A Membrane-Bound Cytochrome c_3: A Type II Cytochrome c_3 from *Desulfovibrio vulgaris* Hildenborough

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A new tetraheme cytochrome c_3 was isolated from the membranes of Desulfovibrio vulgaris *Hildenborough (*Dv*H). This cytochrome has a molecular mass of 13.4 kDa and a pI of 5.5 and contains four heme* c *groups with apparent reduction potentials of −170 mV, −235 mV, −260 mV and −325 mV at pH 7.6. The complete sequence of the new cytochrome, retrieved from the preliminary data of the* Dv*H genome, shows that this cytochrome is homologous to the "acidic" cytochrome c_3 from* Desulfovibrio africanus *(*Da*). A model for the structure of the* Dv*H cytochrome was built based on the structure of the* Da *cytochrome. Both cytochromes share structural features that distinguish them from other cytochrome c_3 proteins, such as a solvent-exposed heme 1 surrounded by an acidic surface area, and a heme 4 which lacks most of the surface lysine patch proposed to be the site of hydrogenase interaction in other cytochrome c_3 proteins. Furthermore, in contrast to previously discovered cytochrome c_3 proteins, the genes coding for these two cytochromes are adjacent to genes coding for two membrane-associated FeS proteins, which indicates that they may be part of membrane-bound oxidoreductase complexes. Altogether these observations suggest that the* Dv*H and* Da *cytochromes are a new type of cytochrome c_3 proteins (Type II: TpII-c_3) with different redox partners and physiological function than the other cytochrome c_3 proteins (Type I: TpI-c_3). The* Dv*H TpII-c_3 is reduced at considerable rates by the two membrane-bound [NiFe] and [NiFeSe] hydrogenases, but catalytic amounts of TpI-c_3 increase these rates two- and fourfold, respectively. With the periplasmic [Fe] hydrogenase TpII-c_3 is reduced much slower than TpI-c_3, and no catalytic effect of TpI-c_3 is observed.*

KEYWORDS:

cytochromes · electron transfer · heme proteins · hydrogenases · membrane proteins

Introduction

Sulfate-reducing bacteria are anaerobes that can grow by oxidation of hydrogen or organic compounds with sulfate as a terminal electron acceptor. The reduction of sulfate is a true respiratory process, which leads to oxidative phosphorylation through a still poorly understood electron-transfer pathway. A characteristic feature of this electron-transfer pathway is the involvement of multiheme cytochrome *c* proteins of low redox potential.[1] Several elements of this family of electron-transfer proteins have been identified in *Desulfovibrio*, the most studied genus of sulfate-reducing bacteria. The tetraheme cytochrome c_3 ($M_r \approx 13\,000$)[2] is the most abundant member, and is the only one present in all the species studied so far. In addition, it is also present in other genera of sulfate-reducing bacteria such as *Desulfomicrobium*,[3] *Desulfobulbus*[4] and *Thermodesulfobacterium*.[5] Other members are the Split – Soret cytochrome that is a dimer of a diheme subunit,[6] the octaheme cytochrome c_3 ($M_r \approx 26\,000$), a dimer of a tetraheme subunit,[7] the monomeric nine-heme cytochrome *c* (9Hcc)[8][**] and the high molecular weight cytochrome *c* which is a monomer containing 16 hemes (16Hcc).[9] *Desulfovibrio* species contain different cytochrome compositions: For example, in *Desulfovibrio gigas* (*Dg*) the tetraheme cytochrome c_3, the octaheme cytochrome c_3 (26 000) and the 16Hcc were described, whereas in *Desulfovibrio desulfuricans* ATCC 27774 (*Dd*27k) the tetraheme cytochrome c_3, the Split-Soret cytochrome and the 9Hcc were reported. In *Desulfovibrio vulgaris* Hildenborough (*Dv*H) the tetraheme cytochrome c_3 and 16Hcc were isolated, but this bacterium also contains a monoheme cytochrome called cytochrome c_{553}.[10]

The physiological function of some of these cytochromes is still unclear, as is the need for several similar cytochromes in the same bacterium. The ubiquitous tetraheme cytochrome c_3 is generally considered to act as the physiological partner for hydrogenases, which are also always present in these bacteria, and has been proposed to play a central role in the metabolism

[**] *A list of abbreviations can be found in ref. [66].*

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by acting as an energy-transducing device which converts high-energy electrons (low redox potential) and low-energy protons (high pK_a), which result from the periplasmic oxidation of hydrogen, into lower-energy electrons for the reduction of sulfate and higher-energy protons for the production of ATP.[11–13] The involvement of hydrogen as a necessary intermediate in the sulfate respiration process has been proposed,[14] but the generality of this model is still a matter of controversy.[15] Nevertheless, many *Desulfovibrio* species can use H_2 as sole energy source.[16] All these bacteria contain one or more hydrogenases of the [Fe], [NiFe] and [NiFeSe] types. A screening of 25 *Desulfovibrio* species showed that only the genes encoding the [NiFe] hydrogenase are present in all.[17] In a situation analogous to that of the cytochromes, different species contain different hydrogenase compositions and it is not clear why more than one type should be necessary for some bacteria. For example, *Dv*H contains a periplasmic [Fe] hydrogenase,[18] and two membrane-bound [NiFe][19] and [NiFeSe] hydrogenases.[20]

Despite the low sequence homology between cytochrome c_3 proteins of different species, the several crystal structures which have been determined[21–26] show that the general folding is maintained, as is the spatial arrangement of the four-heme core. This four-heme structural motif seems to be a common feature of this family of cytochromes, as it is also observed in octaheme cytochrome c_3 (26 000) proteins,[7, 27] in the 9Hcc,[8] and most probably also in 16Hcc since its sequence shows it is organised in four c_3-like domains[28] (the first one being an incomplete domain with only three hemes) and that the last two domains have a strong sequence similarity to the 9Hcc, which suggests a similar structural organisation.[8] This indicates that the four-heme structural motif may have a specialised functional role.

The genes coding for 16Hcc and the related 9Hcc[8] are both part of operons encoding transmembrane redox complexes[29, 30] proposed to perform the electron transfer between the periplasmic oxidation of hydrogen, carried out by the hydrogenase/cytochrome c_3 couple, and the cytoplasmic reduction of sulfate.[29, 31] Although 16Hcc is not a hydrophobic protein, it is found in higher amounts in the membrane than in the soluble fraction, in both *Dv*H and *Dg*.[9] The reduction of *Dv*H 16Hcc by the [Fe] and [NiFe] hydrogenase is mediated by cytochrome c_3,[31] as is also the case for 9Hcc and [NiFe] hydrogenase of *Dd*27k,[8] and the octaheme cytochrome c_3 (26 000) and [NiFeSe] hydrogenase of *Desulfomicrobium norvegicum* (*Dmn*).[32]

In our attempts to study membrane proteins of *Dv*H that may be involved in the electron-transport pathway for the reduction of sulfate, we have isolated a new tetraheme cytochrome c_3 with a lower pI than the well-characterised and more abundant cytochrome c_3. The new cytochrome is not detected in the soluble fraction, whereas some of the previously characterised cytochrome c_3 was also found to be associated with the membrane fraction. The coexistence of two different tetraheme cytochromes c_3 in one organism was previously reported only for *Desulfovibrio africanus* (*Da*),[33, 34] but both were isolated from the soluble fraction. In this case, the authors opted to name the two cytochromes as "acidic" and "basic",[33] a nomenclature that may be misleading as the pI value of a cytochrome c_3 will, by itself, not reveal to which type the cytochrome belongs. Indeed, cytochrome c_3 from *Dg* which is as acidic as *Dv*H TpII-c_3 (pI of 5.2[35]), does not share the structural features of the TpII-c_3 proteins, but is similar to other TpI-c_3 proteins. As an alternative classification we name the "acidic" cytochrome c_3 proteins of *Dv*H and *Da* as Type II cytochrome c_3 proteins (TpII-c_3s), and the well-characterised and more abundant "basic" cytochrome c_3 proteins as Type I cytochrome c_3 proteins (TpI-c_3s), since structural and genetic evidence (see below) indicates that these cytochromes belong to separate families.

