Structural adaptations of lactate dehydrogenase isozymes

(AMINO ACID SEQUENCES/X-RAY CRYSTALLOGRAPHY/PROTEIN STRUCTURE AND FUNCTION)

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ABSTRACT The three-dimensional structures of dogfish M4 (muscle) and pig H4 (heart) lactate dehydrogenase (L-lactate:NAD+ oxidoreductase, EC 1.1.1.27) have been determined and correlated with the amino acid sequences of the dogfish M4, pig M4, pig H4, chicken M4, and chicken H4 lactate dehydrogenase isozymes. These results have been related to the known differences of physicochemical properties between the M and H lactate dehydrogenase isozymes. By far the largest structural alterations occur in the transition between the "apo-" and "ternary complex" conformational states of the enzyme rather than between species or isozymes. The major catalytic difference can be explained by a replacement of alanine (in the M chain) with a glutamine (in the H chain) in the vicinity of the binding site of the coenzyme phosphates. The known immunological differentiation of the M and H isozymes is consistent with the differences in their amino acid sequences.

The subunits of lactate dehydrogenase (LDH; L-lactate:NAD+ oxidoreductase, EC 1.1.1.27) exist as two major structural forms, usually referred to as M (muscle) and H (heart) or A and B, respectively, which give rise to five different isozymes of the tetrameric molecule (1, 2) in higher vertebrates. The differences in the properties of the LDH isozymes are dependent on their subunit composition and are most exaggerated between the homotetramers M4 (LDH-5) and H4 (LDH-1). The most important of these differences are (independent of species): (i) The turnover number of the M4 isozyme is generally about twice that for the H4 isozyme (3-5). (ii) The activity of the H4 isozyme is more readily diminished by modification of the carboxyamide group on the S position of the nicotinamide (6). For instance, acetylpyridine adenine dinucleotide reduces the rate of catalysis by at least five when compared to NAD in the H4 enzyme, whereas the M4 isozyme is not especially sensitive to this difference of the NAD molecule (7). (iii) The forward reaction lactate-to-pyruvate is inhibited to a far greater extent by high concentrations of pyruvate in the H4 than in the M4 isozyme (5, 7-9). (iv) Both the enzymic activity and the affinity of the substrate for enzyme are reduced to a greater extent in the M4 isozyme as the length of the aliphatic chain of the α-keto acid increases (10-12). (v) The H4 isozyme binds oxidized or reduced coenzyme better than the M4 isozyme (13-20). (vi) An antiserum induced in rabbit against the M4 isozyme of one species will crossreact with the M4 isozyme from several closely related species but not with the H4 isozyme of the same species (4, 21-25). Some appreciation of these differences is now possible from a correlation of recent studies on three-dimensional and primary structures of the H4 and M4 LDH isozymes. The tertiary structure of dogfish M4 LDH has been determined independently for the apo-enzyme and for a complex of LDH, coenzyme, and substrate analogue. The apo-enzyme has been studied at 2.0-Å resolution, while a series of isomorphous tertiary complexes have been investigated to 3.0-Å resolution. The structural differences between these two conformational states of the enzyme have been discussed previously in terms of the function of the enzyme (26). Eventoff et al. (27) have shown that, at low resolution (6.0 Å), conformational differences between a ternary complex with dogfish M4 LDH and a ternary complex with pig H4 LDH are very small. This has been confirmed at 2.5-Å resolution (W. Eventoff and M. G. Rossmann, unpublished results). Similarly, at low resolution there is no detectable difference between the apo-enzyme structures of dogfish M4 LDH and the sperm-specific LDH-X from mouse (28). It must, therefore, be concluded that the major differences between LDH tertiary structures are not between species or isozymes, but in the conformational changes during catalysis. Differences in the catalytic properties of the H and M isozymes must thus arise due to differences in the amino acid side chains decorating the highly conserved structure of the polypeptide backbone.

About two-thirds of the amino acid sequence of dogfish M4 LDH was presented by Taylor et al. (29), where a complete but tentative sequence (S. S. Taylor, unpublished results) was discussed by Holbrook et al. (30). Recently Taylor and coworkers have published their definitive results (31, 32). Kiltz et al. have independently determined the amino acid sequences of pig H4 and M4 LDH (33). In addition, H. J. Torff and his colleagues have almost completed the sequences of chicken H4 and M4 LDH, which are shown aligned with respect to the pig H4 and M4 sequences (Fig. 1).

The numbering system used in previous publications on LDH had been based on the initial building of a backbone without knowledge of sequence. Errors were subsequently found in a few positions where an extra amino acid was either erroneously included or where an amino acid was omitted. The numbering system thus has gaps or insertions. For the sake of the comparisons used in this paper and with reference to the well-recognized numbering of important residues, we retain the numbering (N) based on the "species" established by the early work. In addition, as dogfish M4 LDH has been most studied, its sequential numbering (M) is given in parentheses.†

Active center residues

The important residues that are involved in the active center have been described previously (26, 30) (Fig. 2). However, a variety of new facts and revisions has emerged with knowledge of five LDH amino acid sequences.

