CHAPTER - I

INTRODUCTION

Enzymes form a unique group of proteins which catalyse a variety of chemical reactions. It is generally accepted that the specific chemical functions carried out by enzymes are mediated by special localised regions on the enzyme molecule that are called active sites (1,2,3). The active sites are the three dimensional structure of amino acid residues specially adapted to their specific ligands. The identification of the amino acid residues which form the active site of an enzyme and the elucidation of the mechanism by which the enzyme performs its specific action have been the object of several investigations over the last few decades.

In discussing the structure of proteins, it is customary to use the following nomenclature to describe the structure at various levels of organisation; (a) primary structure, (b) secondary structure, (c) tertiary structure and (d) quaternary structure(4). The primary structure refers to the sequence of the amino acid residues in a protein molecule. Todate the primary structure of ten to twelve proteins has been established (5-14). The primary structure appears to be of considerable importance in determining the properties of a protein molecule (15). It is also believed that the primary structure of a protein has a decisive role in determining its secondary and tertiary structures (16).

The secondary structure refers to the hydrogen bonded structure of proteins which leads to helical or other ordered structures. The backbone of a protein molecule consists of peptide

linkages. The C=O group of the peptide can form hydrogen bond with N-H group of another peptide group either in the same molecule or from the adjacent molecule. Although several ordered structures are possible, the two important types are (1) structure in which hydrogen bonds between the C=O and N-H groups are formed between parallel peptide chains and (2) those in which the hydrogen bonds are formed between C=O and N-H groups of different residues in the same peptide chain. The first type of structure is referred to as pleated sheet structure. In the second type the most important is the α-helical structure which many proteins of biological importance possess. The α-helix first described by Corey and Pauling (17) consists of a repeat pattern in which each NH group is hydrogen bonded to a C=O group three residues beyond it in the chain and each C=O to an N-H three residues away. The NH---OC distance varies between 2.7 and 2.9 Å, and the helix makes a complete turn for every 3.6 - 3.7 residues.

Although hydrogen bonding appears to be the primary force for the ordered structure of a protein molecule, other types of bonds may also be involved. One such type of bond may be the hydrophobic bond between the nonpolar side chains of a protein molecule. Many globular proteins are known to possess a helical structure. However, they may not consist entirely of a helical structure; they may have segments of helix and unorganised structure (random coil). It is known both from X-ray and Optical rotatory dispersion (O.R.D.) data that hen egg white lysozyme contains α-helix, random coil and pleated sheet structures (18,19).

The tertiary structure of a protein refers to the folds and bends in the protein molecule which gives a three dimensional

structure. The chemical bonds which lead to such folds appear to be disulphide linkages, salt linkages between COOH groups of aspartyl and glutamyl residues and NH_2 groups of lysine residues, and tyrosyl carboxylate linkages (20).

A number of proteins are known to consist of more than one polypeptide chain per molecule. These chains can be dissociated from each other without drastic chemical reaction. Apparently the forces responsible for the association of the polypeptide chains are weak. The assemblage of the polypeptide chains (subunits) to give the biologically functional macromolecule is referred to as quaternary structure (4).

Active sites of proteins:

The characterisation of the amino acid residues which form the active site of an enzyme has been a major goal of protein chemists. The active site of an enzyme may be divided into two structural components - the catalytic site and the binding site. The latter site is called regulatory site also. The binding of the specific ligands to the binding or regulatory site on the protein molecule affects the catalytic function of the enzyme indirectly (3,21,22).

In the active site the amino acid residues may be categorised into two groups namely contact and non-contact amino acid residues. Contact amino acid residues may be defined as those whose atoms come within a bond distance of a substrate or an inhibitor. The non-contact amino acid residues are those whose atomsare farther than about 2 Å from the ligand. Interaction between contact and non-contact residues may occur when the enzyme binds the substrate (or inhibitor) (3).

The active site of an enzyme may not be a rigid three dimensional structure in a protein molecule and small changes in the conformation of the active site may occur in the presence of a ligand. It has been postulated that an enzyme in solution may exist in several conformations and that in the presence of the substrate the most appropriate conformation to carry out the catalytic reaction may be stabilised. Thus the substrate molecule itself appears to have an effect on the conformation of the protein molecule. This theory is referred to as the "induced-fit" theory (23-26).

The concept of a flexible enzyme ~~(26)~~ conformation (26) is very useful in explaining the regulation of enzyme activity. Although conformational changes are not necessary to explain all phenomena associated with regulatory control, such changes are certainly visualised in the most common type of regulation, namely, feed back inhibition (26-28). Feed back inhibition has been shown to be caused by the last product in a metabolic pathway acting on the first enzyme in that pathway. It has been demonstrated that a site separate from active site (catalytic site) is present on the protein molecule at which the inhibitor molecule is bound. This site which lacks catalytic activity and which is topologically separate from the catalytic site can however, exert regulatory control over the catalytic site. This site is referred to as allosteric site (29). The presence of binding sites as distinct from active sites has been clearly demonstrated in the case of a few enzymes such as aspartyl transcarbamylase (21), glutamine synthetase (26,30,31) and ribonucleoside-diphosphate reductase (32-34). For example, Gerhart and Schachman (22) were

able to separate the subunits of aspartyl transcarb-amylase and demonstrate that there were two types of poly-peptide chains. One of these had a molecular weight of 1×10^5 and was found to contain a catalytic site. This could by itself give a catalytically active protein which however lacked regulatory control. The other had a molecular weight of 2.7×10^4 and contained the allosteric site (binding site) which was found to bind cytosine triphosphate (CTP). This sub-unit lacked catalytic activity. Further, by measurements of chemical reactivity and sedimentation in an ultracentrifuge Gerhart et al (35, 36) were able to observe conformational changes induced by the substrate namely carbamyl phosphate, and ligands which resembled the substrate. They also demonstrated the conformational effects induced by the allosteric effectors CTP and bromo CTP.

