NOVEL METHODS OF PROTEIN EVOLUTION

FIELD OF THE INVENTION

[0001] In a particular aspect, the present invention is relevant to proteins and to their generation by protein evolution.

BACKGROUND

[0002] Protein engineering via site-directed mutagenesis and, more recently, molecular evolution has been successfully employed to improve enzymatic properties in industrial applications and therapeutic properties in antibodies. Characteristics such as thermostability, pH optimum, enantioselectivity, specificity and binding affinity have all been altered to better adapt proteins and antibodies for specific purposes.

[0003] Since its inception, many different methods for molecular evolution have been described and applied to improve characteristics of the target protein. For example, sets of single point mutants can be generated and screened for upmutants. Beneficial single amino acid substitutions can then be recombined and screened to further optimize the desired characteristics in the target molecule.

[0004] However, the successful evolution of a target molecule starting with single point mutations requires that the (sometimes) subtle changes in performance can be accurately measured to identify the upmutants. In cases where a sensitive assay does not exist, single point mutations cannot be successfully screened. Simultaneous mutations of several sites can be done, however the number of combinations created, increases very quickly and reaches the limits of cloning efficiency and screening capability.

SUMMARY

[0005] The present disclosure relates to comprehensive methods of identifying and mapping mutant polypeptides formed from, or based upon, a template polypeptide. Typically, the polypeptide will comprise n amino acid residues, wherein the method comprises (a) generating n separate sets of polypeptides, each set comprising member polypeptides having X number of different predetermined amino acid residues at a single predetermined position of the polypeptide; wherein each set of polypeptides differs in the single predetermined position as confirmed by sequencing or other technique; assaying each set for at least one and preferably

two predetermined properties, characteristics or activities; (b) for each member identifying any change in said property, characteristic or activity relative to the template polypeptide; and (c) creating a functional map reflecting such changes. The number of different member polypeptides generated is equivalent to n x X

[0006] In the alternative, the method comprises generating a single population comprising the sets of mutated polypeptides. In this embodiment, the entire population is sequenced, tested for expression and screened for a function, the individual members identified, and, preferably, the functional map generated.

[0007] Typically, where each naturally occurring amino acid is used, X will be 19 (representing the 20 naturally occurring amino acid residues and excluding the particular residue present in a given position of the template polypeptide). However, any subset of amino acids may be used throughout, and each set of polypeptides may be substituted with all or a subset of the total X used for the entire population.

[0008] Any mutational or synthetic means may be used to generate the set of mutants. In one embodiment, the generation of polypeptides comprises (i) subjecting a codon-containing polynucleotide encoding for the template polypeptide to polymerase-based amplification using a 63-fold degenerate oligonucleotide for each codon to be mutagenized, wherein each of the 63-fold degenerate oligonucleotides is comprised of a first homologous sequence and a degenerate N,N,N triplet sequence, so as to generate a set of progeny polynucleotides; and (ii) subjecting the set of progeny polynucleotides to clonal amplification such that polypeptides encoded by the progeny polynucleotides are cloned, sequenced, expressed and screened.

[0009] In one embodiment, the entire polypeptide is subjected to comprehensive mutagenesis. In another embodiment, one or more regions are selected for comprehensive mutagenesis. In such case, n represents a subset or region of the template polypeptide. For example, where the polypeptide is an antibody, the entire antibody or one or more complementarity determining regions (CDRs) of the antibody are subjected to comprehensive mutagenesis.

[0010] The disclosure thus includes methods of mapping a set of mutant antibodies formed from a template antibody having at least one, and preferably six, complementarity determining regions (CDRs), the CDRs together comprising n amino acid residues, the method comprising (a) generating n separate sets of antibodies, each set comprising member antibodies having X

number of different predetermined amino acid residues at a single predetermined position of the CDR; wherein each set of antibodies differs in the single predetermined position; and the number of different member antibodies generated is equivalent to n x X; (b) confirming by sequencing or other method that each member antibody has been made; (c) expressing each member antibody; (d) assaying each set for at least one predetermined property, characteristic or activity; (e) for each member identifying any change in a property, characteristic or activity relative to the template polypeptide; and (f) creating a structural positional map of such changes. For antibodies, the predetermined property, characteristic or property may be binding affinity and/or immunogenicity. As set forth above, in the alternative a single population comprising all sets of mutated antibodies may be generated.

[0011] In addition, provided are methods of producing a set of mutant antibodies formed from a template antibody having at least one complementarity determining region (CDR), the CDR comprising n amino acid residues, the method comprising: (a) generating n separate sets of antibodies, each set comprising member antibodies having X number of different predetermined amino acid residues at a single predetermined position of the CDR; wherein each set of antibodies differs in the single predetermined position; and the number of different member antibodies generated is equivalent to n x X. In another embodiment, antibody comprises six CDRs, and together the CDRs comprise n amino acid residues.

