Structure of L-3-hydroxyacyl-coenzyme A dehydrogenase: Preliminary chain tracing at 2.8-Å resolution

(nucleotide binding domain/fatty acid oxidation/NAD structure/B-side dehydrogenase/x-ray diffraction)

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ABSTRACT The conformation of L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) has been derived from electron-density maps calculated at 2.8-Å resolution with phases obtained from two heavy-atom derivatives and the bound coenzyme, NAD. Like other dehydrogenases, 3-hydroxyacyl-CoA dehydrogenase is a double-domain structure, but the bilobal nature of this enzyme is more pronounced than has been previously observed. The amino-terminal domain, which comprises approximately the first 200 residues, is responsible for binding the NAD cofactor and displays considerable structural homology with the dinucleotide binding domains observed in other NAD-, NADP-, and FAD-dependent enzymes. The carboxyl-terminal domain, comprising the remaining 107 residues, appears to be all α -helical and bears little homology to other known dehydrogenases. The subunit-subunit interface in the 3-hydroxyacyl-CoA dehydrogenase dimer is formed almost exclusively by residues in the smaller helical domain. A difference map between the apo and holo forms of the crystalline enzyme has been interpreted in terms of the NAD molecule being bound in a typically extended conformation. The location of the coenzyme binding site, along with the structural homology to other dehydrogenases, makes it possible to speculate about the location of the binding site for the fatty acyl-CoA substrate.

In most organisms, the metabolic breakdown of long-chain fatty acids proceeds through the β -oxidation pathway. In this process, a thioester is formed between CoA and the fatty acid, and the alkyl chain is then degraded by the sequential removal of two-carbon units. The mitochondrial β -oxidation cycle involves four enzymes; one of these is L-3-hydroxyacyl-CoA dehydrogenase [(S)-3-hydroxyacyl-CoA:NAD⁺ oxidoreductase, EC 1.1.1.35]. This enzyme utilizes NAD as a cofactor and L-3-hydroxyacyl-CoA as substrate in the following reaction, which *in vivo* proceeds exclusively to the right:

The specificity of the enzyme is quite broad, as 3-hydroxy fatty acyl derivatives of CoA containing four or more carbons are oxidized at about the same rate (1). While the nucleotide moiety of the CoA was found not to be essential for catalysis, K_m is increased and V_{max} is reduced using S-acyl pantotheine derivatives as substrates (2). Transfer of a hydride ion between substrate and the nicotinamide ring of the cofactor by L-3-hydroxyacyl-CoA dehydrogenase is "B-side"-specific (3). This is the same specificity found for glyceralde-

hyde-3-phosphate dehydrogenase but differs from that of other enzymes of known structure, such as malate, lactate, and liver alcohol dehydrogenases, which all have "A-side" specificity.

In eukaryotic cells, L-3-hydroxyacyl-CoA dehydrogenase is encoded by a nuclear gene and is synthesized in the cytoplasm as a precursor form that appears to contain an additional 30–35 amino acids (4). Although the mature form of the enzyme can be isolated easily from a variety of tissues, the form from pig heart has been particularly well characterized (2, 3, 5). Porcine heart L-3-hydroxyacyl-CoA dehydrogenase is a dimeric protein of molecular weight 67,000 composed of two identical subunits, each with 307 amino acids of known sequence (6). Some heterogeneity at the amino terminus has been reported, which may be the result of proteolytic processing accompanying the translocation of the enzyme into the mitochondrial matrix (6).

The enzyme has been crystallized in a number of different forms (7). The orthorhombic form obtained from polyethylene glycol 6000 at a pH near 8 proved usable for x-ray analysis (8). Under these conditions, crystals belonging to the space group C222₁ are formed and the following unit-cell dimensions are observed: $a = 227.2$ Å, $b = 82.1$ Å, and $c = 124.7$ Å. There are eight equivalent positions in the unit cell.

Analysis of electron-density maps based on x-ray diffraction data extending to 5.25-Å resolution indicated that the protein subunits are packed in an unusual fashion in the crystal lattice. One enzyme dimer is located in a general position and has a noncrystallographic 2-fold rotation axis. A second dimer is located with its molecular dyad axis coincident with a crystallographic 2-fold axis. The low-resolution x-ray diffraction study showed that each subunit of the enzyme had a distinctly bilobal appearance (8). The cofactor NAD was bound to the larger of the two lobes near the interface with the small lobe. The x-ray diffraction data have now been extended to 2.8-Å resolution and electron-density maps have been calculated. The conformation of the polypeptide chain has been obtained and was used to determine the structural relationship between L-3-hydroxyacyl-CoA dehydrogenase and other enzymes that utilize NAD as a cofactor. It is now possible to describe the NAD-enzyme interactions and, in addition, to suggest possible modes of interactions between fatty acyl-CoA and the enzyme.

