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Isolation and characterization of a high molecular weight cytochrome from the sulfate reducing bacterium *Desulfovibrio gigas*

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Abstract

A high molecular weight *c*-type cytochrome (Hmc) was purified and characterized from *Desulfovibrio gigas*. The molecular weight was estimated to be 67 kDa by SDS-PAGE and its N-terminus is homologous to those of the 16 hemes containing high molecular weight cytochrome *c* from *Desulfovibrio vulgaris* strains Hildenborough and Miyazaki. The purified hemoprotein shows *c*-type cytochrome absorption spectrum with ε_{553}(red) = 368 $mM^{-1} \cdot cm^{-1}$. A band at 640 nm, characteristic of high-spin hemes, was detected. The EPR spectra show the presence of two high-spin heme species, plus several non-equivalent low-spin hemes. The heme reduction potentials, at pH 7.6, range from −50 mV to −315 mV. In contrast to what has been described for *D. vulgaris* Hmc, the protein isolated from *D. gigas* directly accepts electrons from hydrogenase and further reduces other redox proteins.

Key words: Cytochrome *c*; Multiheme cytochrome; *Desulfovibrio gigas*; N-Terminal sequence; Electron transfer; Electron paramagnetic resonance

1. Introduction

Bacteria belonging to the *Desulfovibrio* genus are sulfate-reducers that can use sulfate or sulfite as respiratory substrates in the presence of various carbon sources. Several types of cytochromes of the *b* or *c* types have been isolated from them. *D. gigas* contains at least five different cytochromes: cytochrome *b* [1], tetraheme cytochrome c_3 [2], octaheme cytochrome c_3 [3], a partially identified membrane-bound cytochrome *c* [4], and a new type of flavohemo protein, a rubredoxin-oxygen oxidoreductase [5].

A high-molecular weight cytochrome *c* (Hmc), containing 16 hemes per molecule was purified and characterized from *D. vulgaris* strains Hildenborough [6–9] and Miyazaki [10,11]. Although Pollock et al. [6] failed to detect any cross-reaction in *D. gigas* with a DNA probe of *D. vulgaris* Hildenborough Hmc, we show in this report that a similar hemoprotein is present in *D. gigas* and describe its purification and characterization.

2. Materials and methods

2.1. Purification of Hmc

D. gigas (ATCC 19364) cells and soluble extract were obtained as in [12]. All purification procedures were performed at pH 7.6 and 4°C. The purity of Hmc was determined by the ratio of A_{553}(red)/A_{280}(ox) and SDS-PAGE analysis. The dialyzed soluble extract from 1 kg of *D. gigas* frozen cells was loaded onto a DEAE-cellulose column (10 × 40 cm) equilibrated with 10 mM Tris-HCl buffer (buffer I). After loading, the column was washed with 1 l buffer I. A linear Tris-HCl buffer gradient (0.01–0.5 M) of a total volume of 8 l was applied. A fraction containing cytochromes was collected at 0.15 M, which was then applied to an hydroxyapatite column (4.5 × 30 cm) equilibrated with buffer I. An ascending linear gradient (0.001–0.15 M) of potassium phosphate buffer (pH 7.6) was applied with a total volume of 2 l. The eluted Hmc (at ~30 mM phosphate buffer) was dialyzed against water overnight and then loaded onto a DEAE-Biogel column (4 × 30 cm) equilibrated with buffer I. Hmc was eluted at 60–80 mM after application of a linear gradient of Tris-HCl buffer (0.01–0.2 M) with a total volume of 1.5 l. This partially purified Hmc was subjected to an electroelution procedure. The partially purified Hmc was loaded on a 7.5% PAGE gel and submitted to electrophoresis. The red Hmc band was then cut and loaded on to an electroelution chamber (V = 100 mV) and eluted with 50 mM NH_4HCO_3 overnight. After this step, the purified Hmc had an A_{553}(red)/A_{280}(ox) ratio of 2.1. The yield of Hmc is 10 mg/kg frozen cells.