The gap between the loop and the rigid part of the molecule is found to be lined by a ring of negative charges. These are

† Conversion between the two numbering systems is given by M = N + p, in which p = 0 for 1 ≤ N ≤ 20; p = −1 for 21 ≤ N ≤ 81; p = −2 for 82 ≤ N ≤ 103; p = −3 for 104 ≤ N ≤ 132A; p = −2 for 132B ≤ N ≤ 210A; p = −1 for 210B ≤ N ≤ 299; and p = −2 for 300 ≤ N ≤ 331. In order to avoid confusion with previous publications, all residue numbers will be given in the form N(M).

ABBRiVATION: LDH, lactate dehydrogenase.

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FIG. 1 Alignment of the dogfish M, pig M, chicken M, pig H, and chicken H LDH sequences. These sequences are referred to as DM, PM, CM, PH, and CH, respectively. Residues are numbered according to the previously established system (N). Some revisions (32) of the tentative dogfish M, LDH sequence (30) affect some of the active center residues. The peptide 134–148, which had been noted for its bad agreement with the electron density maps, has been interleaved with part of the sequence in the region 210–250. The exchanged sequences both terminate.
provided by glutamic and aspartic acid residues 197(195), 234(233), and 238(237) in the apo structure (Fig. 2). Residue 251(250) contributes charge to this ring in some of the sequences. This ring is particularly obvious in the ternary complex structure, where the closing of the loop adds Glu 107(104) to the ring. The positive charge on the guanidinium group of Arg 106(105) must pass through this ring during the conformational change between the apo and ternary complex structures. The presence of this positive charge in the active center may be a contributing factor to the weaker binding of NAD$^+$ as compared to NADH. Tyr 237(236) within the ring of negative charges is particularly noteworthy. Di Sabato (34) and Jeckel et al. (35) have shown by chemical modifications that there is at least one tyrosine residue close to the coenzyme binding site, and that there is a change of $pK$ for the phenolic hydroxy group when NAD is bound to the modified enzyme. Furthermore, modification of Tyr 237(236) has been shown to be associated with a loss of activity (36).

Revision of the sequence eliminated the special function of residue 250(249), which had been assigned as a lysine and is now found to be an isoleucine. The possible hydrogen bonding between a lysine and the carboxyamide group of nicotinamide had been assumed to give rise to the A side specificity of the coenzyme. Inspection of the electron density maps shows hydrogen bonding possibilities of the carboxyamide group with Ser 163(161) or with the carbonyl of residue 139(137). Ser 163(161) is probably favored in the M form (Fig. 2), where the nicotinamide ring is further in the active center pocket. Because serine can act as both a hydrogen bond donor and acceptor, it is impossible to determine the orientation of the carboxyamide side chain. Revision of the sequence has also increased the hydrophobic character on the B side of the nicotinamide binding site due to the presence of Val 138(136) and Ile 250(249).

The hydrogen bonds and polar interactions among groups on, or associated with, the loop are radically altered between the two conformational states. Some of these still need to be confirmed by refinement of the known LDH structures. Suffice it to mention that Gln 102(100) hydrogen bonds to Asn 140(139) when the loop is down, whereas it hydrogen bonds to the main chain carboxyl of residue 106(103) when the loop is up.

**Differences in the active center of H and M isozymes**

Table 1 shows the major residues involved in the active center and in catalysis. As might be expected, only a very few of these residues are changed between isozymes and even fewer between the same isozyme from different species. Those changes that do occur are thus likely to provide the principal causes for the differing physical and chemical properties of the LDH isozymes.

The changes in the hydrophobic adenosine binding pocket (residues 96(94), 119(116), and 55(54)) tend to reduce the size of the amino acid side chains in the H$_4$ isozyme, increasing the volume within the pocket. Thus, in the initial binding of the cofactor (38), the adenosine will bind with reduced affinity to the H$_4$ as compared to the M$_4$ molecule.

One substantial difference is the presence of Gln 31(30) in the heart enzyme and Ala in the muscle enzyme. The glutamine could form a hydrogen bond with the nicotinamide phosphate (thus increasing the energy of binding for the heart isozyme) and move the nicotinamide end of the coenzyme out of the active center by at least 1 Å. Unfortunately, the evidence for this important change is not clear in the electron density maps. Conformation must await refinement of the present structures. The relative position of the substrate and the nicotinamide would favor the formation of the NAD-pyruvate covalent adduct between the methyl group of pyruvate and carbon 4 of nicotinamide in the H$_4$ isozyme. Thus, the inhibition by pyruvate due to the formation of the abortive ternary complex LDH: NAD-pyruvate (19, 39) can occur more easily.