MATERIALS AND METHODS

The procedures used for the purification and crystallization of 3-hydroxyacyl-CoA dehydrogenase were identical to those

Abbreviation: MIR, multiple isomorphous replacement.

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previously described (8, 9). In the low-resolution study, three heavy-atom derivatives were used for phase determination, and two of these, methylmercury(II) chloride and K_2PtCl_6 were usable for the high-resolution work.

Diffraction data extending to a maximum resolution of 2.7 Å were collected on the University of California at San Diego Mark II area-detector system equipped with two detectors (10–12). Data processing included Lorentz and polarization corrections and an internal scaling procedure that adjusts for radiation damage and absorption (12, 13). One crystal was used to collect complete data sets for each of the apo and holo forms of the enzyme and for the platinum derivative. Due to crystal decay, two crystals were used for data collection from the mercury derivative. Bijvoet pairs were not merged for the crystals containing heavy-atom derivatives. A total of 500,000 x-ray reflections were measured, leading to 110,000 unique reflections. The merging R-factors (13) ranged from 0.046 for the holoenzyme data to 0.073 for the platinum derivative.

Positions of heavy atoms, already known from the low-resolution studies, were confirmed using difference Patterson and cross Fourier methods. Full use was made of the anomalous data. In addition to the two heavy-atom derivatives, the bound cofactor, NAD, was used as a "pseudo"-heavy-atom derivative (14) in a manner identical to that described in the low-resolution studies (8). Positional parameters as well as occupancies and temperature factors for the heavy-atom substituents including the pseudo heavy atoms representing the bound NAD molecules were first refined by using the origin-removed difference Patterson method (15). In the final cycles of the phase calculations, only the occupancies were allowed to vary and the refinement was based on the lack of closure error. The correct handedness of the phases was determined from an analysis of anomalous difference Fourier maps (16). Solvent flattening of the multiple isomorphous replacement (MIR) electron-density maps was done by a local implementation (17) of the procedure according to Wang (18). The procedures of Bricogne (19) were used for the skewing and averaging of electron-density maps.

Map interpretation and model building (α -carbon model) were done on mini-maps and on an MMS-X interactive graphics system (27) using the program NEWNIP (28). The fitting of the polyalanine model was done on a Silicon Graphics (Mountain View, CA) IRIS 3000 using TOM (C. M. Cambillau, Centre National de la Recherche Scientifique, Marseilles, France), a version of FRODO (29) implemented on the IRIS.

RESULTS

X-Ray Data. Preliminary inspection of the diffraction patterns on the multiwire area detector confirmed what had previously been observed from still and oscillation photographs—namely, that the crystals of L-3-hydroxyacyl-CoA dehydrogenase do not diffract much beyond about 2.7-Å resolution. The two heavy-atom derivatives showed a marked reduction in intensities at resolutions higher than 3.0 Å, which consequently was used as the limit for these two data sets. For both the holo and the apo form of the crystals, data extending to 2.7 Å were collected.

Analysis of the heavy-atom phasing parameters (Table 1) shows that beyond 3.5-Å resolution, the lack of closure error for the two heavy-atom derivatives is relatively large, and beyond 3.0 Å only the pseudo-heavy-atom derivative was used. The platinum derivative had just one binding site per asymmetric unit, and the mercury derivative had six binding sites, three major and three minor. The positions of the major sites turned out to be in identical locations in each of the three unique subunits.

Table 1. MIR phase calculations for L-3-hydroxyacyl-CoA dehydrogenase

Mean resolution, Å	No. of observations*	rms ΔF /rms $\epsilon^\dagger$			Figure of merit [‡]
		CH ₃ Hg	PtCl ₆	Apo – holo	
7.3	4329	1.5	1.3	1.3	0.69
4.8	3624	1.3	1.0	1.3	0.59
4.1	4243	1.4	0.76	1.3	0.56
3.6	4671	1.2	0.53	1.4	0.49
3.3	5006	1.0	0.41	1.3	0.35
3.0	5121	0.86	0.33	1.2	0.24
2.8	3208	ND	ND	1.1	0.12

*Total, 30,202.

