Control of the Redox Potential in *c*-Type Cytochromes: Importance of the Entropic Contribution

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ABSTRACT: The enthalpic and entropic components of the redox free energy variation of cytochrome c_{553} from *Desulfovibrio vulgaris* Hildenborough and its mutant Y64V, flavocytochrome b_2 from *Saccharomyces cerevisiae*, and the different hemes of cytochromes c_3 from *Desulfovibrio vulgaris* Miyazaki and *Desulfovibrio desulfuricans* Norway have been determined in 0.1 M Tris−HCl pH 7.0 (7.6 for cytochromes c_3) at 25 °C by using *nonisothermal* potentiometric titrations. The set of available experimental data demonstrates that the entropic component plays an important role in the control of the redox potential in *c*-type and *b*-type cytochromes. The variation of the entropic component within the class of cytochromes characterized by a positive value of $E^{\circ\prime}$ is proposed to be mainly determined by the variation of the exposure of the heme propionates to the solvent. In the case of tetraheme cytochromes c_3, the thermodynamic characteristics vary largely among the hemes belonging to the same molecule, which reflects the environmental peculiarities of each heme and also the heme−heme redox interactions. This study substantiates the existence of compensatory effects between large and opposite contributions to $E^{\circ\prime}$ predicted by all the current theoretical models which are based on electrostatic free energy calculations.

c-Type cytochromes are hemoproteins characterized by the presence of one or several mesohemes covalently bound to the protein through one or two thioether linkages. They are involved as electron carriers in a wide variety of bioenergetic processes, and their redox potentials $E^{\circ\prime}$ (pH 7.0)[1] vary in the range −400 mV to +400 mV, the lower limit corresponding to the tetraheme cytochromes c_3 of sulfate reducing bacteria and the upper limit being encountered in the case of the cytochromes that reduce the photosynthetic reaction center in purple bacteria (Meyer et al., 1983; Dolla et al., 1994). People have been trying for a long time to use the available structural information to define several factors, assumed more or less independent, that may explain these large variations, like the polarity of the heme environment (Kassner, 1973), the accessibility of the heme to the solvent (Stellwagen, 1978), the strength of the axial ligand field (Moore & Williams, 1977; Valentine et al., 1979), and the electrostatic interactions between the heme and its propionates (Moore, 1983).

More recently, this issue has been dealt with by exploiting the wide possibilities offered by chemical modifications (Rees, 1980; Wallace & Proudfoot, 1987; Hall et al., 1989; Wallace & Clark-Lewis, 1992) and site-directed mutagenesis techniques (Caffrey & Cusanovich, 1994; Dolla et al., 1991). However, such modifications may lead to structural changes which are difficult to predict, so that a full interpretation of these experiments is not straightforward in the absence of detailed structural information about the modified proteins (Mauk, 1991). In addition, according to electrostatic free energy calculations based on the three-dimensional structure of some cytochromes, the redox free energy change $\Delta G^{\circ\prime}$ results from large variations of many contributions that largely cancel (Churg & Warshell, 1986; Cutler et al., 1989; Gunner & Honnig, 1991; Langen et al., 1992; Zhou, 1994). This finding seems to preclude any analysis in terms of independent factors and to invalidate any interpretation of chemical modification or site-directed mutagenesis experiments which are not based on a full free energy calculation.

The redox free energy change $\Delta G^{\circ\prime}$ is made of enthalpic and entropic components whose values may result from contributions of rather different origin. Some information about the nature of these contributions in the *c*-type cytochrome family should then be obtained by measuring the enthalpic and entropic contributions to $\Delta G^{\circ\prime}$ and by studying their variations in this family. In particular, the measurement of $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ should prove very useful for studying the compensatory effects predicted by theoretical free energy calculations. In addition, the values of $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ are explicitely needed for determining the reorganization energy involved in electron transfer processes from the experimental activation energy (Bertrand, 1991).

From the experimental point of view, the measurement of $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ is most often based on the analysis of the temperature dependence of E'°, as given by a *nonisothermal* electrochemical or potentiometric cell arrangement. The advantage of this method is that it provides a temperature coefficient that is directly proportional to the standard entropy change $\Delta S^{\circ\prime}_{rc}$ of the studied redox couple (Yee et al., 1979).

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[1] $E^{\circ\prime}$ is the half-reduction potential at a given pH, for which activity (ox) = activity (red).

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In contrast, an *isothermal* cell arrangement can give useful information only when the electrodes are in thermal equilibrium, and when the experimental temperature coefficient is corrected for the temperature dependence of the reference electrode potential (Yee et al., 1979). *Nonisothermal* experiments have already been carried out on several hemoproteins, including monoheme high potential *c*-type cytochromes (Taniguchi et al., 1980), bovine liver cytochrome b_5 (Reid et al., 1982), and sperm whale myoglobin (Crutchley et al., 1985). Temperature coefficients for the two high potential hemes of the cytochrome subunit of the *Rhodopseudomonas viridis* reaction center (Gao et al., 1990) and for cytochrome c_{553} of *Desulfovibrio vulgaris* Hildenborough (Verhagen et al., 1994) have also been measured according to an *isothermal* cell arrangement.

In order to obtain a consistent set of data for the whole range of redox potentials covered by *c*-type cytochromes, we have performed *nonisothermal* potentiometric experiments on cytochromes c_{553} from *D. vulgaris* Hildenborough and its mutant Y64V. Previous experiments have shown that the Y64V mutation does not affect the redox potential of this protein, and it has been suggested that this might arise from compensatory effects of several contributions (Blanchard et al., 1994). The measurement of the thermodynamic parameters of the mutated protein was carried out to ascertain this suggestion. The range of $E^{\circ\prime}$ values covered by *c*-type cytochromes was widen by measuring the thermodynamic parameters of the four hemes of two cytochromes c_3, from *Desulfovibrio desulfuricans* Norway and from *Desulfovibrio vulgaris* Miyazaki. In order to enlarge the comparison between *c*-type and *b*-type cytochromes, the published data concerning cytochrome b_5 have been included and *nonisothermal* experiments were also carried out on flavocytochrome b_2 from *Saccharomyces cerevisiae*. Finally, horse heart and bovine heart cytochromes *c* were studied as controls. A preliminary account of the work carried out on cytochrome c_3 of *D. vulgaris* Miyazaki has already been published (Bertrand et al., 1994).

MATERIALS AND METHODS

Protein Preparation. Horse heart and bovine heart cytochromes *c* were purchased from Sigma and used without further purification. Cytochrome c_{553} from *D. vulgaris* Hildenborough and its mutant Y64V were prepared as previously described (Blanchard et al., 1994). Recombinant *S. cerevisiae* flavocytochrome b_2 and cytochrome c_3 from *D. desulfuricans* Norway were purified as described by Black et al. (1989) and Bruschi et al. (1977), respectively.

