The Three-dimensional Structures of Methanol Dehydrogenase from Two Methylotrophic Bacteria at 2.6-Å Resolution*

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The structures of methanol dehydrogenase (MEDH) from two closely related methylotrophic bacteria, *Methylophilus methylotrophus* and *M. methylophilus*, have been determined at 2.6-Å resolution. The molecule, a quinoprotein of molecular mass of about 138 kDa, contains two heavy (H) and two light (L) subunits of unknown sequence and two molecules of noncovalently associated pyrroloquinoline quinone. The two enzymes crystallize isomorphously in space group P2₁, with one H₂L₂ heterotetramer in the asymmetric unit. The electron density map of the *M. methylophilus* enzyme was obtained by multiple isomorphous replacement with anomalous scattering and improved by solvent leveling and electron density averaging. For model building, the amino acid sequence of MEDH from *Paracoccus denitrificans* for the H subunit and from *Methylobacterium extorquens* AM1 for the L subunit were used to represent the unknown amino acid sequence. At the present time, 579 and 57 amino acid residues for the large and small subunits, respectively, have been fitted into the map. The phases for MEDH from *M. methylophilus* were used directly to analyze the W3A1 structure, and both structures were refined to R-factors (where $R = \frac{\sum |F_o - F_c|}{\sum |F_o|}$) of 0.277 and 0.266, respectively. The L subunit contains a long α-helix and an extended N-terminal segment, both lying on the molecular surface of the H subunit. The H subunit contains eight antiparallel β-sheets, each consisting of four strands arranged topologically like the letter W. The eightWs are arranged circularly, forming the main disc-shaped body of the subunit, with some short helices and loops connecting the consecutive Ws, as well as some excursions within and between some of the Ws. The pyrroloquinoline quinone prosthetic group is located in the central channel of the large subunit near the surface of the molecule. The topology of the eight-W folding unit is similar to those of the six- and seven-W folding units previously reported for three other proteins, neuraminidase, methylamine dehydrogenase, and galactose oxidase.

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The atomic coordinates, structure amplitudes, and phases have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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1 The abbreviations used are: MEDH, methanol dehydrogenase; MIR, multiple isomorphous replacement; MIRAS, multiple isomorphous replacement with anomalous scattering; PQQ, pyrroloquinoline quinone; TTQ, tryptophan tryptophylquinone; H, heavy; L, light.

Methanol dehydrogenase (MEDH, EC 1.1.99.8) is a bacterial quinoprotein that catalyzes the oxidation of methanol to formaldehyde (1). Other primary alcohols, as well as formaldehyde are also substrates (2). The reaction catalyzed is RCHO $\rightarrow$ RCHO + 2H⁺ + 2e⁻. The enzyme is located in the periplasmic space of many methylotrophic bacteria, which are capable of utilizing single carbon compounds, such as methanol, as the sole source of carbon and energy (3). For a number of years, MEDH was thought to be a dimer of identical 62-kDa subunits. More recently, the enzyme has been shown to contain an additional pair of identical 8-kDa peptides, making the enzyme an H₂L₂ heterotetramer of molecular mass of approximately 138 kDa (4).

MEDH contains two molecules of noncovalently bound pyrroloquinoline quinone (PQQ) (Fig. 1) per tetramer (5, 6). The primary electron acceptor in vivo for MEDH is a cytochrome c, sometimes called cytochrome c₅₅, an acidic protein of approximately 20 kDa (7). Electrons are subsequently transferred to a membrane-bound α₅₂-type cytochrome oxidase via a second carrier, sometimes called cytochrome c₃₉, a basic protein similar to soluble mitochondrial or bacterial c₅₂-type cytochromes (8).

