Structural Model of the Fe-Hydrogenase/Cytochrome c553 Complex Combining Transverse Relaxation-optimized Spectroscopy Experiments and Soft Docking Calculations*

Received for publication, December 8, 1999, and in revised form, March 15, 2000 Published, JBC Papers in Press, March 27, 2000, DOI 10.1074/jbc.M909835199

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Fe-hydrogenase is a 54-kDa iron–sulfur enzyme essential for hydrogen cycling in sulfate-reducing bacteria. The x-ray structure of Desulfovibrio desulfuricans Fe-hydrogenase has recently been solved, but structural information on the recognition of its redox partners is essential to understand the structure-function relationships of the enzyme. In the present work, we have obtained a structural model of the complex of Fe-hydrogenase with its redox partner, the cytochrome c553, combining docking calculations and NMR experiments. The putative models of the complex demonstrate that the small subunit of the hydrogenase has an important role in the complex formation with the redox partner; 50% of the interacting site on the hydrogenase involves the small subunit. The closest contact between the redox centers is observed between Cys-38, a ligand of the distal cluster of the hydrogenase and Cys-10, a ligand of the heme in the cytochrome. The electron pathway from the distal cluster of the Fe-hydrogenase to the heme of cytochrome c553 was investigated using the software Greenpath and indicates that the observed cysteine/cysteine contact has an essential role. The spatial arrangement of the residues on the interface of the complex is very similar to that already described in the ferredoxin-cytochrome c553 complex, which therefore, is a very good model for the interacting domain of the Fe-hydrogenase-cytochrome c553.

Sulfate-reducing bacteria of the genus Desulfovibrio utilize hydrogen or organic substrates as electron donors and exhibit a strictly anaerobic mode of growth based on the reduction of sulfate as the terminal electron acceptor (1). These microorganisms couple energy generation to the dissimilatory reduction of sulfate via electron transfer-linked phosphorylation (2). Dissimilatory sulfate reduction with hydrogen is a transmembrane redox process in which $H_2$ oxidation and sulfate reduction take place on opposite sides of the cytoplasmic membrane (3). Sulfate-reducing bacteria have been investigated for many years as a model for respiratory electron transfer chains. The structures of many compounds in this system have been obtained using x-ray crystallography. Among these metalloproteins, the structurally unrelated NiFe- and Fe-hydrogenases (4) catalyze the oxidation of molecular hydrogen or proton reduction according to the following reaction (5):

$$H_2 \leftrightarrow 2H^+ + 2e^- \quad (Eq. \ 1)$$

Both enzymes are periplasmic and use c-type cytochromes as electron transfer partners (6). One of the oxidoreduction partners of Fe-hydrogenase is the periplasmic low potential cytochrome c553. The monohemicytochrome c553 is homologous to mitochondrial cytochromes (7). The relative high exposure of the heme to the solvent is the structural parameter modulating the low oxidoreduction potential of this cytochrome (20 mV) (8). Kinetic experiments (6) have shown that cytochrome c553 has a rather high affinity for hydrogenase ($K_m$ = 46 $\mu$M) and that the electron transfer rate constant ($k_{cat}$ = 710 s$^{-1}$) is comparable to those generally reported in bimolecular electron transfer complexes.

The x-ray structure of Fe-hydrogenase is essential in understanding the reactivity of the enzyme (9). D. desulfuricans ATCC 7757 Fe-hydrogenase, which is identical to the homologous enzyme from D. vulgaris on the basis of its amino acid sequence and spectroscopic properties (10, 11), comprises two different subunits of 42.5 and 11 kDa. The large subunit contains a ferredoxin-like domain composed of two [4Fe-4S] clusters and an unusual active Fe-S center, termed the H cluster. From x-ray studies, it has been shown that the H cluster is constituted of a typical [4Fe-4S] cubane bridged to a binuclear active site. The observed spatial arrangement of the three [4Fe-4S] clusters separated by center-to-center distances of 12 Å provides one plausible electron transfer pathway going from the buried binuclear active site to the distal [4Fe-4S] cluster located close to the molecular surface. Furthermore, the calculated electrostatic potential at the molecular surface surrounding the distal cluster is negative (9). These observations indicate that this region might be involved in electrostatic interactions with cytochrome c553 (see Fig. 1), which exhibits a high content of basic residues around the heme pocket (12). The mature small subunit, which is preceded by a N-terminal signal peptide, is probably involved in the export of the enzyme to the periplasm (11). It shows an unusual topology, which sheds some light on the evolutionary pathway of Fe-hydrogenase.

