQ ID NOs: 10, 29 or 37, preferably SEQ ID NO: 10. The CaM-BP may comprise or consist of the sequence of any one of SEQ ID NOs: 10, 29 or 37, or variants thereof, preferably SEQ ID NO: 10 or a variant thereof. Variants typically comprise sequences having deletions or insertions of between 1-5 amino acids relative to the sequence of any one of SEQ ID NOs: 10, 29 or 37. Variants preferably comprise sequences comprising between 1-10 mutations, preferably 1-5 mutations, relative to the sequence of any one of SEQ ID NOs: 10, 29 or 37. Variants retain binding to calmodulin. In some instances the CaM-BP may have a reduced binding affinity for calmodulin, *i.e.*, have a binding affinity for a calmodulin protein that is lower than that of wild type CaM-BP (such as a peptide having the sequence of SEQ ID NO: 29) for said calmodulin protein. In such instances the CaM-BP may preferably comprise, or consist essentially of, the sequence of SEQ ID NO: 37, or a variant thereof. Alternatively, in such instances the CaM-BP may comprise, or consist essentially of, the sequence of any one of SEQ ID NOs: 45-60, or variants thereof. In such instances, variants typically comprise sequences having deletions or insertions of

between 1-5 amino acids relative to the specified sequence, and/or 1-10 mutations, preferably 1-5 mutations, relative to the specified sequence.

In preferred instances, the heterologous amino acid sequence is a calmodulin protein or a variant or functional fragment thereof, and the target molecule is a peptide, preferably a calmodulin-binding peptide. Thus, in some instances the present invention provides an oxidoreductase enzyme, preferably GDH, comprising a calmodulin protein or a functional fragment thereof, wherein binding of a peptide, preferably a CaM-BP, to the calmodulin protein or functional fragment thereof reversibly regulates the catalytic activity of the enzyme. In some instances the oxidoreductase enzyme comprising a calmodulin protein or a functional fragment thereof comprises or consists of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or at least 100% sequence identity to, preferably at least 80% sequence identity to SEQ ID NO: 1 or 23. In some instances the oxidoreductase enzyme comprising a calmodulin protein or a functional fragment thereof comprises or consists of a sequence of SEQ ID NO: 1 or 23, or a variant thereof.

The present inventors have surprisingly found that calcium sensitivity of a calmodulin-based oxidoreductase biosensor may be improved by selecting an appropriate insertion location for calmodulin in the oxidoreductase enzyme sequence. Preferred insertion sites are described above, but more generally an appropriate location is any location which allows for operation under physiological calcium concentrations. Thus, in some instances, the present invention provides an oxidoreductase enzyme comprising a calmodulin protein or a functional fragment thereof, wherein binding of a peptide to the calmodulin protein or functional fragment thereof reversibly regulates the catalytic activity of the enzyme, wherein binding of the peptide is capable of reversibly regulating the catalytic activity of the enzyme in the presence of physiological calcium concentrations. Physiological calcium concentrations are typically between 500 μ M to 5 mM calcium (*i.e.*, Ca^{2+}). Thus, in some instances, binding of the peptide is capable of reversibly regulating the catalytic activity of the enzyme in the presence of 500 μ M to 5 mM calcium, preferably in the presence of 1 mM to 2 mM calcium. In some instances, binding of the peptide is capable of reversibly regulating the catalytic activity of the enzyme in the presence of at least 0.5 mM, 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM, 3.5 mM, 4 mM, 4.5 mM or at least 5 mM

calcium, or between 0.5-5 mM, 1-5 mM, 2-5 mM, 1-3 mM, 1-4 mM, 2-3 mM, 2-4 mM, 3-4 mM, 3-5 mM or 4-5 mM calcium. In preferred instances, binding of the peptide is capable of reversibly regulating the catalytic activity of the enzyme in the presence of at least 1 mM calcium, preferably in the presence of 1 mM to 2 mM calcium. In some instances, changes in the calcium concentration between 0.5-5 mM do not appreciably affect the catalytic activity of the enzyme. Preferably, changes in the calcium concentration between 1 mM to 2 mM do not appreciably affect the catalytic activity of the enzyme. Calcium concentrations of between 0.5-5 mM, preferably at least 1 mM, do not activate the catalytic activity of the enzyme, such that the activity of the enzyme may be reversibly regulated by binding of the peptide. Thus, the oxidoreductase enzyme does not display regulation of catalytic activity by the calmodulin protein or functional fragment thereof in the presence of calcium alone, absent the peptide or the peptide and the target molecule of the biosensor.

15 Binding Moieties and Target Molecules of the Biosensors

In some instances, the enzymes, oxidoreductase enzymes, polypeptides, target molecules of the heterologous amino acid sequence, peptides or proteases of the invention comprise a binding moiety. In some instances, the enzymes/oxidoreductase enzymes comprise a first binding moiety. In some instances, the target molecule of the heterologous amino acid sequence or the peptide comprises a second binding moiety.

As generally used herein a binding moiety or binding moieties refer to one or a plurality of molecules or biological or chemical components or entities that are capable of recognizing and/or binding each other, or one or more other target molecules. Binding moieties may be proteins, nucleic acids (*e.g.* single-stranded or double-stranded DNA or RNA), sugars, oligosaccharides, polysaccharides or other carbohydrates, lipids or any combinations of these such as glycoproteins, PNA constructs *etc* or molecular components thereof. By way of example only, binding moieties may be, or comprise: (i) an amino acid sequence of a ligand binding domain of a receptor responsive to binding of a target molecule such as a cognate growth factor, cytokine, a hormone (*e.g.* insulin), neurotransmitters *etc*; (ii) an amino acid sequence of an ion or metabolite transporter capable of, or responsive to,

binding of a target molecule such as an ion or metabolite (*e.g.* a Ca^{2+} -binding protein such as calmodulin or calcineurin or a glucose transporter); (iii) a zinc finger amino acid sequence responsive to zinc-dependent binding a DNA target molecule; (iv) a helix-loop-helix amino acid sequence responsive to binding a DNA target molecule; (v) a pleckstrin

5 homology domain amino acid sequence responsive to binding of a phosphoinositide target molecule; (vi) an amino acid sequence of a Src homology 2- or Src homology 3-domain responsive to a signaling protein; (vii) an amino acid sequence of an antigen responsive to binding of an antibody target molecule; or (viii) an amino acid sequence of a protein kinase or phosphatase responsive to binding of a phosphorylatable or phosphorylated target

10 molecule; (ix) ubiquitin-binding domains; (x) proteins or protein domains that bind small molecules, drugs or antibiotics such as rapamycin-binding FKBP and FRB domains; (xi) single- or double-stranded DNA, RNA or PNA constructs that bind nucleic acid target molecules, such as where the DNA or RNA are coupled or cross-linked to an amino acid sequence or other protein-nucleic acid interaction; and/or (xii) an affinity clamp such as a

15 PDZ-FH3 domain fusion; inclusive of modified or engineered versions thereof. Exemplary binding moieties described in the Examples of the present application include FKBP (SEQ ID NO: 7), FRB (SEQ ID NO: 11), calcineurin alpha and beta subunits (SEQ ID NOs: 15 and 16), human serum albumin (HAS) GA binder (SEQ ID NO: 65), cyclophilin (SEQ ID NO: 68), antibody fragments, specifically α -amylase binding antibody VHH fragments

20 VHH1 (SEQ ID NO: 20) and VHH2 (SEQ ID NO: 22) and HAS antibody VHH binder (SEQ ID NO: 66); and variants thereof. Variants are typically functionally binding variants for the relevant respective binding moiety. The skilled person is able to generate antibodies and antibody (VHH) fragments targeting any number of known target molecules for use as binding moieties in the enzymes/oxidoreductase enzymes/biosensors of the

25 present invention. It will also be appreciated that binding moieties may be modified or chemically derivatised such as with binding agents such as biotin, avidin, epitope tags, lectins, carbohydrates or lipids.

In some instances, the binding moieties may be or comprise an antibody or antibody

30 fragment, inclusive of monoclonal and polyclonal antibodies, recombinant antibodies, Fab and Fab'₂ fragments, DARPins, diabodies and single chain antibody fragments (*e.g.* scVs).

Suitably, the first and second binding moieties may be or comprise respective antibodies or antibody fragments that bind a target molecule

In some instances, the binding moieties respectively are, or comprise, amino acid sequences of an affinity clamp. The affinity clamp preferably comprises a recognition domain and, optionally, an enhancer domain. The recognition domain is typically capable of binding one or more target molecules, such as described in (i)-(ix) above. Recognition domains may include, but are not limited to, domains involved in phospho-tyrosine binding (*e.g.* SH2, PTB), phospho-serine binding (*e.g.* UIM, GAT, CUE, BTB/POZ, VHS, UBA, RING, HECT, WW, 14-3-3, Polo-box), phospho-threonine binding (*e.g.* FHA, WW, Polo-box), proline-rich region binding (*e.g.* EVH1, SH3, GYF), acetylated lysine binding (*e.g.* Bromo), methylated lysine binding (*e.g.* Chromo, PHD), apoptosis (*e.g.* BIR, TRAF, DED, Death, CARD, BH), cytoskeleton modulation (*e.g.* ADF, GEL, DH, CH, FH2), ubiquitin-binding domains or modified or engineered versions thereof, or other cellular functions (*e.g.* EH, CC, VHL, TUDOR, PUF Repeat, PAS, MH1, LRR1, IQ, HEAT, GRIP, TUBBY, SNARE, TPR, TIR, START, SOCS Box, SAM, RGS, PDZ, PB1, LIM, F-BOX, ENTH, EF-Hand, SHADOW, ARM, ANK). The enhancer domain typically increases or enhances the binding affinity for at least one or the target molecules. In some embodiments, the affinity may be increased by at least 10, 100 or 1000 fold compared to that of the recognition domain alone. The affinity clamp may further comprise linker connecting the recognition domain and the enhancer domain.

In some instances, the affinity clamp comprises a recognition domain that comprises at least a portion or fragment of a PDZ domain and an enhancer domain that comprises at least a portion or fragment of a fibronectin type III domain. The PDZ domain may be derived from a human Erbin protein. Erbin-PDZ (ePDZ) binds to target molecules such as the C-termini of p120-related catenins (such as δ -catenin and Armadillo repeat gene deleted in Velo-cardio-facial syndrome (ARVCF)). Preferably, this instance of the affinity clamp further comprises the tenth (10th) type III (FN3) domain of human fibronectin as an enhancer domain. In some instances, the affinity clamp may comprise one or more connector amino acid sequences. For example, a connector amino acid sequence may connect the protease amino acid sequence (such as comprising a protease amino acid sequence) to the Erbin-PDZ

domain, the Erbin-PDZ domain to the FN3 domain and/or the FN3 domain to the inhibitor. Reference is also made to WO2009/062170, Zhuang & Liu, 2011, Comput. Theoret. Chem. **963** 448, Huang *et al*, 2009, J. Mol. Biol. **392** 1221, Huang *et al.*, 2008, PNAS (USA) **105** 6578, and Koide^{1,*} and Huang Methods Enzymol. 2013; 523: 285–302 for a more detailed
5 explanation of affinity clamp structure and function, and of particular affinity clamps that may be used in accordance with the invention.

In some instances, the first binding moiety is capable of directly interacting with the second binding moiety. In some instances, respective binding moieties may directly bind, interact
10 or form a complex. The first binding moiety and the second binding moiety may comprise molecules that can directly bind or interact. In preferred instances, the respective binding moieties are capable of binding, interacting or forming a complex with a target molecule. Typically, the respective binding moieties are capable of binding, interacting or forming a complex with the same target molecule. It will also be appreciated that the “same” target
15 molecule can have respective, different moieties, subunits, domains, ligands or epitopes that can be bound by the respective binding moieties to thereby co-localize the first and second binding moieties and hence the further components of the biosensors of the invention. Accordingly, the direct binding interaction between the target molecule and the binding moieties suitably facilitates co-localization of the two components of the biosensors of the
20 present invention.

The target molecule may be any ligand, analyte, ion (e.g., calcium, Ca²⁺), small organic molecule, epitope, domain, fragment, subunit, moiety or combination thereof. The target molecule may be a protein, for example including antibodies and antibody fragments,
25 antigens, enzymes such as α -amylase, human, serum albumin, phosphoproteins, glycoproteins, lipoproteins and glycoproteins. The target molecule may be lipids, phospholipids, carbohydrates including simple sugars, disaccharides and polysaccharides; nucleic acids, nucleoprotein. The target molecule may be a small molecule, chemical entity or any other analyte, including drugs, such as immunosuppressive drugs including
30 rapamycin (*i.e.*, sirolimus), cyclosporine, and tacrolimus (*i.e.*, FK506) and other pharmaceuticals including antibiotics, vitamins, banned substances, illicit drugs or drugs of addiction, chemotherapeutic agents and lead compounds in drug design and screening,

molecules and analytes typically found in biological samples such as biomarkers, tumour and other antigens, receptors, DNA-binding proteins inclusive of transcription factors, hormones, neurotransmitters, growth factors, cytokines, receptors, metabolic enzymes, signalling molecules, nucleic acids such as DNA and RNA, membrane lipids and other
5 cellular components, pathogen-derived molecules inclusive of viral, bacterial, protozoan, fungal and worm proteins, lipids, carbohydrates and nucleic acids. As described above, the same target molecule may be bound by different, respective binding moieties.

Exemplary binding moieties and target molecules are described in the Examples. In some
10 instances, the target molecule is an enzyme such as α amylase. In such instances, the first and second binding moieties may be antibodies therefor, such as exemplified camelid antibodies VHH1 and VHH2 (SEQ ID NOs: 20 and 22) or variants thereof. In some instances, the target molecule is a small organic molecule such as rapamycin. In such instances, the first and second binding moieties may be, respectively FKBP and FRB (SEQ
15 ID NOs: 7 and 11), or variants thereof. In some instances, the target molecule is a small organic molecule such as FK506 (i.e., tacrolimus). In such instances, the first and second binding moieties may be, respectively, an FKBP and a Calcineurin alpha/beta complex (SEQ ID NOs: 7, 15 and 16), or variants thereof. In some instances, the target molecule is human serum albumin (HAS). In such instances, the first and second binding moieties may be,
20 respectively, a HAS GA binder and a HAS-specific VHH (SEQ ID NOs: 65 and 66) or variants thereof. In some instances, the target molecule is cyclosporine. In such instances, the first and second binding moieties may be, respectively, cyclophilin (SEQ ID NO: 68) and a Calcineurin alpha/beta complex (SEQ ID NOs: 15 and 16), or variants thereof.

25 **Biosensors**

The present invention also provides biosensors comprising the oxidoreductase enzymes as described herein. Thus, a biosensor of the present invention may comprise in a first component an oxidoreductase enzyme comprising a heterologous amino acid sequence,
30 preferably a calmodulin protein or a functional fragment thereof, preferably provided as an insert within the amino acid sequence of the enzyme, wherein binding of a peptide to the calmodulin protein or functional fragment thereof reversibly regulates the catalytic activity

of the enzyme, wherein binding of the peptide is capable of reversibly regulating the catalytic activity of the enzyme in the presence of physiological calcium concentrations, as described herein. In preferred aspect, the heterologous amino acid insert, preferably a calmodulin protein or functional fragment thereof, is inserted within the amino acid sequence of the oxidoreductase enzyme in a location corresponding to the loop connecting beta-sheets 5 and 6 of a PQQ-GDH. In such instances, the oxidoreductase enzyme comprises a first binding moiety that is capable of interacting with a second binding moiety on the peptide, wherein interaction between the binding moieties regulates catalytic activity of the enzyme. Preferably, interaction of the binding moieties is dependent on the presence of a target molecule. The binding moieties may be any suitable binding moieties as described herein.

Thus, the second component of the biosensor may comprise the peptide, preferably a calmodulin binding peptide as described herein, and a second binding moiety. Typically, the peptide is engineered to bind the calmodulin protein or functional fragment thereof with an affinity insufficient to enhance catalytic activity in the absence of an interaction between the binding moieties. Thus, binding of the peptide, preferably a CaM-BP, to the calmodulin protein or functional fragment thereof is dependent on the presence of the target molecule. In the presence of the target molecule, the two binding moieties interact, co-localising the two components of the biosensor. This binding interaction brings the peptide into close proximity with the calmodulin protein or functional fragment thereof inserted in the oxidoreductase enzyme. The peptide binds to the calmodulin protein or functional fragment thereof thereby activating the catalytic activity of the enzyme. Thus, the catalytic activity of the enzyme is activated or enhanced in the presence of the target molecule.

In preferred instances, a biosensor of the present invention may comprise a first component comprising a GDH enzyme comprising a calmodulin protein insert and a first binding moiety and a second component comprising a calmodulin binding peptide and a second binding moiety. In an exemplary instance, the biosensor is configured for detection of rapamycin and the biosensor comprises a first component comprising a GDH enzyme comprising a calmodulin protein insert and an FKBP binding moiety and a second component comprising a calmodulin binding peptide and a FRB binding moiety. In some instances, the biosensor for detection of rapamycin comprises a first component comprising or consisting of a

sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 6 or 26; and a second component comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 9. In an exemplary instance, the biosensor is configured for detection of tacrolimus and the biosensor comprises a first component comprising a GDH enzyme comprising a calmodulin protein insert and an FKBP binding moiety and a second component comprising a calmodulin binding peptide and a calcinurin alpha/beta binding moiety. In some instances, the biosensor for detection of tacrolimus comprises a first component comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 6 or 26; and a second component comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 13. In an exemplary instance, the biosensor is configured for detection of α -amylase and the biosensor comprises a first component comprising a GDH enzyme comprising a calmodulin protein insert and an VHH1 binding moiety and a second component comprising a calmodulin binding peptide and a VHH2 binding moiety. In some instances, the biosensor for detection of α -amylase comprises a first component comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 19; and a second component comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 21. In an exemplary instance, the biosensor is configured for detection of HSA and the biosensor comprises a first component comprising a GDH enzyme comprising a calmodulin protein insert and an GA binding moiety and a second component comprising a calmodulin binding peptide and a HSA specific VHH binding moiety. In some instances, the biosensor for detection of HSA comprises a first component comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 63; and a second component comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 64. In an exemplary instance, the biosensor is configured for detection of cyclosporine and the biosensor comprises a first component

comprising a GDH enzyme comprising a calmodulin protein insert and an cyclophilin binding moiety and a second component comprising a calmodulin binding peptide and a calcinurin alpha/beta binding moiety. In some instances, the biosensor for detection of cyclosporine comprises a first component comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 68; and a second component comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 13.

As described above, such a biosensor is insensitive to changes in calcium concentration across the physiological range, which is typically between 0.5 mM-5 mM calcium. Thus, such a biosensor is specifically adapted for detection of target molecules in physiological conditions. This biosensor architecture may be modified by incorporation of any of the further improvements described herein below, including engineered linkers, caged CaM-BPs and variant CaM-BPs.

The biosensors may further comprise, for example as a third component, a polypeptide comprising a calmodulin protein or functional fragment thereof, wherein the calmodulin protein or functional fragment thereof comprises one or more modifications resulting in a reduced binding affinity for the calmodulin binding peptide as compared to a corresponding wild type calmodulin protein or functional fragment thereof, which is as described further herein. Such calmodulin proteins reduce the background signal of the biosensors and enhance the signal-noise ratio. For example, the calmodulin protein as the third component of the biosensor may be configured to bind to the second component of the biosensor comprising the calmodulin binding peptide, to sequester the second component of the biosensor and prevent activation of the enzyme of the first component of the biosensor, until the target molecular of the biosensor co-localises the two components. In the presence of the target molecule, the two binding moieties interact, co-localising the two components of the biosensor. This binding interaction brings the peptide into close proximity with the calmodulin protein or functional fragment thereof inserted in the enzyme of the biosensor, which has a higher affinity for the peptide. The peptide dissociates from the calmodulin protein, or functional fragment thereof, having a reduced binding affinity for the calmodulin

binding peptide and binds instead to the calmodulin protein, or functional fragment thereof, inserted in the enzyme of the biosensor, thereby activating the catalytic activity of the enzyme.

A further biosensor of the present invention may comprise a GDH enzyme comprising a
 5 circularly permuted GDH amino acid sequence comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 79, or may be any other variant sequence thereof as described above. The GDH enzyme may further comprise a calmodulin-binding protein or functional fragment thereof, and comprise an amino acid sequence comprising or consisting
 10 of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 80, or may be any other variant sequence thereof as described above. The GDH enzyme may further comprise particular first and second binding moieties exemplified herein, and comprise an amino acid sequence comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%,
 15 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 78, 81, 84 or 85, or may be any other variant sequence thereof as described above. The biosensor may further comprise a CaM-BP comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to any one of SEQ ID NOs 10, 29 or 37, or may be any other variant sequence
 20 thereof as described above.

An additional biosensor of the present invention may comprise (i) a GDH enzyme comprising a calmodulin-binding protein or functional fragment thereof, comprising an amino acid sequence comprising or consisting of a sequence having at least 60%, 70%, 80%,
 25 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 23, or may be any other variant sequence thereof as described above, and further comprising a first interaction domain; (ii) a polypeptide comprising a second interaction domain capable of interacting with said first interaction domain and a second binding moiety; and (iii) a peptide binding to the calmodulin-binding protein or functional fragment
 30 thereof and comprising a first binding moiety capable of interacting with said second binding moiety, wherein the polypeptide and peptide act to reversibly regulate catalytic activity of the GDH enzyme. The GDH enzyme of (i) may further comprise any particular first

interaction domain described herein and may comprise an amino acid sequence comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 72, or may be any other variant sequence thereof as described above. The polypeptide of (ii) may comprise any
5 second interaction domain capable of interacting with the first interaction domain, and any second binding moiety capable of interacting with the first binding moiety, and may comprise an amino acid sequence comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 75, or may be any other variant sequence thereof as described above.
10 The peptide of (iii) may comprise a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to any one of SEQ ID NOs 10, 29 or 37, or any other variant sequence thereof described above and additionally any first binding moiety capable of interacting with the second binding moiety. The peptide of (iii) may comprise a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%,
15 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 77, or may be any other variant sequence thereof as described above.

Engineered Linkers

As described above, the present invention provides an enzyme comprising a heterologous
20 amino acid sequence which is responsive to a target molecule, wherein binding of the target molecule to the heterologous amino acid sequence reversibly regulates catalytic activity of the enzyme. The present inventors have identified a mechanism whereby the sensitivity of such enzymes may be enhanced, by biasing the conformation of the enzyme to the activated state upon binding of the target molecule.

25

The present invention provides an enzyme comprising a heterologous amino acid sequence which is responsive to a target molecule, wherein binding of the target molecule to the heterologous amino acid sequence reversibly regulates catalytic activity of the enzyme; wherein the heterologous amino acid sequence is provided as an insert within the amino
30 acid sequence of the enzyme flanked on either side by linkers, wherein binding of the target molecule to the heterologous amino acid sequence brings the linkers into proximity with each other;

and wherein interaction between the linkers enhances activation of the catalytic activity of the enzyme, optionally wherein said interaction maintains the linkers in proximity with each other.

- 5 As described above, the heterologous amino acid sequence is preferably provided as an insert within the amino acid sequence of the enzyme flanked on either side by linkers. Thus, the heterologous amino acid sequence is joined to the enzyme amino acid sequence by a linker at the N-terminus of the heterologous amino acid sequence and by a linker at the C-terminus of the heterologous amino acid sequence. Binding of the target molecule to
10 the heterologous amino acid sequence typically results in the structural rearrangement or conformational change in the heterologous amino acid sequence, as described above, which may in some instances, bring the linkers into proximity with each other. Proximity between the linkers may be understood to mean that a portion of each of the linkers are within less than 50 Å, 40 Å, 30 Å, 20 Å, 15 Å, 10 Å or preferably less than 5 Å of each
15 other. Proximity between the linkers may be further understood to mean that the distance between the two linkers is short enough that the two linkers can interact.

- The interaction between the linkers enhances activation of the catalytic activity of the enzyme, optionally wherein said interaction maintains the linkers in proximity with each
20 other. The interaction between the linkers maintains the linkers in proximity with each other and thereby maintains the enzyme in the activated conformation that it adopts upon binding of the target molecule to the heterologous amino acid sequence. Thus, the interaction between the linkers biases the activated conformation of the enzyme and hence enhances activation of the catalytic activity of the enzyme.

- 25 The interaction between the linkers may be reversible or irreversible. The interaction between the linkers may comprise the formation of a non-covalent, or preferably a covalent bond. Suitable non-covalent bonds would be high-affinity interactions, *e.g.*, the biotin-streptavidin interaction. In some instances, the interaction between the linkers may
30 comprise a homo- or hetero-condensation reaction or, preferably, the formation of a disulphide bond.

In some instances, the linkers comprise amino acid sequences. In some instances, the linkers are 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids in length, or between 1-5, 1-10, 1-15 or 1-20 amino acids in length, preferably 1-10 amino acids in length. Typically the linkers may be G/S-rich linkers, *i.e.*, linkers comprising at least 50%, 60%, 70% or 80% glycine and/or serine amino acids. Each linker may comprise corresponding chemical reactive groups that are capable of forming a chemical bond, optionally spontaneously, when the linkers are brought into proximity with each other. In some instances, the linkers may comprise unnatural amino acids capable of homo- or hetero- condensation. In some instances, the linkers may comprise unnatural amino acids comprising a chemical reactive group. Suitable chemical reactive groups include, for example, carbodiimide, NHS ester, imidoester, haloacetyl (*e.g.*, bromo- or iodo-), pyridyldisulfide, thiosulfonate, vinylsulfone, hydrazide, alkoxyamine, diazirine or aryl azide. In some preferred instances the linkers comprise selenocysteine, or preferably cysteine, and binding of the target molecule to the heterologous amino acid sequence brings the linkers into proximity resulting in the formation of a disulphide. In some instances, each linker may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 cysteine residues, such as between 1-5, preferably 1 or 2 cysteine residues. In some instances, the linkers may comprise a total number of 2, 3, 4, 5, 6, 7, 8, 9 or 10 cysteine residues, such as between 2-4, preferably 2 cysteine residues. The linkers may comprise or consist of the sequence of SEQ ID NOs: 24 and 25, or a variant thereof.

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The cysteine residues in the linkers may react to form a disulphide bond when the linkers are in proximity, optionally under reducing conditions. Thus, binding of the target molecule to the heterologous amino acid results in a conformational change in the heterologous amino acid sequence which brings the linkers into proximity, and the cysteine residues in the linkers react to form a disulphide bond, which fixes the conformation of the enzyme in a catalytically active state, thereby enhancing activation of the catalytic activity of the enzyme. In some instances, the enzymes or oxidoreductase enzymes comprising the linkers wherein interaction between the linkers enhances activation of the catalytic activity of the enzyme as described herein, have an enhanced or improved dynamic range as compared to a corresponding enzyme or oxidoreductase enzyme of the invention that does not comprise the linkers wherein interaction between the linkers enhances activation of the catalytic activity of the enzyme as described herein. The dynamic range provides a

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measure of the maximal achievable signal-to-noise ratio of the biosensor under optimal conditions for detection. The larger the dynamic range the better the signal-to-noise ratio of the biosensor, *i.e.*, the better the sensitivity of the biosensor, and the lower the concentration of the target molecule that may be detected by the biosensor. The dynamic

5 range of the enzymes or oxidoreductase enzymes of the invention may be calculated by comparing the activity of the biosensor in the absence of the target molecule of the biosensor to the activity of the biosensor when the target molecule of the biosensor is present in saturating concentrations, *i.e.*, a concentration of the target molecule of the biosensor where further increases in concentration do not increase the activity of the

10 biosensor any further. The dynamic range of the enzyme or oxidoreductase enzyme of the invention comprising the linkers wherein interaction between the linkers enhances activation of the catalytic activity of the enzyme may be at least 10 fold, 20 fold, 30 fold, 40 fold, 50 fold, 60 fold, 70 fold, 80 fold, 90 fold or at least 100-fold, preferably at least 80 fold. In comparison, the dynamic range of a corresponding enzyme comprising the same

15 heterologous amino acid sequence but that does not comprise said linkers whose interaction enhances activation of the catalytic activity of the enzyme, may be between 1-10 fold, 1-8 fold, 1-5 fold, 1-4 fold, 1-3 fold or 1-2 fold, typically 1-5 fold.

This mechanism for enhancing the catalytic activity by interaction of the linkers when in

20 proximity may be applied to any enzyme where the catalytic activity of the enzyme may be reversibly regulated by binding of a target molecule to a heterologous amino acid sequence provided as an insert within the amino acid sequence of the enzyme, as described herein. In preferred instances, the present invention provides an oxidoreductase enzyme comprising a calmodulin protein or a functional fragment thereof, wherein binding of a

25 peptide to the calmodulin protein or functional fragment thereof reversibly regulates the catalytic activity of the enzyme, wherein binding of the peptide is capable of reversibly regulating the catalytic activity of the enzyme in the presence of physiological calcium concentrations comprising linkers as described above. Thus, the present invention provides an oxidoreductase enzyme comprising a sequence having at least 60%, 70%,

30 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to SEQ ID NO: 23. In some instances, the oxidoreductase enzyme may comprise or consist of the sequence of SEQ ID NO: 23, or a variant thereof.

Caged Peptides

The present inventors have also identified a mechanism whereby the signal-to-noise ratio
5 enzymes/oxidoreductase enzymes used in biosensors may be improved and the background
signal from such enzymes/oxidoreductase enzymes may be reduced. Thus, the present
invention provides a polypeptide comprising a calmodulin protein or functional fragment
thereof, wherein the calmodulin protein or functional fragment thereof comprises one or
more modifications resulting in a reduced binding affinity for the calmodulin binding
10 peptide as compared to a corresponding wild type calmodulin protein or functional
fragment thereof. The present invention further provides a polypeptide comprising a
calmodulin binding peptide as described herein and a calmodulin protein or functional
fragment thereof, wherein the calmodulin protein or functional fragment thereof comprises
one or more modifications resulting in a reduced binding affinity for the calmodulin
15 binding peptide as compared to a corresponding wild type calmodulin protein or functional
fragment thereof.

The polypeptide is provided as a contiguous amino acid sequence comprising each of the
components specified herein as a fusion protein. The calmodulin binding peptide as
20 described herein may be positioned at the N- or C-terminus of the amino acid sequence of
the calmodulin protein or functional fragment thereof. The polypeptide may further
comprise a linker between the calmodulin binding peptide and the calmodulin protein or
functional fragment thereof. The linker is engineered so that the calmodulin binding
peptide may bind to the calmodulin protein or functional fragment thereof in the same
25 polypeptide without steric hindrance. The linker may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12,
13, 14, 15, 16, 17, 18, 19 or 20 amino acid residues in length, optionally between 1-5, 1-10,
1-15, 1-20, 5-10, 5-15, 5-20, 10-15 or 10-20 amino acid residues in length, preferably
between 5-20 amino acid residues in length. The linker may be a G/S-rich linker, i.e., an
amino acid sequence comprising at least 60%, 70%, 80%, 85%, 90%, 95% or about 100%
30 glycine and serine residues. The linker may comprise a sequence having at least 60%,
70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or at least 100% sequence identity to,

preferably at least 80% sequence identity to SEQ ID NO: 40. The linker may comprise or consist of the sequence of SEQ ID NO: 40, or a variant thereof.

The calmodulin binding peptide may comprise any amino acid sequence that is capable of specifically binding to calmodulin. The CaM-BP may be an activating CaM-BP, such as a wild type CaM-BP, which CaM-BP may comprise, or consist essentially of, the sequence of SEQ ID NO: 29, or a variant thereof. The CaM-BP may be an activating CaM-BP having a higher binding affinity than the wild type CaM-BP for a calmodulin protein, which CaM-BP may comprise, or consist essentially of, the sequence of SEQ ID NO: 10, or a variant thereof. The CaM-BP may be an activating CaM-BP having a lower binding affinity than the wild type CaM-BP for a calmodulin protein, which CaM-BP may comprise, or consist essentially of, the sequence of SEQ ID NO: 37, or a variant thereof. The CaM-BP may comprise a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or at least 100% sequence identity to, preferably at least 80% sequence identity to, any one of SEQ ID NOs: 10, 29 or 37, preferably SEQ ID NO: 29. The CaM-BP may comprise or consist of the sequence of any one of SEQ ID NOs: 10, 29 or 37, or variants thereof, preferably SEQ ID NO: 29 or a variant thereof. Variants typically comprise sequences having deletions or insertions of between 1-5 amino acids relative to the sequence of any one of SEQ ID NOs: 10, 29 or 37. Variants preferably comprise sequences comprising between 1-10 mutations, preferably 1-5 mutations, relative to the sequence of any one of SEQ ID NOs: 10, 29 or 37. Variants retain binding to calmodulin.

The calmodulin protein or functional fragment thereof comprises one or more modifications resulting in a reduced binding affinity for the calmodulin binding peptide as compared to a corresponding wild type calmodulin protein or functional fragment thereof. The modifications may comprise any changes to the amino acid sequence that result in a reduced binding affinity for the calmodulin binding peptide as compared to a corresponding wild type calmodulin protein or functional fragment thereof. The binding affinity may be measured by any suitable technique known in the art. The skilled person is able to select a suitable technique and compare binding affinities of the modified calmodulin protein or functional fragment thereof and a wild type calmodulin protein or

functional fragment thereof. Suitable techniques include, for example, surface plasmon resonance (SPR), enzyme-linked immunosorbent assay (ELISA) and microscale thermophoresis (MST). In some instances, the calmodulin protein or functional fragment thereof comprising one or more modifications has a binding affinity for the calmodulin binding peptide that is at least 5 fold, 10 fold, 20 fold, 30 fold, 40 fold, 50 fold, 60 fold, 70 fold, 80 fold, 90 fold or at least 100 fold, preferably at least 20 fold, less than the binding affinity of the corresponding wild type calmodulin protein or functional fragment thereof for the same calmodulin binding peptide. The skilled person is able to identify the corresponding wild type calmodulin protein or functional fragment. An exemplary wild type calmodulin protein sequence is provided in SEQ ID NO: 2.

Modifications may include truncations, insertions, deletions, and mutations (*e.g.*, point mutations or substitutions). In some instances, the calmodulin protein or functional fragment thereof may comprise the modification of the deletion of between 1-5, 1-10, 1-15, 1-20, 1-30, 1-40 or 1-50, preferably between 1-10, amino acids from either the N- and/or the C-terminus. In some instances, the calmodulin protein or functional fragment thereof may comprise the modification of deletion of between 1-5, 1-10, 1-15, 1-20, 1-30, 1-40 or 1-50, preferably between 1-5, amino acids from within the sequence of the calmodulin protein or functional fragment thereof. The deletion may comprise a contiguous sequence of amino acids or multiple single amino acid deletions. In some instances, the calmodulin protein or functional fragment thereof may comprise the modification of insertion of between 1-5, 1-10, 1-15, 1-20, 1-30, 1-40 or 1-50, preferably between 1-5, amino acids into the sequence of the calmodulin protein or functional fragment thereof. This may comprise the insertion of a contiguous sequence of amino acids or multiple single amino acids.

In some preferred instances, the calmodulin protein or functional fragment thereof may comprise the modification of one or more amino acid mutations. In some preferred instances, the calmodulin protein or functional fragment thereof may comprise the modification of 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid mutations relative to the sequence of the wild type calmodulin protein or corresponding functional fragment thereof, preferably between 1-5 mutations and most preferably 1 or 2 mutations. A wild type

calmodulin protein sequence is provided in SEQ ID NO: 2. The calmodulin protein or functional fragment thereof may comprise mutations at any of amino acid positions corresponding to positions 79, 88, 101 and/or 137 of the sequence of SEQ ID NO: 2. In some instances the calmodulin protein or functional fragment thereof may comprise

5 mutations at amino acid positions corresponding to positions 88 and/or 137 of the sequence of SEQ ID NO: 2. In some instances the calmodulin protein or functional fragment thereof may comprise mutations at amino acid positions corresponding to positions 79, 88, 101 and 137 of the sequence of SEQ ID NO: 2. The calmodulin protein or functional fragment thereof may comprise a mutation selected from the group consisting of: E79S, F88A,

10 L101A and F137A as compared to SEQ ID NO: 2. The calmodulin protein or functional fragment thereof may comprise one or more mutations selected from the group consisting of: E79S, F88A, L101A and/or F137A as compared to SEQ ID NO: 2. In some instances the calmodulin protein or functional fragment thereof may comprise the mutations F88A and/or F137A as compared to SEQ ID NO: 2. In some instances the calmodulin protein or

15 functional fragment thereof may comprise the mutations E79S, F88A, L101A and F137A as compared to SEQ ID NO: 2. In some instances, the calmodulin protein or functional fragment thereof comprising one or more modifications resulting in a reduced binding affinity for the calmodulin binding peptide as compared to a corresponding wild type calmodulin protein or functional fragment thereof comprises a sequence having at least

20 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to, preferably at least 80% sequence identity to SEQ ID NOs: 27 or 62, preferably SEQ ID NO: 27. In some instances, the calmodulin protein or functional fragment thereof comprising one or more modifications resulting in a reduced binding affinity for the calmodulin binding peptide as compared to a corresponding wild type calmodulin protein

25 or functional fragment thereof comprises or consists of the sequence of SEQ ID NOs: 27 or 62, preferably SEQ ID NO: 27, or variants thereof.

In some preferred instances, the polypeptide of the present invention comprise a calmodulin protein or functional fragment thereof comprising or consisting of a sequence

30 having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or at least 100% sequence identity to, preferably at least 80% sequence identity to SEQ ID NOs: 27 or 62, preferably SEQ ID NO: 27, and a calmodulin binding peptide comprising or consisting of a

sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or at least 99% sequence identity to, preferably at least 80% sequence identity to SEQ ID NO: 29. In some preferred instances, the polypeptides of the invention comprise or consist of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or at least 99% 100% sequence identity to, preferably at least 80% sequence identity to SEQ ID NO: 28. In some preferred instances, the polypeptides of the invention comprise or consist of the sequence of SEQ ID NO: 28 or a variant thereof.

