Biochimica et Biophysica Acta, 1058 (1991) 61–66

ADONIS 000527289100130J

BBABIO 43406

Structural and functional approach toward a classification of the complex cytochrome *c* system found in sulfate-reducing bacteria

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(Received 5 February 1991)

Key words: Cytochrome *c*; Mono-multiheme; Axial ligand; Sulfate reducer; (*Desulfovibrio*); (Spectroscopy)

Following the discovery of the tetraheme cythocrome c_3 in the strict anaerobic sulfate-reducing bacteria (Postgate, J.R. (1954) Biochem. J. 59, xi; Ishimoto et al. (1954) Bull. Chem. Soc. Japan 27, 564–565), a variety of *c*-type cytochromes (and others) have been reported, indicating that the array of heme proteins in these bacteria is complex. We are proposing here a tentative classification of sulfate- (and sulfur-) reducing bacteria cytochromes *c* based on: number of hemes per monomer, heme axial ligation, heme spin state and primary structures (whole or fragmentary). Different and complementary spectroscopic tools have been used to reveal the structural features of the heme sites.

Monoheme-cytochromes (methionine – heme – histidine)

Cytochrome *c*-553, a low-molecular-mass (9 kDa) protein, contains a single covalently attached heme-*c* group with methionine and histidine as axial ligands and a mid-point redox potential quite negative (around 0 mV [3,4]) when compared with other methionine histinide-ligated monoheme cytochromes [5]. This cytochrome was first isolated from *Desulfovibrio (D.) vulgaris* strain Hildenborough [6] and in several other strains [7–9]. Preliminary crystallographic data were reported for the monoheme protein isolated from *D. vulgaris* Miyazaki [10]. An analogous cytochrome has been purified from *D. desulfuricans* 27774 and its N-terminus amino-acid sequence analyzed. EPR and NMR spectroscopies have been utilized to characterize the structure around the heme [11].

Cytochrome *c*-553(550) (split α) is another monoheme protein found in *Desulfomicrobium (Dm.) baculatus* strains Norway 4 and 9974 (DSM 1743) [12] (former *Desulfovibrio baculatus*). The N-terminus amino-acid sequence of this protein shows some homology with both cytochromes *c*-553 from *D. vulgaris*, which are extremely homologous [13] (see Table I). Because of the location close to the N-terminus, these cytochromes belong to the Ambler's class I and can be related to mitochondrial cytochrome *c*. Cytochrome *c*-553 was proposed as a natural electron acceptor for the formate dehydrogenase system in *D. vulgaris* Miyazaki [14].

Diheme proteins

From the soluble fraction of *D. desulfuricans* 27774 grown under sulfate or nitrate-respiring conditions a new heme-protein was isolated. The dimeric cytochrome (named 'split-Soret') is a protein with a molecular mass of 52 kDa [15]. Two hemes *c* are present per 26 kDa subunit having bis-histidinyl axial coordination with distinct midpoint potentials (−168 and −330 mV) (our unpublished results). Its N-terminus structure shows no homology with any other known cytochrome, including the monomeric, diheme *c*-552 found in the denitrifying bacterium, *Pseudomonas stutzeri* [16] (see Table I).

Multiheme cytochromes c_3 (histidine – heme – histidine)

These proteins are members of Ambler's class III. The current state of characterization is summarized in Table II.

Tetraheme proteins (13 kDa)

Until recently, the tetraheme protein was thought to be characteristic of the genus *Desulfovibrio*. However,

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TABLE I

Comparison of N-terminus amino-acid sequences of Desulfovibrionaceae cytochromes c

–, deletion; ( ), not identified; the arrow indicates the end of the tetraheme cytochrome leader sequence.

A. **Monoheme cytochromes**

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1        S G D L G A E ( ) Y A K D ( ) K
2          A D - G A A  L  Y - K S  C  I G  C  H
3 G D K E E D - G A K  L  Y T A L ( ) A S ( ) H
  1        5          10           15           20
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1 *Dm. baculatus*
2 *D. vulgaris* Hildenborough
3 *D. desulfuricans* 27774

B. **Diheme 'split Soret'**

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  G E A Q P A S G R F D Q V G ( ) A F G ( ) K P H
  1        5          10          15          20
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C. **Multiheme cytochromes c_3**

