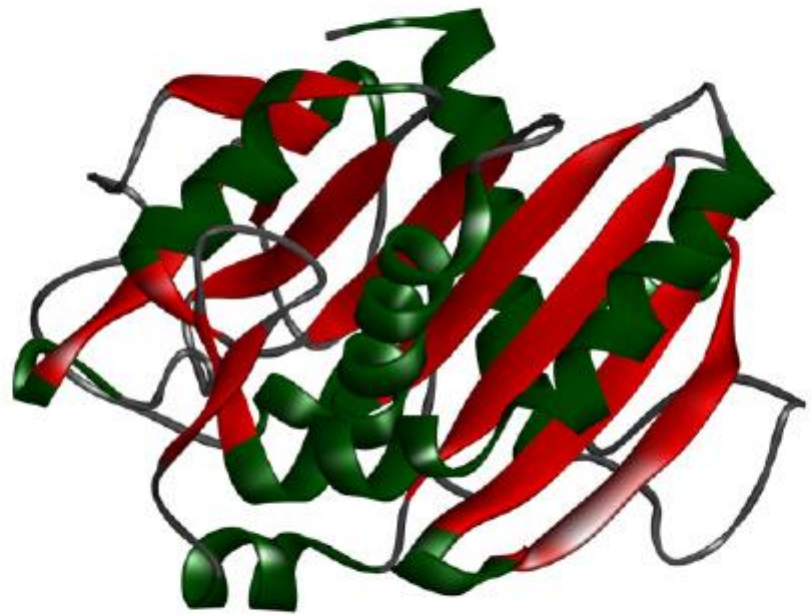
# Protein engineering

Activity

Enantioselectivity

Substrate Specificity



Solvent

Stability

Substrate/Product Tolerance

**Table 1.** List of the enzymes engineered by protein engineering.

| Enzyme | Organism | Improved property | Method | Application | Reference |
|---|---|---|---|---|---|
| Hydantoinase | *Arthrobacter* sp. | Enantioselective hydantoinase and 5-fold more productivity | Saturation mutagenesis, screening | Production of L-Met (L-amino acids) | [7] |
| Cyclodextrin glucanotransferase | *Bacillus stearothermophilus* ET1 | Modulation of cyclizing activity and thermostability | Site-directed mutagenesis | Bread industry | [8] |
| Lipase B | *Candida antarctica* | 20-fold increase in half-life at 70 °C | epPCR | Resolution and desymmetrization of compound | [9] |
| Tagatose-1,6-Bisphosphate aldolase | *E. coli* | 80-fold improvement in $k_{cat}/K_m$ and 100-fold change in stereospecificity | DNA shuffling and screening | Efficient syntheses of complex stereoisomeric products | [10] |
| Xylose isomerase | *Thermotoga neapolitana* | High activity on glucose at low temperature and low pH | Random Mutagenesis and screening | Used in preparation of high fructose syrup | [11] |
| Amylosucrase | *Neisseria polysaccharea* | 5-fold increased activity | Random mutagenesis, gene shuffling, and directed evolution | Synthesis or the modification of polysaccharides | [12] |
| Galactose oxidase | *F. graminearum* | 3.4–4.4 fold greater $V_{max}/K_m$ and increased specificity | epPCR and screening | Derivatization of guar gum | [13] |
| Fructose bisphosphate aldolase | *E. coli* | Increased thermostablity and stability to treatment with organic solvent | DNA shuffling | Use in organic synthesis | [14] |
| 1,3-1,4-α-D-glucanase | *Fibrobacter succinogenes* | 3–4-fold increase in the turnover rate (k) | PCR-based gene truncation | Beer industry | [15] |
| Lipase | *P. aeruginosa* | 2-fold increase in amidase activity | Random mutagenesis and screening | Understanding lipase inability to hydrolyze amides | [16] |
| Protease BYA | *Bacillus* sp. Y | Specific activity1.5-fold higher | Site-directed mutagenesis | Detergents products | [17] |
| *p*-Hydroxybenzoate hydroxylase | *Pseudomonas fluorescens* NBRC 14160 | Activity, reaction specificity, and thermal stability | Combinatorial mutagenesis | Degrading various aromatic compounds in the environment | [18] |
| Endo-1,4-β-xylanase II | *Trichoderma reesei* | Increased alkali stability | Site-directed mutagenesis | Sulfate pulp bleaching | [19] |
| Xylose isomerase | *Thermotoga neapolitana* | 2.3-fold increases in catalytic efficiency | Random mutagenesis | Production of high fructose corn syrup | [11] |

