Medium and Temperature Effects on the Redox Chemistry of Cytochrome *c*

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Cytochromes *c* (cytc) are ubiquitous heme-containing metalloproteins that shuttle electrons in a variety of electron-transport chains, most often central to the production of the chemical energy necessary for cell life. The reduction potential ($E^{\circ\prime}$) of the $Fe^{3+/2+}$ couple is central to the physiological role of these species in that it influences the thermodynamic and kinetic features of electron-exchange reactions with redox partners. In the last two decades, voltammetric techniques exploiting the heterogeneous electron exchange between cytc and solid electrodes have proved to be particularly valuable for the determination of $E^{\circ\prime}$ values for these species and for characterizing the mechanistic and kinetic aspects of the redox process for the various cytc conformers under a variety of solution conditions. The understanding of how, and to what extent, different molecular factors control the $E^{\circ\prime}$ value in these species has been the subject of much debate. First coordination sphere effects on the heme iron and the interactions of the heme group with the surrounding polypeptide chain and the solvent are the main factors affecting $E^{\circ\prime}$ in cytc. These interactions are sensitive to medium effects such as the pH and the nature and ionic composition of the solvent. $E^{\circ\prime}$ is also strongly affected by the temperature. This article summarizes the authors' work on the effects on the selective stabilization of the two redox states of class I cytochromes *c* exerted by acid-base equilibria, general ionic strength effects, specific anion binding, the presence of non-aqueous solvents, and the temperature. The temperature dependence of $E^{\circ\prime}$ allows the determination of the enthalpy and entropy changes that accompany protein reduction. These parameters have proved to be informative with regard to the interplay between first coordination sphere effects and electrostatics at the heme–protein interface, including solvent dipoles, which mainly affect the reduction enthalpy, and solvent reorganization effects and differences in protein dynamics between the two oxidation states, which control the reduction entropy instead.

Introduction

Biological electron-transport (ET) chains in photosynthetic and aerobic and anaerobic respiratory processes are the main source of chemical energy in living systems.[1,2] Electron flow occurs among membrane-bound multi-com-

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Gianantonio Battistuzzi obtained his Ph.D. in Chemistry from the University of Modena (Italy) in 1996, under the supervision of Prof. M. Sola, working on the purification and the characterization of the solution behavior of electron-transfer metalloproteins. After a period in the laboratories of Professors H. Witzel and B. Krebs at the University of Münster, he held a postdoctoral position at the University of Modena, where he was appointed Assistant Professor of Inorganic Chemistry in 1999. His main research interest lies in the understanding of the molecular and solution factors influencing the thermodynamics of the reduction of electron-transfer metalloproteins, in particular cytochromes c and blue copper proteins, through a combination of electrochemical and spectroscopic techniques.

Marco Borsari obtained his Ph.D. in Chemistry from the University of Modena (Italy) in 1992, working with Prof. G. Gavioli on the characterization of the electrochemical behavior of sulfur-containing organic molecules and the determination of the nature and stability of metal complexes in solution through voltammetric and polarographic techniques. In 1992, he was appointed Assistant Professor of Physical Chemistry at the University of Modena and Reggio Emilia (Italy) and started working on the electrochemistry of metalloproteins with Marco Sola. He also established collaborations with the groups of I. Bertini and H. B. Gray. His main research activity has been directed towards the study of the redox properties of electron-transport proteins and redox enzymes, with a particular focus on the thermodynamics of the electron-transfer processes in cytochromes, blue copper, and iron–sulfur proteins.

Marco Sola obtained his Ph.D. in Chemistry from the University of Parma (Italy) in 1987. Between 1985 and 1991 he was a member of the group of I. Bertini and C. Luchinat in Florence working on NMR of paramagnetic metalloproteins. He spent 1988 as a research associate at the California Institute of Technology, Pasadena, CA, with H. B. Gray, working on iron–sulfur proteins involved in biological electron transport. From 1992, he independently pursued the characterization of the redox properties of electron-transport proteins, and with M. Borsari initiated the bio-electrochemistry group at the University of Modena. In 1992, he was also appointed Associate Professor of Chemistry at the University of Basilicata, Potenza, Italy. He then moved to the University of Bologna (1994–1997) and afterwards returned to the University of Modena, where he became Full Professor of General and Inorganic Chemistry in 2000. His main research interests have focused on structure–function relationships in redox metalloproteins and, more recently, on the issue of how the protein matrix and medium effects control the reduction potentials of cytochromes, blue copper, and iron–sulfur proteins.

MICROREVIEWS: This feature introduces the readers to the authors' research through a concise overview of the selected topic. Reference to important work from others in the field is included.

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ponent protein complexes that feature a variety of mono- or polynuclear metal centers (heme, type I and type II copper, and Fe-S) capable of switching between two stable oxidation states. Electrons are shuttled between these complexes by mobile carriers, which may be either organic molecules (e.g. the ubiquinone/ubihydroquinone system) or small metalloproteins such as cytochromes *c*, blue copper proteins, and iron-sulfur proteins.[1,2]

Cytochromes *c* (cytc) are ubiquitous species: their remarkable stability in vitro and ease of purification[2−5] made them the system of choice for an impressive amount of biophysical research on biological ET.[4,5] In their native form, these monomeric proteins contain one or more heme groups covalently bound to the polypeptide chain through two thioether bonds involving two cysteines; in addition, two residues (either His,Met or His,His) serve as axial ligands to the heme iron. The latter thus resides in a six-coordinate (distorted octahedral) geometry and has a typical low-spin state. Cytochromes *c* can be classified into four subclasses on the basis of their structural properties.[4] The most widely studied species belong to class I, found in both eukaryotes and prokaryotes, which are small positively charged globular proteins (M_r = 10−15 kDa) possessing a His,Met pair of axial heme iron ligands and are characterized by a largely preserved polypeptide folding. These species constitute the main focus of this article. The mitochondrial species shuttle electrons between the last two membrane-bound components of the electron-transfer chain of oxidative phosphorylation, namely ubiquinol-cytochrome *c* reductase and cytochrome *c* oxidase, whereas the species from photosynthetic bacteria, generally denoted as cytochromes c_2, act as electron donors to the photooxidized photosynthetic reaction center.[2−5]

Class III cytochromes *c* are multiheme proteins of bacterial origin featuring bis(histidine) coordination.[4−6] Classes II and IV include cytochromes *c*′, in which the heme iron is high-spin pentacoordinate with an axial histidine ligand, and bacterial tetraheme proteins containing either bis(His) or His,Met-coordinated heme.[4,5]

As for any other redox protein, the reduction potential ($E^{\circ\prime}$) is a key property of cytc. On the one hand, it regulates the biological function influencing both the thermodynamic and kinetic aspects of electron-exchange reactions with redox partners, as a result of which extensive work has been devoted to unravelling the complex interplay of molecular factors and medium effects that modulate it. On the other hand, $E^{\circ\prime}$ can be profitably exploited as a tool for the investigation of several aspects of the solution chemistry of cytc. In fact, the reduction potential of cytc is remarkably sensitive to any changes in the properties of the heme and the protein matrix, and can nowadays be measured with relative ease thanks to the development of direct electrochemical (voltammetric) techniques for redox proteins.

This article summarizes the authors' work on both the above aspects, which are closely related, focusing mainly on how the $E^{\circ\prime}$ of cytc is influenced by the ionic composition and pH of the aqueous medium, by the presence of organic solvents, and on temperature effects. The temperature dependence of $E^{\circ\prime}$ allows the determination of the enthalpy and entropy changes that accompany reduction of oxidized cytc: these parameters have proved to be informative with regard to understanding the molecular factors that control the reduction potential in these species. This aspect will also be covered in detail. No details are given here of the theoretical and experimental aspects of the voltammetric techniques or of the operational conditions that allow the attainment of fast heterogeneous electron transfer between the freely diffusing protein in solution and the solid electrode, for which the reader is referred to the original literature.[7−11] It is worth mentioning, however, that under the conditions of variable pH, temperature, and solvent composition employed in these studies, the electrochemical processes involving cytc at surface-modified gold electrodes were invariably found to be one-electron, electrochemically reversible or quasi-reversible, and diffusion-controlled (as an example, a typical cyclic voltammogram of freely diffusing native cytochrome *c* obtained on a 4-mercaptopyridine surface modified gold electrode at pH = 7 is shown in Figure 1, a). These conditions warrant that the electron transfer between the protein and the electrode is fast and that no protein adsorption and denaturation occurs at the electrode surface. Therefore, the half-wave potentials obtained from the voltammograms can be confidently assumed to represent the thermodynamic $E^{\circ\prime}$ values.