* Supported by the French Embassy and by the ICCTI (Project 316C2). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1e08) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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Indeed, this heterodimeric periplasmic enzyme is very similar to Clostridium pasteurianum Fe-hydrogenase and may have evolved from a common ancestral monomeric enzyme. However, the structural role of the small subunit in Fe-hydrogenase is not yet well understood. Although a wealth of biological and structural data have been gathered on Fe-hydrogenase and cytochrome c553, the various types of interaction involved in the recognition and the binding of the two electron transfer partners as well as the interacting molecular surfaces of both redox partners have not been investigated. Furthermore, the detailed mechanisms of intra- and intermolecular electron transfer between the two redox partners still require extensive research.

In the present work, we develop a structural approach to study the cytochrome c553-Fe-hydrogenase complex using protein docking calculations and heteronuclear NMR experiments. Docking algorithms are currently being developed for protein-ligand complex analysis. For protein-protein interactions the use of such algorithms is more difficult, and it is essential that experimental data be used to select the best model. We have recently described an experimental ab initio approach using the software BiGGER in which we have implemented an NMR filter (13). This approach developed for small complexes was carried out using heteronuclear single quantum coherence experiments. Recently, Pervushin et al. (14) introduced transverse relaxation-optimized spectroscopy (TROSY),1 in which constructive interference between dipolar relaxation and relaxation due to chemical shift anisotropy was exploited to reduce the line width in both dimensions in high magnetic fields. The method has clear advantages for structural investigation of high molecular weight complexes when compared with the regular heteronuclear single quantum coherence experiment. Therefore, we have carried out TROSY experiments on 15N-labeled cytochrome in the absence and presence of Fe-hydrogenase. On the basis of proton and nitrogen chemical shift variations, a mapping of the interacting site on the cytochrome has been obtained. These NMR data were used as a filter to choose the best model. We have recently demonstrated (13, 16) that the use of NMR filters necessitates the labeling of both proteins so that NMR constraints are applied on both partners of the complex. In the present work, the labeling of hydrogenase is not possible because of the poor expression of the enzyme in the sulfate-reducing bacteria. Moreover, if 15N labeling allows structural studies of high molecular weight proteins, double labeling (15N and 13C) would be necessary here, to accomplish the full assignment of the Fe-hydrogenase. For this reason, we have first analyzed a complex where ferredoxin from Desulfomicrobium norvegicum was used as a model of the ferredoxin-like domain of the Fe-hydrogenase (16). In the present work, we have developed a new approach for high molecular weight complexes, where NMR constraints are only deduced from 1H and 15N chemical shifts observed on the smaller, labeled protein. The similarities of the model complex and the physiological complex are analyzed.

**MATERIALS AND METHODS**

**NMR Samples**—The cytochrome c553, from D. vulgaris Hildenborough was expressed and purified from *D. desulfuricans* G200 as described previously (17). 15N-Cytochrome c553 was obtained as previously reported (16) and was concentrated in 10 mM Tris-HCl buffer, pH 7.0, on an Amicon microconcentrator (PM 30) to a 0.5 mM final concentration. *D. desulfuricans* ATCC 7757 hydrogenase was purified as reported by Hatchikian et al. (10). Hydrogenase was concentrated under argon atmosphere in Tris-HCl buffer (10 mM), pH 7.0, on an Amicon microconcentrator (PM 30) to a 0.4 mM final concentration. NMR experiments were performed with 0.1 mM cytochrome and 1 or 2 equivalents of hydrogenase.

**NMR Experiments**—The two-dimensional [1H-15N]-TROSY pulse sequence uses single transition to single transition polarization transfer (18) where it affords a sensitivity enhancement for kinetically stable amide groups in proteins. NMR spectra were recorded at 296 K on a Bruker Avance DRX 500 spectrometer operating at 11.7 teslas, equipped with an HCN probe and self-shielded triple axis gradients. Spectra were acquired accumulating 32 scans per free induction decay. 128 complex points in F1 and 1K complex points in F2 were recorded. The spectral width was 6 and 2 KHz in F2 and F1, respectively. 1H chemical shifts were referenced with the H2O resonance calibrated at 4.792 ppm at 296 K. 15N chemical shifts were referenced indirectly by setting the zero point of the nitrogen frequency scale equal to 0.101329118 times the proton zero frequency (19). Processing and spectra analysis were done on Unix workstations using xwinnmr and Aurelia software provided by Bruker.

**Protein Structures**—The protein coordinates of *D. vulgaris* Hildenborough cytochrome c553 and *D. desulfuricans* ATCC 7757 Fe-hydrogenase were obtained from the Protein Data Bank from file 1dvh and 1hfe, respectively.

