The cytochrome c_3 superfamily: amino acid sequence of a dimeric octahaem cytochrome c_3 (M_r 26 000) isolated from *Desulfovibrio gigas*

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Cytochrome c_3 (M_r 26 000) isolated from *Desulfovibrio gigas* is a dimeric cytochrome consisting of two identical subunits of 109 amino acids, each of which contains four haem groups. On the basis of its amino acid sequence, this cytochrome clearly belongs to the cytochrome c_3 superfamily, and will be classified in class III of the c -type cytochromes as defined by Ambler [(1980) in *From Cytochromes to Cytochromes* (Robinson, A. B. and Kaplan, N. O., eds.), pp. 263–279, Academic Press, London]. It contains ten cysteine and nine histidine residues in each subunit, and eight cysteines and eight histidines linked to the four haem groups were found to be invariant on alignment of all known cytochrome c_3 sequences. Two intermolecular disulphide bridges have been determined between cysteine residues 5 and 46 of the two monomers. Cytochrome c_3 (M_r 26 000) from *D. gigas* is clearly different from cytochrome c_3 (M_r 13 000) from the same strain,

with which it shows only 27 % sequence identity. Compared with cytochrome c_3 (M_r 26 000) from *D. desulfuricans* Norway, the three-dimensional structure of which has been determined, 26.95 % of the residues have been conserved. In the enzyme from *D. desulfuricans* Norway, hydrophobic interactions have been described across the dimer interface. Residues involved in similar interactions seem to be well conserved in the equivalent *D. gigas* cytochrome. This sequence provides structural data to allow specification of this new subclass of polyhaem cytochromes. Furthermore, *D. gigas* cytochrome c_3 (M_r 26 000) is the first polyhaem cytochrome shown to contain two disulphide bridges linking two identical subunits, which could induce more rigid folding. The folding and the evolution of this family of polyhaem cytochromes are discussed.

INTRODUCTION

Subsequent to the discovery of an increasing number of cytochromes c in sulphate and sulphur bacteria, various classifications, based on the number of haem groups per monomer, haem axial ligation, haem spin state and primary structures, have been proposed [1]. Ambler [2] has classified the multihaem proteins, including trihaem, tetrahaem and octahaem proteins characterized by a bis-histidiny iron co-ordination, in class III of the c -type cytochromes. On the basis of the amino acid sequence of a high- M_r cytochrome c containing 16 haems [3] and X-ray crystallographic comparisons between the tetrahaem cytochrome c_3 (M_r 13 000) and the octahaem cytochrome c_3 (M_r 26 000) [4,5], we have proposed that all of these cytochromes may belong to the cytochrome c_3 superfamily, and that they may have a common ancestral origin [6]. These cytochromes show no structural similarities with the other cytochrome c classes, and are associated with the most divergent respiratory modes present in sulphur and sulphate reducing bacteria, which are able to utilize elemental sulphur or oxidized sulphur compounds as terminal acceptors.

Cytochrome c_3 (M_r 13 000) is a periplasmic protein which is uniformly present in *Desulfovibrio* and acts as the natural electron donor and acceptor with periplasmic hydrogenase. Several three-dimensional structures have been determined which show that the arrangement of the four haem groups is highly conserved [7–11]. Each haem exhibits a distinct redox potential in the range –200 to –400 mV, and has His–His iron axial ligands. *Desulfuromonas acetoxidans* cytochrome c_3 contains three haem groups and is related to cytochrome c_3 (M_r 13 000), but about 30 % of the molecule, containing one haem group, is absent [12]. Cytochrome c_3 (M_r 26 000) has been isolated only from *Desulfovibrio gigas* [13] and *D. desulfuricans* Norway [14], and recently from *D. africanus* [15]. These enzymes are composed of two

identical subunits, which differ from those of the tetrahaem cytochrome c_3 present in the same species. Based on the three-dimensional structure of the *D. desulfuricans* Norway cytochrome [14], it has been established that the monomeric unit shows the characteristic cytochrome c_3 folding pattern.