Methods for elucidating the active sites of an enzyme:

The information on the amino acid residues which form the active site of an enzyme has mostly been obtained using two major experimental approaches, namely (1) X-ray crystallography and (2) Chemical labelling methods. First we shall discuss the X-ray crystallographic method.

X-ray crystallographic method.

The elucidation of the three dimensional structure of proteins using X-ray diffraction method is one of the major achievements of modern science. Todate, the three dimensional structure of 10-12 crystalline proteins has been determined. This has become possible because of the discovery of the isomorphous

replacement method by Perutz (37). In this technique bulky atoms or groups are attached (tagged) to specific groups, such as SH, in a protein molecule. From the X-ray diffraction patterns given by the tagged and untagged protein, the three dimensional structure is arrived at. It is beyond the scope of this review to deal with the details of X-ray crystallographic method. The discussion is restricted to enumerating the type of information obtainable from X-ray studies. These will be illustrated with a few examples. The three dimensional structure of ribonuclease A (RNase-A) (38) and ribonuclease S (RNase-S) (39) has been elucidated. RNase-A is a protein of molecular weight 13,683 and consists of 124 amino acids. It contains 4 disulphide bonds. Ribonuclease splits ribonucleic acid at the phosphodiester linkages. RNase-S is obtained by cleavage of RNase-A by the bacterial enzyme subtilisin. This splits the peptide bond between the 20 and 21 amino acid residues giving RNase-S and a peptide of 20 amino acid residues. The two proteins (RNase-A and RNase-S) have very similar structures. The salient features of RNase-S are that it has 15% α-helical structure and that it has a striking hydrophobic core which forms about 15% of the total structure. In the three dimensional structure of the enzyme, the residues near the nucleotide binding site have been identified. This was aided by the knowledge of the amino acid sequence of the protein (40). His-12 and 119 are close to the uridine-2-phosphate binding site. The uracil moiety fits into a groove bounded by the hydrophobic side chains Val-43 and Phe-120 and the OH function of Thr-45. Other nearby residues are Glu-11, Asn-44 and Asp-121. These results are in good agreement with the chemical data on the

nature of the active site of RNase. The side chains of Lys-7 and 41 are not clearly defined. The binding of small molecules to RNase-S does not cause any conformational change.

Chymotrypsin.

The three dimensional structure of α-chymotrypsin at 2 Å resolution has been obtained by Matthews et al (41) and of π, δ and γ- chymotrypsins at 5 Å resolution by Kraut et al (42). The amino acid sequence has also been determined. Chymotrypsin has a molecular weight of 25,000 and consists of a single polypeptide chain with 241 amino acids. The polypeptide chain consists of three portions which have been designated as A, B, C chains. These chains are held together by two of the three disulphide linkages. Chymotrypsin is a serine active enzyme. As deduced by the three dimensional structure, His-57 and Asp-194 are adjacent to the active site Ser-195, while Met-192 is nearby. The interaction of Asp-194 with the positively charged α-amino group of Ileu-16 is striking. The involvement of Ser-195; His-57; Ileu-16 in the active centre had been predicted by chemical studies. Thus X-ray crystallographic studies corraborate the chemical findings. Unlike some other proteins, chymotrypsin does not have a deep cleft at its active site.

The above examples show that it is possible to identify from the three dimensional structure of an enzyme the amino acid residues involved in the active site and the mutual interaction between the various residues. The other proteins whose three dimensional structure has been elucidated are myoglobin (43,44), hemoglobin (44-48), lysozyme (18, 49-51), carboxypeptidase (52-54), carbonic anhydrase (55), papain (56), cytochrome-C (57) and

elastase (58, 59). Since the present investigation concerns lysozyme, the three dimensional structure of this enzyme will be discussed later.

Chemical labelling method:

This chemical approach to identify the amino acid residues in the active site of an enzyme has been used extensively. The principle of this method is as follows:-

First a labelled chemical is covalently attached to some amino acid residue(s) within the active site(s) of an enzyme. Then the labelled enzyme is degraded by chemical or enzymic methods and the labelled peptide fragment(s) originating from the active site are isolated and characterised. When such a covalent labelling is achieved, the (reversible) binding of the specific ligand to that site may be expected to be sterically or otherwise inhibited. Thus if for every mole of labelled compound covalently attached per mole of protein a stoichiometric loss of activity occurs, then this is a strong evidence that the label is attached to a residue in the active site. For a homogeneous protein complete inactivation should occur when the number of moles attached per mole of protein equals the number of active sites per protein molecule. This is an important criterion of active site labelling.

However, this technique is not entirely free of ambiguity for the following reasons:- (1) a loss of activity is not necessarily an all-or-none phenomena. If catalytic activity is being measured kinetically, a reduction in rate and an apparent loss in activity may occur because of an increase in the Michaelis constant of the modified enzyme for the particular substrate.

This may be due to a partial impairment of all the active sites or complete impairment of a fraction of them, (2) the attached group may sterically interfere with the binding of the substrate, although it might not be attached to a group in the active site, (3) In certain circumstances the attachment of a label in the contact residue of active site may not lead to inactivation. If the label is a small atom or group and does not seriously interfere sterically with the binding of the specific ligand or perturb the chemical properties of active site, then limited or no inactivation may occur (3).

