دانشگاه علوم پزشکی و خدمات بهداشتی،درمانی کرمانشاه
Kermanshah University Of Medical Sciences

BOOK REVIEW (CLARK BIOTECHNOLOGY)

Protein Engineering

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Faculty of Medicine, class No.3 [Shahid Nowroznezhad] 12:30 p.m

دوشنبه 2 مرداد 1402 ساعت 12:30

Protein Engineering

Presented by: Maryam Mehrang

Supervaiser: Dr.Bahrami

Contents

Introduction

A variety of enzymes have been in industrial use since before genetic engineering appeared.

These proteins are used under relatively harsh conditions and are exposed to oxidizing conditions not found inside living cells.

Table 11.1 Important Proteins Used Industrially

Protein	Function
Amylases	Hydrolysis of starch for brewing
Lactase	Hydrolysis of lactose in milk processing
Invertase	Hydrolysis of sucrose
Cellulase	Hydrolysis of cellulose from plant materials
Glucose isomerase	Conversion of glucose to fructose for high-fructose syrups
Pectinase	Hydrolysis of pectins to clarify fruit juices, etc.
Proteases (ficin, bromelain, papain)	Hydrolysis of proteins for meat tenderizing and clarification of fruit juices
Rennet	Protease used in cheese making
Glucose oxidase	Antioxidant in processed foods
Catalase	Antioxidant in processed foods
Lipases	Lipid hydrolysis in preparing cheese and other foods

Increasing the range of industrial enzymes has three facets.

First, modern biology has identified many novel enzyme-catalyzed reactions that may be of industrial use.

Second, it is now possible to produce desired proteins in large amounts because of gene cloning and expression systems.

Third, the sequence of the protein itself may be altered by genetic engineering to improve its properties.

This is known as protein engineering is the subject of the present chapter

ENGINEERING DISULFIDE BONDS

1- The introduction of extra disulfide bonds is a relatively straightforward way to increase the stability of proteins.

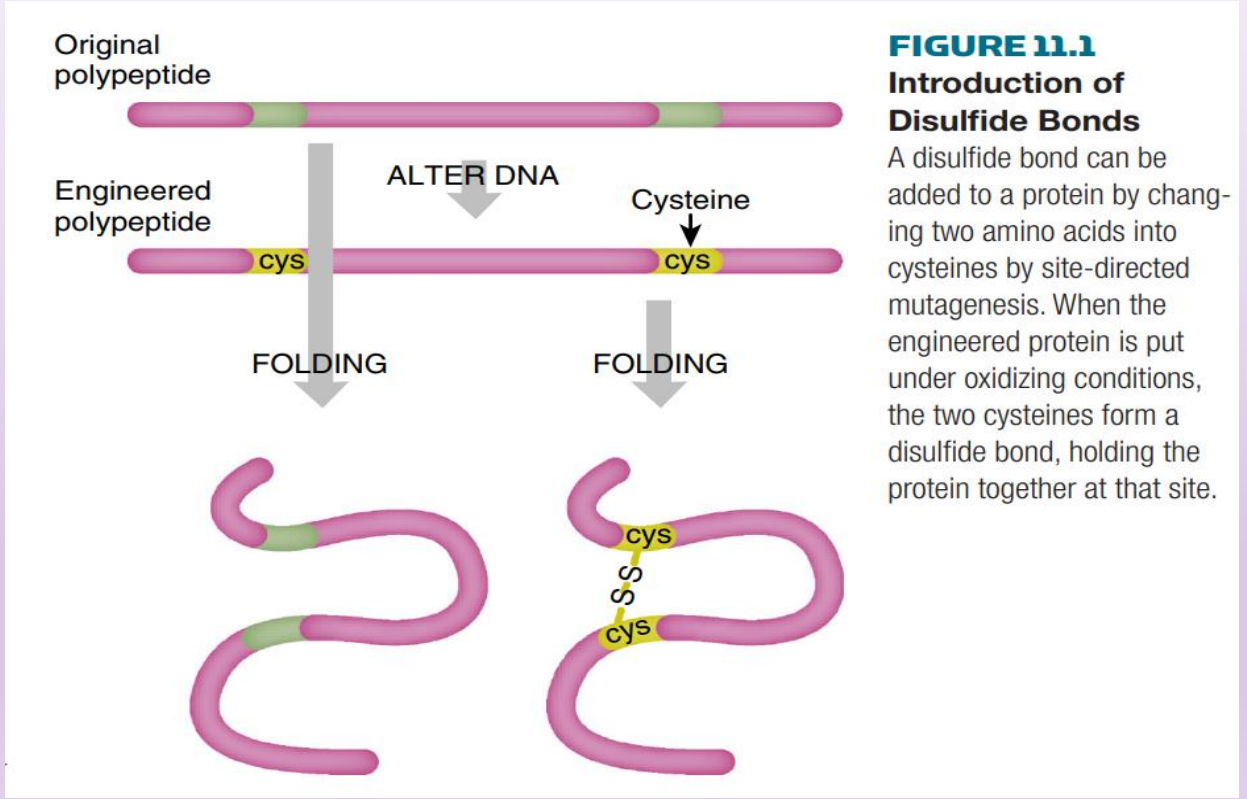


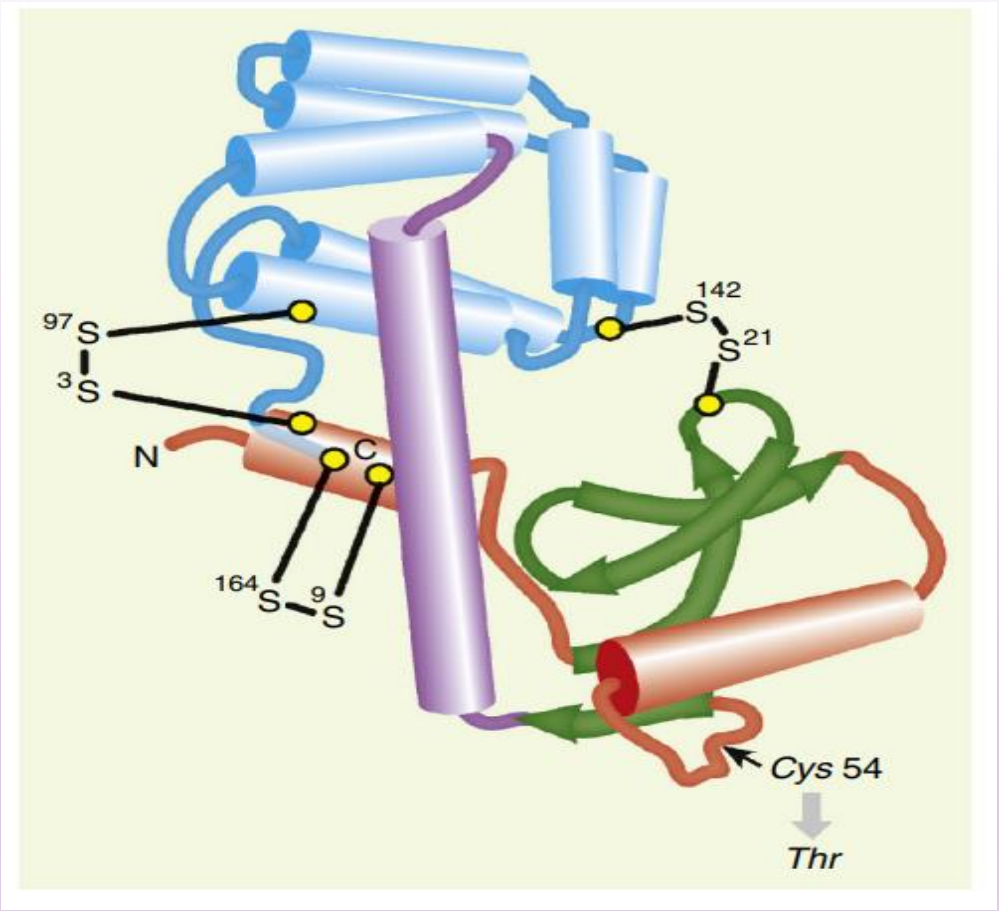
FIGURE 11.1
Introduction of Disulfide Bonds
A disulfide bond can be added to a protein by changing two amino acids into cysteines by site-directed mutagenesis. When the engineered protein is put under oxidizing conditions, the two cysteines form a disulfide bond, holding the protein together at that site.

