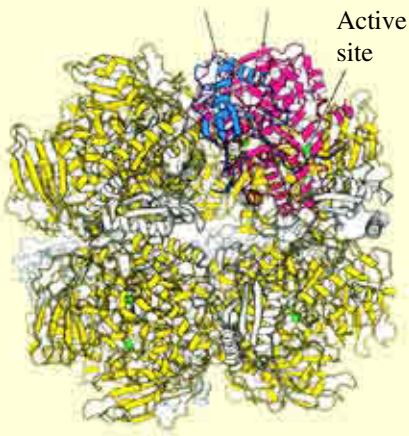
CONTENTS

CHAPTER

17

Enzymes–II

Characteristics and 3 'D' Structure



Ribbon model of the tertiary structure of ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco)

The enzyme comprises eight large subunits (one shown in the red and the others in yellow) and eight small subunits (one shown in blue and the others in white). The active sites lie in the large subunits.

CHEMICAL NATURE

All the enzymes are essentially proteins and possess properties characteristic to these. Dixon and Webb (1964) have stressed the protein nature of an enzyme by defining it as "*a protein with catalytic properties due to its power of specific activation*".

Evidences Proving the Protein Nature of the Enzymes

(*a*) **Elementary composition.** In their elementary composition, the enzymes show the usual proportion of C, H, N and S, as found in the proteins. Some crystalline enzymes, however,also contain minute quantities of P or metal ions such as Cu^{2+}, Mg^{2+}, Zn^{2+} etc. On hydrolysis, the crystalline enzymes yield the amino acids.

(*b*) **Identical action of some enzymes over other enzymes and the proteins.** Enzymes are subjected to the action of those enzymes which are specifically meant for the breakdown of peptide bonds of proteins.

(*c*) **Amphoteric nature.** Like other proteins, the enzymes behave as ampholytes in an electric field. The isoelectric point (*pl*) for various enzymes has also been determined.

(*d*) **Denaturation.** Enzymes, like other proteins, also undergo denaturation. If the crystalline *proteinase chymotrypsin* is subjected to an unfavourable *p*H, some part of protein becomes denatured. This percentage of denatured protein is usually found to be equal to the per cent loss in enzymic activity, thus

proving a sort of correlation between the enzymes and the proteins..

(*e*) **Formation of antibodies.** Many purified enzymes, on injection into animal body, produce the specific antibodies. Since many nonprotein materials have been shown to serve as antigens, this cannot be treated as an evidence in support of the protein nature of enzymes but simply a further support to it.

Chemically, the enzymes may be divided into 2 categories :

1. **Simple-protein enzymes.** These contain simple proteins only *e.g., urease, amylase, papain* etc.
2. **Complex-protein enzymes.** These contain conjugated proteins *i.e.,* they have a protein part called *apoenzyme* (*apo* = away from) and a nonprotein part called *prosthetic group* associated with the protein unit. The two parts constitute what is called a *holoenzyme*, *e.g.*, *catalase, cytochrome c* etc.

The activity of an enzyme depends on the fact that the non-proteinaceous prosthetic group is intimately associated with the proteinaceous apoenzyme. But sometimes the prosthetic group is loosely bound to the protein unit and can be separated by dialysis and yet indispensable for the enzyme activity. In that case, this dialyzable prosthetic group is called as a coenzyme or cofactor. Thus :

$$\text{Conjugated-protein enzyme} \rightleftharpoons \text{Protein part} + \text{Prosthetic group}$$

or

$$\text{Holoenzyme} \rightleftharpoons \text{Apoenzyme} + \text{Coenzyme}$$

Coenzymes are thermostable, dialyzable organic compounds. They may be either attached to the protein molecules or may be present in the cytoplasm. *The coenzyme accounts for about 1% of the entire enzyme molecule.* Sometimes, a distinction is made between coenzymes and cofactors : the former includes the organic prosthetic groups and the latter the metal ions (Fairley and Kilgour, 1966).

CHARACTERISTICS

The enzymes possess many outstanding characteristics. These are enumerated below :

1. Colloidal Nature. Enzyme molecules are of giant size. Their molecular weights range from 12,000 to over 1 million. They are, therefore, very large compared with the substrates or functional group they act upon (Fig. 17–1).

It has been observed that the molecular weights of many enzymes prove to be approximately an *n*-fold multiple (where *n* is an integer) of 17,500 which is found to be an unit in most proteins (Table. 17–1).

On account of their large size, the enzyme molecules possess extremely low rates of diffusion and form colloidal systems in water. Being colloidal in nature, the enzymes are nondialyzable although some contain dialyzable or dissociable component in the form of coenzyme.

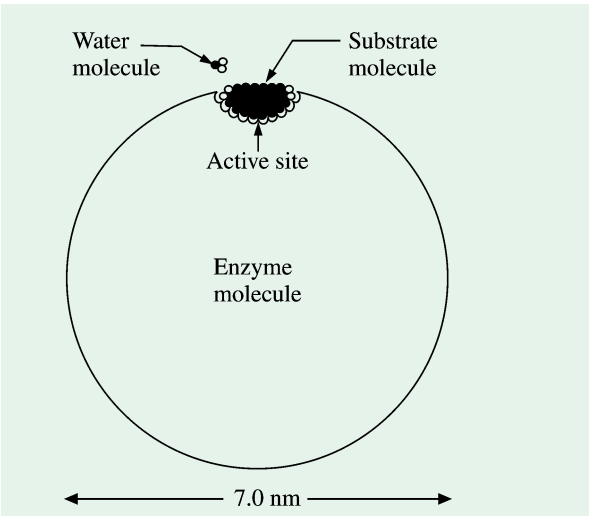


Fig. 17–1. Relative dimensions of a medium-sized enzyme molecule (MW 1,00,000 ; diameter 7 nm) and a typical substrate molecule (MW 250 ; diameter 0.8 nm)
[Note that the active site occupies only a small fraction of the surface area of the enzyme molecule. Also shown for comparison is a water molecule.]

2. Catalytic Nature or Effectiveness. *An universal feature of all enzymatic reactions is the virtual absence of any side products.* Therefore, just as hemoglobin is precisely tailored to transport

oxygen, an enzyme is precisely adapted to catalyze a particular reaction. They act catalytically and accelerate the rate of chemical reactions occurring in plant and animal tissues. They do not normally participate in these reactions or if they do so, at the end of the reaction, they are recovered as such without undergoing any qualitative or quantitative change. This is the reason why they, in very small amounts, are capable of catalyzing the transformation of a large quantity of substrate. Thus, *the catalytic potency of enzymes is exceedingly great.*

Table 17–1. Molecular weight of some enzymes

Enzyme	*Molecular weight*	*n**
Pepsin	35,500	2
Catalase	250,000	14
Urease	480,000	27

**n* = an integer, which is a multiple of 17,500

The catalytic power of an enzyme is measured by the **"turnover number"** (a term devised by Wechselzahl) or **molecular activity** (a term devised by **Norman Arthur Edwards** and **Kenneth Arnold Hassall,** 1980) which is defined as– *the number of substrate molecules converted into product per unit time, when the enzyme is fully saturated with substrate*. For example, a single molecule of *catalase* can convert 50,00,000 H_2O_2 molecules into H_2O and O_2 in a minute (Sumner and Somers, 1947). The value of turnover number varies with different enzymes and depends upon the conditions in which the reaction is taking place. However, *for most enzymes, the turnover numbers fall between 1 to 10^4 per second* (refer Table 17–2). The turnover number of 600,000 sec^{-1} for *carbonic anhydrase* is one of the largest known. Carbonic anhydrase (Fig. 17-2) catalyzes the hydration of carbon dioxide to produce 3,60,00,000 molecules of carbonic acid per minute. This catalyzed reaction is 6 $\times 10^7$ times faster than the uncatalyzed one.

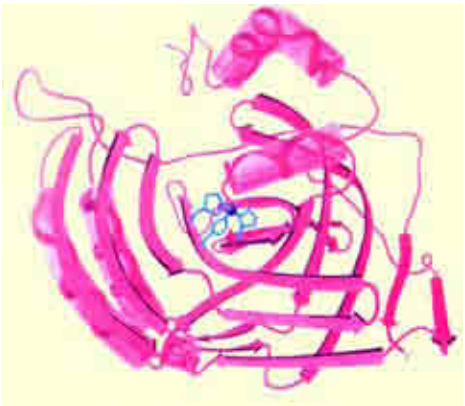


Fig. 17-2. Ribbon model of the tertiary structure of human carbonic anhydrase

The α helices are represented as cylinders and each strand of β sheet is drawn as an arrow pointing towards the polypeptide's C-terminus. The grey ball in the middle represents a Zn^{2+} ion that is coordinated by three His side chains (*blue*). Note that the C-terminus is tucked through the plane of a surrounding loop of polypeptide chain so that carbonic anhydrase is one of the rare native proteins in which a polypeptide chain forms a knot.

(Courtesy: Kannan KK et al, 1971)

$$CO_2 + H_2O \xrightarrow{\text{Carbonic anhydrase}} H_2CO_3$$

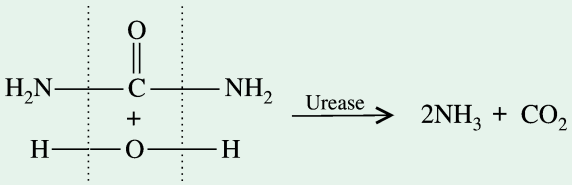
Table 17–2. Maximum turnover numbers of some enzymes

Enzyme	*Turnover number (per second)*
1. Lysozyme	0.5
2. Tryptophan synthetase	2
3. DNA polymerase I	15
4. Phosphoglucomutase	20.5

5. Chymotrypsin	100
6. β–galactosidase	208
7. Lactate dehydrogenase	1,000
8. Penicillinase	2,000
9. β–amylase	18,333
10. Acetylcholinesterase	25,000
11. Carbonic anhydrase	600,000

3. Specificity of Enzyme Action. With few exceptions, the enzymes are specific in their action. Their specificity lies in the fact that they may act (*a*) on one specific type of substrate molecule or (*b*) on a group of structurally-related compounds or (*c*) on only one of the two optical isomers of a compound or (*d*) on only one of the two geometrical isomers. Accordingly, four patterns of enzyme specificity have been recognized :

A. Absolute specificity. Some enzyme are capable of acting on only one substrate. For example, *urease* acts only on urea to produce ammonia and carbon dioxide.



Similarly, *carbonic anhydrase* brings about the union of carbon dioxide with water to form carbonic acid.

$$H_2O + CO_2 \xrightarrow{\text{Carbonic anhydrase}} H_2CO_3$$

B. Group specificity. Some other enzymes are capable of catalyzing the reaction of a structurally-related group of compounds. For example, *lactic dehydrogenase* (LDH) catalyzes the interconversion of pyruvic and lactic acids and also of a number of other structurally-related compounds.