In this report we describe the purification and characterisation of the TpII-c_3 from the membranes of *Dv*H, as well as a study of its reactivity with the [Fe], [NiFe] and [NiFeSe] hydrogenases from the same organism.

Results and Discussion

The repeated washing of the *Dv*H membranes ensured that all soluble and loosely-bound proteins were removed, as confirmed by the visible spectrum, in which the peak of desulfoviridin at 630 nm was used as a marker for the presence of soluble proteins. The detergent extract obtained after solubilisation of the membrane proteins with SB12 contained a high amount of cytochromes. After purification of this extract it was possible to separate four heme proteins. The nonsticking fraction from the diethylaminoethyl cellulose (DEAE) column contained the two high-pI cytochromes 16Hcc[9] and TpI-c_3, which was identical to that obtained from the soluble fraction and was identified by its N-terminal sequence. A more acidic fraction contained the heme *c* nitrite reductase[36] and an unknown cytochrome of small molecular mass that, after purification and characterisation, proved to be a low-pI tetraheme cytochrome c_3 (TpII-c_3). The TpII-c_3 could not be found upon purification of the *Dv*H soluble extract, which suggests that its association with the cytoplasmic membrane is quite strong. The finding of the TpI-c_3 in the membrane extract, even after thorough washing of the membranes, suggests that this periplasmic cytochrome also interacts with membrane-bound proteins. The amount of the TpI-c_3 purified from the membrane fraction was significant, being about half of that usually obtained from the soluble fraction. The distribution of a cytochrome between soluble and membrane extracts was previously described for the 16Hcc of *Dv*H and *Dg*,[9] but these cytochromes are found in much higher amounts in the membrane than in the soluble fraction. The TpII-c_3 was found exclusively in the membrane fraction, and the amount purified was similar to that of the membrane-associated TpI-c_3 (7 mg versus 10 mg). In *Da* the TpII-c_3 was isolated from the soluble fraction.[33] However, since no reference was given to purification of membrane proteins, it is possible that in this bacterium the TpII-c_3 is distributed between the soluble and membrane fractions.

Molecular characteristics

The new TpII-c_3 was judged to be pure by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) which displayed only one band. The molecular mass determined from the gel was 14 kDa, in good agreement with the value calculated from the

amino acid sequence (see below). To determine whether the new cytochrome was a monomer or a dimer, it was dialysed to remove the detergent and the molecular mass was determined by gel filtration; this yielded a value of approximately 17 kDa, which reveals that it exists in solution as a monomer. The isoelectric point of the new cytochrome is 5.5, as determined by isoelectric focussing. The number and type of hemes present were determined by the pyridine hemochrome which showed that it contained four heme *c* groups per molecule.

Besides the TpI-c_3 and TpII-c_3 there is at least one other tetraheme cytochrome *c* associated with the membranes of *Dv*H, which was purified as one of the subunits of the nitrite reductase.[36] The sequence of this cytochrome can be retrieved from the preliminary *Dv*H genome data and shows that it is part of the NapC/NirT family of cytochrome *c* proteins that are proposed to receive electrons directly from the quinone pool.[37, 38]

Spectroscopic characterisation

The UV/Vis spectrum of the TpII-c_3 is typical of cytochrome c_3 proteins (Figure 1). The absence of an absorption band at 695 nm indicates there are no methionine-bound hemes. The millimolar extinction coefficients of the bands observed in the

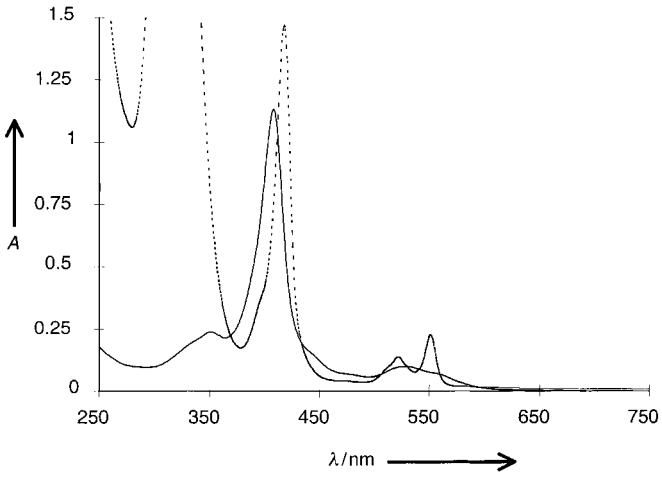

Figure 1. *UV/Vis spectra of the oxidised (full line) and reduced with dithionite (dashed line)* Dv*H TpII-*c_3.

oxidised spectrum are: 280 nm: 49.3 $mM^{-1}cm^{-1}$; 408: 555.7; 527.5: 48.6; and in the reduced spectrum: 417.5 nm: 729.3 $mM^{-1}cm^{-1}$; 522: 65.7; 551: 107.1. These extinction coefficients are similar to those reported for the TpII-c_3 of *Da*.[33] The purity index of the TpII-c_3 (A_{551}(red) $-$ A_{570}(red)/A_{280}(ox)) was 2.2, a value that is lower than that usually observed for TpI-c_3s (2.9 – 3.8). This is apparently due to a broader (and thus reduced in height) 551 nm peak in the TpII-c_3, compared to that of the TpI-c_3s. A similar situation is observed for the *Da* TpII-c_3.[33]

The electron paramagnetic resonance (EPR) spectrum of the oxidised *Dv*H TpII-c_3 is a rhombic spectrum typical of low-spin hemes (Figure 2). Three of the hemes in this cytochrome

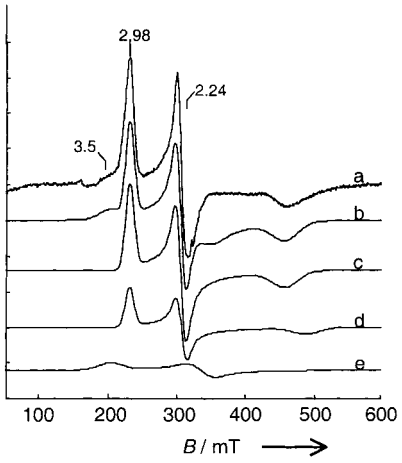

Figure 2. *Trace a: EPR spectrum of oxidised* Dv*H TpII-*c_3. *Temperature: 14 K; modulation amplitude: 0.9 mT; microwave power: 0.75 mW; microwave frequency: 9.63 GHz. Trace b: Simulation of trace a obtained by adding three theoretical spectra with* g *values of:* $g_z = 2.98$, $g_y = 2.24$ *and* $g_z = 1.50$ *(2 hemes; trace c),* $g_z = 2.98$, $g_y = 2.24$ *and* $g_z = 1.40$ *(1 heme; trace d) and* $g_z = 3.5$ *and* $g_y = 2.0$ *(1 heme; trace e).*

have *g* values of $g_z = 2.98$, $g_y = 2.24$ and $g_x = 1.5$, which indicates that the angle between the planes of the two axial histidines is very similar for these hemes and close to 0°.[39, 40] The fourth heme has a g_z value of 3.5, which suggests that in this case the angle between the two histidine planes is close to 90°. In the *Da* TpII-c_3 a similar resonance is observed at 3.7,[34] and the crystal structure confirmed that heme 4 has an angle between the two histidine planes close to 90°.[41] The EPR spectrum of the *Dv*H TpII-c_3 (Figure 2, trace a) was simulated (trace b) by adding three theoretical spectra with *g* values of: $g_z = 2.98$, $g_y = 2.24$ and $g_x = 1.50$ (2 hemes; trace c), $g_z = 2.98$, $g_y = 2.24$ and $g_x = 1.40$ (1 heme; trace d) and $g_z = 3.5$ and $g_y = 2.0$ (1 heme; trace e).

