The Presence of a Histidine-Aspartic Acid Pair in the Active Site of 2-Hydroxyacid Dehydrogenases

X-RAY REFINEMENT OF CYTOPLASMIC MALATE DEHYDROGENASE*

Jens J. Birktoft and Leonard J. Banaszak
From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri 63110

(Received for publication, March 9, 1982)

The structure of cytoplasmic malate dehydrogenase has been partially refined by crystallographic least squares methods. Using x-ray phases based on the refined coordinates, analysis of the resultant electron density maps has led to a new model of cytoplasmic malate dehydrogenase and a tentative "x-ray sequence." The two crystallographically independent subunits comprising the dimeric enzyme are nearly identical in structure and are related to each other by roughly 2-fold rotational symmetry. The best fit of the molecular structure of cytoplasmic malate dehydrogenase to that of lactate dehydrogenase has been obtained by least squares methods. The active sites of these two enzymes contain similarly oriented His-Asp pairs linked by a hydrogen bond which may function as a proton relay system during catalysis. This pair could also provide an explanation for the more strongly binding by cytoplasmic malate dehydrogenase and lactate dehydrogenase of NADH versus NAD. Similar His-Asp pairs have been observed in the serine proteases, thermolysin, and phospholipase A2, and the His-Asp pair may play a similar functional role in all of these enzymes.

The enzymes that utilize the nicotinamide adenine dinucleotide as cofactors in hydride transfer reactions form a diverse group of proteins. Structural studies on many of these enzymes have revealed that their three-dimensional structures can be divided into two well defined domains with one domain primarily involved in coenzyme binding and the other mainly providing the residues responsible for catalysis and substrate binding. Furthermore, comparative studies of the three-dimensional structures of these enzymes have demonstrated that the coenzyme binding domains are highly homologous whereas the catalytic domains show more structural variability (Rossmann et al., 1975). Only cytoplasmic malate dehydrogenase and lactate dehydrogenase are homologous in both the coenzyme binding and the catalytic domains (Banaszak and Bradshaw, 1975). In contrast, the catalytic domains in horse liver alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase show neither apparent similarity in structure to each other nor to the catalytic domains that occur in lactate dehydrogenase and cytoplasmic malate dehydrogenase (Rossmann et al., 1975). It should be pointed out that the substrates utilized by lactate dehydrogenase and cytoplasmic malate dehydrogenase are closely related, both being 2-hydroxyacids, whereas those used by liver alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase are acyl or alkyl alcohols and a phosphorylated aldehyde, respectively.

Studies on the binding of coenzymes, coenzyme fragments, and analogs of and abortive ternary complexes have demonstrated that binding of NAD (NADH) occurs by similar, if not identical, NAD-enzyme interactions in all of these enzymes (Rossmann et al., 1975; Bränden and Eklund, 1980; Birktoft et al., 1982a). Thus, the positioning of the two aromatic rings of NAD is stabilized through numerous nonpolar interactions, whereas hydrogen bonds and ionic interactions are responsible for the locations of the ribose and phosphate moieties.

Hydrogen bonding involving the carboxamide group of the nicotinamide moiety appears to be responsible for providing the correct stereospecificity for the transfer reaction of a hydride ion and from carbon 4 of the pyridine ring (Rossmann et al., 1975). In cytoplasmic malate dehydrogenase, lactate dehydrogenase, and liver alcohol dehydrogenase, the transfer occurs to the so-called A side of the pyridine ring while in glyceraldehyde-3-phosphate dehydrogenase, the transfer occurs to the opposite side of the ring, the B side. Although in all of these enzymes the NAD and substrates are in nearly the same relative steric positions, the pyridine ring is found in two different orientations related by a torsional rotation of 180° around the glycosidic bond (for a review see Bränden and Eklund, 1980).

In contrast to the substantial information concerning NAD-enzyme interactions, little direct information exists on the binding of substrates as well as on the intermediates which occur during a catalytic cycle. Most of our current understanding of catalytic intermediates is based on model building experiments using electron density maps from crystalline forms of binary and abortive ternary complexes. These studies have been supplemented by the observation of an anion believed to be located at a position in the active site normally occupied by the 2-hydroxyacid substrates (Adams et al., 1973; Webb et al., 1973). In the ternary complex of lactate dehydrogenase, the 2-hydroxy group of the substrate is hydrogen bonded to a histidine residue and the carboxylate of lactate is fixed by a guanidino group from Arg 171 (Holbrook et al., 1975). In similar ternary complexes of liver alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, the substrates are believed to be held in the correct orientation by somewhat different groups and the most recent discussion of both A and B side specific coenzyme binding as well as substrate binding can be found in a review by Bränden and Eklund (1980).
In spite of the similarities in the binding of coenzyme, it seems reasonable to expect that the detailed catalytic mechanisms of liver alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and lactate dehydrogenase/cytoplasmic malate dehydrogenase are different from each other. Thus, in liver alcohol dehydrogenase, a zinc atom plays a central role in catalysis and the glyceraldehyde-3-phosphate dehydrogenase reaction involves the formation of a covalent thioacyl intermediate followed by both oxidation and phosphorylation of the substrate. None of these chemical features are shared by the lactate dehydrogenase/cytoplasmic malate dehydrogenase system.

