Interfacial Properties of the Polyheme Cytochrome c_3 Superfamily from *Desulfovibrio*

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ABSTRACT: In order to compare the interfacial behavior of the polyheme cytochromes *c* which belong to the cytochrome c_3 superfamily, the monomolecular film technique was used to determine whether and how these metalloproteins interact with (phospho)lipids. Measurements of the variations of surface pressure and surface potential versus time have shown differences in their penetration capacity into phosphatidylcholine, dicaprin, and phosphatidylglycerol films. The *Desulfovibrio vulgaris* Hildenborough cytochrome with 16 hemes (Hmc) and *Desulfovibrio desulfuricans* Norway tetra- and octaheme cytochromes c_3, which have been assumed to be soluble periplasmic molecules, may be considered as extrinsic membrane proteins, unlike the *D. vulgaris* Hildenborough cytochrome c_3 (M_r 13 000). The interfacial properties are discussed in terms of the available three-dimensional structural data, the electrostatic potential calculation, and the results obtained by hydrophobic cluster analysis of the cytochrome sequences. The very different behavior of the two cytochromes c_3 (M_r 13 000) enlightens the role of a particular surface loop in the interaction with a model membrane. A functional interpretation is proposed assuming that the *D. vulgaris* Hildenborough Hmc and both cytochromes c_3 (M_r 13 000) and (M_r 26 000) from the Norway strain might provide the link between periplasmic hydrogen oxidation and cytoplasmic sulfate reduction.

Sulfate-reducing bacteria are strict anaerobes which are able to use oxidized sulfur compounds such as sulfate or thiosulfate as the terminal electron acceptors of a complex electron-transfer chain in which either organic compounds or hydrogen acts as the initial electron donor. Some striking differences in comparison with other respiratory processes center on the negative redox potentials involved and the presence of several polyheme cytochromes *c*.

Cytochrome c_3 (M_r 13 000) is a periplasmic protein which is ubiquitously present in *Desulfovibrio*. It has been reported to act as the natural electron donor and acceptor for periplasmic hydrogenase (LeGall & Fauque, 1988). The three-dimensional structures of the *Desulfovibrio desulfuricans* Norway (Haser *et al.*, 1979; Pierrot *et al.*, 1982; Czjzek *et al.*, 1994a) and *Desulfovibrio vulgaris* Miyazaki (Higuchi *et al.*, 1984) and Hildenborough (Morimoto *et al.*, 1991) cytochromes c_3 have been determined: the spatial arrangement of the four hemes in the above cytochromes is highly conserved. The hemes have a bishistidinyl iron coordination and exhibit distinct redox potentials in the −200 to −400 mV range. Cytochrome c_3 (M_r 26 000) has been purified and characterized only from *D. gigas* (Bruschi *et al.*, 1969) and *D. desulfuricans* Norway (Guerlesquin *et al.*, 1982). It is a dimer of two identical tetraheme cytochromes which differ from cytochrome c_3 (M_r 13 000) in the amino acid sequence (Bruschi *et al.*, 1994) and EPR spectra (Le Gall *et al.*, 1971). The 3-D structure of the cytochrome from *D. desulfuricans* Norway has been solved (Czjzek *et al.*, 1994b): the monomeric subunit has the c_3 folding. The purification of a high molecular mass cytochrome *c* (Hmc)[1] has been reported in *D. vulgaris* strains Miyazaki (Tasaka *et al.*, 1991) and Hildenborough (Higuchi *et al.*, 1987) and in *D. gigas* (Cheng *et al.*, 1994). By cloning and sequencing the gene encoding the Hmc from *D. vulgaris* Hildenborough, it was demonstrated that this cytochrome is periplasmic and that the apoprotein contains 514 amino acids, with 16 heme binding sites similar to those detected in cytochrome c_3 (Pollock *et al.*, 1991). The existence of 16 hemes per cytochrome has been demonstrated by performing iron analysis and EPR studies (Bruschi *et al.*, 1992). Comparisons of the arrangement of the heme binding sites and the coordinating histidines in the amino acid sequences between cytochromes c_3 and Hmc have shown that the 16 hemes are distributed in four cytochrome c_3-like domains (Pollock *et al.*, 1991).

The cytochrome c_3 (M_r 13 000) seems to constitute the structural basic unit of this class of proteins which could be defined as the cytochrome c_3 superfamily. They have no structural similarities with the mitochondrial-type cyto-

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[1] Abbreviations: *D., Desulfovibrio; D.v.*H.; *Desulfovibrio vulgaris* Hildenborough; *D.d.*N, *Desulfovibrio desulfuricans* Norway; Hmc, high molecular mass cytochrome *c*; PC, phosphatidylcholine; PG, phosphatidylglycerol; dicaprin, *rac*-1,2-didecanoylglycerol; HCA, hydrophobic cluster analysis.

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chromes *c* (class I), and all belong to class III of the *c*-type cytochromes (Ambler, 1991). All the above-mentioned cytochromes react with the periplasmic hydrogenase; however, a recent model built on genetic data has proposed a specific role for each type of cytochrome c_3. The *hmc* gene was found to belong to an operon which comprises six open reading frames encoding membrane-bound proteins, some of which might be electron-transfer proteins. This operon might therefore constitute a putative transmembrane redox channel. This finding illustrates a poorly characterized aspect of the sulfate respiration: the link between periplasmic hydrogen oxidation and cytoplasmic sulfate reduction (Rossi *et al.*, 1993). According to this gene organization, Hmc would be associated with the outer face of the cytoplasmic membrane where it could donate electrons to the membrane electron transport system (Rossi *et al.*, 1993).

