CHAPTER - V

DISCUSSION
UDPglucose 4-epimerase may be regarded as the model oxido-reductase in many ways. A fairly large number of epimerases are known to need NAD for catalysis (167, 170) but it is only for this epimerase that a specific oxidation-reduction mechanism has been worked out. Also, only for this enzyme from *E. coli* or *S. fragilis* the subunit structure, the stability of the quaternary structure, number of catalytic sites and exact reaction intermediates are known. Mechanistically, this type of epimerase has been shown to be very similar to several other enzymes with no apparent similarity in the overall reactions. Thus, a decarboxylase, that leads to the formation of UDPxylulose from UDPglucuronic acid (172) or an enzyme that converts TDPglucose to TDP rhamnose (169) are known to have similar keto sugar and reduced pyridine nucleotide as reaction intermediates.

Epimerase, therefore, emerges as an important model for all these wide varieties of enzymes and a detailed study of its active site may throw considerable light on the evolution of the tertiary structure of various oxido-reductases and dehydrogenases.

For clarity of understanding, the active site of the native epimerase can be divided into three major regions (Fig. 29). Region A represents the substrate binding domain. Region B represents the catalytic domain constituting the sugar moiety of the substrates and the pyridine moiety of the.
Fig. 29. Depicted model for the different regions of the active site for the binding of substrate and coenzyme.

A - Substrate binding domain
B - Catalytic domain
C - Coenzyme binding domain.
bound coenzyme. In analogy with the dehydrogenases, for which knowledge is extensive (74), the pyridine nucleotide may be assumed to remain in a stretched form and this domain or region C is the coenzyme binding domain.

Any modification that is monitored by the pyridine fluorophore in yeast epimerase (excitation maxima 353 nm; emission maxima, 435 nm) can be assumed to be taking place in the catalytic domain. Based on this principle, Ray and Bhaduri were able to identify at least two conformationally vicinal sulphydryl groups in this region (180,181,187). With a suitable oxidizing agent like diamide, a diisene dicarboxylic acid derivative they could show that a single disulphide bond across the subunits was formed which resulted in the simultaneous and coordinated loss of both coenzyme fluorescence and activity. Reduction of the disulphide bond with 2-mercaptoethanol resulted in nearly complete restoration of both activity and fluorescence. Further studies with N-ethylmaleimide indicated that the formation of the fluorophore is probably dependent on one of these sulphydryl groups and this is not needed for catalysis. The other is involved in catalysis and its modification leads to inactivation of the enzyme.

Since a single molecule of the coenzyme is tightly but probably noncovalently bound to the dimeric apoenzyme (137, 145), properties and binding features of this region could be
studied only after dissociating the holoenzyme and then reconstituting the enzyme with suitable analogues of NAD that then serve as probes for this region. Employing etheno-NAD, a fluorescent analogue of NAD in such a reconstituted system, Samanta and Bhaduri could show the presence of a hydrophobic pocket on the enzyme surface for the adenosine subsite of the coenzyme (193). Presence of elements of a dinucleotide fold could also be guessed, since cibachron blue was shown to bind very strongly in competition with NAD to such a reconstituted apoenzyme (193). With this emerging picture of the active site of UDPglucose 4-epimerase from \textit{S.fragilis} we initiated our modification studies on the enzyme.

With the advent of phenylglyoxal (220) and of more specific arginine modifying reagents like 2,3-butanedione (219) and 1,2-cyclohexanedione (200,202), a large number of enzymes have been examined for the involvement of arginine residues in the various stages of catalytic mechanism (204,215-217,229-238). By using these reagents in concert or independently, it was shown that arginyl residues are involved in the binding of substrates (204,215-217,229), in binding of coenzyme (230), in catalytic activity (231, 232), in energy conservation (233), in the binding of allosteric modulators to regulatory site (239,240) and in the binding of proteins to membranes (234). One plausible
function of active site arginines is its participation in electrostatic binding of the anionic ligand to the active site. Since the substrate of UDPglucose 4-epimerase is negatively charged at cellular pH, we attempted to explore the possible involvement of arginine residues in the function of this enzyme with the available dicarbonyl reagents.

