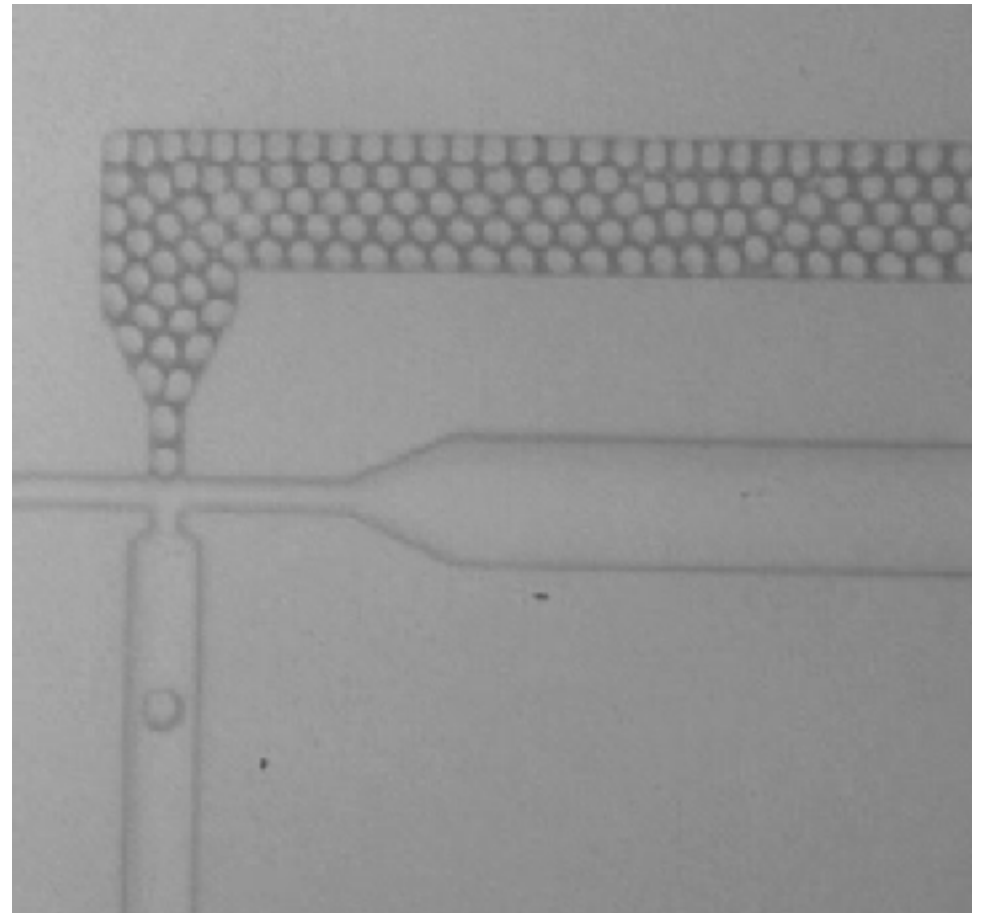# Biotechnologie Moléculaire
3ème année

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Tel: 01 40 79 45 39
Email: andrew.griffiths@espci.fr
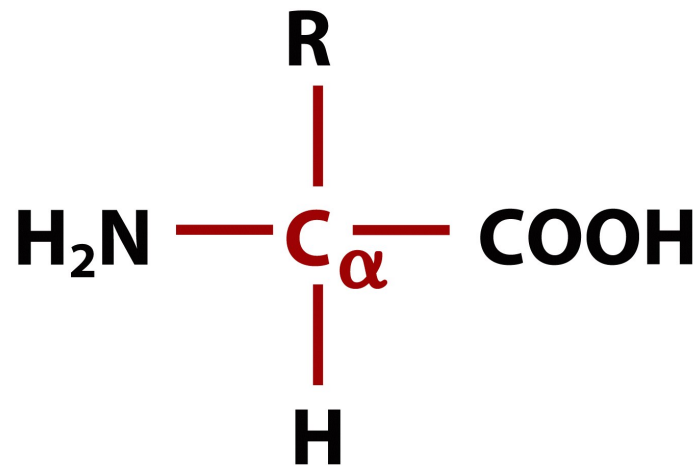
Protein Engineering

From engineered genes to engineered proteins

Proteins

What are they and what is their natural role?

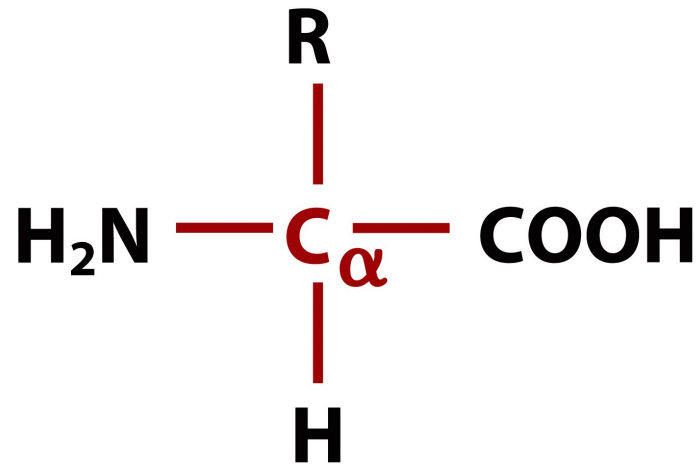# Amino Acids
Amino acids are the building blocks of proteins

$$H_2N - C_\alpha - COOH$$

R (top), H (bottom)

All proteins are composed of 20 "standard" amino acids with different R groups (side chains)

Figure 4-1

Amino Acids
Amino acids are the building blocks of proteins

All proteins are composed of 20 "standard" amino acids with different R groups (side chains)
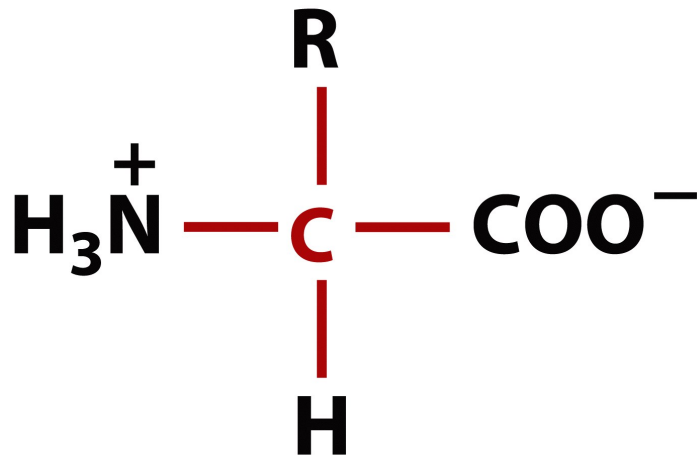
At physiological pH (~7.4) the amino groups are protonated and the carboxylic acid deprotonated (in the carboxylate form)

They are zwitterions – neutral molecules with a positive and negative charge

Figure 4-1

Amino Acids
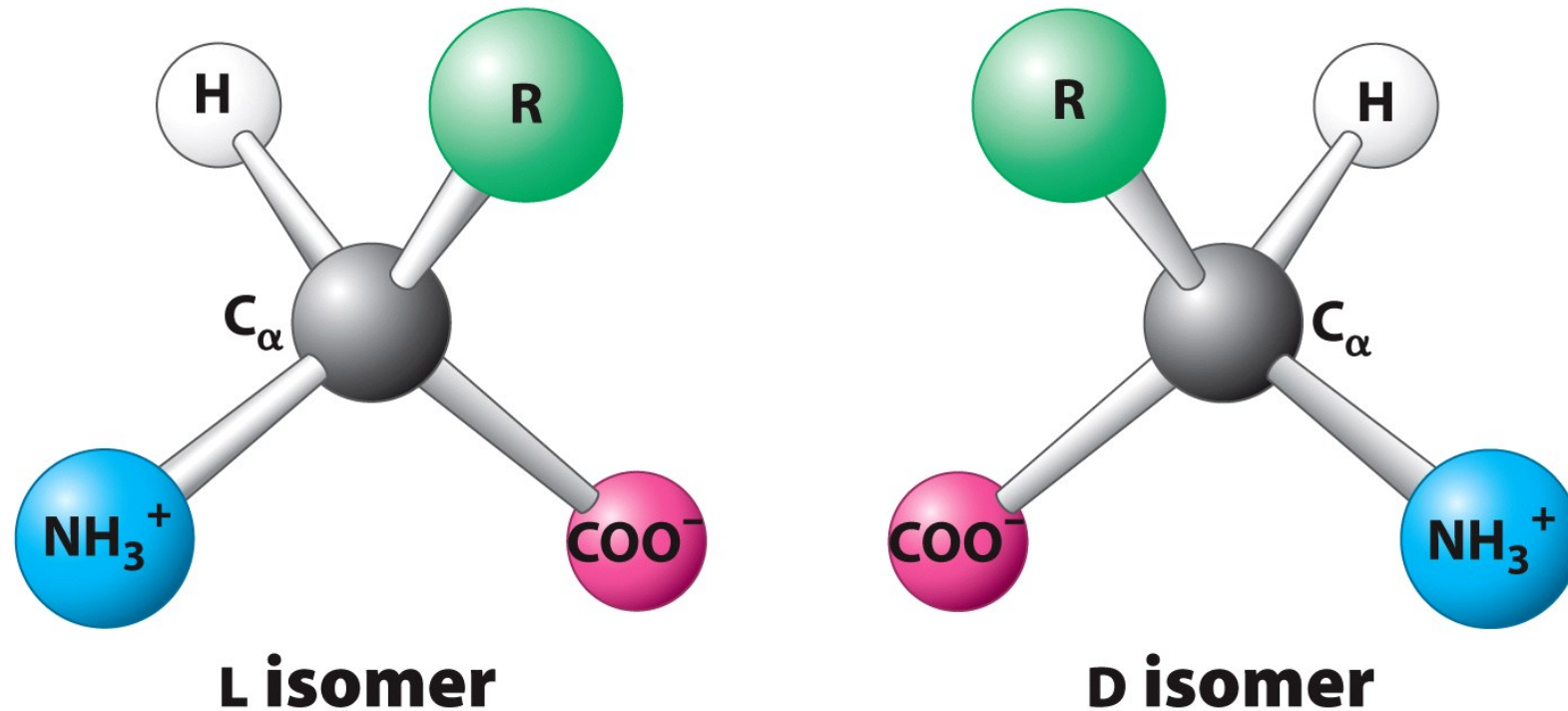## The L and D isomers of amino acids

Figure 3.1
Biochemistry: A Short Course, **First Edition**
© 2010 W. H. Freeman and Company

The isomers are mirror images of each other

Only L amino acids are constituents of proteins

Amino Acids
Polymerization of amino acids
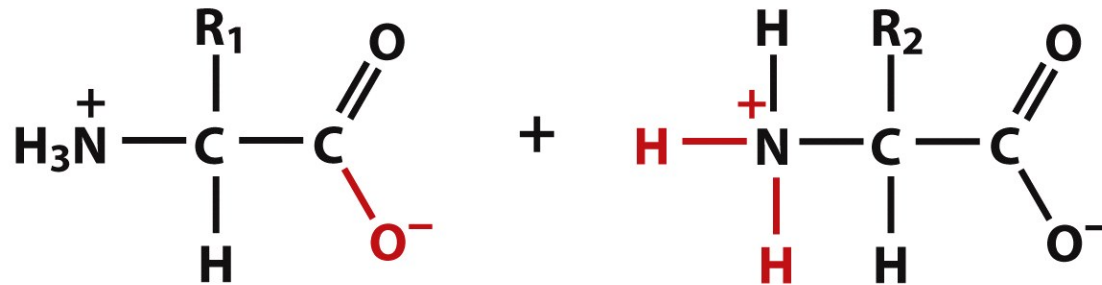
Amino acids can be polymerized to form chains (polypeptides)

The amino and carboxylate groups react to form a peptide bond in a condensation reaction (bond formation with the elimination of a water molecule)
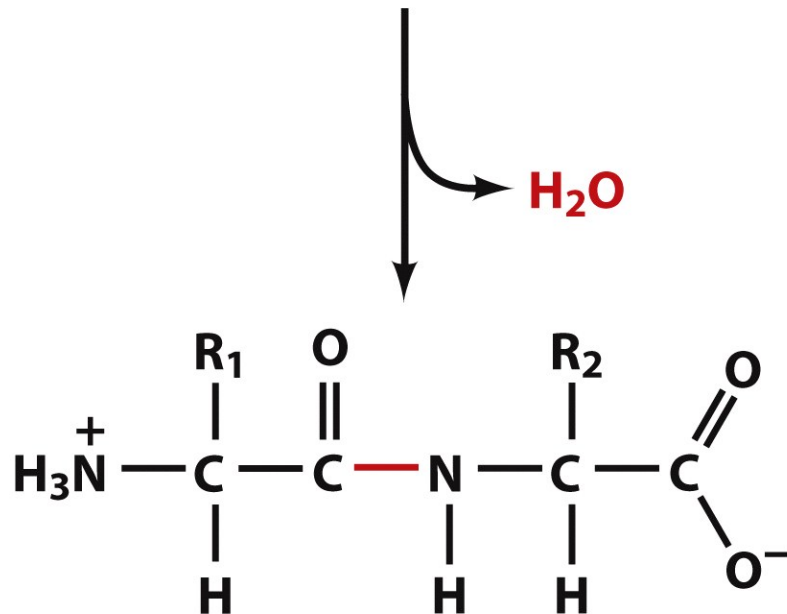
Condensation of two amino acids

Amino Acids
Polymerization of amino acids
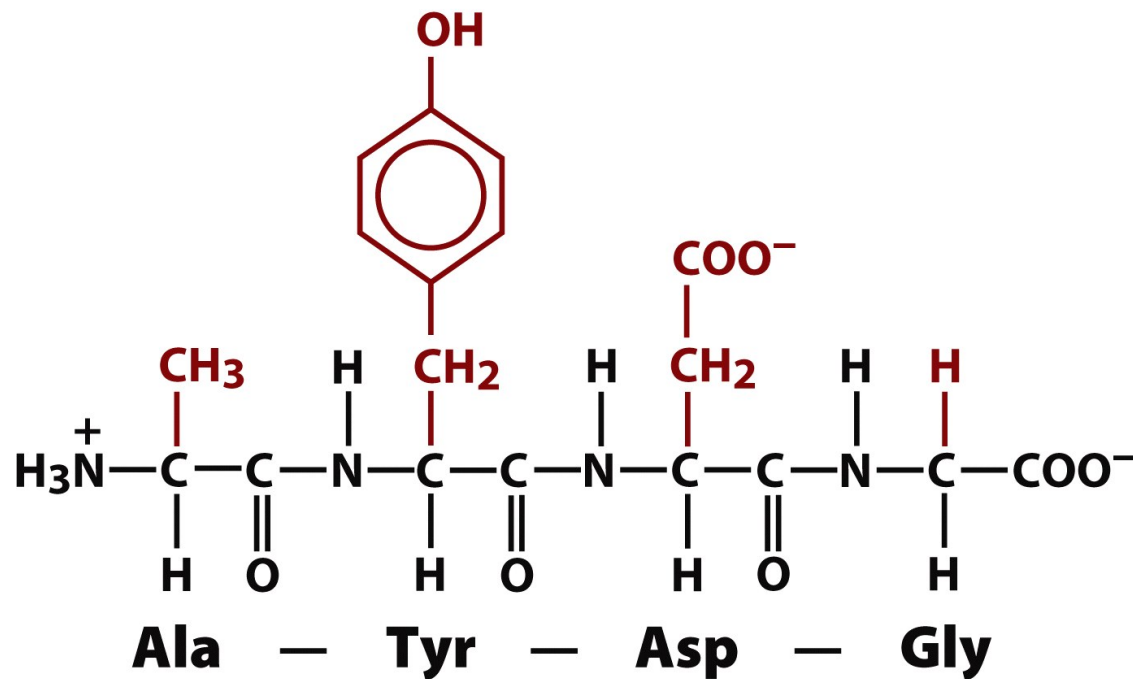
A tetrapeptide

Amino acids can be polymerized to form chains (polypeptides)

The polypeptides can have different lengths and sequeces of amino acids

Protein Structure
Levels of protein structure

(a)   – Lys – Ala – His – Gly – Lys – Lys – Val – Leu – Gly - Ala –

        Primary structure (amino acid sequence in a polypeptide chain)

Protein Structure
Levels of protein structure

(a) **– Lys – Ala – His – Gly – Lys – Lys – Val – Leu – Gly - Ala –**

Primary structure (amino acid sequence in a polypeptide chain)

(b)

**Secondary
structure
(helix)**

Protein Structure
Levels of protein structure

(a) – Lys – Ala – His – Gly – Lys – Lys – Val – Leu – Gly - Ala –

Primary structure (amino acid sequence in a polypeptide chain)

(b)

(c)

β

**Tertiary structure:
one complete protein chain
(β chain of hemoglobin)**

**Secondary
structure
(helix)**

Protein Structure
Levels of protein structure

(a) – **Lys – Ala – His – Gly – Lys – Lys – Val – Leu – Gly - Ala –**

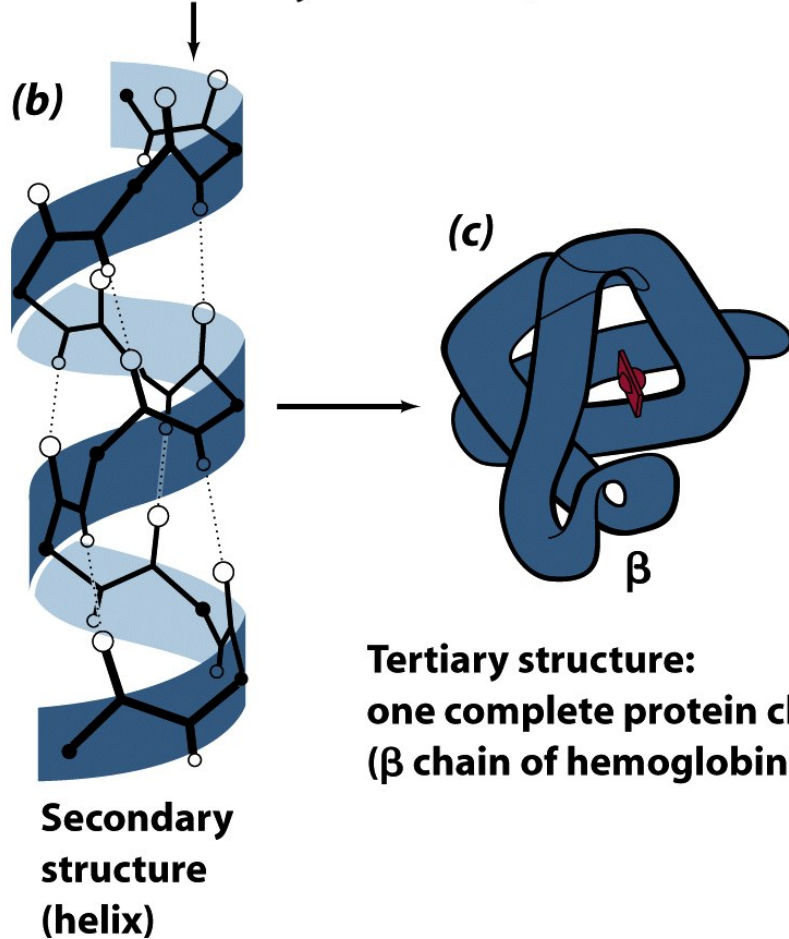**Primary structure (amino acid sequence in a polypeptide chain)**