The methods of active site labelling may be broadly classified into three groups (1) unique labelling (2) differential labelling and (3) affinity labelling. The principle of unique group labelling is as follows:- The catalytic sites of many enzymes generally contain chemically unique or unusually reactive amino acid residue. The unique seryl residue in the active sites of esterases is a familiar example (3). It is possible to take advantage of the unique reactivity of such groups to tag them in a number of ways. The tagging can be done either with a substrate or a substance which resembles the specific substrate or a non-specific reagent. For example, the enzyme phospho-glucomutase which catalyses the glucose-1-phosphate to glucose-6-phosphate can be labelled with phosphate (60). It has been shown with ^{32}P-labelled substrate that phosphoryl serine could be isolated from the tagged enzyme (61). If the covalent intermediate formed between the active site residue and the group of specific substrate is too unstable, a corresponding intermediate may be formed with a

group of a substance which resembles the substrate but differs from it in many important respects. An outstanding example of this type of labelling is the reaction of di-isopropyl fluorophosphate (DFP) and similar compounds with certain esterase enzymes. The studies of Balls et al showed that inactivation of chymotrypsin and trypsin with DFP was stoichiometric (62,63). It has subsequently been shown that DFP and similar compounds form a stable phosporyl bond with unique serine residues of a large number of esterases. In the category of labelling by non specific reagents, the particular amino acid residue involved in the active site may be labelled by a reagent which is neither a specific substrate nor does it resemble the substrate. The modification of groups like cysteinyl, tyrosyl, tryptophyl, histidyl, lysyl and carboxyl groups by a variety of reagents to determine their role in active site of an enzyme is an important approach by itself and this procedure will be discussed in detail in a later section.

Differential labelling.

The one advantage of the differential labelling method is that it can be used not only for labelling the active site but also the regulatory site. The principle of the method is as follows:- The protein is treated with a non-specific reagent in the presence or absence of an excess of the competitive inhibitor of the protein active site. First the exhaustive modification of all similar residues on the outside of active site is achieved with the reagent in the presence of the inhibitor; then the inhibitor is removed. The radio active form of the same reagent is added and the reaction carried out. This labelled reagent should react preferentially with the residue in the active

sites that had been formerly protected. Although the method of differential labelling appears to be straight forward it has several theoretical and practical limitations. The protective effect which is a very important aspect of the method is not absolute and depends on a number of parameters. Clearly the protective action depends upon the equilibrium (association) constant for the formation of the reversible complex between the enzyme and inhibitor. If this constant has a low value, in other words the strength of binding is low, then the protective action is poor. Also the mere binding of the inhibitor to the active site may induce a conformational change in protein molecule which may conceivably deactivate a group which may not be in the active site. The differential labelling technique has been used in identifying the antibody active sites of several proteins (64).

Affinity labelling.

In this technique a labelling reagent for a particular active site is designed by virtue of its steric complimentarity of the active site. The reagent first combines with the active site specifically and reversibly and by virtue of a suitably small reactive group on the reagent it can then react with one or more amino acid residues in the active site to form irreversible covalent bond. The formation of the initial reversible complex increases the local concentration of the labelling reagent in the site as compared to its concentration in free solution and the reaction with a group in the site is markedly favoured. An example of the use of affinity labelling is the reaction of chymotrypsin with chloromethyl ketone derived from N-tosyl-L-phenylalanine (65). The native enzyme was modified to the extent of 1 mole of reagent per mole of protein. The labelled enzyme

was completely inactive. This affinity labelling technique has been used in identifying the antibody active sites of several proteins (66).

Labelling with non-specific reagents.

One of the approaches that has been widely used to identify the groups that are necessary for the activity of an enzyme is the chemical modification of the various groups and to study the effect of such modification on the enzyme activity. The first step in such a study is the preparation of a chemically modified enzyme. After specifically modifying a group its effect on the enzyme activity may be estimated. Table 1 gives the chemical modification to which each group can be subjected and the reagents that have been used in the chemical modification of these groups (3, 67, 68).

It would be beyond the scope of this review to describe in detail the various chemical modifications to which different enzymes have been subjected to and the effect of such modification on the activity of these enzymes. Therefore only the work on hen egg white lysozyme will be described.

L Y S O Z Y M E

Lysozyme.

Hen egg white lysozyme is a protein of a molecular weight of approximately 15000. It is composed of 129 amino acid residues joined together in a single polypeptide chain having lysine as N-terminal and leucine as the C-terminal amino acid residues (69). It contains no prosthetic group and does not require metal ions or cofactors for its action. The entire catalysis and specificity must therefore be affected through the amino acid residues. It has

TABLE No. 1

NON-SPECIFIC LABELLING REAGENTS FOR PROTEINS

Amino acid	Reactive group	MODIFYING REAGENT	
		Monofunctional reagents	Bifunctional reagents
Asp; Glu; and C-terminal amino acid	COO^- groups	Methanol HCl; Carbodimide nucleophilic procedure	
Lys* and N-terminal amino acid	Amino groups	Ethyl acetamidate; succinic anhydrides; N-carboxy-α-amino anhydride; propylene oxide; O-methyl iso-urea; acetic anhydride; phenyl isocyanate; iodo-acetamide; formaldehyde acetamide; iodo acetic acid; FDNB; N-ethyl maleimide (NEM)	Bifunctional isocyanates. Bifunctional acylating reagents. Bifunctional imidoesters. Bifunctional aryl halides. Bifunctional alkyl halides.
His*	Imidazole group	Iodine; oxidation by H_2O_2; photo-oxidation (in the presence of methylene blue); N-bromo-succinamide (NBS); FDNB; NEM	Bifunctional alkyl halides. Bifunctional aryl halides.
Arg	Guanidyl and Amide groups	Formaldehyde + acetamide; Formaldehyde + alanine	
Cystine and Cysteine*	S-S and SH groups	NBS; P-mercuribenzoate (PMBG); FDNB; propylene oxide; P-chloromercuri-benzoate (PCMB); iodine; iodo-acetic acid; NEM; FDNB; H_2O_2; acetic anhydride; Phenyl isocyanate.	N-substituted maleimide derivatives. Bifunctional alkyl halides Bifunctional aryl halides

