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Characterization of the Cytochromes C from *Desulfovibrio desulfuricans* G201

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A monoheme cytochrome c_{553} and a hexadecaheme high molecular weight cytochrome (Hmc) have been isolated and characterized from the sulfate-reducing bacteria *Desulfovibrio desulfuricans* G201, in addition to the tetraheme cytochrome c_3 (Mr 13000) that has been previously described. Both cytochromes are homologous with respect to several biochemical properties to the corresponding cytochromes found in other *Desulfovibrio* species. However, they are acidic proteins while the corresponding molecules, isolated from other *Desulfovibrio* species, are relatively more basic. The *D. desulfuricans* cytochrome content appears identical to that of *D. vulgaris* Hildenborough. Isolation of these cytochromes from a *Desulfovibrio desulfuricans* strain is of great interest in order to get more insight on the physiological function of these molecules.

Sulfate reducing bacteria of the genus *Desulfovibrio* are strict anaerobes which derive energy from the dissimilatory reduction of sulfate by hydrogen or organic substrates. The electron transport chain catalyzing this reaction involves periplasmic hydrogenases, several cytochromes and other both membrane bound and cytoplasmic redox enzymes, some of which have been extensively studied (1). In particular, several various soluble c-type cytochromes have been described in terms of structural and physicochemical properties as well as genetic organization. The tetraheme cytochrome c_3 (Mr 13000) (2-5), the octaheme cytochrome c_3 (Mr 26000) (6-8) and the hexadecaheme high molecular weight cytochrome (Hmc) (9-10) are all multihemic cytochromes characterized by a negative redox potential and a His/His coordination of the heme iron atoms. They show no structural similarity to other cytochromes c classes, and belong to class III of the c-type cytochromes as defined by Ambler (11). The structural analysis of these cytochromes suggests that they can all be classified in the cytochrome c_3 superfamily for which, the basic unit would be of cytochrome c_3 (Mr 13000) type (12). Both cytochrome c_3 (Mr 13000) and cytochrome c_3 (Mr 26000) have been proposed to interact with the hydrogenases in the sulfate reducing pathway (13-14). Genetic data on Hmc have allowed to postulate a function of this cytochrome in the coupling between the periplasmic hydrogen oxidation by hydrogenases and the cytoplasmic sulfate reduction (15). The cytochrome c_{553} is distinguished from the others in that it has the lowest molecular weight (Mr 9000), a single heme group with a His-Met coordinated heme iron and a redox potential of +20 m V (16-18). It is expected to have a function in the formate reduction pathway (19). However, the presence of a cytochrome c oxidase-like gene in the same operon as the *D. vulgaris* Miyazaki cytochrome c_{553} gene suggests a possible other function (20). A "split soret" cytochrome has been isolated only from the sulfate- and nitrate-reducing bacterium *D. desulfuricans* ATCC27774. It is an homodimer that binds two heme c groups per subunit with a bis-histidinyl iron axial coordination. The function of this cytochrome, which has neither nitrate nor nitrite reduction activities, is unknown (21).

Although cytochrome c_3 (Mr 13000) is a periplasmic protein uniformly present in *Desulfovibrio,* the content of the other cytochromes seems variable, depending on the *Desulfovibrio* species considered. The monoheme cytochrome c_{553} has been described only in *D. vulgaris* Hildenborough (16), Miyazaki (22) and in *D. desulfuricans* Norway (17). The high molecular mass cytochrome (Hmc) was isolated from *D. vulgaris* Hildenborough (9-10) and Miyazaki (23) and, more recently, from *D. gigas* (24-25). The octaheme cytochrome c_3 (Mr 26000) has been purified and characterized from only three different species, *Desulfovibrio desulfuricans* Norway (6, 14), *Desulfovibrio gigas* (26) and more recently *Desulfovibrio africanus* (27).