Two-component biosensors comprising a Caged Peptide

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In some instances, the polypeptides of the invention comprising a calmodulin binding peptide and a calmodulin protein or functional fragment thereof, as described above, wherein the calmodulin protein or functional fragment thereof comprises one or more modifications resulting in a reduced binding affinity for the calmodulin binding peptide as compared to a corresponding wild type calmodulin protein or functional fragment thereof, may be incorporated into biosensors of the invention. In some instances, the polypeptide of the invention may further comprise a binding moiety, as described herein, so that they may be incorporated into biosensors of the present invention.

20 In some exemplary instances, the polypeptides of the invention comprise a binding moiety. Typically, the binding moiety and the polypeptide are provided as a single contiguous amino acid sequence. The polypeptide may be connected to the binding moiety by a linker, preferably an amino acid linkers. Suitable linkers are described herein. Typically, the linkers are between 1-20 amino acids in length and are G/S-rich. The linker may 25 comprise the sequence of SEQ ID NO: 41 or a variant thereof. The binding moiety may be FRB, FKBP, VHH1, VHH2, HAS-specific VHH, GA binder, cyclophilin or calcinurin alpha/beta. In some instances, the binding moiety is FRB, a binding moiety suitable for detection of rapamycin. In some instances, the polypeptide comprising a binding moiety may comprise an FRB binding moiety, a calmodulin protein or functional fragment 30 thereof, wherein the calmodulin protein or functional fragment thereof comprises one or more modifications resulting in a reduced binding affinity for the calmodulin binding peptide as compared to a corresponding wild type calmodulin protein or functional

fragment thereof and a calmodulin binding peptide. In some preferred instances, the FRB-calmodulin-CaM-BP polypeptide of the invention may comprise or consist of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to the sequence of SEQ ID NOs: 30 or 61, preferably
5 SEQ ID NO: 30. The the FRB-calmodulin-CaM-BP polypeptide may comprise or consist of a sequence of SEQ ID NOs: 30 or 61, preferably SEQ ID NO: 30, or variants thereof.

In some instances, the polypeptide as described herein above, comprising a calmodulin binding peptide and a modified calmodulin protein or functional fragment thereof having a
10 reduced binding affinity for the calmodulin binding peptide, may further comprise a binding moiety that is capable of interacting with a binding moiety on an oxidoreductase enzyme of the invention as described herein, wherein interaction between the binding moieties regulates the catalytic activity of the enzyme. In this way the polypeptides may be incorporated into two-component biosensors of the present invention.

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In some instances the biosensors of the invention comprise a first component which comprises an oxidoreductase enzyme comprising a calmodulin protein or a functional fragment thereof, wherein binding of a peptide to the calmodulin protein or functional fragment thereof reversibly regulates the catalytic activity of the enzyme, wherein binding
20 of the peptide is capable of reversibly regulating the catalytic activity of the enzyme in the presence of physiological calcium concentrations, optionally wherein the oxidoreductase enzyme comprises the calmodulin protein or functional fragment thereof as an insert within the amino acid sequence of the oxidoreductase enzyme in a location corresponding to the loop connecting beta-sheets 5 and 6 of a PQQ-GDH. In some preferred instances, the
25 calmodulin protein or functional fragment thereof provided as an insert within the amino acid sequence of the oxidoreductase enzyme is flanked on either side by linkers, wherein binding of the peptide to the calmodulin protein or functional fragment thereof brings the linkers into proximity with each other and wherein interaction between the linkers enhances activation of the catalytic activity of the oxidoreductase enzyme, optionally
30 wherein said interaction maintains the linkers in proximity with each other, as described herein. Preferably, these linkers each comprise a cysteine residue which form a disulphide bond when the peptide binds to the calmodulin protein or functional fragment thereof to

maintain the oxidoreductase in the activated conformation. The second component of the biosensor may be in some preferred instances a polypeptide of the invention as described herein. The polypeptide typically comprises a binding moiety that is capable of interacting with the binding moiety on the oxidoreductase enzyme of the first component of the biosensor, a calmodulin binding peptide and a calmodulin protein or functional fragment thereof, wherein the calmodulin protein or functional fragment thereof comprises one or more modifications resulting in a reduced binding affinity for the calmodulin binding peptide as compared to a corresponding wild type calmodulin protein or functional fragment thereof, as described herein.

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Typically, the binding moiety on the polypeptide as described herein interacts with the binding moiety on the oxidoreductase enzyme of first component of the biosensor, thereby co-localising or bringing into proximity the oxidoreductase enzyme and the polypeptide of the invention. This interaction may be dependent on the presence of the target molecule of the biosensor as described herein. The calmodulin protein or a functional fragment thereof comprised within the oxidoreductase enzyme as an insert has a higher affinity for the calmodulin-binding peptide bound to the modified calmodulin protein or functional fragment thereof of the polypeptide. The calmodulin-binding peptide thus dissociates from the modified calmodulin protein or functional fragment thereof of the polypeptide and binds preferentially to the calmodulin protein or a functional fragment thereof comprised within the oxidoreductase enzyme of the first component of the biosensor, thereby regulating, preferably activating, the catalytic activity of the enzyme. Thus, the oxidoreductase enzyme may only be activated in the presence of the target molecule of the biosensor. The biosensor has a reduced background, giving an improved signal-to-noise ratio because the caged calmodulin binding peptide is prevented from activating the oxidoreductase enzyme in the absence of the target molecule of the biosensor bringing the oxidoreductase enzyme and the polypeptide into proximity. Furthermore, such biosensors can be operated over a much larger concentration range than the two component biosensors that rely on the “uncaged” calmodulin binding peptide.

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In an exemplary instance, the target molecule of the biosensor is rapamycin. Preferably, the first component of the biosensor comprises the polypeptide of the invention comprising

an FRB binding moiety, a calmodulin protein or functional fragment thereof, wherein the calmodulin protein or functional fragment thereof comprises one or more modifications resulting in a reduced binding affinity for the calmodulin binding peptide as compared to a corresponding wild type calmodulin protein or functional fragment thereof and a

5 calmodulin binding peptide. Preferably, the second component of the biosensor comprises an oxidoreductase enzyme comprising a calmodulin protein or a functional fragment thereof, wherein binding of a peptide to the calmodulin protein or functional fragment thereof reversibly regulates the catalytic activity of the enzyme, wherein binding of the peptide is capable of reversibly regulating the catalytic activity of the enzyme in the

10 presence of physiological calcium concentrations, optionally wherein the oxidoreductase enzyme comprises the calmodulin protein or functional fragment thereof as an insert in a location corresponding to the loop connecting beta-sheets 5 and 6 of a PQQ-GDH and an FKBP binding moiety. Preferably, the biosensor comprises a first component comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%,

15 98%, 99% or 100%, preferably 80%, sequence identity to the sequence of SEQ ID NOs: 30 or 61, preferably SEQ ID NO: 30, and a second component comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to the sequence of SEQ ID NO: 6 or 26. In some preferred instances, the biosensor comprises a first component comprising or consisting of

20 the sequence of SEQ ID NOs: 30 or 61, preferably SEQ ID NO: 30, or variants thereof and a second component comprising or consisting of the sequence of SEQ ID NO: 6 or 26 or variants thereof.

Protease-based biosensors comprising a Caged Peptide

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The caged CaM-BP may also form part of a protease-based biosensor. Thus, in some instances, the polypeptide as described herein above, comprising a calmodulin binding peptide and a modified calmodulin protein or functional fragment thereof having a reduced binding affinity for the calmodulin binding peptide, may further comprise a binding moiety

30 that is capable of interacting with a binding moiety on a protease.

A protease is a protein which displays, or is capable of displaying, an ability to hydrolyse or otherwise cleave a peptide bond. Like terms include proteinase and peptidase.

Proteases include serine proteases, cysteine proteases, metalloproteases, threonine proteases, aspartate proteases, glutamic acid proteases, acid proteases, neutral proteases,

5 alkaline proteases, exoproteases, aminopeptidases and endopeptidases although without limitation thereto. Proteases may be purified or synthetic (*e.g.* recombinant synthetic) forms of naturally-occurring proteases or may be engineered or modified proteases which comprise one or more fragments or domains of naturally-occurring proteases which, optionally, have been further modified to possess one or more desired characteristics,
10 activities or properties.

The protease may be any protease for which a protease cleavage site is known. For example, the protease may be a protease involved in blood coagulation such as thrombin, plasmin, factor VII, factor IX, factor X, factor Xa, factor XI, factor XII (Hageman factor)
15 and other proteases such as kallikreins (*e.g.* kallikrein III, P-30 or prostate specific antigen), matrix metalloproteinases (such as involved in wounds and ulcers; *e.g.* MMP7 and MMP9), adamalysins, serralysins, astacins and other proteases of the metzincin superfamily, trypsin, chymotrypsin, elastase, cathepsin G, pepsin and carboxypeptidase A as well as proteases of pathogenic viruses such as HIV protease, West Nile NS3 protease,
20 dengue virus protease, Tobacco Etch Virus (TEV) protease, Tobacco mosaic virus (TMV) protease and tobacco vein mottling virus (TVMV) protease. In some instances, the protease is TEV protease or TVMV protease, preferably TVMV protease. The protease may be a TVMV protease and comprise or consist of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity
25 to the sequence of SEQ ID NO: 32. The protease may be a TVMV protease and comprise or consist of a sequence of SEQ ID NO: 32 or a variant thereof. The protease may further comprise a binding moiety, as described herein, which is capable of interacting with the first binding moiety on the polypeptide. The protease may further comprise an inhibitor of the protease.

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The inhibitor may be any molecule which at least partly, or substantially or completely suppresses or inhibits the protease activity of the amino acid sequence of the protease. The

inhibitor may be a protein, preferably a peptide, or a non-protein organic molecule such as a small organic molecule, a lipid, a carbohydrate or a nucleic acid. Preferably, the protease inhibitor is an inhibitory peptide. Suitably, the inhibitory peptide comprises an amino acid sequence which binds the active site of a protease without being cleaved by the protease.

- 5 Preferably, the inhibitory peptide competitively at least partly inhibits binding and cleavage of one or more protease substrates by the protease. The inhibitory peptide may comprise an amino acid sequence that corresponds to at least a fragment of a substrate of the protease, but not an amino acid sequence of a protease cleavage site. In this regard, the inhibitory peptide may comprise an amino acid sequence of a protease cleavage site
- 10 modified or engineered to resist cleavage by the protease. In some instances, the protease is TVMV protease and the inhibitor is a peptide comprising or consisting of the sequence of SEQ ID NO: 33, or a variant thereof. In preferred instances, the protease further comprising an inhibitor of the protease is a TVMV protease comprising the inhibitory peptide comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%,
- 15 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to the sequence of SEQ ID NO: 42. In some preferred instances, the protease further comprising an inhibitor of the protease is a TVMV protease comprising the inhibitory peptide comprising or consisting of the sequence of SEQ ID NO: 42 or a variant thereof. The skilled person is able to identify suitable inhibitors, such as inhibitory peptides for use with the specific
- 20 protease included in the biosensor.

- Thus, typically the protease comprises a second binding moiety and optionally an inhibitory peptide. Typically, these components are provided as a single contiguous amino acid sequence. Typically, the protease is connected to the binding moiety and the inhibitor
- 25 by linkers, preferably amino acid linkers. Suitable linkers are described herein. Typically, the linkers are between 1-20 amino acids in length and are G/S-rich. The linker may comprise the sequence of SEQ ID NO: 41 or a variant thereof. In some instances, the binding moiety is FKBP, a binding moiety suitable for detection of rapamycin or tacrolimus, and the protease is TVMV protease. In some preferred instances, the binding
- 30 moiety is FKBP, a binding moiety suitable for detection of rapamycin or tacrolimus, and the protease is TVMV protease and the protease component of the biosensor comprises or consists of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%,

99% or 100%, preferably 80%, sequence identity to the sequence of SEQ ID NO: 31. In some preferred instances, the binding moiety is FKBP, a binding moiety suitable for detection of rapamycin or tacrolimus, and the protease is TVMV protease and the protease component of the biosensor comprises or consists of the sequence of SEQ ID NO: 31 or a variant thereof.

In instances where the polypeptides of the invention are incorporated into a protease-based biosensor, the linker between the calmodulin binding peptide and the calmodulin protein or functional fragment thereof typically comprises an amino acid sequence cleavable by the protease, *i.e.*, a protease cleavage site. The skilled person is capable of selecting a suitable amino acid sequence that is cleavable by the specific protease included in the biosensor. Typically, the amino acid sequence cleavable by the protease comprises a linear peptide epitope of between 1-20, 1-15, 1-10, 1-8 or 1-6 amino acids, preferably 1-10 amino acids. In some instances, the protease included in the biosensor is TVMV protease and the linker between the calmodulin binding peptide and the calmodulin protein or functional fragment comprises an amino acid sequence cleavable by TVMV protease, preferably wherein the amino acid sequence cleavable by TVMV protease comprises or consists of the sequence of SEQ ID NO: 35, or a variant thereof. Thus, in some instances the protease included in the biosensor is TVMV protease and the linker between the calmodulin binding peptide and the calmodulin protein or functional fragment comprises or consists of the sequence of SEQ ID NO: 43 or a variant thereof. In some preferred instances, the polypeptide of the present invention comprises calmodulin, a linker comprising a TVMV protease cleavage site and a calmodulin binding peptide, preferably wherein the sequence of the polypeptide comprises or consists of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to the sequence of SEQ ID NO: 44. In some preferred instances, the sequence of the polypeptide comprises or consists of the sequence of SEQ ID NO: 44 or a variant thereof. In some instances, the polypeptide comprises a first binding moiety. In some instances, the binding moiety may be FRB, which is suitable for detecting the target molecule rapamycin. Thus, in some instances the polypeptide of the invention comprises an FRB binding moiety, calmodulin, a linker comprising a TVMV protease cleavage site and a calmodulin binding peptide, preferably wherein the sequence of the polypeptide comprises or consists of a sequence

having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to the sequence of SEQ ID NO: 34. In some preferred instances, the sequence of the polypeptide comprises or consists of the sequence of SEQ ID NO: 34 or a variant thereof.

5

The polypeptides of the present invention may form part of a protease-based biosensor. In such instances, the binding moiety of the polypeptide is capable of interacting with the second binding moiety on the protease, as described above, wherein interaction between the binding moieties brings the protease into proximity with the amino acid sequence
10 cleavable by the protease in the linker between the calmodulin binding peptide and the calmodulin protein or functional fragment thereof. In instances where the protease comprises an inhibitor, such as an inhibitory peptide, the amino acid sequence cleavable by the protease displaces the inhibitor when the protease and the amino acid sequence cleavable by the protease are brought into proximity. Thus, interaction between the
15 binding moieties results in cleavage of the linker between the calmodulin binding peptide and the calmodulin protein or functional fragment thereof by the protease, typically cleavage occurs at the amino acid sequence cleavable by the protease comprised in the linker. In preferred instances, interaction of the binding moieties is dependent on presence of a target molecule of the biosensor, as described herein, such that cleavage of the linker
20 between the calmodulin binding peptide and the calmodulin protein or functional fragment thereof is dependent on the presence of the target molecule. In an exemplary instance, the target molecule of the biosensor is rapamycin. Preferably, the first component of the biosensor comprises the polypeptide of the invention comprising an FRB binding moiety, a calmodulin protein or functional fragment thereof, wherein the calmodulin protein or
25 functional fragment thereof comprises one or more modifications resulting in a reduced binding affinity for the calmodulin binding peptide as compared to a corresponding wild type calmodulin protein or functional fragment thereof, a calmodulin binding peptide and a linker between the calmodulin protein or functional fragment thereof and the calmodulin binding peptide comprising a TVMV cleavage site. Preferably, the second component of
30 the biosensor an FKBP binding moiety a TVMV protease and an inhibitory peptide of the TVMV protease. Preferably, the biosensor comprises a first component comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%,

98%, 99% or 100%, preferably 80%, sequence identity to the sequence of SEQ ID NO: 34 and a second component comprising or consisting of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to the sequence of SEQ ID NO: 31. In some preferred instances, the biosensor comprises a
5 first component comprising or consisting of the sequence of SEQ ID NO: 34 or a variant thereof and a second component comprising or consisting of the sequence of SEQ ID NO: 31 or a variant thereof.

Variant Calmodulin Binding Peptides

10

The present invention further provides a variant calmodulin binding peptide (CaM-BP) which is capable of binding to a calmodulin protein or a functional fragment thereof provided as an insert within the amino acid sequence of an enzyme (as described herein), wherein the calmodulin protein or functional fragment thereof activates the catalytic
15 activity of the enzyme on binding of a corresponding activating CaM-BP, optionally a wild-type CaM-BP (as described herein), and wherein binding of the variant CaM-BP to said calmodulin protein or functional fragment thereof inhibits binding of the corresponding activating CaM-BP, optionally the wild type CaM-BP, thereby preventing activation of said enzyme. An activating CaM-BP may be understood to mean any CaM-
20 BP that is capable of binding to a calmodulin protein or a functional fragment thereof provided as an insert within the amino acid sequence of an enzyme (as described herein) wherein binding of the activating CaM-BP to the calmodulin protein or functional fragment thereof activates the catalytic activity of the enzyme, as described herein. Suitable activating CaM-BPs are described herein. In some instances, the activating CaM-
25 BP may be a wild-type CaM-BP. In some instances, the activating CaM-BP may comprise or consist of the sequence of SEQ ID NOs: 10, 29 or 37, preferably SEQ ID NO: 29, or a variant thereof. In some instances, the activating CaM-BP may be a natural or synthetic peptide.

30 Typically, the variant calmodulin binding peptide is capable of binding, preferably specifically binding, to a calmodulin protein or a functional fragment thereof. Typically, the variant calmodulin binding peptide is capable of binding to a calmodulin protein or a

functional fragment thereof at the same site that a wild-type calmodulin binding peptide binds the calmodulin protein or functional fragment thereof. In some preferred instances, the binding affinity of the variant CaM-BP for the calmodulin protein or functional fragment thereof is less than the binding affinity of the corresponding wild-type CaM-BP therefor. In some instances, the binding affinity of the variant CaM-BP for the calmodulin protein or functional fragment thereof is essential the same as the binding affinity of the corresponding wild-type CaM-BP therefor. In some instances, the binding affinity of the variant CaM-BP for the calmodulin protein or functional fragment thereof is greater than the binding affinity of the corresponding wild-type CaM-BP therefor. The skilled person is able to identify a suitable wild type calmodulin binding peptide sequence and also a suitable calmodulin protein or functional fragment thereof to use for testing the binding affinities of the variant calmodulin binding peptides. An exemplary wild type calmodulin binding peptide sequence is provided in SEQ ID NO: 29 and a sequence of a suitable calmodulin protein or functional fragment thereof is provided in SEQ ID NO: 2.

The variant calmodulin binding peptide (CaM-BP) is preferably capable of binding to a calmodulin protein or a functional fragment thereof provided as an insert within the amino acid sequence of an enzyme, preferably an oxidoreductase enzyme, wherein the calmodulin protein or functional fragment thereof activates the catalytic activity of the enzyme on binding of a corresponding activating CaM-BP, optionally a wild-type CaM-BP, as described herein. However, in some instances, binding of the variant CaM-BP to said calmodulin protein or functional fragment thereof does not activate the catalytic activity of the enzyme. The catalytic activity of the enzyme upon binding of the variant CaM-BP may be substantially less than the catalytic activity activated by binding of a corresponding activating CaM-BP, optionally a wild-type CaM-BP, to the calmodulin protein or functional fragment thereof. The catalytic activity of the enzyme upon binding of the variant CaM-BP may be negligible or essentially none. The catalytic activity of the enzyme upon binding of the variant CaM-BP may be none. Preferably, the catalytic activity of the enzyme upon binding of the activating CaM-BP is substantially greater than the catalytic activity of the enzyme upon binding of the variant CaM-BP. The catalytic activity of the enzyme may be measured by any suitable method known in the art. In preferred instances, where in the enzyme is an oxidoreductase enzyme, preferably GDH,

the catalytic activity of the enzyme may be measured as described herein, for example, in the Examples.

In preferred instances, binding of the variant CaM-BP to said calmodulin protein or
 5 functional fragment thereof inhibits binding of the corresponding activating, optionally wild type, CaM-BP, thereby preventing activation of said enzyme. Thus, the variant CaM-BP may preferably bind at the same site as the corresponding activating, optionally wild type, CaM-BP on the calmodulin protein or functional fragment thereof. Optionally, binding of the variant CaM-BP may result in a structural rearrangement or conformational
 10 change that prevent or inhibits binding of the activating, optionally wild type, CaM-BP.

In some instances, the variant CaM-BP is between 1-50, 10-50, 10-45, 10-40, 15-40, 20-50, 20-40, or 20-30 amino acids in length, preferably between 10-50 amino acids in length. The variant CaM-BP may be a peptide or a synthetic peptide, for example the variant
 15 CaM-BP may comprise natural and/or non-natural amino acids. In some instances, the variant CaM-BP may comprise at least one modification relative to a corresponding wild type CaM-BP. As described above, modifications may include truncations, insertions, deletions, and mutations (e.g., point mutations or substitutions). In some instances, the variant CaM-BP may comprise the modification of the deletion of between 1-2, 1-5, 1-10,
 20 1-15 or 1-20, preferably between 1-10, amino acids from either the N- and/or the C-terminus of the peptide as compared to the corresponding wild type CaM-BP. In some instances, the variant CaM-BP may comprise the modification of deletion of between 1-2, 1-5, 1-10, 1-15, or 1-20, preferably between 1-5, amino acids from within the sequence of the CaM-BP as compared to the corresponding wild type CaM-BP. The deletion may
 25 comprise a contiguous sequence of amino acids or multiple single amino acid deletions. In some instances, the variant CaM-BP may comprise the modification of insertion of between 1-2, 1-5, 1-10, 1-15, or 1-20, preferably between 1-5, amino acids into the sequence of the calmodulin protein or functional fragment thereof. This may comprise the insertion of a contiguous sequence of amino acids or multiple single amino acids and the
 30 insertion may be at the N- or C-termini of the peptide. The variant CaM-BP may comprise one or more amino acid mutations as compared to the sequence of the corresponding wild type CaM-BP. In some preferred instances, the variant CaM-BP may comprise 1, 2, 3, 4,

5, 6, 7, 8, 9 or 10 amino acid mutations relative to the sequence of the corresponding wild type CaM-BP, preferably between 1-10 mutations and most preferably between 1-5 mutations. In some instances, the variant CaM-BP comprises or consists of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or at least 100% sequence identity to, preferably at least 80% sequence identity, to the sequence of any one of SEQ ID NOs: 45-60, preferably to any one of SEQ ID NOs: 3, 5, 6, 7, 9, 10, 12, 13 or 15, and most preferably to SEQ ID NO: 12. In some instances, the variant CaM-BP comprises or consists of the sequence of any one of SEQ ID NOs: 45-60, preferably any one of SEQ ID NOs: 3, 5, 6, 7, 9, 10, 12, 13 or 15, and most preferably SEQ ID NO: 12, or variants thereof. In some instances, the variant CaM-BP comprises or consists of the sequence of any one of SEQ ID NOs: 45-60 or variants thereof. In some instances, the variant CaM-BP comprises or consists of the sequence of any one of SEQ ID NOs: 3, 5, 6, 7, 9, 10, 12, 13 or 15 or variants thereof. In some instances, the variant CaM-BP comprises or consists of the sequence of SEQ ID NO: 12 or a variant thereof.

15

Biosensors comprising Variant Calmodulin Binding Peptides

The variant calmodulin binding peptides (CaM-BPs) described herein may be used in biosensors of the present invention. Thus, the present invention provides an enzyme as described herein, comprising (i) a calmodulin protein or functional fragment thereof provided as an insert within the amino acid sequence of the enzyme, as described herein, and (ii) a variant CaM-BP, wherein the calmodulin protein or functional fragment thereof activates the catalytic activity of the enzyme on binding of a corresponding activating CaM-BP, optionally a wild-type CaM-BP, wherein binding of the variant CaM-BP to the calmodulin protein or functional fragment thereof inhibits binding of said corresponding activating, optionally wild type, CaM-BP, and wherein binding of the variant CaM-BP to the calmodulin protein or functional fragment thereof prevents activation of the catalytic activity of the enzyme, as described herein.

30 In some instances, the enzyme further comprises a linker. Suitable linkers are described herein. Typically, the linkers are between 1-20 amino acids in length, preferably between 1-10 amino acids in length, and are G/S-rich. The linker may comprise the sequence of

SEQ ID NO: 41 or a variant thereof. Typically, the linker is located between the enzyme and the variant CaM-BP. In some instances the linker comprises an amino acid sequence cleavable by a protease, *i.e.*, a protease cleavage site, for example as described above. Typically, the amino acid sequence cleavable by a protease comprises a linear peptide
5 epitope of between 1-20, 1-15, 1-10, 1-8 or 1-6 amino acids, preferably 1-10 amino acids.

In some instances the enzyme comprising the variant CaM-BP and the linker comprising a protease cleavage site may comprise a single-component biosensor where the target molecule of the biosensor is a protease. In this instance, the protease cleavage site in the
10 linker is selected to be an amino acid sequence that is cleavable by the protease that is the target of the biosensor. The target protease can be any protease for which a protease cleavage site is known, as described herein. The target protease may be selected from any of the proteases described herein above. In the presence of the protease that is the target of the biosensor, the protease would cleave the linker between the enzyme and the variant
15 CaM-BP. In some instances, the variant CaM-BP is engineered to bind the calmodulin protein or functional fragment thereof with an affinity such that in the absence of the linker the variant CaM-BP either dissociates from the calmodulin protein or functional fragment thereof or is displaced from the calmodulin protein or functional fragment thereof by the binding of an activating, optionally wild type, CaM-BP. Thus, in the presence of both the
20 protease that is the target of the biosensor and an activating or a wild type CaM-BP, as described herein, the activating, optionally wild type, CaM-BP would displace the variant CaM-BP and the activating, optionally wild type, CaM-BP would bind to the calmodulin protein or functional fragment thereof provided as an insert within the amino acid sequence of the enzyme, thereby activating the catalytic activity of the enzyme. Thus, cleavage of
25 the linker between the enzyme and the variant CaM-BP is dependent on the presence of the protease that is the target of the biosensor. Thus, the activation of the catalytic activity of the enzyme is dependent on the presence of the protease that is the target of the biosensor.

In some preferred instances the enzyme comprising the variant CaM-BP and the linker
30 comprising a protease cleavage site may form part of a two-component biosensors where the target molecule of the biosensor may be any molecule, as described herein. In this instance, the enzyme further comprises a first binding moiety, as described herein, that is

preferably capable of interacting with a second binding moiety on a protease. The protease may be any protease as described herein. Typically, the protease is linked to the second binding moiety by a linker, such as any of those linkers described herein. Thus, in some instances the biosensor comprises a first component comprising the enzyme comprising the
5 variant CaM-BP, the first binding moiety and the linker comprising a protease cleavage site. The biosensor preferably comprises a second component comprising a protease and a second binding moiety, optionally further comprising a linker. In such instances, the protease cleavage site in the linker between the enzyme and the variant CaM-BP may be cleavable by the protease in the second component of the biosensor. The skilled person is
10 capable of selecting a suitable protease cleavage site (*i.e.*, amino acid sequence) that is cleavable by the specific protease to be included in the second component of the biosensor.

In some preferred instances, the protease of the second component of the biosensor is selected from TVMV protease, Tobacco Etch Virus (TEV) protease, Hepatitis C virus
15 (HCV) protease and small ubiquitin-like modifier (SUMO) protease (*i.e.*, Ulp). In some instances, the protease of the second component of the biosensor is TEV protease and the linker between the enzyme and the variant CaM-BP comprises an amino acid sequence cleavable by TEV protease. In some instances, the protease of the second component of the biosensor is HCV protease and the linker between the enzyme and the variant CaM-BP
20 comprises an amino acid sequence cleavable by HCV protease. In some instances, the protease of the second component of the biosensor is SUMO protease and the linker between the enzyme and the variant CaM-BP comprises an amino acid sequence cleavable by SUMO protease. In some preferred instances, the protease of the second component of the biosensor is TVMV protease and the linker between the enzyme and the variant CaM-
25 BP comprises an amino acid sequence cleavable by TVMV protease. Preferably, the TVMV protease comprises or consists of a sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, preferably 80%, sequence identity to the sequence of SEQ ID NO: 32. Preferably, the amino acid sequence cleavable by TVMV protease (*i.e.*, the protease cleavage site) comprises or consists of the sequence of SEQ ID
30 NO: 35, or a variant thereof.

Thus, in some instances, interaction between the first binding moiety on the enzyme and the second binding moiety on the protease brings the two components of the biosensor into proximity with each other and brings the protease into proximity with the linker comprising the protease cleavage site and results in cleavage of the linker between the enzyme and the variant CaM-BP by the protease. In preferred instances, interaction of the binding moieties is dependent on presence of a target molecule, such that cleavage of the linker between the enzyme and the variant CaM-BP is dependent on the presence of the target molecule. Wherein the target molecule may be any target molecule as described herein. Following cleavage of the linker between the enzyme and the variant CaM-BP the variant CaM-BP dissociates from the calmodulin protein or functional fragment thereof or the variant CaM-BP may be displaced from the calmodulin protein or functional fragment thereof by the binding of an activating, optionally wild type, CaM-BP. The wild type CaM-BP binds to the calmodulin protein or functional fragment thereof and thereby activates the catalytic activity of the enzyme. Thus, catalytic activity of the enzyme may be dependent on the presence of the target molecule of the biosensor and the activating CaM-BP.

Circularly permuted oxidoreductase enzymes

The present invention also provides circularly permuted oxidoreductase enzymes, in particular an oxidoreductase enzyme comprising a heterologous amino acid sequence that releasably maintains the enzyme in a catalytically inactive state by allosteric regulation and which is responsive to a molecule to release said regulation, a first binding moiety and a second binding moiety, wherein the amino acid sequence of the enzyme is circularly permuted, and wherein (i) release of the allosteric regulation by the heterologous amino acid sequence and (ii) interaction between the binding moieties, reversibly regulates the catalytic activity of the enzyme.

The oxidoreductase enzyme may be any oxidoreductase enzyme described herein. The heterologous amino acid sequence may be any heterologous amino acid sequence described herein. The molecule to which it is responsive may be any target molecule for such a heterologous amino acid sequence as described above. The heterologous amino acid

sequence may be introduced or inserted in the amino acid sequence of the oxidoreductase enzyme at any location as described above. Thus, the heterologous amino acid sequence may be inserted in the oxidoreductase enzyme in a location capable of regulating the position of the catalytic residues of the oxidoreductase enzyme. Preferably, the

5 heterologous amino acid sequence may be inserted in the oxidoreductase enzyme in a location capable of regulating the position of the catalytic residues of the oxidoreductase enzyme corresponding to Arg406 and/or Arg408 of PQQ-GDH. The heterologous amino acid sequence may be inserted into a loop region of the oxidoreductase enzyme. Preferably, the heterologous amino acid sequence may be inserted in a location

10 corresponding to the loop connecting beta-sheets 5 and 6 of a PQQ-GDH.

Preferably, the heterologous amino acid sequence is a calmodulin protein or functional fragment thereof and the molecule to which it is responsive is a peptide, typically a calmodulin binding peptide (CaM-BP). Thus, the invention provides an oxidoreductase

15 enzyme comprising a calmodulin protein or a functional fragment thereof, a first binding moiety and a second binding moiety, wherein the amino acid sequence of the enzyme is circularly permuted, and wherein (i) binding of a peptide (preferably a CaM-BP) to the calmodulin protein or functional fragment thereof and (ii) interaction between the binding moieties, reversibly regulates the catalytic activity of the enzyme.

20

The calmodulin protein or functional fragment thereof, and the peptide binding thereto (typically a CaM-BP) may be selected from any calmodulin protein or functional fragment thereof and any peptide binding thereto or CaM-BP described herein. The peptide is typically capable of binding to the calmodulin protein or functional fragment thereof and

25 effecting a conformational change that assists in activation of the catalytic activity of the oxidoreductase enzyme. The conformational change in combination with the interaction between the binding moieties typically provides for activation of the catalytic activity of the enzyme. The peptide may comprise a sequence having at least 80 % sequence identity to SEQ ID NO: 10, 29 or 37, or be any other variant thereof (or comprise the specific

30 sequence thereof) as described herein.

The first and second binding moieties comprised in the oxidoreductase enzyme may be selected from any interacting binding moieties described herein. Specific examples are provided by the binding moieties incorporated in SEQ ID NOs 78, 81, 84 and 85. The binding moieties may thus be FKBP and FRB; calcineurin alpha/beta and FKBP;

- 5 calcineurin alpha/beta and cyclophilin or first and second antibodies for a target molecule or antigen-binding fragments thereof. Other suitable pairs of binding moieties may be selected from any pairs described herein.

- 10 Interaction of the binding moieties activates the catalytic activity of the enzyme when in the presence of the peptide. Interaction of the binding moieties is preferably dependent on presence of a target molecule, such that the presence of the target molecule activates the catalytic activity of the enzyme. The target molecule may be any target molecule described herein bound by first and second binding moieties and may for example be rapamycin, FK506, cyclosporine A or amylase.

15

- The oxidoreductase enzyme, heterologous amino acid sequence (such as a calmodulin protein or functional fragment thereof), the first binding moiety and the second binding moiety typically form a contiguous amino acid sequence, optionally connected by linkers. The linkers may be any linkers described herein. The calmodulin protein or functional
20 fragment thereof is typically provided as an insert within the amino acid sequence of the oxidoreductase enzyme, flanked on either side by a linker.

- The amino acid sequence of the oxidoreductase enzyme (the wild-type or native enzyme sequence prior to insertion of the heterologous amino acid sequence) is circularly
25 permuted. Circular permutation as a technique is known in the art (as described for example in Chen et al (Protein Sci 2016, vol 25: 1483-91) and in the context of the present invention typically comprises the creation of new N- and C-termini for the oxidoreductase enzyme by internal cleavage, together with linkage of the wild-type N- and C-termini (those of the native enzyme), typically using a linker, which may be any linker described
30 herein. The cleavage is made at a position other than that of the heterologous amino acid sequence. The cleavage is made at a position that disrupts enzyme activity, with enzyme activity then being restorable by re-association of the respective enzyme portions, through

interaction of the first and second binding moieties of the enzyme (typically in the presence of the target molecule).

The N-terminus of the circularly permuted oxidoreductase enzyme amino acid sequence
5 may be defined as any amino acid located C-terminally in the wild-type amino acid
sequence to the amino acid forming the N-terminus of the circularly permuted
oxidoreductase enzyme. The C-terminus of the circularly permuted oxidoreductase
enzyme may be defined as any amino acid located N-terminally in the wild-type amino
acid sequence to the amino acid forming the N-terminus of the circularly permuted
10 oxidoreductase enzyme.

The circularly permuted oxidoreductase enzyme sequence may be represented by the
following general formula X-C-linker-N-Y, wherein X-C is the wildtype/native C-terminal
portion of the enzyme, and N-Y is the wildtype/native N-terminal portion of the enzyme. X
15 and Y represent respectively the N- and C-terminus of the circularly permuted
oxidoreductase enzyme sequence. The linker links the wildtype N- and C-termini and may
be any linker described herein.

Preferably, the N-terminus of the circularly permuted oxidoreductase enzyme may be any
20 amino acid located between the positions (or positions corresponding to) S140 to A170 of
PQQ-GDH, such as PQQ-GDH of SEQ ID NO:3. The C-terminus of the circularly
permuted oxidoreductase enzyme may be any amino acid located between the positions
(or positions corresponding to) L138 to Q168 of PQQ-GDH, such as PQQ-GDH of SEQ
ID NO:3. In a particularly preferred aspect, the N-terminus of the circularly permuted
25 oxidoreductase enzyme is the amino acid (or amino acid corresponding to) Q155 of PQQ-
GDH and the C-terminus of the circularly permuted oxidoreductase enzyme is the amino
acid (or amino acid corresponding to) P153 of PQQ-GDH. Selection of positions and
corresponding positions is made as described above, and such that the circularly
permuted enzyme generated has disrupted enzyme activity, which is further restorable on
30 reassociation of the respective enzyme portions on interaction of binding moieties.

The first binding moiety is typically linked to (optionally via a linker) or located proximally to the N-terminus of the circularly permuted oxidoreductase amino acid sequence and the second binding moiety is typically linked to (optionally via a linker) or located proximally to the C-terminus of the circularly permuted oxidoreductase amino acid sequence. The first and binding moieties are located such that interaction between the binding moieties is capable of re-association of enzyme portions comprising the N- and C-termini of the circularly permuted oxidoreductase amino acid sequence, allowing for restoration of enzyme activity.

The circularly permuted oxidoreductase enzyme amino acid sequence may have at least 80 % sequence identity to the sequence of SEQ ID NO: 79. The oxidoreductase enzyme may comprise a sequence having at least 80 % sequence identity to any one of SEQ ID NOs: 78 81, 84, 85, or 86. The circularly permuted oxidoreductase enzyme amino acid sequence or the sequence comprised in the oxidoreductase enzyme may be any other variant of the above sequences (or comprise the specific sequence thereof) as described herein.

Three-component biosensors based on oxidoreductase enzymes

The present invention further provides an oxidoreductase enzyme comprising a heterologous amino acid sequence which is responsive to a peptide comprising a first binding moiety, and a first interaction domain capable of interacting with a second interaction domain of a polypeptide, wherein the polypeptide further comprises a second binding moiety capable of interacting with said first binding moiety, and wherein binding between the first and second binding moieties and interaction of the first interaction domain of the enzyme with the second interaction domain of the polypeptide act to colocalise the enzyme and the peptide, wherein binding of the peptide to the heterologous amino acid sequence reversibly regulates catalytic activity of the enzyme.