The amino acid sequences of the H subunit of MEDH found in *Paracoccus denitrificans* (9), *Methylobacterium extorquens* AM1 (10), and *Methylobacterium organophilum* XX (11) have been obtained by DNA sequencing methods and

![Chemical structure of PQQ.](image-url)

Fig. 1. Chemical structure of PQQ.
Crystal Structure of Methanol Dehydrogenase

MATERIALS AND METHODS

Crystallization—We previously reported the crystallization and preliminary x-ray crystallographic study of MEDH from two bacteria, M. methylotrophus and W3A1 (15, 16). In the present study, both bacterial forms of the enzyme crystallize isomorphously in space group P2₁ with unit cell parameters a = 124.9 Å, b = 62.7 Å, c = 85.0 Å, and β = 93.4°. The crystals contain one full heterotetramer per asymmetric unit, giving Vₚ = 2.41 Å³/Da and a solvent content of about 60%, based on a protein molecular mass of 135 kDa (14). They diffract beyond 2.4 Å resolution. The crystals of the M. methylotrophus enzyme were prepared by macroseeding using seeds obtained by the hanging drop method. The conditions of crystallization were 0.5% protein, 13.5% polyethylene glycol 8000, 0.05 M Na/K phosphate buffer, pH 8.25. Under very similar conditions, crystals in space group C₂ have been prepared (15) that are very close in cell parameters to the P₂₁ crystal form. The crystal of MEDH from bacterium W3A1 used in this study was grown directly without seeding, from a solution containing 0.26% protein, 13% polyethylene glycol 8000, and 0.04 M Na/K phosphate buffer at pH 8.25.

Structure Determination—In the MEDH crystal structure investigation reported here, the enzyme from M. methylotrophus was studied first and determined entirely by the MIRAS method. The structure of the enzyme from W3A1 was analyzed afterwards either by applying the M. methylotrophus phases directly to the W3A1 data collection statistics are summarized in Tables I and II, respectively. Using the ROCKS crystallographic computing program (17) together with the ROCKS crystallographic computing program (17), the heavy atom derivatives KAuCl₄, (NH₄)₃IrCl₆, and K₂PtCl₄ of the heavy atom derivatives of the gold derivative by including anomalous scattering using both the refinement program HEAVY (21). Heavy atom positions were obtained by alternative difference Fourier syntheses and heavy atom refinement. The anomalous scattering information was also used in this study was grown directly without seeding, from a solution range of 5.0 to 2.6 Å, where R = Z (Fₒ – F – [Σ])/ΣF and Fₒ and F are the observed and calculated structure factors, respectively. Similar refinement using the x-ray data from bacterium W3A1 gave a final R-factor of 0.290. The root mean square deviation from ideal bond lengths was 0.021 and 0.022 Å, respectively, for the two structures. In each case, the deviation between equivalent Cα atoms in the two subunits was about 0.5 Å.

Further refinement of the two types of MEDH was carried out by simulated annealing followed by positional and temperature factor refinement using XPLOR (29). A slow cooling procedure (30) from 300 K to 120 K was used. The simulated annealing and data between 10 and 2.6-Å resolution were included. However, the two halves of the molecule in the asymmetric unit were restrained to obey noncrystallographic symmetry so that the root mean square deviation between equivalent Cα positions remained between 0.02 to 0.03 Å. The resulting R-factors were 0.277 for the M. methylotrophus enzyme and 0.282 for the W3A1 enzymes; the root mean square deviation from ideal bond lengths was 0.025 and 0.019 Å, respectively. Further refinement of the W3A1 enzyme by the same procedure, first with PQQ included in the model (see below) and then with nine substituted amino acids in each half of the molecule, yielded a final R-factor of 0.286.