I. The Reduction Potential of the Native Form of Class I Cytochromes *c*

The native His,Met-ligated form (state III) of class I cytochromes *c* from mitochondrial and bacterial sources has reduction potentials in the range of +0.2 to +0.38 V (vs. SHE) (all the potential values reported herein are referred to the standard hydrogen electrode, SHE).[4,12] Two main factors have been recognized as contributing to these remarkably positive $E^{\circ\prime}$ values. One is the π-electron accepting character of the thioether sulfur atom of the methionine axially bound to the iron, which stabilizes the reduced (Fe^{2+}) state.[12−14] The other is the poor accessibility of the solvent to the heme and its burial within a hydrophobic pocket, which induce a further significant enthalpic stabilization of the ferrous over the ferric state.[12] Against this background, electrostatic interactions of the charge at the redox center with net charges and polar groups on the protein and solvent dipoles are responsible for the fine-tuning of $E^{\circ\prime}$.[4,5,15−18] Theoretical and experimental approaches have been directed towards an understanding of how and to what extent these interactions modulate $E^{\circ\prime}$ in cytochromes *c*.[4,5] Models for the calculation of the electrostatic interaction energies of the heme with polar and charged residues and solvent dipoles were able to reproduce the differences in $E^{\circ\prime}$ among cytc from different sources.[18−20] Evidence has been obtained from experiments involving chemical modification, site-directed mutagenesis, and specific anion binding[4,21−25] that modification of internal net charges, such as those of the heme propionates, induces variations in $E^{\circ\prime}$ (50−65 mV per charge) that

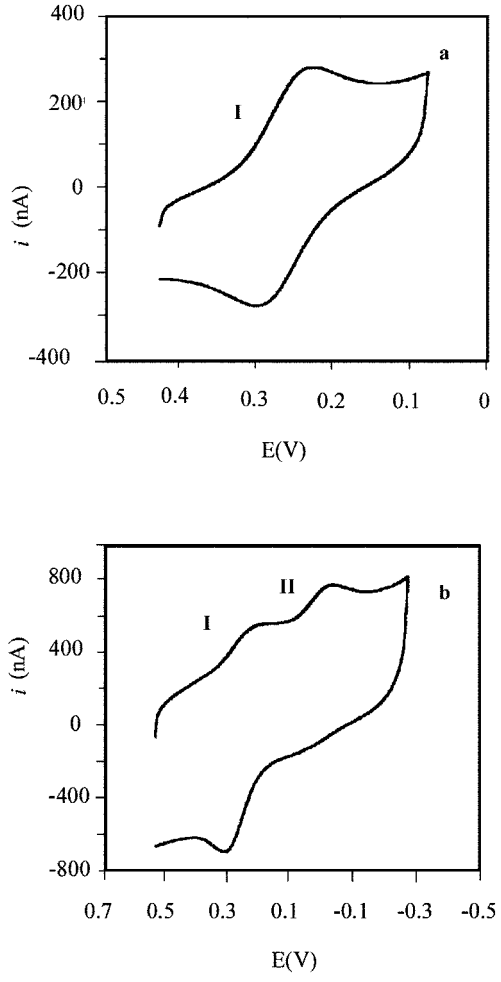

Figure 1. Cyclic voltammograms of beef heart cytochrome *c* at a 4-mercaptopyridine surface modified gold disc electrode: (a) pH = 7, $T = 293$ K, sweep rate 50 mV s^{-1}, protein concentration 0.17 mM; (b) pH = 9.13, $T = 293$ K, sweep rate 50 mV s^{-1}, protein concentration 0.25 mM; I and II refer to the waves of the native and alkaline conformers, respectively; supporting electrolyte 0.1 M sodium acetate (reproduced by permission from G. Battistuzzi et al., *Biochemistry* **1999**, *38*, 7900−7907; copyright American Chemical Society, 1999)

are more pronounced than those due to surface charges (10−30 mV per charge). The overall effect depends on several factors, such as the heme−charge distance and the dielectric properties of the intervening medium, which can be described by an "effective dielectric constant"; the latter is different for each particular interaction and involves contributions from both the solvent and the protein.[4,16,21−23,25]

Our recent work on the thermodynamics of cytc reduction, carried out by means of variable-temperature $E^{\circ\prime}$ measurements (see Section II), has elucidated the role of entropic effects in determining the reduction potential. Reduction entropy is likely to be largely controlled by differences in dynamic and solvation properties between the two redox states, although no detailed understanding of the factors involved at a molecular level has yet been achieved. However, it is clear that this thermodynamic contribution strongly affects $E^{\circ\prime}$[12] and may contribute to the differences in $E^{\circ\prime}$ between cytc from different sources, as found for other classes of electron-transport metalloproteins.[26,27]

A wealth of data is now available on the way in which the pH, temperature, ionic composition, and dielectric constant of the solvent influence the electrostatic interactions at the heme−protein/solvent interface, axial coordination to the heme iron, and the dynamic and solvation properties of both redox forms, and hence how they ultimately affect $E^{\circ\prime}$. These effects, which constitute the focus of this review, indicate that the properties of the medium exert a critical influence on the redox behavior of cytochrome *c* in vitro and, in principle, may also affect its function in vivo.

II. Temperature Dependence of $E^{\circ\prime}$: Reduction Thermodynamics

The redox behavior of class I cytochromes *c* is temperature-sensitive. The reduction potential of the conformers for which the voltammetric signal due to the Fe^{3+}/Fe^{2+} couple falls in the potential range accessible in diffusion-controlled direct electrochemistry experiments at surface-modified gold or graphite electrodes (from +1.3 to −0.60 V), namely the native His,Met-ligated form (state III) and the alkaline His,Lys-ligated form (state IV), invariably decreases with increasing temperature between 5 and 65 °C. However, the $E^{\circ\prime}/T$ profiles are pH-dependent and differ slightly for species from different sources.[12,28−37] In particular, the $E^{\circ\prime}$ of the native form of cytc from mammalian mitochondria shows a monotonic linear decrease with increasing temperature at slightly acidic and neutral pH values (Figure 2, a), whereas at higher pH values the $E^{\circ\prime}$ vs. T plot clearly becomes biphasic with a transition point at approximately 50 °C (Figure 2, b). This behavior has been ascribed to a reversible conformational transition between two states of the oxidized native form involving fractional proton loss on going from the low-T to the high-T conformer,[33] which is indeed consistent with the decrease in the transition point temperature observed with increasing pH.[12] Spectroscopic studies indicate that the low-T and high-T native conformers are not significantly different in the surroundings of the heme.[12] An additional effect of increasing temperature is a depression of the pK_a for the alkaline transition (see Section III.2).

The $E^{\circ\prime}/T$ profiles for the native form of class I cytc from plant mitochondria and photosynthetic bacteria are similar to those outlined above, but show some interesting differences (Figure 3).[12,28] In particular: (i) the transition between the low-T and high-T native forms occurs at lower pH values and lower temperatures (35−40 °C), (ii) the temperature of the transition point decreases with increasing pH, (iii) the T-induced decrease in the pK_a for the alkaline transition is more marked as compared to that of their mammalian analogues (for these species, formation of the alkaline form can be observed at pH values as low as 7−7.5 at temperatures of approximately 35−40 °C).[12,28]

The temperature dependence of $E^{\circ\prime}$ can be profitably exploited to measure the thermodynamic parameters of cytc

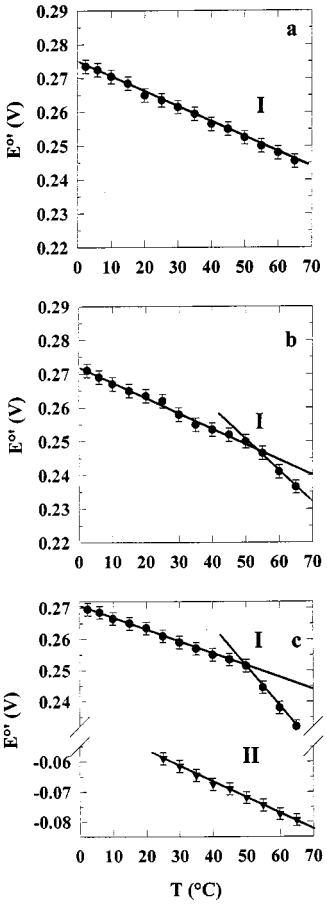

Figure 2. Temperature dependence of the reduction potential of beef heart cytochrome *c* at pH = 6.9 (a), 7.5 (b), and 8.3 (c); supporting electrolyte 0.1 M sodium chloride; solid lines are least-squares fits to the data points; I and II refer to the waves of the native and alkaline conformers, respectively (reproduced by permission from G. Battistuzzi et al., *Biochemistry* **1997**, *36*, 16247−16258; copyright American Chemical Society, 1997)

