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Structure of Liver Alcohol Dehydrogenase at 2.9-Å Resolution

(crystallographic structure/zinc/coenzyme binding)

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ABSTRACT **The conformation of the polypeptide chain in horse liver alcohol dehydrogenase (EC 1.1.1.1), as well as the binding sites for some inhibitor molecules, have been determined from x-ray crystallographic data to a resolution of 2.9 Å. Each subunit of the dimeric molecule is organized into two parts unequal in size and separated by a wide and deep active-site cleft. The adenosine moiety of the coenzyme is bound within the smaller region. Interactions between these coenzyme-binding substructures define the subunit contact area of the molecule. The "catalytic" zinc atoms are bound at the bottom of the clefts about 20 Å from the surface of the molecule. The coenzyme binding region has a main-chain conformation very similar to a corresponding region in lactate and malate dehydrogenase. It is suggested that this substructure is a general one for binding of nucleotides and, in particular, the coenzyme NAD^+.**

The structures of a family of related enzymes, the dehydrogenases, are emerging, and striking structural homologies within this group of proteins have become apparent. The three-dimensional structure of lactate dehydrogenase (LDH) has been determined to a resolution of 2.5 Å by Rossmann *et al.* (1). More recently, Hill *et al.* (2) showed that the conformation of the malate dehydrogenase (MDH) subunit is very similar to that of LDH. We now show that part of the subunit of liver alcohol dehydrogenase (LADH), and by inference yeast alcohol dehydrogenase (YADH), have structures that are homologous to both LDH as well as MDH. In particular, our data as well as those in the accompanying paper by Jörnvall (3), show that the conformation of the coenzyme-binding portions of all four dehydrogenase subunits are conserved, while other portions of the subunit chains have been free to evolve in divergent directions.

LADH (EC 1.1.1.1) has the following properties. It is an NAD-requiring enzyme that catalyzes the oxidation of various primary and secondary alcohols to the corresponding aldehydes. The active enzyme has a molecular weight of 80,000 and is a dimer of two identical subunits. The sequence of the 374 amino acids of the subunit has been determined by Jörnvall (4). Each subunit binds firmly two zinc atoms (5) and has one main coenzyme-binding site (6). An obligatory binding order of coenzyme followed by substrate has been established (7). This order is consistent with the finding that binding of coenzyme is accompanied by a conformational change of the protein (8, 9).

The apoenzyme of LADH crystallizes in space-group $C222_1$ with one subunit per asymmetric unit and cell dimensions $a = 56.0$ Å, $b = 75.2$ Å, and $c = 181.6$ Å (10). The crystallographic 2-fold axis relating the two subunits of the apoenzyme molecule is not present in crystals of complexes between apoenzyme and coenzyme, which suggests that the coenzyme may induce a structural asymmetry in the chemically identical subunits. An electron density map to 5-Å resolution of the apoenzyme molecule has been described (11). Here we report the structure of this molecule as deduced from an electron-density distribution at a resolution of 2.9 Å. Most important is the analysis of the binding sites for some inhibitor molecules, which permits us to identify functional attributes of the enzyme structure.

MATERIALS AND METHODS

The enzyme was isolated from fresh horse livers (Åkeson, Å. & Lundqvist, G., to be published). Preparation of crystals and heavy-atom derivatives suitable for x-ray studies has been described (10). Methods of isomorphous replacement similar to those used for other protein-structure determinations were applied to obtain the electron density map. The crystallographic data were measured at +4° on a computer-controlled Philips-Stoe four-circle diffractometer equipped with a 32 K disc storage. Data were collected to a resolution of 2.9 Å from crystals of the native protein, three heavy-metal derivatives [$K_2Pt(CN)_4$, $KAu(CN)_2$, and $K_2Pt(CN)_4$ + $KAu(CN)_2$], and one inhibitor complex [adenosine diphosphate ribose (ADP-ribose)]. Intensities within 4.5 Å were also measured on two other inhibitor complexes: 8-Br-ADP-ribose and 1,10-phenanthroline. A skeletal model of the main chain was built with the Kendrew-type models, with an optical comparator (12).

CONFORMATION OF THE SUBUNIT

In this section we describe briefly the conformation of the polypeptide chain and some details of the subunit interaction and binding of inhibitor molecules as deduced from our electron-density maps. In the next section we will discuss some implications of this structure.

