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 conditionally active biologic proteins.

[0005] 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

[0006] 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.

[0006a] In one aspect, there is provided a method of preparing a conditionally active biologic protein, wherein the conditionally active biologic protein is a protein which exhibits a lower activity in an assay under a normal physiological condition within a normal range at a site of administration of the conditionally active biologic protein, or at a tissue or organ at a site of action of the conditionally active biologic protein in a subject, compared to the activity of the conditionally active biologic protein in an assay under an aberrant condition that deviates from the normal range at the site of administration of the conditionally active biologic protein, or at the tissue or organ at the site of action of the conditionally active biologic protein in the subject, the method comprising a step of:

selecting the conditionally active biologic protein from a library of wild-type proteins wherein the conditionally active biologic protein exhibits a lower activity in the assay under the normal physiological condition compared to the activity of the conditionally active biologic protein in the assay under the aberrant condition,

wherein the assay under the normal physiological condition and the assay under the aberrant condition are performed in assay solutions containing at least one component at substantially the same concentration and capable of binding to both the mutant protein and a binding partner of the mutant protein, said at least one component being selected from:

(a) an inorganic compound selected from boric acid, calcium chloride, calcium nitrate, di-ammonium phosphate, magnesium sulfate, mono-ammonium phosphate, mono-potassium phosphate, potassium sulfate, copper sulfate, iron sulfate, manganese sulfate, zinc sulfate, calcium chelate, copper chelate, iron chelate, manganese chelate, zinc chelate, ammonium molybdate, ammonium sulphate, calcium carbonate, magnesium phosphate, potassium bicarbonate, potassium nitrate, hydrochloric acid, carbon dioxide, sulfuric acid, phosphoric acid, carbonic acid, uric acid, hydrogen chloride, and urea;

(b) an ion selected from ammonium ion, an iron ion, a zinc ion, a phosphorus ion, a sulfuric ion, a copper ion, magnesium ion, sulfate ion, bisulfate ion, carbonate ion, bicarbonate ion, nitrate ion, nitrite ion, phosphate ion, hydrogen phosphate ion, dihydrogen phosphate ion, persulfate ion, monopersulfate ion, and borate ion; and

(c) an organic molecule selected from an amino acid, a sugar and an organic acid.

[0006b] In another aspect, there is provided a method of preparing a conditionally active biologic protein, wherein the conditionally active biologic protein is a protein which exhibits a decrease in activity in an assay at a normal physiological condition within a normal range at a site of administration of the conditionally active biologic protein, or at a tissue or organ at a site of action of the conditionally active biologic protein in a subject compared to the same activity in an assay under an aberrant condition that deviates from the normal range at the site of administration of the conditionally active biologic protein, or at the tissue or organ at the site of action of the conditionally active biologic protein in the subject, the method comprising steps of:

i. selecting a parent polypeptide;

ii. evolving a DNA which encodes the parent polypeptide using one or more evolutionary techniques to create mutant DNAs;

iii. expressing the mutant DNAs to obtain mutant proteins at least one of which exhibits a decrease in the activity in the assay at the normal physiological condition compared to in the same activity in the assay under the aberrant condition; and

iv. selecting the conditionally active biologic protein from the mutant proteins that exhibits a decrease in the activity in the assay at the normal physiological condition compared to the same activity in the assay under the aberrant condition,

wherein the assay under the normal physiological condition and the assay under the aberrant condition are performed in assay solutions containing at least one component at substantially the same concentration and capable of binding to both the mutant protein and a binding partner of the mutant protein, said at least one component being selected from:

(a) an inorganic compound selected from boric acid, calcium chloride, calcium nitrate, di-ammonium phosphate, magnesium sulfate, mono-ammonium phosphate, mono-potassium phosphate, potassium sulfate, copper sulfate, iron sulfate, manganese sulfate, zinc sulfate, calcium chelate, copper chelate, iron chelate, manganese chelate, zinc chelate, ammonium molybdate, ammonium sulphate, calcium carbonate, magnesium phosphate, potassium bicarbonate, potassium nitrate, hydrochloric acid, carbon dioxide, sulfuric acid, phosphoric acid, carbonic acid, uric acid, hydrogen chloride, urea,