**Table 1.** *Cont.*

| Enzyme | Organism | Improved property | Method | Application | Reference |
|---|---|---|---|---|---|
| α-Amylase | *Bacillus* sp. TS-25 | 10 °C enhancement in thermal stability | Directed evolution | Baking industry | [20] |
| Xylanase | | Tm improved by 25 °C | Gene site-saturation mutagenesis | Degradation of hemicellulose | [21] |
| Fructosyl peptide oxidase | *Coniochaeta* sp | 79.8-fold enhanced thermostability | Directed evolution and site-directed mutagenesis | Clinical diagnosis | [22] |
| Endo-β-1,4-xylanase | *Bacillus subtilis* | Acid stability | Rational protein engineering | Degradation of hemicellulose | [23] |
| Subtilase | *Bacillus* sp. | 6-fold increase in caseinolytic activity at 15–25 °C | Directed evolution and site-directed mutagenesis | Detergent additives and food processing | [24] |
| CotA laccase | *B. subtilis* | 120-fold more specific for ABTS | Directed evolution | Catalyze oxidation of polyphenols | [25] |
| Pyranose 2-oxidase | *Trametes multicolor* | Altered substrate selectivity for D-galactose, D-glucose | Semi-rational enzyme engineering approach | Food industry | [26] |
| Xylanase XT6 | *Geobacillus stearothermophilus* | 52-fold enhancement in thermostability; increased catalytic efficiency | Directed evolution and site-directed mutagenesis | Degradation of hemicellulose | [27] |
| Lipase | *Bacillus pumilus* | Thermostability and 4-fold increase in $k_{cat}$ | Site-directed mutagenesis | Chemical, food, leather and detergent industries | [28] |
| Bgl-licMB | *Bacillus amyloliquefaciens* (Bgl) and *Clostridium thermocellum* (licMB) | 2.7 and 20-fold higher $k_{cat}/K_m$ than that of the parental Bgl and licMB, respectively | Splicing-by-overlap extension | Brewing and animal-feed industries | [29] |
| β-agarase AgaA | *Zobellia galactanivorans* | Catalytic activity and thermostability | Site-directed mutagenesis | Production of functional neo-agarooligosaccharides | [30] |
| Prolidase | *Pyrococcus horikoshii* | Thermostability | Random mutagenesis | Detoxification of organophosphorus nerve agents | [31] |

# Improving thermal Stability

- Protein stability can be increased by creating a molecule which will **not readily unfold** under unfavorable conditions.
- Protein stability can be improved by:
  * Adding **disulphide bonds**
  * Replacing **labile amino acids**
  * Reducing the number of **free S-H** (sulphydryl) groups.

# Adding Disulphide Bonds

- **Disulphide bonds** can significantly **stabilize** the native structure of proteins.
- This effect is presumed to be due to the **decrease in configuration chain entropy** of the unfolded polypeptide.
- **Wild type lysozyme** has **2 cysteine** residues and **no disulphide bonds**.
- Site-directed mutagenesis was used to **introduce new cysteine residues** and new **internal S-S bonds** between amino acids:
  - 3 and 97  9 and 164  21 and 142

# Mutagenesis of Lysozyme

- After mutagenesis each mutant gene was **expressed** in *E. coli*.
- The modified enzymes were purified and tested for **enzyme activity and thermostability**.
- The results showed that the **thermal stability increased** with the presence of **disulphide bonds**.
- The most thermostable mutant was the one with 3 S-S bonds.
- Those mutants which had S-S bonds between amino acids 21 and 142 lost 100% of their activity.
- Can you guess why?