The kinetic method of Levy et al (218) has been extensively utilized in recent years to determine number of reactive amino acid residues involved in enzyme activity. Theoretical limitation of this method was discussed by Hayman and Colman (241). In cases, where a slow reaction of one essential residue is followed by a much more rapid reaction of other essential residues, this type of kinetic analysis may record only the minimal kinetic order and hence the minimal number of residues that can be correlated with loss of activity. Notwithstanding this limitation, the kinetic method provides a powerful tool for the initial identification of active aminoacid residues. The result of such kinetic analysis (Fig. 1, Fig. 3 and Fig. 4) show that all the dicarbonyl reagents are highly effective in inactivating the enzyme and in each case a strictly pseudo-first order kinetics is obtained. Though phenylglyoxal occasionally lacks in specificity, the other two reagents are highly specific for arginine (206,214). The partial but
definite regeneration of activity of 1,2-cyclohexanone inactivated enzyme in nucleophilic buffer and of 2,3-butane-dione inactivated enzyme on removal of borate by dilution (Table 1) provides strong confirmatory evidence in favour of a specific modification of arginine residues on the enzyme surface. Such reversibilities had earlier been demonstrated for other enzymes (200, 216). The reactivity of these reagents vary widely (Fig.5) but in all cases the end product after complete modification is an enzyme that still retains 15% of the native activity (Fig.2). There are some interesting and wide difference in their reactivity towards the active arginine residues and this is reflected in the values of the second order rate constants (Fig.5). Such differences have also been observed for several other enzymes (200, 216).

The reaction order with respect to phenylglyoxal was close to two (Fig.1, Inset) and that with respect to other two dicarbonyl reagents was close to unity (Fig.3, Fig.4, Insets) thereby suggesting the presence of at least one essential arginine residue in the yeast enzyme. The preferential labelling experiment with isotopic phenylglyoxal (Table 4, Tube C) also demonstrates fairly unambiguously that two moles of the reagent are consumed for each mole of the dimeric protein when essential arginine residue(s) is modified. This important difference in stoichiometry between phenylglyoxal and the value obtained by purely kinetic
method for the other two dicarbonyl reagents may result from the variable reactivity of phenylglyoxal to arginine residues on enzyme surface. Some authors have reported 1:1 stoichiometry (204, 215, 216) but Takahashi (220) had shown that the reaction product of phenylglyoxal with free arginine is di (phenylglyoxal) arginine and this 2:1 stoichiometry was also reported by some workers for other enzymes (221, 222). It is likely, therefore, that the 2:1 stoichiometry found for the reaction of phenylglyoxal with the enzyme (Table 4), is also indicative of the presence of only one essential arginine residue in the epimerase. However, the possibility that kinetic analysis for cyclohexanedione and butanedione, fails to detect a second active residue can not be totally excluded and a final definitive answer has to await a careful aminoacid analysis. Considering that the dimeric enzyme has 26 arginine residues, this would be a formidable analytical task and facilities for such work is not available with us at the moment. Inspite of this uncertainty, presence of at least one essential arginine residue for enzyme activity can be clearly inferred from these data.

Direct estimation of NAD content (Table 2) as also the gel-filtration data for the modified enzyme showing a molecular weight of 120,000 (Text) clearly showed that the structural integrity of the holoenzyme remained unaffected and the loss of activity was exclusively due to the
modification of the arginine residue on the enzyme surface. Where such modifications lead to a change in tertiary structure, the direct cause for inactivation remains uncertain. The S. fragilis enzyme was earlier shown to become inactive either due to ejection of the coenzyme from the dimeric apoenzyme on controlled heating (179) or due to dissociation of the dimer on treatment with p-chloromercuribenzoate (174, 177).