The EPR spectrum of the *Dv*H TpI-c_3 is different from that of the TpII-c_3 and displays several resonances with g_z values of 2.77, 2.82, 2.97 and 3.12.[42]

Heme reduction potentials

The redox behaviour of the *Dv*H TpII-c_3 at pH 7.6 was probed by a redox titration in the presence of redox mediators (Figure 3). The experimental points were fitted with four noninteracting Nernst equations (n = 1) with reduction potentials of -170 mV, -235 mV, -260 mV and -325 mV. These reduction potentials are similar to those commonly observed for TpI-c_3s (including that from *Dv*H[42]), and have in common the fact that one of the hemes displays a considerably higher reduction potential than the other three. This was not observed for the TpII-c_3 of *Da*, for which the four reduction potentials obtained by cyclic voltammetry were quite close (-210 mV, -240 mV, -260 mV and -270 mV).[33]

Sequence analysis

The N-terminal sequence of the TpII-c_3 was determined up to residue 48, to confirm that the new cytochrome is distinct from

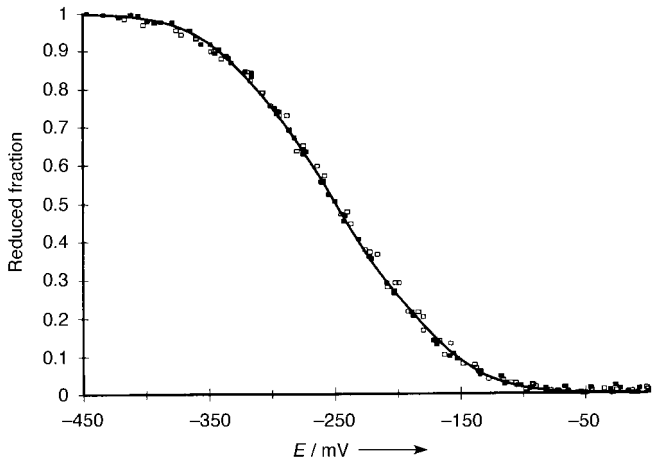

Figure 3. *Redox titration of* Dv*H TpII-c*$_3$ *obtained by following absorption changes at 552 nm (pH 7.6; ■ reduction, □ oxidation). The experimental points are from three different experiments. The full line corresponds to a theoretical simulation obtained by assuming reduction of four hemes with redox potentials of − 170 mV, − 235 mV, − 260 mV and − 325 mV.*

TpI-c_3. This N-terminal sequence was used to retrieve the complete sequence of the TpII-c_3 gene from the preliminary data of the *Dv*H genome, which is being sequenced at The Institute for Genomic Research. The gene reveals that the TpII-c_3 is synthesised as a precursor protein with a signal peptide, which indicates that it will be exported to the periplasmic side of the membrane. The signal peptide (MFRRIGTVMLAFATLALLLAVAFA) contains 24 residues, with two positive residues at the beginning (R3,R4), followed by a string of hydrophobic residues. The sequence obtained for the TpII-c_3 shows that this is not a hydrophobic protein and that it contains no transmembrane helices. This indicates that the membrane attachment of this cytochrome is not likely to be due to protein – lipid interactions, but rather to interactions of the cytochrome with other membrane-bound proteins.

The sequence of the *Dv*H TpII-c_3 is homologous to that of *Da* TpII-c_3, having 46% identity and 62% similarity. The two sequences can be aligned with only one amino acid insertion (Figure 4 A). The molecular mass of the *Dv*H TpII-c_3 obtained from the sequence, including the four hemes, is 13445 Da.

The crystal structure of the *Da* TpII-c_3 revealed that this cytochrome has some features that distinguish it from other cytochrome c_3 proteins.[41] In order to investigate the presence of such features in the newly discovered cytochrome c_3, as well as to search for other conserved motifs, a model of the three-dimensional (3D) structure of this cytochrome was generated with the Swiss-Model program,[43] with the structures of the oxidised and reduced *Da* TpII-c_3[41] (Protein Data Bank (PDB) accession numbers: 3CAOA and 3CARA, respectively) used as templates. This model and the structures of several other cytochrome c_3 proteins (*Da* TpII-c_3,[41] *Dv*H TpI-c_3,[24] *Desulfovibrio vulgaris* Miyazaki (*Dv*M) cytochrome c_3,[22] *Dg* cytochrome c_3,[26] *Dd*27k cytochrome c_3[24] and *Dmn* cytochrome c_3[25]) were structurally aligned with the program MODELLER 4[44] (Figure 4 B). This structural alignment yields a multisequence alignment that is generated by tridimensional proximity rules. If two residues from different proteins are aligned, this means that the distance between their C^{α} is below a certain cut-off. The cut-off used in this case was 4 Å. This alignment and structural

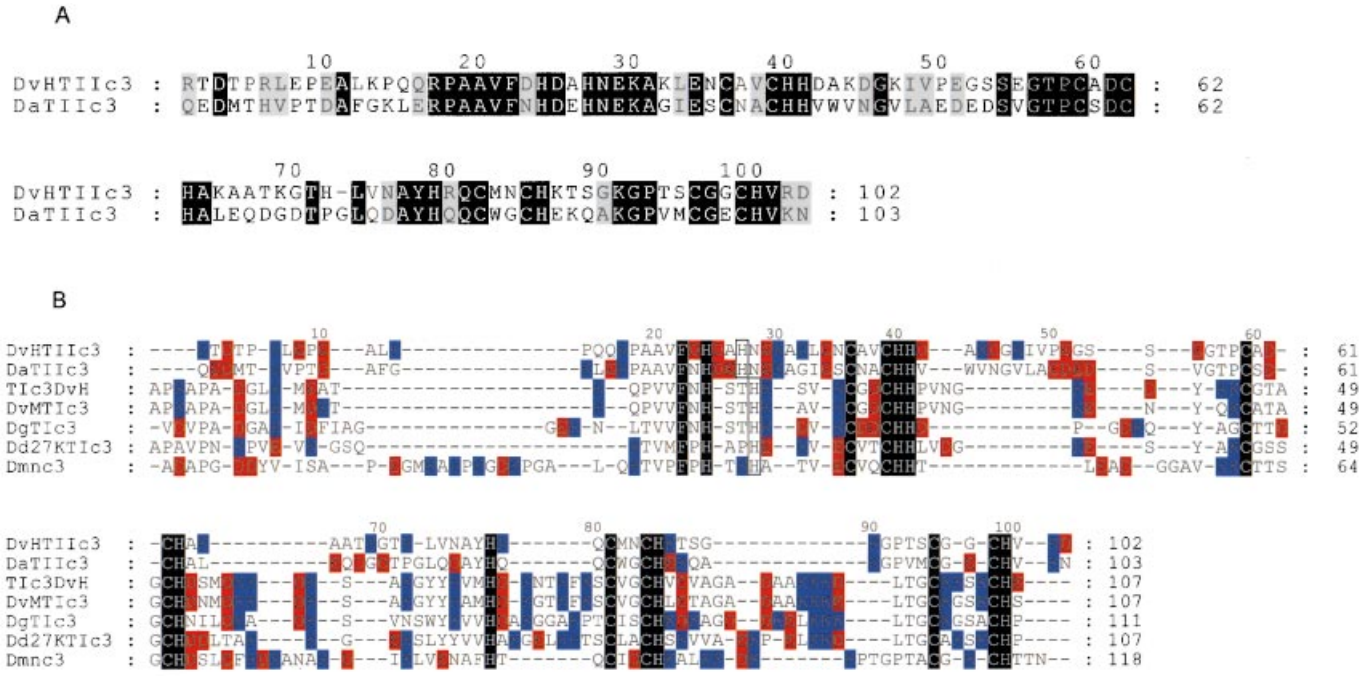

Figure 4. *A) Sequence alignment of the TpII-c*$_3$*s from* Dv*H and* Da*: Identical residues are coloured black, and similar residues are coloured grey. B) Structure-based alignment of the sequences of* Dv*H TpII-c*$_3$*,* Da *TpII-c*$_3$*,* Dv*H TpI-c*$_3$*,* Dv*M TpI-c*$_3$*,* Dg *TpI-c*$_3$*,* Dd*27k TpI-c*$_3$ *and* Dmn *cytochrome c*$_3$ *obtained with the MODELLER 4 program.*[44] *Fully conserved residues are coloured black, acidic residues are coloured red and basic residues coloured blue. The boxed histidine residue (ligand to heme 3) does not align in the TpII-c*$_3$*s with the corresponding residue in the other cytochromes because their* C^{α} *is quite distant, although the side-chain ring is actually very close to that of the other cytochrome c*$_3$ *proteins. The numbering in both alignments corresponds to the sequence of* Dv*H TpII-c*$_3$*.*

comparison of all the cytochromes shows that several of the structural features unique to the *Da* TpII-c_3[41] are also present in the *Dv*H TpII-c_3, and that altogether they justify the separation of these cytochromes from other *Desulfovibrio* cytochrome c_3 proteins.

