

ENZYMOLGY

Mechanism of enzyme action

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Keywords

Enzyme mechanism; Coenzyme/Cofactor functions; Enzyme catalysis; Chymotrypsin; Lysozyme; Carboxypeptidase; Ribonuclease

Role of cofactors in enzyme catalysis

The non-proteinous, low molecular weight organic compounds required for the activity of some enzymes have been referred to as prosthetic groups or coenzymes, depending upon the affinity of their binding to the enzyme protein. Coenzymes always participated in the chemical reaction accepting or donating a group, hydrogen atoms, or electrons from or to the substrate. In the overall metabolic changes, another reaction regenerates the coenzymes by transfer of these groups to or from another metabolite. Thus, the coenzyme functions as links between different metabolic chains besides participating in individual enzyme reactions.

One of the coenzymes of addition-elimination reactions is *thiamine pyrophosphate* (TPP) (Fig. 1), which is derived from thiamine (Vitamin B₁). Thiamin consists of a substituted pyrimidine joined by a methylene bridge to a substituted *thiazole*. An ATP-dependent thiamin diphosphotransferase is responsible for the conversion of thiamin to its active form, thiamin diphosphate (pyrophosphate). There are two types of reactions in which the coenzyme participates: (i) an oxidative decarboxylation of α -keto acids (e.g., α -ketoglutarate, pyruvate, and the α -keto analogs of leucine, isoleucine, and valine); and (ii) transketolase reaction (e.g., pentose phosphate pathway). In each case, the thiamin diphosphate provides a reactive carbon on the thiazole that forms a carbanion (Fig. 2), which is then free to add the carbonyl group of, for example, pyruvate. The additional compound then decarboxylates eliminating CO₂. This reaction occurs in a multienzyme complex known as the pyruvate dehydrogenase (PDH) complex. Similarly, an enzyme complex structurally similar to PDH complex catalyzes the oxidative decarboxylation of α -ketoglutarate to succinyl-CoA and CO₂. Here again, the thiamin diphosphate provides a stable carbanion to react with the α -carbon of α -ketoglutarate. The role of thiamin diphosphate as a coenzyme in the transketolase reaction is similar to that described above for the oxidative decarboxylations.

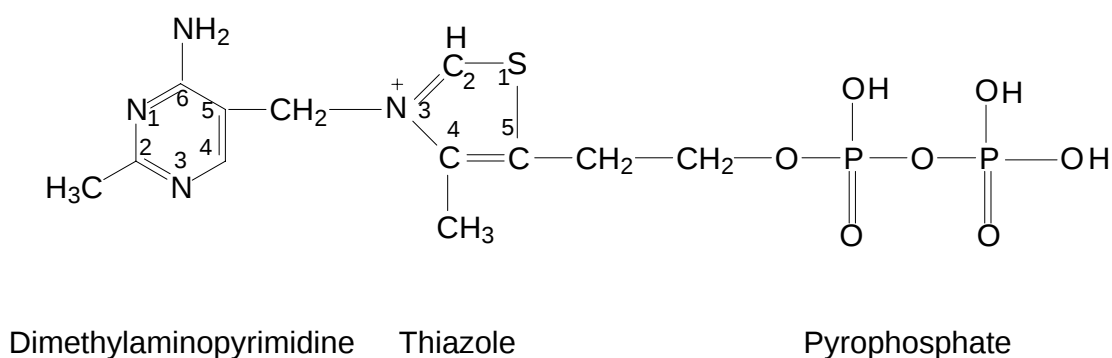


Fig. 1: Structure of thiamin pyrophosphate

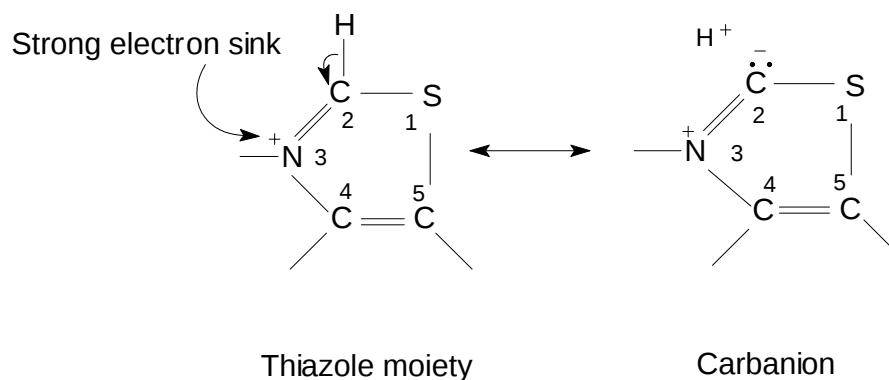


Fig. 2: Mechanism of biochemical action of thiamin pyrophosphate

Two coenzymes containing a nicotinamide moiety (Fig. 3) participate in oxidation-reductions reactions. They are nicotinamide-adenine-dinucleotide (NAD^+) and its phosphate (NADP^+). In both these coenzymes, the nicotinamide moiety participates in the redox reactions, i.e., in the exchange of hydrogen with the substrate. These oxido-reductases are specific for the substrate as well as for the coenzyme (NAD^+ or NADP^+). Generally, NAD^+ -linked dehydrogenases or reductases are often found in pathways (e.g., TCA cycle), whereas NADP^+ -linked dehydrogenases or reductases are often found in pathways concerned with reductive syntheses (e.g., the pentose phosphate pathway). The mechanism of oxido-reduction (Fig. 4) involves a reversible addition of a hydride ion (H^-) to the pyridine ring plus the generation of a free hydrogen ion (H^+) e.g.,

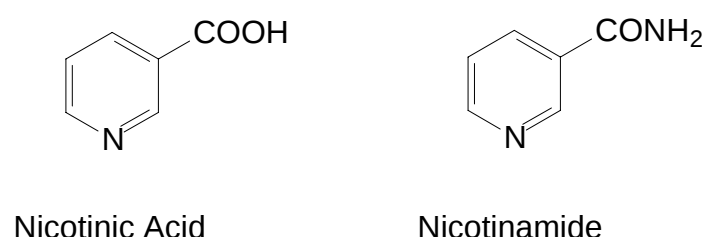


Fig. 3: Structures of nicotinic acid and nicotinamide

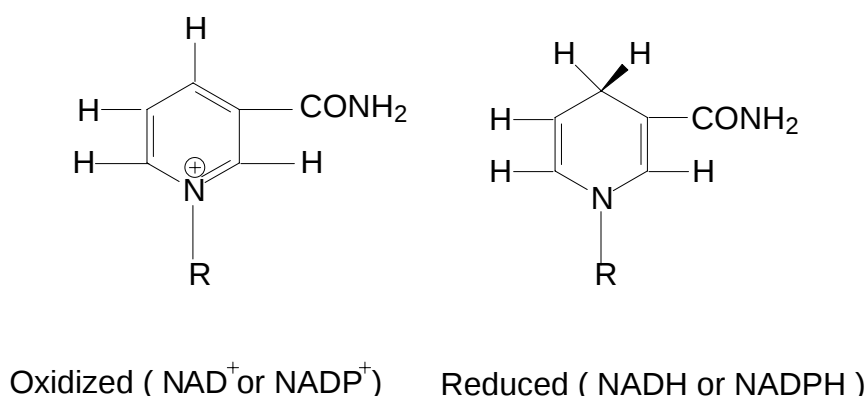


Fig. 4: Oxidized and reduced forms of NAD^+ or NADP^+

The absorption spectra of the reduced and oxidized forms of nicotinamide nucleotides are quite different; the reduced form shows an extra band at 340 nm called 'Racker's Band' (Fig. 5). By measuring the change in the absorption of light at 340 nm during the course of reaction, it is possible to follow the reduction or oxidation of the coenzyme. The NAD^+ - and NADP^+ -dependent dehydrogenases catalyze at least six different types of reactions: simple hydride transfer, deamination of an amino acid to form an α -keto acid, oxidation of β -hydroxy acids followed by decarboxylation of the β -keto acids intermediate, reduction of isolated double bonds, oxidation of carbon-nitrogen bonds and oxidation of aldehydes.

Another pair of coenzymes, which participate in oxidation-reduction reactions, is derived from riboflavin. These are flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Fig. 6). FMN is formed by ATP-dependent phosphorylation of riboflavin, whereas

FAD is synthesized by a further reaction with ATP in which the AMP moiety of ATP is transferred to FMN. Succinate dehydrogenase and fatty acyl coenzyme A dehydrogenase require flavin nucleotides (mostly FAD) for their activity. Reduced flavin coenzymes are reoxidized by cytochromes and cytochrome oxidases. These enzymes are known as *flavoproteins* (Fp). The prosthetic groups are usually tightly bound but not covalently bound to their apoproteins. Many flavoprotein enzymes contain one or more metals e.g., molybdenum and iron as essential cofactors and are known as *metalloflavoproteins*. In their role as coenzymes, flavoproteins undergo reversible reduction to yield the reduced forms FMNH₂ and FADH₂ (Fig. 7).