(b) ion selected from ammonium ion, an iron ion, a zinc ion, a phosphorus ion, a sulfuric ion, a copper ion, magnesium ion, sulfate ion, bisulfate ion, carbonate ion, bicarbonate ion, nitrate ion, nitrite ion, phosphate ion, hydrogen phosphate ion, dihydrogen phosphate ion, persulfate ion, monopersulfate ion, borate ion, and

(c) an organic molecule selected from an amino acid, a sugar and an organic acid.

[0006c] Further disclosed is a method of preparing a conditionally active biologic protein, wherein the conditionally active biologic protein is a protein which exhibits both: (a) a decrease in activity in an assay at a normal physiological condition compared to a wild-type biologic protein, and (b) an increase in activity in the assay under an aberrant condition compared to the wild-type biologic protein, the method comprising steps of:

i. selecting a fragment of a wild-type protein, a mutant protein or a fragment of a mutant protein with a desired property;

ii. evolving a DNA which encodes the protein or protein fragment selected in step (i) using one or more evolutionary techniques to create mutant DNAs;

iii. expressing the mutant DNAs to obtain mutant proteins at least one of which exhibits both (a) a decrease in activity in the assay at the normal physiological condition compared to the reference protein, and (b) an increase in activity in the assay under the aberrant condition compared to the reference protein; and

iv. selecting a mutant protein exhibits both (a) a decrease in activity in the assay at the normal physiological condition compared to the reference protein, and (b) an increase in activity in the assay under the aberrant condition compared to the reference protein.

[0006d] In various embodiments, the normal physiological condition is selected from one or more of temperature, pH, osmotic pressure, osmolality, oxidative stress and electrolyte concentration. In a particular embodiment, 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 embodiments, the conditionally active biologic protein is reversibly or irreversibly inactivated at the wild type normal physiological conditions. In one specific embodiment, 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 embodiments, 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 (VTP), vasopressin, and angiostatin.

WHAT IS CLAIMED IS:

1. A method of preparing a conditionally active biologic protein, wherein the conditionally active biologic protein is a protein which exhibits a lower activity in an assay under a normal physiological condition within a normal range at a site of administration of the conditionally active biologic protein, or at a tissue or organ at a site of action of the conditionally active biologic protein in a subject, compared to the activity of the conditionally active biologic protein in an assay under an aberrant condition that deviates from the normal range at the site of administration of the conditionally active biologic protein, or at the tissue or organ at the site of action of the conditionally active biologic protein in the subject, the method comprising a step of:

selecting the conditionally active biologic protein from a library of wild-type proteins wherein the conditionally active biologic protein exhibits a lower activity in the assay under the normal physiological condition compared to the activity of the conditionally active biologic protein in the assay under the aberrant condition,

wherein the assay under the normal physiological condition and the assay under the aberrant condition are performed in assay solutions containing at least one component at substantially the same concentration and capable of binding to both the mutant protein and a binding partner of the mutant protein, said at least one component being selected from:

(a) an inorganic compound selected from boric acid, calcium chloride, calcium nitrate, di-ammonium phosphate, magnesium sulfate, mono-ammonium phosphate, mono-potassium phosphate, potassium sulfate, copper sulfate, iron sulfate, manganese sulfate, zinc sulfate, calcium chelate, copper chelate, iron chelate, manganese chelate, zinc chelate, ammonium molybdate, ammonium sulphate, calcium carbonate, magnesium phosphate, potassium bicarbonate, potassium nitrate, hydrochloric acid, carbon dioxide, sulfuric acid, phosphoric acid, carbonic acid, uric acid, hydrogen chloride, and urea;

(b) an ion selected from ammonium ion, an iron ion, a zinc ion, a phosphorus ion, a sulfuric ion, a copper ion, magnesium ion, sulfate ion, bisulfate ion, carbonate ion, bicarbonate ion, nitrate ion, nitrite ion, phosphate ion, hydrogen phosphate ion, dihydrogen phosphate ion, persulfate ion, monopersulfate ion, and borate ion; and

(c) an organic molecule selected from an amino acid, a sugar and an organic acid.

