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(54) Title: MIRAC PROTEINS

(57) Abstract: This disclosure relates to a method of generating conditionally active biologic proteins from wild type proteins, in particular therapeutic proteins, which are reversibly or irreversibly inactivated at the wild type normal physiological conditions. For example, evolved proteins are virtually inactive at body temperature, but are active at lower temperatures.

MIRAC PROTEINS

RELATED APPLICATION INFORMATION

[0001] This application is being filed on 09 March 2010, as a PCT International Patent application in the name of BioAtla, LLC, a U.S. limited liability corporation, applicant for the designation of all countries except the US, and Jay M. Short, Hwai Wen Chang, both citizens of the U.S., and Gerhard Frey, a citizen of Germany, applicants for the designation of the US only, and claims priority to United States Provisional Patent Application Serial No. 61/209,489 filed March 9, 2009, the entire contents of which are incorporated herein by this reference.

FIELD OF THE DISCLOSURE

[0002] This disclosure relates to the field of protein evolution and activity. Specifically, this disclosure relates to a method of generating conditionally active biologic proteins from wild type proteins, in particular therapeutic proteins, and which are reversibly or irreversibly inactivated at the wild type normal physiological conditions. For example, evolved proteins are virtually inactive at body temperature, but are active at lower temperatures.

BACKGROUND OF THE DISCLOSURE

[0003] There is a considerable body of literature describing the potential for evolving proteins for a variety of characteristics, especially enzymes for example, to be stabilized for operation at different conditions. For example, enzymes have been evolved to be stabilized at higher temperatures, with varying activity. In situations where there is an activity improvement at the high temperature, a substantial portion of the improvement can be attributed to the higher kinetic activity commonly described by the Q10 rule where it is estimated that in the case of an enzyme the turnover doubles for every increase of 10 degrees Celsius. In addition, there exist examples of natural mutations that destabilize proteins at their normal operating conditions, such as wild-type temperature activity of the molecule. For temperature mutants, these mutants can be active at the lower temperature, but typically are active at a reduced level compared to the wild type molecules (also typically described by a reduction in activity guided by the Q10 or similar rules).

[0004] It is desirable to generate useful molecules that are conditionally activated, for example virtually inactive at wild-type conditions but are active at other than wild-type conditions at a level that is equal or better than at wild-type conditions, or that are activated or inactivated in certain microenvironments, or that are activated or inactivated over time. Besides temperature, other conditions for which the proteins can be evolved or optimized include pH, osmotic pressure, osmolality, oxidation and electrolyte concentration. Other desirable properties that can be optimized during evolution include chemical resistance, and proteolytic resistance.

[0005] Many strategies for evolving or engineering molecules have been published. However, engineering or evolving a protein to be inactive or virtually inactive (less than 10% activity and especially 1% activity) at its wild type operating condition, while maintaining activity equivalent or better than its wild type condition at new conditions, requires that the destabilizing mutation(s) co-exist with activity increasing mutations that do not counter the destabilizing effect. It is expected that destabilization would reduce the protein's activity greater than the effects predicted by standard rules such as Q10, therefore the ability to evolve proteins that work efficiently at lower temperature, for example, while being inactivated under their normal operating condition, creates an unexpected new class of proteins we refer to as Mirac Proteins.

[0006] Throughout this application, various publications are referenced by author and date. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the disclosure described and claimed herein.

SUMMARY OF THE DISCLOSURE

The disclosure provides a method of preparing a conditionally active biologic protein, the method comprising: selecting a wild-type biologic protein; evolving the DNA which encodes the wild-type biologic protein using one or more evolutionary techniques to create a mutant DNA; expressing the mutant DNA to obtain a mutant protein; subjecting the mutant protein and the wild-type protein to an assay under a normal physiological condition and the assay under an aberrant condition; and selecting the conditionally active biologic protein from those mutant proteins which exhibit both (a) a decrease in activity in the assay at the normal physiological condition compared to the wild-type protein, and (b) an increase in activity in the assay under the aberrant condition

compared to the wild-type protein. In various aspects, the normal physiological condition is selected from one or more of temperature, pH, osmotic pressure, osmolality, oxidation and electrolyte concentration. In a particular aspect, the normal physiological condition is temperature; wherein the conditionally active biologic protein is virtually inactive at the normal physiological temperature, but is active at an aberrant temperature less than the normal physiological temperature. In other aspects, the conditionally active biologic protein is reversibly or irreversibly inactivated at the wild type normal physiological conditions. In one specific aspect, the protein is reversibly inactivated at the wild type normal physiological conditions. Alternatively, conditionally active biologic proteins are selected from those proteins which exhibit changes in activity, reversibly or irreversibly, in two or more different physiological conditions.

[0007] In one embodiment, the wild-type biologic protein is an enzyme. In certain aspects, the wild-type biologic protein is selected from the group consisting of tissue plasminogen activator, streptokinase, urokinase, renin, and hyaluronidase.

[0008] In another embodiment, the wild-type biologic protein is selected from calcitonin gene-related peptide (CGRP), substance P (SP), neuropeptide Y (NPY), vasoactive intestinal peptide(VIP), vasopressin, and angiostatin.

[0009] In another embodiment, the biologic protein is an antibody.

[0010] In another embodiment, the disclosure provides a method of preparing a conditionally active biological response modifier, the method comprising: selecting an inflammatory response mediator; identifying a wild-type antibody to the mediator; evolving the wild-type antibody; screening differentially for mutants that exhibit decreased binding to the mediator relative to the wild-type antibody at a first condition, and exhibit increased binding affinity to the mediator at a second condition to identify up-mutants; and recombining the heavy chains and the light chains of the up-mutants to create recombined up-mutants; and screening the recombined up-mutants for mutants that exhibit decreased binding to the mediator relative to the wild-type antibody at the first condition, and show increased binding affinity to the mediator at the second condition to identify the conditionally active biological response modifier. In one aspect, the inflammatory response mediator is selected from IL-6, IL-6 receptor, TNF-alpha, IL-23 and IL-12. In another aspect, the first and second conditions are selected from conditions of pH, osmotic pressure, osmolality, oxidation and electrolyte concentration.

[0011] In another embodiment, the disclosure provides a pharmaceutical composition comprising a conditionally active biologic protein, and a pharmaceutically acceptable carrier.

DETAILED DESCRIPTION

[0012] In order to facilitate understanding of the examples provided herein, certain frequently occurring methods and/or terms will be described.

[0013] As used herein in connection with a measured quantity, the term "about" refers to the normal variation in that measured quantity that would be expected by the skilled artisan making the measurement and exercising a level of care commensurate with the objective of the measurement and the precision of the measuring equipment used. Unless otherwise indicated, "about" refers to a variation of +/- 10% of the value provided.

[0014] The term "agent" is used herein to denote a chemical compound, a mixture of chemical compounds, an array of spatially localized compounds (e.g., a VLSIPS peptide array, polynucleotide array, and/or combinatorial small molecule array), biological macromolecule, a bacteriophage peptide display library, a bacteriophage antibody (e.g., scFv) display library, a polysome peptide display library, or an extract made from biological materials such as bacteria, plants, fungi, or animal (particular mammalian) cells or tissues. Agents are evaluated for potential enzyme activity by inclusion in screening assays described herein below. Agents are evaluated for potential activity as conditionally active biologic therapeutic enzymes by inclusion in screening assays described herein below.

[0015] An "ambiguous base requirement" in a restriction site refers to a nucleotide base requirement that is not specified to the fullest extent, i.e. that is not a specific base (such as, in a non-limiting exemplification, a specific base selected from A, C, G, and T), but rather may be any one of at least two or more bases. Commonly accepted abbreviations that are used in the art as well as herein to represent ambiguity in bases include the following: R=G or A; Y=C or T; M=A or C; K=G or T; S=G or C; W=A or T; H=A or C or T; B=G or T or C; V=G or C or A; D=G or A or T; N=A or C or G or T.

[0016] The term "amino acid" as used herein refers to any organic compound that contains an amino group (--NH_2) and a carboxyl group (--COOH); preferably either as

free groups or alternatively after condensation as part of peptide bonds. The "twenty naturally encoded polypeptide-forming alpha-amino acids" are understood in the art and refer to: alanine (ala or A), arginine (arg or R), asparagine (asn or N), aspartic acid (asp or D), cysteine (cys or C), gluatamic acid (glu or E), glutamine (gln or Q), glycine (gly or G), histidine (his or H), isoleucine (ile or I), leucine (leu or L), lysine (lys or K), methionine (met or M), phenylalanine (phe or F), proline (pro or P), serine (ser or S), threonine (thr or T), tryptophan (trp or W), tyrosine (tyr or Y), and valine (val or V).

[0017] The term "amplification" means that the number of copies of a polynucleotide is increased.

[0018] A molecule that has a "chimeric property" is a molecule that is: 1) in part homologous and in part heterologous to a first reference molecule; while 2) at the same time being in part homologous and in part heterologous to a second reference molecule; without 3) precluding the possibility of being at the same time in part homologous and in part heterologous to still one or more additional reference molecules. In a non-limiting embodiment, a chimeric molecule may be prepared by assembling a reassortment of partial molecular sequences. In a non-limiting aspect, a chimeric polynucleotide molecule may be prepared by synthesizing the chimeric polynucleotide using plurality of molecular templates, such that the resultant, chimeric polynucleotide has properties of a plurality of templates.

[0019] The term "cognate" as used herein refers to a gene sequence that is evolutionarily and functionally related between species. For example, but not limitation, in the human genome the human CD4 gene is the cognate gene to the mouse 3d4 gene, since the sequences and structures of these two genes indicate that they are highly homologous and both genes encode a protein which functions in signaling T cell activation through MHC class II-restricted antigen recognition.

[0020] A "comparison window," as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may

be conducted by the local homology algorithm of Smith (Smith and Waterman, 1981 ,"Comparison of biosequences", Adv Appl Math, 2:482-489; Smith and Waterman, 1981, "Overlapping genes and information theory", J Theor Biol,91:379-380; Smith and Waterman, J Mol Biol, "Identification of common molecular subsequences",1981, 147:195-197; Smith et al., 1981, ""Comparative biosequence metrics", J Mol Evol, 18:38-46), by the homology alignment algorithm of Needleman (Needleman and Wunsch, 1970, "A general method applicable to the search for similarities in the amino acid sequence of two proteins" J Mol Biol, 48(3):443-453), by the search of similarity method of Pearson (Pearson and Lipman, 1988, "Improved tools for biological sequence comparison", Proc Nat Acad Sci USA, 85:2444-2448), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected.

[0021] The term "conditionally active biologic protein" refers to a variant, or mutant, of a wild-type protein which is more or less active than the parent wild-type protein under one or more normal physiological conditions. This conditionally active protein also exhibits activity in selected regions of the body and/or exhibits increased or decreased activity under aberrant, or permissive, physiological conditions. Normal physiological conditions are those of temperature, pH, osmotic pressure, osmolality, oxidation and electrolyte concentration which would be considered within a normal range at the site of administration, or at the tissue or organ at the site of action, to a subject. An aberrant condition is that which deviates from the normally acceptable range for that condition. In one aspect, the conditionally active biologic protein is virtually inactive at wild-type conditions but is active at other than wild-type conditions at a level that is equal or better than at wild-type conditions. For example, in one aspect, an evolved conditionally active biologic protein is virtually inactive at body temperature, but is active at lower temperatures. In another aspect, the conditionally active biologic protein is reversibly or irreversibly inactivated at the wild type conditions. In a further aspect, the wild-type protein is a therapeutic protein. In another aspect, the conditionally active biologic protein is used as a drug, or therapeutic agent. In yet another aspect, the

protein is more or less active in highly oxygenated blood, such as, for example, after passage through the lung or in the lower pH environments found in the kidney.

[0022] "Conservative amino acid substitutions" refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

[0023] The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference "TATAC" and is complementary to a reference sequence "GTATA."

[0024] The term "degrading effective" amount refers to the amount of enzyme which is required to process at least 50% of the substrate, as compared to substrate not contacted with the enzyme.

[0025] As used herein, the term "defined sequence framework" refers to a set of defined sequences that are selected on a non-random basis, generally on the basis of experimental data or structural data; for example, a defined sequence framework may comprise a set of amino acid sequences that are predicted to form a .beta.-sheet structure or may comprise a leucine zipper heptad repeat motif, a zinc-finger domain, among other variations. A "defined sequence kernal" is a set of sequences which encompass a limited scope of variability. Whereas (1) a completely random 10-mer sequence of the 20 conventional amino acids can be any of $(20)^{10}$ sequences, and (2) a pseudorandom 10-mer sequence of the 20 conventional amino acids can be any of $(20)^{10}$ sequences but will

exhibit a bias for certain residues at certain positions and/or overall, (3) a defined sequence kernal is a subset of sequences if each residue position was allowed to be any of the allowable 20 conventional amino acids (and/or allowable unconventional amino/imino acids). A defined sequence kernal generally comprises variant and invariant residue positions and/or comprises variant residue positions which can comprise a residue selected from a defined subset of amino acid residues, and the like, either segmentally or over the entire length of the individual selected library member sequence. Defined sequence kernels can refer to either amino acid sequences or polynucleotide sequences. Of illustration and not limitation, the sequences $(NNK)_{10}$ and $(NNM)_{10}$, wherein N represents A, T, G, or C; K represents G or T; and M represents A or C, are defined sequence kernels.

[0026] "Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 microgram of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 microliters of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 micrograms of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37 degrees C. are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a gel to isolate the desired fragment.

[0027] "Directional ligation" refers to a ligation in which a 5' end and a 3' end of a polynuclotide are different enough to specify a preferred ligation orientation. For example, an otherwise untreated and undigested PCR product that has two blunt ends will typically not have a preferred ligation orientation when ligated into a cloning vector digested to produce blunt ends in its multiple cloning site; thus, directional ligation will typically not be displayed under these circumstances. In contrast, directional ligation will typically be displayed when a digested PCR product having a 5' EcoR I-treated end and a 3' BamH I is ligated into a cloning vector that has a multiple cloning site digested with EcoR I and BamH I.

[0028] The term "DNA shuffling" is used herein to indicate recombination between substantially homologous but non-identical sequences, in some embodiments DNA shuffling may involve crossover via non-homologous recombination, such as via cer/lox and/or flp/frt systems and the like. DNA shuffling can be random or non-random.

[0029] The term "drug" or "drug molecule" refers to a therapeutic agent including a substance having a beneficial effect on a human or animal body when it is administered to the human or animal body. Preferably, the therapeutic agent includes a substance that can treat, cure or relieve one or more symptoms, illnesses, or abnormal conditions in a human or animal body or enhance the wellness of a human or animal body.

[0030] An "effective amount" is an amount of a conditionally active biologic protein or fragment which is effective to treat or prevent a condition in a living organism to whom it is administered over some period of time, e.g., provides a therapeutic effect during a desired dosing interval.

[0031] As used herein, the term "electrolyte" is used to define a mineral in the blood or other body fluids that carries a charge. For example, in one aspect, the normal physiological condition and aberrant condition can be conditions of "electrolyte concentration". In one aspect, the electrolyte concentration to be tested is selected from one or more of ionized calcium, sodium, potassium, magnesium, chloride, bicarbonate, and phosphate concentration. For example, in one aspect, normal range of serum calcium is 8.5 to 10.2 mg/dL. In this aspect, aberrant serum calcium concentration may be selected from either above or below the normal range. In another example, in one aspect, normal range of serum chloride is 96-106 milliequivalents per liter (mEq/L). In this aspect, aberrant serum chloride concentration may be selected from either above or below the normal range. In another example, in one aspect, a normal range of serum magnesium is from 1.7-2.2 mg/dL. In this aspect, an aberrant serum magnesium concentration may be selected from either above or below the normal range. In another example, in one aspect, a normal range of serum phosphorus is from 2.4 to 4.1 mg/dL. In this aspect, aberrant serum phosphorus concentration may be selected from either above or below the normal range. In another example, in one aspect, a normal range of serum, or blood, sodium is from 135 to 145 mEq/L. In this aspect, aberrant serum, or blood, sodium concentration may be selected from either above or below the normal range. In another example, in one aspect, a normal range of serum, or blood, potassium is from 3.7

to 5.2 mEq/L. In this aspect, aberrant serum, or blood, potassium concentration may be selected from either above or below the normal range. In a further aspect, a normal range of serum bicarbonate is from 20 to 29 mEq/L. In this aspect, aberrant serum, or blood, bicarbonate concentration may be selected from either above or below the normal range. In a different aspect, bicarbonate levels can be used to indicate normal levels of acidity (pH), in the blood. The term "electrolyte concentration" may also be used to define the condition of a particular electrolyte in a tissue or body fluid other than blood or plasma. In this case, the normal physiological condition is considered to be the clinically normal range for that tissue or fluid. In this aspect, aberrant tissue or fluid electrolyte concentration may be selected from either above or below the normal range.

[0032] As used in this disclosure, the term "epitope" refers to an antigenic determinant on an antigen, such as an enzyme polypeptide, to which the paratope of an antibody, such as an enzyme-specific antibody, binds. Antigenic determinants usually consist of chemically active surface groupings of molecules, such as amino acids or sugar side chains, and can have specific three-dimensional structural characteristics, as well as specific charge characteristics. As used herein "epitope" refers to that portion of an antigen or other macromolecule capable of forming a binding interaction that interacts with the variable region binding body of an antibody. Typically, such binding interaction is manifested as an intermolecular contact with one or more amino acid residues of a CDR.

[0033] As used herein, an "enzyme" is a protein with specific catalytic properties. Factors such as, for example, substrate concentration, pH, temperature and presence or absence of inhibitors can affect the rate of catalysis. Typically, for a wild type enzyme, Q10 (the temperature coefficient) describes the increase in reaction rate with a 10 degree C rise in temperature. For wild type enzymes, the Q10 = 2 to 3; in other words, the rate of reaction doubles or triples with every 10 degree increase in temperature. At high temperatures, proteins denature. At pH values slightly different from an enzymes optimum value, small changes occur in the charges of the enzyme and perhaps the substrate molecule. The change in ionization can affect the binding of the substrate molecule. At extreme pH levels, the enzyme will produce denaturation, where the active site is distorted, and the substrate molecule will no longer fit.

[0034] As used herein, the term "evolution", or "evolving", refers to using one or more methods of mutagenesis to generate a novel polynucleotide encoding a novel polypeptide, which novel polypeptide is itself an improved biological molecule &/or contributes to the generation of another improved biological molecule. In a particular non-limiting aspect, the present disclosure relates to evolution of conditionally active biologic proteins from a parent wild type protein. In one aspect, for example, evolution relates to a method of performing both non-stochastic polynucleotide chimerization and non-stochastic site-directed point mutagenesis disclosed in U.S. patent application publication 2009/0130718, which is incorporated herein by reference. More particularly, the present disclosure provides methods for evolution of conditionally active biologic enzymes which exhibit reduced activity at normal physiological conditions compared to a wild-type enzyme parent molecule, but enhanced activity under one or more aberrant conditions compared to the wild-type enzyme.

[0035] The terms "fragment", "derivative" and "analog" when referring to a reference polypeptide comprise a polypeptide which retains at least one biological function or activity that is at least essentially same as that of the reference polypeptide. Furthermore, the terms "fragment", "derivative" or "analog" are exemplified by a "pro-form" molecule, such as a low activity proprotein that can be modified by cleavage to produce a mature enzyme with significantly higher activity.

[0036] A method is provided herein for producing from a template polypeptide a set of progeny polypeptides in which a "full range of single amino acid substitutions" is represented at each amino acid position. As used herein, "full range of single amino acid substitutions" is in reference to the 20 naturally encoded polypeptide-forming alpha-amino acids, as described herein.

[0037] The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

[0038] "Genetic instability", as used herein, refers to the natural tendency of highly repetitive sequences to be lost through a process of reductive events generally involving sequence simplification through the loss of repeated sequences. Deletions tend to involve the loss of one copy of a repeat and everything between the repeats.

[0039] The term "heterologous" means that one single-stranded nucleic acid sequence is unable to hybridize to another single-stranded nucleic acid sequence or its complement. Thus areas of heterology means that areas of polynucleotides or polynucleotides have areas or regions within their sequence which are unable to hybridize to another nucleic acid or polynucleotide. Such regions or areas are for example areas of mutations.

[0040] The term "homologous" or "homeologous" means that one single-stranded nucleic acid sequence may hybridize to a complementary single-stranded nucleic acid sequence. The degree of hybridization may depend on a number of factors including the amount of identity between the sequences and the hybridization conditions such as temperature and salt concentrations as discussed later. Preferably the region of identity is greater than about 5 bp, more preferably the region of identity is greater than 10 bp.

[0041] The benefits of this disclosure extend to "industrial applications" (or industrial processes), which term is used to include applications in commercial industry proper (or simply industry) as well as non-commercial industrial applications (e.g. biomedical research at a non-profit institution). Relevant applications include those in areas of diagnosis, medicine, agriculture, manufacturing, and academia.

[0042] The term "identical" or "identity" means that two nucleic acid sequences have the same sequence or a complementary sequence. Thus, "areas of identity" means that regions or areas of a polynucleotide or the overall polynucleotide are identical or complementary to areas of another polynucleotide.

[0043] The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or enzyme present in a living animal is not isolated, but the same polynucleotide or enzyme, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or enzymes could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

[0044] The term "isolated nucleic acid" is used to define a nucleic acid, e.g., a DNA or RNA molecule, that is not immediately contiguous with the 5' and 3' flanking sequences with which it normally is immediately contiguous when present in the naturally occurring genome of the organism from which it is derived. The term thus

describes, for example, a nucleic acid that is incorporated into a vector, such as a plasmid or viral vector; a nucleic acid that is incorporated into the genome of a heterologous cell (or the genome of a homologous cell, but at a site different from that at which it naturally occurs); and a nucleic acid that exists as a separate molecule, e.g., a DNA fragment produced by PCR amplification or restriction enzyme digestion, or an RNA molecule produced by in vitro transcription. The term also describes a recombinant nucleic acid that forms part of a hybrid gene encoding additional polypeptide sequences that can be used, for example, in the production of a fusion protein.

[0045] As used herein "ligand" refers to a molecule, such as a random peptide or variable segment sequence, that is recognized by a particular receptor. As one of skill in the art will recognize, a molecule (or macromolecular complex) can be both a receptor and a ligand. In general, the binding partner having a smaller molecular weight is referred to as the ligand and the binding partner having a greater molecular weight is referred to as a receptor.

[0046] "Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Sambrook et al., (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory, Cold Spring Harbor, NY., p. 146; Sambrook et al., Molecular Cloning: a laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory Press, 1989). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 micrograms of approximately equimolar amounts of the DNA fragments to be ligated.

[0047] As used herein, "linker" or "spacer" refers to a molecule or group of molecules that connects two molecules, such as a DNA binding protein and a random peptide, and serves to place the two molecules in a preferred configuration, e.g., so that the random peptide can bind to a receptor with minimal steric hindrance from the DNA binding protein.

[0048] As used herein "microenvironment" means any portion or region of a tissue or body that has constant or temporal, physical or chemical differences from other regions of the tissue or regions of the body.

[0049] As used herein, a "molecular property to be evolved" includes reference to molecules comprised of a polynucleotide sequence, molecules comprised of a polypeptide sequence, and molecules comprised in part of a polynucleotide sequence and

in part of a polypeptide sequence. Particularly relevant--but by no means limiting--examples of molecular properties to be evolved include protein activities at specified conditions, such as related to temperature; salinity; osmotic pressure; pH; oxidation, and concentration of glycerol, DMSO, detergent, &/or any other molecular species with which contact is made in a reaction environment. Additional particularly relevant--but by no means limiting--examples of molecular properties to be evolved include stabilities--e.g. the amount of a residual molecular property that is present after a specified exposure time to a specified environment, such as may be encountered during storage.

[0050] The term "mutations" means changes in the sequence of a wild-type nucleic acid sequence or changes in the sequence of a peptide. Such mutations may be point mutations such as transitions or transversions. The mutations may be deletions, insertions or duplications.

[0051] As used herein, the degenerate "N,N,G/T" nucleotide sequence represents 32 possible triplets, where "N" can be A, C, G or T.

[0052] The term "naturally-occurring" as used herein as applied to the object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally occurring. Generally, the term naturally occurring refers to an object as present in a non-pathological (un-diseased) individual, such as would be typical for the species.

[0053] As used herein, "normal physiological conditions", or "wild type operating conditions", are those conditions of temperature, pH, osmotic pressure, osmolality, oxidation and electrolyte concentration which would be considered within a normal range at the site of administration, or the site of action, in a subject.

[0054] As used herein, a "nucleic acid molecule" is comprised of at least one base or one base pair, depending on whether it is single-stranded or double-stranded, respectively. Furthermore, a nucleic acid molecule may belong exclusively or chimerically to any group of nucleotide-containing molecules, as exemplified by, but not limited to, the following groups of nucleic acid molecules: RNA, DNA, genomic nucleic acids, non-genomic nucleic acids, naturally occurring and not naturally occurring nucleic acids, and synthetic nucleic acids. This includes, by way of non-limiting example,

nucleic acids associated with any organelle, such as the mitochondria, ribosomal RNA, and nucleic acid molecules comprised chimerically of one or more components that are not naturally occurring along with naturally occurring components.

[0055] Additionally, a "nucleic acid molecule" may contain in part one or more non-nucleotide-based components as exemplified by, but not limited to, amino acids and sugars. Thus, by way of example, but not limitation, a ribozyme that is in part nucleotide-based and in part protein-based is considered a "nucleic acid molecule".

[0056] In addition, by way of example, but not limitation, a nucleic acid molecule that is labeled with a detectable moiety, such as a radioactive or alternatively a non-radioactive label, is likewise considered a "nucleic acid molecule".

[0057] The terms "nucleic acid sequence coding for" or a "DNA coding sequence of" or a "nucleotide sequence encoding" a particular enzyme--as well as other synonymous terms--refer to a DNA sequence which is transcribed and translated into an enzyme when placed under the control of appropriate regulatory sequences. A "promotor sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The promoter is part of the DNA sequence. This sequence region has a start codon at its 3' terminus. The promoter sequence does include the minimum number of bases where elements necessary to initiate transcription at levels detectable above background. However, after the RNA polymerase binds the sequence and transcription is initiated at the start codon (3' terminus with a promoter), transcription proceeds downstream in the 3' direction. Within the promotor sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1) as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

[0058] The terms "nucleic acid encoding an enzyme (protein)" or "DNA encoding an enzyme (protein)" or "polynucleotide encoding an enzyme (protein)" and other synonymous terms encompasses a polynucleotide which includes only coding sequence for the enzyme as well as a polynucleotide which includes additional coding and/or non-coding sequence.

[0059] In one preferred embodiment, a "specific nucleic acid molecule species" is defined by its chemical structure, as exemplified by, but not limited to, its primary sequence. In another preferred embodiment, a specific "nucleic acid molecule species" is

defined by a function of the nucleic acid species or by a function of a product derived from the nucleic acid species. Thus, by way of non-limiting example, a "specific nucleic acid molecule species" may be defined by one or more activities or properties attributable to it, including activities or properties attributable to its expressed product.

[0060] The instant definition of "assembling a working nucleic acid sample into a nucleic acid library" includes the process of incorporating a nucleic acid sample into a vector-based collection, such as by ligation into a vector and transformation of a host. A description of relevant vectors, hosts, and other reagents as well as specific non-limiting examples thereof are provided hereinafter. The instant definition of "assembling a working nucleic acid sample into a nucleic acid library" also includes the process of incorporating a nucleic acid sample into a non-vector-based collection, such as by ligation to adaptors. Preferably the adaptors can anneal to PCR primers to facilitate amplification by PCR.

[0061] Accordingly, in a non-limiting embodiment, a "nucleic acid library" is comprised of a vector-based collection of one or more nucleic acid molecules. In another preferred embodiment a "nucleic acid library" is comprised of a non-vector-based collection of nucleic acid molecules. In yet another preferred embodiment a "nucleic acid library" is comprised of a combined collection of nucleic acid molecules that is in part vector-based and in part non-vector-based. Preferably, the collection of molecules comprising a library is searchable and separable according to individual nucleic acid molecule species.

[0062] The present disclosure provides a "nucleic acid construct" or alternatively a "nucleotide construct" or alternatively a "DNA construct". The term "construct" is used herein to describe a molecule, such as a polynucleotide (e.g., an enzyme polynucleotide) which may optionally be chemically bonded to one or more additional molecular moieties, such as a vector, or parts of a vector. In a specific--but by no means limiting--aspect, a nucleotide construct is exemplified by DNA expression constructs suitable for the transformation of a host cell.

[0063] An "oligonucleotide" (or synonymously an "oligo") refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides may or may not have a 5' phosphate. Those that do not will not ligate to another oligonucleotide without adding

a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated. To achieve polymerase-based amplification (such as with PCR), a "32-fold degenerate oligonucleotide that is comprised of, in series, at least a first homologous sequence, a degenerate N,N,G/T sequence, and a second homologous sequence" is mentioned. As used in this context, "homologous" is in reference to homology between the oligo and the parental polynucleotide that is subjected to the polymerase-based amplification.

[0064] As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame.

[0065] A coding sequence is "operably linked to" another coding sequence when RNA polymerase will transcribe the two coding sequences into a single mRNA, which is then translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequences are ultimately processed to produce the desired protein.

[0066] As used herein the term "parental polynucleotide set" is a set comprised of one or more distinct polynucleotide species. Usually this term is used in reference to a progeny polynucleotide set which is preferably obtained by mutagenization of the parental set, in which case the terms "parental", "starting" and "template" are used interchangeably.

[0067] The term "patient", or "subject", refers to an animal, for example a mammal, such as a human, who is the object of treatment. The subject, or patient, may be either male or female.

[0068] As used herein the term "physiological conditions" refers to temperature, pH, osmotic pressure, ionic strength, viscosity, and like biochemical parameters which are compatible with a viable organism, and/or which typically exist intracellularly in a viable cultured yeast cell or mammalian cell. For example, the intracellular conditions in a yeast cell grown under typical laboratory culture conditions are physiological conditions.

Suitable in vitro reaction conditions for in vitro transcription cocktails are generally physiological conditions. In general, in vitro physiological conditions comprise 50-200 mM NaCl or KCl, pH 6.5-8.5, 20-45 degrees C and 0.001-10 mM divalent cation (e.g., Mg^{++}, Ca^{++}); preferably about 150 mM NaCl or KCl, pH 7.2-7.6, 5 mM divalent cation, and often include 0.01-1.0 percent nonspecific protein (e.g., BSA). A non-ionic detergent (Tween, NP-40, Triton X-100) can often be present, usually at about 0.001 to 2%, typically 0.05-0.2% (v/v). Particular aqueous conditions may be selected by the practitioner according to conventional methods. For general guidance, the following buffered aqueous conditions may be applicable: 10-250 mM NaCl, 5-50 mM Tris HCl, pH 5-8, with optional addition of divalent cation(s) and/or metal chelators and/or non-ionic detergents and/or membrane fractions and/or anti-foam agents and/or scintillants. Normal physiological conditions refer to conditions of temperature, pH, osmotic pressure, osmolality, oxidation and electrolyte concentration in vivo in a patient or subject at the site of administration, or the site of action, which would be considered within the normal range in a patient.

[0069] Standard convention (5' to 3') is used herein to describe the sequence of double standed polynucleotides.

[0070] The term "population" as used herein means a collection of components such as polynucleotides, portions or polynucleotides or proteins. A "mixed population" means a collection of components which belong to the same family of nucleic acids or proteins (i.e., are related) but which differ in their sequence (i.e., are not identical) and hence in their biological activity.

[0071] A molecule having a "pro-form" refers to a molecule that undergoes any combination of one or more covalent and noncovalent chemical modifications (e.g. glycosylation, proteolytic cleavage, dimerization or oligomerization, temperature-induced or pH-induced conformational change, association with a co-factor, etc.) en route to attain a more mature molecular form having a property difference (e.g. an increase in activity) in comparison with the reference pro-form molecule. When two or more chemical modifications (e.g. two proteolytic cleavages, or a proteolytic cleavage and a deglycosylation) can be distinguished en route to the production of a mature molecule, the reference precursor molecule may be termed a"pre-pro-form" molecule.

[0072] As used herein, the term "pseudorandom" refers to a set of sequences that have limited variability, such that, for example, the degree of residue variability at another position, but any pseudorandom position is allowed some degree of residue variation, however circumscribed.

[0073] "Quasi-repeated units", as used herein, refers to the repeats to be re-assorted and are by definition not identical. Indeed the method is proposed not only for practically identical encoding units produced by mutagenesis of the identical starting sequence, but also the reassortment of similar or related sequences which may diverge significantly in some regions. Nevertheless, if the sequences contain sufficient homologies to be reasserted by this approach, they can be referred to as "quasi-repeated" units.

[0074] As used herein "random peptide library" refers to a set of polynucleotide sequences that encodes a set of random peptides, and to the set of random peptides encoded by those polynucleotide sequences, as well as the fusion proteins that contain those random peptides.

[0075] As used herein, "random peptide sequence" refers to an amino acid sequence composed of two or more amino acid monomers and constructed by a stochastic or random process. A random peptide can include framework or scaffolding motifs, which may comprise invariant sequences.

[0076] As used herein, "receptor" refers to a molecule that has an affinity for a given ligand. Receptors can be naturally occurring or synthetic molecules. Receptors can be employed in an unaltered state or as aggregates with other species. Receptors can be attached, covalently or non-covalently, to a binding member, either directly or via a specific binding substance. Examples of receptors include, but are not limited to, antibodies, including monoclonal antibodies and antisera reactive with specific antigenic determinants (such as on viruses, cells, or other materials), cell membrane receptors, complex carbohydrates and glycoproteins, enzymes, and hormone receptors.

[0077] "Recombinant" enzymes refer to enzymes produced by recombinant DNA techniques, i.e., produced from cells transformed by an exogenous DNA construct encoding the desired enzyme. "Synthetic" enzymes are those prepared by chemical synthesis.

[0078] The term "related polynucleotides" means that regions or areas of the polynucleotides are identical and regions or areas of the polynucleotides are heterologous.

[0079] "Reductive reassortment", as used herein, refers to the increase in molecular diversity that is accrued through deletion (and/or insertion) events that are mediated by repeated sequences.

[0080] The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence," "comparison window," "sequence identity," "percentage of sequence identity," and "substantial identity."

[0081] A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing, or may comprise a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity.

[0082] "Repetitive Index (RI)", as used herein, is the average number of copies of the quasi-repeated units contained in the cloning vector.

[0083] The term "restriction site" refers to a recognition sequence that is necessary for the manifestation of the action of a restriction enzyme, and includes a site of catalytic cleavage. It is appreciated that a site of cleavage may or may not be contained within a portion of a restriction site that comprises a low ambiguity sequence (i.e. a sequence containing the principal determinant of the frequency of occurrence of the restriction site). Thus, in many cases, relevant restriction sites contain only a low ambiguity sequence with an internal cleavage site (e.g. G/AATTC in the EcoR I site) or an immediately adjacent cleavage site (e.g./CCWGG in the EcoR II site). In other cases, relevant restriction enzymes [e.g. the Eco57 I site or CTGAAG(16/14)] contain a low ambiguity sequence (e.g. the CTGAAG sequence in the Eco57 I site) with an external

cleavage site (e.g. in the N.sub.16 portion of the Eco57 I site). When an enzyme (e.g. a restriction enzyme) is said to "cleave" a polynucleotide, it is understood to mean that the restriction enzyme catalyzes or facilitates a cleavage of a polynucleotide.

[0084] In a non-limiting aspect, a "selectable polynucleotide" is comprised of a 5' terminal region (or end region), an intermediate region (i.e. an internal or central region), and a 3'terminal region (or end region). As used in this aspect, a 5' terminal region is a region that is located towards a 5' polynucleotide terminus (or a 5' polynucleotide end); thus it is either partially or entirely in a 5' half of a polynucleotide. Likewise, a 3' terminal region is a region that is located towards a 3' polynucleotide terminus (or a 3' polynucleotide end); thus it is either partially or entirely in a 3' half of a polynucleotide. As used in this non-limiting exemplification, there may be sequence overlap between any two regions or even among all three regions.

[0085] The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. This "substantial identity", as used herein, denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence having at least 80 percent sequence identity, preferably at least 85 percent identity, often 90 to 95 percent sequence identity, and most commonly at least 99 percent sequence identity as compared to a reference sequence of a comparison window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison.

[0086] As known in the art "similarity" between two enzymes is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one enzyme to the sequence of a second enzyme. Similarity may be determined by

procedures which are well-known in the art, for example, a BLAST program (Basic Local Alignment Search Tool at the National Center for Biological Information).

[0087] The members of a pair of molecules (e.g., an antibody-antigen pair or a nucleic acid pair) are said to "specifically bind" to each other if they bind to each other with greater affinity than to other, non-specific molecules. For example, an antibody raised against an antigen to which it binds more efficiently than to a non-specific protein can be described as specifically binding to the antigen. (Similarly, a nucleic acid probe can be described as specifically binding to a nucleic acid target if it forms a specific duplex with the target by base pairing interactions (see above).)

[0088] "Specific hybridization" is defined herein as the formation of hybrids between a first polynucleotide and a second polynucleotide (e.g., a polynucleotide having a distinct but substantially identical sequence to the first polynucleotide), wherein substantially unrelated polynucleotide sequences do not form hybrids in the mixture.

[0089] The term "specific polynucleotide" means a polynucleotide having certain end points and having a certain nucleic acid sequence. Two polynucleotides wherein one polynucleotide has the identical sequence as a portion of the second polynucleotide but different ends comprises two different specific polynucleotides.

[0090] "Stringent hybridization conditions" means hybridization will occur only if there is at least 90% identity, preferably at least 95% identity and most preferably at least 97% identity between the sequences. See Sambrook et al., Molecular Cloning: a laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory Press, 1989, which is hereby incorporated by reference in its entirety.

[0091] Also included in the disclosure are polypeptides having sequences that are "substantially identical" to the sequence of an enzyme polypeptide. A "substantially identical" amino acid sequence is a sequence that differs from a reference sequence only by conservative amino acid substitutions, for example, substitutions of one amino acid for another of the same class (e.g., substitution of one hydrophobic amino acid, such as isoleucine, valine, leucine, or methionine, for another, or substitution of one polar amino acid for another, such as substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine).

[0092] Additionally a "substantially identical" amino acid sequence is a sequence that differs from a reference sequence or by one or more non-conservative substitutions,

deletions, or insertions, particularly when such a substitution occurs at a site that is not the active site of the molecule, and provided that the polypeptide essentially retains its behavioural properties. For example, one or more amino acids can be deleted from an enzyme polypeptide, resulting in modification of the structure of the polypeptide, without significantly altering its biological activity. For example, amino- or carboxyl-terminal amino acids that are not required for enzyme biological activity can be removed. Such modifications can result in the development of smaller active enzyme polypeptides.