A high- M_r cytochrome c (Hmc) containing 16 haem groups per molecule has been characterized in both *D. vulgaris* strains Hildenborough [16] and Miyazaki [17] and *D. gigas* [18]. On the basis of sequence identity [3], this protein was taken to be a multidomain protein in which the 16 haems are distributed among four cytochrome c_3 -like domains. *D. gigas* is the only bacterium described so far in which the four-, eight- and 16-haem cytochromes are all present.

With a view to elucidating the structural features of the cytochrome c_3 (M_r 26 000) group, of which only *D. desulfuricans* Norway cytochrome c_3 (M_r 26 000) has so far been fully characterized, and to comparing them with the cytochromes c_3 (M_r 13 000), we determined the characteristics and amino acid sequence of cytochrome c_3 (M_r 26 000) from *D. gigas*. Determination of the structures of members of this family should help us to understand the mechanisms involved in the folding and evolution of the polyhaem cytochromes, and throw some light on the physiological roles of the various cytochromes in redox processes.

MATERIALS AND METHODS

Cytochrome c_3 (M_r 26 000) was isolated from *Desulfovibrio gigas* (NCIMB 9332) as described previously [13]. The purity coefficient, expressed as the ratio between the absorbances at the given wavelengths (in nm), i.e. $C = (A_{553}^{\text{red}} - A_{570}^{\text{red}}) / A_{280}^{\text{ox}}$, was found to be 3.2 in the case of the purified protein.

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Optical absorption spectra

Visible and UV absorption spectra were determined with a Philips PU 8820 spectrometer. Molar absorption coefficients at the absorption maxima were obtained from these spectra using protein concentrations based on amino acid analysis data.

Isoelectric point measurements

The isoelectric point of the protein was determined by performing isoelectric focusing using a Phast System apparatus from Pharmacia LKB Biotechnology. Phast Gel IEF 3–9, which operates in the pH range 3–9, and Ampholine polyacrylamide gel plates from Pharmacia (pH range 3.5–9.5) were used together with a Pharmacia broad-range pI calibration kit containing proteins with various isoelectric points ranging from 3 to 10.

M_r determination

The M_r of the protein was determined by performing SDS/PAGE under reducing conditions on a Pharmacia Phast System with Phast Gel 12 % polyacrylamide and Phast Gel SDS buffer strips. MS analysis was carried out on a Perkin–Elmer Sciex API III triple quadrupole mass spectrophotometer equipped with a nebulizer-assisted electrospray (ion-spray) source. The spectra were recorded as described previously [19].

Analysis of iron and haem content

The iron content was determined using a Unicam SP 1900 atomic absorption spectrometer. Fe_2O_3 , first heated and dissolved in conc. HCl and then diluted in distilled water, was used as a standard. The total number of haem units was determined using the pyridine ferrohaemochromogen test. A known mass of the protein (determined by hydrolysing an aliquot of protein solution and performing quantitative amino acid analysis) was added to an aqueous alkaline (7.5 mM NaOH) pyridine (25 %) solution and reduced by adding a few crystals of sodium dithionite. The haem content was determined from the pyridine ferrohaemochrome spectrum, using a molar absorption coefficient of $29.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 550 nm with the cytochrome c_3 derivatives [20].