The membrane compositions of *D. desulfuricans* Norway and *D. vulgaris* exhibit a typical Gram-negative phospholipid pattern which consists of phosphatidylethanolamine, phosphatidylglycerol, cardiolipin, and lysophosphatidylserine (Makula & Finnerty, 1974). We used the monomolecular film technique as a membrane model in order to check the nature and the specificity of the interactions of cytochrome c_3 (M_r 13 000), cytochrome c_3 (M_r 26 000), and Hmc with self-organized glycerolipids. With a view to identifying the type of interactions involved, three kinds of lipid films, bearing various electrical charges, were used: the neutral dicaprin, the zwitterionic phosphatidylcholine, and the acidic phosphatidylglycerol.

MATERIALS AND METHODS

Protein Purification. Cytochromes c_3 (M_r 13 000) and c_3 (M_r 26 000) were isolated from *D. desulfuricans* Norway (NCIB 8310) as described by Bruschi *et al.* (1977) and Guerlesquin *et al.* (1982), respectively. Cytochrome c_3 (M_r 13 000) and high molecular mass cytochrome *c* (Hmc) from *D. vulgaris* Hildenborough (NCIB 8303) were purified as previously described (Le Gall *et al.*, 1965; Loutfi *et al.*, 1989).

Lipids. Chicken egg yolk phosphatidylcholine (1,2-diacyl-*sn*-phosphatidylcholine), dicaprin (*rac*-1,2-didecanoylglycerol), and L-α-phosphatidylglycerol were obtained from Sigma. Lipids were first dissolved in chloroform at a concentration of 1 mg/mL and then gently deposited at the air/water interface with a microsyringe in order to form a lipid monolayer. The initial surface pressure is adjusted by successive additions of the lipid solution on a constant film area.

Measurements of Surface Parameters. The surface pressure was measured using the Wilhelmy method with a thin platinum plate (perimeter 3.94 cm) attached to a Beckman electromicrobalance (model LM 600). A cylindrical Teflon trough (surface 7 cm^2, volume 5 mL) was used to measure the adsorption at the air/water interface and the penetration into the preformed lipid monolayers. The cytochrome solution was injected at a final protein concentration of 2 μg/mL into the subphase. The aqueous subphase (0.02 M Tris-HCl, pH 7.0, with or without 0.15M NaCl) was continuously stirred with a magnetic rod. The surface potential was measured using a gold-coated Am^{241} ionizing electrode with an electrometer VA-J-51 (GDR) connected to a chart recorder. The accuracy was ±15 mV for the initial surface potential of a clean air/water interface.

Comparison between Protein Sequences. The protein sequences of *D. vulgaris* Hildenborough cytochrome c_3 (M_r 13 000), *D. desulfuricans* Norway cytochromes c_3, and the four c_3-like domains of *D. vulgaris* Hildenborough Hmc have been analyzed and compared by performing hydrophobic clusters analysis (Gaboriaud *et al.*, 1987; Lemesle-Varloot *et al.*, 1990).

Electrostatic Potential Calculation. The electrostatic potentials were calculated using the procedure of Getzoff and co-workers (Getzoff *et al.*, 1983; Roberts *et al.*, 1991). The charges used were those of the X-PLOR force field. The calculation procedure was implemented in a series of programs [CHARGES and POTENTIAL (Tegoni *et al.*, 1993)]. The electrostatic field and potentials are calculated on surface points (program MS) located at 11 Å from the C_α atoms using a distance-dependent (four radii) dielectric constant. Ten sets of electric field arrows were generated according to a sorting procedure based on increasing values of the electrostatic potential. The different electrostatic field surfaces are visualized using the program TURBO-FRODO (Roussel & Cambillau, 1989). The program allows vizualization (and coloring) of each set of arrows independently.

RESULTS AND DISCUSSION

Cytochrome/Lipid Interactions: Kinetic Aspects. In order to investigate the interfacial properties of the various polyheme cytochromes from Desulfovibrio, two protocols were used: adsorption at the air/water interface or penetration into lipid/water interfaces. During the adsorption process, we observed (i) a decrease in the measured surface tension of the aqueous solution, which is equivalent to an increase in the surface pressure ($\Delta\pi$), and (ii) a reorganization of the electrical dipoles existing at the interface, including the water molecules, which creates variations in the surface potential (ΔV).

First, the intrinsic amphipathic character of the cytochromes was investigated through their ability to adsorb from an aqueous solution to the air/water interface. The variations with time in the surface pressure and surface potential, after the injection of cytochromes into the subphase, are shown in Figure 1. The *D. vulgaris* Hildenborough cytochrome c_3 (M_r 13 000) does not display tensioactive properties (Figure 1A), although it shows a tendency to affect the electrical potential of the air/water interface, as can be seen from the ΔV recordings (Figure 1B). On the contrary, injection of *D. desulfuricans* Norway cytochromes c_3 and *D. vulgaris* Hildenborough Hmc into the water subphase produced an immediate increase in the surface pressure (Figure 1A), and the surface potential became more positive (Figure 1B). The changes with time in the surface pressure ($\Delta\pi$) and potential (ΔV) occurred simultaneously. Therefore, these latter water-soluble metalloproteins seem to have more marked hydrophobic character than *D. vulgaris* Hildenborough cytochrome c_3 (M_r 13 000). However, their surface activity at the air/water interface is rather weak as compared with those of other proteins such as colipase—$\Delta\pi$ = 15 $mN.m^{-1}$ (de La Fournière *et al.*, 1994)—and mellitin, one of the strongest natural surface active agents at the air/water interface—$\Delta\pi$ = 24.5 $mN.m^{-1}$ (Sessa *et al.*, 1969; Bougis *et al.*, 1981).

