

Enzymology

 Course	 Essential Protein Structure and Function
 Confidence	Confident
 Next Review	@April 27, 2024
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Introduction to enzymes

Importance of enzyme

- Physiology: virtually all chemical reactions in cells are catalysed by enzymes
- Medicine: i) explain how defective enzymes cause disease. ii) use enzymes for diagnoses
- Pharmacology: an explanation of how drugs act.
- Chemistry: the design and synthesis of new drugs
- Economics: how to exploit enzymes in industry

What are enzymes?

Enzymes are catalysts:

- E accelerate reactions by rate factors between 10^6 to 10^{17} .
- They do this by reducing the activation energy required to form the transition state ES if the reaction
- Enzymes bring substrate S together in orientations favorable for the formation of the transition state
- The active site of an enzyme E contains the catalytic groups that interact with the substrate S.

The enzymes may achieve this through proximity:

- By bringing the two substrates together, there is a very high local concentration within the enzyme

- As the reaction rates increase with concentration of the reagents
- Complementary shapes and charges between the catalytic groups of the active site and substrate allow the favorable orientations

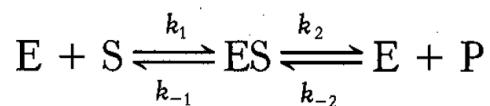
Six groups of enzymes

1. Oxidoreductases	$A_{\text{red}} + B_{\text{ox}} \rightleftharpoons A_{\text{ox}} + B_{\text{red}}$	Involved in oxidation / reduction reactions. Use O_2 , H_2O_2 , or NAD^+ ... as an electron acceptor
2. Transferases	$AB + C \rightleftharpoons A + BC$	Transfer a functional group
3. Hydrolases	$AB + H_2O \rightleftharpoons A-H + B-OH$	Transfer H_2O_2 for hydrolytic cleavage of C-O, C-N, C-C and some other bonds
4. Lyases	$A=B + X-Y \rightleftharpoons A(X)-B(Y)$	Add or remove H_2O , CO_2 or NH_4^+ to break or form double bonds
5. Isomerases	$A \rightleftharpoons B$	Catalyse geometric or structural changes within one molecule
6. Ligases	$A + B + XTP \rightleftharpoons AB + XDP + P_i$	Join together two molecules coupled with the hydrolysis of a diphosphate bond in ATP, GTP ...
7. Translocases		Translocate hydrogen ions, inorganic cations and anions, amino acids, carbohydrates, or other compounds (usually across cell membranes)
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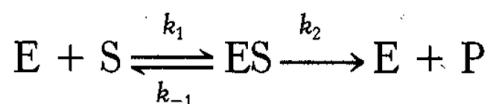
- Enzymes are classified into six groups on the basis of the type of reaction catalysed. These groups are divided into subgroups which are further subdivided.
1. Oxidoreductase - the large group of enzymes catalysing redox reactions
 2. Transferase - enzymes which transfer a group from one substrate to another
 3. Hydrolase - hydrolyse bonds
 4. Lyase - Cleave C-C, C-O, C-N, etc. bonds by elimination thus leaving double bonds (or which add groups to double bond)
 5. Isomerase - Catalyse changes within a single molecule
 6. Ligase - join two substrates in a reaction using ATP (or other nucleoside triphosphate)

Initial Velocity

- An enzyme E converts the substrate S to a produce product P
- Eventually an equilibrium is reached

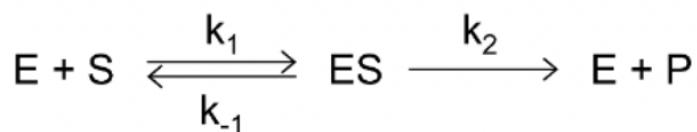


- Enzyme kinetics are simplified if the back reaction (k_{-2}) is ignored. Use initial velocities $v(0)$ to simplify the equations



- $v(0)$ is measured before any P has accumulated to complicate the analyses.
- This illustrates the importance of initial velocity.
 - Here we have a true initial velocity ($v(0)$ at $t(0)$) and other velocities based on a measurement of the product [P] at $t(1)$ and $t(2)$.
 - Only $t(0)$ measurements show the expected behaviour of linearity between rate and enzyme amount.

Michaelis-Menten Equation (1913)



$$V_0 = \frac{V_{max}[S_0]}{[S_0] + K_m} \quad \text{with} \quad V_{max} = k_2[E_0] \quad \text{and} \quad K_m = \frac{k_{-1} + k_2}{k_1}$$

- The M-M equation explains teh appearance of the rate graph of many enzymes
 - Low initial substrate concentration, most of the enzyme is in the [E] form.

$[S_0] \ll K_m$, $[S_0]$ insignificant in the denominator

$$V_0 \approx \frac{V_{max} [S_0]}{K_m}$$

$\therefore \frac{V_{max}}{K_m}$ is a constant

$$\therefore [S_0] \propto V_0$$

↳ First order region

- High initial substrate concentration, most of the enzyme is in the $[ES]$ form.

$[S_0] \gg K_m$, K_m insignificant

$$V_0 \approx \frac{V_{max} [S_0]}{[S_0]} \approx V_{max}$$

↳ Zero order region

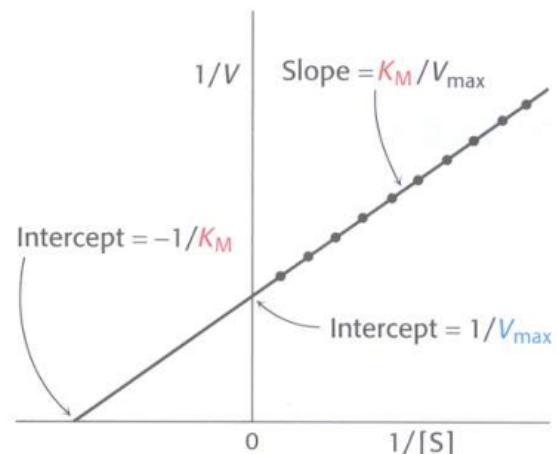
- When $[S] = K_m$, $V(0) = V_{max}/2$

Determine V_{max} and K_m

- At best, V_{max} can only be roughly estimated from a $v(0)$ vs $[S]$ plot.
 - Lineweaver-Burk or double reciprocal plot

$$V_0 = V_{max} \frac{[S]}{[S] + K_M}$$

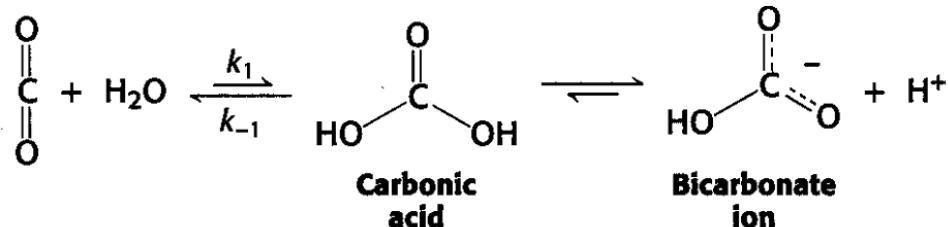

$$\frac{1}{V_0} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}} \cdot \frac{1}{[S]}$$