**Protein Docking**—Molecular interaction simulations were performed using the docking program BiGGER (15). This algorithm performs a complete and systematic search in the binding space of both molecules. A population of 1000 candidate protein-protein-docked geometries is generated and selected, based on the geometric complementarity and amino acid pairwise affinities between the two molecular surfaces. In this process, the algorithm enables implicit treatment of molecular flexibility. In a subsequent step, the putative docked structures are ranked using an interaction scoring function, which combines several interaction terms that are thought to be relevant for the stabilization of protein complexes: geometric packing of the surfaces, explicit electrostatic interactions, desolvation energy, and pairwise propensities of the amino acid side chains to contact across the molecular interface. In the *ab initio* simulations, the entire molecular surface was searched using absolutely no additional information regarding the binding sites.

**NMR Filtering**—Chemical shift variations observed in TROSY experiments of cytochrome c553 in the presence and absence of hydrogenase have been considered to be correlated to the complex formation. These variations were translated into distance parameters (an amide (NH) affected is within 4 Å of any atom belonging to the other protein). The 1000 solutions obtained using the program BiGGER were analyzed using the SHAKE constraints. The solutions were then ranked according to the NMR constraint violations (Table I). The *ab initio* solution clustering was not necessary, because only two families of structures were obtained after the NMR ranking.

**Molecular Dynamics and Minimization of the Complex Structures**—The 10 best structures of the complex, as obtained by the docking algorithm filtered by NMR data, were first minimized in a preparation step, using the conjugate gradient method by Powell (20), with X-PLOR 3.851. Then a molecular dynamics calculation (AMBER force field) was performed on the selected complex structures with a heat bath temperature of 280 K and initial velocities from a Maxwell-Boltzmann distribution at 280 K (integration of the motion equations at 0.001-ps time steps). The SHAKE constraints (21) were applied for all metal centers, i.e. the bi-atomic catalytic site, the FeSx clusters of the hydrogenase, and the heme group of the cytochrome with a tolerance of 0.0004. The coordinates of the “best” structure (family 1 (family 1 solution 3) have been deposited at the Protein Data Bank (code: 1e08).

**Electron Transfer Pathway**—The software Greepath (22) was used to propose an electron transfer pathway from the electron donor (the hydrogenase distal cluster) to the electron acceptor (the cytochrome heme).

**RESULTS**

**Ionic Strength Dependence of the Cytochrome c553-Fe-Hydrogenase**—Cytochrome c has been reported to present a strong dipolar moment induced by the high content of basic residues found around the heme pocket. These lysine residues have been demonstrated to be highly conserved in this class of proteins (7). Complex formation between cytochrome c553 and Fe-hydrogenase has been evaluated in terms of electrostatic interactions. The drastic effect of the ionic strength (0.5 mM NaCl) on the
kinetic measurements of cytochrome c553 reduction by Fe-hydrogenase, which we have observed, establishes that electrostatic interactions are the driving force of the complex formation.

Fig. 1 shows electrostatic isopotential curves for cytochrome c553 and Fe-hydrogenase. The dipolar moment is easily observable for cytochrome c553, and three zones of various charges are observable for hydrogenase. The heme environment of cytochrome c553 and the H cluster environment of the Fe-hydrogenase are found to be positively charged, and the environment of one of the two clusters of the ferredoxin-like domain (the distal cluster) is negatively charged. Considering the electrostatic properties of the two molecules and the ionic strength dependence of the complex formation, one can expect that the distal cluster situated at the extreme end of the domain is the interacting site with cytochrome c553.

NMR Mapping of the Interacting Site—TROSY experiments have been recorded on 0.1 mM cytochrome c553 solution at 296 K (Fig. 2). Out of the 78 amino acids, 58 NH groups were assigned. The mapping of the interacting site was obtained from the induced chemical shifts observed on the cytochrome c553 NH, in the presence of Fe-hydrogenase in the TROSY experiments (Fig. 3A). Nine NH groups undergo notable 15N chemical shift variations from 0.1 to 0.45 ppm and 1H from 0.015 to 0.66 ppm. Analysis of these chemical shifts gives the mapping of the interacting site on the cytochrome represented in Fig. 3B. The interacting site on the cytochrome is the same with either ferredoxin (16) or hydrogenase, involving at least six conserved residues, i.e. 10, 14, 15, 16, 24, and 59.