(b)

(c)

(d) β_2 β_1

β

α_2 α_1
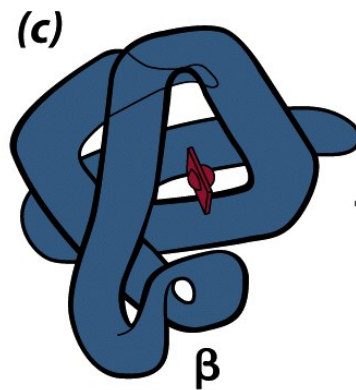
**Tertiary structure:
one complete protein chain
(β chain of hemoglobin)**
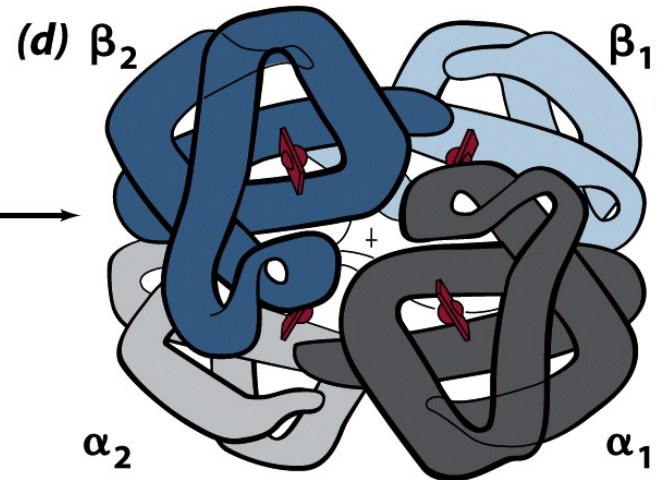
**Quaternary structure:
the four separate chains
of hemoglobin assembled
into an oligomeric protein**

**Secondary
structure
(helix)**

Protein Structure
Levels of protein structure
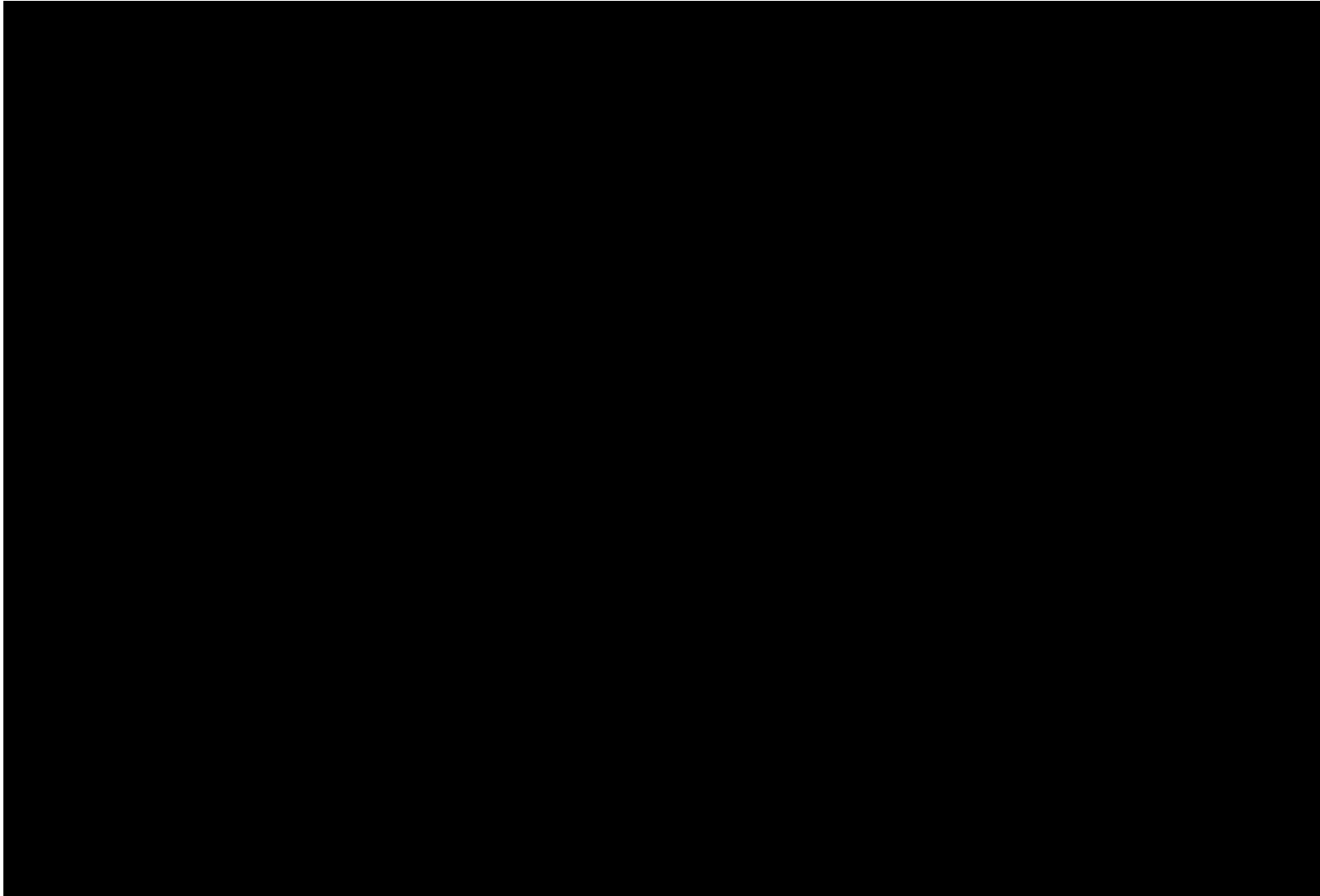
Protein Structure
Different ways to visualise protein structure
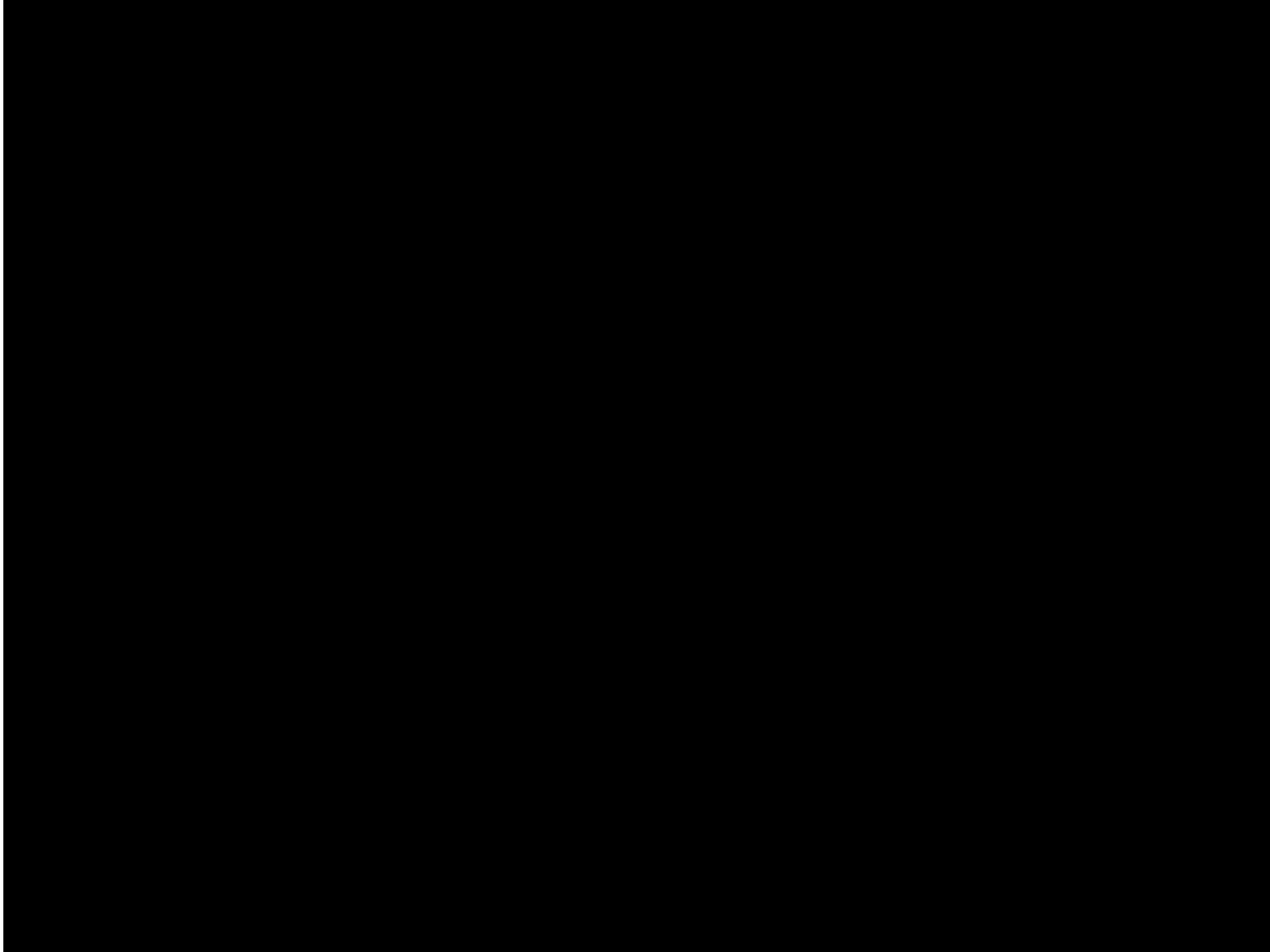
Proteins
Natural Role

Enzymes: catalyse chemical reactions

Hormones: messengers that regulate bodily function

Storage proteins: make essential substances readily available

Transport proteins: carry substances around the body

Structural proteins: support and maintain the shape of the cell

Protective proteins: provide defense against foreign matter/organisms

Contractile proteins: do mechanical work

Signal transduction proteins: transfer information

Channel proteins: allow molecules to cross biological membranes

Proteins

Industrial and biomedical applications

Industrial Enzymes
The 2012 enzyme business and biobusiness sales by industry

1%

8%

13%

16%

34%

28%

- Biopharma
- Household Care Enzymes
- Food & Beverages Enzymes
- Bioenegy Enzymes
- Feed and Other Technical Enzymes
- Microorganisms
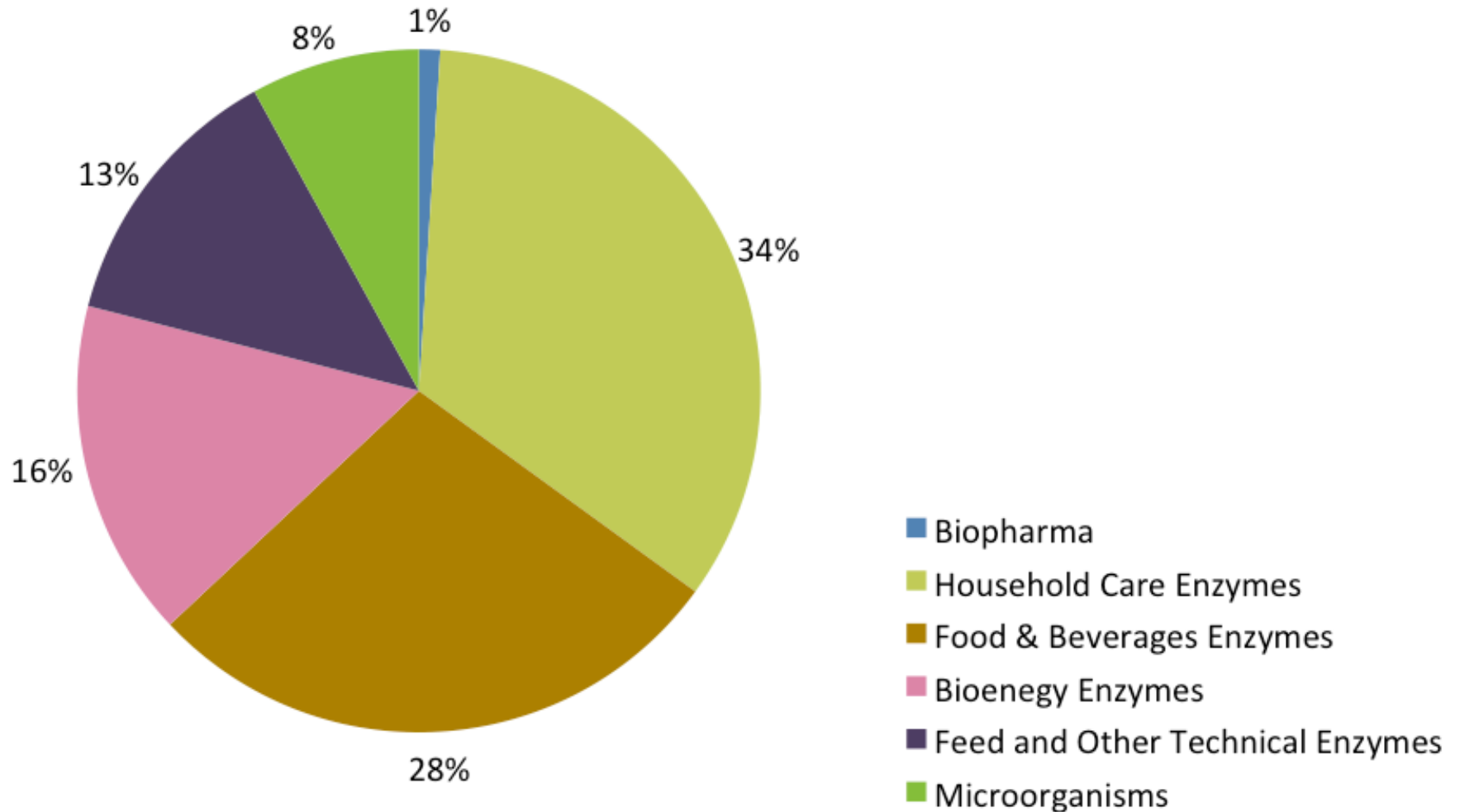
Total market of € 3.3 billion per year

Therapeutic Proteins
US sales by class in 2012
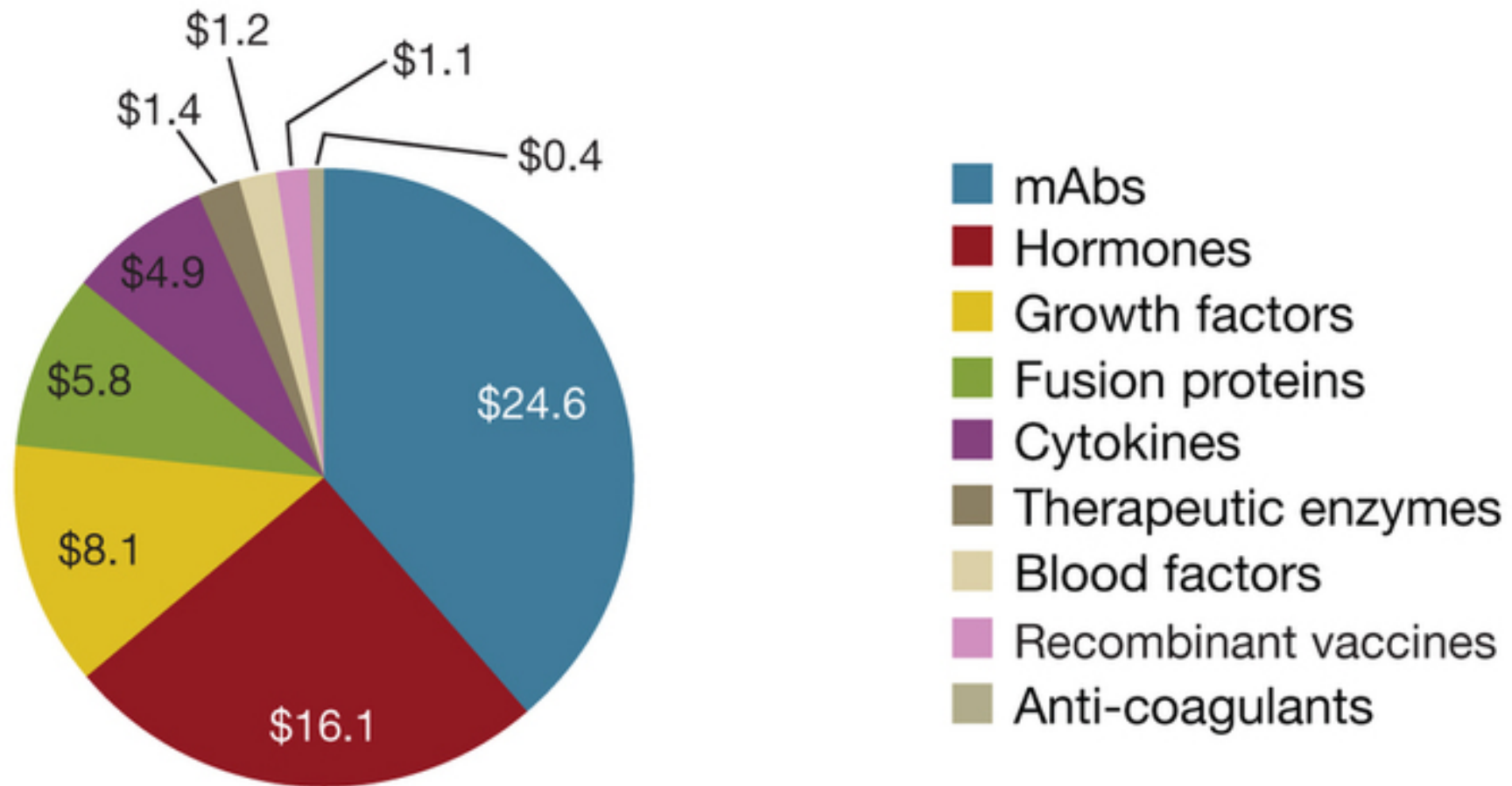
Total US sales of € 63.6 billion per year

