Eur. J. Biochem. *162*, 547 – 554 (1987)
© FEBS 1987

Characterization of the cytochrome system of a nitrogen-fixing strain of a sulfate-reducing bacterium: *Desulfovibrio desulfuricans* strain Berre-Eau

Isabel MOURA [1], Guy FAUQUE [2, 3], Jean LeGALL [3], Antonio V. XAVIER [1] and José J. G. MOURA [1]

[1] Centro de Quimica Estrutural, Universidade Nova de Lisboa
[2] A.R.B.S., Centre d'Etudes Nucléaires, Cadarache
[3] Department of Biochemistry, University of Georgia, Athens

(Received August 11/October 8, 1986) – EJB 86 0897

Two *c*-type cytochromes were purified and characterized by electron paramagnetic resonance (EPR) and nuclear magnetic resonance (NMR) spectroscopic techniques, from the sulfate-reducer nitrogen-fixing organism, *Desulfovibrio desulfuricans* strain Berre-Eau (NCIB 8387). The purification procedures included several chromatographic steps on alumina, carboxymethylcellulose and gel filtration. A tetrahaem and a monohaem cytochrome were identified. The multihaem cytochrome has visible, EPR and NMR spectra with general properties similar to other low-potential bis-histidinyl axially bound haem proteins, belonging to the class of tetrahaem cytochrome c_3 isolated from other *Desulfovibrio* species. The monohaem cytochrome c_{553} is ascorbate-reducible and its EPR and NMR data are characteristic of a cytochrome with methionine-histidine ligation. Their properties are compared with other homologous proteins isolated from sulfate-reducing bacteria.

Since the discovery by Postgate [1] and Ishimoto et al. [2] of tetrahaem cytochrome c_3 in the strict anaerobic sulfate-reducing bacteria, other *c*-type cytochromes have been reported in *Desulfovibrio* species. It is now known that several kinds of *c*-type cytochromes can be isolated from different *Desulfovibrio* species. A classification based on the number of haems per molecule, rather than their molecular masses, has recently been proposed [3] as follows.

Monohaem cytochromes (methionine – haem-iron – histidine)

Cytochrome c_{553} is a low-molecular-mass protein and contains a single haem group with methionine and histidine as axial ligands. This cytochrome was only isolated from *Desulfovibrio* (*D.*) *vulgaris* strains Hildenborough and Miyazaki [4, 5]. It has a midpoint redox potential of approximately + 20 mV [6, 7] which is a low value compared with most other methionine-histidine-ligated monohaem cytochromes.

Cytochrome $c_{553\,(550)}$ is a haem protein found in *D. baculatus* strains Norway 4 [8] (formerly called *D. desulfuricans* strains Norway 4 [NCIB 8310] [3]) and 9974 (DSM 1743) [9]. This cytochrome has a N-terminal sequence showing little homology with *D. vulgaris* strain Hildenborough cytochrome c_{553}. EPR and NMR spectroscopies have been utilized to characterize the structure around the haem [10].

Tetrahaem cytochrome c_3 *(histidine – haem-iron – histidine)*

This is present in all *Desulfovibrio* species so far examined. The four haems, mesoporphyrins, are covalently bound to the polypeptide chain through thioether linkages provided by cysteinyl residues on either a -Cys-$(Xaa)_2$-Cys-His- sequence or a -Cys-$(Xaa)_4$-Cys-His- sequence. The axial ligands are two hystidinyl residues. The three-haem-containing cytochrome $c_{551.5}$ (c_7) isolated from the sulfur-reducing bacterium *Desulfuromonas* (*Drm.*) *acetoxidans* (strain 5071) is a close relative to this class of haem protein [11, 12].

Several tetrahaem cytochromes c_3 isolated from different strains of *Desulfovibrio* have been sequenced: *D. gigas, D. vulgaris* strains Hildenborough and Miyazaki, *D. baculatus* strain Norway 4, and *D. salexigens* strain British Guiana as well as cytochrome $c_{551.5}$ (c_7) from *Drm. acetoxidans* [11, 13 – 15]. Even when deletions are allowed to maximize homology, only about 30% of the amino-acid residues are conserved throughout the above series of proteins. They account mainly for the residues involved in the haem-attachment sites. There are only eight conserved residues not involved in binding the haem groups. This difference in amino-acid composition results in a wide variation of the physico-chemical properties.

Structural studies by X-ray crystallography have been reported for tetrahaem cytochromes c_3 from *D. vulgaris* strain Miyazaki [16] and *D. baculatus* strain Norway 4 [17, 18] at 0.26-nm resolution and a new sequence alignment has been proposed [19]. The relative positions and orientations of the haems are very similar for both proteins. Some of the features of interest coming from these structures are: the haem groups have different solvent exposition, the four haems are not parallel and they are attached in a compact cluster with iron-iron distances ranging over 1.09 – 1.73 nm.

Several physico-chemical techniques, mainly Mössbauer spectroscopy [20], circular dichroism (CD) [21], electron

Correspondence to J. J. G. Moura, Centro de Quimica Estrutural, Universidade Nova de Lisboa, Avenida Rovisco Pais, P-1096 Lisboa, Portugal

Abbreviations. EPR, electron paramagnetic resonance; SDS, sodium dodecyl sulphate.