Injecting Hmc in the aqueous subphase, below monomolecular films of various lipid classes spread at variable initial surface pressures, produced increases in surface pressure ($\Delta\pi$) up to quasiequilibrium values reached after

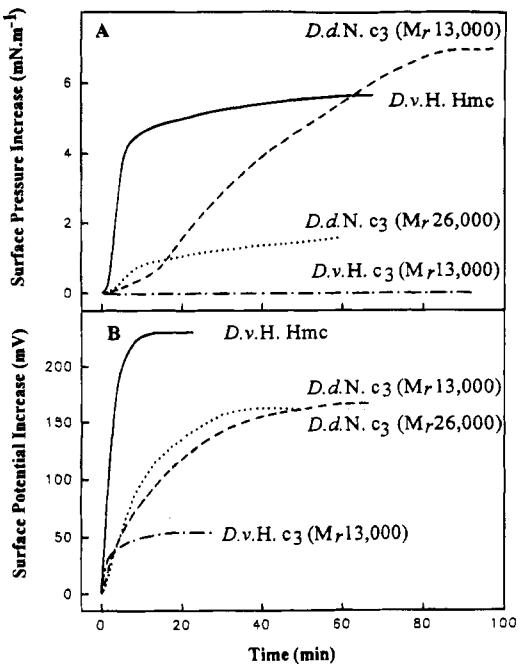

FIGURE 1: Kinetics of the adsorption at the air/water interface of *D.v.*H. Hmc (—), *D.v.*H. c_3 (M_r 13 000) (— • —), *D.d.*N. c_3 (M_r 13 000) (— — —) and *D.d.*N. c_3 (M_r 26 000) (• • •). Increase with time (A) in surface pressure (mN·m^{-1}), and (B) in surface potential (mV). The final protein concentration was 2 μg/Ml, in 0.02 M Tris-HCl, pH 7.0, in a cylindrical Teflon trough (surface 7 cm^2, volume 5 mL).

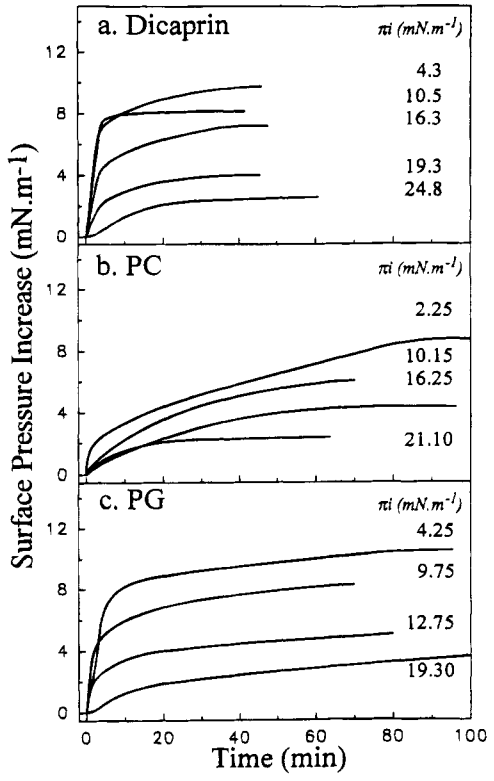

FIGURE 2: Kinetics of the increase in surface pressure upon injection of *D.v.*H. Hmc (2 μg/mL final concentration, in 20 mM Tris-HCl, pH 7.0) below a monolayer of (a) dicaprin, (b) phosphatidylcholine, and (c) phosphatidylglycerol, at variable initial surface pressures.

10−20 min (Figure 2). However, in some cases we observed a slow continuous drift of the surface pressure, probably indicative of a slow unfolding process. These interfacial behaviors were classically interpreted by the penetration of some parts of the polypeptide chain into the lipid monolayer (Macritchie, 1978; Graham & Phillips, 1979; Piéroni *et al.*, 1990). To complete our comparative study, *D. vulgaris* Hildenborough cytochrome c_3 (M_r 13 000) and *D. desulfuricans* Norway cytochromes c_3 (M_r 13 000) and c_3 (M_r 26 000) were also tested to determine their ability to penetrate the same lipid monolayers. The interfacial parameters—maximal surface pressure and surface potential increases and initial rate of surface pressure increase—which are characteristic of the interaction of the above cytochromes with various kinds of lipid monolayers spread at an identical initial surface pressure (10 ± 1 mN·m^{-1}), are summarized in Table 1. Generally, the presence of phospholipid monolayers enhanced the surface activity of the proteins, compared to the surface pressure variations measured at the air/water interface, presuming some specific interactions. Little or no meaningful effects on the surface pressure of lipid films were observed with *D. vulgaris* Hildenborough cytochrome c_3 (M_r 13 000). In contrast, under the same experimental conditions, *D. desulfuricans* Norway cytochrome c_3 (M_r 13 000) has the highest increases in pressure. However, the parameters reflecting its capacity of adsorption were drastically lowered down at high ionic strength—presuming an electrostatic contribution during the interaction with lipids—contrary to what occurred with *D. desulfuricans* Norway cytochrome c_3 (M_r 26 000) which interfacial parameters were not sensitive to the presence of NaCl. High ionic strength inhibited the penetration capacity of Hmc only in electrically charged films (Table 1). Paradoxically and interestingly, *D. desulfuricans* Norway cytochromes c_3 and *D. vulgaris* Hildenborough Hmc induced significant increases of the surface pressure of PC monolayers whereas no variations were observed in their surface potential (Table 1). In fact, the increase of the surface potential reflects a higher resistance to the electron flow in the interfacial region. This process, due to the adsorption of proteins, is certainly counterbalanced by the electrical conductivity of the above-mentioned cytochromes. In accordance with such a finding, it has been shown that solid films of reduced cytochrome c_3 (M_r 13 000) exhibit a very high conductivity (Kimura *et al.*, 1979; Nakahara *et al.*, 1980).