lysozyme from bacterial virus T4.

structure of this enzyme has been solved by X-ray crystallography.
164 amino acids folds into two domains and has two cysteines
Cys54, Cys97

Extensive analysis of possible locations for disulfides was carried out.

Stability was measured by thermal denaturation; the melting
temperature, Tm, is the temperature at which 50% of the protein is denatured.



Although all three disulfides increased stability of the protein, the engineered T4 lysozyme with the 21–142 disulfide had lost its enzyme activity.

Table 11.2 Disulfide Stabilization of T4 Lysozyme

	Disulfide Bonds Present				
Protein	3–97	9–164	21–142	Stability as Tm	Activity (%)
Original	–	–	–	41.9	100
1	+	–	–	46.7	96
2	–	+	–	48.3	106
3	–	–	+	52.9	0
4	+	+	–	57.6	95
5	–	+	+	58.9	0
6	+	+	+	65.5	0

IMPROVING STABILITY IN OTHER WAYS

2 - **Glycine**, whose R-group is just a hydrogen atom, has more **conformational freedom** than any other amino acid residue. In contrast, with its rigid ring, the proline has the **least conformational freedom**.

3- Because hydrophobic residues tend to exclude water, these residues tend to cluster in the center of proteins and avoid the outer surface. If cavities exist in the hydrophobic core, filling them should increase **protein stability**.

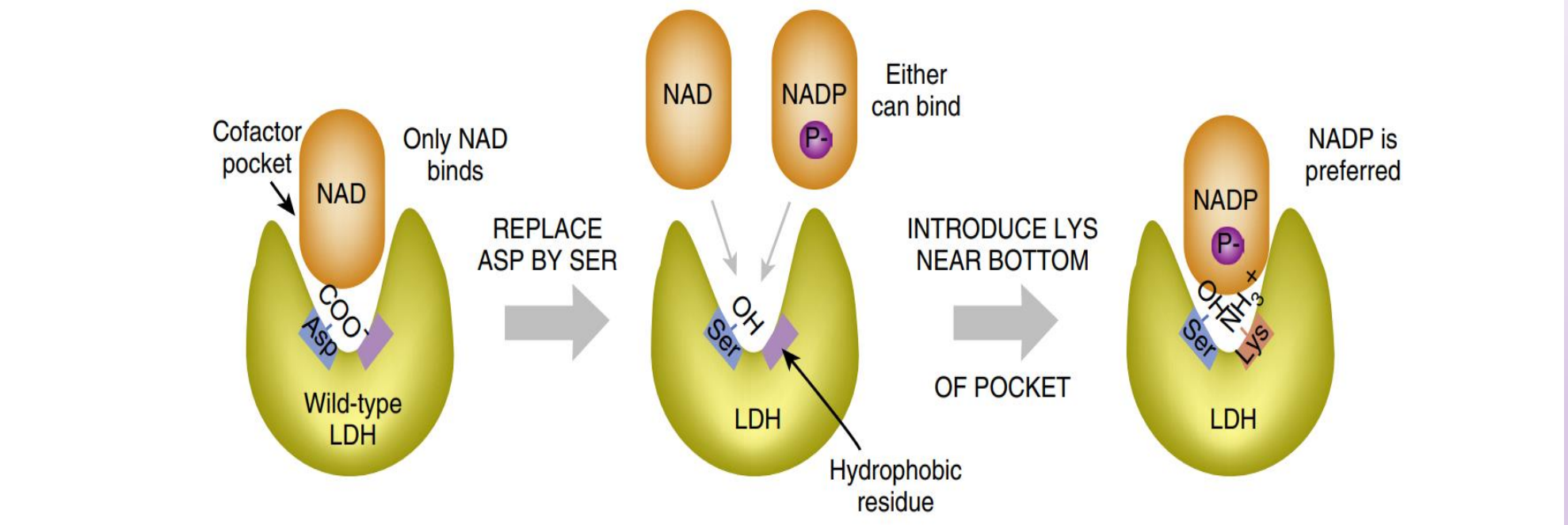
This may be done by replacing small hydrophobic residues, which are already in or near the core, with larger ones. For example, changing Ala to Val or Leu to Phe will achieve this

the alpha helix is actually a dipole with a slight positive charge at its N-terminal end and a slight negative charge at its C-terminal end.

The presence of amino acid residues with the corresponding opposite charge close to the ends of an alpha helix promotes stability.

CHANGING BINDING SITE SPECIFICITY

The most straightforward alterations are those that change the binding specificity for the substrate or a cofactor but do not disrupt the enzyme mechanism.



The specificity of LDH for its substrate can be altered in a similar way. The natural substrate lactate is a three-carbon hydroxy acid. It is possible to alter several residues surrounding the

substrate-binding site without impairing the enzyme reaction mechanism.