Example of applying M-M reasoning: Carbonic anhydrase

- CO₂ is a key end metabolite excreted by the body

- This enzyme accelerates the reaction between CO₂ and H₂O to form carbonic acid
- For example, this enables bicarbonate in your blood to be dehydrated to form CO₂ as the blood passes through the lungs.



K_m

- Note that very often K_m approximates the concentration of substrate inside the cell
- For most enzymes, K_m varies between 100,000 uM (weak) to 0.1 uM (strong)
- K_m depends on the substrate, and on the pH, temperature, and ionic strength
 - If k₂ is much smaller than k₋₁, K_m is the dissociation constant of the ES complex
 - A large value is a weak complex
 - A small value is a strongly bound complex
 - $K_m = (k_{-1} + k_2)/k_1$
- Carbonic anhydrase K_m=8000 uM

V_{max}

- Determining the maximal rate V_{max} gives us the turnover number
- This is the number of S molecules converted into P by an enzyme that is fully saturated by S (when [S] is much greater than K_m)
- The turnover number equals k₂, which is also called k_{cat}
 - Number of substrate molecules converted to product in a given unit of time on a single enzyme molecule when the enzyme is saturated with substrate

- If $[E](T)$ is known, $V_{max} = k_2 \cdot [E](T)$, so k_2 is calculated
- Turnover numbers typically range between 1 to 10^5 per sec.
- Carbonic anhydrase: $600,000 \text{ sec}^{-1}$

Enzyme efficiency

- k_{cat} / K_m
- Neither k_{cat} nor K_m are suitable individually to compare catalytic function since the former neglects the substrate binding and the latter neglects the catalysis
- The upper limit of this ratio is between 10^8 to 10^9 /sec/M which is the diffusion-controlled encounter of E with S
- Carbonic anhydrase $k_{cat}/K_m = 8 \cdot 10^7 \text{ /sec/M}$, very efficient enzyme

Rate acceleration

- The ratio of k_{cat} divided by the rate constant for the reaction in the absence of the enzyme

$$\text{Rate enhancement} = \frac{k_{cat}}{k_{uncat}}$$

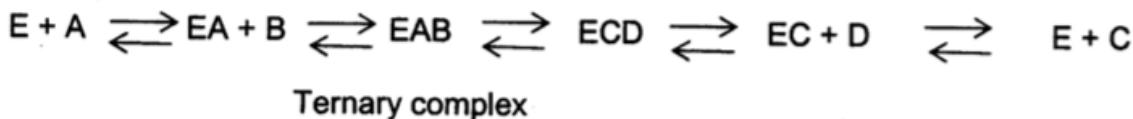
- This is not easy to determine in many cases, as most cellular reactions are extremely slow in the absence of enzyme
- Carbonic anhydrase
 - Non-enzymatic rate is 0.1 sec^{-1}
 - $k_{cat} = 10^6 \text{ sec}^{-1}$
 - Rate acceleration = 10^7

Substrate and Inhibitors

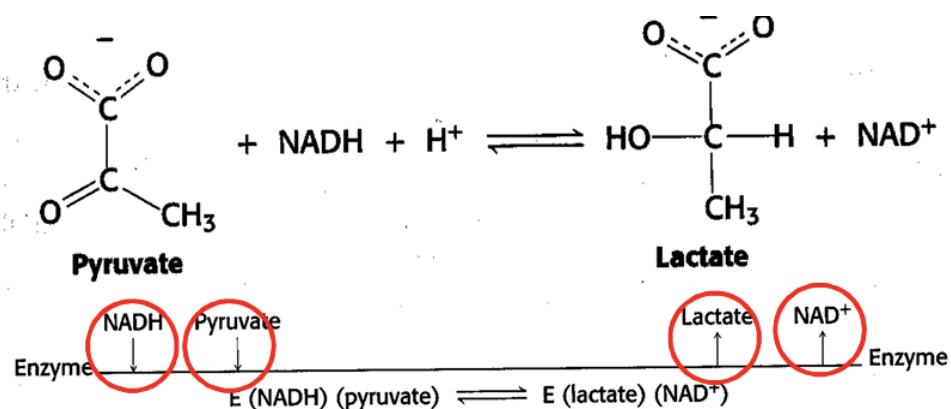
Two substrate enzymes

- About 60% of enzyme reactions have two substrates and two products
- There are two broad types of mechanisms for these called **ternary complex** and **substituted enzyme**

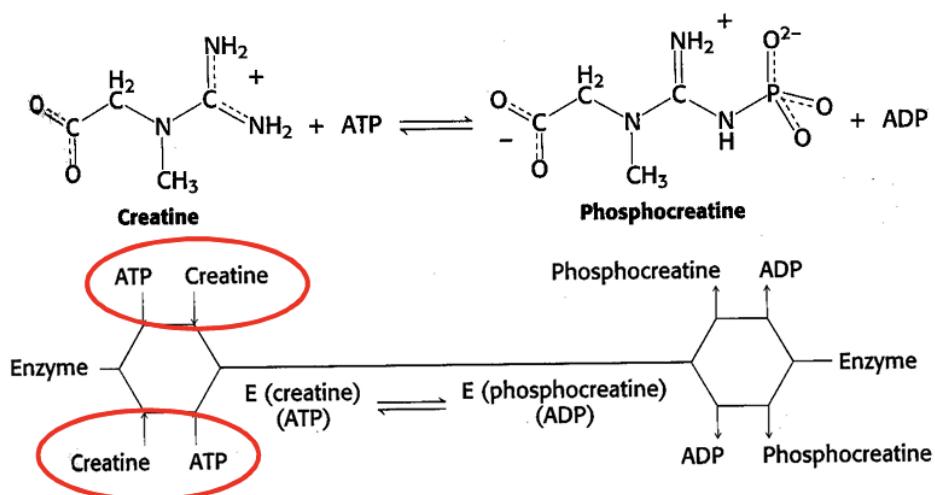
Ternary complex mechanism



- Note that the ternary complex is formed
- The order or reaction of the components can be compulsory.