The oxidoreductase enzyme and heterologous amino acid sequence responsive to a peptide may be selected from any oxidoreductase enzyme and heterologous amino acid sequence responsive to a peptide described herein, The heterologous amino acid sequence is typically a calmodulin protein or functional fragment thereof and the peptide is typically a

calmodulin binding peptide (CaM-BP). The calmodulin protein or functional fragment thereof and CaM-BP may be selected from any described herein. The peptide may comprise a sequence having at least 80 % sequence identity to SEQ ID NO: 10, 29 or 37, or be any other variant thereof (or comprise the specific sequence thereof) as described
5 herein.

Thus, the invention provides an oxidoreductase enzyme comprising a calmodulin protein or functional fragment thereof wherein binding of a peptide (typically a CaM-BP) to the calmodulin protein or functional fragment thereof of the enzyme reversibly regulates the
10 catalytic activity of the enzyme, and further comprising a first interaction domain capable of interacting with a second interaction domain of a polypeptide, wherein the polypeptide further comprises a second binding moiety capable of interacting with said first binding moiety, and wherein binding between the first and second binding moieties and interaction of the first interaction domain of the enzyme with the second interaction domain of the
15 polypeptide act to colocalise the enzyme and the peptide.

The first and second binding moieties present in the peptide and polypeptide may be selected from any interacting binding moieties described herein. Specific examples are
20 provided by the binding moieties incorporated in SEQ ID NOs 75 and 77. The binding moieties may be FKBP and FRB; calcineurin alpha/beta and FKBP; calcineurin alpha/beta and cyclophilin or first and second antibodies for a target molecule or antigen-binding fragments thereof. Other suitable pairs of binding moieties may be selected from any pairs described herein. Interaction of the binding moieties acts to colocalise the enzyme and
25 peptide (via the polypeptide) and allows for activation of the catalytic activity of the enzyme. Interaction of the binding moieties is preferably dependent on presence of a target molecule, such that the presence of the target molecule activates the catalytic activity of the enzyme. The target molecule may be any target molecule described herein bound by first and second binding moieties and may for example be rapamycin, FK506, cyclosporine
30 A or amylase.

The first and second interaction domains may comprise interacting binding moieties described herein. The first and second interaction domains represent a different pair of binding moieties as compared to the binding moieties present in the peptide and polypeptide. Interaction of the interaction domains acts to colocalise the enzyme and polypeptide and thereby allow for colocalisation of the peptide by interaction of the first and second binding moieties and thus allows for activation of the catalytic activity of the enzyme. Interaction of the interaction domains is preferably dependent on presence of an interaction ligand which may correspond to any target molecule described herein able to mediate interaction of two binding moieties, The interaction ligand may be rapamycin, FK506, cyclosporine A or alpha-amylase.

The above oxidoreductase enzyme for use in a three component biosensor may comprise a sequence having at least 80 % sequence identity to SEQ ID NO: 72. A biosensor is also provided herein comprising any of the above oxidoreductase enzymes, peptides and polypeptides in combination. The biosensor may comprise an oxidoreductase enzyme comprising a sequence having at least 80 % sequence identity to SEQ ID NO: 72, a peptide comprising a sequence having at least 80 % sequence identity to any of SEQ ID NOs: 10, 29, 37 and 77, and a polypeptide comprising a sequence having at least 80 % sequence identity to SEQ ID NO: 75. The oxidoreductase enzyme, peptide or polypeptide may comprise a sequence which is any other variant of the above sequences (or comprise the specific sequence thereof) as described herein.

Compositions, Kits and Detection Devices

As described herein, the present invention also provides biosensors comprising the enzymes or oxidoreductase enzymes described herein, optionally in combination with the polypeptides and variant calmodulin binding peptides as described herein. Furthermore, the present invention provides a composition or kit comprising one or more of the biosensors described herein. The present invention also provides a composition or kit comprising one or more of the enzymes or oxidoreductase enzymes described herein, optionally in combination with one or more of the polypeptides and/or variant calmodulin binding peptides as described herein.

The composition or kit may further comprise a target molecule of the heterologous amino acid sequence, which is preferably peptide, as described herein, acting to regulate catalytic activity of the enzyme or oxidoreductase enzyme, which is typically comprised within a biosensor of the invention. In preferred instances, the composition or kit comprises a calmodulin binding peptide, which acts to regulate the catalytic activity of the oxidoreductase enzyme comprising a calmodulin protein or functional fragment thereof as an insert. In some instances, said peptide or calmodulin binding peptide may be comprised within a component of one of the biosensors of the present invention, which forms part of the composition or kit.

The composition or kit may further comprise a polypeptide comprising a calmodulin protein or functional fragment thereof, wherein the calmodulin protein or functional fragment thereof comprises one or more modifications resulting in a reduced binding affinity for the calmodulin binding peptide as compared to a corresponding wild type calmodulin protein or functional fragment thereof, as described herein. Such calmodulin proteins may be added to the biosensors of the invention to reduce the background and enhance the dynamic range and signal-noise ratio of the biosensor. The calmodulin protein may be configured to bind to the calmodulin binding peptide of the biosensor preventing its spontaneous association with the calmodulin inserted in the enzyme of the biosensor until the presence of the target molecule brings the components of the biosensor into close proximity.

The composition or kit may further comprise a substrate molecule for the enzyme or oxidoreductase enzyme. In some instances where the composition or kit comprises a biosensor of the invention, the composition or kit may further comprise a substrate molecule of the enzyme or oxidoreductase enzyme of the biosensor. Suitable substrate molecules for particular enzymes are described herein above. In preferred instances, the enzyme may be a GDH enzyme and the substrate molecule may be glucose. In some instances, the present invention provides a composition or kit comprising one or more of the biosensors described herein in combination with one or more substrate molecules.

The present invention further provides, a detection device that comprises a cell or chamber that comprises one or more of the enzymes, oxidoreductase enzymes or biosensors as described herein. Suitably, a sample may be introduced into the cell or chamber to thereby facilitate detection of a target molecule. In certain instances, the detection device is capable
5 of providing an electrochemical, acoustic and/or optical signal that indicates the presence of the target molecule. In some instances, the detection device may comprise an electrode. In some instances the detection device may comprise a semiconductor device. In some instances the detection device is a device adapted for amperometry. The device may comprise screen printed electrodes, preferably layered with a dry mixture comprising the
10 biosensor of the invention and preferably an electron mediator.

The detection device may further provide a disease diagnosis from a diagnostic target result by comprising: a processor and a memory coupled to the processor, the memory including computer readable program code components that, when executed by the processor, perform
15 a set of functions including analysing a diagnostic test result and providing a diagnosis of the disease or condition.

The detection device may further provide for communicating a diagnostic test result by comprising: a processor and a memory coupled to the processor, the memory including
20 computer readable program code components that, when executed by the processor, perform a set of functions including: transmitting a diagnostic result to a receiving device; and optionally receiving a diagnosis of the disease or condition from the or another receiving device.

25 **Methods**

The present invention further provides a method of detecting a target molecule, said method including the step of contacting one or more of the biosensors described herein with a sample under conditions suitable for detection of the presence or absence of the
30 target molecule in the sample. The present invention also provides a method of detecting a target molecule, said method including the step of contacting one or more of the enzymes

or oxidoreductase enzymes described herein with a sample under conditions suitable for detection of the presence or absence of the target molecule in the sample.

Preferably, the sample is a biological sample. Biological samples may include organ
5 samples, tissue samples, cellular samples, fluid samples or any other sample obtainable, obtained, derivable or derived from an organism or a component of the organism. For example, the biological sample can comprise a fermentation medium, feedstock or food product such as for example, but not limited to, dairy products. Suitably, the enzyme activity of the biosensor is not substantially inhibited by components of the sample (*e.g.*
10 serum proteins, metabolites, cells, cellular debris and components, naturally-occurring protease inhibitors *etc*).

In some instances, the biological sample is obtainable or obtained from a mammal, preferably a human. In preferred instances, the biological sample may be a fluid sample
15 such as blood, serum, plasma, urine, saliva, tears, sweat, cerebrospinal fluid or amniotic fluid. In some instances, the sample may be a tissue sample such as a tissue or organ biopsy or may be a cellular sample such as a sample comprising red blood cells, lymphocytes, tumour cells or skin cells. A particular type of biological sample is a pathology sample. Preferably, the sample is a blood, saliva, serum or urine sample from a
20 human subject. In some preferred instances, the sample is a blood or saliva sample obtained from a human. In some preferred instances, the sample is a serum or urine sample from a human subject. In some instances, the human is a patient. In some instances, the human may have or may be suspected of having a disease for which the target molecule is a marker or biomarker.

25 In some instances, the biosensor and/or methods of use may be applicable to drug testing such as for detecting the use of illicit drugs of addiction (*e.g* cannabinoids, amphetamines, cocaine, heroin *etc.*) and/or for the detection of performance-enhancing substances in sport and/or masking agents that are typically used to avoid detection of performance-enhancing
30 substances. This may be applicable to the detection of banned performance-enhancing substances in humans and/or other mammals such as racehorses and greyhounds that may be subjected to illicit “doping” to enhance performance.

The biosensors of the invention may also be used to screen for proteins that bind to specific target molecules. For example, in some instances, the sample may comprise a known purified target molecule, such as a target protein, a target peptide or a target small
5 molecule. A plurality of biosensors may be provided comprising a panel of different first and second binding moieties to be screened for binding to the target molecule. As described herein, specific binding of the binding moieties to the target molecule would result in activation of the biosensor.

- 10 In a further aspect, the present invention provides a method of diagnosis of a disease or condition in an organism, said method comprising the step of contacting one or more enzymes or oxidoreductase enzyme as described herein, or one or more biosensors as described herein, with a sample obtained from the organism under conditions suitable for detection of the presence or absence of a target molecule in the sample, wherein the
15 presence or absence of the target molecule in the sample is indicative of whether the organism has, or is at risk of having, said disease or condition. In some instances, determination of the presence or absence of the target molecule facilitates diagnosis of the disease or condition. The organism may include plants and animals inclusive of fish, avians and mammals such as humans. In some instances, the organism is a mammal,
20 preferably a human.

- The disease or condition may be any disease or condition where detection of a target molecule assists diagnosis. As described above, target molecules or analytes may include one or more of blood coagulation factors such as previously described, kallikreins
25 inclusive of PSA, matrix metalloproteinases, viral and bacterial proteases, antibodies, glucose, triglycerides, lipoproteins, cholesterol, tumour antigens, lymphocyte antigens, autoantigens and autoantibodies, drugs, salts, creatinine, blood serum or plasma proteins, pesticides, uric acid, products and intermediates of human and animal metabolism and metals. This preferred instance of the invention may be adapted to be performed as a
30 “point of care” method whereby determination of the presence or absence of the target molecule may occur at a patient location which is then either analysed at that location or transmitted to a remote location for diagnosis of the disease or condition.

Diagnostic aspects of the invention may also be in the form of a kit comprising one or a plurality of different biosensors as described herein capable of detecting one or a plurality of different target molecules. In this regard, a kit may comprise an array of different
5 biosensors as described herein capable of detecting a plurality of different target molecules. The kit may further comprise one or a plurality of suitable substrates of the enzyme(s) of the biosensor, as described herein. The kit may further comprise one or more amplifier molecules, deactivating molecules and/or labeled substrates, as described herein. The kit may also comprise additional components including reagents such as buffers and
10 diluents, reaction vessels and instructions for use.

In some instances, the enzymes, oxidoreductase enzyme or biosensors as described herein may be used to assay for protein-protein or protein-small molecule binding interactions. Thus, the present invention provides a method of assaying protein-protein or protein-small
15 molecule interactions comprising contacting the enzymes, oxidoreductase enzyme or biosensors as described herein with a sample under conditions suitable for detection of the presence or absence of an interaction between the binding moieties or between the binding moieties and a target molecule. The sample typically comprises a suitable substrate molecule for the enzyme, oxidoreductase enzyme or the biosensor.

20 Thus, the enzyme, oxidoreductase enzyme or biosensor as described herein may comprise a first binding moiety comprising a first protein or small molecule of interest and a second binding moiety comprising a second protein or small molecule of interest, wherein the catalytic activity of the enzyme is dependent upon a specific protein-protein or protein-
25 small molecule interaction between the first and second binding moieties. Such a biosensor may be used to assay for a direct interaction between the first protein or small molecule of interest and the second protein or small molecule of interest in the first and second binding moieties respectively. A direct interaction between the proteins/small molecules of interest in the first and second binding moieties would co-localise the two
30 components of the biosensor, thereby activating the catalytic activity of the enzyme as described here, producing a detectable read-out as described herein. Such a biosensor may be further used to assay for activators and inhibitors of the said interaction between the

proteins/small molecules of interest in the first and second binding moieties. Thus in some instances the sample may further comprise putative activators and inhibitors, *i.e.*, molecules to be assayed for their ability to activate or inhibit the interaction between the first and second proteins of interest. Activators that enhance the interaction between the first and second proteins of interest would result in increased catalytic activity of the enzyme. Inhibitors that inhibit, prevent or reduce the interaction between the first and second proteins of interest would result in reduced catalytic activity of the enzyme.

Nucleic Acids

10

The present invention also provides a nucleic acid, typically an isolated nucleic acid, encoding an enzyme, an oxidoreductase enzyme, a polypeptide or a biosensor as described herein according to any of the instances of the invention as described herein. The nucleic acid may encode any of SEQ ID NOs: 1-62, or a variant thereof. The nucleic acid may encode any of SEQ ID NOs: 1-71, or a variant thereof. Preferably the nucleic acid may encode any of SEQ ID NOs: 1, 6, 9, 13, 19, 21, 23, 26, 27, 28, 30, 31, 34, 36, 44, 45-60, 61, 62, 63, 64, 67 or 70, or a variant thereof. Most preferably, the nucleic acid may encode any of SEQ ID NOs: 1, 6, 9, 13, 19, 21, 23, 26, 27, 30, 31, 34, 44, 61, 62, 63 or 64.

20 The present invention also provides a genetic construct comprising the isolated nucleic acid of the invention. The present invention also provides a host cell comprising the genetic construct of the invention. The term "*nucleic acid*" as used herein designates single- or double-stranded mRNA, RNA, cRNA, RNAi, siRNA and DNA inclusive of cDNA, mitochondrial DNA (mtDNA) and genomic DNA. Preferably, the nucleic acids of the invention are DNA. The invention also provides variants and/or fragments of the isolated nucleic acids. Variants may comprise a nucleotide sequence at least 70%, at least 75%, preferably at least 80%, at least 85%, more preferably at least 90%, 91%, 93%, 94%, 95%, 96%, 97%, 98% or 99% nucleotide sequence identity with any nucleotide sequence disclosed herein. In other instances, nucleic acid variants may hybridize with any nucleotide sequence described herein, under high stringency conditions. Fragments may comprise or consist of up to 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95-99% of the contiguous nucleotides present in any nucleotide sequence

described herein. Fragments may comprise or consist of up to 20, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900 950, 1000, 1050, 1100, 1150, 1200, 1350 or 1300 contiguous nucleotides present in any nucleotide sequence described herein.

5

The invention also provides genetic constructs that comprise one or more isolated nucleic acids, variants or fragments thereof as described herein, operably linked to one or more additional nucleotide sequences. As generally used herein, a genetic construct is an artificially created nucleic acid that incorporates, and/or facilitates use of, an isolated nucleic acid disclosed herein. In particular instances, such constructs may be useful for recombinant manipulation, propagation, amplification, homologous recombination and/or expression of said isolated nucleic acid.

Thus, the present invention further provides a method of producing a recombinant protein biosensor or a component thereof or an oxidoreductase enzyme or GDH enzyme of the invention or a polypeptide or variant CaM-BP of the invention, said method including the step of producing the recombinant protein biosensor or a component thereof in the host cell of the invention. As used herein, a genetic construct used for recombinant protein expression is referred to as an expression construct, wherein the isolated nucleic acid to be expressed is operably linked or operably connected to one or more additional nucleotide sequences. in an expression vector. An expression vector may be either a self-replicating extra-chromosomal vector such as a plasmid, or a vector that integrates into a host genome.

The one or more additional nucleotide sequences are typically regulatory nucleotide sequences. By operably linked or operably connected is meant that said regulatory nucleotide sequence(s) is/are positioned relative to the nucleic acid to be expressed to initiate, regulate or otherwise control expression of the nucleic acid.

Regulatory nucleotide sequences will generally be appropriate for the host cell used for expression. Numerous types of appropriate expression vectors and suitable regulatory sequences are known in the art for a variety of host cells. One or more regulatory nucleotide sequences may include, but are not limited to, promoter sequences, leader or signal

- sequences, ribosomal binding sites, transcriptional start and termination sequences, translational start and termination sequences, splice donor/acceptor sequences and enhancer or activator sequences. Constitutive or inducible promoters as known in the art may be used and include, for example, nisin-inducible, tetracycline-repressible, IPTG-inducible, alcohol-inducible, acid-inducible and/or metal-inducible promoters. In one instances, the expression vector comprises a selectable marker gene. Selectable markers may be useful for the purposes of selection of transformed bacteria (such as *bla*, *kanR*, *ermB* and *tetR*) or transformed mammalian cells (such as hygromycin, G418 and puromycin resistance).
- 10 Suitable host cells for expression may be prokaryotic or eukaryotic, such as bacterial cells inclusive of *Escherichia coli* (DH5 α for example), yeast cells such as *S. cerevisiae* or *Pichia pastoris*, insect cells such as SF9 cells utilized with a baculovirus expression system, or any of various mammalian or other animal host cells such as CHO, BHK or 293 cells. Introduction of expression constructs into suitable host cells may be by way of techniques including, for example, electroporation, heat shock, calcium phosphate precipitation, DEAE dextran-mediated transfection, liposome-based transfection (*e.g.* lipofectin, lipofectamine), protoplast fusion, microinjection or microparticle bombardment, as are well known in the art.
- 20 Purification of the recombinant biosensor molecule may be performed by any method known in the art. In preferred instances, the recombinant biosensor molecule comprises a fusion partner (preferably a C-terminal His tag) which allows purification by virtue of an appropriate affinity matrix, which in the case of a His tag would be a nickel matrix or resin. The resulting, engineered mutant is preferably expressed in bacteria such as *E. coli* as an epitope-tagged protein and is purified by affinity chromatography.
- 25

The invention may be better understood by reference to the following Examples.

EXAMPLES

Materials and methods

- 5 Rapamycin, tacrolimus, cyclosporine A and human α -amylase were purchased from Sigma-Aldrich. Recombinant Il-23 was purchased from ProSpec. Samples of human biological fluids were collected under the human ethics approval IBC/244B/imb/2018.

Chimeric gene construction and protein expression and purification

- 10 The fusion constructs/chimeric proteins described herein were generated by Gibson Assembly™ method according the manufacturer's instruction (New England Biolab) and cloned into PET28a vector. The gene fragments for the assembly were made either by PCR or by Gblcok gene synthesis from IDT (Integrated DNA Technologies). The protein expression and purification were described by Olsthoorn & Duine (15). The GDH
- 15 biosensors containing the cysteine "ratchet" R β 5/6GDH-CaM chimera lacked the signal peptide sequence and were expressed in the cytosol of *E. coli*. Proteins were purified by Ni-NTA chromatography, and the pooled protein-containing fractions were dialyzed against buffer containing 20 mM Tris/HCl pH 7.2, and 100mM NaCl for 10 hours. The constructs containing VHH domains were additionally purified by size exclusion
- 20 chromatography on Superdex 200 column (GE Healthcare) to remove oligomers. The purified β 5/6-CaM-GDH was reconstituted by adding PQQ with 1:1.5 ratio. This ratio for reconstitution of GDH and PQQ was also used in all other experiments using PQQ-GDH enzymes described herein.
- 25 The biosensor components not containing GDH domain were cloned into either PET28a or pOPINE vectors and expressed in cytosol of *E. coli* BL21(DE3)RIL cells, or into periplasm for the constructs containing VHH fragment and calcineurin A/B complex. Protein purification was performed as described above. The proteins of tacrolimus biosensor were purified as described previously (<http://www.pnas.org/content/99/21/13522>). After Ni-
- 30 NTA purification the pooled enzyme-containing fractions were dialyzed against the buffer containing 20 mM Tris pH7.2 and 100mM NaCl.

Analysis of GDH enzymatic activity

The GDH enzyme assay was performed as described by Yu *et al.* Briefly, the 1-mL assay system consisted of 20 mM glucose, 0.6 mM phenazine methosulfate, 0.06 mM 2,6-dichlorophenol (DCPIP), 10 mM MOPS (pH 7.0), and corresponding concentration of
5 CaCl₂ and enzyme. Alternatively, reactions comprised 20 mM glucose, 0.6 mM phenazine methosulfate (PMS), 0.06 mM 2,6 dichlorophenylindophenol (DCPIP), 20mM Tris-HCl pH 7.2, 20mM NaCl, and defined concentration of CaCl₂ and enzyme. Reactions were carried out in polystyrol cuvettes (SARSTEDT). The enzymatic assay was performed at 25°C by monitoring the reduction in the absorbance of 2,6-dichlorophenol at 600 nm. In
10 some cases the assays were performed in 50µl volume and changes in absorption were detected using plate reader systems.

The GDH enzymatic assays for the three component amylase biosensor were conducted as follows. The protein GDH-CaM-SH3-FKBP was reconstituted with PQQ in 1:1.5 ratio.
15 GDH activity of 10nM of GDH-CaM-SH3-FKBP, 20nM of FRB-SH3L-VHH1, 100nM of VHH2-CaMBP in response to α-Amylase was measured in 1ml reaction mixture. The activity of the enzyme was monitored by changes in absorption (at 600nm) of electron accepting dye dichlorophenolindophenol in the presence of 0.6 mM electron mediator phenazine methosulphate, 20mM of glucose, 1mM CaCl₂, 250nM Rapamycin with 15min
20 pre-incubation time.

The GDH enzymatic assays for the single component biosensors were conducted as follows. The protein sensor was reconstituted with PQQ in 1:1.5 ratio. GDH activity of 2.5 nM, 5 nm or 10 nM of the biosensor, as indicated, in response to the target analyte
25 (e.g., rapamycin, FK506, cyclosporine A, α-amylase) was measured in 1 ml reaction mixture. The activity of the enzyme was monitored by changes in absorption (at 600 nm) of electron accepting dye dichlorophenolindophenol (DCPIP) in the presence of 0.6 mM electron mediator phenazine methosulphate (PMS), 20 mM of glucose, 2.5 mM CaCl₂, 250 nM or 500 nM M13 Calmodulin binding peptide, as indicated, and 1 µM PQQ
30 with 15 min or 30 min pre-incubation time as indicated.

In the assays that utilize high concentrations of biosensors the PMS was omitted and the concentration of PQQ was reduced to 1nM or as indicated. This was done to bring the rate of dye reduction into the observable time frame. The exponential phase of the recorded and the exponential phase of the curves was fitted as single exponential to obtain K_{obs} . In order to obtain the K_d of the interaction the K_{obs} data were plotted against the concentration of the ligand and the data were fitted to the explicit solution of the quadratic equation describing the $E + S \rightleftharpoons ES$ binding equilibrium, where K_d is defined as $K_d = [E] \cdot [S] / [EL]$. $[E_0]$ and $[L_0]$ refer to the total enzyme and ligand concentration (free and bound) in the cuvette. Under these conditions the fluorescence is described by: $F = K_{obs (min)} + (K_{obs (max)} - K_{obs (min)}) / (([E_0] + [L_0] + K_d) / 2 - ([E_0] + [L_0] + K_d)^2 / 4 - [E_0] \cdot [L_0])^{1/2} / [L_0]$

K_{obs} represents the measured rate, while $K_{obs (min)}$ and $K_{obs (max)}$ refer to the minimal and maximal rates observed, respectively. A least-squares fit of the data to equation 1 using the software package Grafit 7.0 (Erithacus software) was used to determine the K_d value.

15 *Construction of a two-component tacrolimus biosensor*

As both rapamycin and tacrolimus share a common binding partner FKBP, we used CaM-GDH-FKBP as a reporter biosensor component. Initially calcinurin A/B-CaM BP complex was obtained by co-expressed native calcinurin A and calcinurin B-CaM BP in *E. coli* as described previously (22). To enhance solubility and the expression yield of the fusion, a solubility SUMO tag was added at the N-terminus of calcinurin A. The resulting SUMO-calcinurin A/B complex was then tagged with the modified version of CaM-BP. The resulting ORFs were cloned into pET28a vectors and expressed in the periplasm of *E. coli*. After Ni-NTA purification, the pooled enzyme-containing fractions were dialyzed against buffer containing 20mM Tris-buffer, pH 7.2, with 0.1M NaCl and stored frozen at -80°C.

25 To simply the expression and purification of the calcinurin A/B complex, we fused two subunits into a continuous open reading frame by joining them with a flexible linker GSGSGSGGG.

Construction of a two component human serum albumin (HSA) biosensors.

30 We searched the PDB databank for protein binders of human serum albumin and identified peptostreptococcal albumin-binding protein (PDB:1TF0) (5) and the VHH fragment (PDB:5VNW) that constitute a non-overlapping binding pair for albumin. Subsequently, we

identified immunoglobulin G binding protein G (PDB:1GJS) as a homolog of the peptostreptococcal albumin-binding protein that displays higher binding affinity toward human serum albumin (6). We fused the Immunoglobulin G binding protein G to the N-terminal of cysteine “ratcheted” R β 5/6GDH-CaM chimera, cloned the construct into pET28a
 5 and expressed it in cytosol in *E. coli*. The VHH fragment with a signal peptide sequence was fused to N-terminal of truncated CaM binding peptide and the fusion open reading frame was cloned into pET28a vector and expressed in *E.coli* periplasm. After Ni-NTA purification both proteins were further purified by size exclusion chromatography on a Superdex 200 column (GE Healthcare) equilibrated with buffer containing 20mM Tris-
 10 buffer, pH 7.2, with 0.1M NaCl. The fractions corresponding to the monomeric proteins were concentrated, snap frozen in liquid nitrogen and stored at -80°C.

Construction and analysis of two component Cyclosporine A biosensor.

The biosensor of Cyclosporine A was constructed by fusing “ratcheted” CaM-GDH switch
 15 module to human Cyclophilin gene that product forms quaternary complex with Cyclosporine A and Calcenurin A and B. To simplify the expression and purification of the calcinurin A/B complex, we fused two subunits into one continuous polypeptide using a flexible linker and prefaced it with a soluble SUMO tag. The resulting SUMO- calcineurin A/B complex was then tagged with the modified version of CaM-BP. The performance of
 20 the biosensor was tested in buffer (Fig. 9A,B) and in 25% human serum (Fig. 9C,D).

Quantification of HSA in serum samples.

To determine the concentration of human serum albumin in patient serum, we first diluted the samples with assay buffer containing 20mM Tris pH7.2 and 20mM NaCl to 1:10000
 25 fold. The assays were performed in 1ml reaction volume containing 100 μ l diluted serum sample, 60 μ M electron accepting dye dichlorophenolindophenol in the presence of 0.6mM electron mediator phenazine methosulphate, 1mM CaCl₂, 10nM of cysteine “ratcheted” globulin binding protein G CaM-GDH fusion and 100nM of VHH-CaM-BP. The mixture was incubated at 25°C for 30 minutes without glucose. Upon addition of glucose to the
 30 final concentration of 20mM, change in absorption at 600nm was monitored. The observed reaction rates were obtained by linear fitting of absorption changes during the first 60 seconds. The k_{obs} was then used to determine albumin concentration by correlating

it with the calibration curve. The calibration curve was obtained by titration of the sensor with the known amount of human serum albumin (Sigma-Aldrich).

Quantification of HSA in urine samples.

- 5 The concentration of human serum albumin in samples of human urine from healthy and diabetic individuals covered a large concentration range from low nM to mid μ M. To determine its concentration in patient urine, we first diluted the human urine sample with assay buffer containing 20mM Tris pH 7.2 and 20mM NaCl to 1:5, 1:50 and 1:500 fold. The assays were performed in 1ml reaction volume containing 100 μ l of the diluted urine
10 samples and assay components described above. The obtained k_{obs} values of the individual dilutions were compared and the highest dilution displaying reactivity were selected for determination of the albumin concentration by correlating it with the calibration curve.

Quantification of HSA in human serum using Beckman Coulter Synchron DxC800

- 15 *chemistry analyser.*

The samples of human serum were analysed using bromocresol dye using the kit and according to the instructions of the manufacturer.

Quantification of HSA in human urine using Beckman Coulter Synchron DxC800

- 20 *chemistry analyser.*

The samples of human serum were analysed using the immune-turbidimetric method using the kit and according to the instructions of the manufacturer.

Quantification of Cyclosporin A in serum samples.

- 25 First, we tested the effect of human serum on the performance of the two component cyclosporine A sensor based on “ratcheted” CaM- 5/6 GDH chimera. We found that addition of 25% of human serum into the assay had no significant effect on sensor performance. A standard calibration curve was generated using a serial dilution of Cyclosporin A in assay buffer containing 25% of human serum. The test samples were
30 prepared by spiking cyclosporine A into human serum to a final concentration ranging from 5nM to 105nM. The assays were performed in 1ml reaction volume containing 250 μ l serum cyclosporine samples, 60 μ M electron accepting dye dichlorophenolindophenol in

the presence of 0.6mM electron mediator phenazine methosulphate, 20mM of glucose, 2mM CaCl₂, 5nM of cysteine “ratcheted” CaM GDH- -Cyclophilin and 30nM of CalA/CalB-CaM-BP. The mixture was incubated at 25°C for 30 minutes without glucose and then the change of 600 nm absorption was monitored upon glucose addition. The observed reaction rate was obtained by linear fitting the absorption trace during the initial 60 seconds of reaction. The k_{obs} was plotted against drug concentration and the K_d was calculated using the above described quadratic equation via Grafit 7 (Erithacus software). The absolute concentration of cyclosporine A in the serum sample was determined by correlating the obtained values with the calibration curve.

Electrochemical measurements.

GDH activity of the sensor was measured electrochemically as depletion of a 2mM 1-methoxy phenazinium methylsulfate mediator (mPMS; Sigma-Aldrich) in 20mM Tris, 20mM NaCl, 1mM CaCl pH 7.2 buffer. Depletion of oxidized mPMS (instead of appearance of reduced mPMS) was monitored to avoid interference from precipitation formed by the reduced mPMS. Reactions were sized at 50µl final volume and contained either 10µM or 0µM FK506 ligand (+/-FK506) and 0.4% DMSO as a ligand vehicle. Both (+/- FK506) reactions contained 10µM E83S, F92A, L105A and F141A calmodulin mutant and 2µM CalA/CalB-CaM-BP. After the final addition of 1µM CaM-GDH-FKBP premixed with 1.5µM pyrroloquinoline quinone (PQQ), the complex was allowed to assemble over 20 minutes at room temperature in a PCR tube. Simultaneously, triplicate reactions were set up omitting the sensor components and FK506 (‘zero signal’), and also with excess wild type GDH instead of the two sensor components (‘max signal’). After the 20 minute complex assembly incubation, sensor activity was commenced with addition of 20mM glucose and 2mM oxidized mPMS mediator, followed by gassing under nitrogen to minimize re-oxidation of the mediator by atmospheric oxygen. The reaction was allowed to proceed for 10 minutes before the tube was opened and the entire 50µl droplet pipetted onto a DropSens screen printed gold electrode (C220BT) with working electrode polarized to -0.3V versus silver reference via a Digi-Ivy DY2100 potentiostat. 5s of chronoamperometric data was taken (20µs sample time), with the first time interval taken for plotting. As the directly measured signal was related to the oxidized mPMS remaining from the initial 2mM pool, both (+/- FK506) current values had the triplicate

averaged 'zero signal' subtracted to yield a μA value proportional to the mPMS reduced by the sensor. The 'max signal' triplicate average was used to verify that the 10 minute incubation with mediator was not long enough to fully reduce the 2mM mPMS pool for either the (+/- FK506) reactions (thus saturating and invalidating the measurement).

5

Biosensor stability testing.

For stability testing 20 μl solutions of protein biosensors were freeze-dried in 1.5ml plastic tubes using Gamma 2-16 LSCplus, Martin Christ® lyophilizer. The dried proteins were stored in closed tubes at room temperature for 1 week and then reconstituted with distilled
10 water to original concentration and used in the activity assays. The parental control samples were kept at -80°C for the duration of the aging trial.

Analysis of ability of different CaM-BPs to activate CaM-GDH fusions.

The assays were performed in duplicates using 200 μl reaction volumes in 96 well
15 microtiter plates. The reaction buffer contained 40mM Tris-HCl, 50mM NaCl, 5nM of CaM-GDH and 200nM of the respective peptide. The mixture was incubated for 20 minutes at room temperature and a stock solution containing DCPIP and PMS was added to bring their final concentration to 0.06mM and 0.6mM respectively. Finally, the glucose stock solution was added simultaneously to all wells to achieve 20mM final concentration
20 and the changes in DCPIP absorption were followed over 10 minutes using a plate reader. The reaction mixtures containing the chimera of CaM inserted in the position 330 of GDH contained 50 μM CaCl_2 , while the chimera with the insertion site 403 was assayed in the presence of 1mM of CaCl_2 . The slope of the observed curve was fitted to a single exponential and used as a measure of enzyme activity (Fig. 18).

25

Construction and activity analysis of DHFR-Calmodulin chimera

Calmodulin was inserted in the site between residues G86 and V88 of the DHFR gene that was previously used for construction of a split enzyme (23). The gene was generated by Gibson Assembly method according the manufacturer's instruction (New England Biolabs)
30 and cloned into pET28a vector. The protein expression and purification were performed as previously described (4). After Ni-NTA purification of both wild type DHFR and CalM-

DHFR, the pooled enzyme-containing fractions were dialyzed against buffer containing 20mM KH_2PO_4 pH7.5 and 100mM NaCl overnight and stored frozen at -80°C .

The DHFR activity was quantified using an established calorimetric assay according to the instruction of the manufacturer (Sigma-Aldrich). Briefly, the reactions were carried out in
 5 1.5 mL volume containing 10nM enzyme, 80 μM NADPH and 67 μM dihydrofolic acid in a buffer containing 20mM Tris/HCl pH7.5 and 20mM NaCl, 1mM CaCl_2 , and chosen concentrations of Calmodulin binding peptide. The assay was performed at 25°C by monitoring the decrease in absorbance of NADPH at 340 nm. The K_{obs} ranging from 0.0001 min^{-1} in the absence of ligand to 0.0341 min^{-1} at 50nM of CaM-BP were used to
 10 obtain the K_d value.

Mass spectrometric analysis of “ratcheted” CaM-GDH biosensor

Plastic tubes containing 20 μl samples containing 4 μM “ratcheted” GaM-GDH in 50 mM Tris-HCl pH7.4 buffer, 50mM NaCl, and 1mM CaCl_2 , 6 μM PQQ with or without 8 μM
 15 CaM-BP peptide were incubated at room temperature for 10 minutes. Analysis of 10 μl samples was performed with SCIEX Triplex TOF 5600 MALDI-TOF and the data was analyzed with Analyst® TF 1.6 Software.

20 Example 1 - Effect of Ca^{2+} on the performance of CaM-GDH-based biosensors

Our previously developed two-component electrochemical biosensor architecture is generally applicable, as it is composed of two interchangeable modules: the binders responsible for the capture of the analyte and the allosteric reporter that converts the
 25 binding event into a biochemical activity. However, one consideration for this design is the carryover of functions associated with the individual building modules into the final assembly. While the binding domains and the oxidoreductase enzyme (e.g., GDH) reporter are not known to possess multiple activities or functions, the calmodulin is often referred to as a “molecular jack-knife” due to the plethora of functions and interactions it is known
 30 to engage into (12). Clearly Ca^{2+} -binding is one of the most prominent activities of calmodulin that is known to regulate its interaction with the majority of its putative 300 peptide ligands. Ca^{2+} -induced conformational changes in calmodulin structure were

explored by others and us to construct multiple Ca^{2+} biosensors with diverse outputs (1, 6, 7, 11). The influence of Ca^{2+} on biosensor performance could perhaps limit their utility as it would require precise control of Ca^{2+} concentrations that vary rapidly and significantly inside and outside of the cell. Hence, we wanted to test what effect different Ca^{2+} concentrations would have on the performance of the developed two-component biosensors. This is particularly important for defining their utility for *in vitro* and *in vivo* applications as Ca^{2+} concentrations may be tightly controlled in the *in vitro* experiments they vary rapidly and significantly inside and outside of the cell.

To analyze the effect of Ca^{2+} experimentally we tested the performance of our tacrolimus (FK506) biosensors at different Ca^{2+} concentration. Ca^{2+} was required for obtaining the response to the ligand, but the optimal signal-to-noise ratio was achieved around between 50 and 75 μM of Ca^{2+} . Below this concentration the ligand addition stimulated low or no GDH activity while the higher concentrations of Ca^{2+} led to progressively increasing background activity and reduced the dynamic range of the biosensor. The concentrations above 500 nM resulted in constitutive activation of the biosensor in ligand-independent fashion (Fig. 1B). In order to ascertain that the observed effects were caused by the CaM-GDH fusion and not by the rest of the biosensor assembly we repeated the experiments by titrating FKBP-CaM-GDH assembly with CaM-BP at different Ca^{2+} concentrations. These experiments confirmed that the excess of Ca^{2+} ions could fully activate the CaM-GDH module and render it insensitive to CaM-BP (Fig. 1C). One can try to rationalize these observations based on the assumed CaM-GDH functional model where the Ca^{2+} -bound extended conformation of CaM pushes the strands of the host loop apart thereby dislodging the catalytic residues from the active site of GDH. Binding of the CaM-BP to the CaM brings the ends of the host loop closer together restoring the functionality of the GDH molecule. Yet, the exact positions of the biosensor's functional elements and their dynamics are unknown and the above-described experiments suggest that the peptide induced conformational change may also be phenocopied by structural changes occurring at high Ca^{2+} concentrations in the absence of the peptide ligand.

