Role of the Tetrahemic Subunit in *Desulfovibrio vulgaris* Hildenborough Formate Dehydrogenase

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ABSTRACT: In the anaerobic sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough (*Dv*H), the genome sequencing revealed the presence of three operons encoding formate dehydrogenases. *fdh1* encodes an $\alpha\beta\gamma$ trimeric enzyme containing 11 heme binding sites; *fdh2* corresponds to an $\alpha\beta\gamma$ trimeric enzyme with a tetrahemic subunit; *fdh3* encodes an $\alpha\beta$ dimeric enzyme. In the present work, spectroscopic measurements demonstrated that the reduction of cytochrome c_{553} was obtained in the presence of the trimeric FDH2 and not with the dimeric FDH3, suggesting that the tetrahemic subunit (FDH2C) is essential for the interaction with this physiological electron transfer partner. To further study the role of the tetrahemic subunit, the *fdh2C* gene was cloned and expressed in *Desulfovibrio desulfuricans* G201. The recombinant FDH2C was purified and characterized by optical and NMR spectroscopies. The heme redox potentials measured by electrochemistry were found to be identical in the whole enzyme and in the recombinant subunit, indicating a correct folding of the recombinant protein. The mapping of the interacting site by 2D heteronuclear NMR demonstrated a similar interaction of cytochrome c_{553} with the native enzyme and the recombinant subunit. The presence of hemes *c* in the γ subunit of formate dehydrogenases is specific of these anaerobic sulfate-reducing bacteria and replaces heme *b* subunit generally found in the enzymes involved in anaerobic metabolisms.

In prokaryotes the formate, produced from pyruvate during anaerobic respiration, serves as the major electron donor to a variety of respiratory pathways that use terminal acceptors other than oxygen. Formate dehydrogenases (FDHs)[1] are metalloenzymes which catalyze the oxidation of formate to CO_2 and H^+. These enzymes contain either Mo or W associated to molybdopterin guanidine dinucleotide (MGD) cofactors and a conserved selenocysteine (SeCys) residue at their active site. Formate dehydrogenases isolated from anaerobic organisms are structurally related, but their contents in subunits and prosthetic groups are highly diversified. The structures of three bacterial enzymes have been solved by X-ray: FDH-H and FDH-N from *Escherichia coli* (*1*, *2*) and FDH from *Desulfovibrio gigas* (*3*). FDH-H is the constitutive formate dehydrogenase of *E. coli* which is part of the anaerobic formate hydrogen-lyase complex. This monomeric enzyme is organized in four main domains and contains a selenocysteine (SeCys), a molybdenum atom coordinated by two molybdopterin guanine dinucleotide (MGD) cofactors, and an iron−sulfur cluster at the active site (*1*). The FDH-N from *E. coli* is a nitrate-inducible enzyme expressed in anaerobic conditions. This molybdoenzyme contains three subunits: the catalytic α subunit coordinates a bis-MGD cofactor and one iron−sulfur (FeS) cluster, the β subunit holds four (FeS) clusters, and the membrane-bound γ subunit incorporates two *b*-type hemes (*2*). *D. gigas* FDH (*Dg*W-FDH) is a tungsten-containing enzyme composed of two subunits: the α subunit, which includes the W binding site and one (FeS) cluster, and the β subunit, which contains three (FeS) clusters.

Trimeric periplasmic formate dehydrogenases have been isolated from both *Desulfovibrio vulgaris* Hildenborough (*4*) and *Desulfovibrio desulfuricans* ATCC7757 (*5*). These enzymes include, in addition to the α and β subunits, a γ subunit which holds four *c*-type hemes. The genome of *D. vulgaris* Hildenborough (*Dv*H) has been recently sequenced and reveals the presence of three operons encoding different formate dehydrogenases (*6*). The *fdh1* operon contains three genes encoding an $\alpha\beta\gamma$ trimer. The γ subunit (FDH1C) includes 11 heme *c* binding sites. The *fdh2* operon corresponds to the well-characterized enzyme (*4*), which contains a tetrahemic subunit (FDH2C) homologous to the soluble tetrahemic cytochromes found in all the *Desulfovibrio* species (*7*). The third operon, *fdh3*, encodes an $\alpha\beta$ dimer, homologous to *DgW*-FDH subunits. We have in previous work isolated the three enzymes and analyzed their expression in *D. vulgaris* Hildenborough (*4*, *8*).

Cytochromes c_3 are low oxidoreduction potential multihemic cytochromes only found associated to the anaerobic metabolism of *Desulfovibrio* and *Shewanella*. This class of cytochromes is characterized by the presence of four bis-histidinyl axial coordinated hemes. The structures of cytochromes c_3 from various *Desulfovibrio* species have been solved by X-ray, and despite the low sequence identity, their three-dimensional structures are highly conserved, containing

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[1] Abbreviations: NMR, nuclear magnetic resonance; FDH, formate dehydrogenase; *Dv*H, *Desulfovibrio vulgaris* Hildenborough; HSQC, heteronuclear single-quantum correlation.