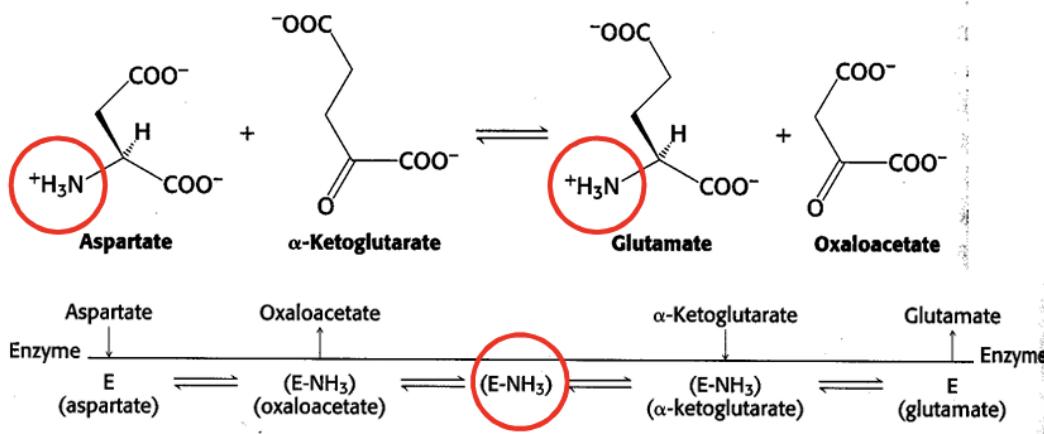
- Or random order



Substituted Enzyme Mechanism

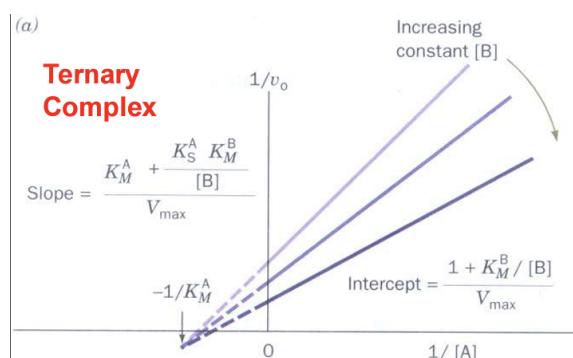
- No ternary complex formed
- The first substrate "modifies" or "substitutes" the enzyme, then this substrate departs before second substrate binds
- Also known as the "double-displacement" or "Ping-Pong" mechanism

- **Transaminase** (amino transferases) are a good example
 - The amino agroup (NH_3) is moved onto the enzyme, then off it.

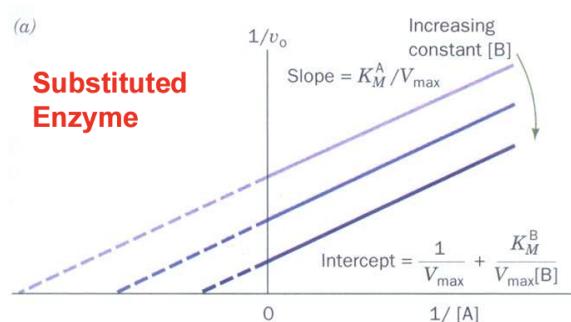


Kinetics of Two substrate enzymes

- Kinetics allows us to distinguish these two mechanisms and to determine V_{\max} and $K(MA)$ and $K(MB)$.
 - Perform a series of experiments by varying $[A]$ in the usual way, over a range of constant $[B]$ values



- Converging lines indicate the ternary complex mechanism



- Parallel lines indicate the substituted Enzyme mechanism

Enzyme inhibition

- Enzyme inhibition comes in several flavors. They have medical or industrial significance (e.g. drug design)

1. Irreversible

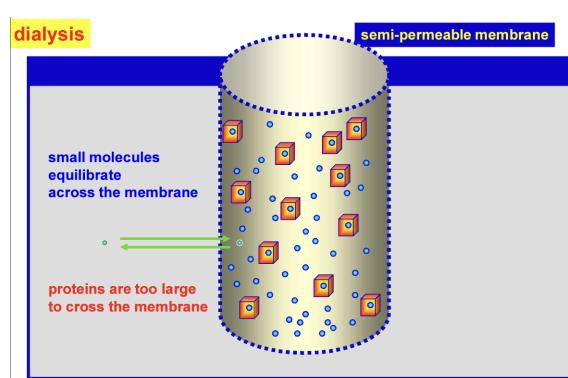
- Inhibitor cannot be removed by dialysis or gel filtration.
- Usually forms a covalent bond with an amino acid side chain at the active site

2. Reversible

- Inhibitor binds reversibly to enzyme, i.e., an equilibrium is set up.
- Can be removed by dialysis or gel filtration. This can be either
 - a. Competitive
 - b. Uncompetitive
 - c. Noncompetitive

How to determine if an inhibitor is reversible or irreversible

Dialysis

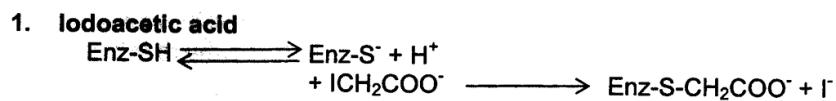


- Enzyme-inhibitor mixture is placed into semi-permeable bag
- Small molecules are able to equilibrate across the membrane while proteins (enzymes) are too large to cross the membrane

- Reversible inhibitor: After dialysis, inhibitors are removed and the enzymatic activity restored
- Irreversible inhibitor: Inhibitors bound to enzymes are not removed and the activity is not restored