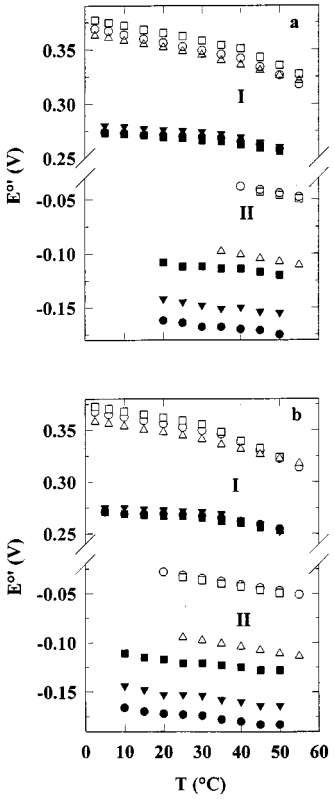

Figure 3. Temperature dependence of the reduction potentials of bacterial and plant cytochromes *c*: (a) pH = 7.5 (plant), 7 (bacterial); (b) pH = 8.5;[12,28] plant cytochromes *c*: spinach (■), cucumber (●), sweet potato (▲); bacterial cytochromes c_2: *Rps. palustris* (□), *Rb. sphaeroides* (○), *Rb. capsulatus* (Δ); supporting electrolyte 0.1 M NaCl in 10 mM phosphate buffer; least-squares fits to the data points are omitted for clarity; I and II refer to the reduction potentials of wave I (native cytc) and wave II (alkaline cytc), respectively

reduction. In particular, the use of a so-called "non-isothermal" electrochemical cell, in which the reference electrode (SCE) is kept at constant temperature while the half-cell containing the working electrode and the junction to the reference electrode is under thermostatic control,[38] allows determination of the standard entropy ($\Delta S^{\circ\prime}_{rc}$) and enthalpy ($\Delta H^{\circ\prime}_{rc}$) changes associated with reduction of the oxidized protein.[12,28,30−37] In particular, with this experimental configuration, the reaction entropy for reduction of the oxidized form is given by the following Equation.[34,38−40]

$$\Delta S^{\circ\prime}_{rc} = S^{\circ\prime}_{red} - S^{\circ\prime}_{ox} = nF\,(dE^{\circ\prime}/dT)$$

Thus, $\Delta S^{\circ\prime}_{rc}$ can be determined from the slope of the plot of $E^{\circ\prime}$ versus temperature, which turns out to be linear under the hypothesis that $\Delta S^{\circ\prime}_{rc}$ is constant over the limited temperature range investigated. With the same assumption, the enthalpy change ($\Delta H^{\circ\prime}_{rc}$) can be obtained from the Gibbs−Helmholtz equation, namely from the slope of the $E^{\circ\prime}/T$ versus $1/T$ plot.

Analysis of the thermodynamic parameters of protein reduction for several mitochondrial and bacterial class I cytochromes *c* (Table 1) allowed further insight to be gained into the interplay between the enthalpic and entropic contributions to the reduction potential (see ref.[12] for an overview on the available thermodynamic data for cytc reduction determined by several authors using a variety of techniques under different solution conditions). It can clearly be seen that the high reduction potentials of these species are largely enthalpic in origin, arising from the remarkably negative $\Delta H^{\circ\prime}_{rc}$ values, which can confidently be ascribed to the stabilization of the ferroheme by ligand binding interactions and the hydrophobicity of the heme environment.[12,28−30] Conversely, the entropic term invariably disfavors protein reduction ($\Delta S^{\circ\prime}_{rc} < 0$),[12,32] yielding a negative contribution to the $E^{\circ\prime}$ values. The entropic term is generally smaller than the enthalpic term, but it nevertheless plays an important role in the modulation of the reduction potential in these species.[12,28−30] Solvent reorganization effects and changes in protein flexibility, the latter expressed as the sum of the conformational (vibrational, torsional) degrees of freedom, associated with reduction of

cytc play a major role in determining the $\Delta S^{\circ\prime}_{rc}$ values.[3,12,28–31] In particular, the entropy loss upon reduction is consistent with the decreased flexibility of the reduced state as compared to the ferri form determined by NMR in solution.[41–45] However, it is still the case that the contribution of the reduction-induced changes in the H-bonding network within the hydration sphere of the molecule remains largely uncharacterized.

Table 1. Reduction potentials for the native and alkaline forms of class I cytochromes *c*, and $\Delta H^{\circ\prime}_{rc}$ [kJ·mol^{-1}] and $\Delta S^{\circ\prime}_{rc}$ [J·mol^{-1}·K^{-1}] values determined from the temperature dependence of the reduction potential (from refs.[12,28])

cyt *c*	pH	$E^{\circ\prime}_{N}$[a]	$E^{\circ\prime}_{A}$[a]	$\Delta S^{\circ\prime}_{rc}$[b] native	$\Delta S^{\circ\prime}_{rc}$[b] alkaline	$\Delta H^{\circ\prime}_{rc}$[b] native	$\Delta H^{\circ\prime}_{rc}$[b] alkaline
beef heart	6.9	+0.263		−44		−38	
	7.5	+0.262		−43		−38	
				−96		−55	
	8.3	+0.261	−0.075[c]	−37	−50	−36	−9
				−125		−65	
spinach	7.0	+0.268		−29		−35	
				−58		−43	
	7.5	+0.268	−0.112	−27	−33	−34	+1
				−58		−43	
	8.4	+0.267	−0.121	−20	−40	−32	0
				−58		−43	
cucumber	7.0	+0.271		−23		−33	
				−58		−44	
	7.5	+0.270	−0.164	−19	−38	−32	+4
				−64		−46	
	8.5	+0.268	−0.173	−13	−41	−30	+4
				−64		−46	
sweet potato	7.0	+0.274		−31		−36	
				−77		−50	
	7.6	+0.274	−0.145	−24	−41	−34	+2
				−79		−51	
	8.4	+0.272	−0.153	−17	−47	−31	+1
				−106		−59	
cyt c_2							
Rps. palustris	7	+0.362		−67	−62	−55	−14
				−151		−81	
	8.5	+0.359	−0.056	−60	−62	−52	−13
				−152		−80	
Rb. sphaeroides	7	+0.354		−69	−63	−55	−13
				−152		−81	
	9	+0.353	−0.054	−63	−61	−53	−13
				−154		−82	
Rb. capsulatus	7	+0.347		−66	−60	−52	−9
				−94		−61	
	9	+0.345	−0.095	−58	−61	−50	−9
				−89		−60	

[a] Subscripts N and A denote native and alkaline forms, respectively. – [b] At 25 °C. Measurements were performed in 10 mM phosphate buffer, 0.1 M NaCl. Values in the upper and lower rows for the native species refer to the low-T (N_1) and high-T (N_2) conformers, respectively. Average errors in the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values are ±2 kJ·mol^{-1} and ±6 J·mol^{-1}·K^{-1}, respectively. – [c] $E_{1/2}$ value at 55 °C for an electrochemically quasi-reversible wave (ΔE_p = 0.07 V).

A similar balance between the enthalpic and entropic contributions to the $E^{\circ\prime}$ value of cytc has been observed for all the other classes of high-potential electron-transport metalloproteins, namely blue copper proteins and high-potential iron–sulfur proteins (HiPIPs).[26,27,46,47] Therefore, it is apparent that Nature uses the same strategy to control the reduction potential of these species; the reasons for this are still unclear and define a fascinating field for future investigations.

The alkaline conformer(s) of cytc are characterized by lower (negative) $E^{\circ\prime}$ values as compared to the native conformers (Table 1). This decrease in $E^{\circ\prime}$ is almost entirely enthalpic in origin, since the $\Delta S^{\circ\prime}_{rc}$ values for this form are found to be comparable to those for the native form, whereas the $\Delta H^{\circ\prime}_{rc}$ values are much less negative (Table 1) (see Section III.2.a for details).[12,48]

III. Acid-Base Equilibria

Acid-base equilibria involving ionizable groups such as axial ligands, heme propionates, and distal residues influence the electronic properties of the heme, axial heme ligation, and polypeptide folding. Therefore, the reduction potential of class I cytc is remarkably pH-dependent.[4,5,28,49,50]

III.1. Equilibria Occurring Between Acidic and Neutral pH Values

The native His,Met-ligated form (state III) transforms into a low-pH conformer (state II) with an apparent pK_a of 2.5. In this conformer, the axial ligands are protonated and are detached from the iron (which indeed becomes high-spin): the free coordination positions are most likely occupied by water molecules.[4,5] The $E^{\circ\prime}$ of this form cannot be determined owing to the worsening of the electrochemical response at low pH caused by protonation of the promoter adsorbed onto the electrode surface and the probable disruption of the adsorbed layer.[49]

At intermediate pH values (approximately from pH = 5 to 8), the pH dependence of the reduction potential of native cytc is species-dependent. In particular, no acid/base equilibria affect the redox properties of mitochondrial (mammalian and plant) cytc in this pH range. In fact, His26 (present in all eukaryotic cytc) and the heme propionate-7 are characterized by low pK_a values. In particular, the former residue, which controls the accessibility of the solvent to the hydrophobic core of cytc influencing the global protein stability and that of the ligating Met80,[51] has a pK_a value lower than 3.6 in horse heart cytc, probably due to its non-polar environment,[4,51,52] which is maintained also in the plant proteins.[53] The pK_a of the heme propionate-7 in oxidized mammalian cytochromes *c* is lower than 4.5.[4,50] In contrast, the $E^{\circ\prime}$ values of some bacterial cytochromes c_2 have been found to be sensitive to the pro-

