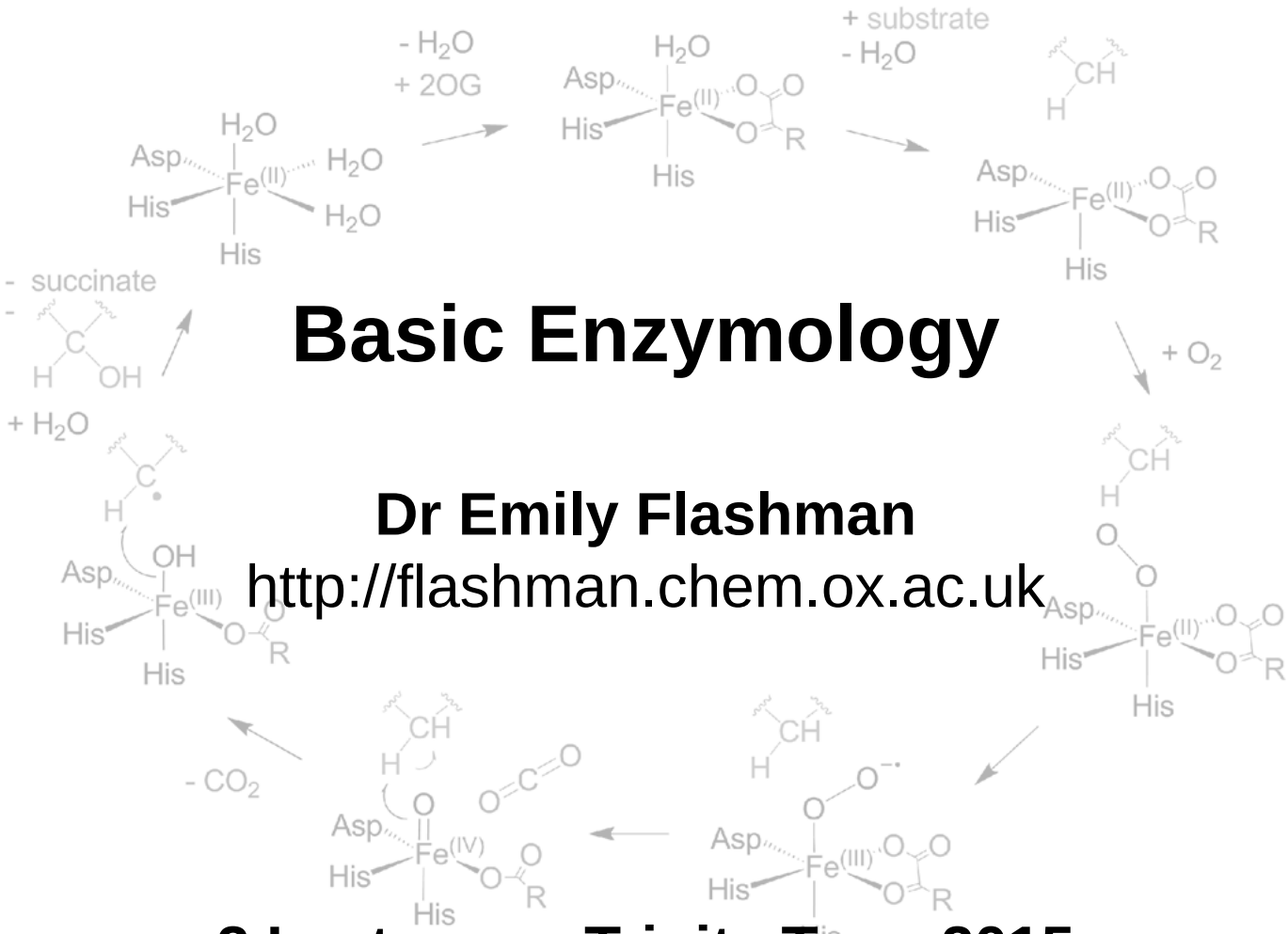
UNIVERSITY OF OXFORD

DEPARTMENT OF CHEMISTRY



Basic Enzymology

Dr Emily Flashman

http://flashman.chem.ox.ac.uk

2 Lectures – Trinity Term 2015

Lecture 1: **Introduction to enzymes**

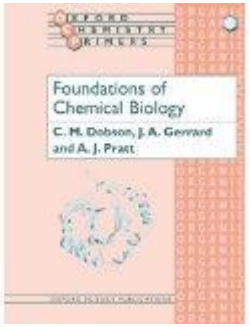
The importance of enzymes in catalysing the chemical reactions of life.
How enzymes promote catalysis
Typical enzyme-catalysed reactions
Enzyme efficiency: selectivity, co-factors and control.

Lecture 2: **Principles of enzyme catalysis**

Thermodynamics of enzyme-catalysed reactions
Transition state stabilisation
Case Study - Triose phosphate isomerase

No prior knowledge other than content of Oxford Chemistry course.

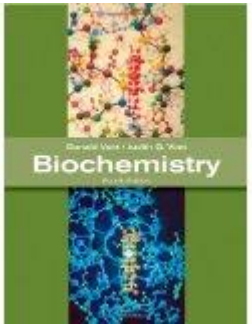
No text books are essential but the following are useful:



Foundations of Chemical Biology, Oxford, Chemistry Primer
(Dobson, Gerrard, Pratt, OUP)

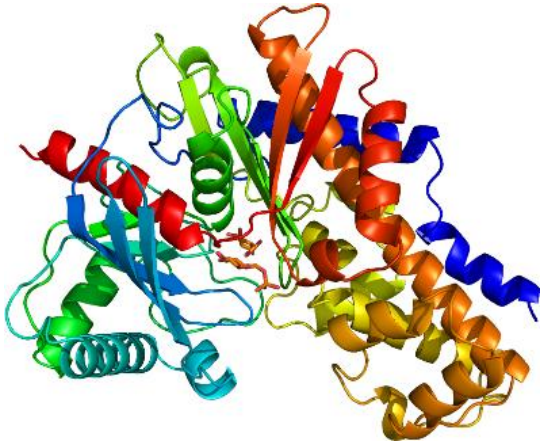
Access to a modern Biochemistry textbook:

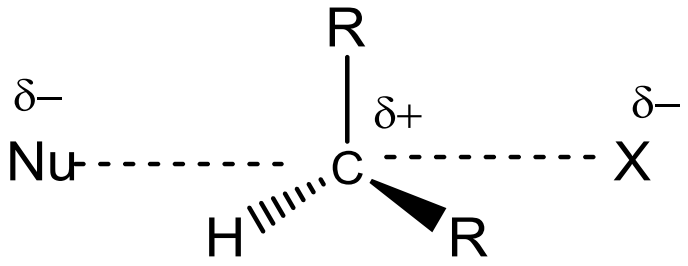
Voet and Voet, *Biochemistry* (4th Edition, Wiley)



- **To provide an introduction to enzyme catalysis and related protein functions, focusing on chemical principles**

- **To connect mechanistic synthetic chemistry with biology**





- **To demonstrate that chemical principles underlie biology and that understanding and manipulating this chemistry is fascinating and the basis of multiple applications.**

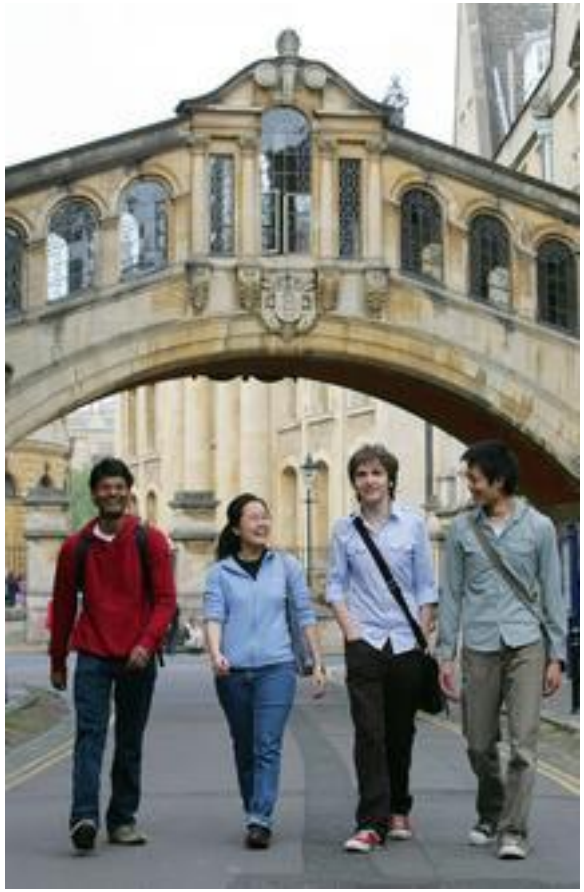
This course is NOT about remembering complex structures

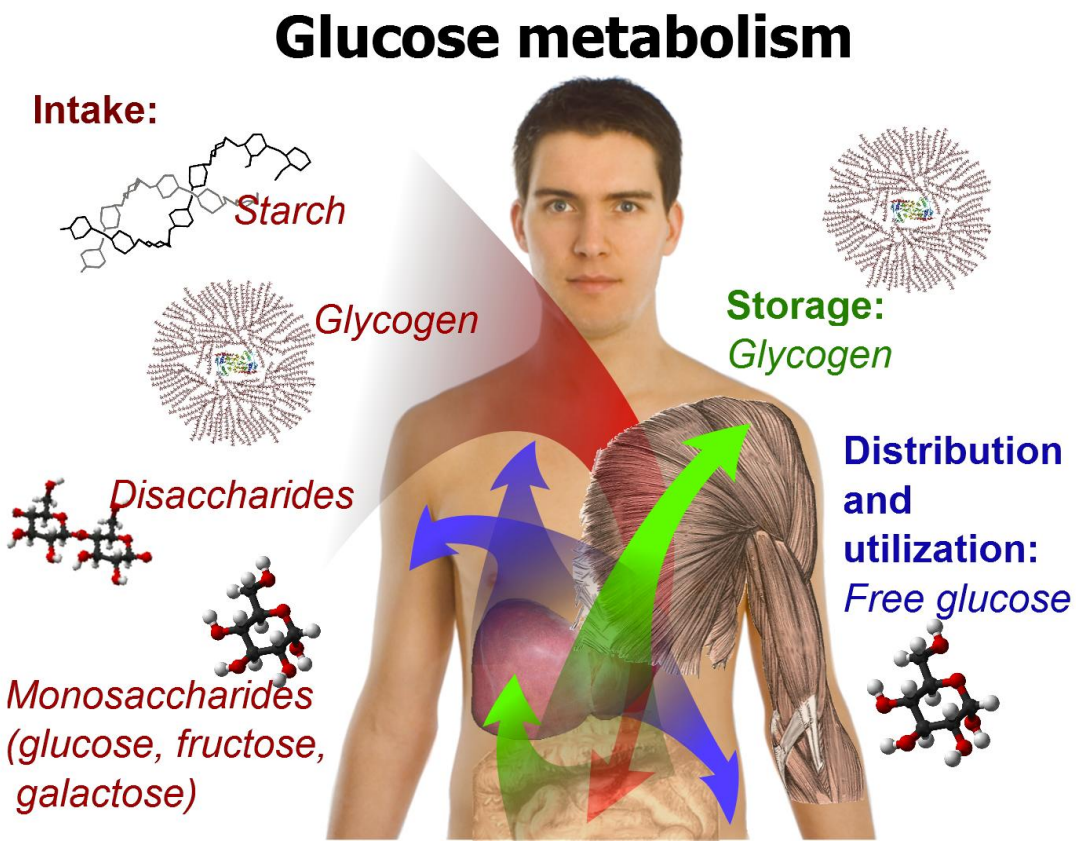
But

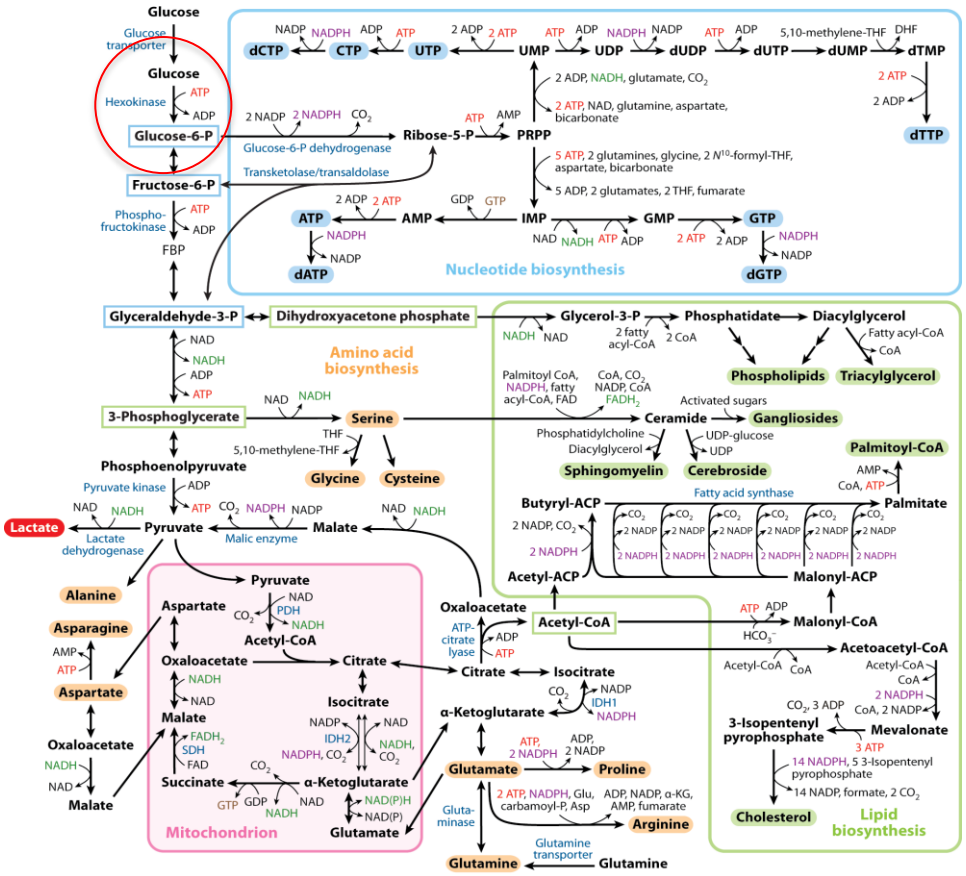
About understanding the chemical principles that control biology

"Enzyme catalysis: not different just better"(Jeremy Knowles)

- One of the most important targets for pharmaceuticals
- Intrinsic in human biology - manipulation
- Fermentation of pharmaceuticals and fine chemical
- Useful in synthesis, especially for resolutions / asymmetric reactions, e.g. resolutions by enantiospecific -amino acid acylase
- Wide ranging applications in society, e.g. food industry, washing powders
- Bioremediation
- An understanding of enzyme catalysed reactions may help us to design useful unnatural 'biomimetic' catalysts, based on knowledge of enzyme structures / mechanisms.