Amino acid analysis and protein sequencing

Haem was removed by the method of Ambler [21], and the resulting apoprotein was isolated by gel filtration on Sephadex G25 in 5 % (v/v) formic acid. *S*-Carboxymethylated cytochrome was prepared by dissolving the apoprotein pellet in 0.5 M Tris/HCl (pH 9.0), 8 M urea and 20 mM EDTA, and treating it with iodoacetic acid as described by Crestfield et al. [22]. Methionine peptides were produced by reacting carboxymethylated cytochrome in 70 % formic acid with a 300-fold excess of CNBr for 24 h at room temperature in the dark. For the enzymic digestions, the carboxymethylated cytochrome was dissolved in 200 mM ammonium acetate (pH 8.5) and left to react at 37 °C for 3 h with chymotrypsin at a 1:50 enzyme/substrate ratio (Worthington) at pH 8.5, or with Arg C endopeptidase at a 1:1000 enzyme/substrate ratio at 37 °C for 18 h. The resulting peptides were separated by reverse-phase HPLC on a C_{18} Ultrapac-TSK-ODS 120T column (LKB) with a linear gradient from 0 to 40 % acetonitrile in 0.1 % trifluoroacetic acid. Elution was monitored by absorbance at 215 nm.

Protein and peptide samples were hydrolysed in 200 μl of 6 M HCL at 110 °C for 18 h in sealed evacuated tubes. Tryptophan was analysed using the method developed by Peuke et al. [23].

Sequence determinations were performed with a gas-phase sequencer (models 470A and 473A; Applied Biosystems). Quantitative determination of phenylthiohydantoin derivatives was carried out by HPLC (Waters Associates) monitored by a data-and chromatography-control station (Waters 840). Typically, the sample (1–5 pmol of protein; 200–2000 pmol of peptide) was loaded on to a micro-sample cartridge filter washed with trifluoroacetic acid and treated with 3 mg of Polybrene in water containing 0.2 mg/ml NaCl. The phenylthiohydantoin derivatives were separated with a gradient system, using a microbore HPLC apparatus connected on-line to the sequencer.

RESULTS

The apparent M_r of the enzyme deduced from SDS/PAGE analysis was about 28 000 for the native form, and 14 000 when the sample had been treated with 6 M guanidine hydrochloride in 10 mM Tris/HCl buffer (pH 7.6) overnight.

The results of the amino acid analysis and sequence determination studies showed that cytochrome c_3 (M_r 26 000) from *D. gigas* contains two identical subunits each of 109 amino acids, giving a calculated M_r of 12 223 in the case of the apoprotein and 14 695 when the four haem groups are included (Table 1). The mass spectra of the native form showed a peak at 29 385 mass units. These results are in good agreement with the M_r of 29 390 calculated for the dimeric form. Upon comparing the amino acid compositions and sequences of cytochrome c_3 (M_r 26 000) and cytochrome c_3 (M_r 13 000), both extracted from the same organism *D. gigas*, some striking differences were found to exist, particularly as regards the presence of eight cysteine and eight histidine residues in cytochrome c_3 (M_r 13 000), which is just sufficient to link four haem groups and is characteristic of all the cytochromes c_3 (M_r 13 000). Cytochrome c_3 (M_r 26 000), however,

Table 1 Amino acid composition of *D. gigas* cytochrome c_3 (M_r 26 000)

The results were obtained by analysing 24, 48 and 72 h hydrolysates, as described in the Materials and methods section. Values in parentheses are the numbers of residues per molecule obtained on the basis of the amino acid sequence. The values given for threonine and serine were obtained by extrapolation from the hydrolysis times.

Amino acid	Content (mol of residues)
Aspartic acid	10.0 (10)
Threonine	1.9 (2)
Serine	6.7 (7)
Glutamic acid	11.7 (12)
Proline	5.0 (5)
Glycine	9.0 (9)
Alanine	8.0 (8)
Cysteine*	9.0 (10)
Valine	6.0 (6)
Methionine	2.8 (3)
Isoleucine	3.9 (4)
Leucine	2.0 (2)
Tyrosine	2.0 (2)
Phenylalanine	4.0 (4)
Histidine	9.0 (9)
Lysine	9.0 (9)
Arginine	6.0 (6)
Tryptophan†	0.9 (1)
Total	109

* Analysed as carboxymethylcysteine.

† Analysed according to [21].

Figure 1 Amino acid sequence of *D. gigas* cytochrome c_3 (M_r 26000)

The half-arrows indicate the automatic degradation of the whole protein and of the various peptides.