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Table 1: Strains and Plasmids for *fdh2C* Gene Expression

strain or plasmid	genotype, comment, and/or ref
E. coli TG1	*E. coli* K12 Δ(*lac-pro*)*supE thi hsdD5* (*F′traD36proA*$^+$*B*$^+$ *lacI*^{q} *lacZDM15*)
D. vulgaris Hildenborough NCIMB 8303	isolated from clay soil near Hildenborough, U.K. (*24*)
D. desulfuricans G201	spontaneous Nal^r derivative of *D. desulfuricans* G100A
pJ800	contains *cyc* gene of *D. vulgaris* Hildenborough on a 640-bp *Eco*RI-*Hin*dIII insert in pUC8 (*25*)
pBMK7	broad-host range vector; Km^r (*26*)
p800fdh2C	pJ800 containing the *cyc-fdh2C* gene fusion (this study)
pK800fdh2C	contains the *cyc-fdh2C* gene fusion on a 694-bp *Eco*RI-*Hin*dIII fragment insert in pBMK7 (this study)

a typical heme core arrangement (*7*). The *DvH* genome contains numerous genes encoding multihemic *c*-type cytochromes (*6*). In the present work, we have shown that the tetrahemic subunit (FDH2C) is necessary to reduce cytochrome c_{553}, the physiological redox partner of *Dv*H formate dehydrogenase (*4*). We have cloned and expressed the FDH2C subunit and investigated, by heteronuclear NMR, its role in the complex formation with *Dv*H cytochrome c_{553}.

MATERIALS AND METHODS

Strains, Vectors, and Media. The bacterial strains and plasmids used in this study are described in Table 1. Growth of *E. coli* was carried out using tryptone−yeast extract medium at 37 °C supplemented with the appropriate antibiotic (0.27 mM ampicillin or 0.17 mM kanamycin) when needed. *D. vulgaris* Hildenborough as well as wild-type and recombinant *D. desulfuricans* G201 strains was cultured in medium C (*9*) at 32 °C in an anaerobic COY chamber (10% CO_2, 5% H_2, 85% N_2) supplemented with kanamycin (0.28 mM) when required.

Cloning and Expression of the fdh2C Gene. Two phosphorylated primers, CYC32ATG (5′P-AGATACCTGGTCATCTCCCT3′) and CYC32STOP (5′P-CTATTTCTTCTCCGGACGCC3′), were designed in order to PCR-amplify, from *D. vulgaris* Hildenborough genomic DNA, the gene encoding FDH2C (DVU2809) (*6*). The resulting 432-bp DNA fragment was named *fdh2C*. Circular plasmid pJ800 containing the *cyc* gene (Table 1) was amplified by PCR with primers C3ATG (5′CATGATCGAACTACCTCC3′) and C3STOP (5′GCAGTACAGACTGACGCA3′) using the Expand high-fidelity PCR system (Roche). The resulting 2.89-kb fragment was gel-purified and ligated with the 432-bp *fdh2C* fragment to give p800fdh2C. The sequence of the resulting *cyc-fdh2C* gene fusion was verified by DNA sequencing. Plasmid p800fdh2C was digested with both *Eco*RI and *Hin*dIII, and the resulting 694-bp fragment was subcloned into pBMK7 digested with the same two enzymes. The resulting plasmid (pK800fdh2C) was used for electrotransformation of *D. desulfuricans* G201 as previously described (*10*). The recombinant *D. desulfuricans* G201 (pK800fdh2C) strain was selected using kanamycin.

Protein Purification. The cytochrome FDH2C was purified from recombinant *D. desulfuricans* G201 (pK800fdh2C) cultured in 300 L of medium C (*9*) supplemented with 0.28 mM kanamycin. Cells (300 g wet weight) were incubated for 30 min at 37 °C in 0.1 M Tris-HCl and 0.1 M EDTA (pH 9.0). After centrifugation (10000*g*, 1 h, 4 °C), the supernatant which contains the periplasmic proteins was dialyzed against 10 mM Tris-HCl buffer (pH 7.6). The periplasmic extract was then loaded onto a DEAE-52 column equilibrated with 10 mM Tris-HCl buffer (pH 7.6) and eluted using a step gradient. The FDH2C cytochrome subunit was eluted with 50 mM Tris-HCl buffer (pH 7.6) whereas the cytochrome c_3 from *D. desulfuricans* G201 was eluted with 100 mM Tris-HCl buffer (pH 7.6) as previously reported (*11*). The FDH2C-containing fraction was then loaded onto a hydroxyapatite column. The FDH2C subunit was eluted in 140 mM phosphate buffer. Purity of the protein was checked by electrophoresis on polyacrylamide gel under denaturing conditions. The purity index, defined as $(A_{red}{}^{553} - A_{red}{}^{570})/A_{ox}{}^{280}$, was 1.7.