RESULTS AND DISCUSSION

Structure Analysis—For the H subunit, two amino acid sequences were examined after the initial chain tracing and Cα placement; one was from P. denitrificans and the other was from M. extorquens AM1. In general, the P. denitrificans sequence corresponded better to the electron density and was used for subsequent model building and refinement. For the L subunit, only the AM1 sequence was available at the time of the model building. In both subunits, good to excellent correspondence was achieved in over 90% of the cases between the side chain density and the models for the six distinctive types of residues Trp, Phe, Tyr, His, Pro, and Gly. The validity of using the M. extorquens sequence to fit the L subunit of M. methylotrophus has been substantiated by the table:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Crystal number</th>
<th>Concentration</th>
<th>Soak time</th>
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<td></td>
<td></td>
<td>mM</td>
<td>days</td>
</tr>
<tr>
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<td>3</td>
<td>4.5</td>
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<tr>
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<td></td>
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<td>1</td>
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<tr>
<td>K₂PtCl₄</td>
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<td>1</td>
<td>8</td>
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</table>
were made in the and could result from thermal motion or from changes in the atomic model. In fitting the MIR electron density of an amino acid. None of these latter changes were incorporated into the model. For about nine other side chains, the electron density was larger than expected, at the end of the XPLOR refinement. For about nine other residues followed by guest on November 14, 2017 http://www.jbc.org/ Downloaded from

recent finding that their sequences are 84% identical for the first 31 residues (13). The partial M. methylotrophus sequence has subsequently been incorporated into the atomic model of the L subunits for both M. methylotrophus and W3A1.

The major structural feature of the H subunit is a superbarrel consisting of a collection of eight $\beta$-sheetlets that are arranged about a pseudo 8-fold axis of symmetry, as shown in the full molecule.

Structure Description—The dimensions of the MEDH molecule are about 110 x 60 x 60 Å, with the noncrystallographic 2-fold axis of symmetry relating a pair of heterodimers inclined by about 5° from the crystallographic $\beta$ axis. The H subunit has been fitted with 579 amino acid residues. The L subunit, 57 residues have been fitted. Each H subunit has a predominantly $\beta$-structure and consists of a single disc-like domain shaped like an inverted cup with an average diameter of approximately 45 and a thickness of about 50 Å, except for one or two loops that extend beyond the disc. In the heterotetramer, the two H subunits come into contact across the noncrystallographic 2-fold axis (Fig. 3). The interface is about 1700 Å² in area, with contact being made by approximately 40 residues in four polypeptide segments from each subunit. In contrast, the L subunits make no contact with each other in the full molecule.

During model building, two disulfide bridges were located; one was in the H subunit, and the other was in the L subunit. In addition, two residues identified as Ser (at positions 150 and 174) in the P. denitrificans sequence (9) for the H subunit were found to be joined by a bridge of high electron density (Fig. 2), strongly suggesting that these are both Cys in M. methylotrophus and form an additional disulfide bond. Two other notable sequence changes, close to the active site, were apparent in the MIR map or in subsequent difference maps during refinement. These are Gly-175, which resembles Ser and Gly-110, which resembles Arg. These four changes, along with those in the L subunit, were incorporated into the model at the end of the XPLOR refinement. For about nine other side chains, the electron density was larger than expected, suggesting minor changes such as Leu replacing Val or Tyr or Trp replacing Phe; in addition, a number of other disagreements between the sequence and the density were found, but for these the side chain density was smaller than expected and could result from thermal motion or from changes in amino acid. None of these latter changes were incorporated into the atomic model. In fitting the MIR electron density of the M. methylotrophus MEDH, no insertions or deletions were made in the P. denitrificans sequence of the H subunit or the M. extorquens AM1 sequence for the L subunit, except for the 20 and 19 residues omitted from the H and L C termini, respectively.

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<th>Number of observations</th>
<th>$R_{ave}$</th>
<th>$\Delta F_{ave}$</th>
<th>Completeness</th>
<th>$I/\sigma(I)'$</th>
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<td>%</td>
<td>Overall</td>
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<td>99.5</td>
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<th>Crystal</th>
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$\Delta F_{ave}$ is the mean isomorphous change in structure factor. It was calculated at 3.0-Å resolution for all derivatives except for iridium 3, which was collected to only 3.2-Å resolution.

$I/\sigma(I)'$ is the signal-to-noise ratio. The second column indicates the value in a thin shell of data at the highest resolution.

This native data set and all derivative data were from enzyme isolated from M. methylotrophus.

Native data from methylotroph W3A1.