[†]The root-mean-square difference in the observed heavy-atom difference amplitudes divided by the root-mean-square lack-of-closure error in the protein phase determination.

[‡]Overall, 0.43.

Electron-Density Maps and Their Interpretation. The center of the dimer located in the general crystallographic position is at (0.15, 0.20, 0.25) and this molecule will be called the A–B dimer. A second dimeric molecule is centered around the crystallographic 2-fold axis that is parallel to the y-axis and passes through the point $x = 0.5$, $z = 0.25$. This dimer will be referred to as the C–C dimer and has its molecular center at approximately (0.50, 0.35, 0.25). The reader should recall that all monomers are equivalent except for crystal packing considerations; hence in this nomenclature $A \equiv B \equiv C$.

Three different electron-density maps were used for the model fitting described here: (i) a MIR map based on three derivatives, (ii) the MIR map that had been subjected to eight cycles of density modification, and (iii) the MIR map that had been both density-modified and averaged around the local 2-fold axis. In several regions of the MIR map, helical structures as well as segments of extended polypeptide chain, later found to be component strands of a β -sheet, could be easily identified although other parts of the MIR map were more difficult to interpret. After eight cycles of density modification, a noticeable improvement of the electron-density map in those regions associated with the A–B dimer could be discerned, but the electron density associated with the C–C dimer did not appear to improve significantly. In a second stage, the electron-density map from the density-modification calculations was interpreted in terms of an α -carbon model that was both crude and incomplete. Markers for 140 α -carbon atoms were placed in each of the A and B subunits, and the two sets of α -carbon coordinates were used to derive a transformation matrix relating the A and B subunits. The matrix derived in this manner proved to be superior to those derived from rotation function analysis and from analysis of heavy-atom positions as judged from inspection of electron-density maps. Note that averaging only included the map volume encompassing the A–B dimer.

Initially, an α -carbon model accounting for 252 residues was obtained, utilizing primarily the MMS-X graphics system but with occasional reference to mini-maps. This initial chain tracing was followed by fitting of a polyalanine model, again using the electron-density map, which was both density-modified and averaged. Hence the model consists of an "averaged" interpretation. During this fitting, the unaveraged MIR map was also consulted, and it is clear that some small conformational differences probably exist between the two subunits of the A–B dimer. Each polypeptide chain of porcine heart L-3-hydroxyacyl-CoA dehydrogenase is known to contain 307 residues, and of these, 288 have been accounted for in each of the A–B subunits.

Initially, an attempt was made to interpret the density associated with the C–C dimer in the MIR map without making any reference to the model derived for the A–B

dimer. Although it was possible to follow short stretches of continuous polypeptide chain, numerous breaks and discontinuities in the electron density made it a difficult task to obtain an unambiguous and independent chain-tracing. However, those model segments that were fitted to the electron density for the C-C dimer correlated well with corresponding elements of the A-B dimer. The crystallographic relationship between the C-C and A-B dimers was also defined by the similar appearance of the difference electron densities attributable to the three NAD molecules. A third source of information used to interpret the electron density for the C-C dimer was provided by the location of the binding sites for the methylmercury(II) chloride used as a heavy-atom derivative. Combining this information, the α -carbon model of the A-B molecule was positioned in the electron density belonging to the C-C dimer. Overall, the correlation between the rotated model and the electron density was only marginal. In order to further optimize the model-map fit, the coordinates were systematically rotated around, as well as translated along, the crystallographic 2-fold axis in the region of the C-C dimer, and a correlation coefficient was calculated. An optimal position was found. However, the model-map correlation was not as good as was observed for the A-B dimer. One explanation for the weaker electron density in the region of the C-C dimer is some form of conformational variability caused by crystal packing. Different forms of crystalline disorder have been observed for other proteins, including instances where entire domains are disordered (20).