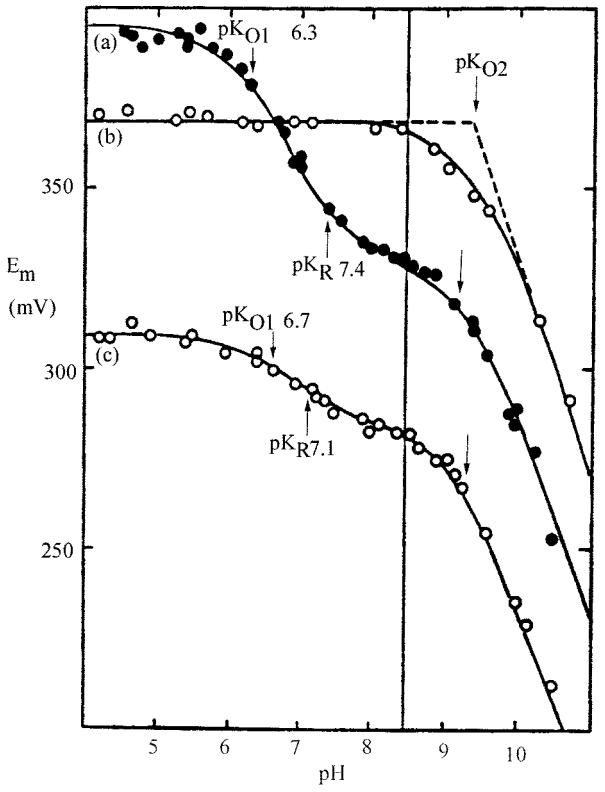

Figure 4. pH dependence of the reduction potentials of cytochromes c_2 from: (a) *Rm. vannielii*, (b) *Rps. viridis*, (c) *Rb. capsulatus* (reproduced by permission from G. R. Moore, G. W. Pettigrew *Cytochromes c: Evolutionary, Structural and Physicochemical aspects*, Springer-Verlag, Berlin, **1990**)

tonation state of a non-ligated histidine residue[4,16,22,50,54] or heme propionate, the pK_a of which falls in the range 6−8 (Figure 4).[4,50] The deprotonation of an ionizable residue induces the typical sigmoidal decrease in $E°'$ because of the electrostatic stabilization of the oxidized form due to the decrease of the overall positive charge of the protein: the extent of the change in $E°'$ depends on the solvation properties of the residue, the distance from the heme, and the properties of the intervening medium.

III.2. The Alkaline Transition

The moderate affinity of the axial methionine sulfur for the ferric ion in the native form of cytochrome *c* renders this residue susceptible to substitution by endogenous ligand(s) at high pH, leading to the "alkaline" isomer(s) (state IV), in which the iron retains the low-spin ferric state.[4,5,12,28−30,48,49,55−60] The spectroscopic and redox changes induced by this equilibrium have been exploited to determine the thermodynamics and kinetics of the isomerization, to identify the nature of the substituting ligands, and to determine the role of the residues neighboring the heme in the relative stabilization of the two conformers. The overall reaction turns out to be a two-step process, in which a fast residue deprotonation triggers a slow and thermodynamically favored conformational change leading to the replacement of the methionine by a surface lysine in axial heme ligation, with an apparent pK_a in the range 8.5−9.5 depending on the species.[4,5] The ionizable residue has not yet been unambiguously identified, and no information is available on the structural features of the alkaline conformers. The existence of two alkaline isomers in a pH-dependent ratio suggests that at least two Lys residues can replace the methionine at high pH.[28,30,48,56−59]

The alkaline conformers of cytc give rise to a single voltammetric wave (wave II) (Figure 1, b), which is diffusion-controlled and chemically irreversible at the potential sweep rates employed for detection of the voltammetric wave for native cytc (wave I) (20−200 mVs^{-1}). The reduction potentials of the alkaline isomers of various cytc's are lower than those of the native forms by 0.3−0.5 V (falling in the range −0.05 to −0.18 V) (Figure 2, c, and Figure 3).[12,28,29,48,49,55] It has been found that this remarkable decrease in the reduction potential is mainly due to the different coordination properties of methionine and lysine: the latter is a stronger donor ligand that selectively stabilizes the ferric form.[12] The chemical irreversibility of wave II is due to the fact that the reduced alkaline conformer is unstable and tends to transform rapidly into the reduced native form through the uptake of a proton. With increasing sweep rate, the reduced alkaline isomer has no time to transform prior to re-oxidation, and indeed the intensity of the anodic counterpart of wave II becomes comparable to that of the cathodic peak using scan rates higher than 0.6 V s^{-1} (for beef heart cytc).[12]

Upon increasing the pH in the range 7.5−10.5, the cathodic peak current of wave II increases to the detriment of that of native cytc, while the sum of the two currents remains constant. Moreover, at each pH value, the cathodic peak currents of both waves (as well as their $E°'$ values) are independent of the potential sweep rate in the range 0.02−1 Vs^{-1}.[48] Individual peak intensities simply depend on the root square of the scan rate, as expected for diffusion-controlled processes.[48] Thus, waves I and II turn out not to be "kinetic waves" (in the range of potential sweep rates used) and, at each pH value, their currents are those determined by the chemical equilibrium between the native and alkaline forms.[48,61] Therefore, the measured currents can be used to determine the apparent equilibrium constants. The current intensity (*i*) of the cathodic peak of wave I was measured as a function of pH at different temperatures from 5 to 65 °C.[48] The fit of the sigmoidal decrease of *i* with increasing pH to a one-proton equilibrium equation yields the apparent pK value (pK_{app}) for the alkaline transition at a given temperature (Figure 5). This value was found to decrease from 9.8 to 8.6 on increasing the temperature from 5 to 65 °C at 0.1 M ionic strength (acetate).[48] The latter effect is a direct consequence of the endothermicity of the overall process of the alkaline isomerization (see Section III.2.a).

It is worthy of note that the alkaline transition is also greatly influenced by the properties of the medium. The pK_{app} decreases with decreasing dielectric constant of the medium, as noted in mixed water/dimethyl sulfoxide solutions (see Section V).[29] This effect is of potential relevance

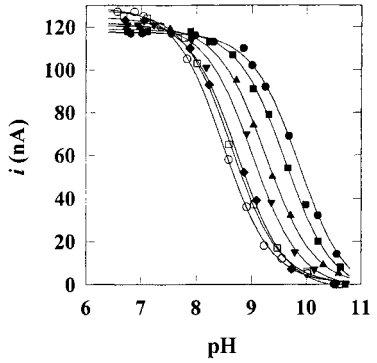

Figure 5. pH dependence of the current intensity of the cathodic peak (*i*) of wave I for beef heart cytochrome *c* at different temperatures in sodium acetate at I = 0.1 M; T [°C] = 5 (●), 10 (■), 20 (▲), 30 (▼), 40 (◆), 50 (□), and 60 (○); the pH values were corrected for temperature; solid lines are fits to a conventional one-proton equilibrium equation (reproduced by permission from G. Battistuzzi et al., *Biochemistry* **1999**, *38*, 7900−7907; copyright American Chemical Society, 1999).

from a physiological point of view since it has been reported that there is a decrease in the dielectric constant near the surface of phospholipid membranes and that the alkaline form of cytochrome *c* is involved in the interaction with synthetic anion phospholipid vesicles.[62,63] Interestingly, monoclonal antibody binding studies indicate that only the native form is present in live cells, where cytochrome *c* acts as an electron shuttle near the inner mitochondrial membrane, whereas the presence of the alkaline isomers has been detected in apoptotic and necrotic cells.[64] The alkaline isomers are formed in the early stages of apoptosis and necrosis, possibly as a result of the translocation of cytochrome *c* through the mitochondrial membrane (or of its association with membranes altered during the process of death). It is therefore possible that the phospholipid-induced conformational changes associated with the formation of the alkaline isomers are connected with the signaling role of cytochrome *c* during apoptosis, although experimental evidence seems to indicate that these isomers are not involved in the interaction with Apaf-1 to form apoptosomes.[63,64]

The pK_{app} for the alkaline transition also increases with increasing ionic strength (see Section III.2.a).[48] This is likely to be one of the factors that hampers separation of the alkaline form in the crystal state from the usually high ionic strength solutions (ammonium sulfate) used in the crystallization procedure.

Upon increasing the pH beyond 10, another state (state V) becomes accessible, in which a hydroxide ion is probably axially bound to the heme iron.[56] The remarkably low reduction potential of this species is outside the potential window accessible with the present techniques.