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In order to improve the knowledge and the comparison of these different classes of cytochrome c, we describe and characterize in the present paper the cytochrome content of an other *Desulfovibrio* species, *Desulfovibrio desulfuricans* G201.

MATERIALS AND METHODS

Growth conditions. *Desulfovibrio desulfuricans* G201 was grown in Postgate medium C (28). Cells were cultured at 37°C in a 200-L fermenter, starting with a 40-L inoculum of fresh culture (Chemap), for 48 hours.

Optical absorption spectra. Visible and ultraviolet absorption spectra of the proteins were determined with a Beckman DU 7500 spectrophotometer. Molar extinction coefficients at the absorption maxima were obtained from these spectra using protein concentrations based on amino acid analysis.

Isoelectric point measurements. Isoelectric points were determined by performing isoelectric focusing using both a Phast Gel apparatus from Pharmacia LKB Biotechnology and a Multiphor II system from Pharmacia. Phast Gel IEF 3-9, which operates in the 3-9 pH range, and ampholine polyacrylamide gel plates from Pharmacia (pH range 3.5-9.5) were used together with a Pharmacia broad-range pI calibration kit containing proteins with various isoelectric points ranging from 3 to 10.

Molecular mass determination. The molecular mass of the protein was determined by performing sodium dodecyl sulfate polyacrylamide gel electrophoresis under denaturing conditions on a Pharmacia Phast System with Phast Gel 12 % polyacrylamide and Phast Gel SDS buffer strips. Mass Spectrometry analyses were carried out on a Perkin-Elmer Sciex API III+ triple quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray (ionspray) source. The spectra were recorded in the 1000-2000 range of mass-to-charge (m/z) ratios in steps of 0.5 m/z with a 2ms dwell time. The signal was averaged over 6 scans. The ionspray probe tip was held at 5 kV. Samples were infused into the source using a Harvard 22 syringe pump at a flow rate of 5 μl/min and a Valco C6W injector equipped with a 1 μl internal loop. The reconstructed molecular mass profiles were obtained by using a deconvolution algorithm (Perkin-Elmer Sciex).

Analysis for iron and heme content. The iron content was determined by performing plasma emission spectroscopy using a Jobin Yvon model JY 38 apparatus.

The total number of heme units was determined by means of the pyridine ferrohemochromogen test. A known mass of the protein (determined by hydrolysis of an aliquot of protein solution and performing quantitative amino acid analysis) was added to an aqueous alkaline (7.5 mM NaOH / 25 %) pyridine solution and reduced by adding a few crystals of sodium dithionite. The heme content was determined from the pyridine ferrohemochrome spectrum, using the millimolar absorbance coefficient of 29.1 M^{-1} cm^{-1} at 553 nm of the cytochrome derivative (29).

Electrochemical techniques. Cyclic voltammetry (CV) and square-wave voltammetry (SWV) were carried out using an EG&G 273A potentiostat modulated with EG&G PARC M270/250 software. The CV and SWV scan rates were 5mV s^{-1} and the SWV curves (difference current i_f-i_r) were obtained using a SW frequency of 2.5Hz, a scan increment of 2mV and a pulse height amplitude of 25mV. A three-electrode cell consisting of a Metrohm Ag/AgCl (saturated NaCl) reference electrode (with a potential of 0.21V *versus* NHE), a platinum wire auxiliary electrode and a pyrolytic graphite electrode were used throughout. Before the experiments began, the working electrode was polished with ultrafine emery paper, then with 0.05μm alumina slurry, and washed with distilled water. Oxygen was purged from solutions by bubbling with high-purity nitrogen. The individual reduction-reoxidation potentials of the hemes in the cytochrome were determined using the EG&G PARC COOL M271 kinetic analysis software.