2.2. Analytical methods

The protein concentration of Hmc was measured by the Lowry assay [13] with pure *D. gigas* tetraheme cytochrome c_3 as a standard. The molecular mass of Hmc was determined by 12.5% SDS-PAGE [14]. The heme content was determined from pyridine-ferrohemochrome spectrum, using the extinction coefficient of 29.1 $mM^{-1} \cdot cm^{-1}$ at 550 nm from horse heart cytochrome *c* [15]. Total iron was measured by plasma emission spectroscopy using a Jarrell-Ash Model 750 atom comp spectrometer. UV/visible spectra were obtained using a Shimadzu spectrophotometer (UV 265). N-Terminal sequence was determined by the method of Edman and Begg [16] with an Applied Biosystem sequenator (Model 470A). EPR spectra were obtained as in [17]. Parallel-mode spectra were obtained with a Bruker ER 4116 Dual Mode cavity. Visible redox titrations were performed as described in [18].

3. Results and discussion

3.1. Chemical characterization

The purified Hmc shows a single band (67 kDa) on 12.5% SDS-PAGE. Although the pyridine-ferrohemo-

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Abbreviations: DEAE, diethyl aminoethyl; Hmc, high molecular weight *c*-type cytochrome; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; Tris, tris (hydroxymethyl) aminomethane.

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                    1     5        10       15       20       25       30
                    |     |        |        |        |        |        |
D.v.Hmc             K-A-L-P-E-G-P-G-E-K-R-A-D-L-I-E-I-G-A-M-E-R-F-G-K-L-D-L-P-K-V-A-F-R-

D.g.Hmc           A-T-P-G-P-A-S-T-A-E-P-K-V-I-L-I-V-G-E-L-A-V-G-F-G-K-L-E-M-P-F-V-M-F-D

D.v.L-peptide         P-L-P-G-A-T-G-E-Q-R-A-D-L-V-E-I-G-V-M-A-K-F-T-N-L-E-L-P-K-V
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Fig. 1. Alignment of N-terminal sequences of Hmc isolated from *D. gigas* and *D. vulgaris* strains Hildenborough [6] and Miyazaki [11]. *D.v.* Hmc, *D. vulgaris* Hildenborough Hmc; *D.g.* Hmc, *D. gigas* Hmc; *D.v.* L-peptide, *D. vulgaris* Miyazaki L-peptide of Hmc. A colon indicates two aligned residues are identical; a dot indicates that two aligned residues are similar. The alignment was performed using PC/Gene software package, version 6.7 [29].

chrome spectrum indicated that *D. gigas* Hmc contains 16 hemes of the *c*-type, only 13.4 atoms of iron per molecule were found by plasma emission. The lower iron content of *D. gigas* Hmc measured by plasma emission could have resulted from technical inaccuracy; however, a definitive heme content will only be established after full amino acid sequencing. It is to be noted that *D. vulgaris* Miyazaki Hmc was reported to have 11 hemes [10], a value that was suggested to be due to its high oxygen sensitivity [8]. The N-terminal sequence of *D. gigas* Hmc (Fig. 1) has 31.4% identity plus 8.6% similarity to *D. vulgaris* Hildenborough Hmc, and 28.6% identity plus 14.3% similarity to *D. vulgaris* Miyazaki Hmc L-peptide. *D. vulgaris* Hildenborough Hmc was suggested to be a periplasmic protein because a signal peptide was found in the sequence of the gene [6]. In contrast, the same Hmc was isolated from *D. vulgaris* Hildenborough cytoplasmic fractions (our unpublished result).

3.2. Electronic spectra

The UV/visible spectrum of oxidized Hmc shows peaks at 280 nm, 409 nm (Soret, $\varepsilon = 1616\ mM^{-1} \cdot cm^{-1}$), 529 nm and ~640 nm (Fig. 2), indicating the presence of high- and low-spin hemes. In the reduced form the Soret band shifts to 419 nm ($\varepsilon = 2470\ mM^{-1} \cdot cm^{-1}$) and the α and β peaks appear at 523 ($\varepsilon = 201\ mM^{-1} \cdot cm^{-1}$) and 553 nm ($\varepsilon = 368\ mM^{-1} \cdot cm^{-1}$), respectively (Fig. 2). A shoulder at ~429 nm, typical of high-spin ferrohemes [19], is also observed upon reduction. Even in highly concentrated samples (up to 100 mM), no peak is observed at the wavelength of 695 nm, characteristic of methionine bound hemes [19]. By using the usual molar absortivities for high- and low-spin ferri-hemes [19], the contribution at 640 nm accounts for aproximately 10% of the the total heme content. The extinction coefficient of reduced *D. gigas* Hmc at 553 nm is approximately 4 times that of tetraheme cytochrome c_3 (ε_{553}(red) = $109\ mM^{-1} \cdot cm^{-1}$) from the same organism, indicating that the new protein may contain 16 hemes. *D. gigas* Hmc has a very low absorption at 280 nm, in agreement with its high heme content and suggesting that relatively few aromatic amino acid residues such as tryptophan are present.