Table 1. *Present knowledge of cytochromes characterization in sulfate-reducing bacteria of the genus* Desulfovibrio
This table was compiled from references indicated in text. Preliminar crystallographic data was recently reported on *D. vulgaris* Miyazaki cytochrome c_{553} [65]. S = sequenced; NMR, EPR, X-ray refer to spectroscopic characterization; P = purified; PNP = present but not purified; – = not reported

Desulfovibrio species	Tetrahaem cytochrome c_3	Octahaem cytochrome c_3	Monohaem cytochrome c_{553}
D. gigas	S, NMR, EPR	P	–
D. vulgaris Hildenborough	S, NMR, EPR	P	S, NMR, EPR
D. vulgaris Miyazaki	S, X-ray	–	P, X-ray
D. baculatus strain 9974	P, NMR, EPR	–	P[a]
D. baculatus Norway 4	S, NMR, EPR, X-ray	S	P, NMR, EPR[a]
D. desulfuricans ATCC 27774	P, NMR, EPR	–	–
D. desulfuricans Berre-Eau	P, NMR, EPR	–	P, NMR, EPR
D. desulfuricans El Algheila Z	S, NMR, EPR	P	–
D. salexigens British Guiana	S, NMR	PNP	PNP
D. desulfuricans Berre-Sol	P, NMR, EPR	–	P, NMR

[a] Refers to monohaem cytochrome $c_{553\,(550)}$.

paramagnetic resonance (EPR) [22–25], nuclear magnetic resonance (NMR) [26–32], cyclic voltametry, differential pulse polarography and pulse radiolysis [33–39] have also been applied to elucidate structural features and the mechanism of electron transfer in cytochromes from *Desulfovibrio* spp.

The midpoint redox potentials of the four haems have been measured by a wide range of techniques [24, 25, 33, 34, 40].

Octahaem cytochrome c$_3$ *(histidine – haem-iron – histidine)*

This cytochrome has been found in several *Desulfovibrio* species (see Table 1). Recently, Guerlesquin et al. [41] characterized the octahaem cytochrome c_3 from *D. baculatus* strain Norway 4. By removal of the haems they demonstrated that two identical subunits of molecular mass 13.5 kDa are obtained. Although the monomer form of octahaem cytochrome c_3 has a molecular mass somewhat similar to that of the tetrahaem cytochrome c_3 isolated from the same bacterium, the amino acid composition and the N-terminal sequence are different, confirming the presence of a different cytochrome [41].

Table 1 contains information about the purification and characterization of *c*-type cytochromes from these bacteria. The physiological role of cytochromes in sulfate-reducing organisms is far from being fully understood. Tetrahaem cytochrome c_3 plays an important role in relevant metabolic pathways of Desulfovibriones, namely in its direct interaction with the hydrogenase system and with the electron transfer chain to the terminal reductases involved in the reduction of sulfur compounds. Octahaem cytochrome c_3 seems to be an electron carrier for the electron transport chain of thiosulfate reduction in the *D. gigas* enzymatic systems [42] and cytochrome c_{553} was identified as a natural electron acceptor for the formate dehydrogenase systems in *D. vulgaris* strain Miyazaki [43].

Other *c*-type cytochromes were also detected in some *Desulfovibrio* spp. In *D. desulfuricans* (ATCC 27774) a sulfate-reducing bacterium that can grow on nitrate as terminal electron acceptor, the nitrite reductase is a hexahaem *c*-type cytochrome [44]. Another *c*-type cytochrome called 'split-soret' was also purified from this organism. It is a trimeric protein with subunit molecular masses of 26.4 kDa with two haems *c* per monomer [45].

In the present communication we report the purification and some properties of tetrahaem cytochrome c_3 and cytochrome c_{553} isolated from *D. desulfuricans* strain Berre-Eau. Another *c*-type cytochrome was also detected for which preliminary properties are presented. *D. desulfuricans* strain Berre-Eau is able to grow while fixing N_2 as has been reported for some strains of *Desulfovibrio* and *Desulfotomaculum* [46–48].

MATERIALS AND METHODS

Analytical procedures and instrumentation

Molecular masses were estimated by gel filtration on a Sephadex G-50 column, according to the method of Whitaker [49], using the following standards: chymotrypsin (M_r = 25000), *D. gigas* ferredoxin II (M_r = 24000), horse heart cytochrome *c* (M_r = 12500) and *D. gigas* rubredoxin (M_r = 6000) and also in the presence of sodium dodecyl sulfate (SDS) using the procedure of Weber and Osborn [50] with the following standards: soybean trypsin inhibitor (M_r = 21000), horse heart cytochrome *c* (M_r = 12500), and *D. vulgaris* strain Hildenborough cytochrome c_{553} (M_r = 9000).

The isoelectric point was determined by isoelectric focusing [51] on a LKB Multiphor apparatus.

Absorption spectra were obtained on a Beckman spectrophotometer, model 35.

For NMR measurements, the cytochromes were desalted and lyophilised three times from ^2H_2O and dissolved to the required concentration (1–2 mM). Reduction of the proteins was achieved by adding small amounts of solid sodium dithionite under an N_2 atmosphere. High-resolution proton NMR spectra were recorded on a Bruker CXP 300 spectrometer (300 MHz) equipped with an Aspect 2000 computer in which mathematical manipulations were carried out. The temperature was controlled to $\pm$ 0.5°C with a standard Bruker B-VT-1000 variable temperature control unit.

Selective Nuclear Overhauser effects were obtained by the TOE method [52]. Typically, one free induction decay was acquired with a gated irradiation pulse on the frequency chosen, and the next with the same gated irradiation pulse, but on an empty region of the spectrum. The spectra were subtracted in order to minimize external effects, the sequence was repeated 1000 times to obtain a good signal/noise ratio. All chemical shift values are quoted in parts per million (ppm)

from internal sodium 3-trimethylsilyl-(2,2,3,3,$^2H_4$)proprionate, positive values referring to low-field shifts.

EPR spectra were recorded in a Bruker ER-200 tt spectrometer equipped with an Oxford Instruments continuous helium flow cryostat interfaced to a Nicolett 1180 computer.

Growth of organisms and purification of D. desulfuricans *strain Berre-Eau cytochromes*

D. desulfuricans strain Berre-Eau (NCIB 8387) was grown in the medium of Starkey [53] on lactate/sulfate at 37 °C and harvested as previously described [54].

All the purifications steps were performed at + 4 °C using potassium phosphate and Tris/HCl buffers, pH 7.6, of appropriate molarity. The frozen cells (600 g, wet weight) were thawed and suspended in 1.4 l of 10 mM Tris/HCl buffer, containing a few deoxyribonuclease crystals. The cell suspension was treated in a French pressure cell: then the cell-free extract was centrifuged for 45 min at 12000 rev./min.

