The Active Site Structure of the Calcium-Containing Quinoprotein Methanol Dehydrogenase†,‡

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ABSTRACT: Pyrroloquinoline quinone (PQQ), widely found in nature, serves as the redox cofactor in bacterial methanol dehydrogenase (MEDH), a heterotetrameric enzyme that oxidizes methanol to formaldehyde. The refined structure of MEDH at 2.4-Å resolution, based on recently obtained amino acid sequence data, reveals that the PQQ, located in a central channel of the disk-shaped protein, is sandwiched between a Trp side chain and a very unusual vicinal disulfide. A Ca^{2+} ion forms a bridge between PQQ and the protein molecule, very close to a putative substrate binding pocket. The vicinal disulfide may form during PQQ incorporation and possibly act to hold the latter in place.

Methanol dehydrogenase (MEDH),[1] a quinoprotein of molecular mass 140 kDa located in the periplasm of many gram negative methylotrophic bacteria (Anthony, 1993), catalyzes the oxidation of methanol and other primary alcohols to their corresponding aldehydes. It is an $\alpha_2\beta_2$ tetramer of approximate subunit molecular masses 62 and 8 kDa, respectively, and contains 2 mol of the prosthetic group pyrroloquinoline quinone (PQQ) per tetramer. The amino acid sequences of both subunits from *Paracoccus denitrificans* (van Spanning et al., 1991) and *Methylobacterium extorquens* AM1 (Anderson & Lidstrom, 1988) and of the large subunit from *Methylobacterium organophilum* XX (Machlin & Hanson, 1988) have been reported. The enzyme also contains tightly bound calcium (Richardson & Anthony, 1992; Adachi et al., 1990). The natural electron acceptor is cytochrome c_L, a class of *c*-type cytochrome which is distinctive in having a low isoelectric point (p*I* = 3–4), large size (17-kDa molecular mass), and little sequence similarity to other bacterial or eukaryotic *c*-type cytochromes (Anthony, 1991). Both MEDH and cytochrome c_L are encoded by structural genes located in the *mox* gene cluster (Lidstrom, 1990) and are induced by growth of the organism on methanol or methylamine.

The three-dimensional structures of MEDH from both *Methylophilus methylotrophus* and *Methylophilus* W3A1 have been determined at 2.6-Å resolution (Xia et al., 1992). At the time, no primary sequence information was available for the large subunits, and only limited information (30 amino acids) was available for the small subunits. The electron density was interpreted on the basis of the published sequences for MEDH from *P. denitrificans* for the large subunit and from *M. extorquens* AM1 for the unknown portion of the small subunit. The structures showed that each large subunit consists of a single disk-shaped superbarrel (Branden & Tooze, 1991) composed of eight four-stranded antiparallel β-sheets arranged about a pseudo-8-fold symmetry axis in a propeller-like fashion. Each small subunit consists of an extended N-terminal peptide segment and C-terminal helix which are wrapped around a portion of the outside surface of the large subunit. The prosthetic group, PQQ, was located in a funnel-shaped cavity centered on the pseudo-8-fold symmetry axis. In the tetramer, a pair of $\alpha\beta$ subunits is packed so that the pseudo-8-fold axes are roughly perpendicular and the active sites are separated by about 45 Å.

On the basis of new sequence data and further crystallographic refinement, we now report the active site structure of MEDH which differs drastically from the previous interpretation. These results provide a clear definition of the orientation of PQQ and its interaction with a calcium ion and with several adjacent amino acid side chains.

EXPERIMENTAL PROCEDURES

X-ray data from a crystal of MEDH from *M.* W3A1 were collected to 2.4-Å resolution on an area detector as reported previously (Xia et al., 1992). The deduced amino acid sequence of the large subunit of MEDH from *M.* W3A1 (Boyd & Mathews, unpublished experiments) was fitted to the 2.6-Å resolution MIR electron density map reported earlier (Xia et al., 1992). The sequence of 571 amino acids is approximately 65% identical to that from *P. denitrificans* used in the original chain tracing. The major differences between them are a six residue deletion in the M. W3A1 sequence at position 118 of the mature sequence and the elimination of the last 20 amino acids corresponding to residues 580–599 of *P. denitrificans*. For the small subunit, 57 of the 70 residues from the closely related MEDH from *M. methylophilus* (Anthony et al., 1993) were built into the model. Use of the *M. methylotrophus* sequence at this stage is justified since the two organisms have very similar properties and crystals of MEDH from both are isomorphous.