Docking of the Complex—An ab initio docking of the complex was calculated on the basis of the Protein Data Base files from cytochrome c553 and Fe-hydrogenase. 1000 putative structures of the complex were obtained from the software BiGGER. These structures were first evaluated and ranked by an ab initio procedure, which uses an interaction score function (Fig. 4A). The next step was to use the NMR filter, explained above, in order to rule out solutions incompatible with the NMR data and to retain a reduced population of plausible structures (Fig. 4B). Eight NH groups of the residues affected by the complex formation were used as a filter for the docking solutions, i.e. 5, 10, 14, 15, 16, 24, 32, 54 and 59. This procedure eliminates the set of solutions in which the cytochrome would bind at the second negatively charged region on the hydrogenase (solutions on the lower left of Fig. 4A). The remaining solutions were ranked according to the level of agreement with the NMR constraints. The 50 top solutions (with fewer than three constraints violated out of eight) involve the binding of the cytochrome at two distinct sites in the surface of the hydrogenase (Fig. 4B). Each site involves, respectively, the distal and the medial cluster of the ferredoxin-like domain of hydrogenase and corresponds to family1 and family2 solutions. However, it has been reported that the hydrogenase-cytochrome c553 complex has a 1:1 stoichiometry (6), so only one of these two families of solutions is relevant. At this level, a reciprocal mapping of the interacting site on labeled hydrogenase would be helpful, but unfortunately, due to the poor expression of Fe-hydrogenase in sulfate-reducing bacteria, to date, it is not possible to carry out the labeling of Fe-hydrogenase. Therefore, we did not discard any of these two families at this stage of the analysis. Of the 50 best solutions, 10 models have a heme/cluster distance smaller than 20 Å. Considering that a maximum distance of 20 Å is necessary for an effective electron transfer, the 10 resulting solutions were energy-minimized. These 10 solutions are equally distributed in two families (fam1 and fam2) and are numbered according to the related family (Table I).

Energy Minimization of the Docking Solutions—All 10 solutions were minimized using X-PLOR, and a molecular dynamics calculation was performed to optimize side chain conformations and interactions on the interface. The resulting properties of the complexes after minimization are reported in Table I. The results indicate that the five solutions close to the distal cluster (fam1) can be grouped in a unique solution after minimization. The optimal distances between the distal cluster and the heme for these five solutions range from 12.3 to 14.3 Å.
and the final energy level varies by less than 0.5%. The solutions on the opposite side of the ferredoxin-like domain of the hydrogenase, closest to the medial cluster (fam2), represents five different solutions, because the orientations vary by more than 20° and different residues are involved in intermolecular interactions. Furthermore, the optimal distances obtained after minimization between the medial cluster and the heme group are clearly less favorable than the solutions of fam1, which bring the distal cluster closest to the heme of cytochrome c553. Therefore, we propose that the electron transfer involves

![Graphical representation](image.png)

**Fig. 3.** A, ^1^H and ^15^N chemical shift variations of cytochrome c553 NH groups observed in TROSY experiments. B, mapping of the hydrogenase interacting site on the cytochrome c553 obtained by heteronuclear experiments. In the top figure, the heme is colored in red, the NH groups whose resonances undergo chemical shift variations are in green, unassigned residues are in blue, and unaffected residues are in white. The bottom figure shows a 180° rotation along the x axis and represents the “back side” of the molecule.

![Graphical representation](image.png)

**Fig. 4.** Docking of the cytochrome c553-hydrogenase complex. A, the 1000 first ab initio docking solutions. B, the 50 best solutions chosen using NMR filtering. Only the hydrogenase backbone is represented; the center of mass of the cytochrome is represented by a small sphere.
the distal cluster as the direct interaction site with the cytochrome (fam1).

Within fam1, analysis of the five solutions involving the distal cluster indicates that it is the same region with small modifications of interacting residues that forms the interface. The choice of one representative structure was made with respect to the results of site-directed mutagenesis obtained on cytochrome \(c_{553}\). We have already suggested that Lys-63 and Tyr-64 are essential for electron transfer with the redox partner (12, 23). On the basis of these data, fam1 solution 3 seemed to be the best representative structure.