Glucose metabolism
Intake:
Starch
Glycogen
Disaccharides
Monosaccharides
(glucose, fructose,
galactose)
Storage:
Glycogen
Distribution
and
utilization:
Free glucose

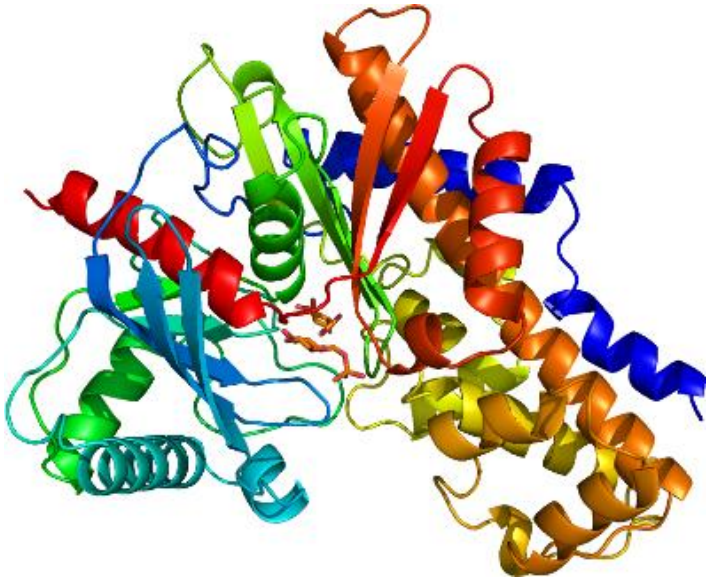


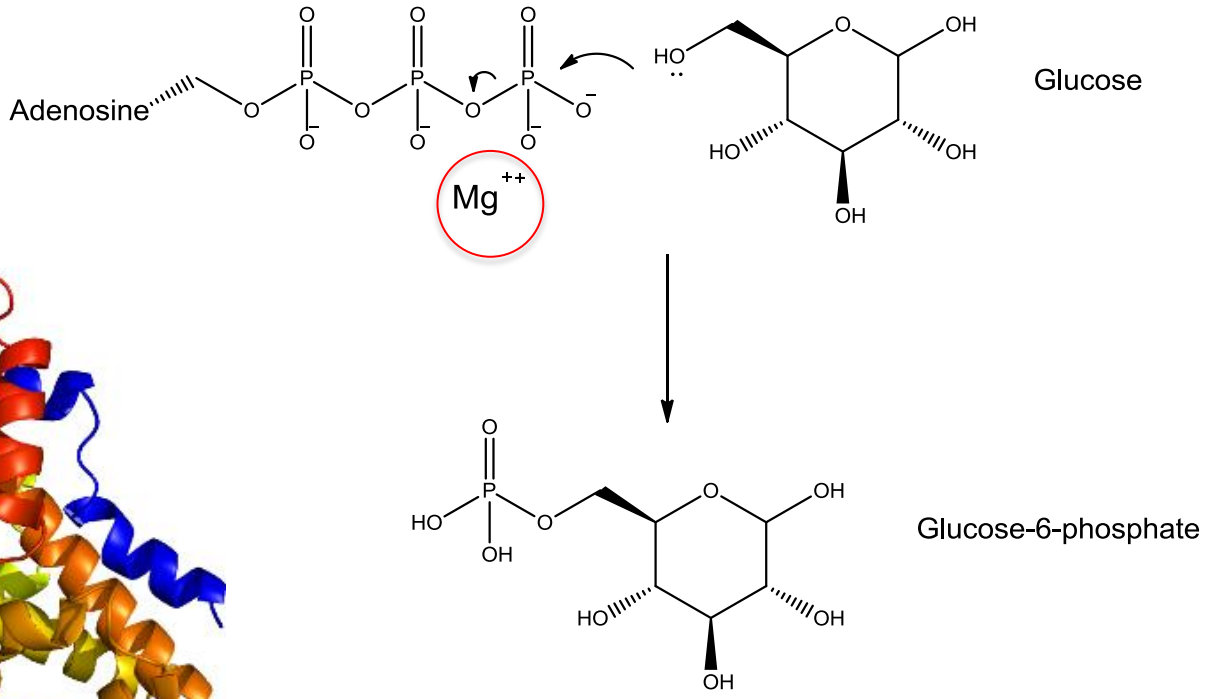
ABBREVIATIONS:

ACP	Acyl carrier protein	**dUTP**	Deoxyuridine triphosphate	**PDH**	Pyruvate dehydrogenase
ADP	Adenosine diphosphate	**FAD**	Flavin adenine dinucleotide	**PRPP**	Phosphoribosyl pyrophosphate
Asp	Aspartate	**$FADH_2$**	Flavin adenine dinucleotide, reduced	**SDH**	Succinate dehydrogenase
ATP	Adenosine triphosphate	**Glu**	Glutamate	**THF**	Tetrahydrofolate
CoA	Coenzyme A	**GMP**	Guanosine monophosphate	**UDP**	Uridine diphosphate
CTP	Cytidine triphosphate	**GTP**	Guanosine triphosphate	**UMP**	Uridine monophosphate
dATP	Deoxyadenosine triphosphate	**IDH1**	Isocitrate dehydrogenase 1	**UTP**	Uridine triphosphate
dCTP	Deoxycytidine triphosphate	**IDH2**	Isocitrate dehydrogenase 2	**α-KG**	α-Ketoglutarate
dGTP	Deoxyguanosine triphosphate	**IMP**	Inosine monophosphate		
DHF	Dihydrofolate	**NAD**	Nicotinamide adenine dinucleotide		
dTMP	Deoxythymidine monophosphate	**NADH**	Nicotinamide adenine dinucleotide, reduced		
dTTP	Deoxythymidine triphosphate	**NADP**	Nicotinamide adenine dinucleotide phosphate		
dUDP	Deoxyuridine diphosphate	**NADPH**	Nicotinamide adenine dinucleotide phosphate, reduced		
dUMP	Deoxyuridine monophosphate	**P**	Phosphate		

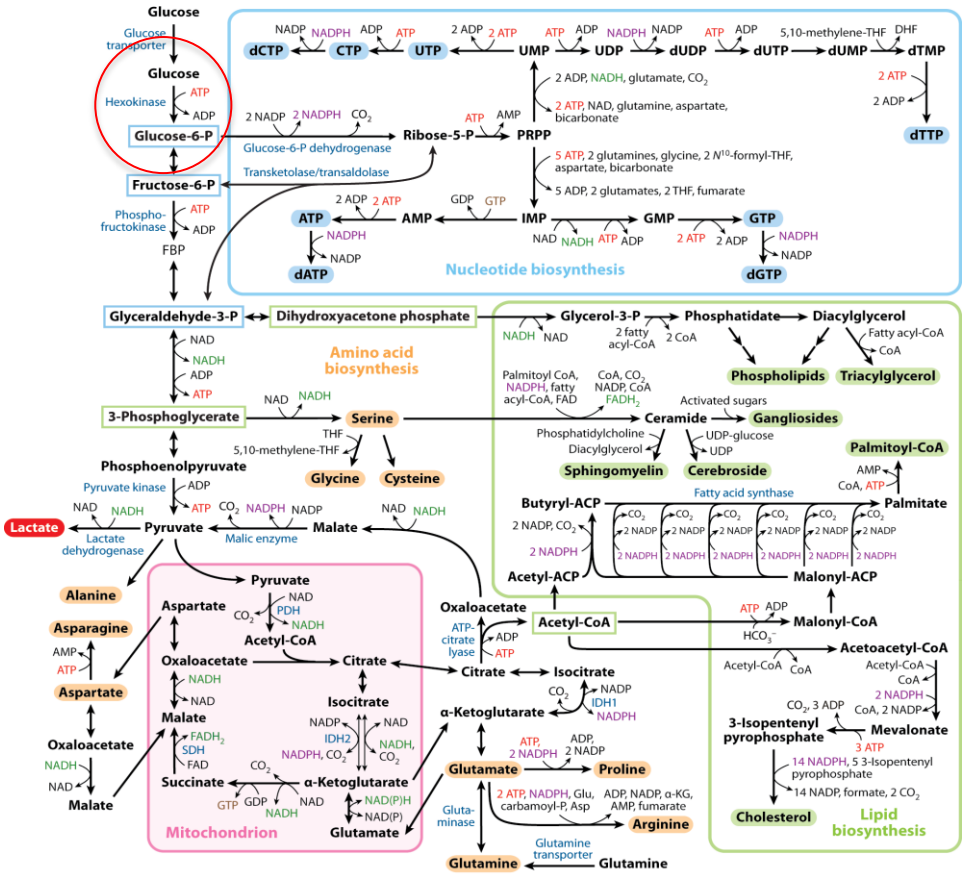
Lunt SY, Heiden MGV. 2011.
Annu Rev. Cell Dev. Biol. 27:441–64

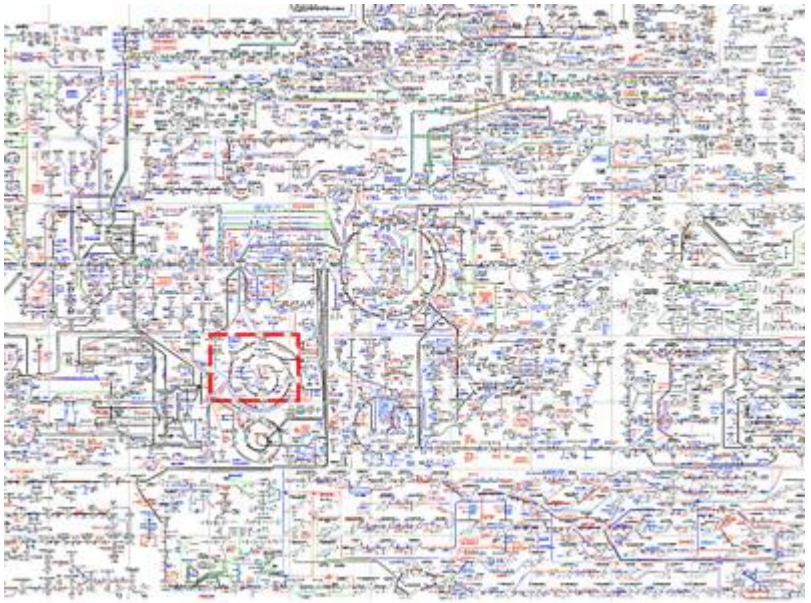
Hexokinase creates right conditions for nucleophilic attack of C6-OH on ATP





The enzyme promotes an otherwise unfavourable reaction that is vital in energy-generating glycolysis process.





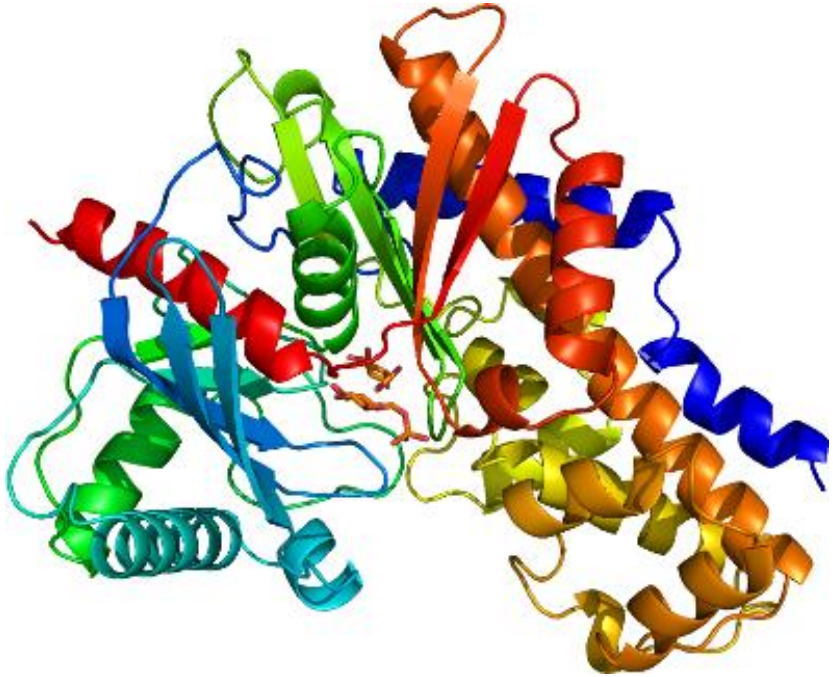
Enzymes are important!

ABBREVIATIONS:

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dATP	Deoxyadenosine triphosphate	IDH1	Isocitrate dehydrogenase 1	UTP	Uridine triphosphate
dCTP	Deoxycytidine triphosphate	IDH2	Isocitrate dehydrogenase 2	α-KG	α-Ketoglutarate
dGTP	Deoxyguanosine triphosphate	IMP	Inosine monophosphate		
DHF	Dihydrofolate	NAD	Nicotinamide adenine dinucleotide		
dTMP	Deoxythymidine monophosphate	NADH	Nicotinamide adenine dinucleotide, reduced		
dTTP	Deoxythymidine triphosphate	NADP	Nicotinamide adenine dinucleotide phosphate		
dUDP	Deoxyuridine diphosphate	NADPH	Nicotinamide adenine dinucleotide phosphate, reduced		
dUMP	Deoxyuridine monophosphate	P	Phosphate		

Lunt SY, Heiden MGV. 2011.
Annu Rev. Cell Dev. Biol. 27:441–64

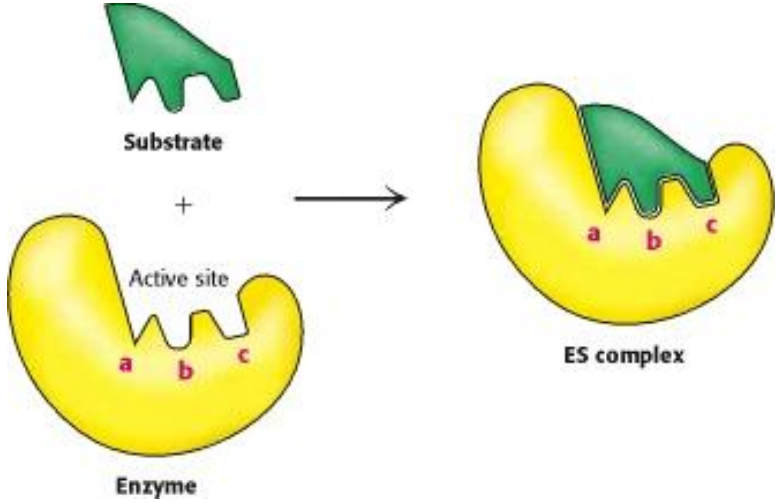
Enzymes are protein-based catalysts – they facilitate chemistry



- **'Free enzymes' are often globular proteins, but enzymes can be part of large complexes or embedded in membranes.**

- **We will focus on 'simple' enzymes that catalyse 'simple' reactions, but the same principles of catalysis apply in all cases.**

Bringing enzyme and substrate(s) together in a favourable conformation to promote the reaction.

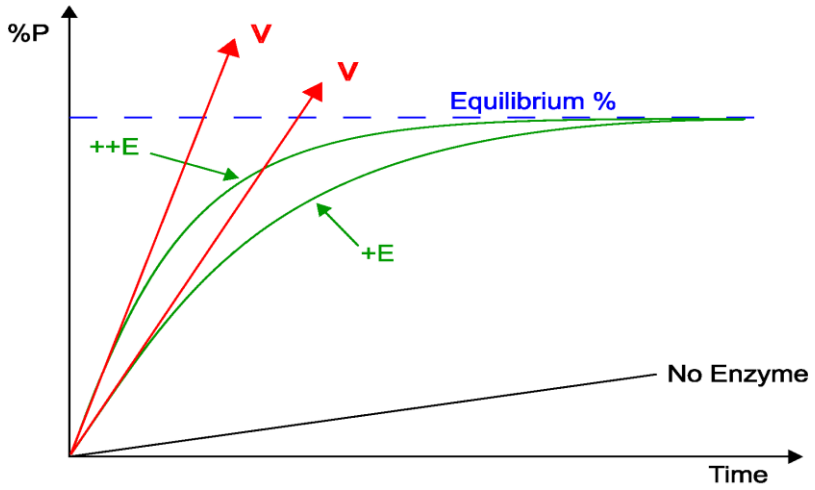


Enzymes have **ACTIVE SITES**

- a 3D cleft or crevice with precisely defined arrangement of atoms
- relatively small area of enzyme
- substrates bind via multiple weak interactions

Enzymes are biological catalysts:

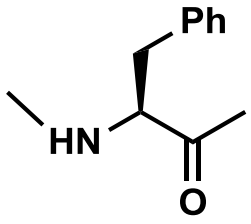
Increase the rate at which a reaction reaches equilibrium



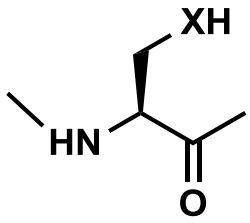
Stabilise the transition state of a reaction relative to the uncatalysed reaction

Enzymes are finely tuned for specificity in substrate binding and optimal arrangement of catalytic groups

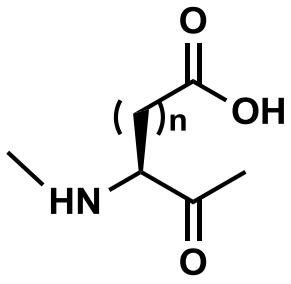
Amino acid side chains (and the peptide backbone) provide a repertoire of functional groups for catalysis and binding



