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# Biochemical studies of the *c*-type cytochromes of the sulfate reducer *Desulfovibrio africanus*. Characterization of two tetraheme cytochromes $c_3$ with different specificity

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**Abstract**

Three *c*-type cytochromes were isolated and characterized from the sulfate reducer *Desulfovibrio africanus*. A basic tetraheme cytochrome $c_3$ of molecular mass 16 kDa was previously described and we have extended its characterization. Two other $c_3$-type cytochromes, not previously observed, have also been characterized. These include an acidic tetraheme cytochrome $c_3$ of molecular mass 15 kDa and an octaheme dimeric cytochrome $c_3$ with a native size of 35 kDa. This is the first report of the presence of two distinct tetraheme cytochromes $c_3$ in a *Desulfovibrio* species. The physico-chemical properties of the three cytochromes, including optical properties, iron content, cysteine and histidine content, N-terminal amino sequence and redox properties, are characteristic of cytochrome $c_3$ family. The acidic tetraheme cytochrome $c_3$ exhibited similar midpoint potential values for all four hemes ($E_{m1} = -210$ mV; $E_{m2} = -240$ mV; $E_{m3} = -260$ mV; $E_{m4} = -270$ mV), whereas in the basic tetraheme cytochrome $c_3$ one heme had a much more positive potential than the others ($E_{m1} = -90$ mV; $E_{m2} = -260$ mV; $E_{m3} = -280$ mV; $E_{m4} = -290$ mV). The acidic tetraheme cytochrome $c_3$ exhibited unique properties including amino-acid composition and poor reactivity towards hydrogenase. However, it is readily reduced by this enzyme in the presence of the basic cytochrome $c_3$. The weak reactivity of the acidic tetraheme cytochrome $c_3$ towards hydrogenase has been correlated with its low content of basic residues.

*Keywords:* Cytochrome $c_3$; Molecular property; Biological activity; Perm-selective-membrane electrode; Redox potential; (*D. africanus*)

## 1. Introduction

The sulfate-reducing bacteria are strict anaerobes that derive energy for metabolic processes from sulfate respiration. The bacteria belonging to the *Desulfovibrionacea* family are characterized by the presence of a tetraheme *c*-type cytochrome, termed cytochrome $c_3$, exhibiting a low redox potential [1]. The structure and the properties of the cytochromes $c_3$ from *Desulfovibrio* species have been extensively studied. This tetraheme cytochrome appears to be located in the periplasm [2,3], and is the potential electron donor and acceptor of the periplasmic hydrogenase [4–6]. The four hemes, mesoporphyrins, are covalently bound to the polypeptide chain through thioether linkage provided by cysteinyl residues in either a Cys-$(Xaa)_2$-Cys-His- sequence or a Cys-$(Xaa)_4$-Cys-His- sequence. These cytochromes differ from other classes of heme *c* proteins by the iron coordination, which is of the bis-histidinyl type. The four hemes generally exhibit non-identical midpoint redox potentials within the approximate range $-120$ to $-400$ mV [7], very low compared with the $+290$ mV for mitochondrial cytochrome *c* [8,9]. To date, three-dimensional structures of three tetraheme cytochromes $c_3$ have been determined at a high resolution by X-ray diffraction [10–12]. The most striking feature of the structure of these proteins is the compact organization of the four heme groups with a relatively high degree of solvent exposure. Despite the small degree of sequence identity among these cytochromes, no significant differences in the overall structure and spatial arrangement of the four heme groups have been observed [13].

Following the discovery of cytochrome $c_3$ in *Desulfovibrio* species, various other *c*-type cytochromes have

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been found, indicating that the cytochrome equipment of these microorganisms is complex. Four different *c*-type cytochromes have been described in various species of *Desulfovibrio*: the monoheme cytochrome *c*-553 ($M_r$ 9000), which has been proposed as the natural electron acceptor of the formate dehydrogenase system [14], the tetraheme cytochrome $c_3$ ($M_r$ 13000–15000) [15], the octaheme cytochrome $c_3$ ($M_r$ 26000–30000) comprising two identical subunits [16–18] and a high-molecular-weight cytochrome *c* ($M_r$ 65000) termed Hmc, containing sixteen hemes per molecule [19]. The tetraheme cytochrome $c_3$ is the only *c*-type cytochrome to have been detected in all species of *Desulfovibrio* examined [1].

Recent studies showed that the redox transfer chains of sulfate-reducing bacteria are much more complex and diverse than previously thought [20]. Therefore, understanding of the bioenergetics of these microorganisms requires an inventory of their electron-transfer components. Various redox proteins have recently been characterized from the sulfate reducer *Desulfovibrio africanus*, which was found on the basis of its rRNA sequences to be significantly different from *Desulfovibrio vulgaris* Hildenborough, *Desulfovibrio gigas* and *Desulfovibrio desulfuricans* Norway 4 [21]. These proteins include a rubredoxin [22], three distinct ferredoxins [23–28], a [NiFeSe] hydrogenase [29] and a pyruvate-ferredoxin oxidoreductase [30]. However, the *c*-type cytochromes of this organism have not been extensively characterized.

In this paper, we report, to our knowledge for the first time, the presence of two distinct tetraheme cytochromes $c_3$ in a *Desulfovibrio* species. *Desulfovibrio africanus* contains a basic tetraheme cytochrome $c_3$ previously described [31] and an acidic cytochrome $c_3$ which exhibits unique properties. The molecular, electrochemical and biological properties of these two cytochromes as well as those of an octaheme cytochrome $c_3$, also present in this microorganism, have been thoroughly investigated.

## 2. Materials and methods

### 2.1. Organisms and culture conditions

*Desulfovibrio africanus* strain Benghazi (NCIB 8401) was grown at 37°C in a basic lactate-sulfate medium [32]. Large-scale cultures were grown anaerobically in a 300 l fermentor and the cells were harvested at the stationary phase after 48 h growth.

### 2.2. Preparation of cell extracts

Freshly thawed cells of *D. africanus* (700 g wet weight) previously stored at −80°C were passed once through a French pressure cell at 100 MPa and the soluble extract that contained the *c*-type cytochromes was prepared as reported previously [30].

### 2.3. Purification of D. africanus c-type cytochromes

In this study, the tetraheme cytochrome $c_3$ previously described [31] is referred to as basic tetraheme cytochrome $c_3$ and the newly discovered cytochrome $c_3$ is referred to as acidic tetraheme cytochrome $c_3$. Unless otherwise noted, all operations were performed at 4°C and the buffers used were at pH 7.6.

The soluble protein extract was treated with DEAE-cellulose (Whatman, DE-52) equilibrated with 10 mM Tris-HCl (pH 7.6) by a batchwise technique as previously described [22]. The unadsorbed protein fraction contained the basic cytochrome $c_3$, whereas the acidic proteins adsorbed on DEAE-cellulose and eluted with 1 M Tris-HCl contained ferredoxins, rubredoxin, desulfoviridin and two acidic *c*-type cytochomes, namely the acidic tetraheme cytochrome $c_3$ and the octaheme cytochrome $c_3$ as shown below.