Protein Engineering
The majority of industrial and therapeutic proteins are engineered

Biological washing powder

Amylases digest **starch** and break it down into sugars

Proteases digest **proteins** and break them down into amino acids

Lipases digest **fats** and break them down into fatty acids and glycerol

Natural enzymes have not evolved to work efficiently in washing machines

Therefore all commercial detergent enzyme are **engineered**

Modifying Proteins

Protein Chemistry

Chemical Modification of Amino Acid Residues
Classical protein chemistry

Protein Chemistry

Can modify amino and carboxyl terminus

Can modify some of the side chains, e.g.
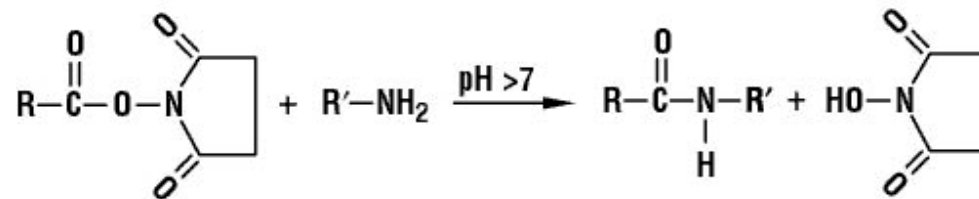
NHS-ester reacting with amine
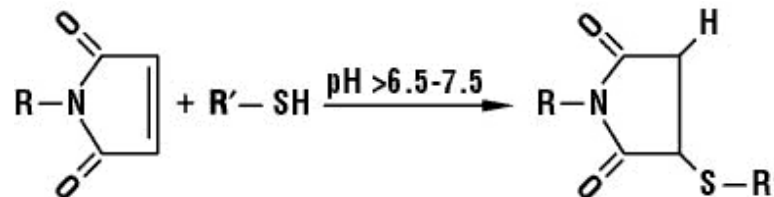
Maleimide reacting with thiol

Chemical Modification of Amino Acid Residues
Classical protein chemistry

Disadvantages of using chemical modification

- Can only modify chemically reactive side-chains
  e.g. not aliphatics - Gly, Ala, Val, Leu, Ile

- Often multiple residues with same reactive group in same protein:
  therefore cannot target the modification of a single amino-acid

- Often non-specific

- Frequently involves making significant changes that:
  - i) add steric bulk, or
  - ii) grossly alter the chemical properties of the side chain

Recombinant DNA Technology

Precise engineering of proteins by engineering the genes that encode them

Genes Direct RNA and Protein Synthesis
The central dogma of molecular biology

replication

transcription

translation

DNA — RNA — protein
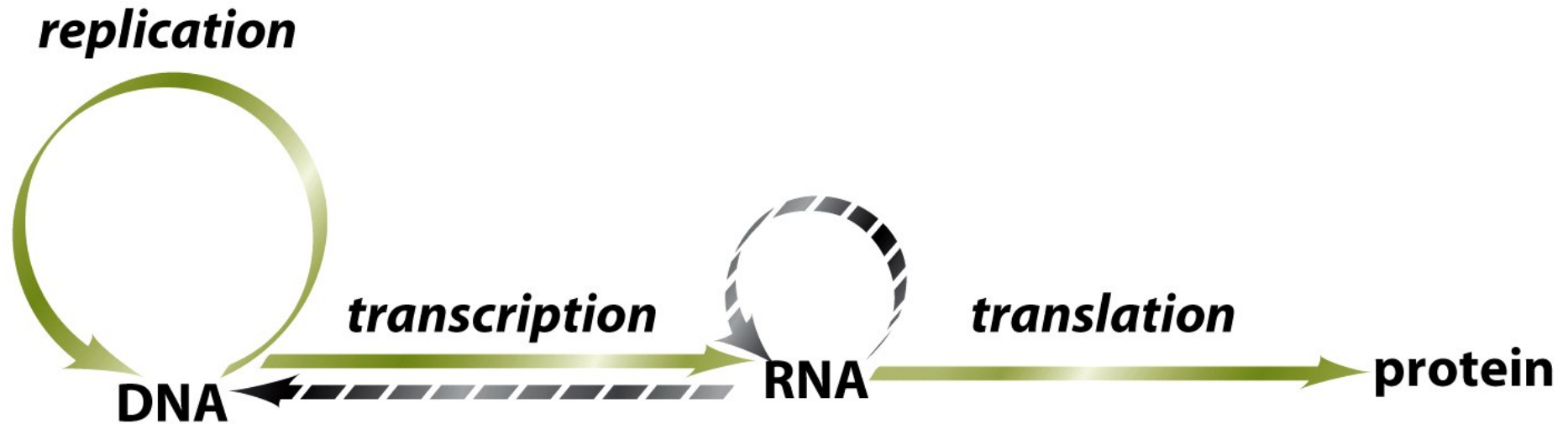
The central dogma of molecular biology was formulated by Francis Crick in 1958.

Solid arrows indicate information transfers that occur in all organisms

Dashed arrow indicate information transfers that only occur in certain organisms

Genes Direct RNA and Protein Synthesis
The central dogma of molecular biology

DNA
Learning
Center

CSH Cold
Spring
Harbor
Laboratory

Directed Mutagenesis and Protein Engineering

Oligonucleotide-Directed Mutagenesis
Changing a single amino acid residue at a specific site in a protein
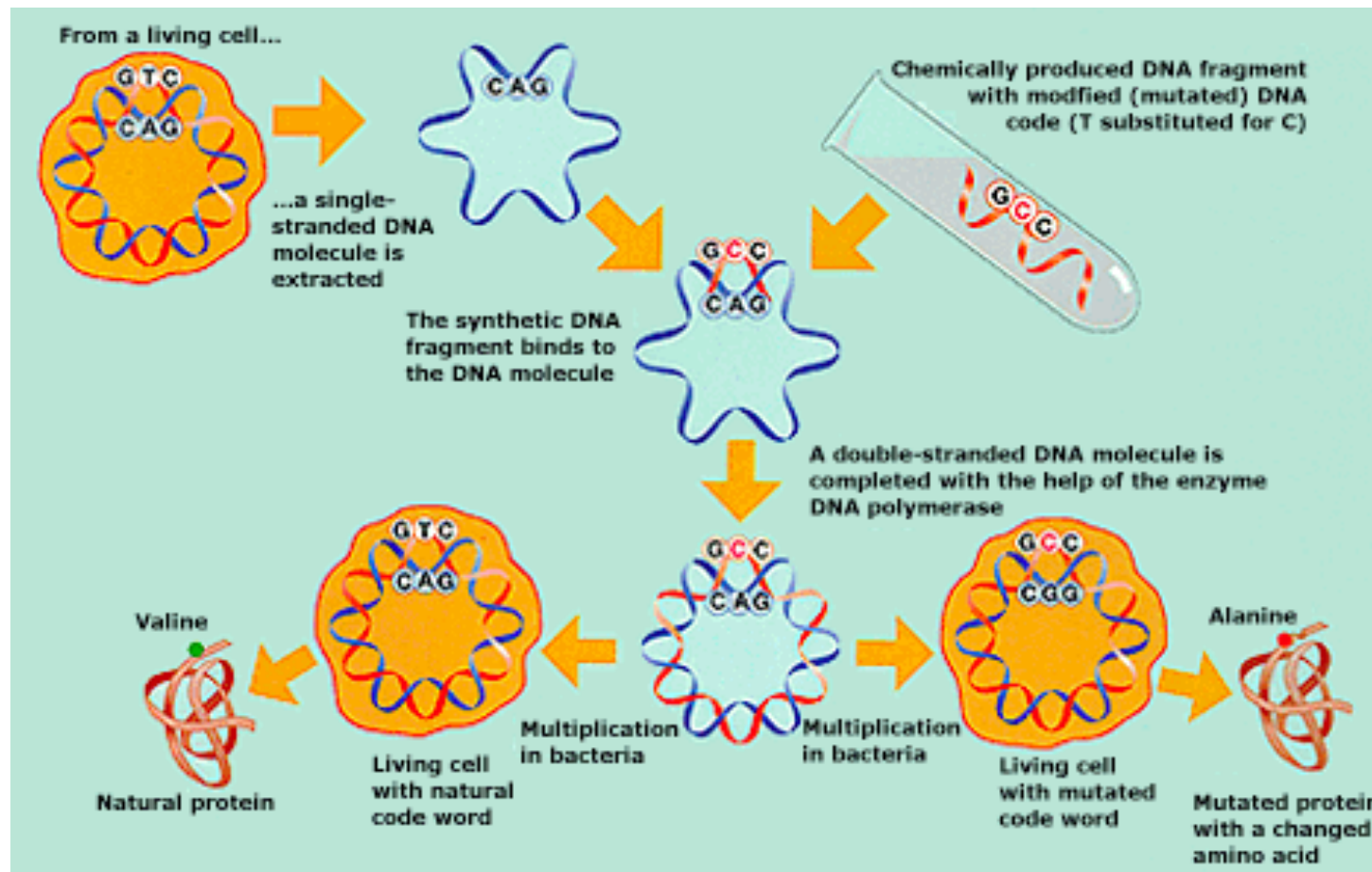
The original scheme for oligonucleotide-directed mutagenesis.

Zoller, M.J. and Smith, M. (1982). Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA. Nucleic Acids Res. 10: 6487-6500.

Oligonucleotide-Directed Mutagenesis
Changing a single amino acid residue at a specific site in a protein
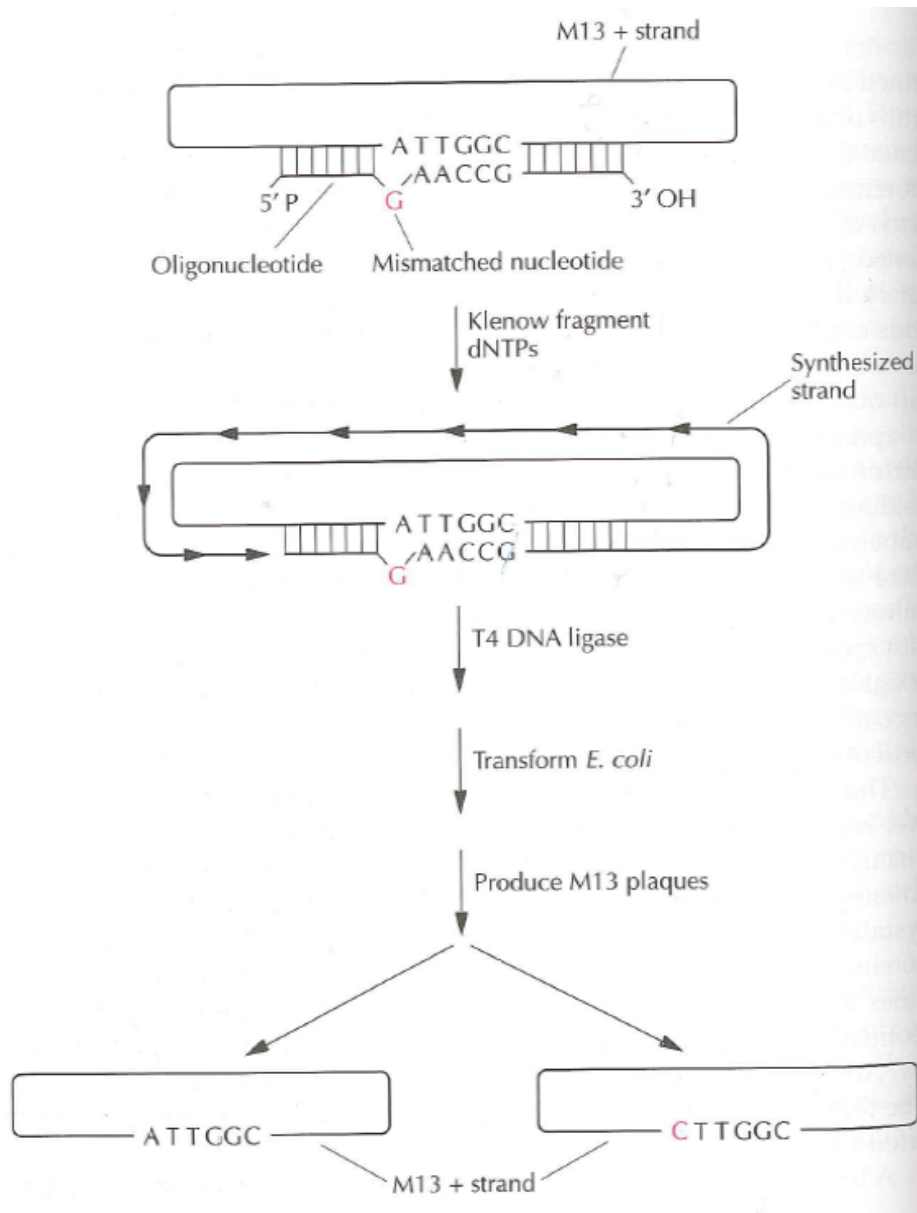
The original scheme for oligonucleotide-directed mutagenesis.

Zoller, M.J. and Smith, M. (1982). Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA. Nucleic Acids Res. 10: 6487-6500.

An oligonucleotide containing a single mismatched base is annealed to single-stranded bacteriophage M13 DNA (M13 + strand) containing the cloned gene to be mutated.

In theory should create 50% M13 phage containing the mutation and 50% wild-type

In practice, one typically gets only 1 to 5 % mutant phage

Oligonucleotide-Directed Mutagenesis
Enrichment of mutated M13 by passage through a *dut ung* strain of *E. coli*
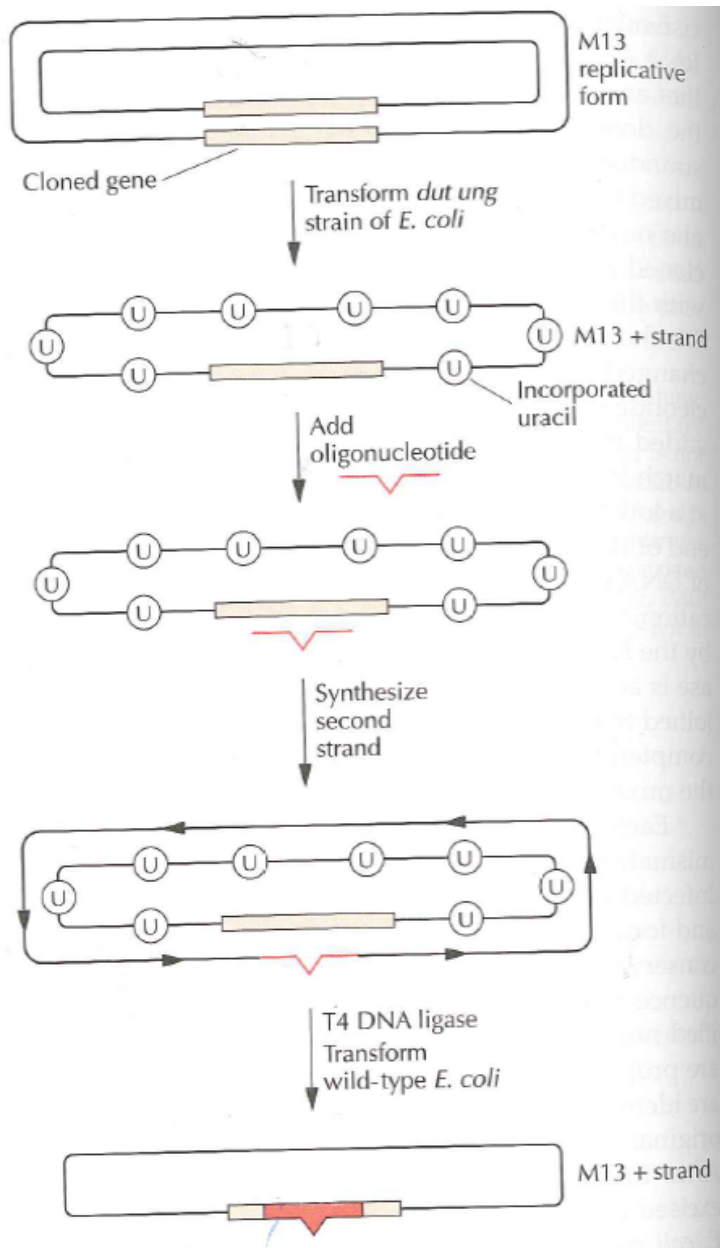
The target gene is cloned into the double-stranded replicative form of phage M13

This DNA is used to transform a *dut ung* strain of *E. coli*

The *dut* mutation causes intracellular dUTP concentration to be elevated - leading to incorporation of a few dUTP residues (U) into the single-stranded M13 DNA

The *ung* mutation prevents removal of incorporated uracil residues

Thus the wild-type template strand contains uracils and much of it is degraded in wild-type *E. coli* due to the activity of the wild-type *ung* gene product (uracil N-glycosylase)