[0093] The present disclosure provides a "substantially pure enzyme". The term "substantially pure enzyme" is used herein to describe a molecule, such as a polypeptide (e.g., an enzyme polypeptide, or a fragment thereof) that is substantially free of other proteins, lipids, carbohydrates, nucleic acids, and other biological materials with which it is naturally associated. For example, a substantially pure molecule, such as a polypeptide, can be at least 60%, by dry weight, the molecule of interest. The purity of the polypeptides can be determined using standard methods including, e.g., polyacrylamide gel electrophoresis (e.g., SDS-PAGE), column chromatography (e.g., high performance liquid chromatography (HPLC)), and amino-terminal amino acid sequence analysis.

[0094] As used herein, "substantially pure" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other individual macromolecular species in the composition), and preferably substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 to 90 percent of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species. Solvent species, small molecules (<500 Daltons), and elemental ion species are not considered macromolecular species.

[0095] The term "treating" includes: (1) preventing or delaying the appearance of clinical symptoms of the state, disorder or condition developing in an animal that may be afflicted with or predisposed to the state, disorder or condition but does not yet experience or display clinical or subclinical symptoms of the state, disorder or condition;

(2) inhibiting the state, disorder or condition (i.e., arresting, reducing or delaying the development of the disease, or a relapse thereof in case of maintenance treatment, of at least one clinical or subclinical symptom thereof); and/or (3) relieving the condition (i.e., causing regression of the state, disorder or condition or at least one of its clinical or subclinical symptoms). The benefit to a patient to be treated is either statistically significant or at least perceptible to the patient or to the physician.

[0096] As used herein, the term "variable segment" refers to a portion of a nascent peptide which comprises a random, pseudorandom, or defined kernal sequence. A "variable segment" refers to a portion of a nascent peptide which comprises a random pseudorandom, or defined kernal sequence. A variable segment can comprise both variant and invariant residue positions, and the degree of residue variation at a variant residue position may be limited: both options are selected at the discretion of the practitioner. Typically, variable segments are about 5 to 20 amino acid residues in length (e.g., 8 to 10), although variable segments may be longer and may comprise antibody portions or receptor proteins, such as an antibody fragment, a nucleic acid binding protein, a receptor protein, and the like.

[0097] The term "variant" refers to polynucleotides or polypeptides of the disclosure modified at one or more base pairs, codons, introns, exons, or amino acid residues (respectively) of a wild-type protein parent molecule. Variants can be produced by any number of means including methods such as, for example, error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, in vivo mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-specific mutagenesis, gene reassembly, saturation mutagenesis and any combination thereof. Techniques for producing variant proteins having reduced activity compared to the wild-type protein at a normal physiological condition of e.g., one or more conditions of temperature, pH, osmotic pressure, osmolality, oxidation and electrolyte concentration; and enhanced activity at an aberrant condition, are disclosed herein. Variants may additionally be selected for the properties of enhanced chemical resistance, and proteolytic resistance, compared to the wild-type protein.

[0098] As used herein, the term "wild-type" means that the polynucleotide does not comprise any mutations. A "wild type protein", "wild-type protein", "wild-type biologic

protein", or "wild type biologic protein", refers to a protein which can be isolated from nature that will be active at a level of activity found in nature and will comprise the amino acid sequence found in nature. The terms "parent molecule" and "target protein" also refer to the wild-type protein.

[0099] The term "working", as in "working sample", for example, is simply a sample with which one is working. Likewise, a "working molecule", for example is a molecule with which one is working.

[00100] The present disclosure is directed to methods of engineering or evolving proteins to generate new molecules that are reversibly or irreversibly inactivated at the wild type condition, but active at non-normal conditions at the same or equivalent level as the wild-type condition. These new proteins are referred to as "Mirac" proteins herein. Mirac proteins are particularly valuable for development of novel therapeutics that are active for short or limited periods of time within the host. This is particularly valuable where extended operation of the protein at the given dose would be harmful to the host, but where limited activity is required to perform the desired therapy. Examples of beneficial applications include topical or systemic treatments at high dose, as well as localized treatments in high concentration. Inactivation under the physiological condition can be determined by a combination of the dosing and the rate of inactivation of the protein. This condition based inactivation is especially important for enzyme therapeutics where catalytic activity cause substantial negative effects in a relatively short period of time.

[00101] The present disclosure is also directed to methods of engineering or evolving proteins to generate new molecules that are different from wild type molecules in that they are reversibly or irreversibly activated or inactivated over time, or activated or inactivated only when they are in certain microenvironments in the body, including in specific organs in the body (such as the bladder or kidney).

[00102] Target Wild-type Proteins

[00103] Any therapeutic protein can serve as a target protein, or wild-type protein, for production of a conditionally active biologic protein. In one aspect, the target protein is a wild-type enzyme. Currently used therapeutic enzymes include urokinase and streptokinase, used in the treatment of blood clots; and hyaluronidase, used as an adjuvant to improve the absorption and dispersion of other drugs. In one aspect, the

wild-type protein selected for generation of a conditionally active biologic protein can be a currently used therapeutic enzyme, in order to avoid or minimize deleterious side effects associated with the wild-type protein or enzyme. Alternatively, an enzyme not in current usage as a therapeutic can be selected for generation of a conditionally active biologic protein. Certain non-limiting examples will be discussed in further detail below.

[00104] Therapeutic proteins are those which can be used in medicine either alone or in conjunction with other therapies to treat various diseases or medical conditions. The conditionally active biologic proteins of the disclosure could be appropriate for use in one or more indications including the treatment of circulatory disorders, arthritis, multiple sclerosis, autoimmune disorders, cancer, dermatologic conditions and use in various diagnostic formats. Depending on the protein and indication, the conditionally active biologic enzyme protein could be administered in parenteral, topical or oral formulations as discussed below.

[00105] Circulatory Disorders-Thrombosis and thrombolytic therapy.

[00106] A thrombus (blood clot) is defined as a solid mass derived from blood constituents that forms in the circulatory system. The thrombus is formed by a series of events involving blood coagulation factors, platelets, red blood cells, and interactions with the vessel wall. A platelet is an intravascular aggregation of platelets, fibrin and entrapped blood cells which can cause vascular obstruction. By obstructing or blocking blood flow, the thrombus deprives downstream tissue of oxygen supply. Fragments (emboli) of the thrombus may break away and obstruct smaller vessels. Arterial thrombus formation is precipitated by any of a variety of factors including an underlying stenosis-atherosclerosis, a low flow state-cardiac function, hypercoagubility as in cancer or a coagulation factor deficiency, or a foreign body such as a stent or catheter. A thrombus leading to arterial ischemia can result in limb or tissue injury, acute myocardial infarction (AMI), stroke, amputation, or bowel infarction. Major causes of morbidity and mortality are the formation of arterial thrombi (coronary arterial thrombi and cerebral arterial thrombi) and pulmonary thrombi. Venous thrombus formation can occur due to endothelial injury such as trauma, stasis due to e.g. immobility, or hypercoagulability, but atherosclerosos is not a factor. Treatment strategies include mechanical thrombectomy, pharmacomechanical thrombectomy and thrombolysis. Thrombotic therapy is used to minimize formation and aid in removal of thrombi.

[00107] Thrombotic therapy includes the use of antiplatelet agents which inhibit platelet activation, anticoagulant therapies, and/or thrombolytic therapy to degrade blood clots. Examples of antiplatelets include aspirin, dipyridamole, and ticlopidine. Examples of anticoagulants include heparin, warfarin, hirudin, and activated human protein C. Examples of thrombolytics include tissue plasminogen activator (tPA)/tPA variants, urokinase and streptokinase. The thrombolytics display a catalytic mode of action.

[00108] Thrombolytic therapy in acute myocardial infarction is well established. Use of thrombolytic agents has become standard emergency treatment. Although effective, these products achieve complete reperfusion in only about 50% of patients and side effects include risk of hemorrhage (in particular intracranial bleeding) as well as hypertension. The degradation of blood clots from a damaged or diseased vessel is termed "fibrinolysis" or the "fibrinolytic process". Fibrinolysis is a proteolytic process, by a plasminogen activator which activates the protein plasminogen, thereby forming plasmin. Plasmin proteolytically degrades the fibrin strands of the blood clot to dissolve the clot. Fibrin specific plasminogen activators include tissue plasminogen activators or variants. Non-specific plasminogen activators can include streptokinase and urokinase.

[00109] Certain commonly used thrombolytic therapies utilize one of several available tissue plasminogen activator (tPA) variants. For example, tPA based product variants which have been previously approved for use are Alteplase (rt-PA), Reteplase (r-PA) and Tenecteplase (TNK). Approved uses for tPA variants include, for example, acute myocardial infarction for the improvement of ventricular function following AMI, the reduction of incidence of congestive heart failure, and reduction of mortality associated with AMI, management of ischemic stroke in adults for improving neurological recovery and reducing incidence of disability, management of acute massive pulmonary embolism in adults for the lysis of acute pulmonary emboli, and for the lysis of pulmonary emboli accompanied by unstable hemodynamics.

[00110] Another commonly used thrombolytic therapy utilizes urokinase. Urokinase is a standard lytic agent used in the management of peripheral vascular disease.

[00111] Streptokinase is a protein secreted by several species of streptococci that can bind and activate human plasminogen. Complexes of streptokinase with human plasminogen can hydrolytically activate other unbound plasminogen by activating

through bond cleavage to produce plasmin. The usual activation of plasminogen is through the proteolysis of the Arg561-Val562 bond. The amino group of Val562 then forms a salt-bridge with Asp740, which causes a conformational change to produce the active protease plasmin. Plasmin is produced in the blood to break down fibrin, the major constituent of blood clots.

[00112] Streptokinase is used as an effective clot-dissolving medication in some cases of myocardial infarction (heart attack), pulmonary embolism (lung blood clots), and deep venous thrombosis (leg blood clots). Streptokinase belongs to a group of medications called fibrinolytics. Streptokinase is given as soon as possible after the onset of a heart attack to dissolve clots in the arteries of the heart wall and reduce damage to the heart muscle. Streptokinase is a bacterial product, so the body has the ability to build up immunity against the protein. Therefore, it is recommended that this product should not be given again after four days from the first administration, as it may not be as effective and cause an allergic reaction. For this reason it is usually given only after a first heart attack, and further thrombotic events are typically treated with tissue plasminogen activator (TPA). Streptokinase is also sometimes used to prevent post-operative adhesions.

[00113] Side effects of streptokinase include bleeding (major and minor), hypotension, and respiratory depression as well as possible allergic reaction. In addition, anticoagulants, agents that alter platelet function (e.g. aspirin, other NSAIDs, dipyridamole) may increase risk of bleeding.

[00114] Administration of the thrombolytics is generally by infusion or by bolus intravenous dose; or by a mechanical infusion system. Adverse effects can include serious intracranial, gastrointestinal, retroperitoneal, or pericardial bleeding. If bleeding occurs the administration must be discontinued immediately.

[00115] In certain embodiments of the disclosure, tPA, streptokinase or urokinase is selected as the target, or wild-type protein.

[00116] In one embodiment, the methods of the disclosure are used to select for a conditionally active recombinant or synthetic streptokinase variant with high activity at aberrant temperature conditions below normal physiological conditions; and substantial deactivation or inactivation at normal physiological conditions (e.g. 37 degrees C). In one aspect, the aberrant temperature condition is room temperature, e.g. 20-25 degrees C.

In another aspect, the disclosure provides a method of treating a stroke or heart attack, the method comprising administering a high dose of the conditionally active streptokinase variant to stroke or heart attack victims in order to clear clots, yet allow for rapid inactivation of the streptokinase variant to avoid excessive bleeding.

[00117] Circulatory Disorders-Renin/Angiotensin

[00118] The renin-angiotensin system is a hormone system that regulates blood pressure and water (fluid) balance. The kidneys secrete renin when the blood volume is low. Renin is an enzyme which hydrolyzes angiotensinogen secreted from the liver into the peptide angiotensin I. Angiotensin I is further cleaved in the lungs by endothelial-bound angiotensin converting enzyme (ACE) into angiotensin II, the most vasoactive peptide. Angiotensin II causes the blood vessels to constrict, resulting in increased blood pressure. However, angiotensin II also stimulates the secretion of the hormone aldosterone from the adrenal cortex. Aldosterone causes the tubules of the kidneys to increase the resorption of sodium and water. This increases the volume of fluid in the body, which also increases blood pressure. An over-active renin-angiotensin system leads to vasoconstriction and retention of sodium and water. These effects lead to hypertension. There are many drugs which interrupt different steps in this system to lower blood pressure. These drugs are one of the main ways to control high blood pressure (hypertension), heart failure, kidney failure, and harmful effects of diabetes.

[00119] Hypovolemic shock is an emergency condition in which severe blood and/or fluid loss makes the heart unable to adequately perfuse the body's cells with oxygenated blood. Blood loss can be from trauma, injuries and internal bleeding. The amount of circulating blood may drop due to excessive fluid loss from burns, diarrhea, excessive perspiration or vomiting. Symptoms of hypovolemic shock include anxiety, cool clammy skin, confusion, rapid breathing, or unconsciousness. Examination shows signs of shock including low blood pressure, low body temperature, and rapid pulse, which may be weak or thready. Treatment includes intravenous fluids; blood or blood products; treatment for shock; and medication such as dopamine, dobutamine, epinephrine and norepinephrine to increase blood pressure and cardiac output.

[00120] In one embodiment, the disclosure provides a method of selecting for a conditionally active recombinant renin variant to be reversibly deactivated at normal physiological temperature, but reactivated at the aberrant lower temperatures in a patient

with hypovolemic shock. The conditionally active protein can be used to treat hypovolemic shock to help increase the volume of fluid in the body, and increase blood pressure.

[00121] Circulatory Disorders-Reynaud's phenomenon

[00122] Reynaud's phenomenon (RP) is a vasospastic disorder causing discoloration of the fingers, toes and occasionally other extremities. Emotional stress and cold are classic triggers of the phenomenon. When exposed to cold temperatures, the extremities lose heat. The blood supply to fingers and toes is normally slowed to preserve the body's core temperature. Blood flow is reduced by the narrowing of small arteries under the skin of the extremities. Stress causes similar reaction to cold in the body. In Reynaud's, the normal response is exaggerated. The condition can cause pain, discoloration, and sensations of cold and numbness. The phenomenon is the result of vasospasms that decrease the blood supply to the respective regions. In Reynaud's disease (Primary Raynaud's phenomenon), the disease is idiopathic. In Raynaud's syndrome (Secondary Reynaud's), the phenomenon is caused by some other instigating factor. Measurement of hand-temperature gradients is one tool to distinguish between the primary and secondary forms. The primary form can progress to the secondary form, and in extreme cases, the secondary form can progress to necrosis or gangrene of the fingertips.

[00123] Raynaud's phenomenon is an exaggeration of responses to cold or emotional stress. Primary RP is essentially mediated by microvascular vasospasm. Hyperactivation of the sympathetic system causes extreme vasoconstriction of the peripheral blood vessels, leading to hypoxia. Chronic, recurrent cases can result in atrophy of the skin, subcutaneous tissue, and muscle. It can also rarely result in ulceration and ischemic gangrene.

[00124] Traditional treatment options for Reynaud's phenomenon include prescription medication that dilates blood vessels and promotes circulation. These include calcium channel blockers, such as nifedipine or diltiazem; alpha blockers, which counteract the actions of norepinephrine, a hormone that constricts blood vessels, such as prazosin or doxazosin; and vasodilators, to relax blood vessels, such as nitroglycerin cream, or the angiotensin II inhibitor losartan, sildenafil, or prostaglandins. Fluoxetine, a selective serotonin reuptake inhibitor and other antidepressant medications may reduce the

frequency and severity of episodes due to psychological stressors. These drugs may cause side effects such as headache, flushing and ankle edema. A drug may also lose effectiveness over time.

[00125] The regulation of cutaneous vasoconstriction and vasodilation involves altered sympathetic nerve activity and a number of neuronal regulators, including adrenergic and non-adrenergic, as well as REDOX signaling and other signaling such as the RhoA/ROCK pathway. Vasoconstriction of vascular smooth muscle cells (vSMC) in the skin is thought to be activated by norepinephrine mediated by alpha1 and alpha2 adrenoreceptors. Alpha2C-ARs translocate from the trans Golgi to the cell surface of the vSMC where they respond to stimulation and signaling of these responses involves the RhoA/Rhokinase (ROCK) signaling pathway. Cold stimulation in cutaneous arteries results in the immediate generation of reactive oxygen species (ROS) in the vSMC mitochondria. ROS are involved in the REDOX signaling through the RhoA/ROCK pathway. RhoA is a GTP-binding protein whose role is the regulation of actin-myosin dependent processes such as migration and cell contraction in vSMC. Non-adrenergic neuropeptides with known function in vasculature with possible involvement in RP include calcitonin gene-related peptide (CGRP), Substance P (SP), Neuropeptide Y (NPY), and vasoactive intestinal peptide(VIP). Fonseca et al., 2009, "Neuronal regulators and vascular dysfunction in Raynaud's phenomenon and systemic sclerosis", Curr. Vascul. Pharmacol. 7:34-39.

[00126] New therapies for RP include alpha-2c adrenergic receptor blockers, protein tyrosine kinase inhibitors, Rho-kinase inhibitors and calcitonin gene related peptide.

[00127] Calcitonin gene related peptide (CGRP) is a member of the calcitonin family of peptides and exists in two forms; alpha-CGRP and beta-CGRP. Alpha-CGRP is a 37-amino acid peptide formed from alternative splicing of the calcitonin/CGRP gene. CGRP is one of the most abundant peptides produced in peripheral and central neurons. It is a potent peptide vasodilator and can function in the transmission of pain. Migraine is a common neurological disorder that is associated with an increase in CGRP levels. CGRP dilates intracranial blood vessels and transmits vascular nociception. CGRP receptor antagonists have been tested as treatments for migraines. Arulmani et al., 2004, "Calcitonin gene-related peptide and it role in migraine pathophysiology", Eur. J. Pharmacol. 500(1-3): 315-330. At least three receptor subtypes have been identified and

CGRP acts through G protein-coupled receptors whose presence and changes in function modulate the peptide's effect in various tissues. CGRP's signal transduction through the receptors is dependent on two accessory proteins: receptor activity modifying protein 1 (RAMP1) and receptor component protein (RCP). Ghatta 2004, Calcitonin gene-related peptide: understanding its role. Indian J. Pharmacol. 36(5): 277-283. One study of the effects of intravenous infusion of three vasodilators: endothelium-dependent vasodilator adenosine triphosphate (ATP), endothelium-independent vasodilator prostacyclin (epoprostenol; PGI2), and CGRP, to patients with Reynaud's phenomenon, and a similar number of age and sex matched controls, using laser Doppler flowmetry (LDF) showed CGRP induced flushing of the face and hands by a rise in skin blood flow in the Reynaud's patients, whereas in controls CGRP caused flushing only in the face. PGI2 caused similar rises in blood flow in hands and face of both groups. ATP did not cause any significant changes in blood flow in hands or face of the patients, but increased blood flow to the face of controls. Shawket et al., 1989, "Selective suprasensitivity to calcitonin-gene-related peptide in the hands in Reynaud's phenomenon". The Lancet, 334(8676):1354-1357. In one aspect, the wild-type protein target molecule is CGRP.

[00128] In one embodiment, the disclosure provides methods of selecting for conditionally active recombinant protein variants of proteins associated with Reynaud's syndrome to be reversibly deactivated at normal physiological temperature, but reactivated at the aberrant lower temperatures in digits. The conditionally active proteins can be used to treat Reynaud's phenomenon, to prevent or reduce loss of digit function due to low circulation.

[00129] Circulatory disorders-Vasopressin

[00130] Arginine vasopressin (AVP, vasopressin, antidiuretic hormone (ADH)) is a peptide hormone found in most mammals that controls reabsorption of molecules in the tubules of the kidney by affecting tissue permeability. One of the most important roles of vasopressin is to regulate water retention in the body. In high concentrations it raises blood pressure by introducing moderate vasoconstriction. Vasopressin has three effects which result in increased urine osmolality (increased concentration) and decreased water excretion. First, vasopressin causes an increase in the permeability of water of the collecting duct cells in the kidney allowing water resorption and excretion of a smaller volume of concentrated urine (antidiuresis). This occurs through insertion of aquaporin-

2 water channels into the apical membrane of the collecting duct cells. Secondly, vasopressin causes an increase in the permeability of the inner medullary portion of the collecting duct to urea, allowing increased reabsorption urea into the medullary interstitium. Thirdly, vasopressin causes stimulation of sodium and chloride reabsorption in the thick ascending limb of the loop of Henle by increasing the activity of the Na^+-K^+-$2Cl^-$-cotransporter. NaCl reabsorption drives the process of countercurrent multiplication, which furnishes the osmotic gradient for aquaporin mediated water reabsorption in the medullary collecting ducts.

[00131] The hypertonic interstitial fluid surrounding the collecting ducts of the kidney provides a high osmotic pressure for the removal of water. Transmembrane channels made of proteins called aquaporins are inserted in the plasma membrane greatly increasing its permeability to water. When open, an aquaporin channel allows 3 billion molecules of water to pass through each second. Insertion of aquaporin-2 channels requires signaling by vasopressin. Vasopressin binds to receptors (called V2 receptors) on the basolateral surface of the cells of the collecting ducts. Binding of the hormone triggers a rising level of cAMP within the cell. This "second messenger" initiates a chain of events culminating in the insertion of aquaporin-2 channels in the apical surface of the collecting duct cells. The aquaporins allow water to move out of the nephron, increasing the amount of water re-absorbed from the forming urine back into the bloodstream.

[00132] The main stimulus for the release of vasopressin from the pituitary gland is increased osmolality of the blood plasma. Anything that dehydrates the body, such as perspiring heavily increases the osmotic pressure of the blood and turns on the vasopressin to V2 receptor to aquaporin-2 pathway. As a result, as little as 0.5 liters/day of urine may remain of the original 180 liters/day of nephric filtrate. The concentration of salts in urine can be as high as four times that of the blood. If the blood should become too dilute, as would occur from drinking a large amount of water, vasopressin secretion is inhibited and the aquaporin-2 channels are taken back into the cell by endocytosis. The result is that a large volume of watery urine is formed with a salt concentration as little as one-fourth of that of the blood.

[00133] Decreased vasopressin release or decreased renal sensitivity to AVP leads to diabetes insipidus, a condition featuring hypernatremia (increased blood sodium concentration), polyuria (excess urine production), and polydipsia (thirst).

[00134] High levels of AVP secretion (syndrome of inappropriate antidiuretic hormone, SIADH) and resultant hyponatremia (low blood sodium levels) occurs in brain diseases and conditions of the lungs (Small cell lung carcinoma). In the perioperative period, the effects of surgical stress and some commonly used medications (e.g., opiates, syntocinon, anti-emetics) lead to a similar state of excess vasopressin secretion. This may cause mild hyponatremia for several days.

[00135] Vasopressin agonists are used therapeutically in various conditions, and its long-acting synthetic analogue desmopressin is used in conditions featuring low vasopressin secretion, as well as for control of bleeding (in some forms of von Willebrand disease) and in extreme cases of bedwetting by children. Terlipressin and related analogues are used as vasoconstrictors in certain conditions. Vasopressin infusion has been used as a second line of management in septic shock patients not responding to high dose of inotropes (e.g., dopamine or norepinephrine). A vasopressin receptor antagonist is an agent that interferes with action at the vasopressin receptors. They can be used in the treatment of hyponatremia.

[00136] In one embodiment, the disclosure provides methods to select for conditionally active biologic recombinant or synthetic protein variants of proteins involved in the vasopressin response to be reversibly deactivated at normal physiological osmotic pressure, but reactivated at aberrant osmotic pressure in the blood. In another embodiment, variants of proteins involved in the vasopressin response are activated under hyponatremic conditions, but inactivated at normal serum sodium concentrations. In one aspect, hyponatremic conditions are those where serum sodium < 135 mEq/L.

[00137] Cancer-Angiostatin

[00138] Angiostatin is a naturally occurring protein in several animal species. It acts as an endogenous angiogenesis inhibitor (i.e., it blocks the growth of new blood vessels). Angiostatin is able to suppress tumor cell growth and metastasis through inhibition of endothelial cell proliferation and migration. Angiostatin is a 38 kD fragment of plasmin (which is itself a fragment of plasminogen). Angiostatin comprises the kringles 1 to 3 of plasminogen. Angiostatin is produced, for example, by autolytic cleavage of

plasminogen, involving extracellular disulfide bond reduction by phosphoglycerate kinase. Angiostatin can also be cleaved from plasminogen by different matrix metalloproteinases (MMPs) including MMP2, MMP12 and MMP9, and serine proteases (neutrophil elastase, prostate-specific antigen (PSA)). In vivo angiostatin inhibits tumor growth and keeps experimental metastasis in a dormant state. Angiostatin is elevated in animals with primary tumors and other inflammatory and degenerative diseases.

[00139] Angiostatin is known to bind many proteins including angiomotin and endothelial cell surface ATO synthase, but also integrins, annexin II, C-met receptor, NG2-proteoglycans, tissue-plasminogen activator, chondroitin sulfate glycoproteins, and CD26. One study shows that IL-12, a TH1 cytokine with potent antiangiogenic activity, is a mediator of angiostatin's activity. Albin"., J. Translational Medicine. Jan. 4, 2009, 7:5. Angiostatin binds and inhibits ATP synthase on the endothelial cell surface. ATP synthase also occurs on the surface of a variety of cancer cells. Tumor cell surface ATP synthase was found to be more active at low extracellular pH; a hallmark of tumor microenvironment. Angiostatin was found to affect tumor cell surface ATP synthase activity at acidic extracellular pH (pHe). At low extracellular pH, angiostatin was directly anti-tumorigenic. At low pH, angiostatin and anti-beta-subunit antibody induce intracellular acidification of A549 cancer cells, as well as a direct toxicity that is absent in tumor cells with low levels of extracellular ATP synthase. It was hypothesized that the mechanism of tumor cytotoxicity is dependent on intracellular pH deregulation due to inhibition of cell surface ATP synthase. Chi and Pizzo, "Angiostatin is directly cytotoxic to tumor cells at low extracellular pH: a mechanism dependent on cell surface-associated ATP synthase", Cancer Res., 2006, 66(2): 875-82.

[00140] In one embodiment, the disclosure provides a method for identification of conditionally active angiostatin variant which is less active than wild-type angiostatin at normal physiological blood pH, but exhibits enhanced activity at low pH. Low pH is defined as being less than normal physiological pH. In one aspect, low pH is less than about pH 7.2. In a particular aspect, low pH is about pH 6.7.

[00141] In one aspect, the conditionally active angiostatin variant can be formulated and utilized as an anticancer agent.

[00142] Enhancement of tissue permeability-Hyaluronidase

[00143] Hyaluronidases are a family of enzymes that degrade hyaluronic acid. By catalyzing the degradation of hyaluronic acid, a major constituent of the interstitial barrier, hyaluronidase lowers the viscosity of hyaluronic acid, thereby increasing tissue permeability. It is used in medicine in conjunction with drugs to speed their dispersion and delivery. The most common application is in ophthalmic surgery, used in combination with local anesthetics. Animal derived hyaluronidase include Hydase™ (PrimaPharm Inc.; Akorn Inc.), Vitrase (ISTA Pharmaceuticals) and Amphadase (Amphastar Pharmaceuticals). Human Recombinant Hyaluronidase is currently approved as an adjuvant to increase absorption of other drugs; hypodermocyclis (subcutaneous infusion of fluids); adjunct in subcutaneous urography to improve resorption of radioopaque agents. (Hylenex; Halozyme Therapeutics, Inc.; Baxter Healthcare Corp.) In one embodiment, hyaluronidase can serve as a wild-type protein (parent molecule) for preparation of a conditionally active biologic protein. Hyaluronidases may play a role in cancer metastasis and perhaps angiogenesis; therefore overexposure to these enzymes could be deleterious. In one aspect, a conditionally active biologic hyaluronidase protein would become irreversibly or reversibly inactivated at normal physiological temperature, but would be active at a level equal to or exceeding that of the wild-type hyaluronidase at certain temperature ranges below that of normal physiological temperature.

[00144] Autoimmune diseases-Conditionally active biological response modifiers

[00145] Rheumatoid arthritis is an autoimmune disease characterized by aberrant immune mechanisms that lead to joint inflammation and swelling with progressive destruction of the joints. RA can also affect the skin, connective tissue and organs in the body. Traditional treatment includes non-steroidal anti-inflammatory drugs (NSAIDS), COX-2 inhibitors, and disease-modifying anti-rheumatic drugs (DMARDS) such as methotrexate. None of the traditional treatment regimes is ideal, especially for long term use.

[00146] Biological response modifiers, which target inflammatory mediators, offer a relatively new approach to the treatment of rheumatoid arthritis and other autoimmune diseases. Such biological response modifiers include antibodies, or active portions thereof, against various inflammatory mediators such as IL-6, IL-6 receptor, TNF-alpha, IL-23 and IL-12.

[00147] Some of the first biological response modifiers were medications targeting tumor necrosis factor alpha (TNF-a), a pro-inflammatory cytokine involved in the pathogenesis of RA. Several anti-TNF-alpha medications are currently marketed for the treatment of RA. For example, Enbrel® (etanercept, Amgen) is a TNF-alpha blocker. Etanercept is a dimeric fusion protein consisting of the extracellular ligand-binding portion of the human 75 kilodalton (p75) tumor necrosis factor receptor (TNFR) linked to the Fc portion of human IgG1. The Fc component of etanercept contains the CH2 domain, the CH3 domain and hinge region, but not the CH1 domain of IgG1. Etanercept is produced in a Chinese hamster ovary (CHO) mammalian cell expression system. It consists of 934 amino acids and an apparent molecular weight of about 150 kilodaltons. Enbrel® is used to treat rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis and plaque psoriasis. Serious side effects of Enbrel® include infections including tuberculosis, fungal infection, bacterial or viral infection due to opportunistic pathogens. Sepsis can also occur. Lymphoma, or other malignancies have also been reported.

[00148] Remicade® (infliximab) is a chimeric anti-TNF-alpha IgGk1 monoclonal antibody composed of human constant and murine variable regions. Remicade is administered by intravenous injection and is used to treat rheumatoid arthritis, psoriasis, Crohn's disease, ulcerative colitis, and ankylosing spondylitis. Side effects of Remicade include serious infection or sepsis, and rarely certain T-cell lymphomas. Other side effects include hepatotoxicity, certain severe hematologic events, hypersensitivity reactions and certain severe neurological events.

[00149] Other biological response modifiers include humanized anti-interleukin-6 (IL-6) receptor antibodies. IL-6 is a cytokine that contributes to inflammation, swelling and joint damage in RA. One humanized anti-IL-6 receptor antibody, Actemra (tocilizumab, Roche), is approved by the FDA and European Commission to treat adult patients with rheumatoid arthritis. Actemra is also approved in Japan for treatment of RA and juvenile idiopathic arthritis (sJIA). Phase III studies showed that treatment with Actemra as a monotherapy, or a combination with MTX or other DMARDs, reduced signs and symptoms of RA compared with other therapies. Actemra is a humanized anti-human IL-6 receptor monoclonal antibody that competitively blocks the binding of IL-6 to its receptor. Thus, it inhibits the proliferative effects of IL-6, which lead to synovial thickening and pannus formation in RA. Serious side effects of Actemra, include serious infections and hypersensitivity reactions including a few cases of anaphylaxis. Other side

effects include upper respiratory tract infection, headache, nasopharyngitis, hypertension and increased ALT.

[00150] Another common autoimmune disease is psoriasis. An overactive immune system can lead to high levels of IL-12 and IL-23, two cytokine proteins that have been found in psoriatic skin plaques. IL-12 and IL-23 are involved in inflammatory and immune responses such as natural killer cell activation and CD4+ T-cell differentiation and activation.

[00151] One treatment for moderate or severe psoriasis involves subcutaneous injection of Stelara™ (ustekinumab, Centocor Ortho Biotech, Inc.) a humanized IgG1k monoclonal antibody against the p40 subunit of the IL-12 and IL-23 cytokines. Stelara has been shown to provide relief from certain symptoms associated with psoriatic plaques, such as plaque thickness, scaling and redness. The formulation for Stelara includes L-histidine and L-histidine monohydrochloride monohydrate, polysorbate 80, and sucrose in aqueous solution. Use of Stelara™ affects the immune system, and may increase chances of infection, including tuberculosis, and infections caused by bacteria, fungi or viruses; as well as increase the risk of certain types of cancer.

[00152] Side effects of the biological response modifiers are significant and are caused in part by high levels following injection into patients renders patients susceptible to serious infection or death. This is a major side effect associated with this important class of drugs. One challenge is avoiding the high initial level of activity from the dose of antibody required to provide a long treatment effect following injection.

[00153] In one embodiment, the disclosure provides a method to prepare a conditionally active biological response mediator, or fragment thereof, that avoids the high level of activity from the dose of antibody required to provide a long treatment effect following injection. The method of the disclosure can be used to design antibodies to inflammatory mediators such as IL-6, IL-6 receptor, TNF-alpha, IL-23 and IL-12 that are inactive at dosing conditions such as room temperature, but slowly refold (reversibly or irreversibly) at body temperature. These antibodies or fragments thereof would be inactive upon initial injection, but would refold or reactivate over a period of hours to days when exposed to blood following injection. This could allow higher dosing, and a longer half-life (or periods between dosing) with reduced side effects.

[00154] In one aspect, the disclosure provides a method for preparation of a conditionally active antibody to an inflammatory mediator, or fragment thereof, that is inactive at dosing conditions such as room temperature, but slowly refold (reversibly or irreversibly) at body temperature. The method comprises the following steps. Selecting an inflammatory mediator. Screening to identify an antibody to the inflammatory mediator via hybridoma. Humanizing the anti- inflammatory mediator antibody. Evolving the anti-inflammatory mediator antibody and screening differentially for binding at two or more conditions, for example, two or more temperature conditions such as at room temperature and at 37°C or higher; selecting for mutations that are inactive at a first condition, relative to wild type, but show increased activity (e.g. binding) relative to the wild type antibody activity (binding) at a second condition. The up-mutants identified in the heavy and light changes are then recombined within the heavy and light chains, as well as through combinatorial association of the heavy and light chains. Screening of these recombined heavy and light chains is repeated at the two conditions, for example, room temperature and at 37°C or higher. In addition, the recombined antibodies or fragments can be screened for activity and stability under storage and physiological conditions.

[00155] Alternatively, the wild-type antibody to the inflammatory mediator is a known antibody or variant or active fragment thereof.

[00156] In one aspect, the first and second conditions are selected from conditions of pH, osmotic pressure, osmolality, oxidation and electrolyte concentration. In another aspect, the inflammatory mediator is selected from IL-6, IL-6 receptor, TNF-alpha, IL-23 and IL-12.

[00157] In another aspect, the disclosure provides a method for preparation of a conditionally active antibody to IL-6, or fragment thereof, that is inactive at dosing conditions such as room temperature, but slowly refold (reversibly or irreversibly) at body temperature. The method comprises the following steps. Screening a fully human library for an antibody to IL-6. Evolving the IL-6 antibody and screening differentially for molecules at room temperature and at 37°C or higher; selecting for mutations that are inactive at room temperature, relative to wild type, but show increased activity (e.g. binding) relative to the wild type antibody activity (binding). The up-mutants identified in the heavy and light changes are then recombined within the heavy and light chains, as

well as through combinatorial association of the heavy and light chains. Screening of these recombined heavy and light chains is repeated at room temperature and the higher temperature. In addition, the recombined antibodies or fragments are tested for activity and stability under storage and physiological conditions.

[00158] The conditionally active anti-IL-6 antibodies thus identified and produced can be used in a method to treat an autoimmune disease, such as rheumatoid arthritis or psoriasis, by administration of an effective amount to a patient in need thereof, with a reduction in the severity of side effects compared to administration of a traditional biological response modifier anti-IL-6 antibody. One advantage of this method is that it allows for smoothing or leveling of the drug quantity over the period of treatment relative to the current high level of biological response modifier drug followed by half-life clearance over weeks or months.

[00159] One or more mutagenesis techniques are employed to evolve the DNA which encodes the wild-type protein to create a library of mutant DNA; the mutant DNA is expressed to create a library of mutant proteins; and the library is subjected to a screening assay under a normal physiological condition and under one or more aberrant conditions. Conditionally active biologic proteins are selected from those proteins which exhibit both (a) a decrease in activity in the assay at the normal physiological condition compared to the wild-type protein, and (b) an increase in activity in the assay under the aberrant condition compared to the wild-type protein. Alternatively, conditionally active biologic proteins are selected from those proteins which exhibit changes in activity, reversibly or irreversibly, in two or more different physiological conditions.

[00160] GENERATION OF EVOLVED MOLECULES FROM PARENT MOLECULE

[00161] Mirac Proteins can be generated through a process of mutagenesis and screening for individual mutations for a reduction in activity at the wild-type condition with activity at non wild-type conditions remaining the same or better than the activity at the wild-type condition.

[00162] The disclosure provides for a method for generating a nucleic acid variant encoding a polypeptide having enzyme activity, wherein the variant has an altered biological activity from that which naturally occurs, the method comprising (a) modifying the nucleic acid by (i) substituting one or more nucleotides for a different

nucleotide, wherein the nucleotide comprises a natural or non-natural nucleotide; (ii) deleting one or more nucleotides, (iii) adding one or more nucleotides, or (iv) any combination thereof. In one aspect, the non-natural nucleotide comprises inosine. In another aspect, the method further comprises assaying the polypeptides encoded by the modified nucleic acids for altered enzyme activity, thereby identifying the modified nucleic acid(s) encoding a polypeptide having altered enzyme activity. In one aspect, the modifications of step (a) are made by PCR, error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, in vivo mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-specific mutagenesis, gene reassembly, gene site saturated mutagenesis, ligase chain reaction, in vitro mutagenesis, ligase chain reaction, oligonuclteotide synthesis, any DNA-generating technique and any combination thereof. In another aspect, the method further comprises at least one repetition of the modification step (a).

[00163] The disclosure further provides a method for making a polynucleotide from two or more nucleic acids, the method comprising: (a) identifying regions of identity and regions of diversity between two or more nucleic acids, wherein at least one of the nucleic acids comprises a nucleic acid of the disclosure; (b) providing a set of oligonucleotides which correspond in sequence to at least two of the two or more nucleic acids; and, (c) extending the oligonucleotides with a polymerase, thereby making the polynucleotide.