#### 2.3.1. Purification of the tetraheme and octaheme acidic cytochromes $c_3$

After dialysis, the acidic proteins were loaded onto a column of DEAE-cellulose (3.5 × 25 cm) equilibrated with 20 mM Tris-HCl. The acidic cytochromes were eluted with a discontinuous gradient (200 mM-340 mM, total volume 500 ml) of the same buffer, at about 320 mM Tris-HCl buffer. After dialysis of the cytochrome fraction, the two *c*-type cytochromes were purified on a Q-Sepharose Fast Flow (Pharmacia) column (2.6 × 20 cm) which was eluted with a linear NaCl gradient (50–500 mM, total volume 500 ml) in 20 mM Tris-HCl. The tetraheme and the octaheme cytochromes were eluted respectively at about 220 mM and 290 mM NaCl, respectively. The acidic tetraheme cytochrome $c_3$ was then filtered through a Sephadex G-50 column (5 cm × 100 cm) equilibrated with 10 mM Tris-HCl. Finally, the protein was further purified by preparative HPLC using an UltroPAC TSK DEAE-5PW column (2.15 cm × 15 cm) (LKB). The tetraheme cytochrome $c_3$ was applied to the column equilibrated with 20 mM Tris-HCl (pH 7.6) and was eluted with a linear gradient (160–330 mM NaCl, total volume 200 ml) in the same buffer, using a flow rate of 1.5 ml/min. The resulting acidic cytochrome *c* was judged to be pure from SDS-PAGE and exhibited a purity index ($A_{552red} - A_{570red}/A_{280ox}$) of 2.6 [32].

The fraction containing the octaheme cytochrome $c_3$ was also filtered through a Sephadex G-50 column in the same conditions as reported above. It was then loaded onto a column of Q-Sepharose Fast Flow (2.6 × 15 cm) equilibrated with 20 mM Tris-HCl buffer and eluted with a linear gradient (80–360 mM NaCl, total volume 220 ml) in 20 mM Tris-HCl. As a last step, the octaheme cytochrome was purified by preparative HPLC using an UltroPac TSK DEAE-PW5 column as indicated above. After adsorption on the column, the cytochrome was eluted with a linear gradient (100–420 mM NaCl, total volume 250 ml) in 20

mM Tris-HCl. The resulting octaheme cytochrome $c_3$ was judged to be pure from SDS-PAGE and had a purity index of 2.7.

*2.3.2. Purification of the basic tetraheme cytochrome $c_3$*

After the treatment of the soluble protein extract with DEAE-cellulose, the unadsorbed protein fraction containing the basic tetraheme cytochrome was brought to 50% saturation with ammonium sulfate and the resulting precipitate was discarded. The supernatant was brought to 67% saturation and the resulting supernatant was dialysed. It was then loaded onto an anion-exchange column (S-Sepharose Fast Flow, Pharmacia) (2.6 × 20 cm) equilibrated with 10 mM potassium phosphate buffer (pH 6.5). The basic cytochrome $c_3$ was eluted with a linear gradient (10–250 mM potassium phosphate (pH 6.5), total volume 400 ml) at about 200 mM. Finally, the cytochrome fraction was filtered through a Sephadex G-50 column as previously described. The resulting basic cytochrome $c_3$ was judged to be pure from SDS-PAGE and had a purity index of 2.8.

*2.4. Purification of D. africanus hydrogenase*

The periplasmic [NiFeSe] hydrogenase of *D. africanus* was used to study the biological activity of the three cytochromes [29]. Partial purification of the hydrogenase was performed using the cell wash fraction, which contains mostly the periplasmic proteins including hydrogenase and the *c*-type cytochromes. Freshly thawed cells of *D. africanus* (240 g wet weight) previously stored at −80°C were suspended in 200 ml of 30 mM Tris-HCl (pH 8) −5 mM EDTA and stirred for 20 min at room temperature. The suspension was centrifuged at 30 000 × *g* for 30 min and the supernatant corresponding to the wash fraction was subsequently used. Three chromatography steps were then carried out, including ion-exchange chromatography on DEAE-cellulose and Q-Sepharose FF and a gel-filtration on Ultrogel AcA 34 (Sepracor). At this stage, the hydrogenase fraction exhibited a specific activity of 150 units/mg protein in the $H_2$ uptake assay and was devoid of cytochrome. Before use for enzyme assays, *D. africanus* hydrogenase was activated in reducing conditions as described previously [29].

*2.5. Analytical procedures and molecular mass determination*

The molecular mass of the native *c*-type cytochromes was determined by analytical ultracentrifugation as previously reported [30]. Analytical gel electrophoresis was performed using the method of Davis [33], whereas SDS/PAGE was carried out following the method of Laemmli [34]. The molecular mass of the subunit of the octaheme cytochrome $c_3$ was estimated using this latter procedure, with the following markers: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

The iron content of the cytochromes was estimated by plasma emission spectroscopy using a Jobin-Yvon model JY38 apparatus. The protein concentration of the pure proteins was determined by quantitative amino-acid analysis using a Beckman model system 6300 amino-acid analyzer. Heme was removed using the method of Ambler [35] and cysteine was determined as *S*-carboxymethylcysteine [36]. Amino terminal sequence analyses of the *S*-carboxymethylated apoproteins were performed by stepwise Edman degradation using a gas-phase sequencer (Models 470 A and 473 A, Applied Biosystems). In order to elucidate the sequence of the blocked N-terminal of the tetraheme acidic cytochrome $c_3$, the *S*-carboxymethylated protein was tentatively treated with a mixture of methanol/HCl [37] as well as with 5-oxopropyl-peptidase according to [38]. Finally, the amino-acid sequence of the N-terminal region of the protein was obtained after cyanogen bromide cleavage of 1 mg of *S*-carboxymethylated apocytochrome in 70% TFA [37]. The fragments resulting from cyanogen bromide cleavage were isolated by reverse-phase HPLC on a $C_{18}$ 10 μm Vidac column (4.6 mm × 250 mm) with a linear gradient from 0 to 90% of acetonitrile/TFA (0.06%) in water/TFA (0.1%). The peptide eluted with 36–38% of acetonitrile and corresponding to the N-terminal region of the tetraheme acidic cytochrome $c_3$ was subjected to amino-acid sequence analysis.

*2.6. Electrochemistry*

Cyclic voltammetry (CV) and square-wave voltammetry (SWV) experiments were carried out as previously reported [39], using the permselective-membrane electrode as the working electrode [40]. The scan rate was 10 mV $s^{-1}$ (CV) or 2 mV $s^{-1}$ (SWV). Unless otherwise specified, all potentials reported are referred to the Ag/AgCl (saturated NaCl) reference electrode. Potentials vs. the standard hydrogen electrode (SHE) can be obtained by adding 210 mV. The individual oxido-reduction potentials of the hemes in the cytochromes were determined by using the EG&G PARC COOL M271 kinetic analysis software. All experiments were carried out at room temperature (about 23°C), using 0.5 M Tris-HCl buffer (pH 7.6) as supporting electrolyte and the solutions were deoxygenated by bubbling with high-purity nitrogen.

*2.7. Enzyme assays*

Reduction of cytochromes by reactivated hydrogenase was measured spectrophotometrically at 553 nm under hydrogen [41].The reaction mixture contained in a cuvette with a serum stopper (total volume 1 ml), *D. africanus* hydrogenase, pure electron carrier and 50 μmol of Tris-HCl

buffer (pH 8.5). The reaction mixture was bubbled with purified hydrogen (Air Liquide N55) for 20 min and the cells were incubated at 30°C. The reactions were started by injecting the enzyme.