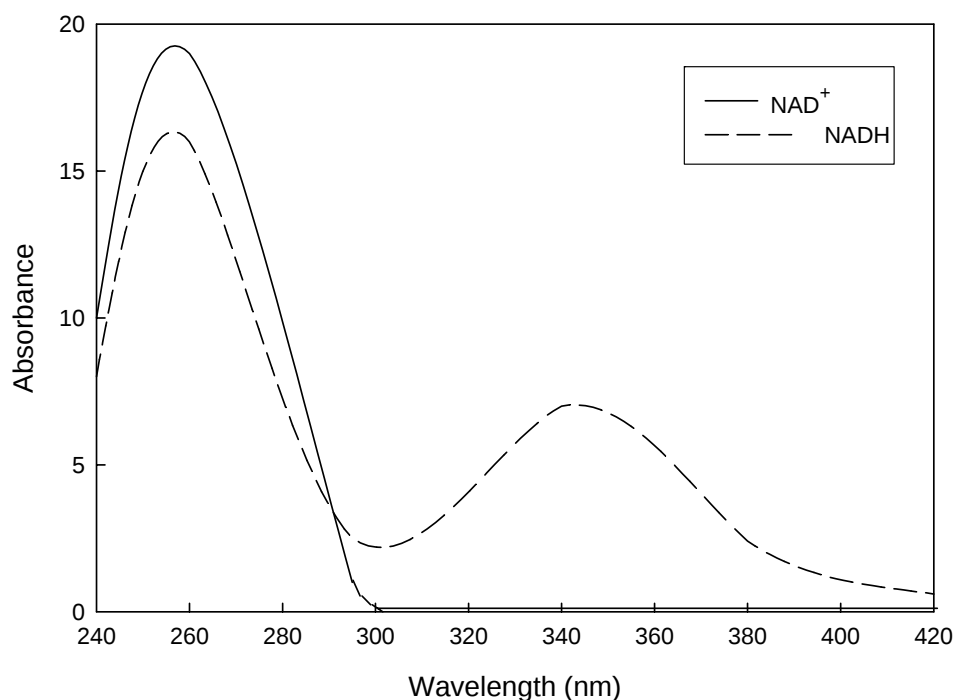


Fig. 5: Absorption spectra of the oxidized and reduced nicotinamide nucleotides

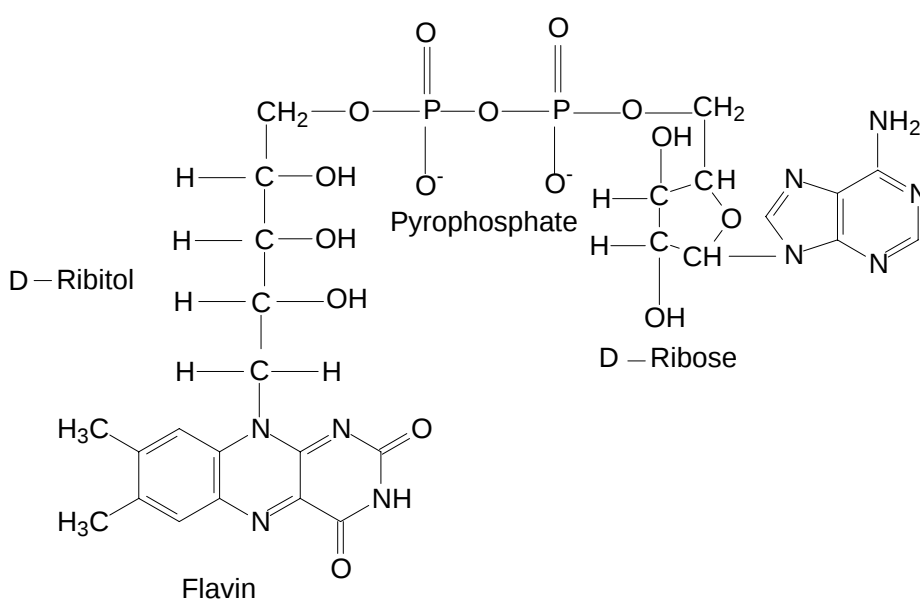


Fig. 6: Structure of FAD

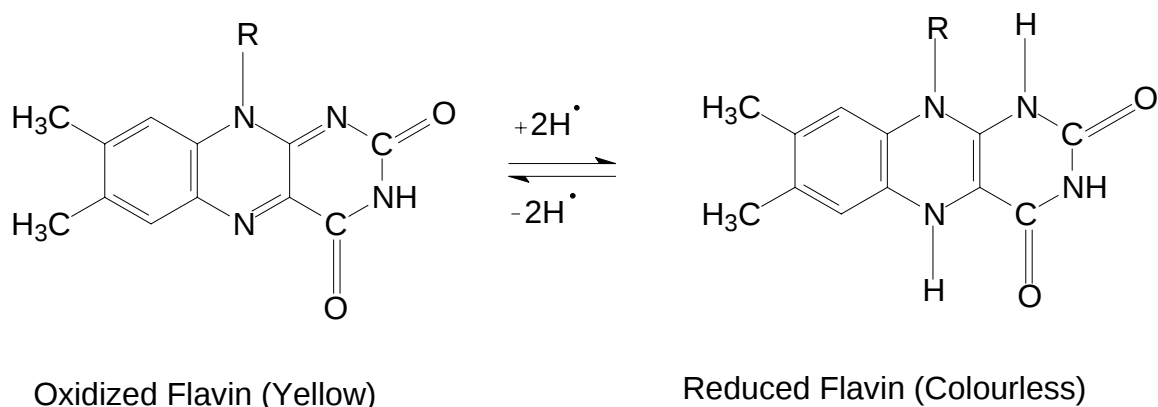


Fig. 7: Oxidation-reduction mechanism of FMN/FAD

Pantothenic acid is formed by combination of pantoic acid and β -alanine (Fig. 8). Addition of cysteine and removal of its carboxyl group results in the net addition of thioethanolamine, generating 4'-phosphopantotheine, the prosthetic group of both CoA and Acyl Carrier Protein (ACP). Coenzyme A serves as a carrier of acyl groups. It accepts acyl groups from the points of their formation (as in the breakdown of carbohydrates and fatty acids) and feed them to other metabolic chains for their ultimate utilization. In each case the acyl group is attached to the terminal SH group to give rise to an acyl-CoA. Thioesters exhibit considerable carbonyl character in which a fractional positive charge may be represented on the carboxyl carbon; the carboxyl oxygen therefore exhibits a partial negative charge. With the fractional positive charge on the carboxyl carbon, the hydrogen atom on the adjacent α -carbon will tend to dissociate as a proton leaving a fractional negative charge on that α -carbon. These two possibilities are responsible for the electrophilic character of the carboxyl carbon atom in thioesters as well as the nucleophilic character of the α -carbon atom (Fig. 9).

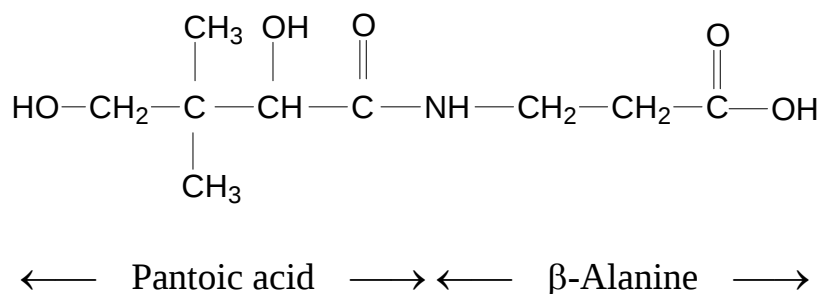


Fig. 8: Structure of pantothenic acid

Vitamin B₆ consists of three closely related pyridine derivatives: pyridoxine, pyridoxal, and pyridoxamine (Fig. 10) and their corresponding phosphates. All three have equal vitamin activity, as they can be interconverted in the body. Most tissues contain the enzyme pyridoxal kinase, which is able to catalyze the phosphorylation by ATP of the unphosphorylated forms of the vitamin to their respective phosphate esters (Fig. 11). While pyridoxal phosphate is the major coenzyme expressing vitamin B₆ activity, pyridoxamine phosphate may also act as an active coenzyme. By entering into a Schiff base (imine bond) combination between its aldehyde group and the amino group of an α -amino acid, pyridoxal phosphate can facilitate changes in the three remaining bonds of the α -amino carbon to allow transamination, decarboxylation, or threonine aldolase activity, respectively (Fig. 12). The coenzyme is an integral part of the mechanism of action of phosphorylase, the enzyme mediating the

breakdown of glycogen. In this action, it also forms an initial Schiff base with an ϵ -amino group of a lysine residue of the enzyme.