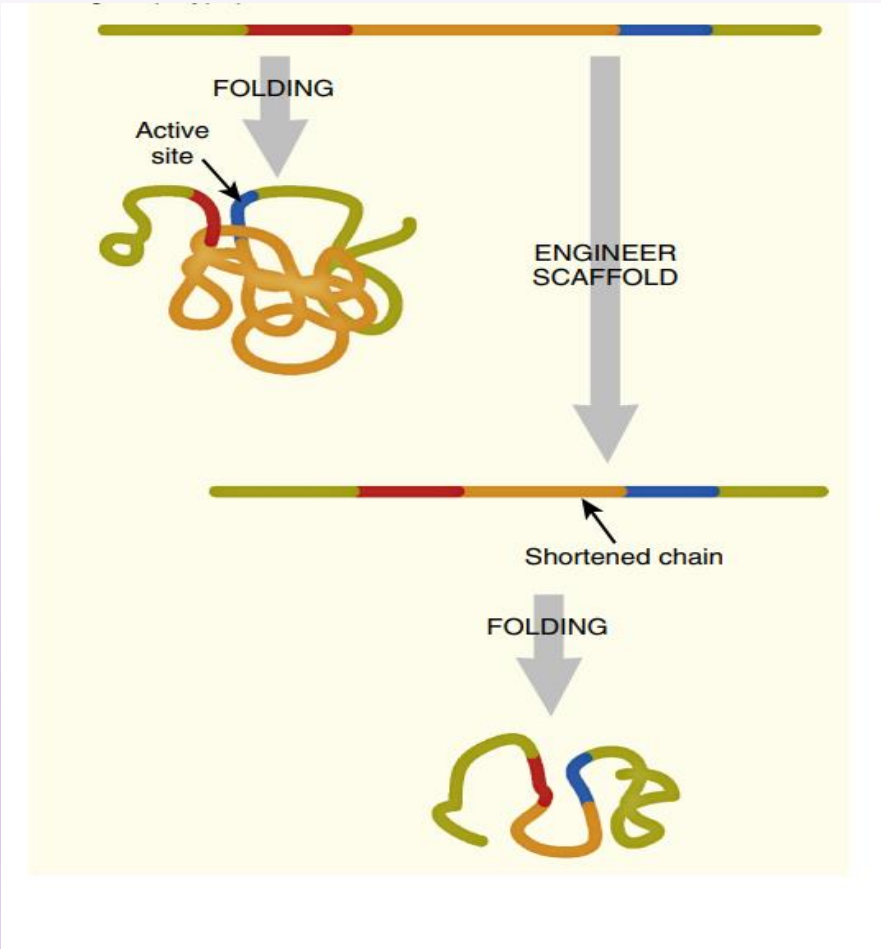
When a pair of alanines are replaced with glycines, the **binding site can be made larger.**

When hydrophilic residues (Lys, Gln) are replaced with hydrophobic ones (Val, Met), the site becomes more hydrophobic. Alteration of multiple residues gives an engineered LDH that accommodates five or six carbon analogs of lactate and uses them as substrate.

STRUCTURAL SCAFFOLDS

Relatively few of the amino acid residues in a protein are actually involved in the active site.
Quite often the scaffold is much larger than really necessary. For example, the β-galactosidase of Escherichia coli (LacZ protein) has approximately 1000 amino acids, whereas most simple hydrolytic enzymes have only 200 to 300.
From an industrial viewpoint, such a smaller protein would obviously be more efficient.

ex: antibody engineering , phage display
Such techniques will allow the engineering of smaller proteins whose biosynthesis consumes less energy and material



DIRECTED EVOLUTION

Directed evolution is a powerful technique to alter the function of an enzyme without the need for exhaustive structural and functional data.

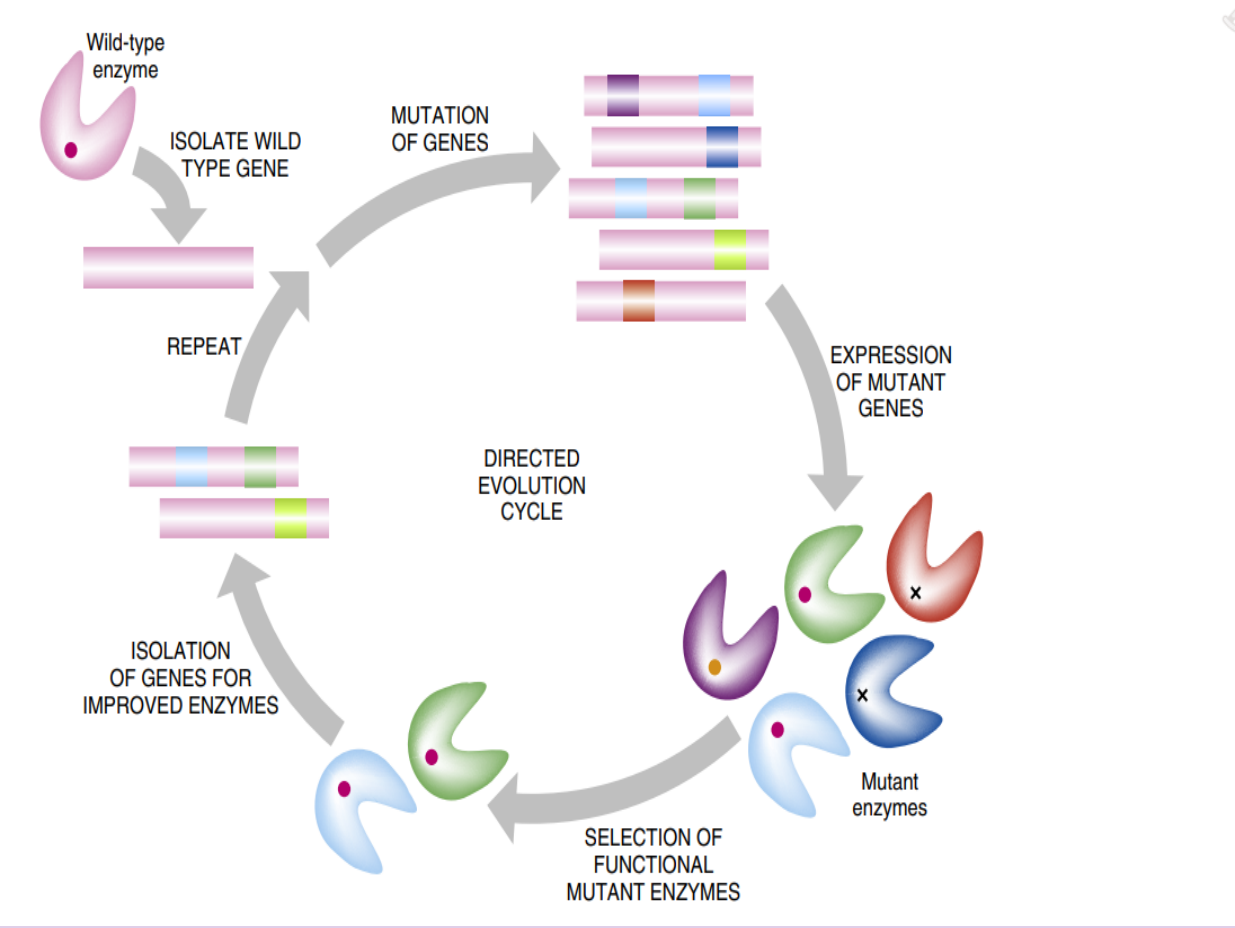
Directed evolution can be used to change substrate specificity, either changing the enzyme to recognize a totally different substrate or making subtle changes in cases in which the substrate is slightly different.

The main premise of directed evolution is the random mutagenesis of the gene of interest, followed by a selection scheme for the new desired function.

Directed evolution screens for new enzyme activities by constructing a library of different enzymes derived from the same original protein.

Random mutagenesis during amplification of the gene creates a library of slightly different genes and, when expressed, slightly different proteins.

Recombination (homologous or nonhomologous) can also be used to create different permutations of the enzyme.



The gene is randomly mutated throughout the entire sequence using **error-prone PCR**.

Different methods exist to induce errors during PCR amplification. The most straightforward is to add **MnCl2** to the PCR reaction. Taq polymerase has a fairly high rate of incorporating the wrong nucleotide, and MnCl2 **stabilizes the mismatched bases.**

Adding nucleotide analogs such as 8-oxo-dGTP and dITP, which form mismatches on the opposite strand, can also enhance the PCR error rate.