# Mutagenesis of Lysozyme

***Table 8.2*** Properties of T4 lysozyme and six engineered variants

| Enzyme | Amino acid at position: | | | | | | | No. of -S-S- | % Activity | $T_m$ (°C) |
|---|---|---|---|---|---|---|---|---|---|---|
| | 3 | 9 | 21 | 54 | 97 | 142 | 164 | | | |
| wt | Ile | Ile | Thr | Cys | Cys | Thr | Leu | 0 | 100 | 41.9 |
| pwt | Ile | Ile | Thr | Thr | Ala | Thr | Leu | 0 | 100 | 41.9 |
| A | Cys | Ile | Thr | Thr | Cys | Thr | Leu | 1 | 96 | 46.7 |
| B | Ile | Cys | Thr | Thr | Ala | Thr | Cys | 1 | 106 | 48.3 |
| C | Ile | Ile | Cys | Thr | Ala | Cys | Leu | 1 | 0 | 52.9 |
| D | Cys | Cys | Thr | Thr | Cys | Thr | Cys | 2 | 95 | 57.6 |
| E | Ile | Cys | Cys | Thr | Ala | Cys | Cys | 2 | 0 | 58.9 |
| F | Cys | Cys | Cys | Thr | Cys | Cys | Cys | 3 | 0 | 65.5 |

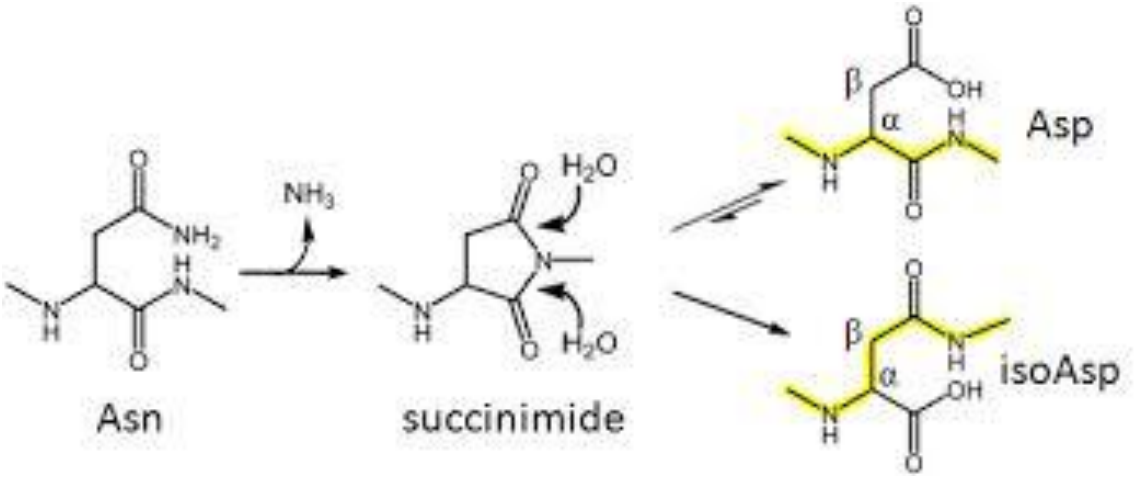
Adapted from Matsumura et al., *Nature* **342**:291–293, 1989.

wt, wild-type T4 lysozyme; pwt, pseudo-wild-type enzyme; A through F, six engineered cysteine variants; -S-S-, disulfide bonds; $T_m$, "melting" temperature (a measure of thermostability).

# Changing Labile Amino Acids

- When proteins are exposed to high temperatures deamidation occurs.
- **Deamidation** $\rightarrow$ release of $NH_3$
- Asparagine $\rightarrow$ Asparatic acid
- Glutamine $\rightarrow$ Glutamic acid
- The loss of the **amide groups** may result in the

lost of activity of the affected enzymes.