The crude extract was passed over a column (37 × 5.5 cm) of DEAE-cellulose (DE-52) equilibrated with 10 mM Tris/HCl. The fraction not adsorbed on this column (1500 ml) contained most of the cytochromes and was passed over an alumina column (18 × 4.5 cm) equilibrated with 10 mM Tris/HCl. A discontinuous gradient of potassium phosphate buffer (10 – 500 mM) was performed. Two main fractions containing cytochromes were eluted between 40 – 100 mM and at 400 mM. The first fraction contained an ascorbate-reducible cytochrome (cytochrome c_{553}), The second fraction contained mostly a cytochrome not reducible by ascorbate but dithionite-reducible (tetrahaem cytochrome c_3).

During the purification a purity coefficient will be defined as [A_{553} (red)-A_{570} (red)]/A_{280} (oxid).

Cytochrome c$_{553}$

The fraction containing cytochrome c_{553} coming from alumina (V = 350 ml with a purity coefficient of 0.15) was dialysed overnight against 20 l distilled water. After dialysis this cytochrome was adsorbed on a column (19 × 4.5 cm) of carboxylmethycellulose (CMC-52) equilibrated with 10 mM Tris/HCl buffer and eluted with a discontinuous gradient of Tris/HCl buffer (10 – 250 mM). The cytochrome c_{553} with a coefficient of 0.82 in a total volume of 310 ml was dialysed again and a second step of purification was performed on another CMC-52 column with a similar elution gradient. At 25 – 50 mM Tris/HCl buffer the cytochrome c_{553} was eluted in a volume of 190 ml with a purity coefficient of 1.02. A last step of purification was done in column (5 × 3 cm) of hydroxyapatite (HTP) equilibrated with 50 mM Tris/HCl buffer. Cytochrome c_{553} was not retained but eluted in a volume of 150 ml having a purity coefficient of 1.2. This cytochrome was completely reduced by ascorbate.

Tetrahaem cytochrome c$_3$

The fraction containing mostly a cytochrome not reducible by ascorbate eluting from the first alumina column was dialyzed overnight against 20 l distilled water. This fraction, in a volume of 450 ml and with a purity coefficient of 0.19, was adsorbed on another alumina column (17 × 4 cm) equilibrated with 10 mM Tris/HCl buffer. During a discontinous gradient of phosphate buffer (10 – 700 mM), a 360-ml cytochrome fraction was collected at 400 mM. This cytochrome was not reducible by ascorbate and the purity coefficient was 1.88.

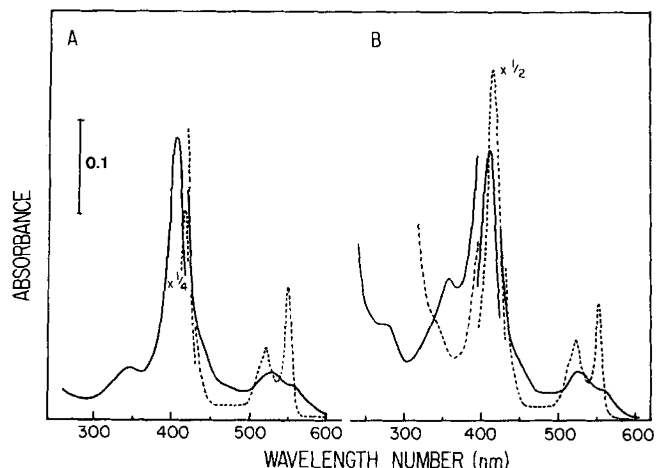

Fig. 1. *Ultraviolet and visible absorption spectra of* D. desulfuricans *strain Berre-Eau cytochromes.* (A) Tetrahaem cytochrome c_3 (1.25 μM): (——) oxidized form; (– – – –) dithionite-reduced form. (B) Monohaem cytochrome c_{553} (4.33 μM): (——) oxidized form; (– – – –) ascorbate-reduced form

After dialysis this cytochrome was adsorbed on an HTP column (11 × 4 cm) equilibrated with 10 mM Tris/HCl. A discontinous gradient in phosphate was performed (1 – 500 mM). Tetrahaem cytochrome c_3 was eluted between 200 – 400 mM in a volume of 175 ml with a purity coefficient of 1.95. After dialysis, the material was adsorbed onto a CMC-52 column (20 × 4.5 cm) and subjected to a discontinuous Tris/HCl gradient (10 – 170 mM). Tetrahaem cytochrome c_3 was eluted at 60 – 100 mM in a volume of 180 ml with a purity coefficient of 2.8 1. Another similar step on CMC-52 increased the purity coefficient to 2.85 and finally tetrahaem cytochrome c_3 was passed on a Sephadex G-50 column (90 × 4.5 cm) equilibrated with 10 mM Tris/HCl. Tetrahaem cytochrome c_3 was obtained in a volume of 70 ml with a purity coefficient of 3.22.

RESULTS

Homogeneity and molecular masses

Tetrahaem cytochrome c_3 and cytochrome c_{553} were judged to be pure by polyacrylamide gel electrophoresis at pH 8.9.

The molecular mass of cytochrome c_{553} was estimated to be 9 kDa by SDS gel electrophoresis. This value is similar to the molecular mass of three other monohaem cytochromes isolated from *Desulfovibrio* species [4, 5, 8, 9].

The molecular mass of tetrahaem cytochrome c_3 determined by gel filtration on Sephadex G-50 and on SDS gel was estimated to be 13.5 kDa. The isoelectric points were determined by isoelectric focusing to be 9.2 and 8.6 respectively for cytochromes c_{553} and c_3.

