Studies of Crystalline Trimethylamine Dehydrogenase in Three Oxidation States and in the Presence of Substrate and Inhibitor*

(Received for publication, January 26, 1989)

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Crystals of trimethylamine dehydrogenase have been examined by difference Fourier methods at 6.0-Å resolution after partial reduction by substrate and by dithionite in the presence of inhibitor. Similar studies of the inhibited oxidized enzyme and of the enzyme reduced fully by dithionite alone were also carried out. In all cases ligand binding at the active site occurred. In addition, there were small structural changes, possibly side chain movements, in the inhibited oxidized enzyme and somewhat larger changes in the partially reduced crystals. The largest changes occurred with the fully reduced enzyme. However, in no cases were subunit or domain movements observed nor were changes observed in the positions of the FMN or [4Fe-4S] cofactors.

Parallel studies of crystalline trimethylamine dehydrogenase were carried out by EPR spectroscopy. The results show that the electronic states of the crystalline enzyme under the conditions of the difference Fourier studies are comparable to those which occur in solution under similar conditions.

Trimethylamine dehydrogenase is an iron-sulfur flavoprotein found in the methylotrophic bacterium W3A1. It catalyzes the oxidative N-demethylation of trimethylamine to formaldehyde and formaldehyde (Steenkamp and Malinson, 1976). The protein is a symmetrical dimer of M, 166,000 (Lim et al., 1982; Kasprzak et al., 1982). Each subunit contains one [4Fe-4S] center and one FMN cofactor. The latter is bound covalently through the 6-position of the flavin ring to a cysteine side chain (Steenkamp et al., 1978a). The natural electron acceptor is an electron transferring flavoprotein of M, 57,000 (Steenkamp and Gallup, 1978), but phenazine methosulfate will serve as an artificial acceptor in vitro.

The crystal structure of trimethylamine dehydrogenase has been analyzed at 2.4-Å resolution and an α-carbon backbone fitted to the electron density (Lim et al., 1986). Each monomer is composed of three domains. The largest domain consists of the N-terminal portion of the polypeptide chain, about half the molecule, and contains the covalently bound flavin. It is folded into a parallel βαβ barrel similar to two other FMN-containing enzymes, flavocytochrome b₅ (Xia et al., 1987) and glycolate oxidase (Levandts and Branden, 1986). The FMN is bound to all three enzymes in a very similar manner, with the flavin ring situated at the C-terminal ends of the parallel β strands making up the β barrel. The other two smaller domains each contain a central 5-stranded parallel β-sheet flanked in one case by α-helices on both sides and in the other case by α-helices on one side and an antiparallel 3-stranded β-sheet on the other. The geometry and topology of folding of the latter two domains are similar to the FAD- and NADPH-binding domains of glutathione reductase, respectively (Thieme et al., 1981). The iron-sulfur cluster is located between the first and second domains and is in van der Waals contact with the 8α-methyl group of the FMN. Both the FMN and the [4Fe-4S] center are buried deeply in the protein interior, each approximately 20 Å from the surface of the molecule. The amino acid sequence of trimethylamine dehydrogenase is unknown except for a 12-residue peptide which contains the covalently bound flavin (Steenkamp et al., 1978a) and two tryptophan-containing peptides each about 9 residues in length (Mathews and Lim, 1987).

The electron density function at 2.4-Å resolution has been improved by the solvent-flattening procedure (Wang, 1985). A polypeptide backbone chain model of 729 residues and an estimated side chain model based on shape have been fitted to the electron density function (Mathews and Lim, 1987). This has resulted in a hypothetical "x-ray sequence." It was also discovered that the electron density of the flavin ring was distinctly nonplanar and could be fitted better by introducing a 20° bend about a line connecting atoms N-5 and N-10 of the flavin ring.

An α-carbon diagram of the large C-terminal domain is shown in Fig. 1. The positions of the FMN and iron-sulfur cluster are also shown. The cluster is bound through four cysteine side chains to an αβ segment. This segment is connected to the domain past the C-terminal end of the eighth β-strand and lies on the surface of the βαβ barrel. The Cys-Sy of one of the ligands to the [4Fe-4S] cluster is in van der Waals contact with the 8α-methyl of the flavin ring. An enlarged view of the active site is shown in Fig. 2.

