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

[0004a] In a first aspect, there is provided a method of providing a deimmunized variant polypeptide by masking immunogenicity with glycosylation while maintaining function as compared to the template polypeptide, the method comprising:

a. preparing a library of novel glycosylated variant polypeptides from a template polypeptide by:

i. identifying fully mutable sites in the template polypeptide as target positions utilizing a functional map capable of identifying one or more of the group consisting of (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 having one or more improved properties, characteristics or activities compared to the template polypeptide;

ii. generating a codon variant library by introducing a codon which codes for either Asn, Ser or Thr at one or more of the target positions;

iii. preparing the library of novel glycosylated variant polypeptides in any expression system which has the ability to glycosylate utilizing the codon variant library,

b. assaying the library for at least one predetermined property, characteristic or activity relative to the template polypeptide;

c. testing the immunogenicity of the variant polypeptides in vitro by T cell proliferation assay, compared to the immunogenicity of the template polypeptide in the same assay; and

d. selecting a variant polypeptide with at least one predetermined property, characteristic or activity improved relative to the template polypeptide and with reduced immunogenicity relative to the template polypeptide.

[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 example, 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 example, 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 example, the entire polypeptide is subjected to comprehensive mutagenesis. In another example, 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 example, antibody comprises six CDRs, and together the CDRs comprise n amino acid residues.

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

[0013] In an additional example, 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 example, 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.

Claims

What is claimed is:

1. A method of providing a deimmunized variant polypeptide by masking immunogenicity with glycosylation while maintaining function as compared to the template polypeptide, the method comprising:

a. preparing a library of novel glycosylated variant polypeptides from a template polypeptide by:

i. identifying fully mutable sites in the template polypeptide as target positions utilizing a functional map capable of identifying one or more of the group consisting of (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 having one or more improved properties, characteristics or activities compared to the template polypeptide;

ii. generating a codon variant library by introducing a codon which codes for either Asn, Ser or Thr at one or more of the target positions;

iii. preparing the library of novel glycosylated variant polypeptides in any expression system which has the ability to glycosylate utilizing the codon variant library,

b. assaying the library for at least one predetermined property, characteristic or activity relative to the template polypeptide;

c. testing the immunogenicity of the variant polypeptides in vitro by T cell proliferation assay, compared to the immunogenicity of the template polypeptide in the same assay; and

d. selecting a variant polypeptide with at least one predetermined property, characteristic or activity improved relative to the template polypeptide and with reduced immunogenicity relative to the template polypeptide.

2. The method of claim 1, wherein the one or more target positions are selected from non-surface residues of the template polypeptide.

3. The method of claim 1, wherein step (c) comprises using an analytic technology selected from Mass Spectroscopy and Dynamic Light Scattering.

4. The method of claim 1, wherein step (c) comprises bioinformatics analysis.

5. The method of claim 1, wherein in step (c) a variant polypeptide is to be administered in patients to determine whether antibodies are generated against the variant polypeptide.

6. The method of claim 1, wherein variant polypeptides in the codon variant library are confirmed by sequencing or other methods.

7. The method of claim 1, wherein step (d) selects the variant polypeptide with at least two predetermined properties, characteristics or activities improved relative to the template polypeptide and one said improved property, characteristic or activity is selected from reduction of protein-protein aggregation, improvement of protein solubility, optimization of pharmacokinetics, optimization of protein secondary and tertiary structure, and deimmunization of antigenic sites.

8. The method of claim 1, further comprising:

e. selecting at least two different variant polypeptides;

f. combining two or more mutations of target positions from the at least two different selected variant polypeptides to create a set of combinatorial polypeptides;

g. assaying each combinatorial polypeptide for immunogenicity; and

g. selecting a combinatorial polypeptide with a reduced immunogenicity compared to the selected variant polypeptides.

9. The method of claim 1, further comprising:

e. performing bioinformatic analysis on the selected variant polypeptide to identify target positions of potential interest for further modification; and

f. mutating positions identified in step (e).

10. The method of claim 9, wherein said bioinformatic analysis is generation of a functional map.

11. The method of claim 7, wherein one of said at least two predetermined properties, characteristics or activities is modified expression.

12. The method of claim 11, wherein the modified expression is improved expression.

13. The method of claim 1, wherein at least one said predetermined property, characteristic or activity is modified expression.

14. The method of claim 13, wherein the modified expression is improved expression.

15. The method of claim 1, wherein the at least one predetermined property, characteristic or activity is selected from reduction of protein-protein aggregation, enhancement of protein stability, increased protein solubility, increased protein pH stability, increased protein temperature stability, increased protein solvent stability, increased selectivity, decreased selectivity, introduction of conjugation sites, enhancement of protein expression, increase in antigen affinity, decrease in antigen affinity, change in binding affinity, change in catalytic activity, pH optimization, or enhancement of specificity.

16. The method of claim 15, wherein the at least one predetermined property, characteristic or activity is increased selectivity.

17. The method of claim 15, wherein the at least one predetermined property, characteristic or activity is introduction of conjugation sites.

18. The method of claim 15, wherein the at least one predetermined property, characteristic or activity is increased antigen affinity.