These analogs in combination with MnCl2 can induce a wide variety of different mutations along the length of a gene.

alternative approach to forming novel enzymes

1 - RECOMBINING DOMAINS

2- DNA SHUFFLING

RECOMBINING DOMAINS

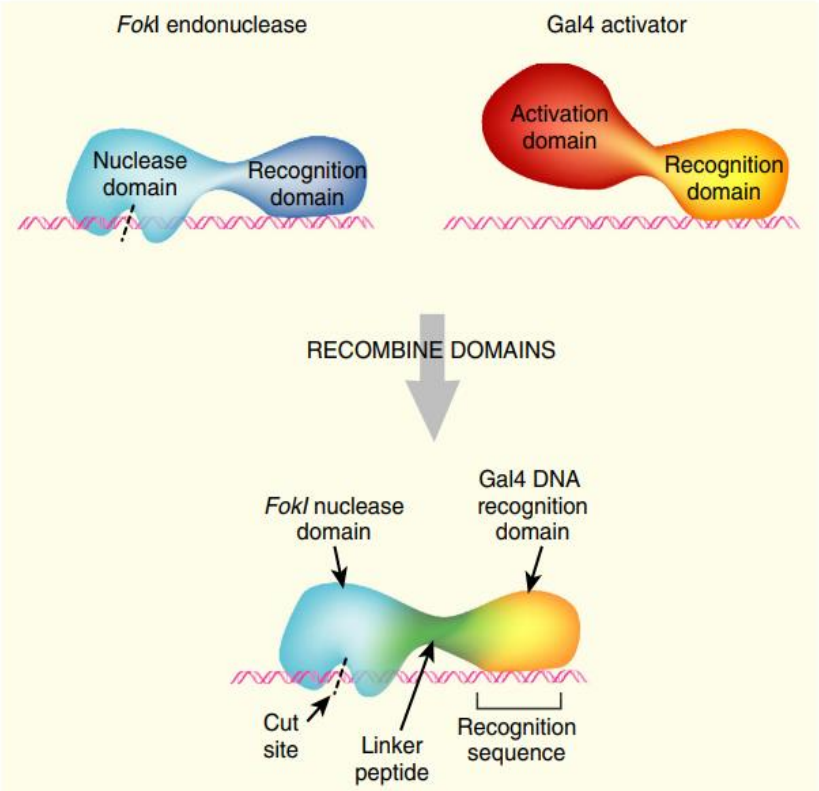
An example is the creation of novel restriction enzymes by linking the cleavage domain from the restriction enzyme FokI with different sequence-specific DNA-binding domains.

FokI is a type II restriction enzyme with distinct N-terminal and C-terminal domains that function in DNA recognition and DNA cutting, respectively.

By itself, the endonuclease domain cuts DNA nonspecifically.

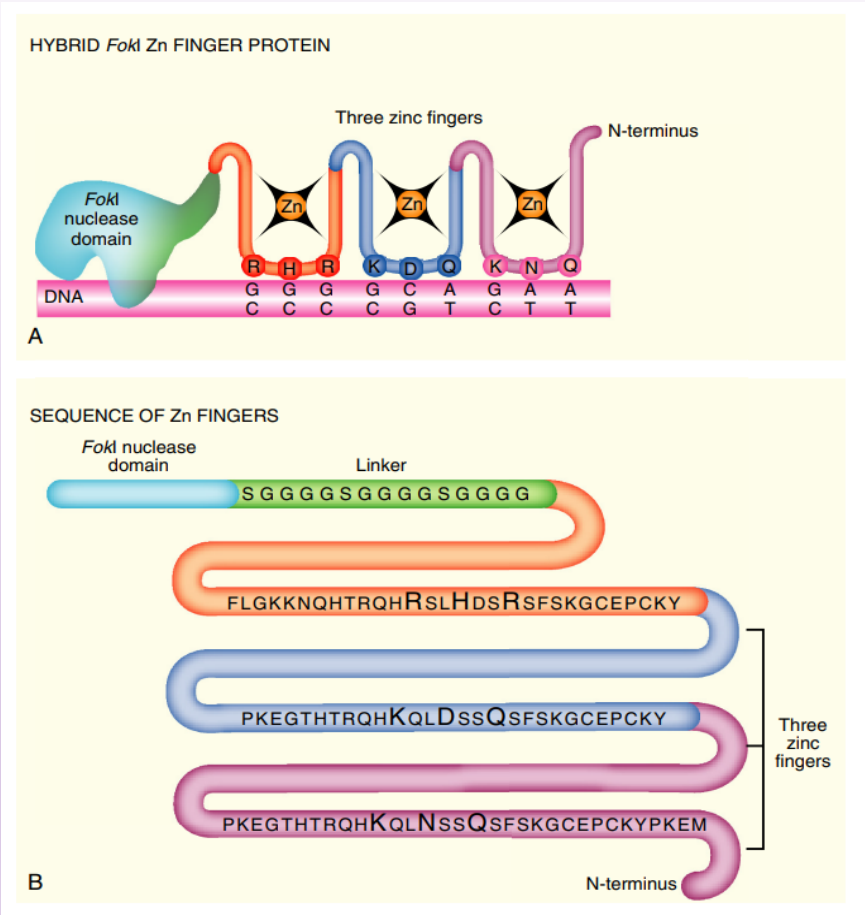
The FokI endonuclease has separate nuclease and sequence recognition domains. Using genetic engineering, the recognition domain of FokI can be replaced with a Gal4 recognition domain, which binds to a different DNA sequence. The two domains are joined with an artificial linker peptide. The new hybrid enzyme now cuts DNA at different locations from the original FokI protein

The two domains may be joined via a sequence encoding a linker peptide such as (GlyGlyGlyGlySer)3.



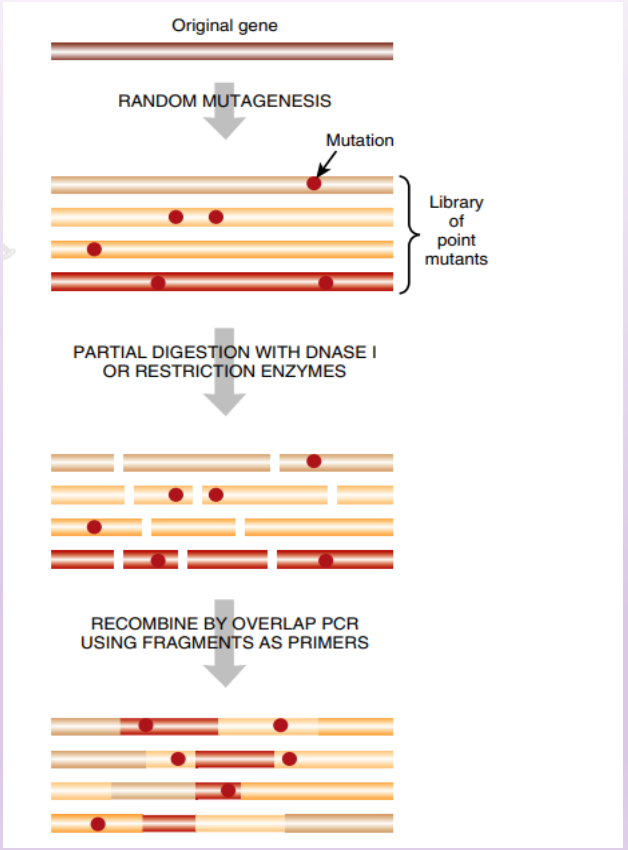
Zinc finger domains have also been joined to the nuclease domain of FokI.