In order to compare more quantitatively the surface behavior of all the cytochromes, their respective penetration powers into the lipid films were determined through surface pressure measurements. When the initial surface pressure of the lipid monolayer was increased, the maximum value of the surface pressure increase ($\Delta\pi_{max}$) was reduced. This is shown in detail in Figure 3, which gives $\Delta\pi_{max}$ as a function of the initial surface pressure (πi) of the film under which the protein was injected. The variations of the $\Delta\pi_{max}$ observed with all the cytochromes decreased linearly over the entire range of π_i. When the π_i was higher than a characteristic threshold value, the protein could no longer penetrate ($\Delta\pi = 0$). These critical pressures for penetration (π_c) into the differently charged lipid films have been estimated by extrapolation to $\Delta\pi = 0$. The *D. vulgaris* Hildenborough cytochrome c_3 (M_r 13 000) showed the weakest penetration capacity of all the tested cytochromes. There is, however, a small rise in surface pressure with electrically charged monolayers (PC and PG) at low π_i and no further increase in surface pressure beyond a π_i of 15 mN·m^{-1} (Figure 3). The interactions may be considered as nonspecific and due to the numerous lysine residues present in the cytochrome. The *D. desulfuricans* Norway cytochrome c_3 (M_r 26 000) exhibited a high selectivity for egg

Table 1: Parameters Reflecting the Adsorption of Polyhemic Cytochromes *c* from *Desulfovibrio* at either the Air/Water Interface or Preformed Lipid Monolayers[a]

		air/water interface		dicaprin		PC		PG	
		−NaCl	+NaCl	−NaCl	+NaCl	−NaCl	+NaCl	−NaCl	+NaCl
*D.v.*H. c_3 (M_r 13 000)	$\Delta\pi_{max}$	0	0	0	1.6	4	0	2.6	0
	$(d\pi/dt)_{t=0}$	0	0	0	0.027	0.16	0	0.14	0
	$\Delta(\Delta V)$	50	nd[b]	14	0	0	0	32	0
*D.v.*H. Hmc	$\Delta\pi_{max}$	3.6	2.8	8.25	6.3	6.1	4.3	8.35	3.25
	$(d\pi/dt)_{t=0}$	1.25	0.625	2.1	2.25	0.31	0.125	2.7	0.125
	$\Delta(\Delta V)$	230	nd	62	19	0	0	30	5.9
*D.d.*N. c_3 (M_r 13 000)	$\Delta\pi_{max}$	6.6	8.12	14.25	2.5	7.7	2.5	11.3	0.75
	$(d\pi/dt)_{t=0}$	0.08	0.06	3	0.1	0.48	0.03	0.55	0.075
	$\Delta(\Delta V)$	170	nd	50	12.2	0	0	32	17.2
*D.d.*N. c_3 (M_r 26 000)	$\Delta\pi_{max}$	1.6	2.4	1.0	2.3	6.7	5.5	1	2
	$(d\pi/dt)_{t=0}$	0.09	0.06	0.07	0.05	0.07	0.06	0.07	0.035
	$\Delta(\Delta V)$	160	nd	20	0	0	5.4	0	0

[a] Initial surface pressure, 10 ± 1 mN·m−1; subphase, 0.02 M Tris-HCl, pH 7.0, with or without 0.15 M NaCl. The maximal surface pressure ($\Delta\pi_{max}$) and surface potential [$\Delta(\Delta V)$] increases are expressed in mN·m^{-1} and mV, respectively. The initial rate of surface pressure increase $(d\pi/dt)_{t=0}$ is in mN·m^{-1}·min^{-1}. [b] nd, not determined.

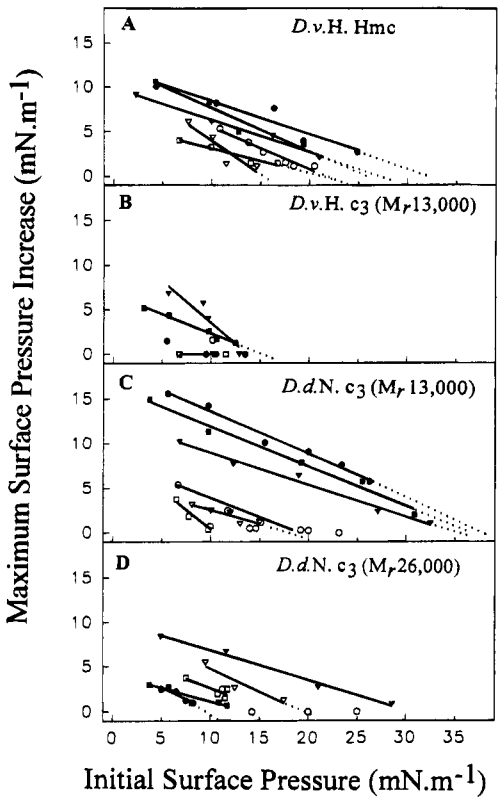

FIGURE 3: Maximum values of the surface pressure increase of various lipid films as a function of their initial surface pressure after injection of the cytochromes into the subphase (2 μg/mL final concentration in 0.02 M Tris-HCl, pH 7.0) (filled symbols) and the same buffer with 0.15 M NaCl (open symbols). Dicaprin (●, ○), phosphatidylcholine (▼, ▽) and phosphatidylglycerol (■, □) films were used. Panels: (A) *D.v.*H. Hmc, (B) *D.v.*H. c_3 (M_r 13 000), (C) *D.d.*N. c_3 (M_r 13 000), and (D) *D.d.*N. c_3 (M_r 26 000).