[0011a] In a first aspect, there is provided a method of mapping mutant polypeptides formed from a template polypeptide wherein n is the number of amino acid residues in the template polypeptide, the method comprising steps of:

a. generating n-1 separate sets of mutant polypeptides, each set comprising mutant polypeptides having X number of different predetermined amino acid residues at a single predetermined position of the template polypeptide; wherein each set of mutant polypeptides differs in the single predetermined position; and the number of different mutant polypeptides generated is equivalent to [n-1] x X, wherein X is 19 and represents the 19 naturally occurring amino acid residues not present in a predetermined position of the template polypeptide;
b. assaying each set of mutant polypeptides for at least one predetermined property, characteristic or activity;

c. for each mutant polypeptide identifying any change in said property, characteristic or activity relative to the template polypeptide; and
d. creating a functional map that is is used to identify (a) positions and mutations which do not affect the activity of the mutant polypeptide compared to the template polypeptide; (b) fully mutable sites compared to the template polypeptide; and (c) positions and mutations which result in an up-mutant compared to the template polypeptide.

[0011b] In a second aspect, there is provided a method of mapping a population of mutant antibodies formed from a template antibody having at least one complementary determining region (CDR), said at least one CDR together have a total of n amino acid residues, the method comprising:

a. generating a population comprising n sets of mutant antibodies, each set comprising mutant antibodies having X number of different predetermined amino acid residues at a single predetermined position of the CDR; wherein each set of mutant antibodies differs in the single predetermined position; and the number of different mutant antibodies generated is equivalent to n x X, wherein X is 19;

b. assaying the population for at least one predetermined property, characteristic or activity;
c. for each mutant antibody identifying any change in said property, characteristic or activity relative to the template antibody; and
d. creating a structural positional map that is used to identify (a) positions and mutations which do not affect the activity of the mutant polypeptide compared to the template antibody; (b) fully mutable sites compared to the template antibody; and (c) positions and mutations which result in an up-mutant compared to the template antibody.

[0011c] In a third aspect, there is provided a method of producing a library of multi-site mutant polypeptides formed from a template polypeptide wherein n is the number of amino acid residues in the template polypeptide, the method comprising:

a. generating n-1 separate sets of point mutation polypeptides, each set comprising point mutation polypeptides having X number of different predetermined single amino acid substitutions at a single predetermined site of

the template polypeptide; wherein each set of point mutation polypeptides differs in the single predetermined site; and the number of different point mutation polypeptides generated is equivalent to [n-1] x X, wherein X is 19;

b. screening each set of point mutation polypeptides for at least one predetermined property, characteristic or activity;
c. identifying any change in said property, characteristic or activity relative to the template polypeptide for each point mutation polypeptide;
d. categorizing each amino acid point mutation as one of deactivating, non-deactivating or activating;
e. creating a functional map of such changes categorizing each site as one of non-mutable; partially mutable; or fully mutable;
f. utilizing the functional map to select non-deactivating amino acid point mutations within partially mutable sites that are adjacent to non-mutable sites; and
g. recombining permutations of two to twenty amino acid point mutations selected in step (f) simultaneously to generate a library of multi-site mutant polypeptides.

[0011d] In a fourth aspect, there is provided a method of selecting two or more sites in a template polypeptide for multi-site mutation, the method comprising:

a. obtaining a functional map of mutant polypeptides formed from the template polypeptide wherein n is the number of amino acid residues in the template polypeptide, said obtaining step comprising the steps of:
    i. generating n-1 separate sets of mutant polypeptides, each set comprising mutant polypeptides having X number of different predetermined amino acid residues at a single predetermined position of the template polypeptide; wherein each set of mutant polypeptides differs in the single predetermined position; and the number of different mutant polypeptides generated is equivalent to [n-1] x X, wherein X is 19;
    ii. assaying each set for at least one predetermined property, characteristic or activity;
    iii. for each mutant polypeptide identifying any change in said property, characteristic or activity relative to the template polypeptide; and

iv. creating a functional map of such changes identifying positions and mutations which do not affect the activity of the mutant polypeptide compared to the template polypeptide;

b. utilizing the functional map to select partially mutable sites that are adjacent to non-mutable sites for multi-site mutation.

[0011e] In a fifth aspect, there is provided a method of mapping mutant polypeptides formed from a template polypeptide wherein n is the number of amino acid residues in the template polypeptide, the method comprising:

a. generating n-1 (n-2 in the case where the initial residue is methionine) separate mutant polypeptides, wherein each mutant polypeptide differs from the template polypeptide in that one amino acid is deleted at a single predetermined position;

b. assaying the population of mutant polypeptides for at least one predetermined property, characteristic or activity;

c. for each mutant polypeptide identifying any change in said property, characteristic or activity relative to the template polypeptide; and

d. creating a functional map that is is used to identify those deletions which result in (a) deletions which do not affect the activity of the mutant polypeptide compared to the template polypeptide; (b) deletions which result in a down-mutant compared to the template polypeptide; and (c) deletions which result in an up-mutant compared to the template polypeptide.