The newly fitted model of MEDH, lacking the PQQ prosthetic group and with portions of the N-terminus omitted,

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[1] Abbreviations: MEDH, methanol dehydrogenase; MIR, multiple isomorphous replacement; PQQ, pyrroloquinoline quinone; RMS, root mean square.

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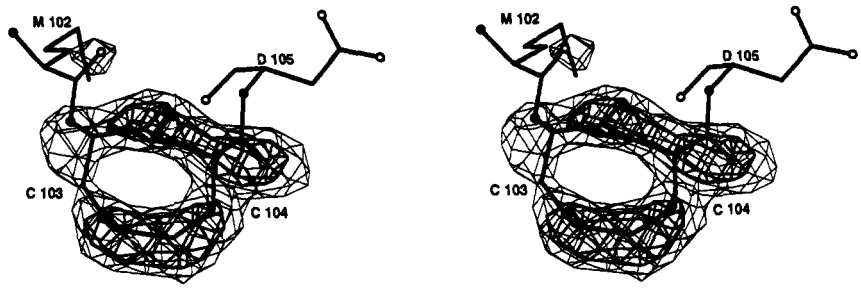

FIGURE 1: Simulated annealing omit map for methanol dehydrogenase from *M.* W3A1. Residues Cys 103 and Cys 104 were omitted from both subunits. Electron density contours are at 2σ (light) and 4σ (dark).

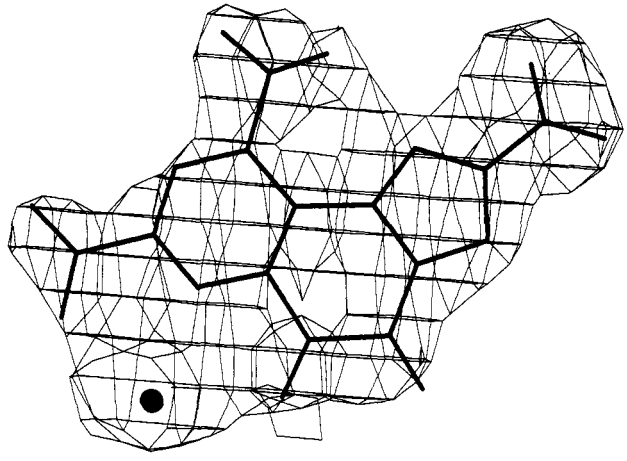

FIGURE 2: Simulated annealing omit map for methanol dehydrogenase from *M.* W3A1. The PQQ moiety and all atoms within a 3-Å sphere of all PQQ atoms in both subunits were omitted from the calculation. The filled circle represents the calcium ion.

was refined by simulated annealing at 2.6-Å resolution using X-PLOR Version 2.1 (Brunger et al., 1987) using parameters from Engh & Huber (1991) and adjusted on a molecular graphics system using TURBO-FRODO (Cambillau & Horjales, 1987). The resolution was extended to 2.4 Å by energy-restrained positional refinement, yielding a crystallographic *R*-factor of 0.210, where $R = \Sigma|F_o - F_c|/\Sigma F_o$, summed over all reflections, and F_o and F_c are the observed and calculated structure factors, respectively.

At this point the $2F_o - F_c$ electron density map clearly indicated the position of the two PQQ molecules and defined a unique orientation for each of them.[2] Located just above the PQQ moiety, a pair of adjacent cysteine residues was observed to be in a disulfide linkage. This -Cys-Cys- disulfide was confirmed in a simulated annealing omit map (Figure 1). The orientation of the PQQ was also verified in the same way (Figure 2). After further positional refinement ($R = 0.207$) an $F_o - F_c$ difference map indicated the presence of a solvent site in each subunit coordinated to 2 oxygen atoms and one nitrogen atom of PQQ and three oxygen atoms from protein side chains, at an average distance of 2.53 Å. Because of its strong difference density and oxygen-rich environment, this site has been interpreted as a calcium binding site. The difference electron density for the Ca^{2+} site was 5 and 7 times background in subunits 1 and 2, respectively, the latter being the highest peak in the difference map. An anomalous difference map for the native protein indicated a strong anomalous signal at this site in subunit 2, comparable to that of various sulfur atoms. In subunit 1, the anomalous peaks for the Ca^{2+} site and for the disulfide bridge were weaker and the map more noisy, possibly indicating lower occupancy or systematic bias in the anomalous X-ray data. Further refinement and difference Fourier calculations with Ca^{2+} included in the model revealed many clearly defined solvent molecules, three of which are located close to PQQ. After refinement, the thermal *B*-factors of the two Ca^{2+} positions were 16 and 13 Å^2, respectively, while those for all other atoms within 5 Å of the two Ca^{2+} ions were 10 and 6 Å^2, respectively. The present *R*-factor, for 50 907 reflections in the 10.0–2.4-Å resolution range, is 20.05% for a model with 11 912 non-hydrogen atoms contained in 1256 amino acid residues, 2 molecules of PQQ, and 2 Ca^{2+} ions. The RMS deviations from ideal bond lengths and angles are 0.009 Å and 1.14°, respectively, and only 8 of the 1256 residues correspond to energetically unfavorable regions of the Ramachandran diagram.

