

LOOK-THROUGH MUTAGENESIS

Related Applications and Information

This application claims priority to U.S. Provisional Application No. 60/483282,
5 filed on June 27, 2003, the entire contents of which are incorporated by reference herein.
The entire contents of all other patents, patent applications, and references cited
throughout the following specification also are incorporated by reference herein in their
entireties.

Background of the Invention

Mutagenesis is a powerful tool in the study of protein structure and function.
Mutations can be made in the nucleotide sequence of a cloned gene encoding a protein
of interest and the modified gene can be expressed to produce mutants of the protein.
By comparing the properties of a wild-type protein and the mutants generated, it is often
15 possible to identify individual amino acids or domains of amino acids that are essential
for the structural integrity and/or biochemical function of the protein, such as its binding
and/or catalytic activity. The number of mutants that can be generated from a single
protein, however, renders it difficult to select mutants that will be informative or have a
desired property, even if the selected mutants that encompass the mutations are solely in
20 putatively important regions of a protein (e.g., regions that make up an active site of a
protein). For example, the substitution, deletion, or insertion of a particular amino acid
may have a local or global effect on the protein.

Previous methods for mutagenizing polypeptides have been either too restrictive,
too inclusive, or limited to knocking out protein function rather than to gaining or
25 improving function. For example, a highly restrictive approach is selective or site-
directed mutagenesis which is used to identify the presence of a particular functional site
or understand the consequences of making a very specified alteration within the
functional site. A common application of site directed mutagenesis is in the study of
phosphoproteins where an amino acid residue, that would ordinarily be phosphorylated
30 and allow the polypeptide to carry out its function, is altered to confirm the link between
phosphorylation and functional activity. This approach is very specific for the
polypeptide and residue being studied.

Conversely, a highly inclusive approach is saturation or random mutagenesis that
is designed to produce a large number of mutations encompassing all possible alterations
35 within a defined region of a gene or protein. This is based on the principle that, by
generating essentially all possible variants of a relevant protein domain, the proper
arrangement of amino acids is likely to be produced as one of the randomly generated
mutants. However, in practice, the vast number of random combinations of mutations

generated can prevent the capacity to meaningfully select a desired candidate because of the presence of the so-called "noise" of so many undesired candidates.

Another approach, referred to as "Walk Through" mutagenesis (see, *e.g.*, U.S. Patent Nos: 5,830,650; 5,798,208) has been used to mutagenize a defined region of a polypeptide by synthesizing a mixture of degenerate oligonucleotides that, statistically, contain a desired set of mutations. However, because degenerate polynucleotide synthesis is employed, Walk-Through mutagenesis yields a number of undesired alterations in addition to the desired set of mutations. For example, to sequentially introduce a mutation across a defined region of only five amino acid positions, a set of over 100 polynucleotide must be made (and screened) (see, *e.g.*, Fig. 6). Accordingly, to make and screen, for example, two or three regions becomes increasingly complex, *i.e.*, requiring the making and screening of 200 to over 300 polynucleotides, respectively, for the presence of only 10 to 15 mutations.

In yet another approach which has been used to mutagenize proteins is alanine scanning mutagenesis, where an alanine residue is "scanned" through a portion of a protein to identify positions where the protein's function is interrupted. However, this approach only looks at loss of protein function by way of substituting a neutral alanine residue at a given position, rather than gain or improvement of function. Thus, it is not a useful approach for generating proteins having improved structure and function.

Accordingly, a need remains for a systematic way to mutagenize a protein for new or improved function.

Summary of the Invention

The invention pertains to a method of mutagenesis for the generation of novel or improved proteins (or polypeptides) and to libraries of polypeptide analogs and specific polypeptides generated by the methods. The polypeptide targeted for mutagenesis can be a natural, synthetic or engineered polypeptide, including fragments, analogs and mutant forms thereof.

In one embodiment, the method comprises introducing a predetermined amino acid into essentially every position within a defined region (or several different regions) of the amino acid sequence of a polypeptide. A polypeptide library is generated containing polypeptide analogs which individually have no more than one predetermined amino acid, but which collectively have the predetermined amino acid in every position within the defined region(s). The method can be referred to as "look-through" mutagenesis because, in effect, a single, predetermined amino acid (and only the predetermined amino acid) is substituted position-by-position throughout one or more defined region(s) of a polypeptide. Thus, the invention allows one to "look-through" the structural and functional consequences of separately substituting a

predetermined amino acid at each amino acid position within a defined region of the polypeptide, thereby segregating a specific protein chemistry to the defined region without any interference or “noise” from the generation of unwanted polypeptide analogs (*i.e.*, analogs containing amino acid substitutions other than those that follow the “look-through” scheme) (see, for example, Fig. 6).

Accordingly, the present invention allows for highly efficient and accurate systematic evaluation of the role of a specific amino acid change in one or more defined regions of a polypeptide. This becomes particularly important when evaluating (by mutating) two or more defined regions, such that the number of polypeptide analogs required greatly increases and, thus, the presence of undesired analogs also increases. The present invention obviates this problem by completely eliminating undesired analogs and, thus, the potential that any changes in protein structure or function observed are the result of anything but substitution of the predetermined amino acid. Thus, the effect of segregating a specific protein chemistry to even multiple regions with a protein can be studied with high accuracy and efficiency. Importantly, this includes studying how mutagenesis can effect the interaction of such regions, thereby improving the overall structure and function of the protein.

In a particular embodiment of the invention, the library of polypeptide analogs is generated and screened by first synthesizing individual polynucleotides encoding a defined region or regions of a polypeptide where, collectively, the polynucleotides represent all possible variant polynucleotides according to the look-through criteria described herein. The variant polynucleotides are expressed, for example, using *in vitro* transcription and translation and/or using a display technology, such as ribosome display, phage display, bacterial display, yeast display, arrayed display or any other suitable display system known in the art.

The expressed polypeptides are then screened and selected using functional assays, such as binding assays or enzymatic/catalytic assays. In one embodiment, the polypeptides are expressed in association with the polynucleotide that encodes the polypeptide, thereby allowing for identification of the polynucleotide sequence that encodes the polypeptide. In yet another embodiment, the polypeptides are directly synthesized using protein chemistry.

Thus, the present invention provides a method of mutagenesis that can be used to generate libraries of polypeptide analogs that are of a practical size for screening, in part, because the libraries are devoid of any undesired analog polypeptides or so-called noise. The method can be used to study the role of specific amino acids in polypeptide structure and function and to develop new or improved polypeptides such as antibodies, binding fragments or analogs thereof, single chain antibodies, catalytic antibodies, enzymes, and ligands. In addition, the method can be performed with the benefit of *a*

priori information, *e.g.*, via computer modeling, that can be used to select an initial subset of polypeptide analogs to be produced and studied using “look through” mutagenesis.

Other advantages and aspects of the present invention will be readily apparent
5 from the following description and Examples.

Brief Description of the Figures

Figure 1 illustrates exemplary defined regions (or D regions) that can be examined using Look-Through mutagenesis (LTM) and functional assays for identifying
10 desired polypeptide analogs from such D regions.

Figure 2 illustrates the use of LTM within three defined regions in an antibody variable region (*i.e.*, CDR1, CDR2, and CDR3 of an antibody heavy chain variable region). The light chain variable region can be similarly explored, either alone or in combination with the heavy chain variable region. For convenience in subsequent
15 screening assays, the heavy chain variable region can be explored in the context of a single chain antibody (sFv) as shown.

Figure 3 illustrates the use of LTM within a defined region (*i.e.*, positions 31-25 of CDR1) of a heavy chain variable region.

Figure 4 illustrates the use of LTM within a defined region (*i.e.*, positions 55-68
20 of CDR2) of a heavy chain variable region.

Figure 5 illustrates the use of LTM within a defined region (*i.e.*, positions 101-111 of CDR3) of a heavy chain variable region.

Figure 6 illustrates the advantages of LTM as compared to Walk-Through mutagenesis. LTM of a representative defined region, *i.e.*, CDR1 of an antibody heavy
25 chain variable region, results in the sequential alteration of each amino acid position through the defined region, without introducing any undesired amino acid residues or so-called noise.

Figure 7 illustrates the integration of three defined regions of a protein back into an overall protein context following Look-Through mutagenesis, in particular, the
30 integration of all three CDRs of an antibody heavy chain variable region into a single chain antibody format following Look-Through mutagenesis.

Figure 8 illustrates the use of polymerase chain reaction (PCR) to build defined regions of an antibody heavy and light chain subjected to LTM into a larger gene context.

35 **Figure 9** illustrates exemplary diversity formats of each of the CDRs in an antibody variable region for obtaining a catalytic site comprising, *e.g.*, a serine, histidine, and/or aspartic acid and how they can be arrayed.

Figure 10 illustrates the integration of all six CDRs of a binding region of an antibody that have been subjected to LTM and the resultant diversity if one predetermined amino acid residue or twenty different predetermined amino acid residues are used.

5 **Figure 11** illustrates the integration of all six CDRs of a binding region of an anti-TNF single chain antibody (sFv) that have been subjected to LTM and the resultant diversity if one predetermined amino acid residue or three different predetermined amino acid residues are used.

10 **Figure 12** illustrates an arrayed library representing some of the possible polypeptide analogs of the six CDRs of an antibody binding region that can be achieved using LTM.