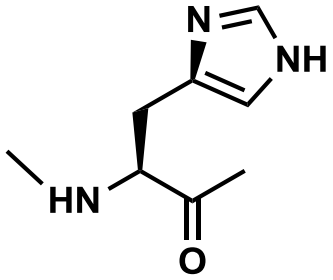
Phenylalanine



X=O, serine
X=S, cysteine

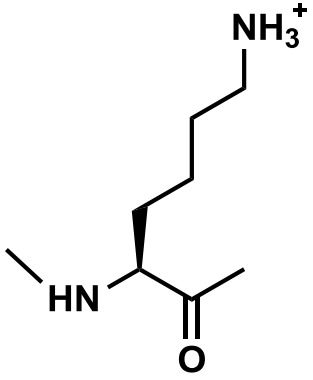


n=1, Aspartate
n=2, Glutamate

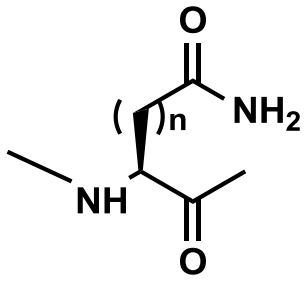


Histidine
(pKa = 7)

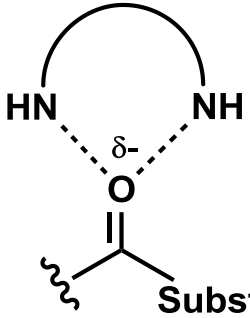
commonly involved in acid/base catalysis, electrostatic interactions, metal binding



Lysine



n=1, Asparagine
n=2, Glutamine



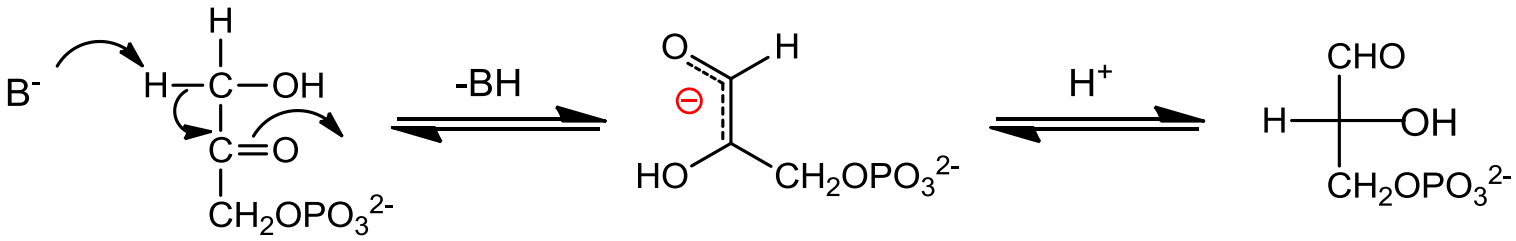
protein backbone can polarise substrate carbonyl groups

Enzymes are more efficient than chemical catalysts:

1. Higher reaction rates – by several orders of magnitude
2. Milder reaction conditions – low temps, atm pressure, neutral pH
3. Greater reaction specificity – no side products
4. Capacity for control – catalytic activity can vary in response to local conditions

Enzymes catalyse both simple reactions and reactions that are 'impossible' for synthetic chemistry.

Triose phosphate isomerase ('easy' reaction)

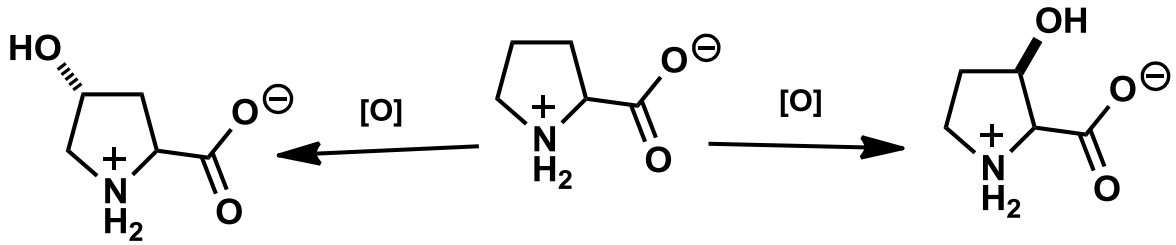


DHAP =dihydroxyacetone phosphate

enolate intermediate

GAP =glyceraldehyde-3-phosphate

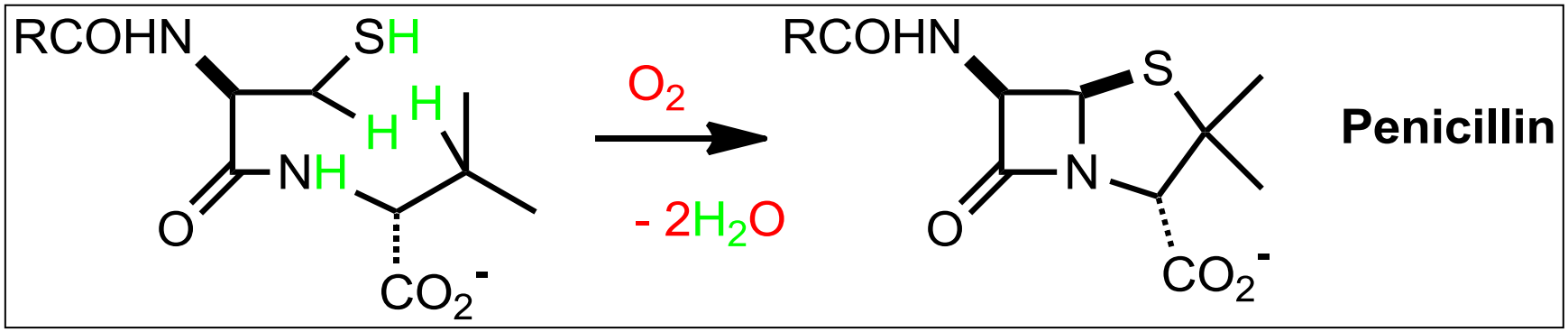
Proline hydroxylase ('difficult' reaction)



trans-4-hydroxyproline

cis-3-hydroxyproline

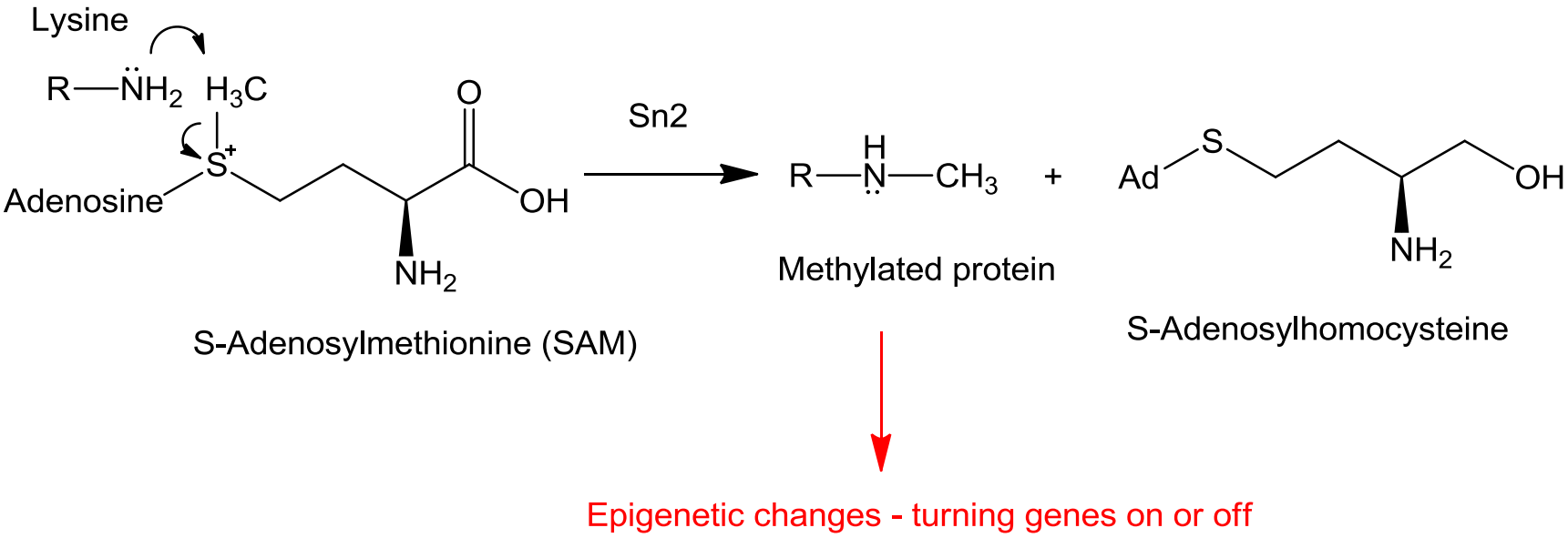
Penicillin biosynthesis catalysed by isopenicillin N synthase



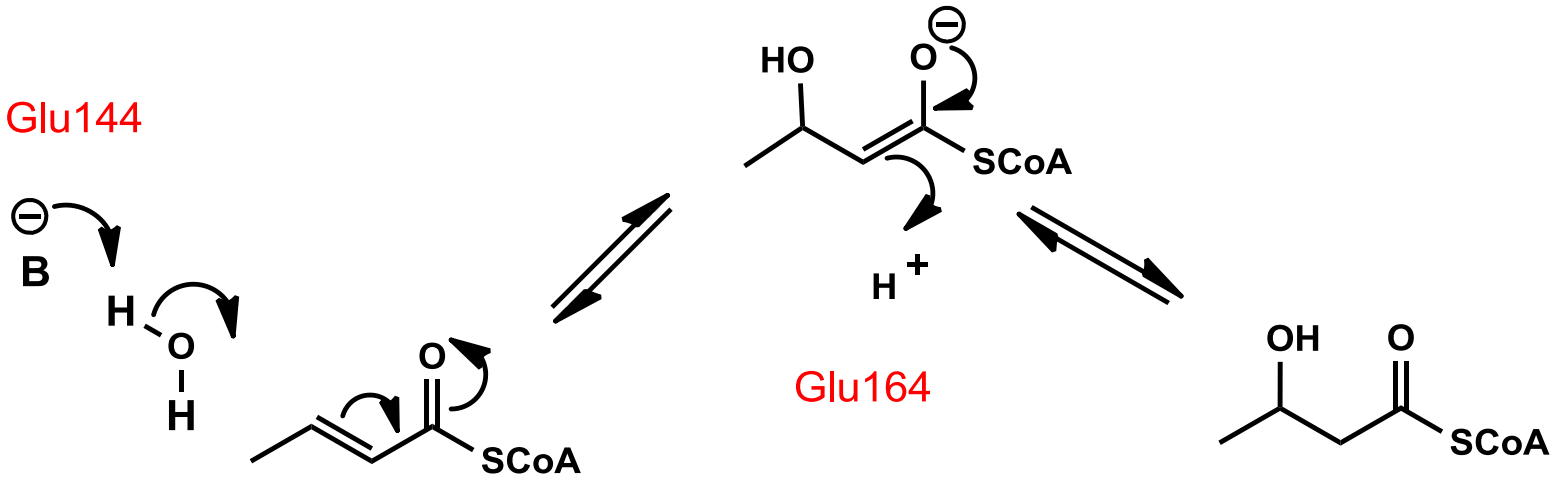
- Fermented on a ton-scale by fermentation
- Fermented pencillins are used directly and others are produced by modification of fermented penicillins
- Single step reaction into highly functionalised penicillin
- Organic synthesis is not competitive (<1% multistep route)

Enzymes catalyse (most) of the 'fundamental' reactions of organic synthesis

Example 1 – the S_N2 reaction by a methyltransferase enzyme



Example 2 – the Michael reaction (conjugate addition) by enoyl CoA hydratase



Enoyl CoA hydratase, CoA=Coenzyme A

How do enzymes manage to be such efficient catalysts?

1. Substrate specificity

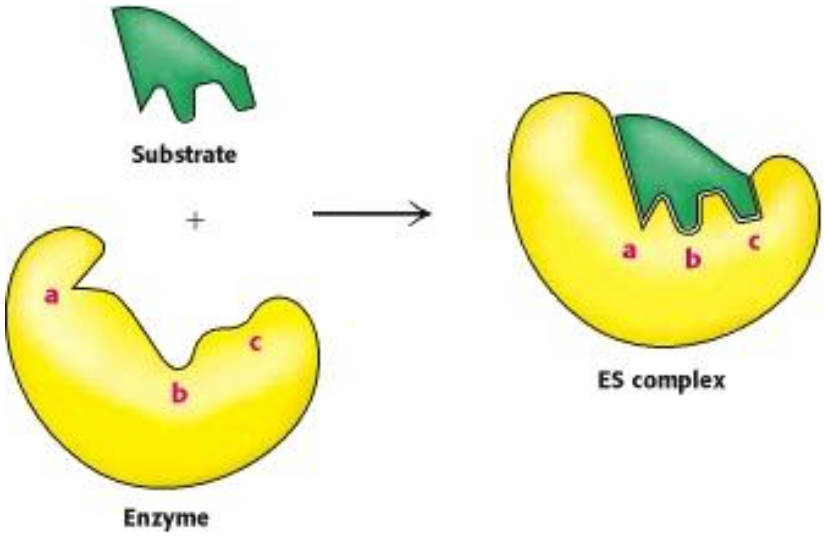
- **Stereospecificity**
- **Geometric specificity**

2. Coenzymes

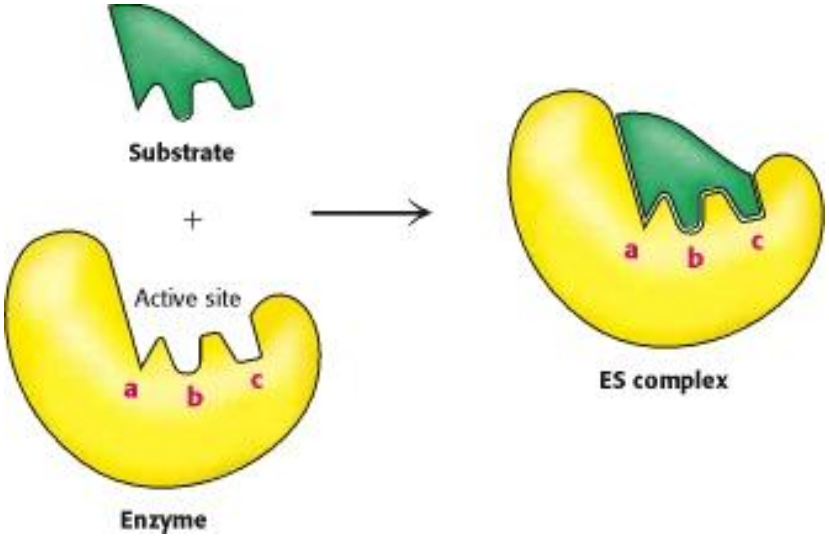
3. Control of activity: regulating activity in time and space

Substrates interact with enzymes via van der Waals, electrostatic, hydrogen bonding and hydrophobic interactions

Induced fit model



Lock and key model



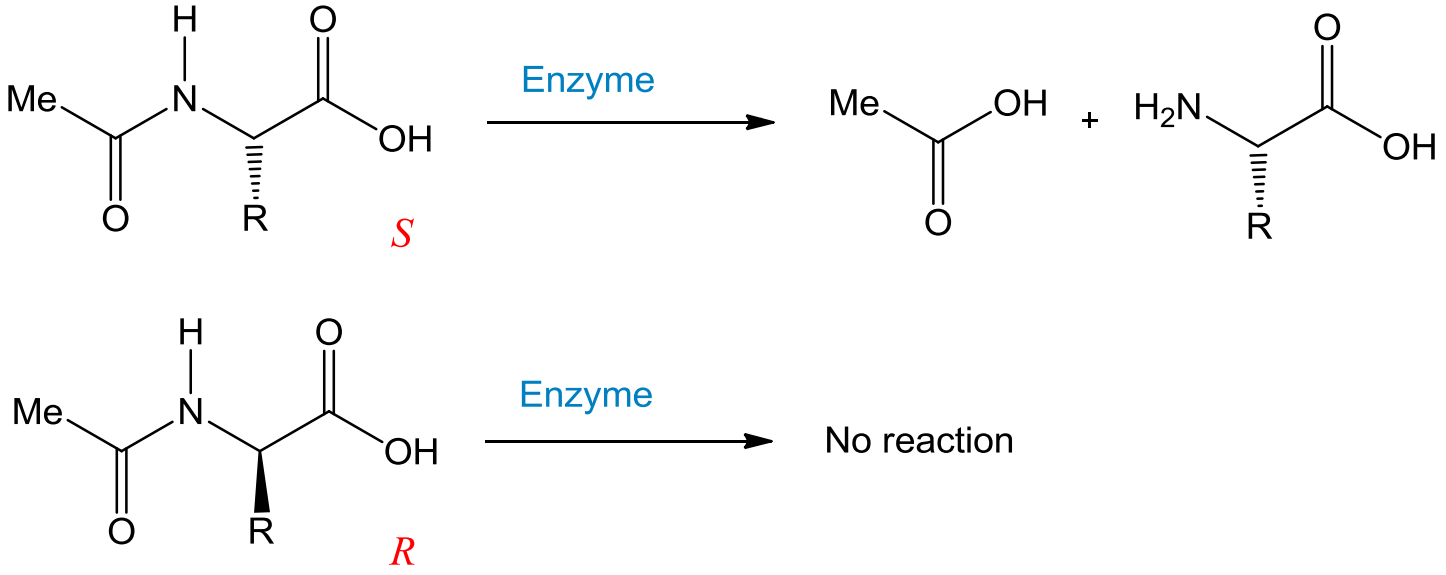
Substrates have geometric and electronic complementarity with their binding site on the enzyme

Enzymes consist of naturally-occurring L-amino acids

= they form assymetric active sites and only catalyse reactions with substrates with complementary chirality

e.g. hexokinase only catalyses phosphorylation of D-glucose, chymotrypsin only catalyses hydrolysis of L-amino acids.

