Structural Differences between Apo- and Holoenzyme of Horse Liver Alcohol Dehydrogenase*

Hans Eklund and Carl-Ivar Branden
From the Department of Chemistry, Uppsala University, Sweden

The three-dimensional structure of a ternary complex of horse liver alcohol dehydrogenase with reduced nicotinamide adenine dinucleotide and the inhibitor dimethyl sulfoxide has been determined to 4.5 Å resolution independently of the apoenzyme structure. The electron density maps of both structures have been compared. The two coenzyme binding domains which form the center of the dimer molecule have retained their conformation and orientation within the molecule whereas the catalytic domains rotate and narrow the cleft between the domains. The active site becomes shielded from the solution by a combination of this rotation, local movements of a loop from residues 53 to 57 and coenzyme and substrate binding. Both subunits bind coenzyme and inhibitor to the same extent. The nicotinamide ring of the coenzyme is positioned close to the active zinc atom and the inhibitor is bound to this zinc atom. The difference between the two crystallographically independent subunits is small. The proposed mechanisms of action for the enzyme based on the apoenzyme structure are confirmed by the present investigation.

* The abbreviations used are: MeS0, dimethyl sulfoxide; NMN, nicotinamide mononucleotide; Tes, N- [tris(hydroxymethyl) methyl-2-amino] ethanesulfonic acid.

Horse liver alcohol dehydrogenase (EC 1.1.1.1) usually crystallizes in three different crystal forms: orthorhombic C2221, monoclinic P21, and triclinic P1 (1). In the absence of coenzyme we always obtain orthorhombic crystals and in the presence of coenzyme the other two modifications are usually formed, sometimes in the same batch. The relationships among the crystal forms have been described earlier (2, 3).

The structure of the orthorhombic modification has been determined to 2.4 Å resolution (4). Furthermore, the binding of a number of inhibitors, coenzyme analogs, and other molecules has been studied in this modification by difference Fourier techniques (5-10). It has not been possible to study the binding of substrates or substrate analogs in the orthorhombic crystals due to weak binding which is probably related to the ordered mechanism of the enzyme (11) and the high concentration of the alcohol precipitant. In order to study coenzyme binding and related conformational changes, possible nonequivalence between subunits, and substrate interactions with the enzyme, a crystallographic investigation of the triclinic form was started.

In this communication we describe the structure determination to a resolution of 4.5 Å of a triclinic ternary complex of enzyme, reduced coenzyme, and the inhibitor MeS0 first described by Perlman and Wolff (12). A number of different ternary complexes crystallize in this crystal modification (3) but we have chosen the complex which reproducibly gave the best quality triclinic crystals. These crystals diffract beyond 2 Å resolution and are more stable than the orthorhombic apoenzyme crystals.

MATERIALS AND METHODS

Horse liver alcohol dehydrogenase, kindly supplied by Dr. Å. Akeson, was crystallized from 14% protein solutions in the presence of NADH and MeS0 (5%) by dialysis against 2-methyl-2,4-pentane diol of increasing concentration in 0.05 M Tes/NH4 buffer at pH 7.0. The crystals are triclinic with the cell dimensions a = 51.2 Å, b = 44.5 Å, c = 94.3 Å, α = 104.5°, β = 102.0°, and γ = 70.9°. The unit cell (which is the asymmetric unit) contains the dimer of molecular weight 82,000.

Three derivatives were used: uranyl oxalate (10-3 M), methyl mercurothiosalicylate (10-3 M, in the absence of MeS0) and Hg(CN)2 (1 to 3 x 10-4 M).

4746 independent x-ray intensities corresponding to a resolution of 4.5 Å were measured on a Stoe-Philips four-circle diffractometer and processed as described earlier (4). The derivative data were scaled by minimizing the sum of the differences between their F-values and the native ones. Anisotropic temperature factors were applied to each derivative crystal in the scaling procedure.

The main heavy atom sites of the derivatives were located from difference Fourier maps using phases calculated from the apoenzyme model. The coordinates of this model were oriented and positioned in the triclinic cell by the rotation function (13) and two mercury positions common to both structures. Additional sites were found by difference and double difference Fourier maps, using isomorphous phase angles. The heavy atom parameters were subjected to about 30 cycles of the usual least squares refinement procedure (14). The present refinement parameters are given in Tables I and II. A mean figure of merit for the 4591 reflexions included in the refinement was 0.73, using best phases during the refinement (15).