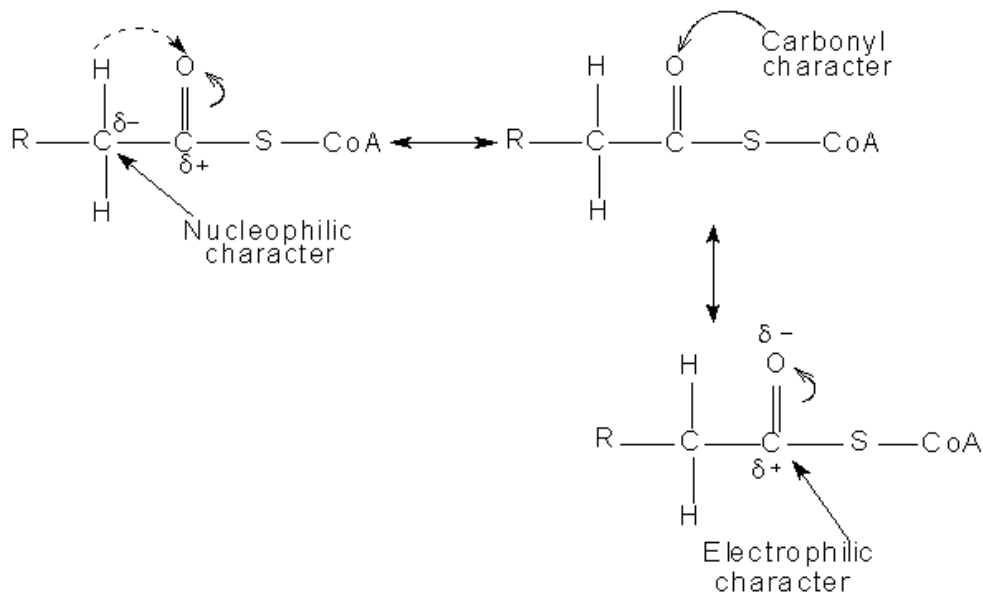


Fig. 9 Electrophilic and nucleophilic character of thioester

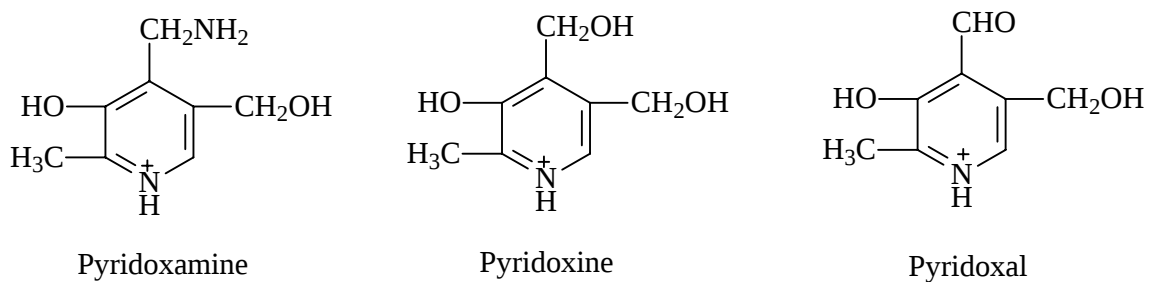


Fig. 10: Naturally occurring forms of Vitamin B₆

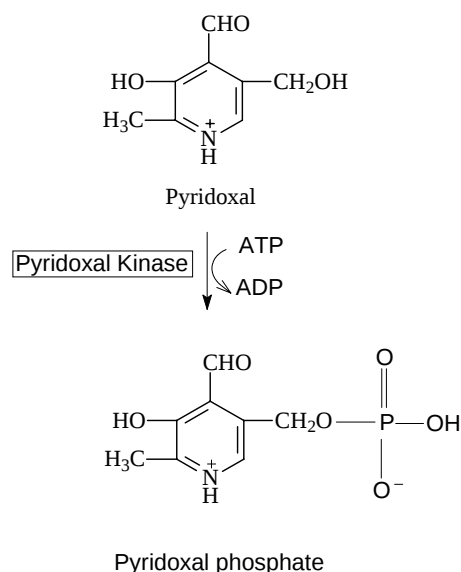


Fig. 11: Formation of pyridoxal phosphate by phosphorylation of pyridoxal carried out by pyridoxal kinase

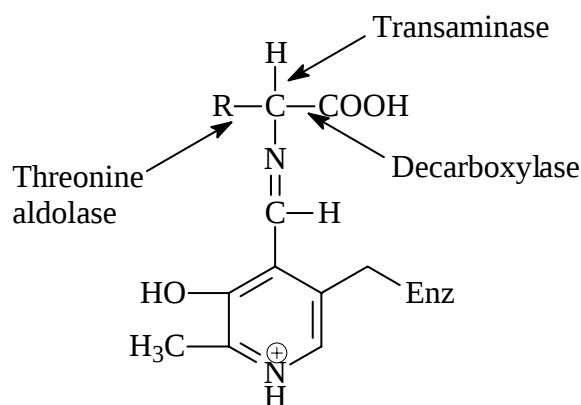


Fig. 12: The covalent bonds of an α -amino acid, which can be made reactive by its binding to various pyridoxal phosphate-specific enzymes

Biotin is an imidazole derivative widely distributed in natural foods (Fig. 13). A large portion of the human requirement for biotin is met by synthesis from intestinal bacteria. Biotin functions as a component of specific multisubunit enzymes that catalyze carboxylase reactions. Each unit is a multienzyme complex containing three components on one polypeptide chain, comprising a biotin carrier protein, biotin carboxylase, and a transcarboxylase. A carboxylate ion is attached to the N^1 of the biotin, generating an activated intermediate, carboxybiotin, attached to the biotin carrier protein. This step requires HCO_3^- , ATP, Mg^{2+} , and acetyl-CoA. The activated carboxyl group is then transferred to the substrate of the reaction, e.g., pyruvate. Consumption of raw eggs cause biotin deficiency as egg white contains a heat-labile protein, avidin, which binds very tightly with biotin, preventing its absorption and inducing deficiency.

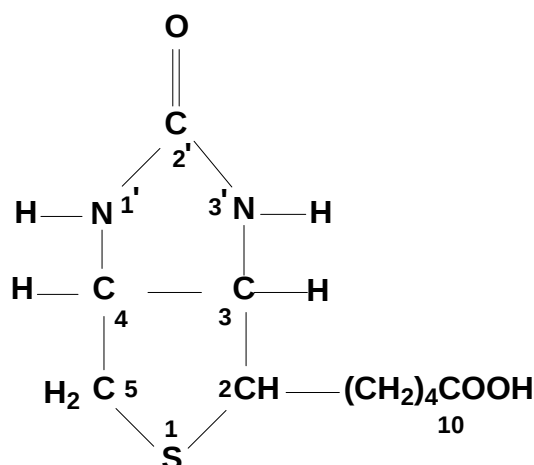


Fig. 13: Structure of biotin

Folacin represents the folic acid and related substances having the biochemical activity of folic acid. Folic acid consists of the base, pteridine attached to one molecule each of *p*-aminobenzoic acid (PABA) and glutamic acid (Fig. 14). Animals are not capable of synthesizing PABA, and therefore, require folate in their diet. Active form of folate is tetrahydrofolate. Folate derivatives in the diet are cleaved/reduced by specific intestinal enzymes (folate reductase), which uses NADPH as donor of reducing equivalents (Fig. 15). The one-carbon units carried by H_4 folate represent a series in various states of oxidation, namely, methyl, methylene, methynyl, formyl, and formimino. All are metabolically

interconvertible. Serine is the major source of one-carbon unit in the form of a methylene group, which it transfers reversibly to H₄ folate to form glycine and N⁵, N¹⁰-methylene-H₄ folate, which plays a central role in one-carbon unit metabolism. It can be reduced to N⁵-methyl-H₄ folate, which has an important role in methylation of homocysteine to methionine involving methylcobalamin as a cofactor.

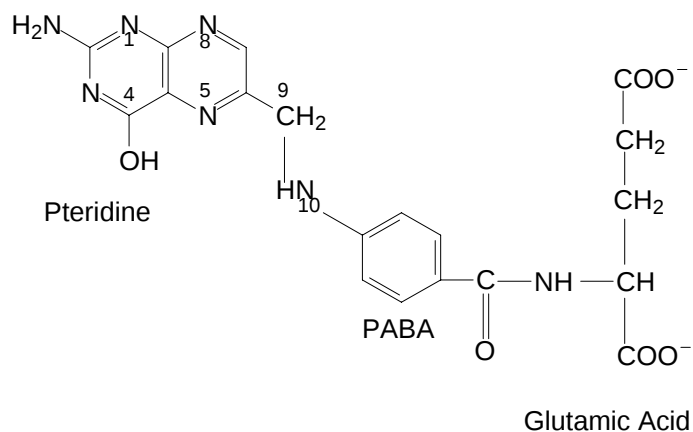


Fig. 14: Structure of folic acid

Lipoic acid is a cofactor of the multienzyme complexes pyruvic dehydrogenase (PDH) and α -ketoglutaric dehydrogenase (α -KDH). There is no evidence of a requirement by man who presumably can synthesize it in the amounts required. Lipoic acid exists in both oxidized and reduced forms due to the ability of the disulfide linkage to undergo reduction (Fig. 16). Lipoate is covalently bound to lysine as ϵ -N-lipoyl-L-lysine (Fig. 17). In the reactions of PDH and α -KDH, the lipoyl containing enzymes catalyze the generation and transfer of acyl groups and, in this process, undergo reduction followed by reoxidation.