RESULTS AND DISCUSSION

The active site of MEDH is shown in Figures 3 and 4. The ring system of PQQ is in a hydrophobic environment sandwiched between Trp 237, below, and the Cys 103–Cys 104 disulfide bridge, above (Figure 3). The 9A-carboxyl oxygen forms a salt bridge with Arg 109 (Figure 4), and both groups are thoroughly shielded from bulk solvent by the disulfide. This hydrophobic arrangement is in agreement with ENDOR experiments (Duine et al., 1984) which also predicted the involvement of the 9-carboxylate group in PQQ binding. The 2A-carboxyl oxygen is located 2.7 Å away from a carboxyl oxygen of Glu 55. Since both acidic groups are shielded from solvent, their proximity to each other suggests that one or both of them are protonated, thus stabilizing their interaction through hydrogen bond formation. The putative Ca^{2+} is six-coordinate with bonds to O5, N6, and carboxyl oxygen O7A of PQQ, the two carboxyl oxygen atoms of Glu 171, and the side-chain oxygen of Asn 255. Somewhat farther away are the side chains of Asp 297 and Arg 324, which form a salt bridge. The latter is also hydrogen bonded to O4 and O5 of PQQ. Several additional hydrogen bonds to PQQ, including an internal one from N1 to O9B, are shown in Figure 4 and listed in Table I.

Of the three solvent molecules in contact with PQQ, the one of greatest significance, W1, is within hydrogen-bonding distance to O5 of PQQ and to carboxyl oxygen atoms of Asp 297 and of Glu 171 (Figure 3). Otherwise, the site is rather hydrophobic, being surrounded by W259, W531, L547, and the C103–C104 cystine bridge. Since this site is easily within reach of the bulk solvent along the axis of the superbarrel, it is a reasonable candidate for substrate binding. Although we have tentatively identified this peak as a water molecule, it might be methanol or formaldehyde, since the crystals were

[2] The orientation of PQQ defined in the present analysis differs from that presented previously [Figure 9 in Xia et al. (1992)] by a 180° rotation about a line approximately joining the 2 and 7 carboxylate groups of PQQ.

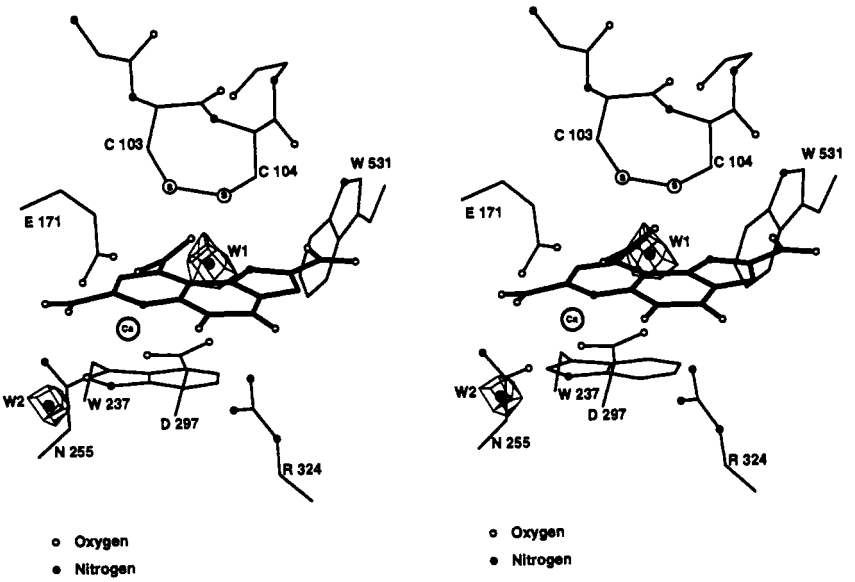

FIGURE 3: Stereoscopic view of the active site of methanol dehydrogenase from *M.* W3A1 with PQQ seen nearly edge on. Two water molecules (W1 and W2) in the active site are surrounded by the $F_o - F_c$ difference density contoured at 4σ.