Amino acid analysis and protein sequencing. For the amino acid analysis, protein samples were hydrolyzed in 200 μl of 6 M HCl at 110°C for 18, 24, and 72 h, in sealed evacuated tubes, and analyzed with a Beckman amino acid analyzer (System 6300). The hemes were removed using Ambler's method (30) and the resulting apoprotein was isolated by gel filtration on Sephadex G 25 in 5 % (by vol.) formic acid. Sequence determinations were carried out with an Applied Biosystems A470 gas-phase sequenator. Quantitative determination of phenylthiohydantoin derivatives was performed using high-pressure liquid chromatographic procedures (Waters Associates, Inc.), monitored with a data and chromatography control station (Waters 840).

S-carboxymethylated protein was prepared by dissolving the apoprotein in 0.5 M Tris-HCl pH 9.0, 8 M urea and 20 mM EDTA, and performing an iodoacetic acid treatement, as described by Crestfield *et al*, 1963 (31).

Western blot analysis. Following the electrophoresis on 12% polyacrylamide gel under denaturing conditions, the proteins were transferred to nitrocellulose membrane using a Semi-dry Biometra Fast blot apparatus for 15 min at a current intensity of 1 mA/cm^2 of membrane. The immunodetection was performed according to Sambrook et al (32) using an anti-*D. v.* Hildenborough cytochrome c_{553} anti-serum from rabbit.

RESULTS

Characterization of the D. desulfuricans G201 cytochrome c_{553}

Two hundred seventy grams wet cells of *Desulfovibrio desulfuricans* were harvested, resuspended in 600 ml of 100 mM Tris-HCl, 100 mM EDTA, 1 mM Phenyl methyl sulfonyl fluoride (PMSF) buffer (pH 9) and stirred for 30 min at 37°C in a water bath. The mixture was then centrifuged (6,700 g, 1h, 4°C) and the resulting supernatant was dialyzed overnight against 10 mM Tris-HCl, 1mM PMSF, 5mM EDTA buffer (pH 7.6) at 4°C. This periplasmic fraction was centrifuged for 1h at 27,000 g (4°C) and then was loaded onto a column of DEAE-Cellulose (Whatman DE 52) equilibrated with 10 mM Tris-HCl, 1mM PMSF, 5 mM EDTA buffer (pH 7.6). During the purification procedure, all steps were performed at 4°C and all buffers (pH 7.6) contained 1 mM PMSF and 5 mM EDTA. The unadsorbed fraction was loaded onto a hydroxyapatite (Bio-Rad) column equilibrated with 10 mM Tris-HCl buffer. The unadsorbed cytochrome containing fraction was applied to a carboxymethyl-cellulose column equilibrated with 10 mM Tris-HCl buffer and the cytochrome c_{553} was eluted with 50 mM Tris-HCl buffer. This cytochrome was found to be pure by electrophoresis on polyacrylamide gels under denaturing conditions (Phast Gel apparatus from Pharmacia). Its absorbance index, defined as c = (A553 − A570)red./(A280)ox., was found to be 1.3. Its UV/Visible spectrum showed typical peaks in the dithionite reduced state at 553, 524 and 418 nm. The Soret peak in the oxidized state is at 412 nm. It should be noted that this cytochrome is also reduced by ascorbic acid. Its molecular weight determined by electrophore-

TABLE 1

Amino Acid Compositions of *D. d.* G201 Cytochromes c_{553}, c_3 and High Molecular Mass (Hmc)

	Cytochrome c_{553} amino acid mol residues (mol)	Cytochrome c_3 amino acid mol residues (mol)	Hmc
Aspartic acid	4	11	56
Threonine[a]	2	5	27
Serine[a]	4	8	23
Glutamic acid	7	6	60
Proline	2	6	28
Glycine	9	6	41
Alanine	10	10	62
Cysteine[b]	2	8	(32)
Valine	3	5	37
Methionine	1	3	13
Isoleucine	7	3	20
Leucine	9	5	37
Tyrosine	5	3	11
Phénylalanine	3	2	14
Histidine	2	8	30
Lysine	12	13	41
Arginine	3	1	21
Tryptophane	N.D.	N.D.	N.D.
Number of residues	85	103	553

The results were obtained by analyzing 24, 48, and 72 hours hydrolysates.