[00164] Any technique of mutagenesis can be employed in various embodiments of the disclosure. Stochastic or random mutagenesis is exemplified by a situation in which a parent molecule is mutated (modified or changed) to yield a set of progeny molecules having mutation(s) that are not predetermined. Thus, in an in vitro stochastic mutagenesis reaction, for example, there is not a particular predetermined product whose production is intended; rather there is an uncertainty--hence randomness--regarding the exact nature of the mutations achieved, and thus also regarding the products generated. Stochastic mutagenesis is manifested in processes such as error-prone PCR and stochastic shuffling, where the mutation(s) achieved are random or not predetermined. The variant forms can be generated by error-prone transcription, such as an error-prone PCR or use of a polymerase which lacks proof-reading activity (see, Liao (1990) Gene 88:107-111), of the first variant form, or, by replication of the first form in a mutator

strain (mutator host cells are discussed in further detail below, and are generally well known). A mutator strain can include any mutants in any organism impaired in the functions of mismatch repair. These include mutant gene products of mutS, mutT, mutH, mutL, ovrD, dcm, vsr, umuC, umuD, sbcB, recJ, etc. The impairment is achieved by genetic mutation, allelic replacement, selective inhibition by an added reagent such as a small compound or an expressed antisense RNA, or other techniques. Impairment can be of the genes noted, or of homologous genes in any organism.

[00165] Current mutagenesis methods in widespread use for creating alternative proteins from a starting molecule are oligonucleotide-directed mutagenesis technologies, error-prone polymerase chain reactions (error-prone PCR) and cassette mutagenesis, in which the specific region to be optimized is replaced with a synthetically mutagenized oligonucleotide. In these cases, a number of mutant sites are generated around certain sites in the original sequence.

[00166] In oligonucleotide-directed mutagenesis, a short sequence is replaced with a synthetically mutagenized oligonucleotide. In oligonucleotide-directed mutagenesis, a short sequence of the polynucleotide is removed from the polynucleotide using restriction enzyme digestion and is replaced with a synthetic polynucleotide in which various bases have been altered from the original sequence. The polynucleotide sequence can also be altered by chemical mutagenesis. Chemical mutagens include, for example, sodium bisulfite, nitrous acid, hydroxylamine, hydrazine or formic acid. Other agents which are analogues of nucleotide precursors include nitrosoguanidine, 5-bromouracil, 2-aminopurine, or acridine. Generally, these agents are added to the PCR reaction in place of the nucleotide precursor thereby mutating the sequence. Intercalating agents such as proflavine, acriflavine, quinacrine and the like can also be used. Random mutagenesis of the polynucleotide sequence can also be achieved by irradiation with X-rays or ultraviolet light. Generally, plasmid polynucleotides so mutagenized are introduced into E. coli and propagated as a pool or library of hybrid plasmids.

[00167] Error-prone PCR uses low-fidelity polymerization conditions to introduce a low level of point mutations randomly over a long sequence. In a mixture of fragments of unknown sequence, error-prone PCR can be used to mutagenize the mixture.

[00168] In cassette mutagenesis, a sequence block of a single template is typically replaced by a (partially) randomized sequence. Reidhaar-Olson J F and Sauer R T:

Combinatorial cassette mutagenesis as a probe of the informational content of protein sequences. Science 241(4861):53-57, 1988.

[00169] Alternatively, any technique of non-stochastic or non-random mutagenesis can be employed in various embodiments of the disclosure. Non-stochastic mutagenesis is exemplified by a situation in which a parent molecule is mutated (modified or changed) to yield a progeny molecule having one or more predetermined mutations. It is appreciated that the presence of background products in some quantity is a reality in many reactions where molecular processing occurs, and the presence of these background products does not detract from the non-stochastic nature of a mutagenesis process having a predetermined product. Site-saturation mutagenesis and synthetic ligation reassembly, are examples of mutagenesis techniques where the exact chemical structure(s) of the intended product(s) are predetermined.

[00170] One method of site-saturation mutagenesis is disclosed in U.S. patent application publication 2009/0130718, which is incorporated herein by reference. This method provides a set of degenerate primers corresponding to codons of a template polynucleotide, and performs polymerase elongation to produce progeny polynucleotides, which contain sequences corresponding to the degenerate primers. The progeny polynucleotides can be expressed and screened for directed evolution. Specifically, this is a method for producing a set of progeny polynucleotides, comprising the steps of (a) providing copies of a template polynucleotide, each comprising a plurality of codons that encode a template polypeptide sequence; and (b) for each codon of the template polynucleotide, performing the steps of (1) providing a set of degenerate primers, where each primer comprises a degenerate codon corresponding to the codon of the template polynucleotide and at least one adjacent sequence that is homologous to a sequence adjacent to the codon of the template polynucleotide; (2) providing conditions allowing the primers to anneal to the copies of the template polynucleotides; and (3) performing a polymerase elongation reaction from the primers along the template; thereby producing progeny polynucleotides, each of which contains a sequence corresponding to the degenerate codon of the annealed primer; thereby producing a set of progeny polynucleotides.

[00171] Site-saturation mutagenesis relates to the directed evolution of nucleic acids and screening of clones containing the evolved nucleic acids for resultant activity(ies) of

interest, such nucleic acid activity(ies) &/or specified protein, particularly enzyme, activity(ies) of interest.

Mutagenized molecules provided by this technique may have chimeric molecules and molecules with point mutations, including biological molecules that contain a carbohydrate, a lipid, a nucleic acid, &/or a protein component, and specific but non-limiting examples of these include antibiotics, antibodies, enzymes, and steroidal and non-steroidal hormones.

[00172] Site saturation mutagenesis relates generally to a method of: 1) preparing a progeny generation of molecule(s) (including a molecule that is comprised of a polynucleotide sequence, a molecule that is comprised of a polypeptide sequence, and a molecule that is comprised in part of a polynucleotide sequence and in part of a polypeptide sequence), that is mutagenized to achieve at least one point mutation, addition, deletion, &/or chimerization, from one or more ancestral or parental generation template(s); 2) screening the progeny generation molecule(s)--preferably using a high throughput method--for at least one property of interest (such as an improvement in an enzyme activity or an increase in stability or a novel chemotherapeutic effect); 3) optionally obtaining &/or cataloguing structural &/or and functional information regarding the parental &/or progeny generation molecules; and 4) optionally repeating any of steps 1) to 3).

[00173] In site saturation mutagenesis, there is generated (e.g. from a parent polynucleotide template)--in what is termed "codon site-saturation mutagenesis"--a progeny generation of polynucleotides, each having at least one set of up to three contiguous point mutations (i.e. different bases comprising a new codon), such that every codon (or every family of degenerate codons encoding the same amino acid) is represented at each codon position. Corresponding to--and encoded by--this progeny generation of polynucleotides, there is also generated a set of progeny polypeptides, each having at least one single amino acid point mutation. In a preferred aspect, there is generated--in what is termed "amino acid site-saturation mutagenesis"--one such mutant polypeptide for each of the 19 naturally encoded polypeptide-forming alpha-amino acid substitutions at each and every amino acid position along the polypeptide. This yields--for each and every amino acid position along the parental polypeptide--a total of 20 distinct progeny polypeptides including the original amino acid, or potentially more than

21 distinct progeny polypeptides if additional amino acids are used either instead of or in addition to the 20 naturally encoded amino acids.

[00174] Other mutagenesis techniques can also be employed which involve recombination and more specifically a method for preparing polynucleotides encoding a polypeptide by a method of in vivo re-assortment of polynucleotide sequences containing regions of partial homology, assembling the polynucleotides to form at least one polynucleotide and screening the polynucleotides for the production of polypeptide(s) having a useful property.

[00175] In another aspect, mutagenesis techniques exploit the natural property of cells to recombine molecules and/or to mediate reductive processes that reduce the complexity of sequences and extent of repeated or consecutive sequences possessing regions of homology.

[00176] Various mutagenesis techniques can be used alone or in combination to provide a method for generating hybrid polynucleotides encoding biologically active hybrid polypeptides with enhanced activities. In accomplishing these and other objects, there has been provided, in accordance with one aspect of the disclosure, a method for introducing polynucleotides into a suitable host cell and growing the host cell under conditions that produce a hybrid polynucleotide.

[00177] Chimeric genes have been made by joining 2 polynucleotide fragments using compatible sticky ends generated by restriction enzyme(s), where each fragment is derived from a separate progenitor (or parental) molecule. Another example is the mutagenesis of a single codon position (i.e. to achieve a codon substitution, addition, or deletion) in a parental polynucleotide to generate a single progeny polynucleotide encoding for a single site-mutagenized polypeptide.

[00178] Further, in vivo site specific recombination systems have been utilized to generate hybrids of genes, as well as random methods of in vivo recombination, and recombination between homologous but truncated genes on a plasmid. Mutagenesis has also been reported by overlapping extension and PCR.

[00179] Non-random methods have been used to achieve larger numbers of point mutations and/or chimerizations, for example comprehensive or exhaustive approaches have been used to generate all the molecular species within a particular grouping of mutations, for attributing functionality to specific structural groups in a template

molecule (e.g. a specific single amino acid position or a sequence comprised of two or more amino acids positions), and for categorizing and comparing specific grouping of mutations.

[00180] Any of these or other methods of evolving can be employed in the present disclosure to generate a new population of molecules (library) from one or more parent molecules.

[00181] Once formed, the constructs may, or may not be size fractionated on an agarose gel according to published protocols, inserted into a cloning vector, and transfected into an appropriate host cell.

[00182] EXPRESSION OF EVOLVED MOLECULES

[00183] Once a library of mutant molecules is generated, DNA can be expressed using routine molecular biology techniques. Thus, protein expression can be directed using various known methods.

[00184] For example, briefly, a wild type gene can be evolved using any variety of random or non-random methods such as those indicated herein. Mutant DNA molecules are then digested and ligated into vector DNA, such as plasmid DNA using standard molecular biology techniques. Vector DNA containing individual mutants is transformed into bacteria or other cells using standard protocols. This can be done in an individual well of a multi-well tray, such as a 96-well tray for high throughput expression and screening. The process is repeated for each mutant molecule.

[00185] Polynucleotides selected and isolated as described are introduced into a suitable host cell. A suitable host cell is any cell which is capable of promoting recombination and/or reductive reassortment. The selected polynucleotides are preferably already in a vector which includes appropriate control sequences. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or preferably, the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (e.g. Ecker and Davis, 1986, Inhibition of gene expression in plant cells by expression of antisense RNA, Proc Natl Acad Sci USA, 83:5372-5376).

[00186] As representative examples of expression vectors which may be used, there may be mentioned viral particles, baculovirus, phage, plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes, viral DNA (e.g., vaccinia, adenovirus, foul pox virus, pseudorabies and derivatives of SV40), P1-based artificial chromosomes, yeast plasmids, yeast artificial chromosomes, and any other vectors specific for specific hosts of interest (such as bacillus, aspergillus and yeast). Thus, for example, the DNA may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences. Large numbers of suitable vectors are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example; Bacterial: pQE vectors (Qiagen), pBluescript plasmids, pNH vectors, (lambda-ZAP vectors (Stratagene); ptrc99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene), pSVK3, pBPV, pMSG, pSVLSV40 (Pharmacia). However, any other plasmid or other vector may be used so long as they are replicable and viable in the host. Low copy number or high copy number vectors may be employed with the present disclosure.

[00187] The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct RNA synthesis. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda PR, PL and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-1. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. Promoter regions can be selected from any desired gene using chloramphenicol transferase (CAT) vectors or other vectors with selectable markers. In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

[00188] Therefore, in another aspect of the disclosure, novel polynucleotides can be generated by the process of reductive reassortment. The method involves the generation of constructs containing consecutive sequences (original encoding sequences), their

insertion into an appropriate vector, and their subsequent introduction into an appropriate host cell. The reassortment of the individual molecular identities occurs by combinatorial processes between the consecutive sequences in the construct possessing regions of homology, or between quasi-repeated units. The reassortment process recombines and/or reduces the complexity and extent of the repeated sequences, and results in the production of novel molecular species. Various treatments may be applied to enhance the rate of reassortment. These could include treatment with ultra-violet light, or DNA damaging chemicals, and/or the use of host cell lines displaying enhanced levels of "genetic instability". Thus the reassortment process may involve homologous recombination or the natural property of quasi-repeated sequences to direct their own evolution.

[00189] In one aspect, the host organism or cell comprises a gram negative bacterium, a gram positive bacterium or a eukaryotic organism. In another aspect of the disclosure, the gram negative bacterium comprises Escherichia coli, or Pseudomonas fluorescens. In another aspect of the disclosure, the gram positive bacterium comprise Streptomyces diversa, Lactobacillus gasseri, Lactococcus lactis, Lactococcus cremoris, or Bacillus subtilis. In another aspect of the disclosure, the eukaryotic organism comprises Saccharomyces cerevisiae, Schizosaccharomyces pombe, Pichia pastoris, Kluyveromyces lactis, Hansenula plymorpha, or Aspergillus niger. As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Streptomyces, Salmonella typhimurium; fungal cells, such as yeast; insect cells such as Drosophila S2 and Spodoptera Sf9; animal cells such as CHO, COS or Bowes melanoma; adenoviruses; and plant cells. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

[00190] With particular references to various mammalian cell culture systems that can be employed to express recombinant protein, examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described in "SV40-transformed simian cells support the replication of early SV40 mutants" (Gluzman, 1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA

sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

[00191] The cells are then propagated and "reductive reassortment" is effected. The rate of the reductive reassortment process may be stimulated by the introduction of DNA damage if desired. In vivo reassortment is focused on "inter-molecular" processes collectively referred to as "recombination" which in bacteria, is generally viewed as a "RecA-dependent" phenomenon. The disclosure can rely on recombination processes of a host cell to recombine and re-assort sequences, or the cells' ability to mediate reductive processes to decrease the complexity of quasi-repeated sequences in the cell by deletion. This process of "reductive reassortment" occurs by an "intra-molecular", RecA-independent process. The end result is a reassortment of the molecules into all possible combinations.

[00192] Host cells containing the polynucleotides of interest can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

[00193] Protein expression can be induced by a variety of known methods, and many genetic systems have been published for induction of protein expression. For example, with appropriate systems, the addition of an inducing agent will induce protein expression. Cells are then pelleted by centrifugation and the supernatant removed. Periplasmic protein can be enriched by incubating the cells with DNAse, RNAse, and lysozyme. After centrifugation, the supernatant, containing the new protein, is transferred to a new multi-well tray and stored prior to assay.

[00194] Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract is retained for further purification. Microbial cells employed for expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents. Such methods are well known to those skilled in the art. The expressed polypeptide or fragment thereof can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose

chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the polypeptide. If desired, high performance liquid chromatography (HPLC) can be employed for final purification steps.

[00195] The clones which are identified as having the desired activity may then be sequenced to identify the polynucleotide sequence encoding an enzyme having the enhanced activity.

[00196] The polypeptides that are identified from such libraries can be used for therapeutic, diagnostic, research and related purposes, and/or can be subjected to one or more additional cycles of shuffling and/or selection. The disclosure provides for a fragment of the conditionally active biologic protein which is at least 10 amino acids in length, and wherein the fragment has activity.

[00197] The disclosure provides for a codon-optimized polypeptide or a fragment thereof, having enzyme activity, wherein the codon usage is optimized for a particular organism or cell. Narum et al., "Codon optimization of gene fragments encoding Plasmodium falciparum merzoite proteins enhances DNA vaccine protein expression and immunogenicity in mice". Infect. Immun. 2001 December, 69(12):7250-3 describes codon-optimization in the mouse system. Outchkourov et al., "Optimization of the expression of Equistatin in Pichia pastoris, protein expression and purification", Protein Expr. Purif. 2002 February; 24(1):18-24 describes codon-optimization in the yeast system. Feng et al., "High level expression and mutagenesis of recombinant human phosphatidylcholine transfer protein using a synthetic gene: evidence for a C-terminal membrane binding domain" Biochemistry 2000 Dec. 19, 39(50):15399-409 describes codon-optimization in E. coli. Humphreys et al., "High-level periplasmic expression in Escherichia coli using a eukaryotic signal peptide: importance of codon usage at the 5' end of the coding sequence", Protein Expr. Purif. 2000 Nov. 20(2):252-64 describes how codon usage affects secretion in E. coli.

[00198] The evolution of a conditionally active biologic protein can be aided by the availability of a convenient high throughput screening or selection process.

[00199] Once identified, polypeptides and peptides of the disclosure can be synthetic, or be recombinantly generated polypeptides. Peptides and proteins can be recombinantly

expressed in vitro or in vivo. The peptides and polypeptides of the disclosure can be made and isolated using any method known in the art. Polypeptide and peptides of the disclosure can also be synthesized, whole or in part, using chemical methods well known in the art. See e.g., Caruthers (1980) "New chemical methods for synthesizing polynucleotides", Nucleic Acids Res. Symp. Ser. 215-223; Horn (1980), "Synthesis of oligonucleotides on cellulose. Part II: design and synthetic strategy to the synthesis of 22 oligodeoxynucleotides coding for Gastric Inhibitory Polypeptide (GIP)[1]", Nucleic Acids Res. Symp. Ser. 225-232; Banga, A. K., Therapeutic Peptides and Proteins, Formulation, Processing and Delivery Systems (1995) Technomic Publishing Co., Lancaster, Pa. For example, peptide synthesis can be performed using various solid-phase techniques (see e.g., Roberge (1995) "A strategy for a convergent synthesis of N-linked glycopeptides on a solid support", Science 269:202; Merrifield (1997) "Concept and early development of solid-phase peptide synthesis", Methods Enzymol. 289:3-13) and automated synthesis may be achieved, e.g., using the ABI 431A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer.

[00200] The peptides and polypeptides of the disclosure can also be glycosylated. The glycosylation can be added post-translationally either chemically or by cellular biosynthetic mechanisms, wherein the latter incorporates the use of known glycosylation motifs, which can be native to the sequence or can be added as a peptide or added in the nucleic acid coding sequence. The glycosylation can be O-linked or N-linked.

[00201] The peptides and polypeptides of the disclosure, as defined above, include all "mimetic" and "peptidomimetic" forms. The terms "mimetic" and "peptidomimetic" refer to a synthetic chemical compound which has substantially the same structural and/or functional characteristics of the polypeptides of the disclosure. The mimetic can be either entirely composed of synthetic, non-natural analogues of amino acids, or, is a chimeric molecule of partly natural peptide amino acids and partly non-natural analogs of amino acids. The mimetic can also incorporate any amount of natural amino acid conservative substitutions as long as such substitutions also do not substantially alter the mimetic's structure and/or activity. As with polypeptides of the disclosure which are conservative variants, routine experimentation will determine whether a mimetic is within the scope of the disclosure, i.e., that its structure and/or function is not substantially altered.

[00202] Polypeptide mimetic compositions of the disclosure can contain any combination of non-natural structural components. In alternative aspect, mimetic compositions of the disclosure include one or all of the following three structural groups: a) residue linkage groups other than the natural amide bond ("peptide bond") linkages; b) non-natural residues in place of naturally occurring amino acid residues; or c) residues which induce secondary structural mimicry, i.e., to induce or stabilize a secondary structure, e.g., a beta turn, gamma turn, beta sheet, alpha helix conformation, and the like. For example, a polypeptide of the disclosure can be characterized as a mimetic when all or some of its residues are joined by chemical means other than natural peptide bonds. Individual peptidomimetic residues can be joined by peptide bonds, other chemical bonds or coupling means, such as, e.g., glutaraldehyde, N-hydroxysuccinimide esters, bifunctional maleimides, N,N'-dicyclohexylcarbodiimide (DCC) or N,N'-diisopropylcarbodiimide (DIC). Linking groups that can be an alternative to the traditional amide bond ("peptide bond") linkages include, e.g., ketomethylene (e.g., --C(.dbd.O)--CH.sub.2-- for --C(.dbd.O)--NH--), aminomethylene (CH.sub.2--NH), ethylene, olefin (CH.dbd.CH), ether (CH.sub.2--O), thioether (CH.sub.2--S), tetrazole (CN.sub.4--), thiazole, retroamide, thioamide, or ester (see, e.g., Spatola (1983) in Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Vol. 7, pp 267-357, "Peptide Backbone Modifications," Marcell Dekker, N.Y.).

[00203] A polypeptide of the disclosure can also be characterized as a mimetic by containing all or some non-natural residues in place of naturally occurring amino acid residues. Non-natural residues are well described in the scientific and patent literature; a few exemplary non-natural compositions useful as mimetics of natural amino acid residues and guidelines are described below. Mimetics of aromatic amino acids can be generated by replacing by, e.g., D- or L-naphylalanine; D- or L-phenylglycine; D- or L-2 thieneylalanine; D- or L-1,-2, 3-, or 4-pyreneylalanine; D- or L-3 thieneylalanine; D- or L-(2-pyridinyl)-alanine; D- or L-(3-pyridinyl)-alanine; D- or L-(2-pyrazinyl)-alanine; D- or L-(4-isopropyl)-phenylglycine; D-(trifluoromethyl)-phenylglycine; D-(trifluoromethyl)-phenylalanine; D-p-fluoro-phenylalanine; D- or L-p-biphenylphenylalanine; D- or L-p-methoxy-biphenylphenylalanine; D- or L-2-indole(alkyl)alanines; and, D- or L-alkylanines, where alkyl can be substituted or unsubstituted methyl, ethyl, propyl, hexyl, butyl, pentyl, isopropyl, iso-butyl, sec-isotyl, iso-pentyl, or a non-acidic amino acids. Aromatic rings of a non-natural amino acid

include, e.g., thiazolyl, thiophenyl, pyrazolyl, benzimidazolyl, naphthyl, furanyl, pyrrolyl, and pyridyl aromatic rings.

[00204] Mimetics of acidic amino acids can be generated by substitution by, e.g., non-carboxylate amino acids while maintaining a negative charge; (phosphono)alanine; sulfated threonine. Carboxyl side groups (e.g., aspartyl or glutamyl) can also be selectively modified by reaction with carbodiimides (R'--N--C--N--R') such as, e.g., 1-cyclohexyl-3(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3(4-azonia-4,4-dimetholpentyl) carbodiimide. Aspartyl or glutamyl can also be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions. Mimetics of basic amino acids can be generated by substitution with, e.g., (in addition to lysine and arginine) the amino acids ornithine, citrulline, or (guanidino)-acetic acid, or (guanidino)alkyl-acetic acid, where alkyl is defined above. Nitrile derivative (e.g., containing the CN-moiety in place of COOH) can be substituted for asparagine or glutamine. Asparaginyl and glutaminyl residues can be deaminated to the corresponding aspartyl or glutamyl residues. Arginine residue mimetics can be generated by reacting arginyl with, e.g., one or more conventional reagents, including, e.g., phenylglyoxal, 2,3-butanedione, 1,2-cyclo-hexanedione, or ninhydrin, preferably under alkaline conditions. Tyrosine residue mimetics can be generated by reacting tyrosyl with, e.g., aromatic diazonium compounds or tetranitromethane. N-acetylimidizol and tetranitromethane can be used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Cysteine residue mimetics can be generated by reacting cysteinyl residues with, e.g., alpha-haloacetates such as 2-chloroacetic acid or chloroacetamide and corresponding amines; to give carboxymethyl or carboxyamidomethyl derivatives. Cysteine residue mimetics can also be generated by reacting cysteinyl residues with, e.g., bromo-trifluoroacetone, alpha-bromo-beta-(5-imidozoyl) propionic acid; chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide; methyl 2-pyridyl disulfide; p-chloromercuribenzoate; 2-chloromercuri-4 nitrophenol; or, chloro-7-nitrobenzo-oxa-1,3-diazole. Lysine mimetics can be generated (and amino terminal residues can be altered) by reacting lysinyl with, e.g., succinic or other carboxylic acid anhydrides. Lysine and other alpha-amino-containing residue mimetics can also be generated by reaction with imidoesters, such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitro-benzenesulfonic acid, O-methylisourea, 2,4, pentanedione, and transamidase-catalyzed reactions with glyoxylate. Mimetics of methionine can be generated by reaction with,

e.g., methionine sulfoxide. Mimetics of proline include, e.g., pipecolic acid, thiazolidine carboxylic acid, 3- or 4-hydroxy proline, dehydroproline, 3- or 4-methylproline, or 3,3,-dimethylproline. Histidine residue mimetics can be generated by reacting histidyl with, e.g., diethylprocarbonate or para-bromophenacyl bromide. Other mimetics include, e.g., those generated by hydroxylation of proline and lysine; phosphorylation of the hydroxyl groups of seryl or threonyl residues; methylation of the alpha-amino groups of lysine, arginine and histidine; acetylation of the N-terminal amine; methylation of main chain amide residues or substitution with N-methyl amino acids; or amidation of C-terminal carboxyl groups.

[00205] A residue, e.g., an amino acid, of a polypeptide of the disclosure can also be replaced by an amino acid (or peptidomimetic residue) of the opposite chirality. Thus, any amino acid naturally occurring in the L-configuration (which can also be referred to as the R or S, depending upon the structure of the chemical entity) can be replaced with the amino acid of the same chemical structural type or a peptidomimetic, but of the opposite chirality, referred to as the D-amino acid, but also can be referred to as the R- or S-form.

[00206] The disclosure also provides methods for modifying the polypeptides of the disclosure by either natural processes, such as post-translational processing (e.g., phosphorylation, acylation, etc), or by chemical modification techniques. Modifications can occur anywhere in the polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also a given polypeptide may have many types of modifications. Modifications include acetylation, acylation, PEGylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of a phosphatidylinositol, cross-linking cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristolyation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, and transfer-RNA mediated addition of amino acids to protein such as arginylation. See, e.g., Creighton, T. E., Proteins--Structure and Molecular

Properties 2nd Ed., W. H. Freeman and Company, New York (1993); Posttranslational Covalent Modification of Proteins, B. C. Johnson, Ed., Academic Press, New York, pp. 1-12 (1983).

[00207] Solid-phase chemical peptide synthesis methods can also be used to synthesize the polypeptide or fragments of the disclosure. Such methods have been known in the art since the early 1960's (Merrifield, R. B., "Solid-phase synthesis.I. The synthesis of a tetrapeptide", J. Am. Chem. Soc., 85:2149-2154, 1963) (See also Stewart, J. M. and Young, J. D., Solid Phase Peptide Synthesis, 2nd Ed., Pierce Chemical Co., Rockford, Ill., pp. 11-12)) and have recently been employed in commercially available laboratory peptide design and synthesis kits (Cambridge Research Biochemicals). Such commercially available laboratory kits have generally utilized the teachings of H. M. Geysen et al., "Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid," Proc. Natl. Acad. Sci., USA, 81:3998 (1984) and provide for synthesizing peptides upon the tips of a multitude of "rods" or "pins" all of which are connected to a single plate. When such a system is utilized, a plate of rods or pins is inverted and inserted into a second plate of corresponding wells or reservoirs, which contain solutions for attaching or anchoring an appropriate amino acid to the pin's or rod's tips. By repeating such a process step, i.e., inverting and inserting the rod's and pin's tips into appropriate solutions, amino acids are built into desired peptides. In addition, a number of available FMOC peptide synthesis systems are available. For example, assembly of a polypeptide or fragment can be carried out on a solid support using an Applied Biosystems, Inc. Model 431A™ automated peptide synthesizer. Such equipment provides ready access to the peptides of the disclosure, either by direct synthesis or by synthesis of a series of fragments that can be coupled using other known techniques.

[00208] The synthetic polypeptide or fragment thereof can be recovered and purified by known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the polypeptide. If desired, high performance liquid chromatography (HPLC) can be employed for final purification steps.

[00209] The disclosure provides for a conditionally active protein variant preparation or formulation which comprises at least one of the protein variants, wherein the preparation is liquid or dry. The protein formulation optionally includes a buffer, cofactor, second or additional protein, or one or more excipients. In one aspect the formulation is utilized as a therapeutic conditionally active biologic protein which is active under aberrant or non-physiological conditions, but less active or inactive under normal physiological conditions of, e.g., temperature, pH, or osmotic pressure, oxidation or osmolality.

[00210] Standard purification techniques can be employed for either recombinant or synthetic conditionally active biologic proteins.

[00211] SCREENING OF MUTANTS TO IDENTIFY REVERSIBLE OR NON-REVERSIBLE MUTANTS

[00212] Identifying desirable molecules is most directly accomplished by measuring protein activity at the permissive condition and the wild type condition. The mutants with the largest ratio of activity (permissive/wild type) can then be selected and permutations of the point mutations are generated by combining the individual mutations using standard methods. The combined permutation protein library is then screened for those proteins displaying the largest differential activity between the permissive and wild type condition.

[00213] Activity of supernatants can be screened using a variety of methods, for example using high throughput activity assays, such as fluorescence assays, to identify protein mutants that are sensitive at whatever characteristic one desires (temperature, pH, etc). For example, to screen for temporally sensitive mutants, the enzymatic or antibody activity of each individual mutant is determined at lower temperatures (such as 25 degrees Celsius), and at temperatures which the original protein functions (such as 37 degrees Celsius), using commercially available substrates. Reactions can initially be performed in a multi well assay format, such as a 96-well assay, and confirmed using a different format, such as a 14 ml tube format.

[00214] The disclosure further provides a screening assay for identifying a enzyme, the assay comprising: (a) providing a plurality of nucleic acids or polypeptides; (b) obtaining polypeptide candidates to be tested for enzyme activity from the plurality; (c) testing the candidates for enzyme activity; and (d) identifying those polypeptide candidates which

exhibit elevated enzyme activity under aberrant or non-physiological conditions, and decreased enzyme activity compared to the wild-type enzyme protein under normal physiological conditions of, e.g., temperature, pH, oxidation, osmolality, electrolyte concentration or osmotic pressure.

[00215] In one aspect, the method further comprises modifying at least one of the nucleic acids or polypeptides prior to testing the candidates for conditional biologic activity. In another aspect, the testing of step (c) further comprises testing for improved expression of the polypeptide in a host cell or host organism. In a further aspect, the testing of step (c) further comprises testing for enzyme activity within a pH range from about pH 3 to about pH 12. In a further aspect, the testing of step (c) further comprises testing for enzyme activity within a pH range from about pH 5 to about pH 10. In a further aspect, the testing of step (c) further comprises testing for enzyme activity within a pH range from about pH 6 to about pH 8. In a further aspect, the testing of step (c) further comprises testing for enzyme activity at pH 6.7 and pH 7.5. In another aspect, the testing of step (c) further comprises testing for enzyme activity within a temperature range from about 4 degrees C to about 55 degrees C. In another aspect, the testing of step (c) further comprises testing for enzyme activity within a temperature range from about 15 degrees C to about 47 degrees C. In another aspect, the testing of step (c) further comprises testing for enzyme activity within a temperature range from about 20 degrees C to about 40 degrees C. In another aspect, the testing of step (c) further comprises testing for enzyme activity at the temperatures of 25 degrees C and 37 degrees C. In another aspect, the testing of step (c) further comprises testing for enzyme activity under normal osmotic pressure, and aberrant (positive or negative) osmotic pressure. In another aspect, the testing of step (c) further comprises testing for enzyme activity under normal electrolyte concentration, and aberrant (positive or negative) electrolyte concentration. The electrolyte concentration to be tested is selected from one of calcium, sodium, potassium, magnesium, chloride, bicarbonate and phosphate concentration. In another aspect, the testing of step (c) further comprises testing for enzyme activity which results in a stabilized reaction product.

[00216] In another aspect, the disclosure provides for a purified antibody that specifically binds to the polypeptide of the disclosure or a fragment thereof, having enzyme activity. In one aspect, the disclosure provides for a fragment of the antibody that specifically binds to a polypeptide having enzyme activity.

[00217] Antibodies and Antibody-based Screening Methods

[00218] The disclosure provides isolated or recombinant antibodies that specifically bind to an enzyme of the disclosure. These antibodies can be used to isolate, identify or quantify the enzymes of the disclosure or related polypeptides. These antibodies can be used to isolate other polypeptides within the scope the disclosure or other related enzymes. The antibodies can be designed to bind to an active site of an enzyme. Thus, the disclosure provides methods of inhibiting enzymes using the antibodies of the disclosure.

[00219] The antibodies can be used in immunoprecipitation, staining, immunoaffinity columns, and the like. If desired, nucleic acid sequences encoding for specific antigens can be generated by immunization followed by isolation of polypeptide or nucleic acid, amplification or cloning and immobilization of polypeptide onto an array of the disclosure. Alternatively, the methods of the disclosure can be used to modify the structure of an antibody produced by a cell to be modified, e.g., an antibody's affinity can be increased or decreased. Furthermore, the ability to make or modify antibodies can be a phenotype engineered into a cell by the methods of the disclosure.

Methods of immunization, producing and isolating antibodies (polyclonal and monoclonal) are known to those of skill in the art and described in the scientific and patent literature, see, e.g., Coligan, CURRENT PROTOCOLS IN IMMUNOLOGY, Wiley/Greene, NY (1991); Stites (eds.) BASIC AND CLINICAL IMMUNOLOGY (7th ed.) Lange Medical Publications, Los Altos, Calif. ("Stites"); Goding, MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE (2d ed.) Academic Press, New York, N.Y. (1986); Kohler (1975) "Continuous cultures of fused cells secreting antibody of predefined specificity", Nature 256:495; Harlow (1988) ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publications, New York. Antibodies also can be generated in vitro, e.g., using recombinant antibody binding site expressing phage display libraries, in addition to the traditional in vivo methods using animals. See, e.g., Hoogenboom (1997) "Designing and optimizing library selection strategies for generating high-affinity antibodies", Trends Biotechnol. 15:62-70; and Katz (1997) "Structural and mechanistic determinants of affinity and specificity of ligands discovered or engineered by phage display", Annu. Rev. Biophys. Biomol. Struct. 26:27-45.

[00220] Polypeptides or peptides can be used to generate antibodies which bind specifically to the polypeptides, e.g., the enzymes, of the disclosure. The resulting antibodies may be used in immunoaffinity chromatography procedures to isolate or purify the polypeptide or to determine whether the polypeptide is present in a biological sample. In such procedures, a protein preparation, such as an extract, or a biological sample is contacted with an antibody capable of specifically binding to one of the polypeptides of the disclosure.

[00221] In immunoaffinity procedures, the antibody is attached to a solid support, such as a bead or other column matrix. The protein preparation is placed in contact with the antibody under conditions in which the antibody specifically binds to one of the polypeptides of the disclosure. After a wash to remove non-specifically bound proteins, the specifically bound polypeptides are eluted.

[00222] The ability of proteins in a biological sample to bind to the antibody may be determined using any of a variety of procedures familiar to those skilled in the art. For example, binding may be determined by labeling the antibody with a detectable label such as a fluorescent agent, an enzymatic label, or a radioisotope. Alternatively, binding of the antibody to the sample may be detected using a secondary antibody having such a detectable label thereon. Particular assays include ELISA assays, sandwich assays, radioimmunoassays, and Western Blots.

[00223] Polyclonal antibodies generated against the polypeptides of the disclosure can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to a non-human animal. The antibody so obtained will then bind the polypeptide itself. In this manner, even a sequence encoding only a fragment of the polypeptide can be used to generate antibodies which may bind to the whole native polypeptide. Such antibodies can then be used to isolate the polypeptide from cells expressing that polypeptide.

[00224] For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique, the trioma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique (see, e.g., Cole (1985) in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

[00225] Techniques described for the production of single chain antibodies (see, e.g., U.S. Pat. No. 4,946,778) can be adapted to produce single chain antibodies to the polypeptides of the disclosure. Alternatively, transgenic mice may be used to express humanized antibodies to these polypeptides or fragments thereof. Antibodies generated against the polypeptides of the disclosure may be used in screening for similar polypeptides (e.g., enzymes) from other organisms and samples. In such techniques, polypeptides from the organism are contacted with the antibody and those polypeptides which specifically bind the antibody are detected. Any of the procedures described above may be used to detect antibody binding.

[00226] Screening Methodologies and "On-line" Monitoring Devices

[00227] In practicing the methods of the disclosure, a variety of apparatus and methodologies can be used to in conjunction with the polypeptides and nucleic acids of the disclosure, e.g., to screen polypeptides for enzyme activity, to screen compounds as potential modulators, e.g., activators or inhibitors, of an enzyme activity, for antibodies that bind to a polypeptide of the disclosure, for nucleic acids that hybridize to a nucleic acid of the disclosure, to screen for cells expressing a polypeptide of the disclosure and the like.

[00228] Arrays, or "Biochips"

[00229] Nucleic acids or polypeptides of the disclosure can be immobilized to or applied to an array. Arrays can be used to screen for or monitor libraries of compositions (e.g., small molecules, antibodies, nucleic acids, etc.) for their ability to bind to or modulate the activity of a nucleic acid or a polypeptide of the disclosure. For example, in one aspect of the disclosure, a monitored parameter is transcript expression of an enzyme gene. One or more, or, all the transcripts of a cell can be measured by hybridization of a sample comprising transcripts of the cell, or, nucleic acids representative of or complementary to transcripts of a cell, by hybridization to immobilized nucleic acids on an array, or "biochip." By using an "array" of nucleic acids on a microchip, some or all of the transcripts of a cell can be simultaneously quantified. Alternatively, arrays comprising genomic nucleic acid can also be used to determine the genotype of a newly engineered strain made by the methods of the disclosure. Polypeptide arrays" can also be used to simultaneously quantify a plurality of proteins. The present disclosure can be practiced with any known "array," also referred to as a "microarray" or "nucleic acid

array" or "polypeptide array" or "antibody array" or "biochip," or variation thereof. Arrays are generically a plurality of "spots" or "target elements," each target element comprising a defined amount of one or more biological molecules, e.g., oligonucleotides, immobilized onto a defined area of a substrate surface for specific binding to a sample molecule, e.g., mRNA transcripts.

[00230] In practicing the methods of the disclosure, any known array and/or method of making and using arrays can be incorporated in whole or in part, or variations thereof, as described, for example, in U.S. Pat. Nos. 6,277,628; 6,277,489; 6,261,776; 6,258,606; 6,054,270; 6,048,695; 6,045,996; 6,022,963; 6,013,440; 5,965,452; 5,959,098; 5,856,174; 5,830,645; 5,770,456; 5,632,957; 5,556,752; 5,143,854; 5,807,522; 5,800,992; 5,744,305; 5,700,637; 5,556,752; 5,434,049; see also, e.g., WO 99/51773; WO 99/09217; WO 97/46313; WO 96/17958; see also, e.g., Johnston (1998) "Gene chips: Array of hope for understanding gene regulation", Curr. Biol. 8:R171-R174; Schummer (1997) "Inexpensive Handheld Device for the Construction of High-Density Nucleic Acid Arrays", Biotechniques 23:1087-1092; Kern (1997) "Direct hybridization of large-insert genomic clones on high-density gridded cDNA filter arrays", Biotechniques 23:120-124; Solinas-Toldo (1997) "Matrix-Based Comparative Genomic Hybridization: Biochips to Screen for Genomic Imbalances", Genes, Chromosomes & Cancer 20:399-407; Bowtell (1999) "Options Available--From Start to Finish--for Obtaining Expression Data by Microarray", Nature Genetics Supp. 21:25-32. See also published U.S. patent applications Nos. 20010018642; 20010019827; 20010016322; 20010014449; 20010014448; 20010012537; 20010008765.