3.3. EPR spectroscopy

The EPR spectra of oxidized *D. gigas* Hmc (Fig. 3) show clearly two high-spin components, with g_{max} at 6.54 and 6.1, corresponding to species with slightly different rhombic distortions (E/D ~0.02 and ~0.005, respectively, see Fig. 3D,E). These two species are present in a 1:1 ratio in the pH range 6–11. Several low-spin components are also observed, with g_{max} values spreading from 3.45 (particularly well observed in the low temperature spectrum, Fig. 3A) to 2.93. Since a complete deconvolution of each species is not possible at present, the low-spin components were simulated in order to obtain an overall representation of the total spectral envelop, which could be used for the spectral integration (see below and Fig.

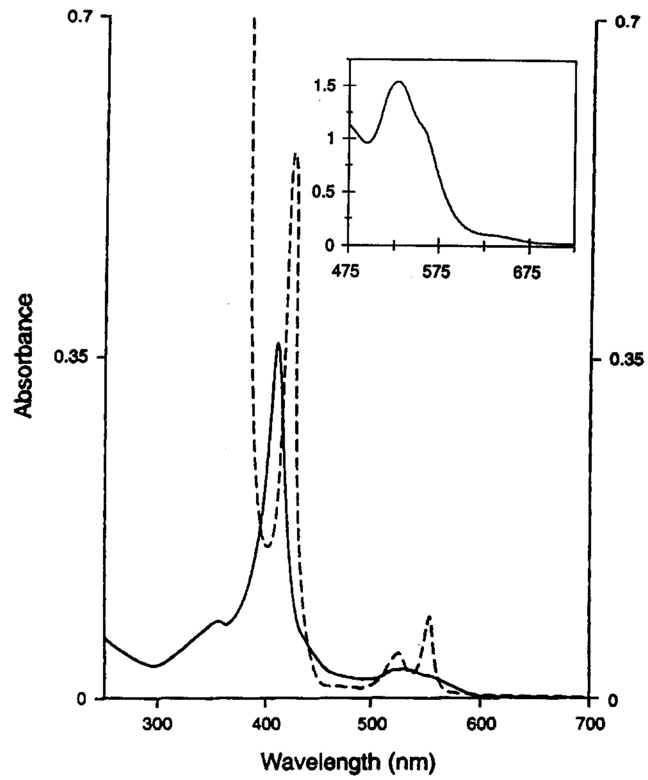

Fig. 2. The UV/visible spectra of *D. gigas* Hmc. Solid line, 0.24 μM oxidized Hmc. Dash line, 0.24 μM Hmc reduced by dithionite. Inserted curve, 12 μM oxidized Hmc in 100 mM Tris buffer (pH 7.6).