III.2.a The Thermodynamics of the Alkaline Transition

The thermodynamic parameters of the alkaline transition have been investigated in some detail.[48,65−67] We have measured the apparent equilibrium constant (K_{app}) of the alkaline transition (AT) of beef heart cytochrome *c* by means of pH titrations of the current intensities in cyclic voltammetry experiments. These were carried out at different temperatures (from 5 to 65 °C) and at different ionic strengths (I = 0.01−0.2 M).[48] The ionic strength of the solution was varied using acetate, which has been found not to bind specifically to beef (and horse) heart cytc, but rather to simply exert an ionic atmosphere effect (see Section IV).[23]

The temperature profile of the pK_{app} values was found to be biphasic (Figure 6). Two distinct sets of $\Delta H^{\circ\prime}_{AT}$ and $\Delta S^{\circ\prime}_{AT}$ values were obtained below and above approximately 40 °C (Table 2). In the low-temperature range, the process is endothermic and is accompanied by a small positive entropy change, whereas at higher temperatures it becomes less endothermic and involves a moderate entropy loss. Ligand substitution should not be responsible for the endothermicity of the process, given the higher affinity of the lysine nitrogen atom for the ferric ion as compared to the methionine sulfur atom. Therefore, other factors, such as transition-induced changes in the hydrogen-bond network (also including ordered water molecules on the protein surface) and in the electrostatic interactions between the metal site and the protein environment and the solvent must be responsible for the positive $\Delta H^{\circ\prime}_{AT}$ values. The slightly positive or negative $\Delta S^{\circ\prime}_{AT}$ values cannot be easily rationalized. In fact, this isomerization has been proposed to involve a number of processes, which would increase the number of degrees of freedom of the molecule, such as the net release of a proton, the formation of multiple conformers,[56−59] the opening of the heme crevice through the disruption of two key stabilizing interactions on the protein surface,[67] and the probable disruption of a hydrogen-bond network involving the internal water molecule and protein residues due to the detachment of Met80.[68] Therefore, other opposing (structure-making) processes must be operative, which most probably involve a rearrangement of the solvation sphere of the protein (in a way

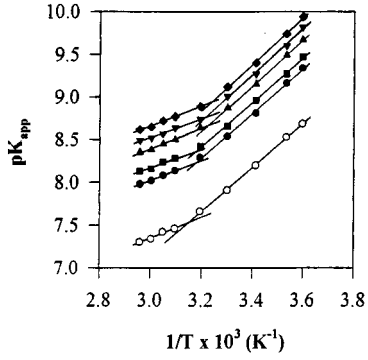

Figure 6. Apparent pK values for the alkaline transition of beef heart cytochrome *c* as a function of 1/T at various ionic strengths (sodium acetate); I = 0.01 M (●), 0.02 M (■), 0.05 M (▲), 0.1 M (▼), and 0.2 M (◆); the pK values extrapolated to I = 0 (○) are true thermodynamic equilibrium constants; solid lines are least-squares fits to the data points (reproduced by permission from G. Battistuzzi et al., *Biochemistry* **1999**, *38*, 7900−7907; copyright American Chemical Society, 1999)

Table 2. Thermodynamic parameters for the alkaline transition (AT) of beef heart ferricytochrome *c* at various ionic strengths obtained from the pK_{app} vs. $1/T$ plots of Figure 6 (from ref.[48])

I [M][a]	$f(I)$	$\Delta H^{\circ\prime}_{AT}$ [kJ·mol^{-1}][b] low-T[c]	high-T	$\Delta S^{\circ\prime}_{AT}$ [J·mol^{-1}·K^{-1}][b] low-T	high-T
0[d]	0	+49	+23	+12	−70
0.01	0.063	+52	+23	+8	−86
0.02	0.076	+52	+22	+5	−90
0.05	0.096	+52	+24	+1	−88
0.1	0.109	+52	+21	−1	−100
0.2	0.12	+53	+22	0	−98

[a] Sodium acetate. − [b] Average errors in the $\Delta H^{\circ\prime}_{AT}$ and $\Delta S^{\circ\prime}_{AT}$ values are ±2 kJ·mol^{-1} and ±6 J·mol^{-1}·K^{-1}, respectively. − [c] Enthalpy and entropy values in the low-T and high-T ranges refer to the N_1 and N_2 conformers, respectively (see text). − [d] Data obtained from the pK_a values extrapolated at $f(I) = 0$ at various temperatures (see Figure 6). These values are true standard thermodynamic parameters.

which is presently unknown, but that might involve formation of internally hydrogen-bonded ordered molecular arrays within the hydrophobic heme crevice or a net release of water molecules from the hydration sphere of the protein to the bulk solvent) and/or a suppression of vibrational, rotational, and torsional motions in the A form as compared to the N form. However, no clear definition of the molecular details of the processes underlying the entropy change is yet available.

The change in transition thermodynamics observed above approximately 40 °C is most likely the result of the thermal transition of native ferricytochrome *c* from a low-T to a high-T conformer, which occurs at alkaline pH values at a comparable temperature (see Section II). Thus, it is apparent that the transitions of the two native conformers to the corresponding alkaline forms are thermodynamically distinct processes. It is suggested that this difference either arises from peculiar transition-induced changes in the hydration sphere of the protein or is due to the preferential binding of different lysines to the heme iron in the two temperature ranges. Extrapolation of the K_{app} values at zero ionic strength allowed determination of the thermodynamic equilibrium constants (K_a) at each temperature, and hence of the "true" standard thermodynamic parameters of the transition. The pK_a value at 25 °C was found to be 8.0. A pK_{app} value of 14.4 was calculated for the alkaline transition of ferrocytochrome *c* at 25 °C and I = 0.1 M. The much greater relative stabilization of the native state in the reduced as compared to the oxidized form turns out to be almost entirely enthalpic in origin, and is most likely due to the greater affinity of the methionine sulfur for the Fe^{II} ion.[48]

The pK_{app} values for the alkaline transition of cytc are influenced by the ionic strength in a fashion that can be explained, at least qualitatively, by the finite-ion version of the Debye−Hückel theory,[48] analogously to the ionic strength dependence of the reduction potential (see Section IV). In particular, the pK_{app} values measured at different ionic strengths were found to conform to Equation (1).

$$pK_{app} = pK_a + 0.5(z^2_{cytH} - z^2_{cyt^-}) \cdot \frac{\sqrt{I}}{1+6\sqrt{I}} = pK_a + 0.5(z^2_{cytH} - z^2_{cyt^-}) \cdot f(I) \quad (1)$$

Here, z_{cytH} and z_{cyt^-} are the net charges of the neutral and alkaline forms, respectively (Figure 7).[4,69] The slope of the plots (approximately +10) is almost twice that expected from the net charges of the native and alkaline forms, namely $0.5\cdot(6^2 - 5^2) = +5.5$. This difference is most likely due to the intrinsic limits of the Debye−Hückel theory as applied to a complex electrolyte such as a protein (see below), although it might also be due to the specific binding of one acetate ion to a surface site of the alkaline form. In fact, although the acetate ion was shown not to interact specifically with neutral cytc, nothing is known about its interaction with the alkaline conformer.

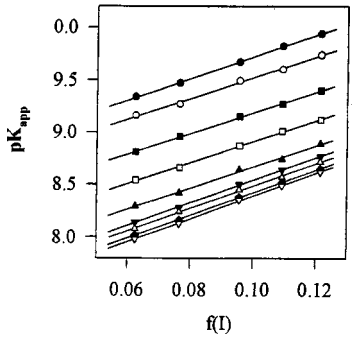

Figure 7. Apparent pK values for the alkaline transition of beef heart cytochrome *c* as a function of $f(I) = \sqrt{I}/(1 + 6\sqrt{I})$ at various temperatures; T [°C] = 5 (●), 10 (○), 20 (■), 30 (□), 40 (▲), 50 (▼), 55 (Δ), 60 (◆), 65 (▽); solid lines are least-squares fits to the data points (reproduced by permission from G. Battistuzzi et al., *Biochemistry* **1999**, *38*, 7900−7907; copyright American Chemical Society, 1999)

Comparison of the $\Delta H^{\circ\prime}_{AT}$ and $\Delta S^{\circ\prime}_{AT}$ values at different ionic strengths (Table 2) indicates that the increase in the pK_{app} values with increasing ionic strength is almost entirely entropic in origin. Thus, the expected selective stabilization of the native conformer (which has one more positive charge than the alkaline form) by the negative ionic atmosphere that surrounds the protein has little or no effect. This result cannot be readily rationalized at present owing to the uncertainty regarding the factors responsible for the transition entropy.