Conformation of L-3-Hydroxyacyl-CoA Dehydrogenase. The stereodiagram in Fig. 1 shows an α -carbon model of the A-B dimer viewed along the molecular dyad. Also included in this figure is the cofactor, NAD. The existence of significant amino acid sequence homology between the amino-terminal end of L-3-hydroxyacyl-CoA dehydrogenase and other NAD-dependent dehydrogenases has made it possible to establish a tentative but plausible relationship between the amino acid sequence and the three-dimensional structure. Using the known three-dimensional structures of several NAD-dependent enzymes and the above-mentioned sequence homology, the amino acid sequence for the first 50 or

so model residues has been established. Based on this sequence assignment, it is concluded that no electron density can be associated with the first two residues, probably due to disorder in this part of the molecule. Analysis of the binding sites for the methylmercury derivative used in the MIR phase determination provides another sequence reference point. In each of the three subunits, the major methylmercury binding site is closest to model residue 204. The single cysteine residue in L-3-hydroxyacyl-CoA dehydrogenase has sequence number 204, suggesting that the 19 missing residues probably belong to the last third of the primary sequence.

The conformation of the enzyme is that of a double-domain structure with a bilobal appearance. The amino-terminal domain, residues 1-200, is the larger domain and is structurally homologous with the NAD binding domains observed in other dehydrogenases (21, 22). As can be seen in Fig. 1, the NAD binding site is indeed found in the amino-terminal domain and consists of an eight-stranded β -sheet flanked by α -helices. The first six strands are parallel to each other, and the last two strands run in the opposite direction. Even though the amino acid sequence is not incorporated into the model at this stage, we are quite certain about the connectivity in this part of the structure. As is the case with other NAD-binding enzymes, the binding site for the coenzyme is at the carboxyl terminus of the parallel β -sheet and at the amino terminus of the α -helices. The smaller lobe constitutes the remainder of the subunit. So far, residues 201-288 have been incorporated into the model. The only type of secondary structure seen in this domain is helical (Fig. 1). Parts of the carboxyl-terminal domain of the A-B dimer are in close proximity to the C-C dimer in the crystal lattice, and some of the 19 residues missing in this domain may eventually be accounted for when a complete independent model is derived for the dimer with crystallographic symmetry.

NAD Binding Site. The electron densities for the two NAD binding sites in the A-B dimer were readily interpretable in terms of molecular models of NAD. The initial interpretation was facilitated by the use of NAD coordinates obtained from the holo structures of other dehydrogenases, particularly glyceraldehyde 3-phosphate dehydrogenase. The NAD moiety can be placed into the difference density in two ways

FIG. 1. Stereodiagram of the L-3-hydroxyacyl-CoA dehydrogenase dimer. The view in this α -carbon diagram is down the molecular dyad axis. The A-B dimer described in the text is shown with the A subunit in the upper half, outlined in the thinner lines, and the B subunit in the lower half, shown with the darker lines. Also shown are the bound NAD molecules as positioned from difference Fourier maps. The numbering system for the A subunit is based on the current molecular model of L-3-hydroxyacyl-CoA dehydrogenase and does not correspond to the amino acid sequence numbering system (6). Residues in the B subunit have not been numbered, to improve the clarity of stereoviewing.

FIG. 2. Holo - apo difference electron density. The electron-density map belonging to the bound NAD at the A subunit of L-3-hydroxyacyl-CoA dehydrogenase is shown. Superimposed on the density is a molecular model of NAD as observed bound to one of the subunits of glyceraldehyde-3-phosphate dehydrogenase (23). The conformation of the NAD was not changed during the fitting and has the adenine ring in the *anti* conformation and the nicotinamide ring in the *syn* conformation.

because the cofactor has crude internal 2-fold symmetry. Similar to what has been observed in other NAD-enzyme complexes, one end of the difference density is noticeably weaker than the other, and this part has always been associated with the nicotinamide moiety. Consequently the nicotinamide ring of NAD was placed in the end of the difference peak with the lower level of electron density. The nicotinamide ring can assume two orientations around its glycosidic bond. In the *syn* conformation as bound to L-3-hydroxyacyl-CoA dehydrogenase, the A side of the nicotinamide ring faces the solvent and the B side is adjacent to the protein surface. In the *anti* conformation the situation is reversed. Since the enzyme is a B-side-specific dehydrogenase, the B side of the nicotinamide ring must face the solvent region where the substrate is bound, and therefore the nicotinamide conformation is believed to be *syn* when NAD is bound to the enzyme. The good correlation between the difference map and the model of NAD is shown for one subunit of the A-B dimer in Fig. 2. When this conformation is shown in relationship to the entire dimer, as is done in Fig. 1, it is very apparent that the active sites in the dimer are widely separated, by 31 Å at the closest approach, and are fully contained within one subunit.

