Structure-Function Relationships in Lactate Dehydrogenase

(amino-acid sequence/crystallographic structure)

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ABSTRACT The binding of coenzyme and substrate are considered in relation to the known primary and tertiary structure of lactate dehydrogenase (EC 1.1.1.27). The adenine binds in a hydrophobic crevice, and the two coenzyme phosphates are oriented by interactions with the protein. The positively charged guanidinium group of arginine 101 then folds over the negatively charged phosphates, collapsing the loop region over the active center and positioning the unreactive B side of the nicotinamide in a hydrophobic protein environment. Collapse of the loop also introduces various charged groups into the vicinity of the substrate binding site. The substrate is situated between histidine 195 and the C4 position on the nicotinamide ring, and is partially oriented by interactions between its carboxyl group and arginine 171. The spatial arrangements of these groups may provide the specificity for the L-isomer of lactate.

In this paper coenzyme and substrate binding to dogfish (*Squalus acanthias*) M₄ lactate dehydrogenase (LDH; EC 1.1.1.27) will be discussed in relation to the known amino-acid sequence, the crystal structure determinations, and the effect of various chemical modifications of the enzyme and coenzyme. A comparison of the preliminary 3.0-Å resolution structure of the abortive LDH:NAD-pyruvate ternary complex (1) with the more complete 2.0-Å resolution structure of the apoenzyme provides information on possible conformational changes during catalysis.

The ternary complexes LDH:NAD:oxalate and LDH:NADH:oxamate are isomorphous with the LDH:NAD-pyruvate ternary complex. The first two complexes are distinguished from the pyruvate complex in that there is no covalent bond between the inhibitor and the coenzyme. Thus, the oxamate and oxalate complexes are probably more representative of the transition state of LDH (2-4). Crystallographic investigation of these ternary complexes could be a close approach to a study of the active ternary intermediate.

The main chain hydrogen bonding in the enzyme and a fuller account of subunit interactions was presented by Adams *et al.* (5). We, therefore, present only the most striking features that the combination of sequence and structural data has immediately clarified.

Abbreviation: LDH, lactate dehydrogenase.

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Everse and Kaplan (4) have recently reviewed many of the properties of LDH. Evidence from kinetic data indicates that there is an obligatory binding order of coenzyme followed by substrate (Fig. 1), at least near neutral pH (6-8). McPherson (9) has presented evidence to show that the adenine moiety of the coenzyme is required for binding of the nicotinamide moiety.

Coenzyme binding

Studies on the conformations of adenosine, AMP, and ADP at 2.8-Å resolution and of NAD⁺ at 5.0-Å resolution, when diffused into crystals of the apoenzyme, are discussed by Chandrasekhar *et al.* (10). Diffraction patterns of the NADH binary complex closely resemble those of the NAD⁺ binary complex. Although the structure of each of these binary complexes differs slightly from the other, as a class, their mode of binding of the coenzyme to the apoenzyme is distinct from that of the coenzyme in the ternary complex (Fig. 2). Fig. 3 demonstrates this by a comparison of the structure of NAD in the ternary complex (in black) with (a) NAD⁺ and (b) AMP in binary complexes. The protein conformation of the apoenzyme differs markedly from that of the ternary complex structure in that the loop (residues 98-114) has folded down over the active center pocket in the ternary complexes. Many smaller conformational changes within the protein are associated with the large movement of the loop and the different position and conformation of the coenzyme.

The adenosine binds in a hydrophobic crevice lined by valine 27, glycine 28, an alanine, glycine and valine in the region 29-33, valine 52, valine 54, methionine 55, alanine 98, glycine 99, as well as aspartate 53 and threonine 97. The adenine may be further oriented in the binary complexes by a hydrogen bond between nitrogen N1 and the hydroxyl of tyrosine 85. This bond is probably not present in the ternary complex. The peptide containing the tyrosine shown to be essential in pig H₄ LDH by Pfeleiderer *et al.* (11) does not have a composition that is likely to be homologous to this region. The adenine ribose appears to form hydrogen bonds between the O3' hydroxyl group and the carbonyl group of residue 29, and between aspartate 53 and the O2' hydroxyl group. The adenosine, AMP, and ADP difference maps show that aspartate 53 moves to make room for the O2' hydroxyl group when adenosine binds in its hydrophobic pocket.

The fact that the adenosine moiety is located in such a pocket could explain the fact that the N'-hydroxyethyl-

The reactive A side of the nicotinamide ring can be recognized by its relationship to the pyruvate position in the ternary complex (Fig. 4). The carbonyl group of glutamate 140 approaches this side of the ring and is available to balance the positive charge of the nicotinamide N1 of the oxidized coenzyme. In view of this arrangement it is not clear why NADH

binds better than NAD⁺. The unreactive B side of the nicotinamide appears to be in a hydrophobic environment that is mainly provided by residue 32; this might be expected from the blue shift in the absorption maximum of the reduced coenzyme (16). The position of the nicotinamide ring in the ternary complex is slightly altered with respect to its position in the binary complex, bringing it closer to the substrate site and to histidine 195 (Fig. 3).