The presence of the essential arginine residue at the substrate-binding region of the active site (Fig. 29) was proved by several techniques. Significant protection, as reflected in the decreased inactivation rates, was observed not only for the substrate (UDPgalactose) but for other phosphorylated uridine compounds (Table 3), all of which were shown to be competitive inhibitors for the enzyme by us (Fig. 7) and also earlier by Pal and Bhaduri (184). Excellent inverse correlation between the affinity of the inhibitors and substrate for the substrate binding site and the pseudo-first order rate constant for inactivation could be observed. Uridine, which does not have a phosphate group, was not a competitive inhibitor for the enzyme and presumably did not have any binding interaction with active site. Consistent with this behaviour, it failed to provide any protection against inactivation with phenylglyoxal (Table 3). The complete failure to radiolabel the enzyme
in presence of UMP (Table 4 (a) & (b)) also helps to locate the arginine residue at the site where UMP interacts, i.e. at the substrate binding site. Finally, the experiments with the extrinsic anionic fluorophore ANS, which was earlier shown by Samanta and Bhaduri (199) to be a substrate-binding site directed ligand also confirmed this contention. The enzyme, after modification by phenylglyoxal showed marked reduction in interaction with ANS when compared with the native situation. Prior protection with UMP, however, resulted in the full enhancement of fluorescence for the probe (Fig.9). All these data strongly indicate that the active arginine residue is physically located in the substrate binding region and is probably directly involved in the binding of the substrate or of the competitive inhibitors that have at least one phosphate group attached to the uridine moiety. Earlier arginine residues were shown to interact with the phosphate portion of the substrate or of the coenzymes for a number of enzymes (202,203,236,242). Interestingly, even after complete modification of the arginine residue by any of these dicarbonyl reagents, 15% of the initial enzyme activity was retained and the \(K_m\) for the substrate for the modified enzyme increased significantly. (Fig.6). Obviously, an weak but partially effective interaction can still take place between the substrate and the enzyme.
even after the arginine residue is modified. Such residual catalytic activity has been demonstrated for several other enzymes (55, 243-245).

Though the arginine residue is placed in the substrate binding region, it must also be fairly close to the coenzyme fluorophore. Introduction of a phenyl group and increased hydrophobicity in the region after modification, may be the cause of the three-fold enhancement of this fluorescence (Fig.10). However, since the nature of the fluorescence spectrum remained unaltered, any direct role of the arginine residue in maintaining the fluorophore structure can be eliminated. Such a role for a thiolate anion was assumed by Ray and Bhaduri (192) from their fluorimetric studies with sulphydryl modifying reagents.

We do not have any adequate explanation for the exclusive reactivity of the arginine residue at the active site (Table 4). The dimeric enzyme has a large number of arginine residues (139) and it was expected that at least a few of them would be exposed outwards and would be fairly reactive. An analogous situation was earlier observed by Takata and Fujioka (222) for S-adenosyl homocysteinase from rat liver, when they could show that only two arginine residues at the active site out of the fourteen available per subunit were reactive to phenylglyoxal.
Interestingly, this enzyme, consisting of four identical subunits, also has a tightly bound NAD as the coenzyme at the active site of each of the monomers (238). As the active site arginine is the only arginine residue that is modified by phenylglyoxal, labelling of this residue followed by proteolytic cleavage, provides for the first time a direct method to sequence portions of the active site of this important enzyme.

We discussed earlier that UDPglucose 4-epimerase is the prototype of a class of racemases that needs pyridine nucleotide as an obligatory coenzyme for catalysis (133,137). Mechanistically, all these enzymes can be regarded as oxidoreductases with NAD acting as the initial oxidant, though such a course of reaction has been unequivocally demonstrated only in case of this epimerase. There are, however, important and interesting questions of reaction mechanism and stereochemistry that remain essentially unresolved for this catalytic pathway. An outstanding question that remains to be answered is how the enzyme functions non-stereospecifically in converting both UDPglucose and UDPgalactose reversibly to the common UDP-4-ketopyranose intermediate. In all cases of dehydrogenase reactions that have been carefully scrutinized, both the pyridine nucleotide and the corresponding cosubstrate act stereospecifically (246,247). UDPglucose


4-epimerase is exceptional in that, although it is B-side specific for hydrogen transfer to the nicotinamide ring of tightly bound NAD \((165, 191, 248)\), it is non-stereospecific with respect to glycosyl C-4 of the nucleotide sugar substrates. The question, therefore, is how the enzyme acts non-stereospecifically at this locus to either of the substrates to produce the same intermediate. Employing fluorescent analogues of UMP as reaction probes with the \textit{E. coli} epimerase, Wong and Frey \((166)\) suggested that the non-stereospecific action results from relatively weak binding and hence comparatively free movement or rotation of the glycosyl moiety at the active site. The uridyl pyrophosphoryl moiety of the substrate on the other hand is very rightly bound and serves as the binding anchor for the epimerization process. In a further extension of this work, these authors postulated the critical presence of a prototropic amino acid residue that serves as general acid-base catalyst in the epimerization process as follows \((249)\).
In analogy with some of the dehydrogenases (203), they suggested an histidine residue to be the general prototrop and developed an active site directed affinity label inhibitor for this purpose. Unfortunately, careful study with the inhibitor revealed a generally non-specific labelling at the active site with the major modification taking place, quite surprisingly, in the adenine moiety of NAD. No specific histidine or other residue could be detected (249,250) as partial prototrop. We assumed the general scheme to be a plausible one and decided to directly identify histidine residues at the active site with diethylpyrocarbonate, a fairly specific modifying reagent for histidine residues in the pH range of 6.0-7.5 and fairly successfully employed for histidine modification in many enzymes in recent years (224-227).