The nuclease domain of FokI can be linked to a zinc finger domain containing three zinc finger motifs. Zinc fingers recognize three nucleotides each; therefore, any nine-base-pair recognition sequence can, in principle, be linked to the nuclease domain. (B) The sequence of the hybrid between the FokI nuclease and the zinc finger domain. The letters represent the amino acid sequence. The amino acids in large letters recognize and bind the DNA sequence.



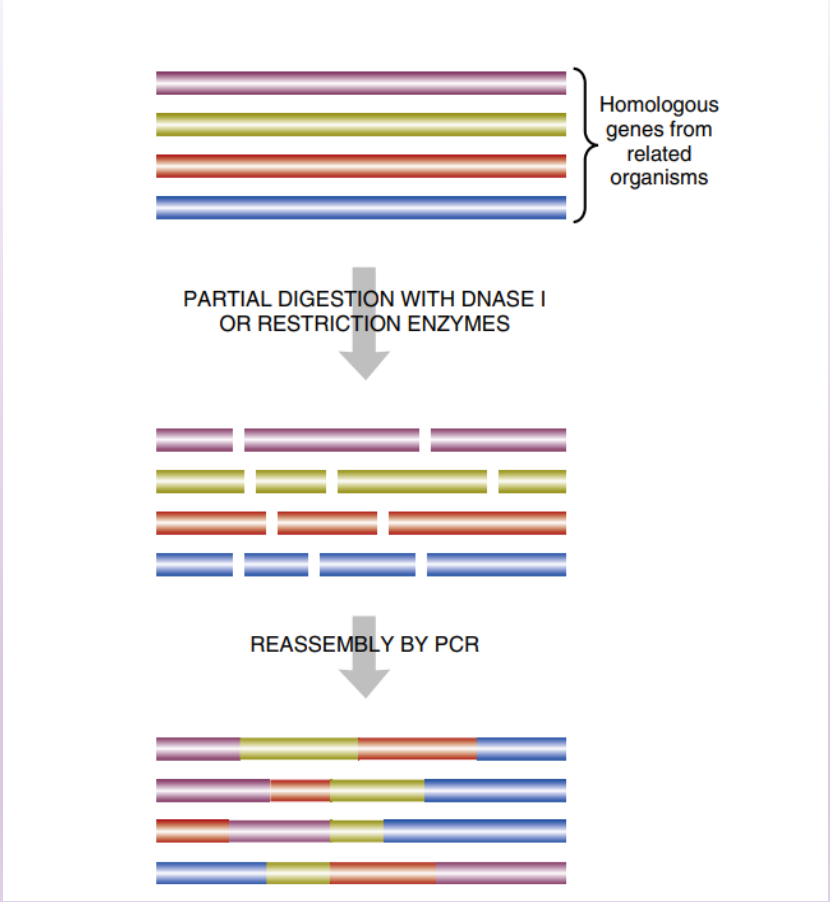
DNA SHUFFLING

DNA shuffling is a method of **artificial evolution** that includes the creation of **novel mutations** as well as **recombination.**



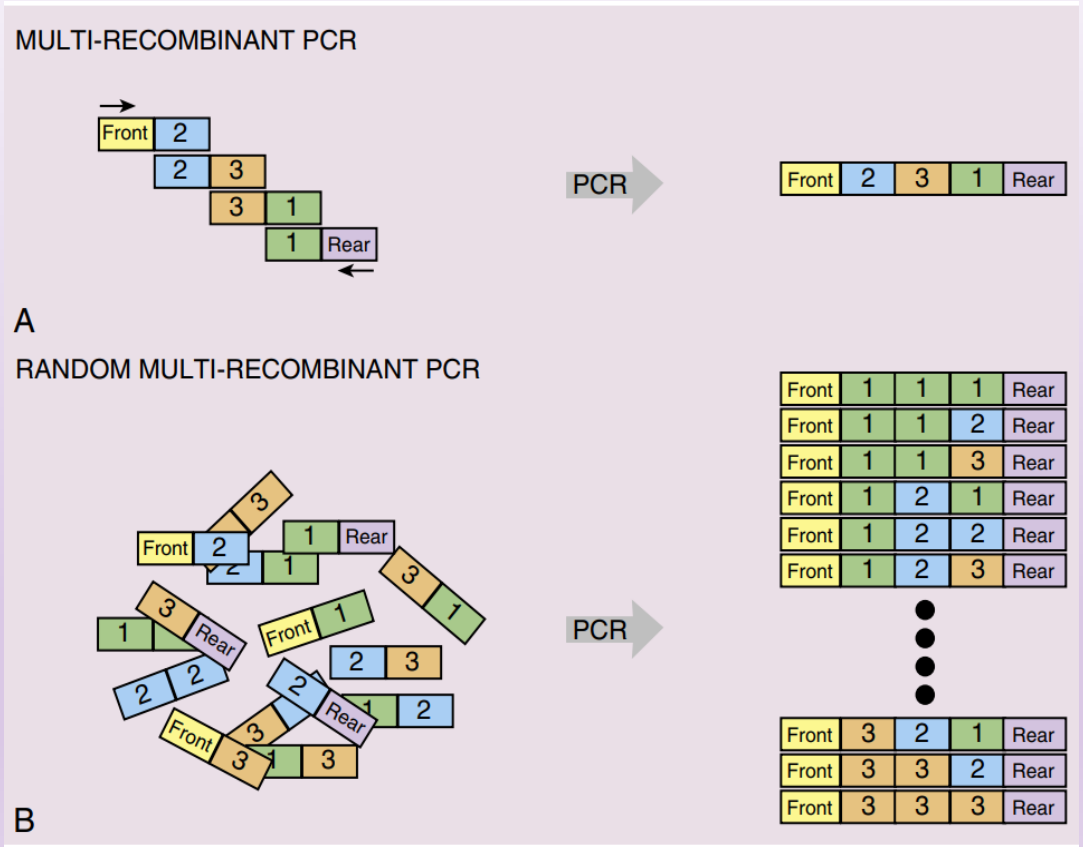
several related β-lactamases from different enteric bacteria have been shuffled.

This approach yielded improved β-lactamases that degraded certain penicillins and cephalosporins more rapidly, making their host cells up to 500-fold more resistant to these β-lactam antibiotics.