The close similarity of the structures of lactate dehydrogenase and cytoplasmic malate dehydrogenase suggested that these two enzymes also share a common catalytic mechanism (Banaszak and Bradshaw, 1975). Recent progress in the crystallographic refinement of cytoplasmic malate dehydrogenase has led to a tentative amino acid sequence and thus an improved description of the molecular structure of this enzyme. In order to facilitate a general description of the active site of the 2-hydroxyacid dehydrogenases, the structures of several lactate dehydrogenases (Birktoft et al., 1976; Mühlethaler and Rossman, 1979) have been compared with that of the refined model of cytoplasmic malate dehydrogenase. Finally, the recently determined amino acid sequence of mitochondrial malate dehydrogenase has been compared with those of the lactate dehydrogenases and it was established that mitochondrial malate dehydrogenase is a closely related member of this family (Birktoft et al., 1982b). The homologous nature of mitochondrial malate dehydrogenase has made it possible to further test proposals suggested by invariant amino acid residues in the active sites of the 2-hydroxyacid dehydrogenases.

### MATERIALS AND METHODS

The initial three-dimensional structure of cytoplasmic malate dehydrogenase was derived from a 2.5 Å resolution electron density map phased by the multiple isomorphous replacement method (Ter-nogou et al., 1972). Since the amino acid sequence of cytoplasmic malate dehydrogenase was unknown, a polyalanine model was constructed in a Richard's box. Subsequently, the multiple isomorphous replacement electron density map was further analyzed and this time an attempt was made to obtain an "x-ray amino acid sequence." To do this, wherever possible one of the standard amino acids compatible with the electron density was incorporated into the model. In ambiguous regions, a linear carbon chain that filled the electron density was assigned. Since there are two identical subunits in the asymmetric unit, in places where the electron density appeared somewhat different, an amino acid most compatible with the electron density in both subunits was selected. In addition, due to the presence of weak electron density at several points in the map, some differences between the subunit models do exist in both the amino acid assignments and in the overall length of the polypeptide chain. Since there was no information which could be used to eliminate this ambiguity, separate models of both subunits were built. Hence, subunit 1 has 321 amino acids and subunit 2 has 324 amino acids. A total of 4127 atoms were assigned to the two subunits or approximately 80% of the number of atoms indicated by the amino acid composition (Wade, 1971).

The coordinates obtained from this model formed the starting set for a restrained crystallographic refinement according to Hendrickson and Konnert (1980). Details of this procedure have been published elsewhere (Birktoft et al., 1982a) and will only be briefly summarized here. After 6 cycles of refinement using x-ray diffraction data in the resolution range 35 Å, the conventional R factor for the x-ray data was reduced to 18%. The total root mean square shift for the 4127 atoms was 0.51 Å. Since a significant portion of the structure was not included in the refinement, it was decided at this stage to re-evaluate the original x-ray data. Electron density maps were calculated using diffraction data in the resolution range between 9.0 and 2.5 Å using as coefficients (N |F_o| - M |F_c| exp(iω_o), with (N,M) = (3,2), (2,1), or (1,1). In the calculation of these electron density maps, the specific segment of the polypeptide chain being analyzed was omitted in the calculation of the coefficients. The resulting electron density maps were examined with the MMS-X interactive graphics system (Barry et al., 1974). Adjustment in the amino acid sequence and in conformation was done using the programs BUILD written by Dr. J. Miller (Miller et al., 1981) and NEWNIP written by Dr. P. F. Bethe (Lederer et al., 1981).

During this re-evaluation of the x-ray sequence of cytoplasmic malate dehydrogenase, reference was made to a number of peptide fragments that have been sequenced by chemical methods. The length of these peptides ranges from 3 to 35 amino acids. We are grateful to R. A. Bradshaw (University of California at Irvine) and his collaborators for this information. Lacking an amino acid sequence determined by chemical methods, the resulting x-ray sequence (Birktoft et al., 1982b) can only be compared with the amino acid composition which is given in Table I (Wade, 1971). With a few exceptions, the number of each amino acid found in the x-ray sequence agrees well with the chemical composition. The largest discrepancy occurs for glutamine/glutamic acid whereas it appears that an extra 12 residues have been assigned to the electron density map. Many examples of this type of residue occur in electron density on the surface of the cytoplasmic malate dehydrogenase molecule and hence in electron density which is more difficult to evaluate.

