Identification of ADP in the Iron-Sulfur Flavoprotein Trimethylamine Dehydrogenase*

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Analysis of the 2.4-Å resolution electron density map of trimethylamine dehydrogenase has revealed the unexpected presence of one molecule of ADP/subunit. This binding has been confirmed chemically. The binding site is located at the analogous position of the ADP moiety of FAD in glutathione reductase, the FAD and NADPH binding domains of which resemble two of the domains of trimethylamine dehydrogenase.

Comparison of the environments of the ADP moieties in the two proteins indicates that 32 residues in 6 peptides are in equivalent positions with a root mean square deviation for C, positions of 1.1 Å. Twelve of these amino acids are identical, based on the electron density-derived “x-ray” sequence of trimethylamine dehydrogenase. Detailed analysis of the environment of the ADP moiety indicates that most of the conserved residues are not in direct contact with the cofactor. Some of them probably represent the “fingerprint” of the βαβ binding fold found in dinucleotide binding proteins, but the remaining conserved residues may indicate a closer evolutionary relationship between these two proteins.

Trimethylamine dehydrogenase from the methylothrophic bacterium W3A1 is an iron-sulfur flavoprotein. It catalyzes the oxidative demethylation of trimethylamine to dimethylamine and formaldehyde and transfers the electrons to an FAD-containing electron transfer flavoprotein (Steenkamp and Mallinson, 1976; Steenkamp and Gallup, 1978). The enzyme is a dimer of identical subunits, with a subunit molecular weight of 83,000 (Lim et al., 1982; Kasprzak et al., 1983).

A three-dimensional model of the enzyme has been determined at 2.4-Å resolution (Lim et al., 1986). It shows that each subunit polypeptide is folded into three distinct domains.

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The largest domain is at the amino terminus and contains the covalently bound FMN and the iron-sulfur cluster. It has a parallel βαβ barrel topology. Two other FMN-containing enzymes, glycolate oxidase (Lindqvist and Branden, 1985) and flavocytochrome b2 (Xia et al., 1987), also have their FMN cofactors in a βαβ barrel domain. The remaining two domains of trimethylamine dehydrogenase contain an α/β parallel β-sheet topology very similar to the FAD and NADPH binding domains of glutathione reductase (Thieme et al., 1981). However, previous biochemical studies have not revealed the presence of these cofactors in trimethylamine dehydrogenase.

The amino acid sequence of trimethylamine dehydrogenase is not known except for a 12-residue peptide containing the cysteine ligand to the covalently bound FMN (Kenney et al., 1978). Attempts to clone and sequence the gene for trimethylamine dehydrogenase are currently in progress in order to interpret the 2.4-Å structure in terms of the amino acid sequence. In parallel with these efforts, we sought to improve the electron density map computed from the x-ray diffraction data. We used an electron density modification procedure (Wang, 1985) to improve the phases by filtering the noise in the solvent region of the electron density function. Although the starting multiple isomorphous replacement phases were fairly well determined, the application of the solvent leveling procedure resulted in an average phase shift of 28° and considerable improvement of the electron density map. The clarity of the map enabled us to place amino acid residues into side chain densities to obtain an “x-ray sequence” (Mathews and Lim, 1987).

RESULTS

In the process of fitting the “x-ray sequence” we have discovered a third cofactor for the enzyme. The new cofactor is an ADP molecule situated between the medium and small domains of trimethylamine dehydrogenase in a location quite analogous to that of FAD in glutathione reductase (Fig. 1). The two phosphate moieties of the ADP were observed in the original multiple isomorphous replacement map as two peaks of high electron density and were ascribed to a possible disulfide linkage, while the adenosine moiety was interpreted as an extra loop containing four peptide residues. However, the density could be matched very closely by a skeletal model of ADP in the noise-filtered map (Fig. 2). We also considered the possibility that FAD might actually be present. However, when we tried to manipulate an FAD molecule into this region we could not find any density which would correspond to the ribitol and flavin moiety of FAD. Therefore, we conclude that it is indeed ADP which is bound in this region in trimethylamine dehydrogenase and not FAD as in glutathione reductase.