Table 2 Amino acid composition, yield and sequence positions of peptides isolated after arginase C digestion of *S*-carboxymethylated *D. gigas* cytochrome c_3 (M_r 26000)

See the Materials and methods section for details. Values in parentheses are the numbers of residues detected in sequence studies.

Amino acid	Content (mol)					
	A-1	A-2	A-3	A-4	A-5	A-6
Aspartic acid	2.0 (2)	—	5.1 (5)	—	—	1.0 (1)
Threonine	0.8 (1)	—	—	—	—	1.0 (1)
Serine	—	0.9 (1)	1.9 (2)	1.0 (1)	2.9 (3)	—
Glutamic acid	2.0 (2)	1.0 (1)	1.0 (1)	1.0 (1)	2.0 (2)	5.0 (5)
Proline	3.7 (4)	—	—	—	—	0.9 (1)
Glycine	1.1 (1)	1.0 (1)	2.1 (2)	—	1.1 (1)	4.1 (4)
Alanine	1.1 (1)	1.0 (1)	1.0 (1)	—	1.0 (1)	3.8 (4)
Cystine (half)*	1.0 (1)	—	4.8 (5)	—	0.9 (1)	2.7 (3)
Valine	2.7 (3)	0.9 (1)	1.9 (2)	—	—	—
Methionine	—	0.9 (1)	0.9 (1)	—	—	0.9 (1)
Isoleucine	0.9 (1)	—	1.0 (1)	—	1.0 (1)	1.0 (1)
Leucine	0.9 (1)	—	—	—	—	1.0 (1)
Tyrosine	—	—	—	0.9 (1)	0.8 (1)	—
Phenylalanine	—	0.9 (1)	1.9 (2)	—	1.0 (1)	—
Lysine	1.0 (1)	2.0 (2)	1.0 (1)	—	1.0 (1)	3.8 (4)
Histidine	0.9 (1)	2.0 (2)	2.9 (3)	—	0.9 (1)	1.9 (2)
Arginine	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	—
Tryptophan	—	—	—	—	1.0 (1)	—
Total	20	12	27	4	15	28
Yield (%)	10	24	17	8	10.5	10
Positions	1–20	21–32	33–59	63–66	67–81	82–109

* Measured as carboxymethylcysteine.

was found to contain ten cysteine and nine histidine residues in each subunit. In order to determine if the two additional cysteines are present as thiol groups or form a disulphide bridge, carboxymethylation of the native protein was performed. Reaction of the cytochrome with an alkylating agent and in denaturing solvents failed to reveal any thiol groups. However, reduction with 2-mercaptoethanol followed by alkylation clearly indicated two half-cysteines per monomer, with 1.86 carboxymethyl cysteines per monomer with a yield of about 90%. Noteworthy differences were found to exist in the number of basic and acidic residues, which was in agreement with the isoelectric points measured, i.e. 5.4 in the case of cytochrome c_3 (M_r 26000) and 5.2 for cytochrome c_3 (M_r 13000).

The iron content of cytochrome c_3 (M_r 26000), as determined by atomic absorption spectrophotometry, was 3.6, which amounts to four atoms of iron per subunit. The number of haem groups per molecule was estimated to be 3.8 per subunit using a molar absorption coefficient of $29.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at the peak of the cytochrome c_3 pyridine ferrohaemochrome. The absorption coefficient at the reduced peak at 553 nm calculated on the basis of the amino acid sequence was $127.83 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ per subunit.

The N-terminal sequence of the reduced *S*-carboxymethylated protein was determined three times. The most efficient degradation process yielded the N-terminal sequence up to the 34th cycle, as shown in Figure 1. A repetitive yield of 82% was calculated with a initial amount of protein of 2.5 pmol.

The complete amino acid sequence given in Figure 1 was determined from the sequence data obtained for the whole protein and for arginase and chymotryptic peptides. The amino acid composition, yield and sequencing positions of each set of peptides are given in Tables 2 and 3.