For the comparison of the refined model of cytoplasmic malate dehydrogenase with other enzymes, the atomic coordinates for dogfish muscle apo- and ternary lactate dehydrogenase and mouse testes apolactate dehydrogenase were obtained from the protein data bank at Brookhaven National Laboratory (Bernstein et al., 1977). The structural similarity between the cytoplasmic malate dehydrogenase structure and those of the apo and holo forms of lactate dehydrogenase was established by utilizing a method employing least squares minimization of the distances between corresponding x-carbon atoms (Rao and Rossman, 1973). To do this, the initial transformation of one molecule into the other was first established by the least squares method. The similarities were then checked with the aid of the MMS-X graphics system. In the next step, atom pairs were omitted from the least squares study when the distance between equivalent x-carbon atoms was more than 1.5 times the mean deviation for all

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1 R. T. Fernley, B. E. Glatthaar, M. R. Sutton, and R. A. Bradshaw, manuscript submitted to J. Biol. Chem.

## Table I

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1 "Wade (1971)."
pairs. The least squares minimization procedure was then repeated. The final coordinate transformation thus generated was used to rotate one structure into another, and the similarities and differences which will be described were further analyzed on the MMS-X graphics system. In these analyses, the two crystallographically independent subunits in cytoplasmic malate dehydrogenase were compared separately with the three different lactate dehydrogenase structures as well as against each other. The detailed comparison of the active centers of the respective structures was then again done on the computer graphics system.

**RESULTS AND DISCUSSION**

With the improved coordinates obtained from the first stage of the least squares refinement, a re-evaluation of several structural features of cytoplasmic malate dehydrogenase was undertaken, the first consisting of a review of the subunit-subunit symmetry. Next, the refined structure of cytoplasmic malate dehydrogenase was compared with lactate dehydrogenase. The third study consisted of a careful re-examination of the active site along with a model building study in which the possible orientations of L-malate were examined in the region of electron density thought to contain an anion in the crystal structure of holo-cytoplasmic malate dehydrogenase. Since it was established that a hydrogen bond-linked His-Asp pair is present near the 2-hydroxyacid substrate, the cytoplasmic malate dehydrogenase coordinates were also compared with several known lactate dehydrogenase structures in order to see if a corresponding arrangement was also present in these homologous enzymes.

**Subunit:Subunit Relationships in Cytoplasmic Malate Dehydrogenase**—In spite of the absence of a complete amino acid sequence, ample chemical and X-ray evidence indicates that cytoplasmic malate dehydrogenase is a dimer consisting of two identical subunits (Banaszak and Bradshaw, 1975). It is to be expected that the subunits comprising the dimer are arranged with 2-fold rotational symmetry. Using the partially refined X-ray coordinates and the least squares procedures, both of which are described under "Materials and Methods," the conformation and the relative orientation of the two subunits of cytoplasmic malate dehydrogenase were studied. The results indicated that in the crystalline enzyme, the two polypeptide chains do not appear to be related by exact 2-fold rotational symmetry (180°) but rather by a 173° rotation and possibly a translation of 0.4 Å along the rotation axis. The results of this superposition are summarized in Fig. 1 which shows the distance between each α-carbon atom in one subunit to the corresponding α-carbon atom in the other subunit after the optimal congruency transformation. Note that for most residues, the refined structures are congruent to within about 0.5 Å. The largest difference occurs in the region of residues 85 to 95. This is the so-called β3αD loop region known to undergo conformation changes in both lactate dehydrogenase and cytoplasmic malate dehydrogenase (Weininger et al., 1977). The nomenclature and labeling system for the dehydrogenase structures is given in the legend to Fig. 1.

Finally, it is possible to estimate the coordinate errors which would be necessary to produce the observed discrepancy from a proper 2-fold rotation axis. Typically, atoms located within a 5-Å radius of the symmetry element would have to be systematically displaced by 0.6 Å, while atoms which are 25 Å from the dyad must be displayed by 3.1 Å. For the refined coordinates employed here, the first value is at the border of experimental error, but the latter value is well beyond the expected experimental limits.

To analyze the larger of the congruency differences in Fig. 1, residues involved in various forms of protein:protein contacts were tabulated and are also shown in Fig. 1. Two principal contacts are found in the subunit interface of cytoplasmic malate dehydrogenase. One is created by residues belonging to the helix, αB, in each of the two subunits. The second contact is formed by the helix αC in one subunit and parts of the helices α2F, α2G, and α3G in the other subunit. A consequence of the different rotational relationships of homologous structural elements participating in subunit:subunit interactions is that only quasi-equivalent interactions are made by homologous residues in the two subunits. This is particularly prevalent in the interactions involving helices, αC in the two subunits. Similar types of asymmetric subunit:subunit interactions have been described for insulin (Blundell et al., 1972), hexokinase (Steitz et al., 1976), and a-chymotrypsin (Birktoft and Blow, 1972; Tulinsky et al., 1973).