[00231] Capillary Arrays

[00232] Capillary arrays, such as the GIGAMATRIX™ Diversa Corporation, San Diego, Calif., can be used in the methods of the disclosure. Nucleic acids or polypeptides of the disclosure can be immobilized to or applied to an array, including capillary arrays. Arrays can be used to screen for or monitor libraries of compositions (e.g., small molecules, antibodies, nucleic acids, etc.) for their ability to bind to or modulate the activity of a nucleic acid or a polypeptide of the disclosure. Capillary arrays provide another system for holding and screening samples. For example, a sample screening apparatus can include a plurality of capillaries formed into an array of adjacent capillaries, wherein each capillary comprises at least one wall defining a lumen for

retaining a sample. The apparatus can further include interstitial material disposed between adjacent capillaries in the array, and one or more reference indicia formed within of the interstitial material. A capillary for screening a sample, wherein the capillary is adapted for being bound in an array of capillaries, can include a first wall defining a lumen for retaining the sample, and a second wall formed of a filtering material, for filtering excitation energy provided to the lumen to excite the sample. A polypeptide or nucleic acid, e.g., a ligand, can be introduced into a first component into at least a portion of a capillary of a capillary array. Each capillary of the capillary array can comprise at least one wall defining a lumen for retaining the first component. An air bubble can be introduced into the capillary behind the first component. A second component can be introduced into the capillary, wherein the second component is separated from the first component by the air bubble. A sample of interest can be introduced as a first liquid labeled with a detectable particle into a capillary of a capillary array, wherein each capillary of the capillary array comprises at least one wall defining a lumen for retaining the first liquid and the detectable particle, and wherein the at least one wall is coated with a binding material for binding the detectable particle to the at least one wall. The method can further include removing the first liquid from the capillary tube, wherein the bound detectable particle is maintained within the capillary, and introducing a second liquid into the capillary tube. The capillary array can include a plurality of individual capillaries comprising at least one outer wall defining a lumen. The outer wall of the capillary can be one or more walls fused together. Similarly, the wall can define a lumen that is cylindrical, square, hexagonal or any other geometric shape so long as the walls form a lumen for retention of a liquid or sample. The capillaries of the capillary array can be held together in close proximity to form a planar structure. The capillaries can be bound together, by being fused (e.g., where the capillaries are made of glass), glued, bonded, or clamped side-by-side. The capillary array can be formed of any number of individual capillaries, for example, a range from 100 to 4,000,000 capillaries. A capillary array can form a micro titer plate having about 100,000 or more individual capillaries bound together.

[00233] Pharmaceutical Compositions

[00234] The present disclosure provides at least one composition comprising (a) a conditionally active biologic protein; and (b) a suitable carrier or diluent. The present disclosure also provides at least one composition comprising (a) a conditionally active

biologic protein encoding nucleic acid as described herein; and (b) a suitable carrier or diluent. The carrier or diluent can optionally be pharmaceutically acceptable, according to known carriers or diluents. The composition can optionally further comprise at least one further compound, protein or composition.

[00235] The conditionally active biologic protein may be in the form of a pharmaceutically acceptable salt. Pharmaceutically acceptable salts means which can be generally used as salts of an therapeutic protein in pharmaceutical industry, including for example, salts of sodium, potassium, calcium and the like, and amine salts of procaine, dibenzylamine, ethylenediamine, ethanolamine, methylglucamine, taurine, and the like, as well as acid addition salts such as hydrochlorides, and basic amino acids and the like.

[00236] The present disclosure further provides at least one conditionally active biologic protein method or composition, for administering a therapeutically effective amount to modulate or treat at least one parent molecule related condition in a cell, tissue, organ, animal or patient and/or, prior to, subsequent to, or during a related condition, as known in the art and/or as described herein. Thus, the disclosure provides a method for diagnosing or treating a condition associated with the wild-type protein in a cell, tissue, organ or animal, comprising contacting or administering a composition comprising an effective amount of at least one conditionally active biologic protein of the disclosure with, or to, the cell, tissue, organ or animal. The method can optionally further comprise using an effective amount of 0.001-50 mg/kilogram of a conditionally active biologic protein of the disclosure to the cells, tissue, organ or animal. The method can optionally further comprise using the contacting or the administrating by at least one mode selected from parenteral, subcutaneous, intramuscular, intravenous, intrarticular, intrabronchial, intraabdominal, intracapsular, intracartilaginous, intracavitary, intracelial, intracelebellar, intracerebroventricular, intracolic, intracervical, intragastric, intrahepatic, intramyocardial, intraosteal, intrapelvic, intrapericardiac, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intrarectal, intrarenal, intraretinal, intraspinal, intrasynovial, intrathoracic, intrauterine, intravesical, bolus, vaginal, rectal, buccal, sublingual, intranasal, or transdermal. The method can optionally further comprise administering, prior, concurrently, or after the conditionally active biologic protein contacting or administering at least one composition comprising an effective amount of at least one compound or protein selected from at least one of a detectable label or reporter, a TNF antagonist, an antirheumatic, a muscle relaxant, a narcotic, a non-steroid

anti-inflammatory drug (NSAID), an analgesic, an anesthetic, a sedative, a local anesthetic, a neuromuscular blocker, an antimicrobial, an antipsoriatic, a corticosteriod, an anabolic steroid, an erythropoietin, an immunization, an immunoglobulin, an immunosuppressive, a growth hormone, a hormone replacement drug, a radiopharmaceutical, an antidepressant, an antipsychotic, a stimulant, an asthma medication, a beta agonist, an inhaled steroid, an epinephrine or analog thereof, a cytotoxic or other anti-cancer agent, an anti-metabolite such as methotrexate, or an anti-proliferative agent.

[00237] The present disclosure further provides at least one conditionally active biologic protein method for diagnosing at least one wild-type protein related condition in a cell, tissue, organ, animal or patient and/or, prior to, subsequent to, or during a related condition, as known in the art and/or as described herein.

[00238] Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions of the present invention. A variety of aqueous carriers can be used, e.g., buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of conditionally active biologic protein in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

[00239] Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the packaged nucleic acid suspended in diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as liquids, solids, granules or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Pharmaceutical compositions and formulations of the invention for oral administration

can be formulated using pharmaceutically acceptable carriers well known in the art in appropriate and suitable dosages. Such carriers enable the pharmaceuticals to be formulated in unit dosage forms as tablets, pills, powder, dragees, capsules, liquids, lozenges, gels, syrups, slurries, suspensions, etc., suitable for ingestion by the patient. Pharmaceutical preparations for oral use can be formulated as a solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable additional compounds, if desired, to obtain tablets or dragee cores. Suitable solid excipients are carbohydrate or protein fillers include, e.g., sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose such as methyl cellulose, hydroxypropylmethyl cellulose, or sodium carboxy-methylcellulose; and gums including arabic and tragacanth; and proteins, e.g., gelatin and collagen. Disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, tragacanth, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscannellose sodium, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically acceptable carriers.

[00240] The invention provides aqueous suspensions comprising a conditionally active biologic protein, in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients include a suspending agent, such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia, and dispersing or wetting agents such as a naturally occurring phosphatide (e.g., lecithin), a condensation product of an alkylene oxide with a fatty acid (e.g., polyoxyethylene stearate), a condensation product of ethylene oxide with a long chain aliphatic alcohol (e.g., heptadecaethylene oxycetanol), a condensation product of ethylene oxide with a partial ester derived from a fatty acid and a hexitol (e.g., polyoxyethylene sorbitol mono-oleate), or a condensation product of ethylene oxide with a partial ester derived from fatty acid and a hexitol anhydride (e.g., polyoxyethylene sorbitan mono-oleate). The aqueous suspension can also contain one or more preservatives such as ethyl or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents and one or

more sweetening agents, such as sucrose, aspartame or saccharin. Formulations can be adjusted for osmolarity.

[00241] Lozenge forms can comprise the active ingredient in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the active ingredient, carriers known in the art. It is recognized that the conditionally active biologic protein, when administered orally, must be protected from digestion. This is typically accomplished either by complexing the conditionally active biologic protein with a composition to render it resistant to acidic and enzymatic hydrolysis or by packaging the conditionally active biologic protein in an appropriately resistant carrier such as a liposome. Means of protecting proteins from digestion are well known in the art. The pharmaceutical compositions can be encapsulated, e.g., in liposomes, or in a formulation that provides for slow release of the active ingredient.

[00242] The packaged conditionally active biologic protein, alone or in combination with other suitable components, can be made into aerosol formulations (e.g., they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like. Suitable formulations for rectal administration include, for example, suppositories, which consist of the packaged nucleic acid with a suppository base. Suitable suppository bases include natural or synthetic triglycerides or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the packaged nucleic acid with a base, including, for example, liquid triglycerides, polyethylene glycols, and paraffin hydrocarbons.

[00243] Dermal or topical delivery compositions of the invention may include in addition to a conditionally active biologic protein, a pharmaceutically acceptable carrier in a cream, ointment, solution or hydrogel formulation, and other compounds so long as the added component does not deleteriously affect delivery of the therapeutic protein. Conventional pharmaceutically acceptable emulsifiers, surfactants, suspending agents, antioxidants, osmotic enhancers, extenders, diluents and preservatives may also be added. Water soluble polymers can also be used as carriers.

[00244] Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions can be administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally. In one aspect, parenteral modes of administration are preferred methods of administration for compositions comprising a conditionally active biologic protein. The compositions may conveniently be administered in unit dosage form and may be prepared by any of the methods well-known in the pharmaceutical art, for example as described in Remington's Pharmaceutical Sciences, Mack Publishing Co. Easton Pa., 18th Ed., 1990. Formulations for intravenous administration may contain a pharmaceutically acceptable carrier such as sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes and the like. Also see and adapt the description in U.S. Pat. No. 4,318,905.

[00245] The formulations of packaged compositions comprising a conditionally active biologic protein can be presented in unit-dose or multi-dose sealed containers, such as ampoules and vials. Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

[00246] The present disclosure also provides at least one conditionally active biologic protein composition, device and/or method of delivery for diagnosing of at least one wild-type protein related condition, according to the present disclosure.

[00247] Also provided is a composition comprising at least one conditionally active biologic protein and at least one pharmaceutically acceptable carrier or diluent. The composition can optionally further comprise an effective amount of at least one compound or protein selected from at least one of a detectable label or reporter, a cytotoxic or other anti-cancer agent, an anti-metabolite such as methotrexate, an anti-proliferative agent, a cytokine, or a cytokine antagonist, a TNF antagonist, an antirheumatic, a muscle relaxant, a narcotic, a non-steroid anti-inflammatory drug (NSAID), an analgesic, an anesthetic, a sedative, a local anesthetic, a neuromuscular

blocker, an antimicrobial, an antipsoriatic, a corticosteriod, an anabolic steroid, an erythropoietin, an immunization, an immunoglobulin, an immunosuppressive, a growth hormone, a hormone replacement drug, a radiopharmaceutical, an antidepressant, an antipsychotic, a stimulant, an asthma medication, a beta agonist, an inhaled steroid, an epinephrine or analog.

[00248] Also provided is a medical device, comprising at least one conditionally active biologic protein of the disclosure, wherein the device is suitable to contacting or administering the at least one conditionally active biologic protein by at least one mode selected from parenteral, subcutaneous, intramuscular, intravenous, intrarticular, intrabronchial, intraabdominal, intracapsular, intracartilaginous, intracavitary, intracelial, intracelebellar, intracerebroventricular, intracolic, intracervical, intragastric, intrahepatic, intramyocardial, intraosteal, intrapelvic, intrapericardiac, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intrarectal, intrarenal, intraretinal, intraspinal, intrasynovial, intrathoracic, intrauterine, intravesical, bolus, vaginal, rectal, buccal, sublingual, intranasal, or transdermal.

[00249] In a further aspect, the disclosure provides a kit comprising at least one conditionally active biologic protein or fragment of the disclosure in lyophilized form in a first container, and an optional second container comprising sterile water, sterile buffered water, or at least one preservative selected from the group consisting of phenol, m-cresol, p-cresol, o-cresol, chlorocresol, benzyl alcohol, phenylmercuric nitrite, phenoxyethanol, formaldehyde, chlorobutanol, magnesium chloride, alkylparaben, benzalkonium chloride, benzethonium chloride, sodium dehydroacetate and thimerosal, or mixtures thereof in an aqueous diluent. In one aspect, in the kit, the concentration of conditionally active biologic protein or specified portion or variant in the first container is reconstituted to a concentration of about 0.1 mg/ml to about 500 mg/ml with the contents of the second container. In another aspect, the second container further comprises an isotonicity agent. In another aspect, the second container further comprises a physiologically acceptable buffer. In one aspect, the disclosure provides a method of treating at least one wild-type protein mediated condition, comprising administering to a patient in need thereof a formulation provided in a kit and reconstituted prior to administration.

[00250] Also provided is an article of manufacture for human pharmaceutical or diagnostic use, comprising packaging material and a container comprising a solution or a lyophilized form of at least one conditionally active biologic protein of the present disclosure. The article of manufacture can optionally comprise having the container as a component of a parenteral, subcutaneous, intramuscular, intravenous, intrarticular, intrabronchial, intraabdominal, intracapsular, intracartilaginous, intracavitary, intracelial, intracelebellar, intracerebroventricular, intracolic, intracervical, intragastric, intrahepatic, intramyocardial, intraosteal, intrapelvic, intrapericardiac, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intrarectal, intrarenal, intraretinal, intraspinal, intrasynovial, intrathoracic, intrauterine, intravesical, bolus, vaginal, rectal, buccal, sublingual, intranasal, or transdermal delivery device or system.

[00251] The present disclosure further provides any disclosure described herein.

[00252] Example 1: General Description of a Multiwall Assay (for example, 96-well assay) for Temperature Mutants:

[00253] Fluorescent substrate is added to each well of a multiwall plate, at both wild-type and new, lower reaction temperatures (for example, either 37°C or 25°C as mentioned above) for an appropriate time period. Fluorescence is detected by measuring fluorescence in a fluorescent plate reader at appropriate excitation and emission spectra (for example, 320 nm exitation/405 nm emission). Relative fluorescence units (RFU) are determined. Supernatant from wild type molecule and plasmid/vector transformed cells are used as positive and negative controls. Duplicate reactions are performed for each sample, reaction temperature, and positive and negative control.

[00254] Mutants that are active at the lower temperature (for example, the mutants active at 25°C) and that have a decrease in activity at the wild type temperature (for example, a 10%, 20%, 30%, 40% or more decrease in activity at 37°C), thus having a ratio of activities greater than or equal to about 1.1 or more (e.g., the ratio of the activities at 25°C or 37°C (25°C/37°C) is greater than or equal to 1.1 or more), can be deemed to be putative primary temperature sensitive hits. These putative primary temperature sensitive hits can then be rescreened, using the same assay, to confirm any primary hits.

[00255] Example 2: General Description of a Different Assay Format for Confirmation of Activity (for example, a 14-mL assay) for Temperature Mutants:

[00256] Mutants that are identified as temperature sensitive primary hits are expressed in 14 ml culture tubes and their enzymatic activity is measured at wild type (for example, 37°C) and the lower temperature (for example, 25°C). Protein is expressed and purified as described above for the multiwall format, with the exception that the expression is performed in different format (14 ml tubes) rather than the multiwall (96-well plate) format.

[00257] Each mutant supernatant is transferred to a multiwall plate, for example a 96-well microplate. Fluorescent substrate is added to each tube at the indicated reaction temperatures (wild-type, lower temperature) for a required period of time. Wild-type molecules are used as a positive control and supernatant from cells transformed with only vector is used as a negative control. Fluorescence is detected by measuring fluorescence in a fluorescent plate reader at the appropriate emission spectra (for example, 320 nm exitation/405 nm emission). Relative fluorescence units (RFU) are determined. Duplicate reactions can are performed for each sample, reaction temperature, and positive and negative control.

[00258] Mutants that are active at the lower temperatures (for example, 25°C) but that demonstrate at least a 30% or more decreased activity at wild type (for example, 37°C), thus have a ratio of activity at lower temperature (for example, 25°C) to wild type temperature (for example, 37°C) equal to or greater than 1.5, are identified as temperature sensitive hits.

[00259] The activities of mutants at the lower temperature (for example 25°C) are compared to the activity of the wild-type molecule at the wild-type temperature (for example 37°C). If molecules are more active than the wild-type molecules at the lower temperature (for example 25°C), as indicated by a residual activity >1, preferably 2 or greater than 2, and if the mutants demonstrate an overall decrease in activity when compared to the wild-type molecule at the wild-type temperature (37°C), the phenotype of the mutants as temperature sensitive mutants can be confirmed.

[00260] Example 3: General Description of Further Evolution of Hits Discovered:

[00261] If desired, a new, combinatorial variant library is generated from all or selected mutant hits previously identified. The new library can be designed to contain

every possible combination of amino acid variants for each of the selected mutants, and rescreened as described for new hits.

[00262] Example 4: General Description of Reversibility of Enzymatic Activity Following Decrease in Temperature:

[00263] Temperature sensitive, evolved mutants can be further assayed to determine whether enzymatic activity at lower temperatures (for example, 25°C) is reversible or irreversible by exposing the mutants to elevated temperatures followed by a return to the lower temperature (for example, 25°C). The temperature sensitive mutants are expressed in any desired format, for example in 14 ml culture tubes, as briefly described. The mutants are tested for their activities under several conditions, including the wild-type temperature (for example, 37°C) as well as other temperatures, and subsequently re-exposure to the requisite lower temperature of (25°C for example). Mutants that are active at lower temperatures, show decreased activity when raised to higher or wild-type temperatures (i.e., the ratio of the activities at lower to higher temperatures is equal to or greater than 1, 1.5, or 2 or higher), and exhibit a baseline activity when lowered again to the lower temperature are scored as "Reversible Hits". Mutants that are active at the lower temperature, show decreased activity when raised to higher or wild-type temperatures (i.e., the ratio of the activities at the lower to higher temperatures is equal to or greater than 1, 1.5 or 2 or higher), and exhibit at least the same amount of decreased activity when lowered again to the lower temperature are scored as "Irreversible Hits".

[00264] Example 5: Materials and methods to screen for conditionally active angiostatin variants. Materials and methods to screen for conditionally active angiostatin variants can be adapted from Chi and Pizzo, "Angiosatin is directly cytotoxic to tumor cells at low extracellular pH: a mechanism dependent on cell surface-associated ATP synthase", Cancer Res. 2006; 66(2):875-882, which is incorporated herein by reference.

[00265] Materials. Wild-type angiostatin kringles 1 to 3, derived from human plasminogen, can be obtained from Calbiochem (Darmstadt, Germany) and reconstituted in sterile PBS. Polyclonal antibodies directed against the catalytic beta-subunit of ATP synthase can be generated and bovine F1 ATP synthase subunit can be purified as previously described (Moser et al., "Angiostatin binds ATP synthase on the surface of human endothelial cells", Proc Natl Acad Sci U S A 1999;96:2811–6; Moser et al.

"Endothelial cell surface F1-F0 ATP synthase is active in ATP synthesis and is inhibited by angiostatin", Proc Natl Acad Sci U S A;2001;98:6656–61). Cariporide can be solubilized in sterile water and sterile filtered.

[00266] Cell culture. A549 (human epithelial cell line derived from a lung carcinoma tissue), or an alternative cancer cell line (DU145, LNCaP, or PC-3 cells) can be obtained from, for example, the ATCC. Human umbilical vein endothelial cells (HUVEC) can be isolated from human umbilical veins as described. (Grant et al., "Matrigel induces thymosin h 4 gene in differentiating endothelial cells", J Cell Sci 1995;108:3685–94.). HUVEC cells can be used as a positive control as a cell line that express ATP synthase on the cell surface. Cells can be cultured in DMEM (Life Technologies, Carlsbad, CA) with 1% penicillin streptomycin and 10% serum replacement medium 3 (Sigma, St. Louis, MO) to minimize the presence of plasminogen. Low-pH (6.7) medium can be prepared by reducing bicarbonate to 10 mmol/L at 5% CO2 and supplementing with 34 mmol/L NaCl to maintain osmolality or incubation of 22 mmol/L bicarbonate medium under 17% CO2 conditions. The method of lowering pH used can be varied by experimental constraints and assay.

[00267] Flow cytometry. To assure ATP synthase is functional on the cell surface of the tumor cell line, flow cytometry experiments can be performed. For example, A549 Cell lines can be cultured in varying pH medium (10, 22, and 44 mmol/L bicarbonate DMEM), under hypoxia (0.5% O2, 5% CO2, N2 balanced) versus normoxia (21% O2, 5% CO2) for 0, 12, 24, 48, and 72 hours. Live cells can be blocked, incubated with anti-β-subunit antibody, washed, blocked, incubated with a secondary goat anti-rabbit antibody-FITC (Southern Biotech, Birmingham, AL), and again washed, with all steps performed at 4 degrees C. Propidium iodide (BD Biosciences, San Jose, CA) can be included with all samples to discriminate cells with compromised membranes. The mean fluorescent intensity of FITC in 10,000 cells can be quantified by FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and cells with propidium iodide uptake can be excluded to eliminate detection of mitochondrial ATP synthase on CELLQuest software (BD Biosciences).

[00268] Cell surface ATP generation assay. A549 or 1-LN cells (60,000 per well) in 96-well plates can be refreshed with medium and treated with angiostatin, angiosatain variant, anti-beta-subunit antibody, rabbit IgG raised to bovine serum albumin (Organon

Teknika, West Chester, PA), piceatannol (a known inhibitor of ATP synthase F1 used as a positive control, Sigma), or medium alone for 30 minutes at 37 degrees C, 5% CO2. Cells can be then incubated with 0.05 mmol/L ADP for 20 seconds. Supernatants can be removed and assayed for ATP production by CellTiterGlo luminescence assay (Promega, Madison, WI) as described (23). Cell lysates can be similarly analyzed to confirm that intracellular pools of ATP did not vary under any conditions. Recordings can be made on the Luminoskan Ascent (Thermo Labsystems, Helsinki, Finland). Data are expressed in moles of ATP per cell based on standards determined for each independent experiment.

[00269] Cell proliferation assay. The effect of angiostatin on cancer cell lines can be assessed with a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)- 2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) proliferation assay in serum-free medium. Relative cell numbers in each well of a 96-well microplate after incubation for 20 hours, 37 degrees C, and 5% CO_2 in the presence or absence of angiostatin can be determined using the AQueous One Cell Proliferation Assay (Promega) per protocol of the manufacturer. Medium pH can be regulated at 5% CO_2 through bicarbonate concentration.

[00270] Assessment of cellular cytotoxicity. To quantify cell death and cell lysis, the activity of lactate dehydrogenase (LDH) released from the cytosol into supernatant can be measured with the Cytotoxicity Detection kit (Roche, Indianapolis, IN). Cancer cells (e.g. A549 cells)(5,000 per well) treated with angiostatin, angiostatin variant, anti-beta-subunit antibody, rabbit IgG, cariporide, and Triton X (a detergent used to permeabilize cells as a positive control) can be incubated at 37 degrees C and 5% CO_2 or 17% CO_2 for 15 hours at neutral and low pH conditions, respectively. An index of cytotoxicity can be calculated by dividing the average absorbance from treated samples in quadruplicate by the average absorbance from untreated samples in quadruplicate corresponding to the same pH medium. Assessment of cellular necrosis and apoptosis. To determine the mode of angiostatin induced cell death a histone-DNA ELISA can be performed. The effects of angiostatin, angiostatin variants, anti-beta-subunit antibody, rabbit IgG, and cariporide on A549 cells (5,000 per well) can be determined using an ELISA apoptosis and necrosis assay (Roche) that is dependent on detection of extranuclear histone-DNA fragments. Apoptosis or necrosis can be determined from, respectively, the cell lysates or supernatants of quadruplicate samples after 15 hours of incubation at 37 degrees C, in the presence or absence of agents. The apoptotic or necrotic indices can be calculated by

dividing the average absorbance from treated samples in quadruplicate by the average absorbance from untreated samples in quadruplicate corresponding to the same pH medium. Medium pH can be regulated by incubation at 5% CO2 or 17% CO_2.

[00271] Intracellular pH (pHi) measurement. pHi can be measured by fluorescence in cells plated on 35-mm microwell dishes with glass coverslips (MatTek, Ashland, MA). Cells can be plated on growth factor–reduced, phenol-red free Matrigel (BD Biosciences). After overnight growth, medium can be changed and cells can be loaded with the pH-sensitive fluorescent dye cSNARF (Molecular Probes, Eugene, OR) for 15 minutes followed by 20 minutes recovery in fresh medium. Cells can then be mounted on a microscope stage at 37 degrees C, 5% CO_2 for 1 hour-long collection of emission spectra from which pHi can be calculated as described from fields containing between 7 and 15 cells each (Wahl ML, Grant DS. "Effects of microenvironmental extracellular pH and extracellular matrix proteins on angiostatin's activity and on intracellular pH", Gen Pharmacol 2002;35:277–85). At the start of spectra collection, medium can be removed from the dish and cells can be challenged with 1 mL of fresh medium in the presence or absence of pH inhibitors angiostatin, anti-beta-subunit, rabbit IgG, or cariporide, a sodium-proton exchange inhibitor. Medium pH can be regulated by bicarbonate concentration, as described above, with fixed %CO_2.

WE CLAIM:

1. A method of preparing a conditionally active biologic protein, the method comprising:

i. selecting a wild-type biologic protein;

ii. evolving the DNA which encodes the wild-type biologic protein using one or more evolutionary techniques to create a mutant DNA;

iii. expressing the mutant DNA to obtain a mutant protein;

iv. subjecting the mutant protein and the wild-type protein to an assay under a normal physiological condition and to an assay under an aberrant condition; and

v. selecting the conditionally active biologic protein from those mutant proteins which exhibit both (a) a decrease in activity in the assay at the normal physiological condition compared to the wild-type protein, and (b) an increase in activity in the assay under the aberrant condition compared to the wild-type protein.

2. The method of claim 1 wherein the wild-type biologic protein is an enzyme.

3. The method of claim 2 wherein the wild-type biologic protein is selected from the group consisting of tissue plasminogen activator, streptokinase, urokinase, renin, and hyaluronidase.

4. The method of claim 1 wherein the wild-type biologic protein is selected from calcitonin gene-related peptide, substance P, neuropeptide Y, vasoactive intestinal peptide, vasopressin, and angiostatin.

5. The method of claim 1, wherein the normal physiological condition is selected from one or more of normal physiological temperature, pH, osmotic pressure, osmolality, oxidation and electrolyte concentration.

6. The method of claim 5, wherein the normal physiological condition is temperature; and wherein the conditionally active biologic protein is substantially

inactive at the normal physiological temperature, and is active at an aberrant temperature less than the normal physiological temperature.

7. A conditionally active biologic protein prepared by the method of claim 1, wherein the protein is reversibly or irreversibly inactivated at the normal physiological condition.

8. The conditionally active biologic protein of claim 7, wherein the protein is reversibly inactivated at the wild type normal physiological conditions.

9. The conditionally active biologic protein of claim 7, wherein the wild-type biologic protein is selected from the group consisting of tissue plasminogen activator, streptokinase, urokinase, renin, and hyaluronidase.

10. The conditionally active biologic protein of claim 7, wherein the wild-type biologic protein is selected from calcitonin gene-related peptide, substance P, neuropeptide Y, vasoactive intestinal peptide, vasopressin, and angiostatin.

11. A pharmaceutical composition comprising an effective amount of the conditionally active biologic protein of claim 7, and a pharmaceutically acceptable carrier.

12. The pharmaceutical composition of claim 11, wherein the conditionally active biologic protein is selected from the group consisting of a tissue plasminogen activator mutant, a streptokinase mutant, a urokinase mutant, a renin mutant, and a hyaluronidase mutant.

13. The pharmaceutical composition of claim 12, wherein the conditionally active biologic protein is selected from the group consisting of a tissue plasminogen activator mutant, a streptokinase mutant, and a urokinase mutant.

14. The pharmaceutical composition of claim 11, wherein the conditionally active biologic protein is selected from the group consisting of a calcitonin gene-related

peptide mutant, a substance P mutant, a neuropeptide Y mutant, a vasoactive intestinal peptide mutant, a vasopressin mutant, and an angiostatin mutant.

15. The pharmaceutical composition of claim 14, wherein the conditionally active biologic protein is selected from the group consisting of a calcitonin gene-related peptide mutant, a vasopressin mutant, and an angiostatin mutant.

16. A method of preparing a conditionally active biological response modifier, the method comprising:

a. selecting an inflammatory response mediator;
b. identifying a wild-type antibody to the mediator;
c. evolving the wild-type antibody;
d. screening differentially for mutants that exhibit decreased binding to the mediator relative to the wild-type antibody at a first condition, and exhibit increased binding affinity to the mediator at a second condition to identify up-mutants; and
e. recombining the heavy chains and the light chains of the up-mutants to create recombined up-mutants; and
f. screening the recombined up-mutants for mutants that exhibit decreased binding to the mediator relative to the wild-type antibody at the first condition, and show increased binding affinity to the mediator at the second condition to identify the conditionally active biological response modifier.

17. The method of claim 16 wherein the inflammatory response mediator is selected from IL-6, IL-6 receptor, TNF-alpha, IL-23 and IL-12.

18. The method of claim 16 wherein the first and second condition are each selected from conditions of pH, osmotic pressure, osmolality, oxidation and electrolyte concentration.

INTERNATIONAL SEARCH REPORT

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PCT/US 10/26611

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68, C12P 21/00; C07K 14/00; A61K 38/17 (2010.01)
USPC - 435/6, 435/69.1, 530/350, 424/133.1, 514/2
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)
IPC(8): C12Q 1/68, C12P 21/00; C07K 14/00; A61K 38/17 (2010.01)
USPC: 435/6, 435/69.1, 530/350, 424/133.1, 514/2

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)
Databases used: WEST (PGPB,USPT,EPAB,JPAB), Google Scholar, Google Patents, esp@cenet
Search terms used: wild type, protein, mutant, enzyme, tissue plasminogen activator, streptokinase, urokinase, renin, hyaluronidase, evolution, interleukin, IL-6, IL-23, IL-12, TNF, Jay Short, Bioatla, Frey, assay, streptokinase, angiostatin, evolution

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2005/0100985 A1 (SHORT) 12 May 2005 (12.05.2005), para [0001], [0002], [0010], [0031],[0033], [0034], [0078], [0094], [0120], [0124], [0132], [0136], [0141], [0142], [0173], [0174], [0175], [0180, [0184], [0269], [0281], [0302], [0307], [0313], [0340], [0367].	1-18
Y	US 2007/0009930 A1 (PATTEN et al.) 11 January 2007 (11.01.2007), abstract; para [0175], [0207], [0239], [0244], [0245], [0269], [0274], [0283], [0324], [0342], Table I.	1-18
Y	US 2008/0131500 A1 (CHANG) 5 June 2008 (05.06.2008), abstract; para [0016], [0082], [0119].	7-15

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"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)
"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
"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
"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
"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
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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权利要求书2页 说明书40页

(54)发明名称

MIRAC 蛋白

(57)摘要

本发明涉及从野生型蛋白中制备在野生型正常生理条件下可逆或不可逆失活的条件活性生物蛋白,特别是治疗性蛋白的方法。例如,演变的蛋白在体温下几乎无活性,但在较低温度下有活性。

CN 105132395 A

1. 一种制备条件活性生物蛋白的方法，该方法包括：

i. 选择一种野生型哺乳动物生物蛋白；

ii. 使用一种或多种演变技术演变编码所述野生型哺乳动物生物蛋白的 DNA，从而产生至少一个突变 DNA；

iii. 表达所述至少一个突变 DNA 以获得至少一种突变蛋白；

iv. 在正常生理条件下和异常条件下对所述至少一种突变蛋白和所述野生型哺乳动物生物蛋白进行测试；以及

v. 从表现出以下两种特性的所述突变蛋白中筛选条件活性生物蛋白：(a) 在正常生理条件下的测试中较野生型哺乳动物生物蛋白活性下降，以及 (b) 在异常条件下的测试中较野生型哺乳动物生物蛋白活性增强。

2. 根据权利要求 1 所述的方法，其中所述野生型哺乳动物生物蛋白是一种酶。

3. 根据权利要求 2 所述的方法，其中所述野生型哺乳动物生物蛋白选自组织型纤溶酶原激活物、链激酶、尿激酶、肾素和透明质酸酶。

4. 根据权利要求 1 所述的方法，其中所述野生型哺乳动物生物蛋白选自降钙素基因相关肽、P 物质、神经肽 Y、血管活性肠肽、血管加压素和血管抑素。

5. 根据权利要求 1 所述的方法，其中所述正常生理条件选自正常的生理温度、pH、渗透压、渗量、氧化和电解质浓度中的一种或多种。

6. 根据权利要求 5 所述的方法，其中所述正常生理条件是温度；且其中所述条件活性生物蛋白在正常生理温度下基本上失活，而在低于正常生理温度的异常温度下有活性。

7. 根据权利要求 1 所述的方法，还包括通过自然方法或化学方法修饰所述条件活性生物蛋白以获得修饰的条件活性生物蛋白的步骤。

8. 根据权利要求 7 所述的方法，其中修饰步骤是使用选自下组的技术进行的化学方法：乙酰化、酰化、聚乙二醇、ADP- 核糖基化、酰胺化、黄素的共价连接、血红素基团的共价连接、核苷酸或核苷酸衍生物的共价连接、脂质或脂类衍生物的共价连接、磷脂酰肌醇的共价连接、交联环化、二硫键形成、去甲基化、形成共价交连、形成半胱氨酸、形成焦谷氨酸、甲酰化、γ- 羧化、糖基化、形成 GPI 锚定、羟基化、碘化、甲基化、豆蔻酰化、氧化、聚乙二醇化、蛋白水解处理、磷酸化、异戊烯化、消旋、蛋白硒化、硫酸盐化及转移 RNA 介导的添加氨基酸蛋白质。

9. 根据权利要求 8 所述的方法，其中所述技术是二硫键形成。

10. 根据权利要求 8 所述的方法，其中所述技术是共价连接。

11. 根据权利要求 7 所述的方法，其中所述自然方法是通过翻译后加工进行的。

12. 根据权利要求 11 所述的方法，其中所述翻译后加工选自磷酸化和酰化。

13. 根据权利要求 1 所述方法制备的条件活性生物蛋白，其中所述蛋白在正常生理条件下不可逆失活。

14. 根据权利要求 13 所述的条件活性生物蛋白，其中所述蛋白在野生型正常生理条件下可逆失活。

15. 根据权利要求 13 所述的条件活性生物蛋白，其中所述野生型哺乳动物生物蛋白选自下组：组织型纤溶酶原激活物、链激酶、尿激酶、肾素和透明质酸酶。

16. 根据权利要求 13 所述的条件活性生物蛋白，其中所述野生型哺乳动物生物蛋白选

自降钙素基因相关肽、P 物质、神经肽 Y、血管活性肠肽、血管加压素和血管抑素。

17. 一种药物组合物，其含有有效量的权利要求 13 所述的条件活性生物蛋白及药用载体。

18. 根据权利要求 17 所述的药物组合物，其中所述的条件活性生物蛋白选自下组：组织型纤溶酶原激活物突变体、链激酶突变体、尿激酶突变体、肾素突变体和透明质酸酶突变体。

19. 根据权利要求 18 所述的药物组合物，其中所述的条件活性生物蛋白选自下组：组织型纤溶酶原激活物突变体、链激酶突变体和尿激酶突变体。

20. 根据权利要求 17 所述的药物组合物，其中所述的条件活性生物蛋白选自下组：降钙素基因相关肽突变体、P 物质突变体、神经肽 Y 突变体、血管活性肠肽突变体、血管加压素突变体和血管抑素突变体。

21. 根据权利要求 20 所述的药物组合物，其中所述的条件活性生物蛋白选自下组：降钙素基因相关肽突变体、血管加压素突变体和血管抑素突变体。

MIRAC 蛋白

[0001] 本申请要求 2009 年 3 月 9 日提交的美国专利申请号 61/209,489 的优先权 ;本申请是 2010 年 3 月 9 日提交的中国专利申请号 2010800114657 的分案申请。

[0002] 相关申请信息

[0003] 本申请作为 PCT 国际专利申请,以申请人 BioAtla, LLC 美国有限责任公司的名义于 2010 年 3 月 9 日提交,指定除美国以外的所有国家,美国公民 Jay M. Short 和 Hwai Wen Chang,以及德国公民 Gerhard Frey 仅作为指定美国的申请人,并要求申请日为 2009 年 3 月 9 日的美国临时专利申请第 61/209,489 号的优先权,其全部内容通过参考并入于此。

技术领域

[0004] 本发明涉及蛋白演变和活性领域。具体而言,本发明涉及从野生型蛋白中制备在野生型正常生理条件下可逆或不可逆失活的条件活性生物蛋白 (conditionally active biologic protein),特别是治疗性蛋白的方法。例如,演变的蛋白在体温下几乎无活性,但在较低温度下有活性。

背景技术

[0005] 有大量文献描述了演变蛋白,特别是例如酶的各种特性的潜力,以在不同条件下稳定工作。例如,酶已经演变为在较高温度下稳定,具有改变的活性。在高温活性增强的情况下,该增强中的大部分属于 Q10 律中普遍描述的较高的动力活性,对于酶来说评估为温度每增加 10℃,其转换率增加一倍。此外,还存在自然突变的例子,其使蛋白在正常工作条件如该分子的野生型温度活性下变得不稳定。对于温度的突变,这些突变可以在较低温度下具有活性,但通常是较野生型分子具有降低水平的活性 (通常也描述为活性下降,该活性受 Q10 或类似规则支配)。

[0006] 人们期望获得有用的有条件地被激活的分子,例如在野生型条件下几乎无活性,但在除野生型条件下之外具有活性,其活性水平与野生型条件相同或比野生型条件更高,或在特定微环境中被激活或被失活,或经过一段时间后被激活或被失活。除了温度,其它能够使蛋白演变或优化的条件包括 pH、渗透压、渗量 (osmolality)、氧化和电解质浓度。其它在演变过程中能够被优化的目标特性包括耐化学性和耐蛋白水解性。