The electron density map was plotted perpendicular to the noncrystallographic molecular 2-fold axis found by the rotation function (κ = 180°, ψ = 30°, and q = 17°). An orthorhombic electron density map at the same resolution was plotted in the same orientation, a carbon coordinates and main chain tracing of the apoenzyme model were plotted in the orthorhombic map to facilitate the interpretation.

Details of the structure determination will be reported in a later paper.

RESULTS AND DISCUSSION

The electron density map obtained was of good quality (Fig. 1) and all significant densities could be assigned to protein, coenzyme, and inhibitor. The highest peaks in the map correspond to a helices, zinc atoms, and the phosphate atoms of the coenzyme. The interpretation was greatly aided by comparison with the known apoenzyme structure. It was possible to follow the main chain through the triclinic electron density even in the most complicated pleated sheet structure of the catalytic domain. There were breaks at some points in the electron density, mostly at glycine residues. Furthermore, many side chains were visible, especially large ones, which made it easy to relate the density to the model of the enzyme.

The ADP part of the coenzyme molecule binds to and
The occupancy is on a relative scale not adjusted to the absolute value. The occupancy factors were not refined, the value B = 15 was used throughout the refinement. The sites with the same letter are symmetry related by the noncrystallographic 2-fold axis. F and F' are MeSO sites with negative occupancy.

### TABLE II

Average values of heavy atom refinement parameters at the present stage

The refinement was done in two parts. The first third of the reflexions are in the left column and the remaining two-thirds in the right column. The division was made due to different occupancy of the Hg(CN)₂⁻ derivative.

### TABLE III

The coordinates of the coenzyme fitted to the mean electron density of both subunits. The coordinates are given in Angstrom units in an orthogonal coordinate system equivalent to the orthorhombic structure (4), and should be considered preliminary.

There is electron density connected with the zinc atom in each substrate pocket. These peaks were interpreted as the direct binding of MeSO to the active site zinc atoms. This interpretation was confirmed by the difference Fourier map of the ethylmercurythiosalicylate derivative, which does not contain Me&SO. In addition to the positive peaks due to the mercury complex there were two negative regions significantly lower than the background, one in each subunit, at the MeSO positions. The binding is illustrated in Fig. 2.

The NMN binding cleft, the active site and the substrate channel of each subunit form two extensive clefts which divide the molecule into three parts: a central core containing the two coenzyme binding domains bound tightly together and the two catalytic domains at each side of this core (4) (Fig. 3). The catalytic domains have two covalent connections with the central core, which form the bottom wall of the cleft. The cleft is accessible to molecules from the solution from the two almost perpendicular directions where nicotinamide and substrate are bound. Between these areas there are van der Waals contacts between the domains, residues 295 to 297 of the NAD binding domain are in contact with residues 53 to 57 of the catalytic domain (Fig. 4). The comparison made here of the orthorhombic and triclinic electron density maps shows that the central parts of the molecule are very similar in the two crystal forms. The coenzyme binding domains, the main subunit interaction area between these domains and parts of the active site clefts are virtually identical. The catalytic domains, however, show differences in their conformations. The gross change can be described as a rotation of these domains with respect to the central core so that the front sides of the domains move toward the core and the back side away from the core (Fig. 5). The central parts of these domains do not move but the front and back parts move considerably, about 6 to 7 Å.

A necessary requirement for this rotation of the catalytic domains is that there is a change in the domain-domain interaction area at the front of the molecule. Since this area does not involve any strong binding it is easily accommodated. We observe that the loop containing residues 295 to 298 is turned down and allows the loop of residues 53 to 57 to move up as seen in Fig. 4. Residue 55 at the tip of this loop is one of the residues which has the largest movement, about 6 Å. Residues 15 to 18 which are in contact with the loop around residue 55 also move in the same direction. The close contacts between these loops are conserved but both loops are displaced by about 6 Å.

A functionally important result of this conformational change is that the clefts between the domains become narrower. The loop around residue 55 and part of the preceding helix, residues 47 to 53, move closer to the bound coenzyme. At the entrance to the substrate channel the loop formed by residues 115 to 118 narrows the entrance by 1 to 2 Å. The
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FIG. 1. Seven consecutive sections of the electron density map cut perpendicular to the noncrystallographic molecular 2-fold axis, which is marked in the center of the figure. The broken line encloses 1 molecule. The 2 zinc atoms of the active site cleft are marked.