30

Example 2 - Development of a CaM-GDH-based biosensor optimized for physiological Ca^{2+} concentrations

The utility of the developed biosensor systems for example for diagnostics or bioengineering, would increase significantly if the conformational switch could be adopted to be tolerant to Ca^{2+} fluctuations in the physiological range that is between 0.5 and 5 mM.

5 Use of different linker sequences is a traditional way of optimizing performance of biosensors based on domain insertions. We, however, decided to test an alternative strategy and explore if a different insertion site could be found that would display lesser sensitivity to the intermediate conformations of CaM:CaM-BP complex. This has an additional benefit of validating the idea that CaM-insertion is a generic way of creating

10 synthetic allosteric switch units that can be assembled in the higher order biosensor architectures. To this end, we reanalyzed the structure of PQQ-GDH and identified a loop connecting β -sheets 5 and 6 (Fig. 1F). This loop carries Arg406 and Arg408 that make ion-pair interactions with the C9 and C2 carboxyl groups of PQQ (Fig. 2A). We conjectured that regulated displacement of these residues would impact the ability of PQQ

15 to engage in glucose catalysis.

Therefore, we constructed a chimeric protein where CaM was inserted between amino acids 403 and 405 of the GDH molecule (further termed $\beta 5/6$ -CaM-GDH) (Fig. 1G). The recombinant chimeric protein showed CaM-BP-dependent CaM-GDH activation that was

20 only marginally influenced by the changes in Ca^{2+} concentration once a threshold concentration of 500 μM of Ca^{2+} had been reached. Encouraged by these results we reconstructed the two-component rapamycin biosensors using the approach described above, where CaM-GDH was fused to FKBP and a modified version of CaM-BP was fused to FRB. In the initial testing the recombinant version of this biosensor showed only

25 modest rapamycin-mediated activation. Therefore, we decided to use a higher affinity version of CaM-BP (KRRWKKNFIAVSAANR (SEQ ID NO: 10)) than was used to construct the earlier version of the switch (KRRWKKNFIAVASASA (SEQ ID NO: 37)). As can be seen in Fig. 2B-2E and 3A this biosensor also showed Ca^{2+} dependence, but increases in Ca^{2+} concentration beyond 500 μM resulted in little further change in the

30 biosensor's activity. We further tested the performance of the biosensor in 50% bovine serum spiked with 5 mM Ca^{2+} and confirmed that neither had an impact on observed rates

or the amplitude (Fig. 2B-2E). Similar results were obtained with tacrolimus biosensor constructed using the same approach (Fig. 3C).

We next used the developed $\beta 5/6$ -CaM-GDH module to construct the FK506 (Tacrolimus) biosensor. As shown in Fig. 3B, the activity of the tacrolimus biosensor increased with the increasing concentration of drug until it came to saturation (Fig. 3B). Increase in Ca^{2+} concentration also had an effect on the assay, but it largely plateaued at 1 mM CaCl_2 concentration (Fig. 3C). The biosensor showed no detectable changes in performance when 50% serum was added to the reaction mix, indicating that it may be suitable for use in serum, plasma and blood samples. However, the biosensor showed higher background activity than the earlier versions of the tacrolimus biosensor and responded slower to the analyte (compare Fig. 1A and Fig. 3B).

In order to demonstrate that the new biosensors could detect analytes in other bodily fluids, we used an α -amylase biosensor comprising VHH1- $\beta 5/6$ -CaM-GDH and VHH2-CaM-BP, wherein VHH1/2 are antibody fragments that bind specifically to α -amylase (Fig. 4A). This α -amylase biosensor was used to measure the concentration of the salivary amylase in collected samples of human saliva (Fig. 4B). In parallel, we assessed the α -amylase concentration using a traditional enzymatic assay. The data from both experiments were compared and showed good correlation (Fig. 4C), similar to the correlation previously reported for assessment of α -amylase concentration by ELISA vs enzymatic activity (13). These experiments showed that even at 50% saliva concentration, the biosensor performed well, thereby confirming its suitability for assessing biomarkers in bodily fluids.

Example 3 - Enhancing the dynamic range of the two-component biosensors.

While we have successfully developed a modular biosensor architecture that was sufficiently stable and sensitive to measure the abundant analytes such as α -amylase in biological fluids, the dynamic range of the system is less than 4 fold. This complicates the use of the system for low abundant analytes and also increases the technical hurdles for transition from plate-based assays onto miniaturized formats that could be used in the Point-of-Care (PoC) setting. The latter is particularly attractive given the broad use of

GDH as a PoC glucose biosensor and the highly advanced manufacturing process for disposable sensory electrodes. Yet, our engineering efforts that resulted in CaM-GDH unit that could operate under the varying Ca^{2+} concentrations appeared to have negatively affected the dynamic range of the sensor, making it less suitable for this purpose.

5

Modification of the linker sequences connecting the switch module with the reporter domain is a well-established approach for optimization of biosensor performance. Changes in length and rigidity of the linker are typically tested to achieve the desired biosensor response. We conjectured that the performance of the switch module may be enhanced if the system is engineered to operate in a non-equilibrium mode, where the activated state is ratcheted or biased, to reduce or prevent relaxation and deactivation of the biosensor. To test this idea, we incorporated a cysteine residue into each of the linkers connecting the CaM domain to GDH. We reasoned that the CaM-BP-induced conformational change might bring the cysteine residues into proximity, increasing the chance of disulfide bond formation. If a bond was formed, it would function as a molecular ratchet, shifting the equilibrium of the biosensor towards the activated state.

To test this idea we designed a variant of the FKBP- β 5/6-CaM-GDH chimera where the CaM domain insert is linked to the GDH using linkers having the sequences GCGG (SEQ ID NO: 24) and GGSCG (SEQ ID NO: 25), respectively. When the construct was produced in the periplasm of *E. coli* cells using our standard expression protocol (as described in (14)), the resulting recombinant protein demonstrated constitutive GDH activity that was not responsive to CaM-BP (data not shown). However, when the protein was produced in the *E. coli* cytosol, presumably in reduced form, the protein was inactive in the ground state and dose-dependently responded to the CaM-BP. The detailed analysis of the data revealed that the biosensor displayed much reduced background activity compared to the parental unit, but displayed remarkable activation of 104 fold in the presence of CaM-BP (Fig. 5A and 6A). To our knowledge, this is the largest dynamic range of a biosensor observed to date. Mass-spectrometric analysis of such “ratcheted” form of CaM-GDH in the absence and presence of CaM-BP detected a mass decrease that matches disulfide bond formation thereby providing evidence for the proposed mechanism (Fig. 7).

Encouraged by these observations we constructed two-component biosensors for rapamycin and FK-506 (tacrolimus) based on the developed “cysteine ratcheted” switch module. As can be seen in Figs 6C-F, the biosensors displayed dynamic ranges of 8 fold
 5 and 20 fold, respectively, which represents an improvement of approximately 3 fold and 5 fold, respectively, with overall activity improved by a factor of 2 compared to the parental biosensors.

We also used the developed switch module to construct a biosensor of human serum albumin (HSA) using Protein G and anti HSA VHH as binding modules. The resulting
 10 biosensor displayed increased GDH activity in response to HSA (Fig. 5B, 8A,B and 17A,B). In order to test the practical utility of this biosensor we collected samples of human serum and quantified the concentration of HSA using our biosensor as well as the established clinical diagnostic platform. As can be seen in the Figure 5C both methods
 15 showed excellent correlation. We subsequently demonstrated that same assay could be used to quantify HSA in human urine correctly identifying patients with albuminuria (Fig. 8C). Encouraged by these results we constructed a biosensor of another macrocyclic immunosuppressant Cyclosporine A (Fig. 5D, 9A-D) that displayed a dynamic range close to 10 fold even in the presence of 25% human serum (Fig. 5D, 9C,D). We tested the
 20 performance of this biosensor in serum samples containing different amounts of cyclosporine. As shown in the Figure 5E the biosensor-based assay correlated well with the concentration of the drug in the samples and the sensitivity of the assay was at least 10 times higher than required for detection of the clinically relevant concentration of the drug in blood (the clinical reference range of cyclosporine is 100-400nM).

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Example 4 - Design and construction of a protease biosensor with a CaM-GDH actuator.

The developed $\beta 5/6$ -CaM-GDH switch allows rapid and sensitive detection of CaM-BP
 30 and conversion of the binding event into electric current. The latest generation of the biosensor (*i.e.*, 5th generation; “cysteine ratcheted”) has an exceptionally large dynamic range (>100 fold). While the $\beta 5/6$ -CaM-GDH switch can be utilized as a part of a two-

component system, the equilibrium nature of the system makes its use at concentrations of target analyte at or below the K_d of the binding moieties potentially problematic (see Fig. 10A and 10B). In an alternative approach, for example visualized in Fig. 10C, binding of the target analyte to the binding or receptor domains of the biosensor results in an irreversible event, such as proteolysis, generating a CaM-BP that can activate the $\beta 5/6$ -CaM-GDH. This approach, however, requires the construction of a CaM-BP precursor that cannot be recognized by the $\beta 5/6$ -CaM-GDH and which can be converted into the active CaM-BP, for example, by proteolysis.

10 To construct such a system, we used the structures of the CaM:CaM-BP complex to design two CaM mutants (CaM*1 and CaM*2) with progressively lower affinities for CaM-BP. These were fused to wild type (wt) CaM-BP as shown in Figure 11. This results in the formation of an autoinhibited module unable to interact with either CaM-BPs or CaM unless they are present at a very high concentration. This is assured by the fact that the k_{on} rate is concentration dependent and the high local concentration of the CaM* and CaM-BP in the fusion shifts the equilibrium towards the complex formation despite the increase in the k_{off} rate resulting from the mutations introduced into the CaM.

We then tested the efficiency of CaM-BP caging in the CaM*-CaM-BP fusion protein by mixing it at different concentrations with CaM-GDH reporter and measuring the extent of the GDH activation. As shown in Figure 12A, the caged CaM*-CaM-BP variant with presumed intermediate affinity mediated only negligible activation of the CaM-GDH reporter up to 2 μ M concentration, indicating an efficient caging of CaM-BP.

25 In subsequent experiments we introduced the TVMV cleavage site into the linker connecting the CaM* and CaM-BP. It was expected that cleavage of the linker would result in dissociation of the complex. The CaM*-TVMV cleavage site-CaM-BP module was fused to FRB. We then used an autoinhibited (AI) version of TVMV protease fused to FKBP developed previously by our group (6). FRB together with FKBP, forms a complex with rapamycin. It was expected that in the absence of rapamycin, the receptor domains would be diffusing in solution independently, resulting in little or no cleavage of the CaM*-CaM-BP (Figs 11 and 12B). However, in the presence of rapamycin both modules

would be brought together, thereby dramatically increasing the local concentrations of the components and leading to intramolecular swap of the TVMV cleavage site and the TVMV inhibitor leading to the cleavage of the CaM*-CaM-BP. This releases a CaM-BP now capable of binding and activating CaM-GDH. This experiment was performed, mixing both components of the rapamycin receptor (FRB-CaM*-CaM-BP and FKBP-TVMV-AI) with CaM-GDH at different rapamycin concentrations. This resulted in rapamycin-dependent activation of GDH activity, confirming that rapamycin-mediated assembly of the complex leads to its proteolysis and the release of the CaM-BP (Figs 11 and 12B). A fit of the data resulted in an affinity of 11 nM, which is reasonably close to the previously determined affinity of the FRB:rapamycin:FKBP complex (Fig. 12C).

Example 5 - Application of caged CaM-BP in non-protease-based two-component systems.

One limitation of the two-component system where the mutant version of CaM-BP is used to activate the CaM-GDH reporter is that it is sensitive to the concentration of the components. At high concentrations of the components, the two-component system may auto-activate due to the shift of the equilibrium toward the bound complex. This could be overcome by the further mutagenesis of the CaM-BP, but this approach is associated with the danger that these will induce suboptimal conformations of the CaM-GDH. Further new mutations need to be made for any concentration range. Another approach to overcome this problem is to use a caged version of the CaM-BP that is efficient in inducing activation of CaM-GDH, but which cannot interact with CaM-GDH unless in close molecular proximity, for example as shown in Fig. 13. This allows the system to operate in a wide range of concentrations.

The FRB-CaM*-CaM-BP described above was tested for background activation levels of a FKBP-GDH-CaM reporter in the absence of rapamycin. Increasing concentrations of FRB-CaM*-CaM-BP were titrated against a constant concentration of the FKBP-GDH-CaM reporter. As shown in Figure 14A, the FRB-CaM*-CaM-BP efficiently caged the CaM-BP and only negligible activation of the FKBP-GDH-CaM was observed in the absence of rapamycin. In contrast, in the presence of saturating concentrations of

rapamycin, activation of the FKBP-GDH-CaM reporter increased as the concentration of FRB-CaM*-CaM-BP increased (Fig. 14B). This demonstrates the independence of the two-component biosensor from the concentration of the caged CaM-BP component concentration, showing only concentration-dependent activation in the presence of rapamycin. Furthermore, this system demonstrates the largest dynamic range as can be seen by comparing rapamycin-mediated activation with activation induced by M13 calmodulin binding peptide.

Furthermore, we then tested stoichiometric ratio of biosensor components FRB-CaM*-CaM-BP and FKBP-GDH-CaM and rapamycin at different concentrations (Fig. 15). These data demonstrate that the two-component caged CaM-BP rapamycin biosensors are capable of maintaining high dynamic range at concentrations varying by at least 20 fold. This allows the system to be deployed over a very large range of analyte concentrations, without losing the dynamic range, and therefore sensitivity.

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Example 6 - Construction of auto-inhibited CaM-GDH module and its activation using calmodulin binding peptides.

We aimed to generate a CaM-BP that binds specifically to calmodulin, but that would not activate the catalytic activity of the GDH enzyme in a CaM-GDH fusion protein. To this end, a library of variant CaM-BPs was generated based on an analysis of the high resolution structures of CaM:CaM-BP complexes. The sequences of the sixteen variant CaM-BP peptides generated is given in SEQ ID NOs 45-60. We then analysed the effect of the synthetic variant CaM-BPs derived from the high resolution structures of CaM:CaM-BP complexes on the activity of the CaM-GDH fusion. In the experiment the FKBP-CaM-GDH was incubated with 200 nM of the respective variant peptide and 600 nM 2-6-dichlorophenyl-Indophenol (DCPIP), 600 nM phenazine methosulfate (PSF), 60 nM PQQ and 20 mM glucose buffered with 1 mM CaCl₂ and 20 mM K₂HPO₄ at pH 7.0. The FKBP-CaM-GDH used in the assay comprised the Q2CaM-GDH (SEQ ID NO: 36), which comprised mutations relative to wild type GDH, but which had been shown to have similar activity (data not shown). The reaction was monitored by measuring the decrease in absorbance at 620 nm in a 96 well plate using a plate reader. As shown in Fig.

16B, the peptides all bound to the CaM, but exhibited a range of abilities to activate the CaM-GDH GDH enzyme. Many of the variant peptides binding to CaM did not activate the GDH activity at all, as compared with the blank. These data demonstrate that variant CaM-BPs may be generated that bind specifically to CaM but that do not activate the enzyme activity upon binding to the CaM-GDH fusion. These variant CaM-BPs may be used to block binding of CaM-BPs that are able to activate the enzyme activity of CaM-GDH fusions as shown in Figs 16C and 16D to create biosensors with reduced signal-to-noise and low background. Again, this allows the system to be deployed over a very large range of analyte concentrations, without losing the dynamic range, and therefore sensitivity.

Example 7 - Generation 5 two component biosensor of Serum Human Albumin (HSA).

As a further example of the applicability of the “cysteine ratcheted” (*i.e.*, generation 5) GDH-based biosensors, we developed a two-component biosensors for Serum Human Albumin (HSA) based on the developed “cysteine ratcheted” switch module. The two-component biosensor comprises GDH-CalM-GA (SEQ ID NO: 63) and VHH-CaM-BP (SEQ ID NO: 64). Fig. 17A shows the GDH activity of the two-component HSA biosensor at different concentrations of purified HSA and Fig. 17B demonstrates that the biosensor had a K_d of 5 nM for HSA. This demonstrates that the cysteine ratcheted biosensors may be adapted for detection of a wide range of target molecules and demonstrates the high sensitivity and high dynamic range of the cysteine ratcheted sensor.

Example 8 – Testing variant CaM-BPs.

In the course of this study we consistently observed that the dynamic range of two component biosensors was smaller than that of its parental switch module. One of the possible explanations lays in the fact that CaM-BP used to design and test the core switch and the CaM-BP used to activate it in the context of a two component system are similar but not identical. This is due to mutations that were introduced to reduce the affinity of peptide for the core switch. While in the context of the ligand-scaffolded biosensor the

equilibrium should be shifted to fully bound CaM-BP, the structure of the complex may be different from the native one. To test the possible effect of CaM-BP structure on the activity of the developed CaM-GDH chimeras created in this study, we tested a collection of the peptides extracted from the available calmodulin:calmodulin binding peptide complexes (SEQ ID NOs: 29, 45, 47-60). As can be seen in the Figure 18 there was a significant difference in the activity response of both CaM-GDH variants to the CaM-BPs. In some cases both switches responded similarly to the peptide (peptides 1 and 4; SEQ ID NOs: 29 and 50)) while peptides had dramatically different effects on each chimera. Interestingly, peptides 5, 7, 8, 13 and 12 (SEQ ID NOs: 51, 53, 54, 59 and 58) displayed inhibitory activity towards one of the chimeras. This can potentially indicate that these peptides stabilize the CaM-GDH chimera in the inactive conformation, thereby reducing the background activity. Based on this data we concluded that small changes in the structure of CaM-GDH:CaM-BP complex may lead to significant changes in its dynamic range (compare effects of peptides 3 and 4 (SEQ ID NOs: 49 and 50) that differ by one residue). This may provide an explanation for reduction of the dynamic range in the two component systems that are operated by CaM-BPs that are structurally different from the parental peptides that were used to identifying the best CaM-GDH designs. Therefore, biosensor architectures that utilize unmodified CaM-BPs may display larger dynamic ranges. Alternatively, one can envision an integrated engineering approach where modification of CaM-BP is done in the context of the two-component system thereby assessing the structure and function linkage more directly.

Example 9 – Suitability of the developed biosensors for amperometry measurements

The simplicity and low cost of chronoamperometric detection of enzyme-generated electron current on disposable screen printed electrodes makes GDH-based biosensors very attractive for PoC applications. Therefore, we tested whether tacrolimus-induced activity change of the tacrolimus biosensor could be detected using standard electrochemistry measurements.

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One attraction of the developed biosensors is the possibility of monitoring their activity using cheap disposable screen-printed electrodes interfaced to portable amperometers. This

approach has been widely adopted for blood glucose monitoring, and expansion of this technology to other biomarkers could be potentially transformative for the diagnostic industry. Therefore, we set out to test the compatibility of the developed two component biosensors with electrochemical detection. One potential caveat of this approach is the sensitivity of the standard solution electrochemical methods. As blood glucose is present at mM concentrations, the methods currently implemented in glucose monitors are less sensitive than the spectroscopic assays used for GDH-based biosensor development. While alternative assay formats with much more efficient electron transfer (and therefore much higher sensitivity) exist, they have not penetrated Point-of-Care (PoC) applications so far and therefore we wanted to test our biosensors under the most commonly used assay.

Performing the assays at micromolar concentrations of the components to meet the sensitivity of standard screen printed electrodes increases the background activation of the biosensor due to the higher fraction of the biosensor complex formed in the absence of the ligand (compare Figures 19D and 19E). This could be overcome by mutagenesis of the biosensor's CaM-BP to further reduce its activity for the CaM-GDH. However, this may have unintended negative consequences for the biosensor performance. Therefore, we devised a more generic strategy for adjusting the biosensor's performance to the desired assay conditions. As the biosensor functions cooperatively with the ligand-binding domains and CaM-CaM-BP contributing to overall affinity of the system, we hypothesized that an additional thermodynamic barrier to binding of CaM-BP to CaM-GDH may be a way of tuning the performance of the biosensor without rebalancing the affinities of the components. Such a barrier could be provided by the addition of the free calmodulin (see, e.g., SEQ ID NOs: 27 and 62) to complex CaM-BP, thus preventing its spontaneous association with CaM-GDH (Fig. 19F). The ligand mediated scaffolding event is to be expected to drive re-distribution of CaM-BP towards CaM-BP (Fig. 19F and 20A). In this case, we would expect the concentration of the calmodulin and the rate of dissociation of CaM-BP and CaM complex to determine the rate of the rearrangement.

In order to ascertain that the system re-equilibrates rapidly and efficiently, we decided to utilize CaM mutants with lower affinity and presumably faster dissociation kinetics. To this end we analyzed the crystal structure of human CaM and M13 CaM-BP complex

(PDB ID: 2BBM) in order to identify the residues in the former that could be mutated without destroying the overall interaction. Based on our analysis we sought to reduce the hydrophobic interaction between CaM and CaM-BP by introducing E83S, F92A, L105A and F141A mutations (see, e.g., SEQ ID NO: 62) next to the Ca²⁺ binding loops preceding EF-3 and succeeding of EF-4 respectively. The resulting mutant was expressed in *E.coli*, produced in recombinant form and purified by Ni-NTA chromatography. We then tested the effect of the mutant on the dynamic range of the assay at different concentrations of the components. As can be seen in Figure 20B, the presence of E83S, F92A, L105A and F141A CaM mutations restored the dynamic range of the assay at high component concentrations (compare with Figure 19E). As the resultant assay conditions were expected to be compatible with simple glucometer-type electrochemical analysis, we performed basic PoC-type amperometric measurements of the reaction mixtures in the presence and absence of tacrolimus. As shown in Figure 20C there was approximately 10 fold signal change in the electron current in the absence and presence of the analyte, indicating that the activity of the developed GDH-based biosensors can be monitored by the conventional PoC-type electrochemical testing. While the glucometer-type electrochemical set up has inherent sensitivity limitations, these stem from the inefficiencies of electron transfer from the biosensor to the electrode and can be overcome by changing the electrode and mediator design. Thus, amperometry could be used to monitor activity of the developed GDH biosensors.

As glucose monitoring systems utilize screen printed electrodes layered with a dry mixture containing the biosensor and the electron mediator we wanted to test if the developed biosensors display sufficient stability to be compatible with such formats. To this end we lyophilized CaM-GDH as well as the two component biosensors of α -amylase, cyclosporine, tacrolimus and human serum albumin described above and tested their activity upon re-hydration and seven day storage at room temperature. The data shown in Figure 21 confirms that even without optimization of drying conditions the proteins retained activity under these conditions.

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Example 10 – Construction and activity analysis of GDH- affinity clamp chimera.

To test if alternative peptide binding receptors can be used to construct synthetic allosteric ON switches, we chose an artificial two domain binder known as the affinity clamp that undergoes a significant conformational transition upon ligand binding (16). We therefore inserted the affinity clamp at the position 330 of PQQ-GDH that we used to construct the first generation of CaM-GDH biosensors. Activity analysis of the resulting recombinant protein revealed that it retained the activity close to that of the wild type, but titration of affinity clamp binding peptide reduced the activity in a dose dependent fashion (Fig. 22A,B). This confirmed that the chosen insertion site could tolerate multiple domain insertions, and the obtained module represents an OFF switch. Attempts to modify the linkers connecting the affinity clamp to the GDH, or the insertion position of the former, to yield an ON switch are ongoing.

Example 11 – Calmodulin insertion is a generic strategy for converting proteins into synthetic allosteric switch units.

The success of calmodulin insertion experiments prompted us to test whether the developed approach could be used to convert other proteins into allosteric switch units that could be compiled into biosensor architectures. We chose dihydrofolate reductase (DHFR) as an example. We used structural analysis to rationally select the calmodulin insertion sites (see Materials and Methods section). As can be seen in the Figure 23A the DHFR chimera demonstrated ON switch behavior in response to the CaM-BP and the CaM-DFFR displayed over 200-fold activity change (Fig. 23A).

We hypothesize that peptide binding induces structural rearrangements in the calmodulin from extended to compact conformation that brings the N- and C- termini closer together by about 10Å (17). Therefore, it is expected that while the extended conformation would introduce significant distortions into the host structure creating an OFF state, these would be relaxed when the peptide-induced conformational transition of calmodulin brings the linkers connecting the domains together, thereby restoring the near native state of the host protein (Fig. 23B,C). We hypothesize that calmodulin is highly suitable for its role as a regulatory domain as its extended apo- form and the compact CaM-BP bound form are highly structured and therefore capable of locking the host protein in two different defined

states. Attempts to replace calmodulin with a synthetic peptide receptor known as affinity clamp are ongoing and so far have produced OFF switches but not the desired ON switches (Fig. 22).

- 5 Unlike split or alternative folding frame systems (18) the introduced distortions are much less likely to compromise the overall stability of the chimeric protein, significantly expanding the number and type of proteins this approach can be used for. Remarkably, our results demonstrate that structurally unrelated reporter domains such as DHFR and GDH can be converted into synthetic allosteric switches using calmodulin insertion by testing
10 just a small number of rationally designed chimeras. We expect that structure and (or) mutagenesis-based optimization of these switches is likely to significantly improve their dynamic range and response kinetics.

- The inherent Ca^{2+} dependence of calmodulin, where four calcium binding sites with
15 different affinities contribute to complexity of the conformational transitions, was found to be dependent on the insertion site, with the insertion site described herein showing little effect of Ca^{2+} at concentrations above 1mM indicating that the complex response landscape of CaM-chimers observed for peptide-induced transitions may also apply to Ca^{2+} induced conformational changes. Moreover, some calmodulin binding peptides are
20 known to induce conformational changes in calmodulin even in the absence of calcium, suggesting that structure-based engineering can resolve the potential limitations of the presented system (19). Structural plasticity of calmodulin has been previously explored to create variants with orthogonal peptide specificity (20,21) and a similar approach could be applied here to create a potentially unlimited number of orthogonal CaM:CaM-BP pairs
25 that could be used to construct an array of orthogonal switches and thereon based signaling circuits.

- We further demonstrated that the developed synthetic switch modules can be integrated into two component biosensors with tunable selectivity. Here, the analyte drives increase
30 of concentration of low affinity CaM-BP in the vicinity of the CaM-operated switch (Fig. 23D). As the system is purely concentration driven, the exact arrangement of the components in space is less important, provided that the linkers connecting the functional

elements are sufficiently flexible. The specificity of the biosensor is encoded by the pair of binding domains that are able to bring both subunits of the biosensor into proximity. While we demonstrated the use of VHH domains and biological targets of macrocyclic compounds any binding domains of sufficient biophysical stability that bind distinct sites of the analyte can be used. We demonstrate that such two component biosensors can be used to measure concentrations of xenobiotics and biomarkers in biological fluids, confirming their suitability for their deployment in diagnostic laboratories and for development of the point-of-care applications. The presented architecture delivers a tool box for rapid construction of orthogonal protein-based signaling circuits that, with some optimization, can be broadly deployed *in vivo* and *in vitro* to construct novel signaling systems of potentially unlimited complexity.

Example 12 – Generation of three component biosensors.

A three component biosensor for detection of amylase was generated by expression of three separate components as follows: GDH-CaM-SH3-FKBP (SEQ ID NO:72); FRB-SH3-VHH1 (SEQ ID NO: 75) ; and VHH2-CaM-BP (SEQ ID NO: 77). A schematic illustrating the biosensor architecture is provided in Figure 24A. GDH activity data and titration to α -amylase to calculate K_d is shown in Figures 24B and C,

Example 13 – Generation of single component biosensors.

In order to generate single component GDH-based biosensors, a circular permuted GDH was generated. The wild type N- and C- termini were joined using a flexible G/S linker sequence and a new N-terminus was generated at position Q155 and a new C-terminus at position P153, resulting in the deletion of amino acid D154 (all residue numbering as in wildtype PQQ-GDH). This resulted in a formation of a circular permuted GDH (cpGDH) (Fig. 25A; SEQ ID NO: 79). The calmodulin protein was inserted via short linker sequences into the insertion site at the loop connecting β -sheets 5 and 6, between amino acids S403 and N405, of the GDH enzyme, resulting in the deletion of N404, to create the conformationally inhibited cpGDH-CaM (SEQ ID NO: 86) (all residue numbering as in wildtype PQQ-GDH). The binding domains, specific for the target

analyte of interest, were then fused to the newly formed N- and C-termini through short linkers. A schematic summarizing the generation of the cpGDH biosensors is provided in Fig. 25A. As shown in Figures 26C and 26D for the cyclosporine A biosensor, the single-component biosensors show reduced noise and provide more accurate and reliable reading of analyte concentration than the corresponding two-component biosensors. Single-component biosensors for detecting α -amylase, rapamycin, FK506 and cyclosporine A were generated. Data demonstrating detection of analytes using these biosensors are shown in Figures 25, 26 and 27. The sequences of these exemplary single component biosensors are provided in SEQ ID Nos 78, 81, 84 and 85.

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SEQUENCE LISTING**SEQ ID NO: 1** - 4th generation *GDH-CaM*:

DVPLIPSQFAKAKSENFDDKKVILSNLNKPHALLWGPDNQIWLTERATGKILRVNPESGSV
 5 KTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKSTDKELPNQTIIRRYT
 YNKSTDTLEKPVDLLAGLPSSKDHQSGRLVIGPDQKIYYTIGDQGRNQLAYLFLPNQAQ
 HTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNGVVSHIYTLGHRNPQGLAFTP
 NGKLLQSEQGPNSSDDEINLIVKGGNYGWPNAVAGYKDDSGYAYANYSAANKTIKDLAQ
 NGVKVAAGVPVTKESSEWTGKNFVPLKTLTYTVQDTYNYNDPTCGEMTYICWPTVAPSS
 10 AYVYKGGKKAITGWENTLLVPSLKRGVIFRIKLDPTYSTTYDDAVPMFKSGSGGTEEQI
 AEFKEAFSLFDKDGDTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDF
 PEFLTMMARKMKDSTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEE
 VDEMIREADIDGDGQVNYEEFVQMMTAGGSGGNRYRDVIASPDGNVLYVLTDTAGN
 VQKDDGSVTNTLENPGSLIKFTYKAKHHHHH

15

SEQ ID NO: 2 – Calmodulin (CaM):

TEEQIAEFKEAFSLFDKDGDTITTKELGTVMRSLGQNPTEAELQDMINEVDADGN
 GTIDFPEFLTMMARKMKDSTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEK
 LTDEEVDEMIREADIDGDGQVNYEEFVQMMTA

20

SEQ ID NO: 3 – PQQ-GDH with no insert (insert location shown in bold and underlined):

DVPLIPSQFAKAKSENFDDKKVILSNLNKPHALLWGPDNQIWLTERATGKILRVNPE
 SGSVKTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKSTDKELPN
 QTIIRRYTYNKSTDTLEKPVDLLAGLPSSKDHQSGRLVIGPDQKIYYTIGDQGRNQL
 25 AYLFLPNQAQHTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNGVVSHIYT
 LGHRNPQGLAFTPNGKLLQSEQGPNSSDDEINLIVKGGNYGWPNAVAGYKDDSGYA
 YANYSAANKTIKDLAQNGVKVAAGVPVTKESSEWTGKNFVPLKTLTYTVQDTYN
 YNDPTCGEMTYICWPTVAPSSAYVYKGGKKAITGWENTLLVPSLKRGVIFRIKLDP
 TYSTTYDDAVPMFK**SN**RYRDVIASPDGNVLYVLTDTAGNVQKDDGSVTNTLEN
 30 PGSLIKFTYKAK

SEQ ID NO: 4 – linker 1: GSGG

SEQ ID NO: 5 – linker 2: GGSGG

SEQ ID NO: 6 – 4th generation Rapamycin and FK506 (Tacrolimus) sensor component 2
(GDH-CaM-FKBP):

5 DVPLIPSQFAKAKSENFDDKKVILSNLNKPHALLWGPDNQIWLTERATGKILRVNPESGSV
KTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKSTDKELPNQTIIRRYT
YNKSTDITLEKPVDLLAGLPSSKDHQSGRLVIGPDQKIYYTIGDQGRNQLAYFLPNQAAQ
HTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNGVVSIIYTLGHRNPQGLAFTP
10 NGKLLQSEQGPNSDDEINLIVKGGNYGWPVNAVAGYKDDSGYAYANYSAAANKTIKDLAQ
NGVKVAAGVPVTKESWTGKNFVPLKTLTYTVQDTYNYNDPTCGEMTYICWPTVAPSS
AYVYKGGKKAITGWENTLLVPSLKRGVIFRIKLDPTYSTTYDDAVPMFKSGSGGTEEQI
AEFKEAFSLFDKDGDTITTKELGTVMRSLGQNPTAEALQDMINEVDADGNGTIDF
PEFLTMMARKMKDSTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEE
15 VDEMIREADIDGDGQVNYEEFVQMMTAGGSGGNRYRDVIASPDGNVLYVLDTAGN
VQKDDGSVTNTLENPGSLIKFTYKAKGGSGGGVQVETISPGDGRTFPKRGQTCVVHY
TGMLLEDGKKFDSSRDRNKPFFKMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDY
AYGATGHPGIIPPHATLVFDVELLKLEKLAAALEHHHHHHH

20 **SEQ ID NO: 7** – FKBP:

GVQVETISPGDGRTFPKRGQTCVVHYTGMLLEDGKKFDSSRDRNKPFFKMLGKQE
VIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEKLA
AALE

25 **SEQ ID NO: 8** – 6 x His-tag

HHHHHH

SEQ ID NO: 9 – 4th and 5th generation Rapamycin sensor component 1 (FRB-BP):

30 AHHHHHHSSGTRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGP
QTLKETSFNQAYGRDLMEAEQWCRKYMKSGNVKDLTQAWDLYYHVFRRISSGGSGGSG
SGSGSGSGGKRRWKKNFIAVSAANR

SEQ ID NO: 10 – higher affinity Calmodulin binding peptide:

KRRWKKNFIAVSAANR

SEQ ID NO: 11 – FRB

- 5 RVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFN
QAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRIS

SEQ ID NO: 12 – linker 3: GSGSGSGSGSGSGSGG

- 10 **SEQ ID NO: 13** – 4th and 5th generation FK506 (Tacrolimus) sensor component 1 (*SUMO-CN alpha/beta-BP*)

- DVPLIPSQFAKAKSENFDDKKGSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIK
KTTPLRRLMEAFKRQKGEMDSLRLFLYDGIRIQADQTPEDLDMEDNDIIEAHREQIGG
GSGSGGAHHHHHHSSGTSEPKAIDPKLSTTDRVVKAVPFPPSHRLTAKEVFDNDG
15 KPRVDILKAHLMKEGRLEESVALRIITEGASILRQEKNLLDIDAPVTVCGDIHGQFF
DLMKLFEVGGSPANTRYLFLGDYVDRGYFSIECVLYLWALKILYPKTLFLLRGNH
ECRHLTEYFTFKQECKIKYSERVYDACMDAFDCLPLAALMNQQFLCVHGGLSPEI
NTLDDIRKLDRFKEPPAYGPMCDILWSDPLEDFGNEKTQEHFTHNTVRGCSYFY
YPAVCEFLQHNNLLSILRAHEAQDAGYRMYRKSQTTGFPSLITIFSAPNYLDVYNN
20 KAAVLKYENNMNIRQFNCSHPYWLPNFMDVFTWSLPFVGEKVTEMLNVNLNI
CSDDELGSEEDGSGSGSGGGNEASYPLEMCSHFDADEIKRLGKRFFKKLDLDNSGS
LSVEEFMSLPELOQNPLVQRVIDIFDTDGNGEVDFKEFIEGVVSQFSVKGDKEQKLR
FAFRIYDMDKDGYSISNGELFQVLKMMVGNNLKDTQLQOQIVDKTIINADKDGRI
SFEFFCAVVGGLDIHKKMVVDVGGSGSGSGSGSGSGSGGKRRWKKNFIAVSAANR

25

SEQ ID NO: 14 – SUMO

IPSQFAKAKSENFDDKKGSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKK
TTPLRRLMEAFKRQKGEMDSLRLFLYDGIRIQADQTPEDLDMEDNDIIEAHREQIG
G

30

SEQ ID NO: 15 – Calcineurin alpha subunit (CN alpha)

SEPKAIDPKLSTTDRVVKAVPFPPSHRLTAKEVFDNDGKPRVDILKAHLMKEGRLE
 ESVALRIITEGASILRQEKNLLDIDAPVTVCGDIHGQFFDLMKLFEVGGSPANTRYL
 FLGDYVDRGYFSIECVLYLWALKILYPKTLFLLRGNHECRHLTEYFTFKQECKIKY
 SERVYDACMDAFDCLPLAALMNQQFLCVHGGLSPEINTLDDIRKLDRFKEPPAYG
 5 PMCDILWSDPLEDFGNEKTQEHFTHNTVRGCSYFYSSYPVCEFLQHNNLLSILRAH
 EAQDAGYRMYRKSQTTGFPSLITIFSAPNYLDVYNNKAAVLKYENNVNMNIRQFNC
 SPHPYWLPNFMDVFTWSLPPFVGEKVTEMLVNVLNICSDDDELGSEED

SEQ ID NO: 16 – Calcinurin beta subunit (CN beta)

10 NEASYPLEMCSHFDADAIEKRLGKRFFKLDLDNSGSLSVEEFMSLPQLQNNPLVQR
 VIDIFDTDGNGEVDFKEFIEGVSQFSVKGDKEQKLRFARIYDMDKDGYSNGELF
 QVLKMMVGNLKDQLQIVDKTIINADKDGGRISFEFECAVVGGLDIHKKMV
 VDV