Subunit-Subunit Interactions. The subunit-subunit interface for the dimer is provided nearly exclusively by residues in the smaller domain found at the carboxyl-terminal end of the polypeptide chain. The most extensive contacts are found between two α -helices comprising residues 204-222 (Fig. 1). These two helices are within 30° of being antiparallel to each

other, and the molecular dyad is perpendicular to their helical axes. A short stretch of β -structure, residues 237-240, also contributes to the subunit-subunit interactions. A third set of interactions are found between the helical residues 228-236 from one subunit and residues 193-197, which are in an extended conformation, from the other subunit.

DISCUSSION

The dinucleotide binding domain of L-3-hydroxyacyl-CoA dehydrogenase resembles the corresponding domains in most other oxidoreductases. Common to these enzyme structures is the presence of a conformational unit consisting of a four-stranded parallel β -sheet and one α -helix (22). Another feature of interest in L-3-hydroxyacyl-CoA dehydrogenase is the occurrence of two α -helices in the crossover connection between β -strands, β B and β C. This arrangement closely resembles the conformation present in the FAD binding domain of glutathione reductase (24). Also, the last two strands of the β -sheet in L-3-hydroxyacyl-CoA dehydrogenase, β G and β H, are found in the opposite orientation from the other sheet components; so in effect, the β -sheet supersecondary structure in the nucleotide binding domain is really a mixed one, although predominantly parallel.

The conformation of NAD that best fits the difference electron-density map between holo and apo forms of L-3-hydroxyacyl-CoA dehydrogenase resembles the structure of the coenzyme when bound to another B-side-specific dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase. The

FIG. 3. Hypothetical ternary complex. The stereodrawing shows a schematic drawing of the molecular model of the A subunit of L-3-hydroxyacyl-CoA dehydrogenase together with a bound NAD. The hypothetical binding mode for the fatty acyl-CoA substrate is also shown. NAD and CoA are shown as ball-and-stick models.

structure of the enzyme and the position of the bound NAD make it possible to speculate about the substrate binding site, although no definitive substrate binding studies have yet been done. The nicotinamide ring is located in the cleft separating the two domains, with the B side of the nicotinamide ring facing the solvent and the A side facing the enzyme. Furthermore, the stereochemistry at the 3-carbon of the fatty acid is the same as at the 2-carbon of malic acid, the substrate for malate dehydrogenases. Therefore, in our modeling of the enzyme-substrate complex, we used the same stereochemical relationship between carbon atoms in the substrate and NAD as has been postulated for cytoplasmic malate dehydrogenase (25). When a L-3-hydroxy fatty acyl-CoA molecule is positioned adjacent to the nicotinamide ring of the NAD such that the hydrogen on the 3-carbon of the fatty acid is near the 4-carbon of the nicotinamide ring, only two general orientations for the substrate seem possible. The more favorable one places the fatty acid moiety in the narrower end of the cleft between the lobes, with the alkyl chain stretched across the surface of a helix comprising residues 251-266. The cleft is visible in both Fig. 1 and Fig. 3. In this arrangement, the hydrocarbon chain of the fatty acid moiety is in contact with protein on all sides except at the furthest end. The CoA moiety is placed in the more open end of the cleft, where it makes relatively few contacts with the enzyme. The structure of the CoA part of the substrate shown in Fig. 3 is similar to that observed in crystalline complexes of citrate synthase (26). However, the 3'-phospho-ADP-pantothenate moiety appears to be quite flexible and it could be in contact with many different parts of the dimer. In an alternative orientation, the two ends of the substrate are interchanged. The adenine end is cramped into the narrower end of the cleft between the lobes and, in fact, would have to change conformation into a more extended form than that shown in Fig. 3. The hydrocarbon chain of the fatty acid makes fewer contacts with protein in this arrangement, and is more exposed to solvent. Finally, the stereochemical arrangement in the catalytic center is not as satisfactory as in the first orientation. Although speculative, the preferred orientation is shown in Fig. 3.

The model of L-3-hydroxyacyl-CoA dehydrogenase that has been presented in this report is still incomplete in that approximately 20 amino acids are still not accounted for and side chains have not been fitted, but we believe that most of the missing residues belong to the carboxyl-terminal domain. The electron density associated with this part of the structure was more difficult to interpret due to the proximity of electron density attributable to other subunits in the crystal lattice, particularly the subunits belonging to the C-C dimer. It should be possible to improve the electron-density map, particularly at higher resolution, by using model phases once the amino acid sequence has been incorporated into the present interpretation.

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