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<th>Number of observations</th>
<th>$R_{ave}$</th>
<th>$\Delta F_{ave}$</th>
<th>Completeness</th>
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TABLE II
Data collection statistics

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<th>$I/\sigma(I)'$</th>
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TABLE III
MIR phase calculation statistics at 3.0-Å resolution

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$F_n$ is native protein structure factor amplitude.

$m$ is figure of merit.

Phasing power = $\langle f_H \rangle / \langle e_H \rangle$, where $\langle f_H \rangle$ is the root mean square calculated heavy atom structure factor and $\langle e_H \rangle$ is the root mean square lack of closure error.
FIG. 2. Electron density of the putative disulfide bond linking positions 150 and 174 of MEDH from \textit{M. methylotrophus}. In \textit{P. denitrificans}, whose sequence was used to solve the structure, these residues are both Ser, as shown in the diagram. The strong electron density at the tips of the side chains strongly suggests that these residues are Cys in \textit{M. methylotrophus}. The two Ser residues and some adjacent residues are shown in thick lines, whereas a nearby chain is shown in thin lines.

FIG. 3. \textit{a}-Carbon drawing of the MEDH dimer. The molecular 2-fold axis is vertical, and the pseudo 8-fold symmetry axes are inclined by about 45° to the 2-fold axis. The H subunits are drawn in thin lines and the L subunits are drawn in thick lines.

in Fig. 5. Each leaflet is composed of four anti-parallel \( \beta \) strands that form a W-like structure. The four strands, A, B, C, and D, of each W fan outward from the central axis, with the innermost strand lying approximately parallel to the axis. Because of the twist of the \( \beta \)-sheet of each leaflet, the outside strands are inclined by about 45° to the central axis. The pseudo 8-fold axes of the two subunits of the heterotetramer are inclined by approximately 45° to the molecular 2-fold axis, as shown in Fig. 3. In Fig. 5, the H subunit is viewed from the top of the disc, with the inside strand of each W extending downward from the N- to C-terminal end.

The folding topology of MEDH is shown schematically in Fig. 6. The N-terminal segment of the H subunit begins in the central channel near the bottom of the disc and proceeds for about 40 residues along the bottom of the disc and then forms the outside strand (D) of leaflet W8. It then goes on to form the inside strand (A) of leaflet W1. Leaflet W8 is completed by the inner three strands from the C terminus.

There are four long excursions of about 40 residues each from the W-like structures in the H subunit. One of them, between W3 and W4, protrudes from the side of the disc and contains three very short helices or helical turns. The other three lie over the top of the disc. One excursion, between strands B and C of W6, contains a long \( \beta \)-hairpin. Another, between W5 and W6, contains a short \( \beta \)-hairpin plus an extended \( \beta \)-strand that lies alongside the hairpin from W6 to form a three-stranded antiparallel \( \beta \)-sheet. The fourth excursion, between strands B and C of W8, contains two short helices. There are also two shorter polypeptide excursions at the top of the disc, about 15–20 residues in length, one between W1 and W2 containing a short \( \alpha \)-helix, and the other between strands B and C of W4.

Location of PQQ.—While building the model of the \textit{M. methylotrophus} enzyme, a prominent feature of unexplained electron density was found in the central channel at the top of the eight-W disc in the averaged 2.6-Å resolution map. This feature was consistent with the size of PQQ, although portions of the PQQ model lay in weak density, and one part of it was rather close to the protein. An electron density map was also calculated for the isomorphous crystal form of MEDH from the bacterium W3A1, using the solvent-leveled phases for \textit{M. methylotrophus} MEDH, and averaged about the molecular 2-fold axis. The shape of the putative PQQ in the W3A1 map was similar to, but slightly better than, that of the \textit{M. methylotrophus} map.

In order to better define the PQQ position, the models of MEDH from both \textit{M. methylotrophus} and W3A1 with the PQQ omitted were refined as described above. After the PROFF refinement, the shape of the electron density for the putative PQQ site in the 2\( F_o - F_c \) map from W3A1 matched the model considerably better than before, especially when the electron density was averaged about the molecular 2-fold axis. The corresponding density for the \textit{M. methylotrophus} enzyme remained weaker, however. The electron density for the W3A1 enzyme, shown in Fig. 7a, is quite flat and continuous over most of the PQQ molecule. When PQQ is placed in the electron density, its position is slightly displaced from that found earlier in the \textit{M. methylotrophus} MIRAS.
FIG. 4. Skeletal diagram of L subunit of MEDH. The 57 residues observed in the electron density are shown.