15 **SEQ ID NO: 17** – linker 4: GGGSGSGG

SEQ ID NO: 18 – linker 5: GSGSGSGG

SEQ ID NO: 19 – 4th generation Amylase sensor component 2 (*GDH-CaM-VHH1*)

20 *DVPLIPSQFAKAKSENFDDKKVILSNLNKPHALLWGPNDQIWLTERATGKILRVNPESGSV*
KTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKSTDKELPNQTIIRRYT
YNKSTDITLEKPVDLLAGLPSSKDHQSGRLVIGPDQKIYYTIGDQGRNQLAYLFLPNQAQ
HTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNGVVSIIYTLGHRNPQGLAFTP
NGKLLQSEQGPNSDDEINLIVKGGNYGWPNAVAGYKDDSGYAYANYSAAANKTIKDLAQ
 25 *NGVKVAAGVPVTKESWTGKNFVPLKTLTYTVQDTYNYNDPTCGEMTYICWPTVAPSS*
AYVYKGGKKAITGWENTLLVPSLKRGVIFRIKLDPTYSTTYDDAVPMFKSGSGGTEEQI
AEFKEAFSLFDKDGDTITTKELGTVMRSLGQNPTAEALQDMINEVDADGNGTIDF
PEFLTMMARKMKDTEDEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEE
VDEMIREADIDGDGQVNYEEFVQMMTAGGSGGNRYRDVIASPDGNVLYVLDTAGN
 30 *VQKDDGSVTNTLENPGSLIKFTYKAKSGSGGDDTTVSEPAPSCVTLYQSWRYSQADN*
GCAETVTVKVVEYEDDTEGLCYAVAPGOITTVGDGYIGSHGHARYLARCLKLAAA
 LEHHHHHH

SEQ ID NO: 20 – VHH1

DTTVSEPAPSCVTLYQSWRYSQADNGCAETVTVKVVYEDDTEGLCYAVAPGQITT
VGDGYIGSHGHARYLARCL

5

SEQ ID NO: 21 – 4th and 5th generation Amylase sensor component 1: *VHH2-BP*

DGHHHHHHGSGQVQLVESGGGTVPAGGSLRLSCAASGNTLCTYDMSWYRRAPGKGR
DFVSGIDNDGTTTYVDSVAGRFTISQGNNAKNTAYLQMDSLKPDDTAMYYCKPSLRYGLP
GCPIIPWGQGTQVTVSSGGSGGSGSGSGSGSGGGKRRWKKNFIAVSAANR

10

SEQ ID NO: 22 – VHH2

QVQLVESGGGTVPAGGSLRLSCAASGNTLCTYDMSWYRRAPGKGRDFVSGIDND
GTTTYVDSVAGRFTISQGNNAKNTAYLQMDSLKPDDTAMYYCKPSLRYGLPGCPII
PWGQGTQVTVSS

15

SEQ ID NO: 23 – 5th generation *GDH-CaM* (cysteine residues in linkers shown in bold and underlined):

DVPLIPSQFAKAKSENFDDKKVILSNLNKPHALLWGPDNQIWLTERATGKILRVNPESGSV
KTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYYISGTFKNPKSTDKELPNQTIIRRYT
20 YNKSTDITLEKPVDLLAGLPSSKDHQSGRLVIGPDQKIYYTIGDQGRNQLAYLFLPNQAQ
HTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNGVVSIIYTLGHRNPQGLAFTP
NGKLLQSEQGPNSSDDEINLIVKGGNYGWPNAVAGYKDDSGYAYANYSAAANKTIKDLAQ
NGVKVAAGVPVTKESEWTGKNFVPLKTLTYTVQDTYNYNDPTCGEMTYICWPTVAPSS
AYVYKGGKKAITGWENTLLVPSLKRGVIFRIKLDPTYSTTYDDAVPMFKSGCGGTEEQI
25 AEFKEAFSLFDKDGDTITTKELGTVMRSLGQNPTAEELQDMINEVDADGNGTIDF
PEFLTMMARKMKDSTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEE
VDEMIREADIDGDGQVNYEEFVQMMTAGGSCGNRYRDVIASPDGNVLYVLDTAGN
VQKDDGSVTNTLENPGSLIKFTYKAKKLAAALEHHHHHH

30 SEQ ID NO: 24 – linker 6: GCGG**SEQ ID NO: 25 – linker 7: GGSCG**

SEQ ID NO: 26 – 5th generation Rapamycin and FK506 sensor component 2 (GDH-CaM-FKBP) (cysteine residues in linkers shown in bold and underlined):

DVPLIPSQFAKAKSENFDDKKVILSNLNKPHALLWGPNDQIWLTERATGKILRVNPESGSV
 5 KTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKSTDKELPNQTIIRRYT
 YNKSTDTLEKPVDLLAGLPSSKDHQSGRLVIGPDQKIYYTIGDQGRNQLAYLFLPNQAQ
 HTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNGVVSHIYTLGHRNPQGLAFTP
 NGKLLQSEQGPNSSDDEINLIVKGGNYGWPNAVAGYKDDSGYAYANYSAANKTIKDLAQ
 NGVKVAAGVPVTKESEWTGKNFVPLKTLTYTVQDTYNYNDPTCGEMTYICWPTVAPSS
 10 AYVYKGGKKAITGWENTLLVPSLKRGVIFRIKLDPTYSTTYDDAVPMFKSGCGGTEEQI
AEFKEAFSLFDKDGGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDF
PEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEE
VDEMIREADIDGDGQVNYEEFVQMMTAGGSCGNRYRDVIASPDGNVLYVLTDTAGN
VQKDDGSVTNTLENPGSLIKFTYKAKGSGGGVQVETISPGDGRTFPKRGQTCVVHY
 15 TGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKL TISPDY
AYGATGHPGIIPPHATLVFDVELLKLEKLAAALEHHHHHH

SEQ ID NO: 27 – Calmodulin having reduced affinity for CaM-BP (CaM*1) (mutations in calmodulin relative to wild type shown in bold and underlined):

20 TEEQIAEFKEAFSLFDKDGDTITTKELGTVMRSLGQNPTEAELQDMINEVDADGN
 GTIDFPEFLTMMARKMKD TDSEEEIREAFRVADKDGNGYISAAELRHVMTNLGEK
 LTDEEVDEMIREADIDG DGQVNYEEAVQMMTA

SEQ ID NO: 28 – caged CaM-BP – *CaM*1* fusion (mutations in calmodulin relative to wild type shown in bold and underlined):

25 TEEQIAEFKEAFSLFDKDGDTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTI
 DFPEFLTMMARKMKD TDSEEEIREAFRVADKDGNGYISAAELRHVMTNLGEKLTDEEV
 DEMIREADIDG DGQVNYEEAVQMMTAGGSGSGSGSGGKRRWKKNFIAVSAANRF
KKISSSGAL

30

SEQ ID NO: 29 – wild type (wt) CaM-BP:
 KRRWKKNFIAVSAANRFKKISSSGAL

SEQ ID NO: 30 – FRB-CaM*1-CaMBP fusion (mutations in calmodulin relative to wild type shown in bold and underlined):

MAHHHHHHSSGTRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMM
 5 ERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRR
ISGGSGSGSGSGSGSGSGGTEEQIAEFKEAFSLFDKDGDTITTKELGTVMRSLGQNPT
EAELQDMINEVDADGNGTIDFPEFLTMMARKMKDSTDSEEEIREAFRVADKDGNGYISAAE
LRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEAVQMMTAGGSGSGSGSGSGGK
RRWKKNFIAVSAANRFKKISSSGAL

10

SEQ ID NO: 31 – FKBP-TVMV-AI: (including thrombin cleavage site)

MDGSGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGK
 QEVIRGWEEGVAQMSVGQRAKLITSPDYAYGATGHPGIIPPHATLVFDVELLKLEGGSG
 GGSGSGSGSGSSKALLKGVRDFNPISACVCLLENSSDGHSERLFGIGFGPYIIANQH
 15 LFRRNNGELTIKTMHGEFKVKNSTQLQMKPVEGRDIIVIKMAKDFPPFPQKLKFRQ
PTIKDRVCMVSTNFQQKSVSSLVSESSHIVHKEDTSFWQHWITTKDGQCGSPLVSII
DGNILGIHSLTHTTNGSNYFVEFPEK FVATYLDAADGWCKNWKFNADKISWGSFI
LWEDAPEDFMSGLVPRGVGREYVRFAPGSKLAAALEHHHHHHH

20 **SEQ ID NO: 32** – TVMV:

SSKALLKGVRDFNPISACVCLLENSSDGHSERLFGIGFGPYIIANQH
 LFRNNGELTIKTMHGEFKVKNSTQLQMKPVEGRDIIVIKMAKDFPPFPQKLKFRQ
 PTIKDRVCMVSTNFQQKSVSSLVSESSHIVHKEDTSFWQHWITTKDGQCGSPLVSII
 DGNILGIHSLTHTTNGSNYFVEFPEK FVATYLDAADGWCKNWKFNADKISWGSFI
 LWEDAPEDFM
 25 S

SEQ ID NO: 33 – autoinhibitory peptide (AI):

REYVRFAP

30 **SEQ ID NO: 34** – FRB-CaM*1-TVMV cleavage site-CaM-BP

MAHHHHHHSSGTRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMER
 GPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRISGGSGG

SGSGSGSGSGGTEEQIAEFKEAFSLFDKDGDTITTKELGTVMRSLGQNPTEAELQD
MINEVDADGNGTIDFPEFLTMMARKMKD TDSEEEIREAFRVADKDGNGYISAAEL
RHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEAVQMMTAGGSGGSETVRF
QSGGSDDVTPCSMSLGS GSGGKRRWKKNFIAVSAANRFKKISSSGAL

5

SEQ ID NO: 35 – TVMV cleavage site: ETVRFQS

SEQ ID NO: 36 – *Q2GDH-CaM* (mutant of 3rd generation GDH (CaM inserted between β -sheets 4 and 5) – K→Q mutations relative to 4th generation GDH shown in bold and underlined)

10

*MGDVPLIPSQFAKAKSENF***DKQ***VILSNLNKPHALLWGPDNQIWLTERATG***Q***ILRVNPES*
*GSVKTVFQVPEIVNDADGQNGLLGFAFHPDF***Q***NNPYIYISGTF***Q***NP***Q***STD***Q***ELPNQTII*
*R***Q***YTYN***Q***STD***Q***TLE***Q***PVDLLAGLPSSKDHQSGRLVIGPDQKIYYTIGDQGRNQLAYLFLP*
*NQAQHTPTQ***Q***ELNG***Q***DYHTYMGKVLRLNLDGSIP***Q***DNPSFNGVVSHIYTLGHRNPQG*
15 *LAFTPNGKLLQ***Q***SE***Q***GPNSDDEINLIVKGGNYGWPNAVAGYKDDSGYAYANYSAANKTIK*
*DLAQNGVKVAAGVPVTKESEWTGKNFVPPLKTLTYTVQ***D***GSGGTEEQIAEFKEAFSLFD*
KDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARK
MKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIREADI
DGDGQVNYEEFVQMMTAGGSGGYNNDPTCGEMTYICWPTVAPSSAYVY**Q****G****Q****Q****A****I**
20 *TGwentllvpslkrGVIFRIKLDPTYSTTYDDAVPMFKSNNRYRDVIASPDGNVLYVLT*
*D***Q***AGNVQKDDGSVTNTLENPGSLIKFTYKAKHHHHHH*

20

SEQ ID NO: 37 – lower affinity CaM-BP

KRRWKKNFIAVASASA

25

SEQ ID NO: 38 – N-terminal PQQ-GDH fragment (insertion site bold and underlined)

DVPLIPSQFAKAKSENF~~DK~~KVILSNLNKPHALLWGPDNQIWLTERATGKILRVNPE
SGSVKTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKSTDKELPN
QTIIRRYTYNKSTD~~TLE~~KPVDLLAGLPSSKDHQSGRLVIGPDQKIYYTIGDQGRNQ
30 *AYLFLPNQAQHTPTQ***Q***ELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNGVVSHIYT*
*LGHRNPQGLAFTPNGKLLQ***Q***SE***Q***GPNSDDEINLIVKGGNYGWPNAVAGYKDDSGYA*
YANYSAANKTIKDLAQNGVKVAAGVPVTKESEWTGKNFVPPLKTLTYTVQDTYN

30

YNDPTCGEMTYICWPTVAPSSAYVYKGGKKAITGWENTLLVPSLKRGVIFRIKLDP
 TYSTTYDDAVPMFKS

SEQ ID NO: 39 – C-terminal PQQ-GDH fragment (insertion site bold and underlined)

5 NRYRDVIASPDGNVLYVLTDTAGNVQKDDGSVTNTLENPGSLIKFTYKAK

SEQ ID NO: 40 – linker in caged CaM-BP – CaM*1 fusion

GGSGGSGSGSGG

10 **SEQ ID NO: 41** – FKBP-TVMV linker

GGSGGGSGSGSGSGSS

SEQ ID NO: 42 – TVMV-AI

SSKALLKGVRDFNPISACVCLLENSSDGHSERLFGIGFGPYIIANQHFRNNGELTI
 15 KTMHGEFVKVKNSTQLQMKPVEGRDIIVIKMAKDFPPFPQKLKFRQPTIKDRVCMV
STNFQQKS VSSLVSESSHIVHKEDTSFWQHWITTKDGQCGSPLVSIIDGNILGIHSLT
HTTNGSNYFVEFPEKFVATYLDAAADGWCKNWKFNADKISWGSFILWEDAPEDFM
SGLVPRGVGREYVRFAPGSKLAAALEHHHHHHH

20 **SEQ ID NO: 43** – TVMV cleavage site containing linker in CaM*1-TVMV cleavage site-
 CaM-BP construct (cleavage site shown in bold and underlined)

GGSGGSE**ETVRFQS**GGSDDVTPCSMSLGS

SEQ ID NO: 44 – CaM*1-TVMV cleavage site-CaM-BP construct

25 *TEEQIAEFKEAFSLFDKDGDTITTKELGTVMRSLGQNPTAEALQDMINEVDADGNGTI*
DFPEFLTMMARKMKD TDSEEEIREAFRVADKDGNGYISAAELRHVMTNLGEKLTDEEV
DEMIREADIDGDGQVNYEEAVQMMTAGGSGGSETVRFQSGGSDDVTPCSMSLGS
GGKRRWKNFIAVSAANRFFKKISSGAL

30 **SEQ ID NO: 45** – variant CaM-BP 1

KRRWKNFIAVSAANRFFKKISSGAL

SEQ ID NO: 46 – variant CaM-BP 1t

KRRWKKNFIAVASASA

SEQ ID NO: 47 – variant CaM-BP 2

5 EVTVGKIFYATFLIQEYFRKFKKRKEQGLVGKPS

SEQ ID NO: 48 – variant CaM-BP 3

EVTVGKIFYATFLIQ

10 **SEQ ID NO: 49** – variant CaM-BP 4

HMGKIYAAMMIMEYYRQSKAKK

SEQ ID NO: 50 – variant CaM-BP 5

HMGKIYAAMMIMEAYRQSKAKK

15

SEQ ID NO: 51 – variant CaM-BP 6

EMRQKIRSHAHALLAANRFMDM

SEQ ID NO: 52 – variant CaM-BP 7

20 NMGQHLDVKLVPSSSYIKVVKAFHHSSLHE

SEQ ID NO: 53 – variant CaM-BP 8

GRNWKNFALVPLLRD

25 **SEQ ID NO: 54** – variant CaM-BP 9

LTEIKKQIPQKEWDKVVNTPNSLEKQKGVTNLLIKYGIER

SEQ ID NO: 55 – variant CaM-BP 10

IRNKIRAIGKMARVF

30

SEQ ID NO: 56 – variant CaM-BP 11

HSMQALSWRKLYLSRAKLKA

SEQ ID NO: 57 – variant CaM-BP 12

ADKLRAACIRIQKTIRGWLLRK

5 **SEQ ID NO: 58** – variant CaM-BP 13

MQRAAITVQRYVRGYQARCYAKFLRRTK

SEQ ID NO: 59 – variant CaM-BP 14

EIRKILRNNLQKTRQRLRSYNRHTLVADPYEEAWNQMLLRRQK

10

SEQ ID NO: 60 – variant CaM-BP 15

RLSFKTVALLVLACVRMKRIAFY

15 **SEQ ID NO: 61** – FRB-CaM*2-CaMBP fusion (mutations in calmodulin relative to wild type shown in bold and underlined):

MAHHHHHHSSGTRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMM
ERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRI
SGSGSGSGSGSGSGSGGTEEQIAEFKEAFSLFDKDGDTITTKELGTVMRSLGQNPTEA
ELQDMINEVDADGNGTIDFPEFLTMMARKMKDSESEIREAFRVADKDGNGYISAAE
 20 ARHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEAVQMMTAGGSGSGSGSGSGGK
RRWKKNFIAVSAANRFKKISSSGAL

SEQ ID NO: 62 – CaM*2 (mutations in calmodulin relative to wild type shown in bold and underlined):

25 TEEQIAEFKEAFSLFDKDGDTITTKELGTVMRSLGQNPTEAELQDMINEVDADGN
 GTIDFPEFLTMMARKMKDSESEIREAFRVADKDGNGYISAAEARHVMTNLGEK
 LTDEEVDEMIREADIDGDGQVNYEEAVQMMTA

30 **SEQ ID NO: 63** – 5th generation human serum albumin (HAS) sensor component 1 (GA
binder-GDH-CalM) (cysteine residues in linkers shown in bold and underlined):
AVDANSLAEAKVLANRELDKYGVSDFYKRLINKAKTVEGVEALKLHILAAALPGGS
GSGSGSGSGSGSGSGSGSGGGDVPLIPSQFAKAKSENFDDKKVILSNLNKPHALLWGP

DNQIWLERATGKILRVNPESGSKTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPIY
ISGTFKNPKSTDKELPNQTIIRRYTYNKSTDTLEKPVDLLAGLPSSKDHQSGRLVIGPDQ
KIYYTIGDQGRNQLAYLFLPNQAQHTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNP
SFNGVVSHIYTLGHRNPQGLAFTPNGKLLQSEQGPNSDDEINLIVKGGNYGWPNAVGY
5 KDDSGYAYANYSAANKTIKDLAQNGVKVAAGVPVTKESEWTGKNFVPPLKTLYTVQD
TYNYNDPTCGEMTYICWPTVAPSSAYVYKGGKKAITGWENTLLVPSLKRGVIFRIKLDPT
YSTTYDDAVPMFKSGCGGTEEQIAEFKEAFSLFDKDGDTITTKELGTVMRS LGQNP
TEAELQDMINEVDADGNGTIDFPEFLTMMARKMKD TDSEEEIREAFRVFDKDGNG
YISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTAGGSCG
10 NRYRDVIASPDGNVLYVLTDTAGNVQKDDGSVTNTLENPGSLIKFTYKAKKLAAALEH
HHHHH

SEQ ID NO: 64 – 5th generation human serum albumin (HAS) sensor component 2 (VHH
binder-Cam-BP) (cysteine residues in linkers shown in bold and underlined):

15 DGHHHHHHHGSQVQLQESGGGLVQAGGSLRLSCAASGYISDAYYMGWYRQAPG
KEREFVATITHGTNTYYADSVKGRFTISRDNAKNTVY LQMNSLKPEDTAVYYCAV
LETRSYSFYWGQGTQVTVSSGGSGSGSGSGSGSGSSGGSGSGSGGGKRRWKKNFI
AVSAANR

20 **SEQ ID NO: 65** – GA binder (comprised within GA binder-GDH-CalM):
 AVDANSLAEAKVLANRELDKYGVSDFYKRLINKAKTVEGVEALKLHILAALP

SEQ ID NO: 66 – VHH binder (comprised within VHH binder-Cam-BP):

25 QVQLQESGGGLVQAGGSLRLSCAASGYISDAYYMGWYRQAPGKEREFVATITHG
TNTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAVLETRSYSFYRW
GQGTQVTVSS

SEQ ID NO: 67 - 5th generation cyclosporine sensor component 1 (*GDH-CalM-Cyclophilin*) (cysteine residues in linkers shown in bold and underlined):

30 DVPLIPSQFAKAKSENFDDKKVILSNLNKPHALLWGPDNQIWLTERATGKILRVNPESGSV
KTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKSTDKELPNQTIHRRYT
YNKSTDTLEKPPVDLLAGLPSSKDHOSGRLVIGPDOKIYYTIGDOGRNOLAYLFLPNOAQ

HTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNGVVSHIYTLGHRNPQGLAFTP
 NGKLLQSEQGPNSSDDEINLIVKGGNYGWPNAVAGYKDDSGYAYANYSAANKTIKDLAQ
 NGVKVAAGVPVTKESWTGKNFVPPLKTLTYTVQDITYNYNDPTCGEMTYICWPTVAPSS
 AYVYKGGKKAITGWENTLLVPSLKRGVIFRIKLDPTYSTTYDDAVPMFKSGCGGTEEQI
 5 AEFKEAFSLFDKDGDTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDF
PEFLTMMARKMKDSTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEE
VDEMIREADIDGDGQVNYEEFVQMMTAGGSCGNRYRDVIASPDGNVLYVLTDTAGN
VQKDDGSVTNTLENPGSLIKFTYKAKGGSGGSGSGSGSGGSMVNPTVFFDIAVDGEP
LGRVSFELFADKVPKTAENFRALSTGEKGFGYKGSCFHRIIPGFMCQGGDFTRHNG
 10 TGGKSIYGEKFEDENFILKHTGPGILSMANAGPNTNGSQFFICTAKTEWLDGKHVV
FGKVKEGMNIVEAMERFGSRNGKTSKKITIADCGQLEKLAAALEHHHHHH

SEQ ID NO: 68 - Cyclophilin (comprised within *GDH-CalM-Cyclophilin*):

MVNPTVFFDIAVDGEPLGRVSFELFADKVPKTAENFRALSTGEKGFGYKGSCFHRI
 15 IPGFMCQGGDFTRHNGTGGKSIYGEKFEDENFILKHTGPGILSMANAGPNTNGSQF
 FICTAKTEWLDGKHVVFGKVKEGMNIVEAMERFGSRNGKTSKKITIADCGQLE

SEQ ID NO: 69 - GDH-affinity clamp chimer (*GDH-Affinity Clamp*)

DVPLIPSQFAKAKSENFDDKKVILSNLNKPHALLWGPNDQIWLTERATGKILRVNPESGSV
 20 KTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKSTDKELPNQTIIRRYT
 YNKSTDTLEKPVDLLAGLPSSKDHQSGRLVIGPDQKIYYTIGDQGRNQLAYLFLPNQAQ
 HTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNGVVSHIYTLGHRNPQGLAFTP
 NGKLLQSEQGPNSSDDEINLIVKGGNYGWPNAVAGYKDDSGYAYANYSAANKTIKDLAQ
 NGVKVAAGVPVTKESWTGKNFVPPLKTLTYTVQDEDAPESGSPELGFSISGGVGGRG
 25 NPERPDDDGIFVTRVQPEGPASKLLQPGDKIIQANGYSFINIEHGQAVSLLKTFQNT
VELIIVREVGNGAKQEIRVRVEKDGGSGGVSSVPTNLEVVAATPTSLLISWDAYRE
LPVSYYRITYGETGGNSPVQFTVPGSKSTATISGLKPGVDYTITVYAHYNYHYYS
SPISINIRGSGPGYNYNDPTCGEMTYICWPTVAPSSAYVYKGGKKAITGWENTLLVPSLK
RGVIFRIKLDPTYSTTYDDAVPMFKSNNRYRDVIASPDGNVLYVLTDAGNVQKDDGSV
 30 TNTLENPGSLIKFTYKAKHHHHHH

SEQ ID NO: 70 – 1st generation *CalM-DHFR* chimer:

ISLIAALAVDRVIGMENAMPWNLPADLAWFKRNTLNKPVIMGRHTWESIGRPLPGRKNII
 LSSQPGTDDRVTWVKSVDIAAACGGSGGTEEQIAEFKEAFSLFDKDGDTITTKELG
 TVMRS LGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKD TDSEEEIREA
 FRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDDEMIREADIDGDGQVNYEEFVQ
 5 MMTAGGSGGVPEIMVIGGGRVYEQFLPKAQKLYLTHIDAEVEGDTHFPDYEPDDWES
 VFSEFHDADAQNSHSYCFEILERRKLAAALEHHHHHHH

SEQ ID NO: 71 – DHFR (comprised within the CalM-DHFR):

ISLIAALAVDRVIGMENAMPWNLPADLAWFKRNTLNKPVIMGRHTWESIGRPLPGRKNII
 10 LSSQPGTDDRVTWVKSVDIAAACGVPEIMVIGGGRVYEQFLPKAQKLYLTHIDAEVEG
 DTHFPDYEPDDWESVFSEFHDADAQNSHSYCFEILERRKLAAALE

SEQ ID NO: 72 – Three component system - amylase sensor - component 1: 5th
 generation GDH-CaM-SH3-FKBP

15 DVPLIPSQFAKAKSENFDDKKVILSNLNKPHALLWGP DNQIWLTERATGKILRVNPESGSV
 KTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKSTDKELPNQTIHRRYT
 YNKSTD TLEKPVDLLAGLPSSKDHQSGRLVIGPDQKIYYTIGDQGRNQ LAYLFLPNQAAQ
 HTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNGV VSHIYTLGHRNPQGLAFTP
 NGKLLQSEQGPNSDDEINLIVKGGNYGWP NVAGYKDDSGYAYANYSA AANKTIKDLAQ
 20 NGVKVAAGVPVTKESEWTGKNFVPPLKTLTYTVQDTYNYNDPTCGEMTYICWPTVAPSS
 AYVYKGGKKAITGWENTLLVPSLKRGVIFRIKLDPTYSTTYDDAVPMFKSGCGGTEEQI
 AEFKEAFSLFDKDGDTITTKELGTVMRS LGQNPTEAELQDMINEVDADGNGTIDF
 PEFLTMMARKMKD TDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEE
 VDEMIREADIDGDGQVNYEEFVQMMTAGGSCGNRYRDVIASPDGNVLYVLTD TAGN
 25 VQKDDGSVTNTLENPGSLIKFTYKAKGSGSGGA EYVRALFDFNGNDEEDLPFKKGDI
 LRIRDKPEEQWWNAEDSEGKRG MIPVPYVEKYGGSGSGSGGVQVETISPGDGRTEP
 KRGQTCVVHYTGMLEDGKKFDSSRDRNKPFK FMLGKQEVIRGWEEGVAQMSVG
 QRAKLTISPDYAYGATGHPGIIPPHATLVFDVELL KLEKLAAALEHHHHHHH

30 **SEQ ID NO: 73** – SH3

AEYVRALFDFNGNDEEDLPFKKGDI LRIRDKPEEQWWNAEDSEGKRG MIPVPYVE
 KY

SEQ ID NO: 74 – FKBP

VQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFFKMLGKQEV
 RGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLE

5

SEQ ID NO: 75 – Three component system - amylase sensor - component 2: FRB-SH3L-VHH1

SHHHHHHGTRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQT
 LKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRISGGSGGSGGP
 10 PPPLPPKRRRGGSGSGDTTVSEPAPSCVTLYQSWRYSQADNGCAETVTVKVYYED
DTEGLCYAVAPGQITTVGDGYIGSHGHARYLARCL

SEQ ID NO: 76 – SH3L

PPPPLPPKRRR

15

SEQ ID NO: 77 – Three component system - amylase sensor - component 3: VHH2-CaMBP

DGHHHHHHGSGQVQLVESGGGTVPAGGSLRLSCAASGNTLCTYDMSWYRRAPGKGR
 DFVSGIDNDGTTTYVDSVAGRFTISQGNAKNTAYLQMDSLKPDDTAMYYCKPSLRYGLP
 20 GCPIIPWGQGTQVTVSSGGSGGSGSGSGSGGKRRWKKNFIAVSAANR

SEQ ID NO: 78 – Single component system - rapamycin sensor - FKBP-GDH-CaM-ERB

DHHHHHHHGQVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFFK
FMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVE
 25 LLKLEGGSGGSGGQKIYYTIGDQGRNQLAYLFLPNQAQHTPTQQELNGKDYHTYMGK
VLRLNLDGSIPKDNPSFNGVVSHIYTLGHRNPQGLAFTPNGKLLQSEQGPNSDDEINLIV
KGGNYGWPNVAGYKDDSGYAYANYSAAANKTIKDLAQNGVKVAAGVPVTKESEWTGK
NFVPPLKTLTYTVQDTYNYNDPTCGEMTYICWPTVAPSSAYVYKGGKKAITGWENTLLVP
SLKRGVIFRIKLDPTYSTTYDDAVPMFKSGSGGTEEQIAEFKEAFSLFDKDGDTITTK
 30 ELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDSTDSEEEI
REAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEE
FVQMMTAGGSGGNRYRDVIASPDGNVLYVLTDTAGNVQKDDGSVTNTLENPGSLIKF

TYKAKGGSGSGSGSGSGSGSGSGGDVPLIPSQFAKAKSENFDDKKVILSNLNKPHALLWG
 PDNQIWLTERATGKILRVNPESGSVKTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYI
 YISGTFKNPKSTDKELPNQTIIRRYTYNKSTDITLEKPVDLLAGLPSSKDHQSGRLVIGPGS
 GSGSGGLWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPOTLKET
 5 SNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFERRIS

SEQ ID NO: 79 – Circularly permuted GDH for single component system; natural N-terminus underlined and in bold; natural N and C terminus joined by a linker (grey box); the Asn residue that is deleted when CaM is inserted is highlighted in grey

10 QKIYYTIGDQGRNQLAYLFLPNQAQHTPTQQELNGKDYHTYMGKVLRLNLDGSIP
 KDNPSFNGVVSHIYTLGHRNPQGLAFTPNGKLLQSEQGPNSDDEINLIVKGGNYG
 WPNVAGYKDDSGYAYANYSAANKTIKDLAQNGVKVAAGVPVTKESEWTGKN
 FVPPLKTLTYTVQDTYNYNDPTCGEMTYICWPTVAPSSAYVYKGGKKAITGWENT
 LLVPSLKRGVIFRIKLDPTYSTTYDDAVPMFKSN¹NR YRDVIASPDGNVLYVLTDTA
 15 GNVQKDDGSVTNTLENPGSLIKFTYKAKGGSGSGSGSGSGSGSGGDVPLIPSQFA
 KAKSENFDDKKVILSNLNKPHALLWGPDNQIWLTERATGKILRVNPESGSVKTVFQ
 VPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKSTDKELPNQTIIRRYTYN
 KSTDITLEKPVDLLAGLPSSKDHQSGRLVIGP

20 **SEQ ID NO: 80** – FRB in single component rapamycin sensor
 LWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPOTLKETSFNQAY
 GRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFERRIS

SEQ ID NO: 81 – Single component system - FK506 sensor - calcineurin alpha/beta-
 25 GDH-CaM-FKBP
 DSSGTSEPKAIDPKLSTTDRVVKAVPFPPSHRLTAKEVFDNDGKPRVDILKAHLMK
 EGRLEESVALRIITEGASILRQEKNLLDIDAPVTVCGDIHGQFFDLMKLFEVGGSPA
 NTRYLFLGDYVDRGYFSIECVLYLWALKILYPKTLFLLRGNHECRHLTEYFTFKQE
 CKIKYSERVYDACMDAFDCLPLAALMNQQFLCVHGGLSPEINTLDDIRKLDRFKE
 30 PPAYGPMCDILWSDPLEDFGNEKTQEHFTHNTVRGCSYFYSPAVCEFLQHNNLL
 SILRAHEAQDAGYRMYRKSQTTGFPSLITIFSAPNYLDVYNNKAAVLKYENNVNMN
 IRQFNCSHPYWLPNFMDVFTWSLPFVGEKVTEMLVNVNLNICSDDDELGSEEDGSG

SGSGGGNEASYPLEMCSHFDADEIKRLGKRFKKLDLDNSGSLSVVEEFMSLPELQQ
 NPLVQRVIDIFD TDGNGEVDFKEFIEGV SQFSVKGDKEQKLRF AFRIYDMDKDG YI
 SNGELFQVLKMMVGNLKD TOLQQIVDKTIINADKDG DGRISFEFECAVVGGLDI
 HKKMVVDVGGSGSGSGGQKIYYTIGDQGRNQ LAYLFLPNQAQHTPTQQELNGKDYH
 5 TYMGKVLRLNLDGSIPKDNPSFNGVVSHIYTLGHRNPQGLAFTPNGKLLQSEQGPNSD
 DEINLIVKGGNYGWP NVAGYKDDSGYAYANY SAAANKTIKDLAQNGVKVAAGVPVTK E
 SEWTGKNFVPPLKTL YTVQDTYNYNDPTCGEMTYICWPTVAPSSAYVYKGGKKAITGW
 ENTLLVPSLKRGVIFRIKLDPTYSTTYDDAVPMFKSGSGSGGTEEQIAEFKEAFSLFDKDG
 DGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMK
 10 DTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGD
 GQVNYEEFVQMMTAGGSGGNRYRDVIASPDGNVLYVLTDTAGNVQKDDGSVTNTLE
 NPGSLIKFTYKAKGSGSGSGSGSGSGSGG DVPLIPSQFAKAKSENFDDKKVILSNL NK
 PHALLWGP DNQIWLTERATGKILRVNPESG SVKTVFQVPEIVNDADGQNGLLGF AFHP
 DFKNNPYIYISGTFKNPKSTDKELPNQTIIRRYTYNKSTD TLEKPVDLLAGLPSSKDHQSG
 15 RLVI GPGSGSSSGSGGGVQVETISPGDGRTFPKRGQTCVVHYTG MLEDGKKFDSSRD
 RNKPKFKFMLGKQE VIRGWEEGVAQMSVGQRAKLTISP DYAYGATGHPGIIPPHAT
 LVFDVELLKLEKLAAALEHHHHHH

SEQ ID NO: 82 – Single component system - FK506 sensor - calcineurin alpha

20 SSGTSEPKAIDPKLSTTDRVVKA VPFPPSHRLTAKEVFDNDGKPRVDILKAHLMKE
 GRLEESVALRIITEGASILRQEKNLLDIDAPVTVC GDIHGQFFDLMKLFEVGGSPAN
 TRYLFLGDYVDRGYFSIECVLYLWALKILYPKTLFLLRGNHECRHLTEYFTFKQEC
 KIKYSERVYDACMDAFDCLPLAALMNQQFLCVHGGLSPEINTLDDIRKLDRFKEPP
 AYGPMCDILWSDPLEDFGNEKTQEHFTHNTVRGCSYFYSYPAVCEFLQHNNLLSIL
 25 RAHEAQDAGYRMYRKSQTTGFPSLITIFSAPNYLDVYNNKAAVLKYENNVNMNIRQ
 FNCSPHPYWLPNFMDVFTWSLPFVGEKVTEMLVNVLNICSDDDELGSEED

SEQ ID NO: 83 – Single component system - FK506 sensor - FKBP

GVQVETISPGDGRTFPKRGQTCVVHYTG MLEDGKKFDSSRD RNKPKFKFMLGKQE
 30 VIRGWEEGVAQMSVGQRAKLTISP DYAYGATGHPGIIPPHATLVFDVELLKLE

SEQ ID NO: 84 – Single component system - Cyclosporine A sensor - calcineurin
alpha/beta-GDH-CaM-cyclophilin

DHHHHHHSSGTSEPKAIDPKLSTTDRVVKAVPFPPSHRLTAKEVFDNDGKPRVDIL
KAHLMKEGRLEESVALRIITEGASILRQEKNNLLDIDAPVTVCGDIHGQFFDLMKLFE
5 VGGSPANTRYLFLGDYVDRGYFSIECVLYLWALKILYPKTLFLLRGNHECRHLTEY
FTFKQECKIKYSERVYDACMDAFDCLPLAALMNQQFLCVHGGLSPEINTLDDIRK
LDRFKEPPAYGPMCDILWSDPLEDFGNEKTQEHFTHNTVRGCSYFYSPAVCEFL
QHNNLLSILRAHEAQDAGYRMYRKSQTTGFPSLITIFSAPNYLDVYNNKAAVLKY
ENNVNMNIRQFNCSPHPYWLPNFMVFTWSLPFVGEKVTEMLVNVLNICSDDELGS
10 EEDGSGSGSGGGNEASYPLEMCSHFDADEIKRLGKRFKKLDLDNSGSLSVEEFMS
LPELOQNPLVQRVIDIFDTDGNGEVDFKEFIEGVSOFSVKGDKEOKLRFARIYDM
DKDGYISNGELFQVLKMMVGNNLKDTQLQQIVDKTIINADKDGDRISFEFFCAV
VGGLDIHKKMVVDVVGSGSGSGSGSGSGSGGGQKIYYTIGDQGRNQLAYLFLPNQA
QHTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNGVVSHIYTLGHRNPQGLAFT
15 PNGKLLQSEQGPNSDDEINLIVKGGNYGWPNAVAGYKDDSGYAYANYSAANKTIKDLA
QNGVKVAAGVPVTKESEWTGKNFVPLKTLTYTVQDTYNYNDPTCGEMTYICWPTVAPS
SAYVYKGGKKAITGWENTLLVPSLKRGVIFRIKLDPTYSTTYDDAVPMFKSGSGSGTTEEQI
AEFKEAFSLFDKDGDTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDF
PEFLTMMARKMKDSTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEE
20 VDEMIREADIDGDGQVNYEEFVQMMTAGGSGSGNRYRDVIASPDGNVLYVLTDTAGN
VQKDDGSVTNTLENPGSLIKFTYKAKGGSGSGSGSGSGSGSGGDVPLIPSQFAKAKS
ENFDKKVILSNLNKPHALLWGPDNQIWLTERATGKILRVNPESGSVKTVFQVPEIVNDA
DGQNGLLGFAPDFKNNPYIISGTFKNPKSTDKELPNQTIIRRYTYNKSTDITLEKPVD
LLAGLPSSKDHQSGRLVIGPGSGSGSGSGSGSGSGSGGMVNPTVFFDIAVDGEPLGR
25 VSFELFADKVPKTAENFRALSTGEKGFGYKGSCHFRIIPGFMCQGGDFTRHNGTGG
KSIYGEKFEDENFILKHTGPGILSMANAGPNTNGSQFFICTAKTEWLDGKHVVFGK
VKEGMNIVEAMERFGSRNGKTSKKITIADCGQLE

SEQ ID NO: 85 – Single component system - Amylase sensor - VHH1-GDH-CaM-VHH2
30 DTTVSEPAPSCVTLYQSWRYSQADNGCAETVTVKVYVEDDTEGLCYAVAPGQITT
VGDGYIGSHGHARYLARCLGGSGSGSGGQKIYYTIGDQGRNQLAYLFLPNQAQHTPT
QQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNGVVSHIYTLGHRNPQGLAFTPNGKL

LQSEQGPNSSDDEINLIVKGGNYGWPNAVAGYKDDSGYAYANYSAANKTIKDLAQNGVK
 VAAGVPVTKESSEWTGKNFVPLKTLTYTVQDTYNYNDPTCGEMTYICWPTVAPSSAYVYK
 GGKKAITGWENTLLVPSLKRGVIFRIKLDPTYSTTYDDAVPMFKSGSGGTEEQIAEFKE
 AFSLFDKDGDTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLT
 5 MMARKMKDSTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEM
IREADIDGDGQVNYEEFVQMMTAGGSGGNRYRDVIASPDGNVLYVLDTAGNVQKD
 DGSVTNTLENPGSLIKFTYKAKGGSGSGSGSGSGSGSGGSDVPLIPSQFAKAKSENF
 KKVILSNLNKPHALLWGPNDQIWLTERATGKILRVNPESGSVKTVFQVPEIVNDADGQN
 GLLGFAFHPDFKNNPYIYISGTFKNPKSTDKELPNQTHIRRYTYNKSTDITLEKPVDLLAG
 10 LPSSKDHQSGRLVIGPGSGSGSGGQVQVLESGGGTVPAGGSLRLSCAASGNTLCTY
DMSWYRRAPGKGRDFVSGIDNDGTTTYVDSVAGRFTISQGNKNTAYLQMDSLK
PDDTAMYYCKPSRLRYGLPGCPIIPWGOGTQVTVSSKLAAALEHHHHHH

SEQ ID NO: 86 – Single component system - *cpGDH-CaM*

15 QKIYYTIGDQGRNQLAYLFLPNQAQHTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDN
PSFNGVVSHIYTLGHRNPQGLAFTPNGKLLQSEQGPNSSDDEINLIVKGGNYGWPNAVAG
YKDDSGYAYANYSAANKTIKDLAQNGVKVAAGVPVTKESSEWTGKNFVPLKTLTYTVQ
DTYNYNDPTCGEMTYICWPTVAPSSAYVYKGGKKAITGWENTLLVPSLKRGVIFRIKLD
TYSTTYDDAVPMFKSGSGGTEEQIAEFKEAFSLFDKDGDTITTKELGTVMRSLGQN
 20 PTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDSTDSEEEIREAFRVFDKDG
NYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTAGGSG
GNRYRDVIASPDGNVLYVLDTAGNVQKDDGSVTNTLENPGSLIKFTYKAKGGSGSGS
GSGSGSGSGGSDVPLIPSQFAKAKSENFDDKKVILSNLNKPHALLWGPNDQIWLTERAT
GKILRVNPESGSVKTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKST
 25 DKELPNQTHIRRYTYNKSTDITLEKPVDLLAGLPSSKDHQSGRLVIGP

CLAIMS

1. An oxidoreductase enzyme comprising a calmodulin protein or a functional
fragment thereof, wherein binding of a peptide to the calmodulin protein or functional
5 fragment thereof reversibly regulates the catalytic activity of the enzyme, wherein binding
of the peptide is capable of reversibly regulating the catalytic activity of the enzyme in the
presence of physiological calcium concentrations.
2. The oxidoreductase enzyme of claim 1, wherein binding of the peptide to the
10 calmodulin protein or functional fragment thereof activates the catalytic activity of the
enzyme.
3. The oxidoreductase enzyme of claim 1 or 2, wherein the calmodulin protein or
functional fragment thereof is provided as an insert within the amino acid sequence of the
15 oxidoreductase enzyme, flanked on either side by a linker.
4. The oxidoreductase enzyme of claim 3, wherein binding of the peptide to the
calmodulin protein or functional fragment thereof brings the linkers into proximity with
each other, optionally within 5 Å of each other.
20
5. The oxidoreductase enzyme of claim 3 or 4, wherein interaction between the
linkers enhances activation of the catalytic activity of the enzyme, optionally wherein said
interaction maintains the linkers in proximity with each other.
- 25 6. The oxidoreductase enzyme of claim 5, wherein a covalent bond is capable of being
formed between the linkers.
7. The oxidoreductase enzyme of any one of claims 3-6, wherein each linker
comprises a cysteine and binding of the peptide to the heterologous amino acid sequence
30 brings the linkers into proximity resulting in the formation of a disulphide bond.