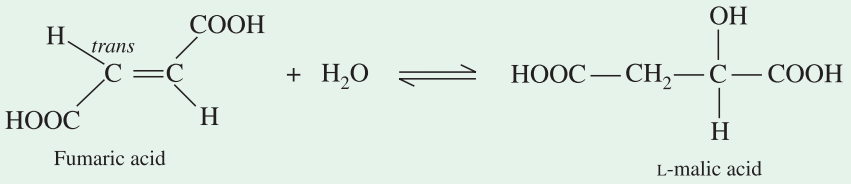
$$\underset{\text{Pyruvic acid}}{CH_3.CO.COOH} + NADH + H^+ \underset{}{\overset{\text{Lactic dehydrogenase}}{\rightleftharpoons}} \underset{\text{Lactic acid}}{CH_3.CHOH.COOH} + NAD^+$$

C. Optical specificity. The most striking aspect of specificity of enzymes is that a particular enzyme will react with only one of the two optical isomers. For example, *arginase* acts only on L-arginine and not on its D-isomer. Similarly, D-*amino acid oxidase* oxidizes the D-amino acids only to the corresponding keto acids.

Although, the enzymes exhibit optical specificity, some enzymes, however, interconvert the two optical isomers of a compound. For example, *alanine racemase* catalyzes the interconversion between L- and D-alanine.

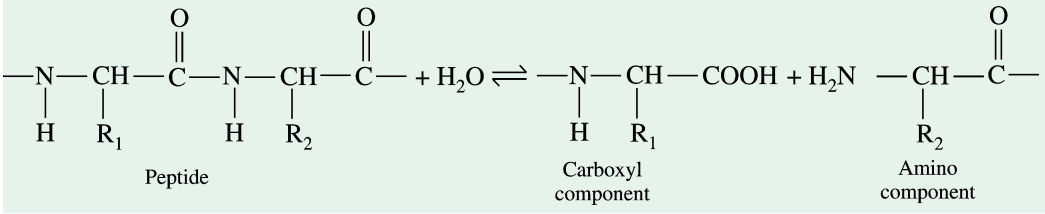
$$\text{L-alanine} \overset{\text{Alanine racemase}}{\rightleftharpoons} \text{D-alanine}$$

D. Geometrical specificity. Some enzymes exhibit specificity towards the *cis* and *trans* forms. As an example, *fumarase* catalyzes the interconversion of fumaric and malic acids :

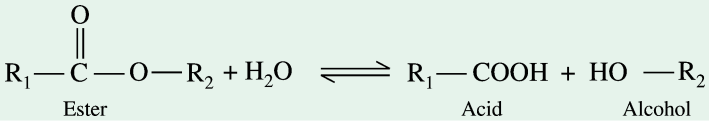


It does not react with maleic acid which is the *cis* isomer of fumaric acid or with D-malic acid.

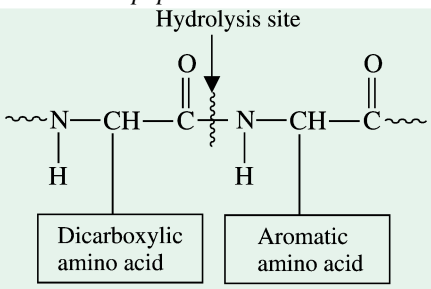
The degree of specificity of the enzymes for substrate is usually high and sometimes virtually absolute. Proteolytic enzymes, for instance, catalyze the hydrolysis of a peptide bond :



Many proteolytic enzymes (pepsin, trypsin, chymotrypsin) catalyze a different but related reaction, namely the hydrolysis of an ester bond.

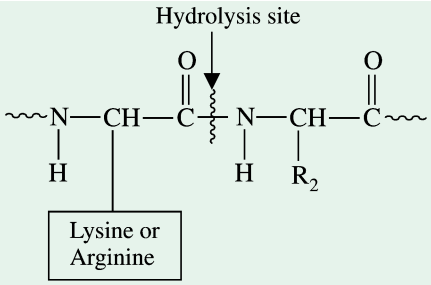


These enzymes vary markedly in their degree of specificity. For example, *subtilisin*, a bacterial enzyme, does not discriminate the nature of the side chains adjacent to the peptide bond to be cleaved. Another enzyme *pepsin* prefers bonds involving the carboxyl and amino groups of dicarboxylic and aromatic amino acids respectively. Since the bonds attached are usually located in the interior of the protein substrate, pepsin is called an *endopeptidase*.



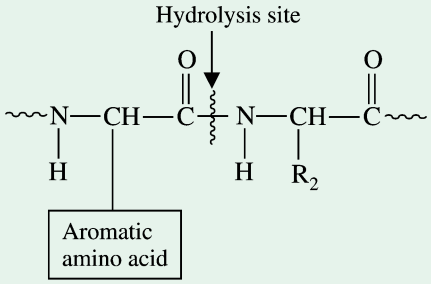
Specificity of pepsin

Trypsin, likewise, is an endopeptidase but is quite specific in that it splits peptide bonds in which carboxylic group is contributed by either lysine or arginine only.



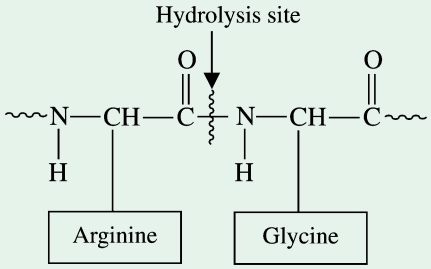
Specificity of trypsin

Chymotrypsin preferentially splits peptide bonds in which the carboxyl group is from an aromatic amino acid.



Specificity of chymotrypsin

Thrombin, an enzyme involved in blood coagulation, is even more specific in that the side chain on the carboxyl side of the susceptible peptide bond must be arginine whereas the one on the amino side must be glycine.



Specificity of thrombin

Alteration of enzyme specificity – The specificity of some enzymes is altered by physiological behaviour. *Lactose synthetase* (Fig. 17–3), for example, catalyzes the synthesis of lactose (a sugar consisting of a galactose and a glucose residue) in the mammary glands. It consists of a catalytic subunit and a modifier subunit. The *catalytic subunit* alone cannot synthesize lactose. Instead, it has a different role of catalyzing the attachment of galactose to proteins that contain a covalently linked carbohydrate chain. The *modifier subunit* alters the specificity of the catalytic subunit so that it links galactose to glucose to form lactose. The level of modifier subunit is under hormonal control. During pregnancy, the catalytic subunit is formed in the mammary glands and very little modifier subunit is formed. But at the time of childbirth (= parturition), the hormonal levels change significantly and the modifier subunit is synthesized in great quantities, thus resulting in the production of large amounts of lactose.

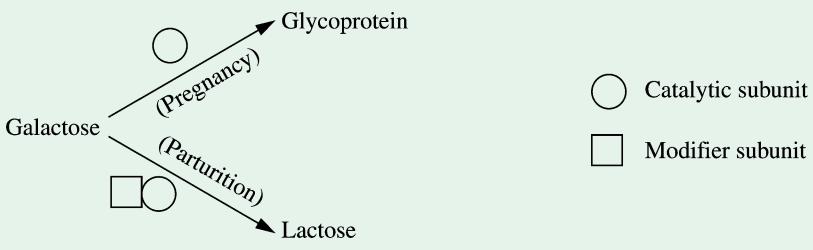


Fig. 17–3. Alternation in enzyme specificity of lactose synthetase

There are, however, instances where one enzyme acts on more than one substrate and conversely a substrate may also be catalyzed by more than one enzyme (Fig. 17–4). For example,

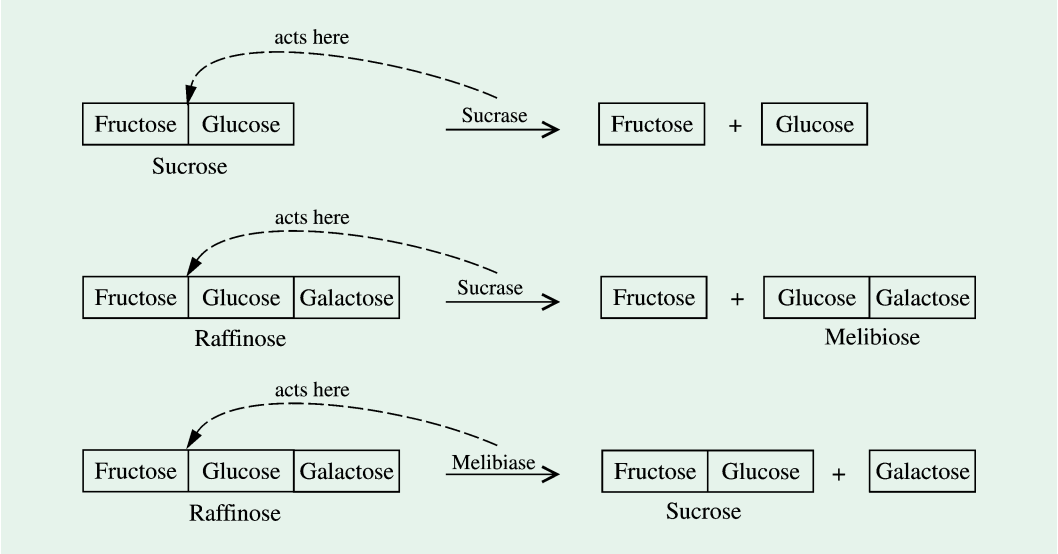


Fig. 17–4. Enzyme specificity of sucrase and melibiase

sucrase acts on both sucrose (a disaccharide sugar, containing one mole of glucose and fructose each) and raffinose (a trisaccharide sugar, containing one mole each of glucose, fructose and galactose). But in both these cases, the enzyme sucrase attacks only the glucose-fructose linkage resulting in the production of fructose and glucose (in the case of sucrose) or fructose and melibiose (in the case of raffinose). However, raffinose is also acted upon by another enzyme, the *melibiase*. But this enzyme, unlike sucrase, breaks up glucose-galactose linkage so that at the end of the reaction sucrose and galactose are produced.

The coenzymes possess much less specificity. For example, among the *hydrolases*, NAD^+ and $NADP^+$ act as common coenzymes. The relative nonspecificity of the coenzymes, in contrast to the absolute specificity of the enzymes, can be visualized by comparing coenzyme to a common hammer, used equally by various apoenzyme workers (ironworker, watchmaker, shoemaker or electrician). Although some differences may occur in the nature of hammers (*e.g.*, between NAD^+ and $NADP^+$), the apoenzyme worker is strictly specific, corresponding to the nature of the substrate concerned.

4. Thermolability (= Heat sensitivity). Being proteinaceous in nature, the enzymes are very sensitive to heat. The rate of an enzyme action increases with rise in temperature, the rate being frequently increased 2 to 3 times for a rise in temperature of 10ºC, *i.e.*, the value of temperature quotient or Q_{10}* is 2 to 3. But at higher temperatures, the value of coefficient does not remain constant and decreases rapidly. Above 60ºC, the enzymes coagulate and thus become inactivated, because there occurs an irreversible change in their chemical structure. The enzymes of dry tissues like seeds and spores, however, can endure still higher temperatures of about 100º to 120ºC.

A temperature difference of 10 °C has become a standard that is used to measure the temperature sensitivity of a biological function. This value, called the **temperature quotient (Q_{10}),** is determined (for temperature intervals of exactly 10 °C) simply by dividing the value of a rate function (such as metabolic rate or the rate of an enzymatic reaction) at the higher temperature by the value of the rate function at the lower temperature. In general, metabolic reactions have Q_{10} values about 2 to 3. Purely physical processes, such as diffusion, have much lower Q_{10} values, usually close to 1.