FIG. 5. Ribbon diagram of the H subunit of MEDH. Those β-sheets contained in the eight β-leaflets (labeled W1-W8) are indicated, as are the N and C termini. The pseudo 8-fold axis is approximately perpendicular to the diagram.

FIG. 6. Folding topology of the H subunit of MEDH. Polypeptide strands in β-sheets are indicated by arrows, and helical segments are indicated by open boxes. Excursions above the main disk of the H subunit described in the text are shown.

There are four orientations of PQQ that are consistent with the overall shape of the electron density. Two of these have the quinone oxygen atoms pointed upward in Fig. 7a, whereas the other two have them pointed downward. In each of these cases, the two alternate orientations differ by 180° rotation about a vertical axis. An orientation was chosen to represent PQQ, which seemed to fit the density best at the current resolution. When this orientation of PQQ was included in the XPLOR refinement of the W3A1 crystal, its atomic parameters were well behaved, and its electron density in a 2F0 - Fc difference map matched the model closely (Fig. 7b). When a model oriented with the quinone oxygen atoms pointed in the opposite direction was tested, the PQQ molecule became distorted, with the carbonyl oxygen atoms twisting out of the plane of the molecule.

The PQQ prosthetic group is located in the funnel-shaped central channel at the top end of the eight-W disc (Fig. 8). The normal of the PQQ plane is tilted by about 30° from the pseudo 8-fold axis. In the orientation shown, the carboxylic acid at position 7 (Fig. 1) appears to be more accessible to solvent, along with the carbonyl oxygen at position 5, in accordance with its reactivity toward the addition of acetone upon extraction from the enzyme (5) and its implied catalytic role in other PQQ-containing enzymes (31). The binding site of PQQ is also consistent with the locations of the active sites in other super barrel structures with similar motif (see below).

Since the amino acid sequence of MEDH from W3A1 is not known and its structure is not fully refined, a complete description of the binding of PQQ to the enzyme is not possible. However, the general features of the PQQ-binding site based on the P. denitrificans sequence are evident at this stage, as indicated in Fig. 9. The PQQ molecule lies on top of Trp-243, making van der Waals contact with it and with Trp-539. In the orientation shown, the central carboxylic acid group, at C9, is closest to Arg-331 and Asn-394. It also is close enough to interact with Asp-105, which is located above the PQQ ring. The other two carboxylic acid groups, on C2 and
C7, appear to interact with the side chains of Glu-55 and Asn-261, respectively. Also close by these carboxylic acid groups are Asp-303 and Trp-475, respectively. The carbonyl oxygen at position 4 of PQQ is close to Thr-159, whereas the one at position 5 appears to interact with Ser-175 (Gly in the P. denitrificans sequence). The side chain of Arg-110 (Gly in the P. denitrificans sequence) extends into the active site region and appears to interact with Thr-159 and is within 4 Å of Glu-55 and the C9 carboxyl group of PQQ. One of the alternate orientations of PQQ would exchange the positions of C2 and C9 but allow most of the equivalent interactions with the protein to be made. In the other alternative orientations, these protein side chains would interact with different portions of the PQQ molecule.

In order to investigate the PQQ density found in the M. methylotrophus enzyme, an XPLOR refinement was carried out with PQQ included in the molecular model. The $2F_o - F$ difference map still showed weaker density, as compared with the W3A1 enzyme, which was reflected by an increase in the corresponding temperature factors for the PQQ. These results suggest the binding site may be only partially occupied, possibly resulting from some loss of PQQ during storage or crystallization. This phenomenon has been observed for aged samples of MEDH from P. denitrificans. Complete verification of the PQQ orientation and a detailed analysis of its environment must await the amino acid sequence and full refinement of the structure.