8. The oxidoreductase enzyme of any one of claims 3-7, wherein each linker is an amino acid sequence of between 1-10 amino acids in length comprising at least one cysteine residue, optionally wherein the pair of linkers comprise respectively the sequences GCGG and GGSCG.

5

9. The oxidoreductase enzyme of any one of claims 1-8, wherein the peptide is a calmodulin binding peptide (CaM-BP).

10. The oxidoreductase enzyme of any one of claims 1-9, wherein the peptide comprises a sequence having at least 80 % sequence identity to SEQ ID NO: 10, 29 or 37.

11. The oxidoreductase enzyme of any one of claims 1-10, wherein the oxidoreductase enzyme comprises the calmodulin protein or a functional fragment thereof in a location capable of regulating the position of the catalytic residues of the oxidoreductase enzyme corresponding to Arg406 and/or Arg408 of PQQ-GDH.

12. The oxidoreductase enzyme of any one of claims 1-11, wherein the oxidoreductase enzyme comprises the calmodulin protein or functional fragment thereof in a location corresponding to the loop connecting beta-sheets 5 and 6 of a glucose dehydrogenase (pyrroloquinoline quinone) (PQQ-GDH).

13. The oxidoreductase enzyme of any one of claims 1-12, wherein the oxidoreductase enzyme is a GDH enzyme, optionally PQQ-GDH.

14. The oxidoreductase enzyme of any one of claims 1-13, comprising a first binding moiety.

15. The oxidoreductase enzyme of any one of claims 1-14, wherein the peptide comprises a second binding moiety.

30

16. The oxidoreductase enzyme of claim 15, wherein the first binding moiety is capable of interacting with the second binding moiety on the peptide, wherein interaction between the binding moieties regulates catalytic activity of the enzyme.
- 5 17. The oxidoreductase enzyme of claim 15 or 16, wherein the peptide is engineered to bind the calmodulin protein or functional fragment thereof with an affinity insufficient to enhance catalytic activity in the absence of said interaction between binding moieties.
18. The oxidoreductase enzyme of any one of claims 15-17, wherein interaction of the
10 binding moieties is dependent on presence of a target molecule, such that catalytic activity of the enzyme is enhanced in the presence of the target molecule.
19. The oxidoreductase enzyme of any one of claims 15-18, wherein in the absence of an interaction between the first and second binding moieties, the peptide is bound by a
15 second calmodulin protein or functional fragment thereof comprising one or more modifications resulting in a reduced binding affinity for the peptide as compared to the calmodulin protein or functional fragment thereof comprised within the oxidoreductase enzyme.
- 20 20. The oxidoreductase enzyme of any one of claims 1-19, wherein the peptide further comprises a linker and a second calmodulin protein or functional fragment thereof comprising one or more modifications resulting in a reduced binding affinity for the peptide as compared to the calmodulin protein or functional fragment thereof comprised within the oxidoreductase enzyme.
- 25 21. The oxidoreductase enzyme of claim 19 or 20, wherein the second calmodulin protein or functional fragment thereof comprises the sequence of SEQ ID NO: 27.
22. The oxidoreductase enzyme of any one of claims 15-21, wherein interaction
30 between the first and second binding moieties brings the peptide and the calmodulin protein or functional fragment thereof into proximity.

23. The oxidoreductase enzyme of any one of claims 15-22, wherein the peptide binds to the calmodulin protein or functional fragment thereof upon interaction between the first and second binding moieties, wherein interaction between the first and second binding moieties regulates the binding of the peptide to the calmodulin protein or functional
5 fragment thereof and regulates the catalytic activity of the enzyme.

24. An oxidoreductase enzyme comprising a heterologous amino acid sequence which is responsive to a target molecule, wherein binding of the target molecule to the heterologous amino acid sequence reversibly regulates catalytic activity of the enzyme,
10 wherein the oxidoreductase enzyme comprises the heterologous amino acid sequence in a location corresponding to the loop connecting beta-sheets 5 and 6 of a PQQ-GDH.

25. An enzyme comprising a heterologous amino acid sequence which is responsive to a target molecule, wherein binding of the target molecule to the heterologous amino acid
15 sequence reversibly regulates catalytic activity of the enzyme;
wherein the heterologous amino acid sequence is provided as an insert within the amino acid sequence of the enzyme flanked on either side by linkers, wherein binding of the target molecule to the heterologous amino acid sequence brings the linkers into proximity with each other;
20 and wherein interaction between the linkers enhances activation of the catalytic activity of the enzyme, optionally wherein said interaction maintains the linkers in proximity with each other.

26. The enzyme of claim 25, wherein said interaction is reversible or irreversible.
25

27. The enzyme of claim 25 or 26, wherein said interaction comprises the formation of a covalent bond between the linkers.

28. The enzyme of any one of claims 25-27, wherein binding of the target molecule to
30 the heterologous amino acid sequence brings the linkers into within 5 Å of each other.

29. The enzyme of any one of claims 25-28, wherein each linker comprises a cysteine and binding of the target molecule to the heterologous amino acid sequence brings the linkers into proximity resulting in the formation of a disulphide bond.
- 5 30. The enzyme of any one of claims 25-29, wherein each linker is an amino acid sequence between 1-10 amino acids in length comprising at least one cysteine, optionally wherein the linkers comprise respectively the sequences GCGG and GGSCG.
- 10 31. A polypeptide comprising a calmodulin protein or functional fragment thereof, wherein the calmodulin protein or functional fragment thereof comprises one or more modifications resulting in a reduced binding affinity for a calmodulin binding peptide as compared to a corresponding wild type calmodulin protein or functional fragment thereof.
- 15 32. A polypeptide comprising a calmodulin binding peptide and a calmodulin protein or functional fragment thereof, wherein the calmodulin protein or functional fragment thereof comprises one or more modifications resulting in a reduced binding affinity for the calmodulin binding peptide as compared to a corresponding wild type calmodulin protein or functional fragment thereof.
- 20 33. The polypeptide of claim 31 or 32, wherein the calmodulin protein or functional fragment thereof comprises mutations at any or all of the amino acid positions corresponding to positions 79, 88, 101 and/or 137 of the sequence of SEQ ID NO: 2.
- 25 34. The polypeptide of any one of claims 31-33, wherein the calmodulin protein or functional fragment thereof comprises the sequence of SEQ ID NO: 27 or 62.
35. The polypeptide of any one of claims 32-34, comprising a linker between the calmodulin binding peptide and the calmodulin protein or functional fragment thereof.
- 30 36. The polypeptide of any one of claims 32-35, comprising a binding moiety that is capable of interacting with the binding moiety on the oxidoreductase enzyme according to

claim 14, wherein interaction between the binding moieties regulates the catalytic activity of the enzyme.

37. The polypeptide of any one of claims 32-35, comprising a binding moiety that is
5 capable of interacting with a second binding moiety on a protease.

38. The polypeptide of claim 37, wherein the protease further comprises an inhibitor of the protease.

10 39. The polypeptide of claim 37 or 38, wherein the linker between the calmodulin binding peptide and the calmodulin protein or functional fragment thereof comprises an amino acid sequence cleavable by the protease.

40. The polypeptide of any one of claims 37-39, wherein the binding moiety is capable
15 of interacting with the second binding moiety on the protease, wherein interaction between the binding moieties results in cleavage of the linker between the calmodulin binding peptide and the calmodulin protein or functional fragment thereof.

41. The polypeptide of any one of claims 37-40, wherein interaction of the binding
20 moieties is dependent on presence of a target molecule, such that cleavage of the linker between the calmodulin binding peptide and the calmodulin protein or functional fragment thereof is dependent on the presence of the target molecule.

42. The polypeptide of any one of claims 37-41, wherein following cleavage of the
25 linker between the calmodulin binding peptide and the calmodulin protein or functional fragment thereof the calmodulin binding peptide dissociates from the calmodulin protein or functional fragment thereof, optionally wherein the dissociated calmodulin binding peptide is capable of activating the catalytic activity of the oxidoreductase enzyme according to any of claims 1-14.

30

43. A variant calmodulin binding peptide (CaM-BP) which is capable of binding to a calmodulin protein or a functional fragment thereof provided as an insert within the amino

acid sequence of an enzyme, wherein the calmodulin protein or functional fragment thereof activates the catalytic activity of the enzyme on binding of a corresponding activating CaM-BP, and wherein binding of the variant CaM-BP to said calmodulin protein or functional fragment thereof inhibits binding of the corresponding activating CaM-BP,
5 thereby preventing activation of said enzyme.

44. The variant CaM-BP of claim 43, wherein the variant CaM-BP comprises a sequence having at least 80% sequence identity to any one of SEQ ID NOs: 45-60, preferably at least 80% sequence identity to any one of SEQ ID NOs: 48, 50, 51, 52, 54,
10 55, 57, 58, 60, most preferably to SEQ ID NO: 57.

45. The variant CaM-BP of claim 43 or 44, wherein the binding affinity of the variant CaM-BP for the calmodulin protein or functional fragment thereof is less than the binding affinity of the corresponding activating CaM-BP therefor.

15

46. An enzyme comprising (i) a calmodulin protein or functional fragment thereof provided as an insert within the amino acid sequence of the enzyme, and (ii) a variant CaM-BP, wherein the calmodulin protein or functional fragment thereof activates the catalytic activity of the enzyme on binding of a corresponding activating CaM-BP, wherein
20 binding of the variant CaM-BP to the calmodulin protein or functional fragment thereof inhibits binding of said corresponding activating CaM-BP, and wherein binding of the variant CaM-BP to the calmodulin protein or functional fragment thereof prevents activation of the catalytic activity of the enzyme.

25 47. The enzyme of claim 46, wherein the variant CaM-BP has a sequence having at least 80% sequence identity to one of SEQ ID NOs: 45-60, preferably to one of SEQ ID NOs: 48, 50, 51, 52, 54, 55, 57, 58, 60, most preferably to SEQ ID NO: 57.

48. The enzyme of claim 46 or 47, wherein the enzyme further comprises a first
30 binding moiety.

49. The enzyme of any one of claims 46-48, comprising a linker between the enzyme and the variant CaM-BP.
50. The enzyme of claim 49, wherein the variant CaM-BP is engineered to bind the calmodulin protein or functional fragment thereof with an affinity such that in the absence of the linker the variant CaM-BP is displaced from the calmodulin protein or functional fragment thereof by the binding of an activating CaM-BP, optionally a wild type CaM-BP.
51. The enzyme of claim 49 or 50, wherein the linker comprises 1-10 amino acids.
52. The enzyme of any one of claims 49-51, wherein the linker comprises a protease cleavage site.
53. The enzyme of any one of claims 49-52, wherein the linker comprises a protease cleavage site that is cleavable by a protease.
54. The enzyme of claim 53, wherein the protease comprises a second binding moiety.
55. The enzyme of claim 54, wherein the first binding moiety on the enzyme is capable of interacting with the second binding moiety on the protease, wherein interaction between the binding moieties results in cleavage of the linker between the enzyme and the variant CaM-BP by the protease.
56. The enzyme of claim 55, wherein interaction of the binding moieties is dependent on presence of a target molecule, such that cleavage of the linker between the enzyme and the variant CaM-BP is dependent on the presence of the target molecule.
57. The enzyme of any one of claims 52-56, wherein following cleavage of the linker between the enzyme and the variant CaM-BP the variant CaM-BP dissociates from the calmodulin protein or functional fragment thereof.

58. The enzyme of any one of claims 52-57, wherein following cleavage of the linker between the enzyme and the variant CaM-BP the variant CaM-BP is displaced from the calmodulin protein or functional fragment thereof by the binding of an activating CaM-BP, optionally a wild type CaM-BP.
59. The enzyme of any one of claims 53-58, wherein the protease comprises an inhibitor of the activity of the protease.
60. An oxidoreductase enzyme comprising a heterologous amino acid sequence that releasably maintains the enzyme in a catalytically inactive state by allosteric regulation and which is responsive to a molecule to release said regulation, a first binding moiety and a second binding moiety, wherein the amino acid sequence of the enzyme is circularly permuted, and wherein (i) release of the allosteric regulation by the heterologous amino acid sequence and (ii) interaction between the binding moieties, reversibly regulates the catalytic activity of the enzyme.
61. The oxidoreductase enzyme of claim 60, wherein said heterologous amino acid sequence is a calmodulin protein or functional fragment thereof.
62. The oxidoreductase enzyme of claim 61, wherein said molecule is a peptide, optionally a calmodulin binding peptide (CaM-BP), and (i) binding of said peptide to the calmodulin protein or functional fragment thereof and (ii) interaction between the binding moieties, activates the catalytic activity of the enzyme.
63. The oxidoreductase enzyme of claim 62, wherein interaction of the binding moieties is dependent on presence of a target molecule, such that the presence of the target molecule activates the catalytic activity of the enzyme.
64. The oxidoreductase enzyme of any one of claims 60-63, wherein the N-terminus of the circularly permuted oxidoreductase enzyme is any amino acid located between positions corresponding to S140 to A170 of PQQ-GDH.

65. The oxidoreductase enzyme of any one of claims 60-63, wherein the C-terminus of the circularly permuted oxidoreductase enzyme is any amino acid located between positions corresponding to L138 to Q168 of PQQ-GDH.
- 5 66. The oxidoreductase enzyme of any one of claims 60-65, wherein the first binding moiety is linked to the N-terminus of the oxidoreductase enzyme and the second binding moiety is linked to the C-terminus of the oxidoreductase enzyme, each optionally linked through a linker, or the second binding moiety is linked to the N-terminus of the oxidoreductase enzyme and the first binding moiety is linked to the C-terminus of the oxidoreductase enzyme, each optionally linked through a linker.
- 10 67. The oxidoreductase enzyme of any one of claims 60-66 which is a GDH enzyme, optionally PQQ-GDH.
- 15 68. The oxidoreductase enzyme of claim 67, wherein the circularly permuted amino acid sequence of the enzyme has at least 80 % sequence identity to the sequence of SEQ ID NO: 79.
- 20 69. The oxidoreductase enzyme of any one of claims 60-68, wherein the peptide, CaM-BP, and/or calmodulin protein or functional fragment thereof are as defined in any one of claims 1-12, 19 or 20.
- 25 70. The oxidoreductase enzyme of any one of claims 60-69, which comprises a sequence having at least 80 % sequence identity to the sequence of any one of SEQ ID NOs: 78, 81, 84, 85 or 86.
- 30 71. An oxidoreductase enzyme comprising a heterologous amino acid sequence which is responsive to a peptide comprising a first binding moiety, and a first interaction domain capable of interacting with a second interaction domain of a polypeptide, wherein the polypeptide further comprises a second binding moiety capable of interacting with said first binding moiety, and wherein binding between the first and second binding moieties and interaction of the first interaction domain of the enzyme with the second interaction domain of the polypeptide act to colocalise the enzyme and the peptide,

wherein binding of the peptide to the heterologous amino acid sequence reversibly regulates catalytic activity of the enzyme.

72. The oxidoreductase enzyme of claim 71, wherein the first and second interaction
5 domains directly interact, bind or form a complex.

73. The oxidoreductase enzyme of claim 71, wherein the first and second interaction domains interact, bind or form a complex through an interaction ligand.

10 74. The oxidoreductase enzyme of any one of claims 71-73, wherein interaction of the binding moieties is dependent on presence of a target molecule, such that the presence of the target molecule activates the catalytic activity of the enzyme.

75. The oxidoreductase enzyme, of any one of claims 71-73, wherein said heterologous
15 amino acid sequence is a calmodulin protein or functional fragment thereof.

76. The oxidoreductase enzyme of claim 74, wherein said peptide is a calmodulin binding peptide (CaM-BP).

20 77. The oxidoreductase enzyme of any one of claims 70-75 which is a GDH enzyme, optionally PQQ-GDH.

78. The oxidoreductase enzyme of any one of claims 70-76, wherein the peptide, CaM-BP, and/or calmodulin protein or functional fragment thereof are as defined in any one of
25 claims 1-12, 19 or 20.

79. The oxidoreductase enzyme of any one of claims 70-78, which comprises a sequence having at least 80 % sequence identity to the sequence of SEQ ID No 72.

30 80. A biosensor comprising:

- (i) the oxidoreductase enzyme of any one of claims 1-23;
- (ii) the oxidoreductase enzyme of claim 24;
- (iii) the enzyme of any one of claims 25-30;

- (iv) the oxidoreductase enzyme of any one of claims 1-23 and a polypeptide of any one of claims 31-42;
- (v) the oxidoreductase enzyme, enzyme, or oxidoreductase enzyme and polypeptide of any of (i) to (iv), or the enzyme of any one of claims 46-59, and further comprising a peptide, wherein binding of the peptide to the calmodulin protein or functional fragment thereof reversibly regulates the catalytic activity of the enzyme of the biosensor, optionally wherein the peptide is as defined in any one of claims 9, 10 or 15-23;
- (vi) the oxidoreductase enzyme of any one of claims 60-70;
- (vii) the oxidoreductase enzyme of any one of claims 71-79;
- (viii) the oxidoreductase enzyme of any one of claims 62-70 and further comprising a CaM-BP, wherein said CaM-BP binds to the calmodulin protein or functional fragment thereof of the enzyme, optionally wherein the peptide is as defined in any one of claims 9, 10 or 19-23; or
- (ix) the oxidoreductase enzyme of any one of claims 71-79 and further comprising (i) a polypeptide comprising a first interaction domain and a second binding moiety and (ii) a peptide comprising a first binding moiety and which binds to the heterologous amino acid sequence of the enzyme to reversibly regulate catalytic activity of the enzyme, optionally as defined in any one of claims 70-74 and 78.

- 81. The biosensor of claim 80, further comprising a variant calmodulin binding peptide according to any one of claims 43-45.
- 82. A composition or kit comprising:
 - (i) the oxidoreductase enzyme of any one of claims 1-23;
 - (ii) the oxidoreductase enzyme of claim 24;
 - (iii) the enzyme of any one of claims 25-30;
 - (iv) the oxidoreductase enzyme of any one of claims 1-23 and a polypeptide of any one of claims 31-42;
 - (v) the enzyme of any one of claims 46-59;
 - (vi) the oxidoreductase enzyme of any one of claims 60-70;

- (vii) the oxidoreductase enzyme of any one of claims 71-79;
- or
- (viii) the biosensor of claim 80 or 81.

5 83. The composition or kit of claim 82, further comprising a said peptide acting to regulate catalytic activity of said enzyme.

84. The composition or kit of claim 83, wherein said oxidoreductase enzyme, said enzyme or said biosensor comprises a binding moiety and said peptide comprises a
10 respective binding moiety, wherein interaction between the binding moieties regulates catalytic activity of the enzyme.

85. The composition or kit of any one of claims 82-84, further comprising a substrate molecule for said enzyme.

15

86. A method of detecting a target molecule, comprising contacting the oxidoreductase enzyme of any one of claims 1-23; the oxidoreductase enzyme of claim 24; the enzyme of any one of claims 25-30; the oxidoreductase enzyme of any one of claims 1-23 and a polypeptide of any one of claims 31-42; the enzyme of any one of claims 46-59; the
20 oxidoreductase enzyme of any one of claims 60-79 or the biosensor of claim 80 or 81; with a sample under conditions suitable for detection of the presence or absence of the target molecule in the sample.

87. A method of diagnosis of a disease or condition in an organism, comprising
25 contacting the oxidoreductase enzyme of any one of claims 1-23; the oxidoreductase enzyme of claim 24; the enzyme of any one of claims 25-30; the oxidoreductase enzyme of any one of claims 1-23 and a polypeptide of any one of claims 31-42; the enzyme of any one of claims 46-59; the oxidoreductase enzyme of any one of claims 60-79 or the biosensor of claim 80 or 81; with a sample obtained from the organism under conditions
30 suitable for detection of the presence or absence of the target molecule in the sample, wherein presence or absence of the target molecule in the sample is indicative of whether the organism has, or is at risk of having, said disease or condition.

88. A method of assaying for protein-protein or protein-small molecule interactions comprising contacting the oxidoreductase enzyme of any one of claims 1-23; the oxidoreductase enzyme of claim 24; the enzyme of any one of claims 25-30; the
5 oxidoreductase enzyme of any one of claims 1-23 and a polypeptide of any one of claims 31-42; the enzyme of any one of claims 46-59; the oxidoreductase enzyme of any one of claims 60-79 or the biosensor of claim 80 or 81; with a sample under conditions suitable for detection of the presence or absence of an interaction between the binding moieties, or between the binding moieties and a target molecule, wherein the binding moieties and/or
10 the target molecule is a protein or a small molecule.

89. A detection device that comprises a cell or chamber that comprises the oxidoreductase enzyme of any one of claims 1-23; the oxidoreductase enzyme of claim 24; the enzyme of any one of claims 25-30; the oxidoreductase enzyme of any one of claims 1-
15 23 and a polypeptide of any one of claims 31-42; the enzyme of any one of claims 46-59; the oxidoreductase enzyme of any one of claims 60-79; or the biosensor of claim 80 or 81.

90. A nucleic acid encoding the oxidoreductase enzyme of any one of claims 1-23; the oxidoreductase enzyme of claim 24; the enzyme of any one of claims 25-30; the
20 oxidoreductase enzyme of any one of claims 1-23 and a polypeptide of any one of claims 31-42; a polypeptide of any one of claims 31-42; the enzyme of any one of claims 46-59; the oxidoreductase enzyme of any one of claims 60-79 or the biosensor of claim 80 or 81.