The observed effect of temperature on enzyme action is the net result of the effect of temperature on the rate of enzyme action and their destruction as well. There will, thus, be obtained an optimum temperature for the enzyme action (Fig. 17–5). The cruve AB represents the effect of temperature on action alone and the curve CD, the temperature effect on enzyme destruction. The observed relation between the temperature and the rate of enzyme action will then be represented by the curve AE.

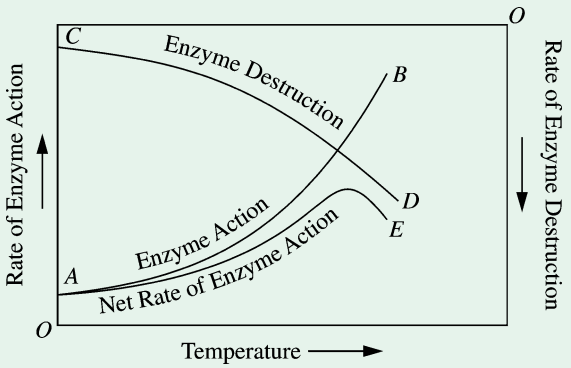


Fig. 17–5. Graph representing the relation between temperature and the rate of enzyme activity
(After Duclaux, 1883)

If, however, the effect of increasing temperature (in terms of three ill-demarcated categories of low, medium and high) on enzyme activity is studied (Fig. 17–6), it may be observed that the initial velocity of the reaction (given by the shape of the curves at $t = 0$) steadily increases with temperaure. However, after a certain temperature is passed, the cessation of activity comes earlier and earlier with the result that less product is formed. There is, thus, a somewhat ill-defined optimum region of temperature which is that at which the two factors of increased initial rate and decreased active life of the enzyme are balanced to produce the most product in a reasonable time. It is not easy to determine the exact value for the optimum temperature because it is somewhat vague concept, and will depend on the length of time over which the measurements are made. However, the approximate values obtained often show a distinct correlation with the body temperatures of the organisms from which the enzyme came. Thus, mammalian enzymes often have optimum temperatures in the range 35–45ºC, while the enzymes from the bacteria that live in volcanic hot springs may have optima of 80ºC.

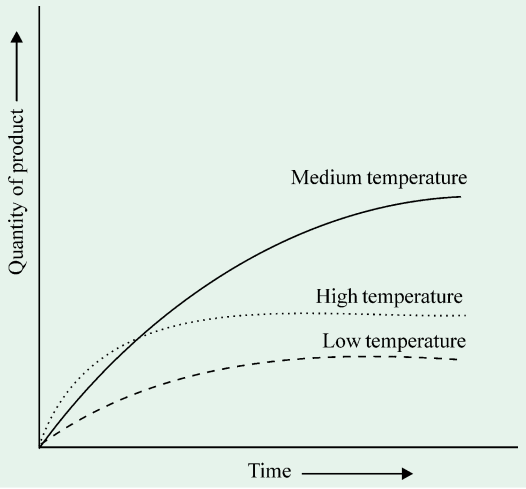


Fig. 17–6. Effect of temperature (coupled with time) on enzyme activity

In general, the optimum temperature range for most enzymes varies between 30 and 45ºC. For

instance, it is 30ºC for *catalase*. Because enzymes are globular proteins, most are thermolabile and begin to denature (indicated by loss of enzyme activity) at temperatures between 45º and 50ºC (Fig. 17–7).

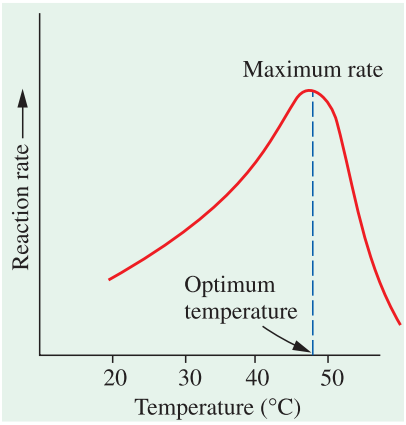


Fig. 17–7. Hypothetical temperature activity profile of an enzyme

At low temperatures, the catalytic activity of the enzyme predominates, although some thermal denaturation takes place during this period. Decreasing temperatures to near or below 0ºC although inactivate the enzyme but this is a reversible type of change and the enzyme regains its catalyzing power upon increasing the temperature to optimum. At higher temperaure, although the catalytic activity of the enzyme increases, yet its denaturation predominates. Henceforth, all the enzyme is denatured in a very short time. The enhanced enzyme activity with the rise in temperature is due to the fact that the energy of molecule becomes greater which, in turn, enhances the inherent reactivity of the molecules and the frequency of their collisions. It is because of the high rate of enzyme destruction at increasing temperatures that an enzyme is stable for weeks at 0ºC, for days at 10ºC, for hours at 30ºC but for fraction of seconds at 70ºC.

The effect of heat also mainfests itself in the preservation of enzyme activity during storage. *The best preservation of enzyme preparations is by refrigeration or quick freezing.* This has been shown by Nord (1932) in the case of *zymase*.

5. Reversibility of a Reaction. The enzymes are capable of bringing about reversion in a chemical reaction. The digestive enzymes catalyze the hydrolytic reactions which are reversible. For instance, *lipase*, which catalyzes the synthesis of fat from glycerol and fatty acid, can also hydrolyze them into their component units.

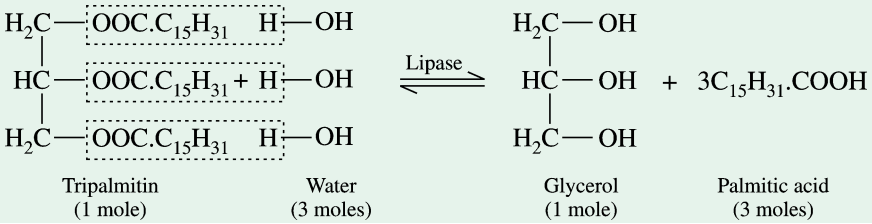


Fig. 17–8 shows the results of experiments on the action of *lipase* from castor on a fat, triolein. The final equilibrium mixture is the same whether one starts with the ester or with its individual components.

The direction in which the reaction proceeds depends upon many factors like –

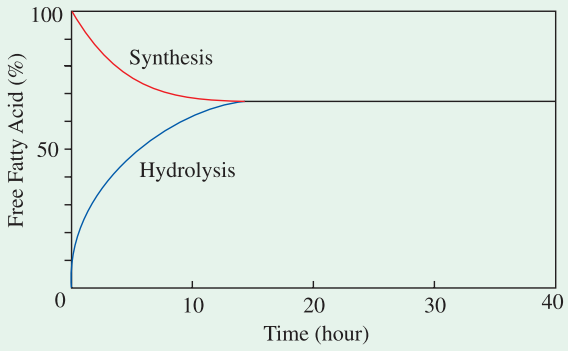


Fig. 17–8. Reversibility of action of lipase (from castor) on triolein

(*a*) the *p*H of the cell sap,

(*b*) the presence of reacting substances, and

(*c*) the accumulation of end products.

It does not, however, necessarily follow that the same enzyme invariably catalyzes both the synthesis and degradation of a given kind of molecule. For instance, urea is synthesized from arginine by the action of the enzyme, *arginase* but is hydrolyzed by action of another enzyme, *urease* to produce ammonia and carbon dioxide.

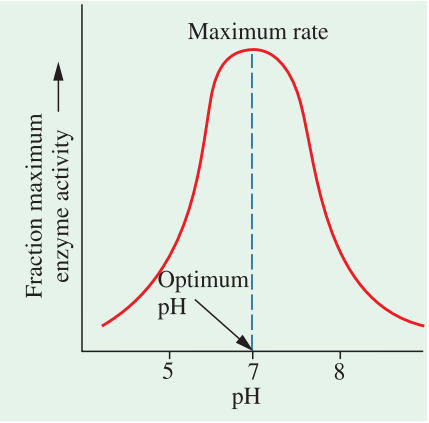


Fig. 17–9. Hypotetical pH activity profile of an enzyme

6. pH Sensitivity. The *p*H value or the H^+ ion concentration of the medium controls the activity of an enzyme to a great extent. This is mainly related to the degree of dissociation, to the electric charge of the enzyme and, through this, to the formation of the enzyme-substrate complex (a discussion of which will follow in the succeeding chapter). Each enzyme, thus, acts best in a certain pH value which is specific to it and its activity slows down with any appreciable change (increase or decrease) in the H^+ ion concentration. In fact, the *p*H will affect the efficiency of an enzyme and usually there will be a *p*H at which the activity is at a maximum. The activity will fall off on either side of this value. Fig. 17–9 depicts the effect of *p*H on an enzyme-catalyzed reaction.

Table 17–3. pH optima for various enzymes

S.No.	*Enzyme*	*Optimum pH of the medium*	*Nature of the medium*
1.	Pepsin	1.5–1.6	Highly acidic
2.	Invertase	4.5	Acidic
3.	Lipase (stomach)	4.0–5.0	Acidic
4.	Lipase (castor oil)	4.7	Acidic
5.	Lipase (pancreas)	8.0	Alkaline
6.	Amylase (malt)	4.6–5.2	Acidic
7.	Amylase (pancreas)	6.7–7.0	Acidic–neutral
8.	Cellobiase	5.0	Acidic
9.	Maltase	6.1–6.8	Acidic
10.	Sucrase	6.2	Acidic
11.	Catalase	7.0	Neutral
12.	Urease	7.0	Neutral
13.	Cholinesterase	7.0	Neutral
14.	Ribonuclease	7.0–7.5	Neutral
15.	Fumarase	7.8	Alkaline
16.	Trypsin	7.8–8.7	Alkaline
17.	Adenosine triphosphatase	9.0	Alkaline
18.	Arginase	10.0	Highly alkaline

Some optimum *p*H values for various enzymes are given in Table. 17–3. A perusal of the table indicates that *the approximate optimum pH value for most enzymes lies near neutrality.* This value depends on many factors such as :

(*a*) the nature of buffer system,
(*b*) the presence of other colloids, activators or inhibitors,
(*c*) the age of the cell tissue, and
(*d*) the nature of the substrate.

Usually maximum enzyme activity is obtained at or near the isoelectric point (*p*l) of the enzymes. Thus *trypsin*, whose *p*l value is 10.1, shows maximum activity at *p*H range between 7 and 9.

The correction between the enzymic activity and the *p*H value for 3 enzymes has been graphically represented in Fig. 17–10.