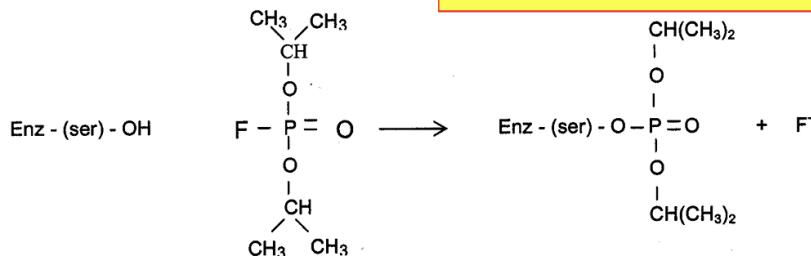
Irreversible enzyme inhibition

- Iodoacetic acid
- Diisopropylphosphofluoridate
 - Reacts with activated serine-OH at active site of many proteases and esterases
 - Nerve gas inhibits acetylcholinesterase ; insecticide

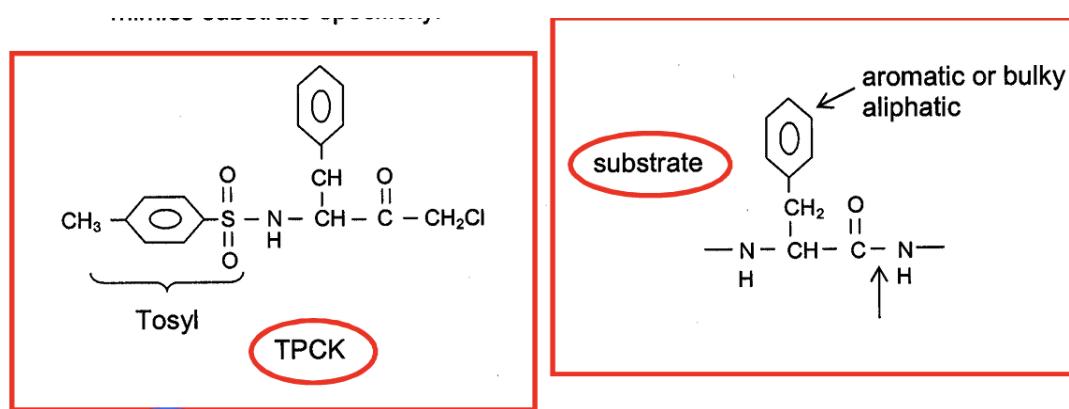


2. Diisopropylphosphofluoridate
reacts with "activated" serine-OH at active site of many proteases and esterases.

Nerve gas - inhibits acetylcholinesterase
Insecticides



- Tosyl-L-phenylalanine chloromethyl ketone (TPCK)
 - React with a histidine at the active site of chymotrypsin as the phenylalanine mimics substrate specificity
 - Tosyl-L-lysine chloromethyl ketone (TLCK) is specific for trypsin



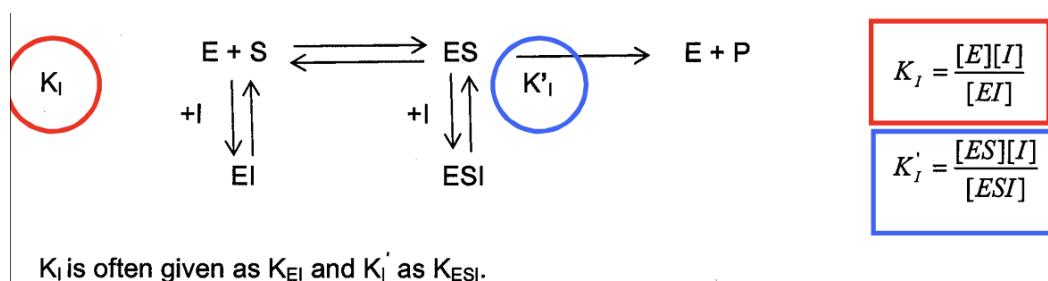
- Suicide inhibitors
 - A suicide inhibitor binds to the enzyme as the normal substrate does and the reaction begins
 - A reactive intermediate is generated which then reacts with a group at the active site inhibiting the enzyme
 - Such inhibitors are highly specific and thus make ideal drugs
 - Fluorouracil is used in cancer chemotherapy. It is a suicide inhibitor of thymidylate synthase.
 - Fluorouracil is converted to fluorodeoxyuridylate. The enzyme normally converts dUMP to dTMP. As part of its mechanism, a

covalent intermediate between the dUMP, the cofactor methylene tetrahydrofolate, and a thiol group at the active site is formed. When FdUMP undergoes this reaction, the intermediate is stable and the enzyme is inhibited.

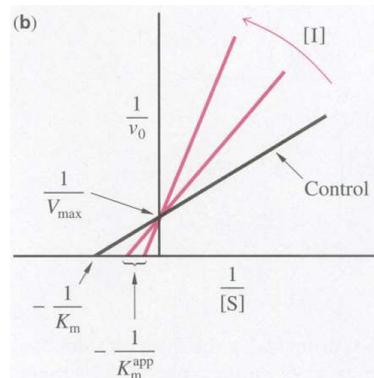
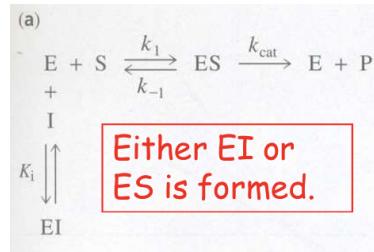
- Monoamine oxidase (MAO) is important in neurotransmitter metabolism. The drug Deprenyl is used in treatment of PD and depression.
 - Monoamine oxidase has a tightly bound FAD cofactor. The drug binds to the enzyme and is oxidised by the flavin. The oxidised drug immediately reacts with the flavin thus inhibiting the enzyme.