In order to better visualise some of the structural differences between TpII-c_3s and TpI-c_3s, the model of the *Dv*H TpII-c_3 and the structure of the *Dv*H TpI-c_3 were superimposed by their heme groups (Figure 5). The list of the differences makes

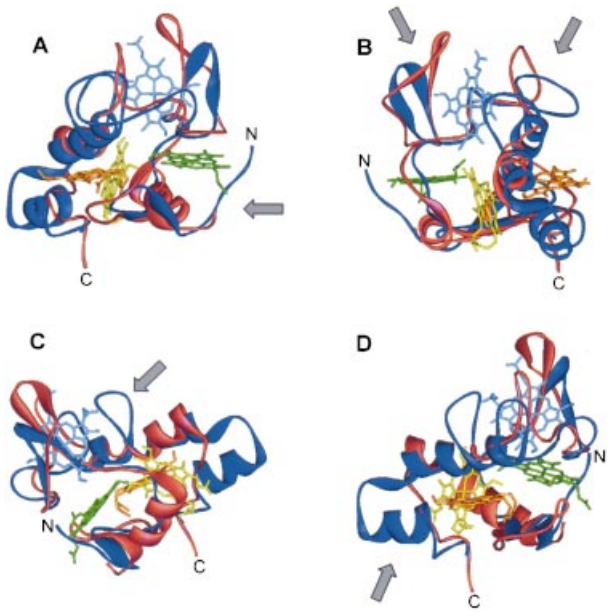

Figure 5. *Superposition of the* Dv*H TpII-*c_3 *model (red) and* Dv*H TpI-*c_3 *structure (blue) obtained by superimposing the heme groups. (Only one set of heme groups is shown: Heme 1 green, heme 2 pale blue, heme 3 yellow, heme 4 orange.) The N terminus of the TpI-*c_3 *and C terminus of the TpII-*c_3 *are identified by N and C. The grey arrows point to the features highlighted in each view: A) Shorter N-terminal region of the TpII-*c_3*; B) two loops covering heme 2 in the TpII-*c_3*; C) loop missing in TpII-*c_3*; D) shorter C-terminal region of TpII-*c_3*.*

compelling evidence for the classification of the two TpII-c_3s as a new type of *Desulfovibrio* cytochrome c_3 protein:

1) All heme binding sites of the two TpII-c_3s are of the form CXXCH, whereas other cytochrome c_3 proteins contain one or two heme binding sites with four residues between the two Cys residues (CXXXXCH).
2) The N-terminal region of the two TpII-c_3s is shorter than that of other cytochrome c_3 proteins by three to four residues[41] (Figure 5 A). These are mostly hydrophobic residues that cover the external edge of heme 1 in TpI-c_3s, and as a result of their absence this heme is more exposed to the solvent in both TpII-c_3s.
3) Several acidic residues are clustered at the surface region around heme 1 of *Dv*H TpII-c_3 (D3, D25, E29, E34, D42, E51 and E55), as was found for *Da* TpII-c_3.[41] This is in contrast with TpI-c_3s where heme 1 is surrounded by a neutral environment.
4) The H27 of the two TpII-c_3s (the axial ligand to heme 3) does not align with the corresponding residue of the other cytochrome c_3 proteins. Inspection of the structures shows that, although the C^{α} of the TpII-c_3s histidine is indeed quite distant from that of the other cytochromes, its side-chain ring (which binds to the Fe of heme 3) is still very close to the side-chain ring of the corresponding histidine in the other cytochrome c_3 proteins.
5) Residues 42 – 51 of the two TpII-c_3s correspond to a loop formed by two β-strands that is shorter in other cytochrome c_3 proteins[41] and which partly covers heme 2 (Figure 5 B).
6) The loop formed by residues 66 – 72 of TpII-c_3s is shifted away from heme 4 towards heme 2, relative to the corresponding loop in TpI-c_3s (residues 54 – 64 in *Dv*H TpI-c_3) which contains two to three lysines that are at the surface of heme 4 (Figure 5 B).
7) Another loop of 6 – 7 residues is missing in the two TpII-c_3s between residues 79 and 80,[41] which in TpI-c_3s interrupts two α-helices and contains several lysine residues (Figure 5 C). This loop is also absent in the *Dmn* cytochrome c_3.
8) The C-terminal region of the TpII-c_3s is shorter and is missing a region of 6 – 8 residues containing several lysines, between residues 89 and 90[41] (Figure 5 D).
9) The three points mentioned directly above and also the absence of lysine residues between the two cysteine residues binding heme 4 mean that both TpII-c_3s have fewer basic residues than other cytochrome c_3 proteins, and particularly so around heme 4 where in TpI-c_3s several lysine residues are concentrated to form a positive surface patch. (In *Dv*H TpIc_3 this patch is formed by lysine residues 15, 57, 58, 60, 72, 94, 95, 101 and 102.[45])

The relative identities and similarities between all the cytochromes obtained from the structure-based alignment (Figure 4) are shown in Table 1. These values support the separation of the *Desulfovibrio* cytochromes into two types. The classification of the *Desulfomicrobium* cytochrome c_3 is

Table 1. *Percentages of identity (first line), similarity (second line) and residues aligned with gap characters (third line) between the several cytochrome* c_3 *proteins, obtained from the structural alignment shown in Figure 4. The values between TpII-*c_3*s and between TpI-*c_3*s are in bold.*

	Da TpII-c_3	*Dv*H TpI-c_3	DvM TpI-c_3	Dg TpI-c_3	*Dd*27 TpI-c_3	*Dmn* c_3
*Dv*H TpII-c_3	**45**	13	14	14	16	16
	62	20	24	21	19	22
	0	47	47	50	49	48
Da TpII-c_3		15	16	13	14	15
		19	21	19	18	23
		46	46	49	48	47
*Dv*H TpI-c_3			**86**	**45**	**42**	21
			93	**53**	**58**	31
			0	**12**	**8**	40
*Dv*M TpI-c_3				**47**	**38**	20
				55	**55**	30
				12	**8**	40
Dg TpI-c_3					**33**	22
					44	26
					15	39
*Dd*27 TpI-c_3						23
						28
						41

presently not straightforward because it has some features of both types. Nevertheless, it has more similarities to type I cytochromes (Table 1); it also acts as the physiological partner for the *Dmn* [NiFeSe] hydrogenase and can mediate the reduction of other multiheme cytochromes by this hydrogenase.[32] This suggests that the *Dmn* cytochrome c_3 is probably a type I cytochrome c_3 more distantly related than the *Desulfovibrio* TpI-c_3s because it originates from a bacterium of a different genus.

In order to better visualise the TpII-c_3s features, the surface electrostatic potentials of the fully oxidised *Dv*H TpII-c_3, *Da* TpII-c_3 and, as a reference, *Dv*H TpI-c_3 were calculated with the GRASP program[46] (Figure 6). Overall, the *Dv*H TpII-c_3 is not as acidic as the *Da* one, as would be expected since it has 13 acidic residues (D and E) and 14 basic ones (K and R), compared to 19 acidic and 6 basic for *Da* TpII-c_3. However, the similarity between the two TpII-c_3s and the contrast with the *Dv*H TpI-c_3 is readily apparent. Looking at the view from the edge of heme 1 (Figure 6 A), it is clear that this heme is indeed surrounded by an acidic surface, more pronounced in the *Da* TpII-c_3[41] than in the *Dv*H one, but completely absent in *Dv*H TpI-c_3. Looking at the structures, it is obvious that the N-terminal residues covering heme 1 in *Dv*H TpI-c_3 are not present in the two TpII-c_3s. These features support the suggestion that heme 1 of the TpII-c_3s plays a role in intermolecular electron transfer,[41] contrary to the case in other cytochrome c_3 proteins where this heme is surrounded by a neutral environment and is protected against solvent by the N terminus.