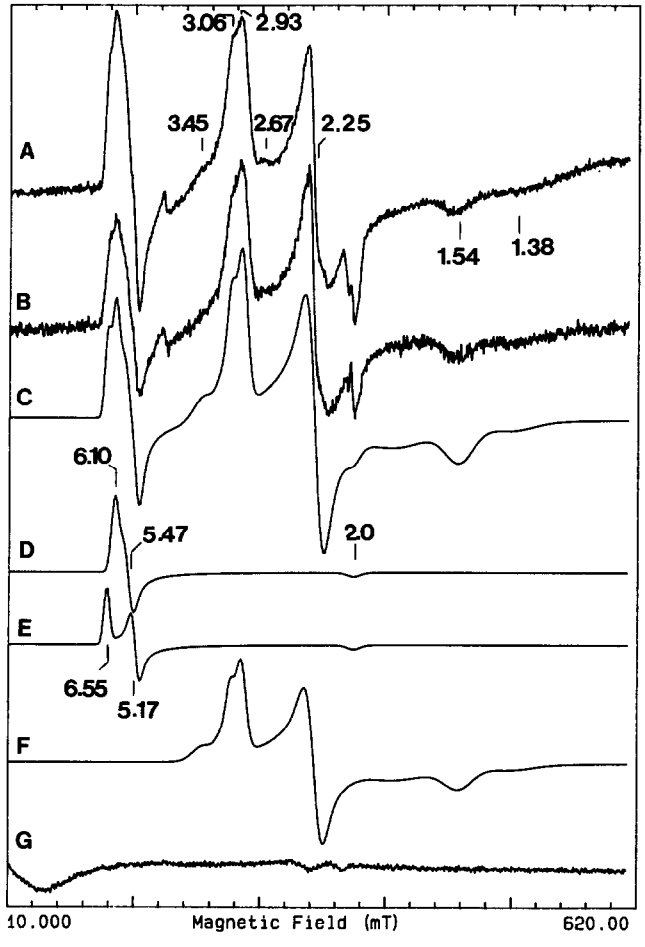

Fig. 3. EPR spectra of *D. gigas* Hmc, oxidized (A,B) and H_2/hydrogenase reduced (G). The full lines correspond to the theoretical simulations, generated using the *g*-values presented below, for the total spectrum (C), the high-spin species (D,E) and the low-spin species (F). Temperature, 4.2 K (A,G) and 19 K (B). Modulation amplitude, 0.9 mT; Microwave power, 2.4 mW; Microwave frequency, 9.63 GHz (A,B) and 9.38 GHz (G, parallel mode spectrum). The following *g*-values were used for the simulation: (i) for the high-spin species, 6.55, 5.17, 2.0 and 6.10, 5.47, 2.0; for the low-spin species, 2.93, 2.26, 1.54 (I), 3.06, 2.25,1.38 (II), 3.16, 2.23,1.0 (III) and 3.45, 1.90, 0.7 (IV). The simulations were added in the ratio 1:1:5:2.5:2.5:4 (see text).

3). The crystal field parameters for the species I–III are within the range found for hemes with a bis-histidine or histidine-methionine axial coordination [17,20] with a rhombic crystal field symmetry. The components with g_{max} = 3.45 have an almost axial ligand field, which, for bis-histidine ligation, indicates an almost perpendicularity of the imidazole planes. In some samples, a species with g_{max} at ~2.67 is observed, accounting for less than 0.2% of the total low-spin heme intensity. This species, also observed in *D. vulgaris* Hmc [8], has a g_{max} value in the range of hydroxide bound hemes [20], but does not increase in intensity upon raising the pH. The proportion of high- to low-spin hemes was determined by using the theoretical simulations of both types of species, adjusted to the experimental spectrum obtained under non-saturating conditions (Fig. 3B). Assuming a zero-field splitting of 10 cm^{-1} for the high-spin hemes [21], the percentage of high- to low-spin hemes was calculated to be approximately 13, which suggests the presence of two high-spin hemes. The overall spectrum of *D. gigas* Hmc is similar to that of *D. vulgaris* Hildenborough Hmc: a closer look at the EPR spectrum of this last protein [8] shows the presence of two distinct high-spin species, apparently identical to those observed for the *D. gigas* protein. The authors referred to this species but did not include it in the simulation, leading to an underestimation of the total high-spin heme content.

D. gigas [NiFe]-hydrogenase can reduce all the Hmc hemes. The EPR spectra of reduced Hmc show only a broad signal at very low magnetic field, particularly well observed using paralell mode EPR (Fig. 3G), indicating the presence of high-spin ferro-hemes [22].

3.4. Redox potentials

The reduction potentials for *D. gigas* Hmc at pH 7.6 were estimated by redox titrations, measuring visible absorbance changes at 419, 429 and 553 nm as a function of the solution redox potential (Fig. 4). The experimental curves were adjusted with 4 Nernst equations, with reduction potentials of −80, −215 −255 and −315 mV, added in the ratio 4:4:4:2. The high-spin heme reduction potentials were estimated to be of about −50 mV, from the increase in absorbance at 429 nm (data not shown).

3.5. Electron transfer properties

It was reported that *D. vulgaris* Miyazaki Hmc could not be reduced by H_2/hydrogenase unless in the presence of cytochrome c_3 [10]. As shown above, this is not the case for *D. gigas* Hmc (see Fig. 3G). Furthermore, under H_2, the hydrogenase/Hmc mixture can induce the rapid reduction of *D. gigas* rubredoxin, which in turn fully reduces *D. gigas* rubredoxin-oxygen oxidoreductase [5] (data not shown). This last reaction is not possible with the tetraheme cytochrome c_3.