Potentiometric Experiments. The potentiometric device used in temperature-dependent experiments was of the *nonisothermal* type. An optical cell containing the protein solution, a platinum electrode, and a calibrated thermocouple was placed in a variable temperature holder. This cell was electrically connected through a flexible KCl (3 M) salt bridge to a second cell containing the Ag/AgCl−KCl (3 M) reference electrode which was kept at 23 °C (+209 mV vs SHE). In the text, all potential values are given with respect to the standard hydrogen electrode. Under these conditions, the temperature coefficient $dE^{\circ\prime}/dT$ of the midpoint potential $E^{\circ\prime}$ measured between the two electrodes is directly proportional to the entropy variation $\Delta S^{\circ\prime}_{rc} = S^{\circ\prime}_{red} - S^{\circ\prime}_{ox}$ of the studied redox couple, if $E^{\circ\prime}$ is corrected from the thermal junction potential (Yee et al., 1979). The stability of the salt bridge was checked in the temperature range 8−50 °C, and the magnitude of the junction potential, as evaluated by replacing the protein solution by a 0.1 M Tris-HCl (pH 7.0) solution, was found to vary between 3 and 6 mV in this range.

Redox titrations of monoheme cytochromes were carried out in 0.1 M Tris-HCl (pH 7.0) buffer. The protein concentration was 25 μM for cytochromes *c* from horse heart and bovine heart, 21 μM for cytochrome c_{553} and its mutant Y64V, and 10 μM for flavocytochrome b_2. Solution potentials were adjusted by addition of stoichiometric amounts of a concentrated solution of sodium dithionite, and the reversibility of the titrations was checked by allowing the reduced protein solution to reoxidize. Equilibration with the electrode was achieved by adding the following mediators, each at 2 μM concentration: horse heart and bovine heart cytochromes *c*:2,6-dichlorophenol indophenol (+220 mV), 1,2 naphthoquinone (+145 mV), phenazine methosulfate (+80 mV); cytochrome c_{553} and flavocytochrome b_2: 1,2-naphthoquinone (+145 mV), phenazine methosulfate (+80 mV), methylene blue (+11 mV), and resorufin (−51 mV). The titration of tetraheme cytochromes c_3 was carried out in 0.1 M Tris-HCl (pH 7.6) buffer with a protein concentration equal to 6 μM, in the presence of the following mediators, each at 1.3 μM concentration: phenosafranine (−255 mV), benzyl viologen (−350 mV), and methyl viologen (−440 mV). For all the titrations, the level of heme reduction was monitored at the maximum of the α band, where the absorption of the mediators was negligible in comparison to that of the protein. In the case of tetraheme cytochromes c_3, such absorbance variation studies yield only the macroscopic redox potentials $E^{\circ\prime}_i$. The determination of the individual (microscopic) potentials of the hemes requires the knowledge of the set of interaction potentials $I_{ij} = e_i - e^j_i$, where e_i is the potential of heme *i* when all other hemes are reduced, and e^j_i is the potential of heme *i* when only heme *j* is oxidized. In the case of cytochrome c_3 from *D. vulgaris* Miyazaki, two different sets of I_{ij} have been proposed from redox titrations monitored by EPR[2] (Benosman et al., 1989) and NMR (Fan et al., 1990) spectroscopies, and the possible origin of these differences has been analyzed (Bertrand et al., 1994). These two different sets of I_{ij} were used to determine the temperature dependence of the individual redox potentials for this cytochrome (Bertrand et al., 1994). In the case of *D. desulfuricans* Norway cytochrome c_3, we used the set of interaction potentials given by a potentiometric titration monitored by EPR (Gayda et al., 1988). In both cases, the interaction potentials were assumed to be temperature independent.

The enthalpy $\Delta H^{\circ\prime}$ and entropy $\Delta S^{\circ\prime}$ changes adjusted to the SHE scale were deduced from the following expressions (Taniguchi et al., 1980):

$$\Delta S^{\circ\prime}_{rc} = \mathcal{F}\frac{dE^{\circ\prime}}{dT}, \quad \Delta S^{\circ\prime} = \Delta S^{\circ\prime}_{rc} - 66.5\ \text{J·mol}^{-1}\text{·K}^{-1} \quad (1)$$

$$\Delta G^{\circ\prime} = -\mathcal{F}E^{\circ\prime}, \quad \Delta H^{\circ\prime} = \Delta G^{\circ\prime} + T\Delta S^{\circ\prime}$$

RESULTS

The temperature dependence of $E^{\circ\prime}$ (pH 7.0, I = 0.1 M) for horse heart and bovine heart cytochromes *c*, cytochrome

[2] Abbreviations: NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance.

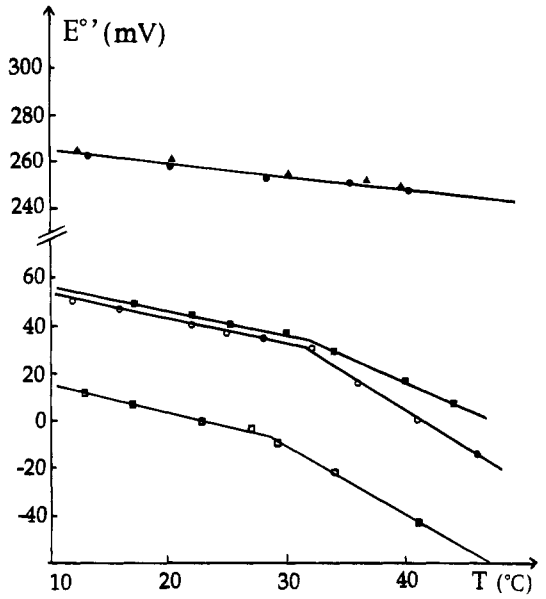

FIGURE 1: Temperature dependence of $E^{\circ\prime}$ (pH 7.0, I = 0.1 M) for (●) horse heart cytochrome c, (▲) beef heart cytochrome c, (○) native form (■) Y54V mutant of cytochrome c_{553} from *D. vulgaris* Hildenborough, and (□) flavocytochrome b_2 from *S. cerevisiae*. The thermodynamic parameters at 25 °C deduced from these data are reported in Table 1.