Tyr*	Phenolic	Propylene oxide; iodine; acetic anhydride; photo-oxidation (in the presence of methylene blue); H_2O_2 NBS; FDNB; succinic anhydride	Bifunctional aryl halides
Ser and Thr	Hydroxyl groups	Di-isopropylfluorophosphate (DFP)	
Trp	Indole groups	Oxidation with H_2O_2; photo-oxidation (in the presence of methylene blue); NBS; Ozonisation	
Met	Thioether groups	H_2O_2; photoxidation (in the presence of methylene blue); NBS; iodoacetic acid	

* Amino acid residues which can be modified by bifunctional reagents.

been shown both by X-ray diffraction studies and optical rotation dispersion techniques that lysozyme consists of α-helix, β-pleated sheet structure and random coil. Particularly striking in the lysozyme structure are the large regions without any ordered secondary structure (18, 19, 70).

Enzymic activity.

Lysozyme acts on the bacterial cell walls and also on low-molecular-weight substrates which contain N-acetyl-D-glucosamine (NAG) and N-acetyl muramic acid (NAM). The first step in lysis of bacterial cells by lysozyme seems to be an interaction between the positive charges of lysozyme and the negative charges on the surface of the bacterial cells. The second step is the hydrolysis of the β-1, 4 glucosaminide linkage in the polysaccharide of the cell wall. The last step is the dissolution of the damaged cell wall (71). Any treatment which increases acidity of a lysozyme (and therefore decreases basicity) decreases lytic activity (72).

Primary structure of lysozyme.

Lysozymes are widely distributed enzymes. They occur in animals, plant tissues, secretions, egg white from different birds and even in viruses (69, 72, 73). Of all the lysozymes, hen egg white lysozyme has been thoroughly studied and its primary, secondary and tertiary structures have been determined. Lysozymes from any of the above mentioned sources split the chemical skeleton of bacterial cell walls by hydrolysing the β(1→4) linkages between NAM and NAG. The amino acid composition of hen egg white is given in Table 2 and the primary structure in

TABLE No. 2

AMINO ACID COMPOSITION OF HEN EGG WHITE LYSOZYME[73]

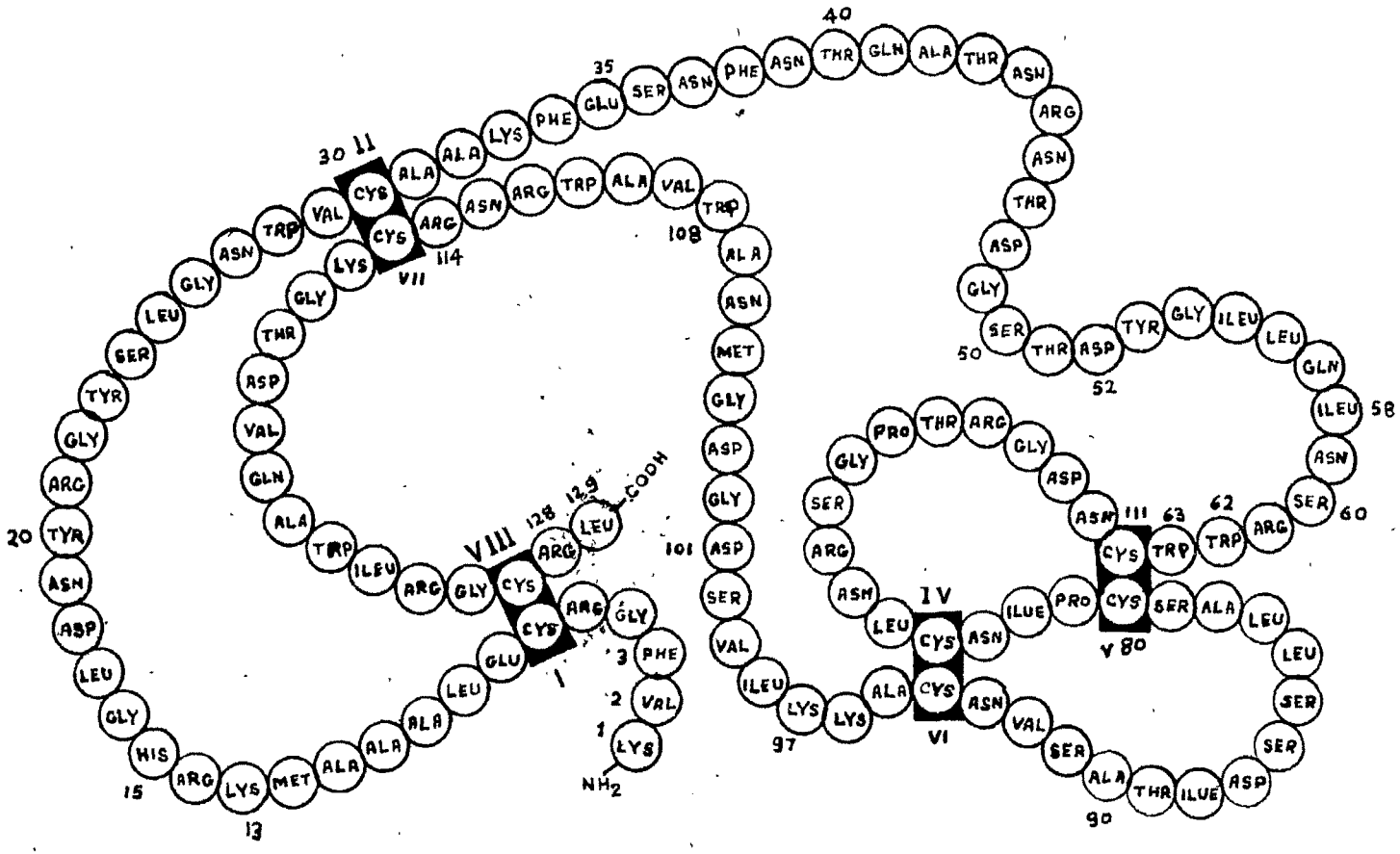
Amino acid	No. of Residues per Mole of lysozyme
Aspartic	21
Threonine	7
Serine	10
Glutamic	5
Proline	2
Glycine	12
Alanine	12
Valine	6
Cysteine	8
Methionine	2
Isoleucine	6
Leucine	8
Tyrosine	3
Phenyl alanine	3
Tryptophan	6
Histidine	1
Arginine	11
Lysine	6