Ultraviolet and visible absorption spectral properties

The absorption spectra of oxidized and reduced forms of purified cytochrome c_{553} and tetrahaem cytochrome c_3 are shown in Fig. 1. Cytochrome c_{553} is completely reduced by ascorbate and cytochrome c_3 is only dithionite-reducible. In contrast with cytochrome c_3, cytochrome c_{553} shows in the oxidized form a peak in the 280-nm region and a weak shoul-

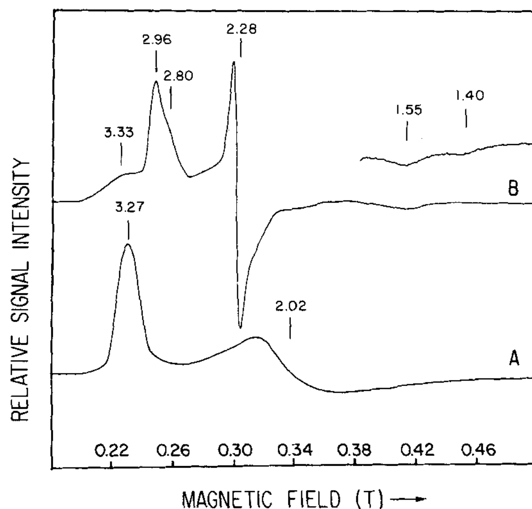

Fig. 2. *EPR spectra of* D. desulfuricans *strain Berre-Eau ferricytochrome* c_{553} *(A) and ferritetrahaem cytochrome* c_3 *(B).* (A) Measured at 5.2 K, microwave power 2 mW, field modulation 1 mT, gain 5×10^4; (B) measured at 4.8 K, other experimental conditions as in A

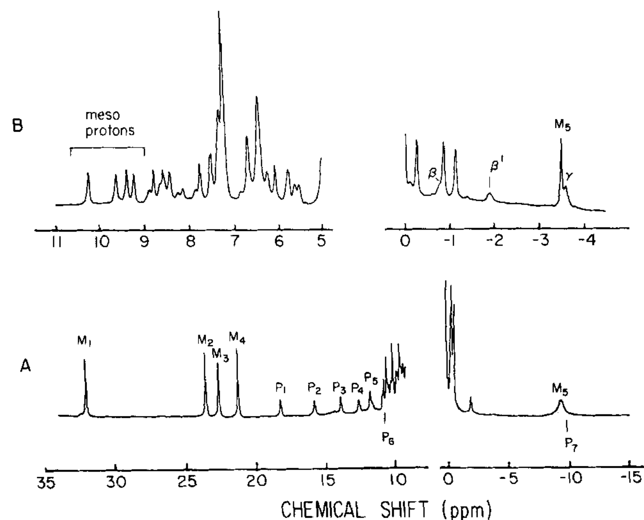

Fig. 3. *300-MHz spectra of* D. desulfuricans *Berre-Eau cytochrome* c_{553} *in (A) reduced form at 313 K and (B) oxidized form at 300 K.* Some relevant resonances are assigned

der at 690 nm (not shown), characteristic of the haem-methionine axial ligation.

The highest purity coefficient found for tetrahaem cytochrome c_3 is 3.22 and for cytochrome c_{553} is 1.20. The values are similar to the ones reported from other Desulfovibrione tetrahaem cytochromes c_3 and monohaem cytochromes c_{553}.

Electron paramagnetic resonance data

Ferricytochrome c_{553}. The EPR spectrum recorded at 6 K (Fig. 2A) contains two main components present at $g = 3.27$ and $g = 2.02$ which are assigned to a low-spin species. The third component of the corresponding rhombic g tensor is not observable, presumably being too broaden in the high field region.

Ferritetrahaemcytochrome c_3. Fig. 2B shows the EPR spectrum recorded at 10 K. The spectrum is quite complex, showing in the g_{max} region several superimposed signals. A very broad feature is detected around 3.33, a prominent feature at 2.96 and a shoulder at 2.80. A derivative peak is observed at 2.28 (probably g_{med}) and two broad signals at high field: 1.55 and 1.40 (g_{min}).

Nuclear magnetic resonance data

Ferricytochrome c_{553}. Fig. 3A shows the NMR spectrum of the low-field region of *D. desulfuricans* strain Berre-Eau ferricytochrome c_{553}. Four haem methyl resonances are observed (M_{1-4}) in a pattern very similar to that observed for *D. vulgaris* strain Hildenborough cytochrome c_{553} [10]. At high field the ε-methyl protons from the axial-ligated methionine can be observed at -9.34 ppm (300 K). A one-proton resonance, presumably from a methylene group of this residue, is underneath the ε-methyl methionine group (P_7) and can be separated when varying the temperature. The temperature dependence of selected resonances of the NMR spectra of ferricytochrome c_{553} (Fig. 4) indicates that the methyl resonances M_2, M_3 and M_4 do not follow a Curie law. Although the chemical shift of ε-methyl methionine resonance decreases

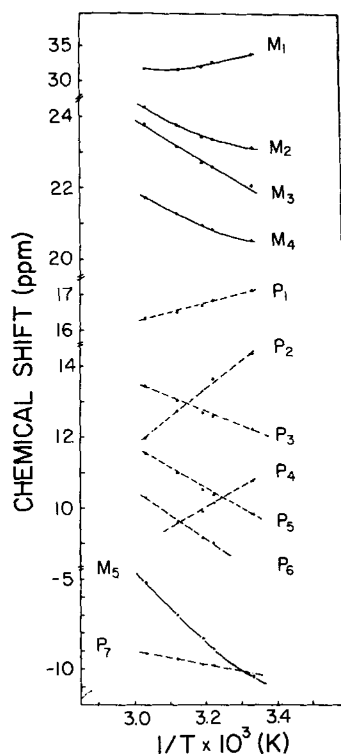

Fig. 4. *Temperature dependence of selected NMR resonances of* D. desulfuricans *strains Berre-Eau ferricytochrome* c_{533} *as indicated in Fig. 3*

when the temperature is increased, the temperature-dependence plot is complex and not linear. This is also observed for methyl resonances M_1, M_2 and M_4.