Figure 13 illustrates the screening of an arrayed expression library using cell-free ribosome display.

15 **Figure 14** illustrates the combinatorial chemistry explored when the binding region of an antibody variable region (*i.e.*, all six CDRs) is subjected to LTM.

Figure 15 shows the sequence of the variable region (in single chain format) of several representative anti-TNF binding molecules that can be subjected to LTM.

Figure 16 shows a schematic for carrying out the protease selection assay for screening catalytic candidates of LTM.

20 **Figure 17** shows a schematic detailing the mechanism (and advantages) of the protease selection assay when carried out in bacterial cells.

Figure 18 shows a flowchart detailing the mechanics of screening gene libraries for catalytic activity, for example, catalytic antibody activity, using either ribosome or yeast display.

25 **Figure 19** shows a schematic for carrying out LTM where *a priori* information (*e.g.*, computer modeling information) and empirical information (assay results) can be coordinated for more efficient molecule design and development. This guided approach of LTM is referred to as “guide-through” mutagenesis.

30 **Figure 20** shows a hypothetical V_H CDR3 wild-type sequence (uppermost shaded ovals) and the resulting sequences in LTM His substitution (in open ovals) library members. The individual LTM His substitutions are encoded by individual oligonucleotides, *e.g.*, oligonucleotides synthesized in a high-throughput fashion. LTM subset libraries for the other (LTM) amino acid substitutions in this CDR domain are constructed in a similar fashion.

35 **Figure 21** illustrates the generation of scFv libraries. On the top row of the x axis and the far left column of the y axes the three digits represent the 3 CDRs on each of the light and heavy chains. A “0” indicates a wild-type CDR sequence, whereas a “1” indicates an LTM mutated CDR. The number on the grid indicates the complexity of the

subset library. For example in the uppermost left corner of the matrix is a "0" where the corresponding x and y axes are "000" and "000" indicating that none of the CDR in either the V_H and V_L are LTM mutated respectively. In moving one row over from the "0" corner to the neighboring "1" grid position would be designated by x-axis "100" and y-axis "000" indicating that V_H CDR1 is mutated while all V_L CDR remain wild type. Thus, in likewise fashion, a grid numbering of "4" would mean that there are four CDRs simultaneously mutated. Initially the seven V_H and seven V_L chains (indicated by the arrows) are made using SOE-PCR. The V_H and V_L chains are then amplified and mixed and matched by mega primer to generate all the remaining V_L-V_H combinations in one step.

Detailed Description of the Invention

In order to provide a clear understanding of the specification and claims, the following definitions are provided below.

Definitions

As used herein the term "analog" refers to a variant or mutant polypeptide (or a nucleic acid encoding such a polypeptide) having one or more amino acid substitutions.

The term "binding molecule" refers to any binding molecule, including proteins, polypeptides, peptides, and small molecules, that bind to a substrate or target. In one embodiment, the binding molecule is an antibody or binding fragment thereof (*e.g.*, a Fab fragment), single domain antibody, single chain antibody (*e.g.*, sFv), or peptide capable of binding a ligand.

The term "defined region" refers to a selected region of a polypeptide.

Typically, the defined region includes all or a portion of a functional site, *e.g.*, the binding site of a ligand, the binding site of a binding molecule or receptor, or a catalytic site. The defined region may also include multiple portions of a functional site. For example, the defined region can include all, a portion, or multiple portions of a complementarity determining region (CDR) or a complete heavy and/or light chain variable region (VR) of an antibody. Thus, a functional site may include a single or multiple defined regions that contribute to the functional activity of the molecule.

The term "library" refers to two or more molecules mutagenized according to the method of the invention. The molecules of the library can be in the form of polynucleotides, polypeptides, polynucleotides and polypeptides, polynucleotides and polypeptides in a cell free extract, or as polynucleotides and/or polypeptides in the context of a phage, prokaryotic cells, or in eukaryotic cells.

The term "mutagenizing" refers to the alteration of an amino acid sequence. This can be achieved by altering or producing a nucleic acid (polynucleotide) capable of

encoding the altered amino acid sequence, or by the direct synthesis of an altered polypeptide using protein chemistry.

The term “polynucleotide(s)” refers to nucleic acids such as DNA molecules and RNA molecules and analogs thereof (*e.g.*, DNA or RNA generated using nucleotide
5 analogs or using nucleic acid chemistry). As desired, the polynucleotides may be made synthetically, *e.g.*, using art-recognized nucleic acid chemistry or enzymatically using, *e.g.*, a polymerase. Typical modifications include methylation, biotinylation, and other art-known modifications. In addition, the nucleic acid molecule can be single-stranded or double-stranded and, where desired, linked or associated (*e.g.*, covalently or non-
10 covalently) to a detectable moiety.

The term “variant polynucleotide” refers to a polynucleotide encoding a corresponding polypeptide analog (or portion thereof) of the invention. Thus, variant polynucleotides contain one or more codons that have been changed to result in expression of a different amino acid.

15 The term “polypeptide(s)” refers to two or more amino acids joined by a peptide bond, *e.g.*, peptides (*e.g.*, from 2 to ~50 amino acid residues), as well as longer peptide sequences *e.g.*, protein sequences which typically comprises amino acid sequences from as few as 50 amino acid residues to more than 1,000 amino acid residues.

The term “pooling” refers to the combining of polynucleotide variants or
20 polypeptide analogs to form libraries representing the Look-Through mutagenesis of an entire polypeptide region. The molecules may be in the form of a polynucleotide and/or polypeptide and may coexist in the form of a sublibrary, as molecules on a solid support, as molecules in solution, and/or as molecules in one or more organisms (*e.g.*, phage, prokaryotic cells, or eukaryotic cells).

25 The term “predetermined amino acid” refers to an amino acid residue selected for substitution at each position within a defined region of a polypeptide to be mutagenized. This does not include position(s) within the region that already (*e.g.*, naturally) contain the predetermined amino acid and, thus, which need not be substituted with the predetermined amino acid. Accordingly, each polypeptide analog generated in
30 accordance with the present invention contains no more than one “predetermined amino acid” residue in a given defined region. However, collectively, the library of polypeptide analogs generated contains the predetermined amino acid at each position within the region being mutagenized. Typically, a predetermined amino acid is selected for a particular size or chemistry usually associated with the side group of the amino
35 acid. Suitable predetermined amino acids include, for example, glycine and alanine (sterically small); serine, threonine, and cysteine (nucleophilic); valine, leucine, isoleucine, methionine, and proline (hydrophobic); phenylalanine, tyrosine, and

tryptophan (aromatic); aspartate and glutamate (acidic); asparagine, glutamine, and histidine (amide); and lysine and arginine (basic).

Detailed Description

5 The study of proteins has revealed that certain amino acids play a crucial role in their structure and function. For example, it appears that only a discrete number of amino acids participate in the binding of an antibody to an antigen or are involved in the catalytic event of an enzyme.

10 Though it is clear that certain amino acids are critical to the activity or function of proteins, it is difficult to identify which amino acids are involved, how they are involved, and what substitutions can improve the protein's structure or function. In part, this is due to the complexity of the spatial configuration of amino acid side chains in polypeptides and the interrelationship of different portions of the polypeptide that contribute to form a functional site. For example, the interrelationship between the six
15 CDRs of the variable heavy and light chain regions of an antibody contribute to the antigen or ligand-binding pocket.

 Previous mutagenesis methods, such as selective (site-directed) mutagenesis and saturation mutagenesis, are of limited utility for the study of protein structure and function in view of the enormous number of possible variations in complex
20 polypeptides. This is especially true given that desirable combinations are often accompanied by the presence of vast amounts of undesirable combinations or so-called noise.

 The method of this invention provides a systematic, practical, and highly accurate approach for evaluating the role of particular amino acids and their position,
25 within a defined region of a polypeptide, in the structure or function of the polypeptide and, thus, for producing improved polypeptides.

1. Selecting a Defined Region

 In accordance with the present invention, a defined region or regions within a
30 protein are selected for mutagenesis. Typically, the regions are believed to be important to the protein's structure or function (see, *e.g.*, Fig. 1). This can be deduced, for example, from what structural and/or functional aspects are known or can be deduced from comparing the defined region(s) to what is known from the study of other proteins, and may be aided by modeling information. For example, the defined region can be one
35 that has a role in a functional site, *e.g.*, in binding, catalysis, or another function. In one embodiment, the defined region is a hypervariable region or complementarity determining region (CDR) of an antigen binding molecule. In another embodiment, the defined region is a portion of a complementarity determining region (CDR). In other

embodiments, two or more defined regions, e.g., CDRs or portions thereof, are selected for mutagenesis.

2. Selecting a Predetermined Amino Acid Residue

5 The amino acid residue chosen for substitution within the defined region(s) is generally selected from those known to be involved in the structure or function of interest. The twenty naturally occurring amino acids differ with respect to their side chain. Each side chain is responsible for chemical properties that make each amino acid unique. For the purpose of altering binding or creating new binding affinities, any of the
10 twenty naturally occurring amino acids generally can be selected. Thus, previous methods of mutagenesis, which created vast numbers of analogs for every substitution, were impractical for evaluating the effect on protein binding of substitution each of the twenty amino acids. In contrast, the methods of the present invention creates a practical number of analogs for each amino acid substitution and, thus, allows for the evaluation
15 of a greater variety of protein chemistries within a segregated region or regions of a protein.

