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Crystal Structure of Quinohemoprotein Alcohol Dehydrogenase from Comamonas testosteroni

STRUCTURAL BASIS FOR SUBSTRATE OXIDATION AND ELECTRON TRANSFER*

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Quinoprotein alcohol dehydrogenases are redox enzymes that participate in distinctive catabolic pathways that enable bacteria to grow on various alcohols as the sole source of carbon and energy. The x-ray structure of the quinohemoprotein alcohol dehydrogenase from Comamonas testosteroni has been determined at 1.44 Å resolution. It comprises two domains. The N-terminal domain has a β-propeller fold and binds one pyrroloquinoline quinone cofactor and one calcium ion in the active site. A tetrahydrofuran-2-carboxylic acid molecule is present in the substrate-binding cleft. The position of this oxidation product provides valuable information on the amino acid residues involved in the reaction mechanism and their function. The C-terminal domain is an α-helical type I cytochrome c with His608 and Met647 as heme-iron ligands. This is the first reported structure of an electron transfer system between a quinoprotein alcohol dehydrogenase and cytochrome c. The shortest distance between pyrroloquinoline quinone and heme c is 12.9 Å, one of the longest physiological edge-to-edge distances yet determined between two redox centers. A highly unusual disulfide bond between two adjacent cysteines bridges the redox centers. It appears essential for electron transfer. A water channel delineates a possible pathway for proton transfer from the active site to the solvent.

Bacteria have versatile metabolic pathways that enable them to adapt to different environmental conditions. Many Gram-negative bacteria, for example, can grow on compounds as different as methylamine, ethanol, and glucose as their sole source of carbon and energy (1–3). The crucial, first step in the catabolism of such compounds is often an oxidation reaction catalyzed by a class of periplasmic enzymes called quinoproteins. Quinoproteins are oxidoreductases that possess one of four different quinone compounds instead of nicotinamide or flavin cofactors (4–7). They oxidize a wide variety of alcohol- and amine-containing substrates to the corresponding aldehydes or ketones. Proteins containing the pyrroloquinoline quinone (PQQ) cofactor form the best characterized and largest quinoprotein subclass (8). Two different types of PQQ-containing alcohol dehydrogenases (ADHs) have been characterized. The first type includes quinoprotein alcohol dehydrogenases (ADHs) from several Pseudomonas species (9–11) and quinoprotein methanol dehydrogenases (MDHs) from methylo trophic bacteria (12). The second type of PQQ-dependent ADHs is the quinohemoprotein alcohol dehydrogenases (QH-ADHs). In addition to PQQ, these latter enzymes contain a covalently bound heme c. Both soluble monomeric QH-ADHs (2, 11, 13–16) and membrane-associated enzymes that consist of several subunits (17–19) have been described.

No three-dimensional structures of QH-ADHs are known. In contrast, several x-ray structures have been reported of type I quinoprotein ADHs (20–24) and a soluble quinoprotein glucose dehydrogenase (sGDH) (25). sGDH-inhibitor (26) and sGDH-substrate (27) complexes have provided detailed insights into the dehydrogenation reaction. A recent MDH structure revealed the presence of a PQQ intermediate in the catalytic mechanism (28). These data have clearly shown how sGDH and MDH react with their substrates and, although no information has so far been available to substantiate this hypothesis, it has been argued that the same mechanism operates in the other quinoprotein ADHs (29). In contrast, much less is known about how these enzymes catalyze the electron transfer reactions from reduced PQQ (PQQH2) to the natural electron acceptors. To obtain more information about these reactions, the QH-ADH from Comamonas testosteroni has been studied extensively (30–33). This enzyme consists of two distinct functional domains. The N-terminal domain binds PQQ and calcium in the active site and is homologous to the PQQ-binding domains of all quinoprotein ADHs. The C-terminal domain has a covalently attached heme c and is similar to some cytochrome c proteins (33). QH-ADH oxidizes primary alcohols and aldehydes in the PQQ-binding domain (31). Subsequently, protons and electrons are removed from PQQH2. The protons are released into the periplasm, thus contributing to the proton motive force. The electrons are transferred one by one to the...
heme c in the C-terminal domain (30, 32); from there they are carried by the blue copper protein azurin to a terminal cytochrome oxidase (34). Alcohol oxidation is thus efficiently coupled to the generation of an electrochemical gradient, which in turn drives ATP synthesis. Further advances in understanding the mechanisms of proton and electron transfer in QH-ADH, and in PQQ-dependent proteins in general, have awaited the determination of the three-dimensional structure of QH-ADH.