Table 3 Amino acid composition, yield and sequence positions of peptides isolated after chymotryptic digestion of S-carboxymethylated *D. gigas* cytochrome c_3 (M_r 26 000)

See the Materials and methods section for details. Values in parentheses are the numbers of residues found in sequence studies.

Amino acid	Content (mol)					
	C-1	C-2	C-3	C-4	C-5	C-6
Aspartic acid	2.0 (2)	1.0 (1)	5.8 (6)	—	—	1.0 (1)
Threonine	1.0 (1)	—	—	—	—	1.0 (1)
Serine	—	1.9 (2)	0.8 (1)	—	2.8 (3)	—
Glutamic acid	3.0 (3)	—	0.9 (1)	1.0 (1)	2.0 (2)	5.1 (5)
Proline	4.1 (4)	—	—	—	—	1.0 (1)
Glycine	1.9 (2)	—	2.0 (2)	1.0 (1)	1.1 (1)	3.1 (3)
Alanine	1.0 (1)	—	1.0 (1)	—	1.0 (1)	3.0 (3)
Cystine (half)*	0.9 (1)	1.9 (2)	2.8 (3)	—	1.9 (2)	1.9 (2)
Valine	3.8 (4)	1.9 (2)	—	—	—	—
Methionine	0.8 (1)	0.9 (1)	—	—	0.9 (1)	—
Isoleucine	1.0 (1)	—	0.8 (1)	—	1.0 (1)	1.0 (1)
Leucine	1.0 (1)	—	—	—	—	1.0 (1)
Tyrosine	—	—	0.8 (1)	0.9 (1)	—	—
Phenylalanine	0.9 (1)	0.9 (1)	0.9 (1)	1.0 (1)	—	—
Lysine	1.8 (2)	—	1.0 (1)	—	0.9 (1)	3.0 (3)
Histidine	0.9 (1)	2.9 (3)	0.9 (1)	—	2.0 (2)	1.0 (1)
Arginine	0.9 (1)	1.0 (1)	1.9 (2)	1.0 (1)	1.0 (1)	—
Tryptophan	—	—	—	—	—	—
Total	26	13	14	5	15	10
Yield (%)	10.5	13	21	19	7	22
Positions	1–26	31–43	44–64	65–69	73–87	88–109

* Measured as carboxymethylcysteine.

The arginase peptides A-1, A-2, A-3, A-4, A-5 and A-6 covered residues 1–20, 21–32, 33–59, 63–66, 67–81 and 82–109 respectively. No peptide containing residues 60–63 was recovered during the peptide purification (Table 2). The overlap between the N-terminal sequence (residues 1–34) and chymotrypsin peptide C-2 (residues 31–43) confirmed the order of peptides A-1, A-2 and A-3. The sequence data lacking between peptides A-3 and A-4 was provided by peptide C-3 (residues 44–64). That no residues were absent between peptides A-4 and A-5 was confirmed by peptide C-4 (residues 65–69), and between A-5 and A-6 by peptide C-5 (residues 73–87). The two peptides A-6 and C-6 (residues 88–109) both covered the C-terminal sequence.

The four Cys(Xaa)_{2/3}-Cys-His sequences described as the haem binding sites are located at positions 36–40, 52–56, 80–84 and 99–104. The first three sites have the standard two-residue separation which is found in all the cytochromes c , but haem site 4 has three amino acids between the cysteine residues instead of the two or four which occur in the other known cytochrome c_3 sequences.

DISCUSSION

On the basis of the amino acid sequence of *D. gigas* cytochrome c_3 (M_r 26 000) established in the present study, this cytochrome clearly belongs to the cytochrome c_3 superfamily, and it will be classified among the class III c -type cytochromes as defined by Ambler [2]. This cytochrome is composed of two identical subunits, with M_r values of 14 689 and 29 390 for the monomeric and dimeric forms respectively. Our comparisons between amino acid sequences provide definite evidence that cytochrome c_3 (M_r 26 000) and cytochrome c_3 (M_r 13 000) isolated from *D. gigas* are two distinct cytochromes, with only 27% sequence identity.