The two highest features in our 2.9-Å electron-density map were roughly spherical in shape and were interpreted as the two zinc atoms of the subunit. From our 5-Å work we already knew the position of one of these zinc atoms and the subunit

Abbreviations: LADH, liver alcohol dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; GDPH, glyceraldehyde-3-phosphate dehydrogenase; YADH, yeast alcohol dehydrogenase; ADP-ribose, adenosine diphosphate ribose.

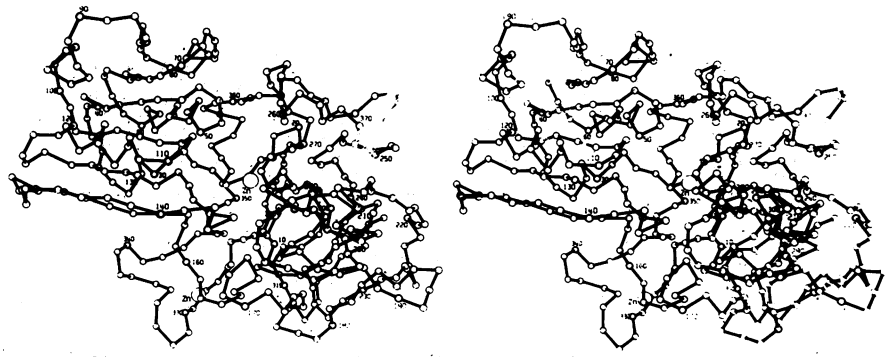

FIG. 1. Stereo drawing of the α-carbon backbone of the LADH subunit. Computer-drawn stereo figure was made by the program OR-TEP of Dr. Carrol Johnson.

boundaries (11). The remaining high density within one subunit could then easily be allocated to one continous chain with corresponding side-chain densities. The correct hand of the map was determined by choosing the orientation that gave right-handedness to all observed helices.

The subunit is organized into two parts unequal in size and separated by a rather wide and deep cleft (Fig. 1). The smaller part, which will be called the coenzyme-binding substructure, binds the adenosine end of the coenzyme.

The first 23 amino acids from the N-terminal are located in the bigger part of the subunit. These residues participate in two regions of pleated sheet in this substructure. Residues 24–27 form a bridge to the coenzyme-binding part, which is built up from six parallel strands of pleated sheet connected by helical regions or loops. The chain is brought to the beginning of the first of these parallel strands by residues 28–39, which contain a two-turn helix. Residues 40–165 are used to build up this structure from the beginning of the first strand to the end of the last strand.

A very similar coenzyme-binding substructure has been found for residues 20–160 in the structure of LDH (1) and more recently also in MDH (2). For the following description of the similarities and differences in these substructures we use the terminology of Hill *et al.* (2). The parallel strands are called βA–βF and the helices αA–αE consecutively from the amino end. The physical sequence of strands from top to bottom of the sheet in Fig. 2 is C-B-A-D-E-F. Fig. 2 is an attempt to present this substructure in two dimensions. We are indebted to B. Furugren who designed this drawing.

Since all six strands of LDH and LADH except βF are of about the same length and all are arranged in the same order, and since the sheets have the same twist of about 90°, we can superimpose the two substructures so that the strands coincide. We then find that the helices αB and αC, as well as the corners joining these helices to their corresponding strands, also roughly coincide on one side of the sheet. The first halves of these substructures in LDH and LADH thus have a very similar conformation. The similarities also extend to the position of the loop regions connecting the remaining strands on the other side of the sheet so that the helices αE are nearly superimposable.

There are, however, significant differences between these loops. The chain segment connecting the strands βD and βE contains a loop region in LDH, comprising about 20 amino acids, that changes its conformation upon coenzyme binding. This part, which includes helix αD, is completely absent in LADH where only three amino acids are used to join strand βD and helix αE. The loops connecting βE and βF have rather different conformations in these two structures. In LADH there is no helical arrangement in this loop, but instead, part of the chain has an extended conformation running about 15 Å from and antiparallel to strand βF. Furthermore, the βF strand is seven residues long in LADH compared to three in LDH.

After completion of the coenzyme-binding substructure by strand βF, the chain crosses over to the larger region on the other side of the subunit. The conformation of this part of the LADH subunit shows no similarities to the remaining parts of the LDH subunit. The main elements of secondary structure within this part are two regions of pleated sheet, containing five and six strands. The strands within these sheets are both parallel and antiparallel. Furthermore, there are several regions of antiparallel β-structure containing two or three strands. Here there are only a few short helices. A more detailed description of this segment of the subunit will be given when the detailed assignment of the side chains has been completed.