Fig. 1 (6). Lysozymes of different origins differ in their primary structure although they exhibit qualitatively the same biological activity. Lysozymes from different organs of the same species also vary in amino acid composition. For example, lysozyme from human saliva and placenta have different amino acid composition (72). These differences exist mainly in the proportion of Glu, Pro, Arg and aromatic amino acid.

In goose egg white lysozyme the amino acids Lys, His, Tyr are more in number whereas Trp residues are less. It has the same molecular weight as hen egg white lysozyme. It is active only towards M. lysodeikticus, but has no ability to hydrolyse a pentasaccharide derived from chitin. Canfield (75) has attributed this to a difference in the structure of the active site of this lysozyme from that of hen egg white lysozyme. All lysozymes have a single polypeptide chain.

X-ray structure and nature of active site of hen egg white lysozyme:

X-ray analysis of lysozyme shows that the inside and outside of the molecule are less easily defined than they are in myoglobin in which the interior of the molecule consists almost exclusively of hydrophobic side chains. The lysozyme molecule appears to have a hydrophobic spine consisting mainly of ~~the~~ six tryptophan side chains. Three of these tryptophan side chains, 62, 63 and 123, protrude however, beyond the molecular boundary and there are in addition a number of strongly hydrophobic side-chains clearly on the molecular surface (for e.g. Val-2, Phe-3, 34 and Leu-17). The parts of the molecule most shielded from contact with surrounding medium appear to include



PRIMARY STRUCTURE OF HEN EGG WHITE LYSOZYME

FIG.1

Ser-91 and Gln-57. All the lysine and arginine side chains are external (76).

The X-ray studies of the enzyme-inhibitor complexes have revealed that NAG and NAM are bound to lysozyme in the pronounced cleft which is a marked feature of the enzyme structure. The other competitive inhibitors di-NAG and NAG-NAM also bind in the cleft nonspecifically. The binding of tri-NAG in the cleft, involves just fits three sugar rings which are linked together and are arranged in a line.

The trisaccharide substrate, lying in the cleft is bound with a number of amino acid residues through specific contacts. There are six important hydrogen bonds which are identified by number (49). The most critical of these interactions appear to involve the acetamide group of sugar residue-C (third from top) whose carbon atom I is not linked to another sugar residue. There are hydrogen bonds from the CO group of this side chain to the main chain NH group of amino acid 59 (Asn) in enzyme molecule (bond No.I) and from its NH group to the main chain CO group of residue 107 (Ala) in the enzyme molecule (bond No.2). Its terminal CH_3 group makes contact with the side chain of residue 108 (Trp). Hydrogen bonds (No.3 and No.4) are also formed between two oxygen atoms adjacent to carbon atoms 6 and 3 of sugar residue C and the side chains of residues 62 and 63 (both Trp). Hydrogen bond (No.5) is formed between the acetamide side chain of sugar residue A and residue 101 (Asp) in the enzyme molecule. From residue 101 there is a hydrogen bond (No.6) to the oxygen adjacent to carbon atom 6 of sugar residue B. These polar interactions are supplemented by nonpolar interactions.

Among nonpolar interactions, however, are those between sugar residue B and the ring system of residue 62. These deserve special mention because they are affected by a small change in the conformation of the enzyme molecule that occurs when the trisaccharide is bound to it. The side chain of residue 62 moves about 0.75 Å towards the position of sugar residue B. Solution studies suggest that the interactions in the crystal phase are similar to those in solution.

The nature of the predicted model for enzyme mechanism.

Model building shows that the cleft in the surface of the lysozyme can accommodate six NAG molecules designated A, B, C, D, E, F. Amino acids surrounding NAG residues A, B and C of the trisaccharide were located by X-ray.

The relationship between these sites and various inhibitors is summarised.

Inhibitor	Binding site
NAG	C and D
NAM	D
Di-NAG	B-C and G-D
NAG-NAM	C-D and G-D
tri-NAG	A B C

Four of these sites are roughly in line and lie in the length of the cleft but the fifth (G) is to one side of this line. Since NAG residues B and C in tri-NAG lysozyme complex are part of the stable complex, hydrolysis between D and E is more likely in the hexamer-lysozyme complex. The bond between D and E residues is surrounded by Glu-35 and Asp-52 residues on either

side of the β (1→4) linkage (between NAG residues D and E). Both side chains have markedly different environments. Glu-35 is found to be in the non-polar region near the bottom of the cleft, its neighbour being Ala-110, Glu-57 and Trp-108. Asp-52 lies in the polar region on the left side of the cleft, and appears to be involved in the complex net work of hydrogen bonds that involve the amino acid residues Asn-46, Ser-50 and Asn-59.