[0007] 已公布了许多演变或改造分子的策略。然而,将蛋白改造或演变为在其野生型工作条件下变为失活或几乎失活 (≤ 10 % 活性,特别是 1 % 活性),而在新的条件下与其野生型条件的活性相当或高于其野生型条件的活性,需要该失稳突变 (destabilizing mutations) 与不对抗该失稳效应 (destabilizing effect) 的活性增强的突变共存。据预期,不稳定降低该蛋白的活性会比标准规则如 Q10 所预测的影响大,因此,演变出在低温下有效工作的蛋白的能力,例如,而在它们的正常工作条件下是失活的,可制备一类意想不到的新的蛋白,我们称之为 Mirac 蛋白。

[0008] 在本申请中,根据作者和日期引用了各种出版物。为了更充分地描述本领域技术人员已知的这些公开物的日期和请求保护的权利要求中所描述的本领域状况,这些出版物

以参阅的方式并入本申请。

[0009] 发明概述

[0010] 本发明提供一种制备条件活性生物蛋白的方法，该方法包括：选择一种野生型生物蛋白；使用一种或多种演变技术演变编码该野生型生物蛋白的DNA，从而产生突变DNA；表达该突变DNA以获得突变蛋白；在正常生理条件下和异常条件下对该突变蛋白和野生型蛋白进行测试；以及从那些表现出以下两种特性的突变蛋白中筛选条件活性生物蛋白：(a)在正常生理条件下的测试中较野生型蛋白活性下降，以及(b)在异常条件下的测试中较野生型蛋白活性增强。在各个方面中，所述正常生理条件选自温度、pH、渗透压、渗量、氧化和电解质浓度中的一种或多种。在一个特定的方面，所述正常生理条件是温度，其中该条件活性生物蛋白在正常生理温度下是完全失活的，而在低于正常生理温度的异常温度下有活性。在其它方面，该条件活性生物蛋白在野生型正常生理条件下是可逆或不可逆失活的。在一个特定的方面，该蛋白在野生型正常生理条件下是可逆失活的。可选择地，条件活性生物蛋白选自那些在两种或两种以上不同生理条件下表现出活性可逆或不可逆变化的蛋白。

[0011] 在一个实施方案中，所述野生型生物蛋白是一种酶。在某些方面，所述野生型生物蛋白选自下组：组织型纤溶酶原激活物、链激酶、尿激酶、肾素和透明质酸酶。

[0012] 在另一个实施方案中，所述野生型生物蛋白选自降钙素基因相关肽(CGRP)、P物质(SP)、神经肽Y(NPY)、血管活性肠肽(VIP)、血管加压素(vasopressin)和血管抑素。

[0013] 在另一个实施方案中，所述生物蛋白是一种抗体。

[0014] 在另一个实施方案中，本发明提供一种制备条件活性生物反应调节剂(biological response modifier)的方法，该方法包括：选择一种炎症反应介质；鉴定该介质的野生型抗体；演变该野生型抗体；差异筛选出具有以下特性的突变体：在第一条件下较野生型抗体与介质的结合下降，以及在第二条件下与介质的结合亲和力增加，从而鉴定出改善型突变体(up-mutant)；重组该改善型突变体的重链和轻链，以产生重组改善型突变体；以及筛选该重组改善型突变体中具有以下特性的突变体：在第一条件下较野生型抗体与介质的结合下降，以及在第二条件下与介质的结合亲和力增加，从而鉴定出条件活性生物反应调节剂。在一个方面，所述的炎症反应介质选自下组：IL-6、IL-6受体、TNF-α、IL-23和IL-12。在另一个方面，所述第一和第二条件选自以下条件：pH、渗透压、渗量、氧化和电解质浓度。

[0015] 在另一个实施方案中，本发明提供一种含有条件活性生物蛋白及药用载体的药物组合物。

[0016] 发明详述

[0017] 为了便于理解本文提供的实例，将对一些频繁出现的方法和/或术语进行描述。

[0018] 本文中使用的与测定量有关的术语“约”是指测定量的正常变化，其可由本领域熟练技术人员进行测量和实施与测量目的和测量仪器精度相称的关注等级(level of care)所预料。除非另有说明，“约”是指所提供值的变化为+/-10％。

[0019] 本文中使用的术语“试剂”是指一种化合物、化合物的混合物、空间定位化合物系列(例如，VLSIPS肽系列、多核苷酸系列和/或组合小分子系列)、生物大分子、噬菌体肽展示文库、噬菌体抗体(例如，scFv)展示文库、多核糖体肽展示文库或从生物材料，如细菌、植物、真菌或动物(特别是哺乳动物)的细胞或组织中获得的提取物。在下面描述的筛选

试验中评价试剂的潜在酶活性。在下面描述的筛选试验中评价试剂作为条件活性生物治疗性酶的潜在活性。

[0020] 限制位点中的“多义碱基需要(ambiguous base requirement)”是指不限定为最大范围的核苷酸碱基需要,即不限于特定的碱基(如,在非限制性的实例中,特定的碱基选自A、C、G和T),而可以是至少两种或两种以上碱基中的任一种。本领域以及本文中使用的普遍接受的缩写,代表以下碱基的多义包括:R=G或A;Y=C或T;M=A或C;K=G或T;S=G或C;W=A或T;H=A或C或T;B=G或T或C;V=G或C或A;D=G或A或T;N=A或C或G或T。

[0021] 本文中使用的术语“氨基酸”是指任何含有氨基($-NH_2$)和羧基(-COOH)的有机化合物;优选为自由基或聚合后作为肽键的一部分。本领域已知的“二十个自然编码的多肽-形成α-氨基酸”是指:丙氨酸(ala或A)、精氨酸(arg或R)、天冬酰胺(asn或N)、天冬氨酸(asp或D)、半胱氨酸(cys或C)、谷氨酸(glu或E)、谷氨酰胺(gln或Q)、甘氨酸(gly或G)、组氨酸(his或H)、异亮氨酸(ile或I)、亮氨酸(leu或L)、赖氨酸(lys或K)、蛋氨酸(met或M)、苯丙氨酸(phe或F)、脯氨酸(pro或P)、丝氨酸(ser或S)、苏氨酸(thr或T)、色氨酸(trp或W)、酪氨酸(tyr或Y)和缬氨酸(val或V)。

[0022] 术语“扩增”是指多核苷酸的拷贝数增加。

[0023] 具有“嵌合特性”的分子是指分子:1)与第一参考分子部分同源和部分异源;且2)同时与第二参考分子部分同源和部分异源;而不具有3)预先排除同时与一种或多种其它参考分子部分同源和部分异源的可能性。在一个非限制性的实施方案中,嵌合分子可以通过组装重新排列的部分分子序列而制得。在一个非限制性的方面,嵌合多核苷酸分子可以通过使用大量分子模板来合成嵌合多核苷酸而制得,使得到的嵌合多核苷酸具有大量模板的特性。

[0024] 本文中使用的术语“同源(cognate)”是指物种间的进化和功能相关的基因序列。例如,但不限于,在人类基因组中人CD4基因是小鼠3d4基因的同源基因,因为这两个基因的序列和结构表明,它们具有较高的同源性,两个基因都编码一种在通过MHC Ⅱ类限制抗原识别而传递T细胞激活信号中发挥作用的蛋白。

[0025] 本文中使用的术语“比较窗口”是指至少20个连续核苷酸的概念片段(conceptual segment),其中多核苷酸序列可以与至少20个连续核苷酸的参考序列进行比较,且比较窗口中的部分多核苷酸序列可包括与参考序列(其不包括添加或缺失)相比20%或低于20%的添加或缺失(即,缺口),使两条序列达到最适比对。用于比较窗口比对的最适比对序列可以按照史密斯局部同源性算法来进行(Smith and Waterman,1981“生物序列的比较(Comparison of biosequences)”,Adv Appl Math,2:482-489;Smith and Waterman,1981,“重叠基因和信息理论(Overlapping genes and information theory)”,J Theor Biol,91:379-380;Smith and Waterman,J MoI Biol,“普通分子序列的确定(Identification of common molecular subsequences)”,1981,147:195-197;Smith等人,1981,“比较的生物序列的排列(Comparative biosequence metrics)”,J MoI Evol,18:38-46),按照Needleman同源性比对算法来进行(Needleman和Wunsch,1970,“两个蛋白氨基酸序列的相似性搜索的一般适用方法(A general method applicable to the search for similarities in the amino acid sequence of two proteins)”J

MoI Biol,48(3):443-453)，按照Pearson相似性搜索的方法来进行(Pearson and Lipman,1988,“用于生物序列比较的改进工具(Improved tools for biological sequence comparison)”,Proc Nat Acad Sci USA,85:2444-2448)，按照这些算法的计算机化运行来进行(GAP,BESTFIT,FASTA,and TFASTA in the Wisconsin Genetics Software Package Release 7.0,Genetics Computer Group,575Science Dr.,Madison,Wis)，或通过检查，根据选择的各种方法产生最佳比对（即，在比较窗口中形成最高同源百分数）。

[0026] 术语“条件活性生物蛋白(conditionally active biologic protein)”是指野生型蛋白的变体或突变体，其在一种或多种正常生理条件下较亲本野生型蛋白活性高或低。这种条件活性蛋白还在身体的选定区域表现出活性，和/或在异常或允许的生理条件下表现出增加的或下降的活性。正常生理条件是温度、pH、渗透压、渗量、氧化和电解质浓度，它们在个体的给药部位或在个体作用部位的组织或器官中被认为是在正常范围内的。异常条件是指偏离了正常可接受范围的条件。在一个方面，条件活性生物蛋白在野生型条件下几乎是无活性的，但在除野生型条件之外是有活性的，其活性与野生型条件的活性相同或高于野生型条件的活性。例如，在一个方面，演变的条件活性生物蛋白在体温下几乎无活性，但在较低温度下有活性。在另一方面，条件活性生物蛋白在野生型条件下是可逆或不可逆失活的。在另一方面，野生型蛋白是一种治疗性蛋白。在另一个方面，条件活性生物蛋白用作药物或治疗剂。在另一个方面，所述蛋白在高含氧血液，例如，在流经肺后或在肾脏中发现的较低pH环境中，或多或少有活性。

[0027] “保守氨基酸替换”是指具有相似侧链残基的可互换性。例如，一组具有脂肪族侧链的氨基酸：甘氨酸、丙氨酸、缬氨酸、亮氨酸和异亮氨酸；一组具有脂肪族羟基侧链的氨基酸：丝氨酸和苏氨酸；一组具有含酰胺侧链的氨基酸：天冬酰胺和谷氨酰胺；一组具有芳香侧链的氨基酸：苯丙氨酸、酪氨酸和色氨酸；一组具有碱性侧链的氨基酸：赖氨酸、精氨酸和组氨酸；以及一组具有含硫侧链的氨基酸：半胱氨酸和蛋氨酸。优选的保守氨基酸替换组为：缬氨酸-亮氨酸-异亮氨酸、苯丙氨酸-酪氨酸、赖氨酸-精氨酸、丙氨酸-缬氨酸和天冬酰胺-谷氨酰胺。

[0028] 本文中使用的术语“对应于”是指多核苷酸序列与全部或部分参考多核苷酸序列同源（即，是相同的、非严格进化相关的），或多肽序列与参考多肽序列是相同的。相比之下，本文中使用的术语“互补”是指其互补序列与全部或部分参考核苷酸序列同源。例如，核苷序列“TATAC”对应于参考序列“TATAC”，与参考序列“GTATA”互补。

[0029] 术语“降解有效”量是指与不接触酶的底物相比，加工至少50％底物所需的酶量。

[0030] 本文中使用的术语“确定的序列框架”是指一组基于非随机选择的具体序列，一般基于实验数据或结构数据；例如，一个确定的序列框架，除其它变体外，可包括预测形成β片层结构的一组氨基酸序列，或可包括一个亮氨酸拉链七肽重复基序(motif)、一个锌指结构域。“确定的序列核”是包括有限范围可变性的一组序列。而(1)20个常规氨基酸的完全随机10-mer序列可以是$(20)^{10}$个序列中的任一条序列，以及(2)20个常规氨基酸的伪随机10-mer序列可以是$(20)^{10}$个序列中的任一条序列，但表现出特定位置和/或整体的特定残基有偏差，(3)如果各残基位被允许是20个常规氨基酸（和/或允许的非常规氨基/亚氨基酸）中的任一个，则确定的序列核是序列的子序列。确定的序列核一般包括变化和未发生变化的残基位，和/或包括变化的残基位，该变化的残基位可包括一个选自氨基酸残基

的确定子集的残基等，或者选自从序列库中筛选出的单独序列的片段或全长中的残基。确定的序列核可以指氨基酸序列或多核苷酸序列。确定的序列核的实例为，但不限于，序列 $(NNK)_{10}$ 和 $(NNM)_{10}$，其中，N 代表 A、T、G 或 C；K 代表 G 或 T；以及 M 代表 A 或 C。

[0031] DNA 的“消化”是指用仅在 DNA 的某些序列发挥作用的限制酶催化 DNA 的裂解 (cleavage)。本文中使用的各种限制酶可市售获得，它们采用的反应条件、辅助因子和其它条件是本领域普通技术人员已知的。为了进行分析，一般向含有 1 微克质粒或 DNA 片段的约 20 微升缓冲液中加入约 2 单位的酶。为了分离用于构建质粒的 DNA 片段，一般在较大体积中用 20 至 250 单位的酶消化 5 至 50 微克的 DNA。合适的缓冲液和用于特定限制酶作用的底物量由制造商指定。通常在 37℃进行孵育，时间约 1 小时，但按照供应商的说明可能会有所不同。消化结束后，反应物直接在凝胶上进行电泳，分离出所需的片段。

[0032] “定向连接”是指其中多核苷酸的 5’端和 3’端不同的连接，足以确定较佳的连接方向。例如，具有两个钝末端的未经处理的和未消化的 PCR 产物，在连接至经消化的多克隆位点处形成钝末端的克隆载体中时，一般没有较佳的连接方向；因而，在这些情况下一般不会出现定向连接。相比之下，一般在具有用 EcoR Ⅰ处理的 5’端和用 BamH Ⅰ处理的 3’端的经消化的 PCR 产物连接至多克隆位点用 EcoR Ⅰ和 BamH Ⅰ消化的克隆载体中时，出现定向连接。

[0033] 本文中使用的术语“DNA 改组 (shuffling)”是指基本上同源但不完全相同的序列之间的重组，在一些实施方案中，DNA 改组可包括通过非同源重组交叉 (crossover)，如通过 cer/lox 和 / 或 flp/frt 系统等。DNA 改组可以是随机或者非随机的。

[0034] 术语“药物”或“药物分子”是指包括一种将其对人体或动物体给药时对人体或动物体产生有利效果的物质的治疗剂。优选地，所述治疗剂包括可以治疗、治愈或缓解人体或动物体中一种或多种症状、疾病或异常情况的物质或可以增进人体或动物体健康的物质。

[0035] “有效量”是指向活的有机体给药一段时间，对于治疗或预防活的有机体内疾病有效的条件活性生物蛋白或片段的量，例如在所需的给药间隔期内，提供治疗的效果。

[0036] 本文中使用的术语“电解质”的含义为在血液或其它体液中带有电荷的矿物质 (mineral)。例如，在一个方面，正常生理条件和异常条件可以是“电解质浓度”的条件。在一个方面，待测电解质浓度选自一种或多种离子化的钙、钠、钾、镁、氯、碳酸氢盐和磷酸盐浓度。例如，在一个方面，血清钙的正常范围为 8.5 至 10.2mg/dL。在这方面，异常的血清钙浓度可以在高于或低于正常范围内选择。在另一个实例中，在一个方面，血清氯的正常范围是每升 96-106 毫当量 (mEq/L)。在这方面，异常的血清氯浓度可以在高于或低于正常范围内选择。在另一个实例中，在一个方面，血清镁的正常范围是从 1.7-2.2mg/dL。在这方面，异常的血清镁浓度可以在高于或低于正常范围内选择。在另一个实例中，在一个方面，血清磷的正常范围是从 2.4 到 4.1mg/dL。在这方面，异常的血清磷浓度可以在高于或低于正常范围内选择。在另一个实例中，在一个方面，正常的血清或血液中钠的范围是从 135 到 145mEq/L。在这方面，异常的血清或血液中钠浓度可以在高于或低于正常范围内选择。在另一个实例中，在一个方面，正常的血清或血液中钾的范围是从 3.7 到 5.2mEq/L。在这方面，异常的血清或血液中钾浓度可以在高于或低于正常范围内选择。在另一个方面，正常的血清碳酸氢盐范围是从 20 到 29mEq/L。在这方面，异常的血清或血液中碳酸氢盐的浓度可以在高于或低于正常范围内选择。在一个不同的方面，碳酸氢盐水平可以用来指示血液中

酸度的正常水平 (pH)。术语“电解质浓度”也可以用来定义除血液或血浆以外的组织或体液中特定电解质的条件。在这种情况下,正常生理条件被认为是该组织或液体的临床正常范围。在这方面,异常的组织或体液的电解质浓度可以在高于或低于正常范围内选择。

[0037] 本文中使用的术语“抗原表位”是指抗原如酶多肽上的抗原决定簇,抗体互补位如酶的特异性抗体结合到抗原决定簇上。抗原决定簇通常是由分子的化学活性表面组合如氨基酸或糖侧链组成的,可具有特定的三维结构特点以及特定的电荷特性。本文中使用的“抗原表位”是指能够形成相互结合作用 (binding interaction) 的抗原或其它大分子的那部分,其与抗体的可变区结合体相互作用。通常情况下,这种相互结合作用表现为与 CDR 的一个或多个氨基酸残基的分子间接触。

[0038] 本文中使用的“酶”是具有特定催化性能的蛋白。例如底物浓度、pH、温度和有无抑试剂存在等因素可以影响催化的速率。通常情况下,对于野生型酶,Q10(温度系数)以温度上升 10℃描述反应速率的增加。对于野生型酶,Q10 = 2 到 3 ;换言之,温度每上升 10℃反应速率提高到两倍或三倍。在高温下,蛋白变性。在 pH 值与酶的最佳 pH 值略有不同时,酶以及也许底物分子所带电荷发生微小变化。离子的变化可以影响底物分子的结合。在极端的 pH 水平,酶会发生变性,其活性位点被破坏,不再适合底物分子。

[0039] 本文中使用的术语“演变 (evolution)”或“演变 (evolving)”是指使用一种或多种诱变方法,产生编码新多肽的新多核苷酸,新多肽本身就是一种改进的生物分子和 / 或有助于另一种改进的生物分子的生成。在一个特定的非限制性方面,本发明涉及从亲本野生型蛋白中演变出条件活性生物蛋白。例如,在一个方面,演变涉及采用美国专利申请公开号 2009/0130718(通过参考并入于此)中公开的非随机的多核苷酸嵌合 (chimerization) 和非随机的定点诱变的方法。更具体地,本发明提供在正常生理条件下较野生型酶亲本分子表现出活性下降,而在一种或多种异常条件下较野生型酶表现出活性增强的条件活性生物酶的演变方法。

[0040] 当术语“片段”、“衍生物”和“类似物”对照参考多肽时,包括保留至少一种至少在本质上与参考多肽相同的生物功能或活性的多肽。此外,术语“片段”、“衍生物”或“类似物”的实例是分子的“前体形式”,如低活性蛋白原 (proprotein),其可通过切割修饰而产生具有显著高活性的成熟酶。

[0041] 本文提供了一种由模板多肽生成一组其中在各氨基酸位表现出“单个氨基酸全方位替换”的后代多肽 (progeny polypeptide) 的方法。本文中使用的“单个氨基酸全方位替换”是关于本文中所描述的 20 个自然编码的多肽形成的 α - 氨基酸。

[0042] 术语“基因”是指参与产生多肽链的 DNA 片段 ;它包括编码区之前和之后的区域(前导区和拖尾区)以及各编码片段(外显子)之间的间隔序列(内含子)。

[0043] 本文中使用的“遗传不稳定”是指在减少事件 (reductive events) 过程中(其一般涉及通过重复序列的丢失使序列简化)高度重复序列被丢失的自然倾向。缺失往往涉及一个重复拷贝的丢失和重复拷贝之间的一切丢失。

[0044] 术语“异源”是指一条单链核酸序列无法与另一条单链核酸序列或其互补序列进行杂交。因而,异源区是指多核苷酸区或多核苷酸的序列中具有无法与另一条核酸或多核苷酸进行杂交的区或区域。这种区域或区例如为突变区。

[0045] 术语“同源 (homologous)”或“部分同源 (homeologous)”是指一条单链核酸序列

可以与单链核酸序列的互补序列进行杂交。杂交的程度可能取决于许多因素，包括序列之间同一性数量和杂交条件如稍后讨论的温度和盐浓度。优选地，同一性区大于约 5bp，更优选地，同一性区大于约 10bp。

[0046] 本发明的益处延伸到“工业应用”（或工业生产过程），该术语用于包括在商业性工业中适当的应用（或简称工业）以及非商业性的工业应用（例如在非营利机构的生物医学研究中）。相关的应用包括那些诊断、医药、农业、制造业和学术界领域。

[0047] 术语“相同”或“同一性 (identity)”是指两条核酸序列有相同的序列或互补序列。因而，“同一性区”，是指核苷酸或整条多核苷酸区域或区是相同的或与另一条多核苷酸序列区互补。

[0048] 术语“分离的”是指该物质被从原来的环境（例如，自然环境，如果它是天然存在的）中移出。例如，存在于活体动物中自然产生的核苷酸或酶是未分离的，但从天然系统中共存的部分或全部物质中分离出的相同的核苷酸或酶是分离的。这种多核苷酸可以是载体的一部分，和 / 或这种多核苷酸或酶可以是组合物的一部分，由于这种载体或组合物不是其天然环境的一部分，其仍然是分离的。

[0049] 使用术语“分离的核酸”用来定义与 5’ 和 3’ 侧翼序列不紧密连接的核酸，例如 DNA 或 RNA 分子，而在衍生其的有机体中天然产生的基因组中通常是紧密连接的。因而，该术语描述为，例如，整合至载体如质粒或病毒载体中的核酸；整合至异源细胞基因组（或同源细胞基因组，但位点不同于其天然发生的位点）中的核酸；以及作为单独分子存在的核酸，例如通过 PCR 扩增或限制酶切产生的 DNA 片段，或通过体外转录产生的 RNA 分子。该术语还描述了形成编码其它多肽序列的杂交基因一部分的重组核苷酸，该其它多肽可用于例如产生融合蛋白。

[0050] 本文中使用的“配体”是指可被特定受体识别的分子，如随机肽或可变区序列。本领域的熟练技术人员应知道，分子（或大分子复合物）既可以是受体也可以是配体。在一般情况下，具有较小分子量的结合体是指配体，具有较大分子量的结合体是指受体。

[0051] “连接”是指两条双链核酸片段之间形成磷酸二酯键的过程 (Sambrook 等人，(1982). 分子克隆：分子克隆手册，冷泉港实验室 (Molecular Cloning:A Laboratory Manual.Cold Spring Harbour Laboratory),Cold Spring Harbor,NY.,p.146；Sambrook 等人，分子克隆：分子克隆手册，第二版 (Molecular Cloning:a laboratory manual,2nd Ed.),Cold Spring Harbor Laboratory Press,1989)。除另有规定外，连接可采用已知的缓冲液和条件来完成，用 10 个单位的 T4DNA 连接酶（“连接酶”）连接每 0.5 微克约等摩尔量的 DNA 片段。

[0052] 本文中使用的“连接序列 (linker)”或“间隔序列 (spacer)”是指连接两个分子如 DNA 结合蛋白和随机肽的分子或分子组合，以使两个分子处于较佳的构型，例如，以使随机肽以最小空间位阻与 DNA 结合蛋白的受体结合。

[0053] 本文中使用的“微环境”是指与身体的组织或区的其它区域具有持续的或暂时的、生理的或化学的差异的组织或身体的任何部位或区域。

[0054] 本文中使用的“要被演变的分子特性”包括含有多核苷酸序列的分子，含有多肽序列的分子，含有部分多核苷酸和部分多肽序列的分子。与被演变的分子特性特别相关的，但绝不限于以下实例，包括：特定条件下蛋白的活性，如涉及温度；盐度；渗透压；pH；氧化以

及甘油、二甲基亚砜、去污剂和/或在反应环境中接触的任何其它分子种类的浓度。其它的与被演变的分子特性特别相关的，但绝不限于以下实例，包括稳定性，例如在经过曝露于特定环境一段时间后表现出的剩余的分子特性，如在存储过程中会遇到的情况。

[0055] 术语“突变(mutation)”是指在野生型核酸序列中序列的变化或在肽序列中序列的变化。这种突变可以是点突变，如转换或颠换。所述突变可以是缺失、插入或重复。

[0056] 本文中使用的简并“N,N,G/T”核苷序列代表32种可能的三联体，其中“N”可以是A、C、G或T。

[0057] 本文中使用的术语“天然存在”所适用的对象所指事实为可以在自然界中发现的对象。例如，存在于生物体（包括病毒）中的多肽或多核苷酸序列为天然存在的，其可以从自然的来源中分离，且未在实验室中被人为地有意改造过。一般而言，术语天然存在是指对象存在于非病理（未病变）的个体中，这在物种中是普遍存在的。

[0058] 本文中使用的“正常生理条件”或“野生型工作条件”是指那些在个体的给药部位或在个体作用部位将被考虑的正常范围内的条件：温度、pH、渗透压、渗量、氧化和电解质浓度。

[0059] 本文中使用的“核酸分子”由至少一个碱基或一个碱基对组成，这取决于它是否分别是单链或双链。此外，核酸分子可能唯一地(exclusively)或嵌合地(chimerically)属于任一组含有核苷的分子，作为实例但不限于以下组的核酸分子：RNA、DNA、基因组核酸、非基因组核酸、天然存在的和非天然存在的核酸以及合成的核酸。通过非限制性举例的方式，这包括与任何细胞器如线粒体、核糖体RNA和由不随天然存在的成分一起天然存在的一种或多种成分嵌合组成的核酸分子相关的核酸。

[0060] 此外，“核酸分子”可部分包含一种或多种基于非核苷的成分，作为实例但不限于氨基酸和糖类。因而，通过举例的方式，但不限于此，核酶是部分基于核苷和部分基于蛋白，被认为是“核酸分子”。

[0061] 此外，通过示例的方式，但不限于此，用可检测到的基团，如放射性的或者非放射性的标记来标记核酸分子，同样被认为是“核酸分子”。

[0062] 术语“编码特定酶的核酸序列”或特定酶的“DNA编码序列”或“编码特定酶的核苷酸序列”，以及其它同义词是指DNA序列被置于适当的调控序列控制下，被转录并翻译成酶。“启动子序列”是能够在胞内结合RNA聚合酶的DNA调控区，并启动下游(3’方向）编码序列的转录。启动子是DNA序列的一部分。在该序列的3’端具有起始密码子。该启动子序列包括最低数目的碱基，这种组成对于在高于背景的可检测到的水平上起始转录是必须的。然而，在RNA聚合酶结合序列且从起始密码子（启动子的3’端）开始转录之后，转录朝着下游的3’方向进行。在该启动子序列内会找到转录起始位点（可方便地由核酸酶S1图谱确定）以及负责结合RNA聚合酶的蛋白结合域（共有序列）。

[0063] 术语“编码酶（蛋白）的核酸”或“编码酶（蛋白）的DNA”或“编码酶（蛋白）的多核苷酸”和其它同义词包括仅含有酶的编码序列的多核苷酸以及含有其它编码和/或非编码序列的多核苷酸。

[0064] 在一个优选的实施方案中，“特定的核酸分子种类”是由其化学结构，例举但不限于，其一级结构来确定的。在另一个优选的实施方案中，特定的“核酸分子种类”是由该类核酸的功能或由衍生自该类核酸的产物的功能来确定的。因而，通过非限制性举例的方式，

“特定的核酸分子种类”可由归属于它的一种或多种活性或性质来确定的，包括归属于它的表达产物的活性或性质来确定的。

[0065] “将工作的核酸样品组装至核酸库中”的即时定义包括将核酸样品整合至基于载体的集合 (collection) 的过程，如通过连接至载体并转化宿主。相关载体、宿主和其它试剂以及它们具体的非限制性实例的说明在下文中提供。“将工作的核酸样品组装至核酸库中”的即时定义还包括将核酸样品整合至非基于载体的库的过程，如通过连接至接头 (adaptor)。优选地，接头能够与 PCR 引物退火，以便于 PCR 扩增。

[0066] 因此，在非限制性的实施方案中，“核酸库”是由一种或几种核酸分子的基于载体的集合组成。在另一个优选的实施方案中，“核酸库”是由核酸分子的非基于载体的集合组成。而在另一个优选的实施方案中，“核酸库”是由部分基于载体和部分基于非载体的核酸的组合集合组成。优选地，根据各核酸分子种类，含有库的分子集合是可查询的且可分离的。

[0067] 本发明提供“核酸构建体”或“核苷酸构建体”或“DNA 构建体”。本文中使用的术语“构建体”是用以描述可任选地与一种或多种其它分子基团如载体或载体进行化学结合的分子，如多核苷酸（例如，酶多核苷酸）。在一个具体的但绝非限制性的方面，核苷酸构建体的实例是适于宿主细胞转化的 DNA 表达构建体。

[0068] “寡核苷酸”（或同义的“寡”）是指可化学合成的单链多聚脱氧核苷酸或两条互补的多聚脱氧核苷酸链。这种合成的寡核核苷酸可以带有或不带有 5’磷酸。在激酶存在下不随 ATP 加入磷酸，那些不带有 5’磷酸的寡核核苷酸不会与另一条寡核苷酸连接。合成的寡核苷酸会与未被去磷酸化的片段连接。为了实现基于聚合酶的扩增（如通过 PCR)，提到“32 倍简并寡核苷酸是连续地 (in series) 由至少第一同源序列、简并 N,N,G/T 序列和第二同源序列组成。”本文中使用的“同源”是关于基于聚合酶扩增的寡核苷酸和亲本多核苷酸之间的同源性。

[0069] 本文中使用的术语“可操作连接的”是指功能关系中的多核苷酸元件之间的连接。当核酸被置于与另一条核酸序列的功能关系中时，它是“可操作连接的”。例如，如果启动子或增强子影响到编码序列的转录，则它与该编码序列是可操作连接的。可操作连接是指被连接的 DNA 序列通常是连续的，且在需要连接两个蛋白质编码区时是连续的并位于阅读框内。

[0070] 编码序列“可操作连接至”另一条编码序列，则 RNA 聚合酶转录这两条编码序列为一条 mRNA，然后其被翻译成一条含有来自两条编码序列的氨基酸的多肽。只要表达的序列被最终加工成所需的蛋白，编码序列不必与另一条编码序列是连续的。

[0071] 本文中使用的术语“亲本多核苷酸组合”是由一种或多种不同的核苷酸组成的组合。通常该词用于关于优选通过诱变处理亲本组合获得的后代多核苷酸组合，在这种情况下，术语“亲本”、“起始物”和“模板”可互换使用。

[0072] 术语“患病体”或“个体”是指动物，例如哺乳动物如人，其作为治疗的对象。所述个体或患病体，可能是雄性或雌性。

[0073] 本文中使用的术语“生理条件”是指温度、pH、渗透压、离子强度、粘度等生化参数，其与活有机体相容和 / 或通常存在于活的酵母培养细胞或哺乳动物细胞的细胞内。例如，在典型的实验室培养条件下生长的酵母细胞的胞内条件是生理条件。用于体外转录混合

物 (cocktails) 的合适体外反应条件一般是生理条件。通常体外生理条件包括 50-200mM NaCl 或 KCl，pH6.5-8.5，20-45℃和 0.001-10mM 二价阳离子（例如 Mg^{++}、Ca^{++}）；优选约 150mM NaCl 或 KCl，pH7.2-7.6，5mM 二价阳离子，而且往往包括 0.01-1.0％的非特异性蛋白（例如，BSA）。往往存在非离子型去污剂（吐温、NP-40、Triton X-100），通常约 0.001-2％，典型地为 0.05-0.2％ (v/v)。特定的水溶液条件由医生按常规方法选择。对于一般性的指导，以下缓冲的水溶液条件可以适用：10-250mM NaCl，5-50mM Tris HCl，pH5-8，任选加入二价阳离子和 / 或金属螯合剂和 / 或非离子型去污剂和 / 或膜组分和 / 或消泡剂和 / 或闪烁材料 (scintillant)。正常生理条件是指病人或个体体内给药部位或作用部位的温度、pH、渗透压、渗量、氧化和电解质的浓度，其被考虑到是在病人体内的正常范围内。

[0074] 本文中采用标准惯例 (Standard convention)(5’到 3’）来描述双链多核苷酸的序列。

[0075] 本文中使用的术语“群 (population)”是指如多核苷酸、多核苷酸的部分或多核苷酸或蛋白成分的集合。“混合的群”是指属于相同的核酸或蛋白家族（即，是相关的）但序列不同（即，不同源）因而生物活性不同的成分的集合。

[0076] 具有“前体形式”的分子是指经过一种或多种共价和非共价化学修饰（例如糖基化、蛋白水解裂解、二聚化或寡聚化、温度诱导或 pH 诱导的构象变化、与辅因子相连等）的任何组合的分子，从而获得较参考前体分子更成熟的性质不同（例如活性增加）的分子形式。当两种或两种以上的化学修饰（例如两种蛋白水解裂解或一种蛋白水解裂解和一种去糖基化）能够被区分，从而产生成熟的分子，所述参考前体分子可被称为“原前体形式 (pre-pro-form)”的分子。

[0077] 本文中使用的术语“伪随机”是指具有有限可变性的一组序列，例如，在另一个位置的残基可变性程度，但任何伪随机位置允许一定程度的残基变化，然而变化是有限的。

[0078] 本文中使用的“准重复单元 (Quasi-repeated units)”是指将被重新排列的重复且被定义为不相同。事实上，该方法的提出不仅用于由相同起始序列诱变形成的几乎相同的编码单元，还用于某些区显著不同的类似或相关序列的重新排列。然而，如果序列中含有足够的可被该方法重新排列的同源序列，它们可被称为“准重复”单元。

[0079] 本文中使用的“随机肽库”是指编码一组随机肽的一组多核苷酸序列和由那些多核苷酸序列编码的随机肽的组合，以及含有那些随机肽的融合蛋白。

[0080] 本文中使用的“随机肽序列”是指由两种或两种以上的氨基酸单体组成且通过随机的 (stochastic) 或随机 (random) 方法构建的氨基酸序列。随机肽可包括含有不可变序列的框架 (framework) 或骨架 (scaffolding) 基序。

[0081] 本文中使用的“受体”是指对给定的配体具有亲和力的分子。受体可以是自然产生或人工合成的分子。受体可以其未改变的状态或与其它种类形成聚集体而应用。受体可直接或通过特定的结合物质共价或非共价连接于结合单元。受体的实例包括但不限于抗体，其包括单克隆抗体且能够与特定的抗原决定簇（如位于病毒、细胞或其它材料上）发生抗血清反应，细胞膜受体、碳水化合物和糖蛋白的复合物、酶和激素受体。

[0082] “重组”酶是指通过重组 DNA 技术生产的酶，即通过将编码所需酶的外源 DNA 构建体转化的细胞产生的酶。“合成”的酶是指那些通过化学合成制备的酶。

[0083] 术语“相关的多核苷酸”是指多核苷酸的区域或区是相同的，且多核苷酸的区域或

区是异源的。

[0084] 本文中使用的“减少重排 (Reductive reassortment)”是指通过由重复序列介导的缺失（和 / 或插入）事件而产生的分子多样性的增加。

[0085] 下列术语用来描述两种或两种以上的多核苷酸序列之间的关系 :“参考序列”、“比较窗口”、“序列同一性”、“序列同一性百分数”和“基本上的同一性”。

[0086] “参考序列”是确定的、用作序列比较基础的序列 ;参考序列可以是一个较大序列的子序列,例如全长 cDNA 中的一段序列或序列表中给定的基因序列,或可包括完整的 cDNA 或基因序列。一般来说,参考序列的长度至少是 20 个核苷酸,通常至少是 25 个核苷酸,往往至少是 50 个核苷酸。由于两个多核苷酸可各自为 (1) 包括两个多核苷酸之间相似的序列（即,完整的多核苷酸序列的一部分）以及 (2) 可进一步包括两个多核苷酸之间不同的序列,两个（或两个以上）多核苷酸之间的序列比较一般是在“比较窗口”中通过比较两个多核苷酸的序列来进行,以确定和比较序列相似的局部区域。

[0087] 本文中使用的“重复指数 (RI)”是指克隆载体中包含的准重复单元 (quasi-repeated unit) 的平均拷贝数。

[0088] 术语“限制位点”是指显示限制酶发生作用所必须的识别序列,其包括催化切割位点。可知切割位点可包含于或不包含于含有低多义性序列 (low ambiguity sequence)（即含有限制位点出现频率的主要决定因素的序列）的限制位点的一部分中。因而,在许多情况下,相关限制位点仅含有内部具有切割位点（例如 EcoR Ⅰ位点的 G/AATTC) 或紧邻切割位点（例如 EcoR Ⅱ位点的 /CCWGG) 的低多义性序列。在其它情况下,相关限制酶 [例如 Eco57 Ⅰ位点或 CTGAAG(16/14)] 包含具有外部切割位点（例如 Eco57 Ⅰ位点的 N.sub.16 部分）的低多义性序列（例如 Eco57 Ⅰ位点中的 CTGAAG 序列）。当提到酶（例如限制酶）“切割”多核苷酸时,它被理解为限制性酶催化或促进多核苷酸的裂解。

[0089] 在一个非限制性的方面,“可选择的多核苷酸”是由 5’ 端区（或末端区域）、中间区域（即内部或中心区）和 3’ 端区（或末端区域）组成的。如在所述的方面中所用,5’ 端区是位于 5’ 多核苷酸末端（或 5’ 多核苷酸尾）方向的区 ;因而它一部分或全部位于多核苷酸 5’ 端的一半核苷酸内。同样,3’ 端区是位于 3’ 多核苷酸末端（或 3’ 多核苷酸尾）方向的区 ;因而它一部分或全部位于多核苷酸 3’ 端一半核苷酸内。如该非限制性的示例中所用,可以是在任两个区或甚至全部三个区之间重叠的序列。