Enzymes can be used to resolve racemic mixtures of compounds, e.g. N-acyl amino acids

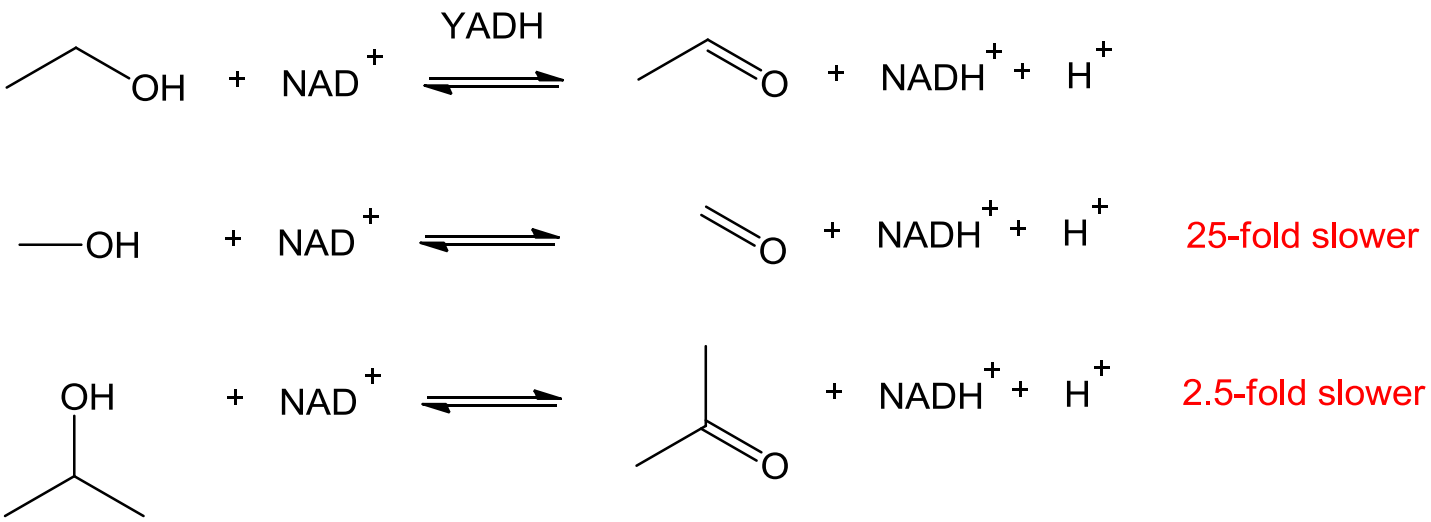


Most enzymes are selective about the chemical groups that will fit into their active sites

Enzymes vary in their degree of specificity

Most enzymes catalyse the reaction of a small range of related reactions

e.g. yeast alcohol dehydrogenase (YADH) catalyses oxidation of small primary and secondary alcohols, but ethanol is most efficient



Enzymes are good at acid/base reactions, transient covalent bonds and charge charge interactions

Not so good at redox reactions and group transfer processes

Need **Cofactors**

Metal ions

e.g. Fe^{2+}

Organic molecules

Transient

e.g. NAD^+ (usually); redox

Prosthetic group

e.g. FAD, haem; redox

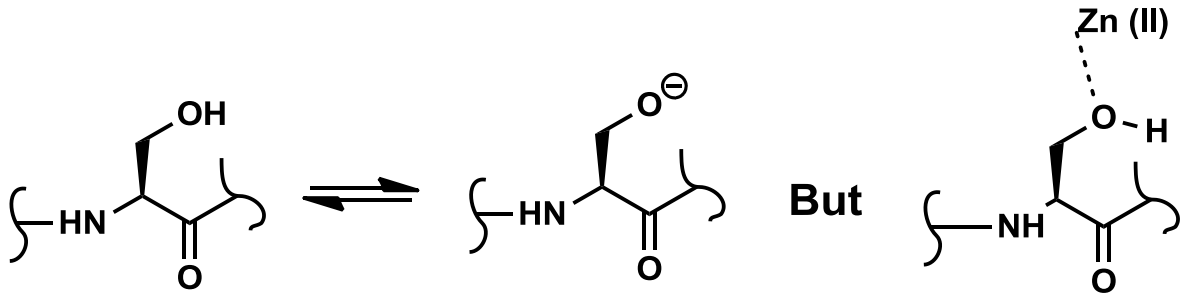
Apoenzyme (inactive) + cofactor



Holoenzyme (active)

Metal cofactors are often transition metals

Cofactor Example I – Complexation with Zn(II) reduces the pKa of alcohols/ water in amide hydrolysis reactions (metallo-proteases are common)



Serine

pKa ~13

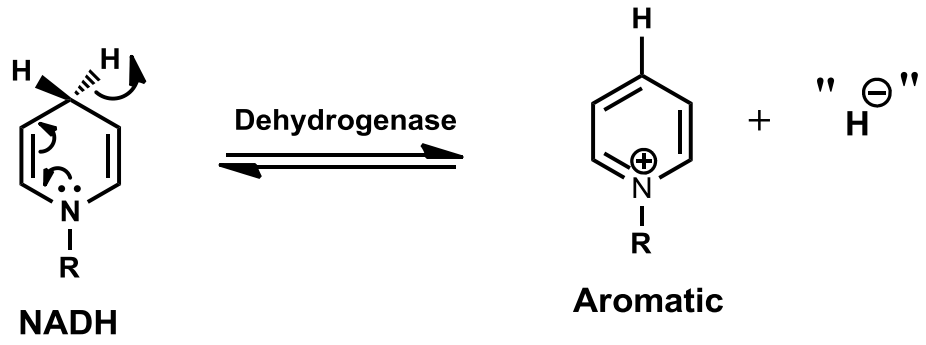
pKa <10

Cofactor Example II Transition metals are used to activate triplet state dioxygen, e.g. superoxide dismutase



Highly active superoxide

Cofactor Example III -The cofactor NADH is a biological equivalent of $NaBH_4$



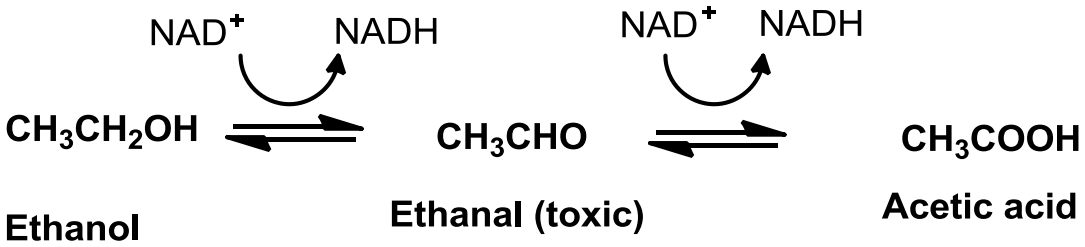
NADH Aromatic

(R maybe phosphorylated -NADPH)

NADH= reduced form of nicotine adenine dinucleotide

Note the Stereoselective H-transfer- which H is lost depends on the enzyme.

This chemistry is used in ethanol metabolism.



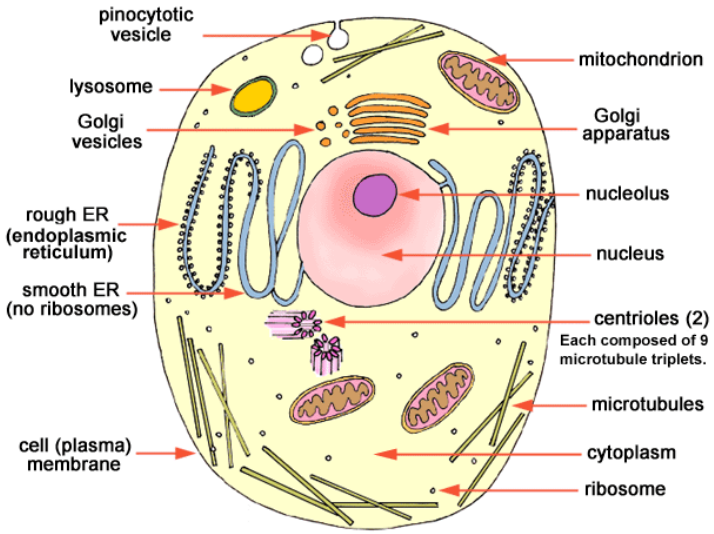
Necessary to coordinate metabolic processes, respond to changes in environment, grow and differentiate

And to prevent inappropriate catalysis – enzymes are powerful catalysts and need to be regulated

Enzymes need to be in the right place at the right time

Control of Enzyme Availability

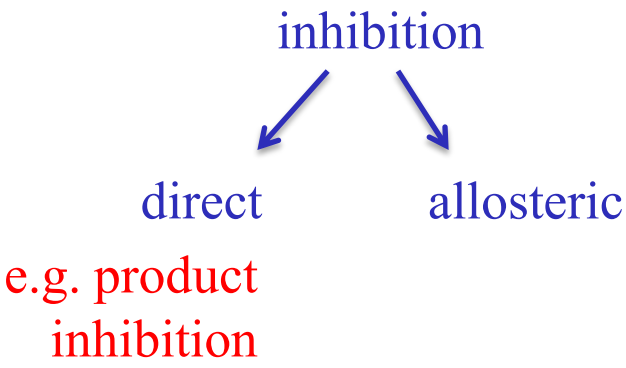
- Rate of enzyme synthesis (genetic control of expression)
- Rate of enzyme degradation
- Compartmentalisation



Control of Enzyme Activity – structural or conformational alterations

- Activation by cleavage of inactive pro-enzymes (e.g. in serine proteases)
- Requirement for co-factors/cosubstrates
- Activation by post translational modifications (of enzyme or of protein substrates)
- Inhibition by small molecules (feedback inhibition is important in metabolism)

an enzyme is only as active as the amount of enzyme:substrate complex



1.Oxidoreductases	**Oxidation and reduction transfer of electrons**	**Dehydrogenase,reductase,oxidase and oxygenases**
2. Transferases	**Transfer of function groups eg. Acetyl, methyl and phosphate**	**Acetyltransferase, methyl transferase, protein kinase and polymerase**
3. Hydrolases	**Hydrolysis reactions where a molecule is split by the addition of water**	**Protease, nuclease and phosphatase**
4. Lyases	**Catalyze the cleavage of C-C, C-O and C-N bonds (not hydrolysis or oxidation)**	**Decarboxylase and aldolase**
5. Isomerases	**Atomic rearrangement within molecules**	**Racemase and mutase**
6. Ligases	**Join the two molecules together (using ATP)**	**DNA ligase, peptide synthase, fatty acid synthase**

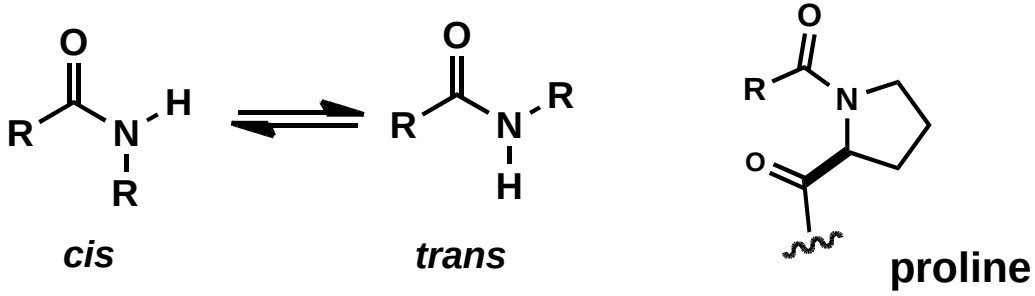
http://www.chem.qmul.ac.uk/iubmb/enzyme

- **Enzymes are not the only biological catalysts –**

- Ribosomes are made of rRNA and catalyse protein synthesis

- Many reactions in cells are probably catalysed by ions (H^+)

- **Enzymes do not only catalyse covalent reactions but also non-covalent 'processes' / conformational changes' e.g. chaperones catalyse protein folding and *cis-trans* prolyl-amide bond isomerases.**



Enzymes are amazing!!

- Super-efficient catalysts
- Catalyse reactions not possible synthetically
- Precise 3D arrangement of amino acids at active site and beyond
- Highly specific
- Often require cofactors
- Activity must be controlled and regulated

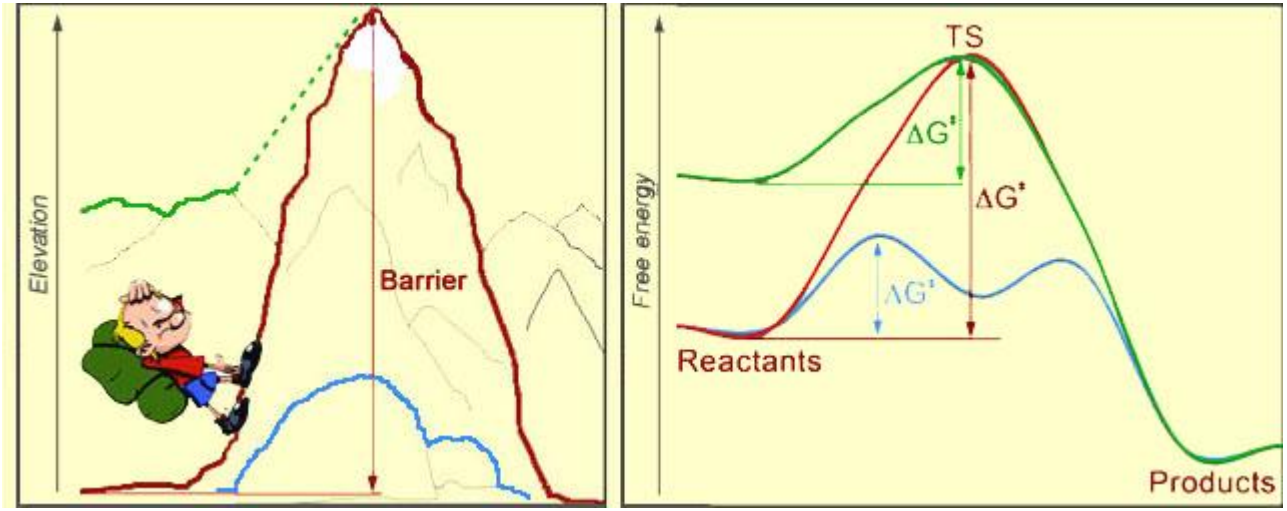
How do they do it?!