[a] The values given for threonine and serine were extrapolated from the hydrolysis times.

[b] Analyzed as carboxymethylcysteine.

sis on polyacrylamide gel under denaturing conditions was the same as that of the *D. vulgaris* Hildenborough cytochrome c_{553} (data not shown).

The heme content per molecule was found to be 1 using the pyridine ferrohemochromogen assay which is in agreement with the aminoacid composition (table 1), in which only two cysteines were found. No peculiar acidic nor basic property of this cytochrome can be deduced from its aminoacid composition; this can be related with the fact that it was unadsorbed either on DEAE-cellulose or CM-cellulose columns.

The N-terminal aminoacid sequence was determined until the tenth residue (figure 1A). This sequence exhibited a large identity with the N-terminal sequence of the *D. vulgaris* Hildenborough cytochrome c_{553} (60%). The homology between the two cytochromes was confirmed by a western blot analysis. Using an anti *D. vulgaris* Hildenborough cytochrome c_{553} anti-serum, an immunoreactive band was detected on the nitrocellulose membrane for both cytochromes (figure 2). This suggests that the homology between *D. desulfuricans* G201 and *D. vulgaris* Hildenborough cytochromes c_{553} is large enough to allow the detection of the former cytochrome by antibodies designed against the latter cytochrome.

Cyclic voltammetry of the *D. desulfuricans* G201 cytochrome c_{553} has shown that the electrochemical system is reversible. The corresponding square-wave voltammogram is given in figure 3, with one peak at $E_p = -182$ mV. Since the electrochemical system is fast, the theoretical treatment allowed to determine a redox potential of + 28 mV ± 10 mV (*versus* NHE). This value is in the same order range as the published value for *D. vulgaris* Hildenborough (33) and *D. desulfuricans* Norway (34) cytochromes c_{553}. By analogy with these cytochromes, the sixth axial ligand to the heme iron atom is expected to be a sulfur atom from a methionine residue. It should be noted that the aminoacid composition revealed the presence of only one methionine residue and two histidine residues, which is compatible with a His/Met axial coordination of the heme iron atom.

The Cytochrome c_3 (Mr 13000)

This tetraheme cytochrome is found in all *Desulfovibrio* species and it has been already purified from *D. desulfuricans* G200 (5). This latter is homologous to the other cytochromes c_3 (Mr 13000), including four heme groups with a bis-histidinyl iron axial coordination. It is an acidic protein with a pI of 5.8. Comparisons of its amino-acid composition and N-terminus sequence with those of the other cytochromes c isolated from the same organism are shown in table 1 and figure 1B, respectively.

Characterization of the D. desulfuricans G201 High Molecular Weight Cytochrome c (Hmc)

The previously described first DEAE-cellulose column was eluted with 1M Tris-HCl buffer and the cytochromes containing fraction, which was further dialyzed overnight against 10 mM Tris-HCl buffer, was then applied to a hydroxyapatite (Bio-Rad) column equilibrated with 10 mM Tris-HCl buffer. A cytochromes containing fraction, eluted with 50 mM Tris-HCl buffer was 10 fold diluted and then loaded onto a DEAE-cellulose column equilibrated with 10mM Tris-HCl buffer. The Hmc was contained in a fraction eluting at 150 mM Tris-HCl buffer. A second cycle of hydroxyapatite/DEAE-cellulose column was performed in the same conditions as described above. The Hmc fraction was then concentrated with a hydroxyapatite (Bio-Rad) column eluted with 200 mM Tris-HCl buffer. The Hmc was found to be pure by electrophoresis on polyacrylamide gels under denaturing conditions with an absorbance index of 2.1. The UV/Visible spectrum of the purified cytochrome exhibited a Soret peak at 410 nm in the oxidized state and peaks at 420, 523 and 553 nm in the dithionite reduced form. The absorption coefficient at 553 nm in the reduced state was 321,875 M^{-1} cm^{-1}. No band at 695 nm, characteristic of a heme