Hydrogen evolution activity of *D. africanus* hydrogenase from dithionite in the presence of *c*-type cytochromes was measured using the manometric method [42]. The reaction mixture contained in a Warburg vessel (total volume 3 ml), *D. africanus* hydrogenase, pure electron carrier and 450 $\mu$mol potassium phosphate buffer (pH 7). The center well contained 0.05 ml of NaOH (10 M).The gas phase was argon and the temperature 30°C. Reactions were started by adding sodium dithionite (5 mg) from the sidearm.

The sulfur reductase activity of the three cytochromes was investigated by the manometric method under $H_2$ as reported by Fauque et al. [43] using pure *Desulfovibrio gigas* hydrogenase [44].

## 3. Results

### 3.1. Homogeneity and molecular mass

The two tetraheme cytochromes $c_3$ and the octaheme cytochrome $c_3$ were judged to be pure both by polyacrylamide gel electrophoresis and by the presence of a single symmetrical peak after ultracentrifugation. The molecular masses of acidic and basic tetraheme cytochromes $c_3$ determined by equilibrium sedimentation were estimated to be $15.4 \pm 0.7$ kDa and $16.5 \pm 0$ 5 kDa, respectively, using a partial specific volume of 0.72 ml $g^{-1}$. The molecular mass of the native octaheme cytochrome $c_3$ determined by analytical ultracentrifugation was estimated to be $34.7 \pm 2$ kDa after dialysis against 2 M NaCl/50 mM Tris-HCl buffer (pH 8.0). The high concentration of salt was necessary for the stability of the protein during ultracentrifugation. SDS-gel (13%) electrophoresis of octaheme cytochrome $c_3$ showed a single band of molecular mass 20 kDa. These results indicate that this cytochrome is composed of two identical subunits.

### 3.2. Spectral properties

The absorption spectra of oxidized and reduced forms of purified acidic and basic tetraheme cytochromes and octaheme cytochrome $c_3$ are similar to homologous cytochromes already described [2,16,45,46]. The lack of the 695 nm absorption band for the oxidized proteins indicates that methionine is not an iron ligand for these *c*-type cytochromes. Millimolar extinction coefficients of the three cytochromes are presented in Table 1. The values found for the eight hemes of the dimeric molecule of the octaheme cytochrome $c_3$ are comparable to the values found for the four hemes in the acidic and basic tetraheme cytochromes $c_3$.

Table 1
Optical spectral data of *D. africanus* *c*-type cytochromes

| Acidic tetraheme cytochrome $c_3$ ox | | Basic tetraheme cytochrome $c_3$ ox | | Octaheme cytochrome $c_3$ ox | |
|---|---|---|---|---|---|
| λ nm | $\epsilon$ ($mM^{-1}$ $cm^{-1}$) | λ (nm) | $\epsilon$ ($mM^{-1}$ $cm^{-1}$) | λ (nm) | $\epsilon$ ($mM^{-1}$ $cm^{-1}$) |
| 280 | 49100 | 280 | 39300 | 280 | 120000 |
| 410 | 472120 | 411 | 482500 | 409 | 1123000 |
| 532 | 43120 | 531 | 42900 | 533 | 101800 |
| acidic tetraheme cytochrome $c_3$ red | | basic tetraheme cytochrome $c_3$ red | | octaheme cytochrome $c_3$ red | |
| 420 | 696400 | 420 | 711200 | 420 | 1539000 |
| 524 | 62120 | 524 | 60700 | 524 | 144600 |
| 553 | 105400 | 553 | 113000 | 553 | 246000 |

### 3.3. Heme content

The absorption value of the pyridine hemochrome of the acidic and basic cytochromes $c_3$ yielded respectively 3.4 and 3.6 heme *c* groups per molecule using a coefficient of 31.18 $mM^{-1}$ $cm^{-1}$ at 550 nm [47]). Moreover, the average values of iron content determined by plasma emission spectroscopy for these two cytochromes are respectively 3.8 and 4.0 iron atoms per protein molecule. These results are indicative of the presence of four hemes per molecule in the two *D. africanus* cytochrome $c_3$ ($M_r$ 15000) The absorption value of the cytochrome $c_3$ ($M_r$ 35000) yields 7.1 heme *c* groups per molecule and the average value of iron content is 9.2 iron atoms per protein molecule, which results are in agreement with the presence of eight hemes in this cytochrome.

### 3.4. Amino-acid composition

Table 2 gives the amino-acid composition of the three *D. africanus* cytochromes. These proteins possess the required number of cysteine (eight or sixteen residues) to bind four or eight hemes per molecule for the tetraheme and the octaheme cytochromes $c_3$, respectively, as well as the required number of histidine residues, since each iron coordination in both tetraheme and octaheme cytochromes $c_3$ is of the bis-histidinyl type [1]. Comparison of the amino-acid composition of these cytochromes is consistent with the presence of three distinct *c*-type cytochromes in *D. africanus*. In particular, comparison of the amino-acid composition of acidic and basic tetraheme cytochromes $c_3$ reveals important differences in the numbers of lysine residues and acidic residues (Table 2). In addition, the data show that the acidic tetraheme cytochrome $c_3$ has a lower content of serine and threonine and contains an arginine residue that is not present in the basic cytochrome $c_3$. The most striking feature of the amino-acid composition of the acidic tetraheme cytochrome $c_3$ is its low content of basic residues. It contains only six residues of lysine + arginine per molecule. This property distinguishes it from all the

Table 2
Amino-acid composition of the three *c*-type cytochromes of *D. africanus*

| Amino acids | No. of amino acids in : | | | |
|---|---|---|---|---|
| | acidic tetraheme cytochrome $c_3$ | basic tetraheme cytochrome $c_3$ | | octaheme cytochrome $c_3$ subunit |
| Lysine | 5 | 15 | 15 [a] | 6 |
| Histidine | 10 | 8 | 8 | 9 |
| Arginine | 1 | 0 | 0 | 6 |
| Tryptophan | n.d. [1] | n.d. | 1 | n.d. |
| Aspartic acid | 15 | 9 | 8 | 10 |
| Threonine | 4 | 9 | 9 | 13 |
| Serine | 3 | 7 | 6 | 12 |
| Glutamic acid | 18 | 10 | 9 | 19 |
| Proline | 5 | 6 | 7 | 12 |
| Glycine | 10 | 7 | 7 | 8 |
| Alanine | 9 | 14 | 13 | 13 |
| Cysteine | 8 | 8 | 8 | 8 |
| Valine | 8 | 5 | 4 | 7 |
| Methionine | 2 | 3 | 3 | 1 |
| Isoleucine | 1 | 2 | 2 | 1 |
| Leucine | 5 | 4 | 4 | 7 |
| Tyrosine | 1 | 1 | 1 | 2 |
| Phenylalanine | 2 | 4 | 4 | 8 |
| Total residues | 107 | 112 | 109 | 136 |
| Molecular weight [b] | 11 616 | 12 009 | 11 790 | 14 925 |

[1] n.d., not determined.
[a] From Singleton et al. [31].
[b] Minimum molecular weight calculated from amino-acid composition.

tetraheme cytochromes $c_3$ as well as the octaheme cytochromes $c_3$ so far characterized [16,48–54]. Moreover, the amino-acid composition of the two tetraheme cytochromes is in keeping with the important difference of the two protein isoelectric points. As shown in Table 2, the amino-acid composition of the basic cytochrome $c_3$ reported in this study is very close to that previously reported [31].