Lecture 1: **Introduction to enzymes**

The importance of enzymes in catalysing the chemical reactions of life.
How enzymes promote catalysis
Typical enzyme-catalysed reactions
Enzyme efficiency: selectivity, co-factors and control.

Lecture 2: **Principles of enzyme catalysis**

Thermodynamics of enzyme-catalysed reactions
Transition state stabilisation
Case Study - Triose phosphate isomerase


Elevation
Barrier
Free energy
TS
ΔG‡
ΔG‡
ΔG'
Reactants
Products

ΔG = free energy change of a reaction

Negative ΔG → spontaneous reaction

ΔG is **energy** difference between reactants and products

ΔG reveals nothing about **rate** of reaction

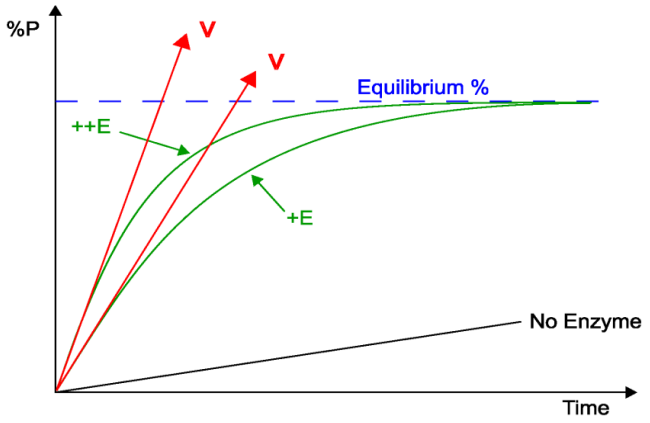
ΔG reveals nothing about **mechanism** of reaction

$A + B \rightleftharpoons C + D$ $\Delta G = \Delta G^\circ + RT \ln \frac{[C][D]}{[A][B]}$ Equilibrium constant $\Delta G^{\circ\prime} = -RT \ln K'_{eq}$

At equilibrium, ΔG = 0

K'_{eq} depends on the concentration of [A] [B] [C] [D]

ΔG therefore dependent on substrate concentration



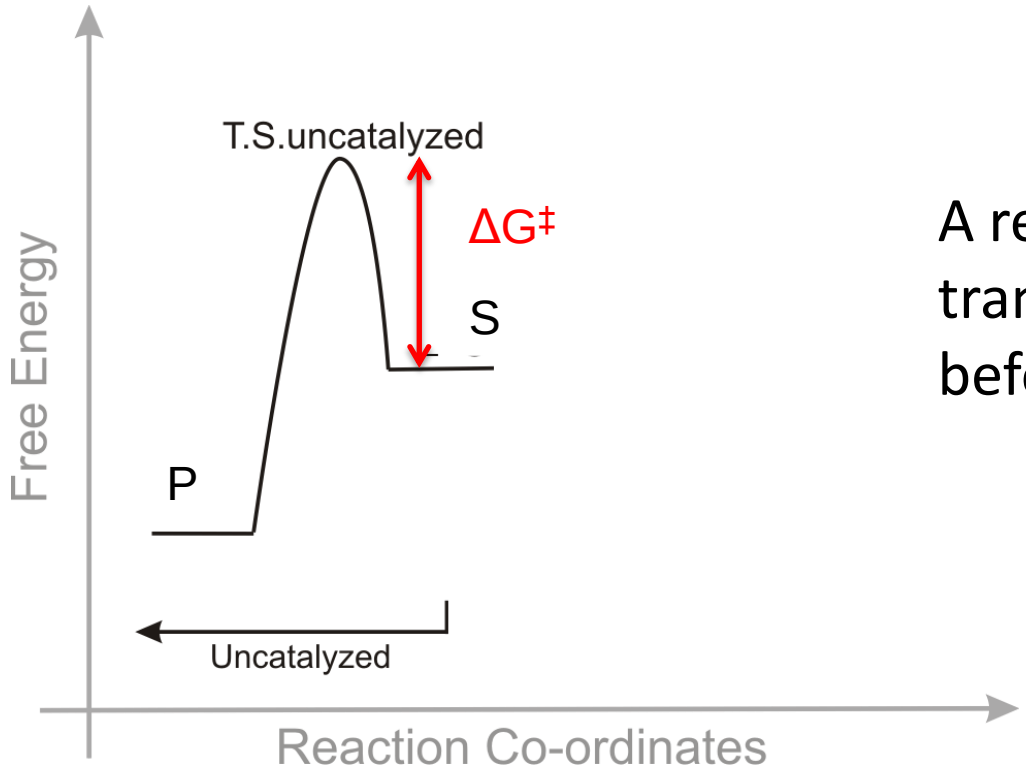
An enzyme cannot alter the equilibrium of a chemical reaction

The amount of product formed will be the same whether or not the enzyme is present

The difference is the rate at which equilibrium is reached and the rate of product formation (seconds compared to hours)

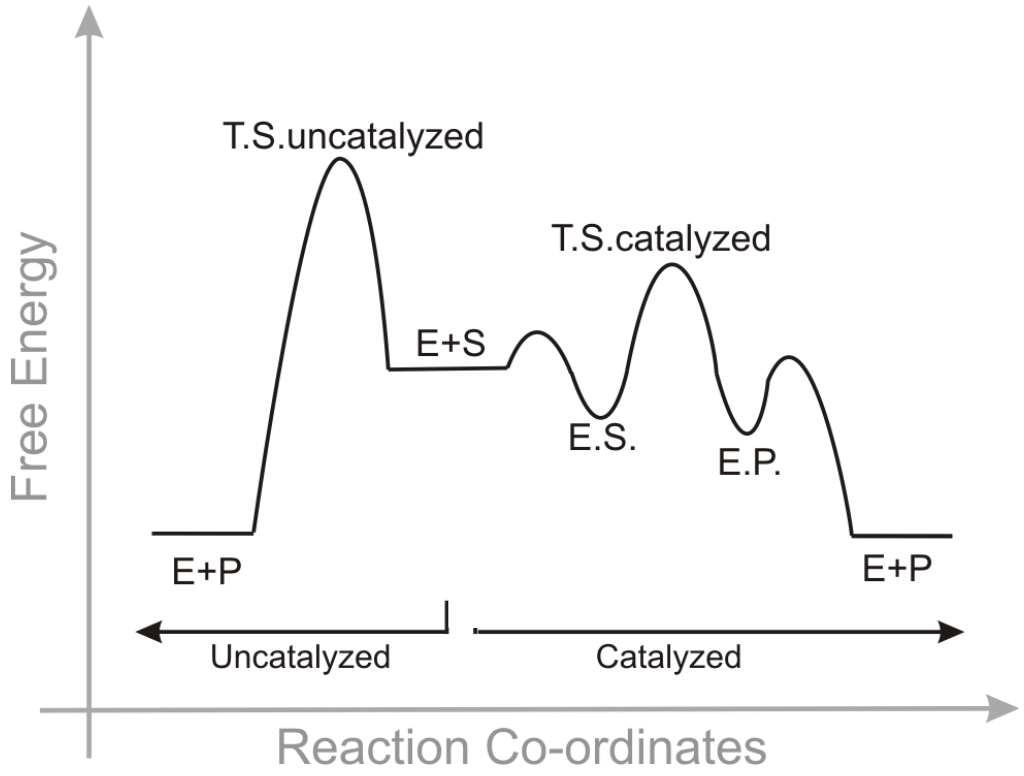
Pathway from substrate to product?

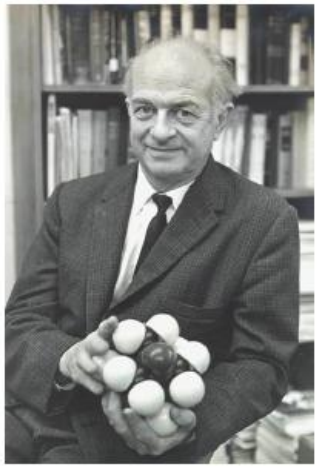
So how do enzymes increase the RATE of a reaction?



A reaction must go through a transition state (high energy) before product formation

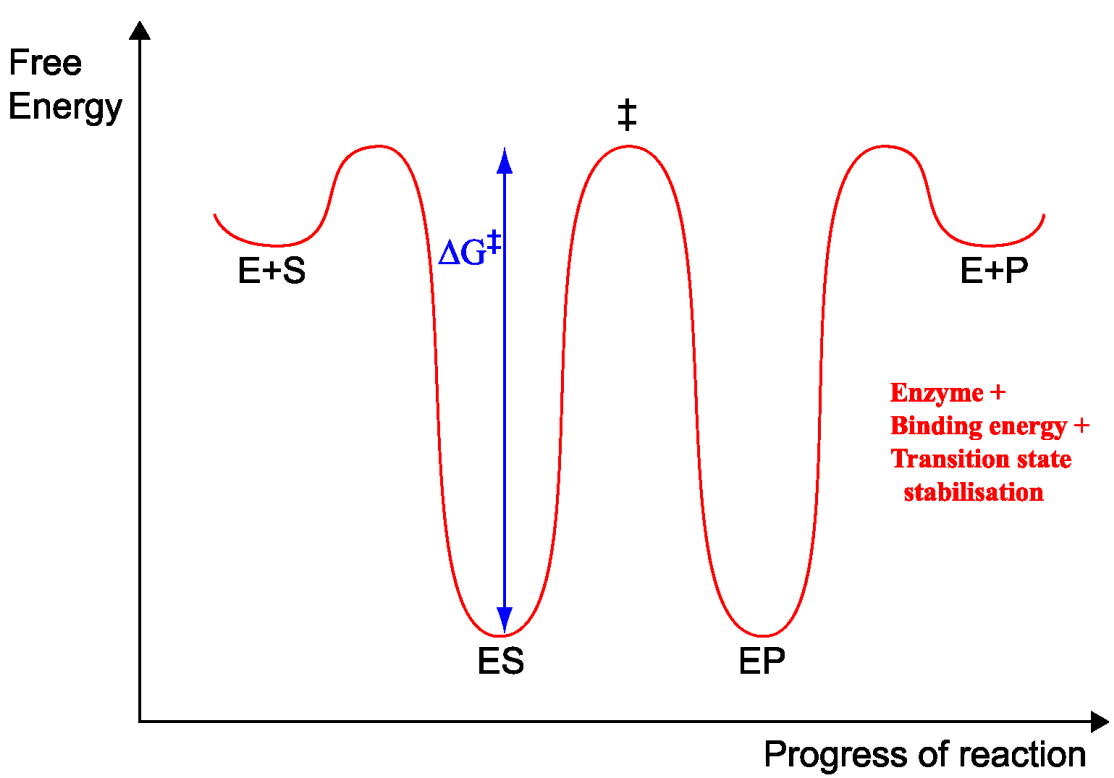
Activation energy ($\Delta G^{\ddagger}$) is that required to reach the transition state

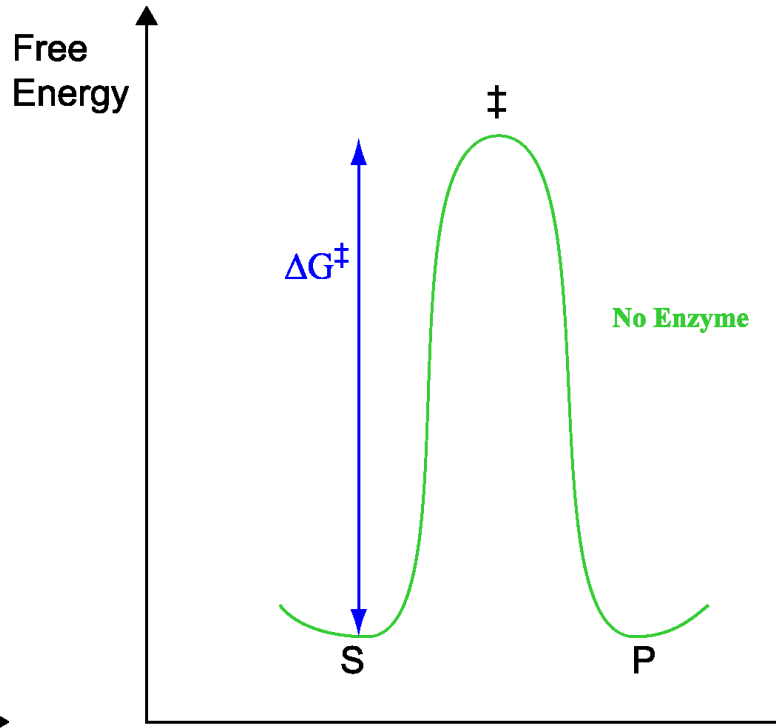




Linus Pauling "An enzyme stabilises the transition state of the catalyzed reaction more than it stabilises the substrate and the product"

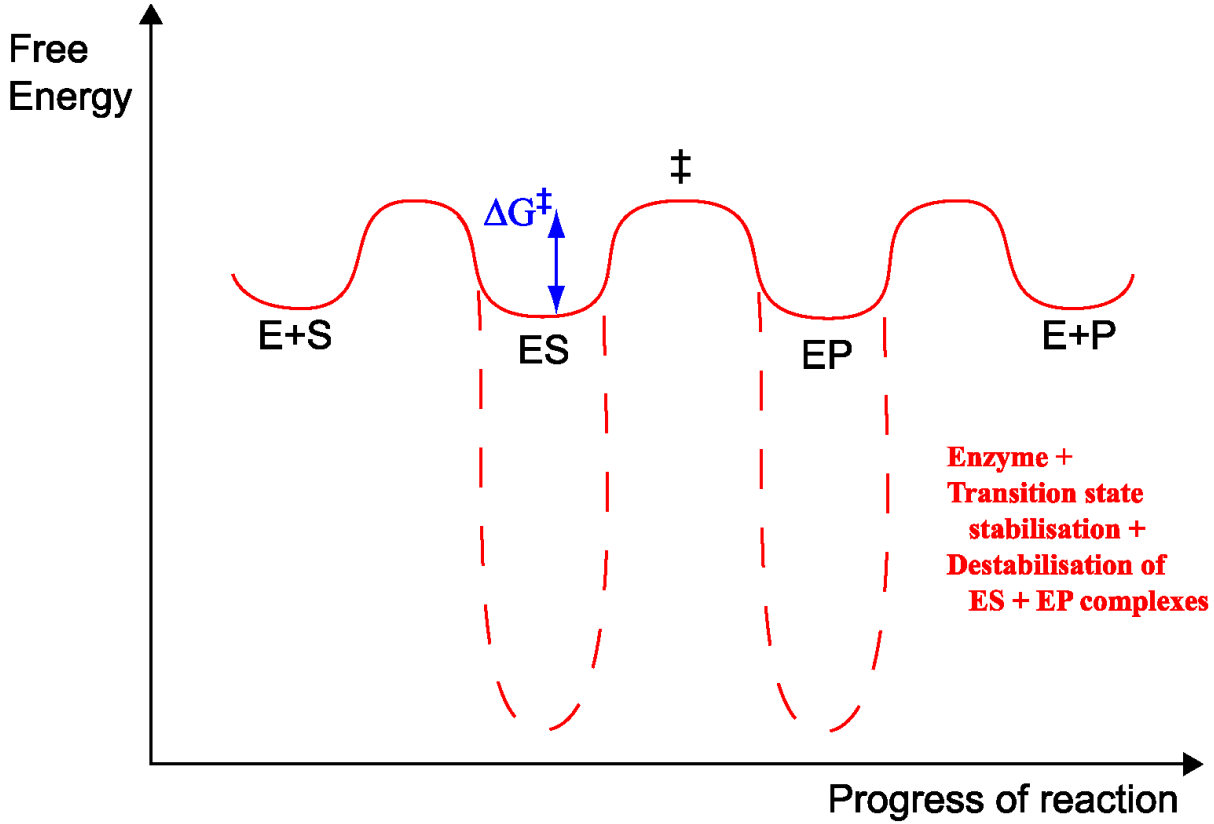
If the ES complex, transition state and EP complex are all stabilised by the same amount then:



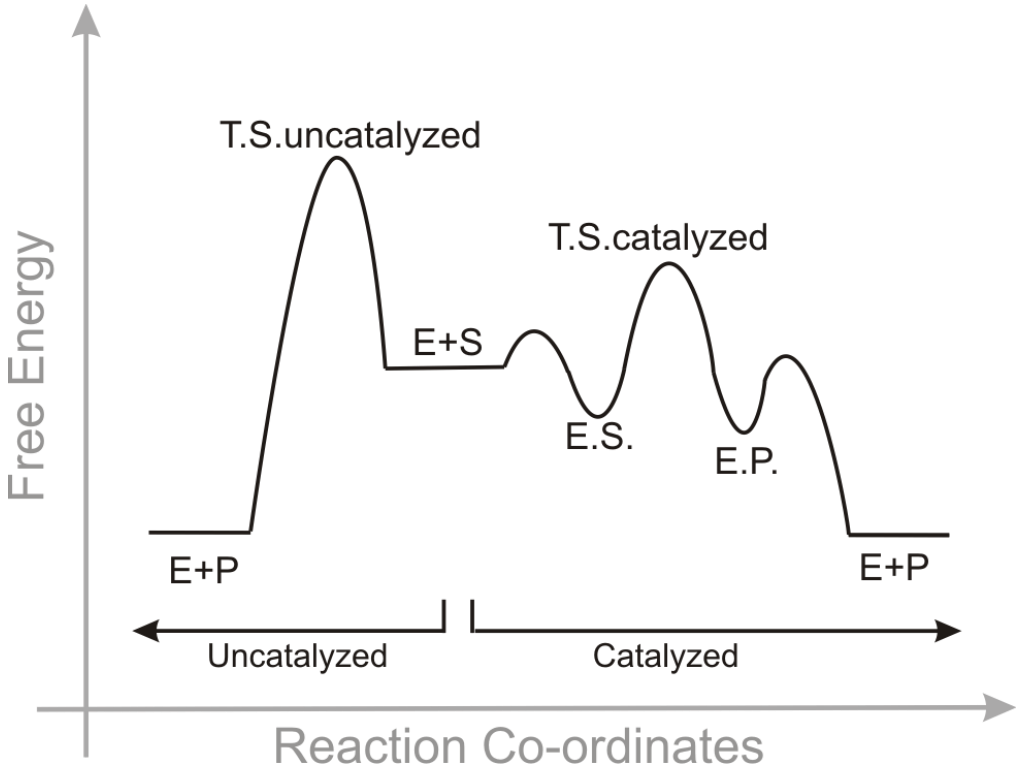


Equal stabilisation results in no change in $\Delta G^{\ddagger}$ therefore no catalysis.