Studies on the role of amino acid residues in the enzyme molecule:

Carboxyl groups.

From electrometric titrations, it has been concluded that three carboxylic groups of glutamic acid are unavailable for titration. It has been suggested that the unavailable carboxylic groups might be involved in intramolecular interaction with lysine residues (77). As described earlier a role for Glu-35 in the lysis of substrate has been predicted (49, 51, 70). X-ray data also show that all the eleven carboxyl groups of lysozyme are exposed on the surface. Further, the involvement of Asp-52 in the catalytic mechanism of lysozyme has also been proposed (49, 51, 70).

Esterification of lysozyme leads to the distortion of the catalytic site but the position of the esterified residues has not been determined (2, 78, 79). Modification of carboxylic groups by carbodimide nucleophillic procedure has shown that all the carboxyl groups except Glu-35 can be modified under normal experimental conditions. The presence of substrate on the enzyme molecule protects the Asp-52 and an enzyme with over 50% enzymic activity is obtained in which all the COO— groups

except Asp-52 and Glu-35 have been converted to $\overset{O}{\overset{\|}{C}}-NHCH_2SO_2^-$ derivative. Subsequent treatment with the reagent after removal of substrate leads to a modifion of Asp-52 and loss of enzyme activity. These results eliminate all COO^- residues except Glu-35 and Asp-52 as potential catalytic or binding groups. These results strongly support the hypothesis of Phillips and coworkers deduced from crystallographic data that a COO^- group is involved in the lytic action of lysozyme. However, in this investigation, the effect of COO^- modification on the conformation of the native enzyme has not been studied(80).

Tyrosine.

Lysozyme has 3 tyrosine residues. In titration studies 2 of the 3 residues titrate normally and the third titrates at very high pH values suggesting that one of the tyrosine residues is burried in the interior of the protein molecule. Iodination studies also indicate that two residues react readily and the third residue becomes accessible only after the protein has been denatured or when the reaction is carried out in the presence of denaturing agent like guanidine hydrochloride (GU-HCl) or urea. Neither enzyme activity nor the conformation of the iodine derivatives appears to have been studied (81-84).

Tryptophan.

Lysozyme has 6 tryptophan residues. With the solvent perturbation technique, it was found that all the tryptophans were exposed on the surface of the molecule; such exposure was not dependent on pH in the range 1.8 to 7.0 or on urea concentration

upto 8M (85). X-ray data indicate that Trp-62, 63 and 108 are situated on the surface of the cleft. Studies on photo-oxidation, iodination, oxidation by hydrogen peroxide, ozonisation and NBS treatment of lysozyme suggest that tryptophan has some essential function in the enzyme activity (biological). Recent studies show that tryptophan residue(s) is involved in the binding of substrate or inhibitor by the enzyme. Hartdegen and Rupley (86,87) and Rupley (88,89) have shown that treatment of lysozyme with iodine results in facile oxidation of Trp-108 (tryptophan to oxi-indole). Extensive iodine-oxidation results in oxidation of Trp-108 as well as Trp-62 (90). Modification of the latter results in inactivation of the enzyme. During ozonisation two indole residues of Trp-108, 111 are modified into formylkynurenine. Oxidation of these indole residues did not affect the enzyme activity, pH optima, extent of inhibition by NAG or heat stability of the enzyme leading to the conclusion that the essential conformation of the molecule is not affected by oxidation of Trp-108 and Trp-111 and that they are not essential for the maintenance of conformation and biological activity (91, 92).

NBS has also been used by many workers in the hope that it may selectively oxidise the indole group moieties of tryptophan residues (69, 93-96). Treatment with NBS resulted in progressive oxidation of tryptophan residues accompanied by a parallel decrease in enzyme activity. In completely-inactivated lysozyme, Trp-62 is oxidised by NBS without any accompanying conformational change thereby suggesting its implication in enzyme activity. (94, 95). However, it is now known that NBS is a non-specific

reagent, and earlier data obtained with NBS would need a re-appraisal (96). X-ray data show that Trp-62, 63 and 108 interact with NAG, di-NAG and tri-NAG (51).

The role of Trp-62 in the activity of the enzyme was further confirmed by the interaction of the enzyme with the cationic detergent, dimethyl-benzylmyristyl ammonium chloride (DBMA) (97). The enzyme readily reacts with 0.05% DBMA at pH 5.6 and is completely inactivated without alteration in its conformation.

Arginine.

Lysozyme contains 11 arginyl residues. X-ray studies show that all arginyl residues are present on the surface of the molecule and that Arg-114 is involved in intermolecular contacts (70). Glyoxal reacts readily with all the 11 arginines. However, neither the enzyme activity nor conformation of the modified enzyme has been studied (98, 99). Modification of 7 out of the eleven arginyl residues with 2,3-butanedione (100) reduced the activity of acetyl lysozyme towards M. lysodeikticus. However, its activity towards the tetramer of NAG was not reduced. Under the same experimental conditions acetyl lysozyme was active both towards M. lysodeikticus and the tetramer of NAG. Since modification of the guanidyl groups of acetyl lysozyme reduces its activity towards the bulky substrate M. lysodeikticus but not towards the low molecular weight substrate it may be concluded that steric hindrance may be the reason for the decrease in activity.