EXPERIMENTAL PROCEDURES

Purification and Crystallization—QH-ADH was purified from C. testosteroni cells grown on ethanol (31). The enzyme was crystallized using polyethylene glycol 6000 as the precipitant (35). A complete data set was collected to 1.44 Å resolution at beam line X11 at the EMBL outstation in Hamburg, Germany and then processed and reduced with the DENZOSCALEPACK package (36) (Table I).

Structure Determination—The orientation and position of the PQQ domain were determined by molecular replacement using the program EMR (37) with the PQQ-dependent EDH from Pseudomonas aeruginosa (38% sequence identity to QH-ADH in 567 amino acids, Rutgers Protein Data Bank accession code 1KB0). The three-dimensional structure of QH-ADH (677 residues) consists of two distinct domains connected by a long linker (residues 567–590), which spans the whole length of the protein (Fig. 1). The respective orientation of the two domains is completely different from that published in a rudimentary homology model (44). The cytochrome domain is located on top of the dehydrogenase. The edge-to-edge distance and the angle between PQQ and heme c are 12.9 Å and 74°, respectively. The surface area buried between the two domains, as calculated with the program Surface (42), is 1028 Å² on each domain. Direct interdomain contacts (ignoring the connecting loop) involve residues 66–67, 109–110, 113, 118, 430–437, 440, 445, 451, and 546–550 from the dehydrogenase and residues 598, 601–607, 609, 614–619, 643–646, 648, and 653 from cytochrome c. Most of these interactions are hydrophobic. In addition, 16 direct and several solvent-mediated hydrogen bonds stabilize the respective orientation of the domains. Two different channels can be identified in the structure: one leads from the solvent to the PQQ-binding site, whereas the other contains a chain of hydrogen-bonded water molecules that connects the bulk solvent to a cavity between the two domains.

PQQ-binding Alcohol Dehydrogenase Domain—The N-terminal domain (residues 1–566) has a β-propeller fold (Fig. 1) and is very similar to the PQQ-binding domains of MDH (21–44, 400, 446, 541, and 546–550 from the dehydrogenase and residues 598, 601–607, 609, 614–619, 643–646, 648, and 653 from cytochrome c. Most of these interactions are hydrophobic. In addition, 16 direct and several solvent-mediated hydrogen bonds stabilize the respective orientation of the domains. Two different channels can be identified in the structure: one leads from the solvent to the PQQ-binding site, whereas the other contains a chain of hydrogen-bonded water molecules that connects the bulk solvent to a cavity between the two domains.

Structure of Quinohemoprotein Alcohol Dehydrogenase

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RESULTS AND DISCUSSION

Overall Structure—The three-dimensional structure of QH-ADH (677 residues) consists of two distinct domains connected by a long linker (residues 567–590), which spans the whole length of the protein (Fig. 1). The respective orientation of the two domains is completely different from that published in a rudimentary homology model (44). The cytochrome domain is located on top of the dehydrogenase. The edge-to-edge distance and the angle between PQQ and heme c are 12.9 Å and 74°, respectively. The surface area buried between the two domains, as calculated with the program Surface (42), is 1028 Å² on each domain. Direct interdomain contacts (ignoring the connecting loop) involve residues 66–67, 109–110, 113, 118, 430–437, 440, 445, 451, and 546–550 from the dehydrogenase and residues 598, 601–607, 609, 614–619, 643–646, 648, and 653 from cytochrome c. Most of these interactions are hydrophobic. In addition, 16 direct and several solvent-mediated hydrogen bonds stabilize the respective orientation of the domains. Two different channels can be identified in the structure: one leads from the solvent to the PQQ-binding site, whereas the other contains a chain of hydrogen-bonded water molecules that connects the bulk solvent to a cavity between the two domains.