**DISCUSSION**

The model, corresponding to fam1 solution 3 of the cytochrome \(c_{553}\)-hydrogenase complex, is presented in Fig. 5. The interacting domain is very similar to the model proposed for the ferredoxin-cytochrome \(c_{553}\) complex. This is not surprising, considering that the root mean square distance between the ferredoxin-like domain of Fe-hydrogenase and the ferredoxin is 1.75 Å and that the distal cluster is the one conserved in the monocluster ferredoxins. The interacting surface is significantly bigger for hydrogenase (2284 Å) than it is for ferredoxin (1037 Å). This is directly correlated with the fact that the interacting site on hydrogenase is not only formed by the ferredoxin-like domain but about half of the protein-protein interface involves the small subunit of Fe-hydrogenase. The heme to Fe-S distance (12.3 Å) is longer in the hydrogenase complex than in the ferredoxin complex (10 Å). However, the distribution of hydrophobic and electrostatic atomic contacts are comparable in both complexes. These data demonstrate that ferredoxin is a good
model for the ferredoxin-like domain of Fe-hydrogenase with respect to its properties when interacting with the cytochrome c553. The use of an isolated functional domain is certainly a good approach for obtaining structural information in high molecular weight complexes.

Another significant aspect provided by the model of the cytochrome c553-hydrogenase complex is the involvement of the small subunit in the interaction site. The N- and C-terminal ends of the hydrogenase small subunit interact with the N-terminal part and the axial histidine-containing sequence (residues 12–17) of the cytochrome. This finding is strengthened by the TROSY data, where residues 14, 15, and 16 are found closest to Ala-397 of the hydrogenase (Fig. 5 part a). An electron transfer pathway was calculated with the Greenpath software. In the “best” pathway, the two cysteines facing each other are the site of electron transfer between the two molecules. The proposed pathway can be described as follows: the electron comes from the distal cluster and passes through the Fe–S bond of hydrogenase Cys-38, via the sigma bond of the cysteine to its carboxylic oxygen, from where it jumps to Cys-10 of the cytochrome. A further jump from the sulfur lone pair of Cys-10 to the histidine (His-14), the axial ligand of cytochrome c553, completes the path. The atoms of this path are closely arranged according to the straight line between the two iron atoms (distal cluster Fe2-heme Fe: 12.34 Å). The path length as calculated with Greenpath is 14 Å. Covalently bound sulfur atoms are indeed known to be involved in electron transfer of metal-organic compounds (25). Interestingly, close cysteine-cysteine contacts, between cysteines covalently bound to heme groups, has also been observed in the dimeric interface of the cytochrome c553 (M, 26,000) (26). Furthermore, a closely related electron transfer pathway, including two residues coordinating the [4Fe-4S] clusters, Cys-287 of formaldehyde ferredoxin oxidoreductase and Asp-14 of ferredoxin from Pyrococcus furiosus has recently been described from the analysis of the structure of the cocystalized complex (27). Such an electron pathway is probably found in many electron transfer complexes. Site-directed mutagenesis would be an appropriate approach to validate the electron pathway. However, in the cytochrome c553-hydrogenase complex, substitution of one of the two cysteine residues (by Ala, Ser, or Met) is difficult to achieve without drastic modifications to the protein folding or redox properties. The substitution of hydrogenase Cys-38 by a selenocysteine would be a better approach, but the barriers of heterologous expression of the selenoprotein gene in bacteria are a limitation of this kind of substitution (28).

The interacting surface includes the environment of the axial methionine (Met-57) of the cytochrome. Residues 54, 59, and 60 were found affected in TROSY experiments. In the model, a hydrogen bond exists between Asn-59 of the cytochrome and the main chain carboxyl groups of residues Ile-36 of the large subunit of hydrogenase. This Ile residue in the ferredoxin-like domain of hydrogenase is conserved in all the ferredoxins (Ile-10). In the present model, as in the ferredoxin-cytochrome c553 model, the main chain CO-group of this Ile residue makes a hydrogen bond with Lys-63 of the cytochrome, and the side chain of Ile-36 of hydrogenase exhibits hydrophobic van der Waals contacts with the side chain of Tyr-64 of the cytochrome. The role of these two residues in the electron transfer process in cytochrome c553 has been previously established (12, 23). The interaction of Tyr-64 of the cytochrome with the conserved Ile-36 residue of the hydrogenase assures the correct spatial arrangement by packing forces to bring the two electron transferring cysteines into the optimal position. Furthermore, Tyr-64 of the cytochrome, having a bulky aromatic side chain, probably blocks the accessibility of the prosthetic group crevice to the solvent molecules, which is certainly essential for the electron transfer reaction. By replacing residue Ile-36 by Ala in hydrogenase, both the importance of the length of the side chain for interaction with Tyr-64 of the cytochrome and the ability to block the access of the electron transfer site to the solvent would be elucidated.

Acknowledgements—We thank Susan D. Wells for reading the manuscript. The NMR equipment was partially provided by the Conseil Général des Bouches-du-Rhône.

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J. Biol. Chem. 2000, 275:23204-23210.
doi: 10.1074/jbc.M909835199 originally published online March 27, 2000

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