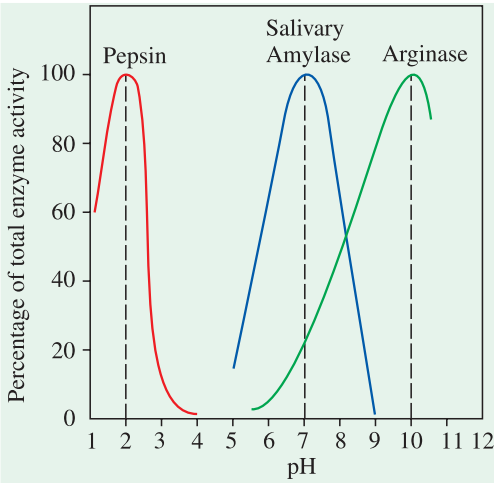


Fig. 17–10. Effect of pH on enzyme action
(Modified from Fruton and Simmonds, 1958)

THREE DIMENSIONAL STRUCTURE OF THE ENZYMES

A single crystal of protein or the protein fibres will deflect x-rays and the resultant image formed on a photographic plate can give certain important clues regarding the structure of the crystal or the fibres. This techinque is called **x-ray crystallography** and has been widely used for the elucidation of protein structure at micro level. X-ray crystallography has so far revealed the structure of many enzymes, namely, *ribonuclease, lysozyme, chymotrypsin,* trypsin etc. The structure of four of them is described below :

1. Ribonuclease (RNase)

Ribonuclease (Fig. 17–11), a small globular protein, is an enzyme secreted by the pancreas into the small intestine, where it catalyzes the hydrolysis of certain bonds in ribonucleic acids present in ingested food.

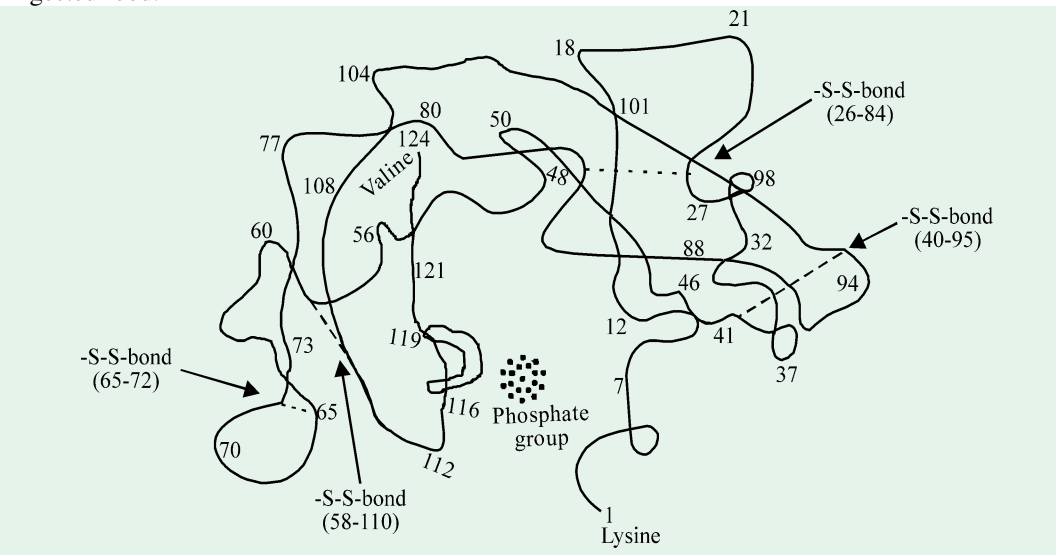


Fig. 17–11. Structure of ribonuclease as determined from x-ray diffraction studies
Numbers refer to specific amino acid residues.
(From Harper and Rodwell, 1973)

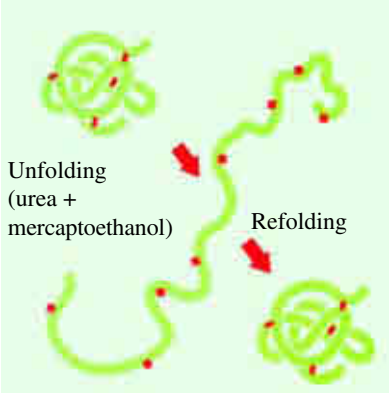


Fig. 17–12. Denaturation and refolding of ribonuclease
A native ribonuclease molecule (with intramolecular disulfide bonds indicated) is reduced and unfolde with β-mercaptoethanol and 8 M urea. After removal of these reagents, the protein undergoes spontaneous refolding. *(From. CJ Epstein, RF Goldberger and CB Anfinsen, 1963).*

The molecule of ribonuclease is reniform (kidney-shaped) and has dimensions of about 3.2, 2.8 and 2.2 nm. Ribonuclease, like myoglobin, contains a tightly packed, highly nonpolar interior. This enzyme-protein (as already described on page 152) consists of a single polypeptide chain of 124 amino acid residues with lysine at the N-terminal and valine at the C-terminal (Hirs, Moore and Stein, 1960). It has a molecular weight of 13,700. There are 8 cysteine residues, thus apparently forming 4 disulfide linkages– 26-84, 40-95, 58-110 and 65-72. These serve to hold the tertiary structure firmly in place. There is very little (26%) α-helix structure ; many of its segments are present in β conformation which amounts to 35%. Only 4 turns of the helix, two each at residues 5-12 and 28-35, are present. The chain assumes a complex configuration with a deep depression in the middle of one side. The active site is believed to be on the edge of this depression and the residues forming the active site are 6-8, 11, 12, 41, 42, 46-48 and 117-119. A phosphate ion is associated directly with the active site of the enzyme. The amino acid residues 12 and 119 (both histidine) are nearest to the phosphate ion.

The bacterial enzyme, *subtilisin*, cleaves the chain into 2 inactive fragments : the shorter one (S-peptide) consisting of first 21 amino acids from N-terminal and the longer one (S-protein) with remaining residues. On reunion of the two segments, the enzyme molecule regains its full activity upon treatment with 8 M urea and mercaptoethanol, the native ribonuclease molecule become reduced and unfolded. After removal of these reagents, the enzyme undergoes spontaneous refolding (Fig. 17–12).

It is interesting to note that there is remarkable similarity in structure between the ribonucleases from cows and humans beings (Fig. 17–13). The strucutural similarity is often followed by functional similarity.

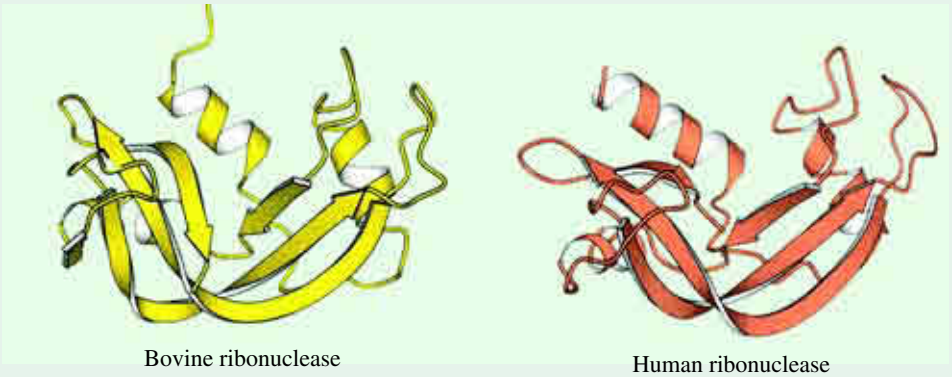


Fig. 17–13. Ribbon diagrams of the structure of ribonucleases from cows and human beings
Structural similarity often follows functional similarity.

2. Lysozyme (= Muramidase)

Lysozyme (Figs. 17–12 and 17–13), another small globular protein, is an enzyme present in tears, nasal mucus, gastric secretions, milk and egg white. Lysozyme is so named because it can

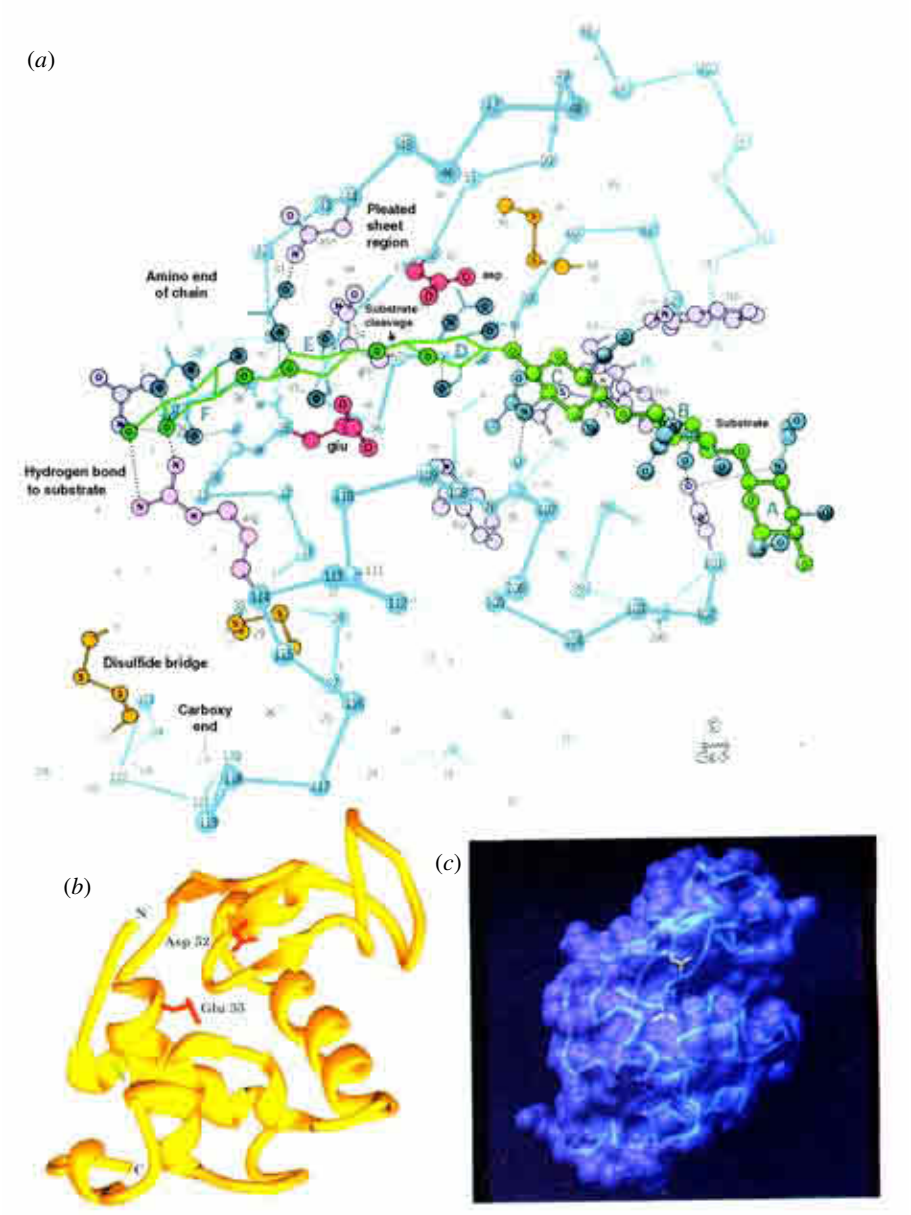


Fig. 14–14. The X-ray structure of hen egg white (HEW) lysozyme

(*a*) **The ball-and-stick model.** Each circle represents a single amino acid residue. Numbers refer to specific amino acid residues. The segment between residues 403 and 54 has a pleated sheet structure. The polypeptide chain is shown with a bound $(NAG)_6$ substrate (*green*). The positions of the backbone C_α atoms are indicated together with those of the side chains that line the substrate binding site and form disulfide bonds. The substarate's sugar rings are designated A, at its nonreducing end (*right*), through F, at its reducing end (*left*). Lysozyme catalyzes the hydrolysis of the glycosidic bond between residues D and E. Rings A, B, and C are observed in the x-ray structure of the complex of $(NAG)_3$ with lysozyme; the positions of rings D, E, and F were inferred from model studies.