The comparison of the amino-acid compositions of acidic tetraheme cytochrome $c_3$ and octaheme cytochrome $c_3$ which is a homodimer, shows that the former cytochrome is not the subunit of the second cytochrome. Indeed, the numbers of arginine, aspartic acid, threonine, serine and phenylalanine residues of the acidic tetraheme cytochrome $c_3$ are clearly different from the corresponding numbers for the octaheme cytochrome $c_3$ subunit.

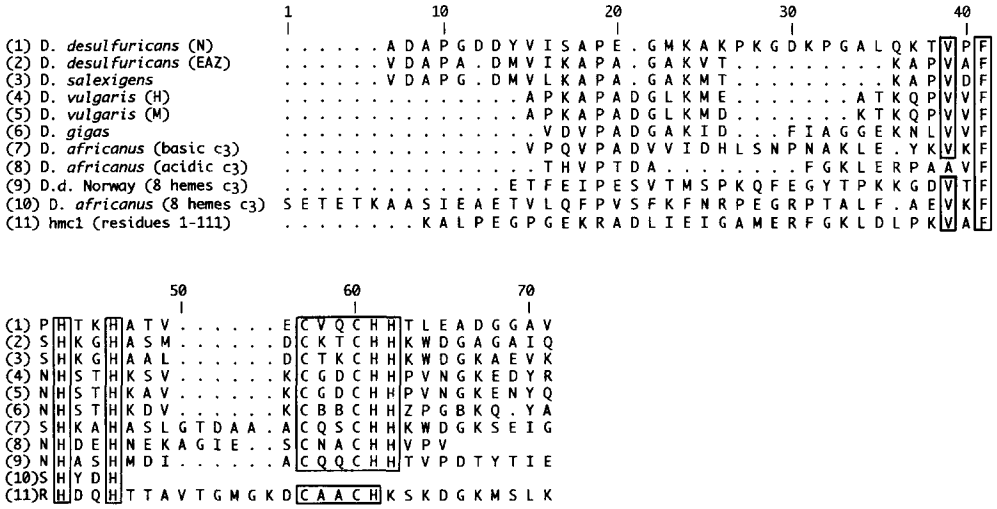


Fig. 1. Alignment of the N-terminal amino-acid sequences of tetraheme cytochrome $c_3$ from *D. desulfuricans* Norway [53] and El Agheila Z [48], *D. salexigens* [49], *D. vulgaris* Hildenborough [50] and Miyazaki [52], *D. gigas* [51], *D. africanus* (basic and acidic cytochromes $c_3$), octaheme cytochrome $c_3$ from *D. desulfuricans* Norway [54] and *D. africanus* and the first domain of cytochrome Hmc from *D. vulgaris* Hildenborough [19]. Residues common to all proteins, heme attachment sites and histidine-containing regions are enclosed in boxes.

The minimal molecular weights calculated from the amino-acid compositions were 14072, 14465 and 17381 for the acidic and the basic tetraheme cytochromes $c_3$ and the octaheme cytochrome $c_3$ subunit, respectively, including four heme groups per molecule or subunit. These values are in good agreement with the values obtained by analytical ultracentrifugation, taking into account that the octaheme cytochrome $c_3$ contains two identical subunits per molecule.

### 3.5. N-terminal sequences

The amino-acid sequences of the N-terminals of the basic tetraheme cytochrome $c_3$ and octaheme cytochrome $c_3$ were determined up to 55 and 45 residues, respectively (Fig. 1). Acidic tetraheme cytochrome $c_3$ exhibits a blocked N-terminal extremity which could not be unblocked by treatments with methanol/HCl [37] and 5-oxopropyl-peptidase [38]. The sequence of the N-terminal region of the acidic tetraheme cytochrome $c_3$ was finally determined after cyanogen bromide cleavage of the apoprotein and purification of a peptide which is homologous to the N-terminal part of the other tetraheme cytochromes. The 40 first residues of this peptide were identified and compared with the N-terminal sequences of the two other cytochromes of *D. africanus* as well as with the homologous cytochromes of the other *Desulfovibrio* species (Fig. 1). The sequences of the *c*-type cytochromes of *D. africanus* can be aligned on the invariant sequences found in the N-terminal extremity of all homologous cytochromes from *Desulfovibrio* species, including the sequence V-F and the histidine cluster -H-X-X-H- involved in iron coordination. In addition, both tetraheme cytochromes $c_3$ for which longer parts of their amino-acid sequences were determined exhibited the consensus heme 1 binding sequence -C-X-X-C-H-H-. The missing sequence at the N-terminal of the acidic tetraheme cytochrome $c_3$ is expected to be approximately 10–15 residues long, from comparison with the known sequences of homologous cytochromes of five different *Desulfovibrio* species (Fig. 1). The data indicate that the octaheme cytochrome $c_3$, which has an N-terminal sequence of 41 residues preceding the histidine cluster, has the longest N-terminal extremity. It is also to be noted that among the amino-terminal sequences of the homologous $c_3$ cytochromes from *Desulfovibrio* species reported in Fig. 1, the acidic tetraheme cytochrome $c_3$ from *D. africanus* shows the highest degree of homology with the first domain of Hmc from *D. vulgaris* Hildenborough.

### 3.6. Electrochemical properties of the three D. africanus c-type cytochromes

As with other $c_3$ cytochromes [55], cyclic voltammetry shows that the three *D. africanus* cytochromes $c_3$ are quasi-reversible electrochemical systems, and so the electrode surface does not need to be promoted for electron-transfer.

#### 3.6.1. Study of basic tetraheme cytochrome $c_3$ at the PME

Typical cyclic and square-wave voltammograms, obtained at the PME for the 255 $\mu$M basic cytochrome $c_3$ solution trapped between the permselective membrane and the pyrolytic graphite surface are shown in Fig. 2A and 2B, respectively. Two steps of oxidation or reduction are observed. The symmetrical shape of the peaks and the peak separation $\Delta E_{p1}$ ($= E_{pa1} - E_{pc1}$) = 6 mV and $\Delta E_{p2}$ ($= E_{pa2} - E_{pc2}$) = 13 mV agree respectively with a diffusionless, thin-layer voltammetry process and a quasi-reversible electrode reaction [56]. The means of anodic ($E_{pa1} = -0.502$, $E_{pa2} = -0.300$) and cathodic ($E_{pc1} = -0.508$, $E_{pc2} = -0.313$) CV peak potentials (Fig. 2A) and SWV peak potentials ($E_{p1} = -0.505$, $E_{p2} = -0.300$ V) Fig. 2B) yield common values of $-510 \pm 10$ mV and $-300 \pm 10$ mV for both step potentials. The same values were obtained for a more diluted (67 $\mu$M) basic cytochrome $c_3$ solution. We can observe that the first step value $-510$ mV (i.e., $-300$ mV vs. SHE) is rather close to the value ($-276$ mV) obtained by Singleton et al. [31] from electrochemical titration with sodium dithionite.