[0011f] In a sixth aspect, there is provided a method of mapping mutant polypeptides formed from a template polypeptide wherein n is the number of amino acid residues in the template polypeptide, the method comprising:

a. generating 20 x (n-1) separate mutant polypeptides, wherein each mutant polypeptide differs from the template polypeptide in that it has inserted after a specific position in the template one each of the 20 naturally occurring amino acids;

b. assaying the population for at least one predetermined property, characteristic or activity;

c. for each mutant polypeptide identifying any change in said property, characteristic or activity relative to the template polypeptide; and

d. creating a functional map that is used to identify those insertions which result in (a) insertions which do not affect the activity of the mutant polypeptide compared to the template polypeptide; (b) insertions which result in a down-mutant compared to the template polypeptide; and (c) insertions which result in an up-mutant compared to the template polypeptide.

[0011g] In a seventh aspect, there is provided a method of providing an optimized protein, the method comprising:

a. selecting a template polypeptide wherein n is the number of amino acid residues in the template polypeptide;
b. generating n-1 separate sets of mutant polypeptides from the template polypeptide, each set comprising mutant polypeptides having X number of different predetermined amino acid residues at a single predetermined position of the template polypeptide; wherein each set of mutant polypeptides differs in the single predetermined position; and the number of different mutant polypeptides generated is equivalent to [n-1] x X, wherein X is 19;
c. assaying each mutant polypeptide for at least one predetermined property, characteristic or activity;
d. identifying any change in said property, characteristic or activity of the mutant polypeptide relative to the template polypeptide;
e. creating a functional map wherein the functional map is used to identify positions and mutations which result in an up-mutant compared to the template polypeptide;
f. combining two or more of each of the relevant positions and mutations identified from mutant polypeptides which result in a up-mutant compared to the template polypeptide to create a set of combinatorial polypeptides;
g. assaying each combinatorial polypeptide for said predetermined property, characteristic or activity; and
h. selecting a combinatorial polypeptide in which said property, characteristic or activity is optimized compared to up-mutant polypeptides.

[0011h] In an eighth aspect, there is provided a method of evolving a template polypeptide encoding a functional protein, the method comprising:

generating and cloning at least 20 or more separate sets of mutant polypeptides, each set comprising mutant polypeptides having 19 different

predetermined amino acid residues at a single predetermined position of the template polypeptide; wherein each set of mutant polypeptides differs in the single predetermined position;

b. confirming that each different mutant polypeptide was generated and cloned;

c. assaying each set of mutant polypeptides for at least one predetermined property, characteristic or activity;

d. performing bioinformatic analysis to identify positions of potential interest in the template polypeptide for further modification; and

e. mutating positions identified in step (d).

[0011i] In a ninth aspect, there is provided a method of evolving a template polypeptide encoding a functional protein and wherein n is the number of amino acid residues in the template polypeptide, the method comprising:

generating and cloning n-1 sets of mutant polypeptides, each set comprising mutant polypeptides having 19 different predetermined amino acid residues at a single predetermined position of the template polypeptide; wherein each set of mutant polypeptides differs in the single predetermined position; and the total number of different mutant polypeptides generated is equivalent to [n-1] x 19;

b. confirming that each different mutant polypeptide was generated and cloned;

c. assaying each set of mutant polypeptide for at least one predetermined property, characteristic or activity;

d. performing bioinformatic analysis to identify positions of potential interest for further modification; and

e. mutating positions identified in step (d).

[0011j] In a tenth aspect, there is provided a method of evolving a polypeptide from a functional protein, the method comprising:

incorporating N,N,N at every codon position in a polynucleotide encoding the functional protein in order to generate a set of predetermined mutant polynucleotides containing every possible codon sequence and cloning each predetermined mutant polynucleotide;

b. confirming by sequencing, or some other method, that each predetermined mutant polynucleotide in the set was generated and cloned;

c. producing a set of predetermined mutant polypeptides from the set of predetermined mutant polynucleotides;

d. assaying each mutant polypeptide for at least one predetermined property, characteristic or activity compared to the functional protein; and

e. creating a functional map that is used to identify (a) positions and mutations which do not affect the activity of the mutant polypeptide compared to the functional protein; (b) fully mutable sites compared to the functional protein; and (c) positions and mutations which result in an up-mutant compared to the functional protein.

[0011k] In an eleventh aspect, there is provided a functional positional map made by the method of the first or second aspect.

[0012] One embodiment of the disclosure includes a functional positional map (EvoMap™) made by the methods described herein.

[0013] In an additional embodiment, certain residues particularly sensitive to change may be so indicated on the EvoMap™. Further optimization may be implemented by making additional mutational changes at positions outside of these sensitive positions.

[0014] In a specific embodiment, the mutations generated in the comprehensive evolution techniques of the disclosure are confirmed by sequencing, or some other method.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Figure 1 illustrates how comprehensive positional evolution (CPE™) is used to generate a molecule specific database (EvoMap™).

[0016] Figure 2 shows an example of a EvoMap™ and how additional optimization may be implemented by Synergy evolution.