The mechanism of action of trimethylamine dehydrogenase has been studied by absorption spectroscopy, stopped flow and freeze-quenched kinetic methods, and by EPR spectroscopy (Steenkamp et al., 1978b; Steenkamp and Beinert, 1982a, 1982b). When the enzyme is reduced by excess substrate (1-
Fig. 1. Stereo view of the α-carbon backbone of the N-terminal domain of trimethylamine dehydrogenase; residues 1–366 are included. Every 20th residue is labeled. The FMN and [4Fe-4S] cofactors are shown in heavy lines. The FMN is bound at the C-terminal end of a parallel β-sheet structure. The [4Fe-4S] cluster is bound to an α/β loop, also shown in heavy lines, located after the eighth α-helix of the βαβ barrel.

2 mM under anaerobic conditions, substrate binding and reduction of the flavin to the hydroquinone form occurs within a few milliseconds. Flavin reduction is followed by formation of a triplet state after transfer of one electron from the reduced flavin to the [4Fe-4S]⁺ center. This triplet state gives rise to a very intense EPR signal. The EPR spectrum has been analyzed in terms of coupling of the two S = 1/2 spin systems on the flavin semiquinone and the reduced [4Fe-4S]⁺ group to form an artificial spin system consisting of S = 1/2 and S = 3/2 (Bricogne et al., 1978). Formation of the triplet state occurs much more slowly, in 300–400 ms; even at low concentrations of substrate (0.1 mM) the half-time for formation of the triplet state is about 20 ms and may be the rate-limiting step during catalysis. Above pH 8.0 the triplet state is achieved by stoichiometric amounts of substrate, but excess substrate is needed at lower pH values.

When trimethylamine dehydrogenase is treated with excess dithionite under anaerobic conditions, it takes up a total of three electrons to form the fully reduced flavin and reduced [4Fe-4S]⁺ center. The EPR signal is typical of an isolated [4Fe-4S]⁺ cluster. However, in the presence of tetramethylammonium chloride, a competitive inhibitor of trimethylamine dehydrogenase, the enzyme takes up only two electrons/subunit, with full development of the triplet state.

To explain the kinetic behavior of trimethylamine dehydrogenase during reduction by substrate and the differences in the stoichiometry of electron uptake between reduction by substrate and by dithionite, a conformational change in the enzyme was proposed (Steenkamp et al., 1978b). This change would be induced by the binding of substrate or of inhibitor. We report here the results of a crystallographic investigation at 6.0-Å resolution of trimethylamine dehydrogenase in three oxidation states in the presence of substrate, inhibitor, and/or reducing agent. In addition we have carried out studies by EPR spectroscopy of single crystals of the enzyme.

### MATERIALS AND METHODS

**X-ray Studies**—Crystals of trimethylamine dehydrogenase were prepared by the technique of macro-seeding of sitting drops as described previously (Lim et al., 1982). Four separate difference Fourier experiments involving one crystal each were carried out. These experiments are summarized in Table I. All crystals were equilibrated against an artificial mother liquor containing 12% polyethylene glycol 8000, 20 mM NaCl, and 60 mM Na/K phosphate buffer, pH 7.5. Each crystal was then transferred to a solution containing the test compound (Table I) and then mounted in a thin walled glass capillary.

| Experiment | Compound | Concentration | Soaking time | Oxidation state |
|------------|----------|---------------|--------------|----------------|
| 1          | TMAC     | 25            | 4 h          | Oxidized       |
| 2          | TMA      | 25            | 1 h          | +2e⁻           |
| 3          | TMAC + DT⁺ | 25 + 25      | Overnight + 1 h | +2e⁻         |
| 4          | DT       | 50            | ½ h          | +6e⁻           |

* DT, dithionite.

**Table II**

| Compound | Major peak heights |
|----------|-------------------|
|          | A     | B     | C     | D     | E     | F     |
| TMAC     | 0.32  | 0.19  | -0.22 | 0.23  |       |       |
| TMA      | 0.42  | 0.24  | -0.27 | 0.53  |       |       |
| TMAC + DT⁺ | 0.38 | 0.21  | -0.30 | 0.18  |       |       |
| DT       | 0.30  | 0.23  | -0.28 | 0.41  | -0.29 |       |

* DT, dithionite.

The crystal in the capillary was totally immersed in the solution and held in place by cotton fibers as described previously (Lim et al., 1984). The ends of the capillary were sealed with wax.

The wax seal was tested for oxygen permeability by partially filling a capillary with a solution containing 50 mM dithionite and 10 ppm methylene blue. The dye turned colorless almost immediately after the capillary was sealed and remained so for several months.

