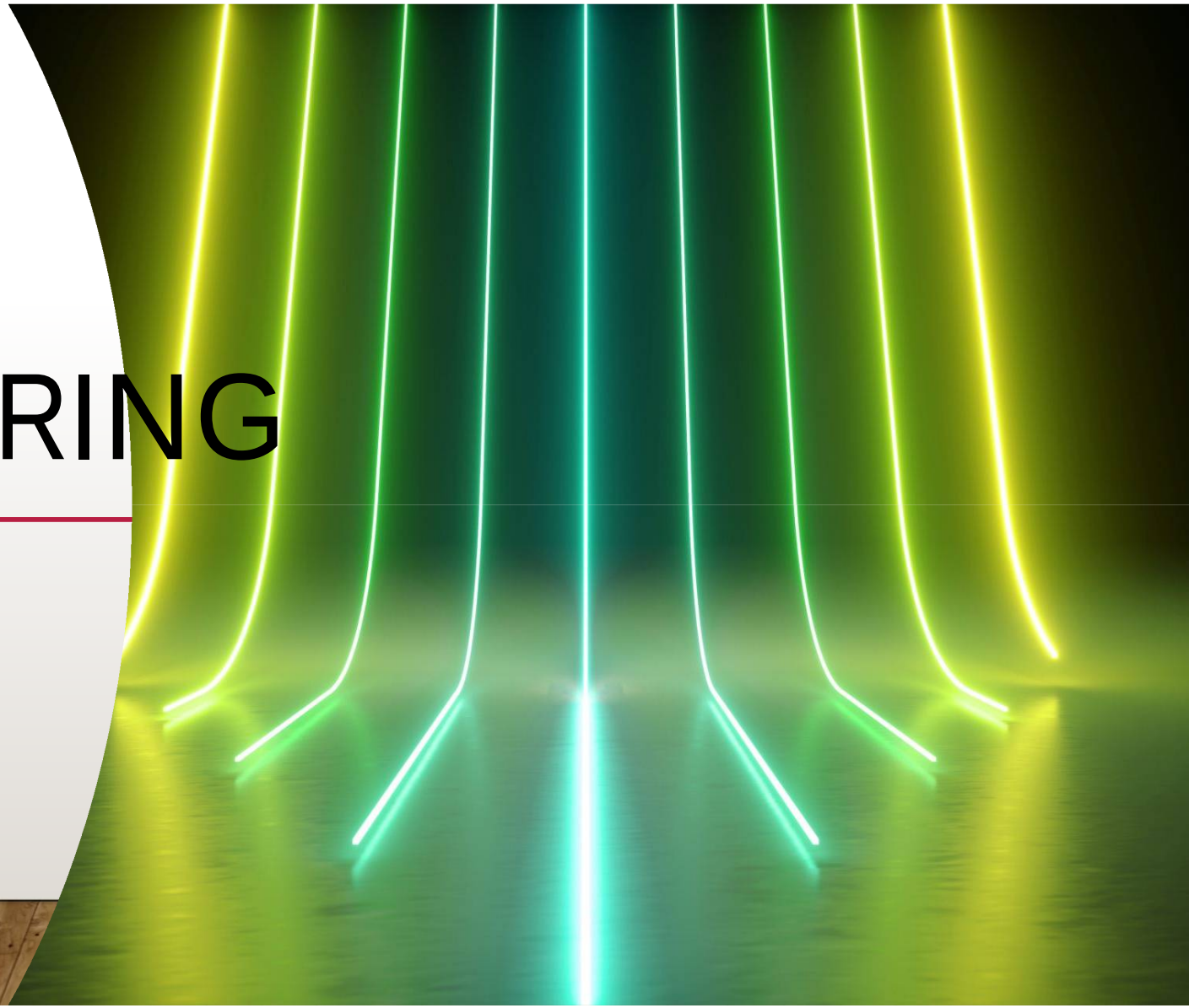
PROTEIN ENGINEERING



BASICS AND DESIGNING

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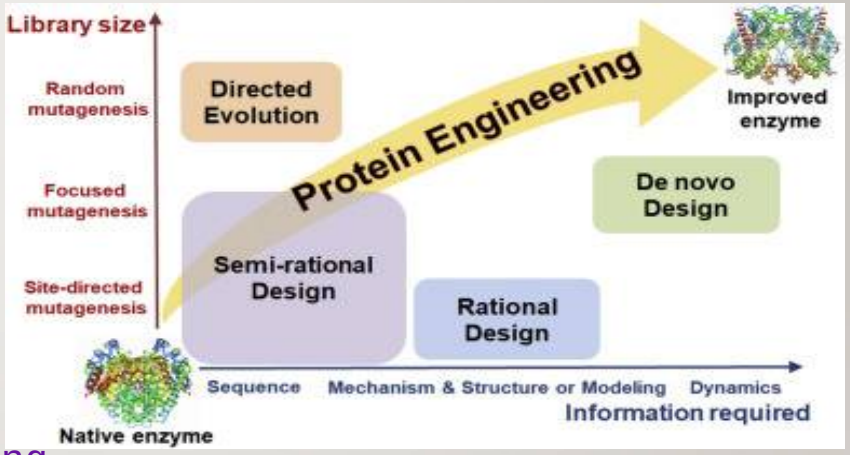
The process for the development of new proteins through directed evaluation and rational protein designing, which can be used as therapeutic agents is protein engineering.



This involves altering the structure proteins by modifying Gene sequences is a powerful and widely used technique that can greatly speed up the identification of novel protein variants.

The desired effect might be alteration of the catalytic activity of an enzyme by modification of the residues around the active site, an improvement in the nutritional status of a storage protein, or an improvement in the stability of a protein used in industry or medicine.

Proteins that have been engineered by the incorporation of mutational changes have become known as **muteins**.

DJA2 Dr Jaya Arora, 01-05-2020

INTRODUCTION

- Proteins possess a broad range of structural and functional properties (enzymes) that are unmatched by any other class of biological molecules.
- Nature has also inspired many scientists and engineers to design and create their own customized proteins.
- These engineered proteins can serve as novel molecular tools for scientific, medical and industrial applications, thus addressing many needs unmet by naturally occurring proteins.
- Protein engineering requires identification of particular amino acid sequences that will result in desired structural and functional properties. Despite recent advances in the field, however, protein engineering remains as much an art as it is a science.