The newly synthesised mutant strand does not contain uracil and is not degraded

Oligonucleotide-Directed Mutagenesis
Mutagenesis by PCR – the two tube method
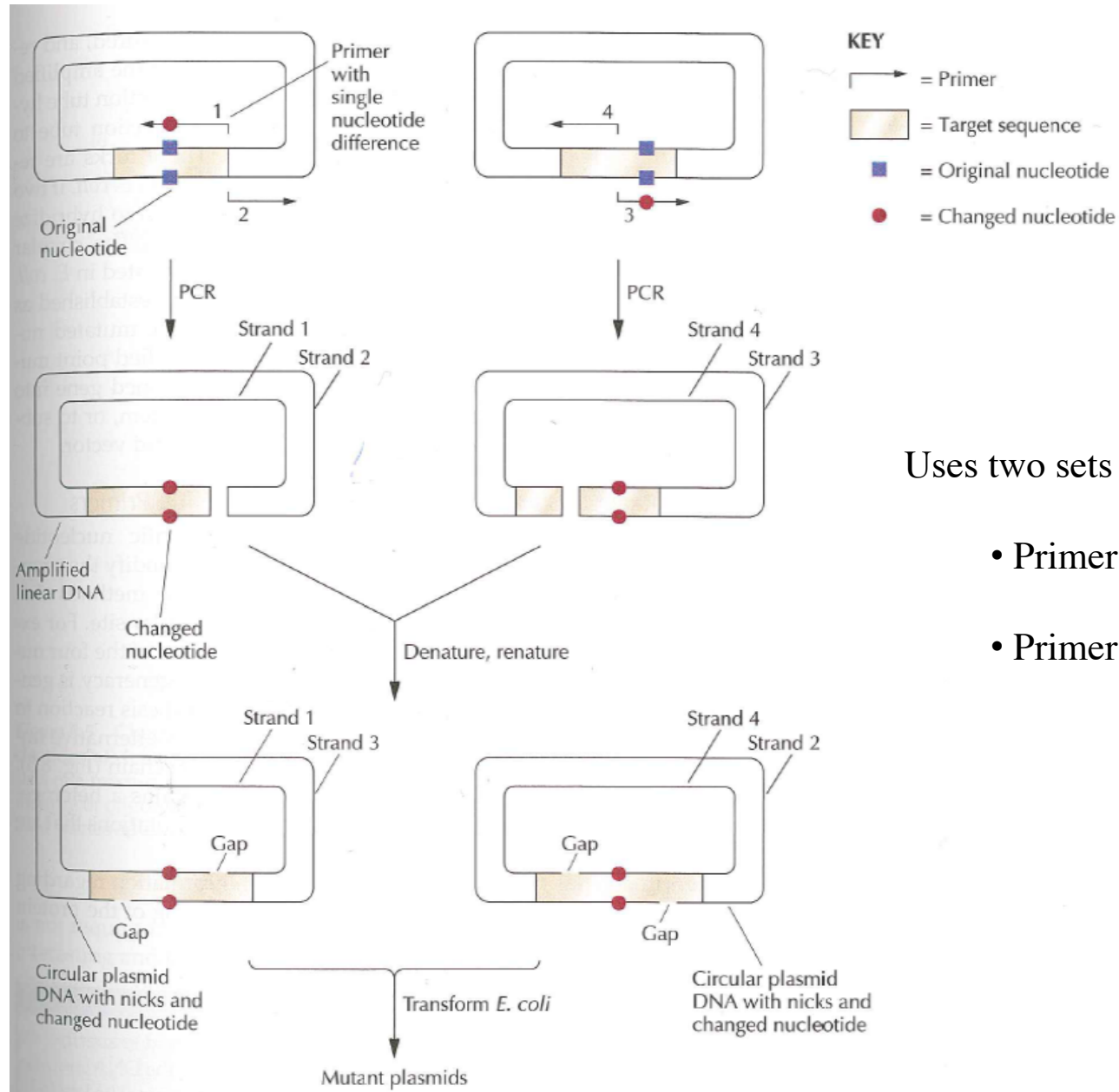
Uses two sets of oligonucleotide primers:

- Primers 1 and 2

- Primers 3 and 4

Mutagenesis using PCR
The "QuikChange" system from Stratagene
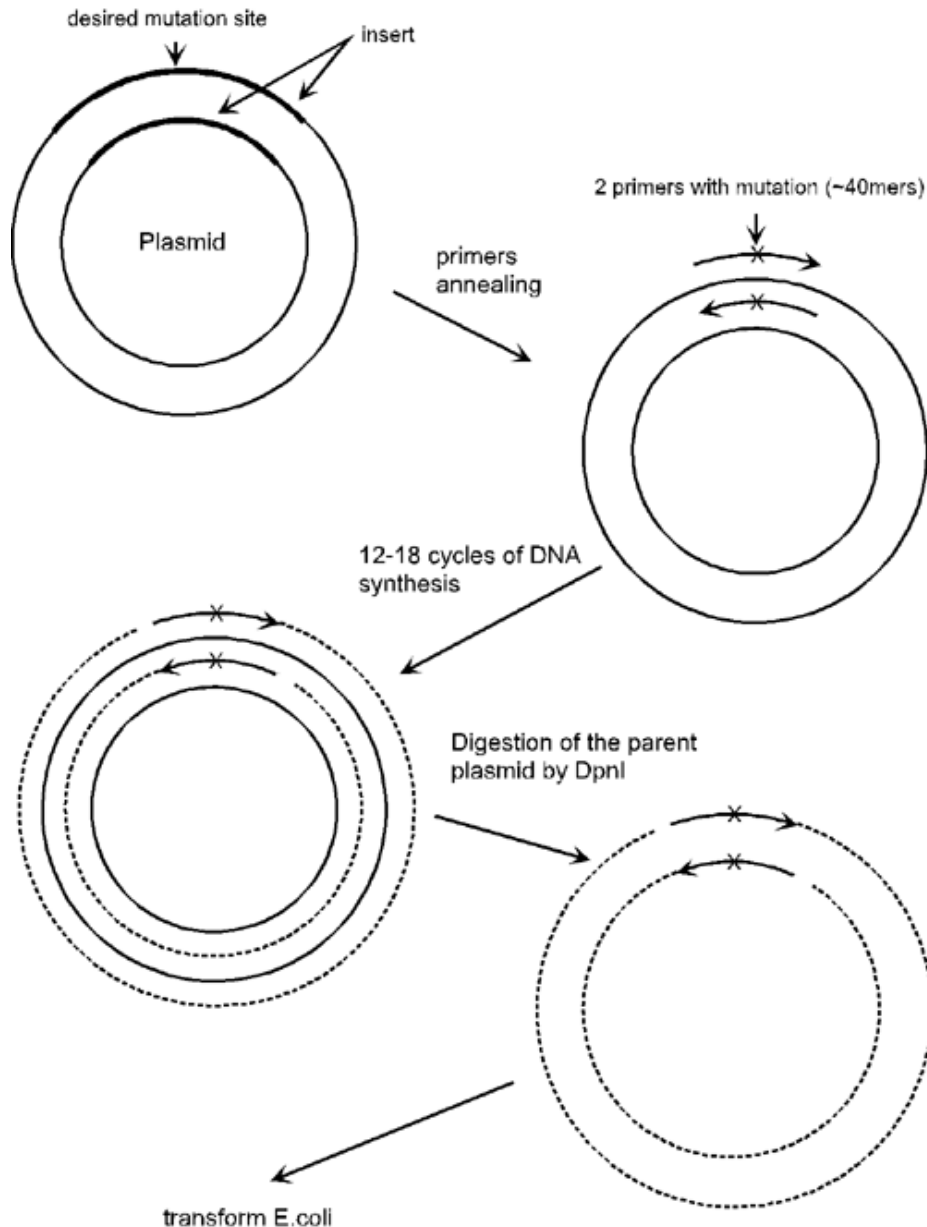
Based on the fact that the restriction endonuclease DpnI will *only* cut methylated DNA strands

Parent DNA is produced in bacteria which methylate the DNA: digested

The new DNA strands made by PCR are unmethylated: survive

Mutagenesis using PCR
The "QuikChange" system from Stratagene

Day 1

Step 1
Plasmid Preparation

Gene in plasmid with target site (◎) for mutation

Step 2
Temperature Cycling

Denature the plasmid and anneal the oligonucleotide primers (✐) containing the desired mutation (×)

Mutagenic primers

Using the nonstrand-displacing action of *PfuTurbo* DNA polymerase, extend and incorporate the mutagenic primers resulting in nicked circular strands

Step 3
Digestion

Digest the methylated, nonmutated parental DNA template with *Dpn* I

Mutated plasmid (contains nicked circular strands)

Step 4
Transformation

Transform the circular, nicked dsDNA into XL1-Blue supercompetent cells

After transformation, the XL1-Blue supercompetent cells repair the nicks in the mutated plasmid

LEGEND

― Parental DNA plasmid

✐ Mutagenic primer

― Mutated DNA plasmid

This is the most commonly used oligonucleotide-directed mutagenesis procedure today

Protein Engineering using Directed Mutagenesis
Goals

1. To understand the way proteins work e.g. to study the mechanism of catalysis

2. To engineer proteins with activities tailored for industrial for therapeutic applications

Protein Engineering

Fundamental studies of enzymatic catalysis

Enzymes
Enzymes are powerful and highly specific catalysts

Catalyst:

A substance, usually used in small amounts relative to the reactants, that increases the rate of a reaction without being consumed in the process.

Catalysts accelerate reactions, but do not change the equilibrium position of the reaction
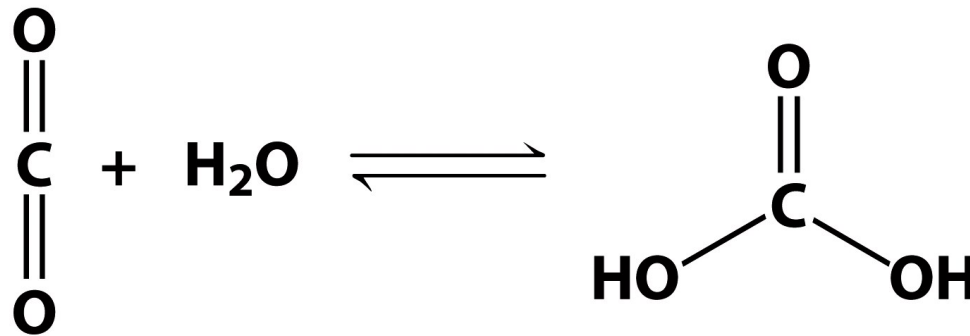
Enzymes
Enzymes are powerful and highly specific catalysts

Catalyst:

A substance, usually used in small amounts relative to the reactants, that increases the rate of a reaction without being consumed in the process.

Catalysts accelerate reactions, but do not change the equilibrium position of the reaction

The enzyme carbonic anhydrase can hydrate 10^6 molecules of CO_2 per second

The catalyzed reaction is 10^7 times as fast as the uncatalyzed one

Enzymes
Enzymes are powerful and highly specific catalysts

Table 5.1 Rate enhancement by selected enzymes

Enzyme	Nonenzymatic half-life		Uncatalyzed rate (k_{un} s^{-1})	Catalyzed rate (k_{cat} s^{-1})	Rate enhancement (k_{cat} s^{-1}/k_{un} s^{-1})
OMP decarboxylase	78,000,000	years	2.8×10^{-16}	39	1.4×10^{17}
Staphylococcal nuclease	130,000	years	1.7×10^{-13}	95	5.6×10^{14}
AMP nucleosidase	69,000	years	1.0×10^{-11}	60	6.0×10^{12}
Carboxypeptidase A	7.3 years		3.0×10^{-9}	578	1.9×10^{11}
Ketosteroid isomerase	7	weeks	1.7×10^{-7}	66,000	3.9×10^{11}
Triose phosphate isomerase	1.9 days		4.3×10^{-6}	4,300	1.0×10^{9}
Chorismate mutase	7.4 hours		2.6×10^{-5}	50	1.9×10^{6}
Carbonic anhydrase	5	seconds	1.3×10^{-1}	1×10^{-6}	7.7×10^{6}

Abbreviations: OMP, orotidine monophosphate; AMP, adenosine monophosphate.
Source: After A. Radzicka and R. Wolfenden, *Science* 267:90–93, 1995.

Enzymes
General properties of enzymes

Enzymatic catalysis differs from ordinary chemical catalysis in many important respects:

1. Higher reaction rates. Reaction rates are typically 10^6-10^{12} times the uncatalyzed reaction.

2. Milder reaction conditions. Enzymatic reactions occur under mild conditions, temperatures <100°C, atmospheric pressure, near neutral pH

3. Greater reaction specificity. Higher specificity for their substrates (reactants) and products

4. Capacity for regulation. Many enzymes can be regulated by substances other than their substrates

Enzymes
Enzymes alter the reaction rate but not the reaction equilibrium
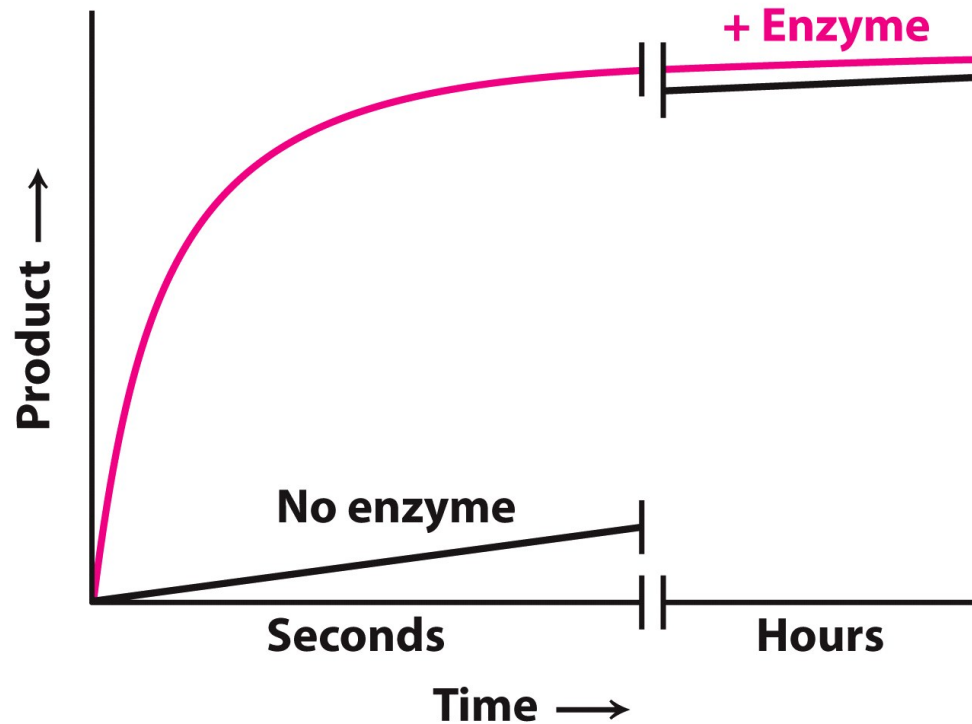
Enzymes are remarkable catalysts, but they cannot alter the laws of thermodynamics

Enzymes accelerate the attainment of equilibrium (improve the kinetics)

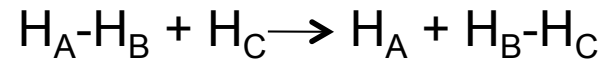But do not change the position of the equilibrium (no change in thermodynamics)

Enzymes
Enzymes facilitate the formation of the transition state

Much understanding of enzymatic catalysis comes from transition state theory, developed in the 1930s, principally by Henry Eyring

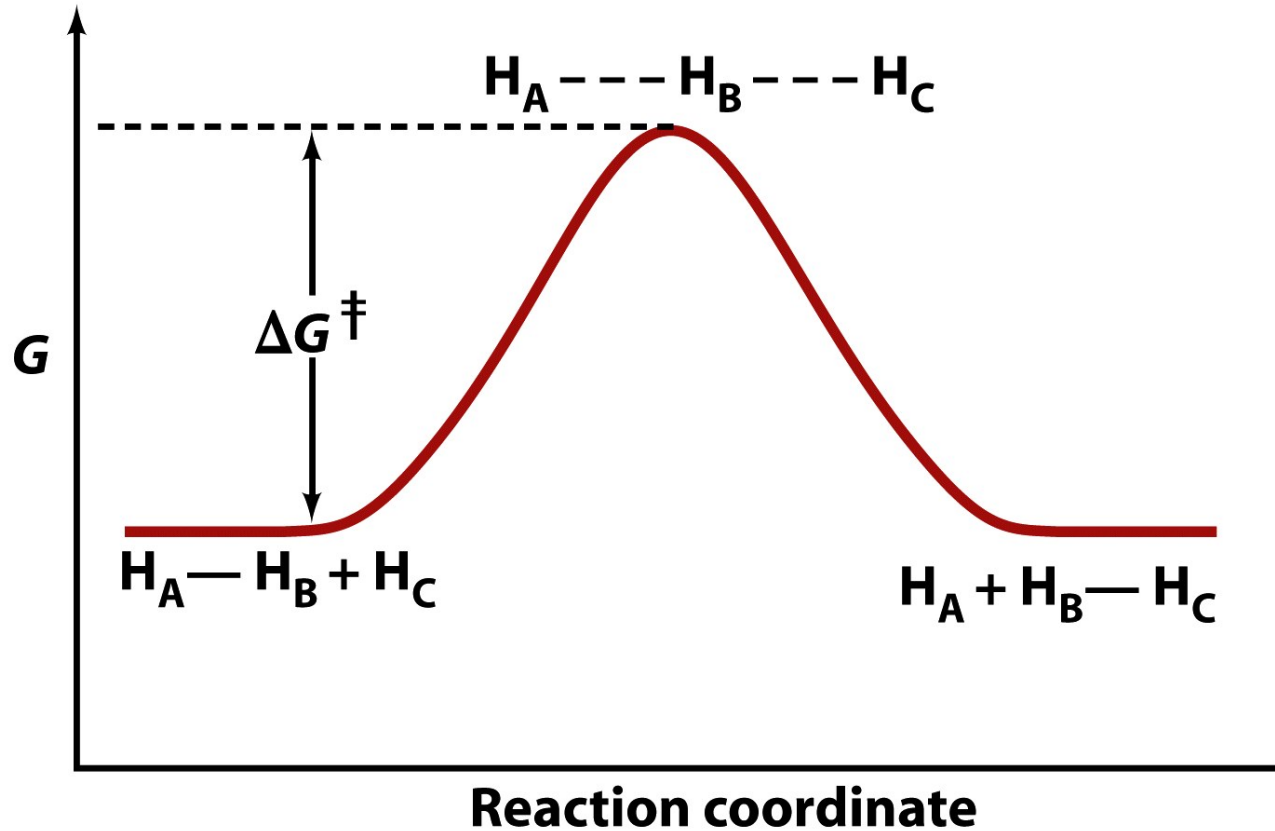Consider the reaction between a hydrogen atom and a diatomic hydrogen:

$$H_A\text{-}H_B + H_C \longrightarrow H_A + H_B\text{-}H_C$$

As H_C approaches $H_A\text{-}H_B$, at some point there exists a high-energy (unstable) complex $H_A\text{---}H_B\text{---}H_C$ in which one bond is forming the other breaking