[0090] 术语“序列同一性”是指在比较窗口中,两个多核苷酸序列是相同的（即,基于核苷酸对核苷酸）。术语“序列同一性百分数”是通过在比较窗口中比较两个最佳对齐的序列而计算得到的,确定在两条序列中出现的相同核酸碱基（例如,A、T、C、G、U 或 I) 位置的数目,以得到匹配位置数,用匹配位置数除以比较窗口中的总位置数（即窗口大小）,结果乘以 100,从而得到序列同一性百分数。本文中使用的“基本上的同一性”表示多核苷酸序列的特征是,与至少 25-50 个核苷酸的比较窗口参考序列相比,所述多核苷酸包括的序列具有至少 80％的序列同一性,优选至少 85％序列同一性,通常 90％至 95％序列同一性,最常见至少 99％序列同一性,其中序列同一性百分数的计算是通过比较参考序列与含有比较窗口中≤ 20％参考序列的缺失序列或添加序列的多核苷酸序列而计算得到的。

[0091] 本领域已知的两种酶之间的“相似性”是通过比较一种酶的氨基酸序列和它的保守氨基酸序列替换与第二种酶的序列来确定的。相似性的确定可采用本领域众所周知的方

法，例如，BLAST 程序（美国国立生物技术信息中心提供的 Basic Local Alignment Search Tool）。

[0092] 如果它们彼此结合的亲和力要高于与其它非特异性分子的亲和力，则该分子对（例如，抗体－抗原对或核酸对）的成员被描述为彼此“特异性结合”。例如，对抗抗原的抗体与该抗原的结合比非特异性的蛋白更有效，可以描述为抗体特异性地结合抗原。（同样，如果核酸探针与靶核酸通过碱基配对作用（见上）形成了特异的二聚体，可以描述为核酸探针特异性地结合靶核酸。）

[0093] 本文中定义的“特异性杂交”是指在第一多核苷酸和第二多核苷酸（例如，具有与第一多核苷酸序列不同，但序列大致相同的多核苷酸）之间形成杂交，其中大致不相关的多核苷酸序列在该混合物中不形成杂交。

[0094] 术语“特定的多核苷酸”是指具有特定的终点和特定的核酸序列的多核苷酸。两个多核苷酸，其中一个多核苷酸具有与第二多核苷酸的一部分相同的序列，但末端不同，构成了两个不同的特定的多核苷酸。

[0095] “严格杂交条件”是指两条序列之间仅当同一性为至少 90％，优选至少 95％，更优选至少 97％时才发生的杂交。见 Sambrook 等人，分子克隆：分子克隆手册第二版，冷泉港实验室出版社，1989 年 (Molecular Cloning:a laboratory manual,2nd Ed.,Cold Spring Harbor Laboratory Press,1989)，以参阅的方式全文并入于此。

[0096] 本发明中还包括具有序列与酶多肽的序列“基本上相同 (substantially identical)”的多肽。“基本上相同”的氨基酸序列是仅由于保守氨基酸替换形成的与参考序列不同的序列，例如，将同一类的一种氨基酸替换为另一种氨基酸（例如，将一种疏水性氨基酸，如异亮氨酸、缬氨酸、亮氨酸或蛋氨酸替换为另一种，或将一种极性氨基酸替换为另一种，如精氨酸替换为赖氨酸、谷氨酸替换为天冬氨酸，或谷氨酰胺替换为天冬酰胺）。

[0097] 其它“基本上相同”的氨基酸序列是与参考序列或由于一种或多种非保守替换、缺失或插入，特别是当替换发生在分子的非活性位点时，所形成的不同的序列，且前提是多肽基本上保留了其行为特性。例如，可以从酶多肽中去掉一个或多个氨基酸，导致多肽结构的改进，而没有显着改变其生物活性。例如，不是酶发挥生物活性所必须的氨基端或羧基端氨基酸可以去掉。这种改进导致开发了更小的活性酶多肽。

[0098] 本发明提供了“基本上纯的酶”。本文中使用的术语“基本上纯的酶”用来描述完全游离于自然情况下与其结合的蛋白、脂类、碳水化合物、核酸和其它生物材料的分子，如多肽（例如，酶多肽或其片段），例如，基本上纯的分子，如多肽，可至少占目标分子的 60％干重。可采用标准方法，包括，例如聚丙烯酰胺凝胶电泳（例如，SDS-PAGE）、柱层析（例如，高效液相色谱 (HPLC)）和氨基末端氨基酸序列分析来确定多肽的纯度。

[0099] 本文中使用的“基本上纯的”表示目标分子是主要存在的分子（即基于摩尔，它在组合物中的比任何其它的大分子多），优选地，基本上纯的部分是指组合物中目标分子占所有大分子的至少 50％（基于摩尔）。一般来说，基本上纯的组合物包括高于组合物中所有大分子约 80％到 90％的目标分子。更优选地，目标分子被纯化至基本均一（采用常规检测方法检测不到组合物中的污染分子），其中组合物主要由单一的大分子组成。溶剂、小分子（<500 道尔顿）和离子元素不视为大分子。

[0100] 术语“治疗”包括：(1) 预防或延缓发生于动物体内的状态 (state)、疾病或病症的

临床症状出现，该动物可能患有或易感于该状态、疾病或病症但尚未经历或表现出该状态、疾病或病症的临床或亚临床症状；(2) 抑制该状态、疾病或病症（即，阻止、减少或延缓疾病的发展，或维持治疗情况下疾病的复发，或至少一种临床或亚临床症状）；和 / 或 (3) 减轻病症（即，引起该状态、疾病或病症的衰退或引起至少其一种临床或亚临床症状的衰退）。对将要进行治疗的病人的好处是在统计学上显著，或至少对病人或医生的好处是可察觉到的。

[0101] 本文中使用的术语“可变区段 (variable segment)”是指包括随机的、伪随机的或确定的核心序列的新生肽的一部分。“可变区段”是指包括随机的、伪随机的或确定的核心序列的新生肽的一部分。可变区段可包括可变的或不可变的残基位，可变的残基位上的残基可变程度可能是有限的：两种选择由医生定夺。通常情况下，可变区段长约 5 至 20 个氨基酸残基（例如，8 至 10），尽管可变区段可以更长，并可能包括抗体部分或受体蛋白，如抗体片段、核酸结合蛋白、受体蛋白等。

[0102] 术语“变体 (variant)”是指在野生型蛋白亲本分子的的一个或多个碱基对、密码子、内含子、外显子或氨基酸残基（分别）被修饰的本发明的多核苷酸或多肽。变体可由任何方法产生，这些方法包括例如，易错 PCR(error-prone PCR)、改组 (shuffling)、寡核苷酸定向诱变、装配 PCR(assembly PCR)、有性 PCR 诱变 (sexual PCR mutagenesis)、体内诱变、盒式突变、循环总体诱变 (recursive ensemble mutagenesis)、指数总体诱变 (exponential ensemble mutagenesis)、特异位点诱变、基因重组、饱和诱变和它们的任意组合。本文公开了用于生产在正常生理条件，例如温度、pH、渗透压、渗量、氧化和电解质浓度中的一种或多种条件，较野生型蛋白活性下降，以及在异常条件下活性增强的变体蛋白的技术。可另外筛选出较野生型蛋白抗化学性和抗蛋白水解性能增强的变体。

[0103] 本文中使用的术语“野生型”是指不包括任何突变的多核苷酸。“野生型蛋白 (wild type protein)”、“野生型蛋白 (wild-type protein)”、“野生型生物蛋白 (wild-type biologic protein)”或“野生型生物蛋白 (wild type biologic protein)”是指可以从自然界中分离的在自然条件下具有一定水平活性的且包含在自然条件下发现的氨基酸序列的蛋白。术语“亲本分子”和“目标蛋白”也指野生型蛋白。

[0104] 术语“工作”，如在“工作样本”中，例如，仅简单地指一个用于工作的样本。同样，“工作分子”，例如，是指用于工作的分子。

[0105] 本发明涉及改造或演变蛋白以产生在野生型条件下可逆或不可逆失活，而在非正常条件下表现出活性与在野生型条件下相同或相当水平的新分子的方法。本文中这些新蛋白是指“Mirac”蛋白。Mirac 蛋白对于发展新疗法特别有价值，其在很短的时间或有限的时期内在宿主中有活性。给定剂量该蛋白的扩展工作 (extended operation) 将对宿主是有害的，这点特别有价值，但为了进行所需的治疗，其活性必须是有限的。有益的应用例包括以高剂量进行局部或全身治疗，以及以高浓度进行局部治疗。在生理条件下的失活可通过给药和蛋白失活速率的组合来确定。催化活性在较短的时间内造成重大的负面影响，这种基于失活的条件对于酶疗法尤为重要。

[0106] 本发明还涉及改造或演变蛋白从而产生不同于野生型分子的新分子的方法，所述新分子随时间可逆或不可逆地活化或失活的或仅在体内特定微环境包括在体内特定器官（如膀胱或肾脏）中才活化或失活。

[0107] 目标野生型蛋白

[0108] 任何治疗性蛋白可以作为用于生产条件活性生物蛋白的目标蛋白，或野生型蛋白。在一个方面，目标蛋白是野生型的酶。目前常用的治疗性酶包括尿激酶和链激酶，用于治疗血液凝块；以及透明质酸酶，作为提高其它药物的吸收和分散的佐剂。在一个方面，为了避免或尽量减少野生型蛋白或酶带来的有害副作用，所选用于生产条件活性生物蛋白的野生型蛋白可以是目前使用的治疗性酶。或者，目前没有作为治疗性酶而使用的酶可选择用于生产条件活性生物蛋白。某些非限制性的实例将在下文进一步详细讨论。

[0109] 治疗性蛋白是那些可以单独用于药物或与其它疗法结合以治疗各种疾病或病症(indications)的蛋白。本发明的条件活性生物蛋白适用于一个或多个病症，包括治疗循环系统疾病、关节炎、多发性硬化症、自身免疫性疾病、癌症、皮肤病，并用于各种诊断形式(diagnostic formats)。取决于蛋白和病症，条件活性生物酶蛋白可以是如下讨论的肠外、外用或口服试剂给药。

[0110] 循环疾病－血栓形成与溶栓治疗

[0111] 血栓（血块）的定义是在循环系统中形成的来自血液成分中的固体块。血栓是由一系列涉及凝血因子、血小板、红细胞以及与血管壁相互作用的事件而形成的。血小板是可能会导致血管阻塞的血小板、纤维蛋白和被捕获(entrapped)的血细胞的血管内聚集。通过阻塞或阻断血流，血栓剥夺了下游组织的氧气供应。血栓的片段（栓塞）可破坏或阻塞较小的血管。动脉血栓的形成是由于各种因素中的任一种因素所导致的沉淀，这些因素包括潜在的狭窄－动脉粥样硬化、低流量心脏功能、癌症或凝血因子缺乏所形成的血凝过快(hypercoagubility)，或异物如支架或导管。导致动脉缺血的血栓可以导致肢体或组织损伤、急性心肌梗死(AMI)、中风、截肢或肠梗塞。发病率和死亡率的主要原因是动脉血栓（冠状动脉血栓和脑动脉血栓）和肺血栓的形成。静脉血栓的形成可能是由于内皮损伤如创伤、不动所导致的瘀积或血凝过快所引起的，但动脉粥样硬化不是一个因素。治疗策略包括机械去除血栓、药物－机械去除血栓及溶栓。血栓治疗用以尽量减少血栓的形成和有助于血栓的去除。

[0112] 血栓治疗包括使用能够抑制血小板活化的抗血小板试剂、抗凝疗法和/或溶栓治疗，以降低血液凝块。抗血小板试剂的实例包括阿司匹林、潘生丁和噻氯匹啶。抗凝血剂的实例包括肝素、华法林、水蛭素和活化的人类蛋白C。溶栓的实例包括组织型纤溶酶原激活物(tPA)/tPA变体、尿激酶和链激酶。溶栓显示了作用的催化模式。

[0113] 急性心肌梗死中的溶栓治疗是公认的。溶栓试剂的使用已成为标准的紧急治疗方法。虽然有效，这些产品仅在约50％的患者中实现完全再灌注(reperfusion)，且副作用包括出血（尤其是颅内出血）以及高血压的风险。受损或病变的血管的血凝块的减少，被称为“纤溶”或“纤溶过程”。纤溶是一个蛋白水解过程，是由纤溶酶原激活剂激活蛋白纤维溶酶原，从而形成纤溶酶。纤溶酶降解血液凝块的纤维蛋白丝以溶解凝块。纤维特异性的纤溶酶原激活剂包括组织型纤溶酶原激活剂或变体。非特异性的纤溶酶原激活剂可以包括链激酶和尿激酶。

[0114] 某些常用的溶栓疗法利用几种可获得的组织型纤溶酶原激活剂(tPA)变体中的一种。例如，先前已批准使用的基于tPA的产品变体是阿替普酶(rt-PA)、瑞替普酶(r-PA)和替奈普酶(TNK)。tPA变体批准使用的用途包括例如，用以改善AMI影响心室功能的急性

心肌梗死，充血性心力衰竭发病率的减少，以及 AMI 所致死亡率的下降，用于促进神经功能的恢复并减少残疾的发病率的成人缺血性中风治疗 (management)，用于急性肺栓塞溶解以及用于伴有不稳定血流动力学的肺栓塞溶解的成人急性大面积肺栓塞治疗。

[0115] 另一种常用的溶栓治疗采用尿激酶。尿激酶是一种用于外周血管疾病治疗的标准溶解剂。

[0116] 链激酶是由几种链球菌分泌的、可以结合并激活人纤溶酶原的蛋白。链激酶与人纤溶酶原的复合物可通过键断裂产生纤溶酶而水解激活其它未结合的纤溶酶原。通常纤溶酶原激活是通过水解 Arg561-Val562 键。然后 Val562 的氨基与 Asp740 形成盐桥，导致构象变化而产生活化的蛋白酶纤溶酶。纤溶酶产生于血液中以分解血液凝块的主要成分－纤维蛋白。

[0117] 链激酶作为一种有效的血块溶解药物用于下列疾病的某些情况：心肌梗死（心脏病发作）、肺栓塞（肺血块）和下肢深静脉血栓（腿部血液凝块）。链激酶属于一组称为纤维蛋白溶解剂的药物。心脏病发作后尽快施以链激酶，可溶解心壁动脉中的血块并减少对心脏肌肉的损害。链激酶是一种细菌产品，因此身体能够形成对该蛋白的免疫力。因此，建议不应在第一次给药四天后再次施用这种产品，因为它可能没有效并会引起过敏反应。出于这个原因，通常只在第一次心脏病发作后给药，进一步的血栓性事件通常用组织型纤溶酶原激活剂 (TPA) 治疗。链激酶有时也用于预防手术后粘连。

[0118] 链激酶的副作用包括出血（主要和次要）、低血压和呼吸抑制以及可能的过敏反应。此外，改变血小板功能的抗凝血剂、试剂（例如阿司匹林、其它 NSAID、潘生丁）可能会增加出血的风险。

[0119] 溶栓的给药一般通过输液或通过静脉单次剂量注射 (bolus intravenous dose)；或通过机械输液系统。不利的影响可包括严重的颅内、消化道、腹膜后或心包出血。如果发生出血，给药必须立即停止。

[0120] 在本发明的特定实施方案中，tPA、链激酶或尿激酶被选作目标或野生型蛋白。

[0121] 在一个实施方案中，本发明的方法被用于选择在低于正常生理条件的异常温度条件下具有高活性，且在正常生理条件（例如 37℃）下完全灭活或失活的条件活性重组的或合成的链激酶变体。在一个方面，异常的温度条件下是室温，例如 20-25℃。在另一个方面，本发明提供治疗中风或心脏病发作的方法，该方法包括对患有中风或心脏病发作的病人施以高剂量的条件活性链激酶变体，但允许链激酶变体的迅速失活以免失血过多。

[0122] 循环疾病－肾素／血管紧张素

[0123] 肾素／血管紧张素系统是一种调节血压和水（流体）平衡的激素系统。在低血容量时，肾脏分泌肾素。肾素是一种酶，其将肝脏分泌的血管紧张素水解为肽血管紧张素 I。血管紧张素 I 进一步在肺中被内皮结合的血管紧张素转化酶 (ACE) 切割成血管紧张素 II，最重要的血管活性肽。血管紧张素 II 引起血管收缩，导致血压升高。然而，血管紧张素 II 也能刺激肾上腺皮质分泌激素醛固酮。醛固酮引起肾脏的肾小管增加了对钠和水的吸收。这增加了体内的液量，也增加了血压。过度活性的肾素－血管紧张素系统导致血管收缩及钠和水的滞留。这些影响导致了高血压。有很多药物可中断这个系统中的不同步骤，以降低血压。这些药物是用来控制高血压（高血压症）、心力衰竭、肾衰竭和糖尿病的有害影响的主要途径之一。

[0124] 低血容性休克(Hypovolemic shock)是一种急性病症,其中严重的血液和/或体液流失使心脏无法充分地带给人体细胞含氧血。创伤、受伤和内部出血可导致失血。由于烧伤、腹泻、过度出汗或呕吐导致的过多的液体流失可形成血液循环的量下降。低血容性休克的症状包括焦虑、皮肤湿冷、神志不清、呼吸急促或失去知觉。检查时可见休克的迹象,包括血压低、体温低和可能微弱的或虚弱的(thready)脉快。治疗包括静脉输液,输血或血制品,休克治疗和药物如多巴胺、多巴酚丁胺、肾上腺素和去甲肾上腺素,以升高血压和提高心输出量。

[0125] 在一个实施方案中,本发明提供了一种用于筛选在正常生理温度下可逆失活,但在低血容量性休克病人的异常较低温度下恢复活性的条件活性重组肾素变体的方法。该条件活性蛋白可用于治疗低血容量性休克,以帮助提高体内的液体量并升高血压。

[0126] 循环疾病-雷诺氏现象

[0127] 雷诺氏现象(RP),是引起手指、脚趾和偶尔其它四肢变色的血管痉挛症。情绪紧张和寒冷经常触发此现象。当暴露在寒冷的气温下,四肢失去热量。手指和脚趾血液供应通常放缓,以保持身体的核心温度。通过四肢皮肤下的小动脉变窄使血流量减少。压力会在体内导致与寒冷类似的反应。雷诺氏病中,正常的反应被扩大了。该病可引起疼痛、变色以及寒冷和麻木的感觉。该现象是血管痉挛的结果,其减少了对各部分的血液供应。雷诺氏病(原发性雷诺氏现象)中,这种疾病是先天的。雷诺氏综合征(继发性雷诺氏病)中,这种现象是由一些其它的促发因素造成的。手的温度梯度测量是一种用来区分原发性和继发性形式的工具。原发性形式可以发展为继发性形式,在极端情况下,继发性形式可以发展为指尖的坏死或坏疽。

[0128] 雷诺氏现象是一种对寒冷或情绪紧张作出的扩大反应。原发性RP基本上是由微血管血管痉挛介导的。交感神经系统过度活跃,会导致外周血管的极度血管收缩,导致缺氧。慢性、反复发作的情况可导致皮肤、皮下组织和肌肉的萎缩。它也很少导致溃疡和缺血性坏疽。

[0129] 雷诺氏现象的传统的治疗方案包括扩张血管和促进血液循环的处方药。这些处方药包括钙通道阻滞剂,如硝苯地平或地尔硫卓;α阻滞剂,其对抗去甲肾上腺素(收缩血管的激素)d的作用,如哌唑嗪或沙唑嗪;以及放松血管的血管扩张剂,如硝酸甘油膏,或血管紧张素II抑试剂氯沙坦、西地那非或前列腺素。由于心理压力,氟西汀(一种选择性的5-羟色胺再摄取抑试剂)及其它抗抑郁药物可能会降低发作频率和严重程度。这些药物可能会引起副作用,如头痛、面色潮红和踝部水肿。药物也可能随着时间的推移失去效力。

[0130] 皮肤血管收缩和血管扩张的调控涉及改变的交感神经活性和许多神经调节剂,包括肾上腺素和非肾上腺素,以及REDOX信号传递和其它信号传递,如RhoA/ROCK通路。皮肤中血管平滑肌细胞(vSMC)的血管收缩被认为是由α1和α2肾上腺素受体介导的去甲肾上腺素激活的。α2C-ARS从反式高尔基体转移至vSMC的细胞表面,然后对涉及RhoA/Rhokinase(ROCK)信号传递通路的这些响应的刺激和信号传递作出反应。皮肤动脉中的冷刺激导致vSMC线粒体中活性氧(ROS)的立即生成。通过RhoA/ROCK通路,ROS参与入REDOX信号传递中。RhoA是一种GTP结合蛋白,其作用是调控依赖于肌动蛋白-肌球蛋白的过程,如vSMC中的迁移和细胞收缩。脉管系统(vascluature)中可能涉及RP已知功能的非肾上腺素神经肽包括降钙素基因相关肽(CGRP)、P物质(SP)、神经肽Y(NPY)和血管活性肠

肽 (VIP)。Fonseca 等人 ,2009, “雷诺氏现象和系统性硬化症中的神经调节剂和血管功能障碍 (Neuronal regulators and vascular dysfunction in Raynaud's phenomenon and systemic sclerosis)”,Curr.Vascul.Pharmacol.7:34-39。

[0131] RP 的新疗法包括 α-2C 肾上腺素受体阻滞剂、酪氨酸蛋白激酶抑试剂、Rho 激酶抑试剂和降钙素基因相关肽。

[0132] 降钙素基因相关肽 (CGRP) 是降钙素家族肽中的一个成员,以两种形式存在 :α-CGRP 和 β-CGRP。α-CGRP 是由降钙素 /CGRP 基因的选择性剪接形成的 37 个氨基酸的肽。CGRP 是外周和中枢神经元中产生的最丰富的肽之一。它是一种强效的肽血管扩张剂,可在疼痛的传递中发挥功能。偏头痛是一种常见的神经系统疾病,其与增加的 CGRP 水平有关。CGRP 扩张颅内血管并传递血管伤害感受 (vascular nociception)。将 CGRP 受体拮抗剂用于治疗偏头痛已经过测试。Arulmani 等人,2004, “降钙素基因相关肽以及它在偏头痛病理生理中的作用 (Calcitonin gene-related peptide and it role in migraine pathophysiology)”,Eur.J.Pharmacol.500(1-3):315-330。至少有三个受体亚型已经确定,CGRP 通过 G 蛋白 - 偶联受体发生作用,该受体的存在和功能的变化调节该肽在各种组织中的效果。CGRP 通过受体的信号转导依赖于两个辅助蛋白 :受体活性修饰蛋白 1(RAMP1) 和受体组成蛋白 (RCP)。Ghatta 2004, 降钙素基因相关肽 :认识其作用 (Calcitonin gene-related peptide:understanding its role).Indian J.Pharmacol.36(5):277-283。一项使用激光多普勒血流计 (LDF) 的有关三种扩血管剂 :内皮依赖性血管扩张剂腺苷三磷酸 (ATP)、不依赖于内皮的血管扩张剂前列环素 (依前列醇 ;PGI2) 和 CGRP 对雷诺的现象患者和相似数量年龄和性别匹配的对照组的影响的研究表明,由雷诺氏症患者的皮肤血流量上升造成 CGRP 诱导脸部和手潮红,而对照组 CGRP 仅引起脸部潮红。PGI2 引起两组中脸部和手相似的血流量上升。ATP 没有引起病人手或脸部血流量的任何大的改变,但增加了对照组的脸部血流量。Shawket 等人,1989, “雷诺氏现象中手的降钙素基因相关肽的选择性过敏症 (Selective suprasensitivity to calcitonin-gene-related peptide in the hands in Reynaud's phenomenon)”,《柳叶刀》(The Lancet),334(8676):1354-1357。在一个方面,野生型蛋白的目标分子是 CGRP。

[0133] 在一个实施方案中,本发明提供筛选与雷诺氏综合征相关的、在正常生理条件下可逆失活但在手指或足趾中较低的异常温度下恢复活性的条件活性重组蛋白变体的方法。该条件活性蛋白可用于治疗雷诺氏现象,以防止或减少由于低循环导致的手指或足趾功能的损失。

[0134] 循环疾病 - 血管加压素

[0135] 精氨酸血管加压素 (AVP、血管加压素、抗利尿激素 (ADH) 是发现于大多数哺乳动物中的一种肽类激素,其通过影响组织通透性控制肾脏的肾小管中分子的重吸收。血管加压素的最重要作用之一,是调节体内保水力 (water retention)。在高浓度下,它通过引入适度的血管收缩提升高血压。血管加压素具有三种效应导致尿渗透压增加 (浓度增加) 并减少水的排泄。首先,血管加压素会使得肾脏集合管细胞 (collecting duct cells) 的水通透性增加,从而允许水的重吸收和较小体积浓缩尿的排泄 (抑制尿分泌 (antidiuresis))。这是通过将水通道蛋白 -2 的水通道插入集合管细胞的顶膜中而产生的。其次,血管加压素引起集合管的内髓部分对尿素的通透性增加,从而允许髓间质增加了对尿素的重吸收。第

三，通过增加 Na^+-K^+-$2Cl^-$ 协同转运蛋白的活性，血管加压素引起在亨利氏环厚上行支血管中对钠和氯重吸收的刺激。NaCl 的重吸收驱动了反流倍增，其为髓质集合管中水通道蛋白介导的水重吸收提供渗透梯度。

[0136] 肾集合管周围的高渗组织间液提供了高渗透压以去除水。由蛋白组成的跨膜通道称为水通道蛋白，它插入质膜中极大地增加了水的渗透性。当通道打开时，水通道蛋白通道每秒可使 3 百万个水分子通过。水通道蛋白 -2 通道的插入需要血管加压素传递信号。血管加压素与集合管细胞的基底表面上的受体（称为 V2 受体）结合。该激素的结合触发了细胞内 cAMP 水平的提高。该“第二信使”启动了一连串事件，最终在集合管细胞的顶端表面的插入水通道蛋白 -2 通道。水通道使水移出肾元，从而增加了从形成的尿液中重吸收而返回血液中的水的量。

[0137] 从脑垂体后叶释放的血管加压素的主要刺激是增加了血浆渗透压。任何使身体脱水的方法，如大量出汗，增加了血液渗透压并打开了血管加压素 -V2 受体 - 水通道蛋白 -2 通路。因此，低至 0.5 升 / 天的尿可能会保持原有 180 升 / 天的肾滤液。尿中的盐浓度可以是血液中的四倍。如果血液变得太稀，喝大量的水会使血液变得太稀，血管加压素分泌受到抑制，水通道蛋白 -2 通道可通过内吞作用进入细胞内。结果是，形成大量的盐浓度仅为血液盐浓度四分之一的水样尿。

[0138] 血管加压素释放的减少或肾对 AVP 敏感度的下降导致尿崩症 (diabetes insipidus)、高钠血症（增加的血钠浓度）、多尿（产生过多的尿）和多饮（口渴）。

[0139] 高水平的 AVP 分泌（抗利尿激素分泌过多综合征，SIADH) 和由此造成的低钠血症（血液中钠含量低），发生于脑部疾病和肺疾病（小细胞肺癌）。在围手术期，手术应激和一些常用的药物（例如，鸦片、催产素、止吐药）的影响，导致类似的血管加压素分泌过度的情况。这可能会导致数天的轻度低钠血症。

[0140] 血管加压素激动剂用于治疗各种病症，其长效的合成类似物去氨加压素 (desmopressin) 用于血管加压素分泌量低的病症，以及控制出血（以某些血管性血友病的形式）和极端案例儿童尿床中。特利加压素 (Terlipressin) 和相关类似物用作特定病症中的血管收缩剂。血管加压素输液已被用来作为第二种治疗对高剂量影响肌力药 (inotropes)（例如，多巴胺或去甲肾上腺素）没反应的感染性休克 (septic shock) 患者的方法。血管加压素受体拮抗剂是干扰血管加压素受体作用的试剂。它们可用于治疗低钠血症。

[0141] 在一个实施方案中，本发明提供了筛选用于涉及血管加压素响应的、在正常生理渗透压下可逆失活但在血液中异常的渗透压下恢复活性的条件活性生物重组的或合成的蛋白变体的方法。在另一个实施方案中，涉及血管加压素响应的蛋白变体在低钠条件下被激活，但在正常血清钠浓度下失活。在一个方面，低钠条件是血清钠 <135mEq/L。

[0142] 癌 - 血管抑素

[0143] 血管抑素是在几种动物中自然产生的蛋白。它作为一种内源性血管生成抑试剂（即，它阻止新血管的生长）。血管抑素能够通过抑制内皮细胞增殖和迁移来抑制肿瘤细胞的生长和转移。血管抑素是纤溶酶的 38kD 片段（其本身是纤溶酶原的片段）。血管抑素包括纤溶酶原可里格尔区 (kringle)1-3。血管抑素产生，例如，通过纤溶酶原的自溶裂解，包括磷酸激酶引起的胞外二硫键还原。血管抑素也可以由不同的基质金属蛋白酶 (MMPs)，包括

MMP2、MMP12 和 MMP9 以及丝氨酸蛋白酶（中性粒细胞弹性蛋白酶、前列腺特异抗原 (PSA)）切割纤溶酶原而得到。在体内，血管抑素抑制肿瘤的生长和使实验性转移 (experimental metastasis) 保持休眠状态。血管抑素在具有原发肿瘤和其它炎症和退行性疾病的动物中是升高的。

[0144] 已知血管抑素结合许多蛋白，包括血管抑素动蛋白 (angiomotin) 和内皮细胞表面 ATO 合酶，还有整合素 (integrin)、膜联蛋白 II(annexin II)、c-met 受体、NG2- 蛋白多糖、组织型纤溶酶原激活物、硫酸软骨素糖蛋白和 CD26。一项研究表明，IL-12，一种具有强效抗血管生成活性的 TH1 因子，是血管抑素活性介质。Albin″.,J.Translational Medicine.Jan.4,2009,7:5。血管抑素结合并抑制内皮细胞表面 ATP 合成酶。ATP 合成酶还出现在多种癌细胞的表面。发现肿瘤细胞表面 ATP 合成酶在胞外低 pH 下更活跃，肿瘤微环境的一个标志。发现在胞外酸性 pH(pHe) 下，血管抑素影响肿瘤细胞表面的 ATP 合成酶活性。在胞外低 pH 下，血管抑素直接抗肿瘤发生。在低 pH 下，血管抑素和抗 -β- 亚基抗体诱导 A549 癌细胞的胞内酸化，以及直接的毒性，该毒性在具有低水平的胞外 ATP 合成酶水平的肿瘤细胞中是不存在的。据推测，肿瘤细胞毒的作用机制依赖于由细胞表面的 ATP 合成酶的抑制引起的胞内 pH 异常。Chi 和 Pizzo，“在胞外低 pH 值下血管抑素对肿瘤细胞的直接细胞毒性 :依赖于细胞表面相关的 ATP 合酶的机制 (Angiostatin is directly cytotoxic to tumor cells at low extracellular pH:a mechanism dependent on cell surface-associated ATP synthase)”,Cancer Res.,2006,66(2):875-82。

[0145] 在一个实施方案中，本发明提供用于确定在正常生理血液 pH 下活性低于野生型血管抑素，但在低 pH 下活性增强的条件活性血管抑素变体的方法。低 pH 被定义为比正常生理 pH 低。在一个方面，低 pH 是≤约 7.2。在一个特定的方面，低 pH 是约 6.7。

[0146] 在一个方面，条件活性血管抑素变体可被配制并用作抗癌剂。

[0147] 组织通透性的增强 - 透明质酸酶

[0148] 透明质酸酶是降解透明质酸的酶家族。通过催化间质屏障 (interstitial barrier) 的主要成分透明质酸的降解，透明质酸酶降低了透明质酸的粘度，从而提高组织的通透性。在医学上，它被用于与其它药物结合以加速其分散和运送。最常见的应用是在眼科手术中，与局部麻醉剂组合使用。动物源性透明质酸酶包括 Hydase™(PrimaPharm Inc. ;Akorn Inc.)、羊型透明质酸酶 (Vitrase)(ISTA Pharmaceuticals) 和牛型透明质酸酶 (Amphadase)(Amphastar Pharmaceuticals)。目前已批准重组人透明质酸酶作为佐剂以增加其它药物的吸收 ;皮下输液（皮下注射液体）;改善造影剂 (radioopaque agent) 吸收的皮下尿路造影中的附属物 (adjunct)。(Hylenex ;Halozyme Therapeutics,Inc. ;Baxter Healthcare Corp.)。在一个实施方案中，透明质酸酶可作为野生型蛋白（亲本分子）用于制备条件活性生物蛋白。透明质酸酶可能在癌症转移和或许在血管生成中发挥作用 ;因此，过度暴露这些酶可能是有害的。在一个方面，条件活性生物透明质酸酶蛋白将会在正常生理温度下不可逆转或可逆失活，但会在特定的低于正常生理温度的温度范围内较野生型透明质酸酶的活性水平相同或高于其活性水平。

[0149] 自身免疫性疾病 - 条件活性生物反应调节剂

[0150] 类风湿关节炎 (RA) 是一种自身免疫性疾病，其特点是导致关节发炎和肿胀并伴随关节逐渐破坏的异常免疫机制。RA 也可影响皮肤、结缔组织和身体中的器官。传统的疗

法包括非甾体抗炎药 (NSAIDs)、COX-2 抑试剂和改善病情抗风湿药 (disease-modifying anti-rheumatic drugs)(DMARDS),如甲氨蝶呤。传统的治疗方案都不理想,尤其是对于长期使用而言。

[0151] 生物反应调节剂,其作用靶标是炎症介质,提供了一种相对新的治疗类风湿关节炎和其它自身免疫性疾病的方法。这种生物反应调节剂包括抗各种炎症介质,如 IL-6、IL-6 受体、TNF-α、IL-23 和 IL-12 的抗体或其活性部分。

[0152] 一些第一生物反应调节剂是靶向肿瘤坏死因子 α (TNF-a),参与 RA 发病机制的一种前炎症细胞因子的药物。目前市面上可获得几种用于治疗 RA 的抗 TNF-α 药物。例如,Enbrel® (依那西普,Amgen) 是 TNF-α 阻滞剂。依那西普是一种二聚体融合蛋白,由连接于人 IgGl Fc 部分的 75kD(p75) 肿瘤坏死因子受体 (TNFR) 的胞外配体结合部分组成。依那西普的 Fc 部分包含 CH2 域、CH3 域和铰链区,但不包含 IgGl 的 CHL 域。依那西普是在中国仓鼠卵巢 (CHO) 哺乳动物细胞表达系统中产生的。它由 934 个氨基酸组成,分子量约 150kD。Enbrel® 用于治疗类风湿关节炎、银屑病关节炎、强直性脊柱炎和牛皮癣。Enbrel®严重的副作用,包括由条件致病菌引起的感染包括结核、真菌感染、细菌或病毒感染。也可发生败血症。淋巴瘤或其它恶性肿瘤也有报道。

[0153] Remicade® (英利昔单抗) 是一种由人恒定区和小鼠可变区组成的嵌合抗 TNF-α IgGkI 单克隆抗体。Remicade 通过静脉注射给药,并用于治疗类风湿关节炎、银屑病、克罗恩病、溃疡性结肠炎和强直性脊柱炎。Remicade 的副作用包括严重感染或败血症,很少为某些 T 细胞淋巴瘤。其它副作用包括肝毒性,某些严重的血液事件、过敏反应和某些严重的神经事件。

[0154] 其它生物反应调节剂包括人源化的抗白细胞介素 -6(IL-6) 受体抗体。IL-6 是一种细胞因子,其有助于消炎、消肿和 RA 中的关节损伤。一种人源化的抗 IL-6 受体抗体,阿克特姆拉 (Actemra)(tocilizumab, Roche),通过了 FDA 和欧盟委员会批准用于治疗患有类风湿关节炎的成人患者。阿克特姆拉也在日本批准用于治疗 RA 和青少年特发性关节炎 (sJIA)。III 期临床研究表明,阿克特姆拉作为单一疗法,或与 MTX 或其它 DMARD 组合治疗,与其它疗法相比减少了 RA 的迹象和症状。阿克特姆拉是人源化的抗人 IL-6 受体单克隆抗体,其竞争性地阻止 IL-6 与其受体的结合。因此,它抑制 IL-6 的细胞增殖作用,从而导致在 RA 中滑膜增厚和血管翳形成。阿克特姆拉的严重副作用包括严重的感染和过敏反应,其包括少数病例的过敏性反应。其它副作用包括上呼吸道感染、头痛、鼻咽炎、高血压和增加的 ALT。

[0155] 另一种常见的自身免疫性疾病是牛皮癣。过于活跃的免疫系统可导致高水平的 IL-12 和 IL-23、两种已在银屑病斑块中发现的细胞因子蛋白。IL-12 和 IL-23 参与炎症和免疫反应,如自然杀伤细胞的活化和 CD4+T 细胞分化和活化。

[0156] 中度或重度牛皮癣的一种治疗包括皮下注射 Stelara™(ustekinumab, Centocor Ortho Biotech, Inc.),一种人源化的抗 IL-12 和 IL-23 因子的 p40 亚基的 IgGIk 单克隆抗体。已证明 Stelara 可减轻与银屑病斑块有关的某些症状,如斑块厚度、脱屑和发红。Stelara 的配方包括 L- 组氨酸和 L- 组氨酸盐酸盐一水合物、聚山梨醇酯 80 和蔗糖,以水配制。Stelara™的使用影响免疫系统,并可能增加感染的机会,包括肺结核和由细菌、真菌或

病毒引起的感染；以及增加某些类型的癌症的风险。

[0157] 生物反应调节剂的副作用很值得注意，部分由于注射患者后的高水平使患者易患严重感染或死亡。这是一种与此类重要药物有关的主要副作用。挑战之一是避免所需抗体剂量的初始高活性水平，从而注射后提供一个长期治疗效果。

[0158] 在一个实施方案中，本发明提供制备能够避免所需抗体剂量的高活性水平，从而注射后提供一个长期治疗效果的条件活性生物反应介质或其片段的方法。本发明的方法可用于设计炎症介质，如 IL-6、IL-6 受体、TNF-α、IL-23 和 IL-12 的抗体，其在给药条件下如室温下是无活性的，但在体温下慢慢地重折叠（可逆或不可逆的）。它们的抗体或片段在初始注射时是无活性的，但注射后接触到血液数小时至数天后，重新折叠或恢复活性。这可以允许较高的给药量和较长的半衰期（或给药期间）并减少副作用。

[0159] 在一个方面，本发明提供制备在给药条件下如室温下无活性，但在体温下缓慢重新折叠（可逆或不可逆的）的炎症介质的条件活性抗体或其片段的方法。该方法包括以下步骤。选择一种炎症介质。通过杂交瘤细胞进行筛选以确定炎症介质的抗体。人源化该抗炎症介质的抗体。演变该抗炎症介质的抗体并差异筛选用于在两种或两种以上条件下的结合，例如，两种或两种以上温度条件，如在室温和在 37℃或更高温度；筛选在第一条件下相对于野生型无活性但在第二条件下显示出相对于野生型抗体活性（如结合）增强了活性（结合）的突变。然后将在重链和轻链变化中确定的改善型突变在重链和轻链内部重新组合，以及通过重链和轻链的组合关联。在两种条件下，例如室温或 37℃或更高温度条件下重复筛选这些重组的重链和轻链。此外，可筛选在储存和生理条件下保持活性和稳定性的重组抗体或片段。