Cytochrome c_3 (M_r 26 000) and cytochrome c_3 (M_r 13 000) isolated from *D. desulfuricans* Norway are also two distinct cytochromes, with 34.6% identity. Alignment of the sequences of the cytochromes c_3 (M_r 26 000) from *D. desulfuricans* Norway and *D. gigas* shows that 26.95% of the residues are conserved. The sequence comparison between the two cytochromes c_3 (M_r 26 000) isolated to date provides the structural data to identify this new subclass of polyhaem cytochromes.

Upon determining the three-dimensional structure of the *D. desulfuricans* Norway cytochrome c_3 (M_r 26 000) [4,5], it was established that the monomeric unit adopts the cytochrome c_3 fold. The two subunits are in close contact with the iron atoms of haem 1 and haem 1', at a distance of 15.1 Å (1.51 nm). It has been reported that 15 residues of each subunit are involved in the formation of the dimer. These regions, which play an important part in the interactions of the dimer, seem to be well conserved, judging from the alignment of the cytochromes from *D. desulfuricans* Norway and *D. gigas* (Figure 2). They are the N-terminal region (residues 1–5; *D. gigas* cytochrome c_3 numbering), Ala-29, and residues 34–36 and 47–59. In the *D. desulfuricans* Norway enzyme, eight hydrogen bonds and interactions between hydrophobic residues have been described across the interface, and no salt bridges have been observed.

We could suppose that a similar folding pattern exists in the *D. gigas* dimer. Sequence comparisons with other cytochromes c_3 indicate that the residues involved in the formation of the dimer interface vary between the cytochromes c_3 in terms of both nature and size. These structural data are in agreement with the finding that these cytochromes may be physiological dimers.

The presence of disulphide bridges in the *D. gigas* cytochrome is the first time that such an interaction has been demonstrated in this class of polyhaem cytochromes. On the basis of sequence comparisons with all the other related cytochromes, we can deduce that the two disulphide bridges involve cysteines 5 and 46 of each monomer and that they are intermolecular, as we have obtained the dimeric form by mass spectra analysis. Using the same conditions of mass spectra analysis, *D. desulfuricans* Norway cytochrome c_3 (M_r 26 000) generates the monomer.

On migration of the *D. gigas* cytochrome c_3 (M_r 26 000) on SDS/PAGE, we obtained the protein only as the dimer, even if the sample was first treated overnight with 6 M guanidine hydrochloride. However, if dithiothreitol was used, we obtained the monomer. Examination of the three-dimensional structure of *D. desulfuricans* Norway cytochrome c_3 [5] shows that the residues (Thr and Glu) at positions corresponding to cysteines-5 and -46 in the *D. gigas* cytochrome face each other, being approx. 10 Å apart.

The thermal stability parameters of some cytochromes c_3 have been characterized [24]. These enzymes are denatured over a wide range of temperature; the *D. vulgaris* Hildenborough cytochrome c_3 (M_r 13 000) is highly thermostable (denaturation temperature 129 °C), unlike the *D. desulfuricans* Norway cytochrome c_3 (M_r 13 000) (denaturation temperature 73 °C). The denaturation temperature of *D. desulfuricans* Norway cytochrome c_3 (M_r 26 000) has been measured to be 73 °C; by comparison, we might suppose that, in the case of *D. gigas* cytochrome c_3 (M_r 26 000), the presence of intramolecular disulphide bridges may induce a more rigid structure and a higher degree of thermostability.

The biochemical role of this class of dimeric molecules may be different from that of cytochrome c_3 (M_r 13 000), and might depend on the high haem content, the strong association in the dimer, the affinity for different redox partners and a possible membrane association [25]. This last property is in agreement with the extremely low solubility of the molecule.