(*b*) **The ribbon model.** It highlights the protein's secondary structure and indicates the positions of its catalytically important side chains. The letters N and C represent the amino and carboxyl terminals of the protein molecule, respectively.

(*c*) **A computer-generated model.** It shows the protein's molecular envelope (*purple*) and C_α backbone (*blue*). The side chains of the catalytic residues, Asp 52 (*above*) and Glu 35 (*below*) are coloured yellow. Note the enzyme's prominent substrate-binding cleft. Parts (*a*), (*b*) and (*c*) have approximately the same orientation)

(Courtesy of (a) Irving Geis, (b) & (c) Arthur Olson.

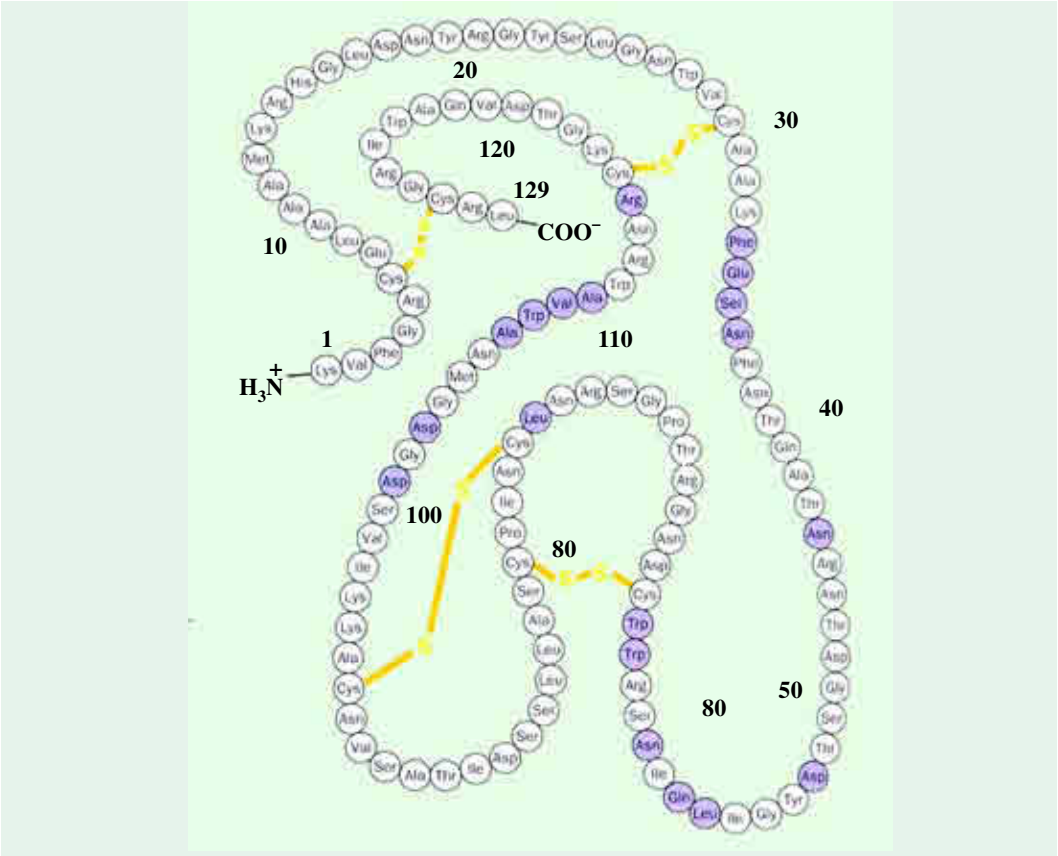


Fig. 17–15. The primary structure of hen egg white lysozyme

The enzyme lysozyme contains 129 amino acids in its primary structure. As may be noted, the first amino acid is not methionine; instead, it is lysine. The first methionine residue in this polypeptide sequence is removed after translation is completed. The removal of the first methionine occurs in many (but not all) polypeptides. The amino acid residues that line the substrate-binding pocket are shown in dark purple.

'lyse', or dissolve, bacterial cell walls and thus serve as a bactericidal agent. This is accomplished by disrupting certain polysaccharide molecules present in the protective cell walls of many gram-positive bacteria. These polysaccharides consist of repeating units of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG), joined by β−1→4 glycosidic linkages. *Lysozyme catalyzes the hydrolysis of glycosidic bond between C-1 of NAM and C-4 of NAG.* The other glycosidic bond, between C-1 of NAG and C-4 of NAM, is not cleaved.

In 1965, David C. Phillips and his colleagues determined the three-dimensional structure of lysozyme. It is a relatively small, compact molecule, roughly ellipsoidal in shape and with dimensions 45 ×30 × 30 Å. It has a molecular weight of 14,600. Its molecule consists of a single polypeptide chain of 129 amino acid residues with 4 intra-chain disulfide linkages— 6-127, 30-115, 64-80 and 76-94. It has lysine at the N-terminal and leucine at the C-terminal. *Lysozyme is devoid of coenzyme or metal ion cofactors* and thus lacks a built-in marker at its active site, in contrast with such proteins as myoglobin and hemoglobin. Like myoglobin and cytochrome c, lysozyme has a compactly-folded conformation and has most of its hydrophobic R groups inside the globular structure, shielded from water, and its hydrophilic R groups outside, facing the aqueous medium. The enzyme has only 12%β conformation and 40% α-helical segments which line a long deep cleft in the side of the molecule. This central cleft is the active site of the enzyme molecule. The interior of lysozyme, like that of

myoglobin and hemoglobin, is almost entirely nonpolar. Hydrophobic interactions evidently play an important role in the folding of lysozyme, as they do for most proteins.

The active site has 6 subsites (A to F ; Fig. 17–16) which bind various substrates or inhibitors. The amino acid residues located at the active sites are 35, 52, 59, 62, 63 and 107. It is, thus, apparent that the active site may include amino acid residues which are distantly placed, as shown in Fig. 17–17. The residues which bring about bond cleavage lie between the subsites D and E, close to the COOH groups of glutamic acid (35) and aspartic acid (52). It is thought that glutamic acid protonates the acetal bond of the substrate while the aspartic acid stabilizes the resulting carbonium ion from the back side (Harper and Rodwell, 1973).

Lysozyme binds 6 of the monomeric units of its polysaccharide substrate, and the strain induced by the binding facilitates the formation of the unstable carbonium ion, $>C^{+}-$, intermediate. The proposed mechanism of lysozyme catalysis employs (Charles J. Flickinger *et al*, 1979) :

1. orientation and approximation through formation of ES complex,
2. strain,
3. general acid-base catalysis, and
4. electrostatic stabilization of a carbonium ion intermediate.

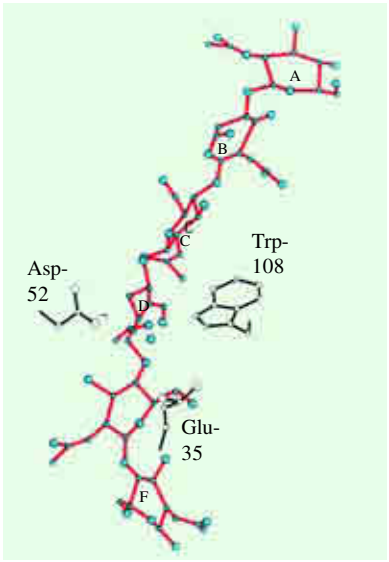


Fig. 17–16. Simplified model of a lysozyme molecule showing a hexasaccharide bound in the cleft of the enzyme

A to F represent the subsites of the enzyme. The locations of key amino acid residues of the enzyme are indicated.

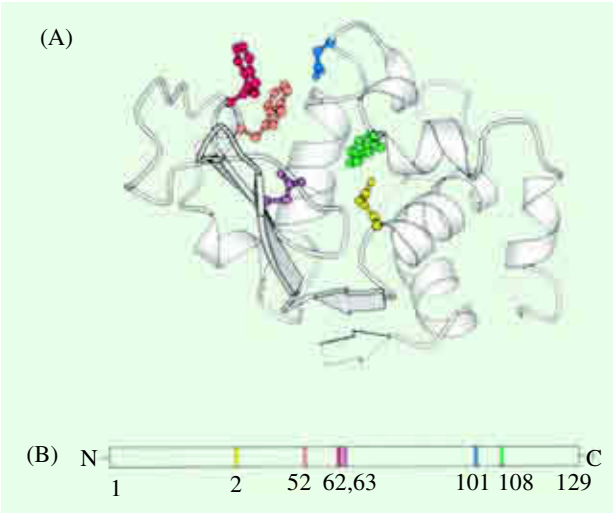


Fig. 17–17. The constituent amino acid residues of the lysozyme active site

(A) Ribbon diagram of the enzyme lysozyme with several components of the active site shown in color.

(B) A schematic of the primary structure of lysozyme showing that the active site is composed of residues that come from different parts of the polypeptide chain.

The importance of the ability of proteins to structure precisely a volume of space is evident.

3. Chymotrypsin

Chymotrypsin, like carboxypeptidase, is a mammalian digestive enzyme which catalyzes the hydrolysis of proteins in the small intestine. Chymotrypsin is highly selective in its action as it catalyzes the hydrolysis of only those peptide bonds which are on the carboxyl side of amino acids with aromatic (phenylalanine, tyrosine, tryptophan) or bulky hydrophobic (methionine) R groups, irrespective of the length or amino acid sequence of the polypeptide chain. Chymotrypsin is synthesized by the exocrine cells of the pancreas as its inactive precursor or zymogen form called **chymotrypsinogen**. The mechanism for lysozyme action, as proposed by David C. Phillips (1965), is presented in Fig. 17–17.