Ferrocytochrome c_{553}. Fig. 3B shows the 300-MHz NMR spectrum of *D. desulfuricans* strain Berre-Eau ferrocytochrome c_{553}. Four one-proton resonances assigned to haem mesoprotons are resolved at 10.24, 9.63, 9.39 and 9.27 ppm in the low-field region of the spectrum at 300 K. In the high-field region the typical pattern of the resonances from the methionine ligated to the haem iron can be observed. The

Table 2. *Chemical shift of axial methionine resonance in* D. desulfuricans *Berre-Eau ferrocytochrome* c_{553} *(313 K)*

Resonance	Chemical shift
	ppm
ε-Methyl	− 3.51
γ′-Methylene	− 3.60
γ-Methylene	− [a]
β′-Methylene	− 1.89
β-Methylene	− 0.76

[a] Not determined. Probably the methionyl γ-proton overlaps the ε-methyl group.

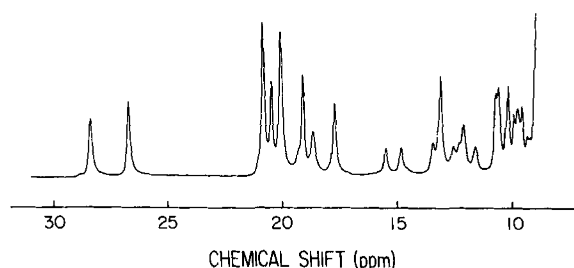

Fig. 5. *300-MHz NMR spectra of the low-field region of* D. desulfuricans *strain Berre-Eau tetrahaem cytochrome* c_3 *in the oxidized form at 313 K*

methyl protons at the ε position of the axial methionine are at − 3.51 ppm, a value lower than those generally observed for methionine-histidine cytochromes (positive redox potential). The assignment of the methionyl β and γ protons was carried out as in [52]. The results are shown in Table 2.

Ferritetrahaemcytochrome c_3. In Fig. 5, the low-field region of the oxidized form of tetrahaem cytochrome c_3 is shown. Several resonances corresponding to three-proton intensity (haem methyl) can be detected in this region as is usual for tetrahaem cytochromes c_3 from other *Desulfovibrio* species. At least 14 haem methyl resonances of the 16 haem methyl groups are observed.

DISCUSSION

Sulfate-reducing bacteria are strict anaerobes that are considered as representative of organisms having an ancestral metabolic process. They are able to carry out the dissimilatory reduction of sulfate, the so-called 'sulfate respiration'. This process involves eight electrons to reduce sulfate up to hydrogen sulfide, coupled with an electron transfer chain.

Cytochromes seem to play an important role in these electron transfer processes, although at the present moment their precise physiological role is still controversial. More research on the cell localization must be undertaken in order to understand fully the involvement of the haem proteins. The localization of tetrahaem cytochrome c_3 is predominantly periplasmic [54] although Odom and Peck [55] have found relevant amounts of *c*-type cytochromes in *D. gigas* cytoplasm. Recently, comparing amino acid sequences of several periplasmic and cytoplasmic proteins from *Desulfovibrio* species, LeGall and Peck [56] proposed that periplasmic proteins have NH_2-terminal amino acid sequences indicative of recognition sites for signal peptidases. This is the case of tetrahaem cytochromes c_3 and supports their previous localization in the periplasmic space.

As indicated in Table 1, tetrahaem cytochromes c_3 are conserved in all the *Desulfovibrio* species analysed so far. It is interesting to note that even when the terminal acceptor is modified, i.e. nitrate by sulfate in *D. desulfuricans* (ATCC 27774) this multihaem cytochrome is conserved [45].

The presence of monohaem cytochromes has not been reported for most of Desulfovibriones (see Table 1).

The general EPR and NMR spectroscopic parameters of the monohaem cytochrome c_{553} isolated from *D. desulfuricans* strain Berre-Eau are very similar to those from *D. vulgaris* strain Hildenborough cytochrome c_{553}. According to the classification of Brautigan et al. [57], *D. desulfuricans* strain Berre-Eau cytochrome c_{553} belongs to the class of cytochromes like yeast iso-2, *Euglena, Rhodospirillum rubrum* and *Pseudomonas denitrificans* that have a major neutral pH form with g_{max} value near 3.2. The tuna, yeast iso-1 and horse cytochromes *c* belong to the other class having at neutral pH a major form with EPR absorption at $g = 3.06$. It was also suggested by Brautigan et al. [57] that in this last class of cytochromes the N-1 from the ligated imidazole is deprotonated or enhanced hydrogen bonding. The NMR pattern of the low-field-shifted methyl haem resonances in the ferric form as well as the chemical shift of the haem-meso proton resonances and the methyl group of the methionine in the ferrous state are identical (see Table 2 and Fig. 3). It was recently shown that in *D. vulgaris* strain Hildenborough cytochrome c_{553}, a different methionine chirality is observed when comparing the oxidized and reduced states of the protein [10]. In the reduced state the axial methionine has an *S* chirality and changes to *R* upon oxidation. The structure is most closely related to that found in cytochromes c_{551}, but it differs from these by a clockwise rotation of approximately 45° around the iron-sulfur bound.

Another monohaem cytochrome was isolated from *D. baculatus* strain Norway 4 [8]. With respect to the chirality of the bound axial methionine, it seems to have the same properties as *D. vulgaris* strain Hildenborough cytochrome c_{553}. However it presents different ring methyl hyperfine shifts in the oxidized form, that are explained as being due to a small rotation of the axial methionine about an axis through the haem iron and the methionine sulfur atom [10]. This cytochrome isolated from *D. baculatus* strain Norway 4 shows a splitting of the α band of the visible spectrum in the reduced state. A similar monohaem cytochrome also showing a split α band was purified from *D. baculatus* strain 9974 [9]. The EPR *g*-values of these two split α cytochromes differ from those observed for *D. vulgaris* strain Hildenborough and *D. desulfuricans* strain Berre-Eau cytochromes c_{553} (Table 3).