Reversible inhibition

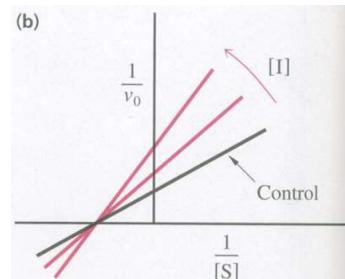
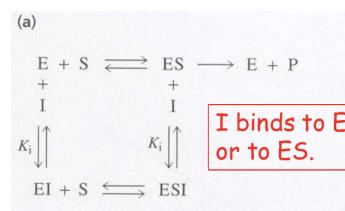
- Depending on the type of inhibition, the inhibitor may bind to either the active site or to the rest of the free enzyme, the ES complex or both



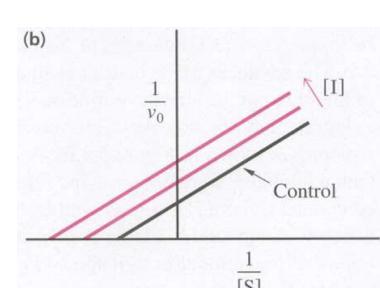
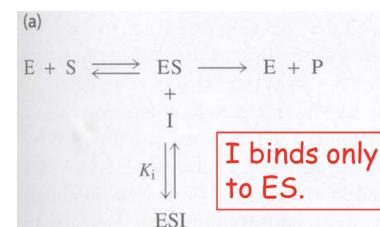
Competitive inhibition



Noncompetitive inhibition



Uncompetitive inhibition



- Km increases because more [S] is needed to achieve the previously observed Vmax.
 - Adding I shift equilibrium from ES to E+S and thus increase Km
- Vmax is unchanged
 - Increase [S] shift the equilibrium to ES
- Benzamidine binding to trypsin - this mimics arginine.
- The most common type of inhibition.
- Km unchanged because I does not interfere with S binding to E.
 - Vmax decreases because active E molecules are removed
 - Increase [S] shift both equilibrium to ES and ESI to the same extent
- Km decreases because the equilibria shifts towards both ES and ESI complex formation.
 - More ES forms ESI as I increase
- Vmax decreases because active ES molecule are removed by conversion to ESI.
 - Increase [S] increases both E+P and ESI
- Usually with multisubstrate reactions.

Regulatory control

Regulation of enzymes

A need to make enzymes catalytically active at the right time in the right place

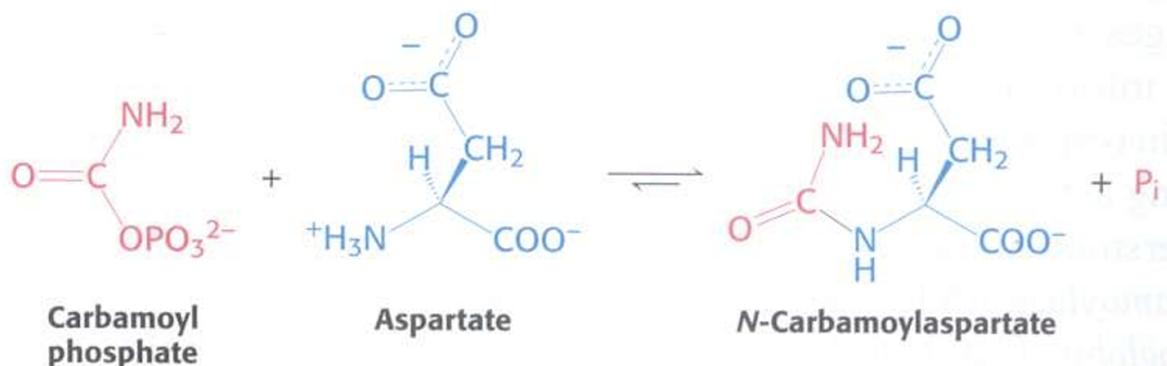
1. Allostericism
 - a. Co-operativity when bound to substrate
 - b. Inhibition by the end product of the pathway
2. Control of enzyme availability - synthesis and degradation
3. Use of isozymes for fine tuning
4. Reversible covalent modification (e.g. by phosphorylation)
5. Activation by proteolytic cleavage - use of a cascade

Allosterism

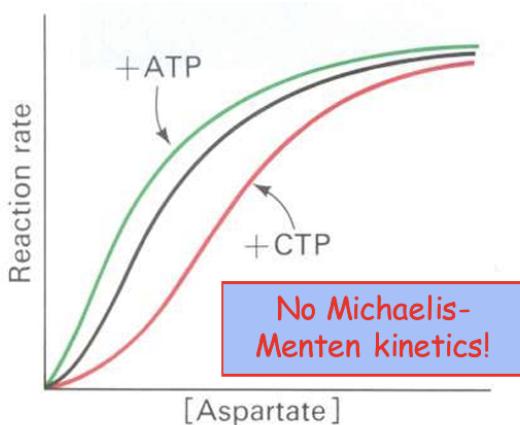
Allosterically-regulated enzymes do not follow Michaelis-Menten kinetics

- Imagine the allosteric enzyme as a mixture of two Michaelis-Menten enzymes
 - One has a high value of K_m that corresponds to the T state
 - The other has a low value of K_m that corresponds to the R state (enzymatically more active)
- As $[S]$ increases, the equilibrium shifts from the T state to R state, giving a steep rise in activity.

Allosterism in ATCase (E. coli)



- Pyrimidine biosynthesis starts with the reaction between carbamoyl phosphate and aspartate.
 - It ends in cytidine triphosphate (CTP)
- This is catalysed by the enzyme aspartate transcarbamoylase (ATCase).
 - ATCase has 6 regulatory and 6 catalytic subunits



- A&G purine, C&T pyrimidine

- The reaction is co-operative and yields a sigmoidal dependence of the reaction velocity on [S].
- ATCase is feedback inhibited by cytidine triphosphate (CTP)
 - I.e., CTP is an inhibitor
- ATCase is an activator of ATCase, i.e., shift the curve to the left
 - When running out of pyrimidine (CTP), purine (ATP) is in excess, and activates pyrimidine synthesis
 - High [ATP] also signals there is sufficient energy for synthesis

Subunits of ATCase

- Catalytic subunit (c3) itself is a trimer of three chains, each of molecular weight 34000
 - 2 catalytic subunits, 3 regulatory subunit
- Regulatory subunit (r2) consists of two chains, 17000 molecular weight each.
 - They reduce the activity of c3.