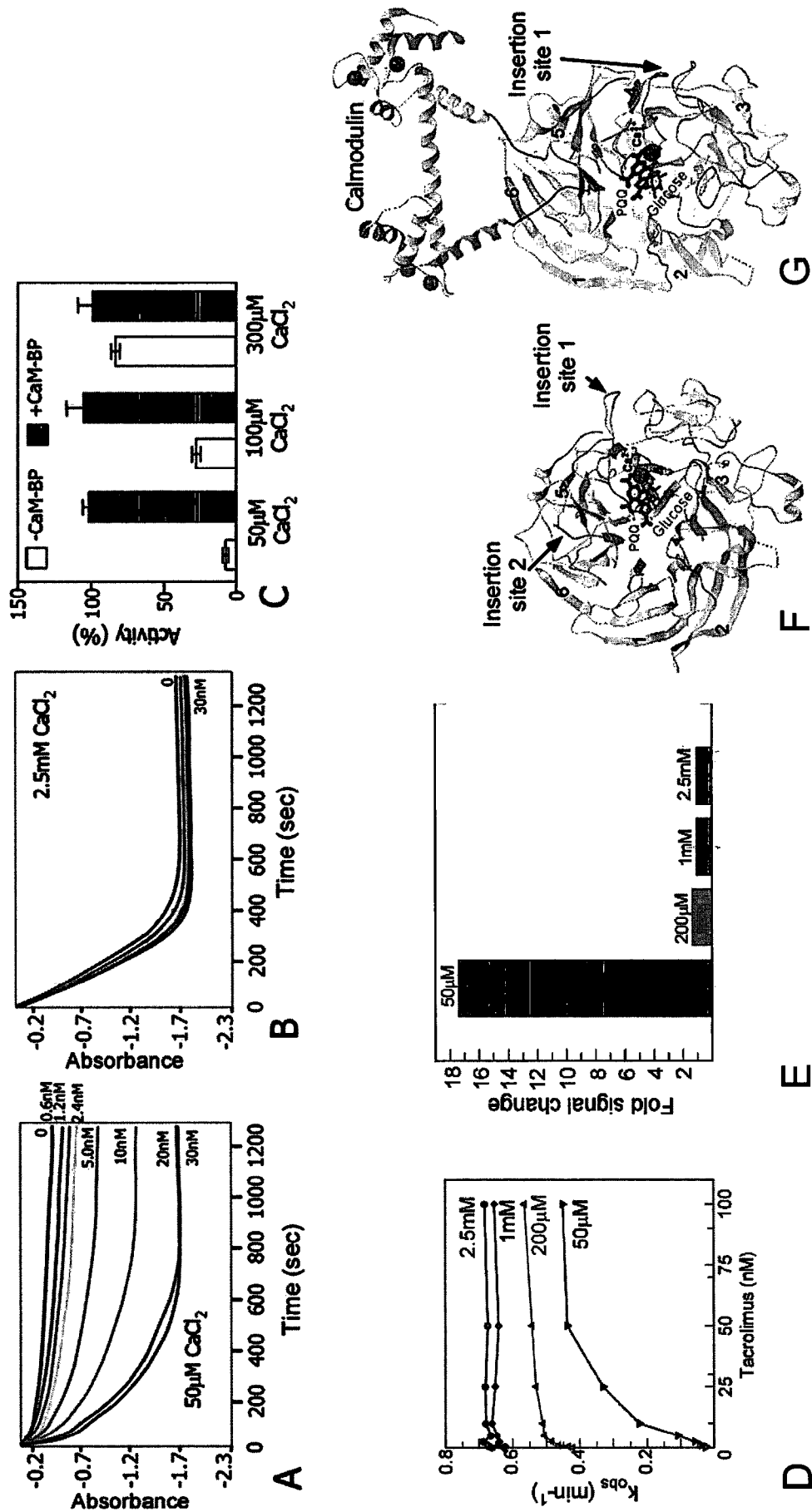


Figure 1

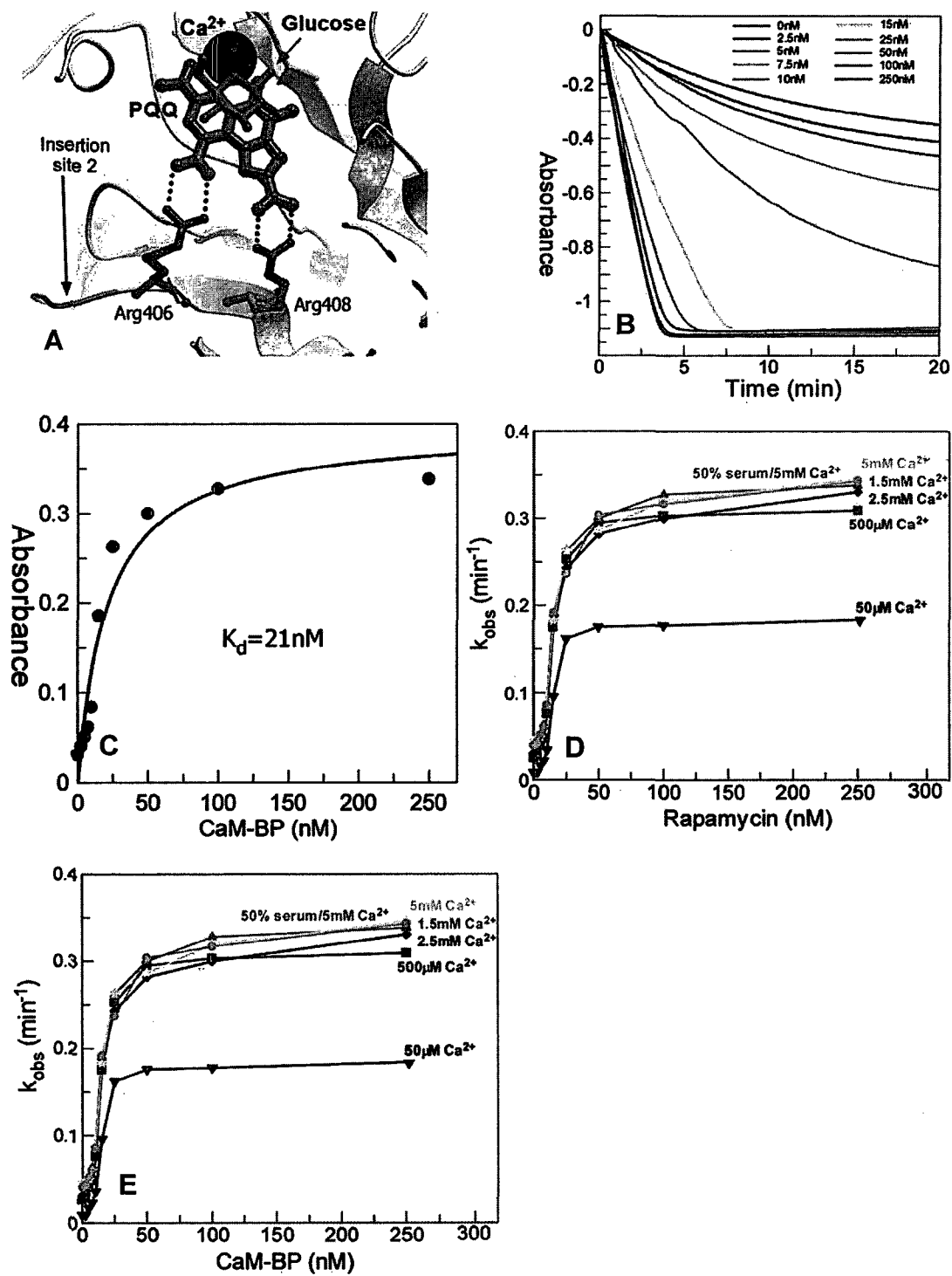


Figure 2

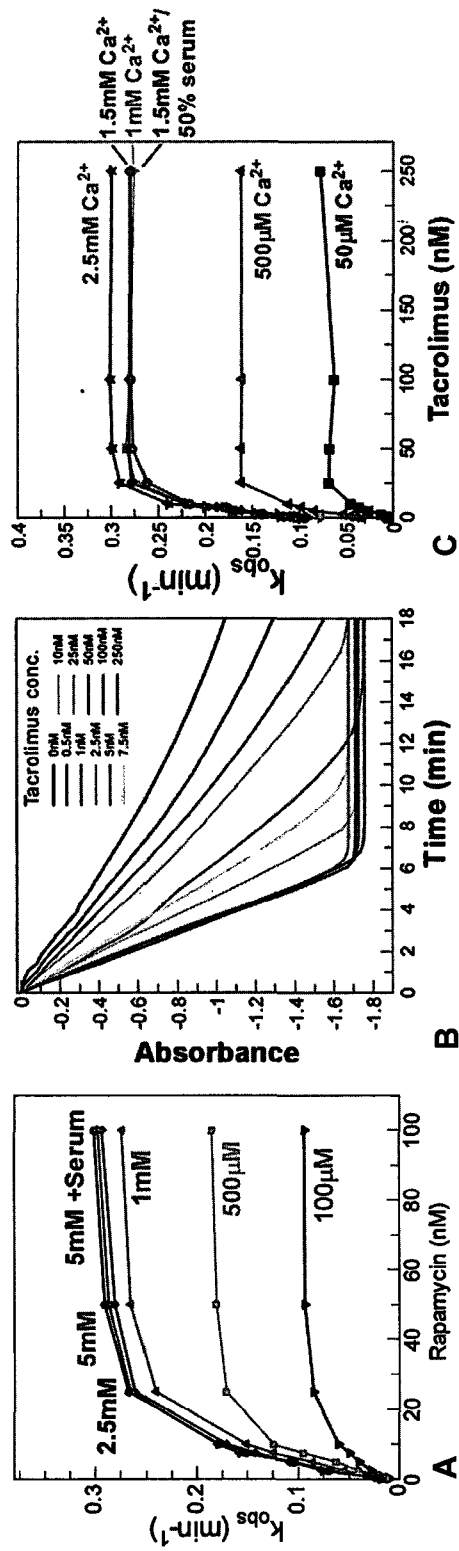


Figure 3

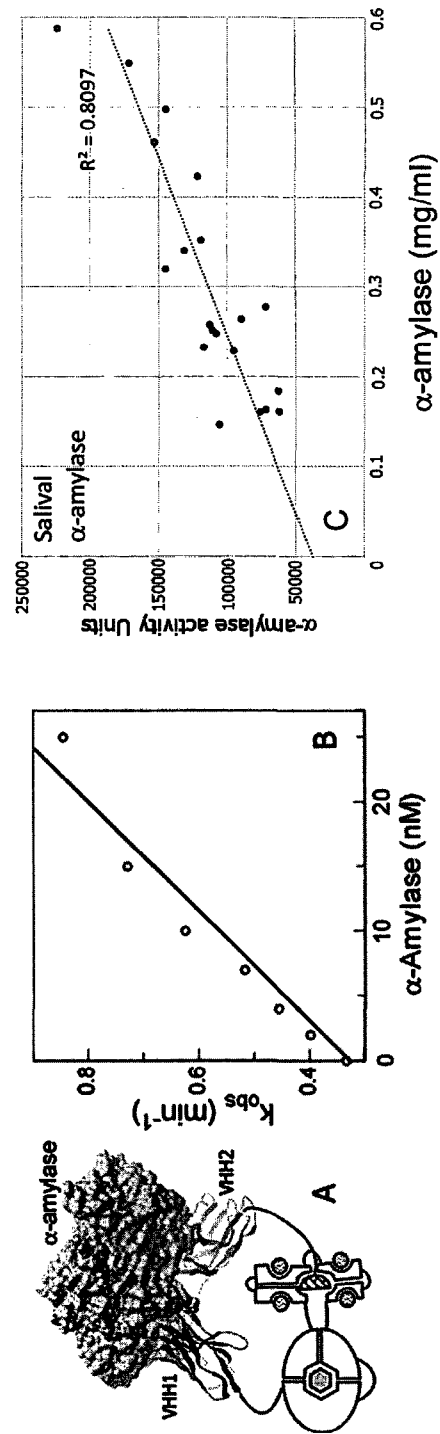


Figure 4

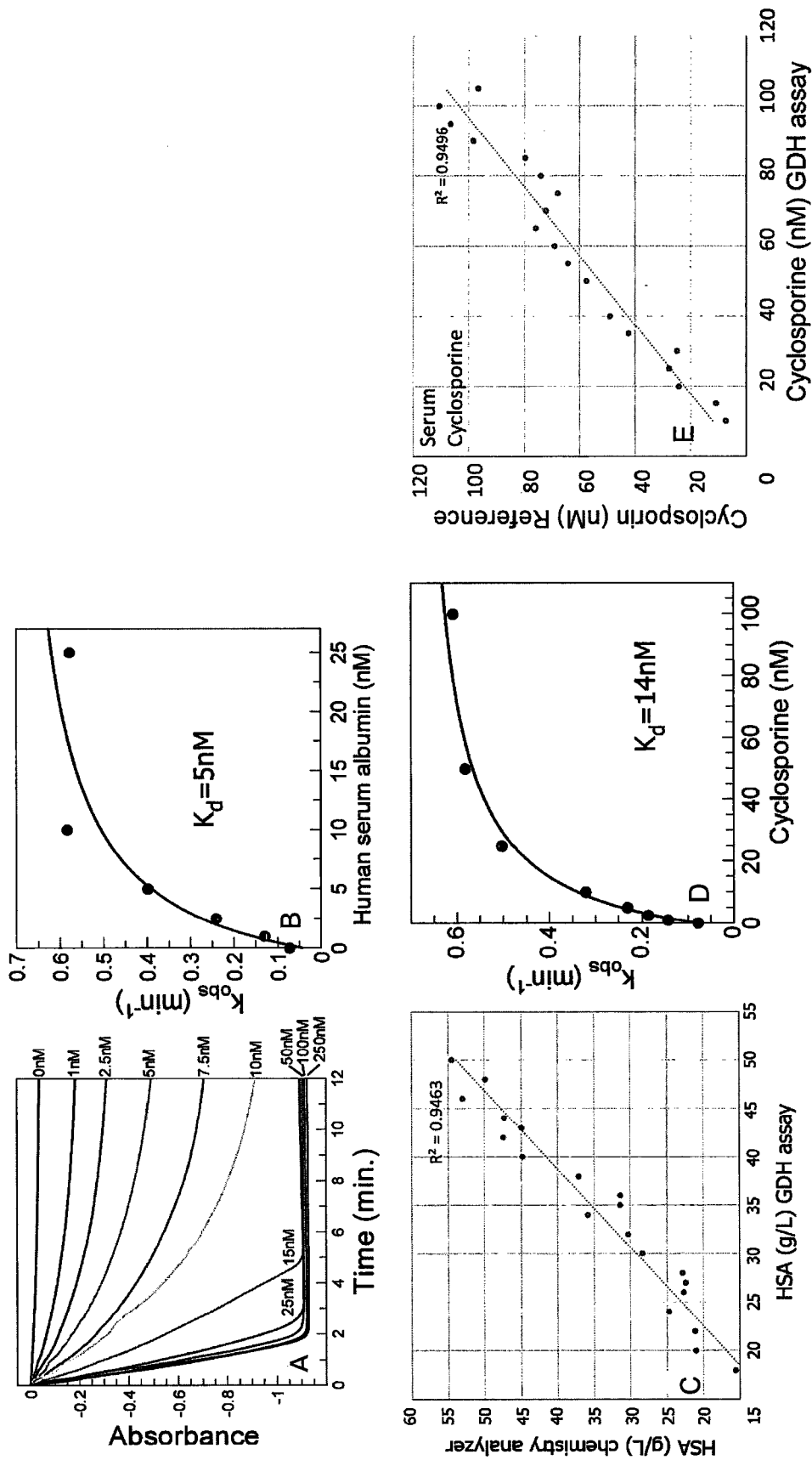


Figure 5

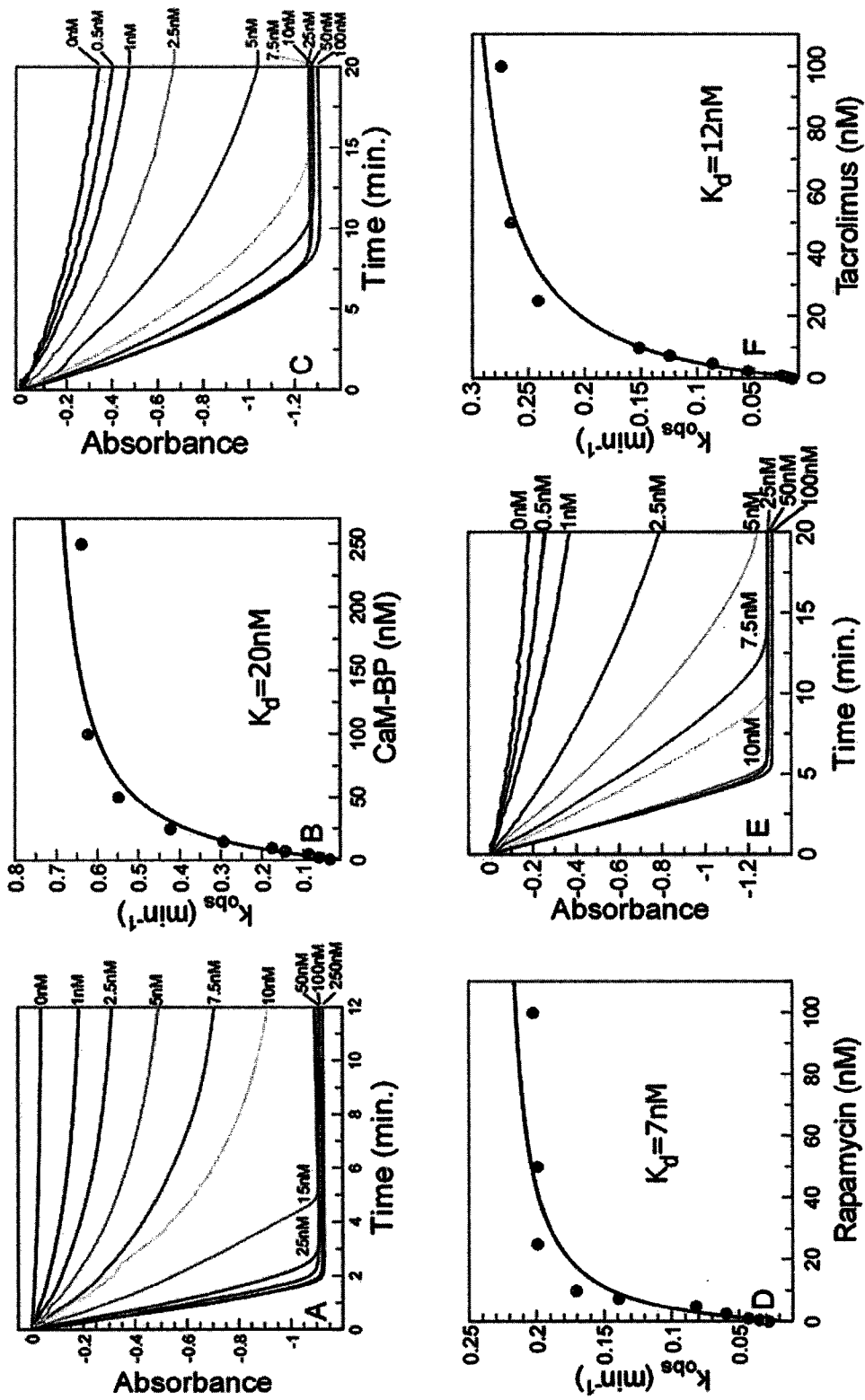


Figure 6

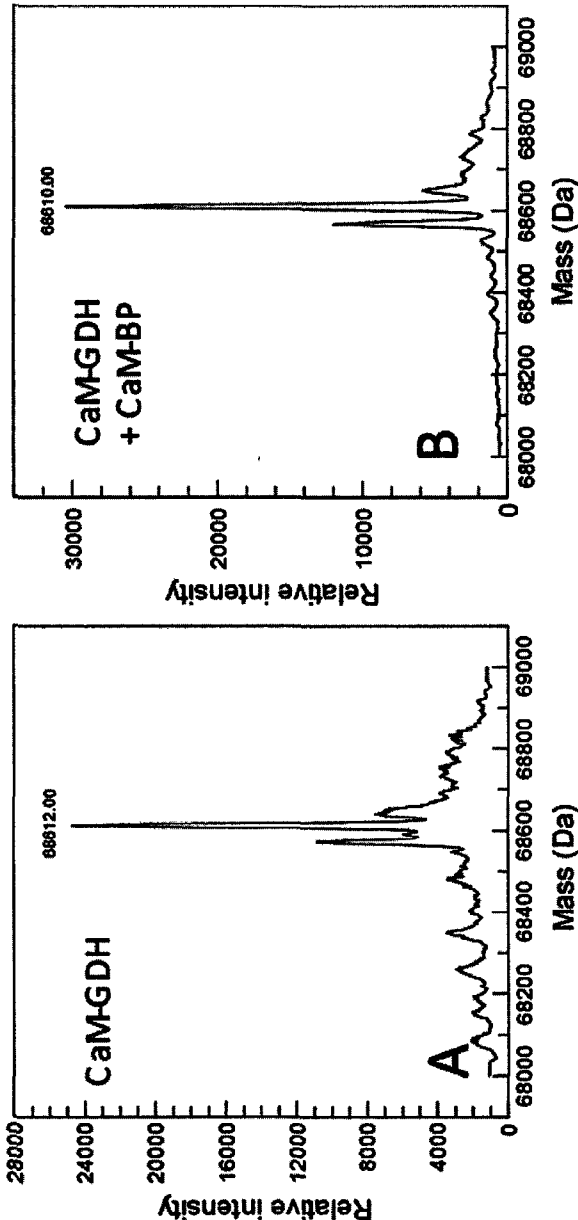


Figure 7

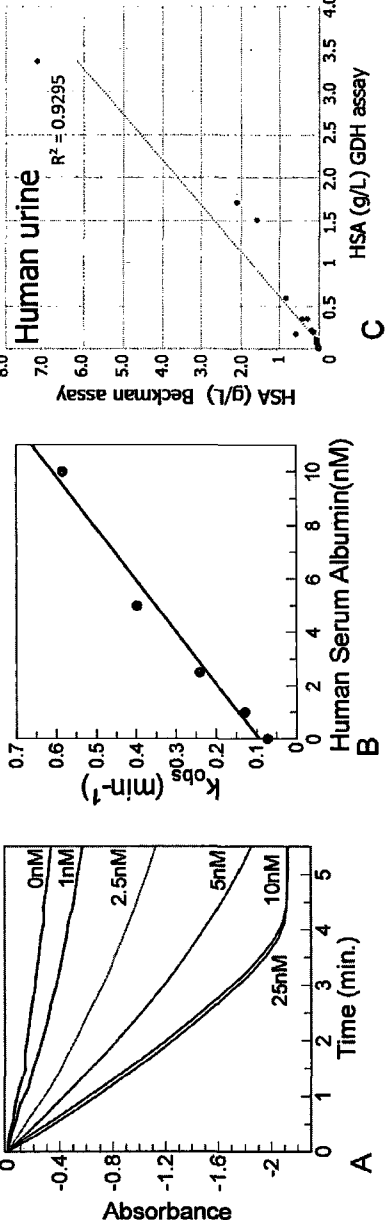


Figure 8

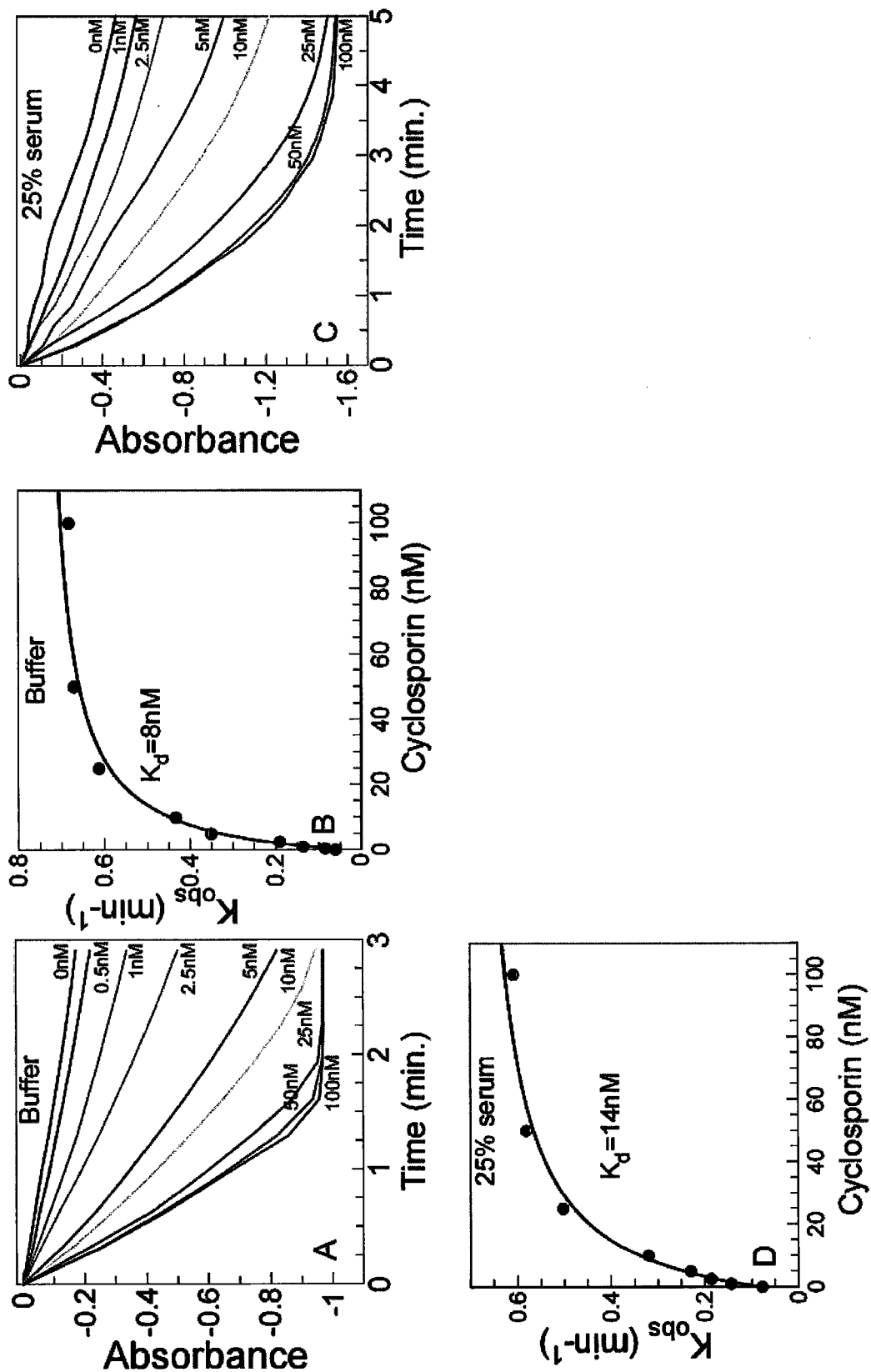


Figure 9

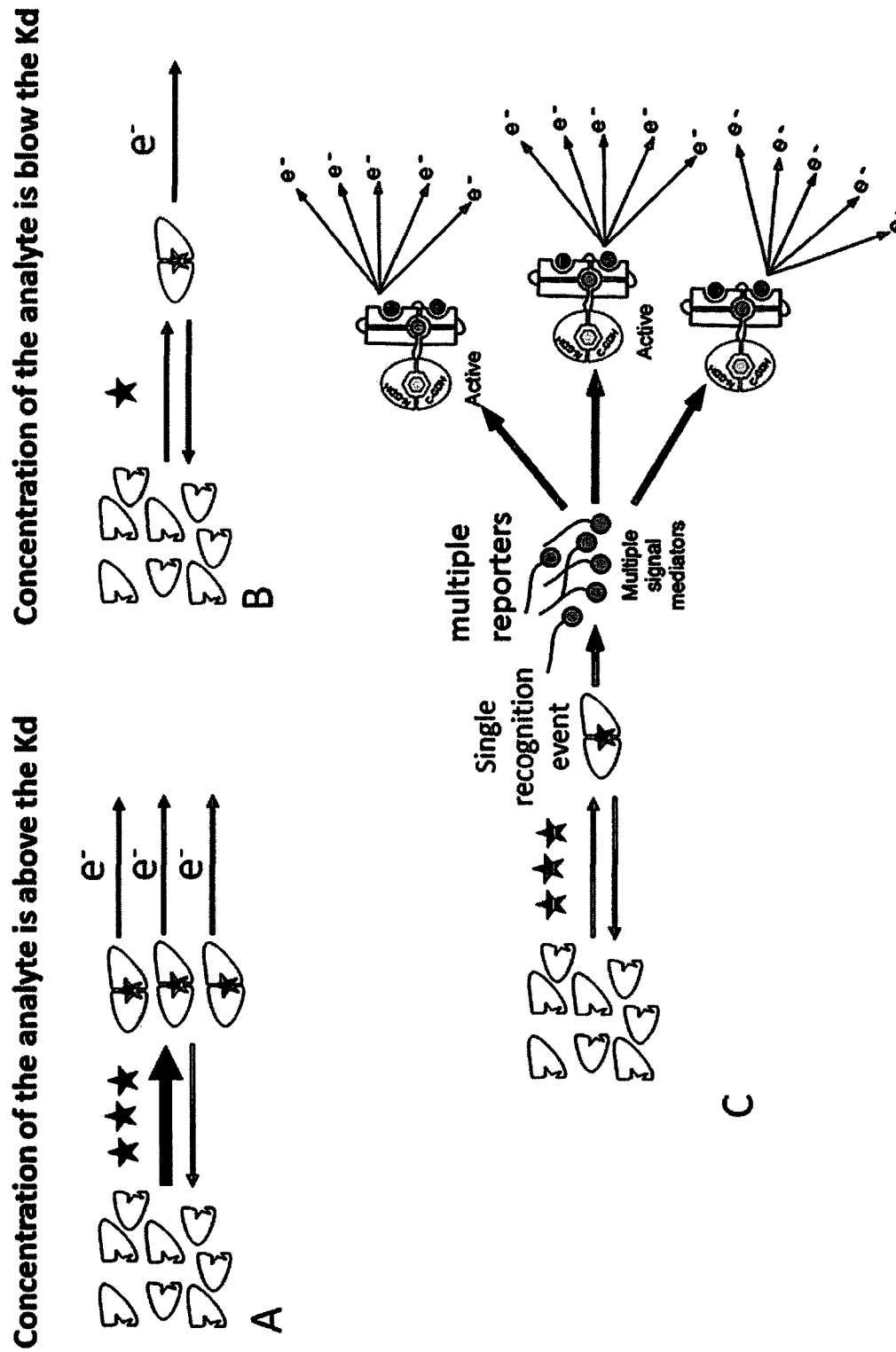


Figure 10

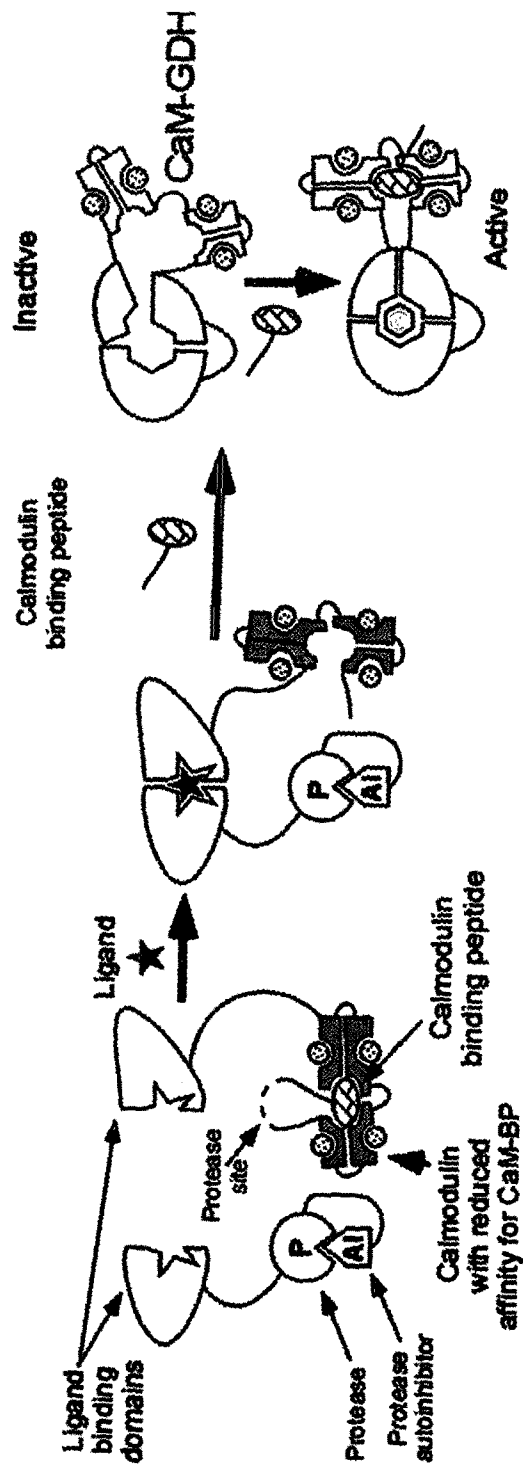


Figure 11

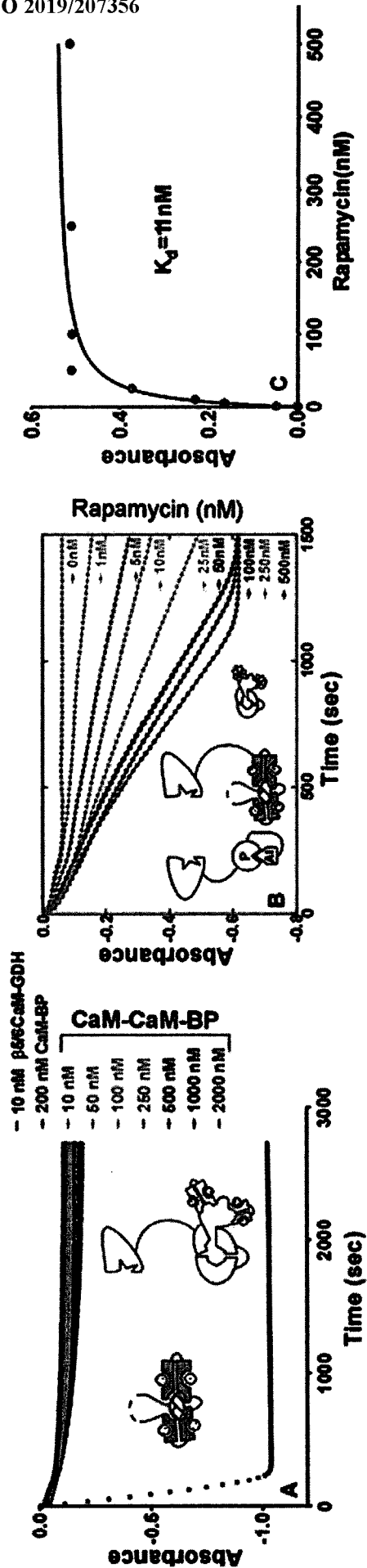


Figure 12

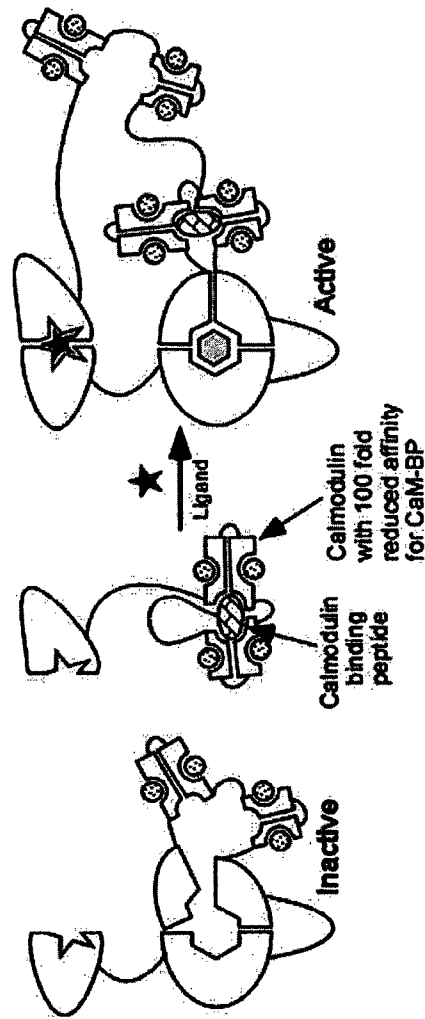


Figure 13

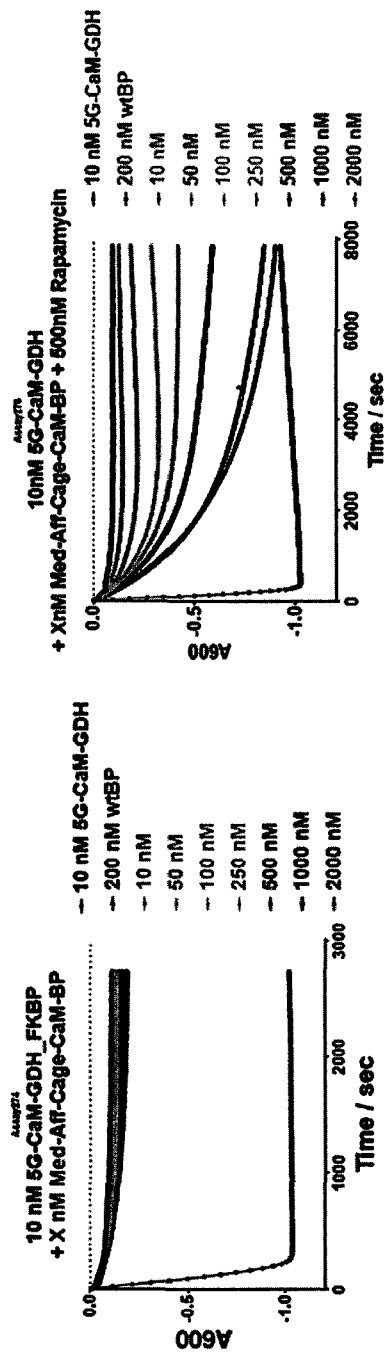


Figure 14

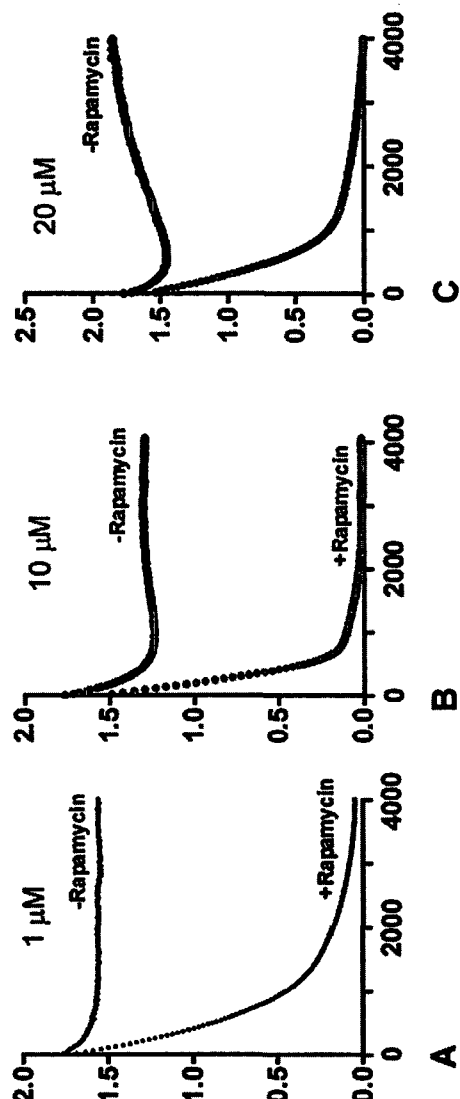
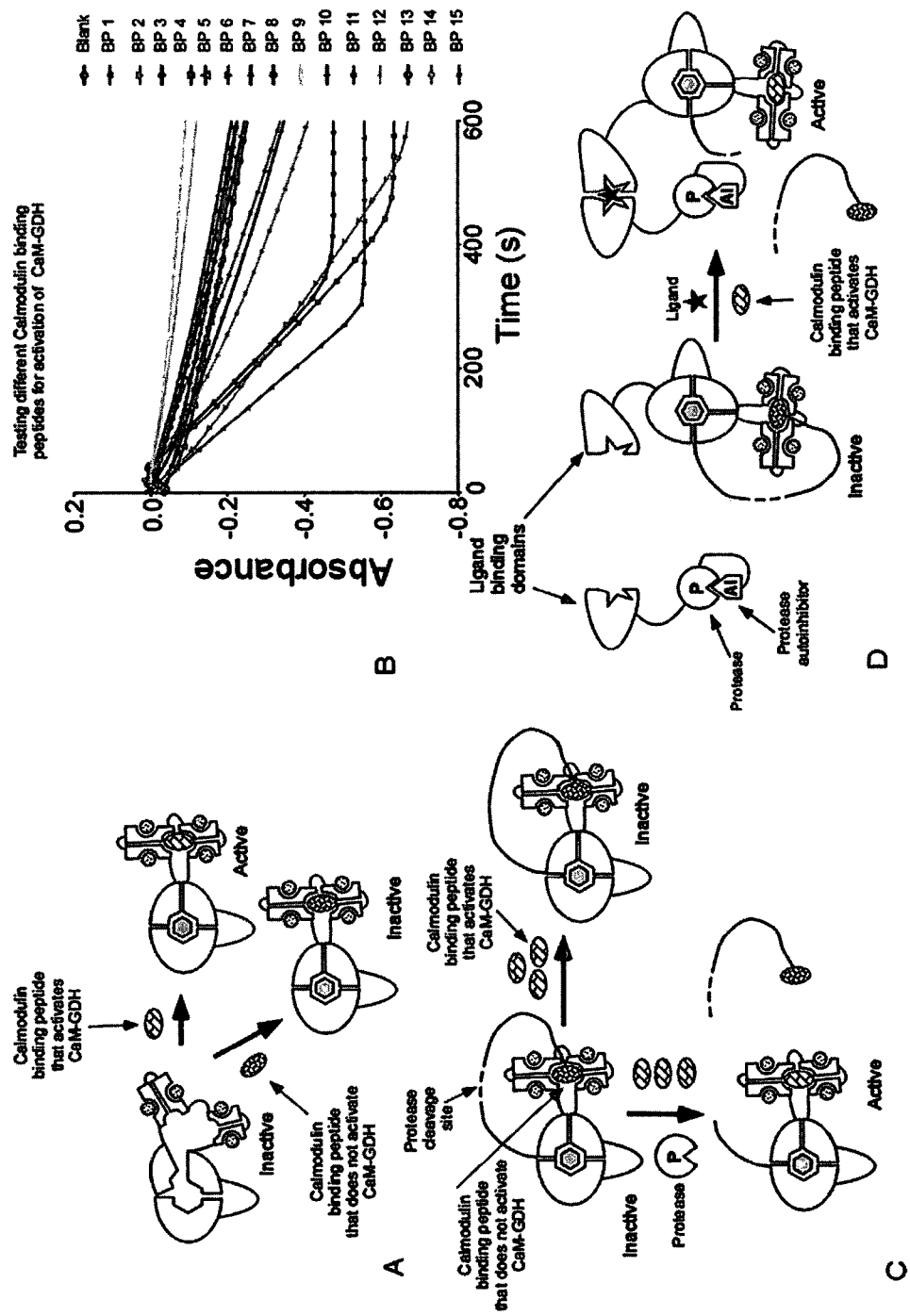