Data from each derivative were recorded to 6.0-Å maximum resolution on a Picker 4-circle diffractometer by the limited step scan procedure as described previously (Lim et al., 1982). Friedel-related reflections were recorded in alternate blocks of 50 and several standard reflections repeated at intervals of 750 to monitor radiation damage. The unit cell parameters varied by less than 0.1%, and the decay was no more than 11% for any crystal during the time required to record approximately 9000 reflections.

Friedel-related reflections for each experiment were scaled locally together (Matthews and Czerniewski, 1975), and the mean structure factors were then locally scaled to the native data. The native data had previously been collected at the University of California, San Diego area detector facility (Lim et al., 1986). Difference Fourier maps were computed and then averaged about the noncrystallographic 2-fold axis (Bricogne, 1978). The difference maps were analyzed by a peak searching algorithm and by displaying them on an MMS-X molecular graphics system (Lederer et al., 1981).

**EPR Measurements**—EPR spectra were recorded from randomly oriented single crystals of trimethylamine dehydrogenase on a Varian Century Line spectrometer operating at 9 GHz. For each spectrum the analog output from several consecutive sweeps was recorded digitally on the TRACOR/Northern NS-570 signal averager. Each sample was sealed in a quartz x-ray capillary in the appropriate medium but without cotton fibers and suspended in a quartz EPR sample tube and maintained at 10–20 K in a liquid helium cryostat. The capillary could be rotated by hand to change the orientation of the crystal with respect to the magnetic field direction. Five single crystals were examined in the EPR spectrometer. For all five crystals the composition of the medium was 12% polyethylene glycol 8000, 20 mM NaCl, and 10 mM Na/K phosphate buffer, pH 7.5. In addition, the medium for the first crystal contained 50 mM dithionite, for the second 25 mM trimethylamine, and for the third and fourth 25 mM tetramethylammonium chloride plus 25 mM dithionite; the fifth crystal was a control without reductant.

### RESULTS

**X-ray Studies**—The results of the four difference Fourier soaking experiments are summarized in Table II. The root mean square difference density of all the maps is about 0.03 e Å⁻³. A minimum threshold of 0.15 e Å⁻³ in magnitude was found to exclude the majority of noise peaks in the map and...
FIG. 2. Stereo view of the active site of trimethylamine dehydrogenase containing the FMN and [4Fe-4S] center. Every third residue is labeled. The FMN is covalently attached to Cys-30. The [4Fe-4S] cluster is attached to Cys-345, Cys-348, Cys-351, and Cys-364. A few amino acid side chains derived from the “x-ray sequence” which point into the active site are also shown. These are Cys-30, “Tyr”-60, “Asx”-69, “Tyr”-169, “Tyr”-174, “Trp”-264, and “Trp”-355.

FIG. 3. Electron density difference map for trimethylamine dehydrogenase soaked with the inhibitor TMAC superimposed on the skeletal model of the active site. Solid contours are at +0.15 e/A³ and dashed contours at −0.15 e/A³. Peaks labeled A–D are discussed in the text.

FIG. 4. Electron density difference map for trimethylamine dehydrogenase soaked with the substrate TMA. Peaks A–D correspond approximately to peaks A–D in Fig. 3. See text for details.

FIG. 5. Difference map for trimethylamine dehydrogenase soaked in dithionite. Contour levels are: +0.15 e/A³, solid and −0.15 e/A³, dashed. Peaks labeled A–C occur in positions corresponding approximately to peaks in TMA, TMAC, and TMAC plus dithionite difference maps. The peak labeled E close to the flavin ring is much larger and more extensive than the peak in a similar position in Fig. 4. The peak labeled F is negative and is located close to “Trp”-264.

Density, extending from peaks B and C, lies close to the benzenoid portion of the flavin ring and might indicate a slight structural change in this area. However, no movement of the flavin ring as a whole, the iron-sulfur center, nor any other large conformational change is observed.

The difference density map for TMAC in the presence of dithionite (not shown) is very similar of that of TMA. Slight differences can be observed, but these probably are not significant at this resolution.

The soak with dithionite alone produces the largest and most numerous features in the difference map (Fig. 5). The highest peak, E, lies between the benzenoid portion of the isoalloxazine ring and the helix linked to the iron-sulfur cluster. This peak overlaps the extension of peak B seen in the TMA and TMAC plus dithionite maps but is much larger. Additional peaks not present in the other maps also occur, including a significant negative peak, F, close to “Trp”-264 (Figs. 2 and 5). In addition, a positive peak, A, occurs approximately in the same location and at the same height as the major peak in the other three soaks, as do the positive and negative pair B and C. The chemical nature of the species binding at site A in the dithionite soak is unknown.