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1 M R K L F F C G V L A L A V A F A L P V V A↓A P K A P A D G L K M E A T K Q P V V F N H
2               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
                1        5          10          15          20          25          30          35
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D. vulgaris multiple-heme c_3:
1 tetraheme
2 hexadecaheme

since the scores of newly discovered sulfate-reducing bacteria have obliged us to reconsider the classification of these organisms, this cytochrome appears to be characteristic of a whole family, the *Desulfovibrionacae*, rather than a simple genus [17]. In addition to *Desulfovibrio*, it is found in genera such as: *Termodesulfobacterium (Td) commune* [18], *Desulfomicrobium (Dm) baculatus* [19] and *Desulfobulbus (Db.) elongatus* [20]. The four hemes are covalently bound to the polypeptide chain by thioether linkages provide by cysteine residues in well-recognized arrangements of the type -CYS-Aaa-Baa-CYS-HIS or -CYS-Aaa-Baa-Caa-Daa-CYS-HIS. The axial ligands are histidines. A large number of amino-acid sequences are available (Ref. 21) and references therein). Even using deletions to maximize homology, only about 30% of the amino-acid residues are conserved [19,22–24]. The conserved residues account mainly for the heme attachment sites. There are only eight residues conserved not directly involved in heme binding (in particular 'strategic' aromatic residues).

Structural studies by X-ray crystallography have been reported for tetraheme cytochromes c_3 from *D. vulgaris* Miyazaki [25], *Dm. baculatus* [26,27] and *D. gigas* [28,29]. The relative position and orientation of the heme groups are fairly conserved when the three available structures are compared. Particular interesting re-

TABLE II

State of art of cytochrome characterization in sulfate-reducing bacteria of the family Desulfovibrionaceae

S, sequence; P, purified; PNP, present not purified; NMR, nuclear magnetic resonance; X, X-ray crystallography; EPR, electron paramagnetic resonance; MB, Mössbauer; –, not reported.

Bacterial species	Tetraheme	Octaheme	Monoheme
D. gigas	S,NMR,EPR,X,MB	P	PNP
D. vulgaris Hildenborough	S,NMR,EPR,MB	X	S,NMR,EPR
D. vulgaris Miyazaki	S,NMR,EPR,X	–	P,X
Dm. baculatus 9974	P,NMR,EPR,MB	–	P [a]
Dm. baculatus Norway 4	S,NMR,EPR,X	S	P,NMR,EPR [a]
D. desulfuricans 27774	P,NMR,EPR,MB	–	P,NMR,EPR
D. desulfuricans Berre Eau	P,NMR,EPR	–	P,NMR,EPR
D. desulfuricans Algheila Z	S,NMR,EPR	P	–
D. salexigens Benghazi	S,NMR,EPR	PNP	PNP
D. desulfuricans Berre Sol	P,NMR,EPR	–	P,NMR,EPR
Drm. acetoxidans	S,NMR,EPR,X [b]	–	–

[a] *c*553(550)
[b] The triheme cytochrome.

sults of the structural analysis are: the different solvent exposure of the hemes, the lack of coplanarity of the hemes and the fact that the hemes form a quite compact arrangment, being close to each other (1.09–1.73 nm).

The four hemes are localized in non equivalent protein environments and each heme has a negative and different mid-point redox potential. The mid-point potentials of the four hemes have been measured by a wide range of different techniques and range from −30 down to −400 mV, a well-documented example of how structural and environmental features control and modulate the redox potential of a given active site [30].

Several physicochemical methods – mainly Mössbauer spectroscopy [31], circular dichroism (CD) [32], magnetic GD [33], electron paramagnetic resonance [34–41], nuclear magnetic resonance [42–49], electrochemical methods [50–55] – have been used in order to elucidate structural features and the mechanisms of electron transfer.

Due to the presence of four paramagnetic centers (in a favorable spin state) wrapped around by a small polypeptide chain (about 30 amino acids per heme) highly resolved proton-NMR spectra were obtained in two stable redox states, reduced ($S = 0$) and oxidized ($S = 1/2$), as well as in intermediate redox states. Cytochrome c_3 was described as a protein containing interacting redox centers after NMR study of the redox equilibria in *D. vulgaris* Hildenborough [46] and Miyazaki [47] and *D. gigas* [48]. In principle, the non-equivalent four-redox-center molecule can have 16 redox states in a multi-redox distribution equilibrium [46,48]. Furthermore, the redox properties of each center may be affected by the redox states of the adjacent centers due to heme–heme interactions. Specialized spectroscopic techniques, namely EPR and NMR, have been used to probe each heme individually and to provide valuable information concerning the interplay between the different redox centers. The proton-NMR spectra of the cytochrome were examined while varying the pH and the redox potential [46,48]. The analysis of the NMR data was based on a model that takes into consideration all the redox species present in solution and showed that the heme–heme interactions resulted in change of the redox properties in a range of −50 to +60 mV. The heme mid-point redox potentials as well as the interacting potentials were shown to be pH-dependent. The NMR analysis of the electron transfer in multiheme cytochromes shows that the intramolecular exchange process is fast but the intermolecular process can range from slow (*D. vulgaris* Hildenborough) to fast (*Dm. baculatus* 9974) in the NMR timescale [44].