The similarities between the monohaem cytochromes from *D. vulgaris* strain Hildenborough and *D. desulfuricans* strain Berre-Eau as well as the similarities between the monohaem cytochromes from *D. baculatus* strains 9974 and Norway 4 are quite noticable [7−10, 58] confirming that two different types of monohaem cytochromes are present in *Desulfovibrio* species.

Table 3 compares some of the data available for monohaem cytochromes from *Desulfovibrio* species. As it was already pointed out the monohaem cytochromes from *Desulfovibrio* species have unusually low oxidation-reduction potentials [6, 7]. This might be correlated with the unusual low position obtained for the ε-methyl group of the axial methionine in the reduced state [59]. A similar value of chemical shift was also reported for the methionyl methyl group of *D. vulgaris* strain Hildenborough ($\delta_{30°C} = 3.62$ ppm)

Table 3. *Physico-chemical data on monohaem cytochromes isolated from sulfate-reducing bacteria of the genus* Desulfovibrio
This table was compiled from references indicated in the text. The midpoint redox potential of *D. vulgaris* Miyazaki was reported by Langone et al. [66]

Monohaem cytochromes c_{553}	Redox potential	EPR values		Isoelectric point	Molecular mass
		g_{max}	g_{med}		
	mV				kDa
D. vulgaris Hildenborough	20 ± 5	3.15	2.065	8.6	8.6
D. vulgaris Miyazaki	−260	–[c]	–[c]	10.2	8.2
D. baculatus Norway 4[a]	≥ -50[b]	3.07	2.24	6.6	9.4
D. desulfuricans Berre-Eau	≥ -50[b]	3.27	2.02	9.2	9.0
D. baculatus strain 9974[a]	≥ -50[b]	3.08	2.25	–[c]	9.0

[a] Refers to monohaem cytochrome $c_{553\ (550)}$.
[b] Ascorbate-reducible.
[c] Not reported.

Table 4. *EPR* g-*values of tetrahaem cytochromes* c_3 *isolated from sulfate-reducing bacteria of the genus* Desulfovibrio

Tetrahaem cytochromes c_3	g_{max}	g_{med}	g_{min}
D. gigas	2.96, 2.85	2.30	1.58, 1.51
D. vulgaris Hildenborough	3.12, 2.97, 2.82	2.29	1.67, 1.57, 1.43
D. baculatus Norway 4	3.36, 3.01, 2.94	2.28	1.51, 1.38
D. baculatus strain 9974	3.36, 3.06, 2.95	2.27	1.51, 1.32
D. desulfuricans Berre-Eau	3.33, 2.96, 2.80	2.28	1.55, 1.40
D. desulfuricans El Algheila Z	2.95	2.28	≈1.51

and *D. baculatus* strain Norway 4 ($\delta_{30^\circ C}$ = 3.60 ppm) ferrocytochromes c_{553} [10].

In Table 4 some of the EPR *g*-values of tetrahaem cytochromes c_3 are compiled. The tetrahaem cytochromes c_3 exhibit quite different EPR characteristics. Tetrahaem cytochrome c_3 from *D. baculatus* strains 9974 and Norway 4 and *D. desulfuricans* strain Berre-Eau (this study) show quite similar EPR characteristics [60]. They all have a broad feature at $g = 3.3$, a resonance around $g = 3.0$ with a shoulder on this peak to lower *g* values. For other tetrahaem cytochromes, like *D. gigas* and *D. desulfuricans* strain El Algheila Z. tetrahaem cytochromes c_3 the broad peak around $g = 3.30$ is missing and only a g_{max} value is prominent around 3.0–2.9, showing in some cases a shoulder. *D. vulgaris* strain Hildenborough tetrahaem cytochrome c_3 seems different in the fact that three g_{max} values are quite discernable at 3.12, 2.97 and 2.82. The g_{med} is sharper when compared to those from other cytochromes c_3 [7, 60].

Recently Palmer and Walker et al. have shown that, in haem model compounds where the two axial imidazoles are perpendicular to each other, the EPR signals are extremely anisotropic with *g* values at around 3.4 [61, 62]. The X-ray structures of tetrahaem cytochromes c_3 from *D. vulgaris* strain Miyazaki and *D. baculatus* strain Norway 4 show that three of the haem groups have the two axial histidines in the same plane in relation to each other. Only one haem in both of these cytochromes has the two axial histidines perpendicular to each other [8, 18]. In this context we can re-examine the EPR spectra of these cytochromes c_3 with *g* values greater than 3. This signal should correspond to the haem with the two histidines perpendicular to each other. This haem has the lowest redox potential (−325 mV) in *D. baculatus* strain Norway 4 [40]. However, in *D. gigas* and *D. desulfuricans* strain El Algheila Z tetrahaem cytochromes c_3, where the X-ray structures are not yet determined, the EPR characteristics are different and the signal with high g_{max} is not present. The tetrahaem cytochrome c_3 purified from *D. desulfuricans* strain Berre-Eau is similar in many aspects to tetrahaem cytochromes c_3 from other sulfate-reducing bacteria of the genus *Desulfovibrio.* It is a tetrahaem protein with a molecular mass of 13.5 kDa. The redox potentials of the four haems are low, since only dithionite, not ascorbate, can reduce the protein. The EPR and NMR characteristics of the tetrahaem cytochrome c_3 are more closely related to the similar proteins isolated from *D. baculatus* strains 9974 and Norway 4. The screening of the EPR and NMR characteristics of tetrahaem cytochromes c_3 would probably permit this class of proteins to be subdivided into sub-groups with more similar properties. This class of cytochromes has been extensively proposed to be involved in the hydrogen metabolism of sulfate-reducing bacteria [3]. It was also shown that in some species of sulfate-reducers, for instance in *D. baculatus* strains 9974 and Norway 4 and *D. gigas*, tetrahaem cytochrome c_3 can act as a sulfur reductase (reduction of elemental sulfur to sulfide), whereas that of *D. vulgaris* strain Hildenborough is rapidly inhibited by sulfide [63, 64].