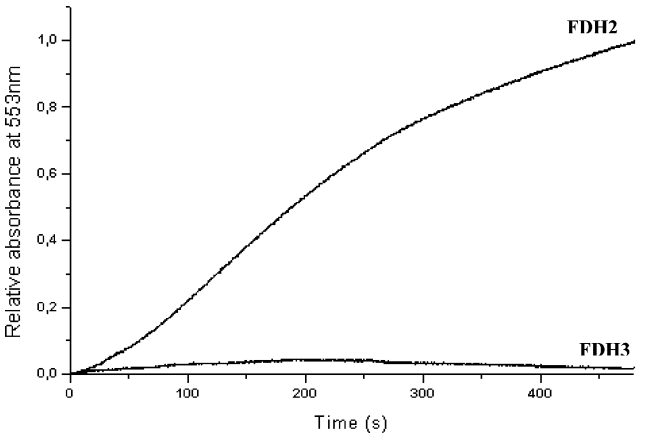

FIGURE 1: Time course of the reduction of cytochrome c_{553} by FDH2 and FDH3 from *Dv*H. The experiments were carried out at 25 °C following the variation of the absorption at 553 nm. The cytochrome c_{553} and FDH concentrations were 10 μM and 1 nM, respectively.

FDH2 and FDH3 were isolated from the *Dv*H extract using a step gradient of Tris-HCl buffer (pH 7.6) performed on a DEAE-52 column; FDH2 was eluted with 100 mM buffer and FDH3 with 400 mM buffer (*4*, *8*). Uniformly ^{15}N-labeled cytochrome c_{553} was expressed and purified from *Dd*G201(pRC41) as previously described (*12*).

Activity Assays. The time course of cytochrome c_{553} reduction by formate dehydrogenase was carried out at 25 °C on a ThermoSpectronic spectrophotometer, using absorption at 553 nm. The reaction mixture contained 10 mM glycine/NaOH buffer at pH 8.3 and 20 mM formate. The cytochrome and formate dehydrogenase concentrations were respectively 10 μM and 1 nM.

Characterization of the FDH2C Subunit. (A) Protein Sequencing. The N-terminal of the FDH2C sequence was performed by stepwise Edman degradation using an automatic Procise 494 sequanator.

(B) Redox Potential Measurements. Cyclic voltammetry (CV) experiments were carried out using an EG&G 273A potentiostat modulated by EG&G PAR M270/250 software. A three-electrode cell consisting of a Metrohm (M/m) Ag/AgCl/saturated NaCl reference electrode (at 210 mV vs normal hydrogen electrode), a gold wire auxiliary electrode, and a working pyrolytic graphite electrode was used. All experiments were done at room temperature in 10 mM potassium phosphate buffer (pH 5.9).