The two subunits are related by a crystallographic 2-fold symmetry-axis in the apoenzyme. The βF strands of each subunit run in opposite directions perpendicular to this axis and are joined together by hydrogen bonds forming two strands of antiparallel β-structure. A similar type of subunit interaction was recently found for concanavalin A (13, 14). The pleated-sheet structure in the coenzyme-binding part of the molecule thus extends through the whole dimeric molecule and comprises 12 strands arranged in two pairs of six parallel strands in each subunit. These pairs are joined together by antiparallel β-binding through the 2-fold symmetry-axis. There is a twist of 180° from the first to the twelfth strand. Side chains from the loop connecting βE and βF also take part in the subunit contact area. The part of this loop that has an extended conformation is furthermore joined by antiparallel β-binding to the corresponding loop in the other subunit. These contacts, which extend over a rather large and flat surface and which seem to be very tight, define the subunit contact area.

The conformational changes induced by binding of coenzyme to LADH are reflected in the different modifications exhibited by the holoenzyme and apoenzyme crystals. In order to locate the coenzyme-binding site we therefore used an analogue that did not cause the conformational change. The obvious choice was ADP-ribose, since this analogue binds at the same site as the coenzyme (15) and the LADH–ADP-ribose complex crystallizes isomorphously with the apoenzyme

(16). A three-dimensional difference-Fourier map with data to 2.9-Å resolution was quite clear and showed beyond doubt the location of the ADP-ribose molecule. In order to orient the ADP-ribose moiety, we decided to study an analogue of ADP-ribose having a substitution of bromine in the 8-position of the adenine ring. This analogue was kindly prepared for us by Abdallah and Biellmann in Strasbourg. The Br position was easily located from a difference-Fourier map of this complex, from data to 4.5-Å resolution. Knowing this position, we could now build a model of ADP-ribose into the difference map and be confident that we had the proper orientation.

The ADP-ribose molecule binds in an extended conformation within the substructure that is similar to LDH. The adenine end lies in a hydrophobic pocket lined by residues from strands βA, βB, and βD and from the loop connecting βD and αE. The phosphates are at the surface in a small depression between the βA αB corner and the βE βF loop. The terminal ribose points into the cleft towards the active-site zinc atom.

FUNCTIONAL SIGNIFICANCE OF THE STRUCTURE

The most striking result of the present structure determination is that there is a coenzyme-binding substructure consisting of about 120 amino acids in each identical subunit of LADH with a polypeptide-chain conformation very similar to a corresponding coenzyme-binding part in the subunits of two other dehydrogenases, LDH and MDH. The degree of structural similarity between these substructures is particularly remarkable in view of the fact that the remaining regions of the subunits are completely different. A comparison of the binding of NAD^+ to LDH with that of ADP-ribose to LADH shows, furthermore, that in both instances the adenosine moiety of the coenzyme molecule is bound in a very similar manner.

We now suggest that this substructure, built up from six parallel strands in a pleated-sheet arrangement and joined by helices or loops, may be a general one for binding of nucleotides and, in particular, the coenzyme NAD^+. This hypothesis is strengthened by Jörnvall's finding (3) of significant sequence homology within the N-terminal thirds of LADH and YADH. Since this is the part of the chain that builds up the coenzyme-binding substructure in LADH, it is very probable that the same structure is present also in YADH. Furthermore, Jörnvall has earlier shown (17) distant evolutionary relation between the amino-acid sequence in LADH and that of glyceraldehyde-3-phosphate dehydrogenase (GDPH). Even slight sequence homology of this kind, provided that it is significant, is strong indication of conserved three-dimensional structure. This finding has been substantiated in this particular instance by the very recent x-ray structure determination (18) of GPDH, where it is found that GPDH indeed has a coenzyme-binding substructure similar to LDH, MDH, and LADH.

The main conformational differences between the two substructures in LDH and LADH reflect functional differences. The loop region in LDH of about 20 amino acids that changes its conformation upon coenzyme-binding is completely absent in LADH. Therefore, the nature of the conformational change in these two dehydrogenases must be quite different. This conclusion is in excellent agreement with the findings that coenzyme analogues like ADPR induce a conformational change in LDH but not in LADH. Adams *et al.* (19) have suggested that the main "trigger" for the change of structure in LDH is an interaction between the adenosine phosphate and an arginine residue within this loop. Since a corresponding loop is absent in LADH, its conformational change must be induced by some other mechanism which in turn must involve the nicotinamide part of the coenzyme (11).