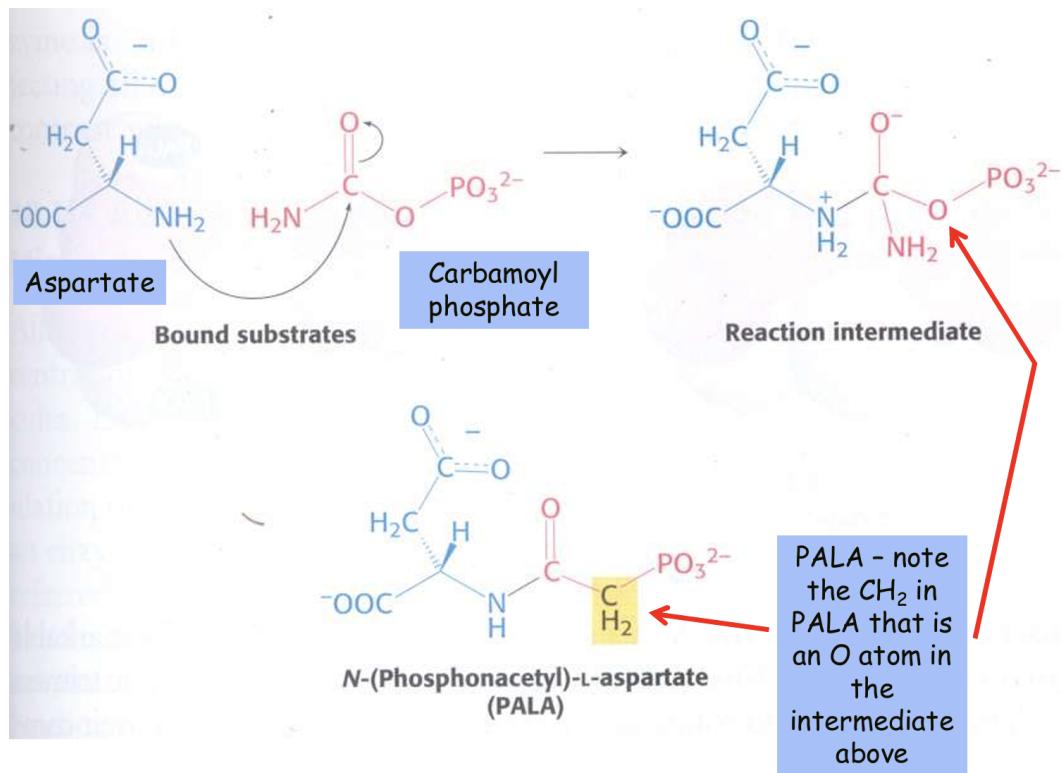
Cooperativity of ATCase

- Mercurials abolish cooperativity by dissociating the catalytic subunits from the regulatory subunits
 - The catalytic subunits are then in the R state
 - *Because mercury binds strongly to the cysteine residues, displacing the zinc and preventing interaction with the c chain*
- In the absence of substrate, ATCase is in the T state.
 - The regulatory subunits hold the two catalytic trimers too close to each other to work properly.

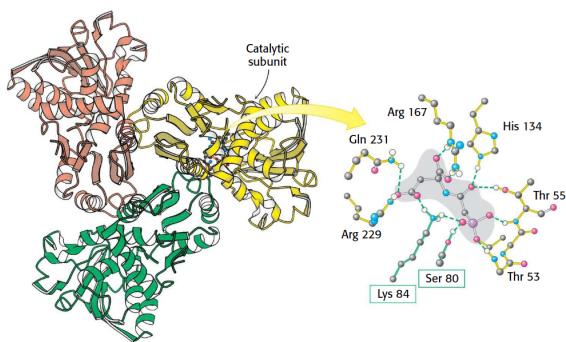
- The binding of substrate shifts the enzyme to the R state.
 - The enzyme expands, the subunits do not sterically clash, and ATCase can not work effectively
- This can be modelled as “concerted or MWC model” - no intermediate states are allowed (in order to retain symmetry) - or as a “sequential model” where a change in one subunit does not necessarily affect the whole enzyme molecule.
 - Noted that ATCase is hexameric rather than tetrameric as Hb.

Inhibitor of ATCase

- The enzyme has an ordered mechanism where carbamoyl phosphate (CAP) binds first.
- N-phosphonacetyl aspartate (PALA) is a **transition state analogue** for the postulated tetrahedral intermediate.
- It is a competitive inhibitor for CAP and a non-competitive inhibitor for aspartate.
- The binding of one PALA to ATCase is sufficient to convert all six catalytic subunits to the R state.
 - Used for X-ray crystallographic studies of ATCase in the R state
 - Thus, at low concentrations, PALA increases the reaction velocity in the presence of substrates until an average of three molecules of PALA are bound per molecule of enzyme. The catalytic subunits unbound by PALA may catalyse the substrate with higher affinity as they are in R state.

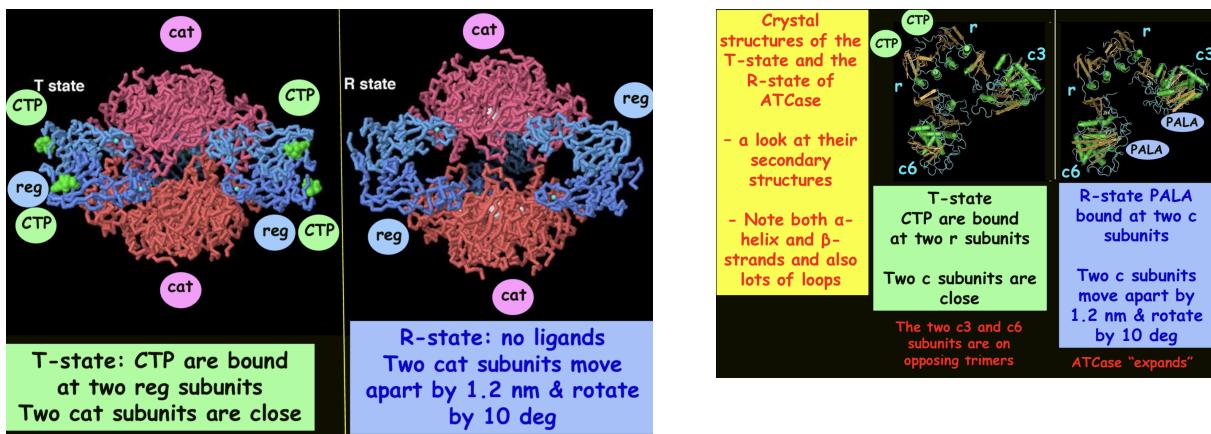


Binding of PALA to the active site



- The ribbon diagram shows one catalytic trimer. Note that two interactions come from the adjacent catalytic subunit within the same trimer.
- CTP-induced changes via the regulatory subunit remove this interaction.

Crystal structures of the compact T-state and the expanded R-state of ATCase



The two catalytic trimers move 12 Å farther apart and rotate approximately 10 degrees about their common threefold axis of symmetry. Moreover, the regulatory dimers rotate approximately 15 degrees to accommodate this motion.