EPR Studies—The EPR spectrum of a trimethylamine dehydrogenase crystal reduced by dithionite is shown in Fig. 6A. Two orientations of the crystal are shown, separated by about 90° rotation of the sample tube in the sample cavity. The observed g values for the rhombic g tensor (approximately 1.92, 1.96, and 2.01) are more closely spaced and shifted to higher values than those in frozen solutions (1.85, 1.92, and 2.00; Stevenson et al., 1986) and are somewhat dependent on orientation. The spectral line widths are also considerably broader than observed in solution.

Difference EPR spectra of a crystal reduced by TMA minus the oxidized form at two orientations of the crystal are shown in Fig. 6B. The difference spectra indicate that TMA can cause reduction of the crystalline enzyme since the resonances occur at positions characteristic of the reduced [4Fe-4S]+.
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**DISCUSSION**

**EPR Spectra**—The EPR spectrum of the dithionite-reduced crystals of trimethylamine dehydrogenase provides a rare example of an [4Fe-4S]+ center in the crystal phase. There are three resonances visible corresponding approximately to the three resonances observed in the frozen aqueous data. Since the coordinate systems of the iron-sulfur clusters are not aligned with the crystal axes, several different aspects of the clusters will be seen by the magnetic field.

The EPR line widths are considerably broader than are observed in frozen solution (Stevenson et al., 1986). This suggests that strain (Hearshen et al., 1986) may be present. Since the iron-sulfur clusters are deeply buried in the protein interior, about 20 Å from the surface, crystal packing forces would not directly affect their microenvironments. However, such forces could affect surface residues, although no deviations of the three-dimensional structure from monoclinic symmetry have been observed as yet. Analysis of the relative thermal motions of interior and exterior side chains and the identification of solvent molecules must await completion of the amino acid sequence and refinement of the structure at high resolution. An alternate explanation for the broader line widths is that the frozen crystal became cracked and the lattice of iron-sulfur clusters was sampled by the magnetic field over a range of orientations.

**Ligand Binding**—All four difference Fourier studies showed a major peak (peak A, Figs. 3-5) at the active site near O-4 and N-5 of the flavin ring. Judging from its size and location, the peak probably corresponds to a bound ligand. At the resolution of the present study it is not possible to identify the nature of the ligand with any certainty. In three of the cases, however, either TMA or TMAC are present, both of which would be expected to have affinity for the enzyme, especially at the high concentrations used. Although the amino acid sequence of trimethylamine dehydrogenase is not known, the proposed “x-ray sequence” enables at least a tentative identification of some prominent side chains in the active site. Fig. 3 shows the positions of several such side chains and part of the α-carbon backbone. Peak A, on the left, appears to be in a hydrophobic environment, surrounded by 5 aromatic side chains. Two of these (tentatively) are

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**Fig. 6.** A, EPR spectra from a crystal of trimethylamine dehydrogenase reduced by dithionite. The two curves correspond to two orientations of the sample tube about 90° apart. B, EPR difference spectra (reduced minus oxidized) of a trimethylamine dehydrogenase crystal reduced by TMA. The two curves correspond to two orientations of the sample tube. C, EPR difference spectra for a crystal of trimethylamine dehydrogenase reduced by dithionite in the presence of TMAC. Two orientations of the sample tube are shown. D, same as C except that a different crystal was used. Running conditions: field modulation, 1 millitesla (mT); microwave power, 1 milliwatt; 4-min sweep.
tryptophan and three are tyrosine. This environment is consistent with the largely hydrophobic nature of the substrate and inhibitor.

An explanation of peaks B and C is more difficult to achieve. Since part of "Trp"-355 lies within the negative volume of peak C, the pair of peaks might indicate movement of this side chain away from the putative ligand, peak A. The consistent nature of these two peaks in all the maps is compatible with this idea.

Triplet State—The difference Fourier maps between the oxidized enzyme and the enzyme in the triplet state do not show any major change in conformation. The substrate and inhibitor ligands appear to bind at the same site, A, and to have associated with them the same structural changes giving rise to peaks B and C. The additional peaks in the maps are relatively small and probably correspond to movements of side chains. Slight perturbation of the geometry of the flavin ring is possible.