The asymmetry in the subunit interface is further supported by an analysis of the positions of heavy atom binding sites. Three binding sites per dimer were observed for the p-hydroxymercuriphenyl sulfonate derivative, and all three are located in the subunit interface (Tserruoglu et al., 1971). Two of the heavy atom sites are symmetrically arranged around the molecular pseudo dyad, whereas the third site is unique. This molecular asymmetry is also reflected in the affinity for

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**Fig. 1. Structural homology between the subunits of cytoplasmic malate dehydrogenase.** The transformation which minimized the root mean square difference between the coordinates of subunit 1 and subunit 2 was used to calculate the congruent sets. Deviation from this congruency (in Ångstroms) are plotted as a function of residue number along the **abscissa.** Below the residue numbers are **bracketed symbols** describing the elements of secondary structure. α and β are used to indicate α-helices and β-structures, respectively. The **second letter** describes the position in the primary structure; hence, βA is the first strand of extended polypeptide chain beginning αC in each of the two subunits. The same nomenclature has been used in the past to describe other dehydrogenases (Rosscaun et al., 1975). To help the unfamiliar reader, the commonly referred to nucleotide binding domain includes approximately residues 1 through 149 and typically the six parallel strands of β-structure in this domain include βA through βF. Specific residues involved in subunit:subunit contacts are shown below the curve by •. Residues in the cytoplasmic malate dehydrogenase dimer which are involved in protein:protein contacts in the crystal lattice are marked on the curve by the larger •. A residue was considered to be involved in a contact if the α-carbon atom of that residue is less than 7.0 Å from an α-carbon atom in another subunit or molecule.
NAD in the crystalline state in that the two subunits bind NAD with different occupancies (Weininger et al., 1977).

The rest of the major structural differences between the two subunits are located on the molecular surface. These are also the locations where differences in the assignment of the number of amino acids differ for the two polypeptide chains. In these instances, segments of the polypeptide chain are often involved in different intermolecular interactions in the crystalline lattice. Residues involved in such protein:protein contacts are also marked in Fig. 1. It seems reasonable to conclude that most, if not all, of the structural differences between the two subunits are caused by crystal packing effects. Solution studies, whether it is kinetic data or chemical modification experiments, do not indicate nonequivalence in the properties of the two subunits (see Weininger et al. (1977) for a discussion).

**Cytoplasmic Malate Dehydrogenase and Lactate Dehydrogenase Homology**—The same method of least squares refinement used to study the subunit:subunit arrangement in cytoplasmic malate dehydrogenase was used to compare the refined coordinates of cytoplasmic malate dehydrogenase with those of the lactate dehydrogenases. After the root mean square deviation between equivalent atoms in the two enzymes was minimized, it was possible to compare the similarities and differences, again using the molecular graphics system. The structural homology in the active site will be described in the next section but a few more general observations are reported here.

The similarity between cytoplasmic malate dehydrogenase and lactate dehydrogenase reported earlier by Rao and Ross- mann (1973), particularly in the nucleotide binding domain, was confirmed. The distances between homologous residues in cytoplasmic malate dehydrogenase and lactate dehydrogenase are shown by the curves in Fig. 2. Overall, the agreement seems to place corresponding atoms within about 2 Å of each other in the two proteins. However, in some instances, the relative positions of elements of secondary structure were observed in the graphics system to differ in a correlated manner. Although the significance of these differences is unknown, a typical example is the case where a translation of a specified helix in the direction of the helical axis is different in the two proteins. Thus, the best fit of cytoplasmic malate dehydrogenase with lactate dehydrogenase appears to place the parallel strands of the β structure in the nucleotide binding domain in a congruent arrangement. However, the four helices interconnecting these strands are in the same orientation but are changed such that the "helical windings" are often shifted in one protein relative to the other. The shift, of course, must be less than the helical pitch of 5.4 Å which would again bring the homologous units into congruence. An example of this is illustrated in Fig. 3, which shows the central parallel β-sheet and helices aB and aC from the nucleotide binding domain of the two enzymes superimposed on each other. The helical shift is readily apparent in helix aC in the left half of the figure.

The dimeric cytoplasmic malate dehydrogenase and the tetrameric lactate dehydrogenase have a 2-fold symmetry axis in common, the Q axis according to the nomenclature of Rossmann et al. (1975). The residues in this region are indicated by a single dot in Fig. 2. Additional subunit:subunit interactions in the tetrameric lactate dehydrogenase takes place along two other molecular dyads, the so-called P and R axes (Rossmann et al., 1975). These are also marked in Fig. 2. The corresponding segments of cytoplasmic malate dehydrogenase are located on the molecular surface and in several instances they display a somewhat larger degree of conformational differences when compared with lactate dehydrogenase. An example of such differences can be seen in Fig. 2 near residues 250 to 255. As is generally the case when homologous structures are compared, the majority of the largest structural differences were located on the molecular surface. In particular, the surface loops that connect elements of the secondary structure show a high degree of structural variability in the two molecules. Most noticeable are the differences seen in Fig. 2 at the beginning of the αG helix and at the end of the α3G helix.