2. The method of claim 1, wherein the selecting step comprises selecting the conditionally active biologic protein which exhibits both: (a) a decrease in the activity in the assay under the normal physiological condition compared to a reference protein, and (b) an increase in the activity in the assay under the aberrant condition compared to the reference protein, wherein the reference protein

has substantially the same activity in both the assay under the normal physiological condition and the assay under the aberrant condition.

3. The method of any one of claims 1-2, wherein the wild-type proteins are wild-type antibodies.

4. The method of any one of claims 1-3, wherein the wild-type protein library is a collection of proteins encoded by cDNAs.

5. The method of any one of claims 1-4, wherein the wild-type protein library is a bacteriophage display library.

6. The method of any one of claims 1-5, wherein the wild-type protein library is a collection of recombinant proteins.

7. The method of any one of claims 1-6, wherein the selected conditionally active biologic protein is subjected to steps of:

i. evolving a DNA which encodes the selected conditionally active biologic protein using one or more evolutionary techniques to create mutant DNAs;

ii. expressing the mutant DNAs to obtain mutant proteins; and

iii. selecting an improved conditionally active biologic protein from the mutant proteins which exhibits both (a) a decrease in activity in the assay under the normal physiological condition compared to the selected conditionally active biologic protein, and (b) an increase in activity in the assay under the aberrant condition compared to the selected conditionally active biologic protein.

8. The method of claim 7, wherein the improved conditionally active biologic protein has an increased ratio of the activity in the assay under the aberrant condition to the activity under the normal physiological condition, as compared to the selected conditionally active biologic protein employed in step (i).

9. The method of claim 7, wherein the improved conditionally active biologic protein has one or more of an improved binding affinity, expression and humanization, as compared to the selected conditionally active biologic protein employed in step (i).

10. A method of preparing a conditionally active biologic protein, wherein the conditionally active biologic protein is a protein which exhibits a decrease in activity in an assay at a normal physiological condition within a normal range at a site of administration of the conditionally active biologic protein, or at a tissue or organ at a site of action of the conditionally active biologic protein in a subject compared to the same activity in an assay under an aberrant condition that deviates from the normal range at the site of administration of the conditionally active biologic protein, or at the tissue or organ at the site of action of the conditionally active biologic protein in the subject, the method comprising steps of:

i. selecting a parent polypeptide;

ii. evolving a DNA which encodes the parent polypeptide using one or more evolutionary techniques to create mutant DNAs;

iii. expressing the mutant DNAs to obtain mutant proteins at least one of which exhibits a decrease in the activity in the assay at the normal physiological condition compared to in the same activity in the assay under the aberrant condition; and

iv. selecting the conditionally active biologic protein from the mutant proteins that exhibits a decrease in activity in the assay at the normal physiological condition compared to the same activity in the assay under the aberrant condition,

wherein the assay under the normal physiological condition and the assay under the aberrant condition are performed in assay solutions containing at least one component at substantially the same concentration and capable of binding to both the mutant protein and a binding partner of the mutant protein, said at least one component being selected from:

(a) an inorganic compound selected from boric acid, calcium chloride, calcium nitrate, di-ammonium phosphate, magnesium sulfate, mono-ammonium phosphate, mono-potassium phosphate, potassium sulfate, copper sulfate, iron sulfate, manganese sulfate, zinc sulfate, calcium chelate, copper chelate, iron chelate, manganese chelate, zinc chelate, ammonium molybdate, ammonium sulphate, calcium carbonate, magnesium phosphate, potassium bicarbonate, potassium nitrate, hydrochloric acid, carbon dioxide, sulfuric acid, phosphoric acid, carbonic acid, uric acid, hydrogen chloride, urea,

(b) an ion selected from ammonium ion, an iron ion, a zinc ion, a phosphorus ion, a sulfuric ion, a copper ion, magnesium ion, sulfate ion, bisulfate ion, carbonate ion, bicarbonate ion, nitrate ion, nitrite ion, phosphate ion, hydrogen phosphate ion, dihydrogen phosphate ion, persulfate ion, monopersulfate ion, borate ion, and

(c) an organic molecule selected from an amino acid, a sugar and an organic acid.

11. The method of claim 10, wherein the at least one component is a component present in a bodily fluid of a mammal at a normal physiologic concentration.

12. The method of claim 11, wherein the at least one component has a concentration in the assay solutions in a range of from 5 % to 500 % of the normal physiologic concentration in the bodily fluid, or from 10 % to 400 % of the normal physiologic concentration in the bodily fluid, or from 30 % to 300 % of the normal physiologic concentration in the bodily fluid, or from 50 % to 200 % of the normal physiologic concentration in the bodily fluid, or from 75 % to 150 % of the normal physiologic concentration in the bodily fluid.

13. The method of claim 10, where the at least one component is selected from one or more of uric acid in concentration range of 2–7.0 mg/dL, calcium ion in a concentration range of 8.2–11.6 mg/dL, chloride ion in a concentration range of 355–381 mg/dL, iron ion in a concentration range of 0.028–0.210 mg/dL, potassium ion in a concentration range of 12.1–25.4 mg/dL, sodium ion in a concentration range of 300–330 mg/dL and carbonic acid in a concentration range of 15–30 mM.

14. The method of claim 10, wherein the at least one component is the amino acid selected from Histidine, Alanine, Isoleucine, Arginine, Leucine, Asparagine, Lysine, Aspartic acid, Methionine, Cysteine, Phenylalanine, Glutamic acid, Threonine, Glutamine, Tryptophan, Glycine, Valine, Pyrrolysine, Proline, Selenocysteine, Serine, and Tyrosine.

15. The method of claim 10, wherein the at least one component is the organic acid selected from citric acid, α-ketoglutaric acid, succinic acid, malic acid, fumaric acid, acetoacetic acid, β-hydroxybutyric acid, lactic acid, pyruvic acid, α-ketonic acid, acetic acid and volatile fatty acids.

16. The method of claim 10, wherein the at least one component is the sugar selected from glucose, pentose, hexose, xylose, ribose, mannose, galactose, lactose, GlcNAcβ1-3Gal, Galα1-4Gal, Manα1-2Man, GalNAcβ1-3Gal and O-, N-, C-, and S-glycosides.

17. The method of claim 10, wherein the at least one component is an ion having a concentration in the range of from 5 % to 500 % of the normal physiologic concentration, or from 10 % to 400 % of the normal physiologic concentration, or from 30 % to 300 % of the normal physiologic concentration, or from 50 % to 200 % of the normal physiologic concentration, or from 75 % to 150 % of the normal physiologic concentration.

18. The method of claim 10, wherein the aberrant condition is a pH that is different from a normal physiological pH.

19. The method of claim 18, wherein the at least one component is an ion having a pKa that is within 1 pH unit from the pH of the aberrant condition, or 0.8 pH unit from the pH of the aberrant condition, or 0.6 pH unit from the pH of the aberrant condition, or 0.5 pH unit from the pH of the aberrant condition, or 0.4 pH unit from the pH of the aberrant condition, or 0.3 pH unit from the pH of the aberrant condition, or 0.2 pH unit from the pH of the aberrant condition.