TABLE I

Data collection and refinement statistics

<table>
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<tr>
<th>Data</th>
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<tr>
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<tr>
<td>Rsym (I) (%), b</td>
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<td>Test set</td>
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a Highest resolution shell in parentheses.

b Rsym(I) = Σh|Ihkl|−<|Ihkl>|/Σh|Ihkl| where <|Ihkl>| is calculated for a randomly chosen 5% of the reflections omitted from refinement.

c Rfree is calculated for a randomly chosen 5% of the reflections omitted from refinement, and Rcryst is calculated for the remaining 95% of the reflections included in the refinement.
(26). PQGH2, has in-plane hydrogen-bonding interactions with the side chains of Glu177, Arg122, Thr167, Asn363, Asp308, Lys535, Asn534, and Trp365, and the carbonyl oxygen atoms of Ala183, Ala184, and Val544 (Fig. 2A). In addition, it ligates the active site calcium with its O5, N6, and O7A atoms in an identical fashion to all other PQQ-dependent proteins of known structure. One side of the tricyclic ring system of PQQ stacks on the side chain of Trp365, and the other side interacts with a disulfide bond. This bond is made between the strictly conserved, adjacent cysteines 116 and 117 (Fig. 2A). The formation of this vicinal disulfide bond creates an eight-membered ring structure and forces the peptide bond in a nonplanar trans configuration. A disulfide bridge between adjacent residues is extremely rare, which suggests that it has an important biological function.

The Cytochrome c Domain — The C-terminal domain (residues 591–677) classifies as a type I cytochrome c. The structure is comprised of five α-helical segments that enclose the c-type heme (Fig. 1), which is covalently attached to Cyg604 and Cyg607 (Fig. 2B). The heme-iron is coordinated by His608 and Met647, and it is in a low-spin hexa-coordinated state, as was shown previously by electron paramagnetic resonance (EPR) and resonance Raman spectroscopy (31, 32). Two of the methyl groups (CMA and CMB) of the heme are surrounded by hydrophobic residues (Cys601, Leu622, Met625, Ile630, Leu633, Tyr629, Phe636, Ala184, and Val544 (Fig. 2A). In addition, it ligates the active site calcium with its O5, N6, and O7A atoms in an identical fashion to all other PQQ-dependent proteins of known structure.

The Substrate-binding Site — The substrate-binding site is located near PQQ in a hydrophobic cavity. This cavity is much larger than that of MDH and EDH and accounts for the relatively broad substrate specificity of QH-ADH. It contains a ring-shaped electron density, which has been interpreted as a ring-shaped electron density map is shown for tetrahydrofuran-2-carboxylic acid. (27). This area of the active site may result from the oxidation of tetrahydrofururyl alcohol (THFA) by QH-ADH. The conversions of THFA to tetrahydrofuran-2-carboxylic acid by the 61% identical, THFA-oxidizing QH-ADH from Ralstonia eutropha (16, 45) and of bulky primary alcohols to the corresponding acids by the Comamonas enzyme (31) have been described. Except for Tyr399, which is replaced by a Phe, all residues that interact with tetrahydrofuran-2-carboxylic acid are identical between the R. eutropha and C. testosteroni enzymes, making it likely that the C. testosteroni enzyme can also degrade THFA. THFA is frequently used as an organic solvent in industry, and therefore it may have contaminated solutions for protein production, purification, and crystallization. Tetrahydrofuran-2-carboxylic acid is tightly bound: the tetrahydrofuran ring makes van der Waals contacts with the hydrophobic wall of the cavity formed by residues Trp267, Pro308, Tyr390, Trp440, Phe446, Val544, and Phe560 (Fig. 3). The carboxylic acid O2A atom is hydrogen-bonded to Asp508 (OD2 atom at 2.3 Å) and Glu185 (OE1 at 3.0 Å), whereas the O2B atom is bound to Glu185 (OE1 at 2.5 Å), Cys116 (SG at 3.0 Å), and Cys117 (SG at 2.9 Å). Because these interactions include direct contacts between several carbon-
ylate groups, at least one of them should be in the carboxylic acid form. Unfortunately, this is not resolved in the present x-ray structure. As the product of two subsequent oxidation reactions catalyzed by QH-ADH, tetrahydrofuran-2-carboxylic acid is the first catalytically relevant molecule to be bound in the active site of a Q-ADH. It therefore provides valuable information about the amino acid residues involved in the oxidation reactions of this class of enzymes and their catalytic function, as will be discussed in the next section.