The yeast enzyme was rapidly inactivated and kinetic analysis showed that at least one histidine residue could be directly correlated with the loss of enzyme activity (Fig. 11). The pk value calculated from the pH dependence of inactivation (Fig. 13), the difference spectrum of the modified enzyme (Fig. 16) and the partial but definite reversal of inhibition by hydroxylamine (Fig.14) suggested that the essential aminoacid residue modified by diethyl pyrocarbonate at pH 7.0 is a histidyl residue. As in case
of arginine modification studies, in this case also, gel filtration analysis of completely inactivated enzyme (detected by coenzyme fluorescence) and also direct determination of protein-bound NAD (Data not shown), clearly showed that the inactivation was not due to any loss of structural integrity or release of the coenzyme from the enzyme surface. Further, polyacrylamide gel electrophoresis of the modified enzyme (Fig. 15) showed identical mobility with the native enzyme. Earlier, Darrow and Rodstrom (139) as also Ray and Bhaduri (179) had shown significantly slower mobility of the dissociated monomers on polyacrylamide gel.

As regards the physical location of the critical histidine residue, some uncertainties exist. The rapid rise in 243 nm spectrum before significant loss of enzymatic activity (Fig. 17) clearly indicates several histidine residues are modified either simultaneously or before the essential one is modified. Presumably, the unfolding that may accompany this process does not significantly affect either the integrity of the holoenzyme or the chemical nature of the fluorophore. These are evident from the following observations. As discussed earlier, molecular weight data and NAD analysis data shows that the basic holoenzyme structure remains unaffected. More significantly, the fluorescence spectrum of the coenzyme fluorophore remained
unaffected (Fig.19). Our studies on trypsinization (Fig.22) as also earlier work by Darrow and Rodstrom (139) with guanidine hydrochloride indicate the fairly stable nature of the fluorophore under conditions of unfolding. The essential histidine residue that is directly related to the enzyme activity (Fig.11) is probably not situated at the substrate binding region of the active site. Failure of the substrate to afford any protection and also the unaltered fluorescence spectrum of the modified enzyme in presence of ANS, the substrate site directed extrinsic fluorophore (199,223), are evidences in this direction. The histidine residue may, however, be located in close proximity to the catalytic region of the active site. This can be inferred from the fact that even though the chemical nature of the fluorophore remains unaffected, it becomes completely inaccessible to chemical reduction after modification with diethylpyrocarbonate (Fig.20). Failure of high concentration of glucose to act as an oxidant in the 'reductive inhibition' process further confirms this inaccessibility of the pyridine moiety of NAD to the reducing agents. Apparently, modification of the critical histidine residue causes a structural or conformational change at the active site that renders the NAD unavailable for the catalytic process.
We mentioned earlier that employing suitable affinity analogues, Wong and Frey (249,250) failed to identify histidine or any other amino acid residue at the active site that might be involved as the prototropic agent in the catalytic process. Our modification experiments with diethylpyrocarbonate on the other hand, has uncovered the necessity for the presence of an unmodified histidine residue during normal catalysis though its specific role in the catalytic process still remains unknown and there remains certain ambiguities regarding its exact location on or near the active site.

Proteolytic cleavage of enzymes or other proteins have often yielded valuable information about in functioning and structural properties of the enzyme or protein under investigation. Defined cleavage of ribonuclease by substilisin or of γ-globulin by papain are classic examples in this direction. Tryptic cleavage of proteins inserted into the membrane bilayer have also proved to be a very powerful tool for study of such protein. Such techniques are very often used even for soluble enzymes. A typical recent example is the limited digestion by trypsin of the dimeric methionyl-tRNA synthetase of identical subunits from E.coli. A nearly 30% reduction in molecular weight takes place, after symmetrical cleavage of the dimer and the crystallizable modified protein remains very similar in properties
to the native enzyme (251-253).