4. Conclusions

The analysis of the *D. vulgaris* Hildenborough Hmc protein sequence deduced from molecular cloning [6] suggested that the heme binding sites and axial ligands are arranged as three complete and one incomplete tetraheme cytochrome c_3-like domains. Actually, the array of the heme binding sites and potential sixth ligand His residues for this so-called incomplete domain is homologous to that of *Desulforomonas acetoxidans* triheme cytochrome $c_{551.5}$ (also known as cytochrome c_7) [23]. Since there is at least one high-spin heme in both redox states of *D. gigas* Hmc and no spectroscopic evidence for a

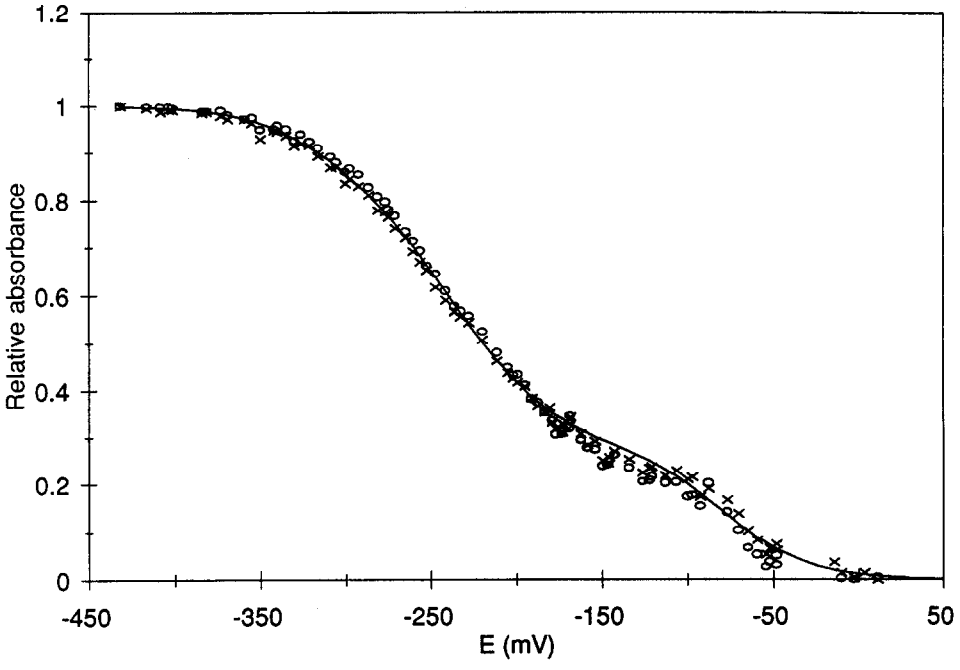

Fig. 4. Redox titration curve of *D. gigas* Hmc, obtained by following the absorption changes at 553 nm (o) and 419 nm (x). The solid line was obtained as described in the text.

methionine-bound heme was found, there is no need to invoke this type of axial ligation [6,8]. Thus, the spectroscopic and chemical data of *D. gigas* Hmc, and the sequence studies of the homologous protein from *D. vulgaris* taken together point to the presence of 14–15 bis-histidinyl coordinated low-spin hemes and 1–2 high-spin hemes in *D. gigas* Hmc. Rossi et al. [9] have suggested that each of the Hmc domains interacts with one of the three hydrogenases found in *D. vulgaris* [24,25]. However, in the same paper, the authors present a scheme in which the [Fe]-hydrogenase of *D. vulgaris* is presented as the only hydrogenase reacting with Hmc, which is proposed to be part of a transmembrane electron transfer complex. As far as *D. gigas* Hmc is concerned, such schemes do not seem to apply since this strain is known to contain only one hydrogenase [26]. However, the possibility still exists that other hydrogenases different from the classical [Fe]-, [NiFe]- and [NiFeSe]-containing enzymes have remained undetected. The electron transfer properties of *D. gigas* Hmc show that, like the tetraheme cytochrome c_3, it can function as an electron acceptor of hydrogenase [27] or as an electron donor to rubredoxin, but that it may play a physiological role other than in *D. vulgaris* because the latter organism does not seem to contain an oxygen-linked rubredoxin-oxygen oxidoreductase (see above).

Our finding that the N-terminus of *D. gigas* Hmc is somewhat different from those of the *D. vulgaris* Hmc's can explain the results of Pollock et al. [6], who failed to detect any cross-reaction in *D. gigas* with a DNA probe of *D. vulgaris* Hildenborough Hmc. As noted by Voordouw [28], this negative result is not a proof that a similar hemoprotein is absent in *D. gigas*.

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