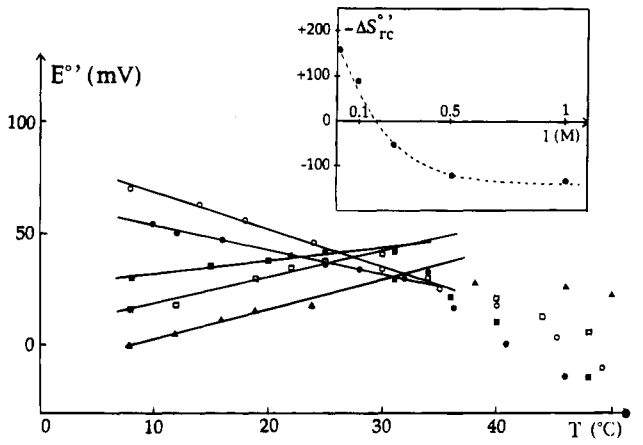

FIGURE 2: Temperature dependence of the redox potential of cytochrome c_{553} from *D. vulgaris* Hildenborough for different concentrations of the Tris-HCl buffer: (○) 0.01 M, (●) 0.1 M, (■) 0.25 M, (□) 0.5 M, (▲) 1 M. The values of $-\Delta S^{\circ\prime}_{rc}$(25 °C) deduced from these data are reported in Table 2 and are represented in the inset as a function of the ionic strength.

c_{553} of *D. vulgaris* Hildenborough and its mutant Y64V, and flavocytochrome b_2 from *S. cerevisiae* are represented in Figure 1. The thermodynamic parameters at 25 °C deduced from these variations are given in Table 1, in which we have reported all the published data concerning other cytochromes that were obtained by *nonisothermal* electrochemical or potentiometric experiments. We have also included the data concerning the high potential hemes H_1 (c_{559}) and H_3 (c_{556}) of the tetraheme cytochrome of the *Rhodopseudomonas viridis* reaction center, which were obtained from isothermal potentiometric experiments (pH 9) in which the temperature coefficient was corrected from the temperature dependence of the reference electrode potential (Gao et al., 1990). The values quoted in Table 1 were deduced from a reanalysis of the data presented in Gao et al. (1990) based on eqs 1 given in the Materials and Methods section.

The results obtained for horse heart and bovine heart cytochromes c are identical to those given by microcalorimetry experiments (George et al., 1968; Watt & Sturtevant, 1969) and agree within experimental errors with those given by previous *nonisothermal* experiments (Taniguchi et al., 1980). Concerning cytochrome c_{553} of *D. vulgaris* Hildenborough, the value $E^{\circ\prime}$ = 37 ± 5 mV measured at 25 °C is more positive than the value $E^{\circ\prime}$ = 20 ± 5 mV measured in a previous redox titration carried out in the same conditions and monitored by EPR (Bertrand et al., 1982), but is in agreement with the value $E^{\circ\prime}$ = 50 mV recently measured by cyclic voltammetry at 23 °C, pH 7.0, I = 0.1 M in Hepes buffer (Verhagen et al., 1994). Verhagen et al. have also studied the temperature dependence of $E^{\circ\prime}$ at pH 7.0, I = 0.02 M, by using an *isothermal* cell arrangement, apparently without correcting the experimental temperature coefficient from the temperature dependence of the reference electrode. In spite of this, they found a temperature dependence very similar to that represented in Figure 1, with the same change of slope occuring at 32 °C, which was attributed by them to a conformational change of the protein. The main difference is an upward shift of about 25 mV which can be attributed in great part to the different ionic strengths used in the two experiments. Unfortunately, these authors have interpreted their data by using erroneous expressions in which $\Delta S^{\circ\prime}$ and $\Delta S^{\circ\prime}_{rc}$ are exchanged in comparison with eqs 1.

Verhagen et al. have pointed out that the $E^{\circ\prime}$ value of cytochrome c_{553} is ionic strength dependent at 23 °C (Verhagen et al., 1994). In order to better understand the meaning of this effect, we have systematically investigated the ionic strength dependence of $\Delta S^{\circ\prime}_{rc}$ and $\Delta H^{\circ\prime}$ for this cytochrome. The temperature dependence of $E^{\circ\prime}$ is represented in Figure 2 for Tris-HCl concentrations ranging from

Table 1: Thermodynamic Parameters of High Potential Cytochromes (25 °C, pH 7.0, I = 0.1 M)

cytochromes	$E^{\circ\prime}$ (mV)	$\Delta S^{\circ\prime}_{rc}$ (J·mol^{-1}·K^{-1})	$T\Delta S^{\circ\prime}$ (kJ·mol^{-1})	$\Delta H^{\circ\prime}$ (kJ·mol^{-1})	ref
R. viridis H1 (c_{559})	+380	−63	−38	−75	Gao et al. (1990)
R. viridis H3 (c_{556})	+330	−60	−37	−69	Gao et al. (1990)
c_2 *R. rubrum*	+324	−40 ± 5	−31.6 ± 1.5	−62.7 ± 2	Taniguchi et al. (1980)
c_{551} *P. aeruginosa*	+276	−67.7 ± 5	−40 ± 1.5	−66.5 ± 2	Taniguchi et al. (1980)
c horse heart	+270	−62 ± 5	−38.2 ± 2	−64 ± 2	Taniguchi et al. (1980)
c horse heart	+257	−53 ± 8	−36 ± 2.5	−60 ± 2.5	this work
c beef heart	+257	−53 ± 8	−36 ± 2.5	−60 ± 2.5	this work
c_{553} (Y64V)	+40	−100 ± 8	−50 ± 2.5	−53 ± 2.5	this work
c_{553}	+37	−100 ± 8	−50 ± 2.5	−54 ± 2.5	this work
b_5 *beef liver*	+5	−90 ± 8	−46.4 ± 1.5	−46 ± 2	Reid et al. (1982)
b_2 *S. cerevisiae*	−2	−115 ± 8	−54 ± 1.5	−54 ± 2	this work

Table 2: Thermodynamic Parameters of c_{553} from *D. vulgaris* Hildenborough at Different Ionic Strengths (25 °C, pH 7.0)

I (M)	$E^{\circ\prime}$ (mV)	$\Delta S_{rc}^{\circ\prime}$ (J·mol^{-1}·K^{-1})	$\Delta H^{\circ\prime}$ (kJ·mol^{-1})
0.01	+43	−160 ± 15	−71 ± 5
0.1	+37	−100 ± 8	−54 ± 3
0.25	+40	+50 ± 8	−9 ± 3
0.5	+37	+115 ± 8	+11 ± 3
1.0	+23	+130 ± 8	+17 ± 3

0.01 to 1.0 M. It appears that the temperature dependence is strongly ionic strength dependent, although the change of slope mentioned above always occurs at about 32 °C (Figure 2). The thermodynamic parameters at 25 °C calculated from these variations are reported in Table 2. These experiments reveal that the moderate $E^{\circ\prime}$ change (about 20 mV) observed in the range 0.01−1 M is due to an almost complete cancellation of large and concomitant variations of $\Delta S_{rc}^{\circ\prime}$ and $\Delta H^{\circ\prime}$ (Table 2). Moreover, $\Delta S_{rc}^{\circ\prime}$ becomes positive and reaches a plateau at high ionic strength (inset of Figure 2), suggesting that it includes components of both signs, the negative one being strongly decreased at high ionic strength where solvation effects are minimized. These findings demonstrate clearly that the redox free energy change $\Delta G^{\circ\prime}$ results from the nearly complete compensation of several very large contributions, as predicted by the current theoretical models (Churg & Warshell, 1986; Cutler et al., 1989; Gunner & Honnig, 1991; Langen et al., 1992; Zhou, 1994). Within experimental errors, the value of $\Delta H^{\circ\prime}$ and $\Delta S_{rc}^{\circ\prime}$ measured for the Y64V mutant of cytochrome c_{553} are identical to those of the wild type protein (Table 1). Thus, although Tyr 64 is believed to play a structural role in this cytochrome (Blanchard et al., 1994), this residue is very likely not involved in the regulation of the redox potential.