phosphatidylcholine films, i.e., for a zwitterionic monolayer (Figure 3). On the contrary, *D. desulfuricans* Norway cytochrome c_3 (M_r 13000) and *D. vulgaris* Hildenborough Hmc interacted with all the phospholipids tested (Figure 3). Their critical surface pressures (π_c) could be compared with those measured with several other proteins (Table 2). These π_c are relatively high: 35−40 mN·m^{-1} and 25−30 mN·m^{-1} in the case of *D. desulfuricans* Norway cytochrome c_3 (M_r 13 000) and Hmc, respectively. The penetration into lipid films of both *D. desulfuricans* Norway cytochrome c_3 (M_r 13 000) and Hmc is of comparable intensity whether electrically charged or neutral films were used. This behavior contrasts with mitochondrial cytochrome *c* (Mustonen *et al.*, 1987) for which lipid binding is drastically enhanced by a negative surface charge density. The association of cytochrome *c* with membranes containing acidic phospholipids involves electrostatic interactions and is sensitive to changes in ionic strength and pH (Nicholls, 1974; Brown & Wüthrich, 1977). In order to evaluate the importance of the electrostatic interactions in the case of the *Desulfovibrio* cytochromes, the surface pressure and surface potential changes were also measured in the presence of 0.15 M NaCl. It appeared that at higher ionic strength the penetration capacity of the cytochromes decreases (Figure 3), whatever the lipid monolayer used. This result is in accordance with an important electrostatic contribution to the cytochrome binding to lipid polar headgroups. However, two kinds of reponse were observed in the presence of NaCl (Figure 3): large reductions of π_c ($\Delta\pi_c$) for *D. desulfuricans* Norway cytochrome c_3 (M_r 13 000) and lower shifts in π_c for *D. desulfuricans* Norway cytochrome c_3 (M_r 26 000) and for *D. vulgaris* Hildenborough Hmc (Table 2). Consequently, for the two latter hemoproteins, the contribution of electrostatic forces would be less determinant in their interactions with lipid monolayers.

D. vulgaris Hildenborough Hmc and *D. desulfuricans* Norway cytochromes c_3 (M_r 13 000) and c_3 (M_r 26 000), which have been described as soluble periplasmic proteins, can therefore be classified as hemoproteins specifically interacting with lipids organized as model membranes. This result presumes a reversible extrinsic association *in vivo* with membranes where peripheral interactions taking place at the membrane surface are primarily regulated by electrostatic forces.

Cytochrome/Lipid Interactions: Structural Data. The protein/lipid interactions may result from (i) covalent modification of the molecule by long-chain fatty acid (Sefton & Buss, 1987), (ii) mediation by an intrinsic structural feature, usually an α-helix, or (iii) primarily electrostatic forces involving a subsequent hydrophobic contribution. In the case of mitochondrial cytochrome *c*, two well-defined patches of basic residues on the surface of this molecule seem to play a role in the electrostatic interaction with lipids (Dickerson *et al.*, 1971). It has been established experimentally, however, that electrostatic binding is followed by partial

Table 2: Critical Surface Pressures (π_c in mN·m^{-1}) for Penetrations of Various Proteins in Differently Charged Lipid Monolayers[a]

	π_c/neutral lipid			π_c/zwitterionic lipid			π_c/acidic lipid			
	−NaCl	+NaCl	$\Delta\pi_c$	−NaCl	+NaCl	$\Delta\pi_c$	−NaCl	+NaCl	$\Delta\pi_c$	references
*D.v.*H. c_3 (M_r 13 000)	0	0	0	12	0	12	15.3	0	15.3	this work
*D.v.*H. Hmc	31.3	21.7	9.6	27.5	15.4	12.1	25.7	21	4.7	this work
*D.d.*N. c_3 (M_r 13 000)	38.4	20.2	18.4	35	18.5	16.5	36.5	10.4	26.1	this work
*D.d.*N. c_3 (M_r 26 000)	10.3	14	3.7	30.4	19.8	10.6	13.7	16.2	2.5	this work
cytochrome *c*				24[b]						Quinn & Dawson, 1969
mellitin					39[c]			42[d]		Bougis *et al.*, 1981
cardiotoxin III					22[c]−28[e]			32[d]−45[f]		Bougis *et al.*, 1981
neurotoxin III				0[e]	0[e]		>15[g]			Bougis *et al.*, 1981
β-lactoglobulin A		36[h]			20[e]					Piéroni *et al.*, 1990
BSA		36[h]			25[e]					Piéroni *et al.*, 1990
ovalbumin		27[h]								Piéroni *et al.*, 1990
myoglobin		37.6[h]								Piéroni *et al.*, 1990
lipase		38[h]			28[e]					Piéroni *et al.*, 1990

[a] −NaCl = 0 M NaCl; +NaCl = 0.15 M NaCl. $\Delta\pi_c$ is the variation in π_c measured at low and hight ionic strengths. [b] π_c for penetration into egg phosphatidylethanolamine monolayer. [c] π_c for penetration into dilauroylphosphatidylcholine. [d] π_c for penetration into dilauroylphosphatidylserine. [e] π_c for penetration into egg phosphatidylcholine. [f] π_c for penetration into dilauroylphosphatidylglycerol. [g] π_c for penetration into phosphatidylserine. [h] π_c for penetration into dicaprin.

insertion into the hydrocarbon chains of cardiolipin-containing bilayers (Brown & Wüthrich, 1977; Szebeni & Tollin, 1988; Spooner & Watts, 1991; Rytömaa *et al.*, 1992).