This conclusion was confirmed by the biochemical detection of ADP in the enzyme by thin-layer chromatography. When trimethylamine dehydrogenase was treated with 0.5 M perchloric acid a strongly UV-absorbing chromophore was found in the supernatant. It has an absorption maximum at 257 nm and a minimum at 233 nm. The supernatant was then neutralized with 0.5 M potassium carbonate and applied to poly-

1 G. Cecchini, personal communication.

2 L. W. Lim and F. S. Mathews, unpublished results.
Identification of ADP in Trimethylamine Dehydrogenase

**FIG. 1.** A, schematic diagram of the two carboxyl-terminal domains of trimethylamine dehydrogenase. Ribbons represent β strands, and cylinders represent helices. The ADP cofactor, solid circles, is attached to the upper domain with the pyrophosphate moiety at the left. B, schematic diagram of the FAD-binding (upper) and NADPH-binding (lower) domains of glutathione reductase. The FAD cofactor is also shown as solid circles. Pictures were produced by a computer program written by A. M. Lesk and K. D. Hardman (Lesk and Hardman, 1982, 1985).

**FIG. 2.** Two orthogonal views of ADP fitted into the 2.4-Å resolution electron density function of trimethylamine dehydrogenase.

An attempt was made to exchange enzyme-bound ADP with [14C]ADP. After incubating trimethylamine dehydrogenase either in the oxidized or substrate reduced form with 200 μM [14C]ADP for 20 h no radiolabel comigrated with trimethylamine dehydrogenase on subsequent gel chromatography on Sephadex G-25. The ADP present in the enzyme as isolated is therefore tightly bound and unable to exchange with free ADP.

We have looked for similarities in the binding sites for the ADP moieties in glutathione reductase (Schulz et al., 1982, 

ethyleneimine cellulose thin-layer plates. The plates were developed with 0.5 M lithium chloride, and the chromophore comigrated with the ADP standard. The neutralized supernatant was also applied to a Hichrom high pressure liquid chromatography anion exchange column (0.46 × 25 cm) and eluted with a linear gradient of 100% A to 100% B (A, 5 mM KH₂PO₄ adjusted to pH 2.5 with HCl; B, 500 mM KH₂PO₄ + 0.912 M KCl adjusted to pH 3.8 with HCl). Again the chromophore was eluted from the column at the same position as ADP.
Identification of ADP in Trimethylamine Dehydrogenase

The tentative amino acid sequence of trimethylamine dehydrogenase is based on the 2.4 Å resolution electron density map. Coordinates at 2.0 Å resolution for glutathione reductase were obtained from the protein data bank (Bernstein et al., 1977). Residues within 4.0 Å of ADP in trimethylamine dehydrogenase and in glutathione reductase were identified using FRODO (Jones, 1985). The Cα atoms of the peptides containing these residues plus one residue on either side were optimally aligned using the program HOMOLOG by M. Rossmann (Rao and Rossmann, 1973).

The conformations of the polypeptide chains and the manner in which the ADP is bound, with one side in contact with protein and the other side open to solvent, are found to be very similar (Fig. 3). Furthermore, the pyrophosphate moieties are located at the NH2 terminus of an α-helix in both proteins. The aligned region contains 32 amino acids located in equivalent positions in the two proteins (Fig. 4). Two of these peptides, of length 4 and 6 amino acids in length, which are identical in trimethylamine dehydrogenase and in glutathione reductase were identified using FRODO (Jones, 1985).

The conformational analysis of the peptides was performed using the program HOMOLOG. The aligned region contains 32 amino acids located in equivalent positions in the two proteins (Fig. 4). Two of these peptides, of length 4 and 6 amino acids in length, which are identical in trimethylamine dehydrogenase and in glutathione reductase were identified using FRODO (Jones, 1985). The Cα atoms of the peptides containing these residues plus one residue on either side were optimally aligned using the program HOMOLOG by M. Rossmann (Rao and Rossmann, 1973).