Almost all of the observed deletions and insertions which are found in the homologous cytoplasmic malate dehydrogenase-lactate dehydrogenase set were in these surface loops. The only exception is near residue 149 in cytoplasmic malate dehydrogenase (155 in lactate dehydrogenase), which is near the junction of the coenzyme binding and the catalytic domains. In this internal position, an additional residue is found in lactate dehydrogenase relative to cytoplasmic malate de-

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**Legend**: The transformation which minimized the RMS difference between dogfish apolactate dehydrogenase and subunit 1 (bottom curve) and subunit 2 (upper curve) of cytoplasmic malate dehydrogenase was used to calculate the congruent sets. Deviations from this congruency (in Angstroms) are plotted as a function of residue number for cytoplasmic malate dehydrogenase along the abscissa. Below the residue number are symbols describing the secondary structure and these are described in more detail in Fig. 1. The numbering system is that of subunit 1 of cytoplasmic malate dehydrogenase: so "inserted" residues in lactate dehydrogenase do not appear on the figure. In addition, residues which are present in cytoplasmic malate dehydrogenase but not in lactate dehydrogenase are shown as breaks in the curves. Residues present in the Q, P, and R 3-fold axes, subunit:subunit interfaces of tetrameric lactate dehydrogenase, are shown by ●, ▲, and ▼, respectively. The criteria used in determining contact residues are the same as described for Fig. 1.
A His-Asp Pair in the Active Site of Malate Dehydrogenase

The Active Site of Cytoplasmic Malate Dehydrogenase—
The x-ray refinement of crystalline cytoplasmic malate dehydrogenase has made it possible to describe the active site in more detail than was previously possible (Webb et al., 1973). The results can be described in terms of the interactions of both the coenzyme and substrate with the enzyme. For the bound NAD, the electron density in difference electron maps using phases calculated from refined model coordinates has led to small readjustments in the conformation of the bound coenzyme (Birktoft et al., 1982a). More important was the fact that amino acid residues interacting with the bound dinucleotide could be identified with more certainty. To give a few examples, the carboxylate side chain of an aspartic acid is found to be hydrogen bonded to the O-2' atom of the adenine ribose in cytoplasmic malate dehydrogenase and in a manner similar to that observed in dogfish lactate dehydrogenase. This residue is Asp 40 in cytoplasmic lactate dehydrogenase and Asp 53 in lactate dehydrogenase (Birktoft et al., 1982a). Similarly, the amido group of Asn residue, 124, has been found to be within hydrogen bonding distance to the hydroxyl group O-2' atom of the nicotinamide ribose and it must be homologous with Asn 140 in lactate dehydrogenase. The side chain of this residue is in both enzymes also near the N-1 atom of the nicotinamide ring of NAD. In general, nearly all of the interactions between cytoplasmic malate dehydrogenase and NAD are homologous with similar groups found in lactate dehydrogenase (Birktoft et al., 1982a).

Interactions between cytoplasmic malate dehydrogenase and bound NAD, with the exceptions of residues 232, 233, and 237, are all formed with amino acids contained in the nucleotide binding domain of cytoplasmic malate dehydrogenase, residues 1 to 149. Model building experiments using the improved electron density maps after least squares refinement have also provided additional information about the binding of the substrate, L-malate. For the most part, this involves residues in the so-called catalytic domain and the results can again be compared with the active site of lactate dehydrogenase.

The substrate binding site for L-malate is depicted in Fig. 4. In this electron density map, a sulfate ion is thought to be present at the substrate site. The 4-carboxylate moiety of L-malate is placed in this ion density and the orientation of the substrate fixed by its known biochemical properties. The molecular arrangement indicated in Fig. 4 shows that the 1-carboxylate of L-malate forms an ion pair with the guanidino group of Arg 155. This ion pair corresponds closely with the lactate:Arg 171 combination found in lactate dehydrogenase (Holbrook et al., 1975). To complete the orientation in L-malate in the anion site, the carbon 2 atom was fixed in a stereochemical arrangement which optimized the distance from the 2-methylene group of L-malate to the carbon 4 atom of the nicotinamide ring. Finally, when the L-malate is in the orientation shown in Fig. 4, the 2-hydroxyl group is located close to the imidazole ring of His 180. The proximity of the 2-hydroxyl group of the substrate to a histidine side chain is similar to the situation in lactate dehydrogenase where the hydroxyl group of L-lactate is believed to be within hydrogen bonding distance to His 195 (Holbrook et al., 1975).