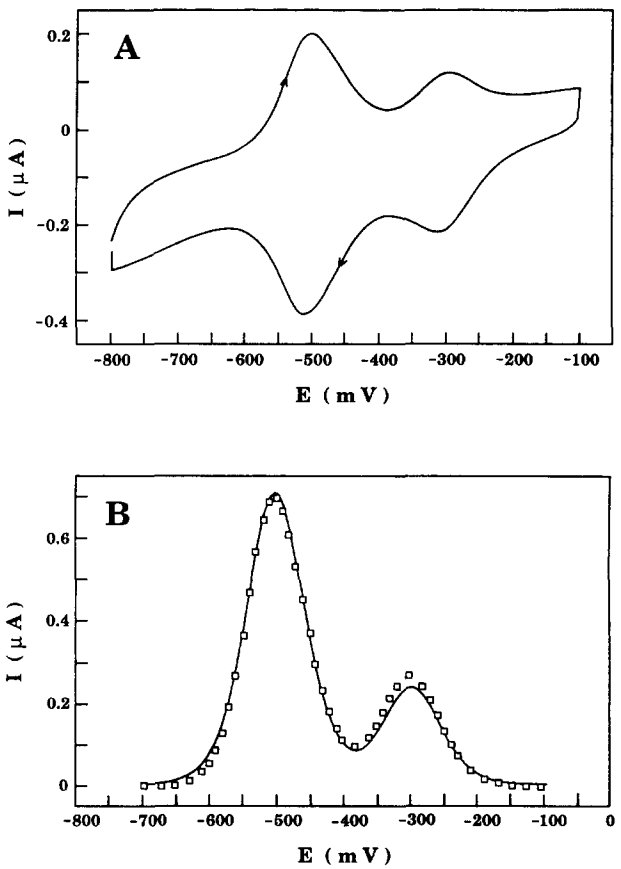


Fig. 2. Cyclic (A) and square-wave (B) voltammograms of 255 $\mu$M basic *D. africanus* tetraheme cytochrome $c_3$. For SWV, the points are experimental data and the curve is calculated using the potential values given in Table 3.

*3.6.2. Study of acidic tetraheme cytochrome $c_3$ at the PME*

Cyclic voltammetry (CV) and square-wave voltammetry (SWV) curves, at the PME, for the 71 μM cytochrome are given in Fig. 3A and 3B, respectively. In contrast to basic cytochrome $c_3$, only one oxido-reduction step is observed. The mean of anodic ($E_{pa} = -467$ mV) and cathodic ($E_{pc} = -500$ mV) CV peak potentials (Fig. 3A) and SWV peak potential ($E_p = -472$ mV) (Fig. 3B) yield a common value of $-480 \pm 10$ mV for the step potential that actually corresponds to oxido-reduction of four hemes that have close potential values.

*3.6.3. Study of octaheme cytochrome $c_3$ at the PME*

Cyclic voltammetry and square-wave voltammetry curves, at the PME, for 30 μM cytochrome are given in Fig. 4A and Fig. 4B, respectively. In this case the presence of a shoulder, more evident in the SWV curve, suggests more marked differences between oxido-reduction potentials than in acidic cytochrome $c_3$. The mean of anodic ($E_{pa} = -526$ mV) and cathodic ($E_{pc} = -535$ mV) CV peak potentials (Fig. 4A) and SWV peak potential ($E_p = -528$ mV) (Fig. 4B) yield a common value of $-530 \pm 10$ mV for the main step potential. The individual oxido-reduction potentials of the three cytochromes, deduced from the simulation of the experimental SWV curves (Fig. 2B, Fig. 3B and Fig. 4B) are collated in Table 3.

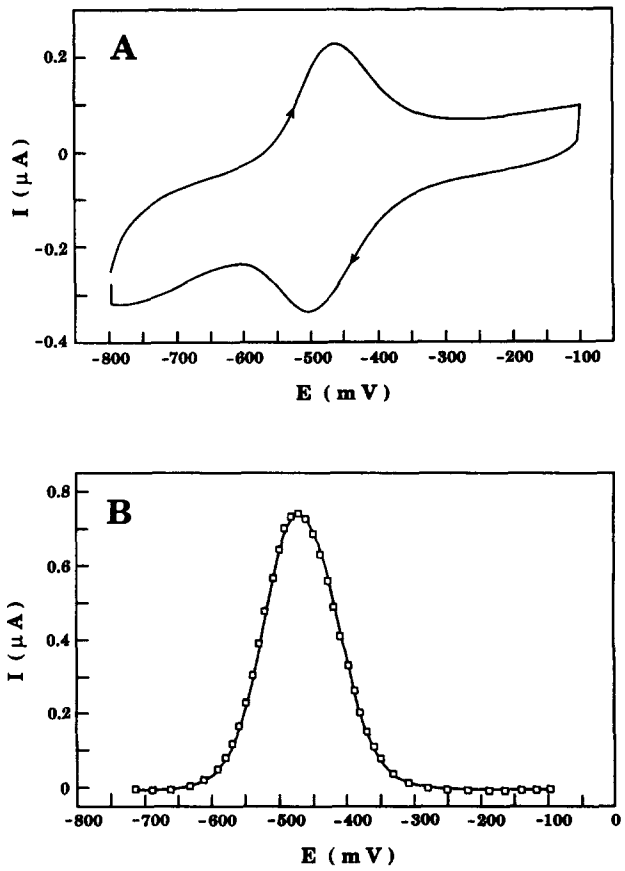


Fig. 3. Cyclic (A) and square-wave (B) voltammograms of 71 μM acidic *D. africanus* tetraheme cytochrome $c_3$. For SWV, the points are experimental data and the curve is calculated using the potential values given in Table 3.

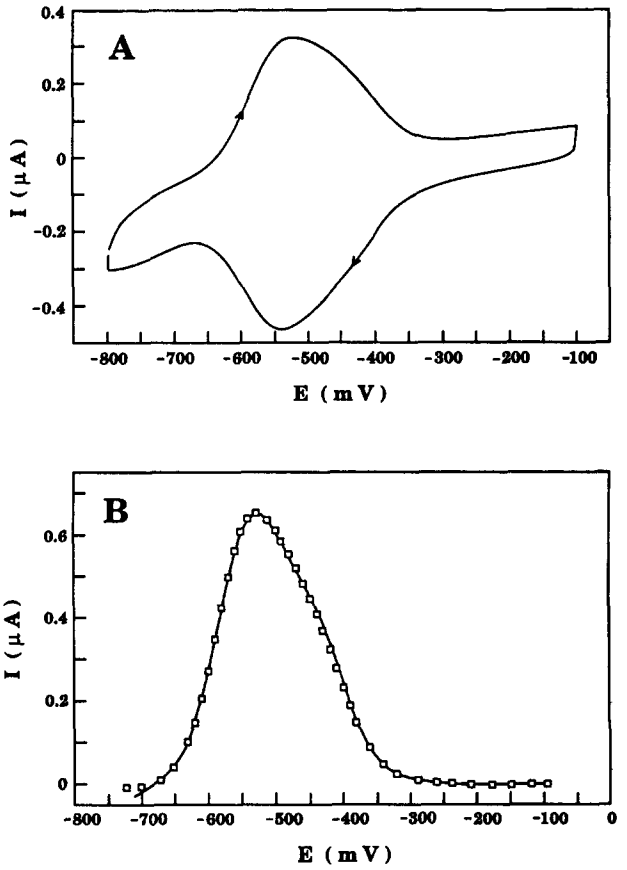


Fig. 4. Cyclic (A) and square-wave (B) voltammograms of 30 μM *D. africanus* octaheme cytochrome $c_3$. For SWV, the points are experimental data and the curve is calculated using the potential values given in Table 3.