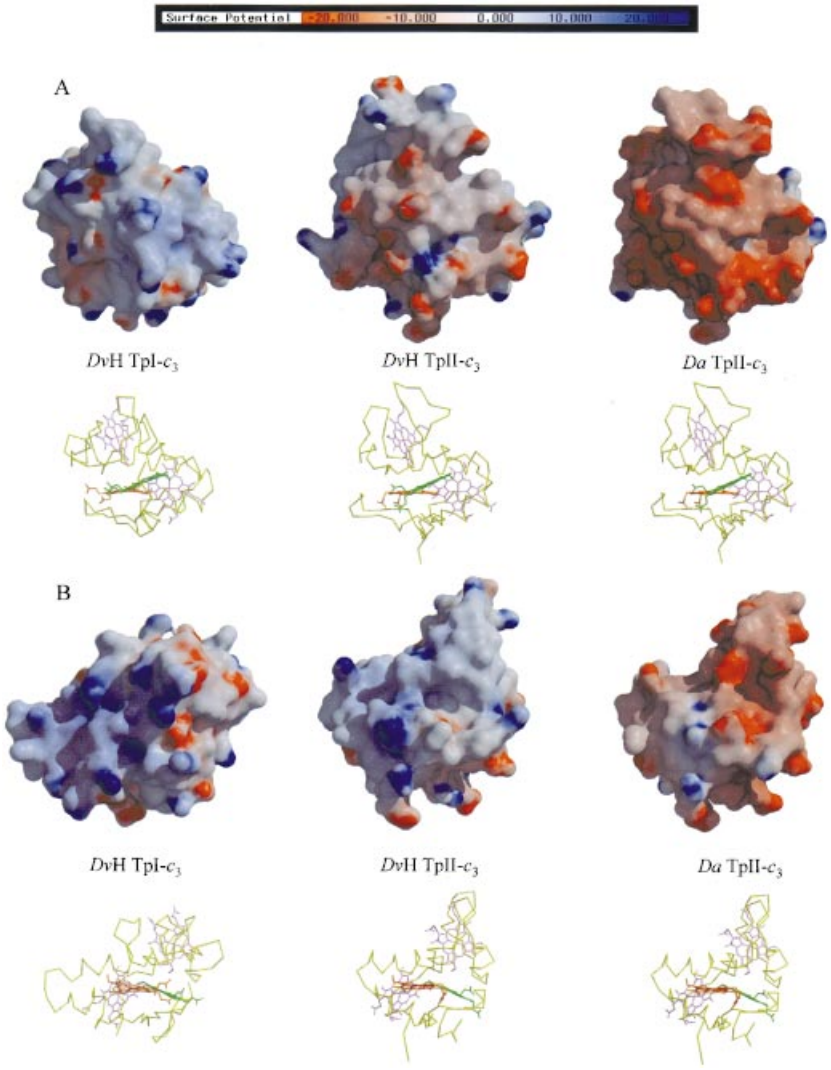

Figure 6. *Representation of the surface electrostatic potential of the fully oxidised* Dv*H TpII-*c_3, Da *TpII-*c_3 *and* Dv*H TpI-*c_3. *Red-coloured zones correspond to negative potentials while blue-coloured zones correspond to positive potentials. The range of potentials spans from* -20 *to 20* kTe^{-1}. *The structures of* Da *TpII-*c_3 *and* Dv*H TpI-*c_3 *and the model of* Dv*H TpII-*c_3 *are shown below in the same orientation as the surface representations. A) View from the edge of heme 1 (coloured green); B) view from the edge of heme 4 (coloured red). The figures were prepared with the GRASP[46] and Raster 3D programs.[57]*

The view from the edge of heme 4 (Figure 6 B) shows that the basic region that can be found close to this heme in *Dv*H TpI-c_3 due to the lysine patch is reduced in *Dv*H TpII-c_3 and even more so in *Da* TpII-c_3.[41] This lysine patch, also present in other *Desulfovibrio* type I cytochrome c_3 proteins, was proposed to be the site of interaction and electron exchange with a negatively charged region of the redox partner hydrogenase.[45, 47, 48] These differences in surface charge distribution indicate that TpII-c_3s may exchange electrons through a different heme (namely heme 1) from that used by TpI-c_3s (probably heme 4).

The structure of the nine-heme cytochrome *c* (9Hcc) from *Dd*27k was also analysed to investigate whether each of its cytochrome c_3 like domains had any resemblances to type I or type II c_3s. Interestingly, several of the TpII-c_3 structural features are observed in both 9Hcc domains, although differences are also present, not least because 9Hcc contains one extra heme. The main similarities between the 9Hcc cytochrome c_3 like domains and the TpII-c_3s are: 1) all heme binding sites of 9Hcc are of the form CXXCH; 2) the first heme of the 9Hcc N-terminal domain is also significantly exposed and surrounded by a negative surface area;[49] 3) in both 9Hcc domains the loop after the second heme binding site is shifted away from heme 4 towards heme 2; 4) in the 9Hcc C-terminal domain the loop before the heme 3 binding site, which interrupts two α-helices in TpI-c_3s, is also absent; 5) heme 4 of the 9Hcc C-terminal domain is not surrounded by a positive surface region. This suggests that, overall, 9Hcc has the same fundamental characteristics as TpII-c_3s, namely, in the N-terminal domain an exposed heme 1 surrounded by an acidic surface region, which is probably the heme involved in intermolecular electron transfer, and in the C-terminal domain a heme 4 (9Hcc heme 9) that lacks the TpI-c_3 characteristic positive surface patch. These similarities may indicate that TpII-c_3s and 9Hcc have analogous reaction modes and/or physiological partners, which is supported by the

evidence that both are part of membrane-bound redox complexes[30] (see below). A similar conclusion may be expected for the 16Hcc since its last two domains have a strong similarity to 9Hcc.[8]

Analysis of the partial sequence obtained from the *Dv*H genome shows that, upstream of the gene coding for TpII-c_3, an open reading frame is present that codes for a protein which has a high similarity (33% identity, 53% similarity) to an FeS protein belonging to the *Dv*H *hmc* operon, HmcF, and which is predicted to contain two $[4Fe-4S]^{2+/1+}$ centres[29, 50] (Figure 7). The *Dv*H *hmc* operon codes for a transmembrane redox complex that is proposed to transfer electrons from the periplasm to the cytoplasm.[29] Although HmcF has no transmembrane sequences, it was shown to be present in the membrane fraction of *Dv*H cells.[50] Genes coding for proteins similar to the FeS protein and HmcF can be found in the genomes of several bacteria and they are usually associated with membrane-bound oxidoreductase complexes. The genome of the sulfate-reducing archaeon *Archaeoglobus fulgidus* contains at least nine genes coding for similar proteins.[51]

The genes coding for the TpII-c_3 and FeS proteins in *Dv*H are located in tandem with an intervening gap of only 25 nucleotides, and the absence of promoter sequences suggests that the two proteins belong to the same operon. The unavailability of nucleotide sequence data upstream of the gene coding for the FeS protein impedes the search for other putative genes and/or promoter sequences. With the BLASTX program, no significant sequence similarity to known genes could be found downstream of the *Dv*H TpII-c_3 gene and the search for typical bacterial terminator sequences was also unsuccessful. Therefore, it is not possible to exclude the hypothesis that other genes may be part of this operon. Remarkably, a similar situation is observed in *Da*, where 15 nucleotides upstream of the region encoding *Da* TpII-c_3[52] an incomplete open reading frame also encodes an FeS protein that shares 67% identity and 79% similarity in the C-terminal sequence available (174 amino acids) to the corresponding sequence of the FeS protein found in the *Dv*H genome (Figure 7). In *Da* the close proximity between the genes for FeS and TpII-c_3 also suggests that they are part of the same operon and questions the proposal that TpII-c_3 is encoded by a monocistronic gene.[34] These observations suggest that the TpII-c_3s and the FeS proteins can be part of membrane-bound oxidoreductase complexes and further support the proposal of considering the two TpII-c_3s as a separate type of cytochrome c_3 protein, since there is no evidence that the genes encoding for the TpII-c_3s are part of polycistronic units.[53] The fact that *Dv*H TpII-c_3 is not a hydrophobic protein and that it is not solubilised upon washing the membranes with buffer also agrees with the existence of a strong interaction between TpII-c_3 and a membrane-bound protein complex. In *Da* this interaction is probably weaker since the TpII-c_3 was found in the soluble fraction.