IV. The Effects of the Ionic Composition of the (Aqueous) Medium on $E^{\circ\prime}$

The ionic composition of the medium is known to affect the interaction of cytc with redox partners[70] and phospholipid membranes.[71] Salt-induced effects on the reduction potential of cytc are of two types: (i) changes in the activity coefficients of both redox states, known as "general" ionic strength effects; (ii) specific protein−anion interactions at surface sites (cytc is positively charged at neutral pH). The former effect is related to the shielding of the protein charge

by the surrounding "ionic atmosphere", which is greater for the oxidized state since it bears the greater positive charge. As a consequence, the oxidized state will be selectively stabilized with respect to the reduced state, leading to a decrease in the reduction potential with increasing ionic strength (see Section IV.2). A combination of the Nernst and extended Debye−Hückel equations, using a model in which the protein behaves as a low dielectric cavity bearing a net charge uniformly distributed on the surface, with no specific ion−protein interactions, yields Equation (2) for the changes in $E^{\circ\prime}$ of cytc induced by the ionic atmosphere.[4,72,73]

$$E^0 = E^0_{I=0} + 2.303 \cdot \frac{RT}{nF} \cdot \left[z^2_{red} - z^2_{ox}\right] \cdot \frac{0.5 \cdot \sqrt{I}}{1 + 0.33 \cdot a \cdot \sqrt{I}} \quad (2)$$

Here, I is the ionic strength, $E^\circ_{I=0}$ is the reduction potential extrapolated to zero ionic strength, z_{red} and z_{ox} are the charges of the reduced and oxidized protein, and a is the "ion-size parameter", defined as the mean distance of closest approach between the protein and an ion of opposite charge belonging to the ionic atmosphere. The value of a lies between the sum of the crystallographic radii and the sum of the solvated radii. An a value of 18 Å has been used for bovine ferricytochrome *c*. By defining Equation (3), Equation (2) becomes Equation (4) which predicts a linear decrease in E° with increasing $f(I)$, with a slope given by $0.059 \cdot (z^2_{red} - z^2_{ox})$.

$$f(I) = (0.5\sqrt{I})/(1 + 0.33a\sqrt{I}) \quad (3)$$

$$E^\circ = E^\circ_{I=0} + 0.059(z^2_{red} - z^2_{ox})f(I) \quad (4)$$

Despite the roughness of the model, especially regarding the dielectric properties of the polypeptide matrix, this approach proved to be surprisingly effective in describing the ionic strength dependence of $E^{\circ\prime}$ for native and chemically modified class I cytochromes *c*.[21−23,69,72−74] This might be ascribed, at least in part, to the facts that these species are roughly spherical, that there are only subtle oxidation state induced structural changes[42,46,75] (which fits with an implicit assumption of the model), and that the metal center is almost totally embedded in the protein matrix. Indeed, we found that the Debye−Hückel theory works less well for the cucumber basic protein (which possesses a type I copper center), in which the metal site is more exposed to the solvent (hence the dielectric constant of the metal environment can no longer be regarded as low and homogeneous) and which shows more pronounced oxidation state induced changes in solvation properties and a stronger asymmetry in charge distribution as compared to cytochrome *c*.[76,77]

Specific binding of anions to surface sites of cytc (intended as more or less localized surface regions of positively charged potential),[78] occurring either on one or both redox states, would result in a decrease in the net positive protein charge, related to the charge of the anion and the number of protein-bound anions. This would alter the slope of the $E^{\circ\prime}/f(I)$ profile as compared to the free protein. Therefore, the above equations were exploited to detect specific anion−cytc interactions and to determine the stoichiometry of binding for a wide variety of anions either occurring in biological systems in vivo (such as Cl^-, HPO_4^{2-}, HCO_3^-), or used as components of buffer systems in vitro (NO_3^-, SO_4^{2-}, citrate^{3-}, oxalate^{2-}).[21−23] Several anions were found to possess peculiar binding properties,[78,79] often including sequential binding due to the presence of multiple protein binding sites with different affinities for the anion.[21−23] In parallel, these studies allowed evaluation of the electrostatic effect on $E^{\circ\prime}$ resulting from anion-induced neutralization of net positive protein surface charge(s). In the following we will briefly illustrate some details of the binding properties of biologically important anions and the relationships between surface charges and $E^{\circ\prime}$ which were obtained with this approach.

IV.1. Specific Anion Binding Effects

IV.1.a. Anion−Protein Binding Stoichiometries

Some examples of the $E^{\circ\prime}$ vs. $f(I)$ plots obtained for bovine and horse heart cytochromes *c* interacting with different anions are shown in Figure 8. Linear plots are invariably obtained, according to Equation (4), showing either a constant slope or biphasic behavior. The latter plots, which yield two sets of protein charges at low and high anion concentrations, are interpreted as being indicative of sequential anion binding. The protein charges obtained from the slopes of the plots allowed an estimate to be made of the number of anions bound to the two redox states of bovine and horse heart cytc, which are listed in Table 3[23] [it is worth noting that evaluation of *two* unknowns, z_{red} and z_{ox}, from only *one* experimental parameter ($z^2_{red} - z^2_{ox}$) can only be achieved by introducing a number of constraints on the charge values, for which the reader is referred to the original literature].[21−23] The main results can be summarized as follows: (i) all anions other than acetate interact specifically with both proteins, (ii) in many cases, the number of anions bound per protein molecule increases with increasing anion concentration, indicating sequential anion binding due to the presence of low and high affinity sites, (iii) chloride and phosphate bind to both species to a greater extent as compared to the other anions and show different binding stoichiometries toward the two closely sequence-related proteins (97% identity). In particular, one less chloride ion binds to the bovine protein as compared to the equine species: it is tempting to attribute this effect to the presence of one less lysine residue in the bovine species as compared to the horse species (a Gly residue in the former replaces Lys60 in the latter). However, since Lys60 is proposed as not being directly involved in the anion binding sites,[69,80] the observed effect is likely to be due to a decrease in the overall positive charge of bovine cytc and to a change in the distribution of the surface electrostatic potential.

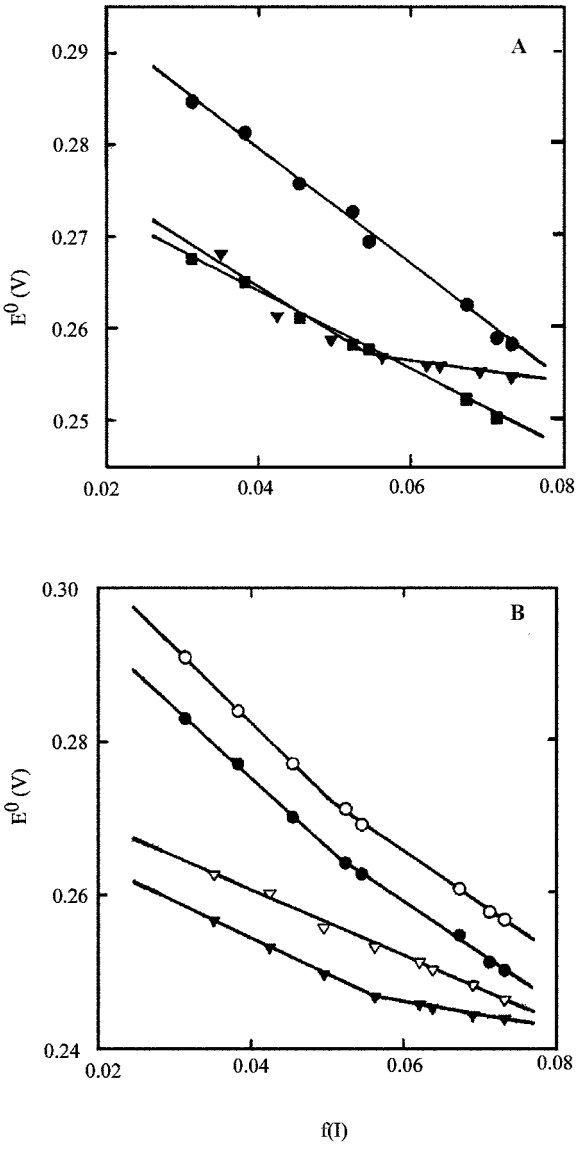

Figure 8. Selected $E^{\circ\prime}$ vs. $f(I)$ plots for horse and bovine heart cytochromes *c* in the presence of various anions: (A) bovine protein with acetate (●), sulfate (▼), bicarbonate (■); (B) bovine protein with chloride (●) and oxalate (▼); horse protein with chloride (○) and oxalate (▽); solid straight lines are fits to Equation (4) (reproduced by permission from G. Battistuzzi et al., *Eur. J. Biochem.* **1996**, *241*, 208−214; copyright Blackwell Science Ltd., 1996)

The presence of two phosphate binding sites on the horse protein at low anion/protein molar ratios was also indicated by different approaches, some of which involved chemical modification of surface lysines.[69,79,81−85] With increasing anion concentration, the electrochemical data indicate the binding of a third phosphate anion (except to the reduced horse protein). As also indicated by ion chromatography data,[69] phosphate displays a distinct binding preference toward the oxidized forms of both proteins as compared to the reduced forms. This is in contrast to chloride, which shows the opposite behavior. This indicates that for both proteins the lysine-based binding sites for the two anions are different and are subject to different reduction-induced changes that affect anion affinity. In addition, phosphate, unlike chloride, binds to a greater extent to the bovine cytochrome, in spite of the smaller positive charge as compared to the horse species. No obvious explanation for this effect can be offered at present. As noted elsewhere,[69] these facts demonstrate that even a few amino acid substitutions perturb the positive surface potential field of the protein in such a way as to significantly alter the number, magnitude, and/or location of the potential energy minima for anion interaction.