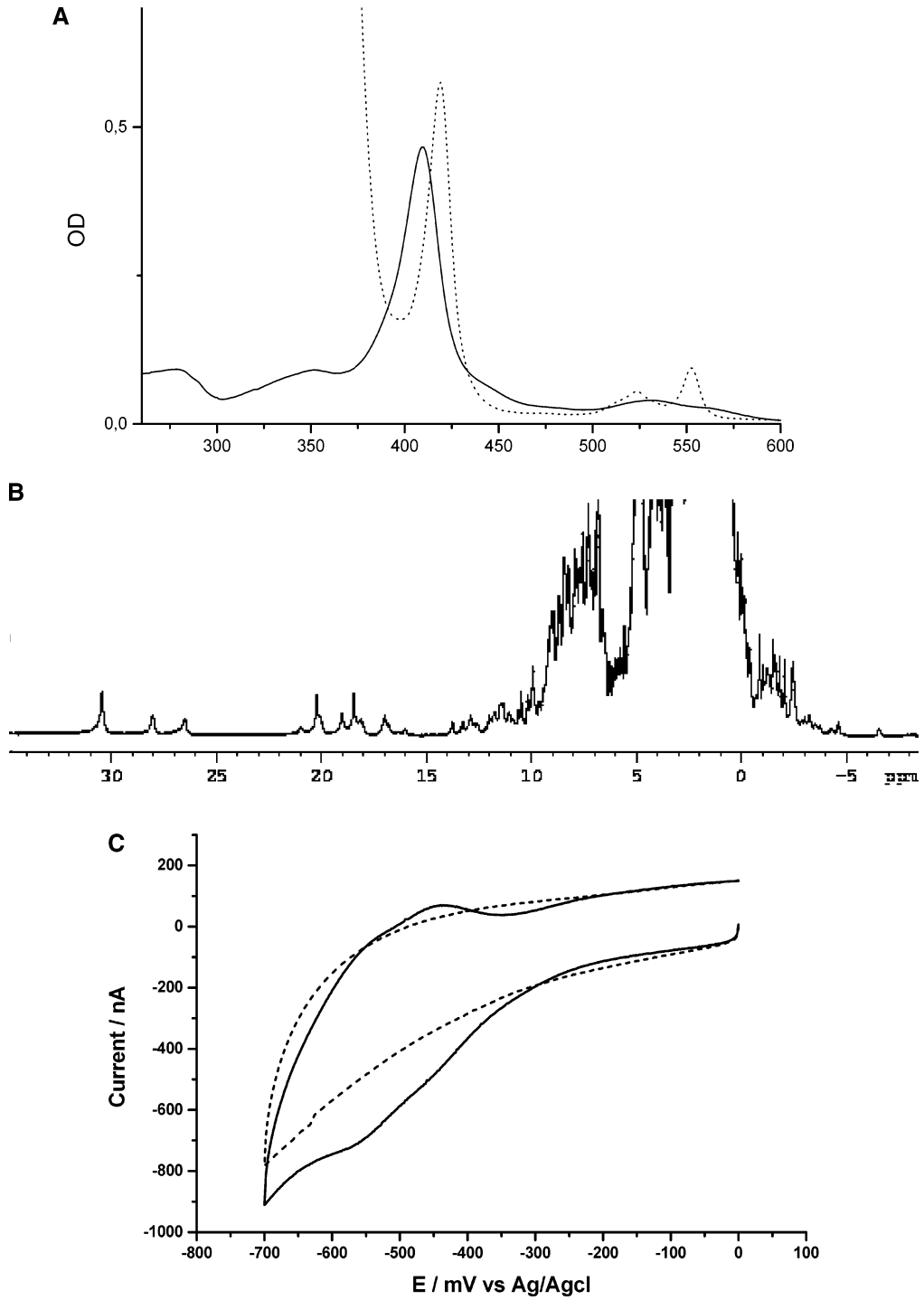

FIGURE 2: (A) UV−visible spectra of FDH2C. The oxidized form corresponds to the continuous line, and the reduced form corresponds to the dotted line. Reduction was obtained by addition of sodium dithionite. (B) 1D NMR spectrum of the FDH2C subunit at 0.4 mM concentration. (C) Cyclic voltammograms at the pyrolytic graphite electrode of the FDH2C subunit (30 μM) in 10 mM potassium phosphate buffer, pH 5.9. The dotted line corresponds to the buffer solution alone. The scan rate was 20 mV·s^{-1}.

(C) Optical Spectrum. Absorption spectra from 260 to 600 nm were recorded on a ThermoSpectronic spectrophotometer.

NMR Experiments. NMR experiments were recorded at 296 K on a Bruker Avance DRX 500 spectrometer. NMR samples were prepared in 10 mM potassium phosphate buffer (pH 7.6) and 10% D_2O. 2D NMR spectra ($^{15}N-^{1}H$ HSQC) were performed on ^{15}N-labeled cytochrome c_{553} at 14 μM concentration. The cytochrome was reduced with 2 equivalents of sodium dithionite. In the presence of an equimolar amount of FDH2, cytochrome c_{553} was reduced by adding sodium formate (50 mM). The spectral widths were 2500 Hz for ^{1}H and 2027 Hz for ^{15}N. A total of 1024 data points were used in t_2, and 64 transients were recorded for each 128 t_1.

RESULTS

Role of FDH2C in the Reduction of the Physiological Partner: Cytochrome c_{553}. In the periplasm of *D. vulgaris* Hildenborough, the FDH enzyme oxidizes formate and transfers the resulting electrons to the monohemic cytochrome c_{553} (*4*, *13*). FDH2 and FDH3 are very similar, sharing 53% and 56% identities between their α and β