A molecule of chymotrypsin (MW = 25,000) consists of 3 short polypeptide chains (A, B, and C) of 13, 131 and 97 amino acid residues respectively, connected by two *interchain* disulfide bonds between 1-122 and 136-201 and three *intrachain* disulfide bonds between 42-58, 168-182 and 191-220 amino acid residues (Fig. 17–18). The 3-dimensional structure of the enzyme was elucidated at 2 Å resolution by the x-ray crystallographic studies of David Blow (Fig. 17–19). The molecule is a compact ellipsoid of dimensions 51× 40 × 40 Å. Chymotrypsin consists of several antiparallel β pleated sheet regions and, unlike myoglobin and hemoglobin, has little α helical structure. All charged

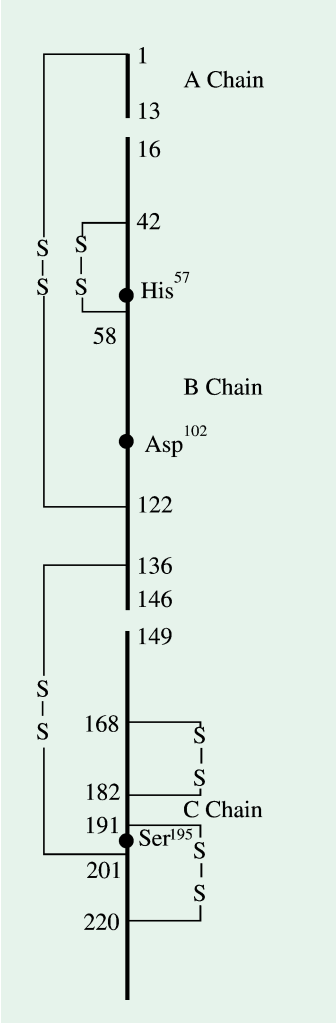
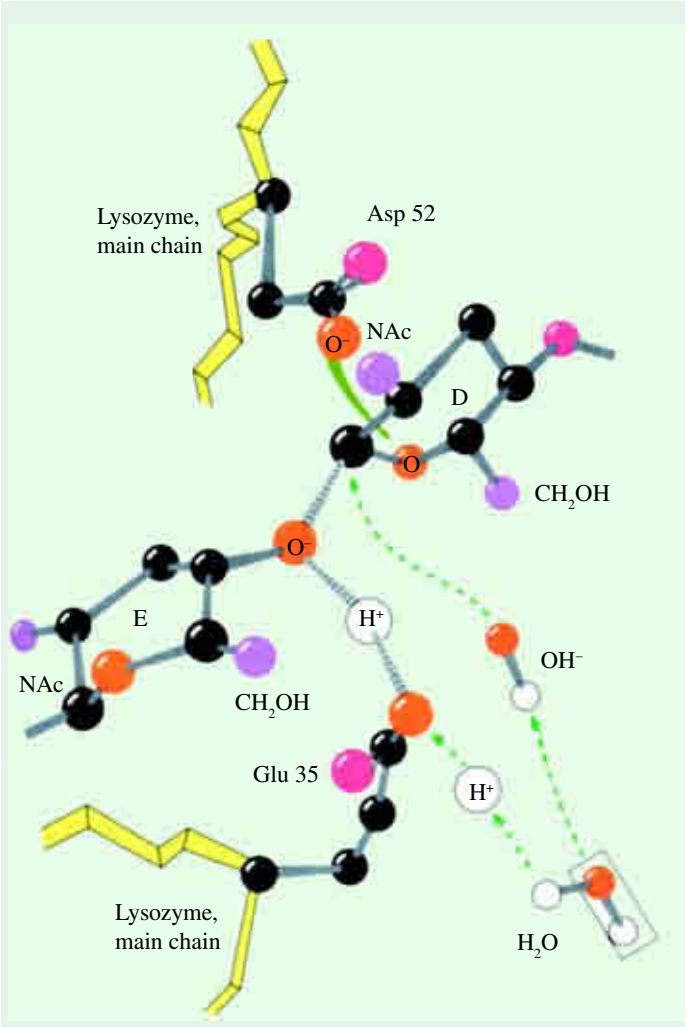


Fig. 17–19. Representation of primary structure of chymotrypsin

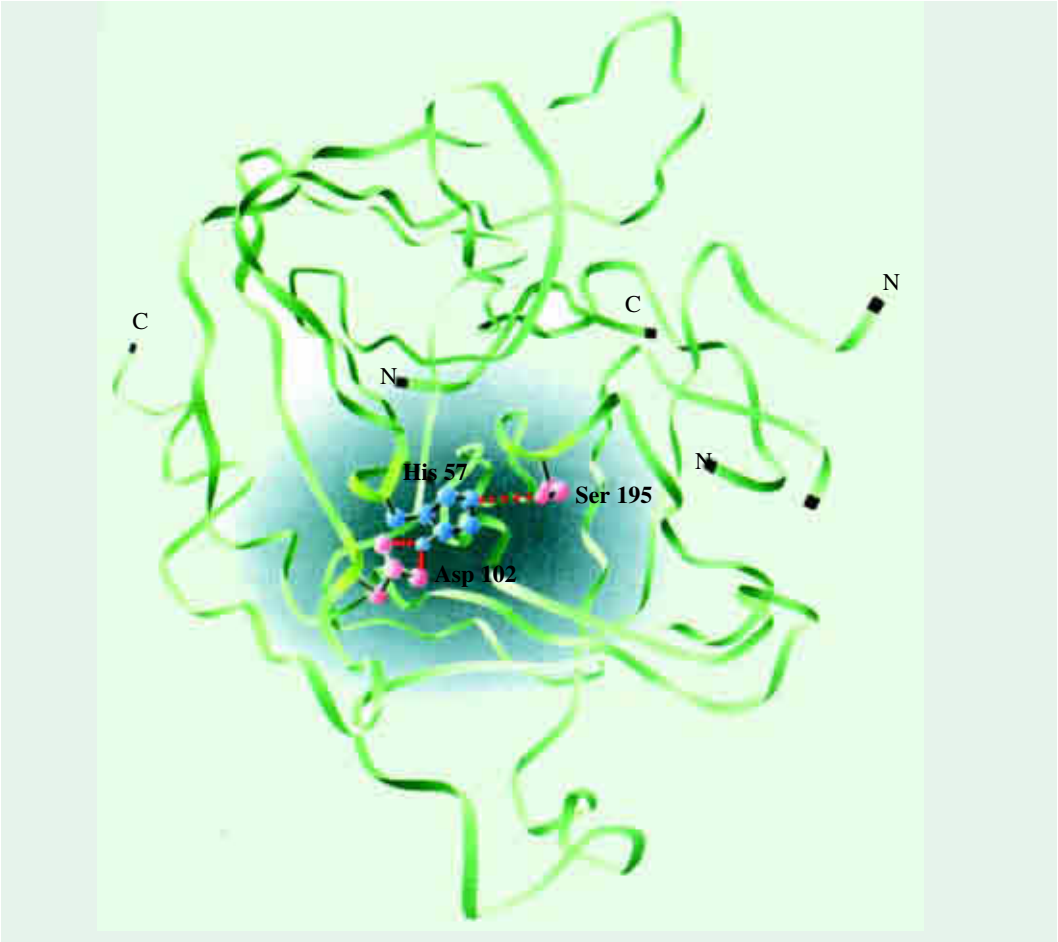
Note the presence of 3 polypeptide chains with 5 disulfide bonds, of which 2 are interchain and 3 are intrachain. Location of 3 amino acid residues forming catalytic triad is shown. The active-site amino acids are found grouped together in the 3-'D' structure.



17–18. The mechanism for lysozyme action as proposed by Phillips

The bond between the fourth (D) and fifth (E) sugar of the hexasaccharide residing in the cleft of the lysozyme molecule is cleaved by acid hydrolysis using a proton donated by the carboxyl group of the closely applied glutamic acid residue (Glu 35). The formation of the positively charged oxocarbonium ion at the C1 position of sugar D is facilitated by the distortion of the sugar shown in the figure and stabilized by the nearby aspartic acid residue (Asp52) of the enzyme. In the final step, the oxocarbonium ion reacts with an OH^- group from the solvent.

(From D Voet and G Voet, 1995)



17–20. Ribbon model of the tertiary structure of chymotrypsin

The amino- and carboxyl-terminals of the 3 constituent chains are labelled as N and C respectively. The tertiary structure of chymotrypsin places the essential amino acid residues close to one another. They are shown as ball-and-stick representations.

(From D Voet and G Voet, 1995)

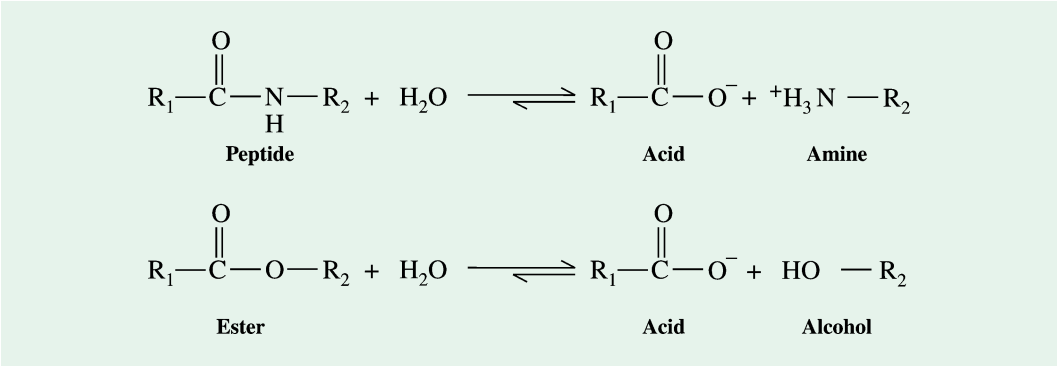
groups are on the surface of the molecule except for three (His^{57}, Asp^{102} and Ser^{195}) that play a critical role in catalysis. A tertiary (or 3-dimensional structure) of chymotrypsin molecule in ribbon form is shown in Fig. 17–20.

Double Displacement Mechanism. Chymotrypsin, like many proteases, hydrolyzes *ester bonds*, in addition to peptide bonds. The hydrolysis (of peptide or *ester bonds*) takes place by a two-step displacement with an amine being produced first, followed by production of an acid. The two steps of this double displacement mechamism are :

First step. *Acylation : Formation of the acetyl-enzyme complex.*

p-mitrophenylacetate (*p*-NPA) combines with chymotrypsin to form an enzyme-substrate (ES) complex. The ester bond of the substrate then cleaves. One of the products, *p*-nitrophenol is released from the enzyme, whereas the acetyl group of the substrate becomes covalently attached to the enzyme.