The kinetic evidence indicates that formation of the triplet state is a slow step relative to flavin reduction by the substrate. One proposed explanation (Steenkamp et al., 1978) was that a kinetically unfavorable conformational change occurred on binding substrate which oriented the flavin and iron-sulfur cluster more favorably for interaction. However, the x-ray results show that the two groups are already very close together in the oxidized form and that no significant changes occur in backbone conformation or cofactor orientation on reduction.

Another explanation for the slow development of the triplet state is that the redox potential of FMNH₂ lies close to that of the [4Fe-4S] cluster so that electron transfer from reduced flavin to the [4Fe-4S]²⁺ cluster would involve a relatively small change in free energy. The rate of electron transfer in biological systems has been shown to be correlated with the negative change in free energy for weakly exothermic reactions (Tollin et al., 1986). Potentiometric titrations of trimethylamine dehydrogenase indicate that the redox potential for electron transfer from reduced flavin to the [4Fe-4S]²⁺ cluster is on the order of 80–100 mV (Stankovich and Steenkamp, 1987) but that there is considerable variability in the potential of the iron-sulfur cluster depending on the redox state of the flavin.

Dithionite Reduction—in the absence of substrate or the TMAC inhibitor, complete reduction of the cofactors by dithionite is achieved. This reduction is accompanied by the largest changes in structure (Fig. 5). However, no major changes in backbone conformation are indicated. Peaks A, B, and C are all present, indicating that a ligand is binding at site A in this experiment as well as the others.

The binding of a ligand at site A in the dithionite-reduced crystals is unexpected. The active reductant species in dithionite is usually thought to be SO₄²⁻. Being negatively charged, it would not be expected to bind at the same site as the substrate or the positively charged competitive inhibitor. However, the peak might be sulfite, an oxidation product of dithionite, bound covalently to the flavin ring. Sulfite is known to form a covalent adduct to flavin in many flavoproteins (Massey et al., 1969) and has been observed in crystalline flavocytochrome b₅ (Tegoni and Mathews, 1988). The structural similarity of trimethylamine dehydrogenase and flavocytochrome b₅ has been noted previously.

The largest peak (Fig. 5, peak E) occupies considerable volume between the benzenoid ring of the flavin and the α-helix above the iron-sulfur cluster. This large peak could indicate a change in conformation of the flavin ring. It could also indicate considerable unresolved side chain movements in the vicinity of the flavin. Another possibility is direct binding of dithionite. The positive and negative density at "Trp"-264 (Fig. 5) could indicate movement of that side chain to accommodate binding of a bulky group.

CONCLUSION

The EPR studies of crystalline trimethylamine dehydrogenase show that the enzyme behaves in essentially the same way in both the crystalline and the solution states with respect to reduction by substrate, dithionite and dithionite in the presence of inhibitor. Optical studies of single crystals reduced by substrate or by dithionite also show that the crystalline enzyme can exist in several oxidation states. In particular, full or partial reduction of the enzyme or development of the triplet state is not inhibited by the forces of the crystal lattice. The difference Fourier studies of the enzyme in the triplet states and fully reduced states as well as in the oxidized state with the inhibitor bound show that no gross changes in structure occur. Relative subunit or domain movements are not observed nor are shifts in the positions of the prosthetic groups. Binding of ligand is observed along with some apparent side chain movement, the greatest of which occurs on reduction by dithionite alone.

The very intense EPR signal which occurs on reduction in the presence of substrate or inhibitor is consistent with the close proximity of the cofactors (Stevenson et al., 1986). The slow development of the triplet state is not the result of a major change in enzyme conformation, as shown by these results. However, it may result from the small magnitude of the free energy change for transfer of an electron from the reduced flavin to the oxidized [4Fe-4S]²⁺ center.

When substrate or inhibitor is present in the active site the transfer of a second electron to the flavin is thermodynamically unfavorable resulting in stabilization of the semiquinone. Modulation of the redox potential by TMAC is not known but can result from the small conformational changes observed on binding and from polarization effects caused by the positive charge of the inhibitor. Modulation of the redox properties of protein-bound flavin by ligand binding has been observed in butyryl-CoA dehydrogenase (Stankovich and Soltysek, 1987) and in flavocytochrome b₅ (Tegoni et al., 1986). In the latter case, subunits with and without bound ligand are found to differ little in conformation (Mathews and Xia, 1987).

In the absence of substrate or inhibitor full reduction of both cofactors becomes thermodynamically favorable. The structural changes accompanying full reduction are considerably greater than for occurrence of the triplet state. The details of control of the redox states of the cofactors by binding of substrate or inhibitor should become better understood when the present studies are extended to high resolution and the complete amino acid sequence has been determined.

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