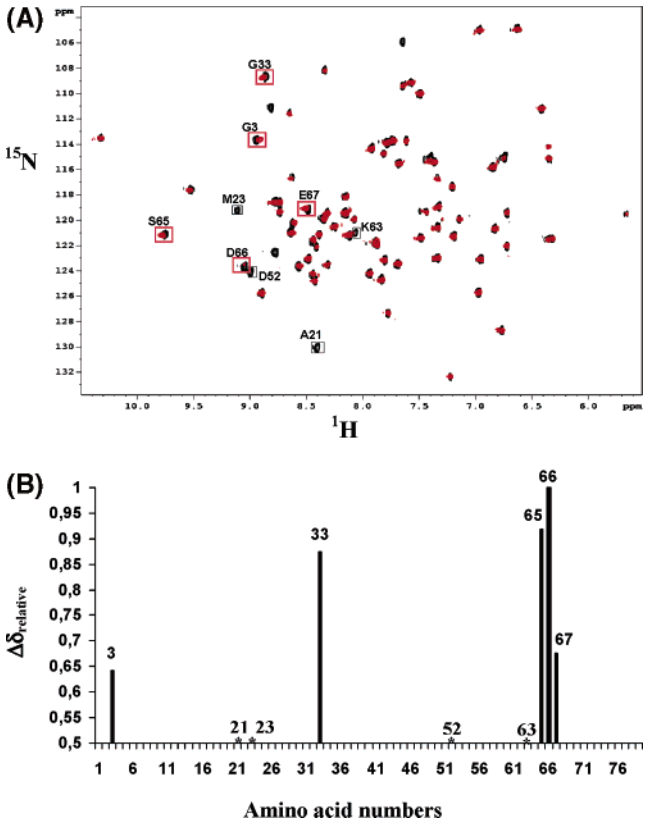

FIGURE 3: Cytochrome c_{553}/FDH2 complex formation. (A) Superimposition of HSQC spectra of ferrocytochrome c_{553} (in black) and ferrocytochrome c_{553} in the presence of 1:1 FDH2 after reduction by formate in anaerobic conditions (in red). Black boxes contain cross-peaks that disappear upon complex formation (residue numbering). Red boxes contain cross-peaks that undergo chemical shift variations during complex formation. (B) Chemical shift variations observed from panel A. The relative variations of ^{1}H and ^{15}N were calculated according to ref *27*. Stars indicate the residues which disappear during the complex formation.

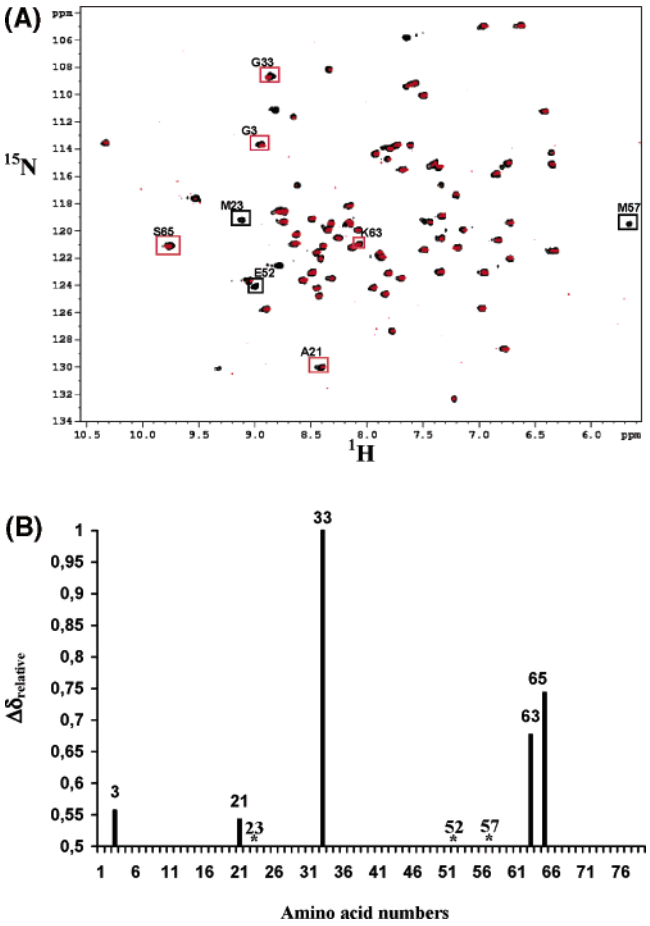

FIGURE 4: Cytochrome c_{553}/FDH2C complex formation. (A) Superimposition of HSQC spectra of ferrocytochrome c_{553} (in black) and ferrocytochrome c_{553} in the presence of 1:1 FDH2C subunit after reduction by sodium diothinite (in red). Black boxes contain cross-peaks that disappear upon complex formation (residue numbering). Red boxes contain cross-peaks that undergo chemical shift variations during complex formation. (B) Chemical shift variations observed from panel A. The relative variations of ^{1}H and ^{15}N were calculated according to ref *27*. Stars indicate the residues which disappear during the complex formation.

subunits, respectively. However, FDH3 is depleted of the γ subunit. We have tested the reduction of cytochrome c_{553} by FDH3 (Figure 1). No reduction of the monohemic cytochrome c_{553} was observed. In contrast, the cytochrome c_{553} reduction was observed in the presence of FDH2. These data suggest that FDH2C, the γ subunit of the formate dehydrogenase, is essential for a productive electron transfer to its physiological partner, the cytochrome c_{553}. To further study the role of the FDH2C subunit, we have produced the recombinant protein in *D. desulfuricans* G201 and investigated its interaction with cytochrome c_{553} using heteronuclear NMR.