In the electron density map of the ternary complex the pyruvate, which is covalently bound to the nicotinamide, is clearly visible (Fig. 2). It is close to histidine 195 and interacts with arginine 171 and possibly arginine 109. Histidine 195 has moved 1–2 Å from its position in the apoenzyme to approach the nicotinamide, while the guanidinium group of arginine 109, a loop residue, has moved 23 Å. The position of the substrate binding site is confirmed by a difference map between the pyruvate ternary complex and the isomorphous complex LDH:NAD:oxalate, which shows the noncovalently bound inhibitor to be slightly further from the coenzyme than the pyruvate. The oxalate forms salt bridges with arginines 109 and 171. This anion site is occupied by a sulfate ion in apoenzyme crystals grown in ammonium sulfate. The sulfate ion is stabilized by arginine 171 and histidine 195 and can be displaced if the crystals are transferred to a solution of sodium citrate, oxamate (17), or pyruvate. The position

FIG. 3. Conformational changes between NAD in the ternary complex (*black*) and (a) NAD^+ or (b) AMP in binary complexes formed by diffusion into grown apoenzyme crystals.

FIG. 4. Diagrammatic representation of coenzyme binding as appears in the LDH:NAD-pyruvate complex.

of the substrate with respect to the C4 position on the nicotinamide ring, histidine 195, and arginine 171 determines the specificity of the enzyme for L-lactate (Fig. 5).

Arginine 171 can interact with the carboxyl group of the substrate. Using phenylglyoxal, Berghäuser and Falderbaum (18) showed an arginine to be involved in substrate binding. Schwert and Yang (19) have used butanedione to implicate as many as three arginines as being essential for the catalytic activity of the enzyme. These are presumably arginines 171, 109, and 101, the last residue being involved in binding of the coenzyme.

The hydroxyl group of the lactate donates a proton to histidine 195 (Fig. 5). A histidine has been suggested as a proton sink or source by Millar and Schwert (20) and by Schwert *et al.* (7). Woenckhaus and coworkers (21-23) demonstrated that a single histidine residue per subunit was essential for enzymatic activity in pig H₄ LDH. On the basis of the sequence of a peptide containing this histidine, Rossmann *et al.* (1) identified it as residue 195. Except for residue 194, the sequence of the Woenckhaus peptide is identical to region 191-203 of dogfish M₄ LDH and confirms that histidine 195 in the dogfish enzyme corresponds to the essential histidine in pig H₄ LDH.

The hydride that is transferred directly to the coenzyme (24, 25) must be oriented towards the A position on the C4 atom of the nicotinamide ring (Fig. 5). The methyl group of the lactate is thus positioned with at least one hydrogen atom pointing towards the outside of the enzyme, a finding consistent with the results of Ray and Bergner (Ray, W. J. Jr. and Bergner, J. W. II, private communication) that phenyl pyruvate is a substrate analogue with the same maximum velocity as pyruvate.

Kaplan and his coworkers (26, 27) have shown that the presence of a substituent at the 3-position of the nicotinamide ring containing a carbon-oxygen, -sulfur, or -nitrogen double bond is essential for activity. The electron density maps of the ternary complexes show the amide to be in the vicinity of the substrate and histidine 195, although the importance of this is not clear. It appears that the importance of the amide group may be primarily to provide the pyridine ring with the appropriate redox potential by means of a conjugated double bond, rather than to provide an additional point of interaction with the enzyme.

A surprising absentee in the catalogue of residues implicated in the binding of the substrate and coenzyme is tryptophan. Since the binding of NADH results in a significant quenching of the protein fluorescence (28, 29), one may have expected to find one or more tryptophan residues to be associated with the binding of the coenzyme. It appears, however, that the change in the quantum yield of the tryptophan residues is a secondary effect, possibly resulting from the conformational changes that occur upon the binding of NADH. These conformational changes may be extended through the protein chain to reach into the region of one or more tryptophan residues.

The "essential" thiol group (cysteine 165) is also not directly involved in binding of the substrate or coenzyme. Modification of this -SH inactivates LDH of most species (30-33). The position of mercurials when bound to cysteine 165 would prevent the movement of histidine 195.

The conformational changes of the loop bring arginine 101 in a position to bind to the coenzyme phosphates, and arginine 109 to approach the substrate site. The folded down loop en-

FIG. 5. Diagrammatic representation of anticipated substrate binding in the active ternary intermediate.

closes the active center, thereby increasing the already large number of charged and hydrophilic groups in this region of the subunit (Fig. 4). These include glutamates 107 and 140, aspartate 168, and arginines 109 and 171, as well as histidine 195 and glutamine 102. The roles of some of these residues have already been discussed. In addition, on binding NAD⁺, glutamate 140 is available to cancel the positive charge of the coenzyme. On reduction, however, the coenzyme loses its charge, which is transferred through the substrate to histidine 195. Arginine 109 could then balance the negative charge of glutamate 140, and aspartate 168 could balance the positive charge of histidine 195. The explanation remains incomplete, since such a charge redistribution is likely to be extended further through the protein.

In general, the features described in this paper provide a framework of interactions necessary, although not in themselves sufficient, for a complete understanding of the mechanism of LDH. The similarities in tertiary structures that are found in LDH and MDH (34) could indicate that the nature of the coenzyme and substrate binding may be similar to that of other dehydrogenases.

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