Examination of the electron density map of cytoplasmic malate dehydrogenase after refinement shows that His 180 is in close contact with the side chain of residue 152. This residue is believed to be an aspartate or asparagine, and is within hydrogen bonding distance of the imidazole ring. The arrangement, as seen in each subunit of cytoplasmic malate dehydrogenase, is shown in the stereodiagrams in Fig. 5. The reliability of the assignment of an aspartic acid/asparagine to position 152 and of histidine to position 180 was aided by the fact that the amino acid sequence of two peptides fit into the electron density in these regions very well. Shown here are only the two tripeptides centered around the pertinent Asp and His residue; the peptides fitted had the sequence Leu-Aax-His-Aax-Arg (Asp 152) and Ile-Gly-Gln-His-Gly-Gln (His 180), respectively. As can be seen by comparing Fig. 5 upper...
A His-Asp Pair in the Active Site of Malate Dehydrogenase

Fig. 4. The anion binding site or cytoplasmic malate dehydrogenase after refinement. The stereodiagram illustrates the electron density in cytoplasmic malate dehydrogenase near the substrate binding site. The electron density map is of the 3Fo-2Fc type and was obtained as described under "Materials and Methods." Shown within the electron density map are the stick models of L-malate, part of the NAD moiety, and the tripeptide Asx 154-Arg 155-Ala 156. The atomic coordinates for these were omitted in the structure factor calculation. The arrow indicates the position of the carbon 4 on the nicotinamide ring of NAD to which a hydrogen is transferred from the substrate, L-malate.

and 5 lower, these amino acid assignments provide a good fit to the electron density in both of the subunits of crystalline cytoplasmic malate dehydrogenase.

An aspartic acid residue is also found in a homologous position in the active site of mitochondrial malate dehydrogenase (Birktoft et al., 1982b) and in all of the lactate dehydrogenases (Eventoff et al., 1977; Pan et al., 1980). In the apo form of mouse testes lactate dehydrogenase, Asp 168 and His 195 are found in nearly the same arrangement as observed in cytoplasmic malate dehydrogenase (Musick and Rossmann, 1979), and a hydrogen bond may be formed between the imidazole and carboxylate in this enzyme as well. This is shown in the stereodiagram in Fig. 6. Based on the best fit of the crystal structures of dogfish apolactate dehydrogenase and a pseudoternary complex of dogfish lactate dehydrogenase to cytoplasmic malate dehydrogenase, it was clear that these two residues are always close to each other in the active site.

In the case of the two subunits of cytoplasmic malate dehydrogenase as shown in Fig. 5, small differences are found in the relative orientations of the planes of the imidazole rings and the carboxyl groups. It should be noted, however, that the His-Asp orientation was arrived at independently in each subunit; no attempt was made to maximize the conformational homology and the congruency differences are well within experimental error.

A more revealing description of the similarities of the His-Asp pairs in the 2-hydroxyacid dehydrogenases can be obtained from the measured distances between the relevant atoms in the crystal structures. Such data is summarized in Table II. Note the relatively good agreement between the two subunits of cytoplasmic malate dehydrogenase for the distances between the α, β, and γ carbon atoms of the active site histidine and aspartate for all the dehydrogenase structures. Using the β-carbons of the His-Asp pair as an example, the mean separation is 6.3 Å with a standard deviation of ±0.7 Å.

Similarly, from Table II, the separation of the centers of the carboxylate and imidazolium moieties is 3.9 ± 0.8 Å with the largest difference occurring for the pseudoternary complex of dogfish lactate dehydrogenase. Of the two possible orientations for the imidazolium ring, the one favored for the active site of cytoplasmic malate dehydrogenase has the NE1 nitrogen closest to the carboxylate of the aspartic acid. For the lactate dehydrogenase, the imidazole ring is in a different orientation and the ND1 nitrogen is closest to the carboxylate of Asp 168. However, this difference in orientation of the imidazole ring is within the bounds of experimental error and may not be of any significance. Although no solvent accessibility studies have yet been carried out, Asp 152 in cytoplasmic malate dehydrogenase appears to be nearly completely shielded from the solvent.

The evidence derived from the electron density maps of cytoplasmic malate dehydrogenase using the refined coordinates support the presence of a His-Asp pair in the active site. Such a molecular arrangement is similar to the catalytically important His-Asp pairs found in a number of other enzymes including all of the serine proteases (Blow et al., 1969; Kraut, 1977), thermolysin (Weaver et al., 1977), and phospholipase A2 (Dijkstra et al., 1981). In addition, the evidence presented in Table II and Fig. 6 shows that a similar arrangement could be formed in the lactate dehydrogenases and that this could occur without any polypeptide backbone rearrangements.

The Catalytic Center of 2-Hydroxyacid Dehydrogenases— The His-Asp pair can be incorporated into a proposed mechanism of action for cytoplasmic malate dehydrogenase and probably other 2-hydroxyacid dehydrogenases. A schematic outline of the more important steps in the catalytic process is shown in Fig. 7. Some aspects of the proposed mechanism are similar to the so-called "oil-water-histidine" mechanism proposed by Parker and Holbrook (1977), as well as that proposed by Bernstein and Everse (1978). However, the distinguishing feature of the catalytic center as shown in Fig. 7 is that in the...
Fig. 5. The His-Asp pair in cytoplasmic malate dehydrogenase. The stereodiagrams show the electron density in the active sites of cytoplasmic malate dehydrogenase. These 3Fo-2Fc maps were calculated using phases determined from the model coordinates after refinement, excluding atoms from residues 152 and 180. The stick models of cytoplasmic malate dehydrogenase include the residues adjacent to residues 152 and 180 and show the molecular structure in this region after refitting to the electron density and clearly indicates the proximity of the side chain of residue 152 to the imidazole ring at position 180. Upper, the residues in the active site of subunit 1; lower, the same region in subunit 2.

active site region of the ternary complexes, the net electrostatic charge is zero throughout the catalytic process. The 4-carboxylate moiety of both L-malate and oxaloacetate is in solvent contact and therefore contributes no net charge to the active site region and is consequently ignored in the following discussion.