[0160] 或者，炎症介质的野生型抗体是已知抗体或变体或其活性片段。

[0161] 在一个方面，第一和第二条件选自以下条件：pH、渗透压、渗量、氧化和电解质浓度。在另一个方面，炎症介质选自 IL-6、IL-6 受体、TNF-α、IL-23 和 IL-12。

[0162] 在另一个方面，本发明提供制备在给药条件如室温下无活性但在体温下缓慢重新折叠（可逆或不可逆的）的 IL-6 的条件活性抗体或其片段的方法。该方法包括以下步骤。在全人体文库中筛选 IL-6 的抗体。演变该抗体，并在室温和 37℃或更高温度条件下差异筛选分子；筛选在室温下相对于野生型无活性但相对于野生型抗体活性（结合）显示出增强的活性（例如结合）。然后将在重链和轻链变化中确定的改善型突变在重链和轻链内部重新组合，以及通过重链和轻链的组合关联。在室温或 37℃或更高温度条件下重复筛选这些重组的重链和轻链。此外，筛选在储存和生理条件下保持活性和稳定性的重组抗体或片段。

[0163] 如此确定并产生的所述条件活性抗 -IL-6 抗体可用于治疗自身免疫性疾病如类风湿关节炎或牛皮癣的方法中，通过对病人施以所需的有效剂量，较施以传统的抗 -IL-6 抗体生物反应调节剂减少了副作用的严重性。这种方法的一个优点是，相对于目前的高水平生物反应调节剂药物在数周或数月后消除 (clearance) 半衰期，它使在治疗一段时间后的药物量保持平缓或维持药物水平。

[0164] 采用一种或多种突变技术来演变编码野生型蛋白的 DNA，以产生突变 DNA 文库；表达突变 DNA 以产生突变蛋白；在正常生理条件和一种或多种异常条件下对文库进行筛选检测。从这些蛋白中筛选出具有以下两种性质的条件活性生物蛋白：(a) 在正常生理条件的测试中，较野生型蛋白活性减少，以及 (b) 在异常条件的测试中，较野生型蛋白活性增强。

或者，条件活性生物蛋白选自那些在两种或两种以上不同的生理条件下，表现出活性可逆或不可逆变化的蛋白。

[0165] 从亲本分子演变出的分子的形成

[0166] Mirac 蛋白质可通过突变和筛选单个突变的过程产生，该单个突变在野生型条件下活性减少，在非野生型条件下保持与野生型条件相同的或具有更高的活性。

[0167] 本发明提供生产编码具有酶活性的多肽的核酸变体的方法，其中所述变体具有较天然存在的改变的生物活性，该方法包括 :(a) 修饰核酸，通过 (i) 将一个或多个核苷酸替换成不同的核苷酸，其中所述核苷酸包括自然的或非自然的核苷酸，(ii) 缺失一个或多个核苷酸，(iii) 添加一个或多个核苷酸，或 (iv) 它们的任意组合。在一个方面，非自然的核苷酸包括肌苷。在另一个方面，该方法进一步包括检测由修饰的核酸编码的多肽的改变的酶活性，以确定该编码具有改变的酶活性的修饰的核酸。在一个方面，修饰的步骤 (a) 由 PCR、易错 PCR、改组、寡核苷酸定向诱变、装配 PCR、有性 PCR 诱变、体内诱变、盒式诱变、循环总体诱变、指数总体诱变、特异位点诱变、基因重组、基因位点饱和诱变、连接酶链式反应、体外诱变、连接酶链式反应、寡核苷酸合成、任何生成 DNA 的技术和它们的任意组合构成。在另一个方面，该方法进一步包括至少一个修饰步骤 (a) 的重复。

[0168] 本发明进一步提供一种从两个或两个以上的核酸生成多核苷酸的方法，该方法包括 :(a) 确定两个或两个以上的核酸之间的相同部分和不同部分，其中至少一个核酸包括本发明的核酸 ;(b) 提供一组与两个或两个以上核酸中的至少两个序列相关的寡核苷酸 ;以及 (c) 用聚合酶延伸该寡核苷酸，由此生成多核苷酸。

[0169] 任何诱变技术可用于本发明的各种实施方案中。随机的 (Stochastic) 或随机 (random) 诱变例举亲本分子突变 (修饰的或改变的) 情形，产生一组具有预先不确定突变的后代分子。因而，在体外随机诱变反应中，例如没有意向中产量的具体预定的产物 ;有关获得的突变的确切性质，还有有关生成的产物是不确定的，因此是随机的。随机诱变出现于例如易错 PCR 和随机改组的方法中，其中获得的突变是随机的和预先不确定的。变体形式可由易错转录如易错 PCR 产生，或使用缺少纠错功能的聚合酶 (见 Liao(1990)Gene 88:107-111)，第一变体形式，或通过在致突变菌株 (mutator strain)(致突变宿主细胞将在下面作进一步详细讨论，其通常是众所周知) 中复制该第一变体形式。致突变菌株可包括在任何错配修复功能受损的生物体中的任何突变。这些突变包括 mutS、mutT、mutH、mutL、ovrD、dcm、vsr、umuC、umuD、sbcB、recJ 等的突变基因产物。损伤是通过加入试剂，如小化合物或表达的反义 RNA 或其它技术产生的基因突变、等位基因置换、选择性抑制而获得的。损伤可以是基因的损伤，或任何生物体同源基因的损伤。

[0170] 目前广泛使用的用于从起始分子产生可替代蛋白的诱变方法是寡核苷酸定向诱变技术、易错聚合酶链式反应 (易错 PCR) 和盒式诱变，其中特定的待优化区被合成的诱变寡核苷酸替换。在这些情况下，许多突变位点围绕起始序列中的某些位点产生。

[0171] 在寡核苷酸定向诱变中，短序列被合成的诱变寡核苷酸替换。在寡核苷酸定向诱变中，使用限制性酶消化，从多核苷酸中去掉多核苷酸的短序列，且其被合成的其中多个碱基相对于起始序列发生变化的多核苷酸替换。多核苷酸序列也可以通过化学诱变改变。化学诱变剂包括，例如，亚硫酸氢钠、亚硝酸、羟胺、肼或甲酸。核苷酸前体的类似物的其它试剂包括亚硝基胍、5- 溴尿嘧啶、2- 氨基嘌呤或吖啶。一般来说，这些试剂被添加到 PCR 反应

中替换核苷酸前体，从而使序列突变。也可使用插入剂如普罗黄素、吖啶黄、奎纳克林等。多核苷酸序列的随机突变也可通过 X 射线或紫外线照射获得。一般来说，如此诱变的质粒多核苷酸被引入大肠杆菌中并作为杂种质粒的池 (pool) 或文库扩繁。

[0172] 易错 PCR 使用低精确度 (low-fidelity) 聚合作用条件在长序列上引入低水平的随机点突变。在未知序列的片段的混合物中，可使用易错 PCR 诱变该混合物。

[0173] 在盒式诱变中，单模板的序列块通常由 (部分) 随机序列替换。Reidhaar-Olson J F 和 Sauer R T: 作为蛋白序列信息内容探针的组合盒式诱变 (Combinatorial cassette mutagenesis as a probe of the informational content of protein sequences) ; Science 241(4861):53-57,1988。

[0174] 此外，任何非随机的 (non-stochastic) 或非随机 (non-random) 诱变技术可用于本发明的各种实施方案中。非随机诱变的示例情况为如亲本分子被突变 (被修饰或改变)，从而产生具有一个或多个预定突变的后代分子。可以理解，现实是在许多发生分子加工的反应中存在一定量的背景产物，这些背景产物的存在不影响具有预定产物的诱变方法的非随机性质。定点饱和诱变和合成连接重组是目标产物确切化学结构预定的诱变技术的实例。

[0175] 一种定点饱和突变的方法公开于美国专利申请公开第 2009/0130718 号，通过参考并入于此。该方法提供了与模板多核苷酸的密码子对应的一套简并引物，进行聚合酶延伸产生包含对应于该简并引物的序列的多核苷酸后代。可表达和筛选该多核苷酸后代以用于定向演变。具体来说，这是产生一组后代多核苷酸的方法，包括步骤 (a) 提供模板多核苷酸拷贝，各含有大量编码模板多肽序列的密码子 ；以及 (b) 对各模板多核苷酸的密码子进行如下步骤 :(1) 提供一套简并引物，其中各引物含有与模板多核苷酸的密码子对应的简并密码子和至少一个与模板多核苷酸密码子的毗邻序列同源的毗邻序列 ;(2) 提供条件使引物与模板多核苷酸的拷贝退火结合 ；以及 (3) 从引物开始沿着模板进行聚合酶延伸反应 ；由此产生多核苷酸后代，各多核苷酸后代含有与退火引物的简并密码子对应的序列 ；由此生成一组多核苷酸后代。

[0176] 定点饱和诱变涉及核酸的定向演变和筛选含有演变的核酸的克隆以获得目标活性，如核酸活性和 / 或特定的蛋白尤其是酶的目标活性。由这一技术提供的突变分子可含有嵌合分子和具有点突变的分子，包括含有碳水化合物、脂类、核酸和 / 或蛋白成分的生物分子，具体的但不限于以下实例，包括抗生素、抗体、酶、甾体和非甾体类激素。

[0177] 定点饱和诱变一般涉及方法 1) 制备分子后代 (包括由多核苷酸序列组成的分子，由多肽序列组成的分子，以及由部分多核苷酸序列和部分多肽序列组成的分子)，从一个或多个祖先或亲代模板，诱变以获得至少一个点突变、添加、缺失和 / 或嵌合 ;2) 筛选后代分子 (优选采用高通量法) 筛选出至少一种目标特性 (如酶活性的提高或稳定性的增加或新的化疗效果) ;3) 任选地获得和 / 或编目结构和 / 或与亲代和 / 或后代分子有关的功能性信息 ；以及 4) 任选地重复步骤 1)- 步骤 3) 的任何步骤。

[0178] 在定点饱和诱变中，产生 (例如从亲代多核苷酸模板) 称为“密码子定点饱和诱变”的多核苷酸后代，各多核苷酸后代含有一套至少多达三个连续的点突变 (即含有新密码子的不同碱基)，从而每个密码子 (或每个编码相同氨基酸的简并密码子家族) 在每个密码子位置被代表。对应于和由该多核苷酸后代编码，也产生一套多肽后代，各自具有至少一个

单独的氨基酸点突变。在一个优选的方面，产生所称的“氨基酸定点饱和诱变”，一种突变的多肽，各 19 个自然编码的多肽，沿着该多肽在每个氨基酸位置上形成 α - 氨基酸替换。如果其它氨基酸被用于替代或加入到 20 个自然编码的氨基酸中，这产生了（在沿着亲代多肽的每个氨基酸位置上）总计 20 个不同的多肽后代，其包括原始的氨基酸或潜在的≥ 21 个不同的多肽后代。

[0179] 也可采用其它诱变技术，包括重组和更具体的通过含有部分同源区的多核苷酸序列的体内重排的方法来制备编码多肽的多核苷酸的方法，重组该多核苷酸以生成至少一个多核苷酸，并筛选该多核苷酸以产生具有有用属性的多肽。

[0180] 在另一个方面，诱变技术利用细胞的自然属性，以重组分子和 / 或介导降低序列复杂性和重复或连续的具有同源区的序列范围的还原过程。

[0181] 各种诱变技术，可单独使用或组合使用，提供制备编码生物活性增强的杂种多肽的杂种多核苷酸的方法。在实现这些和其它目标中，按照本发明的一个方面，提供了向合适的宿主细胞中引入多核苷酸并使该宿主细胞在产生杂种多核苷酸的条件下生长的方法。

[0182] 通过使用由限制性酶产生的相容粘末端连接 2 个多核苷酸片段生成嵌合基因，其中各片段来自单独的祖先（或亲代）分子。另一个实例是亲代多核苷酸的单一密码子位置的诱变（即获得密码子替换、添加或缺失），以产生编码单一位点诱变的多肽的单一多核苷酸后代。

[0183] 此外，已采用体内位点特异性重组系统产生基因杂种，以及体内重组中的随机方法，和同源的但在质粒中截短的基因之间的重组。也有报道通过重叠延伸和 PCR 产生诱变。

[0184] 非随机的方法已被用来获得大量的点突变和 / 或嵌合 (chimerization)，例如全面或详尽的方法已被用来在特定的突变组中产生所有分子，功能归于模板分子中的特定结构群（例如特定的单一氨基酸位置或由两个或两个以上氨基酸位置构成的序列），用以分类和比较特定的突变组。

[0185] 这些或其它演变方法中的任一种方法可用于本发明中，从一个或多个亲代分子产生新的分子群体（文库）。

[0186] 一旦形成，根据已公开的方案 (protocol)，构建体可以或不必在琼脂糖凝胶上区分大小，插入克隆载体，并转染到合适的宿主细胞内。

[0187] 演变分子的表达

[0188] 一旦生成突变分子库，可采用常规分子生物技术表达 DNA。因而，可采用各种已知的方法表达蛋白。

[0189] 例如，简单地说，可采用各种随机的或非随机的方法如本文中描述的那些方法来演变野生型基因。然后采用标准的分子生物技术来消化突变的 DNA 分子并连接至载体 DNA，如质粒 DNA。采用标准方法 (protocol) 将含有单个突变的载体 DNA 转化至细菌或其它细胞中。可在多孔板，如 96- 孔板的单个孔中进行高通量表达和筛选。重复该方法获得各突变分子。

[0190] 将所述筛选和分离的多核苷酸引入合适的宿主细胞内。合适的宿主细胞是任何能够促进重组和 / 或还原重组的细胞。筛得的多核苷酸优选已存在于载体中，该载体包括适当的调控序列。宿主细胞可以是高等的真核细胞如哺乳动物细胞，或低等的真核细胞如酵母细胞，或优选地，宿主细胞可以是原核细胞如细菌细胞。将构建体引入宿主细胞内可以是

磷酸钙转染、DEAE- 葡聚糖介导转染或电穿孔（例如 Ecker 和 Davis,1986, 通过表达反义 RNA 抑制基因在植物细胞中的表达(Inhibition of gene expression in plant cells by expression of antisense RNA),Proc Natl Acad Sci USA,83:5372-5376)。

[0191] 作为有代表性的实例,可使用的表达载体,可提及的是病毒颗粒、杆状病毒、噬菌体、质粒、噬菌粒、粘粒、养粒 (fosmids)、细菌人工染色体、病毒 DNA(如牛痘病毒、腺病毒、禽痘病毒、伪狂犬病和 SV40 的衍生物)、基于 P1 的人工染色体、酵母质粒、酵母人工染色体和其它任何特定的目的宿主（如芽孢杆菌、黑曲霉和酵母菌）。因而,例如,DNA 可包括于任一种用于表达多肽的表达载体中。这种载体包括染色体的、非染色体的和人工合成的 DNA 序列。大量合适的载体是本领域众所周知的且可市售获得。以下载体通过举例的方式提供 :细菌 :pQE 载体 (Qiagen)、pBluescript 质粒、pNH 载体、λ-ZAP 载体 (Stratagene) ; ptrc99a、pKK223-3、pDR540、pRIT2T(Pharmacia) ;真核 :pXT1、pSG5(Stratagene)、pSVK3、pBPV、PMSG、pSVLSV40(Pharmacia)。然而,可使用任何其它质粒或其它载体,只要它们是在宿主中可复制且存活的。本发明中可采用低拷贝量或高拷贝量载体。

[0192] 表达载体中的 DNA 序列可操作地连接至适当的表达调控序列（启动子）来指导 RNA 的合成。特殊命名的细菌启动子包括 lacI、lacZ、T3、T7、gpt、λ PR、PL 和 trp。真核启动子包括 CMV 早期 (immediate early)、HSV 胸苷激酶、早期和晚期 SV40、来自逆转录病毒的 LTR 和小鼠金属硫蛋白 -1。合适的载体和启动子的选择是本领域的普通技术水平。表达载体还含有用于翻译起始和转录终止的核糖体结合位点。载体还包括适当的用于扩增表达的序列。用氯霉素转移酶 (CAT) 载体或其它具有筛选标记的载体,可以从任何目标基因中筛选出启动子区。此外,优选表达载体含有一个或多个选择标记基因,以提供用于筛选转化的宿主细胞的表型性状,如用于真核细胞培养物的二氢叶酸还原酶或新霉素抗性,或如在大肠杆菌中的四环素或氨苄青霉素抗性。

[0193] 因此,在本发明的另一个方面,可通过还原重组的方法产生新的多核苷酸。该方法包括制备含有连续序列（原始编码序列）的构建体,将它们插入合适的载体,随后将它们引入到合适的宿主细胞中。通过在具有同源区的构建体中的连续序列之间或在准重复单元之间的组合方法产生单独分子识别 (molecular identity) 的重排。重排方法重组和 / 或降低了重复序列的复杂性和规模,并产生了新的分子。可采用各种处理来增强重组率。这些可能包括使用紫外光或破坏 DNA 的化学品和 / 或使用显示更高水平的“遗传不稳定”的宿主细胞。因而,重组方法可包括同源重组或指导自身演变的准重复序列的自然属性。

[0194] 在一个方面,宿主生物体或细胞包括革兰氏阴性菌、革兰氏阳性菌或真核有机体。在本发明的另一个方面,革兰氏阴性菌包括大肠杆菌或荧光假单胞菌。在本发明的另一个方面,革兰氏阳性菌包括 Streptomyces diversa、格氏乳杆菌 (Lactobacillus gasseri)、乳酸乳球菌 (Lactococcus lactis)、乳脂链球菌 (Lactococcus cremoris) 或枯草芽孢杆菌。在本发明的另一个方面,真核有机体包括酿酒酵母、裂殖酵母、毕赤酵母、乳酸克鲁维酵母、汉逊酵母或黑曲霉 (Aspergillus niger)。作为合适宿主的有代表性的实例,可提及的是 :细菌细胞,如大肠杆菌、链霉菌、鼠伤寒沙门氏菌 ;真菌细胞,如酵母 ;昆虫细胞,如果蝇 S2 和夜蛾 Sf9 ;动物细胞如 CHO、COS 或黑色素瘤 ;腺病毒和植物细胞。选择合适的宿主被认为是本领域熟练技术人员教导的范围内。

[0195] 特别提到可用于表达重组蛋白的各种哺乳动物细胞培养系统,哺乳动物表达系统

的实例包括猴肾成纤维细胞的 COS-7 系，描述于“SV40 病毒转化的猴细胞支持早期 SV40 病毒突变的复制”(Gluzman，1981)，以及其它能表达相容载体的细胞系，例如，C127、3T3、CHO、HeLa 和 BHK 细胞系。哺乳动物表达载体包括复制起点位点、合适的启动子和增强子，以及任何必要的核糖体结合位点，多聚腺苷酸化位点，剪接供体和受体位点，转录终止序列和 5’侧翼非转录序列。来自 SV40 拼接和多聚腺苷酸位点的 DNA 序列，可用来提供所需的非转录基因元件。

[0196] 然后扩繁细胞和进行“还原重组”。如果需要，可通过引入 DNA 损伤来刺激还原重组率。体内重组集中于“分子间”的方法统称为“重组”，其在细菌中一般被视为“RecA- 依赖”现象。本发明依靠宿主细胞的重组来重组和重排序列，或依靠细胞的能力来介导还原过程，以通过缺失降低细胞中准重复序列的复杂性。这种“还原重组”过程通过“分子内”，RecA- 非依赖的方法发生。最终的结果是分子重组至所有可能的组合中。

[0197] 含有目标多核苷酸的宿主细胞可以在经过修饰的适合于激活启动子、筛选转化子或扩增基因的常规营养培养基中培养。培养条件，如温度、pH 等，是以前所用的筛选宿主细胞表达的条件，对本领域熟练的技术人员而言是明显的。

[0198] 蛋白表达可通过各种已知的方法诱导，许多遗传系统已被公开用来诱导蛋白表达。例如，使用合适的系统，添加诱导剂可诱导蛋白表达。然后离心沉淀细胞，弃上清液。将细胞与 DNA 酶、RNA 酶和溶菌酶孵育，富集周质蛋白。离心后，将含有新蛋白的上清液转移至新的多孔板中，检测前贮存。

[0199] 一般通过离心收集细胞，通过物理或化学方法破坏细胞，所得的粗提物用于进一步纯化。用于表达蛋白的微生物细胞可通过任何方便的方法破坏，包括冻融循环、超声，机械破碎或细胞裂解液的使用。此类方法是本领域熟练的技术人员所熟知的。采用包括硫酸铵或乙醇沉淀、酸提取、阴离子或阳离子交换色谱、磷酸纤维素色谱、疏水层析、亲和层析、羟基磷灰石色谱和凝集素层析的方法，可从重组细胞培养物中回收和纯化表达的多肽或其片段。必要时可使用蛋白复性的步骤，来完成多肽的构型。如果需要，可采用高效液相色谱法 (HPLC) 作为最后的纯化步骤。

[0200] 然后对鉴定出的具有目标活性的克隆进行测序，以确定编码具有增强活性的酶的多核苷酸序列。

[0201] 从这些文库中鉴定出的多肽可用于治疗、诊断、研究和相关的目的，和 / 或可进行一个或多个循环的改组和 / 或筛选。本发明提供至少为 10 个氨基酸大小的条件活性生物蛋白的片段，其中该片段具有活性。

[0202] 本发明提供具有酶活性的密码子优化的多肽或其片段，其中密码子使用针对特定生物体或细胞进行了优化。Narum 等人，“编码恶性疟原虫裂殖子表面蛋白的基因片段的密码子优化，提高了 DNA 疫苗蛋白在小鼠体内的表达和免疫原性 (Codon optimization of gene fragments encoding Plasmodium falciparum merzoite proteins enhances DNA vaccine protein expression and immunogenicity in mice)”. Infect. Immun. 2001December, 69(12):7250-3 描述了在小鼠表达系统中密码子的优化。Outchkourov 等人，“Equistatin 在毕赤酵母中表达的优化，蛋白表达和纯化 (Optimization of the expression of Equistatin in Pichia pastoris, protein expression and purification)”, Protein Expr. Purif. 2002 February ;24(1):18-24 描

述了在酵母表达系统中密码子的优化。Feng 等人,“使用人工合成的基因进行人磷脂转移蛋白的高效表达重组和诱变 :C- 末端膜结合结构域的证据 (High level expression and mutagenesis of recombinant human phosphatidylcholine transfer protein using a synthetic gene:evidence for a C-terminal membrane binding domain)”Biochemistry 2000 Dec.19,39(50):15399-409 描述了在大肠杆菌表达系统中密码子的优化。Humphreys 等人,“使用真核生物信号肽在大肠杆菌中进行高水平的周质表达 :编码序列 5’ 端密码子使用的重要性 (High-level periplasmic expression in Escherichia coli using a eukaryotic signal peptide:importance of codon usage at the 5’ end of the coding sequence)”,Protein Expr.Purif.2000 Nov.20(2):252-64 描述了密码子的使用如何影响在大肠杆菌中的分泌。

[0203] 条件活性生物蛋白的演变可通过方便的高通量筛选或选择的方法来辅助实现。

[0204] 一旦确定,本发明的多肽和肽可以人工合成或重组产生多肽。肽和蛋白可在体外或体内重组表达。本发明的肽和多肽可采用本领域已知的任何方法来制备和分离。采用本领域众所周知的化学方法,本发明的多肽和肽也可以全部或部分是人工合成的。见例如,Caruthers(1980) “用于合成多核苷酸的新的化学方法 (New chemical methods for synthesizing polynucleotides)”,Nucleic Acids Res.Symp.Ser.215-223 ; Horn(1980), “在纤维素上合成寡核苷酸。第二部分 :合成编码抑胃多肽 (GIP)[1]的 22 个寡聚脱氧核苷酸的设计和合成策略 (Synthesis of oligonucleotides on cellulose.Part II:design and synthetic strategy to the synthesis of 22 oligodeoxynucleotides coding for Gastric Inhibitory Polypeptide(GIP)[1])”,Nucleic Acids Res.Symp. Ser.225-232 ;Banga,A.K., 治疗性多肽和蛋白,制备、加工和给药系统 (Therapeutic Peptides and Proteins,Formulation,Processing and Delivery Systems)(1995) Technomic Publishing Co.,Lancaster,Pa。例如,肽的合成可使用各种固相技术 (见例如,Roberge(1995) “在固体支持物上聚合合成 N- 连接糖肽的策略 (A strategy for a convergent synthesis of N-linked glycopeptides on a solid support)”,Science 269:202 ;Merrifield(1997) “固相肽合成的概念和早期发展 (Concept and early development of solid-phase peptide synthesis)”,Methods Enzymol.289:3-13) 以及自动合成,例如采用 ABI 43 IA 多肽合成仪 (Perkin Elmer),按照制造商提供的说明。

[0205] 本发明的肽和多肽也可以被糖基化。糖基化可以利用化学或细胞的生物合成机制在翻译后添加,其中后者采用已知的糖基化基序,该基序可以对序列是原生的,或可作为肽添加或在核酸编码序列中添加。糖基化可以是 O- 连接的或 N- 连接的。

[0206] 如上述所定义,本发明的肽和多肽包括所有的“模拟物”和“模拟肽”形式。术语“模拟物”和“模拟肽”是指人工合成的化合物,其具有本发明多肽的基本上相同的结构和 / 或功能特点。模拟物可以完全由人工合成的、氨基酸的非天然类似物组成,或者是由部分天然肽氨基酸和部分氨基酸的非天然类似物的嵌合分子组成。模拟物还可包括任意数量的氨基酸的保守替换,只要这种替换基本上不改变该模拟物的结构和 / 或活性。本发明的多肽是保守的变体,常规实验可确定是否模拟物在本发明范围内,即它的结构和 / 或功能基本上不变。

[0207] 本发明的多肽模拟物组合物可包含非天然结构成分的任意组合。在另一个方面,

本发明的模拟物组合物包括以下三个结构组中的一种或全部 :a) 残基连接基 (linkage group) 而不是天然的酰胺键 (“肽键”) 连接 ;b) 非天然残基替换天然产生的氨基酸残基 ; 或 c) 诱导二级结构模拟物的残基,即诱导或稳定二级结构,例如,β 转角、γ 转角、β 片层、α - 螺旋构象等。例如,本发明的多肽作为模拟物的特点是,其全部或一部分的残基通过化学方法而不是通过肽键连接。单个肽模拟肽残基的连接可通过肽键、其它化学键或偶联法,例如,戊二醛、N- 羟基琥珀酰亚胺酯、双功能马来酰亚胺、N,N’ - 二环己基碳二亚胺 (DCC) 或 N,N’ - 二异丙基碳二亚胺 (DIC)。可替代传统的酰胺键 (“肽键”) 的连接基 (linking group),例如,酮亚甲基 (例如, --C(.dbd.O)--CH.sub.2--for--C(.dbd.O)--NH--)、氨基甲叉 (aminomethylene)(CH.sub.2--NH)、乙烯、烯烃 (CH.dbd.CH)、乙醚 (CH.sub.2--O)、硫醚 (CH.sub.2--S)、四氮唑 (CN.sub.4--)、噻唑、逆酰胺 (retroamide)、硫代酰胺或酯 (见例如, Spatola(1983)in Chemistry and Biochemistry of Amino Acids,Peptides and Proteins,Vol.7,pp 267-357, “肽骨架修饰 (Peptide Backbone Modifications)”, Marcell Dekker,N.Y)。

[0208] 本发明的作为模拟物的多肽的特点还在于,含有全部或部分的非天然残基替换天然氨基酸残基。非天然残基已描述于科学的和专利文献中 ;几种例举的用作天然氨基酸残基模拟物的非天然组合物和准则 (guidelines) 描述如下。芳香族氨基酸模拟物可通过以如下物质替换产生,例如, D- 或 L- 苯丙氨酸、D- 或 L- 苯甘氨酸、D- 或 L-2 噻吩丙氨酸、D- 或 L-1,-2,3-, 或 4 吡啶基、D- 或 L-3 噻吩丙氨酸、D- 或 L-(2- 吡啶基)- 丙氨酸、D- 或 L-(3- 吡啶基)- 丙氨酸、D- 或 L-(2- 吡嗪)- 丙氨酸、D- 或 L-(4- 异丙基)- 苯甘氨酸、D-(三氟甲基)- 苯甘氨酸、D-(三氟甲基)- 苯丙氨酸、D- 对 - 氟 - 苯丙氨酸、D- 或 L- 对 - 联苯基丙氨酸、D- 或 L- 对 - 甲氧基 - 联苯基丙氨酸、D- 或 L-2- 吲哚 (烷基) 丙氨酸以及 D- 或 L- 烷基胺,其中烷基可以是取代的或未取代的甲基、乙基、丙基、己基、丁基、戊基、异丙基、异丁基、仲 -isotyl、异戊基或非酸性氨基酸。非天然氨基酸的芳香环包括,例如,噻唑、噻吩、吡唑、苯并咪唑、萘、呋喃、吡咯和吡啶芳香环。

[0209] 氨基酸模拟物可通过替换产生,例如被带有负电荷的非羧酸氨基酸、(膦) 丙氨酸、硫酸化苏氨酸所替换。羧基侧基团 (例如,天冬氨酰或谷氨酰) 也可通过与碳化二亚胺 (R’--N--C--N--R”) 反应进行选择性地修饰,例如, 1- 环己基 -3(2- 吗啉基 -4- 乙基) 亚胺或 1- 乙基 -3(4-azonia-4,4-dimethol 戊基) 碳二亚胺。天冬氨酰或谷氨酰也可通过与铵离子反应被转换为天冬酰胺酰基和谷氨酰胺酰基。碱性氨基酸模拟物可被替换产生,例如,(除赖氨酸和精氨酸以外) 氨基酸瓜氨酸、鸟氨酸或 (胍基)- 乙酸或 (胍基) 烷基 - 乙酸,其中烷基如上述定义。丁腈衍生物 (例如,含有 CN- 基团以替换 COOH) 可被替换为天冬酰胺或谷氨酰胺。天冬酰胺酰基和谷氨酰胺酰基可脱氨生成相应的天冬氨酰基或谷氨酰基。精氨酸残基模拟物可由精氨酰与,例如一种或多种常规试剂反应生成,包括例如苯乙二醛、2,3- 丁二酮、1,2- 环己二酮或茚三酮,优选在碱性条件下反应。酪氨酸残基模拟物可由酪氨酰与,例如芳香重氮化合物或四硝基甲烷反应生成。N- 乙酰基咪唑和四硝基甲烷可分别用于生成 O- 乙酰酪氨酸和 3- 硝基衍生物。半胱氨酸残基模拟物可由半胱氨酰与,例如 α - 卤丙酸酯如 2- 氯乙酸或氯乙酰胺和相应的胺反应,以生成羧甲基或甲酰胺基甲基 (carboxyamidomethyl) 衍生物。半胱氨酸残基模拟物还可由半胱氨酰与,例如溴三氟丙酮、α - 溴代 - β -(5- 咪唑基) 丙酸、氯磷酸、N- 烷基马来酰亚胺、3- 硝基 -2- 吡啶基二硫化物、

2- 甲基吡啶二硫化物、对 - 氯汞苯甲酸、2- 氯汞基 -4- 硝基酚或氯 -7- 硝基苯 - 氧 -1,3 二唑反应生成。赖氨酸模拟物可由赖氨酰与，例如琥珀酸或其它羧酸酐反应生成（氨基末端残基可进行修改）。赖氨酸和其它含有 α - 氨基的模拟物也可通过与亚氨酸酯，如甲基吡啶亚胺甲酯、磷酸吡哆醛、吡哆醛、硼氢化氯、三硝基苯磺酸、O- 甲基异脲、2,4- 戊二酮以及转酰胺基酶催化的与乙醛酸的反应生成。蛋氨酸模拟物可由与，例如蛋氨酸亚砜反应生成。脯氨酸模拟物包括，例如哌啶甲酸、噻唑羧酸、3- 或 4- 羟基脯氨酸、二氢脯氨酸、3- 或 4- 甲基脯氨酸或 3,3- 二甲基脯氨酸。组氨酸模拟物可由组氨酰与，例如焦碳酸二乙酯或对溴苯甲酰甲基溴反应生成。其它模拟物包括，例如由脯氨酸和赖氨酸羟化所产生的；由丝氨酰和苏氨酰的羟基的磷酸化所产生的；由赖氨酸、精氨酸和组氨酸的 α - 氨基的甲基化所产生的；由 N- 末端胺的乙酰化所产生的；主链酰胺基的甲基化或 N- 甲基氨基酸的替换所产生的；或 C- 末端羧基的酰胺化所产生的。

[0210] 本发明多肽的残基，例如，氨基酸也可被替换成相反手性的氨基酸（或肽模拟物残基）。因而，任何以 L- 构型天然存在的氨基酸（也可指 R 或 S，这取决于该化学实体的结构）可被替换成相同化学结构类型或肽模拟物但相反手性的氨基酸，可指 D- 氨基酸，也可指 R- 或 S- 形式。

[0211] 本发明还提供通过自然方法，如翻译后加工（例如，磷酸化、酰化等），或通过化学修饰技术修饰本发明多肽的方法。修饰可发生在多肽任何位置，包括肽主链、氨基酸侧链和氨基或羧基末端。可以理解，同一类型的修饰可以相同或不同程度存在于给定多肽中的几个位点。另外给定多肽可能有许多类型的修饰。修饰包括乙酰化、酰化、聚乙二醇、ADP- 核糖基化、酰胺化、黄素的共价连接、血红素基团的共价连接、核苷酸或核苷酸衍生物的共价连接、脂质或脂类衍生物的共价连接、磷脂酰肌醇的共价连接、交联环化、二硫键形成、去甲基化、形成共价交连、形成半胱氨酸、形成焦谷氨酸、甲酰化、γ - 羧化、糖基化、形成 GPI 锚定 (anchor)、羟基化、碘化、甲基化、豆蔻酰化、氧化、聚乙二醇化、蛋白水解处理、磷酸化、异戊烯化、消旋、蛋白硒化 (selenoylation)、硫酸盐化及转移 RNA 介导的添加氨基酸蛋白质，如精氨酰化。见例如，Creighton,T.E., 蛋白 -- 结构和分子性质第二版 (Proteins—Structure and Molecular Properties 2nd Ed.),W.H.Freeman and Company,New York(1993)；翻译后蛋白的共价修饰 (Posttranslational Covalent Modification of Proteins),B.C.Johnson,Ed.,Academic Press,New York,pp.1-12(1983)。

[0212] 固相化学肽合成方法也可用于合成本发明的多肽或片段。该方法在二十世纪六十年代早期就为本领域已知 (Merrifield,R.B.,“固相合成 .I. 四肽的固相合成 (Solid-phase synthesis.I.The synthesis of a tetrapeptide)”,J.Am.Chem.Soc,85:2149-2154,1963)（还见 Stewart,J.M. 和 Young,J.D., 固相肽合成第二版 (Solid Phase Peptide Synthesis,2nd Ed.),Pierce Chemical Co.,Rockford,111.,pp.11-12)），近来也应用于市售获得的实验室肽设计和合成试剂盒 (Cambridge Research Biochemicals)。这类市售可获得的实验室试剂盒已普遍应用了 H.M.Geysen 等人的教导，“应用肽合成探测病毒抗原，用于单个氨基酸的抗原表位 (Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid)”，Proc.Natl.Acad.Sci.,USA,81:3998(1984) 的教导中，提供基于大量连接于单个板的“杆 (rod)”或“棒 (pin)”的尖端 (tip) 的合成肽。当采用这一系统时，一个板的“杆”或“棒”

被反转并插入另一个板的相应孔或池中，所述孔或池含有用于结合或锚定 (anchoring) 适当氨基酸至“棒”或“杆”的尖端的溶液。通过重复这一步骤，即将“杆”和“棒”的尖端反转和插入到适当的溶液中，氨基酸被构建成目标肽。此外，可采用许多 FMOC 肽合成系统。例如，多肽或片段的装配可采用 Applied Biosystems, Inc. Model 431 A™自动肽合成仪在固体支持物上来完成。此类设备易于通过直接合成或合成一系列片段生成本发明的肽，该片段可使用其它已知的技术进行连接。

[0213] 可采用已知方法，包括硫酸铵或乙醇沉淀、酸萃取、阴离子或阳离子交换色谱、磷酸纤维素色谱、疏水层析、亲和层析、羟基磷灰石色谱和凝集素层析来回收和纯化合成的多肽或其片段。必要时可使用蛋白复性的步骤来在完成多肽的构型。如果需要，可采用高效液相色谱法 (HPLC) 作为最后的纯化步骤。

[0214] 本发明提供了包含至少一种蛋白变体的条件活性蛋白变体的制剂 (preparation) 或制剂 (formulation)，其中所述制剂为液态或干燥的。所述蛋白制剂任选包括缓冲液、辅助因子、第二种或更多种蛋白，或一种或多种赋形剂。在一个方面，该制剂用作治疗性的条件活性生物蛋白，其在异常或非生理条件下有活性，但在正常生理条件，例如温度、pH、渗透压、氧化或渗量的条件下活性较低或无活性。

[0215] 标准纯化技术可用于重组或合成条件活性生物蛋白。

[0216] 筛选突变体以确定可逆或不可逆突变

[0217] 确定目标分子主要是通过在允许的条件和野生型条件下测定蛋白活性来直接实现的。然后可筛选出活性比（允许 / 野生型）最大的突变体并采用标准方法通过单个突变的组合生成点突变的排列 (permutation)。然后筛选该组合的排列蛋白文库得到在允许和野生型条件之间显示出最大活性差异的蛋白。

[0218] 可采用多种方法筛选上清液的活性，例如采用高通量活性测定，如荧光检测，鉴定出对想要的特征（温度、pH 等）敏感的蛋白突变体。例如，筛选温度敏感的突变体，使用市售底物，在较低温度（如 25℃）和原始蛋白功能的温度（如 37℃）下测定各突变体的酶活性或抗体活性。反应最初可以是多孔检测的形式，如 96- 孔检测，采用不同的形式，如 14ml 管的形式进行验证。

[0219] 本发明进一步提供用于确定酶的筛选检测，其包括 :(a) 提供大量核酸或多肽 ;(b) 从大量多肽中获得待测酶活性的候选多肽 ;(c) 测试候选多肽的酶活性 ;以及 (d) 确定那些在异常或非生理条件下表现出增加的酶活性，而在正常生理条件，如温度、pH、氧化、渗量、电解质浓度或渗透压下，较野生型酶蛋白的酶活性减少的候选多肽。