The point of highest free energy (G) is called the transition state (TS)

Enzymes
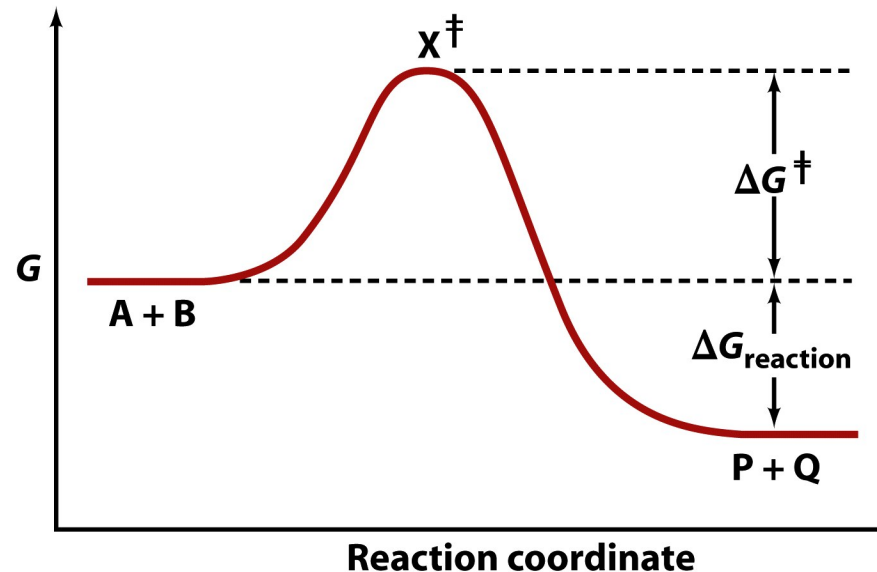## Enzymes facilitate the formation of the transition state

The point of highest free energy (G) is called the transition state (TS)

The difference in free energy between the reactants and the TS is the free energy of activation ($\Delta G^{\ddagger}$)

Enzymes

Enzymes facilitate the formation of the transition state

If the reacting atoms are different, such as in the reaction:

$$A + B \longrightarrow X^{\ddagger} \longrightarrow P + Q$$
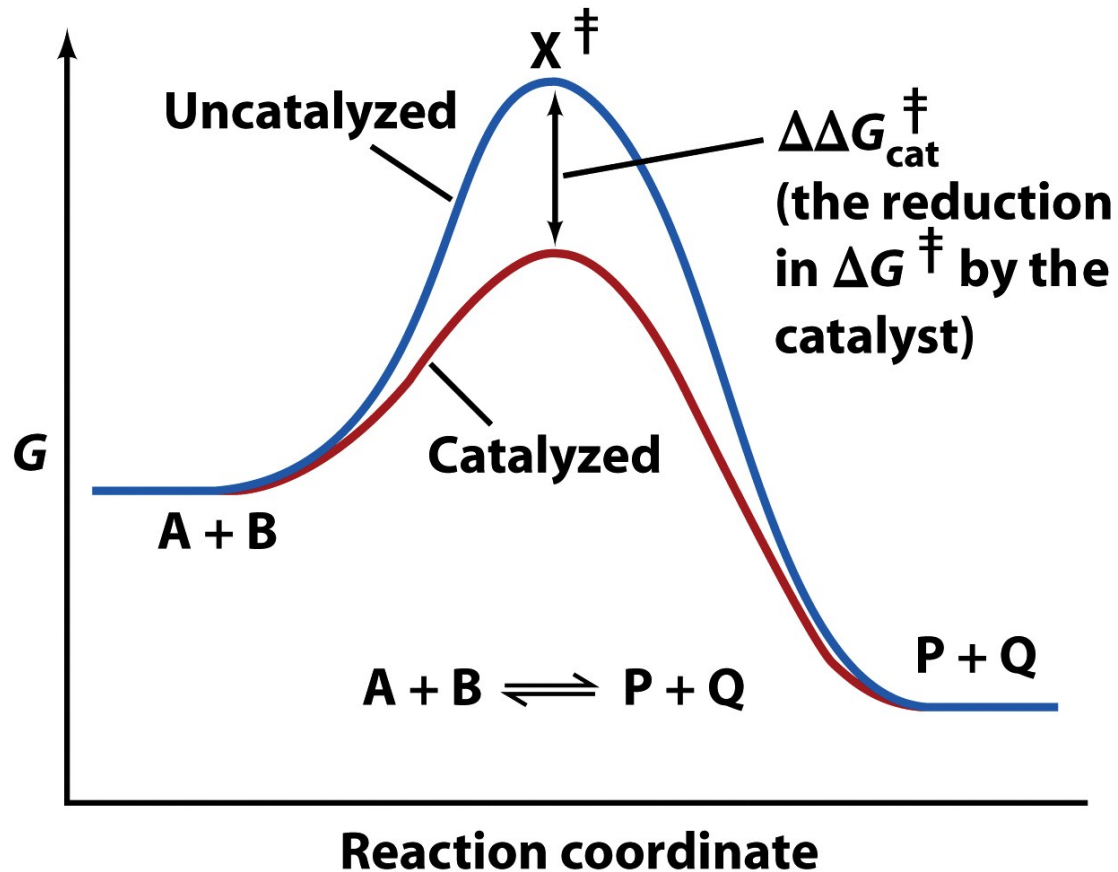
There is a difference in the free energy of the reactants and products ($\Delta G_{reaction}$) and the reaction coordinate is asymmetric.

$\Delta G_{reaction}$ determines the equilibrium position (more negative = more products)

$\Delta G^{\ddagger}$ determines the rate of the reaction

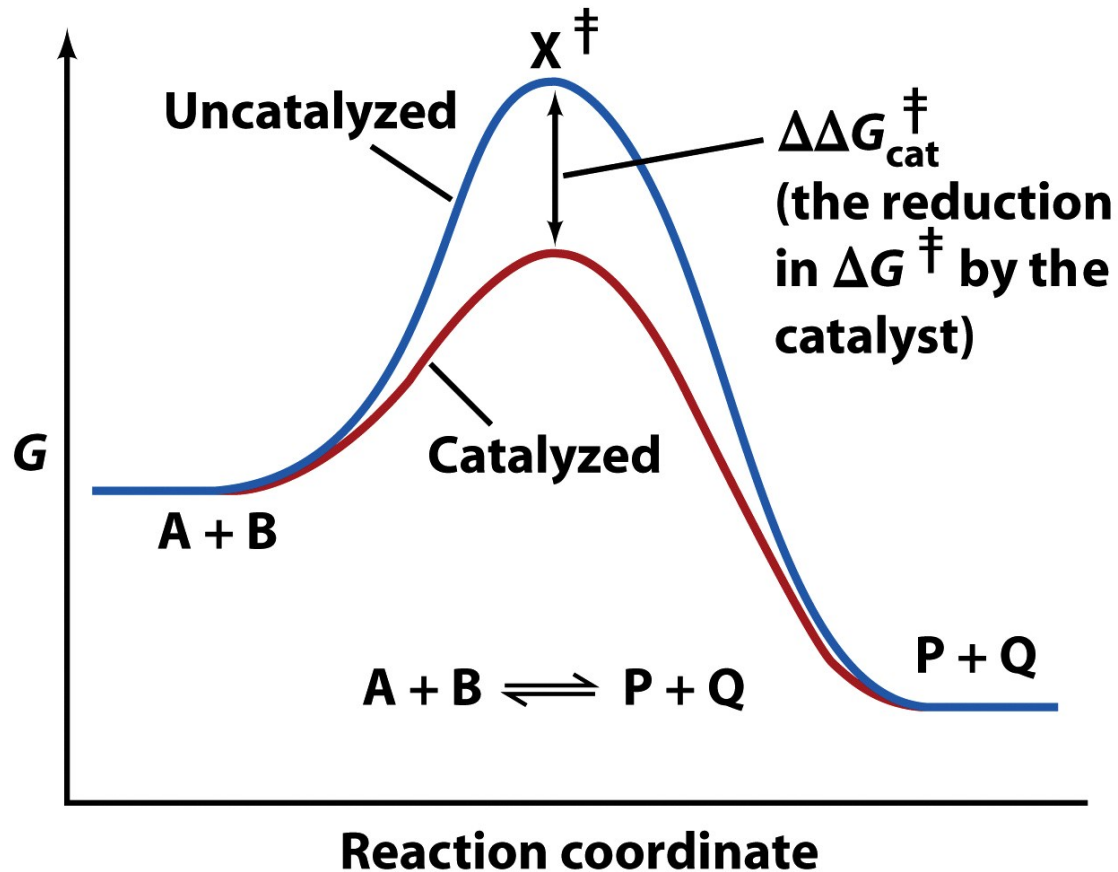# Enzymes
Enzymes facilitate the formation of the transition state

The reaction rate is proportional to $e^{-\Delta G^{\ddagger}/RT}$ (R, gas constant; T, absolute temp)

Lowering $\Delta G^{\ddagger}$ therefore increases the reaction rate

Enzymes
Enzymes facilitate the formation of the transition state

Catalysts provide a reaction pathway whose free energy is lower that that of the uncatalyzed reaction

The difference between $\Delta G^{\ddagger}$ for the catalyzed and uncatalyzed reactions, $\Delta\Delta G^{\ddagger}$, indicates the efficiency of the catalyst
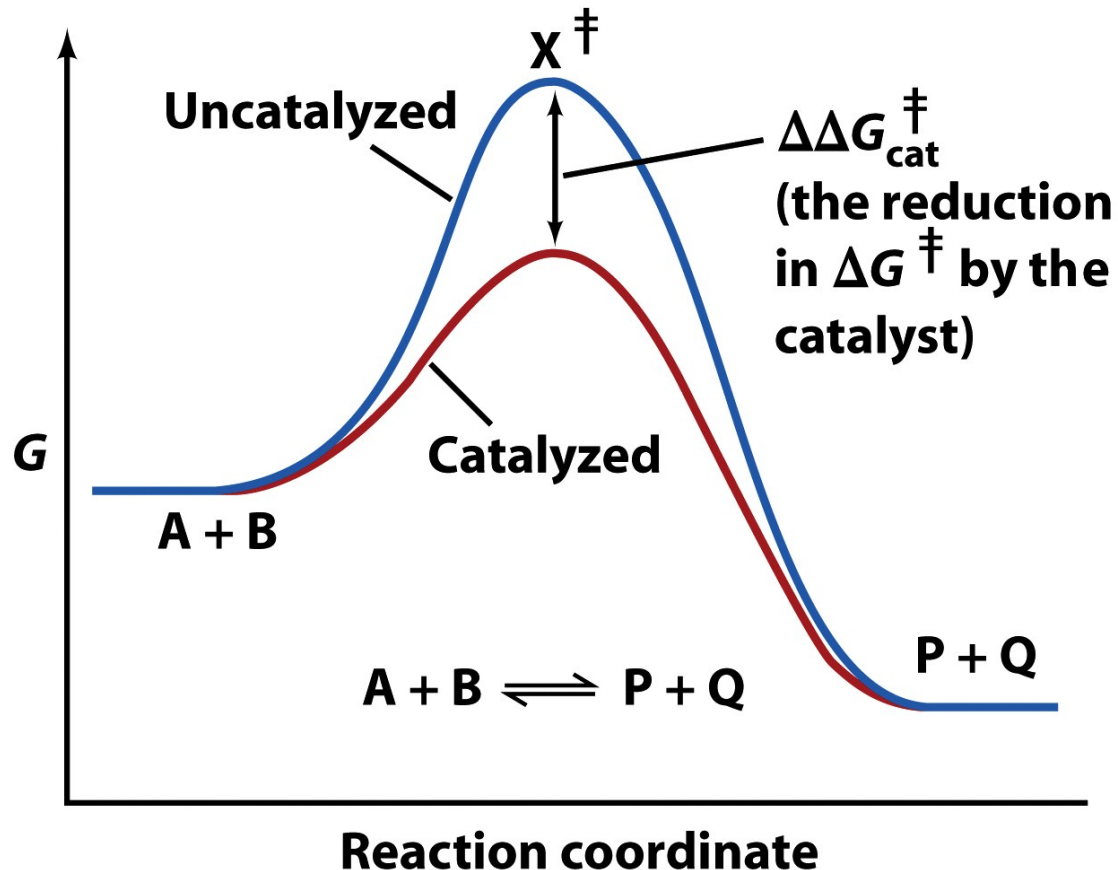
Rate enhancement = catalyzed rate/uncatalyzed rate = $e^{-\Delta\Delta G^{\ddagger}/RT}$

The reaction rate is proportional to $e^{-\Delta G^{\ddagger}/RT}$ (R, gas constant; T, absolute temp)

Lowering $\Delta G^{\ddagger}$ therefore increases the reaction rate

Enzymes
Enzymes facilitate the formation of the transition state

Catalysts provide a reaction pathway whose free energy is lower that that of the uncatalyzed reaction

The difference between $\Delta G^{\ddagger}$ for the catalyzed and uncatalyzed reactions, $\Delta\Delta G^{\ddagger}$, indicates the efficiency of the catalyst

Rate enhancement = catalyzed rate/uncatalyzed rate = $e^{-\Delta\Delta G^{\ddagger}/RT}$

Note: an enzyme must bind the transition state more tightly than the substrates for the reaction rate to be increased.

Enzymes
Enzymes catalyze reactions by preferentially binding the transition state
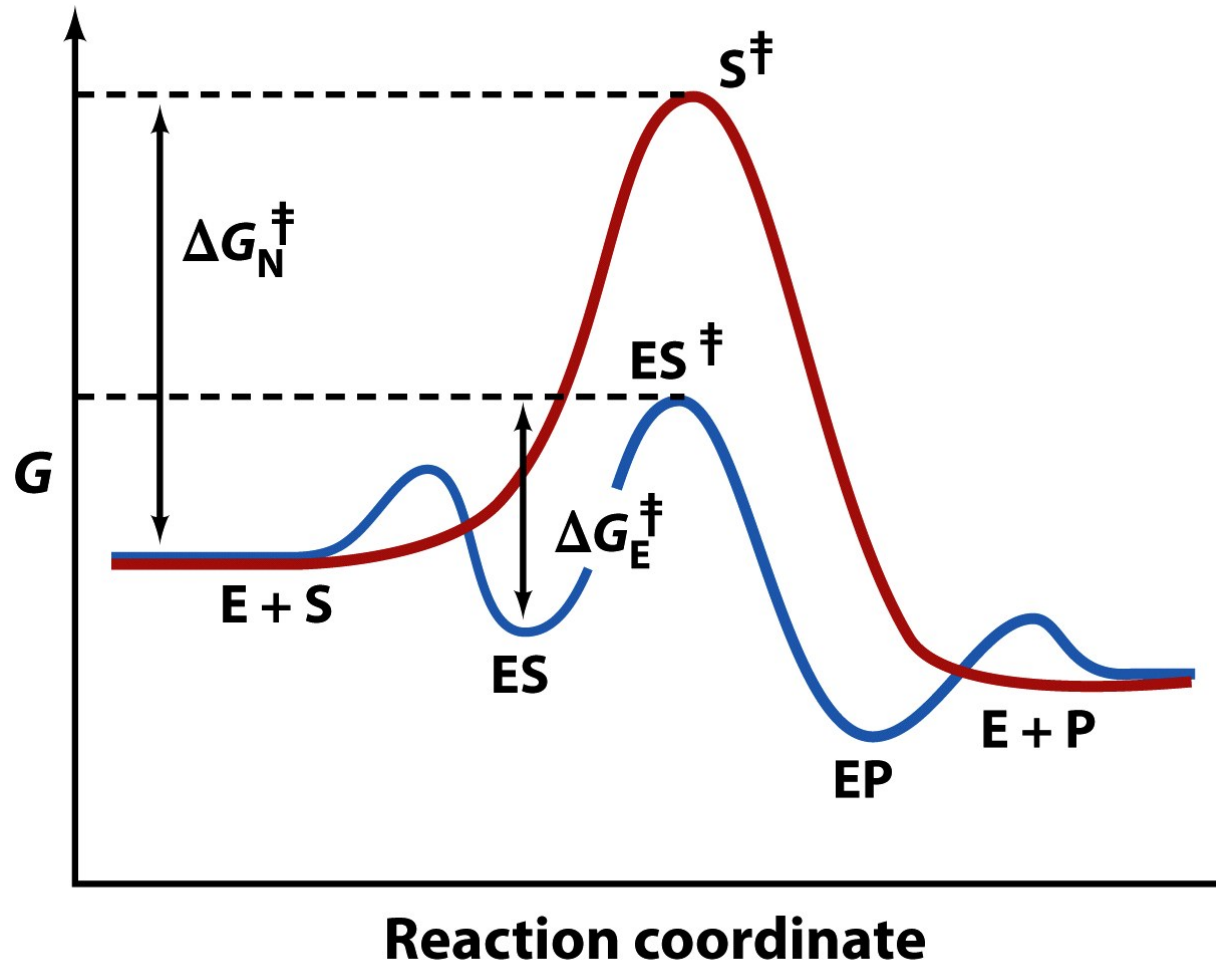
Uncatalyzed reaction = red
Catalyzed reaction   = blue

The $\Delta G^{\ddagger}$ of the catalyzed reaction is lower than the $\Delta G^{\ddagger}$ of the uncatayzed reaction

Enzymes catalyze reactions by binding the transition state with greater affinity than the substrate or product

Understanding Enzyme Mechanism
Using oligonucleotide-directed mutagenesis

What can one learn using protein engineering that cannot be learned from classical enzymology?

The fundamental difference between an enzyme catalysed reaction and the uncatalysed reaction is the use of binding energy between the enzyme, the substrate, and the intermediates in the reaction pathway to provide specificity and rate enhancement.

Thus, to understand an enzymatic reaction one must characterise the complex of the enzyme with:

1. Substrates
2. Intermediates
3. Transition states
4. Products

and determine the interaction energies for each as the reaction proceeds.