# Triose Phosphate Isomerase

- **Triose phosphate isomerase** catalyses the interconversion of dihydroxyacetone and phosphate to glyceraldehyde –3 phosphate during glycolysis.
- The enzyme (*Saccharomyces cerevisiae*) consist of 2 identical subunits and each subunit has **2 asparagine residues** which contributes to its thermal sensitivity.
- Using oligonucleotide directed mutagenesis:
  - Asn 14 $\rightarrow$ Ile
  - Asn 78 $\rightarrow$ Thr
- Resulted in **enhanced thermostability**.
- When both Asn $\rightarrow$ Asp the resulting protein was unstable even at **room temperature**.

# Increasing the stability of Triose Phosphate Isomerase

***Table 8.3*** Stability at 100°C of the yeast enzyme triosephosphate isomerase and its engineered derivatives

| Enzyme | Amino acid at position: | | Half-life (min) |
|---|---|---|---|
| | 14 | 78 | |
| Wild type | Asn | Asn | 13 |
| Variant A | Asn | Thr | 17 |
| Variant B | Asn | Ile | 16 |
| Variant C | Thr | Ile | 25 |
| Variant D | Asp | Asn | 11 |

Adapted from Ahern et al., *Proc. Natl. Acad. Sci. USA* **84**:675–679, 1987.

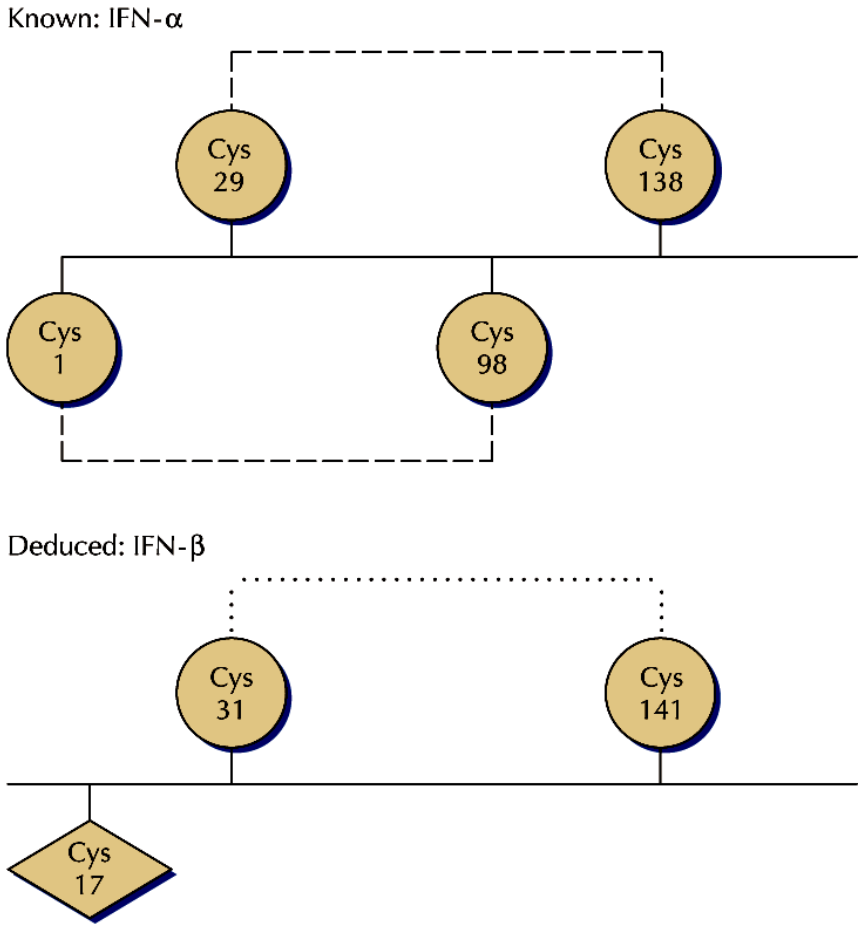
Enzyme stability is expressed as the half-life, or rate of enzyme inactivation, at 100°C. A longer half-life indicates a more stable enzyme.