However, if the ES and EP complexes are destabilised relative to the transition state and/or transition state sufficiently stabilised:

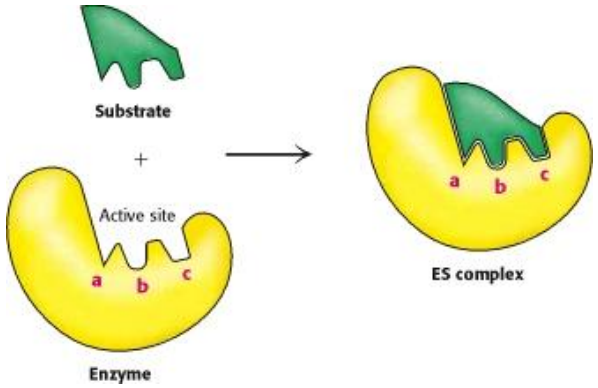


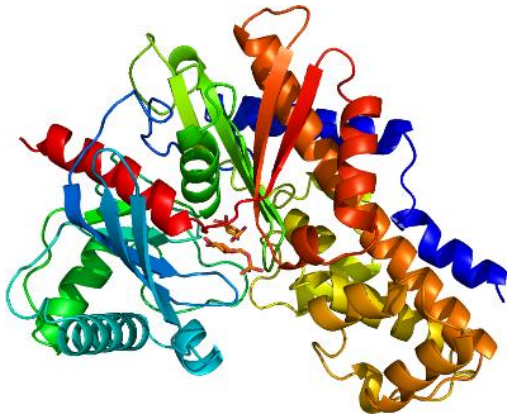
Binding energy is 'used' to lower $\Delta G^{\ddagger}$. ES and EP are not significantly stabilised relative to E+S and E+P.


T.S.uncatalyzed
T.S.catalyzed
E+S
E.S.
E.P.
E+P
E+P
Free Energy
Uncatalyzed
Catalyzed
Reaction Co-ordinates

How do enzymes achieve transition state stabilisation?

Bringing enzymes and substrates together **in a favourable conformation to promote formation of the transition state.**





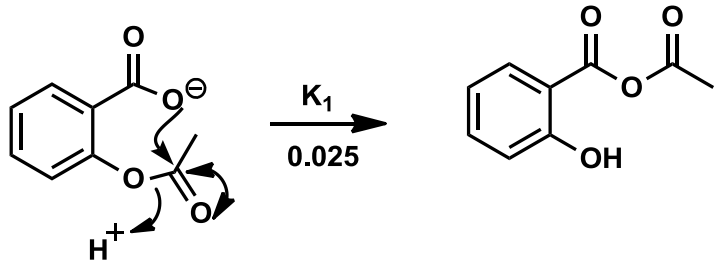
Substrate binds to enzyme **active site**

- a 3D cleft or crevice with precisely defined arrangement of atoms
- relatively small area of enzyme
- substrates bind via multiple weak interactions

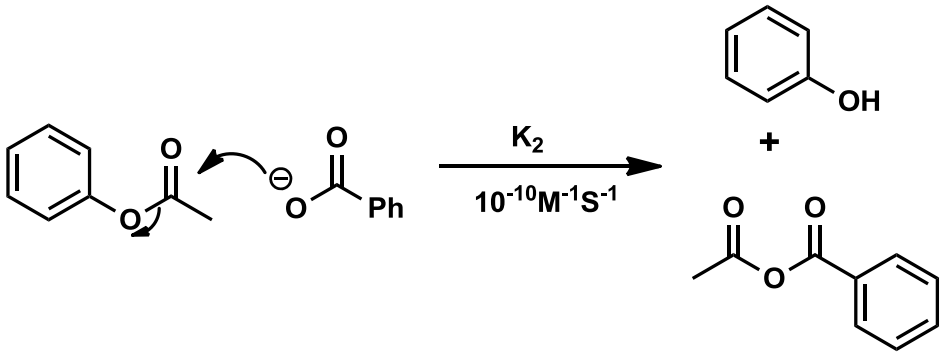
How do enzymes achieve transition state stabilisation?

1. Reduction in entropy – intramolecular reactions

Intramolecular reactions are faster than equivalent intermolecular reactions



K1/K2=2x10^{7}M



Note stereoelectronic geometry requirements must always be met.

How do enzymes achieve transition state stabilisation?

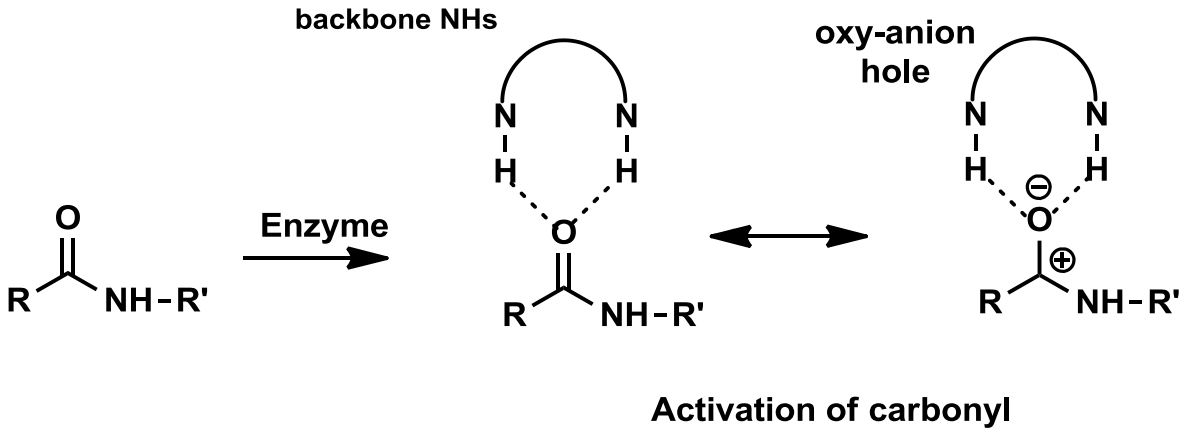
1. Reduction in entropy – intramolecular reactions

2. Optimal orientation of substrates

3. Binding energy provided by interaction between enzyme and substrate

4. Bond strain can be tolerated

Example – In some proteases the 'oxy-anion hole' polarises the pi-bond of the amide carbonyl making it more susceptible to nucleophilic attack.

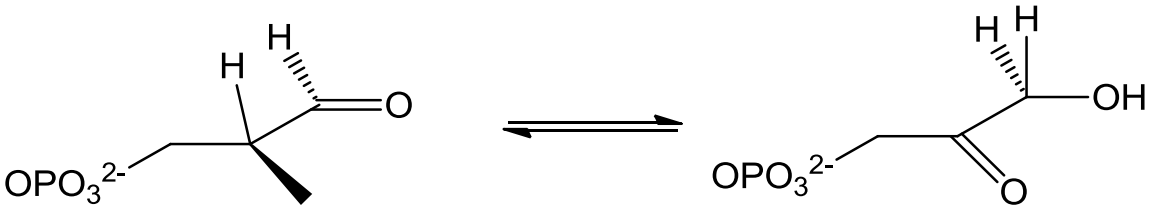


- There is good geometric fit of charge in the transition state

Transition state stabilisation enables catalysis by:

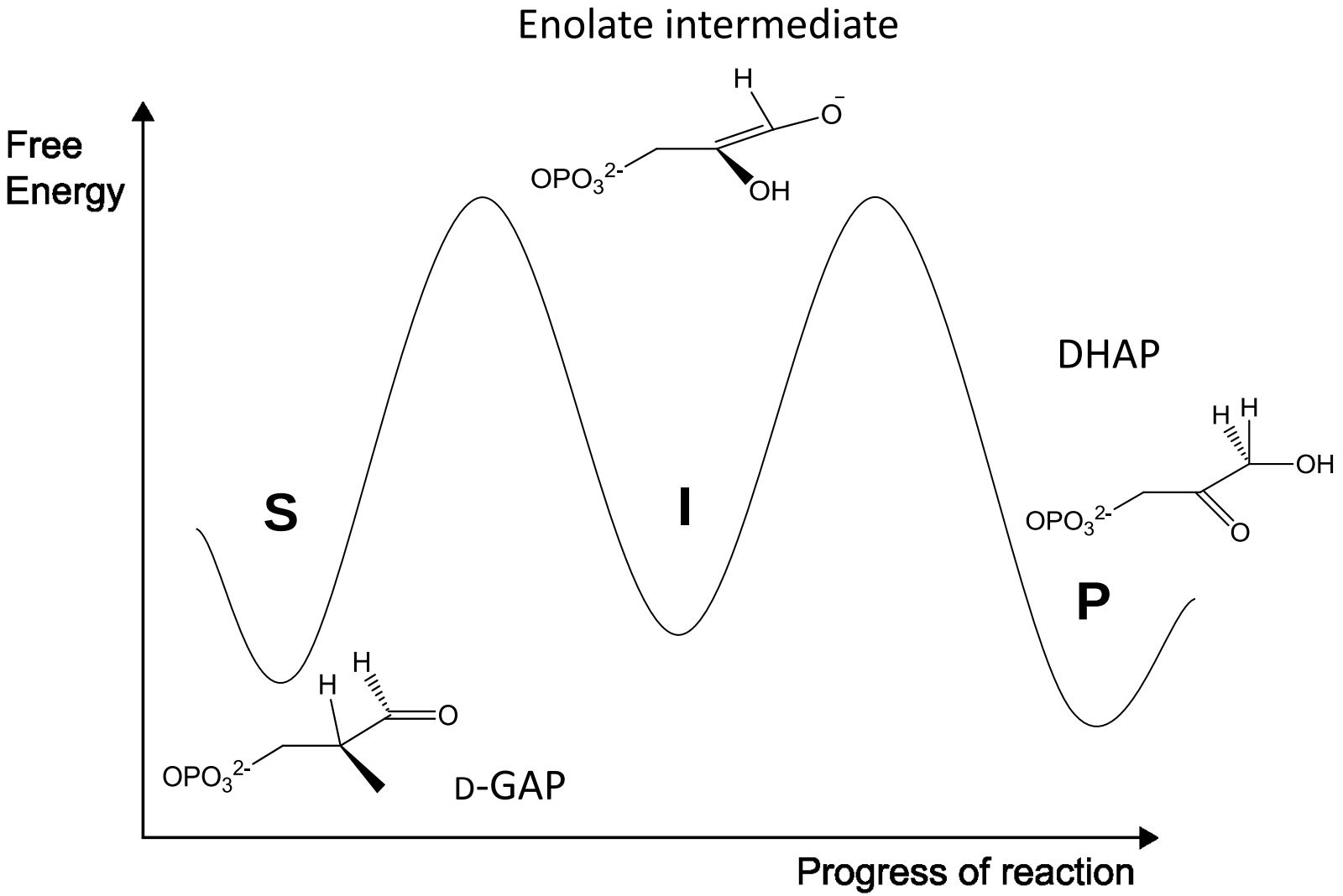
- Hydrophobic interactions
- Electrostatic interactions
- Acid/base interactions
- Metal ion interactions
- Covalent reactions with substrate (covalent catalysis)
- *Cofactors*

- Catalyses the interconversion of D-glyceraldehyde-3-phosphate (GAP) and dihydroxyacetonephosphate (DHAP)
- Keto-enol tautomerisation
- TPI (TIM) is amongst the best understood of all enzymes.
- Good illustration of transition state stabilisation Jeremy Knowles