Second step. *Deacylation : Hydrolysis of the acetyl-enzyme complex.*



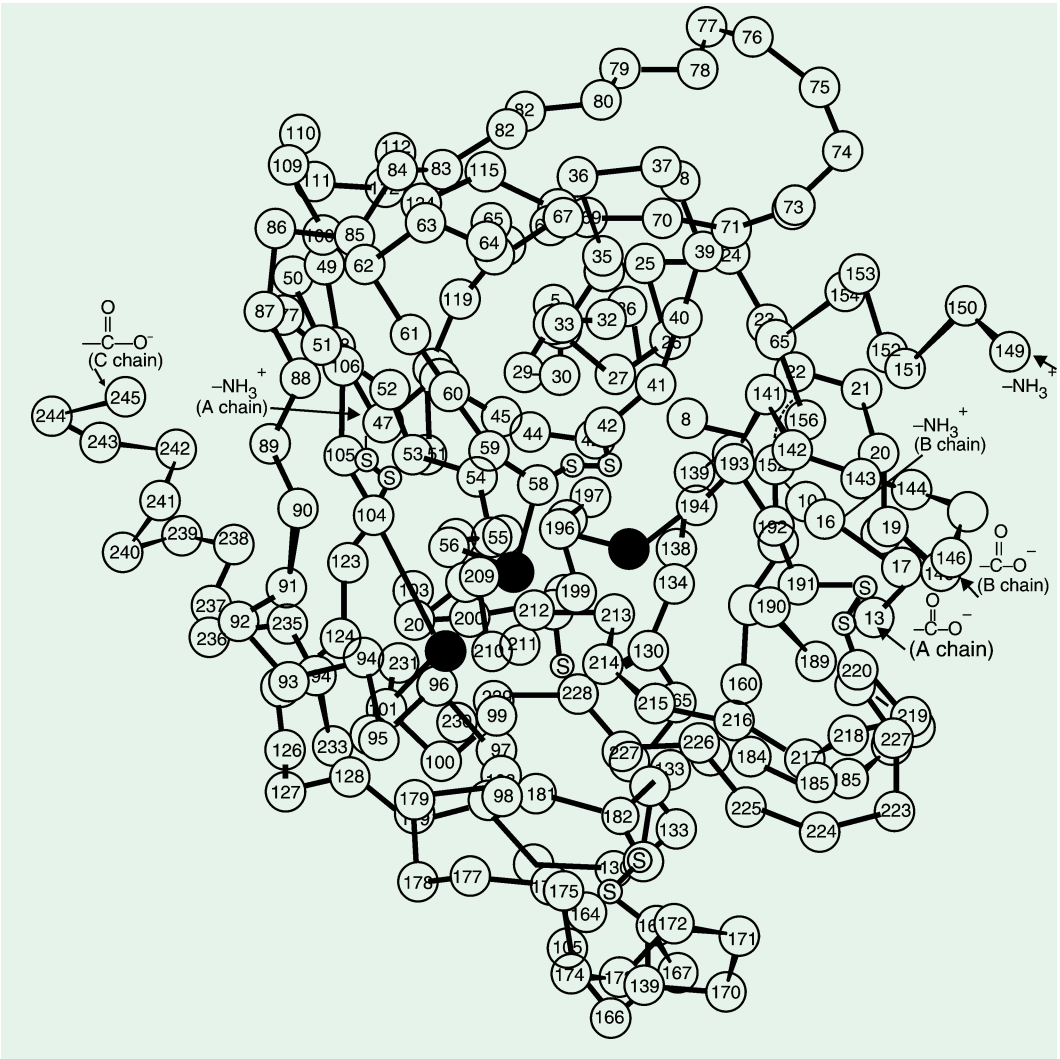
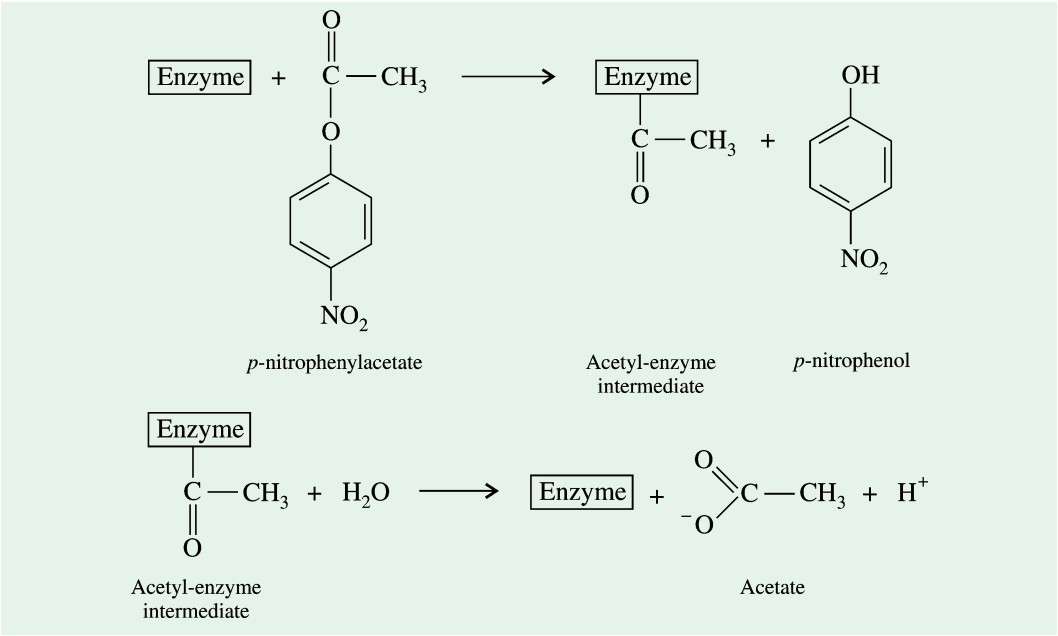


Fig. 17–21. Ball-and-stick model of the three-dimensional structure of α-chymotrypsin

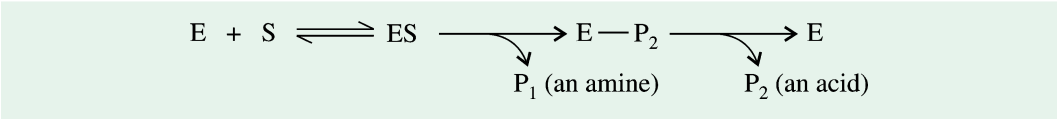
Only the α carbon atoms are shown. Residues of the catalytic triad (His^{57}, Asp^{102} and Ser^{195}) are labelled. The hydrophobic pocket of the substrate is indicated by the dark residues.

(After RE Dickerson and I Geis, 1969)

Water then attacks the acetyl-enzyme complex to yield acetate ion and regenerate the enzyme.



The second step (deacylation) is much slower than the first step (acylation), so that it determines the overall rate of hydrolysis of esters by chymotrypsin. The acetyl-enzyme complex is sufficiently stable to be isolated under proper conditions. The catalytic mechanism of chymotrypsin can, thus, be represented by,



where P_1 is the amine (or alcohol) component of the substrate, E-P_2 is the covalent intermediate, and P_2 is the acid component of the substrate. A distinct feature of this mechanism is the appearance of a covalent intermediate. In the first-step-reaction, an acetyl group is covalently bonded to the enzyme and the group attached to chymotrypsin at E-P_2 stage is an acyl group. Thus, E-P_2 is an acyl-enzyme intermediate.

Catalytic Triad. Proteolytic enzymes containing a highly reactive serine residue are known as **serine proteases**. These enzymes are readily identifiable by their rapid inactivation by DIPF. Chymotrypsin, trypsin and thrombin are noteworthy examples of this clan.

Chymotrypsin contains 28 seryl residues but only one of them (Ser^{195}) is a strong nucleophile. This is due to a specific spatial relationship between three amino acid residues (His^{57}, Asp^{102} and Ser^{195}) which constitute a ***catalytic triad*** and is based on hydrogen bonding (Fig. 17–26). The hydrogen bonding, that occurs between the buried Asp^{102} and His^{57} and between His^{57} and Ser^{195}, establishes an equilibrium that allows for the loss of the proton of the OH group of Ser^{195} (at the catalytic site) to His^{57}. This loss makes the oxygen atom of Ser^{195} residue a strong nucleophile, *i.e.*, makes serine^{195} an active serine. The proton gained by His^{57} converts the side chain of that residue into a positive imidazolium ion that forms an **ion pair** with the negative carboxylate ion of Asp^{102}. Thus, loss of a proton by Ser^{195} and formation of an ion pair by His^{57} and Asp^{102} explain the mechanism behind the functioning of catalytic triad.

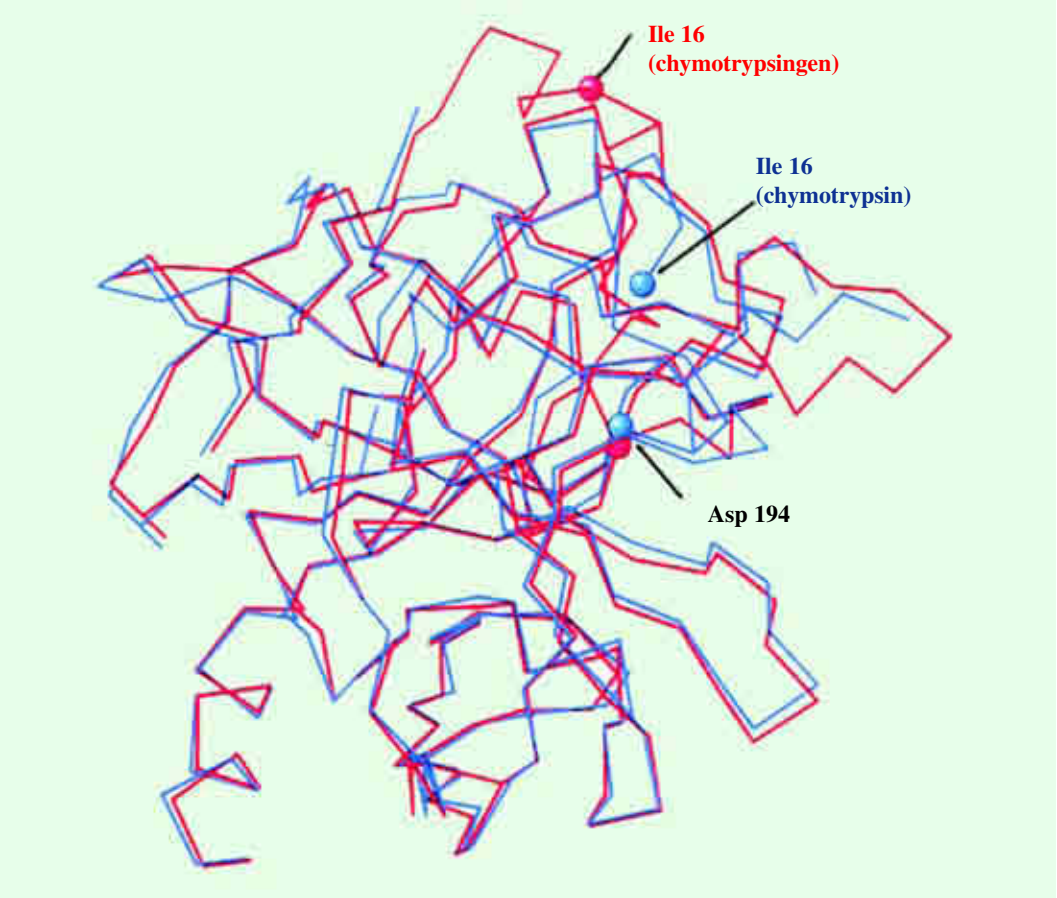
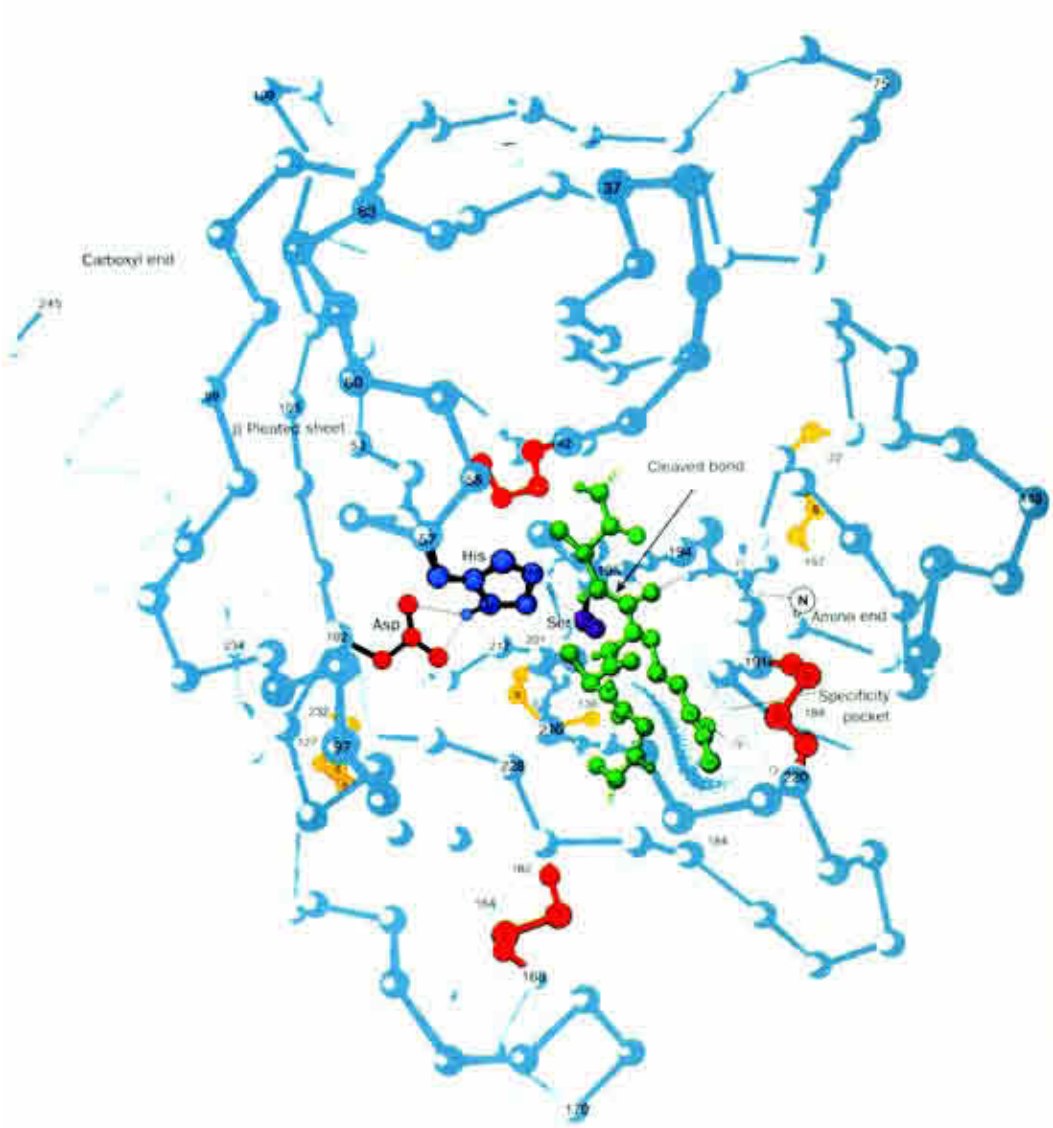