- Engineering an arbitrary protein structure or function remains a formidable challenge, because the rules defining sequence-structure-function relationships are still not well understood. Even with refined quantitative models, the large degrees of freedom present in a typical protein do not easily allow identification of optimal sequences using currently available computational techniques. Furthermore, the complexity of proteins present engineering challenges whose solutions will most likely require a combination of experimental and computational approaches.

IMPORTANCE

- In the agrochemical industry, Protein engineering holds an immense opportunity as engineering in proteins may lead to the generation of enzymes with improved function that may increase the crop yield or facilitate the biofuel production.
- A tool to attain increased crop yield needed to meet future demand is also likely to be played as an important role. The prevalence increasing of protein deficient diseases, adoption of protein drugs over non-protein drugs, demand for alternatives to chemical processes, and rising government funding for protein engineering are the propelling factors in the global protein engineering market.
- A group of disorders that includes marasmus, kwashiorkor is Protein energy malnutrition (PEM). It is increasing in the rural areas of emerging economies. The protein deficient diseases is driving the overall market by increasing. The government initiatives is rising in number such as funding R&D for protein engineering and awareness programs, which have influenced the healthcare industry. Thus, the government is proactively investing in many of the research. Protein Technologies Ltd (PTL) has received funding from the UK government's technology Strategy Board for its innovative research in protein engineering.