Figure 7 presents an alignment of the incomplete sequence of the *Dv*H FeS protein, the incomplete C-terminal sequence of the *Da* FeS protein, the sequence of *Dv*H HmcF (residues 58 – 461), the sequence of a similar FeS protein taken from the genome of *Archaeglobus fulgidus*[51] (gene AF0547; PID: g7448923; resi-

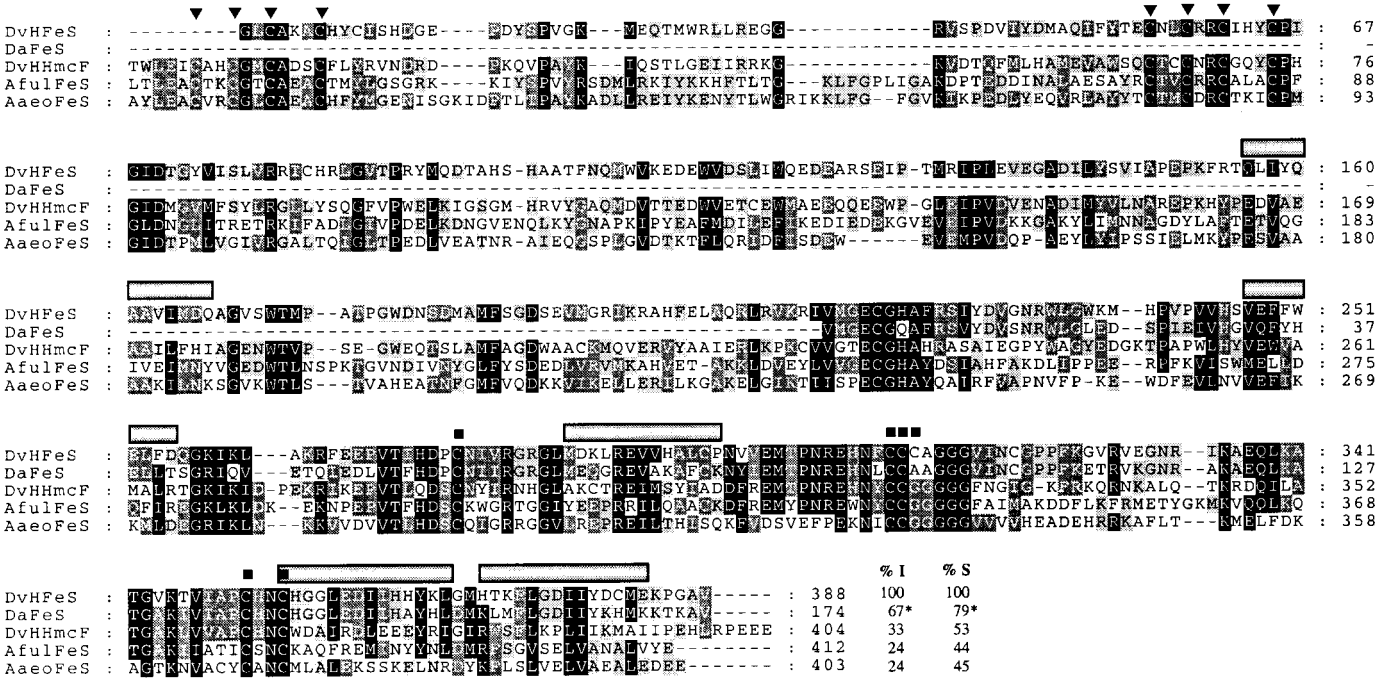

Figure 7. *Alignment of the incomplete sequence of the* Dv*H FeS protein, the incomplete C-terminal sequence of the* Da *FeS protein, the sequence of* Dv*H HmcF (residues 58 – 461), the sequence of a similar FeS protein from* Archaeglobus fulgidus[47] *(gene AF0547; PID: g7448923; residues 66 – 477) and the sequence of a similar FeS protein from* Aquifex aeolicus[49] *(gene AF0543, PID: g7448926; residues 32 – 434). The conserved amphipatic α-helices potentially involved in membrane attachment are represented by boxes over the sequences. %I = % Identity, %S = % similarity, *: the values of identity and similarity for* Da *FeS protein refer to the alignment of the 174 aminoacids available for the sequence of this protein. The cysteine residues binding the two [4Fe-4S]$^{2+/1+}$ centres are marked by ▼ and those belonging to the cysteine motif by ■.*

dues 66 – 477), which is adjacent to a gene coding for a putative membrane-bound γ-subunit of a nitrate reductase (NarI), and the sequence of a similar FeS protein taken from the genome of *Aquifex aeolicus*[54] (gene AF0543, PID: g7448926; residues 32 – 434), which codes for a putative subunit of a heterodisulfide reductase (HrdD). These proteins contain two $[4Fe-4S]^{2+/1+}$ centres and share a considerable similarity that indicates they are homologous proteins (see Figure 7). Although the sequence retrieved from the *Dv*H genome data still lacks the N-terminal part of this FeS protein, the last two cysteine residues of the first iron – sulfur cluster and the cysteine binding motif of the second are conserved. A cysteine motif formed by $CX_{34}CCGX_{39-41}CX_2C$ is also conserved in these proteins, as was observed for other similar FeS proteins from several organisms.[55] All of these FeS proteins are membrane-associated although they all lack obvious transmembrane helices. It was proposed that the membrane binding domain of these FeS proteins could be formed by the hydrophobic face of several amphipatic α-helices,[55] as observed for several other monotopic membrane proteins (see, for example, ref. [56]). The predicted secondary structure of the *Dv*H FeS protein, as well as those of the other proteins aligned in Figure 7, shows that these proteins also contain several conserved amphipatic α-helices in the region of the cysteine motif which could be involved in a similar membrane attachment.

Reduction by DvH hydrogenases

The difference in molecular characteristics between the *Dv*H type I and type II cytochrome c_3 proteins suggests that they will have different redox partners and physiological roles. The TpI-c_3 is generally considered to be the partner for the enzyme hydrogenase and was shown to mediate the reduction of some other cytochromes by hydrogenases, namely *Dv*H 16Hcc,[31] *Dv*H cytochrome c_{553},[31] *Dd*27k 9Hcc[8] and the *Dmn* octaheme cytochrome c_3 (26 000).[32] However, for *Desulfovibrio desulfuricans* Essex 6, it was recently reported that cytochrome c_3 does not affect the reduction rate of the 9Hcc by the [NiFe] hydrogenase.[58] In *Dv*H three different hydrogenases were identified, two of which are membrane-bound (the [NiFe][19] and [NiFeSe] hydrogenases[20]). Thus, it was investigated whether the TpII-c_3 can interact specifically with one of them. For each *Dv*H hydrogenase a comparison between the rates of the two cytochromes was obtained, and the effect of catalytic amounts of TpI-c_3 on the reduction of the TpII-c_3 was also observed. A concentration of TpII-c_3 lower than the Michaelis constant (K_m) was used to ensure that the enzyme was not saturated so that a catalytic effect of TpI-c_3 could be observed.