Vitamin B₁₂ (cobalamin) has a complex structure (*corrin ring*), similar to porphyrin ring to which is added a cobalt ion at its center. The vitamin is synthesized exclusively by microorganisms. Thus, it is absent from plants unless they are contaminated by microorganisms but is conserved in animals in the liver, where it is found as methylcobalamin, adenosylcobalamin, and hydroxocobalamin. The active B₁₂ coenzymes are *methylcobalamin* and *deoxyadenosylcobalamin*. The reducing system is complex in that it involves a NADH-flavoprotein (Fp)-disulfide (S-S) protein system (Fig. 18). The reductant, NADH, transfers its electrons via a flavoprotein to the specific disulfide (S-S) protein to form a dithiol (SH, SH) protein that converts vitamin B₁₂ (Co²⁺) to vitamin B₁₂ (Co¹⁺). This reduced form then becomes the substrate for the alkylation reaction with ATP. Deoxyadenosylcobalamin is the coenzyme for conversion of methylmalonyl-CoA to succinyl-CoA. This is a key reaction in the pathway of conversion of propionate to a member of the tricarboxylic acid cycle. Methylcobalamin is coenzyme for the combined conversion of homocysteine to methionine and of methyltetrahydrofolate to tetrahydrofolate. In this reaction, the methyl group bound to cobalamin is transferred to homocysteine to form methionine, and the cobalamin then removes the methyl group from N⁵-methyltetrahydrofolate to form tetrahydrofolate. The metabolic benefits of this reaction are that stores of methionine are maintained and tetrahydrofolate is made available to participate in purine, pyrimidine and nucleic acid biosyntheses.

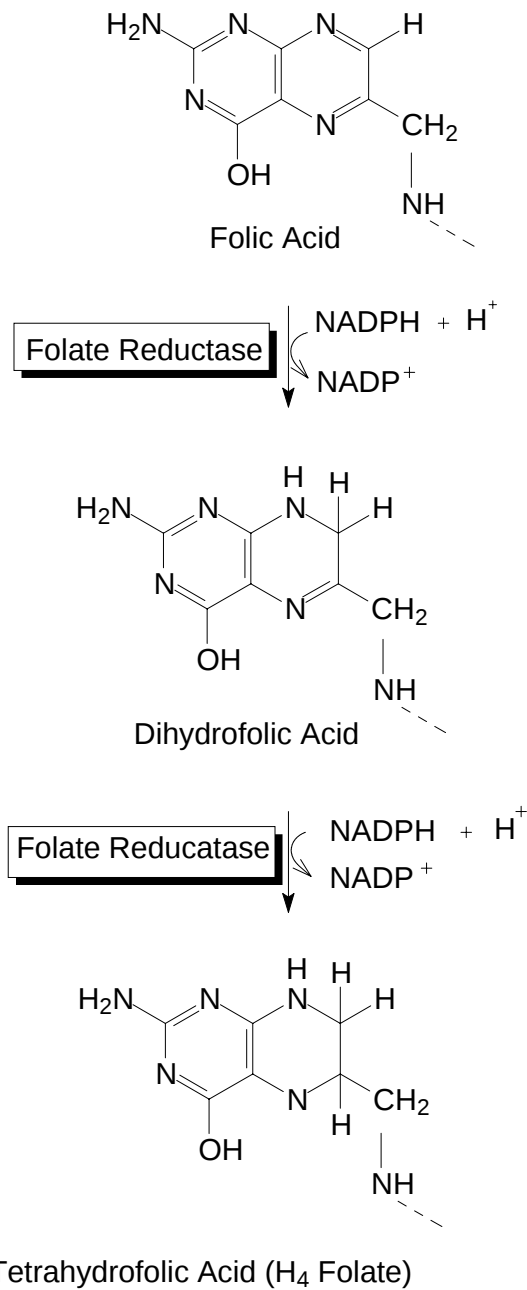


Fig. 15: The reduction of folic acid to dihydrofolate and then to tetrahydrofolate by the action of folate reductase

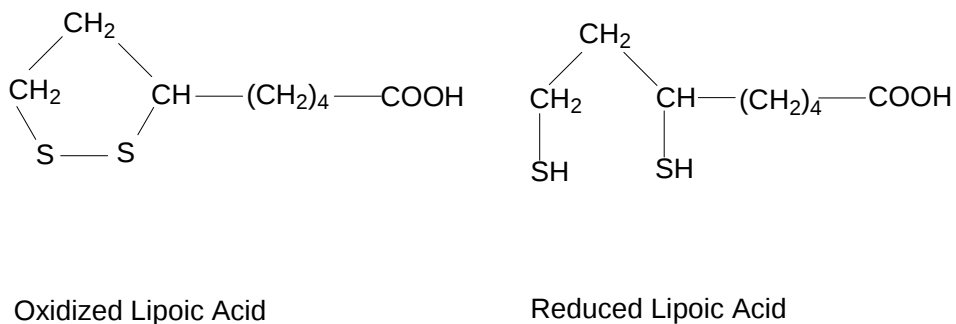


Fig. 16: Structure of oxidized and reduced lipoic acid

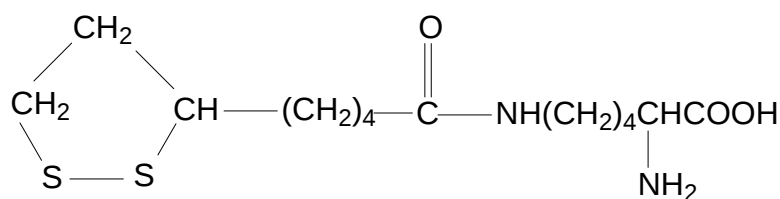


Fig. 17: Structure of biocytin (ϵ -N-biotinyl-L-lysine)

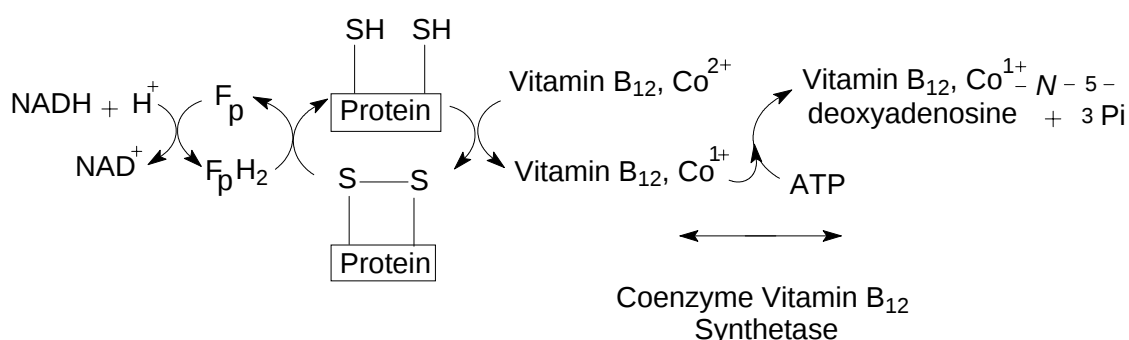


Fig. 18: Reducing system of cobalamin (vitamin B₁₂)

General concepts of mechanism of enzyme action

A common feature of all the enzyme-catalyzed reactions is the formation of an enzyme substrate (ES) complex. In all these reactions very small portion of enzyme comes in close contact with the substrate held by van der Waals contacts (less than 0.4 nm) is called *Active Site* of the enzyme. The amino acid side chains that form part of the active site are generally far removed from each other in the primary structure of the protein. Formation of enzyme substrate complex has greater implications for the catalytic activity as together they are parts of a single molecule. Under such conditions, the enzyme-catalyzed reaction is a case of intra-molecular catalysis, which is known to be more efficient than intermolecular catalysis.

Enzymes as we know cause rate enhancement that are orders of magnitude greater than those of the best chemical catalysts. One may ask, “What makes enzyme such powerful catalysts operating under mild conditions?” This enquiry includes their specificity and catalytic efficiency of binding. Nature has had ample opportunity to fine-tune the performance of most enzymes.

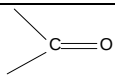
Enzymes normally employ one or more of the following strategies to catalyze specific reactions:

1. Covalent Catalysis
2. Acid – Base Catalysis
3. Metal Ion Catalysis
4. Proximity and Orientation Effects
5. Binding of the Transition-State Analogs

Covalent catalysis

The active site of the enzymes contains a reactive group, usually a powerful nucleophile that becomes temporarily covalently modified during the course of catalysis. The characterization of covalent intermediates in enzyme-mediated reactions is striking proof of ES complexes. These intermediates are not just simple Michaelis complexes, though they are formed by the covalent reaction of a portion of the substrate molecule with a reactive group present in the enzyme. The side chains of amino acids in proteins offer a variety of nucleophilic centers for catalysis, including amines, carboxylates, aryl and alkyl hydroxyls, imidazoles, and thiol groups. These groups readily attack electrophilic centers of substrates, forming covalently bonded E-S intermediates. Typical electrophilic centres in substrates include phosphoryl groups, acyl groups, and glycosyl groups. The covalent intermediates thus formed can be attacked in a subsequent step by a water molecule or a second substrate, giving the desired product. We know of at least hundred enzyme that form covalent intermediates during catalysis. For example, serine proteases like, chymotrypsin, trypsin, subtilisin and elastase have a reactive serine residue at the active site of the protein and form Acyl-Ser covalent intermediates; similarly glyceraldehydes 3-phosphate dehydrogenase is known to have a reactive cysteine, which covalently forms Acyl-Cys intermediate (Table 1).