The four haems of tetrahaem cytochromes c_3 have histidine-histidine ligation and, as shown by EPR and NMR spectroscopies, they are localized in non-equivalent protein environments and each haem has a different redox potential [25, 30, 31, 40]. Although the absolute values vary with the experimental method of measurement, there is a general agreement that the four haem groups exhibit different and negative redox potential values.

By redox studies, followed by monitoring changes in the haem methyl resonance by NMR spectroscopy, we have recently shown that there is a haem-haem redox interaction [31, 32]. The midpoint redox potentials of some of the haems, as well as their interacting potentials, are pH-dependent. From these studies it seems that tetrahaem cytochrome c_3 has the potential of donating or receiving two coupled electrons and can interact with other electron transfer proteins in different

physiological conditions by modulating its mid-point redox potentials [32]. A full characterization of this new tetrahaem cytochrome c_3 in the light of this model of the interaction between the four haems is under study and will probably contribute to a better understanding of the electron transfer mechanism in this class of cytochromes and of their physiological significance.

The cytochrome system in *D. desulfuricans* strain Berre-Eau is mainly constituted by cytochrome c_{553} and tetrahaem cytochrome c_3; a very small amount of another *c*-type cytochrome was also detected. This cytochrome has not yet been fully purified. It does not belong to the octahaem cytochrome c_3 type and is partially reduced by ascorbate. Its molecular mass is between that of tetrahaem cytochrome c_3 and cytochrome c_{553}. NMR and EPR show that it is a multihaem cytochrome with significant differences from tetrahaem cytochrome c_3. A complete study of this cytochrome is underway to elucidate its properties fully.

We thank M. Scandellari and R. Bourelli for growing the bacteria used for the present study. We are indebted to I. Pacheco for her skilful technical help. This research was supported by grants from *Instituto Nacional de Investigação Cientifica, Junta Nacional de Investigação, Cientifica e Tecnologica* and Agency for International Development (J. J. G. Moura) and National Science Foundation Grant DMB-8602789 (J. LeGall). Part of this work has been made possible thanks to a special collaborative arrangement between the University of Georgia, Athens (USA) and the *Centre National de la Recherche Scientifique* and the *Comissariat de l'Energie Atomique* (France).