Protein engineering allows systematic alteration of the interactions between the enzyme and the substrates, intermediates, transition states and products.

Tyrosyl-tRNA Synthetase
The first enzyme to be studies using protein engineering

Tyrosyl- tRNA synthetase from Bacillus stearothermophilus catalyses the aminoacylation of tRNA^{Tyr}

$$E + Tyr + ATP \rightleftharpoons E.Tyr\text{-}AMP + PPi$$

$$E.Tyr\text{-}AMP + tRNA \longrightarrow Tyr\text{-}tRNA + E + AMP$$
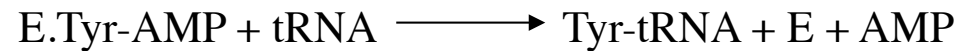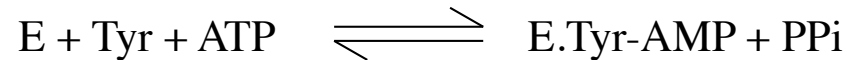
Classical protein chemistry, kinetics and even the crystal structure gave no clues to the reaction mechanism or the groups involved in catalysis
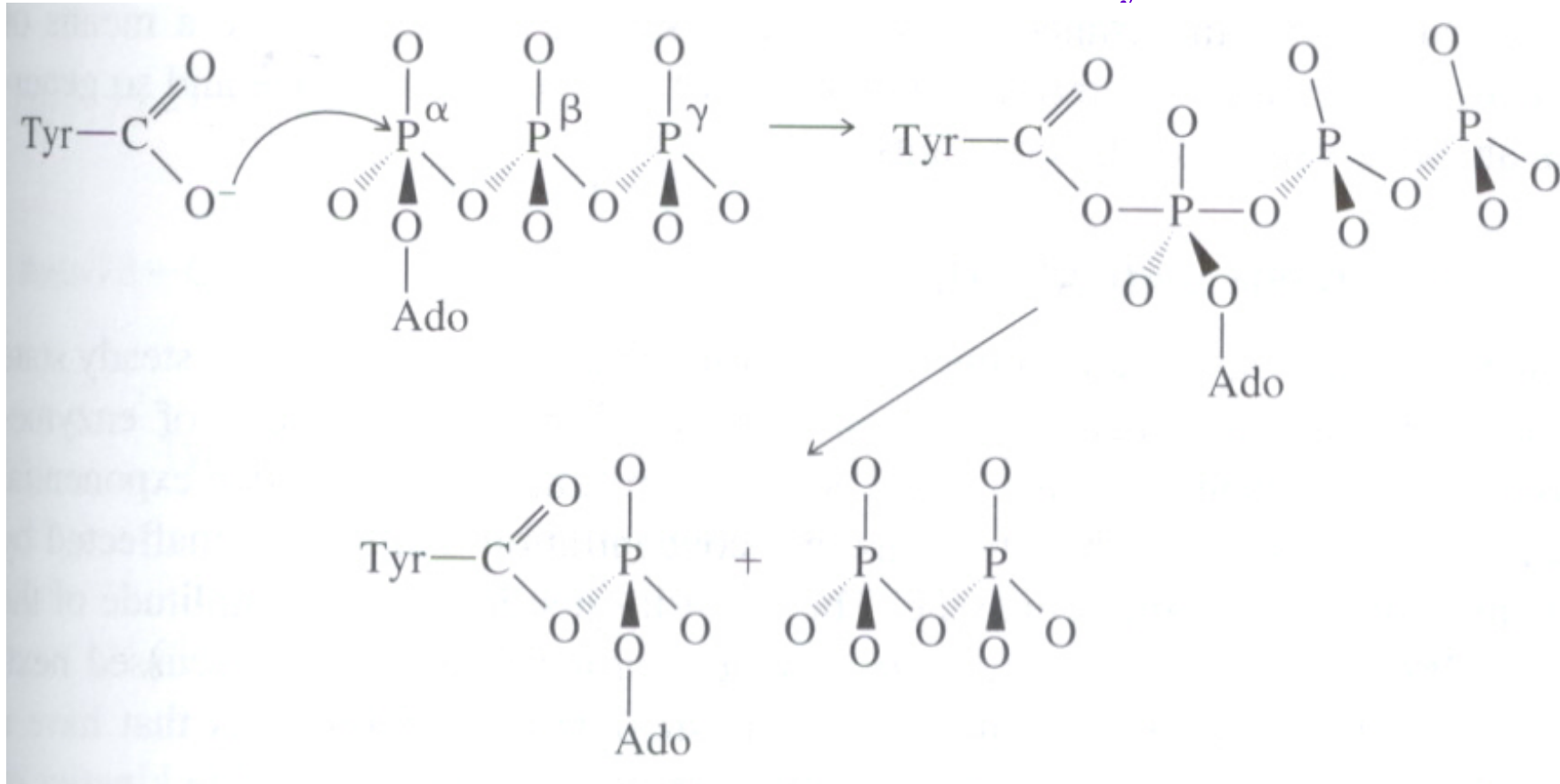
Protein engineering:

- revealed the mechanism

- gave direct experimental evidence about fundamental theories of enzyme catalysis which had previously been untested

Winter, G, Fersht A.R., Wilkinson, A.J., Zoller, M. and Smith, M. Nature 299, 756 (1982)

Tyrosyl-tRNA Synthetase
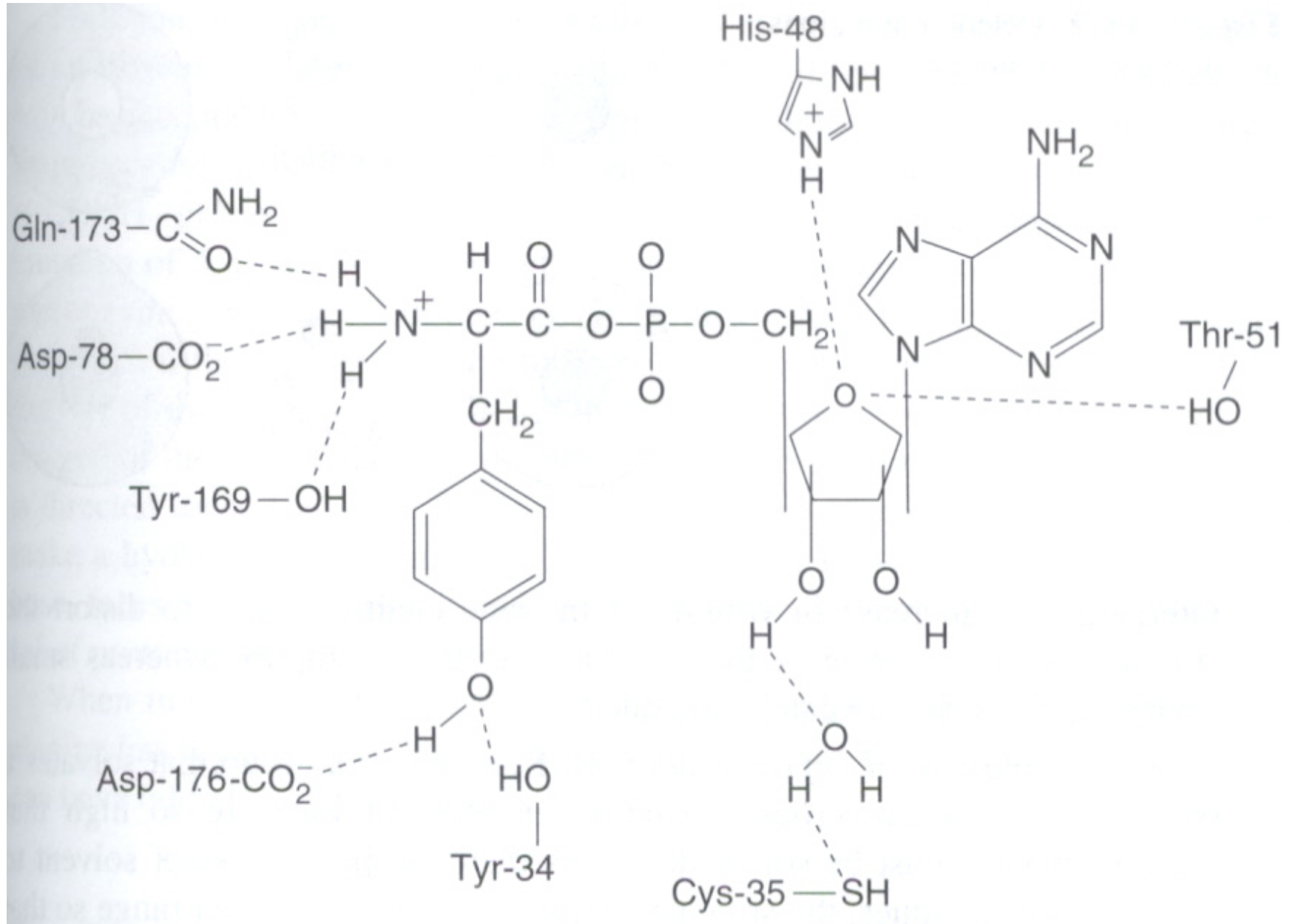## Chemical mechanism of activation of tyrosine

Crystal structures were determined of:

- E
- E.Tyr
- E.Tyr-AMP

The intermediates accumulate in the absence of ATP and tRNA respectively

Tyrosyl-tRNA Synthetase
## Residues that form hydrogen bonds with the tyrosyl adenylate

Tyrosyl-tRNA Synthetase
Choice of mutation

It was impossible to make all possible mutations - there are potentially 19 at any one position

Fortunately there are some simple rules to guide the choice:

1. Reduce rather than increase the size of the side chain - increased volume is liable to distort the structure, whereas small cavities can be tolerated
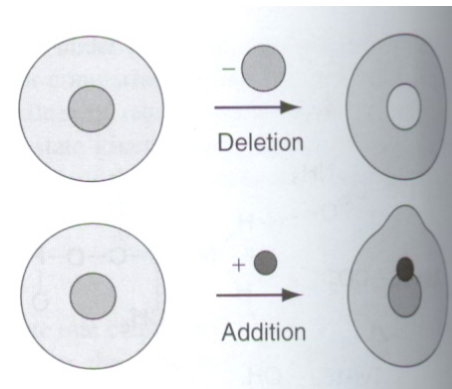
2. Avoid creating buried unpaired charges - if the charged group is not in open access to solvent the structure will rearrange to allow solvation of the charge by water or other groups

3. Delete the minimal number of interactions - analysing the change of just one interaction is difficult enough

4. Do not add new functional groups to side chains - this can cause local reorganisation of structure if the new group makes novel interactions

5. All the previous rules may be disobeyed when appropriate (Alan Fersht)

Tyrosyl-tRNA Synthetase
Nondisruptive deletions

The ideal mutation is a non-disruptive deletion: i.e. one that removes an interaction without causing a disruption or reorganisation of the structure

Ile>Val, Ala>Gly, Thr>Ser :
- loss of -CH$_2$-
- good for probing hydrophobic interactions
- no change in chemistry, tiny cavity created

Ile>Ala, Val>Ala, Leu>Ala :
- bigger cavity, larger loss of energy
- more chance side chains will move or solvent ingress to fill cavity

Ser>Ala, Tyr>Phe, Cys>Ala :
- good for probing hydrogen bonds

His>Asn, His>Gln:
- sometimes suitable as an NH of -CONH$_2$ of Asn can sometimes substitute for N$^\delta$H of imidazole ring,
- or NH of -CONH$_2$ of Gln can substitute for the N$^\epsilon$H of imidazole ring as a hydrogen-bond donor
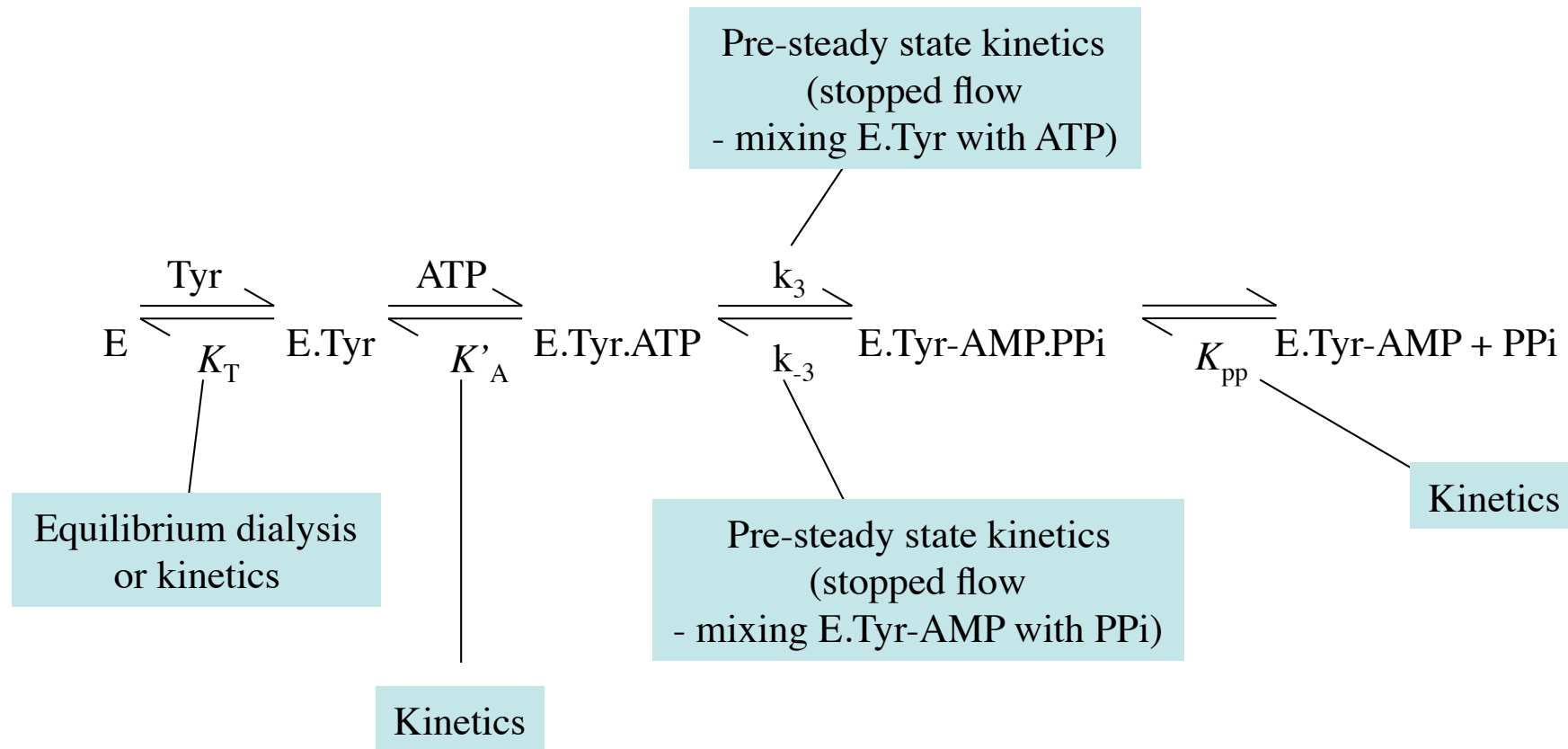
If in doubt - mutate to alanine (not glycine whose wider freedom of conformations can alter the structure)

Tyrosyl-tRNA Synthetase
Strategy: free energy profiles and difference energy diagrams

Mutate side chains that interact with the substrate or intermediates and measure changes in activity
There are many suitable side-chains that interact with Tyr-AMP

Measure complete free energy profiles for wild-type and mutant proteins: by measuring rate and equilibrium constants

Pre-steady state kinetics
(stopped flow
- mixing E.Tyr with ATP)

$$E \xrightleftharpoons[K_T]{Tyr} E.Tyr \xrightleftharpoons[K'_A]{ATP} E.Tyr.ATP \xrightleftharpoons[k_{-3}]{k_3} E.Tyr\text{-}AMP.PPi \xrightleftharpoons[K_{pp}]{} E.Tyr\text{-}AMP + PPi$$

Equilibrium dialysis
or kinetics

Kinetics

Pre-steady state kinetics
(stopped flow
- mixing E.Tyr-AMP with PPi)

Kinetics

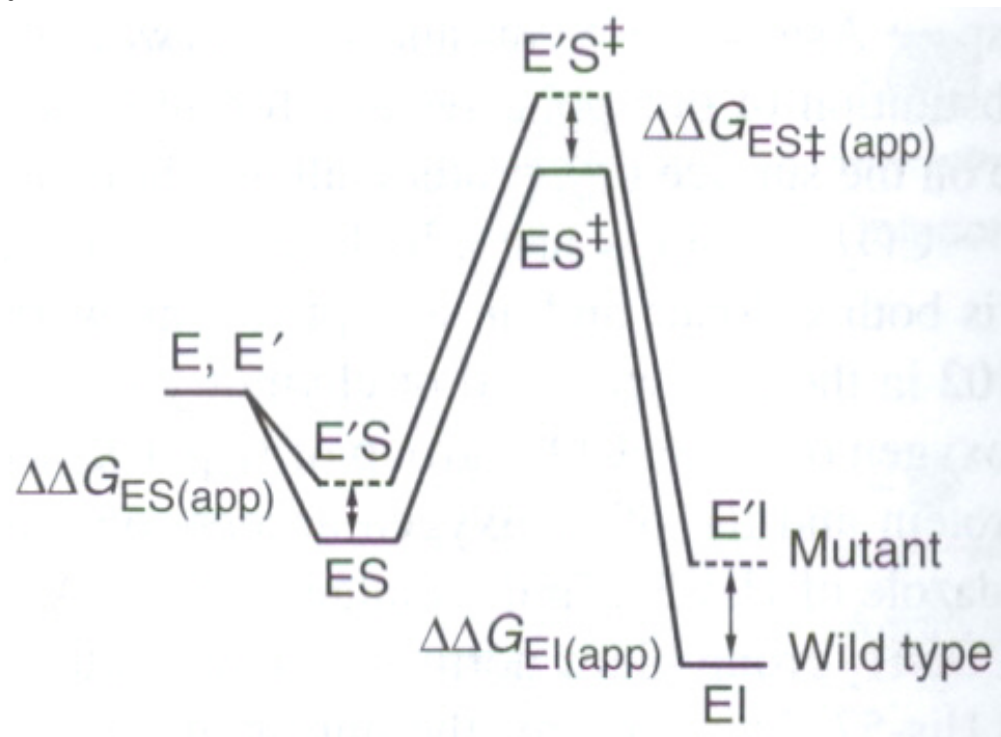Convert equilibrium constant (K) into Standard-State Free Energy (ΔG°): $\Delta G° = - RT \ln K$