FDH2C Production and Purification. The *fdh2* operon is composed of three genes encoding respectively the α, β, and γ subunits of the already characterized *Dv*H formate dehydrogenase (*4*). The γ subunit (FDH2C) encoded by the third gene (DVU2809) is expected to be a *c*-type heme-containing molecule. To produce this single subunit, which is named hereafter FDH2C, the coding region was inserted into an expression vector based on the *cyc* transcription unit from *D. vulgaris* Hildenborough (*14*). The construction was designed so that the coding region of the *cyc* gene was replaced by that of the *fdh2C* gene. The use of the *cyc* transcription unit has been already shown to be highly effective for the heterologous production of multiheme cytochromes in *D. desulfuricans* G201 (*10*).

The purification procedure described in Materials and Methods allowed us to get 3.14 mg of pure FDH2C subunit from 300 L of culture. Only one band was detected on the gels, corresponding to a relative molecular mass of 15 kDa. It is to be noted that while the heterologous production of the tetraheme cytochrome c_3 (M_r 13000) from *Dv*H in *D. desulfuricans* G201 was very efficient (*11*), the production of the FDH2C subunit is much lower. FDH2C is a part of a multimeric enzyme and may be less stable when it is produced as recombinant single protein.

Characterization of the Recombinant FDH2C Subunit. The NH_2-terminal sequence of the recombinant FDH2C subunit was found to be ADAAKA. According to the translated gene sequence of DVU2809 (*6*), the signal sequence is correctly cleaved in *D. desulfuricans* G201. The UV−visible spectrum of the FDH2C subunit presents different absorption bands, characteristics of *c*-type heme-containing proteins (Figure 2A). The oxidized state of the protein shows two absorption maxima at 409 nm (Soret) and 530 nm. Upon reduction by sodium dithionite, the Soret band intensifies and shifts to 418 nm, while the α and β bands appear at 552 and 523 nm, respectively. It should be noticed that the same absorp-

tion bands were found in the UV−visible spectrum of native FDH2 (*4*), indicating that the hemes are correctly inserted in the recombinant subunit. Moreover, in the low-field part of the 1D NMR spectrum of the FDH2C subunit, well-resolved resonances are assigned to the 16 heme methyl lines (Figure 2B). Finally, the shape of the forward and backward traces of cyclic voltammetry curves (Figure 2C) indicates a reversible electrochemical process corresponding to the reduction−reoxidation of the hemic centers. The plots for the native FDH2 and the FDH2C subunit are similar, because iron−sulfur clusters are not electroactive in these conditions as observed for other (FeS) enzymes such as hydrogenases (*15*). The deduced macroscopic oxidoreduction potentials of the four hemes are centered at −220 mV. The values obtained for the recombinant tetrahemic subunit and the native enzyme were quite similar, with a small shift toward lower potential values for the recombinant cytochrome. These data indicate that the α and *β* subunits do not have any drastic effect on the modulation of the heme redox potentials of the FDH2C subunit.

NMR Mapping of FDH2 and FDH2C Interacting Sites. $^{1}H-^{15}N$ HSQC experiments correspond to the fingerprint of the protein where each cross-peak represents the amide group of an amino acid. Complex formation induces ^{15}N or/and ^{1}H chemical shifts of the residues located at the interacting interface. In the NMR spectrum of *Dv*H cytochrome c_{553} already assigned (*12*), nine resonances are affected by the presence of FDH2 (Figure 3). Five resonances (G3, G33, S65, D66, and E67) shift from their position in the free protein to a position corresponding to the bound form, indicating a fast exchange between the two states, at the NMR time scale. The line width of all the resonances increases notably with the size of the binary complex, but due to the fast exchange most of the resonances are still observable. However, four resonances (A21, M23, E52, and K63) shifting at low concentrations disappear with increasing concentrations of FDH2. In the presence of the FDH2C subunit, only eight resonances are affected by the complex formation (Figure 4). The resonances of residues G3, A21, G33, K63, and S65 shift, and three resonances (M23, E52, and M57) disappear upon complex formation. Comparison of the interacting sites (Figure 5) reveals that ~80% of the affected residues on cytochrome c_{553} are common to both complexes. This result indicates that the tetrahemic subunit of FDH2 is the cytochrome c_{553} interacting site and thus corresponds to the electron gate of the FDH2 enzyme.