The slight outward movement of the carboxyamide group in the heart isozyme apparently breaks the hydrogen bond with Ser 163(161) and generates an interaction between the carboxyamide amino group and the carboxyl of residue 139(137). Such an interaction would be disturbed by modifications of the carboxyamide group and correlate with the apparent greater importance of this group in catalysis by the H$_4$ isozyme than...
The differential position of the coenzyme in the M and H isozymes modifies the gap between the loop and the rigid part of the subunit. Model-building studies indicate that the aliphatic chains of \(\alpha\)-keto acid substrates pass through this gap, lined by the ring of negative charge described above. In the \(H_4\) isozyme there is probably less steric hindrance and an environment more favorable to longer hydrophobic substrates, which would result in a lowering of substrate specificity.

**Differences between LDH isozymes other than at the active center**

Table 2 is a similarity matrix between the five known LDH sequences in terms of minimum base changes (MBC) per codon. There are fewer changes between the same isozyme in different species than between different isozymes. This result is consistent with immunological and other data (42, 43) suggesting independent evolution of the M and H genes in higher vertebrates. The number of accepted point mutations among the LDH sequences given in Fig. 1 (about 0.3 MBC/codon) is similar to that found between pig and lobster glyceraldehyde-3-phosphate dehydrogenase (0.33 MBC/codon). Depending on the sequence comparison, between 21 and 38% of the 116 completely external residues are altered, whereas only 6–20% are changed in the 108 completely internal residues. It is perhaps not surprising that two enzymes of the same metabolic pathway have similar rates of evolution.

The differences in amino acid sequence are consistent with the immunological results. Although no sequences of rabbit LDH are available, it can be assumed that rabbit and pig LDH sequences are closely related, since dehydrogenases are very slowly evolving enzymes. Thus, if a foreign LDH is injected into a rabbit, then antibody binding sites might be generated wherever the foreign LDH differs from both \(M_4\) and \(H_4\) rabbit LDH on the molecular surface (44). Inspection of Fig. 1 shows that the dogfish and chicken \(M_4\) LDH sequences are sufficiently different from rabbit LDH to produce almost no similarity of possible antigenic sites. Thus, rabbit antisera to avian \(M_4\) LDH would not be expected to crossreact with fish \(M_4\) LDH or avian \(H_4\) LDH.

The behavior of residues in the subunit–subunit contact area is strikingly different for the three differing types of contact generated by the molecular P, Q, and R axes (30). There are 26, 52, and 44 residues per subunit involved in forming the P, Q, and R axes contact surfaces, respectively. Of these 37, 9, and 46% are changed on the average when the sequences in Fig. 1 are compared. Thus, although there are more residues in the Q-axis contact surface, these residues are far more conserved than in the other two contact regions. The Q-axis association is that which is observed in the soluble malate dehydrogenase dimer (45). Thus the, presumably more recent, P- and R-axis-generated surfaces have evolved more rapidly than the older Q-axis contacts. Casual inspection of the sequence comparisons in Fig. 1 shows that the largest concentrations of changes occur in the amino-terminal arm and in the region 294(293) to 310(308). These two regions complement each other in that they are both the characteristic arm of the M4 isozyme. Other groups within 5.0 Å of the carboxyamide group are Val 32(31), Ser 139(137), Ser 163(161), Ile 250(249), and the substrate, none of which are altered between the M and H isozymes.

The net result of all the changes to the NAD binding site is that, although the coenzyme has more difficulty in finding its orientation in the \(H_4\) isozyme, once oriented it binds more tightly, primarily due to the formation of one extra hydrogen bond. These conclusions are supported by the difference of the NAD dissociation constants for M and H LDH (9 and 0.5 \(\mu\)M, respectively). Because the rate-limiting step of the reaction is the dissociation of the coenzyme from the enzyme (18, 40, 41), the turnover number of the \(H_4\) isozyme will be lower due to both the looser initial fit and the tighter final binding.

The asterisk (*) denotes residues that are not completely conserved. Amino acids are represented by the Dayhoff one-letter code (37).
form the principal R-axis-generated subunit contacts. The concentration of these changes into two extended chain regions, instead of a wide distribution over the length of the polypeptide chain, dramatically demonstrates the high rate of change of amino acids in the R-axis contact surface.

Conclusions

It has been possible to give a qualitative discussion of the principal differences between the M and H isozymes of LDH in terms of the three-dimensional structure of the LDH molecule, five amino acid sequences, and an appreciation of the kinetic properties of the enzyme. The change of a relatively smaller number of amino acids, albeit catalytically important ones, rather than a dramatic conformational alteration is the predominant factor in the differentiation of the LDH isozymes. A similar situation has been observed in the human erythrocyte carbonic anhydrase isozymes B and C (46), and it is tempting to suggest that this may be the general mechanism for differentiation in the majority of other isozyme systems. Verification of these concepts will now be necessary by refinement of the various crystal structures, analysis of other amino acid sequences, and selection of chemical modifications in the study of enzyme kinetics.

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