Only one bicarbonate ion binds to both of these proteins, in agreement with studies of chemical modification of surface lysines, from which it was proposed that lysines 72 and/or 73 and 86 and/or 87 are involved in anion binding.[79] It is possible that one of the nitrate binding sites corresponds to the bicarbonate site, in view of the structural similarity of the two anions.

Sulfate and citrate show the same binding properties. For both proteins and redox states, the number of anions bound per protein molecule increases from one to two with increasing anion concentration. This suggests the presence of two protein sites with different affinities for the two anions in both of the cytochromes, possibly corresponding to those for phosphate, given the close similarity of sulfate and phosphate in terms of geometry, dimensions, and charge. Consistently, citrate has previously been shown to have at least one binding site in common with phosphate.[67,79,86]

Given that a number of anions show different binding stoichiometries toward horse and bovine cytc, which are highly sequence-related, the anion binding data for a bacterial cytochrome c_2 from *Rps. palustris* (which shows only about 38% sequence identity with the mitochondrial species) were expected to be quite distinctive. However, it has been found that, although some anions such as chloride, phosphate (at concentrations below 0.06−0.08 M), acetate, and oxalate do indeed show peculiar binding properties toward different species (e.g. chloride does not bind to the bacterial species), many others, including perchlorate, bicarbonate, nitrate, phosphate (at concentrations above 0.06−0.08 M), sulfate, and citrate exhibit the same binding stoichiometries (Table 3).[21−23] These two groups of anions do not differ in their structural and charge characteristics, or in their ability to form hydrogen bonds, hence intrinsic anion properties are not responsible for this distinct behavior. Therefore, the invariance of the binding data for the latter group of anions is most probably a consequence of the fact that the binding sites are maintained in the different species: this would suggest the existence of (a) more or less extended region(s) of relatively conserved electrostatic potential in the three cytochromes. It is known that the distribution of lysines around the perimeter of the heme crevice at the "front" of the molecule is largely retained in eukaryotic cytochromes *c* and bacterial cytochromes c_2, which also share the main features of the overall polypeptide folding.[87−91] This also appears to be the case for *Rps. palustris* cytochrome c_2.[21] No other regions show analogous similarities in the location of lysine residues. As a consequence, the "frontside" region of these cytochromes,

Table 3. Number *n* of specifically bound anions for horse and bovine heart cytochromes *c* and *Rps. palustris* cyt c_2, determined from the ionic strength dependence of the redox potential in the presence of various anions, according to Equation (2); $T = 25$ °C; measurements for chloride, perchlorate, nitrate, and sulfate were carried out at pH = 7; for the basic anions the pH varied from 8 to 9.3 depending on anion concentration; in the case of sequential anion binding, values in the upper and lower rows refer to low and high anion concentrations, respectively (from refs.[21−23])

Anion	Bovine cyt *c*			Horse cyt *c*			*Rps. palustris* cyt c_2		
	c [M]	n_{ox}	n_{red}	*c* [M]	n_{ox}	n_{red}	*c* [M]	n_{ox}	n_{red}
Cl^-		1	2		2	3		0	0
	> 0.04	2	3	> 0.04	3	4			
ClO_4^-		1	1		1	0		1	1
				> 0.02	1	1			
HCO_3^-		1	1		1	0		1	1
				> 0.02	1	1			
CH_3COO^-		0	0		0	0		1	1
NO_3^-		1	2		1	2		1	2
HPO_4^{2-}		3	2		2	2		1	1
	> 0.08	3	3	> 0.06	3	2	> 0.06	3	3
SO_4^{2-}		1	1		1	1		1	1
	> 0.08	2	2	> 0.08	2	2	> 0.1	2	1 (2)
$C_2O_4^{2-}$		1	1		1	1		2	1 (2)
	> 0.06	2	2				> 0.08	3	3
$citrate^{3-}$		1	1		1	1		1	1
	> 0.1	2	2	> 0.1	2	2	> 0.08	2	2

which has been proposed to correspond to the likely site of interaction with physiological oxidoreductases,[91−94] should also serve as a site of interaction with a number of anions. Accordingly, the proposed binding site for the bicarbonate ion to the horse protein[79] as well as the common binding site for phosphate and citrate (''Phosphate Site I'' formed by Lys86, Lys87, and Lys88)[79,81,82] fall in this region. As a consequence, the sites for chloride and acetate, as well as the additional low-affinity site(s) for phosphate, are probably located elsewhere.

IV.1.b. Relationships between Surface Charges and $E^{\circ\prime}$

The role of net surface charges in determining the reduction potential in redox metalloproteins has been the subject of much debate.[16,18,95−98] In the various protein families, net charges contribute to the $E^{\circ\prime}$ value only by approximately 10−30 mV per charge, depending on their location with respect to the metal center. These data were obtained through mutation or chemical modification of surface residues and pH titration studies. This moderate effect can mainly be attributed to the screening of the coulombic interaction by the dielectric effect of water. However, recent work from this laboratory[99] has shown that enthalpy-entropy compensation phenomena also contribute in minimizing the resulting $E^{\circ\prime}$ change.

Anion binding to specific surface sites of cytochrome *c* may be exploited as an additional tool to monitor the effect of changes in charge on the $E^{\circ\prime}$ value.[21−23] In fact, since NMR and X-ray data indicate that increasing anion concentrations induce only minor structural modifications, which leave the overall protein folding and conformational flexibility of the molecule largely unaffected,[44,100] it is reasonable to assume that the $E^{\circ\prime}$ change caused by specific anion binding is mainly electrostatic in origin, due to the partial neutralization of positive surface charges. The relationships between $E^{\circ\prime}$ and charge were obtained as follows.[21−23] When only one anion is bound per protein molecule, in both redox states, extrapolation of the linear $E^{\circ\prime}$ vs. $f(I)$ plot to $f(I) = 0$ allows determination of the $E^{\circ\prime}$ value at zero ionic strength ($E^{\circ\prime}_{I=0}$) for the protein, the surface charge of which is decreased by that of the bound anion. The value $E^{\circ\prime}_{I=0}$ for the ion-free protein is taken as that extrapolated in the plot for the non-binding anion (acetate and chloride for the mitochondrial and bacterial species, respectively). The difference between $E^{\circ\prime}_{I=0}$ for the ion-free and the electrostatically perturbed protein ($\Delta E^{\circ\prime}_{I=0}$) yields an estimate of the fall in reduction potential due to the neutralization of *n* positive surface charges (at zero ionic strength). The $\Delta E^{\circ\prime}_{I=0}$ and $dE^{\circ\prime}/dq \cdot (dE^{\circ\prime}/dq$ = change in $E^{\circ\prime}$ per unit charge) values obtained for the three cytochromes studied are listed in Table 4. A number of observations can be made. First, the $dE^{\circ\prime}/dq$ values for the multiply charged anions, such as sulfate, oxalate, and citrate, interacting with the mitochondrial species range from 14.6 to 16.5 mV. They are intermediate between the theoretical value of 12 mV predicted by the smeared charge model for a sphere of radius 1.5 nm in water[4] and 23 mV determined from a comparison of the reduction potentials of a series of proteins bearing different charges.[101] They compare very well with the $dE^{\circ\prime}/dq$ value at zero ionic strength of 11−14 mV determined from mutation of surface lysines,[102] while they appear to be somewhat higher than the 5−10 mV estimated from chemical modification and pH titration studies.[4] Second, the $dE^{\circ\prime}/dq$ values for the two monovalent anions, perchlorate and bicarbonate, are somewhat greater, especially those for the latter ion (24 and 25 mV for beef and horse heart cytc, respectively). This may be due to a closer approach of these anions to the heme, consistent with the existence of a specific ''bicarbonate site''.[79]

Table 4. $\Delta E^{\circ\prime}_{I=0}$ and $dE^{\circ\prime}/dq$ values (see text) for beef and horse heart cytochromes *c* and *Rps. palustris* cytochrome c_2 determined upon anion binding to one site in both redox states of the proteins (from refs.[21−23])

Anion	$\Delta E^{\circ\prime}_{I=0}$ [mV]			$dE^{\circ\prime}/dq$		
	horse	bovine	*Rps. palustris* cyt c_2	horse	bovine	*Rps. palustris* cyt c_2
ClO_4^-	21	20	23	21	20	23
HCO_3^-	25	24	33	25	24	33
SO_4^{2-}	31	31	31	15.5	15.5	15.5
$C_2O_4^{2-}$	33	33	–	16.5	16.5	–
$citrate^{3-}$	44	45	41	14.6	15	14
CH_3COO^-	–	–	28	–	–	28
HPO_4^{2-}	–	–	32	–	–	16

Overall, the $dE^{\circ\prime}/dq$ values determined using the present approach are in line with those previously estimated for mutated or chemically modified species. The picture that emerges is that the effect of alteration of one net surface charge of the protein on the reduction potential of the heme, although being small, as discussed above, is measurable and is also significant at physiological ionic strengths. Thus, besides the general ionic strength effects related to the charge shielding of the protein as a whole due to the surrounding ionic atmosphere, the ionic composition of the medium may also induce protein charge neutralization at specific site(s), which determines an additional alteration (decrease) in the reduction potential. Hence, the salt effects on the rate of electron transfer between cytochrome *c* and redox partners may not only be due to changes in orientation and/or distance of the redox centers in the complex, but a contribution may also arise from changes in the driving force.