*3.7. Biological activity of the three D. africanus c-type cytochromes*

*3.7.1. Reactivity with hydrogenase*

The reduction of *D. africanus* *c*-type cytochromes and $H_2$ evolution from reduced cytochromes were measured in the presence of activated [NiFeSe] hydrogenase from the same microorganism (see Materials and Methods). The

Table 3
Oxidoreduction potentials of the three *c*-type cytochromes of *D. africanus*

| Acidic tetraheme cytochrome $c_3$ | Basic tetraheme cytochrome $c_3$ | Octaheme cytochrome $c_3$ |
|---|---|---|
| −210 | −90 | −220 |
| −240 | −260 | −280 |
| −260 | −280 | −310 |
| −270 | −290 | −350 |

Potentials (mV) are referred to SHE. These values are deduced from the simulation of experimental SWV curves (Figs. 2B, 3B and 4B).

Table 4
Reduction of *D. africanus* cytochromes from hydrogen and hydrogen evolution from reduced cytochromes catalyzed by hydrogenase

| Electron carrier added | Amount of carrier ($\mu$M) | | Hydrogenase activity | |
|---|---|---|---|---|
| | (A) | (B) | (A)rate of carrier reduction (units/mg) | (B)rate of $H_2$ evolution (units/mg) |
| None | – | 0 | – | 0.01 |
| Basic tetraheme cytochrome $c_3$ | 5 | 7 | 13.25 | 0.58 |
| Acidic tetraheme cytochrome $c_3$ | 5 | 6 | 0.21 | 0.057 |
| Octaheme cytochrome $c_3$ | 5 | 2 | 8.36 | 1.96 |
| Basic tetraheme cytochrome $c_3$ | 3 | – | | |
| + | | | 11.4 | – |
| Acidic tetraheme cytochrome $c_3$ | 5 | – | | |

The reduction of *D. africanus* under $H_2$ (A) and $H_2$ evolution activity from reduced cytochromes (B) were measured as reported in Materials and Methods. (A) The reaction mixture contained: *D. africanus* hydrogenase, 0.105 $\mu$g, 2.1 $\mu$g and 0.052 $\mu$g protein, with the basic tetraheme cytochrome $c_3$, acidic tetraheme cytochrome $c_3$ and octaheme cytochrome $c_3$, respectively; pure cytochromes, as indicated in the table. (B) The reaction mixture contained: *D. africanus* hydrogenase, 0.125 mg, 2.5 mg and 0.05 mg protein, with the basic tetraheme cytochrome $c_3$, acidic tetraheme cytochrome $c_3$ and octaheme cytochrome $c_3$, respectively; pure cytochromes, as indicated in the table. The control without cytochrome contained 2.5 mg of hydrogenase.

three cytochromes from *D. africanus* were fully reduced by hydrogenase under hydrogen, resulting in the appearance of the $\alpha$ peak of the reduced cytochrome at 553 nm (Table 4). A 1 min lag was observed only with acidic tetraheme cytochrome $c_3$, whose reduction required a high concentration of hydrogenase. The maximum velocities obtained in these experiments were 13.25, 0.21 and 8.36 $\mu$mol $H_2$ consumed per min per mg protein with basic tetraheme cytochrome $c_3$, acidic tetraheme cytochrome $c_3$ and octaheme cytochrome $c_3$, respectively (Table 4A). These rates of *D. africanus* c-type cytochrome reduction were 9-, 571- and 14.3-fold less than the rate of methyl viologen reduction, respectively. Hydrogen evolution activity from reduced cytochromes was significantly lower than $H_2$ uptake activity in the presence of the same electron carriers, especially for the basic tetraheme cytochrome $c_3$ (Table 4B). Comparison of the reactivity of the three cytochromes with hydrogenase in both activities clearly showed that the tetraheme acidic cytochrome $c_3$ exhibited weak coupling activity with hydrogenase as compared to the two other c-type cytochromes. The effectiveness of basic tetraheme cytochrome $c_3$ and octaheme cytochrome $c_3$ in the electron transfer with hydrogenase was 63- and 40-fold higher in the reduction of cytochromes from hydrogen and 10- and 34-fold higher in $H_2$ evolution activity as compared to that of acidic tetraheme cytochrome $c_3$ in the same reactions, respectively (Table 4). However, it is to be noted that the rate of acidic tetraheme cytochrome $c_3$ reduction under hydrogen was greatly increased in the presence of basic tetraheme cytochrome $c_3$ (Table 4A). The data show that the two cytochromes are fully reduced in about 5 min. The kinetics of reduction of both cytochromes is linear in the assay conditions and their rate of reduction (11.4 units/mg) was close to that of the reduction of the basic cytochrome $c_3$ alone (13.25 units/mg) (Table 4A). This result indicated that the basic tetraheme cytochrome $c_3$ is an efficient intermediary electron carrier between hydrogenase and the acidic tetraheme cytochrome $c_3$.

Table 5
Sulfur reductase activity of the *D. africanus* cytochromes $c_3$

| | nmol of cytochromes in the assay | Specific activity [a] | Hydrogen consumed ($\mu$mol) | Sulfide formed ($\mu$mol) |
|---|---|---|---|---|
| Basic tetraheme cytochrome $c_3$ | 20 | 2.8 | 10.2 | 11.4 |
| | 17 | 3 | 12.4 | 15 |
| Octaheme cytochrome $c_3$ | 12 | 4 | 12.2 | 18 |
| | 10 | 4 | 10 | 10 |

[a] One unit of sulfur reductase activity catalyzes the consumption of 1 $\mu$mol of hydrogen used for sulfur reduction in the assay conditions.

The apparent $K_m$ values of *D. africanus* hydrogenase for basic tetraheme cytochrome $c_3$ and octaheme cytochrome $c_3$ calculated from double-reciprocal plots have been found to be 1 and 0.4 $\mu$M at pH 7, respectively, as measured in the electron-carrier-dependent $H_2$ evolution activity. These values are in agreement with those obtained for the homologous cytochromes from *D. gigas* [5]. The $K_m$ for *D. africanus* acidic tetraheme cytochrome $c_3$ in the same reaction was not determined because large amounts of hydrogenase were required for its estimation.

### *3.7.2. Reduction of colloidal sulfur by D. africanus c-type cytochromes*

The sulfur reductase activity of *D. africanus* cytochromes was determined using the manometric method under an $H_2$ atmosphere with pure [NiFe] hydrogenase from *D. gigas* [43]. In these conditions, the acidic tetraheme cytochrome $c_3$, which was not reduced by this enzyme in contrast to the two other cytochromes, exhibited no activity. The results reported in Table 5 indicate that the basic tetraheme cytochrome $c_3$ and the octaheme cytochrome $c_3$ readily reduced colloidal sulfur, although their activities are lower than those obtained with various homologous cytochromes $c_3$ [43]. The amounts of hydrogen consumed and those of sulfide produced during the reaction appeared to be stoichiometric.

## 4. Discussion

We have isolated and characterized three different *c*-type cytochromes from the sulfate reducer *Desulfovibrio africanus*. One of these was previously reported by Singleton et al. [31] and we have extended its characterization. We have confirmed that this cytochrome of molecular mass 16 kDa that exhibits basic properties is a tetraheme cytochrome $c_3$. In addition, an acidic tetraheme cytochrome $c_3$ of molecular mass 15 kDa and an octaheme cytochrome $c_3$ which were not observed in previous work, were described. The physico-chemical properties of the two cytochromes of molecular mass 15–16 kDa, including optical properties, iron content, cysteine and histidine contents, N-terminal amino-acid sequence and redox properties are characteristic of the tetraheme cytochrome $c_3$ family. The third *c*-type cytochrome, which belongs to the octaheme cytochrome $c_3$ family, has a molecular mass of 35 kDa and comprises two identical subunits. The monoheme cytochrome *c*-553 and Hmc have not been detected in the soluble extract of *D. africanus*.