Metabolic significance of the mechanism

- Allosteric cooperativity ensures that CTP synthesis can be amplified quickly when the need for this arises.
 - The K_m drops when ATCase converts into the R state after the binding of substrate
- CTP inhibition of the beginning of the pathway that makes CTP ensures that CTP is not excessively synthesised.
 - Alternatively, if CTP pool is depleted, the ATCase is able to rectify this
- ATP activation of the beginning of the pathway enables the rates of synthesis of purine (A) and pyrimidine (C) to be coordinated for nucleic acid biosynthesis.
 - High ATP levels also imply sufficient energy for DNA replication.

Enzyme availability

The amount of a given enzyme in a cell depends on both its rate of synthesis and its rate of degradation

Both are controlled by the cell. But this is slow - hours or days to take effect.

Examples of synthesis

- E. coli grown without lactose lacks the enzymes needed to metabolise this. On exposure to lactose, E. coli synthesises the relevant enzymes within minutes
- Human cells contain identical genetic information, but different sets of enzymes prevail in the cells of different tissues
- Human: high blood glucose causes increased insulin synthesis, in turn causing increased synthesis of enzymes involved in glucose metabolism

Degradation

- Proteins have lifetimes from minutes to weeks - so excessive enzymes and regulatory proteins can be removed
- The most rapidly degraded enzymes all occupy important metabolic control points
- Attachment of ubiquitin (72 amino acids) signals that the protein is to be destroyed

Use of Isozymes

- Isozymes are enzymes that catalyse that same reaction but differ in their physical properties because of amino acid variations in their sequences
- Different organs often express different proportions of different isozymes, thus measurement will identify the organ that has suffered tissue damage

Creatine kinase (CK)

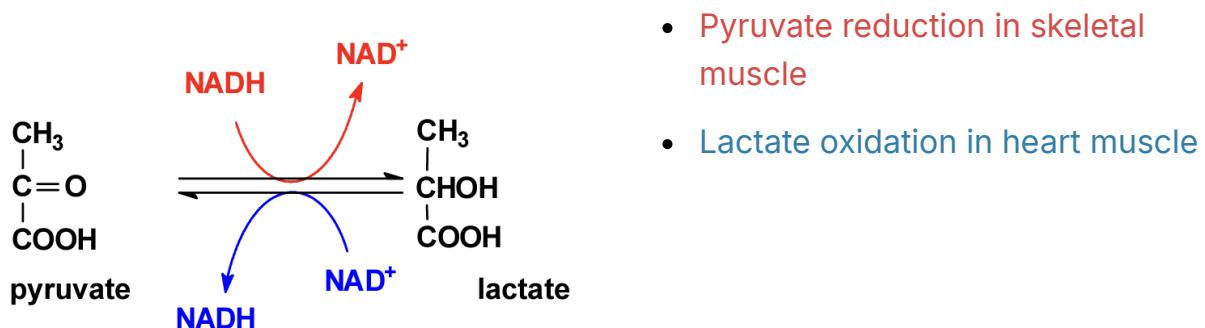
- Exists as a dimer of either B or M subunits
 - BB, MB, and MM combinations
 - They have different electrophoretic mobilities
- Myocardial muscle is the only tissue with >5% MB
 - The appearance of MB 4-8 hours after chest pains start is diagnostic of an acute myocardial infarction
 - Thus confirm that the pain is due to infarction rather than digestive problems

Lactate dehydrogenase (LDH)

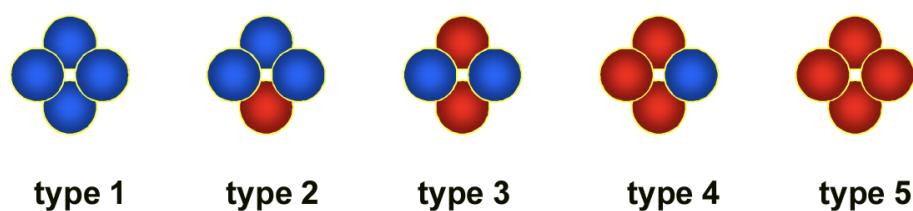
- Functions in anaerobic glucose metabolism and glucose synthesis

- The enzyme is a tetramer. It can comprise either the H4 isozyme (highly expressed in the heart) or the M4 isozyme (in skeletal muscle).
 - Molecular weight 35000 per monomer.
 - Other combinations of subunits M3H, M2H2, MH3 are possible
- H4 has a higher substrate affinity than M4
- High [pyruvate] will allosterically inhibit H4 but not M4
- H4 operates optimally in an aerobic environment while M4 operates optimally in an anaerobic environment

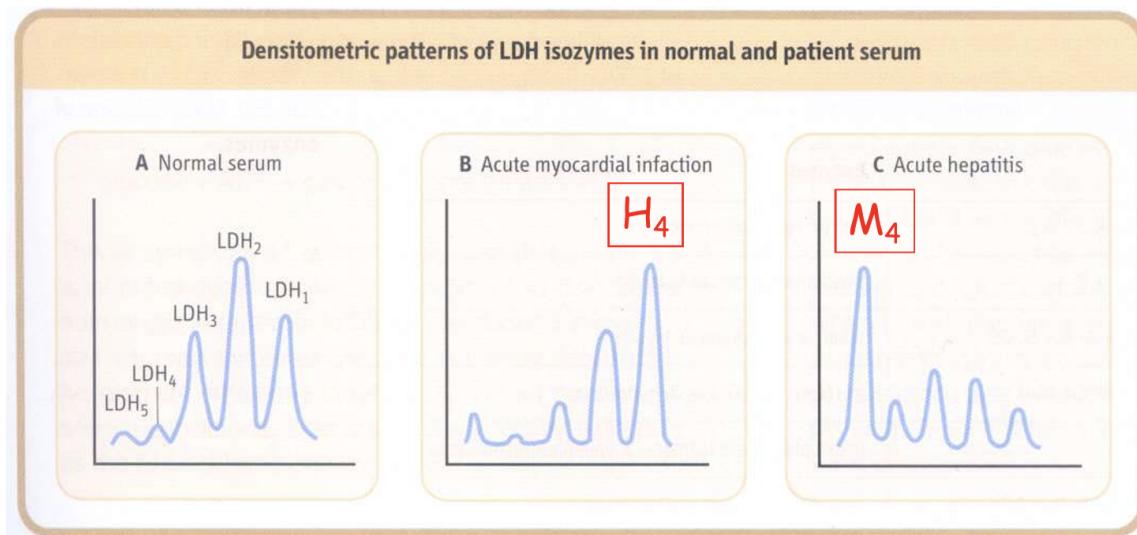
LDH are tissue-specific isoenzymes



- Depending on the chemical reactions need to be promoted, the enzyme may consist of either type of monomer units
 - Blue: H; Red: M