REFERENCES

1. Postgate, J. R. (1954) *Biochem. J. 59*, Xi.
2. Ishimoto, M. K., Kayama, J. & Nagai, Y. (1954) *Bull. Chem. Soc. Japan 27*, 564–565.
3. LeGall, J. & Fauque, G. (1986) in *Biology of anaerobic microorganisms (Environmental microbiology of anaerobes)*, (Zehnder, A. J. B. ed.) in the press.
4. Bruschi, M., LeGall, J. & Dus, K. (1970) *Biochem. Biophys. Res. Commun. 38*, 607–616.
5. Yagi, T. (1979) *Biochim. Biophys. Acta 548*, 96–105.
6. Bianco, P., Haladjian, J., Bruschi, M. & Pillard, R. (1982) *J. Electroanal. Chem. 136*, 129–299.
7. Bertrand, P., Bruschi, M., Denis, M., Gayda, J. P. & Manca, F. (1982) *Biochem. Biophys. Res. Commun. 106*, 756–760.
8. Fauque, G., Bruschi, M. & LeGall, J. (1979) *Biochem. Biophys. Res. Commun. 86*, 1020–1029.
9. Fauque, G. (1979) *These de Doctorat de Specialite*, University of Aix-Marseille I.
10. Senn, H., Guerlesquin, F., Bruschi, M. & Wuthrich, K. (1983) *Biochim. Biophys. Acta 748*, 194–204.
11. Ambler, R. P. (1971) *FEBS Lett. 18*, 351–353.
12. Probst, I., Bruschi, M., Pfennig, N. & LeGall, J. (1977) *Biochim. Biophys. Acta 460*, 58–64.
13. Ambler, R. P. (1973) *Syst. Zool. 22*, 554–565.
14. Shinkai, W., Hase, T., Yagi, T. & Matsuabara, H. (1980) *J. Biochem. (Tokyo) 87*, 1747–1756.
15. Bruschi, M. (1981) *Biochim. Biophys. Acta 1*, 219–226.
16. Higuchi, Y., Bando, S., Kusunoki, M., Matsuma, Y., Yasuoka, N., Kakudo, M., Yamanaka, T., Yagi, T. & Inokuchi, H. (1981) *J. Biochem. (Tokyo) 89*, 1659–1662.
17. Haser, R., Pierrot, M., Frey, M., Payan, F., Astier, J. P., Bruschi, M. & LeGall, J. (1979) *Nature (Lond.) 282*, 806–810.
18. Pierrot, M., Haser, R., Frey, M., Payan, F. & Astier, J. P. (1982) *J. Biol. Chem. 257*, 14341–14348.
19. Higuchi, Y., Kusanaki, M., Yasuaka, N., Kakudo, M. & Yagi, T. (1981) *J. Biochem (Tokyo) 90*, 1571.
20. Ono, K., Keisaku, K. & Kimmer, K. (1975) *J. Chem. Phys. 63*, 1640–1642.
21. Drucker, H. L., Campbell, L. & Woody, R. W. (1970) *Biochimistry 9*, 1519–1527.
22. LeGall, J., Bruschi-Heriaud, M. & DerVartanian, D. V. (1971) *Biochim. Biophys. Acta 234*, 499–512.
23. DerVartanian, D. V. & LeGall, J. (1974) *Biochim. Biophys. Acta 346*, 79–99.
24. DerVartanian, D. V., Xavier, A. V. & LeGall, J. (1978) *Biochimie (Paris) 60*, 315–320.
25. Xavier, A. V., Moura, J. J. G., Moura, I., LeGall, J. & DerVartanian, D. V. (1979) *Biochimie (Paris) 61*, 689–695.
26. McDonald, C. C., Philips, W. D. & LeGall, J. (1974) *Biochemistry 13*, 1952–1959.
27. Dobson, C. M., Hoyle, N. J., Geraldes, C. F., Bruschi, M., LeGall, J., Wright, P. E. & Williams, R. J. P. (1974) *Nature (Lond.) 249*, 425–429.
28. Xavier, A. V. & Moura, J. J. G. (1978) *Biochimie (Paris) 60*, 327–338.
29. Moura, I., Moura, J. J. G., Santos, H. & Xavier, A. V. (1980) *Cienc. Biol. 5*, 189–191.
30. Moura, J. J. G., Santos, H., Moura, I., LeGall, J., Moore, G. R., Williams, R. J. P. & Xavier, A. V. (1982) *Eur. J. Biochem. 127*, 151–155.
31. Santos, H., Moura, J. J. G., Moura, I., LeGall, J. & Xavier, A. V. (1984) *Eur. J. Biochem. 141*, 283–296.
32. Xavier, A. V., Santos, H., Moura, J. J. G., Moura, I. & LeGall, J. (1985) *Rev. Port. Quim. 27*, 149–150.
33. Niki, K., Yagi, T., Inokuchi, H. & Kimura, K. (1977) *J. Electrochem. Soc. 124*, 1979–1991.
34. Niki, K., Yagi, T., Inokuchi, H. & Kimura, K. (1979) *J. Am. Chem. Soc. 10*, 3335–3340.
35. Sokol, W. F., Evans, D. H., Niki, K. & Yagi, T. (1980) *J. Electroanal. Chem. 108*, 107–115.
36. Bianco, P. & Haladjian, J. (1981) *Electrochim. Acta 26*, 1001–1004.
37. Bianco, P. & Haladjian, J. (1979) *Biochim. Biophys. Acta 545*, 86–93.
38. Eddowers, M. J., Elzanouska, H. & Hill, H. A. O. (1979) *Biochem. Soc. Trans. 7*, 735–737.
39. Van Leeuwen, J. W., Van Dijk, C., Grande, H. J. & Veeger, C. (1982) *Eur. J. Biochem. 141*, 283–296.
40. Cammack, R., Fauque, G., Moura, J. J. G. & LeGall, J. (1984) *Biochim. Biophys. Acta 784*, 68–74.
41. Guerlesquin, F., Bovier-Lapierre, G. & Bruschi, M. (1982) *Biochem. Biophys. Res. Commun. 105*, 530–538.
42. Hatchikian, E. C., LeGall, J., Bruschi, M. & Dubourdieu, M. (1972) *Biochim. Biophys. Acta 258*, 701–708.
43. Yagi, T. (1979) *Biochim. Biophys. Acta 548*, 96–105.
44. Liu, M. C. & Peck, H. D. Jr (1981) *J. Biol. Chem. 256*, 13159–13164.
45. Liu, M. C., (1982) Ph. D. Thesis, University of Georgia, Athens GA.
46. Nazina, T. N., Rozanova, E. P. & Kaulininskaya, T. A. (1979) *Mikrobiologiya 48*, 133–136.
47. Lespinat, P. A., Denariaz, G., Fauque, G., Toci, R., Berlier, Y. & LeGall, J. (1985) *C. R. Acad. Sci. (Paris) 301*, 707–710.
48. Postgate, J. R. & Kent, H. M. (1985) *J. Gen. Microbiol. 131*, 2119–2122.
49. Whitaker, J. R. (1963) *Anal. Chem. 35*, 1950–1953.
50 Weber, K. & Osborn, M. (1961) *J. Biol. Chem. 244*, 4406–4412.
51. Vesterberg, O. (1972) *Biochim. Biophys. Acta 247*, 11–19.
52. Senn, H., Keller, R. M. & Wuthrich, K. (1980) *Biochem. Biophys. Res. Commun. 92*, 1362–1369.
53. Starkey, R. L. (1938) *Arch. Mikrobiol. 9*, 268–304.
54. LeGall, J., Mazza, G. & Dragoni, N. (1961) *Biochim. Biophys. Acta 99*, 385–387.
55. Odom, J. M. & Peck, H. D. Jr (1984) *Annu. Rev. Microbiol. 38*, 551–592.
56. LeGall, J. & Peck, H. D. Jr (1986) *FEMS Lett.*, in the press.
57. Brautigan, D. L., Feinberg, B. A., Hoffman, B. A., Margoliash, E., Peisach, J. & Blumberg, W. E. (1977) *J. Biol. Chem. 252*, 574–582.

58. Coutinho, I., Moura, I., Moura, J. J. G., Xavier, A. V., Fauque, G. & LeGall, J. (1984) *7° Encontro Anual da Sociedade Portuguesa de Quimica*, PC 4.
59. Moore, G. R. & Williams, R. J. P. (1977) *FEBS Lett. 79*, 229 – 232.
60. Moura, I., Xavier, A. V., Moura, J. J. G., Fauque, G., LeGall, J., Moore, G. R. & Huynh, B. H. (1985) *Rev. Port. Quim. 27*, 212 – 215.
61. Palmer, G. (1984) *Biochem. Soc. Trans. 13*, 548 – 560.
62. Walker, F. A., Huynh, B. H., Scheidt, W. R. & Osvath, S. R. (1986) *J. Am. Chem. Soc.* (accepted for publication).
63. Fauque, G., Herve, D. & LeGall, J. (1979) *Arch. Microbiol. 121*, 261 – 264.
64. Fauque, G., Barton, L. L. & LeGall, J. (1980) *CIBA Found. Symp. 72*, 71 – 86.
65. Nakagawa, A., Nagashima, E., Higuchi, Y., Kusumoki, M., Matsuura, Y., Yasuoka, N., Katsube, Y., Chichara, H. & Yagi, T. (1986) *J. Biochem. (Tokyo) 99*, 605 – 606.
66. Langone, J. J., Boyle, M. D. P. & Borsos, T. (1978) *J. Immunol. 121*, 327 – 332.