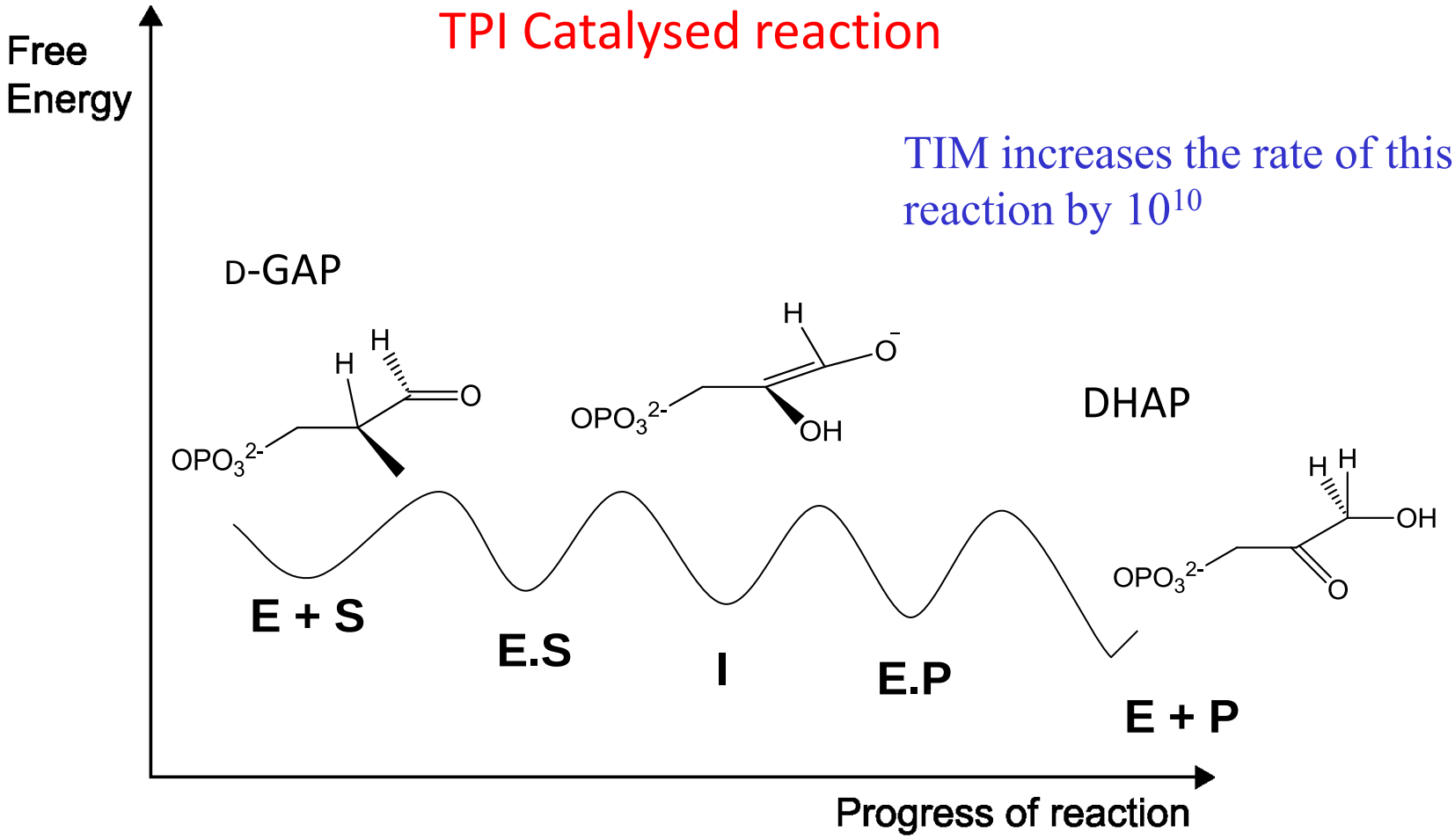
D-GAP DHAP

Uncatalysed reaction

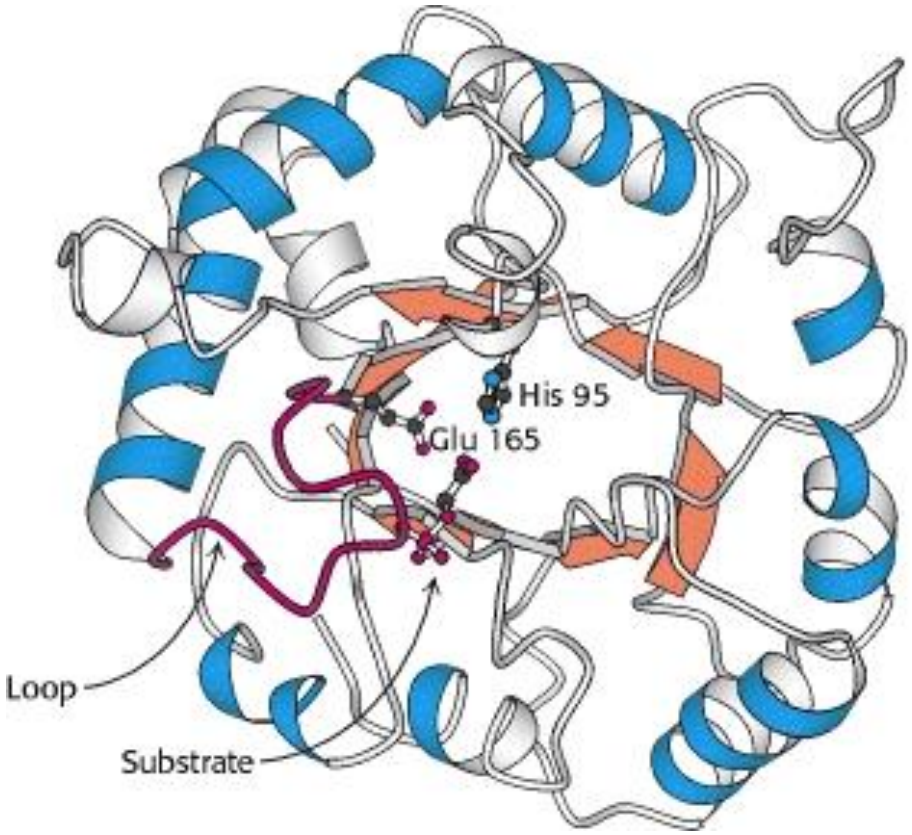


TPI Catalysed reaction

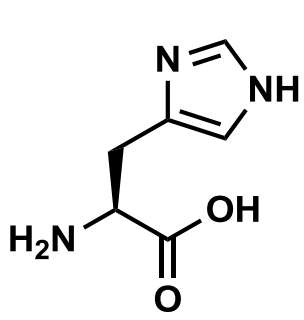
TIM increases the rate of this reaction by 10^{10}



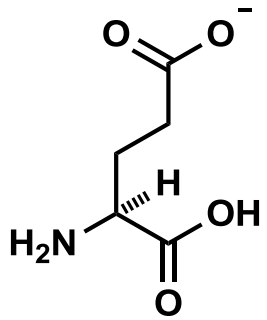
Stabilisation of intermediate = reduced ΔG to reach transition state
Enolate intermediate tightly bound at TIM active site



- α/β barrel
- Same motif found in other glycolytic enzymes, e.g. aldoloase, enolase
- Glu 165 and His 95 essential for catalysis

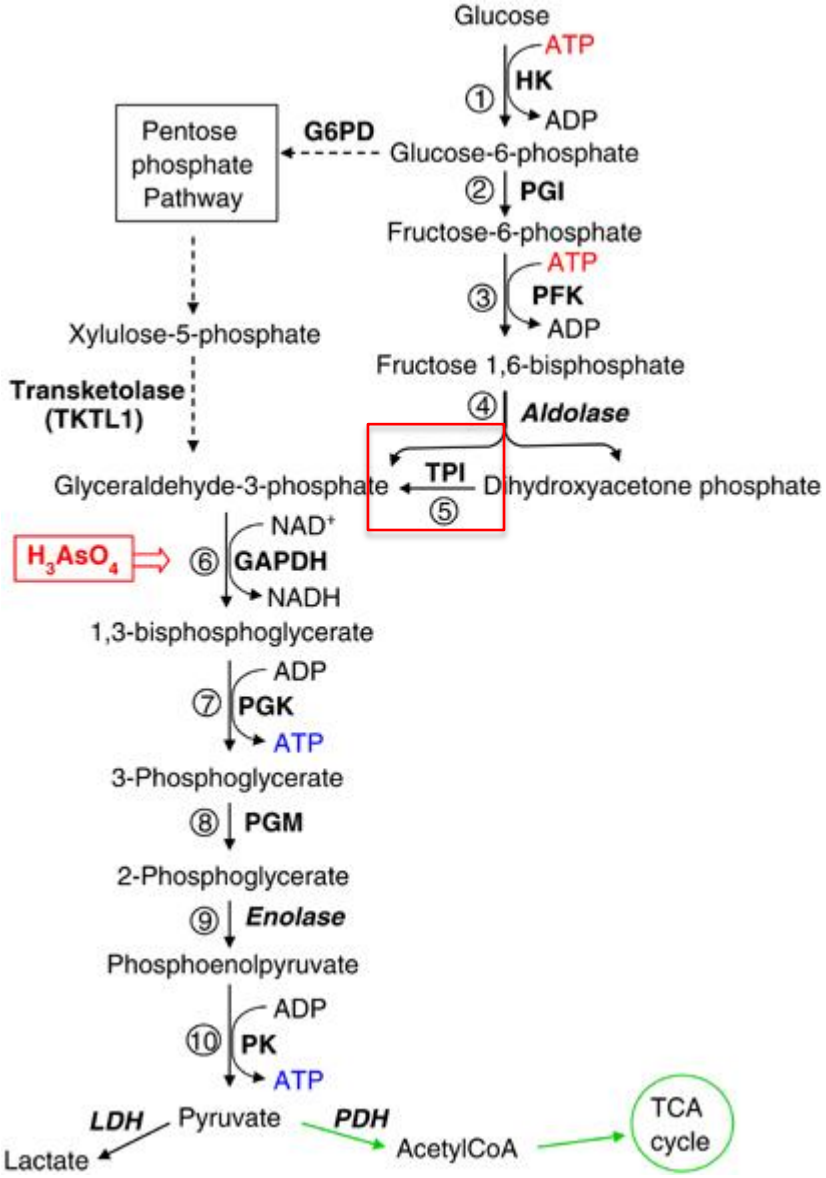


Histidine



Glutamate

- Loop closes on substrate binding



- Important in GLYCOLYSIS
- Glycolysis is the first (anaerobic) pathway in the conversion of glucose to ATP (energy)
- At equilibrium, reaction favours DHAP, but GAP consumed so rapidly that reaction essentially flows in this direction

DHAP $\rightleftharpoons$ GAP $\quad \Delta G = \Delta G^\circ + RT \ln \frac{[GAP]}{[DHAP]} \quad \Delta G^{\circ\prime} = -RT \ln K'_{eq}$

$$\Delta G^{\circ\prime} = -2.303RT \log_{10} K'_{eq}$$

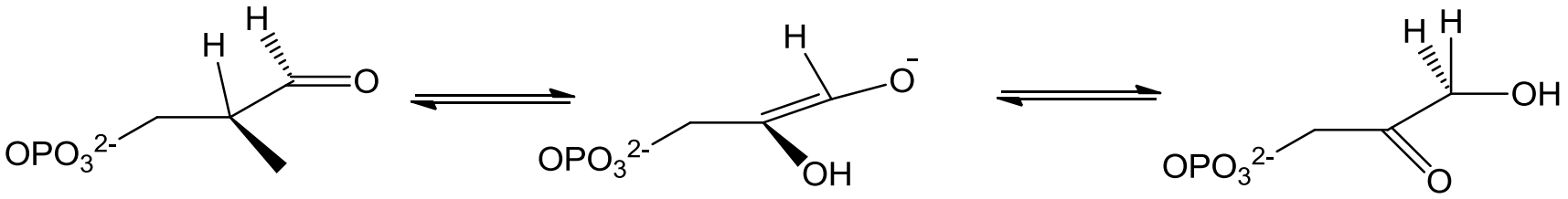
At equilibrium, ratio of GAP:DHAP = 0.0475, i.e. $K'_{eq} = 0.0475$, under standard conditions.

$$\Delta G^{\circ\prime} = -2.303RT \log_{10} K'_{eq}$$

$$\Delta G^{\circ\prime} = +7.53 \text{ kJ/mol}$$ endergonic

If DHAP is present in excess, e.g. 2 x 10^{-4} M compared to GAP at 3 x 10^{-6} M, then K'_{eq} changes and $\Delta G^{\circ\prime} = -2.89$ kJ/mol exergonic

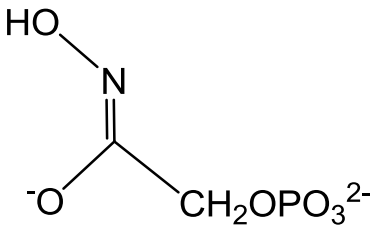
Reaction will only proceed when DHAP is in excess…



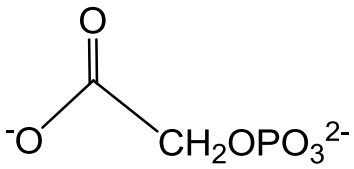
D-GAP Enolate intermediate DHAP

Transition states bind to enzymes more tightly than substrates

Proof of involvement of enolate intermediate from the use of transition state analogues:



Phosphoglycohydroxyamate



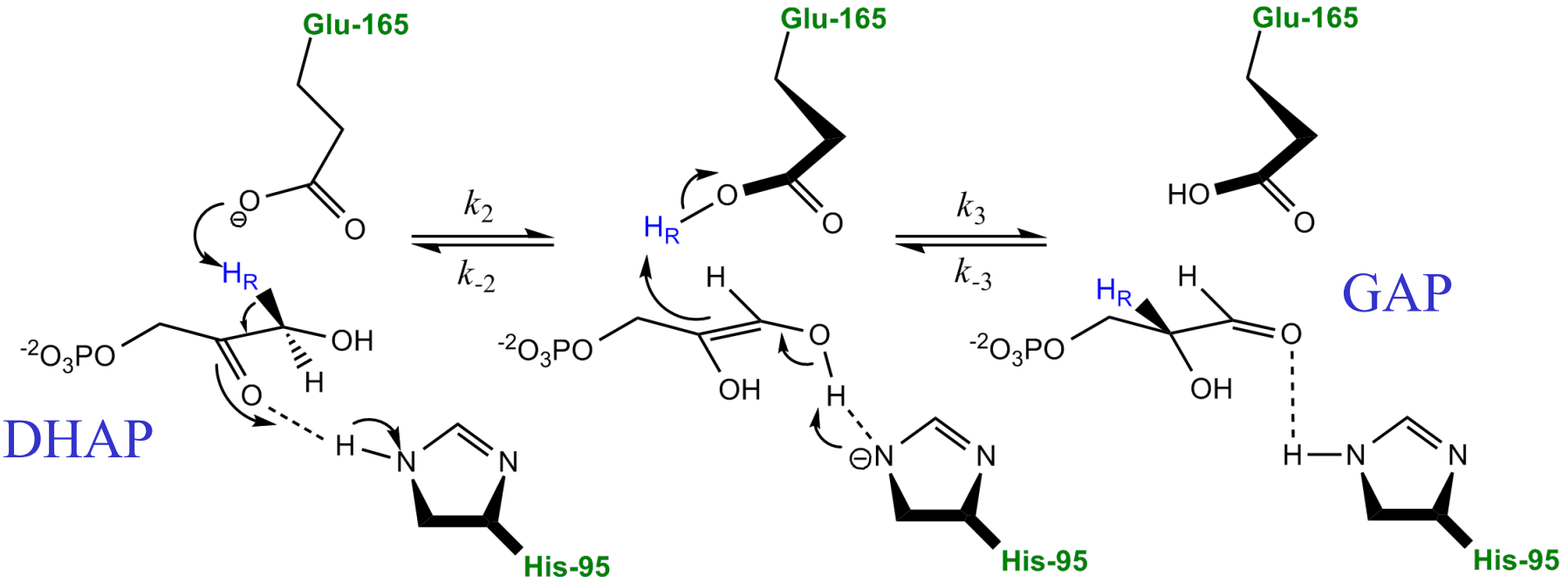
2-Phosphogycolate

Which bind more tightly than substrate, do not act as substrate, and inhibit TPI reaction