Table 1: Examples of covalent ES intermediates

S. No.	Enzyme	Reacting Residue	Covalent Intermediate
1.	Chymotrypsin / Trypsin / Subtilisin / Elastase	Serine (-OH)	Acyl Serine
2.	Glyceraldehyde 3-phosphate dehydrogenase	Cysteine (-SH)	Acyl Cysteine
3.	Alkaline phosphatase/ Phosphoglucomutase	Serine (-OH)	Phosphoserine
4.	Sucrose phosphorylase	Carboxyl (-COO ⁻)	Glucosyl enzyme
5.	Acetoacetate decarboxylase	Lysine (-NH ₂)	
6.	Histidine decarboxylase	Pyruvic acid	
7.	Succinyl CoA decarboxylase	Histidine (imidazoles)	Phosphohistidine
8.	Aldolase / PLP-Enzymes	Amino (-NH ₃ ⁺)	Schiff base
9.	ATPase	Aspartate (-COO ⁻)	Phosphoryl enzyme

PLP: Pyridoxal phosphate

Acid – base catalysis

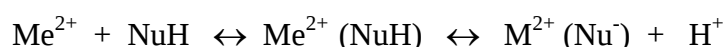
In general acid-base catalysis, a molecule other than water plays the role of proton acceptor or donor. Nearly all enzymes involve some degree of acid or base catalysis. There are two types of acid-base catalysis: (i) specific acid-base catalysis, in which H⁺ or OH⁻ accelerates the reaction; and (ii) general acid-base catalysis, in which an acid or base other than H⁺ or OH⁻ accelerates the reaction. In specific acid or base catalysis, the buffer concentration has no effect. In general acid or base catalysis, however, the buffer may donate or accept a proton in the transition state, k_{obs} (observed reaction rate constant) is dependent on buffer concentration

and thus affect the rate. General acid or general base catalysis may increase reaction rates one to two orders of magnitude.

Metal ion catalysis

Many enzymes require metal ions for maximum activity. If the enzyme binds the metal very tightly usually held by coordinate covalent bonds or requires the metal ion to maintain its stable, native state, it is referred to as *metalloenzymes*. On the other hand if the enzymes bind metal ions more weakly during the catalytic cycle, they are referred to as *metal activated*. One role of metal is to act as electrophilic catalysts, stabilizing the increased electron density or negative charge that can develop during reactions. One classical example of this class is liver alcohol dehydrogenase, which catalyzes the transfer of a hydride ion (H^-) from NADH to acetaldehyde (CH_3CHO), forming ethanol ($\text{C}_2\text{H}_5\text{OH}$). An active site zinc ion stabilizes negative charge development on the oxygen atom of acetaldehyde, leading to an induced partial positive charge on the carbonyl carbon atom. Transfer of the negatively charged hydride ion to this carbon forms ethanol.

Another potential function of metal ions (Me^{2+}) is to provide a powerful nucleophile (Nu) at neutral pH. Coordination to a metal ion can increase the acidity of a nucleophile with an ionizable proton. The reactivity of the coordinated, deprotonated nucleophile is typically intermediate between that of the un-ionized and ionized forms of the nucleophile. The example of this class is carboxypeptidase (containing Zn^{2+}), which facilitates deprotonation of a water molecule (see details in later sections of this chapter).



Proximity and orientation effects

Chemical reactions go faster when the reactants are in proximity, that is, near each other. Enzymes, which have specific binding sites for particular reacting molecules, essentially take the reactants out of dilute solution and hold them close to each other. This proximity of reactants is said to raise the *effective* concentration over that of substrates in solution, and leads to an increased reaction rate. Enzymes not only bring substrates and catalytic groups together, they orient (specific geometric alignment) them in a manner suitable for catalysis as well. Clearly, proximity and orientation play a role in enzyme catalysis, but there is a problem with each for comparisons since we cannot separate true proximity and orientation effects from the effects of entropy loss when molecules are brought together.

Binding of transition-state analogs

Transition-state analogs bind more strongly than a substrate and more strongly than competitive inhibitors that bear no significant similarity to the transition state. Importantly, the transition-state analogs are only approximations of the transition state itself and will never bind as tightly as would be expected for the true transition state. We do not know the exact structures of transition-states, but stable compounds having structures reasonably similar to states have been synthesized. These transition-state analogs bind very tightly to active site (10^{12} - 10^{15} times better than substrates) and are specific in their binding properties. For example, transition state analog, pyrrole-2-carboxylase binds *proline racemase* 160 times

more tightly than L-proline; phosphoglycolohydroxamate binds forty thousand times more tightly to *aldolase* from yeast than its substrate dihydroxyacetone phosphate (DHAP).

Typical enzyme mechanisms

Chymotrypsin

A number of proteolytic enzymes are involved in the breakdown of proteins in the digestive systems of mammals and other organisms. One such enzyme, chymotrypsin, is synthesized in the pancreas as an enzymatically inactive precursor, chymotrypsinogen. In small intestine the activation of chymotrypsinogen is initiated by a proteolytic attack of trypsin, which is followed by the action of some active chymotrypsin already present in the intestine. Two dipeptide fragments are excised from the single polypeptide chain of 245 amino acid chymotrypsinogen. The complete three-dimensional structure of the enzyme was deduced from X-ray crystallographic data by David Blow in 1967.

Chymotrypsin is also a good example of the use of *covalent modification* as a catalytic strategy. In case of chymotrypsin side chain –OH group of a serine residue has been shown to acquire a strong nucleophilic character. The chymotrypsin molecule contains 28 serine residues, out of which only one i.e., Ser 195 reacts and gets phosphorylated by diisopropylfluorophosphate (DIFP, an organofluorophosphate), resulting in a complete loss of enzyme activity (Fig. 19). Free serine does not react with DIFP. This chemical modification reaction suggested that this unusually reactive serine residue plays a central role in the catalytic mechanism of chymotrypsin.

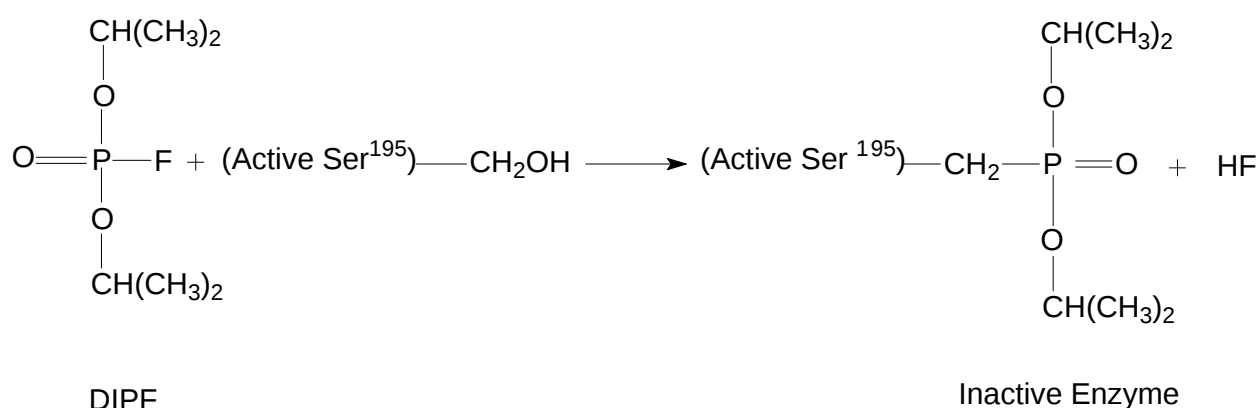


Fig. 19: Mechanism of action of diisopropylfluorophosphate (DIFP) on chymotrypsin

A study of the enzyme's kinetics provides a second clue to chymotrypsin's catalytic mechanisms and the role of Ser 195. Under steady-state conditions, the cleavage of *N*-acetyl-L-phenylalanine *p*-nitrophenyl ester (a chromogenic substrate) obeys Michaelian kinetics with a K_m of 20 μM and a k_{cat} of 77 s^{-1} . There was an initial "burst" phase observed with the help of rapid kinetic technique called "stopped-flow method" followed by a slow steady-state reaction. In the first step, the highly reactive Ser 195 of the enzyme reacts with the substrate to form the acyl-enzyme intermediate, releasing the *p*-nitrophenol. In second step, acyl-enzyme intermediate is hydrolyzed to release the carboxylic acid component of the substrate and regenerate the free enzyme (Fig. 20).

The determination of the three-dimensional structure of the chymotrypsin was a source of further insight into the reactivity of Ser 195 and mechanism of action of the enzyme. Side

chains of two other amino acids, Asp 102 and His 57 (also discovered through affinity labeling studies using tosyl-L-phenylalanine chloromethyl ketone, TPCK) (Fig. 21) were found to be less than 0.30 nm away from the hydroxyl group of serine. Chymotrypsin specifically binds TPCK because of its resemblance to a Phe residue; it reacts with His 57, thereby inactivating the enzyme (Fig. 22). The TPCK reaction is inhibited by β -phenylpropionate (Fig. 23), a competitive inhibitor of chymotrypsin. These observations confirm that His 57 is an essential active site residue of chymotrypsin.