EPR potentiometric titrations have been performed [36–41]. Due to the poor spectral resolution at the g_{max} region, assignments have been difficult. By taking into account the total intensity of the EPR spectra, the four macroscopic redox potentials of the tetraheme cytochrome c_3 were determined [38,40] with corresponding assignment of g-values to each heme and estimation of interacting potentials. Using differential spectroscopy, the four g_{max} resonances were assigned and resolved in *Dm. baculatus* cytochrome c_3 and the four redox potentials were determined, one being a true microscopic-value [41]. Using the available X-ray coordinates, the EPR data were interpreted to suggest that two of the hemes have the histidinyl ligands in a non-coplanar arrangment [41]. The conjunction of X-ray information with EPR data and Molecular Graphics allowed structural correlations to be made between each heme and its spectroscopic and redox properties.

Mössbauer spectroscopy showed that in the native state and in the absence of an external magnetic field a weak heme–heme magnetic interaction can be detected at 4.2 K [56]. A good correlation between EPR g_{max} values and magnetic hyperfine interactions could be achieved. Also, a combined EPR/Mössbauer redox study enabled decomposition of the complex Mössbauer data into the four heme components (our unpublished results in a collaboration with B.H. Huynh).

The heme axial ligands of tetraheme proteins show a certain degree of flexibility: high-spin states can be obtained at low pH values due to histidine axial protonation [57] and reactivity toward external small ligands (CO, NO and hydroxylamine) has been observed [34].

Tetraheme cytochromes have been considered as cofactors of hydrogenase and to be required for electron transfer to other redox partners such as ferredoxin, flavodoxin and rubredoxin, although slow reduction of these components can be achieved directly by the H_2/hydrogenase system in the absence of the tetraheme protein [58]. They have been reported to stimulate the H_2 evolution/consumption in bacterial cell extracts but have never been proven to be direct electron donors or acceptors [59]. In vitro, specific interactions were probed between cytochrome c_3 and other electron carriers, namely ferredoxin, rubredoxin, flavodoxin and hydrogenase [45,60–65]. Protein–protein titrations followed by NMR, as well as redox equilibria studies of binary protein complexes, provided stoichiometry, binding-constant values, rates of electron exchange and definition of docking sites [64,65].

A mechanism of reduction of elemental sulfur was discussed using *Dm. baculatus* Norway 4 cytochrome c_3 [66]. It was noted that the tetraheme cytochrome from *D. vulgaris* Hildenborough, which is not capable of using S^0 [67], was strongly inhibited by S, the product of the reduction of colloidal sulfur.

Triheme proteins

The triheme-containing cytochrome *c*-551.5 (named c_7) isolated from the sulfur-reducing bacterium *Drm. acetoxidans* [68,69] has a molecular mass of 9 kDa. Two

hemes have a midpoint potential of −177 mV and the third −107 mV [70]. The absence of a very low potential heme could well explain the lack of activity of this cytochrome toward sulfur [71]. A particular interest in the triheme proteins stems from its similarities with tetraheme proteins. A comparison of the amino-acid sequence of the triheme cytochrome with the tetraheme proteins clearly shows a high degree of homology, with remarkable shortening of the polypeptide chain in the region of the second sequence of heme attachment -CYS-Aaa-Baa-Caa-Daa-CYS-HIS. X-ray crystallographic data obtained at 0.25 nm resolution indicated heme–heme distances ranging from 1.16 to 1.92 nm [24]. The NMR study supported the presence of histidinyl heme ligands and showed a very fast intermolecular electron transfer rate. High-spin transition could be detected at extreme pH values due to histidine protonation [72]. Curiously, although the bacterium does not contain any hydrogenase, the cytochrome is still reduced by *Desulfovibrio* hydrogenase. These results casts some doubts on the so-called 'specificity' of cytochrome c_3 as a co-factor for hydrogenases.

In the following section, we shall still retain the provisional names 'octaheme', 'dodecaheme' and 'hexadecaheme' cytochromes. However, it is very probable following the work of Loufti et al. [73], that these cytochromes are in fact polymers of a four-heme-containing subunit.

Octaheme proteins

This cytochrome is a low redox potential heme-protein found in different sulfate reducers (see Table II). Preliminary X-ray crystallographic studies have been reported for the octaheme protein isolated from *D. gigas* [74]. Evidence has been put forward indicating that *Dm. baculatus* Norway 4 is composed of two identical subunits of 13.5 kDa and that the hemes may play a role on the building of the dimer [73]. Although the monomer has a molecular mass identical to that of the tetraheme isolated from the same organism, they are distinct in terms of localization, amino-acid composition and N-terminal sequences. The octaheme cytochrome was found to act as an electron carrier in the reduction of thiosulfate from molecular H_2 in *D. gigas* extracts [74].