FIG. 2. A tentative conformation of the coenzyme based on the best fit of the models to the electron density. The nicotinamide ring is placed close to the active site zinc atom, which in the figure is shown with parts of the three protein ligands of cysteine 46, histidine 67, and cysteine 174. The binding of the inhibitor Me₃SO to the zinc atom is shown in a tentative conformation.

FIG. 3. A schematic drawing of a section through the molecule based on the van der Waals radius of the atoms of the orthorhombic structure showing the substrate clefts and the active site zinc atoms. The positions of the coenzyme and the Me₃SO molecule are superimposed onto this section.

FIG. 4. The domain-domain interaction in the triclinic structure with the active site and the coenzyme. The parts of the corresponding orthorhombic structure which have different conformation are shown in broken lines. For the nomenclature of strands and helices, see Eklund et al. (4).

FIG. 5. A schematic drawing of the main conformational changes of the alcohol dehydrogenase molecule in the transition from apoenzyme to ternary complex. The arrows describe the movements of the catalytic domains at the ends of the central core of the two coenzyme binding domains. The molecular 2-fold axis which is crystallographic for the apoenzyme but not for the holoenzyme is shown at the center of the molecule. The front side, which faces the viewer, contains the area which binds the 2 coenzyme molecules. We are indebted to Bo Furugren for the drawing of Figs. 4 and 5.
combined effect of these conformational changes and coenzyme and substrate binding is to shield off the active site zinc atom and its surroundings from the solution. This effect should play an important role in lowering the activation energy for the reaction mechanism.

A loop movement with a similar effect on the active site has been described for lactate dehydrogenase (16). In that enzyme a loop region in the coenzyme binding domain, residues 103 to 115, moves toward the active site. In a comparison of the structural similarities of the coenzyme binding domains of lactate and alcohol dehydrogenase (17) it was noticed that this important loop was absent in the coenzyme-binding domain of alcohol dehydrogenase. It is now interesting to observe that a loop from the catalytic domain of alcohol dehydrogenase moves to approximately the same region in space. Thus a similar functional effect has been produced by the conformational changes of completely different structural regions in these two enzymes.

Since the active sites are very similar in the triclinic holoenzyme structure and the orthorhombic apoenzyme structure the mechanism of action for the enzyme proposed on the basis of the apoenzyme structure (18, 19) are confirmed by the present study. The assumed position of the coenzyme molecule has been confirmed by the present study and the assumed position of the substrate has been confirmed by a recent study of triclinic crystals containing coenzyme and p-bromobenzyl alcohol (20). It is also important to note that compared to the apoenzyme structure no new residues have been brought into the active site. Furthermore, the positions of the protein groups which have been suggested (18) as important for catalysis and substrate binding have changed only marginally with the possible exception of His 51. However, since local small conformational changes of the order of 1 Å may greatly affect the reactivity of the enzyme, we intend to continue this structure determination to high resolution.

Differences in protein fluorescence upon coenzyme binding have been observed and have been related to the conformational change of the enzyme (21). There are only two tryptophans per subunit and they have very different environments (4). Tryptophans 314 are completely buried in the hydrophobic subunit contact area and do not change conformation or environment as observed at this resolution. Tryptophans 15 on the other hand are at the surface of the molecule in the region that moves considerably. The side chains of these tryptophan residues have changed their environment and become more exposed to the solution.

In order to investigate possible differences between the two crystallographically independent subunits we plotted electron density maps of each subunit separately and compared these section by section. The differences found were generally very small, and the subunits are thus very similar. Differences in peak positions of the order of 1 to 2 Å are, however, found at some places including the active site region. These differences are not due to an error in the direction of the 2-fold axis, since all other densities superimpose after rotation around this 2 fold axis. Although these local differences are clearly seen we cannot exclude the possibility that they are due to errors in phasing or to the low resolution.

By these comparisons we can also see that the coenzyme molecules are bound with similar occupancies in both subunits. Thus, for instance, the peak heights on a relative scale for the pyrophosphate moieties are 2047 and 2095, respectively. Similar occupancies of the inhibitor Me$_2$SO molecule are apparent from Table I, where sites F and F' correspond to the removal of Me$_2$SO molecules in the ethylmercurithiosalicylate derivative. These negative occupancies are the same within the limits of the error.

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H Eklund and C I Brändén


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