 In contrast to protein binding, only a subset of amino acid residues typically participate in enzymatic or catalytic events. For example, from the chemical properties of the side chains, only a selected number of natural amino acids preferentially
20 participate in catalytic events. These amino acids belong to the group of polar and neutral amino acids such as Ser, Thr, Asn, Gln, Tyr, and Cys, the group of charged amino acids, Asp and Glu, Lys and Arg, and especially the amino acid His. Other polar and neutral side chains are those of Cys, Ser, Thr, Asn, Gln and Tyr. Gly is also considered to be a borderline member of this group. Ser and Thr play an important role
25 in forming hydrogen bonds. Thr has an additional asymmetry at the beta carbon, therefore only one of the stereoisomers is used. The acid amide Gln and Asn can also form hydrogen bonds, the amido groups functioning as hydrogen donors and the carbonyl groups functioning as acceptors. Gln has one more CH₂ group than Asn which renders the polar group more flexible and reduces its interaction with the main chain.
30 Tyr has a very polar hydroxyl group (phenolic OH) that can dissociate at high pH values. Tyr behaves somewhat like a charged side chain; its hydrogen bonds are rather strong.

 Neutral polar acids are found at the surface as well as inside protein molecules. As internal residues, they usually form hydrogen bonds with each other or with the
35 polypeptide backbone. Cys can form disulfide bridges.

 Histidine (His) has a heterocyclic aromatic side chain with a pK value of 6.0. In the physiological pH range, its imidazole ring can be either uncharged or charged, after taking up a hydrogen ion from the solution. Since these two states are readily available,

His is quite suitable for catalyzing chemical reactions. It is found in most of the active centers of enzymes, for example, serine proteases.

Asp and Glu are negatively charged at physiological pH. Because of their short side chain, the carboxyl group of Asp is rather rigid with respect to the main chain. This
5 may be the reason why the carboxyl group in many catalytic sites is provided by Asp and not by Glu. Charged acids are generally found at the surface of a polypeptide.

In addition, Lys and Arg are found at the surface. They have long and flexible side chains presenting multiple rotamers of similar energies. In several cases, Lys and Arg take part in forming internal salt bridges or they help in catalysis. Because of their
10 exposure at the surface of the polypeptide, Lys is a residue more frequently recognized by enzymes that either modify the side chain or cleave the peptide chain at the carbonyl end of Lys residues.

While the side group chemistry of an amino acid can guide the selection of a predetermined amino acid residue, the lack of a desired side group chemistry can be a
15 criterion for excluding an amino acid residue for use as the predetermined amino acid. For example, sterically small and chemically neutral amino acids, such as alanine, can be excluded from Look-Through mutagenesis for lacking a desired chemistry.

3. Synthesizing Polypeptide Analog Libraries

20 In one embodiment, a library of polypeptide analogs is generated for screening by synthesizing individual oligonucleotides that encode the defined region of the polypeptide and have no more than one codon for the predetermined amino acid. This is accomplished by incorporating, at each codon position within the oligonucleotide either the codon required for synthesis of the wild-type polypeptide or a codon for the
25 predetermined amino acid. This differs from the oligonucleotides produced in saturation mutagenesis, random mutagenesis, or walk-through mutagenesis in that, for each oligonucleotide, only one mutation, as opposed to multiple mutations is made.

The oligonucleotides can be produced individually and then mixed or pooled as desired. When the codon of the wild type sequence and the codon for the predetermined
30 amino acid are the same, no substitution is made.

Accordingly, the number of amino acid positions within the defined region will determine the maximum number of oligonucleotides made. For example, if five codon positions are altered with the predetermined amino acid, then five polynucleotides plus one polynucleotide representing the wild-type amino acid sequence are synthesized.

35 Two or more regions can simultaneously be altered.

The mixture of oligonucleotides for generation of the library can be synthesized readily by known methods for DNA synthesis. The preferred method involves use of solid phase beta-cyanoethyl phosphoramidite chemistry. See U.S. Pat. No. 4,725,677.

For convenience, an instrument for automated DNA synthesis can be used containing specified reagent vessels of nucleotides. The polynucleotides may also be synthesized to contain restriction sites or primer hybridization sites to facilitate the introduction or assembly of the polynucleotides representing, *e.g.*, a defined region, into a larger gene
5 context.

The synthesized polynucleotides can be inserted into a larger gene context of the polypeptide being mutagenized by using standard genetic engineering techniques. For example, the polynucleotides can be made to contain flanking recognition sites for restriction enzymes. See Crea, R., U.S. Pat. No. 4,888,286. The recognition sites are
10 designed to correspond to recognition sites that either exist naturally or are introduced in the gene proximate to the DNA encoding the region. After conversion into double stranded form, the polynucleotides are ligated into the gene by standard techniques. By means of an appropriate vector (including, *e.g.*, phage vectors, plasmids) the genes can be introduced into a cell-free extract, phage, prokaryotic cell, or eukaryotic cell suitable
15 for expression of the mutant polypeptides.

In cases where the amino acid sequence of the polypeptide to be mutagenized is known or where the DNA sequence is known, gene synthesis is a possible approach. For example, partially overlapping polynucleotides, typically about 20-60 nucleotides in length can be designed. The internal polynucleotides are then phosphorylated annealed
20 to their complementary partner to give a double-stranded DNA molecule with single-stranded extensions useful for further annealing. The annealed pairs can then be mixed together and ligated to form a full-length double-stranded molecule (see, *e.g.*, Fig. 8). Convenient restriction sites can be designed near the ends of the synthetic gene for cloning into a suitable vector. The full-length molecules can be cleaved with those
25 restriction enzymes and ligated into a suitable vector. Convenient restriction sites can also be incorporated into the sequence of the synthetic gene to facilitate introduction of mutagenic cassettes.

As an alternative to synthesizing polynucleotides representing the full-length double-stranded gene, polynucleotides which partially overlap at their 3' ends (*i.e.*, with
30 complementary 3' ends) can be assembled into a gapped structure and then filled in with a suitable polymerase to make a full length double-stranded gene. Typically, the overlapping polynucleotides are from 40-90 nucleotides in length. The extended polynucleotides are then ligated. Convenient restriction sites can be introduced at the ends and/or internally for cloning purposes. Following digestion with an appropriate
35 restriction enzyme or enzymes, the gene fragment is ligated into a suitable vector. Alternatively, the gene fragment can be blunt end ligated into an appropriate vector.

In these approaches, if convenient restriction sites are available (naturally or engineered) following gene assembly, the degenerate polynucleotides can be introduced

subsequently by cloning the cassette into an appropriate vector. Alternatively, the degenerate polynucleotides can be incorporated at the stage of gene assembly. For example, when both strands of the gene are fully chemically synthesized, overlapping and complementary degenerate polynucleotides can be produced. Complementary pairs
5 will anneal with each other.

When partially overlapping polynucleotides are used in the gene assembly, a set of degenerate nucleotides can also be directly incorporated in place of one of the polynucleotides. The appropriate complementary strand is synthesized during the extension reaction from a partially complementary polynucleotide from the other strand
10 by enzymatic extension with a polymerase. Incorporation of the degenerate polynucleotides at the stage of synthesis also simplifies cloning where more than one domain or defined region of a gene is mutagenized.

In another approach, the gene of interest is present on a single stranded plasmid. For example, the gene can be cloned into a phage vector or a vector with a filamentous
15 phage origin of replication that allows propagation of single-stranded molecules with the use of a helper phage. The single-stranded template can be annealed with a set of degenerate polynucleotides representing the desired mutations and elongated and ligated, thus incorporating each analog strand into a population of molecules that can be introduced into an appropriate host (Sayers, J. R. et al., *Nucleic Acids Res.* 16: 791-802
20 (1988)). This approach can circumvent multiple cloning steps where multiple domains are selected for mutagenesis.

Polymerase chain reaction (PCR) methodology can also be used to incorporate polynucleotides into a gene. For example, the polynucleotides themselves can be used as primers for extension. In this approach, polynucleotides encoding the mutagenic
25 cassettes corresponding to the defined region (or portion thereof) are complementary to each other, at least in part, and can be extended to form a large gene cassette using a polymerase, *e.g.*, using PCR amplification.

The size of the library will vary depending upon the length and number of regions and amino acids within a region that are mutagenized. Preferably, the library
30 will be designed to contain less than 10^{15} , 10^{14} , 10^{13} , 10^{12} , 10^{11} , 10^{10} , 10^9 , 10^8 , 10^7 , and more preferably, 10^6 polypeptide analogs or less.

The description above has centered on the mutagenesis of polypeptides and libraries of polypeptides by altering the polynucleotide that encodes the corresponding polypeptide. It is understood, however, that the scope of the invention also encompasses
35 methods of mutagenizing polypeptides by direct synthesis of the desired polypeptide analogs using protein chemistry. In carrying out this approach, the resultant polypeptides still incorporate the features of the invention except that the use of a polynucleotide intermediate is eliminated.

For the libraries described above, whether in the form of polynucleotides and/or corresponding polypeptides, it is understood that the libraries may be also attached to a solid support, such as a microchip, and preferably arrayed, using art recognized techniques.