2 V. Davidson, unpublished results.
Comparison with Other Proteins—The β-leaflet motif found in MEDH has been observed in three other proteins, the influenza virus neuraminidase (32), methylamine dehydrogenase (33, 34), and galactose oxidase (35). In all cases, the four stranded W-like β-sheet structures are arranged about a central axis of pseudosymmetry, with the strands of each sheet arranged in the pattern A, B, C, and D, progressing outward from the central axis. However, the number of Ws and type of pseudosymmetry differs among the four proteins, being 6-fold in the neuraminidase, 7-fold in methylamine dehydrogenase and galactose oxidase, and 8-fold in MEDH. The topologies of these four proteins are compared schematically in Fig. 10.

The N-terminal segments of the W-containing domains of these four proteins differ somewhat. In MEDH, the outside strand of W-8, the last W, is contributed by the N-terminal segment before it goes on to form W-1, as described above. This construction is similar to that of the influenza neuraminidase and of galactose oxidase but differs from that of methylamine dehydrogenase, as shown in Fig. 9. In methylamine dehydrogenase, the three outside strands, B, C, and D of the last W (W-7) are formed by the N-terminal segment before the first W is formed.

There are several major structural and functional differences between the four β-leaflet structures. MEDH is an $H_2L_2$ heterotetramer, in which the H subunit consists entirely of the W domain. The L subunits are not folded as independent domains but are appended to the H subunit and make no other intersubunit interactions in the tetramer. Methylamine dehydrogenase is also an $H_2L_2$ heterotetramer in which the H subunit, of about 45 kDa, corresponds entirely to the W domain, except for a 30-residue N-terminal tail that protrudes from the domain (33). However, the L subunit is an independently folded domain of about 15-kDa that interacts with both H and L domains in the heterotetramer.

Like MEDH, methylamine dehydrogenase is a quinopro-
be reported. Because of the considerable sequence homology to other MEDHs and to alcohol dehydrogenase from Aceto-

The enzyme catalyzes the oxidation of primary alcoholic substrates. The active site of the enzyme contains a copperylamine  dehydrogenase, galactose oxidase, and methanol dehydrogenase.

The influenza virus neuraminidase is a homotetramer of about 68 kDa. It consists of three domains, with the central domain corresponding to the 7-W structure, about 375 residues in length. The enzyme catalyzes the oxidation of primary alcoholic substrates. The active site of the enzyme contains a copper atom located at the top of the molecule on the pseudo 7-fold symmetry axis. One of the copper ligands is provided by the third domain, which contains a long loop that passes through the center of the 7-W domain from the bottom. The copper and one of its ligands, a tyrosine side chain that is covalently linked to a cysteine side chain, act as the primary electron acceptor during oxidation to form a coperous ion and a tyrosyl radical. This tyrosine is stacked against a tryptophan side chain that may help stabilize the radical form of the enzyme.

In MEDH, the redox properties of PQQ may also be influenced by the presence of a tryptophan side chain as described above.

Concluding Remarks—The present crystal structure of MEDH is the first structure of a PQQ-containing enzyme to be reported. Because of the considerable sequence homology to other MEDHs and to alcohol dehydrogenase from Aceto-

The function of the L subunit of MEDH is not obvious at the moment. It is located mostly on the outside edge of the H subunit and has little contact with the active site region in the "top" of the H subunit or with the dimeric interface. It had been proposed (4) that the L subunit might form part of the binding site for cytochrome c\textsubscript{L}, the primary electron acceptor from MEDH, because of the high lysine content of this subunit (20% for MEDH from \textit{M. extorquens} AM1) and the acidic nature of the cytochrome. It was subsequently shown that electrostatic interactions do play an important role in the electron transfer process (14), but based on "zero length" cross-linking experiments, the L subunit is not directly involved in formation of the intermolecular electron transfer complex. The position of the L subunit in MEDH is consistent with these observations. The most likely role of the L subunit appears to be structural, somehow maintaining the integrity of the active site and other important aspects of the catalytic process.

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