# Reducing the # of Free S-H Groups

- **Interferons** “interfere” with virus replication.
- They are small protein molecules released from virus infected cells and binds to adjacent cells causing then to produce antiviral proteins which **disrupts viral replication**.
- When β **interferon** was cloned and expressed in *E. coli* it had about **10% of the activity** of the authentic form.
- The *E. coli* expressed interferon was found to existed as **dimers and higher oligomers**.
- Analysis of the DNA of the cloned gene showed that it has **3 cysteine** residues which may be involved in **intermolecular disulphide bonding** resulting in dimers and higher oligomers.

# β Interferon

- It was not know which or if any of the cysteine residues may be involved in intramolecular bonding.
- A similar molecule **α interferon** have 4 Cys residues at amino acid positions **1 , 29, 98 and 138** with S-S bonds between Cys **29 and 138**, which is homologous to Cys **31 and 141 of β INF.**



# β Interferon

- This suggests that **Cys 17 of β INF** was not involved intramolecular S-S bond.
- Therefore Cys 17 was targeted for **mutation to serine.**
- What is the structural relationship between Cys and Ser?
- Ser has an O atom instead of S atom in Cys therefore cannot form S-S bonds.
- Sure enough mutation of Cys 17$\rightarrow$ Ser the resulting β INF has **specific activity** similar to wild type β INF.

Enzyme activity

- Rate of substrate turnover may be limited by diffusion
- Binding and desorption rates

# Modifying Cofactor Requirement

- **Subtilisins** are a class of microbial serine proteases and are widely used as a **biodegradable** cleaning agents in laundry **detergents**.
- Subtilisin binds one or more molecules of **$Ca^{2+}$** which is important for their **stability**.
- Unfortunately subtilisins are used in industrial settings where there are **metal-chelating agents** which will bind $Ca^{2+}$.
- To circumvent this problem directed mutagenesis was used to **abolish the $Ca^{2+}$ binding** capability of subtilisin and to stabilize the modified enzyme.

# Mutagenesis of Subtilisins

- The x-ray crystallography structure of the enzyme and **the amino acids involved in the $Ca^{2+}$ binding** was known.
- Oligonucleotide mutagenesis was used to construct a mutant protein by **deleting amino acids 75-83** that is responsible for $Ca^{2+}$ binding.
- The next thing to do was to stabilize the modified protein.
- aa selected for mutagenesis came from 4 different regions : the **N terminus** (aa 2-5), **omega loop** (aa 36-44), **α helical region** ( aa 63-85) and a **β pleated region** (aa 202-222)
- The mutants were assayed for enzyme activity and stability.

# Mutagenesis of Subtilisins

- Stabilizing mutations were identified **at 7 of the 10** sites.
- These stabilizing mutations were introduced into a single gene.
- How could all seven mutations be introduced into a single gene?
- **The results**:
- The mutant subtilisins did **not require $Ca^{2+}$** as a cofactor.
- The mutant enzyme was **10 times more stable** than the native form in the absence of $Ca^{2+}$ and 50% more stable in presence of $Ca^{2+}$.

# Increasing Enzyme Specificity

- **Tissue plasmogen activator** (tPA) is a protease that is used for the **dissolution of blood clot**.
- Treatment with tPA requires an intravenous infusions (1.5-3.0 hrs) because of the clearance of tPA from the circulation is rapid (**t½~6 min**).
- For tPA to be effective the patient must be given in high initial concentration which can often cause **nonspecific bleeding**.
- Therefore a long life tPA with **increase specificity** for fibrin in blood clot is desirable.
- Directed mutagenesis was used to try achieve these goals.

# Mutagenesis of tPA

- Changing **Thr 103 → Asn** cause tPA to persist in rabbit plasm 10 times longer than the native form ( longer life tPA).
- Changing amino acids **296-299** from:
- Lys-His-Arg-Arg → Ala-Ala-Ala-Ala produced an enzyme with **more fibrin specificity**. (LHAA →A)
- Changing **Asn 117 → Gln** causes the enzyme to retain the enzymatic activity of the native form.
- Combining these three mutations into a single gene allows all three mutations to be expressed in a single protein simultaneously. It remains to be seen if this modified protein will be effective in humans.