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4. Expression and Screening Systems

Libraries of polynucleotides generated by any of the above techniques or other suitable techniques can be expressed and screened to identify polypeptide analogs having desired structure and/or activity. Expression of the polypeptide analogs can be carried
10 out using any suitable expression display system known in the art including, but not limited to, cell-free extract display systems (*e.g.*, ribosome display and arrayed (*e.g.*, microarrayed or macroarrayed) display systems), bacterial display systems, phage display systems, prokaryotic cells, and/or eukaryotic cells (*e.g.*, yeast display systems).

In one embodiment, the polynucleotides are engineered to serve as templates that
15 can be expressed in a cell free extract. Vectors and extracts as described, for example in U.S. Patent Nos. 5,324,637; 5,492,817; 5,665,563, can be used and many are commercially available. Ribosome display and other cell-free techniques for linking a polynucleotide (*i.e.*, a genotype) to a polypeptide (*i.e.*, a phenotype) can be used, *e.g.*, ProfusionTM (see, *e.g.*, U.S. Patent Nos. 6,348,315; 6,261,804; 6,258,558; and 6,214,553).

Alternatively, the polynucleotides of the invention can be expressed in a
20 convenient *E. coli* expression system, such as that described by Pluckthun and Skerra. (Pluckthun, A. and Skerra, A., *Meth. Enzymol.* 178: 476-515 (1989); Skerra, A. et al., *Biotechnology* 9: 273-278 (1991)). The mutant proteins can be expressed for secretion in the medium and/or in the cytoplasm of the bacteria, as described by M. Better and A.
25 Horwitz, *Meth. Enzymol.* 178: 476 (1989). In one embodiment, the single domains encoding VH and VL are each attached to the 3' end of a sequence encoding a signal sequence, such as the *ompA*, *phoA* or *pelB* signal sequence (Lei, S. P. et al., *J. Bacteriol.* 169: 4379 (1987)). These gene fusions are assembled in a dicistronic construct, so that they can be expressed from a single vector, and secreted into the periplasmic space of *E.*
30 *coli* where they will refold and can be recovered in active form. (Skerra, A. et al., *Biotechnology* 9: 273-278 (1991)). For example, antibody heavy chain genes can be concurrently expressed with antibody light chain genes to produce antibody or antibody fragments.

In still another embodiment, the polynucleotides can be expressed in eukaryotic
35 cells such as yeast using, for example, yeast display as described, *e.g.*, in U.S. Patent Nos. 6,423,538; 6,331,391; and 6,300,065. In this approach, the polypeptide analogs of the library are fused to a polypeptide that is expressed and displayed on the surface of the yeast. Other eukaryotic cells for expression of the polypeptides of the invention can also

be used such as mammalian cells, for example myeloma cells, hybridoma cells, or Chinese hamster ovary (CHO) cells. Typically, the polypeptide analogs when expressed in mammalian cells are designed to be expressed into the culture medium, or expressed on the surface of such a cell. The antibody or antibody fragments can be produced, for example, as entire antibody molecules or as individual VH and VL fragments, Fab
5 fragments, single domains, or as single chains (sFv) (see Huston, J. S. et al., Proc. Natl. Acad. Sci. USA 85: 5879-5883 (1988)).

The screening of the expressed polypeptide analogs (or polypeptides produced by direct synthesis) can be done by any appropriate means. For example, binding
10 activity can be evaluated by standard immunoassay and/or affinity chromatography and catalytic activity can be ascertained by suitable assays for substrate conversion. Screening of the polypeptide analogs of the invention for proteolytic function can be accomplished using a standard hemoglobin plaque assay as described, for example, in U.S. Patent No. 5,798,208.

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5. Computer Modeling-Assisted Look Through Mutagenesis

The look-through mutagenesis of the invention may also be conducted with the benefit of structural or modeling information concerning the polypeptide analogs to be generated, such that the potential for generating analogs having the desired improved
20 function is increased. The structural or modeling information can also be used to guide the selection of predetermined amino acid to introduce into the defined regions. Still further, actual results obtained with the polypeptide analogs of the invention can guide the selection (or exclusion) of subsequent polypeptides to be made and screened in an iterative manner. Accordingly, structural or modeling information can be used to
25 generate initial subsets of polypeptide analogs for use in the invention, thereby further increasing the efficiency of generating improved polypeptides.

In a particular embodiment, in silico modeling is used to eliminate the production of any polypeptide analog predicted to have poor or undesired structure and/or function. In this way, the number of polypeptide analogs to be produced can be sharply reduced
30 thereby increasing signal-to-noise in subsequent screening assays. In another particular embodiment, the in silico modeling is continually updated with additional modeling information, from any relevant source, e.g., from gene and protein sequence and three-dimensional databases and/or results from previously tested analogs, so that the in silico database becomes more precise in its predictive ability (Fig. 19).

35 In yet another embodiment, the in silico database is provided with the assay results of previously tested polypeptide analogs and categorizes the analogs, based on the assay criterion or criteria, as responders or nonresponders, e.g., as polypeptide analogs that bind well or not so well or as being enzymatic/catalytic or not so

enzymatic/catalytic. In this way, the guide-through mutagenesis of the invention can equate a range of functional response with particular structural information and use such information to guide the production of future polypeptide analogs to be tested.

Accordingly, the method is especially suitable for screening antibody or antibody
5 fragments for a particular function, such as binding affinity (e.g., specificity), stability (e.g., half life) and/or effector function (e.g., complement activation and ADCC).
Accordingly, mutagenesis of noncontiguous residues within a region can be desirable if it is known, *e.g.*, through in silico modeling, that certain residues in the region will not participate in the desired function. The coordinate structure and spatial interrelationship
10 between the defined regions, *e.g.*, the functional amino acid residues in the defined regions of the polypeptide, *e.g.*, the predetermined amino acid(s) that have been introduced, can be considered and modeling. Such modeling criteria include, *e.g.*, amino acid residue side group chemistry, atom distances, crystallography data, etc.
Accordingly, the number of polypeptide analogs to be produced can be intelligently
15 minimized.

In a preferred embodiment, one or more of the above steps are computer-assisted. The method is also amenable to be carried out, in part or in whole, by a device, *e.g.*, a computer driven device. Accordingly, instructions for carrying out the method, in part or in whole, can be conferred to a medium suitable for use in an electronic device
20 for carrying out the instructions. In sum, the methods of the invention are amendable to a high throughput approach comprising software (*e.g.*, computer-readable instructions) and hardware (*e.g.*, computers, robotics, and chips).

6. Exploring the Combinatorial Chemistry of Multiple Defined Regions

25 The present invention provides the important advantage of allowing for evaluation by mutagenesis of several different regions or domains of a polypeptide simultaneously. This can be done using the same or a different predetermined amino acid within each region, enabling the evaluation of amino acid substitutions in conformationally related regions, such as the regions that upon folding of the
30 polypeptide, are associated to make up a functional site (*e.g.*, the binding site of an antibody or the catalytic site of an enzyme). This, in turn, provides an efficient way to create new or improved functional sites.

For example, as depicted in Fig. 14, the six CDRs of an antibody that make up the unique aspects of the antigen binding site (Fv region), can be mutagenized
35 simultaneously, or separately within the VH or VL chains, to study the three dimensional interrelationship of selected amino acids in this site. In one embodiment, the combinatorial chemistry of three or more defined regions are systematically explored using look-through mutagenesis, and preferably six defined regions, for example, the six

CDRs of an antibody heavy and light chain variable region. For performing look-through mutagenesis on a CDR, typically 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more amino acid positions are altered.

Accordingly, the present invention opens up new possibilities for the design of many different types of novel and improved polypeptides. The method can be used to improve upon an existing structure or function of a protein. For example, a binding site for an antibody or antibody fragment can be introduced or affinity for a pre-existing antigen, effector function and/or stability improved. Alternatively, the introduction of additional "catalytically important" amino acids into a catalytic domain of an enzyme can be performed resulting in a modified or enhanced catalytic activity toward a substrate. Alternatively, entirely new structures, specificities or activities may be introduced into a polypeptide. De novo synthesis of enzymatic activity can be achieved as well. The new structures can be built on the natural or consensus "scaffold" of an existing protein by mutating only relevant regions by the method of the invention.

15

7. Look-Through Mutagenesis for Making New or Improved Antibodies

The method of this invention is especially useful for modifying antibody molecules. As used herein, antibody molecules or antibodies refers to antibodies or portions thereof, such as full-length antibodies, Fv molecules, or other antibody fragments, individual chains or fragments thereof (e.g., a single chain of Fv), single chain antibodies, and chimeric antibodies. Alterations can be introduced into the variable region and/or into the framework (constant) region of an antibody. Modification of the variable region can produce antibodies with better antigen binding properties, and, if desired, catalytic properties. Modification of the framework region can also lead to the improvement of chemo-physical properties, such as solubility or stability (e.g., half life), which are especially useful, for example, in commercial production, bioavailability, effector function (e.g., complement activation and/or ADCC) and binding affinity (e.g., specificity) for the antigen. Typically, the mutagenesis will target the Fv region of the antibody molecule, i.e., the structure responsible for antigen-binding activity which is made up of variable regions of two chains, one from the heavy chain (VH) and one from the light chain (VL). Once the desired antigen-binding characteristics are identified, the variable region(s) can be engineered into an appropriate antibody class such as IgG, IgM, IgA, IgD, or IgE.