Alcohol and Aldehyde Oxidation—The mechanism of alcohol oxidation has been extensively studied for MDH and sGDH (glucose is a secondary alcohol). These enzymes oxidize their substrates according to a mechanism involving base-catalyzed proton abstraction in concert with hydride transfer. However, the hydrogen bond between tetrahydropurran-2-carboxylic acid in the active site is shorter (2.3 Å), and in MDH the equivalent of Asp 308, not of Glu 185, is hydrogen-bonded to a water molecule and the aldehyde C5 atom. This C5 atom is susceptible to nucleophilic addition because of polarization of the C5–O5 bond by calcium (27–29, 46). Because the catalytic machinery of MDH is strictly conserved in QH-ADH, the mechanisms of alcohol oxidation are most likely identical for both enzymes. In QH-ADH, the side chains of Glu 185 and Asp 308 ligate the oxygen atoms of tetrahydrofuran-2-carboxylic acid (Fig. 3). Because both amino acid residues are conserved, either one of them could, in principle, act as the general base to catalyze proton abstraction. However, the hydrogen bond between tetrahydrofuran-2-carboxylic acid and Asp 308 is shorter (2.3 Å) than that between the acid and Glu 185 (3.0 Å), and in MDH the equivalent of Asp 308, not of Glu 185, is hydrogen-bonded to the only active site water molecule (28). Moreover, Asp 308 is in a similar position to His 144, the catalytic base in the sGDH (27). These observations suggest the conserved Asp 308 functions as the catalytic base in the oxidation reactions.

The mechanism of aldehyde oxidation is different from alcohol oxidation because the conversion of an aldehyde into an acid requires the addition of a hydroxyl group. Under the assumption that the same catalytic machinery is used for such a reaction, the following mechanism for the oxidation of aldehydes by QH-ADH may be proposed on the basis of the binding of tetrahydrofuran-2-carboxylic acid in the active site (Fig. 4B): Asp 308 is hydrogen-bonded to a water molecule and the aldehyde substrate is bound with its oxygen atom to the OE1 atom of Glu 185 and the SG atoms of Cys 116 and Cys 117. Asp 308 abstracts a proton from the hydrogen-bonded water, and the resultant hydroxyl ion performs a nucleophilic attack on the aldehyde C1 atom to yield the corresponding acid. This occurs in concert with hydride transfer from the aldehyde C1 to the PQQ C5 atom. This mechanism involves a shift in binding position for aldehydes (oxygen atom is bound to Glu 185), compared with alcohols (oxygen atom close to Asp 308). Kinetic evidence is available to substantiate the existence of such an alternative binding site (30).

Electron Transfer from PQQH2 to Heme c—Because heme c is a one-electron acceptor, the two electrons from PQQH2 must be transferred in two separate steps. The PQQ intermediate between these steps is the free-radical PQQH, which was identified by EPR spectroscopy (31). The redox centers and the intervening protein medium in QH-ADH are shown in Fig. 5. The shortest distance between PQQ and heme c is 12.9 Å. The conserved disulfide bond between Cys 116 and Cys 117 is positioned right between PQQ and heme c. Asp 118 and Arg 67 are also located between the cofactors. Asp 118 is conserved in all PQQ-dependent ADHs, including MDHs, whereas Arg 67 is present in all ADHs with the exception of some MDHs (not shown). Other residues located in the vicinity of the redox sites but not involved in cofactor binding are much less conserved. In addition, several water molecules are located in the interface between the redox centers.