(A) Cytochrome c_{553}

```
                1       5       10
D. d. G201      A E D G A S L Y R M . . .
D. v. H.        A - D G A A L Y K S . . .
```

(B) Cytochrome c_3 (Mr 13000)

```
                        5       10      15      20
D. d. G201        A E A P A D G L K M E N Y K M P V I F N K . . .
D. v. H.        A P K A P A D G L K M E A Y K Q P V V F N H . . .
```

(C) Hmc :

```
                1       5       10      15      20      25      30      35
D. d. G201      M P E P A G A G G G N A D L I R I D V V K Q F D D L E L P P A S F R H . . .
D. v. H.        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 H . . .
```

FIG. 1. Alignments of the N-terminus sequences of cytochromes c_{553} (A), cytochromes c_3 (Mr 13000) (B), and high molecular mass cytochromes (Hmc) (C) from both *D. desulfuricans* G201 (*D. d.* G201) and *D. vulgaris* Hildenborough (*D. v.*H)

iron-methionine sulfur bond, was detected on the Visible spectrum.

The mass spectra showed that this cytochrome is monomeric with a molecular weight of 64,903 Da ±7Da. The isoelectric point of this cytochrome was found to be 4.7 which is in agreement with the low lysine and arginine residue contents compared to the aspartic and glutamic acid residues as determined by aminoacid composition (table 1).

The iron content as determined by performing plasma emission spectroscopy was 16 atoms of iron per molecule, which was in agreement with number of heme determined by the pyridine ferrohemochromogen assay.

The electrochemistry experiments, performed on this cytochrome, did not allow to determine the redox potential of the heme groups. Whatever the conditions and electrodes used, no response was observed on the cyclic voltamograms. The reason for that might be the high molecular weight of the molecule.

The N-terminus aminoacid sequence has been determined up to the 35th residue (figure 1C). This sequence shows some homologies with the aminoacid sequence of the *D. vulgaris* Hildenborough Hmc. However, several

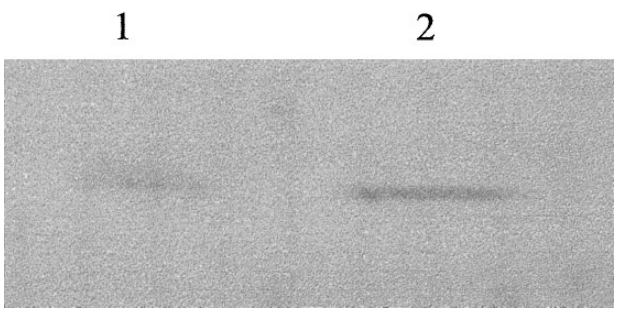

FIG. 2. Western blot analysis of *D. vulgaris* Hildenborough (lane 1) and *D. desulfuricans* G201 (lane 2) cytochromes c_{553}. 1 μg of each cytochrome was loaded on the polyacrylamide gel. The immunoreactive bands were detected using an anti *D. v.* Hildenborough cytochrome c_{553} anti serum from rabbit.