8. Look-Through Mutagenesis for Making / Improving Catalytic / Enzymatic Polypeptides

The method of the invention also is particularly suited to the design of catalytic proteins, particularly catalytic antibodies. Presently, catalytic antibodies can be

prepared by an adaptation of standard somatic cell fusion techniques. In this process, an animal is immunized with an antigen that resembles the transition state of the desired substrate to induce production of an antibody that binds the transition state and catalyzes the reaction. Antibody-producing cells are harvested from the animal and fused with an immortalizing cell to produce hybrid cells. These cells are then screened for secretion of an antibody that catalyzes the reaction. This process is dependent upon the availability of analogues of the transition state of a substrate. The process may be limited because such analogues are likely to be difficult to identify or synthesize in most cases.

The method of the invention provides a different approach that eliminates the need for a transition state analogue. By the method of the invention, an antibody can be made catalytic by the introduction of suitable amino acids into the binding site of an immunoglobulin (Fv region). The antigen-binding site (Fv) region is made-up of six hypervariable (CDR) loops, three derived from the immunoglobulin heavy chain (H) and three from the light chain (L), which connect beta strands within each subunit. The amino acid residues of the CDR loops contribute almost entirely to the binding characteristics of each specific monoclonal antibody. For instance, catalytic triads (comprising of amino acid residues serine, histidine, and aspartic acid) modeled after serine proteases can be created in the hypervariable segments of the Fv region of an antibody with known affinity for the substrate molecule and screened for proteolytic activity of the substrate.

In particular, the method of the invention can be used to produce many different enzymes or catalytic antibodies, including oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. Among these classes, of particular importance will be the production of improved proteases, carbohydrases, lipases, dioxygenases and peroxidases. These and other enzymes that can be prepared by the method of the invention have important commercial applications for enzymatic conversions in health care, cosmetics, foods, brewing, detergents, environment (*e.g.*, wastewater treatment), agriculture, tanning, textiles, and other chemical processes. These include, but are not limited to, diagnostic and therapeutic applications, conversions of fats, carbohydrates and protein, degradation of organic pollutants and synthesis of chemicals. For example, therapeutically effective proteases with fibrinolytic activity, or activity against viral structures necessary for infectivity, such as viral coat proteins, can be engineered. Such proteases could be useful anti-thrombotic agents or anti-viral agents against viruses such as, for example, HIV, rhinoviruses, influenza, or hepatitis. In the case of oxygenases (*e.g.*, dioxygenases), a class of enzymes requiring a co-factor for oxidation of aromatic rings and other double bonds, industrial applications in biopulping processes, conversion of biomass into fuels or other chemicals, conversion of waste water contaminants,

bioprocessing of coal, and detoxification of hazardous organic compounds are possible applications of novel proteins.

The present invention is further illustrated in the following examples, which
5 should not be construed as limiting.

Exemplification

Throughout the examples, the following materials and methods were used unless
10 otherwise stated.

Materials and Methods

In general, the practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, recombinant DNA
15 technology, PCR technology, immunology (especially, *e.g.*, antibody technology), expression systems (*e.g.*, cell-free expression, phage display, ribosome display, and ProfusionTM), and any necessary cell culture that are within the skill of the art and are explained in the literature. See, *e.g.*, Sambrook, Fritsch and Maniatis, *Molecular Cloning: Cold Spring Harbor Laboratory Press* (1989); *DNA Cloning*, Vols. 1 and 2,
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35 al., Yeast surface display for directed evolution of protein expression, affinity, and stability, *Methods Enzymol.*, 328:430-44 (2000); ribosome display as described by Pluckthun et al. in U.S. Patent No. 6,348,315, and ProfusionTM as described by Szostak et al. in U.S. Patent Nos. 6,258,558; 6,261,804; and 6,214,553.

EXAMPLE 1

LOOK-THROUGH MUTAGENESIS OF THREE DEFINED REGIONS IN AN ANTIGEN BINDING MOLECULE

5 In this example, the look-through mutagenesis of three CDRs of an antibody to improve binding and proteolysis of a substrate is described.

 In particular, the "look-through" mutagenesis of three complementarity determining regions (CDRs) of a monoclonal antibody is performed. CDR1, CDR2, and CDR3 of the heavy chain variable region (VH) are defined regions selected for look-
10 through mutagenesis. For this embodiment, the predetermined amino acids selected are the three residues of the catalytic triad of serine proteases, Asp, His and Ser. Asp is selected for VH CDR1, His is selected for VL CDR2, and Ser is selected for VH CDR3. The selection of these three predetermined amino acids allows for the use of a convenient protease assay in order to detect when the three residues are positioned
15 correctly to exhibit a functional activity, *i.e.*, proteolysis of a test substrate.

 An exemplary antibody, MCPC 603, is recognized as a good model for investigating binding and catalysis because the antibody binding region has been well characterized. The amino acid sequence and DNA sequence of the MCPC 603 VH and VL regions are publicly available (see, *e.g.*, Rudikoff, S. and Potter, M., Biochemistry
20 13: 4033 (1974); Pluckthun, A. et al., Cold Spring Harbor Symp. Quant. Biol., Vol. LII: 105-112 (1987)). The CDRs for the MCPC 603 antibody have been identified as shown in Fig. 2. In the heavy chain, CDR1 spans amino acid residue positions 31-35, CDR2 spans positions 50-69, and CDR3 spans positions 101-111. In the light chain, the amino acid residues of CDR1 are 24-40, CDR2 spans amino acids 55-62, and CDR3 spans
25 amino acids 95-103.

 The design of the oligonucleotides for look-through mutagenesis in the CDRs of MCPC 603 is such that polypeptide analogs result having the amino acid sequence for CDR1, CDR2, and CDR3 as shown in Figs. 3-5. It is understood that the oligonucleotides synthesized can be larger than the CDR regions to be altered to facilitate
30 insertion into the target construct as shown in Fig. 7. A single chain antibody format is chosen for convenience in subsequent expression and screening steps. The oligonucleotides can be converted into double-stranded chains by enzymatic techniques (see *e.g.*, Oliphant, A. R. et al., 1986, *supra*) and then ligated into a restricted plasmid as shown in Fig. 8. The restriction sites can be naturally occurring sites or engineered
35 restriction sites.

 Polynucleotides encoding corresponding polypeptide analogs can be expressed in any of the convenient expression systems described herein and screened using the serine protease assay described, *e.g.*, in U.S. Patent No. 5,798,208 (see also, Figs. 16-17).

Briefly, the expressed polypeptide analogs are exposed to a test substrate and examined for proteolysis of the test substrate. The amount of proteolysis, revealed by a zone of clearance of the substrate, indicates a polypeptide analog having the desired functional activity.

5

EXAMPLE 2

LOOK-THROUGH MUTAGENESIS OF SIX DEFINED REGIONS IN AN ANTIGEN BINDING MOLECULE

In this example, the look-through mutagenesis of all six CDRs of an antibody to improve binding and proteolysis of a substrate is described.

In particular, a "look-through" mutagenesis of all six of the hypervariable regions or complementarity determining regions (CDRs) of the above mentioned model antibody (MCPC 603) is performed. In this example, "look-through" mutagenesis is carried out from two to three times with a different amino acid in a given region or domain. For example, Asp, Ser and His are sequentially walked-through the heavy and light chains as shown in Fig. 10.

Mutagenesis of noncontiguous residues within a region can be desirable if it is known, or if one can deduce, that certain residues in the region will not participate in the desired function. In addition, the number of analogs can be minimized. Other considerations in selecting the predetermined amino acid and the particular positions to be altered are that the residues must be able to hydrogen bond with one another. This consideration can impose a proximity constraint on the variants generated. Thus, only certain positions within the CDRs may permit the amino acids of the catalytic triad to interact properly. Thus, molecular modeling or other structural information can be used to enrich for functional variants.

In this case, known structural information was used to identify residues in the regions that may be close enough to permit hydrogen bonding between Asp, His and Ser, as well as the range of residues to be mutagenized. Roberts et al. have identified regions of close contact between portions of the CDRs (Roberts, V. A. et al., Proc. Natl. Acad. Sci. USA 87: 6654-6658 (1990)). This information together with data from the x-ray structure of MCPC 603 is used to select promising areas of close contact among the CDRs targeted for mutagenesis. This type of look-through mutagenesis guided by structural / modeling information can be referred to as "guide-through" mutagenesis.

Look-through mutagenesis is carried out as illustrated in Fig. 10 where each CDR is subjected to one predetermined amino acid resulting 8×10^5 polypeptide analogs and if all twenty amino acids are explored, 5×10^{13} polypeptide analogs.

Polynucleotides can be expressed in any of the convenient expression systems described herein and screened using the serine protease described, *e.g.*, in U.S. Patent No.

5,798,208. Briefly, the expressed polypeptide analogs are exposed to a test substrate and examined for proteolysis of the test substrate. The amount of proteolysis, revealed by a zone of clearance of the substrate, indicates a polypeptide analog having the desired functional activity.

5

EXAMPLE 3

LOOK-THROUGH MUTAGENESIS OF ANTI-TNF BINDING MOLECULES TO IMPROVE FUNCTION

In this example, the look-through mutagenesis of an anti-TNF antibody to improve binding is described.