- Analysis using electrophoresis of serum on cellulose acetate



- An increase in H4 in serum indicates a myocardial infarction, this being optimal after 48 hours from onset of chest pains
 - Note that this is the concentration measured from serum, thus indicating H4 released into the blood because of splitting of heart muscle
- Liver disease is indicated by an increase in M4.
 - Liver fall apart and release M4 into blood, may be due to heavy drinking

Reversible chemical modification

Phosphorylation - kinases and phosphatases

- The covalent attachment of another molecule can modify enzyme activity.
 - Most are **reversible**
- Phosphorylation is the most prevalent reversible covalent modification.
 - Acceptors are inside cells, not outside
 - Kinases: adding phosphate; phosphatase: removing phosphate
- Mediated by protein kinases, of which there are >550 in man.
- The terminal phosphate of ATP is either transferred to Ser/Thr or to a Tyr.
 - Phosphate group add onto OH group
- Kinases are controlled by second messengers (commonly cAMP)

- E.g. GPCR modify kinases (intracellular)
- Enzymes are obligatory - neither reaction occurs spontaneously

Why phosphate?

1. Adding a phosphate group adds **two negative charges** to the protein.
 - a. This can markedly affect substrate binding and catalytic activity.
2. The phosphate groups can form **three or more hydrogen bonds** which can interact with specific donors
3. The **free energy change** of phosphorylation is large.
 - a. This changes any equilibrium between functional states by as much as a factor of 10E4.
4. Phosphorylation can happen in seconds or hours. The **kinetics can be adjusted** to suit the physiological process.
5. Phosphorylation evokes highly **amplified effects** - a single kinase can phosphorylate hundreds of targets.

Proteolytic cleavage

Proteolysis

- Some enzymes are synthesised as inactive precursors that are cleaved at a peptide bond before full enzymatic activity is formed
 - Thus, proteolysis is an activation mechanism
- No ATP is needed
- Proteins outside and inside the cells can be activated in this way
- This process is **irreversible**

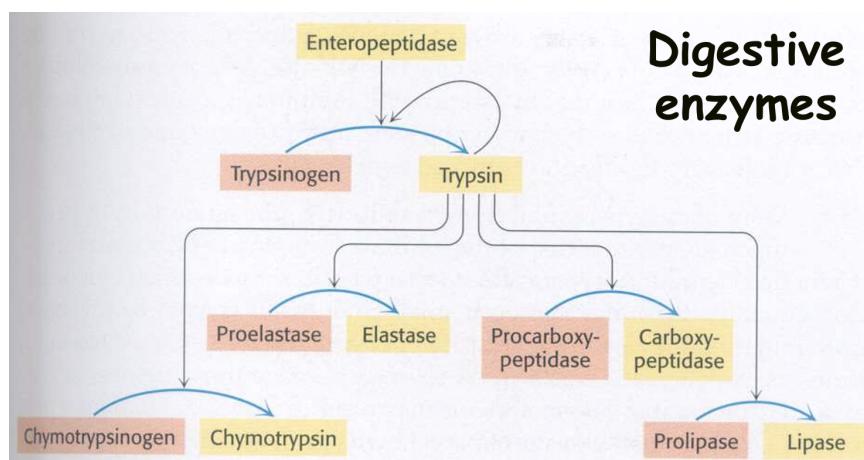
Examples

- Digestive enzyme trypsin and chymotrypsin
 - Precursors: trypsinogen and chymotrypsinogen
- Insulin
 - Proinsulin
- Collagen

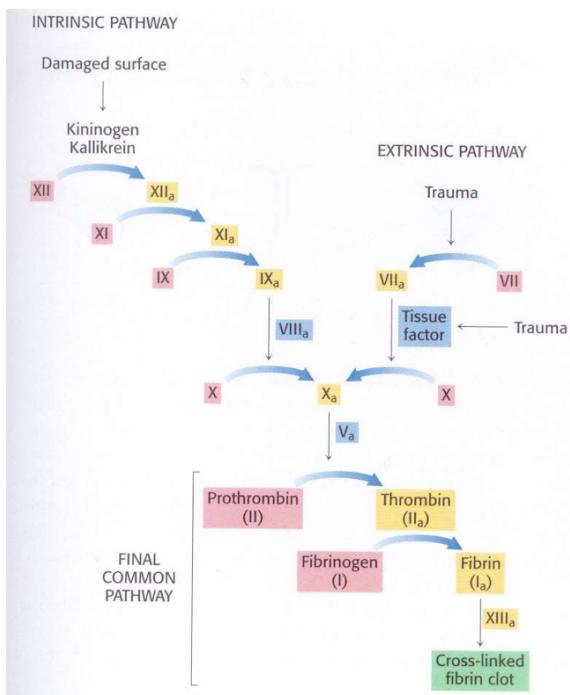
- Procollagen
- Developmental processes and apoptosis are controlled in this way

Activation of chymotrypsinogen

- Cleave between residues 15 and 16
- The Ile16 N-terminus inserts into the protein to form a salt-bridge with Asp194
- Formation of this salt bridge triggers a series of small conformational changes that result in the proper formation of the active site in chymotrypsin
- **Multiple control** of all the digestive enzymes in the gut is achieved using trypsin
 - Enteropeptidase cleaves a small amount of trypsinogen to form trypsin, then trypsin can activate five other proteolytic enzymes (including itself)



Enzymatic cascades



- Cascades generate a rapid response. This is important for blood clotting after trauma (e.g. wounds)
- One protease can activate many protease molecules, which then cleave many more.
 - Six steps will create enough activated proteases to respond effectively to trauma
- A balance between hemorrhages and thrombosis is vital.
 - Protein inhibitors and regulators are essential.
 - Genetic disorders lead to “bleeding disorders”
 - Unwanted clot formation leads to strokes and cardiac infarctions.