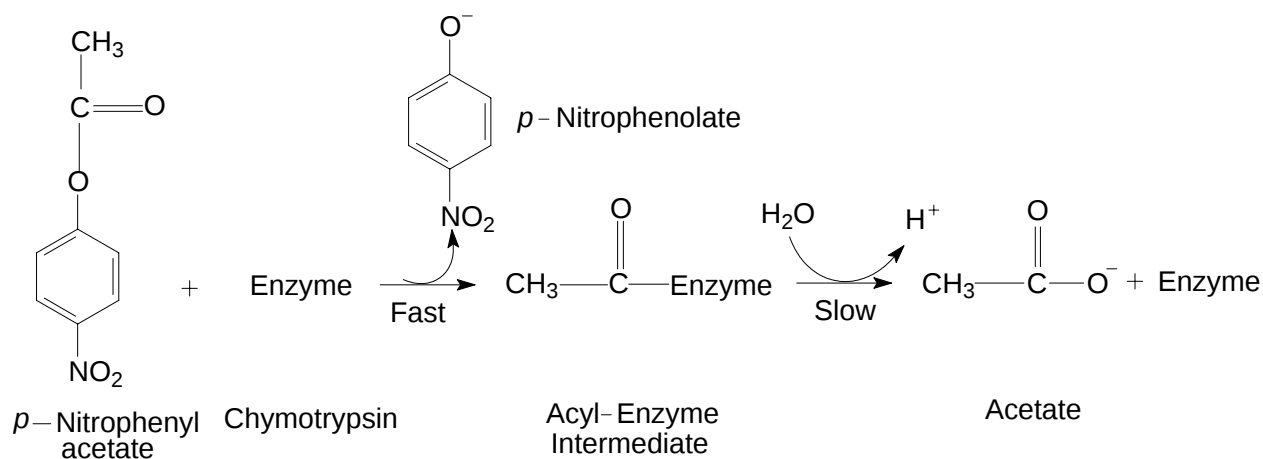
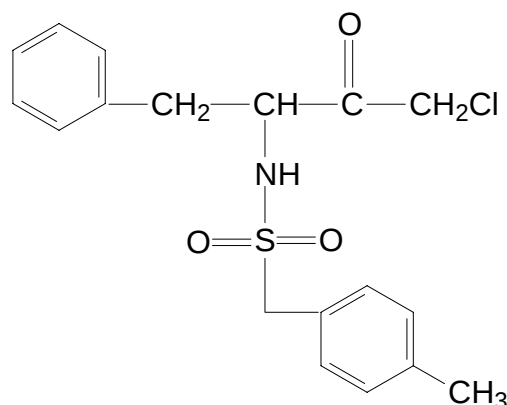


Fig. 20: Biphasic reaction of *p*-nitrophenyl acetate with chymotrypsin



L- 1-tosylamino-2-phenylethylchloromethyl ketone (TPCK)

Fig. 21: Structure of L-tosylamino-2-phenylethylchloromethyl ketone (TPCK)

A *charge relay mechanism* has been proposed to account for the high reactivity of Ser 195. Such active serine residues in a *catalytic triad* of serine, aspartate and histidine (Fig. 24) are part of the active sites of several proteases and esterases. Like the example of serine in the case of chymotrypsin side chain of some other amino acids have been shown to be involved in the formation of covalent intermediates during catalysis. Some enzymes have been classified based on the essential amino acids involved in catalysis. For example, proteases have been classified into two major groups as serine proteases and thiol proteases based on the essential amino acid required for catalysis.

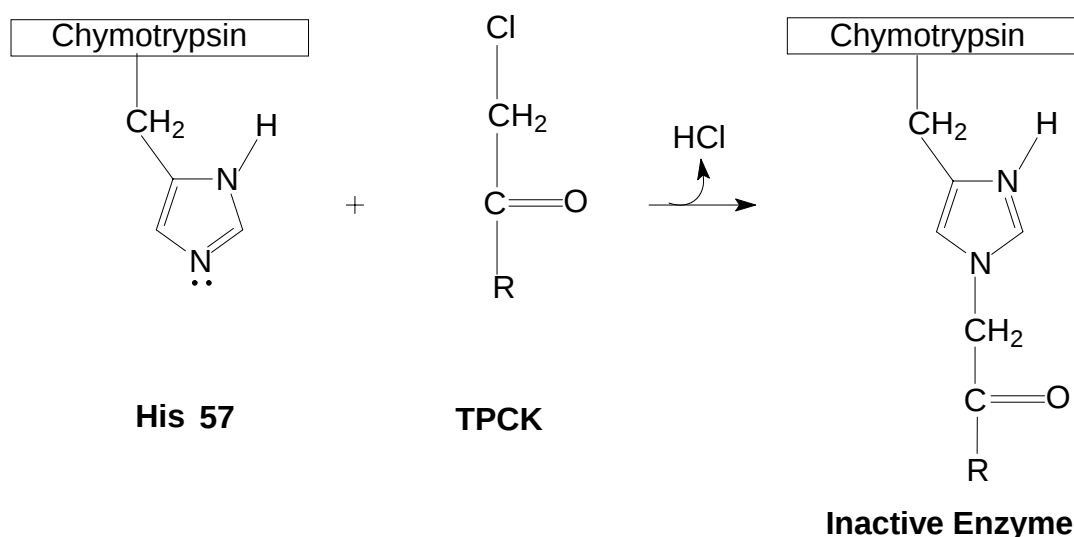


Fig. 22: Reaction mechanism of TPCK with imidazoles residue of chymotrypsin

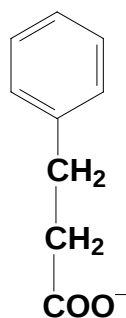


Fig. 23: Structure of β -phenylpropionate

Lysozyme

Lysozyme (a glycosidase) is an enzyme that occurs widely in the cells and degrades the cell walls of some bacteria. It hydrolyzes the $\beta(1\rightarrow4)$ glycosidic linkages from N-acetylmuramic acid (NAM) to N-acetylglucosamine (NAG) (both are derivatives of glucosamine in which the amino group is acetylated) in the alternating NAM-NAG polysaccharides of cell wall peptidoglycans. The X-ray structure of hen egg white lysozyme, elucidated by D. Phillips in 1965, was the second structure of a protein and first of an enzyme. Its most striking feature is a prominent cleft, the substrate-binding site, which traverses one face of the molecule. It is relatively a small protein having a molecular mass of 14.6 kD, which is cross-linked by four disulfide bridges, making it highly stable. From the active site studies, even the location of the active site was not obvious. Therefore active site studies were made in the presence of tri-NAG (NAG)₃, which is a potent inhibitor of lysozyme. Oligomers of NAG consisting of fewer than five residues are hydrolyzed very slowly or not at all (Table 2). However, they do bind to the active site of the enzyme. The finding that tri-NAG filled only half the cleft suggested that three additional sugar residues which would fill the rest of the cleft were required for formation of a reactive ES complex. From the table it is clear that as the number of residues is increased from 4 to 5, the rate of hydrolysis increases strikingly and becomes constant when the sixth residue is added. This finding is consistent with the X-ray data, showing that the six residues fill the active site cleft.