While membrane association has been suggested previously in the case of cytochrome c_3 (M_r 26 000) (Bruschi *et al.*, 1969; Hatchikian *et al.*, 1972) and *D. vulgaris* Hmc (Rossi *et al.*, 1993; Ogata *et al.*, 1993), the very different behaviors of the two cytochromes c_3 (M_r 13 000), with regard to their critical pressure of penetration, is more surprising. In fact, their available three-dimensional structures show that while the heme cores are well conserved, differences have been detected between the polypeptide chains of *D. desulfuricans* Norway cytochrome c_3 (Czjzek *et al.*, 1994a) and *D. vulgaris* Hildenborough and Miyazaki cytochromes c_3 (Morimoto *et al.*, 1991; Higuchi *et al.*, 1984). One major loop of 14 residues (Figures 4 and 5) is deleted in *D. vulgaris* Hildenborough and Miyazaki cytochromes c_3. In *D. desulfuricans* Norway cytochrome, this loop is highly exposed to the surface and contains alternating charged (lysine, glutamate, aspartate) and apolar (methionine, alanine, proline, glycine) residues. The hydrophobic side chains are all oriented toward one side of the amphipathic loop, whereas the charged groups are arranged, on the other side and Asp23 and Lys24 are at the very end of the loop. Furthermore, there exists a relatively surface-exposed phenylalanine 72 close to the hydrophobic side of the loop. Neither the region from residue 20 to residue 26 nor the region around residue 72 has a very well defined density in the structure refinement. The temperature factors are high compared to the rest of the molecule. This indicates that these regions show thermal or structural disorder and can be interpreted by a higher flexibility of these loops. An exposed cluster of charged residues with interspersed electrically neutral amino acids can allow the binding of a significant fraction of a soluble protein to acidic phospholipids of the plasma membrane (Mosior & McLaughlin, 1991). *D. desulfuricans* Norway cytochrome c_3 (M_r 13 000) interacts, however, with neutral, as well as with zwitterionic and negatively charged lipids: the effects of electrostatic interactions may work along with the partitioning of hydrophobic groups from the protein into the lipid film. The deletion of the two particular structural features described above in the *D. desulfuricans* Norway cytochrome c_3 (M_r 13 000) might explain the lack of interaction between *D. vulgaris* Hildenborough cytochrome c_3 and lipids. A similar explanation has been proposed in the case of cardiotoxins and neurotoxins purified from the same venom (Bougis *et al.*, 1981). The latter toxins poorly penetrate lipid films (Table 2) even though both toxin types share high structural homologies. A clear hydrophobicity difference, however, appears between the first loops of these two toxins. In an initial step, electrostatic interactions occur between the negatively charged group of the phospholipids and the basic residues located near this loop in cardiotoxins; then this hydrophobic loop penetrates into the lipid film. In the case of neurotoxins, this last step could not occur, explaining the absence of increase in the surface pressure (Bougis *et al.*, 1981).

The electrostatic potential surface of *D. desulfuricans* Norway cytochromes c_3 (M_r 13 000) and c_3 (M_r 26 000) and *D. vulgaris* HIldenborough cytochrome c_3 (M_r 13 000), calculated *in vacuo* (corresponding to a neutral pH), is represented in Figure 6. For all three proteins the positive electrostatic potentials mapped onto the surfaces have a pronounced maximum, situated around the surface-exposed edge of heme 4. Both cytochromes c_3 (M_r 13 000) and c_3 (M_r 26 000) from *D. desulfuricans* Norway have a marked dipolar character. However, no dipolar character is observed for *D. vulgaris* Hildenborough cytochrome c_3. This is caused by a more regular distribution of compensating positive and negative charges in the case of *D. vulgaris* Hildenborough cytochrome c_3 (M_r 13 000), except in the region around heme 4. One could also notice that the surface loops 13−29 in the case of *D. desulfuricans* Norway cytochrome c_3 (M_r 13 000) and 14−22 in the case of cytochrome c_3 (M_r 26 000) are located in extending neutral regions with small positive maxima, and this neutral region is forming a sort of belt between the two polar extremities. This observation supports the idea that electrostatic interactions in the first place, followed by a partial penetration of the extending loops into the lipid layer, forming hydrophobic contacts, are the reason for the observed protein/lipid inteactions of *D. desulfuricans* Norway cytochromes c_3 (M_r 13 000) and c_3 (M_r 26 000) and the absence of both dipolar character and flexible surface loops prevents *D. vulgaris* Hildenborough cytochrome c_3 (M_r 13 000) from interacting in the same way.

The primary amino acid sequence of Hmc is organized into four c_3-like domains (Pollock *et al.*, 1991). Structural arrangements analogous to that observed in *D. desulfuricans*