In particular, the "look-through" mutagenesis of all six of the hypervariable regions or complementarity determining regions (CDRs) of two different anti-TNF antibodies is performed. Anti-TNF antibodies have general application in the treatment of immune disease in patients having inappropriate levels of the ligand TNF (tumor necrosis factor). Two commercially available anti-TNF antibodies exist. For convenience in performing look-mutagenesis and subsequent screening, the variable light and heavy chain regions (see SEQ ID NOs: 2-4) of these antibodies were converted to a single chain format using a poly Gly-Ser linker (see Fig. 15). The defined regions selected for look through mutagenesis with a predetermined amino acid are identified by the presence of a black bar as shown in Fig. 15. These defined regions correspond to the CDRs of the single chain antibodies.

Polynucleotides representing the six CDR regions and sufficient flanking regions to allow for the assembly of the polynucleotides into the complete single chain sequence shown in Fig. 15 are synthesized as described herein. Predetermined amino acid residues are selected for each CDR region and separately and sequentially introduced into each amino acid position throughout the six CDR regions. The polynucleotides are further engineered to serve as templates capable of supporting the transcription of a corresponding RNA transcript that can then be translated into a polypeptide using ribosome display.

Polynucleotides encoding corresponding polypeptide analogs are expressed using a cell-free transcription and translation extracts. The RNA transcripts are covalently linked to a detectable moiety such as a fluorescent moiety. Alternatively, the sequence of interest can be fused in frame to a fluorescent moiety such as green fluorescent protein (GFP) to allow for the convenient detection of expression and normalization of binders versus non-binders. Preferably, the polynucleotides encoding the polypeptide analogs are at least partially arrayed, *e.g.*, expressed in a well that has multiple polypeptide analogs but can be readily deconvoluted. Accordingly, each well contains a subset of polypeptide analogs now linked to the corresponding transcript linked to a florescent moiety using

ribosome display. The well is probed with target ligand, *i.e.*, TNF and assayed for polypeptide analogs that bind and with what binding affinity as compared to wild-type polypeptide.

Polypeptide analogs that bind better than the wild-type polypeptide are then re-engineered into a full-length IgG antibody format for parallel testing with the analogous commercial antibody for improved binding using standard techniques.

EXAMPLE 4

LOOK-THROUGH MUTAGENESIS OF ANTIBODIES AGAINST BOTULINUM NERVE TOXIN SEROTYPE B (BoNT/B) AND BOTULINUM NERVE TOXIN SEROTYPE A (BoNT/A) TO IMPROVE FUNCTION

In this example, improved antibodies against botulinum nerve toxin serotype B (BoNT/B) and botulinum nerve toxin serotype A (BoNT/A) are generated using look-through mutagenesis (LTM) to improve function. The LTM approach is based on creating single mutations per CDR throughout the binding pocket, based on a subset (the LTM set) of the 20 amino acids that explore size, charge, hydrophobicity, and hydrogen bonding characteristics. The criteria for selecting the amino acids in the LTM set are discussed below.

1. Antibody Purification and Gene Sequencing and Single Chain (scFv) Design

Murine antibody fragments (Fab) with binding affinities to BoNT/B can be obtained as described in Emaneul *et al.* (1996) *Journal of Immunological Methods* 193: 189-197. The *BotFab 5* (SEQ ID NOs:10 and 11, light and heavy chain polypeptides, respectively), the *BotFab 20* (SEQ ID NOs:12 and 13, light and heavy chain polypeptides, respectively) or the *BotFab 22* (SEQ ID NOs:14 and 15, light and heavy chain polypeptides, respectively) antibodies can be used. The foregoing sequences are described in U.S. Patent No. 5,932,449, the contents of which are incorporated herein by reference. The murine BoNT/B antigen (full-length toxin, light chain, and/or heavy chain) can be obtained from Metabionics, Inc., WI.

The anti-BoNT/A antibodies described in Pless *et al.* (2001) *Infect. Immun.* 69:570 can be used with the objective of improving their affinities by at least one order of magnitude. The BoNT/A heavy chain binding domain (BoNT/A Hc) antigen can be obtained from Metabionics, Inc., WI.

The V_L and V_H fragments of the antibody(ies) are cloned and sequenced using standard molecular biology techniques. The variable regions of the molecules are amplified by the polymerase chain reaction (PCR) and linked with a poly-Gly-Ser linker (typically SGGGSGGGSGGGGS (SEQ ID NO:7)) to generate single chain antibodies (scFv). A poly-His tag (HHHHHH (SEQ ID NO:8)) and a myc tag

(EQKLISEEDL (SEQ ID NO:9)) are also appended to the C-terminus of the genes to facilitate purification and detection. These molecules are displayed on any of the well known technologies (*e.g.*, yeast, bacterial or phage based technologies) and tested for their ability to bind BoNT/B or BoNT/A.

- 5 The monovalent scFv version of whole antibodies provide for a good format to undertake mutagenesis studies and are known to generally reproduce the binding mechanism of the whole molecule, with the exception of the avidity effects displayed by multivalent molecules.

10 **2. Antibody Improvement Using Look Through Mutagenesis (LTM) and Gene Design**

- For the Look Through Mutagenesis (LTM), the following nine amino acids and their representative functional characteristics are chosen: Alanine and Leucine (aliphatics), Serine (hydroxyl group), Aspartic Acid (acidic) and Glutamine (amide),
- 15 Lysine and Histidine (basic), Tyrosine (aromatic), Proline (hydrophobic). These amino acids display adequate chemical diversity in size, charge, hydrophobicity, and hydrogen bonding ability to provide meaningful initial information on the chemical functionality needed to improve antibody properties. The choices are also based on the frequency of occurrence of these amino acids in the CDRs of antibodies. For example, given between
- 20 tyrosine and phenylalanine to represent amino acids with aromatic side chains, the former is chosen because of its significantly higher preponderance in antibody CDRs and its ability to hydrogen bond. LTM is initially employed to identify specific amino acids and chemical properties that are beneficial for binding, neutralization and/or any additional properties desired in the final antibody. However, LTM is not limited to these
- 25 nine amino acids or nine total amino acids. LTM analysis can be performed with any combination of amino acids and the LTM subset can be as high as 18 amino acids (1 short of saturation mutagenesis).

2.1 LTM Oligonucleotide Synthesis

- 30 In the primary LTM analysis, the goal is to explore each amino acid's side chain contribution to the overall binding affinity within each CDR. To efficiently generate meaningful diversity, each of the LTM subset amino acid is targeted at every single position within the CDR sequence with only one substitution per CDR. Thus, each individual oligonucleotide encodes for only a single CDR mutation. For instance, in
- 35 order to do LTM Histidine mutagenesis on a hypothetical eleven amino acid V_H CDR3 domain, 11 oligonucleotides are synthesized that encode for all 11 possible single Histidine mutations (see Figure 20). Such an analysis tests the effects of having a bulky amide in each position in the CDR. Therefore to generate a V_H CDR3 LTM library,

only 99 oligonucleotides are synthesized for the LTM analysis (9 LTM amino acids x 11 V_H CDR3 positions). Oligonucleotide sequences are tested for inadvertent stop codons, wild-type duplication, inefficient codon usage, hairpins, loops, and other secondary structures using publicly available software.

5

2.2 Degenerate oligonucleotide synthesis for combinations of beneficial LTM mutations

In employing systematic LTM replacement of individual amino acids within a CDR, the preference of chemical functionalities at each position is uncovered. In order to combine all the mutations from the LTM selections and explore possible additivity and energetic synergy between these, degenerate oligonucleotides encoding for these mutations and the wild-type sequence are synthesized. This degenerate pool of oligonucleotides with 1.6×10^4 variants is subsequently used to generate a second generation library that explores the additive nature of these substitutions.

15

2.3 Computer Assisted Oligonucleotide Design, Library, and Results Database

Software coupled with automated custom-built DNA synthesizers enables rapid oligonucleotide synthesis. The first step involves deciding which target amino acids will be incorporated into the CDRs. The software determines the codon preference (*e.g.*, yeast, bacterial or phage codon preference, depending on the chosen system) needed to introduce the targeted amino acids and also eliminates any duplication of the wild-type sequence that may be generated by this design process. It then analyzes for potential stop codons, hairpins, and loop structures or for other problematic sequences that are subsequently corrected prior to synthesis. The completed LTM design plan is then sent to the DNA synthesizer, which performs an automated synthesis of the oligonucleotides. In this manner, the oligonucleotides needed to create the libraries can be rapidly generated.

An electronic database may store information of all LTM oligonucleotide sequences synthesized, details of the scFv library CDR substitutions, and binding assay results for a target antigen. Archival of the oligonucleotide data allows for rational iterations of the design strategies, and re-use of reagent oligonucleotides.

3. Overall LTM Strategy

LTM is initially employed to identify specific amino acids and chemical properties that are beneficial for binding, neutralization and/or any additional properties desired in the final antibody. It also quickly identifies the regions that do not tolerate any mutagenesis without significant loss in affinity (or any physical property being selected). Therefore, not only is the LTM analysis a good methodology to explore the chemical

requirements at each position in all CDRs of an antibody, but it also rapidly determines the amino acids absolutely required for antigen binding. After the identification of beneficial mutations by LTM, combinatorial mutagenesis schemes may be employed that firstly incorporate all these disparate amino acid mutations to generate multiply
5 mutated CDRs. Additionally or alternatively, "Walk Through" mutagenesis (WTM) may be used to probe the effects of multiple mutations of the same amino acid in a CDR (as described, for example, in U.S. Patent Nos: 5,830,650; 5,798,208).