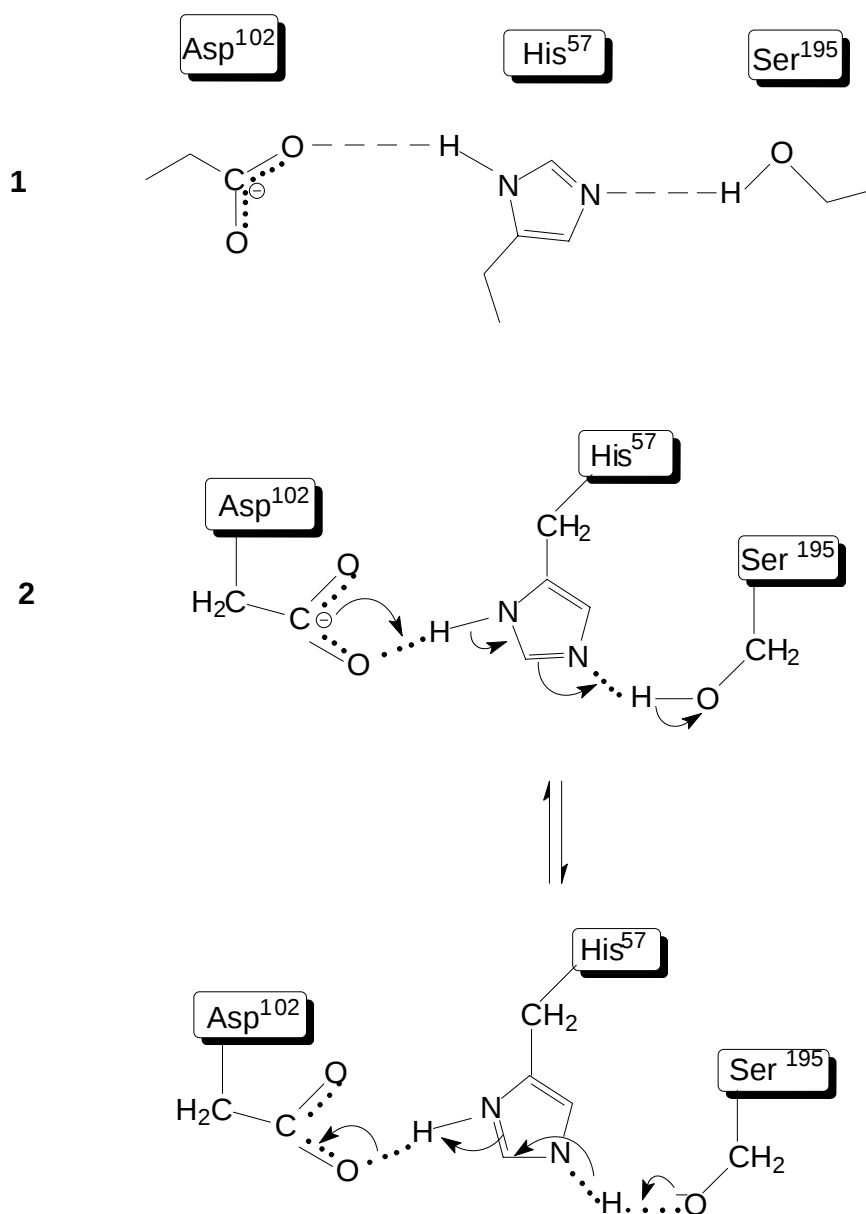


Fig. 24: Catalytic triad and charge relay system of chymotrypsin

Table 2: Effectiveness of oligomers of *N*-acetylglucosamine as substrate

Substrate	Relative Rate of Hydrolysis
(NAG) ₂	0
(NAG) ₃	1
(NAG) ₄	8
(NAG) ₅	4, 000
(NAG) ₆	30, 000
(NAG) ₈	30, 000

Essential steps of the mechanism proposed by Phillips and coworkers involve (Fig. 25): (a) transfer of proton from Glu 35 to the glycosidic oxygen atom between rings D and E; (b) the glycosidic bond is thereby cleaved and a carbonium ion intermediate (transient species) is formed; (c) the dimer of the NAG consisting of residues E and F diffuses away from the

enzyme; (d) the carbonium ion intermediate then reacts with OH^- from the solvent; (e) Glu 35 becomes reprotonated and tetra-NAG, consisting of residues A, B, C, and D diffuses away from the enzyme and the lysozyme is then ready for another round of catalysis.

Many lines of experimental evidences support the proposed mechanism of lysozymes: (a) hexa-NAG is split into tetra-NAG and di-NAG, which confirm the structural data; (b) use of transition state analogs, which bind 3600 times stronger to lysozyme than tetra-NAG itself suggesting that the distortion of the D ring of a normal substrate could accelerate catalysis several thousand fold; (c) chemical modification studies to carry out esterification of Asp 52 leads to complete loss of catalytic activity; (d) lysozyme is active only when Glu 35 is unionized and Asp 52 is ionized, clearly showing dependence of the catalytic rate on pH; (e) X-ray crystallographic structure of NAM-NAG-NAM bound to lysozyme solved at high resolution clearly shows that the bound sugar is forced into a conformation resembling that of the transition state.

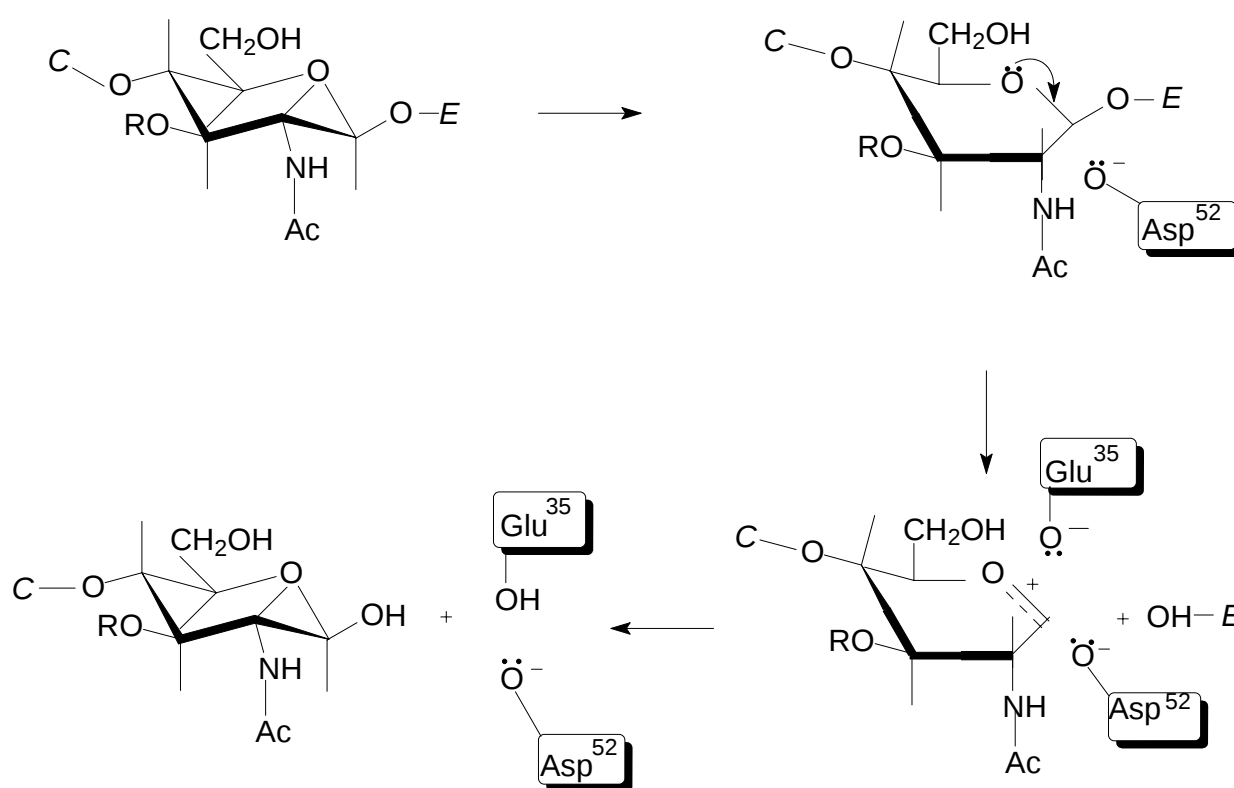


Fig. 25: Postulated mechanism for the hydrolysis of polysaccharides by lysozyme

Ribonuclease

Ribonuclease (RNase) was one of the first enzymes to be crystallized and it was the first enzyme to be completely sequenced. The extreme stability of this enzyme may be disadvantageous to nucleic acid chemists wanting to isolate undegraded RNA, but it is a distinct advantage to enzymologists. Because of this property of RNase, many experiments have been performed on RNase that can be done with few enzymes e.g., easy reversible denaturation.

Ribonuclease catalyzes the hydrolysis of phosphodiester bonds in RNA, which takes place in two steps: a depolymerizing transesterification in which the 2'-hydroxyl group replaces the

leaving 5'-hydroxyl group to form 2',3'-cyclic phosphate; and the subsequent hydrolysis of the cyclic phosphate. Step 1 is more rapid and reversible. Step 2 is slower but not reversible. RNase is specific for phosphodiester bonds in which cyclic 2',3'-phosphates of pyrimidine nucleosides are formed.

Two laboratories (Anfinsen and his colleagues; Hirs, Stein and Moore and their coworkers) were involved in the determination of the sequence of 124 amino acid residues. RNase-A, like lysozyme, has four dithiol bridges making it highly stable. These are formed between residues 26-84, 40-95, 58-110 and 65-72. There are four histidines (His 12, His 48, His 105 and His 119) and two of these are present at the catalytic site. Active-site histidine residues His 12 and His 119 are assigned the pKa values of 5.8 and 6.2, respectively. Crystallographic studies with both ribonuclease-A and ribonuclease-S have been reported. The chemical modification studies suggest that both His 12 and His 119 are catalytically essential and, as verified by X-ray data, are close to each other in the active site.

Of several mechanisms proposed for the action of ribonuclease that suggested by Rabin is most consistent. This mechanism involves the two active-site histidines as acid-base catalysts (Fig. 26). One thought to be His 12, is a general base in the form of the cyclic phosphate, and the other (His 119) is a general acid in this step. The imidazoles of His 12 abstracts a proton from an RNA 2'-OH group, thereby promoting its nucleophilic attack on the adjacent phosphorus atom and formation of pentacovalent phosphorus intermediate. The 2',3'-cyclic intermediate is hydrolyzed through what is essentially the reverse of the first step in which water replaces the leaving group. Thus, His 12 acts as general acid and His 119 as a general base to yield the hydrolyzed RNA and the enzyme in its original state. These roles for His 12 and His 119 are compatible with the three-dimensional models of RNase-S:nucleotide complexes. Calorimetry studies along with other data on RNase, led Biltonen to suggest that the protein provides a positively charged environment at the catalytic site that stabilizes a dianionic penta-co-ordinated phosphate intermediate. Combined with proximity of the substrate to the catalytic residues, the electrostatic effect is sufficient to explain the rate of hydrolysis of 2',3'-cyclic nucleotides catalyzed by RNase.