This is, to our knowledge, the first report of the presence of two distinct tetraheme cytochromes $c_3$ in a *Desulfovibrio* species. These two cytochromes $c_3$ differ in their isoelectric points, redox potentials, amino-acid compositions and N-terminal amino sequences. From the shape of SWV curve (only one reduction peak, Fig. 3B) and from the redox potential values (Table 3), it was found that the acidic tetraheme cytochrome $c_3$ redox midpoint potential values are close to each other, ranging from $-210$ mV to $-270$ mV. In contrast, the basic cytochrome $c_3$ exhibits two well-separated reduction peaks with the lowest redox potential value of $-290$ mV and the highest value of $-90$ mV. This latter value ($-90$ mV) is the highest value so far reported for the midpoint redox potential of a heme group of tetraheme cytochrome $c_3$ [55]. The differences observed in the redox properties of the two tetraheme cytochromes $c_3$ from *D. africanus* could be related to differences in their heme environments and notably to the degree of solvent exposure of the heme groups [57,58]. Octaheme cytochrome $c_3$ shows redox properties which are intermediate between those of the two tetraheme cytochromes with one SWV reduction peak exhibiting a slight shoulder.

The acidic tetraheme cytochrome $c_3$ from *D. africanus* exhibits unique properties including its blocked N-terminus, its low content of basic residues correlating with its acidic properties and its poor reactivity towards hydrogenase as compared to the other tetraheme cytochromes $c_3$ [1,4,41,48–53]. The physiological relevance of the tetraheme cytochrome $c_3$ in *Desulfovibrio* metabolism is not definitely established. Although it can be considered as the natural electron carrier of the periplasmic hydrogenase on the basis of its localization in the same compartment [2,3], its high affinity for hydrogenase [5,6] and the high second-order rate constant of electron transfer between hydrogenase and tetraheme cytochrome $c_3$ [6,59], the octaheme cytochrome $c_3$ exhibits similar properties [5,60]. The effectiveness of acidic tetraheme cytochrome $c_3$ in the electron transfer with *D. africanus* hydrogenase under $H_2$ is 63- and 40-fold lower as compared to the basic cytochrome $c_3$ and the octaheme cytochrome $c_3$, respectively. This indicates that the biological activity of the basic cytochrome $c_3$ is similar to that of the homologous cytochromes $c_3$ [1]. In contrast, the weak reactivity of the acidic tetraheme cytochrome $c_3$ towards hydrogenase suggests that this protein is not involved as electron carrier of hydrogenase.

The low reactivity of acidic cytochrome $c_3$ towards hydrogenase could be related to a possible steric hindrance by its blocked N-terminus at the edge of the interacting heme. However, this hypothesis is not in agreement with the reactivity of this protein with other redox partners. Indeed, the acidic tetraheme cytochrome $c_3$ was readily reduced by hydrogenase in the presence of the basic cytochrome $c_3$. This indicates that efficient electron transfer occurs between the two tetraheme cytochromes. In addition, although it is unlikely that the *c*-type cytochromes can be involved as the natural electron acceptor(s) of *D. africanus* pyruvate:oxidoreductase, as these proteins are not located in the same cellular compartments, their reactivity with this enzyme shows significant differences [30]. The electron transfer rate between pyruvate:oxidoreductase and the acidic cytochrome $c_3$ was found to be 7-fold and 1.6-fold higher than that obtained with the basic cytochrome $c_3$ [30] and the octaheme cytochrome $c_3$ (data not shown), respectively.

It follows from various studies of the interaction between tetraheme cytochromes $c_3$ and acidic redox proteins as ferredoxin, flavodoxin or rubredoxin used as model systems [61–63], that several lysine residues particularly well conserved among the various cytochrome $c_3$ amino-acid sequences are involved in complex formations between the two partners [61]. In addition, the $c_3$-type cytochromes exhibit an asymmetrical distribution of charges producing a dipole moment that could serve for protein recognition [61]. The same type of electrostatic interaction probably occurs between typical tetraheme cytochrome $c_3$, including the basic cytochrome $c_3$ from *D. africanus* with (NiFe) and (NiFeSe) hydrogenases. Furthermore, recent data of the three-dimensional structure of the (NiFe) hydrogenase of *D. gigas* indicate that several acidic residues surrounding the partially exposed His ligand of the distal [4Fe-4S] cluster could be involved in the interaction process with the redox partners [64]. This strongly suggests that the recognition process and the formation of a complex between hydrogenase and the tetraheme cytochrome $c_3$ prior to the electron transfer, involves specific electrostatic interactions between the two redox partners in which the excess of positive charges located at the surface of the electron carrier plays a crucial role. In contrast to the basic cytochrome $c_3$ and the other homologous cytochromes, the acidic cytochrome $c_3$ from *D. africanus* exhibits a signifi-

cant lack of positively charged residues (lysine + arginine) and a large excess of acidic residues (Table 2). Thus, the unique charged residue composition of this protein could explain its poor reactivity with hydrogenase. The complete amino-acid sequence and the determination of the 3-D structure of the protein will be needed to confirm this hypothesis.

The physiological role of this cytochrome is unknown, whereas basic tetraheme cytochrome appears to function as an electron acceptor from hydrogenase. The acidic tetraheme cytochrome $c_3$ could be involved as an electron storage protein through intermolecular electron exchange with the basic cytochrome $c_3$. Alternatively, it could serve as intermediate electron carrier between the basic cytochrome $c_3$ and the membrane-bound electron transfer chain coupled with the cytoplasmic reduction of sulfate. Further studies will be needed to establish which of these hypotheses is correct.