COMBINATORIAL PROTEIN LIBRARIES

*Generation of Random Shuffling Library



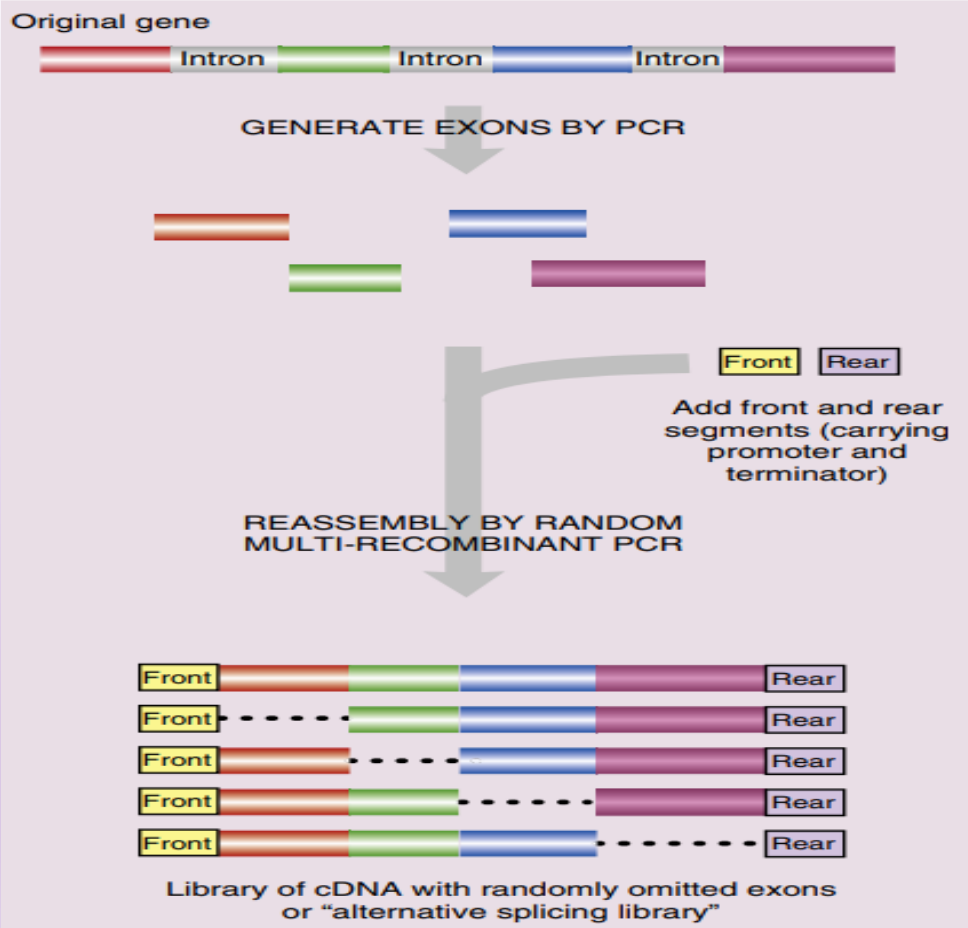
Generation of Alternative Splicing Library (exon shuffling approach)

Two variants exist, depending on the design of the overlap primers for the PCR assembly

random splicing library

alternative splicing library

increases the chances of a functional protein



CREATION OF DE NOVO PROTEINS

The ultimate in generating novel proteins is to create wholly novel polypeptide sequences—de novo proteins.

These proteins are made by first generating random DNA sequences, which are then expressed in a host cell.

The vast majority of random polypeptides will not fold into stable or functional structures. Thus, while de novo proteins are indeed novel, they are not completely random.

The ultimate goal is to learn how to fully predict protein 3D structure given the polypeptide sequence

In four cases (serB, gltA, ilvA, fes), de novo proteins were found
that rescued the mutants.

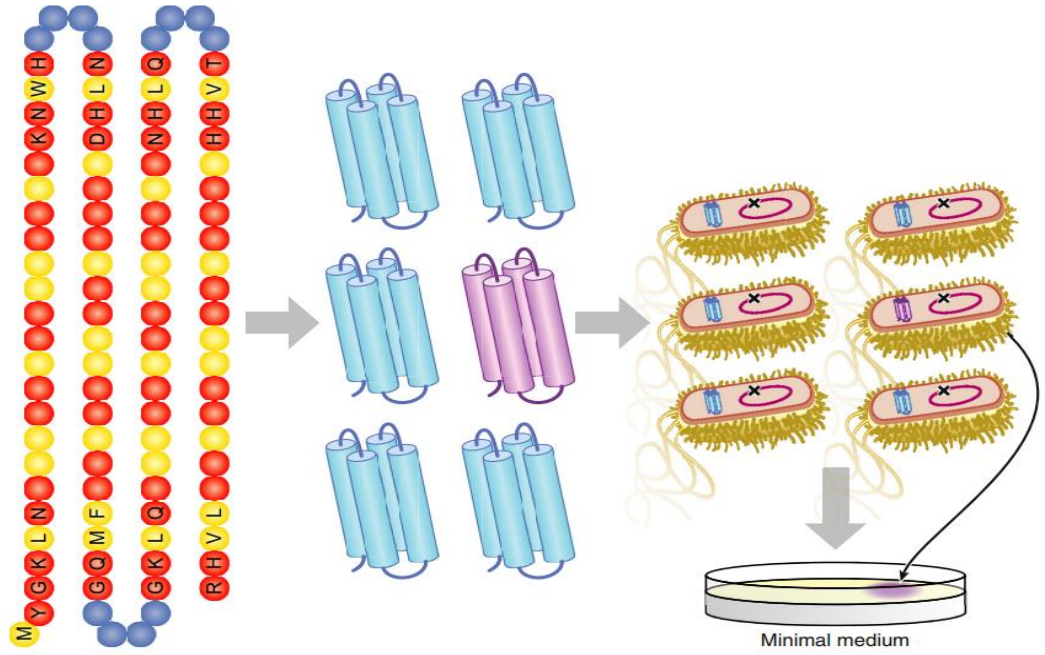
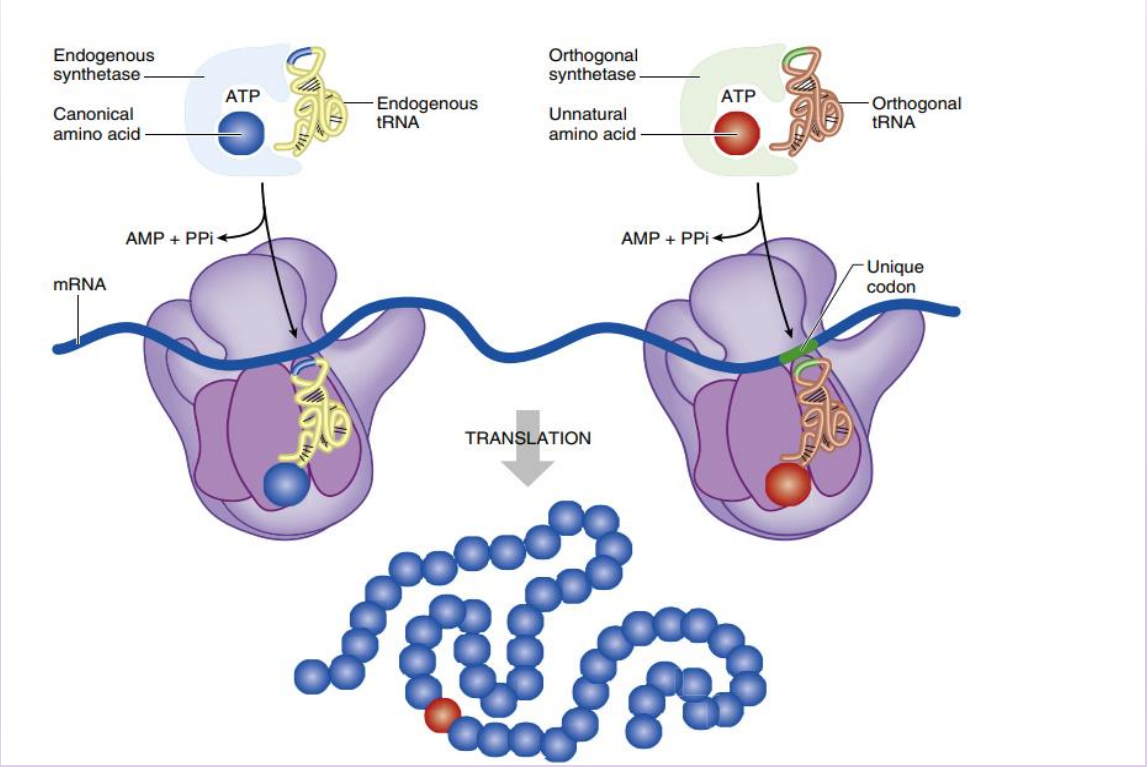