Dodecaheme proteins

The dodecaheme cytochrome (41 kDa) was isolated from *D. desulfuricans* 27774 [15]. Information on heme ligation and heme spin states is scarce. Most probably, histidines are the axial heme ligands. The amino-acid composition is distinct from the other multiheme proteins isolated from this organism, suggesting that they are the products of different gene.

Hexadecaheme proteins

The presence of high-molecular-mass cytochromes (70 kDa) was first reported by Yagi [75] in *D. vulgaris* Miyazaki. More recently, a cytochrome containing 16 hemes was purified from *D. vulgaris* Hildenborough [76]. The heme content per 70 kDa was recently challenged by Tan and Cowan [77], who found a value of three hemes per mole of this protein. However, our own results support the finding of Higushi et al. [76], since a value of 16.7 hemes per 70 kDa was found in our preparations (see Note added in proof).

Hexaheme proteins – nitrite reductase (histidine – heme – histidine) – (high spin/low spin)

When grown in a medium containing nitrate as electron acceptor, nitrate and nitrite reductases are induced in *D. desulfuricans* 27774. The membrane-bound nitrite reductase was isolated and shown to be a hexaheme-containing protein [78]. The enzyme catalyses the six electron reduction step of nitrite to ammonia. EPR and visible redox titrations have indicated that the hemes have a span of redox potentials from +100 to −300 mV, some of the hemes being reduced by ascorbate but others reduced only by dithionite [79]. A conjunction of EPR and Mössbauer measurements indicated that five low-spin ($S = 1/2$) and one high-spin ($S = 5/2$) hemes are present [87]. The system is complex and two pairs of magnetically interacting hemes were detected (low-spin/low-spin and low-spin/high-spin). The high-spin heme was found to bind NO, suggesting that this heme could be the substrate binding site and NO could be an intermediate present in an enzyme-bound form. The low-spin hemes are most probably bishistidinyl coordinated and the unusual large g_{max} values determined for two of the low-spin hemes are indicative of perpendicular alignment of the imidazole plans. This multiple-heme *c*-containing enzyme can be directly related to similar proteins which have been found in *Escherichia coli* [80] and in *Wolinella succinogenes* [81], and may be also related to the hydroxylamine oxido-reductase isolated from *Nitrosomonas europaeae* [82].

Final remarks

We have defined four distinct families of cytochromes *c* which have been found so far in sulfate- and sulfur-reducing bacteria. The axial heme environment is dominated in these proteins by bis-histidinyl coordination, as observed in the diheme and multiheme families. Even in the case of the hexaheme nitrite reductase, the coordination is predominantly of this type, with the exception of one heme that is high-spin and was found to be the enzyme-active site. Methionine-histidine coordination is restricted to the monoheme family.

Little is known concerning the synthesis of these cytochromes, and one of the most puzzling observations is that, although tetraheme cytochromes c_3 are found in the periplasm [83] (indeed, its cloned gene shows the presence of a leader sequence [84]), octa- and hexadecaheme cytochromes are thought to be located in the cytoplasm [85].

If the current view concerning the compartimentalization of cytochromes c_3 in SRB, i.e., tetraheme c_3, cytochrome *c*-553 in the periplasm, nitrite reductase membrane-bound, octaheme in the cytoplasm, is correct, the mechanism of heme insertion will also become fascinating problem. It is difficult to conceive an entirely folded cytochrome c_3 as being able to cross the cytoplasmic membrane and, by extrapolating what is know concerning the mechanism of action of mitochondrial cytochrome heme lyase [86,87], at least two locations of this enzyme should exist: one on the external site of the cytoplasmic membrane to assure heme insertion on the periplasmic apo-cytochromes, another on the internal side of the membrane for the synthesis of the cytoplasmic cytochromes. Further complications may arise from the necessity of specific heme-inserting enzymes for each cytochrome family and eventually for each particular heme in multiple-heme proteins. Whatever solution has been retained, these organisms constitute excellent material for the study of the mechanism of localization of homologous proteins within the bacterial cell.

Acknowledgments

This work was supported by grants from the National Institute of Health GM 41482-02, Instituto Nacional de Investigação Cientifica and Junta Nacional de Investigação Cientifica e Tecnológica.

Note added in proof (Received 4 April 1991)

The gene for the *D. vulgaris* Hildenborough hexadecaheme protein has recently been cloned, confirming the cytochrome to be a single polypeptide chain [88]. Since the gene is preceded by a leader sequence, it should be found in periplasm of *D. vulgaris*, although no evidence for such allocation could be found after gel electrophoresis. This result demonstarates that the report by Loufti et al. [73] of the presence of an octaheme cytochrome in *D. vulgaris* was mistaken, since this protein is identical to the hexadecaheme one.

Also, the three dimensional X-ray structure of *D. vulgaris* strain Hildenborough cytochrome c_3 was recently determined by the method of molecular replacement using the refined structure of the tetraheme protein from *D. vulgaris* strain Miyazaki F [89].

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