Like that of cytochrome c_{553}, the temperature dependence of $E^{\circ\prime}$ for flavocytochrome b_2 exhibits a change of slope, which occurs at about 28 °C (Figure 1). $E^{\circ\prime}$ values measured at different temperatures have been reported for this cytochrome (Capeillere-Blandin et al., 1975; Lederer, 1991 and references therein; Walker & Tollin, 1992; Kay & Lippay, 1992), which agree with those reported in Figure 1 within 10 mV. The value of $\Delta S_{rc}^{\circ\prime}$ deduced from the temperature dependence of $E^{\circ\prime}$ is similar to those measured for cytochrome c_{553} and cytochrome b_5 (Table 1).

We now examine the results obtained for tetraheme cytochromes c_3. As explained in the Materials and Methods section, two different sets of thermodynamic parameters were deduced for the hemes of *D. vulgaris* cytochrome c_3 by using the interaction potentials determined by EPR and NMR (Bertrand et al., 1994). Although these interaction potentials differ significantly, they lead to rather similar thermodynamic parameters (Table 3). In the case of *D. desulfuricans* Norway cytochrome c_3, using the set of interaction potentials determined by EPR, we obtain the temperature dependence of the microscopic potentials represented in Figure 3 and the thermodynamic parameters reported in Table 3. It should be noted that the highest macroscopic potential $E_1^{\circ\prime}$ of this cytochrome is much less negative than the others (Gayda et al., 1988), so that $E_1^{\circ\prime}$ can be identified with the microscopic potential e_1^{234} of the highest potential heme. It follows that, for this heme, the temperature dependence of the microscopic potential is directly obtained from redox titrations monitored by absorbance variations. The same comment applies to some extent to the highest potential heme of *D. vulgaris* Miyazaki cytochrome c_3 (Bertrand et al., 1994).

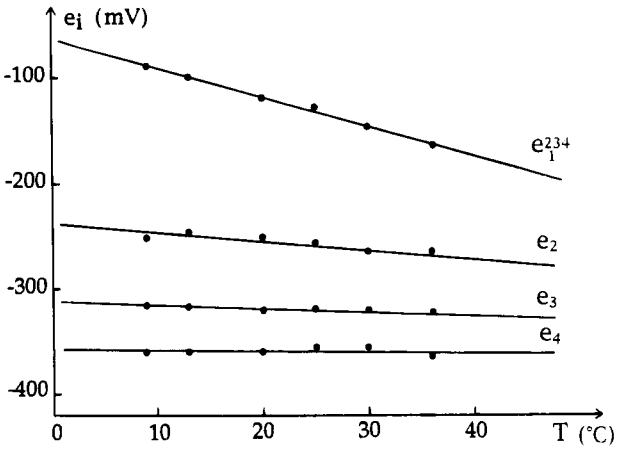

FIGURE 3: Temperature dependence of the hemes' microscopic potentials e_i for *D. desulfuricans* Norway cytochrome c_3 (pH 7.6, 0.1 M Tris−HCl). The e_i values were obtained by fitting the absorbance changes observed during the redox titrations by theoretical curves calculated by using the interaction potentials measured in Gayda et al. (1988).

DISCUSSION

The results of the present study together with those already available in the literature provide a consistent set of data covering the whole range of redox potentials exhibited by *c*-type cytochromes (Tables 1 and 3). In the following, we first analyze the data concerning cytochromes with $E^{\circ\prime}$ values positive with respect to the standard hydrogen electrode, and we consider next the case of tetraheme cytochromes c_3.

Table 3: Thermodynamic Parameters of the Hemes of Tetraheme Cytochromes c_3 (25 °C, pH 7.6, I = 0.1 M)

cytochrome		$E^{\circ\prime}$ (mV)	$\Delta S_{rc}^{\circ\prime}$ (J·mol^{-1}·K^{-1})	$T\Delta S^{\circ\prime}$ (kJ·mol^{-1})	$\Delta H^{\circ\prime}$ (kJ·mol^{-1})
D. v. Miyazaki[a]	e_1^4	−240 ± 10	−330 ± 20	−118 ± 6	−95 ± 6
		(−280 ± 10)	(−280 ± 20)	(−103 ± 6)	(−80 ± 6)
	e_2, e_3	−320 ± 10	0 ± 20	−19 ± 6	+11 ± 6
		(−295 ± 10)	(0 ± 20)	(−19 ± 6)	(+9 ± 6)
	e_4	−340 ± 10	−38 ± 20	−31 ± 6	+2 ± 6
		(−350 ± 10)	(−48 ± 20)	(−34 ± 6)	(−1 ± 6)
D. d. Norway	e_1^{234}	−132	−270 ± 20	−100 ± 6	−87 ± 6
	e_2	−255	−67 ± 20	−40 ± 6	−15 ± 6
	e_3	−320	−30 ± 20	−29 ± 6	+2 ± 6
	e_4	−360	0 ± 20	−20 ± 6	+15 ± 6

[a] Without parentheses: based on the set of interaction potentials reported in Benosman et al. (1989). In parentheses: based on the set of interaction potentials reported in Fan et al. (1990).

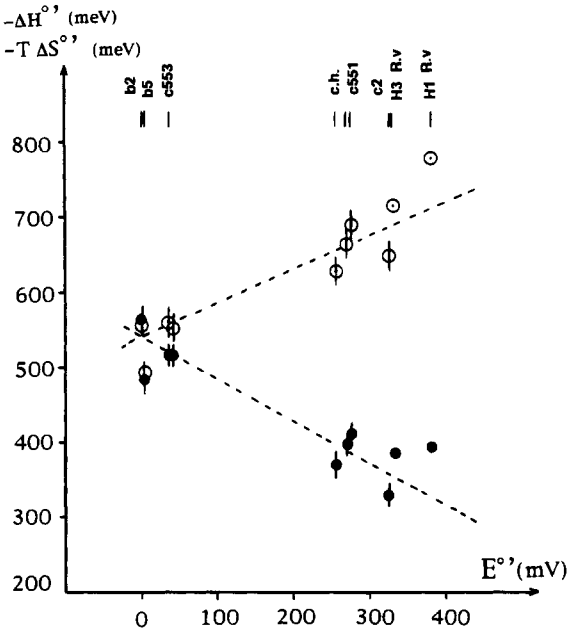

FIGURE 4: Variations of (○) $-\Delta H^{\circ\prime}$ and (●) $-T\Delta S^{\circ\prime}$ expressed in meV as a function of $E^{\circ\prime}$ for the cytochromes quoted in Table 1.