SCOPE OF PROTEIN ENGINEERING

By Product Type	• **Modified Enzymes** • **Insulin** • **Monoclonal Antibodies** • **Coagulation Factors (Blood factors + Tissue plasminogen)** • **Vaccines** • **Growth Factors (Hormones + Cytokine)** • **Other Product Types**
By Technology	• Irrational Protein Design • Rational Protein Design
By End User	• Pharmaceutical and • Biotechnology Companies • Academic Institutions • Contract Research Organizations (CROs)

METHODS

Desired protein can be obtained broadly through two mathods which involve gene (chromosome) level manipulation

a. Rational Design
b. Directed evolution

Some desired changes in protein can be done at its structural level in which one methods Addition of Disulphide bonds is described in this presentation.

RATIONAL DESIGN

- The use of a rational design protocol depends on some detailed information about the protein being available. Typically the target protein may have been characterized biochemically, and its gene cloned and sequenced. Thus, the mRNA coding sequence, predicted or actual amino acid sequence, three-dimensional structure, folding characteristics, and so on may be available. This information is used to predict what the effect of changing part of the protein might be; the change then be made and altered protein is tested to see the desired changes have been made or not.
- A procedure known as mutagenesis in vitro enables specific mutations to be introduced into a gene sequence One variant of this technique is called oligonucleotide directed or site-directed mutagenesis and is elegantly simple in concept.

Mutagenesis in vitro is an elegant technique that is used to introduce defined mutations into a cloned DNA sequence to alter the amino acid sequence of the protein that it encodes can then be made and the altered protein tested to see if the desired changes have been incorporated.

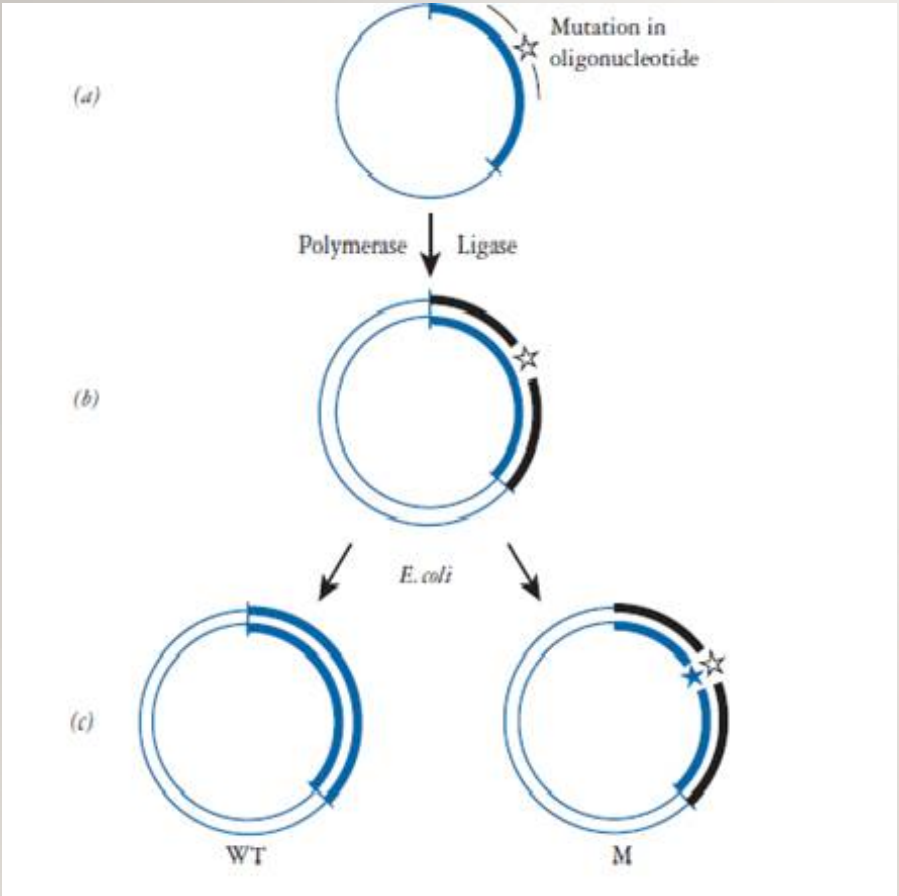
The requirements are a single-stranded (ss) template containing the gene to be altered, and an oligonucleotide (usually 15--30 nucleotides in length) that is complementary to the region of interest.