Fig. 17–22. Conformations of chymotrypsinogen (red) and chymotrypsin (blue)
The electrostatic interaction between the carboxylate of aspartate 194 and the α-amino group of isoleucine 16, essential for the structure of active chymotrypsin, is possible only in chymotrypsin.

4. Trypsin

Trypsin (and another enzyme elastase) are homologues of chymotrypsin. These two have catalytic triads similar to that discovered in chymotrypsin. The catalytic triad in trypsin consists of His 57, Asp 102 and Ser 195. (Fig. 17–23). Their sequences are approximately 40% identical with that of chymotrypsin, and their orverall structures are the same (Fig. 17-24). However, they have very different substrate specificities. Trypsin cleaves at the peptide bond after residues with long, positively charged side chains-namely, arginine and lysine (Fig. 17-25), whereas elastase, cleaves at the peptide bond after amino acids with small side chains-such as alanine and serine. In trypsin, an aspartate residue (Asp 189) is present at the bottom of the S_1 pocket (in place of a serine residue in chymotrypsin). The aspartate residue attracts and stabilizes a positively-charged arginine or lysine residue in the substrate.

Fig. 17–23. Structural similarity of trypsin and chymotrypsin
An overlay of the structure of chymotrypsin (red) on that of tryspin (*blue*) shows the high degree of similarity. Only α-carbon atom positions are shown. The mean deviation in position between corresponding α-carbon atoms is 1.7.



(*a*)

Fig. 17–24. The x-ray structure of bovine trypsin

(*a*) **The ball-and-stick model.** Each circle represents a single amino acid residue. Numbers refer to specific amino acid residues. The drawing of the enzyme with a polypeptide substrate (*green*) that has its Arg side chain occupying the enzyme's specificity pocket (*stippling*). The C_α backbone of the enzyme is shown together with its disulfide bonds and the side chains of the catalytic triad, Ser 195, His 57, and Asp 102.

(*b*) (*next page*)**The ribbon model.** This diagram highlights its secondary structure and indicates the arrangement of its catalytic triad.

(*c*) (*next page*) **A computer-generated model.** It shows the surface of trypsin (blue) superimposed on its polypeptide backbone (purple). The side chains of the catalytic triad are shown in green.

Parts *a*, *b*, and *c* have approximately the same orientation.

(Courtesy : (a) Irving Geis, (b) & (c) Arthur Olson)

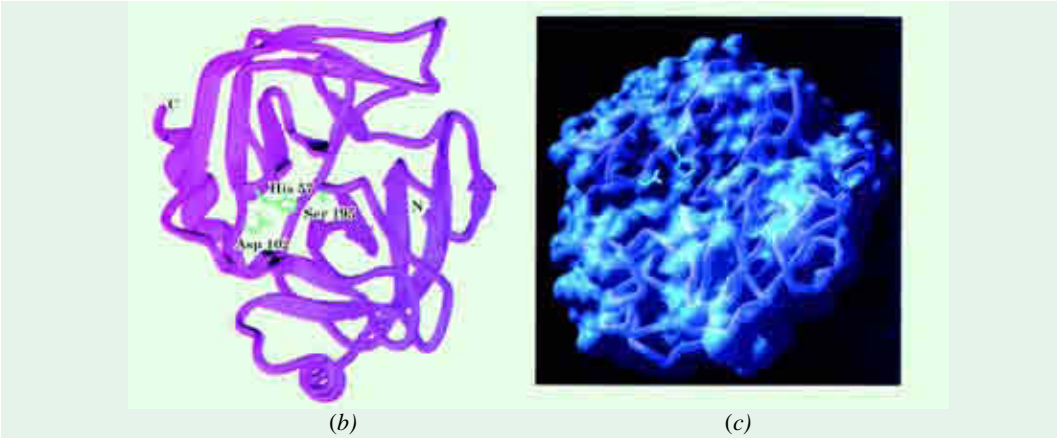


Fig. 17–25. Specificity of trypsin

Trypsin cleaves on the carboxyl side of arginine and lysine residues.

To produce active trypsin, the cells that line the duodenum secrete on enzyme, *enteropeptidase* that hydrolyzes a unique lysine–isoleucine peptide bond trypsinogen as the precursor enters the duodenum from the pancreas. The small amount of trypsin produced in this way activates more trypsinogen and the other zymogens. Thus, *the formation of tryspin by enteropeptidase is the master activation step.*

It is to be noted that in all the three enzymes described above, the active site is present in the groove or depression. The groove is, in fact, the ideal place for the active site as it provides a nonpolar microenvironment in which alone the van der Waal's forces and the hydrogen bond formation can operate between the polar groups of the active site and the substrate. In the case of an `exposed' active site, on the contrary, the water molecules will interfere with such activity.

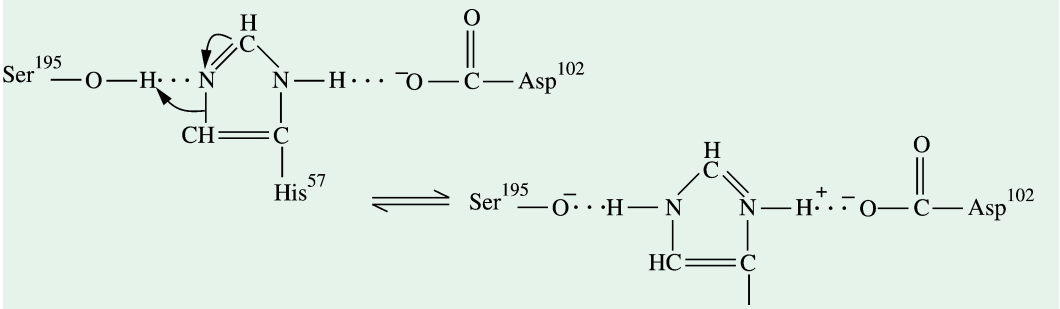


Fig. 17–26. Role of the catalytic triad in chymotrypsin

Note that the catalytic triad is created by the hydrogen-bonding of Ser^{195}, His^{57} and Asp^{102}.

From the foregoing discussion, it may be concluded that the chemical nature of an enzyme depends much on the active site of the enzyme molecule. Apart from the various kinds of constituent amino acid residues comprising an enzyme, its properties further depend on the pattern of '3-D' structure. The typical folding of the polypeptide chain, for example, ensures that the constituent amino acids of the active site are brought closer in the grooves.

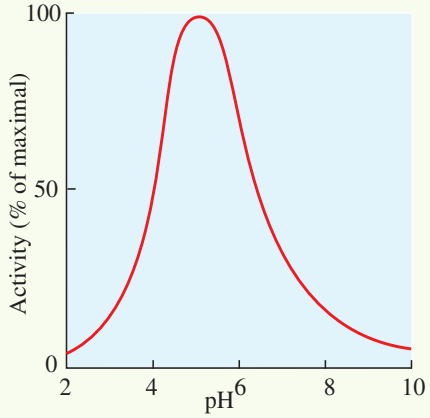
REFERENCES

See list following Chapter 18.

PROBLEMS

1. The sweet taste of freshly picked corn is due to the high level of sugar in the kernels. Store-bought corn (several days after picking) is not as sweet because about 50% of the free sugar of corn is converted into starch within one day of picking. To preserve the sweetness of fresh corn, the husked ears are immersed in boiling water for a few minutes ("blanched") and then cooled in cold water. Corn processed in this way and stored in a freezer maintains its sweetness. What is the biochemical basis for this procedure ?

2. The enzymatic activity of lysozyme is optimal at pH 5.2.



The active site of lysozyme contains two amino acid residues essential for catalysis : Glu^{35} and Asp^{52}. The pK_a values of the carboxyl side chains of these two residues are 5.9 and 4.5, respectively. What is the ionization state (protonated or deprotonated) of each residue at the pH optimum of lysozyme ? How can the ionization states of these two amino acid residues explain the pH-activity profile of lysozyme shown above ?

3. Which of the following statements is not universally applicable to enzymes :

(*a*) they generally work very rapidly.

(*b*) they can catalyze a reaction in both directions

(*c*) they are not used up during a reaction.

(*d*) they will bind one substrate only.

(*e*) they are proteins.

4. Why does a wound on tongue heal faster than those on other parts of the body ?