Tyrosyl-tRNA Synthetase
Free energy profiles

Use measured rate and equilibrium constants to calculate free energy profiles (the change in Gibbs free energy throughout the catalytic cycle) for:
- Wild-type enzyme
- Mutant enzyme(s)

Note: this does not show absolute values of free energy levels, but differences in free energy levels between the wild-type and mutant

The difference energy is sometimes called the apparent binding energy of a group ($\Delta\Delta G_{app}$) = free energy of mutant minus free energy of wild-type

Tyrosyl-tRNA Synthetase
Demonstration of enzymes-transition state complimentarity using difference energy diagrams

Replot the data as a difference energy diagram: plotting the difference in free energy ($\Delta\Delta G_{app}$) between the wild-type and the mutant(s).
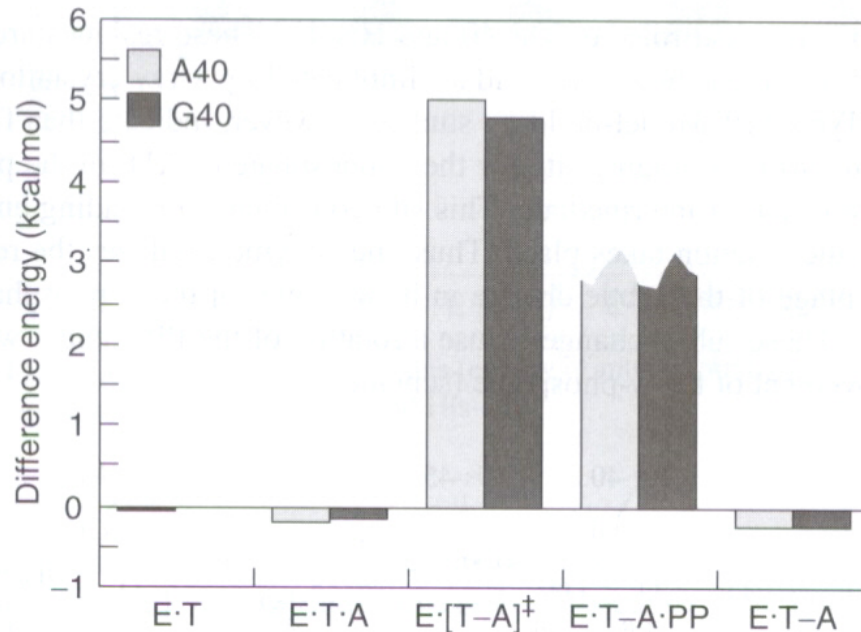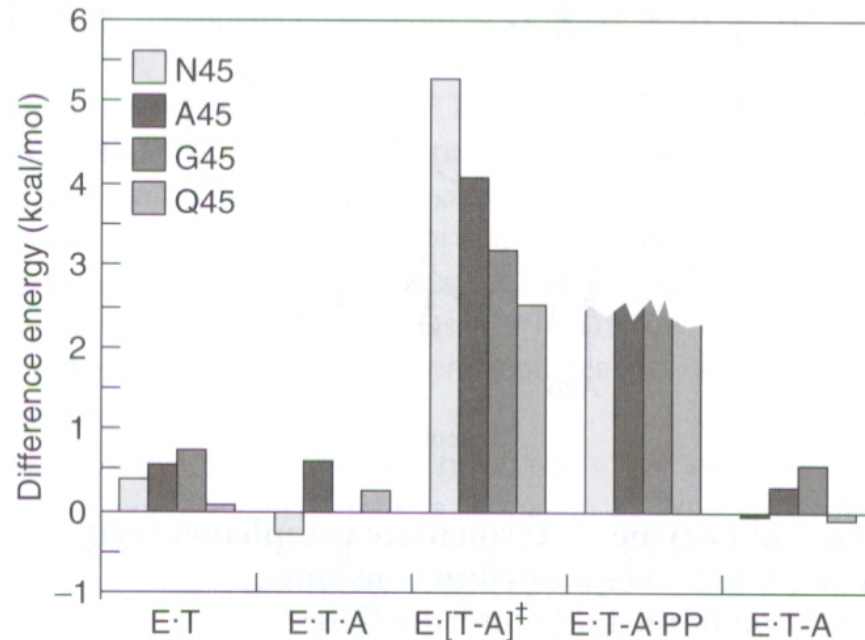

Mutating Thr-40 to Ala or Gly

• Has essentially no effect on the binding energies of Tyr or ATP to the enzyme.
• But there is massive raising of the energy level of the transition state (20 kJ/mol).
• This result shows that Thr-40 binds only binds the transitions state and not the substrates

Tyrosyl-tRNA Synthetase
Demonstration of enzymes-transition state complimentarity
using difference energy diagrams

Replot the data as a difference energy diagram: plotting the difference in free energy ($\Delta\Delta G_{app}$) between the wild-type and the mutant(s).


Mutating His-45 to Ala, Gly, Asn or Gln

Gives very similar results

- Has essentially no effect on the binding energies of Tyr or ATP to the enzyme.
- But there is massive raising of the energy level of the transition state (20 kJ/mol).
- This result shows that His-45 binds only binds the transitions state and not the substrates

This was the first experimental demonstration of the Haldane-Pauling postulate of enzyme - transition state complimentarity - by specifically binding the transition-state they lower its free energy and thereby accelerate the reaction.

Enzymes

Enzymes catalyze reactions by preferentially binding the transition state
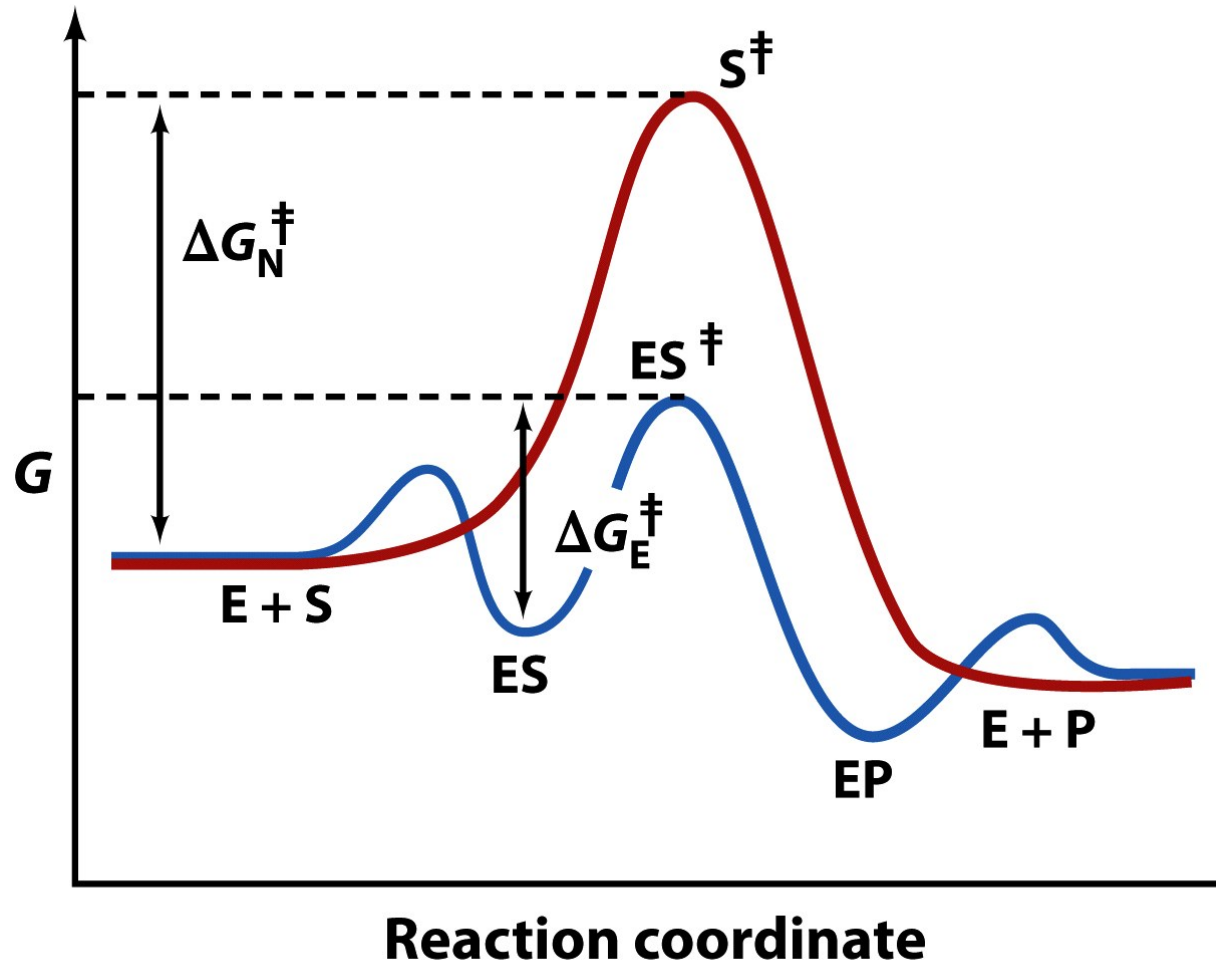
Uncatalyzed reaction = red
Catalyzed reaction   = blue

The $\Delta G^{\ddagger}$ of the catalyzed reaction is lower than the $\Delta G^{\ddagger}$ of the uncatayzed reaction

Enzymes catalyze reactions by binding the transition state with greater affinity than the substrate or product

Tyrosyl-tRNA Synthetase
Thr-40 and His-45 form a binding site for the
γ-phosphate of ATP in the transition state

From the crystal structure Thr-40 and His-45 are not implicated in binding Tyr-AMP

However, model building indicates that they form part of the binding site for the γ-phosphate of ATP in the pentacovalent transition state
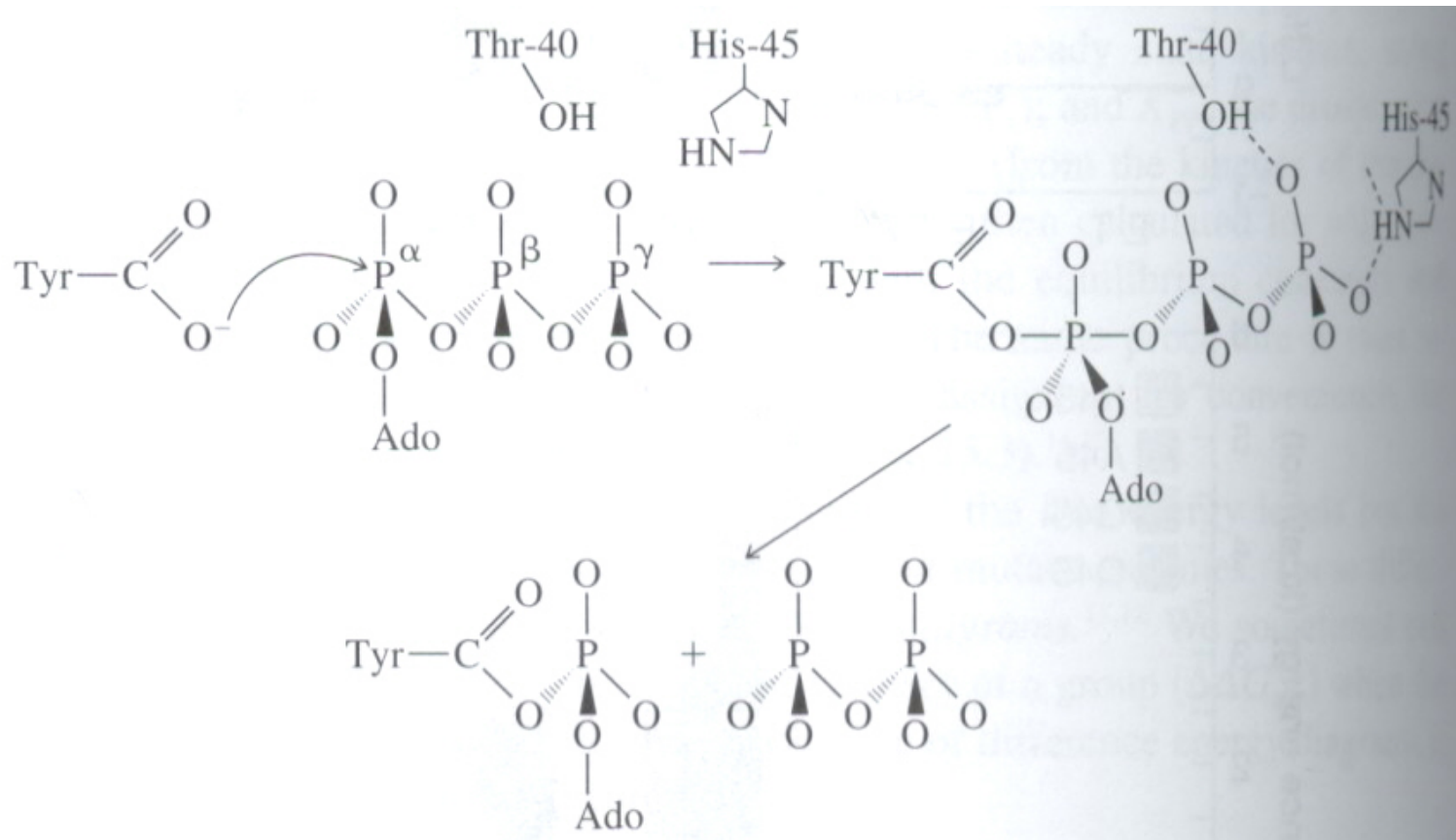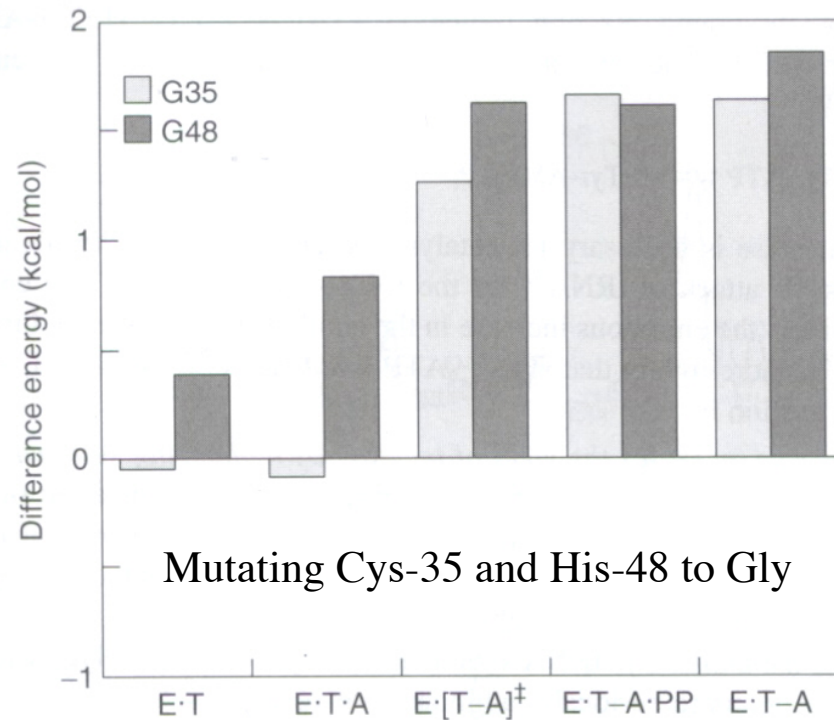
Tyrosyl-tRNA Synthetase
Discovery of enzyme intermediate complementarity
- in apparent contradiction of Haldane and Pauling!

From crystal structure: Cys-35 and His-48 bind to the ribose ring in the E.Tyr-AMP complex

From mutagenesis:
- They contribute little or no binding energy to E.Tyr.ATP ground state complex
- They stabilise ATP in the transition state
- They contribute even more stabilisation energy in the E.Tyr-AMP complex

Mutating Cys-35 and His-48 to Gly

How can stabilising an intermediate be helpful? - It is related to the reaction being multi-step

Tyrosyl-tRNA Synthetase
Discovery of enzyme intermediate complementarity
- in apparent contradiction of Haldane and Pauling!