DISCUSSION

The genome sequence of *D. vulgaris* Hildenborough shows the presence of three gene clusters encoding formate dehydrogenases (*6*). Two of these enzymes contain a multihemic subunit. Multimeric formate dehydrogenases are common in bacteria; some of them, like FDH-N from *E. coli* (*2*), contain membrane-bound *b*-type cytochromes which transfer electrons to quinones. However, the presence of low redox potential *c*-type hemes in formate dehydrogenases is specific to sulfate-reducing bacteria. In the present work, we have established that this hemic subunit, FDH2C, is required to reduce the physiological partner, cytochrome c_{553}. To understand the role of this tetrahemic subunit in *Dv*H formate dehydrogenase, we have produced the FDH2C subunit in *D. desulfuricans* G201. Using 2D NMR mapping on cytochrome c_{553}, we have demonstrated that the interacting sites of the native FDH2 and the recombinant FDH2C are similar (Figure 5) and conclude that FDH2C is the electron gate of the enzyme. The presence of two additional cytochrome c_{553} acidic residues (D66 and E67) in the complex with FDH2 (Figure 5) is probably due to the necessity of a larger

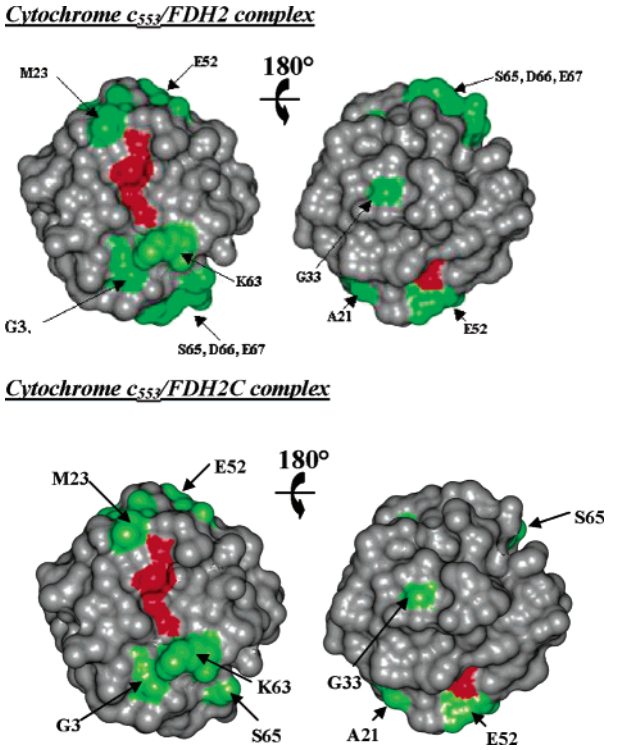

FIGURE 5: Mapping of the FDH2 interacting site (top) and the FDH2C subunit interacting site (bottom) on *Dv*H cytochrome c_{553}. The heme surface is colored in red, and affected residues in the NMR spectra are shown in green.

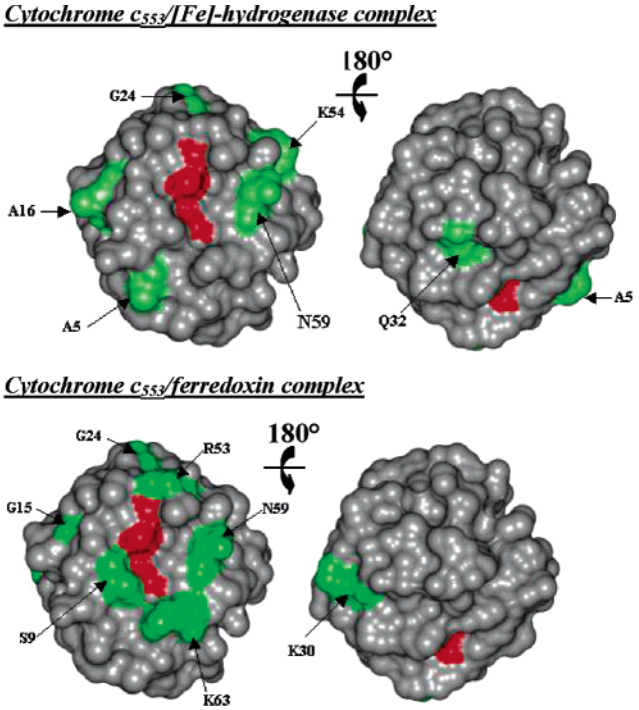

FIGURE 6: Mapping of the [Fe]-hydrogenase interacting site (top) and the ferredoxin interacting site (bottom) on *Dv*H cytochrome c_{553} from refs *18* and *19*. The heme surface is colored in red, and affected residues in the NMR spectra are shown in green.