	1	5	10	15	20	
C3 Dg dimer	L	D	V	P	C	K - V V I T A P E G E D P H P R - -
C3 D d N dimer	E	T	F	E	I	P E S - V T M S P K Q F E - - G Y T - -
C3 D g	V	D	V	P	A	D G A K I D F I A G - - - G E K - -
C3 D d N	A	D	A	P	G	D D Y V I S A P E G M K A K P K G D

	25	30	35	40																						
C3 D g dimer	-	-	-	-	F	G	K	V	E	M	S	H	A	K	H	R	N	V	S	C	V	S	C	H	H	
C3 D d N dimer	-	-	-	P	K	K	G	D	V	T	F	N	H	A	S	H	M	D	I	A	C	Q	Q	C	H	H
C3 D g	-	-	-	-	-	N	L	T	V	V	F	N	H	S	T	H	K	D	V	K	C	D	D	C	H	H
C3 D d N	K	P	G	A	L	Q	K	T	V	P	F	P	H	T	K	H	A	T	V	E	C	V	Q	C	H	H
													H1		H3									Heme 1		H2

	45	50	55	60																				
C3 Dg dimer	M	F	D	G	C	G - D	F	Q	K	C	-	-	A	D	C	H	I	D	R	D	D	R		
C3 D d N dimer	T	V	P	D	T	Y - T	I	E	S	C	M	T	E	G	C	H	D	N	I	K	E	R		
C3 D g	D	P	-	G	D - K	Q	Y	A	G	C	T	T	D	G	C	H	N	I	L	D	K	A		
C3 D d N	T	L	E	A	D	G	G	A	V	K	K	C	T	T	S	G	C	H	D	S	L	E	F	R

Figure 2 Sequence alignment based on the three-dimensional structures of cytochromes c_3 (M_r 13000) from *D. gigas* (C3 D g) and *D. desulfuricans* Norway (C3 D d N) and of cytochrome c_3 (M_r 26000) from *D. desulfuricans* Norway (C3 D d N dimer)

The numbering corresponds to the amino acid sequence of cytochrome c_3 (M_r 26000) from *D. gigas* (C3 D g dimer). Cysteine residues 5 and 46 and residues common to several proteins are emboldened. The haem attachment sites have been boxed and numbered in sequence. The sixth histidine axial ligand is indicated for each haem ('heme'). The numbers below the histidine residues correspond to the haem binding site with which they are presumably associated, according to the three-dimensional structures of *D. desulfuricans* Norway cytochromes c_3 (M_r 13000) and c_3 (M_r 26000).

The exact functions of the cytochromes c_3 (M_r 26000) have not yet been established. The *D. gigas* cytochrome has been reported to stimulate sulphate reduction in a crude extract and to mediate electron transfer between hydrogenase and sulphate reductase, whereas cytochrome c_3 (M_r 13000) is almost inactive in this reaction. However, molecular genetics have shown that dimeric cytochrome is periplasmic whereas the sulphate reductase is cytoplasmic (M. Bruschi, unpublished work).

Measurement of the homogeneous second-order rate constant for electron transfer between [NiFeSe] hydrogenase and the dimeric cytochrome from *D. desulfuricans* Norway yielded a value of $8 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$, as determined by cyclic voltammetry. This compares favourably with the value of $6 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ obtained on reduction of cytochrome c_3 under the same conditions [26].

A high- M_r cytochrome was recently characterized from *D. gigas* [18] and it has been demonstrated that this enzyme can act as an electron acceptor of the [NiFe] hydrogenase from this organism, like the tetrahaem cytochrome c_3 . It can also induce the rapid reduction of *D. gigas* rubredoxin, which in turn reduces *D. gigas* rubredoxin oxygen oxidoreductase [27]. Further studies, including physiological tests, the investigation of interfacial properties with membrane models [25] and comparisons between the three-dimensional structures of the various classes of polyhaem cytochromes, are now required in order to establish their specificity towards their redox partners.

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