Two good reasons for enzyme - intermediate complementarity:

1. Enzyme - product complementarity changes the equilibrium constant for a highly unfavourable reaction (formation of Tyr-AMP from Tyr and ATP)

Tyr + ATP $\rightleftharpoons$ Tyr-AMP + PPi $\qquad$ K = [Tyr-AMP] [PPi] / [Tyr] [ATP] = 3.5×10^{-7}

E.Tyr.ATP $\rightleftharpoons$ E.Tyr-AMP.PPi $\qquad$ K = [E.Tyr-AMP.PPi] / [E.Tyr.ATP] = 2.3

The enzyme increases the equilibrium constant nearly 10^7-fold - by binding Tyr-AMP far more tightly than Tyr + ATP

This increase in equilibrium constant is necessary as *in vivo* the rate limiting step is the attack of $tRNA^{Tyr}$ on the E.Tyr-AMP complex

2. The enzyme increases the yield of reaction by minimising side reactions and sequestering the highly reactive intermediate:

If the highly reactive Tyr-AMP were to diffuse out of the enzyme it would hydrolyse in seconds or aminoacylate reactive side chains of proteins

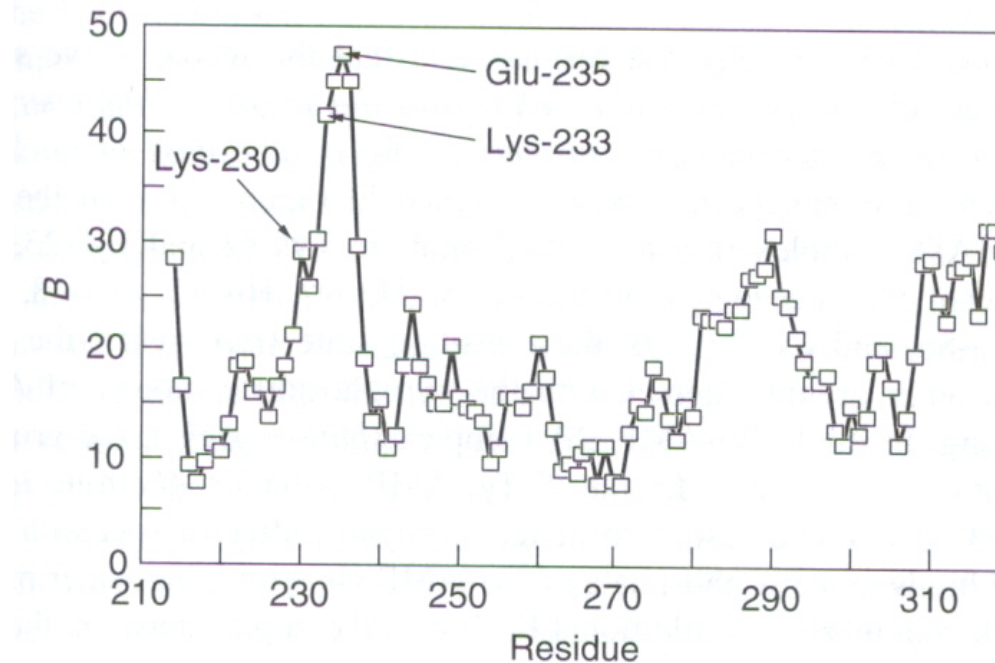# Tyrosyl-tRNA Synthetase
Detection of an induced fit process

All Arginine and Lysine residues were mutated to uncharged side chains (one at a time)

Lys-230 and Lys-233
- too far away to interact with the transition state in the model,
- yet apparently contributed to catalysis by binding to the transition state

Explanation: the loop containing Lys-230 and Lys-233 is highly mobile (seen from the crystallographic temperature factors, B values) and can wrap around the transition state as the reaction occurs.

If this did not occur it would block the access of substrates to the active site

Tyrosyl-tRNA Synthetase
The catalytic mechanism for activation of tyrosine

Many side chains are involved in catalysis - the whole active site contributes to catalysis

Residue	Tyr	ATP	[Tyr-ATP]‡	PP$_i$	Tyr-AMP
Interaction energy of side chains in complex with:[a]					
Tyrosine binding site					
Tyr-34	+	0	+	0	+
Asp-78	++++	++^{b}	++++	++^{b}	++++
Tyr-169	++++	0	++++	0	++++
Gln-173	++++	++^{b}	++++	+^{b}	++++
Nucleotide and pyrophosphate site					
Cys-35	0	0	++	0	+++
Thr-40	0	0	++++	++++	0
His-45	0	0	++++	++++	0
His-48	0	0	+++	0	+++
Thr-51	0	0	0	0	–
Lys-82	0	++	++++	++++	0
Arg-86	0	0	++++	++++	–
Asp-194	0	0	++++	+	+++
Lys-230	0	0	++++	++++	0
Lys-233	0	++++	++++	++++	0

^{a} Apparent stabilization energy from the side chain in kcal/mol (kJ/mol): 0 = –0.5 to +0.5 (–2 to +2); + = 0.5 to 1.0 (2 to 4); ++ = 1.0 to 1.5 (4 to 6); +++ = 1.5 to 2.0 (6 to 8); ++++ = >2.0 (>8); – = –0.5 to –1.0 (–2 to –4).
^{b} Evidence for some disruption of protein structure on mutation.

Catalysis results solely from the use of binding energy

There is no assistance from acid-base or nucleophilic catalysis

Tyrosyl-tRNA Synthetase
Mechanism of transfer step

There are no acidic or basic groups suitably placed to catalyse the attack of the ribose 2'-OH of the tRNA^{Tyr} on the >C=O of Tyr-AMP.

However, the intramolecular attack of the 2'-OH on the extremely activated Tyr-AMP ($t_{1/2}$ for hydrolysis in solution of 1 min) should be very rapid.
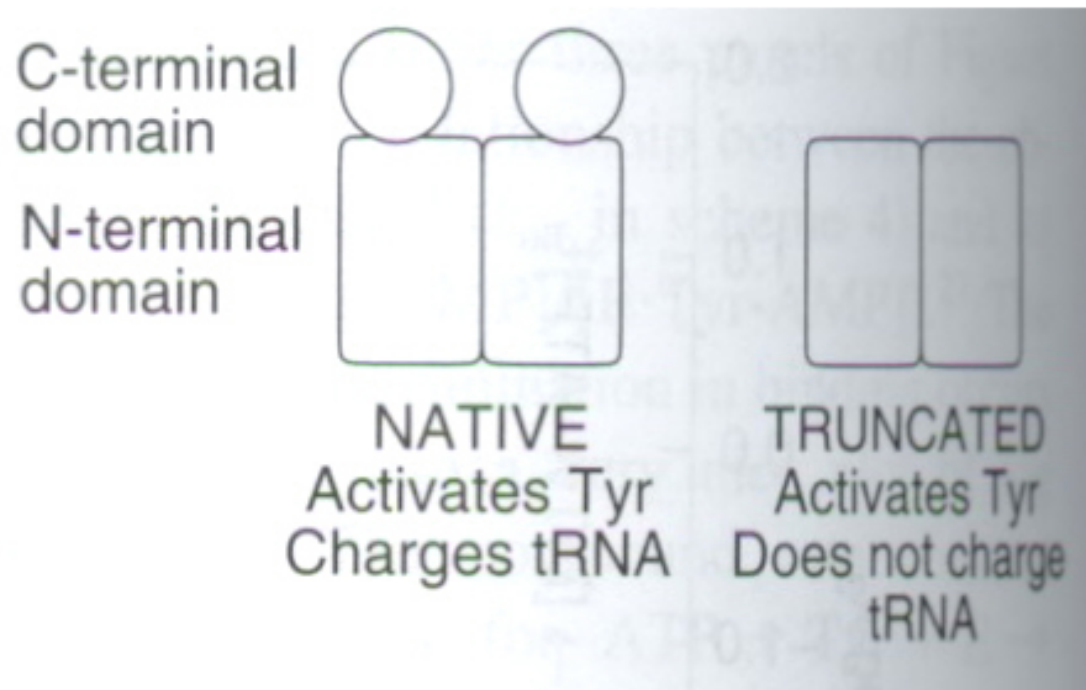
Tyrosyl-tRNA Synthetase
Probing gross structure and symmetry by mutagenesis

Tyrosyl-tRNA synthetase is a homodimer - symmetrical in the crystal

However, it shows "half-of-the sites" activity - forming only one mole of Tyr-AMP per mole of dimer

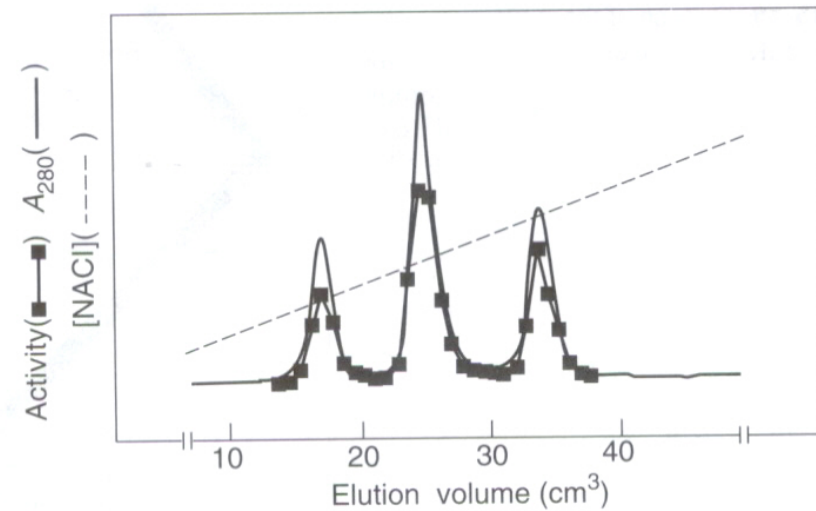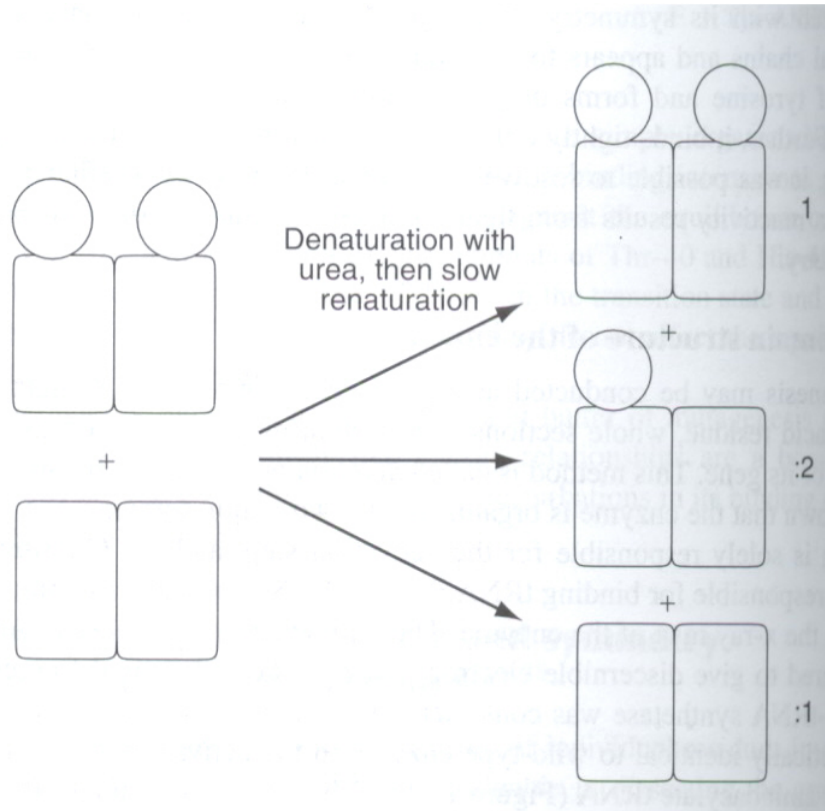Does this result from ligand-induced asymmetry or from pre-existing assymetry?

Can answer question using deletion mutagenesis to remove the C-terminal domain

Tyrosyl-tRNA Synthetase
Probing half-of-the sites activity by constructing heterodimers

Form heterodimers by denaturation and renaturation of full-length and truncated synthetases
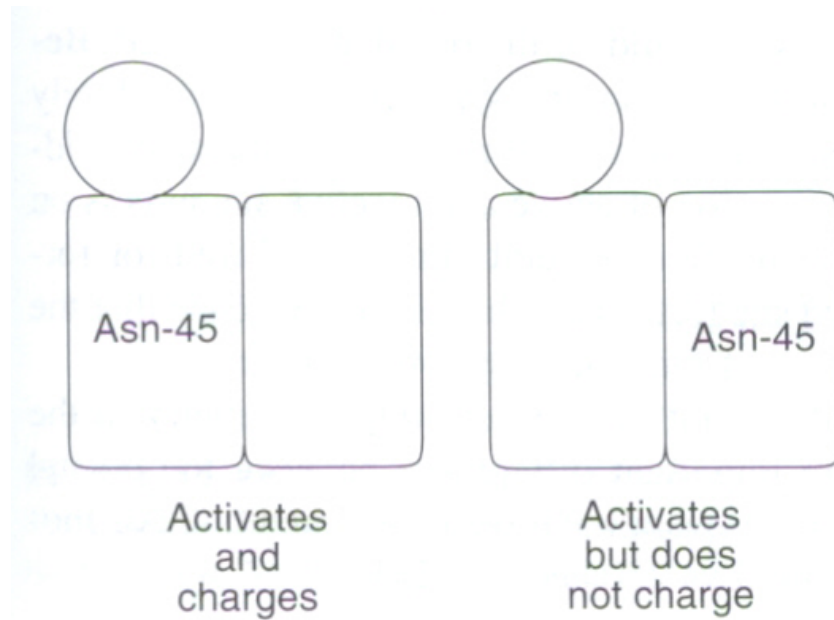

Separate the different species by ion-exchange chromatography with a NaCl gradient
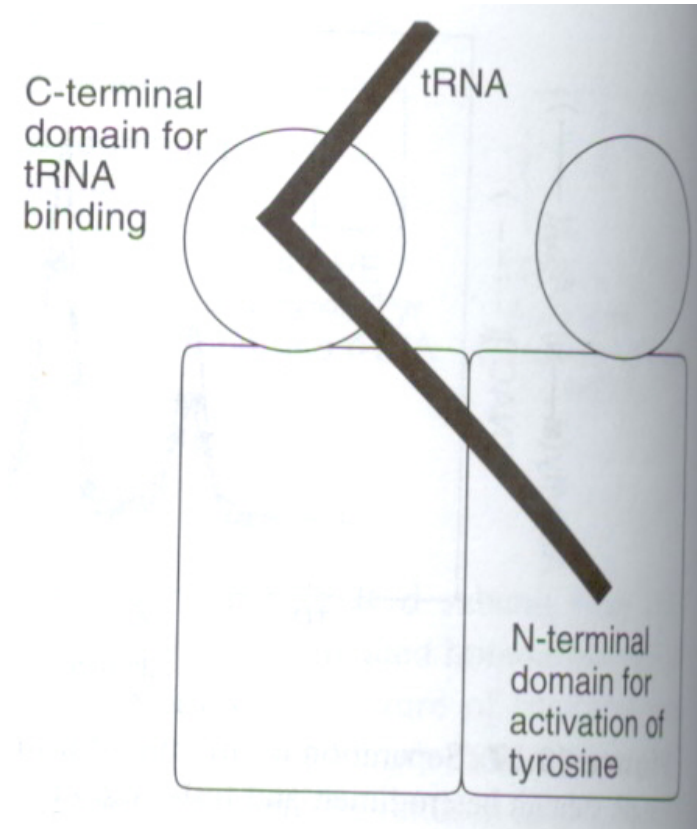
Tyrosyl-tRNA Synthetase
Probing half-of-the sites activity by constructing heterodimers

"Tag" individual subunits with specific mutations

The mutation His ⟶ Asn-45 greatly
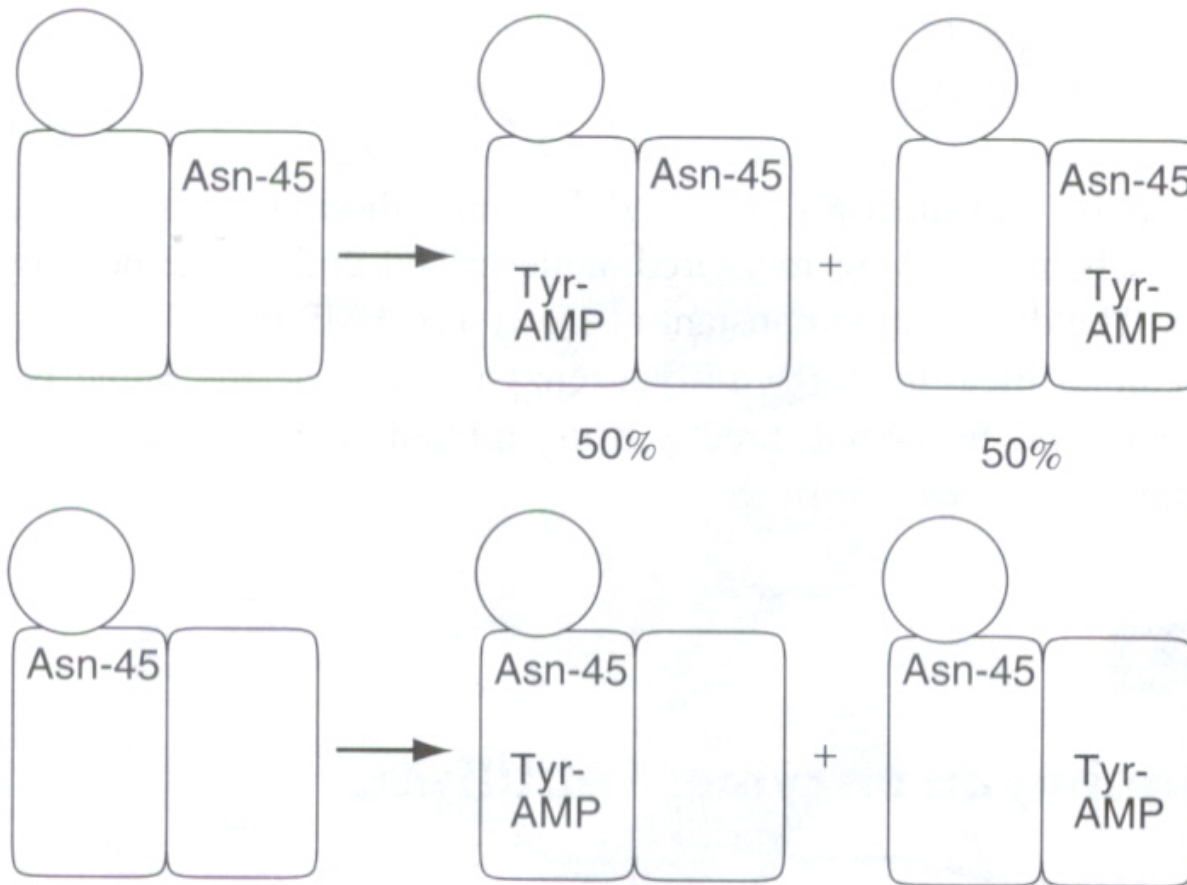slows down the activation in its subunit

Cartoon of mode of binding of tRNA to the dimer

Tyrosyl-tRNA Synthetase
Probing half-of-the sites activity by constructing heterodimers

Heterodimers containing Asn-45 in one subunit form:
- 0.5 mol of Tyr-AMP per mole of dimer rapidly at wild type rate ($t_{1/2}$ = 20 ms)
- 0.5 mol of Tyr-AMP per mole of dimer slowly ($t_{1/2}$ = 200 s)

Thus - the half-of-the sites activity is a result of pre-existing assymetry in solution

Further Background
To study of protein structure and mechanism

**Structure and Mechanism in Protein Science:
A Guide to Enzyme Catalysis and Protein Folding**

Alan Fersht

W.H. Freeman and Company, New York, 1999