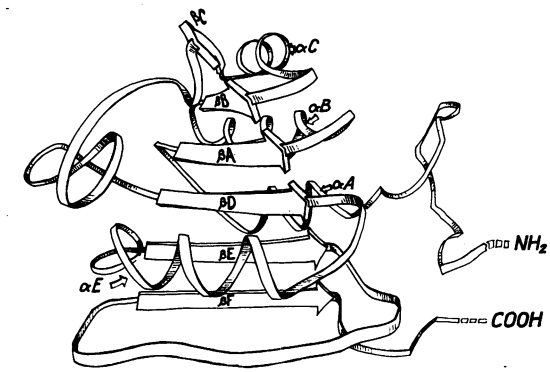

FIG. 2. Schematic drawing of the main-chain conformation of the coenzyme-binding substructure of LADH (by Bo Furugren).

There are also differences in the length of the last of the six parallel strands and in the conformation of the last loop, reflecting differences in subunit interaction. In LADH this region constitutes the main subunit interaction site. In LDH, on the other hand, the corresponding region is entirely within the subunit and does not participate in any kind of subunit binding. If the LADH subunit is rotated so that its coenzyme-binding substructure coincides with the similar substructure of one LDH subunit, then the position of the 2-fold axis of the LADH molecule is quite different from any of the 2-fold axes of the LDH molecule.

Interaction of the subunits in LADH through these coenzyme-binding substructures form a dimeric molecule with the overall shape of a prolate ellipsoid of approximate dimensions 45 × 55 × 110 Å with a coenzyme-binding core in the middle. This core has sites for the binding of two coenzyme molecules. The ends of the LADH molecule form two wings that are separated from the core by wide and deep active-site clefts. Two of the four zinc atoms of the molecule are at the bottom of these clefts, while the other two are bound in neck regions connecting the wings to the core.

From studies of the enzyme in solution it has been suggested (20) that two of the zinc atoms are involved in the catalytic activity of the enzyme and the other two are necessary for the structural stability of the molecule. These results are confirmed by our structure determination. The "catalytic" zinc atom has been characterized in several investigations (15, 21, 22) by its ability to bind 1,10-phenanthroline. This molecule is a competetive inhibitor of both coenzyme and substrate binding (23). In order to correlate such experiments in solution with our crystal structure, we have studied the LADH–phenanthroline complex by x-ray methods to 4.5-Å resolution.

We find that 1,10-phenanthroline binds to the zinc atom in the active-site cleft, and we can conclude that experiments in solution demonstrating functions of the "catalytic" zinc refer to the zinc atom located in the active-site cleft in our crystal structure.

This zinc atom is anchored in the bottom of the cleft by three protein side chains. Phenanthroline is bound in the cleft

from the side opposite the protein ligands and completes the zinc coordination. The cleft is very deep, with a distance of 20–25 Å from the zinc atom to the surface of the molecule. In all probability this cleft constitutes the substrate-binding site. Work is now in progress to test this by locating the binding site for big physiological substrates like 17-hydroxystearic acid (24).

When ADP-ribose is bound to LADH, the terminal ribose of the inhibitor is separated from the "active-site" zinc by a distance of 6–8 Å, and there is no protein density in the space between them. The amide group of the coenzyme would be in the vicinity of the zinc atom if the nicotinamide part is built onto the ADP-ribose molecule with the conformation of NAD^+ that has been found when it binds to LDH (19). Although we have no knowledge of the nature of the coenzyme-induced conformational change, these results provide strong evidence that this zinc atom participates in the catalytic activity.

The second zinc atom of the subunit is bound by four protein ligands within one of the two narrow neck regions that join the two substructures. Although it is bound near the surface of the molecule, this zinc atom is completely surrounded by the protein and is not accessible to chelating agents when the protein is in its native conformation. It is far removed from the active-site cleft. The distance between the two zinc atoms of the subunit is 20 Å. We do not know the function of this zinc apart from the vague notion that it might be required to maintain the proper conformation of the subunit.

Several investigators have suggested (25, 26) that subunit interactions within the LADH dimer play an important role in ordering the course of reaction. From the present structure it is evident that any kinetic model based on direct interaction between the active sites is excluded. The distance between the two active-site zinc atoms of the dimer is 47 Å. However, in this connection it is interesting that the subunits are bound together through their coenzyme-binding substructures and that one wall of each active-site cleft is lined by side chains from these substructures. Indirect interaction between the active sites is thus quite possible. We hope that future determination of the holenzyme structure where the subunits are not crystallographically identical will clarify these problems

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