Cytochromes with Positive Values of $E^{\circ\prime}$. Although the hemes of these cytochromes are all coordinated by a histidine and a methionine residue, their redox potentials vary in the range 0−400 mV. In order to put into evidence the respective part of $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ in these variations, we have represented in Figure 4 the quantities $-\Delta H^{\circ\prime}$ and $-T\Delta S^{\circ\prime}$ expressed in meV as a function of $E^{\circ\prime}$. In this representation, the vertical difference between $-\Delta H^{\circ\prime}$ and $-T\Delta S^{\circ\prime}$ is numerically equal to $E^{\circ\prime}$. For these cytochromes, it appears that $-\Delta H^{\circ}$ and $-T\Delta S^{\circ\prime}$ vary in opposite direction so that the decrease of $E^{\circ\prime}$ is due both to a decreasing of $-\Delta H^{\circ\prime}$ and to an increasing of $-\Delta S^{\circ\prime}$, the two contributions being of similar magnitude. Thus, entropic effects play an important role in the control of the $E^{\circ\prime}$ value in this class of cytochromes. As a matter of fact, the quantity $\Delta S^{\circ\prime}_{rc}$ characteristic of the redox couple (cytochrome ox/cytochrome red) varies by a factor of about 3 in Table 1. In the following, the possible causes of these variations are analyzed. Generally speaking, the entropy of a system can be decomposed into the sum of components only to the extent that the system can be separated into subsystems characterized by independent degrees of freedom (Mark & van Gunsteren, 1994). In the case of transition metal complexes, the $\Delta S^{\circ\prime}_{rc}$ variations have been rationalized by defining electronic, vibrational, and solvent based components (Hupp & Weaver, 1984; Richardson & Sharpe, 1993):

$$\Delta S^{\circ\prime}_{rc} = \Delta S^{\circ\prime}_{rc}(\text{el}) + \Delta S^{\circ\prime}_{rc}(\text{vib}) + \Delta S^{\circ\prime}_{rc}(\text{sol})$$

The same approach can be attempted in the case of *c*-type cytochromes. The component $\Delta S^{\circ\prime}_{rc}$(el) comprises a temperature independent term equal to $-R \ln 2 = -5.8$ J·mol^{-1}·K^{-1} due to the variation of the ground state spin degeneracy and a temperature dependent term due to the population of electronic excited levels, which is expected to be very weak at room temperature in cytochromes. The vibrational component $\Delta S^{\circ\prime}_{rc}$(vib) arises from the frequency shift of the protein vibrational modes induced by the reduction. We first consider the heme vibrational modes, which fall in the range 150−1600 cm^{-1} according to resonance Raman experiments. In mitochrondrial cytochrome *c*, the reduction gives rise to frequency shifts smaller than 2 cm^{-1} in the range 150−900 cm^{-1} and smaller than 10 cm^{-1} in the range 900−1600 cm^{-1} (Hu et al., 1993). Some of these shifts are upward and other downward, so that their contributions to the redox entropy change largely cancel. As a consequence, by using the data reported in Hu et al. (1993) and the expression of $\Delta S^{\circ\prime}_{rc}$(vib) given, for example, in Richardson and Sharpe (1991), we calculated a very small entropy change equal to +0.33 J·mol^{-1}·K^{-1}. Low frequency protein modes may also be sensitive to the heme redox state. For example, it has been proposed that the oxidized form of mitochrondrial cytochrome *c* may undergo low frequency motions of amplitude larger than the reduced form (Eden et al., 1982). This phenomenon should bring a negative contribution to $\Delta S^{\circ\prime}_{rc}$(vib). In contrast, recent ^{1}H NMR experiments have revealed that the opposite situation occurs in cytochrome c_{553} from *D. vulgaris* Hildenborough, for which the oxidized form is more compact than the reduced one (L. Blanchard, M. Blackledge, D. Marion, and F. Guerlesquin, submitted). In this case, this effect would give a positive contribution to $\Delta S^{\circ\prime}_{rc}$(vib). However, the variations of $\Delta S^{\circ\prime}_{rc}$ observed experimentally (Table 1) do not follow this trend, showing that these expected entropic contributions are masked by larger terms.

From the preceding discussion, it appears very likely that the variations of $\Delta S^{\circ\prime}_{rc}$ apparent in Figure 4 are due neither to the electronic nor to the vibrational component, but rather to the $\Delta S^{\circ\prime}_{rc}$(sol) component. The existence of a large negative $\Delta S^{\circ\prime}_{rc}$(sol) component is supported by the strong ionic strength dependence of $\Delta S^{\circ\prime}_{rc}$ observed for cytochrome c_{553} from *D. vulgaris* Hildenborough: increasing the ionic strength leads to less negative and even to positive values of $\Delta S^{\circ\prime}_{rc}$ (inset of Figure 2). A similar albeit less pronounced behavior has been reported in the case of horse heart cytochrome *c* (Margalit & Schechter, 1970), which suggests that $\Delta S^{\circ\prime}_{rc}$(sol) may vary significantly in the series reported in Table 1. The $\Delta S^{\circ\prime}_{rc}$(sol) contribution is due to the interaction of the solvent dipoles with all the charged groups of the protein. Although the surface charged groups are expected to interact strongly with the solvent, their neutralization or even their change of sign by chemical modification or site-directed mutagenesis techniques give rise to very small $E^{\circ\prime}$ changes, whose magnitude is less than 15 mV for mitochrondrial cytochrome *c* (Rees, 1980), cytochrome c_2 from *Rhodobacter sphaeroides* (Hall et al., 1989), as well as for cytochrome b_5 (Rodgers & Sligar, 1991). It would be very interesting to determine the enthalpic and entropic contributions to these small changes in order to assess whether they arise from an enthalpic−entropic compensation. However that may be, it is likely that surface charges do not play an important role in the $\Delta S^{\circ\prime}_{rc}$ variations apparent in Figure 4, since the charge of the cytochromes of Table 1 calculated from their amino acid sequence varies largely and in a way that is not correlated with the $\Delta S^{\circ\prime}_{rc}$ variations: cytochrome from *R. viridis* (−4), cytochrome c_2 from *R. rubrum* (0), cytochrome c_{551} (−2), horse heart cytochrome *c* (+8), cytochrome c_{553} (+1), cytochrome b_5 (−7), cytochrome domain of flavocytochrome b_2 (−3).