Reduction of the TpII-c_3 by the *Dv*H periplasmic [Fe] hydrogenase proceeds at a rate about 30 times slower than that of the TpI-c_3 under the same conditions (see Table 2). When the reduction of the TpII-c_3 is performed in the presence of catalytic amounts of TpI-c_3 (equimolar with the hydrogenase) the rate is only marginally increased. The behaviour of the TpII-c_3 with the [Fe] hydrogenase is very similar to that observed with the monoheme cytochrome c_{553}.[31] The lack of catalytic effect of the TpI-c_3 may indicate that TpII-c_3 will preferentially interact directly with the hydrogenase. In the case of *Da* no reduction of TpII-c_3 by the *Desulfovibrio desulfuricans* ATCC 7757 [Fe] hydrogenase was observed, even in the presence of TpI-c_3, although this last cytochrome was efficiently reduced.[34] However, since the proteins used belong to different organisms no definite conclusion can be drawn from this result, and it should be pointed out that no [Fe] hydrogenase was ever detected in *Da*. The absolute reduction rates observed for the *Dv*H [Fe] hydrogenase can not be directly compared to those obtained with the other two hydrogenases because the experimental procedures used in each case were different,[31] and this will affect the activation state of the hydrogenases.

Table 2. *Rates of reduction of* Dv*H* TpII-c_3 *and* TpI-c_3 *with the three* Dv*H hydrogenases.*[a]

	Reduction rate		
	TpI-c_3	TpII-c_3	TpII-c_3/TpI-c_3
[Fe] H_2ase	700	26	30
[NiFeSe] H_2ase	1100	344	750
[NiFe] H_2ase	1670	205	795

[a] Rates of reduction given in nmol of cytochrome per min per nmol of hydrogenase (nmol Cyt min^{-1} nmol H_2ase^{-1}).

With the membrane-bound [NiFeSe] hydrogenase the reduction of the TpII-c_3 is faster and is only threefold slower than that of the TpI-c_3. A catalytic amount of TpI-c_3 increased the rate of reduction of the TpII-c_3 twofold; this indicates that the electron transfer is more efficient through the TpI-c_3, which may be the physiologically preferred electron acceptor of the hydrogenase. In the case of reduction of the *Da* cytochrome c_3 proteins by the *Da* [NiFeSe] hydrogenase, a much larger difference between the TpII-c_3 and TpI-c_3 was observed, since the latter is reduced with a rate about 60-fold faster than the former.[33] When both cytochromes were present, the rate of reduction was close to that of the TpI-c_3, which also indicates that this is an efficient intermediary electron carrier between the hydrogenase and the TpII-c_3.

The membrane-bound [NiFe] hydrogenase reduces the TpII-c_3 with a rate about eightfold slower than that of the TpI-c_3. As with the [NiFeSe] hydrogenase, a catalytic amount of TpI-c_3 increased the rate of reduction of the TpII-c_3, which indicates again that the electron transfer is more efficient when it is carried out through the TpI-c_3. Again, in *Da* the TpI-c_3 is reduced 90 times faster by the *Dg* [NiFe] hydrogenase than the TpII-c_3, and the presence of small amounts of the TpI-c_3 increases this rate of reduction fivefold.[34]

Altogether, these results show that the *Dv*H TpII-c_3 is less efficient than the TpI-c_3 as an electron acceptor for all three *Dv*H hydrogenases. However, its behaviour is different with each hydrogenase. The [NiFeSe] hydrogenase is that for which the least difference between the two cytochromes was observed, which indicates that TpII-c_3 may preferentially interact with this hydrogenase. However, both for this enzyme and the [NiFe] hydrogenase, the reduction of TpII-c_3 is faster with TpI-c_3 as a mediator. Nevertheless, the two *Dv*H membrane-bound hydrogenases, and in particular the [NiFeSe], can reduce the TpII-c_3 at a

considerable rate, a result which raises the question of whether, in vivo, this cytochrome is able to receive electrons from these hydrogenases. It should also be pointed out that the physiological interaction between these proteins will take place in a bidimensional lipidic phase which can significantly alter the properties of this interaction. By contrast, for the [Fe] hydrogenase, not only is the reduction of TpII-c_3 much slower than that of TpI-c_3, but it is also not affected by the presence of the latter. It is interesting to note that overall the *Dv*H TpII-c_3 reacts better with the hydrogenases than the *Da* one.[33, 34] This may be related to the fact that the surface region around heme 4 is more basic in the *Dv*H TpII-c_3 than in the *Da* one, which may permit a better interaction with the hydrogenases.

In relation to the catalytic effect of the TpI-c_3 on the reduction of the TpII-c_3 by the [NiFe] and [NiFeSe] hydrogenases, it should be noted that for the *Dd*27k 9Hcc, which presents a similar effect,[8] a high probability specific interaction was observed by modelling studies between the negative heme 1 N-terminal region of 9Hcc and the positive heme 4 region of the TpI-c_3.[49] Since heme 1 of the *Dd*27k 9Hcc has similar characteristics to that of the TpII-c_3s (solvent-exposed and surrounded by a negative surface charge[49]), it is likely that an analogous situation may be present in the interaction between the *Dv*H TpII-c_3 and TpI-c_3.

Conclusion

A new tetraheme cytochrome c_3 was isolated from the membranes of *Dv*H. This cytochrome is homologous to the TpII-c_3 from *Da*, which was the first sulfate-reducing bacteria reported to contain two tetraheme cytochromes c_3.[33, 34] The TpII-c_3 from *Da* was isolated from the soluble fraction, whereas the one from *Dv*H was found exclusively in the membrane extract. This membrane association may be an important feature of the TpII-c_3, and may constitute one of the reasons why it has not been found in most other sulfate-reducers studied, since few studies of membrane-bound proteins have been carried out in these bacteria.

The *Dv*H and *Da* TpII-c_3s share structural and genetic characteristics that distinguish them from the other cytochrome c_3 proteins and suggest they belong to a different type of proteins, possibly associated with membrane-bound oxidoreductase complexes and having different redox partners and physiological function. The considerable amount of TpII-c_3 isolated from *Dv*H indicates that it plays an important role in its metabolism.

Experimental Section

Bacterial growth: *Dv*H (DSM 644) cells were grown in lactate/sulfate medium as previously described.[18] The cells (360 g) were suspended in 10 mM tris(hydroxymethyl)aminomethane (Tris)/HCl (Tris-HCl) buffer (350 mL; pH 7.6) and ruptured by passing twice through a Manton–Gaulin press. The resulting extract was centrifuged at a force of 10000 g for 15 min to remove cell debris, and the supernatant was then centrifuged at a force of 100000 g for 2 h.

Preparation of the membrane extract: The pellet (membrane fraction) was washed twice with 10 mM Tris-HCl buffer (pH 7.6) containing 1 mM EDTA by resuspending the membranes in buffer and recentrifuging, in order to remove any remaining soluble components. (Desulfoviridin was used as a marker to ascertain the presence of soluble proteins.)

The detergent Zwittergent 3-12 (*N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propansulfonate; SB12) was used to solubilise the membrane components. The membrane pellet obtained after the final washing was resuspended in 20 mM Tris-HCl buffer (pH 7.6), and SB12 was added to a final concentration of 2% (w/v). The suspension was stirred for 2 h and then centrifuged at a force of 100000 g for 40 min. Two extraction steps were performed.

Protein Purification: All purification procedures were performed at pH 7.6 and 4 °C. The solubilised membrane extract was loaded on a DEAE Sepharose Fast Flow column (5 × 40 cm; Pharmacia), equilibrated with buffer A (20 mM Tris-HCl buffer containing 0.2% SB12 (w/v)). The column was washed with buffer A (400 mL) and a linear gradient from 0–40% buffer B (100 mM Tris-HCl buffer containing 0.2% SB12 and 1 M NaCl; 2.4 L) was applied. After this, a second gradient of 40–100% buffer B (0.75 L) was utilised.