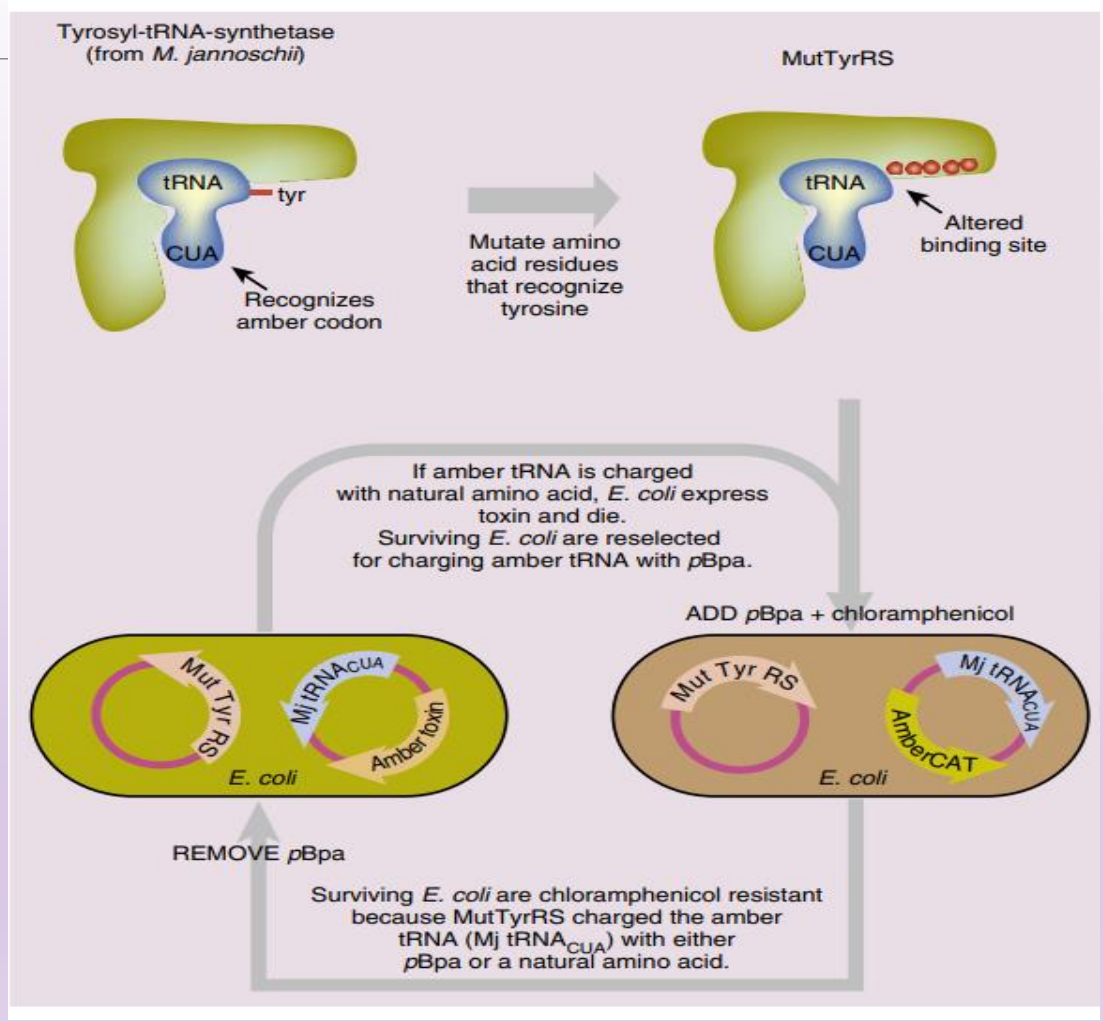
FIGURE 11.13 ***De novo*** **Proteins Rescue** ***E. coli*** **Mutants**
A pattern of polar (red) and nonpolar (yellow) residues was designed to fold into four-helix bundles. Circles with letters show fixed residues, and empty circles show variable positions. The experimental library of 1.5 million synthetic genes cloned on a high-expression plasmid was transformed into strains of *E. coli* from which genes needed for growth on minimal medium had been deleted (black X). Colonies were selected on minimal medium to find novel sequences (purple) that rescue the cells. Modified after Fisher MA et al. (2011). De novo designed proteins from a library of artificial sequences function in *Escherichia coli* and enable cell growth. *PLoS One* 6:e15364.

EXPANDING THE GENETIC CODE

Many non-natural amino acids have different functional groups that are useful in protein engineering or biochemical analysis.



The two selections were alternated numerous times, and finally, a mutant tRNA synthetase was isolated that still recognized the amber tRNA and specifically linked pBpa to it



ROLES OF NON-NATURAL AMINO ACIDS

Table 11.3 Roles for Non-Natural Amino Acids in Proteins

Role	Examples
Analyzing post-translational modification	Serine phosphorylation Lysine acetylation
Reactive chemical groups	Azidohomoalanine
UV activated cross-linking	*p*-Benzoyl-L-phenylalanine
Light-activated functional groups	4,5-Dimethoxy-2-nitrobenzyl-cysteine
Fluorescent probes	Coumarin and dansyl derivatives
Infrared probes	*p*-Azido-L-phenylalanine
Isotopic labels for NMR spectroscopy	Several fluoro-amino acids