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## References

[1] Moura, J.J.G., Costa, C., Liu, M.-Y., Moura, I. and LeGall, J. (1991) Biochim. Biophys. Acta 1058, 61–66.
[2] LeGall, J., Mazza, G. and Dragoni, N. (1965) Biochim. Biophys. Acta 99, 385–387.
[3] Voordouw, G. and Brenner, S. (1986) Eur. J. Biochem. 159, 347–351.
[4] Yagi, T., Honya, M. and Tamiya, N. (1968) Biochim. Biophys. Acta 153, 692–698.
[5] Bell, G.R., Lee, J.P., Peck, H.D. and LeGall, J. (1978) Biochimie 60, 315–320.
[6] Niviere, V., Hatchikian, E.C., Bianco, P. and Haladjian, J. (1988) Biochim. Biophys. Acta 935, 34–40.
[7] Bruschi, M., Loutfi, M., Bianco, P. and Haladjian, J. (1984) Biochem. Biophys. Res. Commun. 120, 384–389.
[8] Dutton, P.L., Wilson, D.F. and Lee, C.P. (1970) Biochemistry 9, 5077–5082.
[9] Denis, M., Neau, E. and Blein, J.P. (1980) Bioelectrochem. Bioenerg. 7, 757–773.
[10] Haser, R., Pierrot, M., Frey, M., Payan, F., Astier, J.P., Bruschi, M. and LeGall, J. (1979) Nature 282, 806–810.
[11] Higuchi, Y., Bando, S., Kusunoki, M., Matsuura, Y., Yasuoka, N., Kakudo, M., Yamanuka, T., Yagi, T. and Inokuchi, H. (1981) J. Biochem. 89, 1659–1662.
[12] Morimoto, Y., Tani, T., Okumura, H., Higuchi, Y. and Yasuoka, N. (1991) J. Biochem. 110, 532–540.
[13] Higuchi, Y., Akutsu, H. and Yasuoka, N. (1994) Biochimie 76, 537–545.
[14] Yagi, T. (1969) J. Biochem. (Tokyo) 66, 473–478.
[15] Bruschi, M. (1981) Biochim. Biophys. Acta 671, 219–226.
[16] Bruschi, M., LeGall, J., Hatchikian, E.C. and Dubourdieu, M. (1969) Bull. Soc. Fr. Physiol. Veg. 15, 381–390.
[17] Guerlesquin, F., Bovier-Lapierre, G. and Bruschi, M. (1982) Biochem. Biophys. Res. Commun. 106, 530–538.
[18] Loutfi, M., Guerlesquin, F., Bianco, P., Haladjian, J. and Bruschi, M. (1989) Biochem. Biophys. Res. Commun. 159,670–676.
[19] Pollock, W.B.R., Loutfi, M., Bruschi, M., Rapp-Giles, B.j., Wall, J.D. and Voordouw, G. (1991) J. Bacteriol. 173, 220–228.
[20] LeGall, J., Payne, W.J., Chen, L., Liu, M.Y. and Xavier, A.V. (1994) Biochimie 76,655–665.
[21] Devereux, R., He, SH., Doyle, CL., Okland, S., Stahl, D.A., LeGall, J. and Whitman, W.B. (1990) J. Bacteriol. 172, 3609–3619.
[22] Hatchikian, E.C., Jones, H.E. and Bruschi, M. (1979) Biochim. Biophys. Acta 548, 471–483.
[23] Hatchikian, E.C. and Bruschi, M. (1981) Biochim. Biophys. Acta 634, 41–45.
[24] Bruschi, M. and Hatchikian, E.C. (1982) Biochimie 64, 503–507.
[25] Hatchikian, E.C., Cammack, R., Patil, D.S., Robinson, A.E., Richards, A.J.M., George, S. and Thomson, A.J. (1984) Biochim. Biophys. Acta 784, 40–47.
[26] Bovier-Lapierre, G., Bruschi, M., Bonicel, J. and Hatchikian, E.C. (1987) Biochim. Biophys. Acta 913, 20–26.
[27] Armstrong, F.A., Cammack, R., George, S.J., Hatchikian, E.C. and Thomson, A.J. (1989) Biochem. J. 264, 265–273.
[28] George, S.J., Armstrong, F.A., Hatchikian, E.C. and Thomson, A.J. (1989) Biochem. J. 264, 275–284.
[29] Nivière, V., Forget, N., Gayda, JP. and Hatchikian, E.C. (1986) Biochem. Biophys. Res. Commun. 139, 658–665.
[30] Pieulle, L., Guigliarelli, B., Asso, M., Dolle, F., Bernadac, A. and Hatchikian, E.C. (1995) Biochim. Biophys. Acta 1250, 49–59.
[31] Singleton, R. Jr., Campbell, L.L. and Hawkridge, F.M. (1979) J. Bacteriol. 140, 893–901.
[32] Moura, I., Fauque, G., LeGall, J., Xavier, A.V. and Moura, J.J.G. (1987) Eur. J. Biochem. 162, 547–554.
[33] Davis, B.J. (1964) Anal. NY Acad. Sci. 121, 404–427.
[34] Laemmli, U.K. (1970) Nature 227, 680–685.
[35] Ambler, R.P. (1963) Biochem. J. 89, 349–378.
[36] Crestfield, A.M., Moore, S. and Stein, W.H. (1963) J. Biol. Chem. 238, 622–627.
[37] Morrisson, J.R., Fidge, N.H. and Grego, B. (1990) Anal. Biochem. 186, 145–152.
[38] Kellerman, J., Lottspeich, F., Henschen, A. and Müller-Esterl, W. (1986) Eur. J. Biochem. 154, 471–478.
[39] Dolla, A., Florens, L., Bianco, P., Haladjian, J., Voordow, G., Forest, E., Wall, J., Guerlesquin, F. and Bruschi, M. (1994) J. Biol. Chem. 269, 6340–6346.
[40] Haladjian, J., Bianco, P., Nunzi, F. and Bruschi, M. (1994) Anal. Chim. Acta 289, 15–20.
[41] Niviere, V., Hatchikian, E.C., Bianco, P. and Haladjian, J. (1988) Biochim. Biophys. Acta 935, 34–40.
[42] Bell, G.R., Lee, J.-P., Peck, H.D., Jr. and LeGall, J. (1978) Biochimie 60, 315–320.
[43] Fauque, G., Herve, D. and LeGall, J. (1979) Arch. Microbiol. 121, 261–264.
[44] Hatchikian, E.C., Bruschi, M. and LeGall, J. (1978) Biochem. Biophys. Res. Commun. 82, 451–461.
[45] Bruschi, M., Hatchikian, E.C., Golovleva, L.A. and LeGall, J. (1977) J. Bacteriol. 129, 30–3813.
[46] Guerlesquin, F., Bovier-Lapierre, G. and Bruschi, M. (1982) Biochem. Biophys. Res. Commun. 105, 530–538.

[47] Bartsch, R.G. (1971) Methods Enzymol. 23, 344–363.
[48] Ambler, R.P., Bruschi, M. and LeGall, J. (1971) FEBS Lett. 18, 347–350.
[49] Ambler, R.P. (1973) System. Zool. 22, 554–565.
[50] Ambler, R.P. (1968) Biochem. J. 109, 47–48.
[51] Ambler, R.P, Bruschi, M. and LeGall, J. (1969) FEBS Lett. 5, 115–117.
[52] Shinkai, W., Hase, T., Yagi, T. and Matsubara, H. (1980) J. Biochem. 87, 1747–1756.
[53] Bruschi, M. (1981) Biochim. Biophys. Acta 671, 219–226.
[54] Bruschi, M., Leroy, G., Guerlesquin, F. and Bonicel, J. (1994) Biochim. Biophys. Acta 1205, 123–131.
[55] Bianco, P. and Haladjian, J. (1994) Biochimie 76, 605–613.
[56] Hubbard, A.T. and Anson, F.C. (1970) Electroanalytical Chemistry, Vol. 4 (Bard, A.J., ed.), pp. 129–214, Marcel Dekker, New York.
[57] Stellwagen, E. (1978) Nature 275, 73–74.
[58] Dolla, A., Blanchard, L., Guerlesquin, F. and Bruschi, M. (1994) Biochimie 76, 471–479.
[59] Halajian, J., Bianco, P., Guerlesquin, F. and Bruschi, M. (1987) Biochem. Biophys. Res. Commun. 147, 1289–1294.
[60] Haladjian, J., Bianco, P., Guerlesquin, F. and Bruschi, M. (1991) Biochem. Biophys. Res. Commun. 179, 605–610.
[61] Cambillau, C., Frey, M., Mosse, J., Guerlesquin, F. and Bruschi; M. (1988) Proteins: Structure, Func. Genet. 4, 63–70.
[62] Stewart, D.E., LeGall, J., Moura, I., Moura, J.J.G., Peck, H.D. Jr., Xavier, A.V., Weiner, P.K. and Wampler, J.E. (1988) Biochemistry 27, 2444–2450.
[63] Guerlesquin, F., Dolla, A. and Bruschi, M. (1994) Biochimie 76, 515–523.
[64] Volbeda, A., Charon, M.H., Piras, C., Hatchikian, E.C., Frey, M. and Fontecilla, J. (1995) Nature 373, 580–587.