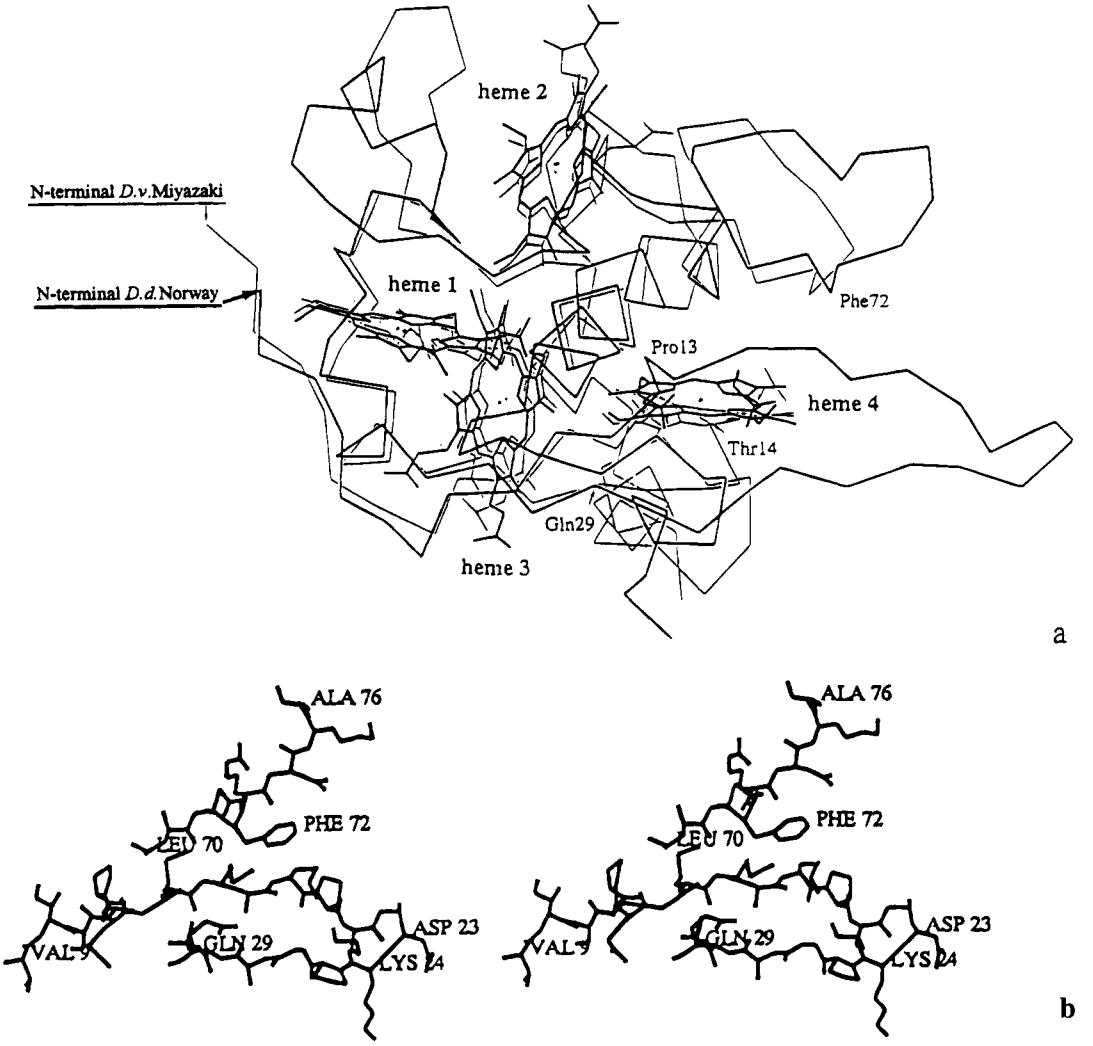

FIGURE 4: (a) Superimposition of a C_α representation of the three-dimensional structures of the cytochromes c_3 (M_r 13 000) of *D. desulfuricans* Norway (thick line) and *D. vulgaris* Miyazaki (thin line), showing the Pro13 to Gln29 hydrophobic loop of *D.d.* Norway and the surface exposed Phe72. (b) Stereoview selecting the two interesting structural features of the *D.d.* Norway c_3 with all the side chains from residues 9 to 29 and from residues 70 to 76.

D.v. H. c3 (M_r13,000)	APKAPADGLKMEA.....T.....KPQ.VVFNHSTH--CHDSMDKKDKS--
D.d. N. c3 (M_r 13,000)	ADAPGDDYVISAPEGMKAKPKGDKPGALQKTVPFPHTKH--CHDSLEFRDKA--
D.v. H. Hmc Domain I	KALPEGPGEKRADLIEIGAMERFGKLDLPK.VAFRHDQH----.MSLKFMRLD--
D.v. H. Hmc Domain II	PSAASSWK.....EIGDKSL.HYRH--CHGEKPVDKRP--
D.v. H. Hmc Domain III	--VPRLDRGQPDAALILPVPGKDAPREMKGTMKPVAFDHKAH--CHTVNGTADSK--
D.v. H. Hmc Domain IV	--IGSIAKEYQPSEFPHRKIVKTLIAGIGEDKLAATF.H.....--CHG.KPFDADR--
D.d. N. c3 (M_r26,000)	ETFEIPESVTMSP.KQ....FEGYTP.KKGDVTFNHATH--CHDNIKERTEI--

FIGURE 5: Partial sequence alignment of the two loops of the cytochromes c_3 from *D. desulfuricans* Norway and *D. vulgaris* Hildenborough and of the Hmc c_3-like domains based on the comparisons between the three-dimensional structures and on the HCA plots. The apolar residues are boxed, and the phenylalanine exposed to the surface, in the *D.d.*N. cytochrome c_3 (M_r 13 000) structure, is marked with an asterisk. The dots and the dashes stand for the deletions and the omitted sequences, respectively.

Norway cytochrome c_3 (M_r 13 000) might be expected to account for the Hmc/lipid monolayer interaction, similar to those observed in *D. desulfuricans* Norway cytochrome c_3 (M_r 13 000). Hydrophobic cluster analysis (HCA) (Gaboriaud *et al.*, 1987; Lemesle-Varloot *et al.*, 1990) has been carried out to determine whether one or more Hmc c_3-like domains show closely homologous folding with cytochromes c_3 (M_r 13 000). In this α-helical 2-D diagram of