TpII-c_3: The cytochrome-containing fraction that eluted from the DEAE column at around 15% buffer B was pooled, concentrated and dialysed against buffer A. This fraction was then passed over a Pharmacia Q-Sepharose high-performance column (Hiload 26/10, flow rate 5 mL min^{-1}) equilibrated with buffer A. A stepwise gradient of 0–1 M NaCl was performed. The cytochrome-containing fraction that eluted at 150 mM NaCl was pooled, concentrated and loaded on a Pharmacia S-75 gel filtration column equilibrated with 50 mM Tris-HCl buffer containing 0.2% SB12 and 100 mM NaCl. The cytochrome-containing fraction from this column was pooled, concentrated and dialysed against buffer A. This fraction was then again passed over a Pharmacia Q-Sepharose high-performance column (Hiload 16/10, flow rate 3 mL min^{-1}) equilibrated with buffer A. A linear gradient was performed (0–200 mM NaCl), and pure TpII-c_3 eluted at 120 mM NaCl. The purity index (A_{551}(red)-A_{570}(red)/A_{280}(ox)) was 2.2 and the amount of TpII-c_3 obtained was 7 mg.

TpI-c_3 from the membrane fraction: The cytochrome-containing fraction that eluted from the DEAE column before the start of the gradient was pooled, concentrated and dialysed against buffer A. This fraction was then passed over a Pharmacia Q-Sepharose high-performance column (Hiload 26/10, flow rate 5 mL min^{-1}) equilibrated with buffer A. A stepwise gradient of 0–1 M NaCl was performed. The fraction that eluted before the start of the gradient was pooled, concentrated and dialysed against 20 mM Tris-HCl buffer. This fraction was then passed again over a Pharmacia S-Sepharose high-performance column (Hiload 16/10, flow rate 3 mL min^{-1}) equilibrated with 20 mM Tris-HCl buffer. A stepwise gradient of 0–1 M NaCl was performed, and pure TpI-c_3 eluted at 250 mM NaCl. The purity index (A_{551}(red)-A_{570}(red)/A_{280}(ox)) was 3.8 and the amount of TpI-c_3 obtained was 10 mg. The identity of this cytochrome was confirmed by determination of its N-terminal sequence.

Analytical methods: Protein concentration was determined with the Bicinchoninic Acid assay from Pierce, with soluble TpI-c_3 as a standard. The pyridine hemochrome was performed according to Berry and Trumpower,[59] with the millimolar absorptivity of $\varepsilon_{r-o,550-535} = 23.97\ mM^{-1}cm^{-1}$ for heme *c*. SDS-PAGE was performed according to Laemmli.[60] Gels were stained with Coomasie Blue for proteins, and tetramethylbenzidine for *c*-type cytochromes.[61] The isoelectric point was determined by analytical isoelectric focussing with a Bio-Rad model 111 mini IEF cell. A pH gradient of 3.5–10 was obtained with carrier ampholites. Protein molecular masses were

determined by SDS-PAGE with BioRad low-range protein standards. Molecular mass determination by native size-exclusion chromatography was performed on a Pharmacia superdex 75 HR column, calibrated with Pharmacia low molecular mass calibration kits. For N-terminal sequencing, the protein was adsorbed onto a poly(vinylidene difluoride) (PVDF) membrane (ProSorb, Perkin-Elmer) and washed repeatedly to remove salts and detergent. The N-terminal sequence was obtained by the method of Edman and Begg[62] with an Applied Biosystem model 470A sequencer.

Spectroscopic methods: UV/Vis spectra were obtained with a Shimadzu (UV 260) spectrophotometer. EPR spectra were recorded on a Brucker ESP 380 spectrometer equipped with an ESR 900 continuous-flow helium cryostat (Oxford Instruments), as previously described.[63] Redox titrations monitored by visible spectroscopy were performed in an anaerobic chamber, in 40 mM Tris-maleate buffer (pH 7.6), following the changes in absorbance at the α band of the reduced hemes, corrected for the corresponding isosbestic points and with buffered sodium dithionite as the reductant and potassium ferricianide as the oxidant. The following redox mediators were used (at a final concentration of 3.5 μM each): Gallocyanine, indigo tetrasulfonate, indigo trisulfonate, indigo disulfonate, 2-hydroxy-1,4-naphthoquinone, anthraquinone-2,7-disulfonate, anthraquinone-2-sulfonate, safranine, neutral red, benzyl viologen and methyl viologen. The reduction potentials are referenced to the standard hydrogen electrode.

Enzymatic activities: For the reduction of cytochromes with hydrogenases, all experiments were performed in a stirred cell, with a hydrogen overpressure of 15 kPa flowing through the cell, and linked with a Shimadzu UV3100 spectrophotometer. The buffer used in all cases was 100 mM Tris-HCl (pH 7.6). The [NiFe] and [NiFeSe] hydrogenases were activated by flushing with hydrogen for about 1 h and then leaving overnight at 4 °C under a hydrogen atmosphere. The [Fe] hydrogenase was preactivated by standing for 1 h inside an anaerobic chamber. The reduction of the cytochromes was measured by following the increase in absorption at 551 nm for TpII-c_3 and 553 nm for TpI-c_3, with the respective absorption coefficients. The rates were measured from the linear portion of the reduction curves. The concentrations used were chosen so that a reasonable rate could be measured, after having assured that the rates were proportional to the hydrogenase concentrations. Each of the experiments, performed as previously described,[31] was repeated at least three times. The same procedure was used for the [NiFe] and [NiFeSe] hydrogenases, with the following concentrations: TpII-c_3/H_2ase: 4 μM TpII-c_3 and 14 nM H_2ase; TpI-c_3/H_2ase: 4 μM TpI-c_3 and 14 nM H_2ase; TpII-c_3/H_2ase/TpI-c_3: 4μM TpII-c_3, 14 nM H_2ase and 14 nM TpI-c_3. The concentrations used in the experiments with the [Fe] hydrogenase were: TpII-c_3/H_2ase: 4μM TpII-c_3 and 28 nM H_2ase; TpI-c_3/H_2ase: 4μM TpI-c_3 and 2.8 nM H_2ase; TpII-c_3/H_2ase/TpI-c_3: 4μM TpII-c_3, 28 nM H_2ase and 28 nM TpI-c_3. At the end of each experiment the cytochromes were reduced with dithionite to check that reduction by the hydrogenase had been complete.

Sequence analysis tools and molecular modelling: Preliminary sequence data from the *Dv*H genome was obtained from The Institute for Genomic Research website at http://www.tigr.org. The nucleotide sequence data were analysed with the Genetics Computer Group (Wisconsin) package provided by the Portuguese EMBnet Node (PEN) and Neural Networks for Eukaryotic Promoter Prediction.[64] A three-dimensional model of *Dv*H TpII-c_3 was generated with the Swiss-Model program,[43] with the structures of the oxidised and reduced *Da* TpII-c_3[41] (PDB accession numbers: 3CAOA and 3CARA, respectively) as templates. The structures of several cytochromes were retrieved from the Protein Data Bank, with the following accession numbers: *Dv*H TpI-c_3: 2CTH;[24] *Dv*M cytochrome c_3: 2CDV;[22] *Dg* cytochrome c_3: 1WAD;[26] *Dd*27k cytochrome c_3: 3CYR;[24] *Dmn* cytochrome c_3: 2CY3.[25] The cytochromes were structurally aligned with the program MODELLER 4.[44] The surface electrostatic potential of the cytochromes was calculated with the program GRASP.[46] Figure 6 was prepared with the GRASP[46] and Raster 3D programs.[57] Sequence alignments not based on structure were performed with the ClustalW program, version 1.8.[65]

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[66] Abbreviations: 16Hcc: 16-heme high molecular weight cytochrome *c*; 9Hcc: nine-heme cytochrome *c*; *Da*: *Desulfovibrio africanus*; *Dv*H: *Desulfovibrio vulgaris* Hildenborough; *Dv*M: *Desulfovibrio vulgaris* Miyazaki; *Dg*: *Desulfovibrio gigas*; *Dd*27k: *Desulfovibrio desulfuricans* ATCC 27774; *Dmn*: *Desulfomicrobium norvegicum*; TpII-c_3: Type II cytochrome c_3; TpI-c_3: Type I cytochrome c_3.

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