Figure 15



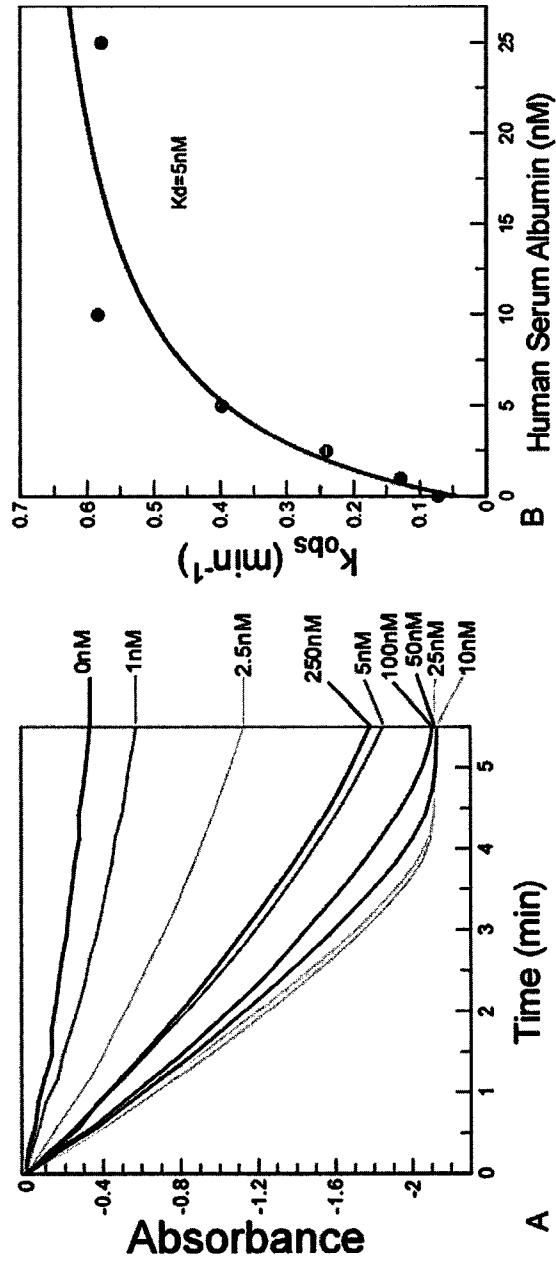


Figure 17

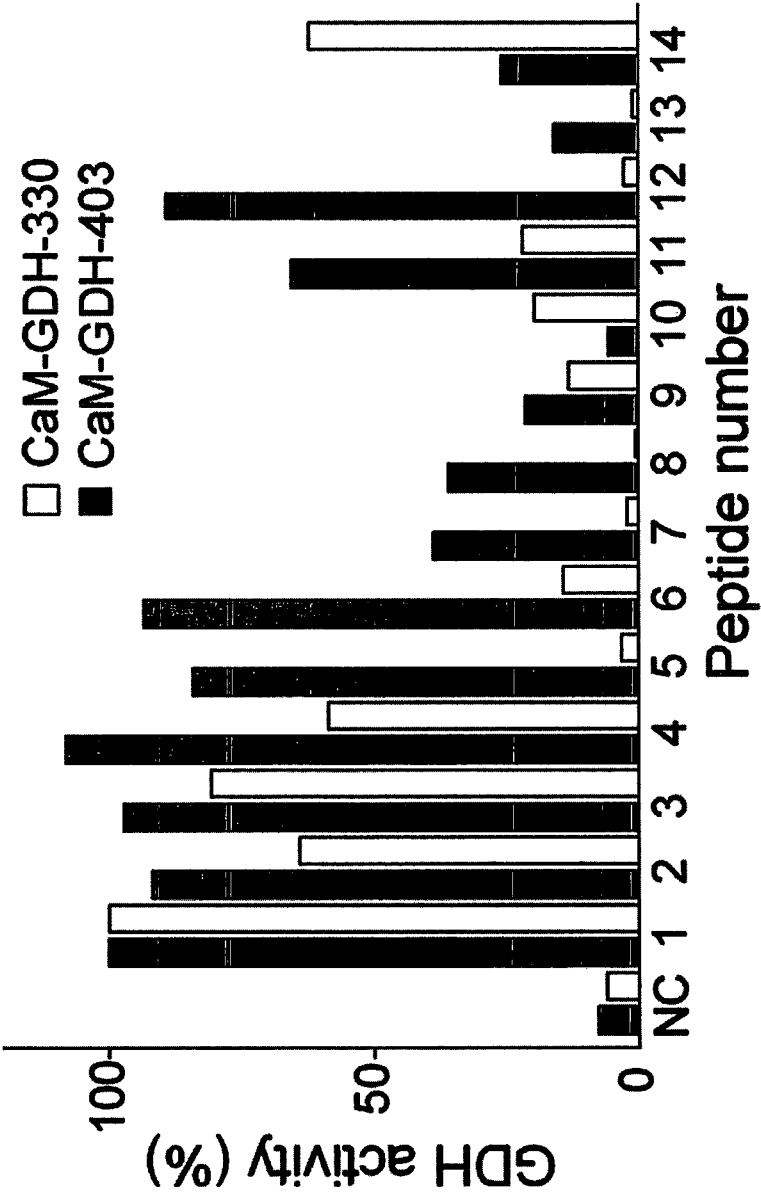


Figure 18

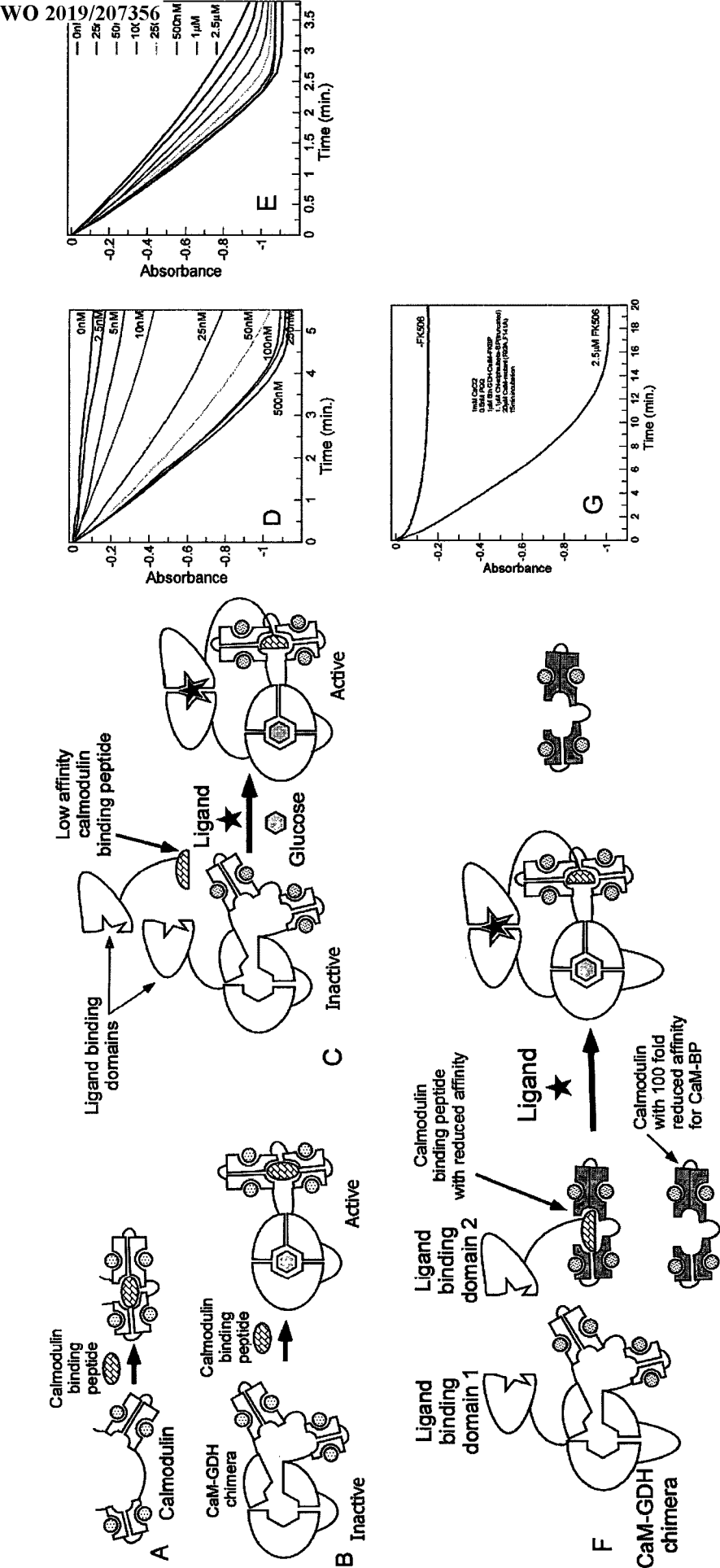


Figure 19

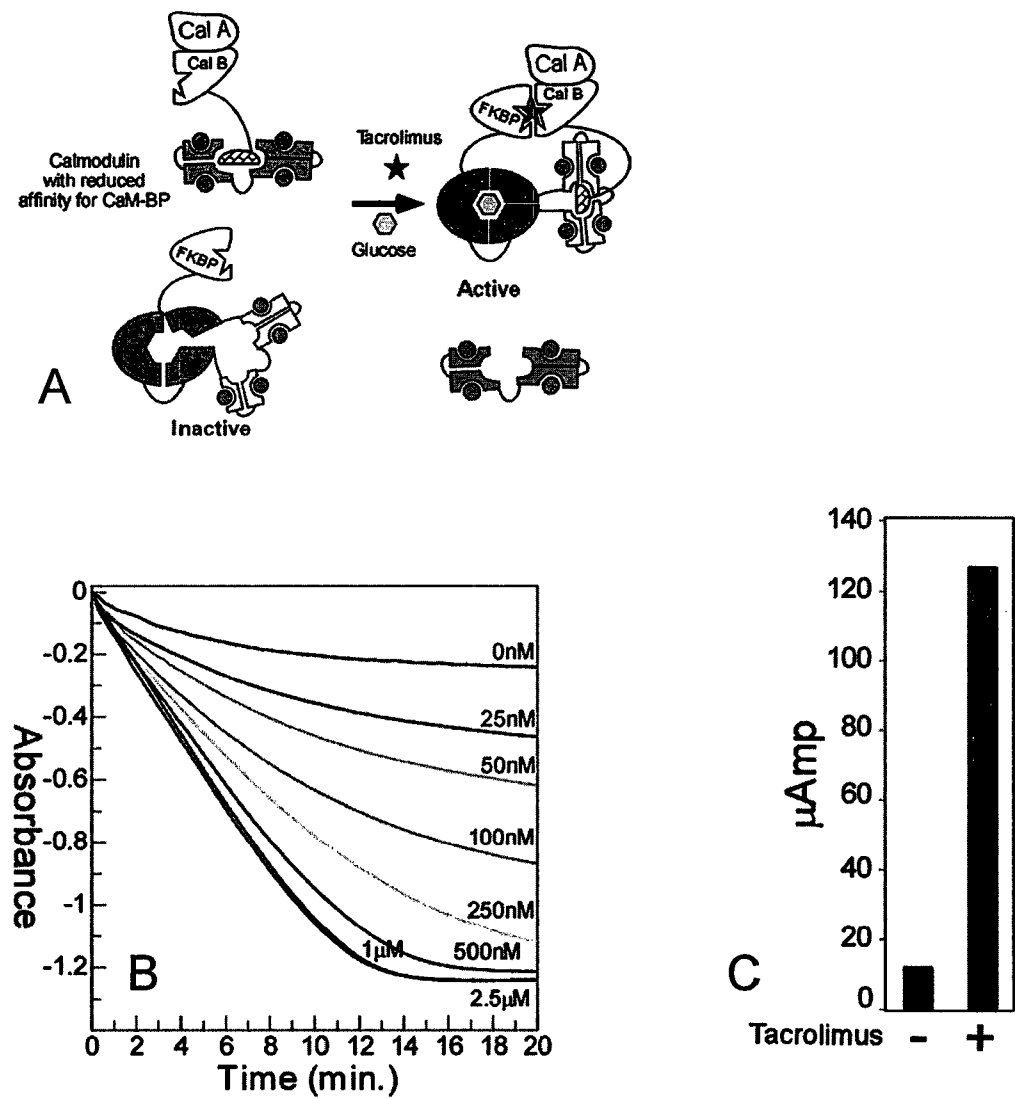


Figure 20

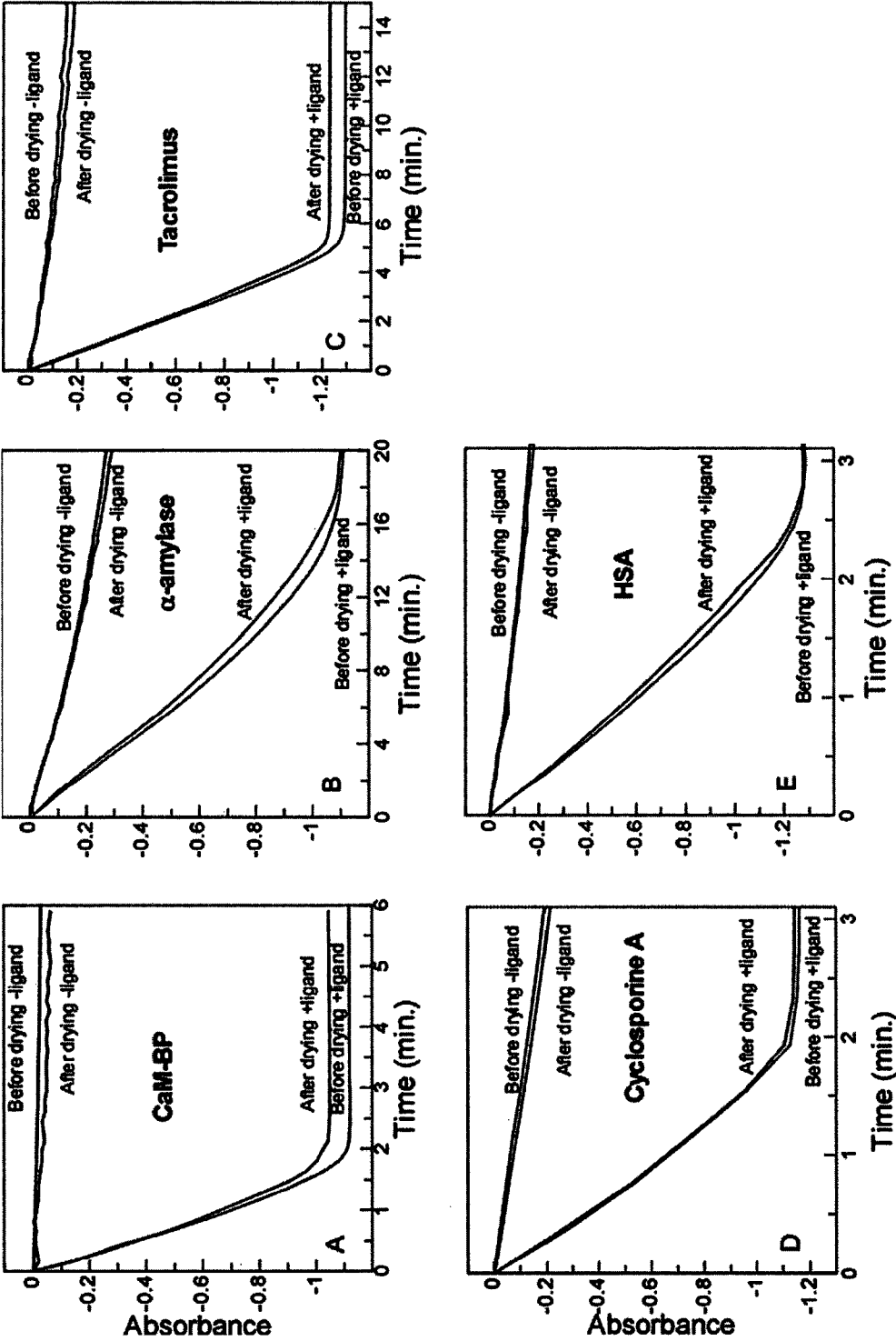


Figure 21

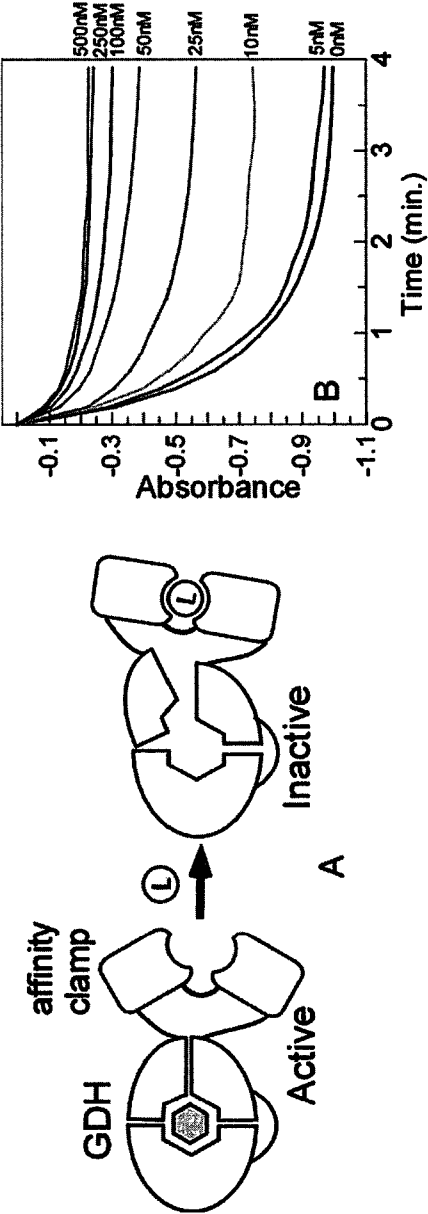


Figure 22

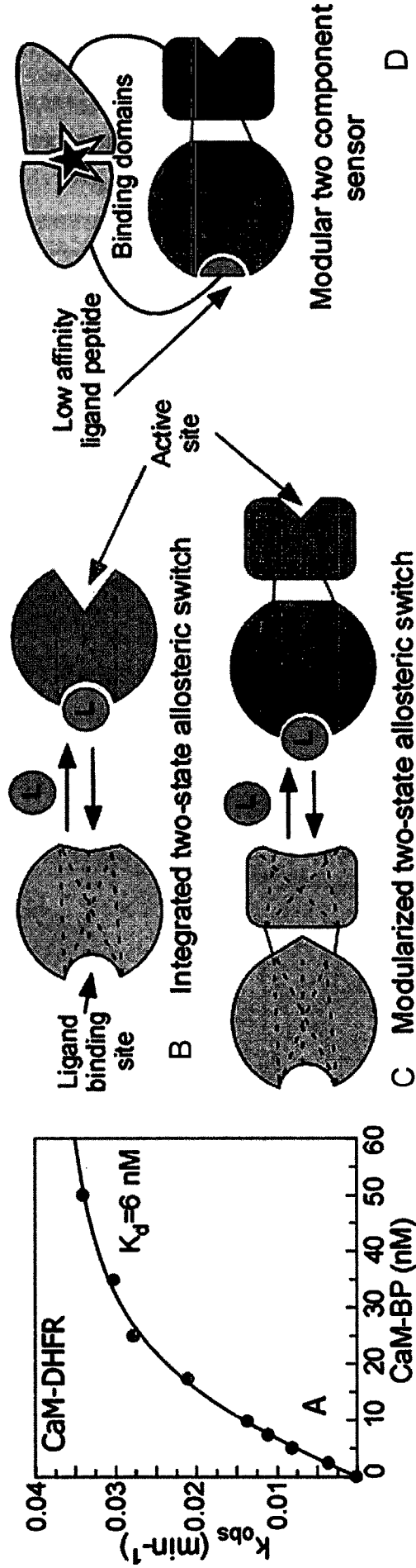


Figure 23

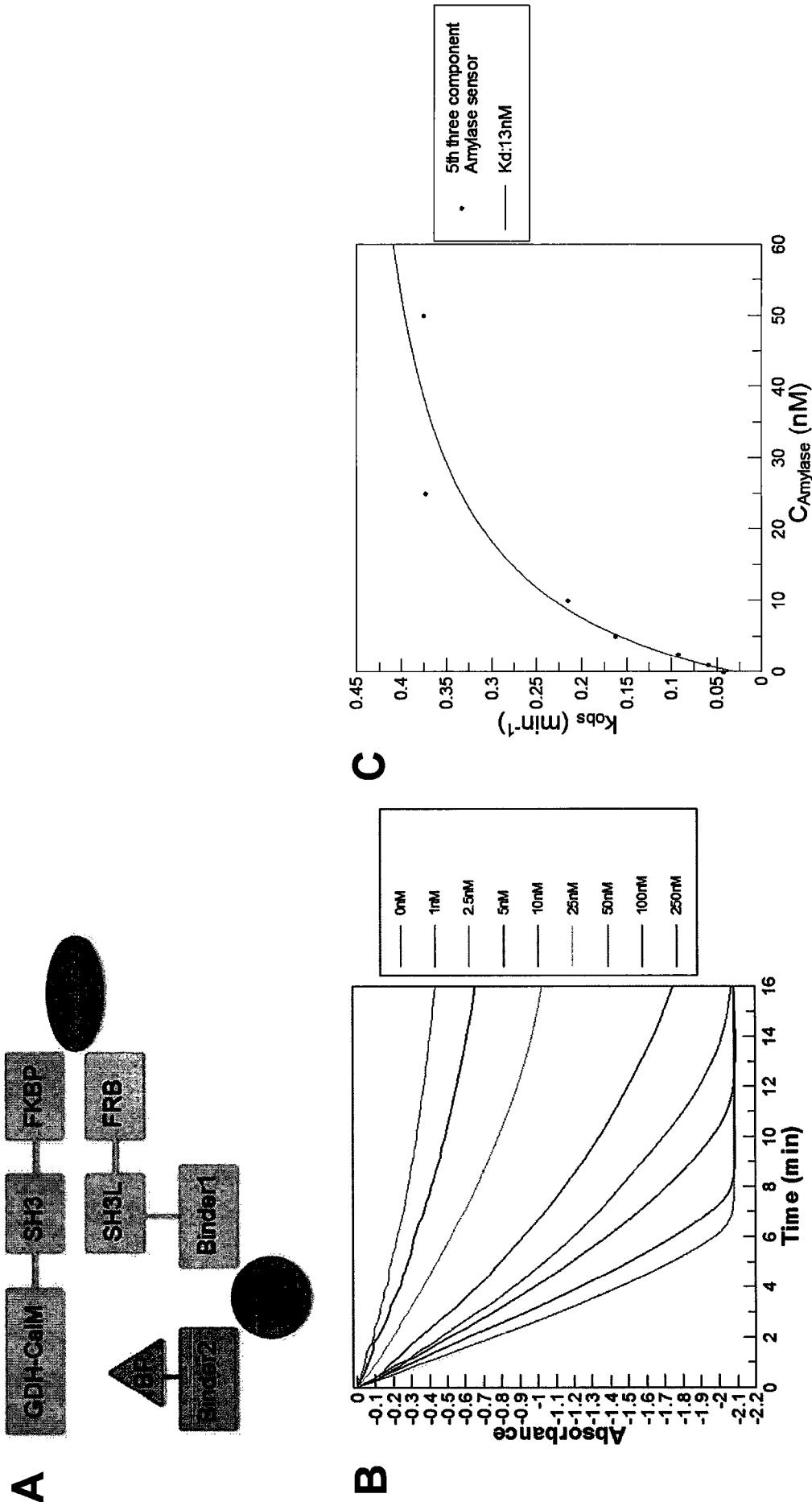


Figure 24

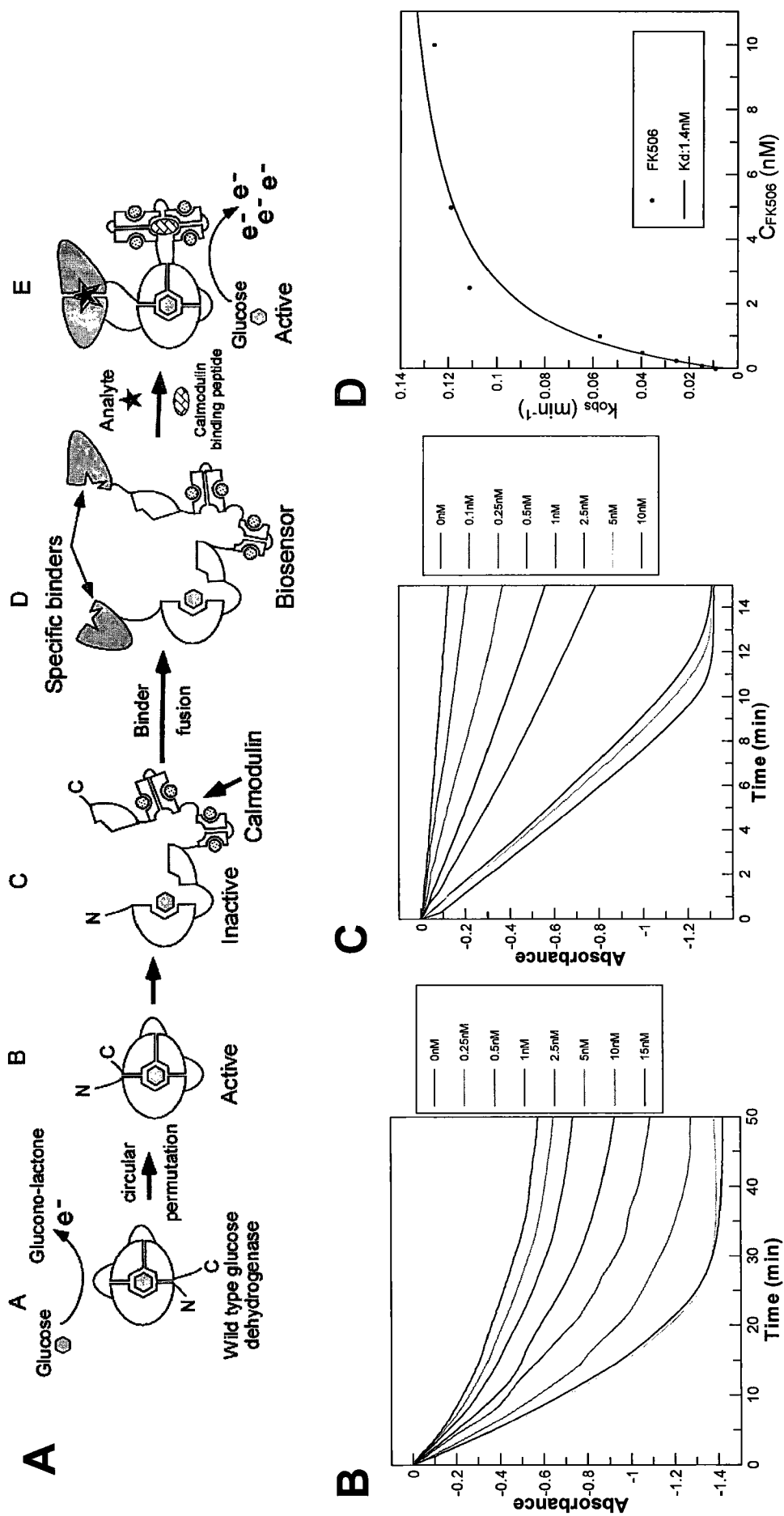


Figure 25

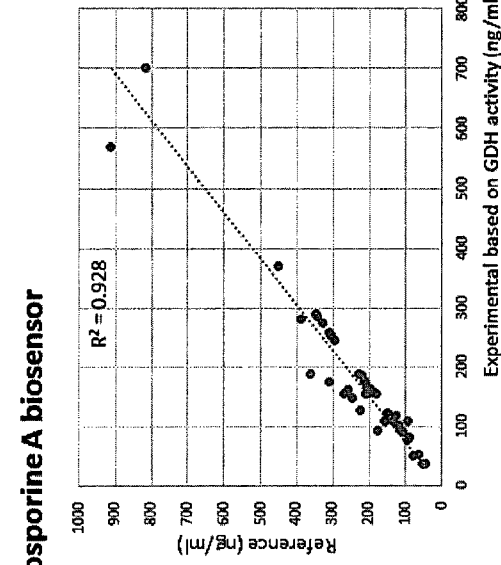
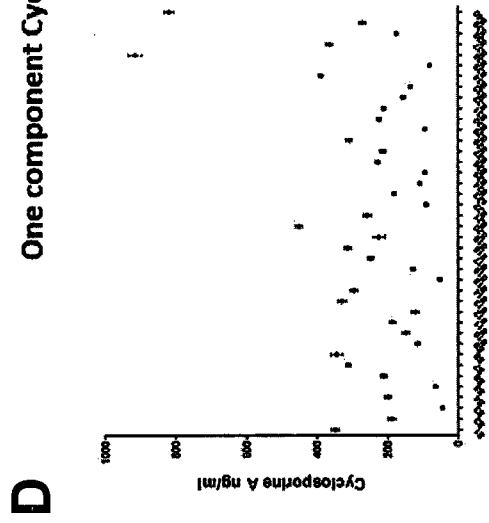
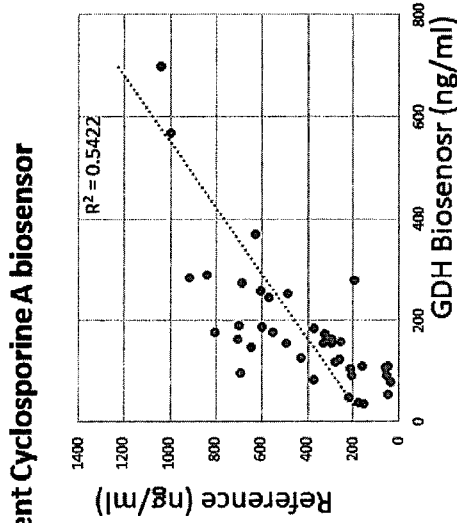
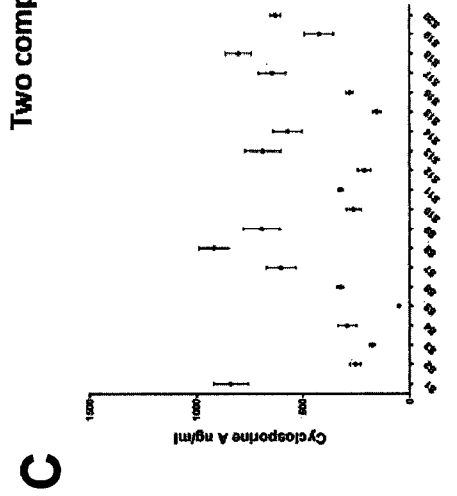
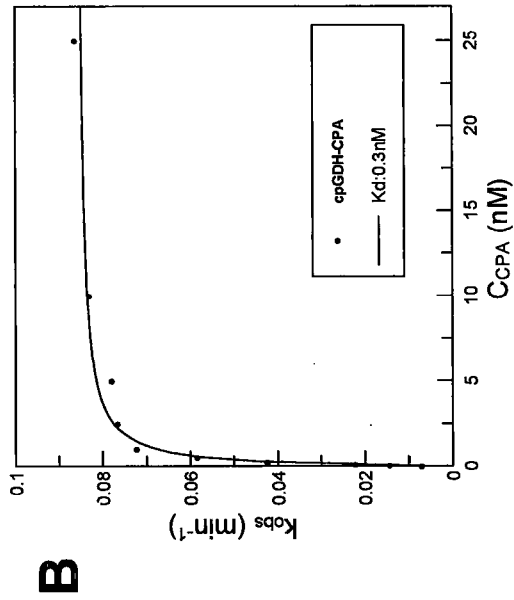
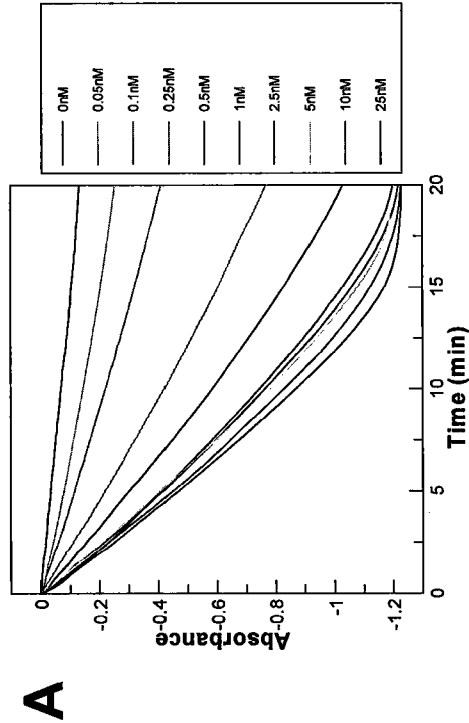
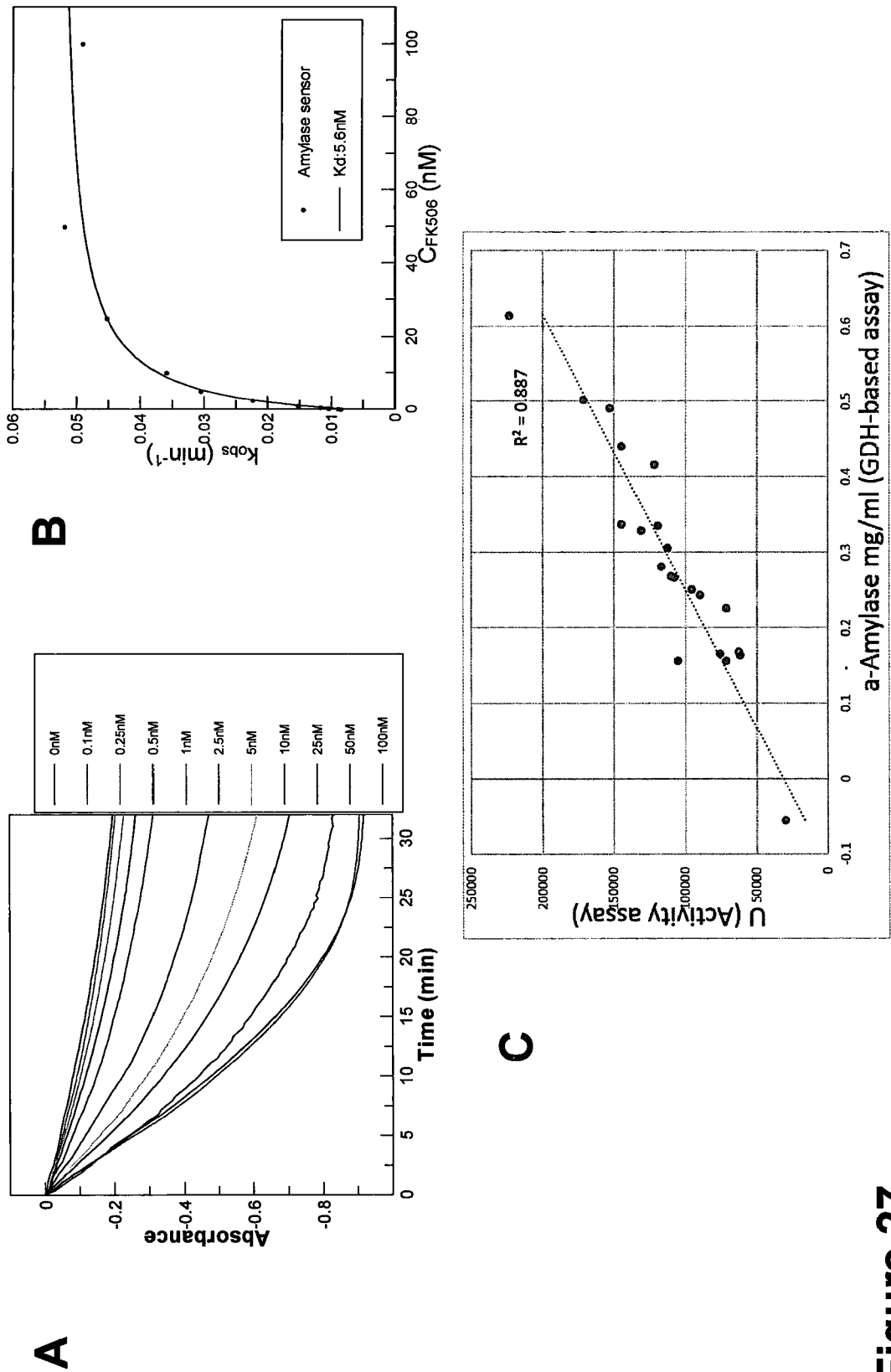


Figure 26



INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2019/000389

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/32 (2006.01) C07K 19/00 (2006.01) G01N 33/542 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PATENW, MEDLINE, CAPLUS, BIOSIS, EMBASE & keywords: biosensor, oxidoreductase, enzyme, glucose dehydrogenase, luciferase, calmodulin, disulphide, split, circularly permuted, allosteric, reconstituted, linker, intramolecular, irreversible CPC/IPC: C12Q1, C12N9/0004, C12Y, C07K14/4728, G01N33/542

PUBMED, ESPACENET, Internal Databases: Inventor and Applicant names

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

☒ Further documents are listed in the continuation of Box C

☒ See patent family annex

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"D" document cited by the applicant in the international application	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
24 September 2019

Date of mailing of the international search report
24 September 2019

Name and mailing address of the ISA/AU

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INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/IB2019/000389
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KAIHARA, A. et al., "Bioluminescent Indicators for Ca ²⁺ Based on Split <i>Renilla</i> Luciferase Complementation in Living Cells", <i>Analytical Sciences</i> , 2008, vol. 24, pages 1405-1408 Abstract; Experimental; Fig. 1	1-4, 9, 10, 14-18, 22, 23, 25, 26, 28, 80, 82-90
Y	Abstract; Experimental; Fig. 1	5-8, 13, 27, 29, 30
Y	WO 2001/051629 A2 (PANORAMA RESEARCH, INC.) 19 July 2001 Abstract, Fig. 1A; Example 5, pages 43-48	5-8, 27, 29, 30
Y	WO 2016/191812 A1 (THE UNIVERSITY OF QUEENSLAND) 08 December 2016 Abstract; page 10 lines 4-11; page 17 lines 8-27; Fig. 3G, Fig. 9	13
P,X	WO 2018/073588 A1 (MOLECULAR WAREHOUSE, LTD) 26 April 2018 Figs. 2B, 2C, 3A, 3C	1-4, 9, 10, 13-18, 22, 23, 80, 82-90

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
3-8 and 25-30 (completely) and 1, 2, 9, 10, 13-18, 22, 23, 80 and 82-90 (in part)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Supplemental Box**Continuation of: Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups:

- Invention 1 is defined by claims 3-8 and 25-30 (completely) and claims 1, 2, 9, 10, 13-18, 22, 23, 80 and 82-90 (in part). The feature of an enzyme comprising a heterologous amino acid sequence that reversibly regulates the catalytic activity of the enzyme, wherein the heterologous amino acid sequence is responsive to a target molecule and comprises a pair of linkers that interact with one another (and in particular embodiments the interaction comprises the formation of a covalent bond between the linkers), is specific to this group of claims.
- Invention 2 is defined by claims 11, 12 and 24 (completely) and claims 1, 2, 9, 10, 13-18, 22, 23, 80 and 82-90 (in part). The feature of a PQQ-GDH enzyme comprising a heterologous amino acid sequence that regulates the catalytic activity of the enzyme and is located between beta sheets 5 and 6 of the enzyme is specific to this group of claims.
- Invention 3 is defined by claims 19-21 and 31-42 (completely) and claims 1, 2, 9, 10, 13-18, 22, 23, 80 and 82-90 (in part). The feature of a calmodulin protein or functional fragment thereof that comprises modifications resulting in a reduced binding affinity for a calmodulin binding peptide, and polypeptides and biosensors comprising said calmodulin polypeptide, is specific to this group of claims.
- Invention 4 is defined by claims 43-59 and 81 (completely) and claims 80 and 82-90 (in part). The feature of a variant CaM-BP that inhibits binding of an activating CaM-BP to a calmodulin protein, preventing activation of the catalytic activity of an enzyme, is specific to this group of claims.
- Invention 5 is defined by claims 60-70 (completely) and claims 80 and 82-90 (in part). The feature of a circularly permuted oxidoreductase comprising a heterologous sequence that allosterically regulates the activity of the polypeptide is specific to this group of claims.
- Invention 6 is defined by claims 71-79 (completely) and claims 80 and 82-90 (in part). The feature of an oxidoreductase comprising a heterologous amino acid sequence comprising the particular arrangement of binding moieties and interaction domains as defined in claim 71 is specific to this group of claims.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. The only feature common to all of the claimed inventions and which provides a technical relationship among them is a calmodulin protein or calmodulin binding polypeptide as part of a biosensor, which may also comprise an enzyme such as an oxidoreductase enzyme. However this feature does not make a contribution over the prior art because it is disclosed in:

WO2016/191812 A1

This document discloses a number of biosensor comprising PQQ-GDH (an oxidoreductase) and a calmodulin polypeptide (Fig. 9).

KAIHARA, A. et al., "Bioluminescent indicators for Ca^{2+} based on split *Renilla* luciferase complementation in living cells", 2008, Analytical Sciences, vol. 24, pages 1405–1408.

This document discloses a bioassay comprising a luciferase enzyme (an oxidoreductase) and heterologous calmodulin and calmodulin binding peptide sequences that allosterically modulate the activity of the enzyme when brought into contact with calcium (Fig 1).

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB2019/000389**Supplemental Box**

Therefore in the light of these documents this common feature cannot be a special technical feature. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a posteriori*.

INTERNATIONAL SEARCH REPORT		International application No.	
Information on patent family members		PCT/IB2019/000389	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
WO 2001/051629 A2	19 July 2001	WO 0151629 A2	19 Jul 2001
		AU 3647501 A	24 Jul 2001
		AU 6045900 A	12 Dec 2000
		AU 2002364250 A1	24 Jul 2003
		CA 2374476 A1	30 Nov 2000
		CA 2397162 A1	19 Jul 2001
		EP 1183347 A1	06 Mar 2002
		EP 1248840 A2	16 Oct 2002
		JP 2003500051 A	07 Jan 2003
		US 2003165825 A1	04 Sep 2003
		US 7544477 B2	09 Jun 2009
		US 2003175836 A1	18 Sep 2003
		US 8148110 B2	03 Apr 2012
		US 2004038317 A1	26 Feb 2004
		WO 0071702 A1	30 Nov 2000
		WO 03058197 A2	17 Jul 2003
WO 2016/191812 A1	08 December 2016	WO 2016191812 A1	08 Dec 2016
		AU 2016269840 A1	30 Nov 2017
		BR 112017025785 A2	07 Aug 2018
		CA 2986446 A1	08 Dec 2016
		CN 107849544 A	27 Mar 2018
		EA 201792296 A1	29 Jun 2018
		EP 3303574 A1	11 Apr 2018
		JP 2018518168 A	12 Jul 2018
		KR 20180023912 A	07 Mar 2018
		MX 2017015094 A	14 Aug 2018
US 2018245121 A1	30 Aug 2018		
WO 2018/073588 A1	26 April 2018	WO 2018073588 A1	26 Apr 2018
		CA 3041185 A1	26 Apr 2018
		EP 3529354 A1	28 Aug 2019
		US 2019264253 A1	29 Aug 2019
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.			