4. LTM scFv Libraries

10 The LTM techniques described above are used to create pools of oligonucleotides with mutations in a single CDR of the light or heavy chain. These oligonucleotides are synthesized to include some of the surrounding framework to facilitate the overlap and hybridization during PCR. These pools of oligonucleotides are utilized to generate all possible V_L and V_H chains in which there are mutations in single,
15 double, and triple CDRs (single, double, and triple combinations of CDR1, 2, and 3) using single overlap extension PCR (SOE-PCR) (as described by Horton *et al.* (1989) *Gene* 77:61-68). SOE-PCR is a fast and simple method for combining DNA fragments that does not require restriction sites, restriction endonucleases, or DNA ligase. In SOE-PCR two regions in the gene are first amplified by PCR using primers designed so that
20 the PCR products share a complementary sequence at one end. Under PCR conditions, the complementary sequences hybridize, forming an overlap. The complementary sequences then act as primers, allowing extension by DNA polymerase to produce a recombinant molecule.

For example, to create the pool of V_H chains in which both CDR-H1 and CDR-
25 H2 are mutated and CDR-H3 is wild-type, which is denoted as "110" (1 denotes a mutant CDR and 0 denotes a wild-type CDR), the CDR-H1 mutant genes are used as templates and SOE-PCR is conducted to link the CDR-H2 oligonucleotides to generate the doubly mutated pool (Figure 21). Considering that each CDR may be either wild-type or mutant, there are seven possible combinations (depicted by arrows in Figure 21)
30 for each of the pools of V_L and V_H chains (not including the wild-type molecule "000"). Combining the seven V_L and eight V_H pools creates 63 V_L - V_H non-wild-type combinations (scFvs), (Figure 21). Each of the 64 V_L - V_H combinations (including the wild-type sequence) is termed as a "subset" of the whole LTM scFv library ensemble. An scFv library ensemble is created for each amino acid selected for substitution.
35 The number of amino acid sequences represented within each subset library depends on the length of the CDR, the amino acid sequence within the CDR, and the LTM oligonucleotide design strategy.

5. High-Throughput Library Screening and Improved Antibody Selection

A variety of methods are available for antibody expression and display. These include bacteriophage, *Escherichia coli*, and yeast. While each of these methods has been used for antibody improvement, the yeast display system affords several advantages (Boder and Wittrup (1997) *Nat. Biotechnol.* 15:553-557). Yeast can readily accommodate library sizes up to 10^7 , with 10^3 - 10^5 copies of each antibody being displayed on each cell surface. Yeast cells are easily screened and separated using flow cytometry and fluorescence-activated cell sorting (FACS) or magnetic beads. Yeast also affords rapid selection and re-growth. The eukaryotic secretion system and glycosylation pathways of yeast allow for a much larger subset of scFv molecules to be correctly folded and displayed on the cell surface than prokaryotic display systems. Yeast display coupled with directed evolution has been used to increase the K_D of an scFv antibody fragment for fluorescein to 48 fM, two orders of magnitude stronger binding than any previously reported monovalent ligand (Boder *et al.* (2000) *PNAS* 97: 10701-10705). The display system utilizes the α -agglutinin yeast adhesion receptor to display proteins on the cell surface. The proteins of interest, in the present case, anti-BoNT/B scFv LTM libraries or anti-BoNT/A scFv LTM libraries, are expressed as fusion partners with the Aga2 protein. These fusion proteins are secreted from the cell and become disulfide linked to the Aga1 protein, which is attached to the yeast cell wall (see Invitrogen, pYD1 Yeast Display product literature). In addition, there are carboxyl terminal tags included which may be utilized to monitor expression levels and/or normalize binding affinity measurements.

Streptavidin coated magnetic beads (SpheroTech) are used to screen and select for antibodies that bind to BoNT/B or BoNT/A with high affinity. This methodology employs high affinity antibody binding to biotinylated antigen which then binds to streptavidin coated beads in order to select for the yeast clones (Yeung and Wittrup (2002) *Biotechnol. Prog* 18:212-220) and Feldhaus *et al.* (2003) *Nature Biotech.* 21:163-170). The BoNT/B or BoNT/A polypeptide (MetabioLogics) is biotinylated using standard protocols (Pierce) and screening is performed using methods well known in the art. Using equilibrium and kinetic based selection, antibodies with improved affinities are selected for from these libraries. The efficacy of each round of selection is monitored by analytical FACS (FACScan). In addition, relative binding affinities of individual molecules displayed on the yeast surface are measured by titrating the antigen. This allows for rapid identification of molecules with improved affinity. The scFv clones are then sequenced to identify beneficial mutations.

6. Generation of Soluble Antibodies for BIAcore Affinity Measurements

Antibodies of interest are sub-cloned into soluble expression systems (*Pichia pastoris* and/or *E. coli*) and soluble protein is generated. There are several commercially available vectors and cell lines for soluble antibody expression, including those from Invitrogen (e.g., pPIC9 for *P. pastoris*) and Novagen (pET20b for periplasmic expression in *E. coli*). These systems are routinely used to generate soluble single chain or full-length antibodies. The *P. pastoris* expression system (Invitrogen) routinely produces 1-5 mg per liter of soluble purified scFv. Purification of proteins is facilitated by the presence of a His-tag at the C-terminus of the molecule, in the case of single chains or by protein A or protein G columns for full-length antibodies. Soluble single chain and full-length antibodies are generated to obtain BIAcore affinity kinetic rate measurements. This step is necessary as high affinity scFv molecules on the yeast clone cell surface must be verified as a cell free soluble molecule.

Soluble single chain and full-length antibodies generated in the foregoing manner may be used to obtain BIAcore affinity measurements, as well as in the neurite outgrowth assay or the mouse lethality assay described below.

7. Screening of Selected Antibodies for *In Vivo* or *In Vitro* Neutralization

A cell-based assay using the neurite outgrowth of primary chick neurons as an indicator of BoNT intoxication and, thus, as a means to quantify toxin neutralization may be used to screen the selected antibodies. Preliminary experiments using dorsal root ganglion explant cultures from fertilized chicken eggs have indicated that there was less axonal outgrowth from explants treated with BoNT/A than the controls.

Alternatively, a chick ciliary ganglion-iris muscle neuromuscular junction assay (as described in Lomneth *et al.* (1990) *Neuroscience Letters* 113:211-216) may be used.

The mouse lethality assay (MLA, as described by, for example, Schantz and Kautter (1978) *J. Assoc. Off. Anal. Chem.* 61:96-99) is another well known and accepted *in vivo* method for testing for BoNT neutralization. The test involves the interperitoneal injection of approximately 0.5 ml of sample preparations of BoNT/B or BoNT/A with and without the antibody into 20 to 30 gm, white ICR strain mice. Lethality due to respiratory failure is noted over 1-4 days. Quantification of neutralization requires serial dilutions of the MAbs with varying levels of mouse BoNT/B or BoNT/A LD₅₀. The MLA may be used to determine the neutralizing ability of the optimized antibodies identified by the *in vitro* screening.

The neutralizing ability of antibodies can also be measured *in vitro* by the mouse protection assay (MPA, Goeschel *et al.* (1997) *Exp. Neurol.* 147:96-102). In the MPA the left phrenic nerve, together with the left hemidiaphragm, is excised from the mouse.

The phrenic nerve is then continuously electrostimulated in a tissue bath. Purified antibodies are incubated with BoNT/B or BoNT/A and added to the tissue bath. Toxin induced paralysis is defined as a 50% reduction in the initial muscle twitch.

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Equivalents

- 10 Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

Claims

1. A method of generating a library of polypeptide analogs in which a predetermined amino acid appears at each position in a defined region of the polypeptide comprising:
 - 5 selecting a defined region of the amino acid sequence of the polypeptide;
 - determining an amino acid residue to be substituted at each amino acid position within the defined region;
 - synthesizing individual polynucleotides encoding the defined region, the polynucleotides collectively representing possible variant polynucleotides according to
 - 10 the following criteria:
 - i) each polynucleotide containing at each codon position in the defined region, either a codon required for the amino acid residue of the polypeptide or a codon for the predetermined amino acid residue, and
 - ii) each polynucleotide containing no more than one codon for the predetermined
 - 15 amino acid residue,
 - thereby generating a library of polynucleotides in which the predetermined amino acid residue appears at each amino acid position within the defined region.
2. The method of claim 1, wherein the polynucleotides are pooled together.
- 20 3. The method of claim 1, wherein two or more defined regions within the polypeptide are mutagenized.
4. The method of claim 3, wherein the same predetermined amino acid is selected for
- 25 substitution within each of the two or more defined regions.
5. The method of claim 3, wherein different predetermined amino acids are selected for substitution within each of the two or more defined regions, respectively.
- 30 6. The method of any one of the previous claims, wherein the defined region or defined regions comprises a functional domain of the polypeptide.
7. The method of claim 6, wherein the functional domain is selected from the group consisting of an antibody binding site, an antibody framework region, an antibody
- 35 effector region, a receptor binding site, and a catalytic site.