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Seven amino acid residues of glutathione reductase make primary hydrogen bond contact with the ADP portion of FAD, as indicated in Fig. 4 and Table I (Karplus and Schulz, 1987). Only one of these, Asp-331 versus "Asx"-674, is identical in trimethylamine dehydrogenase. In addition, 9 residues of glutathione reductase make secondary contact with ADP through one of four water molecules. Six of these residues are contained within the six equivalent peptides, shown in Fig. 4, and four of these are identical in trimethylamine dehydrogenase. Thus, only one of the 12 identical residues is directly linked to ADP, whereas four of the nine residues forming water bridges to ADP in glutathione reductase involve a residue conserved in trimethylamine dehydrogenase. Since the water structure is not yet known in trimethylamine dehydrogenase, similarities in water bridging cannot be compared at this time, although some density suggestive of water is present.

Eleven atoms in the ADP moiety make possible hydrogen bonds with main or side chain atoms of one or the other
Identification of ADP in Trimethylamine Dehydrogenase

protein molecule, as indicated in Table I. Eight of these atoms make such contact in both proteins. Four atoms interact with main chain atoms in both proteins, situated at similar positions, two interact with a main chain atom in glutathione reductase and a side chain atom in trimethylamine dehydrogenase, and two interact only with side chain atoms in both proteins. There is no apparent sequence similarity in these common interactions except in the last two cases where hydrogen bonds are formed from the 2’- and 3’-hydroxyls of the ribose to the polar side chain atoms of Glu-50 in glutathione reductase and “Asx”-419 in trimethylamine dehydrogenase. Thus, although there is considerable sequence similarity between the two proteins, little of this similarity involves side chains directly interacting with the ADP moiety.

The presence of ADP in trimethylamine dehydrogenase is completely unexpected. Previous enzymatic data can be interpreted without invoking this additional cofactor. Perhaps it plays an unknown regulatory role in the molecule. On the other hand, it might also be bound because a vestigial ADP binding pocket is present but has no functional role. It is also possible that FAD was present in the nascent enzyme but was cleaved to ADP during the purification. If FAD were present, the flavin moiety would be in a solvent-filled cavity between the surface of the enzyme and the buried iron-sulfur cluster. It would be in an excellent position to transfer electrons from the cluster to the natural electron acceptor, a large flavoprotein (Steinkamp and Gallup, 1978).

Three features in the molecular structures of trimethylamine dehydrogenase and glutathione reductase strongly suggest that portions of the two enzymes evolved from a common ancestor. These features are 1) the overall structural similarity of the NADPH- and FAD-binding domains of glutathione reductase to the two smaller domains of trimethylamine dehydrogenase (Lim et al., 1986), 2) the presence of an ADP moiety in both proteins at similar sites, and 3) the apparent high degree of sequence identity of 37% (12/32) at these sites. Since most of the conserved residues are not in direct contact with the ADP moiety this conservation may reflect a relatively close evolutionary distance between the two proteins. The sequence similarity must be judged with caution, however, since the sequence of trimethylamine dehydrogenase has not been determined chemically but is based on the electron density map. However, this portion of the map is exceptionally clear so the assignments can be made with considerable confidence. The conservation of the 12 residues in the ADP-binding site appears to be important for maintaining the tertiary structure of the site rather than to provide a means to bind ADP directly, since ADP interacts to a large extent with main chain atoms and almost none of the conserved residues interact directly with it. This conclusion is consistent with the observation that half the conserved residues are glycine, which is often found in regions of sterically constrained structures (Schulz and Schirmer, 1979). In fact, three of the conserved glycines, in the first peptide of Fig. 4, form part of the characteristic “fingerprint” of the general “ADP-binding βαβ fold” found in a number of dinucleotide-binding proteins (Wierenga et al., 1985; Birktoft and Banaszak, 1984). Additional components of the ADP “fingerprint” retained in trimethylamine dehydrogenase are the characteristic distribution of six hydrophobic residues, the presence of an acidic group at position 419, and the dipolar interaction of the pyrophosphate with the α-helix. However, the structural and sequence similarity between trimethylamine dehydrogenase and glutathione reductase, extending over six peptides as shown in Figs. 3 and 4, is considerably more extensive than observed in the “ADP βαβ fold” and consistent with a closer evolutionary relationship than among most dinucleotide binding proteins.

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