The formation of the binary complex of cytoplasmic malate dehydrogenase and NADH is shown in Fig. 7, A and B. In these figures as well as in Fig. 7F, an anion has been added to the active site so that the illustration corresponds to the form of cytoplasmic malate dehydrogenase studied by X-ray analysis (Webb et al., 1973). The binding of NADH to cytoplasmic malate dehydrogenase reduces solvent accessibility to the active site, thereby causing a reduction of the "apparent" dielectric constant within this small volume. The reduced solvent accessibility would cause the electroneutral form of the His-Asp pair to be favored over the negatively charged form, and this results in an increase of the apparent pK of the His-Asp pair. Holbrook and coworkers have shown that the binding of NADH to cytoplasmic malate dehydrogenase causes an active site residue, assumed to be a histidine, to undergo an increase in pK from 6.4 to 7.4 (Lodola et al., 1978). Also in agreement with the above proposal are the observations with cytoplasmic malate dehydrogenase that binding of NADH is associated with proton uptake by the binary complex and that an increase in pH causes a reduction in the affinity for the reduced coenzyme (Johnson and Rupley, 1979). Similar observations have also been reported for lactate dehydrogenase (Johnson and Rupley, 1979).

In the next step in the catalytic cycle, as shown in Fig. 7C,
The interaction between the keto form of the substrate and the His-Asp pair would also help in the polarization of the substrate. This additional enzyme:substrate interaction together with the above mentioned Arg 155:1-carboxylate interaction provides the stereospecificity for the catalytic reaction. The binding of the 2-ketoacid substrate occurs. As in lactate dehydrogenase, a guanidino group interacts with the 1-carboxylate, Arg 155 in cytoplasmic malate dehydrogenase. In addition, the imidazole ring of the now electronneutral His-Asp pair acts as a hydrogen bond donor to the keto oxygen of the substrate. This additional enzymesubstrate interaction together with the above mentioned Arg 155:1-carboxylate interaction provides the stereospecificity for the catalytic reaction. The interaction between the keto form of the substrate and the His-Asp pair would also help in the polarization of the keto moiety. The concomitant development of a partial positive charge on the C-2 carbon atom of the substrate would facilitate the transfer of a hydride ion from NADH to the oxidized form of the substrate.

Whether or not the transfer of the proton from the imidazole ring and of the hydride ion from the nicotinamide ring of NADH to the substrate follows a sequential or concerted mechanism is unknown. In either case, the transfer of the hydride ion from the coenzyme is accompanied by the development of a positive charge on the nicotinamide ring of the coenzyme. Similarly, the transfer of a proton from the His-Asp pair to the substrate is accompanied by an increasing development of a negative net charge in the His-Asp pair. The transition state, as shown in Fig. 70, is similar to that proposed for chicken heart mitochondrial malate dehydrogenase by Bernstein and Everse (1978). They suggested that an electron-deficient area equal to one positive charge existed in the transition state. The presence of Asp 152 causes this excess positive charge to be neutralized, thus maintaining electroneutrality in the desolvated active center during catalysis. The structural data obtained from cytoplasmic malate dehydrogenase shows that a His-Asp pair serves as the acid-base system instead of a single histidine.

After completion of the transfer processes the situation depicted in Fig. 7E may occur. A hydrogen bond still exists between the substrate, now L-malate, and the His-Asp pair, but the latter now acts as the acceptor, as well as carrying a net negative charge. The positive charge originally residing on the imidazole ring is now located on the nicotinamide ring. However, the net charge of the overall system is still zero.

Fig. 7F illustrates the binary adduct formed with NAD. Since the His-Asp pair is present in all forms of the enzyme-substrate complexes, only the charge separation is altered during the catalytic cycle. In the forms of the active center shown in Fig. 7, A and B, the charge separation is the distance between the centers of the imidazole and the carboxylate, i.e., about 3.4 Å (Table II). The distance between the center of the nicotinamide ring and the center of the carboxylate is, on the other hand, about 8.9 Å. If the dielectric constant in the domain of the active site were similar for both cytoplasmic malate dehydrogenase-NAD and cytoplasmic malate dehydrogenase-NADH complexes, energetic factors related to the His-Asp pair would favor the formation of the cytoplasmic malate dehydrogenase-NADH binary complex. This is found experimentally. The binding constant for NADH is always lower than that for NAD.
several hundred-fold greater than for NAD (Holbrook and Wolfe, 1972). Binding of NAD to cytoplasmic malate dehydrogenase would reduce the "apparent" dielectric constant in the vicinity of the His-Asp pair just as is the case with NADH binding as discussed above. However, a positive charge is introduced into the system concomitant with NAD binding. No proton uptake by the system is, therefore, to be expected in this case, and none is observed experimentally (Johnson