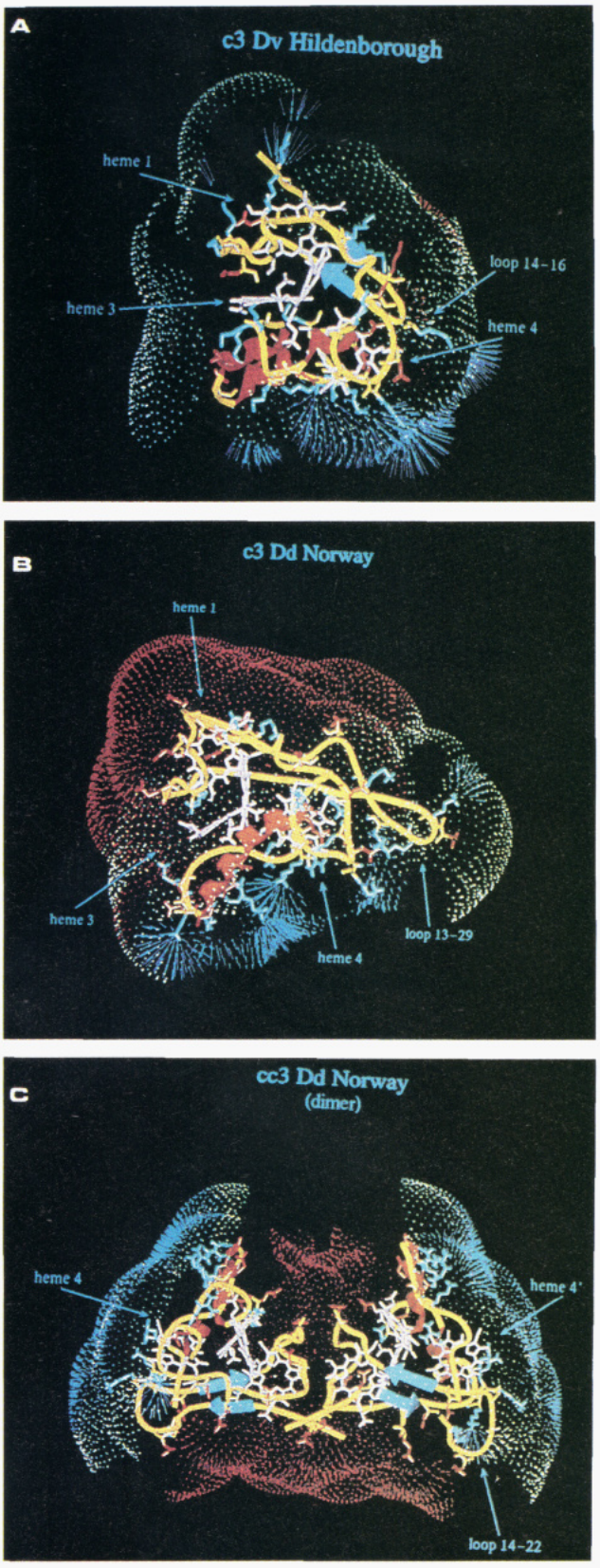

FIGURE 6: Electrostatic potential surfaces, calculated as described above. Ribbon representation of (A) *D.v.*H. c_3 (M_r 13 000), (B) *D.d.*N. c_3 (M_r 13 000), and (C) *D.d.*N. c_3 (M_r 26 000). The side chains of charged residues have been added, colored red when negatively charged and blue when positively charged. The arrows representing the electrostatic field are color-coded according to the magnitude of the field: blue, maximum positive potential arrows: red, minimum negative potential arrows; and white, neutral potential arrows.

the sequences, the hydrophobic residues (V, I, L, F, W, M, and Y) tend to form clusters of various shapes and sizes. Most of these clusters may be linked to regular secondary elements, which constitute the main 3-D folding cores of globular proteins or protein domains. From the deduced sequence alignment given in Figure 5, the region from Ala12 to Leu28 in Hmc domain I may be aligned to the Ala12–Leu28 loop, which is proposed to be the site of interaction with lipids in *D. desulfuricans* Norway cytochrome c_3 and in which hydrophobic and charged residues were found to alternate. In addition, the local environments of Phe72 in the *D. desulfuricans* Norway c_3 sequence and Phe64 in the Hmc domain I sequence are homologous (Figure 5). The region analogous to the Ala12–Leu28 loop in *D. desulfuricans* Norway cytochrome c_3 seemed to be deleted in Hmc domain II, but it is present in the Hmc domains III and IV. According to comparisons between the 3-D structures, the cytochrome c_3 (M_r 26 000) sequence corresponding to the Ala12–Leu28 amphiphilic loop in the *D. desulfuricans* Norway cytochrome c_3 (M_r 13 000) has also been aligned (Figure 5). The corresponding loop is shorter (Pro13 to Pro21) and less hydrophobic residues are present in this region, and they are not segregated into long patches as it is the case in the cytochrome c_3 (M_r 13 000) and in the Hmc domains. The overall hydrophobicity of this loop in the cytochrome c_3 (M_r 26 000) is less marked, which could explain the failure of the interaction with neutral lipids.

Cytochrome/Lipid Interactions: Functional Significance. The present study suggests that *D. desulfuricans* Norway cytochromes c_3 (M_r 13 000) and c_3 (M_r 26 000) and *D. vulgaris* Hildenborough Hmc may be classified as extrinsic membrane proteins, associated with the outer face of the cytoplasmic membrane. The question therefore arises as to what the functional significance of this finding may be.

Many peripherally interacting molecules bind to membranes through association with integral membrane proteins. Cytochrome *c* is the intermediate between the b–c_1 complex and the cytochrome *c* oxidase in the mitochondrial electron-transfer system. Conformational changes in cytochrome *c* induced upon lipid binding have been described (Hildebrandt & Stockburger, 1989a,b; Hildebrandt *et al.*, 1990; Heimburg *et al.*, 1991; Muga *et al.*, 1991; Spooner & Watts, 1991; Pinheiro & Watts, 1994). This membrane-bound intermediate of cytochrome *c* may have functional implications on the efficiency of electron transfer. However, interacting redox partners should not be viewed as structurally stable macromolecular aggregates: the overall electron transport process involves the diffusion of the redox components and random collisions among them. In the case of mitochondrial cytochrome *c*, the collision frequency with its oxidoreductases depends on both the diffusion rate and the concentration of this molecule near the inner membrane surface (Gupte & Hackenbrock, 1988a): at physiological ionic strength, the maximal electron transport activity is mediated by the rapid diffusion of the cytochrome and its low affinity for the inner membrane (Gupte & Hackenbrock, 1988b). A similar process can be assumed to occur during the interactions between Hmc and the potential transmembrane redox complex encoded by the *D. vulgaris* Hildenborough *hmc* operon. No analogous genetic data are available on the *D. desulfuricans* Norway system: the two cytochromes c_3 (M_r 13 000) and c_3 (M_r 26 000) may, however, constitute the missing link between periplasm and membrane. Moreover, it is worth mentioning that the presumed lipid binding site which has been identified in the *D. desulfuricans* Norway cytochrome c_3 (M_r 13 000) surrounds heme 4 (sequential

numbering). Many experiments have shown that this center mediates the interactions between *D. desulfuricans* Norway (Cambillau *et al.*, 1988; Dolla *et al.*, 1991a) and *D. vulgaris* (Stewart *et al.*, 1988; Dolla *et al.*, 1991b, 1994) cytochromes c_3 (M_r 13 000) and their redox partners. Consequently, the Ala12−Leu28 amphiphilic loop and the phenylalanine 72 present in the *D. desulfuricans* Norway cytochrome c_3 (M_r 13 000) may actually have some functional relevance in the interactions with the membrane. The next step will be to identify the membrane-spanning proteins which might be the oxidoreduction partners of these polyhemic cytochromes.

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