The role of Arg-128 in the enzymic activity has been looked into from another angle. Arg-128 is located next to the C-terminal amino acid leucine. The enzymatic cleavage of lysozyme by carboxy

peptidase gives a product which has the same conformation and enzyme activity as the native enzyme suggesting that Arg-128 is not essential either for activity or for maintaining the conformation (69).

Histidine.

Lysozyme contains one histidine residue. Reaction with iodoacetate at alkaline pH values converts it into carboxy methyl histidine (101). This does not cause any loss in enzyme activity suggesting that the single histidine residue is not involved in the catalytic site of the enzyme. X-ray crystallographic studies show that the His-15 residue is far away from the cleft region and is not involved in enzyme activity (51).

It has been shown that lysozyme dimerises in the pH range pH 5 to 9. Dimerisation does not appear to lead to any changes in the conformation of the molecule as could be detected by O.R.D., viscosity and partial specific volume measurements (102-104). However, the enzyme activity of the dimeric form has not been measured. The imidazole group of the histidine residue has been implicated in the dimerisation reaction (73). It may be worth mentioning here that duck egg white lysozyme has no histidine but has 5-6 times more lytic activity than egg white lysozyme (105).

Lysine.

Lysozyme has 6 lysine residues per molecule. From titration studies of native, deaminated and guanidinated lysozyme it has been concluded that some of the lysine residues are involved in interaction with two carboxylic groups within the molecule (77). X-ray studies show that amino groups of Lys-13 forms a salt bridge with the terminal carboxylic group (70). This residue does not appear to be essential for enzymic activity (106).

Carboxymethylation (101) experiments show that 3 lysine residues are easily available for reaction with the reagent while the other ~~3~~ residues become available only after the conformation of the molecule has been drastically altered with a denaturing agent (98, 106-110). Based on this observation, it has been suggested that atleast 3 lysine residues are essential for maintaining the molecular conformation. The derivative in which 3 lysine residues have been carboxymethylated retained full enzyme activity. However when all the six groups were modified the product was inactive. These studies do not reveal whether the loss of activity is due to the break down of the molecular conformation of the enzyme or whether the lysine residues are involved in the enzyme activity. At any rate 3 of the six residues do not appear to have any role either in maintaining the conformation or in the activity.

Guanidination of all lysine residues has no effect on enzyme activity (111). The acetylation of lysozyme has been reported to result in progressive inactivation of enzyme (112). Acetylation of one third of the total lysine residues causes no loss in activity whereas acetylation of all six residues causes total inactivation (71). These results have been interpreted as that the positive charge on lysine residues may be required for interaction of the enzyme with the cell wall of substrate M. lysodeikticus for optimum enzyme activity. However, this interpretation is at variance with other results (101) in which it has been shown that carboxymethylation of lysine residues does not lead to any loss in enzyme.

The effect of polyalanylation of lysine residues on the enzyme activity of lysozyme has been studied. This modification does not alter the conformation of the enzyme as could be detected by O.R.D. and circular dichroism measurements. However it leads to a loss in activity (towards glycol chitin) in proportion to the number and length of side chain of the polyalanine residues attached to the molecule. The loss in activity is due to the inhibition of the enzyme-substrate complex formation by the polyalanyl side chains. These facts suggest that the polyalanyl side chains sterically hinder the interaction of the enzyme molecule with substrate (109). This is supported by the observation that the effect of polyalanyl side chains on enzyme activity of lysozyme toward M. lysodeikticus is more marked than on its activity towards glycol chitin. Incidentaly these results imply that not only the active site but some other sites on the enzyme molecule are also involved in the interaction of lysozyme with M. lysodeikticus.

The hypothesis that the positive charge on lysine residue is of importance in binding the substrate M. lysodeikticus is supported from another investigation also. When the amino groups of lysine residues are modified with ethylacetamidate (100) [a procedure by which the positive charge on amino group is not altered] this does not cause any change in activity of enzyme; if anything it seems to enhance the activity.

The introduction of a new cross link between two lysine residues by reacting lysozyme with phenolic disulphonyl chloride does not appear to affect either the conformation or the activity of the enzyme (113-115). Recently the blocking of

lysine residues of lysozyme with several reversible blocking reagents has been reported (116). Derivatives obtained by complete modification of amino groups by succinic, tetra fluoro-succinic, maleic or citraconic anhydrides or by reaction with dikene were totally inactive. When the modifying groups were removed the enzyme regained its original activity. However, in this study no attempt seems to have been made to determine the effect of progressive modification of various residues on activity.

The role of N-terminal lysine in the enzyme activity has been looked into from another angle. The enzymatic cleavage by amino peptidase (117) gives a product which lacks the three amino acids from the N-terminal, which has same conformation and enzymic activity as the native enzyme suggesting that N-terminal lysine is not essential either for activity or conformation.

Enzyme activity of hen egg white lysozyme after various treatments is summarised in Table No. 3.

THE OBJECT AND THE OUTLINE OF THE PRESENT INVESTIGATION

The preceding discussion shows that the exact role of the six lysines in the activity of the egg white lysozyme has not been clearly established. We therefore desired to investigate this question further and to find out whether or not the lysine residues are essential for the activity of this enzyme. Since lysozyme has two pH optima for its activity, it is conceivable that in the neutral and in the alkaline region different amino acid residues may come to constitute the active centre, particularly because of the intramolecular forces affected by pH.