A clue to the origin of the $\Delta S^{\circ\prime}_{rc}$(sol) variations may be the fact that $\Delta S^{\circ\prime}_{rc}$ reacts to ionic strength changes in the same way in *c*-type cytochromes and in metal complexes with

negatively charged ligands (Fabrizzi et al., 1989). This suggests that the $\Delta S^{\circ\prime}_{rc}$(sol) variations are mainly determined by the interaction of the solvent with negatively charged groups present in all *c*-type cytochromes. Obvious candidates are the heme propionates. As a matter of fact, the accessibility of the propionates to the solvent is known to vary largely in the series reported in Table 1. In the three-dimensional structure of these proteins, this is reflected in the number of hydrogen bonds in which the propionates are engaged (Table 4): the more numerous the hydrogen bonds, the more the propionates are expected to be shielded from the solvent. The variations of the exposure of the propionates to the solvent may modulate $\Delta S^{\circ\prime}_{rc}$(sol) by the following mechanism: the solvent dipoles are highly polarized in the vicinity of the negatively charged propionates, but this polarization is greater when the heme is reduced than when it is oxidized. This effect gives rise to a negative contribution to $\Delta S^{\circ\prime}_{rc}$(sol), which is more negative the more the propionates are exposed (Figure 5), in agreement with the variations of $\Delta S^{\circ\prime}_{rc}$ observed experimentally (Table 4). This effect should even be accentuated by the partial neutralization of shielded propionates engaged in hydrogen bonds. The magnitude of this solvation effect is expected to decrease upon increasing the ionic strength, in agreement with experiment (Figure 2).

In the preceding discussion, the heme propionates were considered as fully ionized. There is evidence that some buried propionates of cytochromes of Table 1 are protonated at pH 7.0. In cytochrome c_2 of *R. rubrum* and cytochrome c_{551} of *P. aeruginosa*, propionate 7 is ionized in the oxidized state and partly protonated in the reduced state, which results in a strong pH dependence of $E^{\circ\prime}$ around pH 7.0 (Moore et al., 1980; Moore, 1983; Rogers et al., 1985). In contrast, propionate 6 is protonated in mitochrondrial ferricytochrome *c* (Hartshorn & Moore, 1989). Whatever their ionization state is, these buried propionates interact weakly with the solvent and hardly contribute to $\Delta S^{\circ\prime}_{rc}$(sol). Therefore, the relation between the heme propionates' exposure and the $\Delta S^{\circ\prime}_{rc}$(sol) value is expected to be little influenced by the protonation state of buried propionates.

If this model is valid, it must also apply to hemoproteins that do not belong to the *c*-type cytochrome family. According to the data reported in Table 1, the $\Delta S^{\circ\prime}_{rc}$ values of cytochrome c_{553}, cytochrome b_5, and flavocytochrome b_2 are very similar. In cytochrome b_5, propionate 6 is relatively buried and participates to two hydrogen bonds with Ser 64, while propionate 7 is fully exposed to the solvent (Mathews, 1985). The overall exposure of the propionates to the solvent is then very similar to that found in cytochrome c_{553} (Table 4). When the two propionates of cytochrome b_5 are neutralized by esterification, the redox potential increases from +5 mV to +69 mV (Reid et al., 1984). This increase is due solely to the entropic component $\Delta S^{\circ\prime}_{rc}$ which varies from −90 to −65 J·mol^{-1}·K^{-1}, while $\Delta H^{\circ\prime}$ remains unchanged (Reid et al., 1984). This experiment demonstrates clearly the great sensitivity of $\Delta S^{\circ\prime}_{rc}$ with respect to the charge carried by the heme propionates. The case of flavocytochrome b_2 from *S. cerevisiae* is also interesting. The crystal structure of this protein exhibits two crystallographically distinguishable subunits per asymmetric unit (Xia & Mathews, 1990; Tegoni & Cambillau, 1994). In the first one, the

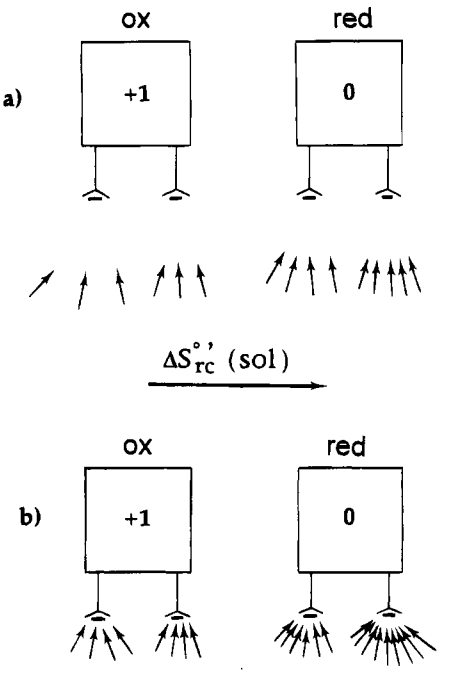

FIGURE 5: Schematic model to explain how the accessibility of the solvent to the heme propionates may modulate the redox entropy change $\Delta S^{\circ\prime}_{rc}$(sol): (a) small accessibility; (b) large accessibility.

Table 4: Hydrogen Bonding Pattern of the High Potential Cytochromes Propionates

cytochromes	$\Delta S^{\circ\prime}_{rc}$ (J·mol^{-1}·K^{-1})	propionate 6 (D)	propionate 7 (A)	ref
c_2	−40	Ser49	Arg38, Tyr48	*a*
R. rubrum		Thr46, Ser49	Trp49	
c horse	−53	Thr49, Thr78, Lys79	Arg38, Tyr48, w125	*b, c*
		Thr49	Gly41, Asn52, Typ49	
R. viridis				
H3	−60	(uncharged)[d]	Tyr89, Tyr102	*e*
R. viridis	−63	Arg272	Val203, Val204	*e*
H1		Arg202, Trp268		
c_{551}	−68	Val55	Arg47	*f*
P. aeruginosa		w2	Trp56	
b_5 beef liver	−90		Ser64	*a*
			Ser64	
c_{553} *D. v.*	−100	exposed	Lys27, Gln32	*g*
Hildenborough			Lys40, Tyr44	
b_2 S. cerevisiae	−115	Lys296, Tyr97	Tyr143, w685	*h*
		w685, w693	w664	

[a] Mathews (1985). [b] Bushnell et al. (1990). [c] w represents a water molecule. [d] Gunner and Honig (1991). [e] From the crystal structure (Brookhaven Data Bank, entry 1 PRC). [f] Matsuura et al. (1982). [g] Blackledge et al. (1995). [h] Xia and Mathews (1990); Tegoni and Cambillau (1994).

cytochrome domain is visible in the protein and the heme propionates participate to a network of hydrogen bonds binding the cytochrome and flavodehydrogenase domains, while in the second subunit the cytochrome is not visible due to a high mobility or to positional disorder. Indeed, NMR experiments indicate that, in solution, the cytochrome domain is mobile with respect to the flavodehydrogenase domain (Labeyrie et al., 1988). Thus, in solution, the propionates of heme b_2 could be much more exposed than expected on the basis of the crystal structure. The value of $\Delta S^{\circ\prime}_{rc}$ measured for flavocytochrome b_2 is fully consistent with this idea and suggests that, in solution, the propionates' exposure is similar to that observed for cytochrome b_5 and cytochrome c_{553} from *D. vulgaris* Hildenborough.