The oligonucleotide is synthesised with the desired mutation as part of the sequence. The ss template is often produced using the M13 cloning system, which produces ss DNA. The template and oligonucleotide are annealed (the mutation site will mismatch, but the flanking sequences will confer stability), and the template is then copied using DNA polymerase. This gives rise to a double-stranded DNA. When this is replicated it will generate two daughter molecules, one of which will contain the desired mutation.

Identification of the mutated DNA can be carried out by hybridization with the mutating oligonucleotide sequence, which is radiolabelled. Non-mutated DNA will retain the original mismatch, whereas the mutant will match perfectly. By washing filters of the suspected mutants at high stringency, all imperfect matches can be removed and the mutants detected by autoradiography. Even a single base-pair change can be picked up using this technique. The mutant can then be sequenced to confirm its identity.

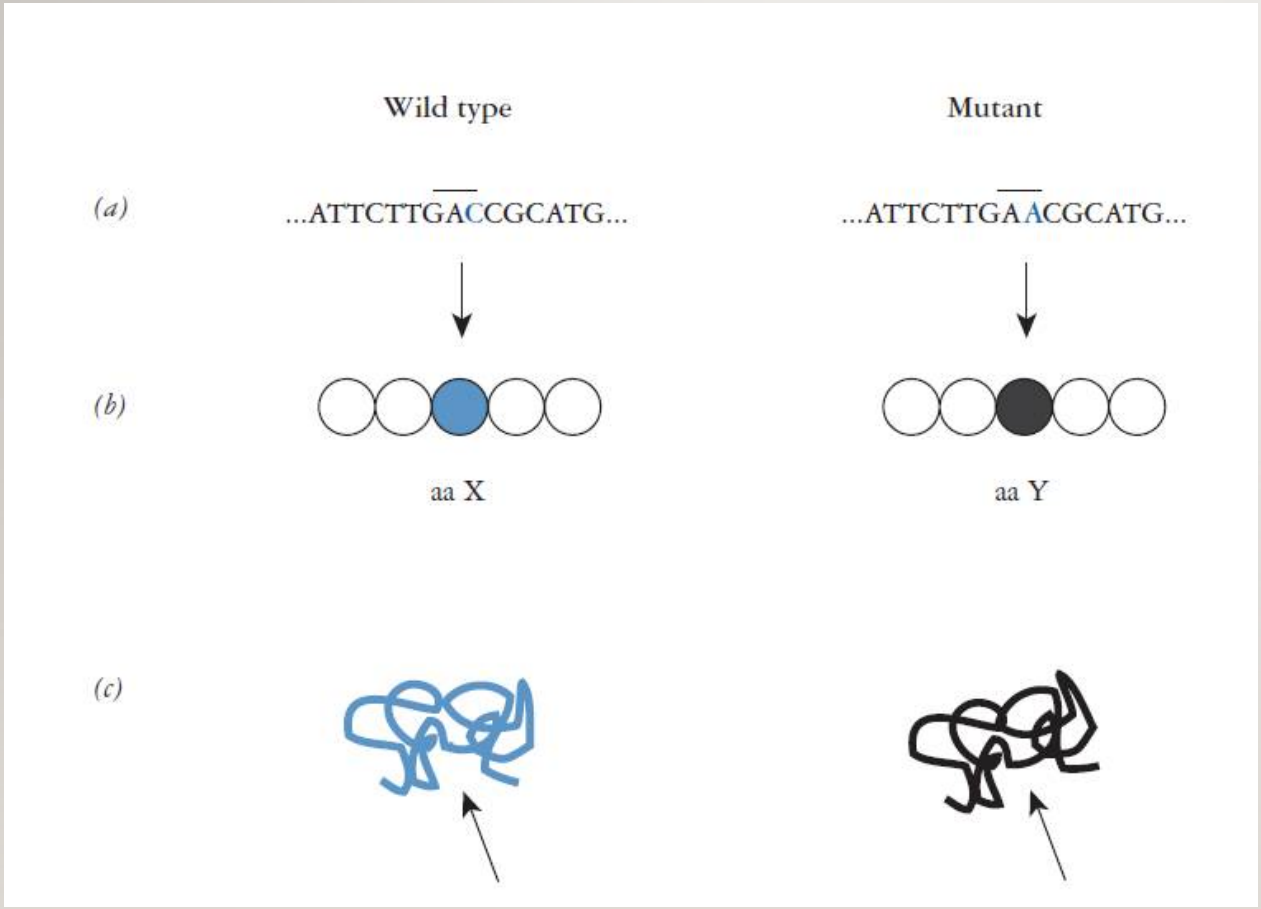
Having altered a gene by mutagenesis, the protein is produced using an expression system. Often a vector incorporating the *lac* promoter is used, so that transcription can be controlled by the addition of IPTG. Alternatively, the *P*L promoter can be used with a temperature-sensitive cI repressor, so that expression of the mutant gene is repressed at 30◦C but is permitted at 42◦C.