The electron transfer rate in biological systems is strongly related to the edge-to-edge distance between the redox centers involved (47). Electrons can travel up to about 14 Å between two redox centers through the protein medium, but transfer over longer distances always involves additional redox sites (48). The 12.9 Å distance between PQQ and heme c is thus one of the longest between functional redox centers determined so far and close to the maximum travel distance of electrons. Using this distance and a calculated value of 0.63 for the atomic density between the two cofactors, a maximum electron transfer rate of 1.0 × 105 s−1 could be predicted (48) with the program Harlem (49). This value is much higher than that of substrate oxidation (keq = 17 s−1), consistent with kinetic data showing that the influence of electron transfer on the kinetic mechanism of QH-ADH is negligible (30).

The intervening protein medium is another important parameter for electron transfer. For PQQ-dependent ADHs, this is stressed by the fact that reduction of the disulfide bond between the active site cysteines in MDH completely abolished electron transfer from MDH to cytochrome c1 (21). The intervening medium may have several different functions in QH-ADH. Given the large separation between PQQ and heme c, close to the maximum distance electrons may travel, one function of the medium may be to reduce that distance by providing an additional redox center. In principle, the disulfide bond...
between cysteines 116 and 117 could act as such by accepting two electrons and two protons. A disulfide bond involved in redox reactions is indeed located close to an iron-sulfur cofactor in the ferredoxin:thioredoxin reductase system (50). However, biochemical experiments indicate that the disulfide bond of quinoprotein MDH is not reduced during the redox cycle (51), indicating that it does not function as a redox center in PQQ-dependent enzymes.

Alternatively, the active site disulfide bridge may ensure conformational rigidity of the loop between PQQ and heme c. This rigidity may be required to maintain the level of nuclear density, which is 0.63 and below the average value of 0.76 (48). Substitution of (one of) the cysteines by other amino acids would thus lead to increased flexibility and possibly to a significantly lower nuclear density, which in turn would have a negative effect on the electron transfer rates.

As a third function, the protein medium could be directly involved in the conduction of electrons. In this case, electron transfer would proceed through specific pathway tubes that may or may not be dynamically controlled (52). The pathways involve covalent bonds, hydrogen bonds, and through-space jumps (53). Optimal electron transfer pathways calculated with Harlem (49) involve atoms of PQQH2, Cys116, Cys117, a water molecule, Cys607, and heme c (Fig. 5A). A second, longer and therefore possibly less efficient, pathway could include PQQH2, Cys117, Asp118, Arg57, and heme c. In conclusion, it appears that the disulfide bond between Cys116 and Cys117, and possibly the side chains of Asp118 and Arg57, are essential for efficient electron transfer from PQQH2 to heme c, whatever their precise function may be.

Proton Transfer from PQQH2 to the Periplasm—Proton pathways usually consist of hydrogen-bonded networks of proton donor and acceptor groups, either water molecules or amino acid side chains (54, 55). Such a network extends from the hydrophobic groups of PQQH2 to the solvent via Lys335, Asp308, Glu185, a water-filled chamber between the two domains, and Arg67 (Fig. 5B). It is disrupted only once by a distance of 4.7 Å between two water molecules. We propose this network as a channel for proton transfer to the solvent, i.e., the periplasm.

Relevance for Other PQQ-dependent Alcohol Dehydrogenases—The physiological electron acceptors of PQQ-dependent ADHs are cytochrome c proteins. The redox centers involved in the electron transfer reactions are thus the same. All quinoprotein ADHs including MDH are homologous to QH-ADH, and the electron transfer reactions are thus the same. All quinoprotein ADHs including MDH are homologous to QH-ADH, and the electron transfer reactions are thus the same.