# Mutagenesis of tPA

*Table 8.6* Stability and activity of various modified versions of tPA

| tPA variant | Modification(s) | Stability in plasma | Fibrin binding | Activity in plasma | Activity vs clots |
|---|---|---|---|---|---|
| 1 | Thr(103)→Asn | 10 | 0.34 | 0.68 | 0.56 |
| 2 | LysHisArgArg(296–299)→AlaAlaAlaAla | 0.85 | 0.93 | 0.13 | 1.01 |
| 3 | Thr(103)→Asn, LysHisArgArg(296–299)→AlaAlaAlaAla | 5.3 | 0.33 | 0.13 | 0.65 |
| 4 | Thr(103)→Asn, Asn(117)→Gln | 3.4 | 1.0 | 1.13 | 1.17 |
| 5 | LysHisArgArg(296–299)→AlaAlaAlaAla, Asn(117)→Gln | 1.2 | 1.33 | 0.16 | 1.38 |
| 6 | Thr(103)→Asn, LysHisArgArg(296–299)→AlaAlaAlaAla, Asn(117)→Gln | 8.3 | 0.87 | 0.06 | 0.85 |

Adapted from Peña et al., *Proc. Natl. Acad. Sci. USA* **91**:3670–3674, 1994.

All of the values shown are normalized to the wild type. Plasma stability is the reciprocal of the time it takes for plasma clearance; larger numbers indicate a more stable derivative. Fibrin specificity is reflected by a high activity versus clots and a low activity in plasma.

# Increasing Enzyme Activity

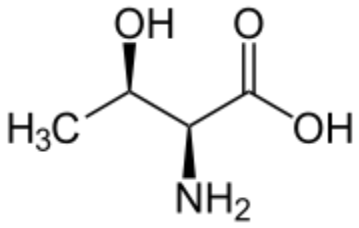
- In addition to stabilizing the enzyme, site-directed mutagenesis may be used to modify its **catalytic activity**.
- To do this detailed geometry of the active site and the amino acids in the active site must be known.
- **Tyrosyl-tRNA synthetase** has been modified for increase **substrate binding** (Km). (If the substrate binding is increased then this increases the rate of the reaction).

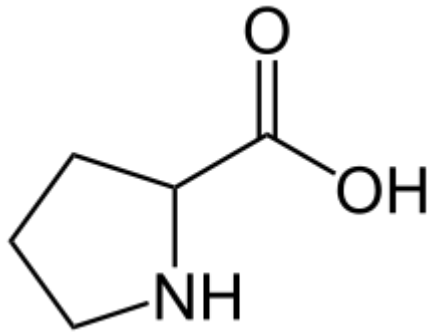
# Tyrosyl-tRNA Synthetase

- **Tyrosyl-tRNA synthetase** catalyses the the transfer of Tyr to $tRNA^{tyr}$.
- This is then added to the growing polypeptide chain.
- Tyr + ATP $\rightarrow$ Tyr-AMP + Ppi
- Tyr-AMP + $tRNA^{tyr}$ $\rightarrow$ Tyr-$tRNA^{tyr}$ + AMP
- The active site of the enzyme was mapped.
- In the crystal structure of the enzyme, the hydroxyl side chain of **Thr 51** form a weak H-bond with AMP of the substrate intermediate of tyrosyl adenylate (Tyr-A).

# Mutagenesis of Tyrosyl-tRNA Synthetase

- Oligonucleotide mutagenesis was used to create 2 mutations at Thr 51:
- **Thr 51 → Ala 51** (removes the H-bond).
- With this mutation the binding affinity (Km) of enzyme for ATP increase 2 fold.
- **Thr 51 → Pro 51**.
- With this mutation ATP is bound 100-fold more tightly.



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