Analysis of the mutant protein is carried out by comparison with the wild-type protein. In this way, proteins can be 'engineered' by incorporating subtle structural changes that alter their functional characteristics.



Oligonucleotide-directed mutagenesis. (*a*) The requirement for mutagenesis *in vitro* is a single-stranded DNA template containing a cloned gene (heavy line). An oligonucleotide is synthesised that is complementary to the part of the gene that is to be mutated (but which incorporates the desired mutation). This is annealed to the template (the mutation is shown as an open star). (*b*) The molecule is made double-stranded in a reaction using DNA polymerase and ligase, which produces a hybrid wild-type/mutant DNA molecule with a mismatch in the mutated region. (*c*) On introduction into *E. coli* the molecule is replicated, thus producing double-stranded copies of the wild-type (WT) and mutant (M) forms. The mutant carries the original mutation and its complementary base or sequence (filled star).

Oligonucleotide directed or site-directed mutagenesis



Protein engineering by the rational design method. In this procedure, some knowledge of the gene and protein sequence is required. A change is then introduced into the gene, as shown in (*a*). In this case the codon GAC is altered to GAA by changing the third base in the codon DNA sequence. This causes a change in the amino acid sequence, shown in (*b*) as a change from aa X to aa Y. This causes a change in the way that the protein folds, shown in (*c*). In this example the active site (shown by the arrows) becomes slightly larger in the altered molecule.

DJA1

DIRECTED EVOLUTION

- The principle of this method is that the gene is mutated, either at a discrete site or at random, and then selection made for a protein variant with the desired property. The improved variant can be subjected to further rounds of mutagenesis and selection, a process known as *directed evolution*. The paradigm for this approach is the enzyme subtilisin

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One of the main disadvantages of the rational design approach to protein engineering is that a very large range of potential structures can be derived by modification of a protein sequence in various ways.

Thus, it is very difficult to be sure that the modification that is being incorporated will have the desired effect -- and the process is labour-intensive.

Directed evolution is a recent development that has increased the range and scope of producing new protein variants.

The technique of directed evolution removes the need for predictive alterations to DNA and protein sequences and is potentially more powerful than site-directed methods.

As the name suggests, Directed Evolution is more like an evolutionary process, rather than the incorporation of a specific alteration in a defined part of the protein. The increase in potential benefit comes not from the fact that any *particular* structural alteration is created, but that a large number of different alterations are generated and the desired variant selected by a process that mimics natural selection.