Carboxypeptidase

Carboxypeptidase, one of the first enzymes to be crystallized, is the most extensively characterized metalloenzyme and the only one for which a clear-cut mechanism of action is available for the zinc. It is an exopeptidase, which catalyzes the hydrolysis of the peptide bond adjacent to a C-terminal free carboxylate ion. Zinc is an essential cofactor for carboxypeptidase A and is important for maintaining the active structure. Carboxypeptidase A shows a preference for C-terminal aromatic or branched aliphatic amino acids. Carboxypeptidase A from beef pancreas is a single polypeptide chain with 307 amino acid residues and one atom of zinc. Inhibition of carboxypeptidase by metal-binding reagents such as cysteine, sulfide and cyanide indicated that the enzyme is a metalloprotein.

X-ray crystallographic data from Lipscomb and coworkers were available before the complete amino acid sequence was determined. The combined data provides a great deal of information about the catalytic site. The molecule is an ellipsoid of about 50 x 42 x 38Å. The active site, comprising of about one-quarter of the molecule, contains the coordinated Zn²⁺, a surface groove for binding of polypeptide substrate. Completion of a three-dimensional model has identified the four ligands bound to the Zn²⁺ as His 69, Glu 72, His 196 and H₂O. These ligands form distorted tetrahedral bonds with the zinc. Vallee proposed the following

mechanism for carboxypeptidase A: (i) a binding area on the protein for the C-terminal side chain; (ii) a positive charge on the protein to bind the carboxylate of the substrate; (iii) coordination of the carbonyl group of the substrate peptide bond to Zn^{2+} ; and (iv) base (nucleophilic catalyst) and acid (proton donor) groups on the enzyme.

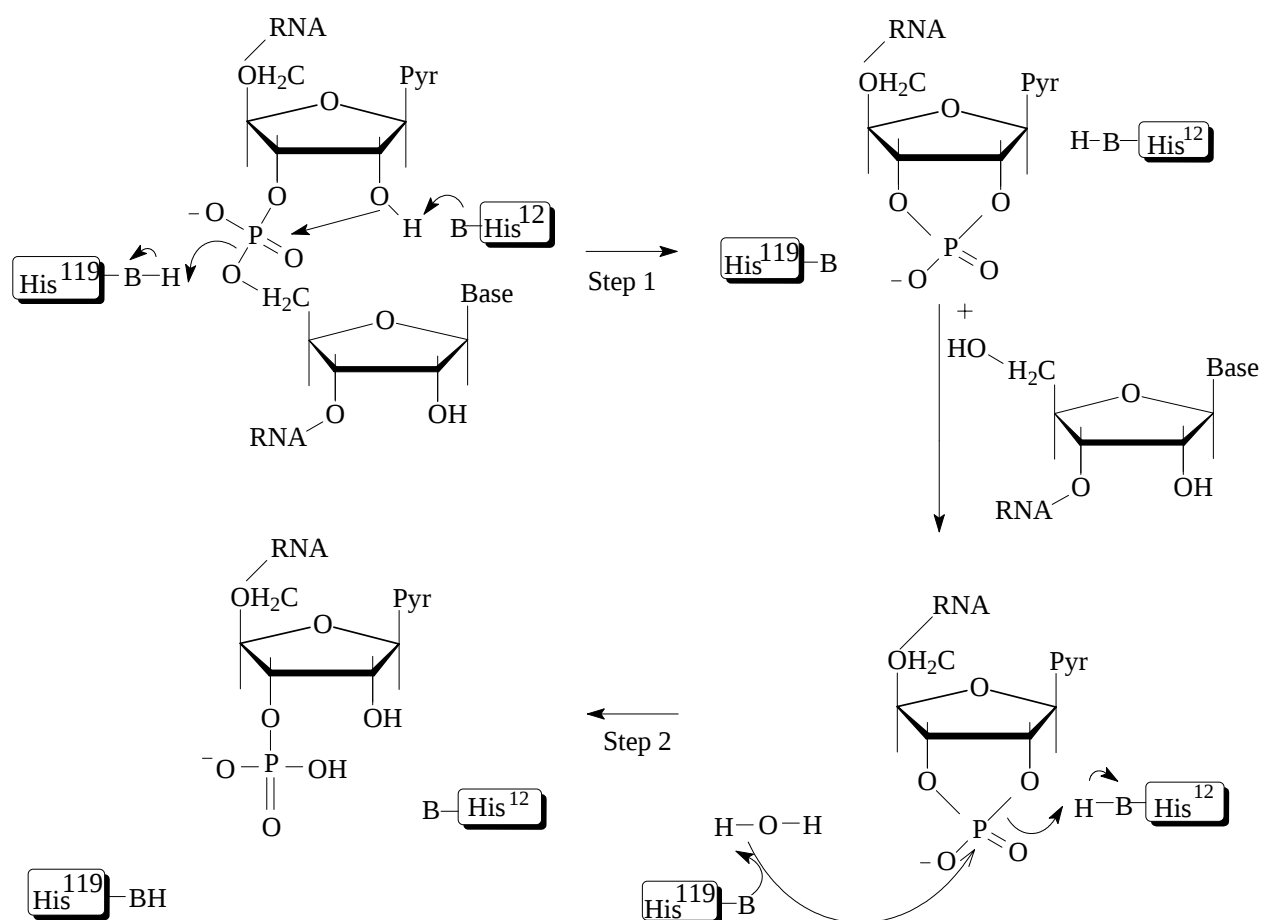


Fig. 26: Outlines of the mechanism postulated for the hydrolysis of a phosphodiester bond in the presence of ribonuclease

Three interactions appear to account for the formation of the active Michaelis complex. First, the C-terminal side chain of a substrate fits into a pocket of relatively low specificity by displacing several molecules of water. Because the pocket is closed, carboxypeptidase has no endopeptidase activity. Second, the terminal carboxylate of the substrate is bound to the positively charged guanidine group of Arg 145. Finally, the carbonyl oxygen of the substrate's terminal peptide bond replaces the water ligand on the zinc. Two additional features appear to be important in catalysis. The phenolic group of Tyr 248 moves close to the peptide bond of the substrate, possibly forming a hydrogen bond with the amide of the peptide bond. This conformational change prevents the entry of water into the side-chain specificity pocket. Models built with larger substrates suggest that the carboxyl group of Glu 270 is the nucleophilic catalyst of the enzyme. Fig. 27 presents these interactions diagrammatically. Examination of the model of carboxypeptidase shows that only Arg 145, the Zinc atom, Glu 270 and Tyr 248 are close enough to the catalytic site to be involved in catalysis. The complexing of the carbonyl oxygen with the zinc probably withdraws electrons from the $-\text{C}=\text{O}$ group. The $-\text{OH}$ of Tyr 248 is suitably located to donate a proton to the amide nitrogen. The carboxyl group of Glu 270 could form a rapidly hydrolyzed anhydride

intermediate as suggested by Valle or it could act as a general base with an intervening molecule of water as the nucleophile (as shown in Fig. 27). In conclusion, in carboxypeptidase the zinc atom is an immobile acidic prosthetic group, and that it most likely behaves in a similar fashion in some other zinc metalloenzymes.

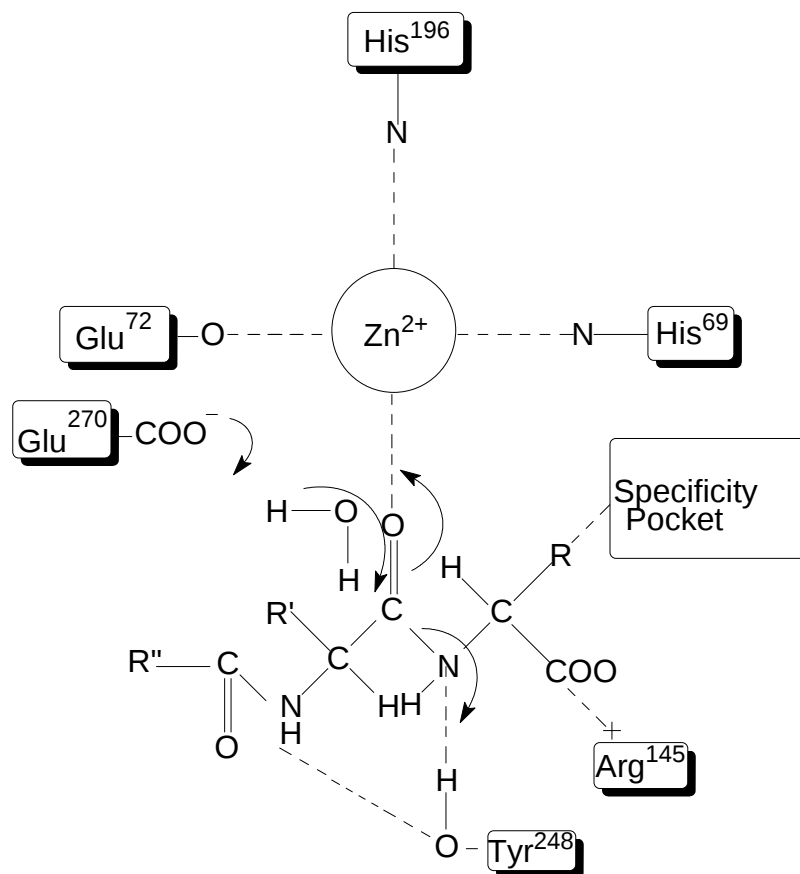


Fig. 27: Diagrammatic representation of the structure of the ES complex formed between carboxypeptidase A and a tripeptide as suggested by the data of Lipscomb and coworkers

Suggested Readings

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