8. The method of claim 7, wherein the antibody binding site or portion thereof comprises a CDR domain selected from the group consisting of CDR1, CDR2, CDR3, CDR4, CDR5, CDR6 and a combination thereof.
- 5 9. The method of claim 7, wherein the antibody framework region comprises a domain selected from the group consisting of FR1, FR2, FR3, FR4 and a combination thereof.
10. The method of claim 7, wherein the antibody effector region comprises a domain selected from the group consisting of a complement binding site and an Fc binding
10 region.
11. The method of claim 1, wherein the predetermined amino acid residue is selected from the group consisting of Ser, Thr, Asn, Gln, Tyr, Cys, His, Glu, Asp, Lys Arg, Ala, Gly, Ile, Leu, Met, Phe, Pro, Trp and Val.
- 15 12. The method of any one of the previous claims, wherein the defined region comprises at least about 3 to 40 amino acids.
13. The method of any one of the previous claims, wherein the polynucleotides are
20 synthesized as expression library.
14. The method of any one of the previous claims, wherein the polynucleotides are synthesized using enzymatic means.
- 25 15. The method of any one of the previous claims, wherein the polynucleotides are synthesized using polymerase chain reaction.
16. The method of any one of the previous claims, wherein the expression library is selected from the group consisting of a phage display library, a ribosome/polysome
30 display library, a yeast display library, a bacterial display library and an arrayed library.
17. A library of polypeptide analogs prepared by the method of claim 1.
18. A method of identifying a polypeptide having a desired structure or function
35 comprising:
selecting a defined region of the amino acid sequence of the polypeptide;
determining an amino acid residue to be substituted at each amino acid position within the defined region;

synthesizing polynucleotides encoding the defined region, the polynucleotides collectively representing possible variant polynucleotides according to the following criteria:

- 5 i) each polynucleotide containing at each codon position in the defined region, either a codon required for the synthesis of the amino acid residue of the polypeptide or a codon for one of the predetermined amino acid residue, and
- ii) each polynucleotide containing no more than one codon for the predetermined amino acid residue,
- 10 thereby generating an expression library containing the polynucleotides; expressing the expression library to produce polypeptide analogs; and screening the polypeptide analogs to select for a polypeptide having a desired structure or function.
- 15 19. The method of claim 18, wherein the method further comprises the step of identifying the polynucleotide that encodes the selected polypeptide analog.
- 20 20. The method of claim 18, wherein the screening comprises, contacting a polypeptide with a target substrate, the polypeptide being associated with the polynucleotide encoding the polypeptide, the polynucleotide further comprising a detectable moiety, such that a variant polypeptide capable of binding a target substrate is detected and thereby identified as encoded by the polynucleotide.
- 25 21. The method of claim 20, wherein the detectable moiety is selected from the group consisting of a fluorescent moiety, a UV moiety, and a visible light absorbing moiety.
22. The method of claim 20, wherein the detectable moiety is selected from the group consisting of a biotin moiety, a GST moiety, and a His tag moiety.
- 30 23. The method of claim 20, wherein the polynucleotide is associated with the polypeptide analog using ribosome display.
24. The method of claim 18, wherein two or more defined regions within the polypeptide are mutagenized.
- 35 25. The method of claim 24, wherein the same predetermined amino acid is selected for substitution within each of the two or more defined regions.

26. The method of claim 24, wherein different predetermined amino acids are selected for substitution within each of the two or more defined regions.
27. The method of claim 18, wherein the polypeptide is a single chain antibody (sFVs).
- 5 28. The method of claim 18, wherein the defined region comprises a functional domain of the polypeptide.
- 10 29. The method of claim 18, wherein the defined region comprises a CDR or portion thereof selected from the group consisting of CDR1, CDR2, CDR3, CDR4, CDR5, CDR6 and a combination thereof.
- 15 30. The method of claim 18, wherein the defined region is an antibody framework region comprising a domain selected from the group consisting of FR1, FR2, FR3, FR4 and a combination thereof.
- 20 31. The method of claim 18, wherein the defined region is an antibody effector region comprising a domain selected from the group consisting of a complement binding site and an Fc binding region.
32. The method of claim 18, wherein the predetermined amino acid residue is selected from the group consisting of Ser, Thr, Asn, Gln, Tyr, Cys, His, Glu, Asp, Lys Arg, Ala, Gly, Ile, Leu, Met, Phe, Pro, Trp and Val.
- 25 33. A library of polynucleotides encoding polypeptide analogs comprising one or more defined regions wherein a predetermined amino acid residue is substituted at each amino acid position within the defined region, the polynucleotides collectively representing all possible variants according to the following criteria:
- 30 i) each polynucleotide contains at each codon position in the defined region, either a codon required for the amino acid residue of the polypeptide or a codon for the predetermined amino acid residue, and
- ii) each polynucleotide contains no more than one codon for the predetermined amino acid residue.
- 35 34. The library of claim 33, wherein two or more defined regions within the polypeptide are mutagenized.

35. The library of claim 33, wherein the same predetermined amino acid is selected for substitution within each of the two or more defined regions.
36. The library of claim 33, wherein different predetermined amino acids are selected
5 for substitution within each of the two or more defined regions.
37. The library of claim 33, wherein the library is an expression library.
38. The library of claim 33, wherein the expression library is selected from the group
10 consisting of a phage display library, a ribosome/polysome display library, a yeast display library, a bacterial display library and an arrayed library.
39. The library of claim 33, wherein the polynucleotides further comprise one or more
15 transcriptional regulatory elements.
40. The library of claim 39, wherein the polynucleotides, when transcribed and translated in vitro, are associated with the polypeptides encoded by the corresponding polynucleotides.
- 20 41. The library of claim 40, wherein the polynucleotides are associated with the polypeptide using ribosome/polysome display.
42. The library of claim 41, wherein the polynucleotides comprise RNA.
- 25 43. The library of claim 42, wherein the polynucleotides further comprise a detectable moiety.
44. The library of claim 43, wherein the detectable moiety comprises a fluorescent moiety.
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45. The library of claim 33, wherein the library comprises at least 10^6 different polynucleotides.
46. The library of claim 33, wherein the library comprises at least $45 \cdot 10^{12}$ different
35 polynucleotides.
47. The library of claim 33, wherein the polypeptide encodes a binding polypeptide.

48. The library of claim 47, wherein the binding polypeptide is selected from the group consisting of a heavy chain variable region (V_H), a light chain variable region (V_L), and a single chain antibody (sFv).
- 5 49. The library of claim 33, wherein the polynucleotide encodes an enzyme.
50. The library of claim 33, wherein the polynucleotide encodes an enzyme inhibitor.
51. The library of claim 33, wherein the polynucleotide encodes a catalytic polypeptide.
- 10 52. The library of claim 33, wherein the library is immobilized on a solid support.
53. The library of claim 33, wherein the solid support is a microchip.
- 15 54. The library of claim 33, wherein the library is an arrayed library.
55. A microchip comprising an array of immobilized polynucleotides according to the library of claim 33.
- 20 56. A polypeptide analog identified according to the library of claim 33, wherein the polypeptide binds to a target molecule and comprises a binding region selected from the group consisting of a heavy chain variable region (V_H), a light chain variable region (V_L), and a single chain antibody (sFv).
- 25 57. The molecule of claim 56, wherein the specified target molecule is $TNF\alpha$.
58. The molecule of claim 56, wherein the polypeptide has at least 70% identity to the amino acid sequence of SEQ ID NO:1, 2, 3, 4, 5 or 6.
- 30 59. A method of identifying a subset of polypeptide analogs having a desired structure or function comprising:
- selecting a defined region of the amino acid sequence of the polypeptide;
- determining an amino acid residue to be substituted at each amino acid position within the defined region;
- 35 synthesizing polynucleotides encoding the defined region, said polynucleotides collectively representing possible variant polynucleotides according to the following criteria:

- i) each polynucleotide containing at each codon position in the defined region, either a codon required for the synthesis of the amino acid residue of the polypeptide or a codon for the predetermined amino acid residue, and
- 5 ii) each polynucleotide containing no more than one codon for the predetermined amino acid residue,
- thereby generating an expression library containing the polynucleotides; exposing the expression library to conditions under which the library is expressed;
- 10 screening the expressed library to identify a polypeptide having a desired structure or function;
- comparing the structure or function of the polypeptide as compared to a control criterion, wherein a polypeptide that corresponds or exceeds the control criterion is categorized as a responder and a polypeptide that fails the control criterion is categorized
- 15 as a nonresponder;
- categorizing responders and nonresponders in a database; and
- querying the database to determine the sequence of a subset polypeptides to be synthesized.
- 20 60. The method of claim 59, wherein one or more of the above steps is computer-assisted.
61. The method of claim 59, wherein the control criterion is selected from the group consisting of binding affinity, stability and effector function.
- 25 62. The method of claim 59, wherein the control criterion is catalytic activity on a specified substrate.
63. The method of claim 59, wherein the polypeptide is selected from the group
- 30 consisting of a heavy chain variable region (V_H), a light chain variable region (V_L), and a single chain antibody (sFv).
64. A medium suitable for use in an electronic device having instructions for carrying out one or more steps of the method of claim 59.
- 35 65. A device for carrying out one or more steps of the method of claim 59.