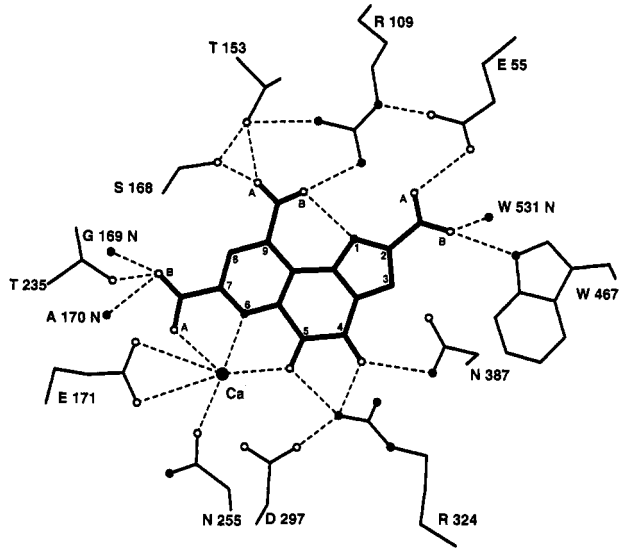

FIGURE 4: Schematic view of the active site of methanol dehydrogenase from *M.* W3A1 parallel to the plane of PQQ. All hydrogen bond interactions between PQQ and its surrounding atoms, except for the three water molecules, are indicated.

grown in the presence of 10 mM methanol (Xia et al., 1989). A second solvent molecule, W2, is hydrogen bonded to W237NE1, which is stacked below PQQ, to the carboxyl oxygen O7A, and to the side-chain oxygen of N255 (Figure 3). The third solvent molecule is hydrogen bonded to carboxyl oxygen O9B and peptide backbone atoms C104N and D105O behind the disulfide (not shown).

The environment and ionization state of PQQ in MEDH is consistent with those observed in crystals of PQQ at pH 5.9 (Ishida et al., 1989). There, the PQQ molecules are closely stacked, at slightly less than van der Waals separation, analogous to the PQQ–tryptophan stacking interaction in MEDH. The 2-carboxylate on the pyrrole ring is protonated while carboxylates 7 and 9 on the pyridine ring are ionized. Furthermore, a sodium ion is coordinated to atoms O5, N6, and carboxyl oxygen O7A of PQQ precisely where the putative Ca^{2+} site is located in MEDH. This site also served to bind gold and platinum ions in the *M. methylophilus* enzyme during the structure analysis by the isomorphous replacement method (Xia et al., 1992).

The functional importance of Ca^{2+} in MEDH is underscored by the fact that at least three gene products of the *mox* gene cluster are required for its incorporation into the enzyme (Richardson & Anthony, 1992). MEDH samples isolated from mutants defective in these genes lack Ca^{2+} but have structural properties identical to those of the wild-type enzyme and contain a full complement of PQQ. However, they are inactive and exhibit perturbed PQQ absorption spectra. Fully active enzyme, indistinguishable from wild type, can be constituted from these inactive proteins by prolonged *in vitro* incubation with high concentrations of Ca^{2+}. The positive charge on the Ca^{2+} in the wild-type enzyme may have an inductive effect on the redox potential of PQQ, helping to stabilize the reduced and semiquinone forms of the enzyme. The absence of Ca^{2+} could allow alternate orientations of the PQQ ring and of some of the protein side chains in the active site, thereby destroying the catalytic ability of the enzyme.

It appears that the presence of Ca^{2+} is a common feature of PQQ-containing enzymes. MEDH has been reported to possess approximately one Ca^{2+} per mole of enzyme (Richardson & Anthony, 1992). Two other dimeric PQQ-dependent enzymes, ethanol dehydrogenase from *Pseudomonas aeruginosa* (Mutzel & Gorisch, 1991) and glucose dehydrogenase from *Acinetobacter calcoaceticus* (Geiger & Gorisch, 1989), have also been shown to possess bound Ca^{2+}, with two and four Ca^{2+}, respectively, per mole of holoenzyme. The latter two enzymes may be prepared as apoenzymes, and for each of them reconstitution of activity by addition of PQQ requires the presence of Ca^{2+}. In view of the wide range of Ca^{2+} values reported for the different quinoproteins, there has been uncertainty as to the correct Ca^{2+} stoichiometry. The current structure shows definitively that Ca^{2+} is present at each active site, although some loss of occupancy may have occurred during crystallization. Likewise, the previous report of only one Ca^{2+} per MEDH molecule possibly reflects some loss of Ca^{2+} in that preparation.