1. H abstraction by Glu165

3. Glu165 protonates enediol

Transition states stabilised by H-bonds

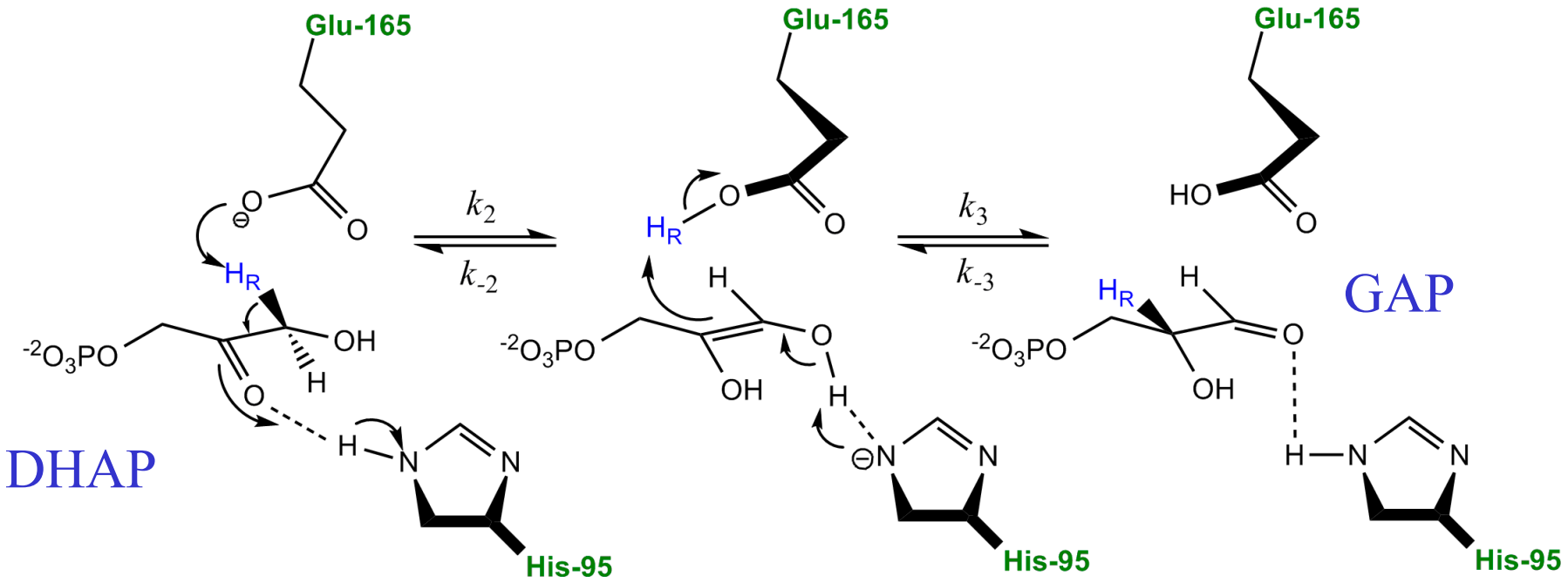


2. Protonation of carbonyl O by His-95

4. H abstraction by His95

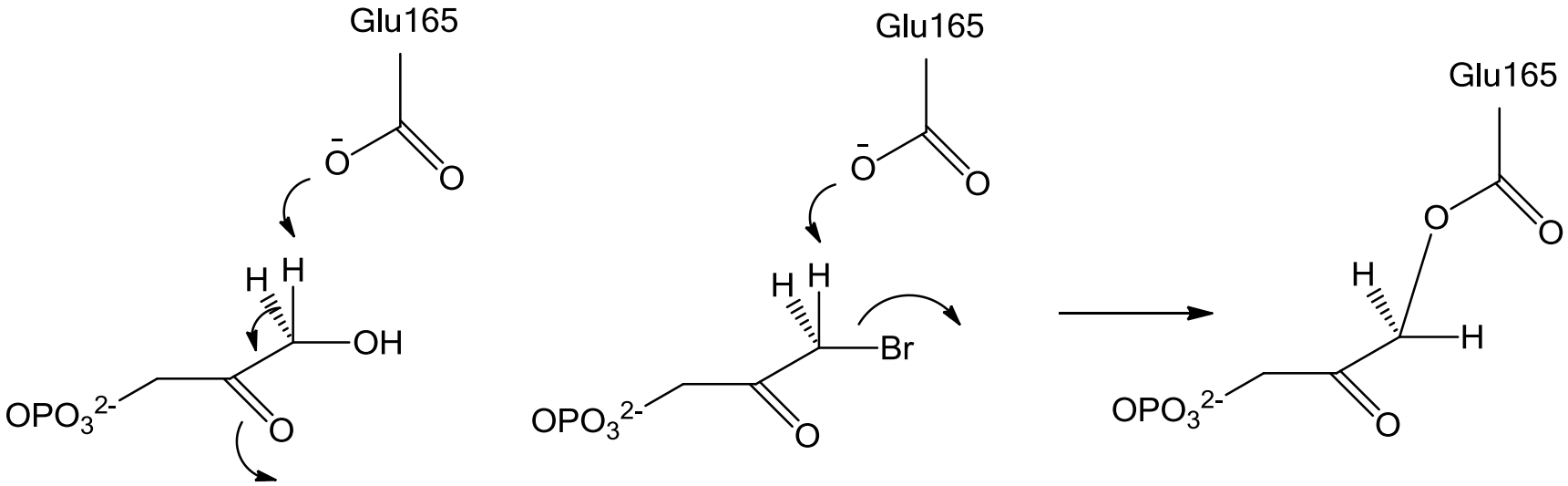
Reaction likely occurs via concerted general acid-base catalysis

- Due to low-barrier hydrogen bonds formed when pK's of H-bond acceptor and donor groups are near equal
- Contribute to stabilisation of transition state



Evidence for role of Glu165:

1. Affinity labelling reagents employed to identify base at active site of TPI:

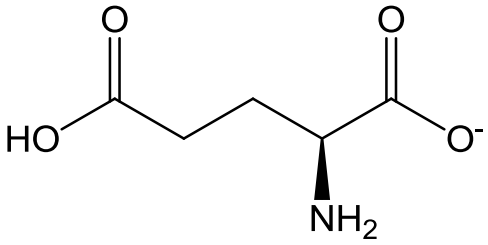


- Detect binding of inhibitor by (i) stoichiometry of radiolabelled compound, (ii) digestion of enzyme and identification of labelled fragment by mass spectrometry.

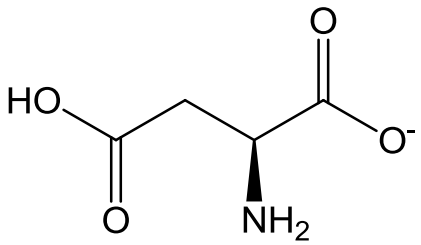
Evidence for Role of Glu165:

2. Mutagenesis, X-ray crystallography and kinetics:

Replace Glu165 with Asp



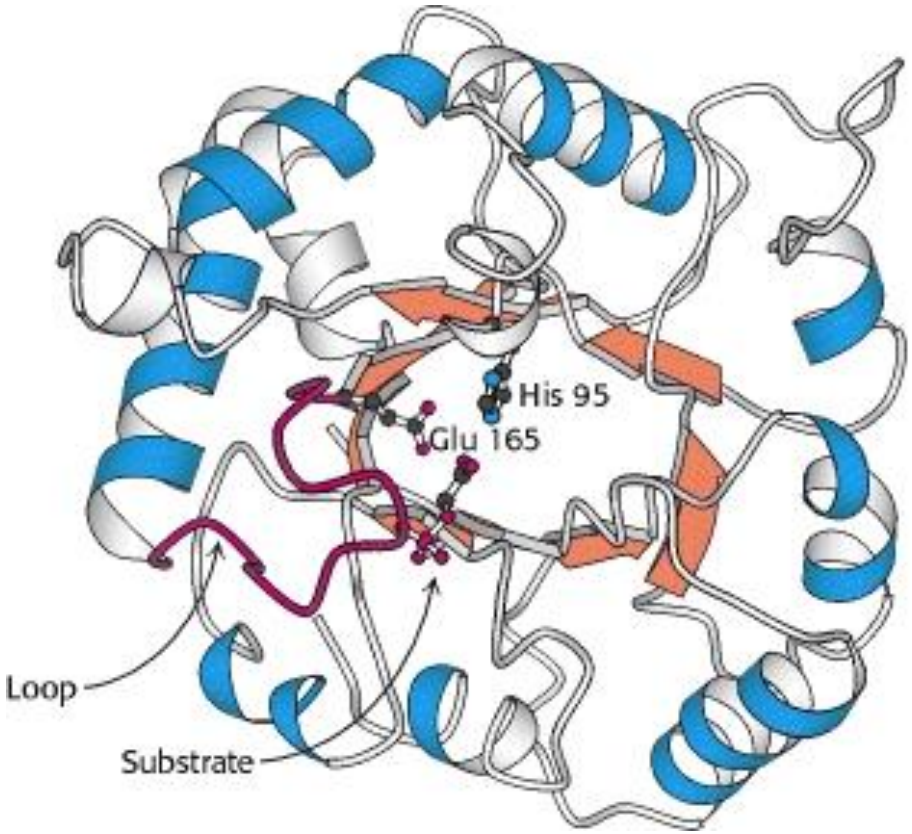
Glutamate



Aspartate

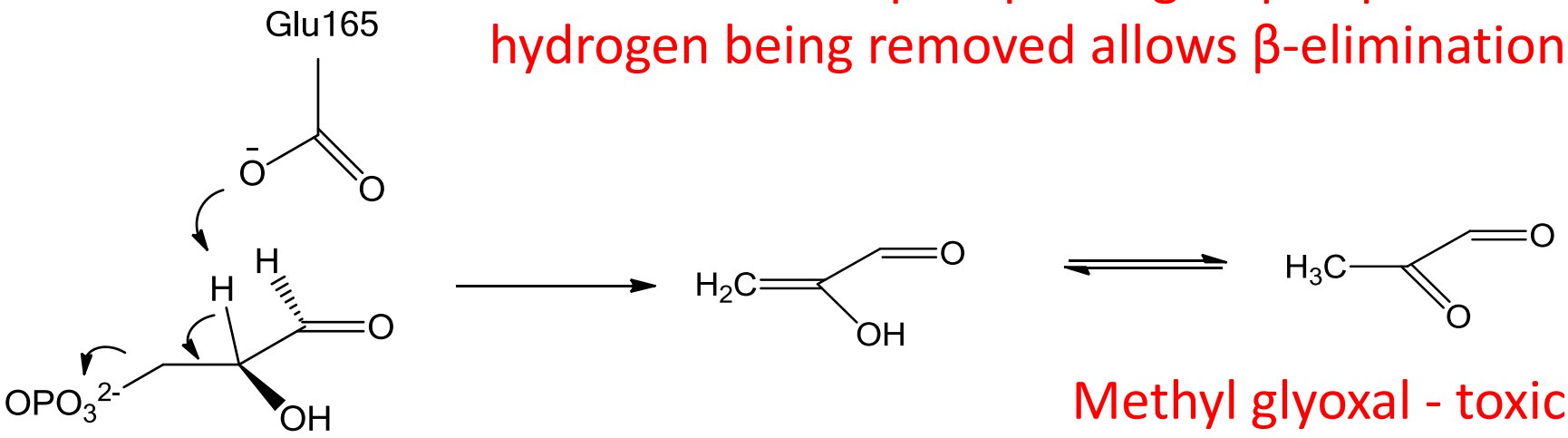
Carboxylate group withdrawn by ~1Å – catalytic efficiency reduced ~1000 fold

Importance of the flexible loop:



- X-ray crystallography comparing E and $E.S_{analogue}$
- Loop acts like a hinged lid
- Lysine residue on loop interacts with phosphate group on substrate
- Loop helps stabilise transition state

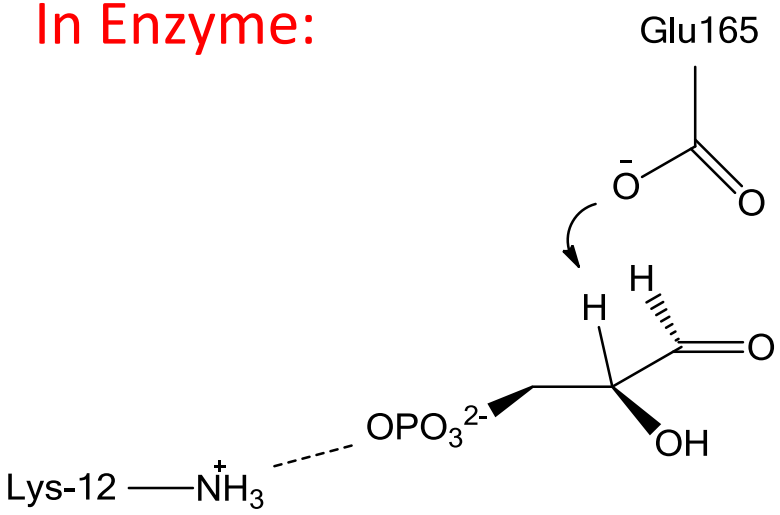
In Solution:



Conformation of phosphate group in plane of hydrogen being removed allows β-elimination

Methyl glyoxal - toxic!

In Enzyme:



Phosphate group held away from the plane, preventing β-elimination and facilitating specific reaction

- Enzymes cannot promote reactions that are not otherwise thermodynamically favourable

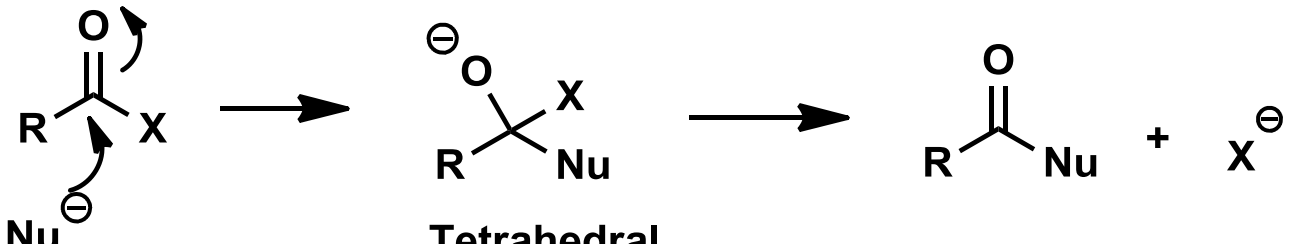
- Enzymes catalyse reactions by stabilising the transition state

- Transition state is stabilised by very specific interactions at the enzyme active site

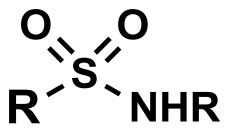
- The well-characterised enzyme triose phosphate isomerase is a good example of transition state stabilisation by:

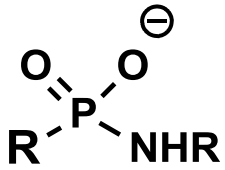
    - acid-base reactions
    - a stabilising loop to confer product selectivity

Can we design a novel biological catalyst?



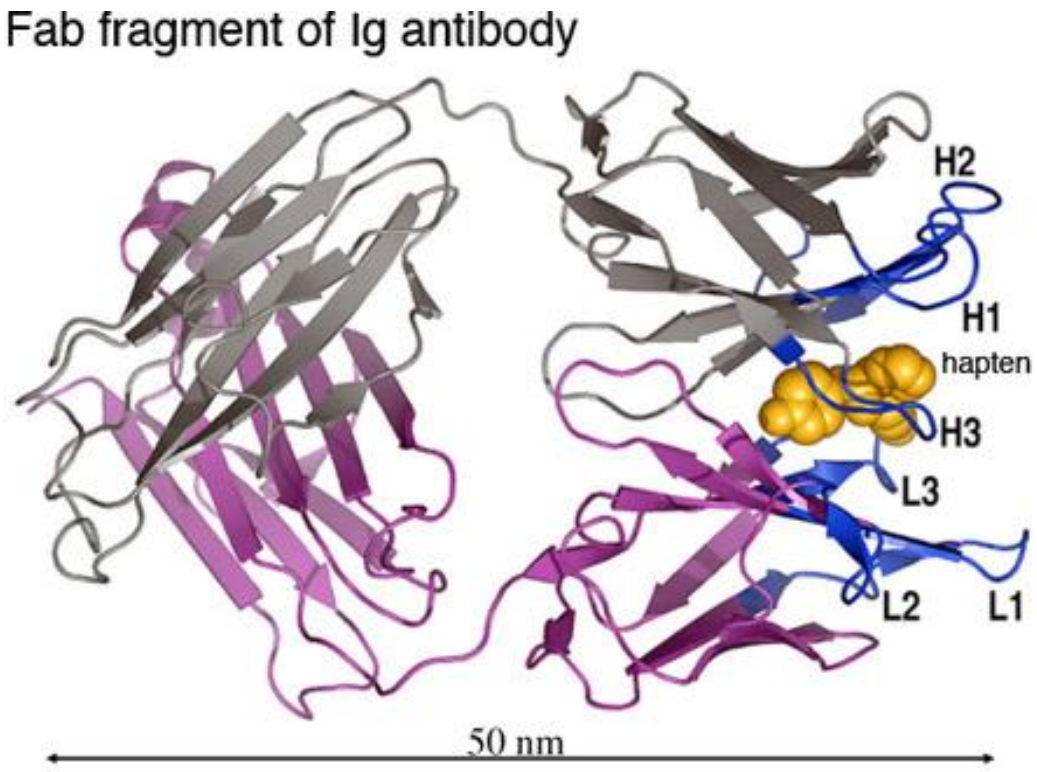
a complementary structure should catalyse the reaction





transition state analogue

Antibodies contain hypervariable regions that bind haptens



If the Pauling theory of enzyme catalysis by stabilisation of transition states is correct, one way to design a *de novo* catalyst for a reaction would be to create a structure complementary to the transition state for the reaction.

1. Synthesis of transition state analogue = hapten
2. Link hapten to carrier protein to give hapten-antigen
3. Immune system
4. Production of antibodies
5. Identification of catalytic antibodies

But catalytic antibodies only enhance rates by ~ $10^3/10^5$ (at best) – enzymes $>10^{10}$….

Why?

Enzymes are more than just rigid template for a single transition state

- *More than one transition state*
- *Catalysis is not simple binding*
- *Product/substrate inhibition need to be avoided*
- *Orientation of substrate in important*
- *Cofactors maybe are required*
- *Antibody substructures are limited by their folds*

Catalytic antibodies do have therapeutic promise, e.g. E-vac, an ‘abzyme’ against HIV (prevents invasion of host cells).

www.abzymeresearchfoundation.org