**Fig. 7.** The active site of cytoplasmic malate dehydrogenase. The schematic drawings are meant to illustrate the possible participation of the His-Asp pair in a catalytic cycle for cytoplasmic malate dehydrogenase and other 2-hydroxycacid dehydrogenases. Although the drawings are largely self-explanatory, they can be described as follows: A and B, enzyme:NADH binary complex; C, enzyme:NADH:oxaloacetate ternary complex; D, putative transition state complex; E, enzyme:NAD-L-malate ternary complex; F, enzyme:NAD binary complex.
and Rupley, 1979). Similarly, the binding of NAD is largely unaffected by pH changes in the pH range of 6.0 to 8.0 (Johnson and Rupley, 1979).

The Role of the His-Asp Pair in Enzymatic Catalysis—While the occurrence in a protein structure of a hydrogen bond-linked His-Asp pair is not unusual, the presence of such a pair in the catalytic center of an enzyme suggests that it may play a central role in the catalytic process. Such a system was first observed to be a part of the active center of a-chymotrypsin where this His-Asp pair together with a serine forms the so-called "charge relay system" (Blow et al., 1969).

This catalytic arrangement was subsequently found to be a characteristic feature of all the so-called serine proteases (Kraut, 1977). More recently, such a catalytically important His-Asp pair has been described in thermolysin, His 231:Asp 226 (Weaver et al., 1977) and in phospholipase A2, His 48:Asp 99 (Dijkstra et al., 1981). The serine proteases, thermolysin, and phospholipase A2 are all hydrolytic enzymes but their respective mechanisms of action are different from each other. The 2-hydroxyacid dehydrogenases are also different in that they catalyze the reversible oxidation of an alcohol to an aldehyde.

Despite these differences in enzymatic properties, the His-Asp pair appears to function in a similar manner in all of these enzyme systems. In every instance, the histidine acts as a general acid-base by either donating or accepting a proton. In the serine proteases, the active serine hydroxyl group acts as a proton donor to the histidine during the acylation step. In deacylation, a water molecule replaces the serine hydroxyl group. In both phospholipase A2 and in thermolysin, a water molecule acts as the proton donor to the histidine. In the 2-hydroxyacid dehydrogenases, water is the proton donor in the direction of reduction, and the hydroxyl group the donor in the opposite direction. In all of the enzyme systems discussed here, the binding of substrate to the active site causes the removal of bulk solvent from the vicinity of the His-Asp system, thus effectively placing the general acid/base, the His-Asp pair, in an environment of decreased polarity.

One might expect that the His-Asp pair in all of these enzymes have many similarities. These can be listed as follows: (A) The proton located between the histidine and aspartate is covalently attached to the histidine as has been demonstrated by neutron diffraction analysis of trypsin (Kossiakoff and Spencer, 1981). (B) The pK of the His-Asp pair is about 7.5, a value determined for the 2-hydroxyacid dehydrogenases (Bernstein and Everse, 1978; Lodola et al., 1978), for the serine proteases (Kraut, 1977), and for thermolysin (Weaver et al., 1977). (C) The His-Asp interaction as seen in all these active sites serves to keep the imidazole ring in a fixed orientation. With a single proton shared by both side chains, a nitrogen atom in the imidazole ring is available to act both as an acid and as a base. In the serine proteases, thermolysin and probably the 2-hydroxyacid dehydrogenases, this is the NE2 nitrogen, in phospholipase A2, it is the ND1 nitrogen. Molecular orbital calculations (Uracyama et al., 1981) show that in the serine proteases, the aspartate plays a significant role in lowering the barrier height for proton transfer to and from the histidine. This effect has been suggested to be due to primarily electrostatic interactions. The role of the His-Asp pair in these enzyme systems appears to be that of a proton relay system (Kraut, 1977).

In conclusion, the refinement studies of cytoplasmic malate dehydrogenase, although still not complete have led to evidence for a His-Asp ion pair in the active site. The close homology with lactate dehydrogenase suggests that a similar arrangement may exist in other 2-hydroxyacid dehydrogenases. Since it was well known that a histidine, e.g. His-195 in lactate dehydrogenase, was present in the active site of these enzymes, the new structural results do not require serious changes in the proposed mechanisms of these enzymes. The structural results do, however, appear to resolve the conceptual problem of introducing a net positive charge when the catalytic reaction involves the positively charged form of the coenzyme, NAD.

Acknowledgments—We wish to thank Dr. J. McAlister for providing programs and advice concerning the use of the MM3-X Graphics System. The excellent assistance by Solveig Storvick-Pollei and Suzanne Winkler in the preparation of this manuscript is gratefully acknowledged.

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A His-Asp Pair in the Active Site of Malate Dehydrogenase

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