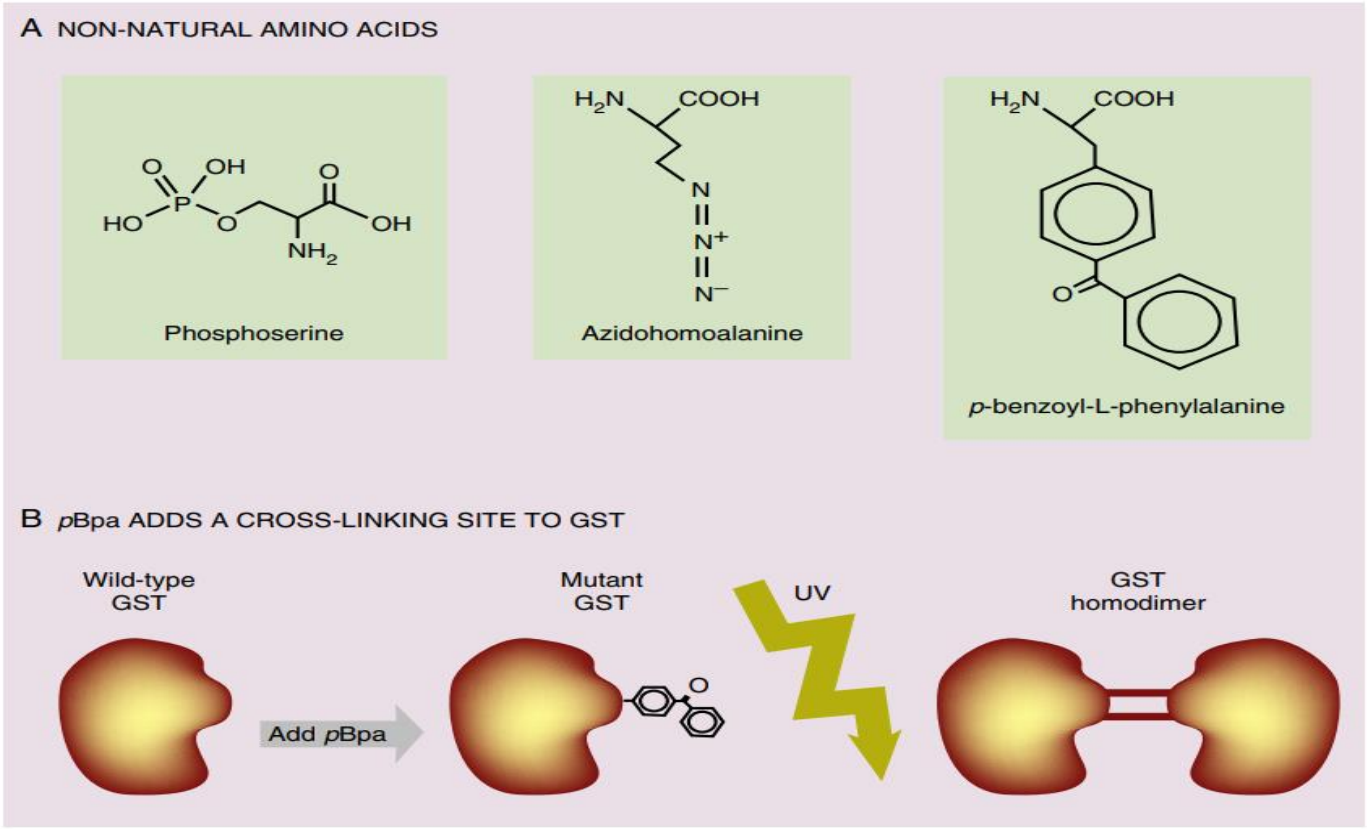


FIGURE 11.16 Adding New Functional Groups to Proteins
(A) Inserting amino acids that are not genetically encoded allows the incorporation of new functional groups. (B) The non-natural amino acid *p*-benzoyl-L-phenylalanine (*p*Bpa) cross-links the GST mutant protein to form a homodimer.

The non-natural amino acid p-benzoyl-L-phenylalanine (pBpa) that was discussed previously is widely used to insert a cross-linking group that can be activated by UV irradiation. Thus, when the protein glutathione-S-transferase (GST) has pBpa inserted, UV irradiation creates a covalently linked homodimer.

UV light converts 4,5-dimethoxy-2-nitrobenzyl-cysteine to cysteine, with the loss of a dimethoxynitrobenzyl group. Incorporation of photo-caged amino acids has been used to make ion channel proteins that are inactive but can be activated by UV light in living nerve cells.

Finally, non-natural amino acids are used as probes for several spectroscopic techniques, including fluorescence, infrared, and NMR spectroscopy.

BIOMATERIALS DESIGN RELIES ON PROTEIN ENGINEERING

In the medical field, biomaterials are crucial for reconstructive surgery, tissue engineering, and regenerative medicine.

Biomaterials include vascular grafts and cartilaginous tissue scaffolds that facilitate the growth of new tissue by providing support and structure.
ELPs possess a repeated peptide sequence such as (VPGZG)n, where Z is any amino acid except proline(Elastin itself contains these repeats, but Z is restricted to Ala, Leu, or Ile).

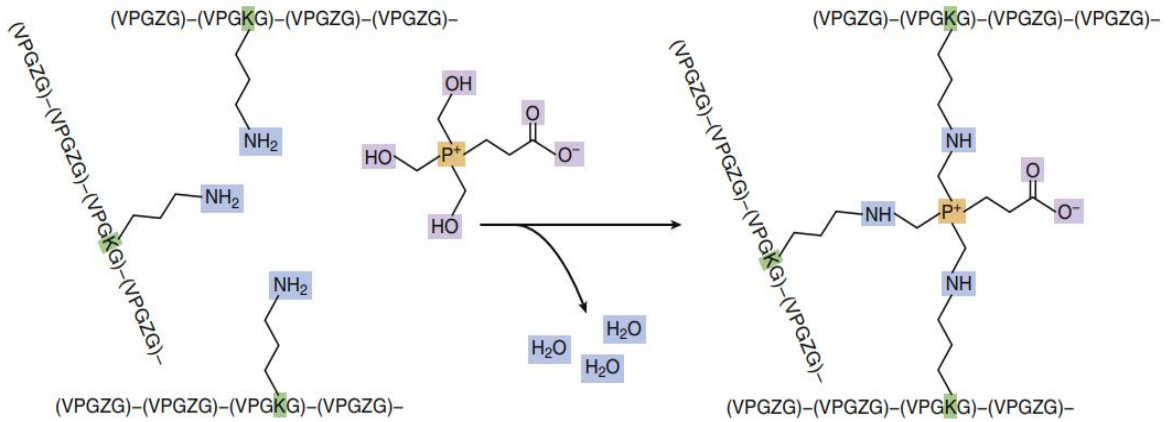


FIGURE 11.17 Chemical Cross-Linking of Elastin-Like Polypeptides

Insertion of lysine (K, green) into the sequence of an elastin-like polypeptide (ELP) made of repeating VPGZG units allows multiple chains to be cross-linked by reaction of the side chain amino groups of lysine with hydroxymethyl phosphine derivatives such as THPP ([Tris(hydroxymethyl)phosphino]propionic acid).

hydroxymethyl phosphine derivatives

ENGINEERED BINDING PROTEINS

antibodies are the most widely used reagents for binding specific target proteins.

However, antibodies require disulfide cross-links to function, and these are often hard to maintain during large-scale manufacture.

To generate novel binding domains, a binding protein with a known structure is chosen and the amino acid residues associated with binding are identified.

The binding protein is modified by mutation of these residues and then screened for new binding partners.

To generate novel binding domains, a binding protein with a known structure is chosen and the amino acid residues associated with binding are identified.

The binding protein is modified by mutation of these residues and then screened for new binding partners.

It is hoped that the targeted directed evolution approach will find new, more easily isolated proteins for targeting drugs to specific target cells within our bodies

THANK YOU