[0220] 在一个方面，该方法进一步包括在测试具有条件生物活性的候选多肽之前，修饰至少一种核酸或多肽。在另一个方面，测试步骤 (c) 进一步包括 :检测在宿主细胞或宿主生物体中多肽的增加表达。在另一个方面，测试步骤 (c) 进一步包括 :在约 pH3- 约 pH12 的 pH 范围内检测酶活性。在另一个方面，测试步骤 (c) 进一步包括 :在约 pH5- 约 pH10 的 pH 范围内检测酶活性。在另一个方面，测试步骤 (c) 进一步包括 :在约 pH6- 约 pH8 的 pH 范围内检测酶活性。在另一个方面，测试步骤 (c) 进一步包括 :在 pH6.7 和 pH7.5 下检测酶活性。在另一个方面，测试步骤 (c) 进一步包括 :在约 4℃ - 约 55℃的温度范围内检测酶活性。在另一个方面，测试步骤 (c) 进一步包括 :在约 15℃ - 约 47℃的温度范围内检测酶活性。在另一个方面，测试步骤 (c) 进一步包括 :在约 20℃ - 约 40℃的温度范围内检测酶活性。在

另一个方面，测试步骤 (c) 进一步包括 :在约 25℃ - 约 37℃的温度范围内检测酶活性。在另一个方面，测试步骤 (c) 进一步包括 :在正常渗透压和异常 (正的或负的) 渗透压的条件下检测酶活性。在另一个方面，测试步骤 (c) 进一步包括 :在正常电解质浓度和异常 (正的或负的) 电解质浓度的条件下检测酶活性。受试电解质浓度选自钙、钠、钾、镁、氯、碳酸氢盐和磷酸盐浓度中的一种，在另一个方面，测试步骤 (c) 进一步包括 :检测形成稳定反应产物的酶活性。

[0221] 在另一个方面，本发明提供特异结合本发明具有酶活性的多肽或其片段的纯化的抗体。在一个方面，本发明提供特异结合具有酶活性的多肽的抗体片段。

[0222] 抗体和基于抗体的筛选方法

[0223] 本发明提供特异结合本发明酶的分离的或重组抗体。这些抗体可用于分离、鉴定或定量本发明的酶或相关多肽。这些抗体可用于在本发明范围内分离其它多肽或其它相关的酶。所述抗体可被设计用来结合酶的活性位点。因而，本发明提供使用本发明的抗体抑制酶的方法。

[0224] 所述抗体可用于免疫沉淀、染色、免疫亲和柱等。如果需要，编码特定抗原的核酸序列的生成，可通过免疫后进行多肽或核酸的分离、扩增或克隆以及将多肽固定于本发明的阵列 (array)。或者，本发明的方法可用于修饰由待修饰细胞产生的抗体的结构，例如，抗体的亲和力可增加或减少。而且，制备或修饰抗体的能力可为通过本发明的方法改造至细胞的表型。免疫方法、制备和分离抗体 (多克隆的和单克隆的) 的方法是本领域熟练的技术人员已知的，并描述于科学和专利文献中，见例如 Coligan, 免疫学流行手册 (CURRENT PROTOCOLS IN IMMUNOLOGY),Wiley/Greene,NY(1991) ;Stites(eds.) 基础和临床免疫学 (第 7 版)(BASIC AND CLINICAL IMMUNOLOGY)(7th ed.)Lange Medical Publications,Los Altos,Calif.("Stites") ;Goding, 单克隆抗体 :原理及操作 (第 2 版)(MONOCLONAL ANTIBODIES:PRINCIPLES AND PRACTICE)(2d ed.)Academic Press,New York,N.Y.(1986) ;Kohler(1975)“分泌特异性抗体的融合细胞的连续培养物 (Continuous cultures of fused cells secreting antibody of predefined specificity)”,Nature 256:495 ;Harlow(1988) 抗体，实验室手册 (ANTIBODIES,A LABORATORY MANUAL),Cold Spring Harbor Publications,New York。除了传统在动物体内的方法以外，抗体也可在体外产生，例如使用重组抗体结合位点表达噬菌体展示库。见例如，Hoogenboom(1997)“生产高亲和力抗体的文库筛选策略的设计和优化 (Designing and optimizing library selection strategies for generating high-affinity antibodies)”,Trends Biotechnol.15:62-70 ;and Katz(1997)“通过噬菌体展示技术发现或改造的配体亲和力和特异性的结构和机械确定 (Structural and mechanistic determinants of affinity and specificity of ligands discovered or engineered by phage display)”,Annu.Rev. Biophys.Biomol.Struct.26:27-45。

[0225] 多肽或肽可用于制备特异结合多肽，例如本发明酶的抗体。得到的抗体可用于免疫亲和色谱法以分离或纯化多肽或确定是否多肽存在于生物样本中。在该方法中，蛋白试剂，如提取物或生物样本与能够特异结合本发明的一种多肽的抗体接触。

[0226] 在免疫亲和法中，所述抗体附着在固体支持物，如珠或其它柱形基质上。所述蛋白试剂在抗体能够特异结合本发明的一种多肽的条件下与抗体接触。洗涤后去除非特异结合

的蛋白，洗脱特异结合的多肽。

[0227] 生物样本中蛋白结合抗体的能力可采用任何一种本领域熟练的技术人员所熟悉的方法来确定。例如，结合可通过用可检测标记，如荧光剂、酶标物或放射性同位素标记抗体而确定。或者，抗体结合样本的能力可利用带有可检测标记的二级抗体来测定。特定的检测包括 ELISA 检测、双抗体夹心法、放射免疫分析和 Western 印迹。

[0228] 产生的作用于本发明多肽的多克隆抗体可通过向动物体内直接注射多肽或通过对非人类的动物施用多肽而获得。然后获得的抗体结合多肽自身。在这种方式下，即使仅编码多肽片段的序列也可用于产生可结合于整个原生多肽的抗体。然后该抗体可用于从表达该多肽的细胞中分离多肽。

[0229] 为了制备单克隆抗体，可采用任何由连续细胞株培养产生抗体的技术。实例包括杂交瘤技术、

[0230] 单克隆抗体的制备，可使用提供由连续的细胞株培养产生的抗体的任何技术。实例包括杂交瘤技术，三体杂交瘤技术、人类 B 细胞杂交瘤技术和 EB 病毒杂交瘤技术（见例如，Cole(1985 年)，单克隆抗体和癌症治疗 (Monoclonal Antibodies and Cancer Therapy)，Alan R.Liss,Inc.,pp.77-96)。

[0231] 所述用于生产单链抗体的技术（见例如，美国专利第 4946778 号）可适用于生产作用于本发明多肽的单链抗体。或者，可用转基因小鼠表达作用于这些多肽或其片段的人源化的抗体。产生的作用于本发明多肽的抗体可用于从其它生物体和样本中筛选类似的多肽（例如，酶）。在这些技术中，来自于生物体的多肽与抗体接触，检测到那些特异结合抗体的多肽。任何以上描述的方法可用于检测抗体结合。

[0232] 筛选方法和“在线”检测装置

[0233] 在本发明方法的操作中，可使用多种仪器和方法用于本发明的多肽和核酸，例如，为了筛选具有酶活性的多肽，为了筛选作为潜在的调节剂，例如酶活性的激活剂或抑试剂的化合物，为了筛选结合本发明多肽的抗体，为了筛选与本发明核酸杂交的核酸，为了筛选表达本发明的多肽的细胞等。

[0234] 阵列或“生物芯片”

[0235] 本发明的核酸或多肽可以被固定于或应用到阵列上。阵列可用于筛选或监测组合物（例如，小分子、抗体、核酸等）文库，针对它们结合或调节本发明核酸或多肽的活性的能力。例如，在本发明的一个方面，监测的参数是酶基因的转录表达。细胞的一种或多种或所有转录物可通过含有细胞转录物，或细胞转录物的核酸代表或其互补核酸的样本的杂交来测定，通过与固定于阵列或“生物芯片”上的核酸杂交来测定。通过使用微芯片上的核酸“阵列”，一些或全部细胞转录物可同时被定量。或者，含有基因组核酸的阵列也可用于测定通过本发明方法新改造的细胞株的基因型。多肽“阵列”也可用于同时定量多种蛋白。本发明可利用任何已知的“阵列”，还被称作“微阵列”或“核酸阵列”或“多肽阵列”或“抗体阵列”或“生物芯片”或它们的变体来操作。一般地，阵列是大量的“点”或“靶元件”，各靶元件包括确定量的一种或多种生物分子，例如，寡核苷酸，固定于底物表面的特定区，用于特异结合样本分子，例如，mRNA 转录物。

[0236] 在本发明方法的操作中，任何已知的阵列和 / 或制作和使用阵列的方法或它们的变体可全文或部分并入于此，描述于例如，美国专利号 6277628、6277489、6261776、

6258606、6054270、6048695、6045996、6022963、6013440、5965452、5959098、5856174、5830645、5770456、5632957、5556752、5143854、5807522、5800992、5744305、5700637、5556752、5434049；也见，例如，WO 99/51773、WO 99/09217、WO 97/46313、WO 96/17958；也见，例如，Johnston(1998)“基因芯片：了解基因调控的希望阵列(Gene chips:Array of hope for understanding gene regulation)”，Curr.Biol.8:R171-R174；Schummer(1997)“用于构建高密度核酸阵列的廉价手持装置(Inexpensive Handheld Device for the Construction of High-Density Nucleic Acid Arrays)”，Biotechniques 23:1087-1092；Kern(1997)“在高密度网格cDNA过滤检测中基因组大克隆插入的直接杂 交(Direct hybridization of large-insert genomic clones on high-density gridded cDNA filter arrays)”，Biotechniques 23:120-124；Solinas-Toldo(1997)“用于基因组失衡筛查的生物芯片：基于基质的比较基因组杂交(Matrix-Based Comparative Genomic Hybridization:Biochips to Screen for Genomic Imbalances)”，Genes,Chromosomes&Cancer 20:399-407；Bowtell(1999)“通过微阵列用于获得从起始到终止的表达数据的选择(Options Available--From Start to Finish--for Obtaining Expression Data by Microarray)”，Nature Genetics Supp.21:25-32。也见公开的美国专利申请号 20010018642、20010019827、20010016322、20010014449、20010014448、20010012537、20010008765。

[0237] 毛细管阵列

[0238] 毛细管阵列，如GIGAMATRIX™Diversa Corporation,San Diego,Calif可用于本发明的方法中。本发明的核酸或多肽可固定于或应用阵列上，包括毛细管阵列。阵列可用于筛选或监测组合物（例如，小分子、抗体、核酸等）文库，针对它们结合或调节本发明核酸或多肽活性的能力。毛细管阵列提供另一个捕捉(holding)和筛选样本的系统。例如样本筛选装置可包括大量毛细管形成相邻的毛细血管阵列，其中各毛细管包括至少一个壁，其限定腔用于保留样本。所述装置可进一步包括置于阵列相邻的毛细管之间的间质材料，并在间质材料内形成一种或多种参考标记。用于筛选样本的毛细管，其中所述毛细管适合于结合在毛细管阵列中，可包括第一个壁，其限定的腔用于保留样本，以及第二壁，其由过滤材料形成，过滤激发能量提供给腔以激发样品。多肽或核酸，例如配体可被引入第一组件中到至少毛细管阵列的一个毛细管的一部分中。毛细管阵列的各毛细管可包括至少一个壁，其限定的腔用于保留第一组件。气泡可被引入在第一组件后面的毛细管中。第二组件可被引入毛细管中，其中第二组件通过气泡与第一组件分开。目标样本可作为用可检测粒子标记的第一液引入毛细管阵列的毛细管中，其中毛细管阵列的各毛细管包括至少一个壁，其限定的腔用于保留第一液和可检测粒子，其中至少一个壁包被有用于使可检测粒子结合到至少一个壁的结合物质。该方法可进一步包括从毛细管中去掉第一液，其中结合的可检测粒子保留在毛细管内，并向毛细管中引入第二液。所述毛细管阵列可包括许多含有至少一个限定腔的外壁的单个毛细管。毛细管外壁可以是一个或多个融合在一起的壁。同样地，所述壁可限定的腔是圆柱形、方形、六角形或任何其它几何形状，只要壁形成保留液体或样本的腔。毛细管阵列的毛细管可以靠近在一起，形成一个平面结构。毛细管可结合在一起，通过融合（例如，其中的毛细血管是由玻璃制成）、粘合、键合或侧壁－侧壁夹紧。毛细管阵列可由任何数量的单个毛细管组成，例如，范围从100到4,000,000的毛细管。毛细管阵列可

由约 100,000 或更多单个毛细管结合在一起形成微滴度板。

[0239] 药物组合物

[0240] 本发明提供至少一种组合物，其包括 (a) 条件活性生物蛋白 ；和 (b) 合适的载体或稀释剂。本发明还提供至少一种组合物，其包括 (a) 由本文所述的核酸编码的条件活性生物蛋白 ；和 (b) 合适的载体或稀释剂。所述载体或稀释剂可任选为药用 (根据已知) 载体或稀释剂。所述组合物可任选进一步包括至少一种化合物、蛋白或组合物。

[0241] 条件活性生物蛋白可以是药用盐的形式。药用盐表示其在制药行业一般可以治疗性蛋白的盐形式使用，包括例如钠盐、钾盐、钙盐等，普鲁卡因、二苄胺、乙二胺、乙醇胺、泛影葡胺、牛磺酸的胺盐等，以及酸加成盐如盐酸盐、碱性氨基酸等。

[0242] 本发明进一步提供至少一种条件活性生物蛋白的制备方法或组合物，用于治疗有效量给药以调节或治疗至少一种在细胞、组织、器官、动物或患者中亲本分子相关疾病，和 / 或在相关的疾病之前、之后或期间的本领域已知的和 / 或描述于本文中的相关疾病。因而，本发明提供诊断或治疗细胞、组织、器官或动物中与野生型蛋白相关的疾病的方法，包括给细胞、组织、器官或动物接触或施以有效量的本发明的至少一种条件活性生物蛋白。该方法可任选进一步包括用有效量的本发明的 0.001-50mg/kg 的条件活性生物蛋白作用于细胞、组织、器官或动物。该方法可任选进一步包括采用至少选自下列模式中的一种接触或给药 ：肠外、皮下、肌肉、静脉、关节内、支气管内、腹腔内、囊内、软骨内、腔内、体腔内、小脑内、侧脑室、结肠内、子宫颈管内、胃内、肝内、心肌内、骨内、骨盆内、心包内、腹腔、胸腔内、前列腺内、肺内、直肠内、肾内、视网膜内、椎管内、滑膜腔内、胸内、胎儿宫内、膀胱内、药丸 (bolus)、阴道、直肠、口腔、舌下、鼻内或透皮吸收。该方法可任选进一步包括在条件活性生物蛋白接触或给药之前、同时或之后，给药含有有效量的至少一种选自至少一种下列可检测标记或报告因子的化合物或蛋白的至少一种组合物 ：TNT 拮抗剂、抗风湿药、肌肉松弛剂、麻醉药、非类固醇消炎药 (NSAID)、止痛药、麻醉剂、镇静剂、局部麻醉剂、神经肌肉阻断剂、抗菌剂、抗牛皮癣剂、皮质类固醇、合成代谢类固醇、促红细胞生成素、免疫接种、免疫球蛋白、免疫抑试剂、生长激素、激素替代药物、放射性药物、抗抑郁药、抗精神病药物、兴奋剂、哮喘药物、β 激动剂、吸入类固醇、肾上腺素或其类似物、细胞毒性或其它抗癌药物、抗代谢药，如甲氨蝶呤或抗增殖剂。

[0243] 本发明进一步提供至少一种条件活性生物蛋白用于诊断至少一种细胞、组织、器官、动物或患者中野生型蛋白相关疾病的方法，和 / 或之前、之后或期间相关的本领域已知的和 / 或本文中所述的疾病。

[0244] 药用载体部分由所施用的特定组合物来确定，以及通过用于给药的特定方法来确定。因此，有多种适合本发明药物组合物的试剂。可施用多种载体溶液，例如，盐缓冲液等。这些溶液是无菌的且一般不含杂质。这些组合物可通过常规的众所周知的杀菌技术进行灭菌。这些组合物可含有大约生理条件所需的药用辅料，如 pH 调节和缓冲液、毒性调节剂等，例如，醋酸钠、氯化钠、氯化钾、氯化钙、乳酸钠等。这些试剂中的条件活性生物蛋白的浓度可以有很大的不同，主要基于液体体积、粘度、体重等，按照选定的给药模式和病人的需求进行选择。

[0245] 适于口服给药的试剂可包括 ：(a) 溶液，如包被有效量的核酸悬浮于稀释剂中，如水、盐水或 PEG 400 ；(b) 胶囊、袋装或片剂，各含有预定量的活性成分，如液体、固体、颗粒

或明胶；(c) 在合适的溶液中的悬浮剂；以及 (d) 合适的乳剂。本发明的用于口服给药的药物组合物和制剂，可采用本领域众所周知的适当的和合适剂量的药用载体进行配制。此类载体能够使药物以单位剂量适合病人摄入形式进行配制，如片剂、丸剂、粉剂、锭剂、胶囊、液体、糖锭、凝胶剂、糖浆、膏剂、悬浮物等。如果需要，在加入合适的其它化合物之后，口服药物制剂可配制成固体赋形剂、任选地研磨产生的混合物和加工颗粒混合物，以得到片剂或锭剂的药芯。合适的固体赋形剂为碳水化合物或蛋白填料包括，例如，糖，包括乳糖、蔗糖、甘露醇或山梨醇；来自玉米、小麦、水稻、马铃薯或其它植物的淀粉；纤维素，如甲基纤维素、羟丙基甲基纤维素或钠羧甲基纤维素；及树胶，包括阿拉伯胶和黄蓍胶；及蛋白，例如，明胶和胶原蛋白。可加入崩解剂或增溶剂，如交联聚乙烯吡咯烷酮、琼脂、褐藻酸或其盐，如海藻酸钠。片剂形式可包括一种或多种乳糖、蔗糖、甘露醇、山梨醇、磷酸钙、玉米淀粉、马铃薯淀粉、黄蓍胶、微晶纤维素、洋槐、明胶、胶体二氧化硅、交联甲羧纤维素钠、滑石粉、硬脂酸镁、硬脂酸和其它赋形剂，着色剂、填充剂、粘合剂、稀释剂、缓冲剂、滋润剂、防腐剂、调味剂、染料、崩解剂和药用载体。

[0246] 本发明提供含有与适于制备悬浮液的赋形剂混合的条件活性生物蛋白的悬浮液。此类赋形剂包括悬浮剂，如羧甲基纤维素钠、甲基纤维素、羟丙基甲基纤维素、海藻酸钠、聚乙烯吡咯烷酮、胶黄蓍胶和胶洋槐悬浮剂，及分散或润湿剂，如自然存在的磷脂（例如，卵磷脂）、环氧烷烃 (alkylene oxide) 与脂肪酸的缩合产物（例如，聚氧乙烯硬脂酸）、环氧乙烷与长链脂肪醇的缩合产物（例如，十七碳乙烯－氧十六醇）、环氧乙烷与来自脂肪酸和己糖醇的部分酯的缩合产物（例如，聚氧乙烯山梨醇单油酸酯），或环氧乙烷与来自脂肪酸和己糖醇酐的部分酯的缩合产物（例如，聚氧乙烯失水山梨醇单油酸酯）。悬浮液也可包含一种或多种防腐剂，如乙基或正丙基对羟基苯，一种或多种着色剂，一种或多种调味剂和一种或多种甜味剂如蔗糖、阿斯巴甜或糖精。制剂可用于调节渗量。

[0247] 含片形式可包括有风味的活性成分，通常是蔗糖和阿拉伯胶或黄蓍胶，以及含有在惰性基质如明胶和甘油或蔗糖和阿拉伯胶乳液、凝胶等中的锭剂，除了活性成分之外，还含有本领域已知的载体。已知当口服给药条件活性生物蛋白时，必须使其免于消化。这一般是通过将条件活性生物蛋白与可使其抵抗酸或酶的水解的组合物混合或将条件活性生物蛋白包被于合适的抵抗载体如脂质体中而实现的。使蛋白免于消化的方法是本领域众所周知的。药物组合物可被封装，例如，在脂质体中，或在制剂中，使活性成分缓慢释放。

[0248] 包被的条件活性生物蛋白，单独或与其它合适的成分组合可制备气雾剂（例如，它们可被“雾化”），通过吸入给药。气雾剂可被放入加压的可接受的推进剂中，如二氯二氟甲烷、丙烷、氮等。适合的直肠给药试剂包括，例如，由栓剂基体包被的核酸组成的栓剂。适合的栓剂基体包括天然或人工合成的甘油三酸脂或石蜡烃。此外，也有可能使用由基质包被的核酸的组合组成的明胶直肠胶囊，所述基质包括，例如液体甘油三酸脂、聚乙二醇和石蜡烃。

[0249] 本发明的皮肤或局部给药组合物可包括，除了条件活性生物蛋白之外，在乳膏、软膏、溶液或水凝胶制剂中的药用载体和其它化合物，只要添加的成分不影响治疗性蛋白的给药。也可加入常规的药用乳化剂、表面活性剂、助悬剂、抗氧化剂、渗透促进剂、填料、稀释剂和防腐剂。水溶性聚合物也可用作载体。

[0250] 适合肠外给药的制剂，例如，通过关节内（关节中）、静脉、肌肉、皮内、腹膜内，及

皮下途径，包括水性和非水性的等渗无菌注射液，它可包含抗氧化剂、缓冲液、硫双二氯酚和使制剂与接受的血液等渗的溶质，以及水性和非水性的无菌悬浮液，其可包括悬剂、增溶剂、增稠剂、稳定剂和防腐剂。在本发明的操作中，组合物可通过，例如静脉输液、口服、外用、腹膜内、膀胱内或鞘内给药。在一个方面，肠外给药模式是优选的含有条件活性生物蛋白的组合物给药方法。所述组合物可方便地以单位剂量形式给药并可通过制药领域任何众所周知的方法来制备，例如，描述于“Remington 药物科学 (Remington's Pharmaceutical Sciences)”，Mack Publishing Co. Easton Pa.，18th Ed.，1990。静脉给药制剂可含有药用载体如无菌水或盐水，聚烷撑二醇如聚乙二醇、植物油、氢化萘等。还可参见和修改美国专利号 4318905 中的描述。

[0251] 含有条件活性生物蛋白的包被的组合物制剂可存在于单剂量或多剂量密封容器中，如安瓶和小瓶。注射液和悬浮液可由前面描述的无菌粉末、颗粒和片剂制备得到。

[0252] 本发明还提供至少一种条件活性生物蛋白组合物、装置和 / 或根据本发明用于诊断至少一种野生型蛋白相关疾病的给药方法。

[0253] 本发明还提供含有至少一种条件活性生物蛋白和至少一种药用载体或稀释剂的组合物。所述组合物可任选进一步包括有效量的至少一种选自至少一种可检测标记或报告因子的化合物或蛋白，细胞毒性或其它抗癌药物，抗代谢药如甲氨蝶呤，抗增殖剂，细胞因子或细胞因子拮抗剂，TNF 拮抗剂，抗风湿药，肌肉松弛剂，麻醉剂，非类固醇消炎药 (NSAID)，镇痛药，麻醉剂，镇静剂，局部麻醉剂，神经肌肉阻滞剂，抗菌剂，抗牛皮癣剂，皮质类固醇，合成代谢类固醇，促红细胞生成素，免疫接种，免疫球蛋白，免疫抑试剂，生长激素，激素替代药物，放射性药物，抗抑郁药，抗精神病药物，兴奋剂，哮喘药物，β - 受体激动剂，吸入类固醇，肾上腺素或类似物。

[0254] 本发明还提供含有至少一种本发明的条件活性生物蛋白的医疗装置，其中所述装置适于接触或给药至少一种条件活性生物蛋白，通过至少一种选自下组的模式 :肠外、皮下、肌肉、静脉、关节内、支气管内、腹腔内、囊内、软骨内、腔内、体腔内、小脑内、脑室内、结肠内、子宫颈管内、胃内、肝内、心肌内、骨内、骨盆内、心包内、腹膜内、胸膜内、前列腺内、肺内、直肠内、肾内、视网膜内、椎管内、滑膜内、胸内、胎儿宫内、膀胱内、药丸、阴道、直肠、口腔、舌下、鼻内或透皮吸收。

[0255] 在另一个方面，本发明提供一种试剂盒，其含有在第一容器中的至少一种本发明的条件活性生物蛋白或其片段的冻干形式，以及任选的第二容器，其含有无菌水、无菌缓冲液或至少一种选自下组的防腐剂 :苯酚、间甲酚、对甲酚、邻甲酚、氯甲酚、苯甲醇、苯基亚硝酸盐、苯氧乙醇、甲醛、三氯叔丁醇、氯化镁、烷基尼泊金酯 (alkylparaben)、苯扎氯铵、苯乙铵氯、钠脱氢和硫柳汞或它们在稀释剂水溶液中的混合物。在一个方面，试剂盒中第一容器中的条件活性生物蛋白或特定部分或变体的浓度与第二容器的成分重组 (reconstituted) 为浓度约 0.1mg/ml 至约 500mg/ml。在另一个方面，第二容器进一步包括等渗剂。在另一个方面，第二容器进一步包括生理可接受的缓冲液。在一个方面，本发明提供治疗至少一种野生型蛋白介导的疾病的方法，包括对有需求的患者施以试剂盒中的制剂，在给药前重配制。

[0256] 本发明还提供用于人类药用或诊断用的制造商的说明，包括包被材料和含有溶液或至少一种本发明的条件活性生物蛋白的冻干形式的容器。制造商的说明可任选包括具有容器作为以下给药途径的装置或系统的组件，所述给药途径为 :肠外、皮下、肌肉、静脉、关

节内、支气管内、腹腔内、囊内、软骨内、腔内、体腔内、小脑内、脑室内、结肠内、子宫颈管内、胃内、肝内、心肌内、骨内、骨盆内、心包内、腹膜内、胸膜内、前列腺内、肺内、直肠内、肾内、视网膜内、椎管内、滑膜内、胸内、胎儿宫内、膀胱内、药丸、阴道、直肠、口腔、舌下、鼻内或透皮吸收。

[0257] 本发明进一步提供任何以下公开的内容。

[0258] 实施例 1 :用于温度突变的多壁检测 (例如,96- 孔检测) 概述

[0259] 荧光底物被添加到多壁板的每一个孔中,在野生型或新的较低反应温度 (例如,上述的 37℃或 25℃) 下保持适当的时间。通过在荧光酶标仪中在适合的激发和发射光谱 (例如,320nm 激发 /405nm 发射) 下测定荧光来检测荧光。测定相对荧光单位 (RFU)。从野生型分子和质粒 / 载体转化的细胞中获得的上清液作为阳性和阴性对照。对每个样品,各反应温度下,以及阳性和阴性对照进行重复反应。

[0260] 在较低的温度下有活性 (例如,突变体在 25℃有活性),且在野生型温度下活性下降 (例如,在 37℃下活性下降 10％、20％、30％、40％或更多),因而活性比高于或等于约 1.1 或以上 (例如,在 25℃或 37℃下的活性比 (25℃ /37℃) 高于或等于 1.1 或以上) 的突变体,可被视为推测的主要温度敏感型苗头突变体 (hit)。然后采用相同的检测方法,重新筛选这些推测的主要温度敏感型苗头突变体,以验证任何主要的苗头突变体 (primary hit)。

[0261] 实施例 2 :用于确定温度突变体活性的不同的检测方法 (例如,14-mL 检测) 概述

[0262] 将确定的主要温度敏感型苗头的突变体在 14ml 培养试管中表达,并在野生型 (例如,37℃) 和较低的温度 (例如,25℃) 下测定它们的酶活性。按照上述多壁法表达蛋白并纯化,除了表达是在不同的形式 (14ml 试管) 中进行,而不是多壁 (96- 孔板) 形式。

[0263] 各突变体的上清液被转移至多壁板中,例如 96- 孔板。在指定的反应温度 (野生型,较低温度) 下向各管中加入荧光底物,保持所需的一段时间。野生型分子用作阳性对照,来自细胞的仅用载体转化的上清液用作阴性对照。通过在荧光酶标仪中在适合的激发和发射光谱 (例如,320nm 激发 /405nm 发射) 下测定荧光来检测荧光。测定相对荧光单位 (RFU)。对每个样品,各反应温度下,以及阳性和阴性对照进行重复反应。

[0264] 在较低温度 (例如,25℃) 下有活性,但在野生型 (例如,37℃) 下显示出活性下降至少 30％或以上,因而在较低温度 (例如,25℃) 下与在野生型温度 (例如,37℃) 下的活性比等于或高于 1.5 的突变体,被确定为温度敏感型苗头突变体。

[0265] 将突变体在较低温度 (例如,25℃) 下的活性与野生型分子在野生型温度 (例如,37℃) 下的活性进行比较。如果分子较野生型分子在较低温度 (例如,25℃) 下的活性高,其表示为剩余活性＞ 1,优选为 2 或高于 2,如果突变体在野生型温度 (37℃) 下较野生型分子显示出总体的活性下降,可确定突变体的表型为温度敏感的突变体。

[0266] 实施例 3 :所发现的苗头突变体的进一步演变概述

[0267] 如果需要,由前面确定的所有或筛选的突变型产生新的组合变体库。新的文库可被设计成含有各筛选突变体的每个可能的氨基酸变体的组合,并如前述重筛得到新的苗头突变体。

[0268] 实施例 4 :温度下降后酶活性的可逆性概述

[0269] 通过将突变体暴露于高温下然后返回到较低温度 (例如,25℃) 下,可进一步检测

温度敏感的演变的突变体，以确定是否酶活性在较低温度（例如，25℃）下是可逆的或不可逆的。温度敏感的突变体表达于任何需要的形式中，例如前面所述的 14ml 培养试管中。检测突变体在几种条件下的活性，包括野生型温度（例如，37℃）以及其它温度，随后再暴露于必须的低温（例如，25℃）。在较低温度下有活性的突变体，当温度上升或升至野生型温度时活性下降（即，在较低和较高温度下的活性比相同或高于 1、1.5 或 2 或更高），当再次降至较低温度时显示出基线 (baseline) 活性，被评分为“可逆型”。在较低温度下有活性的突变体，当温度上升或升高至野生型温度时显示出活性下降（即，在较低和较高温度下的活性比相同或高于 1、1.5 或 2 或更高），当温度再次下降至较低温度时显示出至少等量下降的活性，被评分为“不可逆型”。

[0270] 实施例 5：筛选条件活性血管抑素变体的材料和方法

[0271] 筛选条件活性血管抑素变体的材料和方法可适用 Chi and Pizzo, “在胞外低 pH 值下血管抑素对肿瘤细胞的直接细胞毒性：依赖于细胞表面相关的 ATP 合酶的机制 (Angiosatin is directly cytotoxic to tumor cells at low extracellular pH:a mechanism dependent on cell surface-associated ATP synthase)”, Cancer Res. 2006；66(2):875-882，通过参考并入于此。

[0272] 材料。来自于人纤溶酶原的野生型血管抑素因子 1-3，可从 Calbiochem(Darmstadt, 德国）获得并在无菌 PBS 中重新配制。直接作用于有催化活性的 ATP 合酶 β 亚单位的多克隆抗体可以制备得到，牛 F1ATP 合酶亚单位可如前述方法进行纯化 (Moser 等人，“血管抑素结合人内皮细胞表面的 ATP 合酶 (Angiostatin binds ATP synthase on the surface of human endothelial cells)”, Proc Natl Acad Sci U S A 1999；96:2811-6；Moser 等人，“内皮细胞表面的 Fl-FO ATP 合酶在 ATP 合成中是有活性的并受到血管抑素的抑制 (Endothelial cell surface Fl-FO ATP synthase is active in ATP synthesis and is inhibited by angiostatin)”, Proc Natl Acad Sci U S A；2001；98:6656-ól)。卡立泊来德 (Cariporide) 可溶解于无菌水中并进行无菌过滤。

[0273] 细胞培养。A549（来自肺癌组织的人上皮细胞株）或其它癌细胞株 (DU145、LNCaP 或 PC-3 细胞）可以从例如，ATCC 获得。人脐静脉内皮细胞 (HUVEC) 可以从人脐静脉中分离得到，如所述 (Grant 等人，“在分化中的内皮细胞以基质胶诱导胸腺素 h4 基因 (Matrigel induces thymosin h 4gene in differentiating endothelial cells)”, J Cell Sci 1995；108:3685-94)。HUVEC 细胞可用来作为在细胞表面表达 ATP 合酶的细胞系的阳性对照。细胞可培养于含有 1％青霉素链霉素和 10％血清替代培养基 3(Sigma, St. Louis, MO) 的 DMEM(Life Technologies, Carlsbad, CA) 中，以尽量减少纤溶酶原的存在。低 pH(6.7) 培养基可通过在 5％ CO_2条件下减少碳酸氢钠至 10mmol/L，并补充 34mmol/L NaCl 以保持渗透压，或在 17％ CO_2条件下孵育 22mmol/L 碳酸氢钠培养基来制备。使用的低 pH 的方法可根据试验限制和检测而不同。

[0274] 流式细胞实验。为了保证 ATP 合酶在肿瘤细胞株的细胞表面发挥功能，可采用流式细胞实验。例如，A549 细胞株可培养于不同 pH 培养基 (10、22 和 44mmol/L 碳酸氢钠的 DMEM) 中，在缺氧 (0.5％ O_2、5％ CO_2，N_2平衡）与常氧 (21％ O_2、5％ CO_2) 的条件下培养 1、12、24、48 和 72 小时。活细胞可以被阻滞，与抗 β－亚单位抗体孵育，洗涤、阻滞，与羊抗兔 FITC 标记的二抗 (Southern Biotech, Birmingham, AL) 孵育，再次洗涤，所有步骤于 4℃下

进行。碘化丙啶(BD Biosciences,San Jose,CA)可包含于所有样本中以区别具有受损细胞膜的细胞。10,000个细胞FITC的平均荧光强度可通过FACSCCalibur流式细胞仪(Becton Dickinson,Franklin Lakes,NJ)进行定量,摄入碘化丙啶的细胞可以被排除,以消除线粒体ATP合酶在CellQuest软件(BD Biosciences)中的检测。

[0275] 细胞表面ATP生成检测。96-孔板中的A549或1-LN细胞(每孔60,000个细胞)可加入新鲜的培养基并用血管抑素、血管抑素变体、抗β-亚单位抗体、兔抗牛血清白蛋白IgG(Organon Teknika,West Chester,PA)、苊类化合物(piceatannol)(已知的ATP合酶F1的抑试剂作为阳性对照,Sigma)处理,或单独将培养基置于37℃,5% CO_2中30分钟。然后细胞与0.05mmol/L ADP孵育20秒。移走上清液,采用如(23)描述的CellTiterGlo发光检测(Promega,Madison,WI)检测产生的ATP。细胞裂解液可进行类似的分析以确定细胞内的ATP池在任何条件下无差异。可通过Luminoskan Ascent(Thermo Labsystems,Helsinki,Finland)进行记录。数据是基于各独立试验的标准测定,以每个细胞ATP的摩尔数来表示。

[0276] 细胞增殖检测。血管抑素对癌细胞株的影响可用3-(4,5-二甲基噻唑-2-基)-5-(3-羧基甲氧基苯基)-2-(4-磺苯基)-2H-四唑盐、内盐(inner salt)(MTS)在无血清培养基中的增殖检测来评价。在37℃,5% CO_2中孵育20小时后,在存在或不存在血管抑素情况下,96-孔板的各孔中的相对细胞数可按照制造商的方案(protocol)采用Aqueous单细胞增殖检测(Promega)来测定。培养基的pH值可在5% CO_2条件下通过改变碳酸氢盐浓度来调节。

[0277] 细胞的细胞毒性评估。为了量化细胞死亡和细胞裂解,从细胞质释放到上清液中的乳酸脱氢酶(LDH)的活性,可用细胞毒性检测试剂盒(Roche,Indianapolis,IN)进行测定。用血管抑素、血管抑素变体,抗β-亚单位抗体、兔IgG、卡立泊来德(cariporide)和Triton X(用于透化细胞的去污剂)处理的癌细胞(例如A549细胞)(每孔5,000个细胞)可分别在37℃,5% CO_2或17% CO_2,中性和低pH条件下孵育15小时。细胞毒性指数可用四份处理样本的平均吸收度除以对应于相同pH培养基的四份未处理样本的平均吸收度计算得到。细胞坏死和凋亡的评价。为了确定血管抑素诱导的细胞坏死模式,可进行组蛋白-DNA ELISA。血管抑素、血管抑素变体、抗β-亚单位抗体、兔IgG和卡立泊来德(cariporide)对A549细胞的影响(每孔5,000个细胞)可采用ELISA细胞凋亡和坏死检测(Roche)来确定,取决于核外组蛋白-DNA片段的检测。细胞凋亡或坏死可分别由在含或不含试剂的条件下,在37℃孵育15小时之后四份样本的细胞裂解液或上清液来确定。细胞凋亡或坏死指数可通过四份处理样本的平均吸收度除以对应于相同pH值培养基的四份未处理样本的平均吸收度来计算得到。培养基的pH值可由在5% CO_2或17% CO_2条件下进行孵育来调节。

[0278] 细胞内pH值(pHi)测定。pHi可根据带有盖玻片的35mm微孔盘(MatTek,Ashland,MA)中细胞的荧光来测定。细胞可被置于细胞生长因子减少的、不含酚红的Matrigel(BD Biosciences)中。过夜生长后,更换培养基,并向细胞中加入pH敏感的荧光染料cSNARF(Molecular Probes,Eugene,OR),15分钟后置于新鲜的培养基中20分钟恢复。然后细胞被放入37℃,5% CO_2的显微镜平台(stage)上1小时,收集发射光谱,从各含7个细胞和15个细胞之间的区域中计算得到pHi(Wahl ML,Grant DS.“胞外pH值的微环境和胞外基质蛋白对血管抑素活性和胞内pH值的影响(Effects of microenvironmental

extracellular pH and extracellular matrix proteins on angiostatin's activity and on intracellular pH)",Gen Pharmacol 2002;35:277-85)。在开始收集光谱时,从盘中去掉培养基,将细胞置于 1mL 含或不含 pH 抑试剂血管抑素、抗 β-亚单位、兔 IgG 或卡立泊来德 (cariporide)--一种钠氢交换抑制剂鲜培养基中。培养基的 pH 值可在如上所述固定的 5% CO_2条件下通过改变碳酸氢盐浓度来调节。