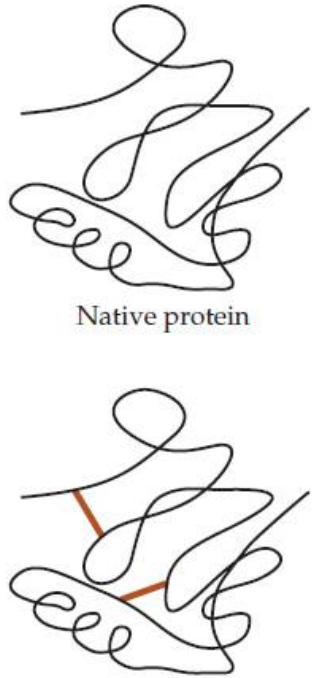
Thus, the need for predictive structural alteration is removed, as the system itself enables large numbers of changes to be generated and screened efficiently.

The process generates a library of recombinants that encode the protein of interest, with random mutagenesis (often using techniques such as **error-prone PCR**) applied to generate the variants. Thus, a large number of different sequences can be generated, some of which will produce the desired effect in the protein. These can be selected by expressing the gene and analysing the protein using a suitable assay system to select the desired variants.

Additional rounds of mutagenesis and selection can be applied if necessary. An extension to this technique called **DNA shuffling** can be used to mix pieces of DNA from variants that show desired characteristcs.This mimics the effect of recombination that would occur *in vivo* and can be an effective way of 'fast-tracking' the directed evolution technique.

ADDING DISULFIDE BONDS

- The thermostability of a protein can be increased by creating a molecule that will not readily unfold at elevated temperatures. In addition, these thermostable enzymes are often resistant to denaturation by organic solvents and non physiological conditions, such as extremes of pH. The addition of disulfide bonds (through the introduction of specifically placed cysteine residues) can usually significantly increase the stability of a protein. The problem is whether extra disulfide bonds perturb the normal functioning of a protein

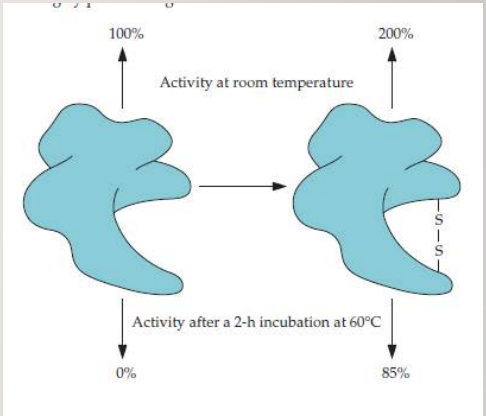


Schematic representation of the engineering of a protein that contains two engineered disulfide bridges (colored lines in the bottom diagram) that hold together and may stabilize regions of the protein that are often separated in the primary amino acid sequence.

Xylanase. In a similar study, the development of a temperature-stable mutant of the enzyme xylanase from *Bacillus circulans* was undertaken.

During the making of paper, wood pulp is chemically treated to remove the hemicellulose that would otherwise contribute to the discoloration of the paper product. Unfortunately, this step results in the creation of large amounts of potentially toxic effluent.

From an environmental perspective, treatment of wood pulp with xylanase, which degrades hemicellulose, is preferred to pulping. Treatment of wood pulp with this enzyme could lower the amount of bleaching chemical that would otherwise be required as a part of this process. However, the step at which xylanase would be added follows the hot-alkali treatment of the pulp. While it is possible to lower the pH of this material by adding acid, current industry practice is directed toward using less water to cool the pulp, so if xylanase were to be used in this process, it must function efficiently at relatively high temperatures. See the figure how protein engineering is used to stabilize the enzyme.



Addition of a disulfide bond between the N and C termini of *B. circulans* xylanase stabilizes the protein. Its activity at room temperature is doubled, and at 60°C it is largely protected against heat inactivation.

Thanks

Resources used for content and figures:

1. An introduction to Genetic Engineering Third edition by Desmond S.T. Nicholl
2. Molecular Biotechnology 4th edition By Bernard R. Glick., Jack J. Pasternak and Cheryl L. Patten