From the analysis developed above, it appears very likely that the accessibility of the heme propionates to the solvent plays a dominant role in the variations of $\Delta S^{\circ\prime}_{rc}$ reported in Table 1. We next consider the origin of the correlation between the $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ variations which is apparent in Figure 4. The value of $\Delta H^{\circ\prime}$ is determined by the electrostatic interactions between the heme, the protein-charged groups, and the dipoles of the protein and of the solvent. The importance of the solvent contribution is demonstrated by the strong effect of the ionic strength on the $\Delta H^{\circ\prime}$ value of cytochrome c_{553} (Table 2). This effect can be explained by considering the electrostatic interactions which take place in the oxidized state between the heme propionates and the charge +1 carried by the ferric iron ion: increasing the solvent ionic strength is expected to decrease the effective dielectric constant for these interactions, giving a more positive $\Delta H^{\circ\prime}$ value, as observed experimentally (Table 2). The electrostatic interactions between the ferric ion and the propionates can also be invoked to correlate the $\Delta H^{\circ\prime}$ variations to the accessibility of the propionates to the solvent: on the one hand, increasing this accessibility is expected to increase the effective dielectric constant, and on the other hand decreasing this accessibility favors the formation of hydrogen bonds between the propionates and the surrounding polar residues, an effect which is also expected to increase the effective dielectric constant. Thus, although it is clear that the degree of exposure of the heme propionates may modulate the enthalpic component $\Delta H^{\circ\prime}$, detailed theoretical calculations are needed to assess whether the balance between these two opposite effects can explain the trend observed in Figure 4.

Alternatively, the protein structural changes responsible for the variation of the propionates' exposure may directly affect the $\Delta H^{\circ\prime}$ value. Such phenomena could be quite subtle and difficult to analyze theoretically. It should be realized that the total $\Delta H^{\circ\prime}$ variation amounts to only 20 kJ·mol^{-1} within this class of cytochromes, which is the order of magnitude of the energy of a hydrogen bond or, alternatively, of a 10% variation of 10 hydrogen bonds. We have seen previously that the oxidized form of mitochrondrial cytochrome *c* is less rigid than the oxidized one and that the opposite is true in cytochrome c_{553} from *D. vulgaris* Hildenborough. These rigidity variations probably reflect slight changes of numerous weak bonds, which may be responsible for the $\Delta H^{\circ\prime}$ variations apparent in Figure 4. Lastly, we observe that the $\Delta H^{\circ\prime}$ value is nearly the same for cytochrome b_5 and flavocytochrome b_2 in which the hemes are axially coordinated by two histidine residues and for cytochrome c_{553} in which the coordination is ensured by a histidine and a methionine residue (Table 1). Thus, in these *b*-type cytochromes, the relative stabilization of the oxidized form provided by the bis-histidine axial coordination is largely compensated by other kinds of interactions.

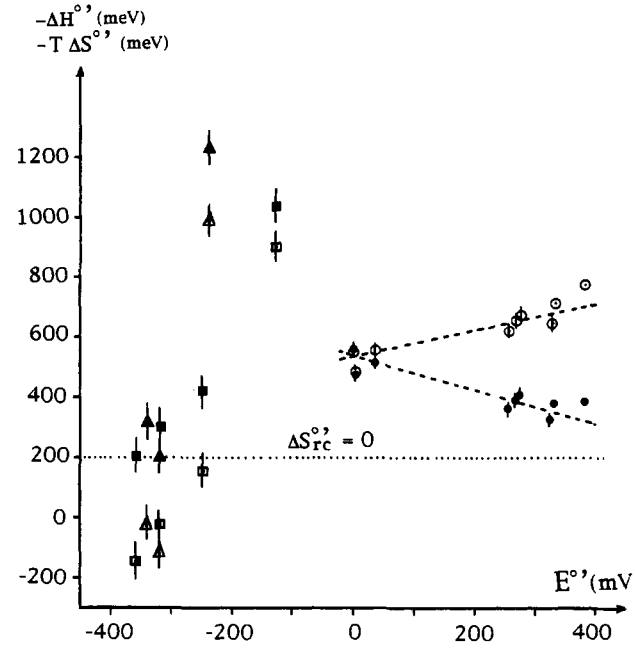

FIGURE 6: Variations of $-\Delta H^{\circ\prime}$ (open symbols) and $-T\Delta S^{\circ\prime}$ (filled symbols) for the different hemes of cytochromes c_3 as a function of their microscopic potentials: (△, ▲) cytochrome c_3 from *D. vulgaris* Miyazaki, (□, ■) cytochrome c_3 from *D. desulfuricans* Norway. The data are taken from Table 3. The data concerning high potential cytochromes are recalled for comparison. The dotted line corresponds to $\Delta S^{\circ\prime}_{rc} = 0$.

Cytochromes c_3. The thermodynamic parameters of these cytochromes differ remarkably from those of cytochromes with positive values of $E^{\circ\prime}$ (Tables 1 and 3). In order to better compare the full set of data, we have represented in Figure 6 the variations of $-\Delta H^{\circ\prime}$ and $-T\Delta S^{\circ\prime}$ expressed in meV as a function of $E^{\circ\prime}$. We first observe that these quantities vary much more within a given cytochrome c_3 than within the whole class of cytochromes with positive values of $E^{\circ\prime}$. In addition, the general trend observed in these cytochromes is not obeyed in cytochromes c_3: in the former, the decreasing of $E^{\circ\prime}$ is due to the decreasing of $-\Delta H^{\circ\prime}$ and to the increasing of $-\Delta S^{\circ\prime}$, while in the latter the decreasing of $E^{\circ\prime}$ is due to a large decreasing of $-\Delta H^{\circ\prime}$ which is almost compensated by a large decreasing of $-\Delta S^{\circ\prime}$ (Figure 6). In the following, we first examine whether the contributions mentioned for cytochromes with positive $E^{\circ\prime}$ values are likely to play an important role in these variations. In this discussion, the heme numbering is that corresponding to the appearance of the binding cysteines in the primary sequence (Mathews, 1985).