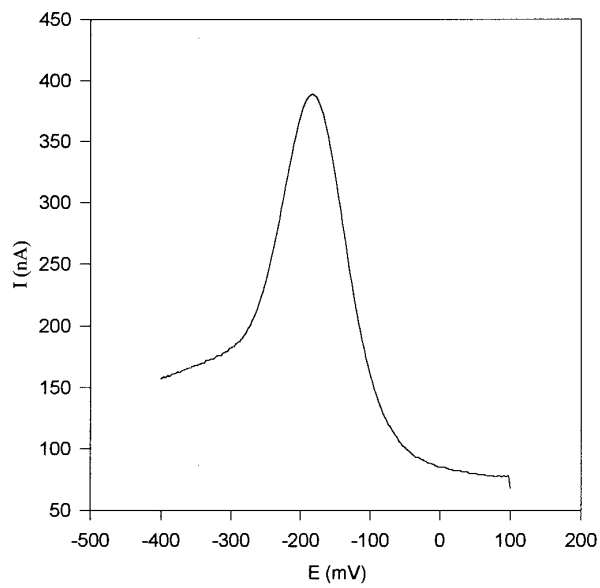

FIG. 3. Square-wave voltammogram at the pyrolytic graphite electrode for 65 μM *D. desulfuricans* G201 cytochrome c_{553} in 0.1M phosphate buffer (pH 7.6).

basic residues in the *D. v.* Hildenborough cytochrome were replaced by non-basic residue in the *D. d.* G201 cytochrome. This is in agreement with the acidic character of this cytochrome (pI 4.7) compared to the *D. v.* Hildenborough Hmc which is basic (pI 9.2) (10).

DISCUSSION

Several various types of soluble c-type cytochromes have been isolated from *Desulfovibrio* species. In this paper, we report the characterization of both a monoheme cytochrome c_{553} and a hexadecaheme Hmc isolated from *Desulfovibrio desulfuricans* G201.

The cytochrome c_{553} appears to be homologous to the various cytochromes c_{553} isolated from other *Desulfovibrio* species; it has the same molecular weight, contains only one heme group and exhibits a redox potential in the same order range. Its folding should be also homologous as anti *D. vulgaris* Hildenborough cytochrome c_{553} anti-serum reacts with *D. deulfuricans* G201 cytochrome c_{553}. A structural analysis is however needed to confirm this structural similarity between the two cytochromes.

The hexadecaheme Hmc isolated from *D. desulfuricans* G201 shares several characteristics with the various high molecular mass cytochromes already isolated. They all are composed of only one subunit of high molecular weight (about 60 kDa) that binds 16 heme groups. The N-terminus sequence of the *D. desulfuricans* Hmc has 41.6% identity to *D. vulgaris* Hildenborough Hmc and 27% identity to *D. gigas* Hmc. However, while the other Hmc's that have been isolated are either neutral or basic proteins, *D. desulfuricans* Hmc has a strong acidic property with an isoelectric point of 4.7.

Both cytochromes were isolated from the periplasmic extract, suggesting thus that these cytochromes could be located in the periplasmic space of the cell. The alanine residue at the N-terminus end of the *D. desulfuricans* G201 cytochrome c_{553} is compatible with a classic peptide signal cleavage site. In the case of the Hmc, the first residue is a methionine. The same case has been already observed in various periplasmic proteins such as the [NiFeSe] hydrogenase from *Desulfovibrio baculatus* (35) and hydrogenases from both nitrogen fixing (36) and photosynthetic bacteria (37).

The cytochrome content of *D. desulfuricans* G201 appears to be identical to that of *D. vulgaris* strains in which the same types of soluble cytochromes have been isolated and characterized. However, the amount of cytochromes in *D. desulfuricans* appears to be much lower than in *D. vulgaris* under the growth conditions we used. It is noteworthy that *D. desulfuricans* is the sole species in which both cytochrome c_3 (Mr 13000) and Hmc are acidic molecules. However, in the case of *D. africanus*, two cytochromes c_3 (Mr 13000) have been characterized, one being a basic molecule and the other acid (27). The same case can be expected for other *Desulfovibrio* species but failure of detection of a cytochrome by either genetic or biochemical methods might be due to a low homology between the molecules or non-optimal growth conditions. An accurate analysis of the *Desulfovibrio desulfuricans* G201 cytochromes content in various growth conditions is a necessary step in order to get a better understanding of the physiological function of the various cytochromes in *Desulfovibrio* cells.

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