TABLE No. 3

ENZYME ACTIVITY OF HEN EGG WHITE LYSOZYME AFTER VARIOUS TREATMENTS

Treatment	Amino acids or groups modified	Activity	Reference
Oxidation	Cys, Met, Trp	Inactivation	112
Photooxidation	His, aromatic amino acids	Reduced activity	118
Ozonisation (slow in formic acid)	2 Trp modified	Active	91
	6 Trp modified	Inactive	92
Reduction	Cys modified	Reversible inactivation	119, 120
Esterification	Free carboxyl groups	Depends on the reagent	112
Acetylation	Free amino groups	Progressive inactivation	111, 112
Guanidination	Lys	Active	111
Polyalanilation	Lys	Inactive	109
Ethyl acetamidate derivative	Lys	Active	100
Succinic and tetra-fluro-succinic etc.	Lys	Inactive	116
Iodoacetic acid	Partially Met, Lys	Formation of closely related active substances	121
Iodination	His, aromatic amino acids	Inactivation reactivation possible	87, 89, 90, 112

Xanthination	Trp	Inactivation	122
NBS	Mainly Trp	Progressive inactivation	87, 95
NBS	62 Trp	Inactivation	94, 95
Photo-oxidation	Trp (position not known)	In enzyme activity	123
H_2O_2	Trp (position not known)	"	124
Iodine oxidation	Trp (position not known)	"	87
Iodine oxidation	108	Substrate binding	86, 89
Iodine oxidation and X-ray analysis	108, 62	"	90
X-ray analysis	62, 63, 108	"	51
Ozonisation	28, 62, 63, 123	"	91, 92
Carboxy peptidase	Release of Arg, Leu. OH	Active	69
Amino peptidase	Release of H. lys-val	Active	117

And probably one or the other out of the six lysine residues may be involved (in either pH range) in the catalytic activity or may be important for maintaining the enzyme conformation. Also, if possible we could determine the number and the position of those residues which may be found participating in the above mentioned functions.

To get detailed information on these aspects we have approached the problem by using chemical modification method. We have modified lysozyme with FDNB and have prepared at two pH values, 7.2 and 9.1, a number of derivatives with varying content of DNP. The two pH regions were chosen for these studies because lysozyme being active in these ranges it was thought that the active site residues may be easily available for participation in the modification reaction. We have then examined systematically the effect of modification on the enzyme activity and on the physicochemical properties of the modified derivatives.

We had other considerations as well for choosing these conditions for preparation of the derivatives. The chemical modification of proteins is usually carried out at alkaline pH in order to facilitate the modification of all the residues in a protein. However, such modification often leads to either complete or partial denaturation of the protein under study. It therefore becomes difficult to know whether the loss of the enzyme activity results from the blocking of a particular residue(s) or it is due to the conformational change in the protein. The implication of the modified residue in the activity or

in the conformation becomes ambiguous; whether the loss of the activity is due to the direct participation of the modified residue in the catalytic function or due to the indirect participation in maintaining the native conformation.

Modifications of the enzymes carried out under mild conditions of pH and temperature, although, lead to the modification of a limited number of residues under such conditions, they usually leave the three dimensional conformation of the enzyme unaltered. And also there is another additional advantage in using mild conditions in that if the modification is carried out with an unspecific reagent, reaction may lead to the modification of a specific residue because of the enhanced reactivity of some of these residues, due to their micro-environment. It was because of the various above considerations modification of lysozyme with FDNB was studied at neutral and alkaline pH values which we thought could provide necessary detailed information regarding the role of the lysine residues in the activity or conformation.

FDNB was chosen for these studies because it easily reacts with several functional groups in an enzyme. Sanger in 1945 first described the use of FDNB for the determination of free amino groups of insulin (125). After this pioneering work, this method has been used in the identification of the N-terminal amino acids of several proteins (126-129). The reaction of FDNB with protein can lead to the modification of any or all of the following functional groups; sulfhydryl, imidazole, and ε-amino and phenol group. Apparently reaction with thioether side chain does not occur. Thus although FDNB and related reagents

appear to be non-specific reagents for protein modification, several reports show that FDNB specifically modifies only the amino residues, provided the reaction conditions are carefully controlled (130-136). Besides, aromatic reagents containing additional activating groups, e.g. nitro-group, ~~which~~ react in two steps; the reaction is slow and therefore is more selective than acylation. Hence by this procedure it is often possible to modify only one out of many reactive groups. For this reason we have used FDNB for the chemical modification of the lysine residues of lysozyme. Dinitrophenylation of proteins in acidic pH is slow in comparison to alkaline pH. Generally mildly alkaline pH's are used for dinitrophenylation.

In this investigation we have studied the effect of various factors such as time, temperature and concentration of the modifying agent on the reaction of lysozyme with FDNB in order to be able to control the reaction and to prepare the derivatives substituted to varying extent. Besides, as mentioned above it was essential to prepare atleast some derivatives in which little or no change in the conformation occurred due to the introduction of DNP groups into the enzyme molecule. To achieve this objective we had to establish appropriate conditions. The resultant DNP-derivatives were analysed for the extent of dinitrophenylation by three different techniques: namely measurement of UV absorption at 360 mμ, using ^{14}C-labelled FDNB and by the chromatographic identification of the dinitrophenylated amino acids obtained by hydrolysis of the DNP-lysozymes. The enzyme activity

was assayed using M. lysodeikticus as substrate. The effect of modification on the enzymatic characteristics has been examined by determining the pH and ionic strength optima, and by the action of inhibitors and physical and chemical denaturing agents on the activity of the derivatives. Effect of modification on the physicochemical properties was investigated by absorption in UV region, ultracentrifugation, gel filtration, tryptic hydrolysis, chromatography on CM-cellulose and on IRP-64 and electrophoresis on starch gel and disc electrophoresis on polyacrylamide gels.