We decided to subject our epimerase to controlled proteolytic digestion mainly because of two reasons. Firstly, the identification of minimally one arginine residue at the active site and its complete protection, with UMP, suggested itself that the enzyme be subjected to graded cleavage by trypsin, a proteolytic enzyme that has exclusive specificity for basic amino acid residues. Secondly, our histidine-modification studies and the earlier guanidine hydrochloride studies of Darrow and Rodstrom (139) suggested unusually stable properties of the coenzyme fluorophore. It was thought that tryptic cleavage may provide a method for investigating the chemical nature of the coenzyme-aminoacid fluorophore. Our preliminary and limited investigation with trypsin that we reported here suggests that controlled proteolytic cleavage of the yeast enzyme may help in providing new information regarding the quaternary structure of the enzyme and the chemical nature of its fluorophore.

Trypsin at high concentration can rapidly inactivate the enzyme (Fig. 21) but the inactivation process was completely protected at high concentration of UMP (Fig. 24). Obviously, a critical basic aminoacid residue at the substrate binding site must be a primary point of attack for inactivation.
We do not know at this stage whether it is the same arginine residue that was shown to be involved for substrate binding in the catalytic process. Most interestingly, the fully active enzyme undergoes a definite reduction in molecular weight after this treatment. The dimeric structure is retained (Fig. 25) and apparently both the subunits are cleaved to equal extent on this protected tryptic digestion (Fig. 26). SDS gel electrophoresis failed to detect any other band corresponding to the smaller fragment of 10,000 M\(^\text{r}\). It is likely that the original peptide was progressively digested to very small fragments during the course of incubation. More controlled experiments with very short periods of incubation need to be done before definitive answer can be given.

Most of the characteristic properties of the modified enzyme were very similar to that of the native enzyme. The coenzyme fluorophore remained totally unaffected with an excitation maxima at 353 nm and emission maxima at 435 nm (Fig. 22). In fact, direct digestion with trypsin in absence of UMP, allows the fluorescence to be retained even when complete inactivation had taken place (Fig. 22). Apparently, a peptide fragment containing the coenzyme fluorophore is generated during this process. pCMB treatment of this fragmented peptides renders the peptide dark or non-fluorescing.
(Fig.23) indicating the involvement of a sulphydryl group in generating the fluorophore. This data essentially confirms the original postulate of Ray and Bhaduri (192) that a thiol group is involved for the formation of the NAD fluorophore.

The basic subunit interaction properties of the enzyme also seem to remain unaffected even though it has undergone a reduction in size. Activation by cations suggests tetramerization of the dimeric holoenzyme (174,176) and particle regeneration of activity on reconstitution with MSH and exogenous NAD, after inactivation with pCMB suggests that the dissociated cleaved monomers can reassociate into dimeric holoenzyme under appropriate conditions.

Fig. 30 depicts the minimal schematic model of the active site of the \textit{S.fragilis} enzyme that has now emerged from the present and earlier studies. Since the dimeric enzyme has only one active site (one coenzyme per dimer) we have assumed the active site to be formed at the interface between the dimers. An alternative possibility of extremenic negative cooperativity can not however be excluded (145). Presence of two thiol groups, each residing on two subunits were earlier shown by Ray and Bhaduri (181,192). At least one of the thiols is probably also involved for generation of the coenzyme fluorophore (192). Our demonstration that
Fig. 30  Active Site of Epimerase

A - Substrate Binding Domain
B - Catalytic Domain
C - Coenzyme Binding Domain
pCMB renders the fluorescent peptide fragment dark, further strengthens this hypothesis. A hydrophobic pocket for adenine subsite was shown by Samanta and Bhaduri (193). We have now demonstrated the presence of at least one arginine residue at the substrate binding site. If the upper value of two arginine residues is accepted $^{14}$C-phenylglyoxal data (Table 4) and phenylglyoxal kinetic data (Fig.1) these will probably be symmetrically placed. Position of histidine residue is uncertain, but it is most possibly located somewhere away from the substrate binding site but close to the catalytic site. The exact site of tryptic cleavage under UMP-protected condition is not known.