According to the recently determined X-ray crystal structure of *D. desulfuricans* Norway cytochrome c_3 at 1.7 Å resolution, two very different heme groups can be defined in this protein with regard to the exposure of the propionates to the solvent: hemes H_2 and H_3 have their two propionates well exposed to the solvent, whereas for H_1 and H_4 the propionates are buried or slightly exposed (Czjzek et al., 1994). In this cytochrome, one heme has a redox potential which is much less negative than the others (Figure 6), and it has been demonstrated by single crystal EPR experiments (Guigliarelli et al., 1990) and confirmed by a ^{1}H NMR study

(Coutinho et al., 1993) that this heme is H_3. Thus, the relative exposure of the propionates of H_3 compared to that of H_1 and H_4 appears qualitatively consistent with the $\Delta S_{rc}^{\circ\prime}$ values reported in Table 3. Although the propionates of H_2 extend into the solvent, they are involved in intermolecular interactions participating to the crystal packing forces (Czjzek et al., 1994) and may adopt a different geometry in solution. Recent experiments have shown that the four macroscopic potentials of this cytochrome react differently with respect to ionic strength changes (G. Chottard, personal communication), and the study of the temperature dependence of this effect could help to clarify this point. The case of cytochrome c_3 from *D. vulgaris* Miyazaki is somewhat different. In this protein, the highest potential heme has been proposed to be H_4 on the basis of ^1H NMR experiments (Fan et al., 1990). Our measurements indicate that the highest potential heme is characterized by a very negative $\Delta S_{rc}^{\circ\prime}$ value (Table 3). It is interesting to note that according to the X-ray crystal structure of this cytochrome at 1.8 Å resolution, H_4 is one of the two hemes in which one propionate is well exposed to the solvent: hemes H_2 and H_3 have their two propionates engaged in hydrogen bonds with main chain or side chain atoms, while for H_1 and H_4 one propionate is well exposed and the other is rather buried (Higuchi et al., 1984). Taken together, the data concerning both cytochromes c_3 are consistent with the magnitude of the redox entropy change of a given heme being influenced by the exposure of its own propionates, but they also suggest that other contributions are involved. The origin of the large variations of $\Delta H^{\circ\prime}$ apparent in Figure 6 is also a difficult issue. All the hemes of tetraheme cytochromes c_3 have a bis-histidine axial coordination, and it might be expected that this characteristic would lead to a systematic lowering of $-\Delta H^{\circ\prime}$ with respect to cytochromes in which the axial coordination is ensured by a histidine and a methionine residue. In both cytochromes c_3, such a lowering is effectively observed for the three lowest potential hemes but not for the highest potential one (Figure 6). Recent site-directed mutagenesis experiments carried out on *Desulfovibrio vulgaris* Hildenborough cytochrome c_3 have shown that replacing the distal histidine by a methionine leads to a large increase of the redox potential (Dolla et al., 1991), and it would be especially interesting to measure the entropic and enthalpic components of these variations.

The difficulties encountered in analyzing the thermodynamic data obtained on cytochromes c_3 may be in great part related to their multiheme character. For example, concerning the $\Delta S_{rc}^{\circ\prime}$ components, the reduction of one heme might affect the solvent polarization around the propionates of other hemes, so that the comparison of the $\Delta S_{rc}^{\circ\prime}$ values of hemes belonging to the same molecule and the comparison with monoheme cytochromes would be difficult. Similarly, the large value of $-\Delta H^{\circ\prime}$ observed for the highest potential heme of both cytochromes c_3 may be due to the fact that, for this particular heme, the quantity $\Delta H^{\circ\prime}$ is measured in a redox state for which other hemes are positively charged, which destabilizes its oxidized form. As a matter of fact, it should be noted that although the redox potential e_1^4 of *D. vulgaris* Miyazaki cytochrome c_3 is nearly equal to e_2 of *D. desulfuricans* Norway, the thermodynamic parameters of the two highest potential hemes are very similar (Table 3). These complex phenomena should be manifested by the existence of interaction potentials. Although large values have been calculated for the interaction potentials in the tetraheme cytochrome of *R. viridis* (Gunner & Honig, 1991), only moderate values have been measured in cytochromes c_3. These moderate values might result from the compensation of large entropic and enthalpic components, whose determination would require the study of the temperature dependence of the interaction potentials. At this point, it is worth recalling that the data reported in Table 3 were obtained by assuming the redox interactions to be temperature independent. If a significant temperature dependence of the I_{ij} could be detected for these cytochromes, this would modify the $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ values of the three lowest potential hemes, but not of the highest potential one whose microscopic potential is well separated from the others.

CONCLUSIONS

The results obtained in the present study emphasize the importance of the entropic component of $\Delta G^{\circ\prime}$ and confirm the necessity of considering explicitely the electrostatic *free energy* in theoretical calculations carried out on cytochromes. These results support an important prediction made by the current theoretical models based on such calculations, namely the existence of large compensatory effects between the different contributions to $\Delta G^{\circ\prime}$. From the available experimental data, it appears that the variations of the $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ components as a function of $E^{\circ\prime}$ are not monotonic in the *c*-type cytochrome family and that the $E^{\circ\prime}$ value results from different contributions whose effects add or cancel, depending on the range of $E^{\circ\prime}$ that is considered. In the case of cytochromes with positive values of $E^{\circ\prime}$, the variations of $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ are correlated and $\Delta S^{\circ\prime}$ appears to be modulated by the exposure of the heme propionates to the solvent. Since the total heme accessibility of these cytochromes is largely determined by the exposure of their propionates, this finding explains the success of the correlation between the $E^{\circ\prime}$ value and the heme accessibility proposed many years ago (Stellwagen, 1978). At the present time, we think that a full understanding of the correlation between $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ apparent in Figure 4 constitutes a challenge for the current theoretical models which are based either on a microscopic (Churg & Warshel, 1986; Langen et al., 1992) or a macroscopic (Gunner & Honig, 1991; Zhou, 1994) description of the protein medium and of the solvent. It should be noted that some cytochromes do not follow this trend: the redox potential of cytochrome c_5 is about +320 mV, and its propionates are well exposed to the solvent (Carter et al., 1985). Conversely, although heme H_4 of the tetraheme cytochrome of *R. viridis* has a histidine/methionine ligation and its propionates are rather buried according to the crystal structure (Brookhaven Data Bank, entry 1PRC), it is characterized by a negative redox potential of about −60 mV (Nitschke & Rutherford, 1994). Thus, it would be especially valuable to determine the enthalpic and entropic components of $\Delta G^{\circ\prime}$ for these cytochromes.

The case of tetraheme cytochrome c_3 appears to be even more complex, since very large variations of $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ are observed among hemes belonging to the same molecule. The analysis of these systems is difficult due to the existence of redox interactions which make the redox properties of a given heme dependent on the redox state of the others. Thus, although the variations apparent in Figure 6 may partly originate from the specific surrounding of each heme, they

are also probably due to the heme−heme redox interactions (Gunner & Honig, 1991). Further work is then needed, regarding in particular the study of the temperature dependence of the interaction potentials, before the redox behavior of these complex systems is fully understood.

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