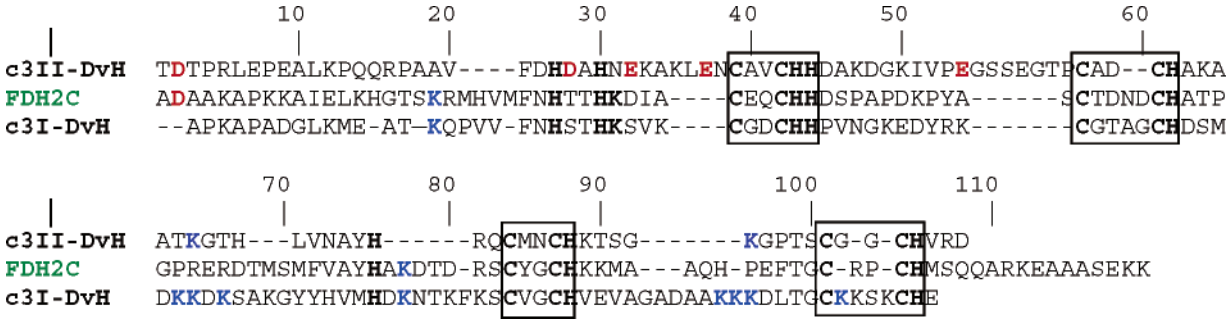

FIGURE 7: Sequence alignment of *D. vulgaris* Hildenborough cytochromes c_3: FDH2C, multihemic subunit of *Dv*H FDH2 formate dehydrogenase; c3I-DvH, type I cytochrome c_3; c3II-DvH, type II cytochrome c_3. Heme binding sites are in black boxes, lysine residues conserved in the type I cytochrome c_3 family are colored in blue, and acidic residues conserved in the type II cytochrome c_3 family are colored in red.

interacting surface with the whole enzyme (124.5 kDa) compared to the recombinant subunit (15 kDa). One can expect that at least one of the two other subunits of the native formate dehydrogenase is involved in the molecular interface.

It has been previously shown by enzymatic analysis that replacement of the K63 residue by a glutamate residue had a drastic effect on the FDH2/cytochrome c_{553} complex formation (*16*). K63 is perturbed in the 2D NMR spectra for both complexes (Figures 3 and 4). This result confirms that K63 is at the interacting surface of the two complexes and plays an important role for the FDH2/cytochrome c_{553} complex formation. Y64 has also been shown to be essential for the electron transfer between FDH2 and cytochrome c_{553} (*17*). The role of this aromatic residue is not yet well understood, but in all of the structural models of electron transfer complexes involving cytochrome c_{553}, Y64 is found at the electron transfer site (*18*, *19*). It is to be noticed that the Y64 NH group is not affected by the presence of FDH2 and FDH2C. However, the resonances of the neighbor residues are very much altered, which is consistent with an involvement of Y64 in the electron transfer process in these two complexes. Figure 6 represents the cytochrome c_{553} interacting surfaces for complexes involving ferredoxin and [Fe]-hydrogenase (*18*, *19*). Most of the residues involved in these complexes are found in the FDH2 complex. However, three additional residues (S65, D66, and E67) are only found at the interface of the complex with FDH2 (Figure 5), which reveals the differences of surface complementarities between cytochrome c_{553} and its physiological partners.

The cytochrome c_3 family is a large class of multihemic soluble cytochromes. Two types of tetrahemic cytochromes c_3 have been characterized, namely, type I and type II cytochromes c_3. Type I cytochrome c_3 acts as an electron shuttle in the periplasm between the cytochrome Hmc and the [Fe]-hydrogenase (*20*, *21*). In these two electron transfer complexes, heme 4 of cytochrome c_3 is the reactive heme bordered by conserved lysine residues. Type II cytochromes c_3 are acidic soluble cytochromes associated to membrane-bound electron transfer complexes (*22*). The three-dimensional structure of the type II cytochrome c_3 from *Desulfovibrio africanus* shows that conserved acidic residues are found around the putative reactive heme 1 (*23*). The tetrahemic subunit of FDH2 contains some of the conserved lysine residues found in the type I cytochromes c_3 and some of the conserved acidic residues characteristic of the type II cytochromes c_3 (Figure 7). Thus, the FDH2C subunit does not belong to any of these two families, and one can propose that it is representative of a new type of cytochrome c_3, namely, type III cytochrome c_3. A survey of the *DvH* genome shows that another member of this family is found associated to the membrane-bound [NiFe]-hydrogenase isoenzyme 2 (DVU2524) (*6*). We suggest that type III cytochromes c_3 constitute a new group of heme *c*-containing proteins corresponding to multihemic subunits as part of multimeric metalloenzymes.

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