Two general mechanisms for substrate oxidation by MEDH have been proposed (Anthony, 1993). One is direct hydride transfer from the substrate α-methyl or methylene group to the PQQ. The second involves covalent addition of the alcohol substrate to PQQ prior to reduction of PQQ, with formation of a hemiketal intermediate. For both mechanisms, acid and base catalysis by the enzyme is required, and the most likely

Table I: Hydrogen Bond Distances in the Active Site of Methanol Dehydrogenase

atom 1	atom 2	distance (Å)[a]
PQQ N1	PQQ O9B	2.5
PQQ O2A	Glu 55 OE1	2.7
PQQ O2A	Arg 109 NH2	3.4
PQQ O2B	Trp 467 NE1	2.8
PQQ O2B	Trp 531 N	3.0
PQQ O4	Arg 324 NH1	2.9
PQQ O4	Asn 387 ND2	3.2
PQQ O5	Arg 324 NH1	3.0
PQQ O7B	Gly 169 N	2.9
PQQ O7B	Thr 235 OG1	2.9
PQQ O9A	Thr 153 OG1	2.7
PQQ O9A	Ser 168 OG1	2.8
PQQ O9B	Arg 109 NH2	3.1
Ca^{2+}	PQQ O5	2.5
Ca^{2+}	PQQ N6	2.4
Ca^{2+}	PQQ O7A	2.4
Ca^{2+}	Glu 171 OE1	2.5
Ca^{2+}	Glu 171 OE2	2.8
Ca^{2+}	Asn 255 OD1	2.6
Arg 109 NE	Glu 55 OE2	3.3
Arg 109 NH2	Thr 153 OG1	2.9
Asp 297 OD2	Arg 324 NH1	3.0

[a] The hydrogen bond distances are averaged over the two independent pairs of $\alpha\beta$ subunits in the asymmetric unit. The RMS difference between equivalent C_α positions is 0.22 Å.

recipient on PQQ for the hydride ion or for covalent addition is the C5 carbon, according to studies of the reactivity of free PQQ (Dekker et al., 1982). The proposed substrate binding site lies close to O5. The nearest candidates for the roles of active site acid or base are Asp 297 and Glu 171. All other potential active site functional groups are considerably farther away and are involved in hydrogen bonds to PQQ or other protein side chains. Both Asp 297 and Glu 171 are inaccessible to bulk solvent and are involved in complex electrostatic interactions since Asp 297 forms a salt bridge to Arg 324 and Glu 171 is a bidentate ligand to the calcium ion. The only other group in the active site which might potentially be involved in catalysis is Cys 104 of the disulfide, but there is no evidence at present for involvement of such a group in the mechanism.

The role of the disulfide is open to speculation. The occurrence of a disulfide bridge between sequentially adjacent cysteine residues is very rare (Sukunaran et al., 1991) and suggests that it may serve an important biological function. Such a bridge has been found in only one other protein structure, mercuric ion reductase (Schiering et al., 1991), and inferred in two other proteins, the acetylcholine receptor α-subunit (Kao & Karlin, 1986) and the γ-subunit of transducin (Ovchinnikov et al., 1985). In mercuric ion reductase a vicinal disulfide is thought to be involved in binding the mercuric ion, while in the acetylcholine receptor the disulfide is believed to be involved in receptor activation. The role of the vicinal disulfide in transducin is unknown. In MEDH, the disulfide might play a redox-active role in catalysis or subsequent electron transfer, as in the case of other enzymes with redox-active disulfides such as glutathione reductase and lipoamide dehydrogenase (Williams, 1992). Another possibility is that the disulfide helps to secure the PQQ to the enzyme after its incorporation. If the two cysteines were in the reduced form, PQQ might bind at the active site and catalyze disulfide bond formation. Free PQQ has been reported to catalyze disulfide bond formation both in model compounds and in proteins with vicinal sulfhydryls (Park & Churchich, 1992). Once formed, the disulfide could serve to hold the PQQ tightly to the enzyme. A third possibility is that the disulfide acts as a regulatory switch, activating the enzyme when it forms. Whatever its role, the functional importance of the vicinal disulfide in MEDH is underscored by the fact that both cysteines are conserved in all four known sequences of the large subunit.

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