IV.1.c. Effects on the Alkaline Transition

Recently, the thermodynamic parameters of the alkaline transition of beef heart ferricytochrome *c* have been measured using the procedure illustrated in Section II.2.a in the presence of various sulfate concentrations.[103] Sulfate is known to bind specifically to cytochrome *c* in a sequential manner at two surface sites.[23] It has been found that the effects of such a specific binding reflect the thermodynamics of the transition and can be satisfactorily interpreted within the frame of the Debye−Hückel theory with simple electrostatic considerations. In particular, the increase in the thermodynamic pK_a values (extrapolated to $I = 0$) upon sequential sulfate binding turns out to be a fully enthalpic effect, which can be accounted for, at least qualitatively, simply by considering the coulombic effects of charge modification due to the formation of ionic couple(s) on the protein surface. In particular, according to Equation (1), the pK_{app} values increase linearly with increasing $f(I)$ (Figure 9). It is worthy of note that: (i) the discontinuity in the pK_{app} vs. $f(I)$ plot corresponds to the sulfate concentration (0.08 M) above which the number of sulfate anions specifically bound to the protein is known to increase from one to two;[23] (ii) the measured slopes are in close agreement with those expected from the decrease in protein charge upon binding of one and two binegative anions to both the alkaline and neutral isomers. These two observations, taken together, clearly indicate that the biphasic profile of the pK_a values as a function of sulfate concentration is the result of the electrostatic effect of sequential anion binding to protein surface sites.

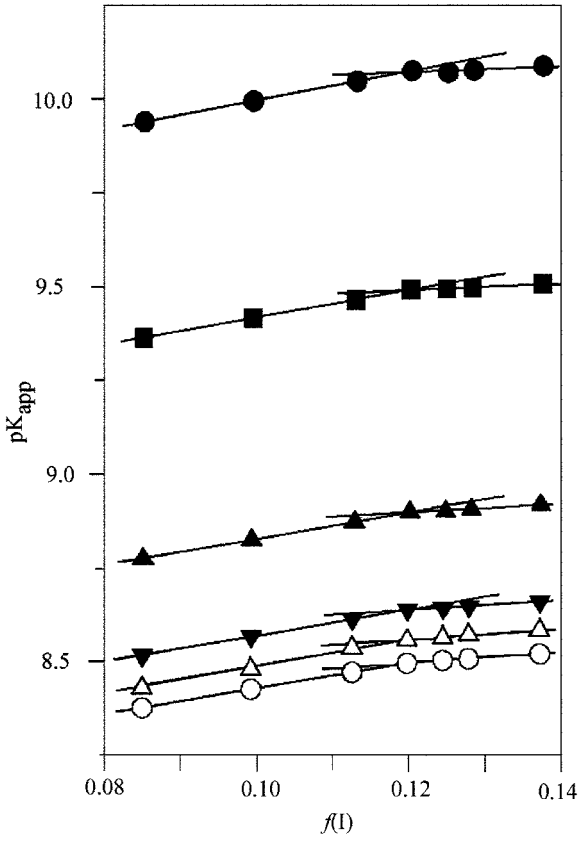

Figure 9. Apparent p*K* values for the alkaline transition of beef heart cytochrome *c* as a function of $f(I) = \sqrt{I}/(1 + 6\sqrt{I})$ (sodium sulfate) at various temperatures; *T* [°C] = 5 (●), 20 (■), 40 (▲), 50 (▼), 55 (Δ), 60 (○); solid lines are least-squares fits to the data points (reproduced by permission from G. Battistuzzi et al., *Arch. Biochem. Biophys.* **2001**, *386*, 177−122; copyright Academic Press, 2001)

IV.2. General Ionic Strength ("Ionic Atmosphere") Effects

The reduction potential of native beef heart cytochrome *c* has been measured as a function of the temperature in the presence of increasing concentrations of the non-binding

anion acetate, in order to quantify the influence of the general ionic strength effect, namely of non-specific electrostatic interactions due to the ionic atmosphere that surrounds the protein, on the thermodynamics of protein reduction.[30] Most of the previous investigations on salt-induced effects on the physicochemical properties of cytochromes *c* were carried out using either phosphate or chloride to adjust the ionic strength of the solution, which, as seen above, do in fact bind specifically to both redox states of cytochrome *c*.[21−23,69,79] The $E^{\circ\prime}/T$ profiles at different ionic strengths and the $E^{\circ\prime}/f(I)$ plots at different temperatures measured at three pH values (7, 8, and 9) are shown in Figure 10 and Figure 11, respectively. At pH = 7 and 8, the reduction enthalpy and entropy become less negative with decreasing ionic strength. The reduction entropy extrapolated to zero ionic strength is approximately zero, indicating that in the absence of the screening effects of the salt ions on the network of the electrostatic interactions at the protein−solvent interface, the solvation properties and the conformational flexibilities of the two redox states are comparable. At the same pH values, the reduction potential of cytc slightly decreases with increasing ionic strength at constant temperature (Figure 11). For example, $\Delta E^{\circ\prime}{}_{IS} = (E^{\circ\prime})_{I=0.1\,M} - (E^{\circ\prime})_{I=0\,M} = -0.035$ V at 25 °C, which corresponds to a $\Delta\Delta G^{\circ\prime}{}_{IS}$ value of +3.3 kJ·mol^{-1}.[30] Since X-ray and NMR studies have shown that salt effects do not alter the overall protein folding and the conformational flexibility of cytc,[100,104−106] the positive $\Delta\Delta G^{\circ\prime}{}_{IS}$ value can mainly be attributed to the selective stabilization of the ferri form by the exclusively electrostatic effects of the salt ions. Such a free energy change turns out to be composed of a $\Delta\Delta H^{\circ\prime}{}_{IS}$ [$= (\Delta H^{\circ\prime})_{I=0.1\,M} - (\Delta H^{\circ\prime})_{I=0\,M}$] term of −8.5 kJ·mol^{-1} and a $T\Delta\Delta S^{\circ\prime}{}_{IS}$ term of −11.8 kJ·mol^{-1} (analogous quantitative relationships between the enthalpic and entropic terms hold at the other pH values and temperatures).[30] Enthalpy-entropy compensation phenomena in the hydration of biopolymers[107−111] must be taken into account to identify the molecular effect responsible for the positive value of $\Delta\Delta G^{\circ\prime}{}_{IS}$. In particular, since the salt-induced changes in the hydration sphere of the two redox states of cytc are likely to be responsible for the negative $T\Delta\Delta S^{\circ\prime}{}_{IS}$ term, a compensating enthalpic effect of the same sign and magnitude should contribute to the measured $\Delta\Delta H^{\circ\prime}{}_{IS}$. Thus, once the compensating enthalpic and entropic effects of the change in solvation properties are factorized out, the measured $\Delta\Delta G^{\circ\prime}{}_{IS}$ value of +3.3 kJ·mol^{-1} turns out to be entirely due to a $\Delta\Delta H^{\circ\prime}$ term attributable to the electrostatic effect of the negatively charged ionic atmosphere that surrounds cytc, which should indeed stabilize the ferri form (which bears a greater positive charge than the reduced state). This would nicely explain why at pH = 7 the $E^{\circ\prime}$ vs. $f(I)$ plot follows the simple electrostatic model of the extended Debye−Hückel equation [Equation (4)], yielding a negative slope that at most temperatures is that expected for the unperturbed protein ($z_{red} = 5+$ and $z_{ox} = 6+$). Notably, at pH = 8, the gradient of such a slope decreases, and at pH = 9 it becomes positive (Figure 11, b, c).[30] We attribute this effect to the specific binding of the hydroxide ion to surface sites of the protein, which results in a decrease in the positive charge of both redox states. The positive slope at pH = 9 would indicate that such a binding occurs preferentially to the oxidized form, as observed elsewhere for a number of anions.[21−23,69]

V. The Effects of Non-Aqueous Solvents

Solvent−solute interactions play a significant role in determining the reduction potentials of redox metalloproteins, affecting both the enthalpic and entropic contributions to the free energy change of the reduction process.[26,27,29] Some theoretical models reproduce the experimental $E^{\circ\prime}$ values of families of homologous ET proteins reasonably well, treating the solvent as a dielectric continuum.[95,112−115]

It has been proposed that there is a decrease in the dielectric constant near the surface of the cell membrane, which induces conformational transitions in some proteins that are central to their physiological role.[116−118] The behavior of cytochrome *c* has been investigated in mixed water/alcohol solutions of low average dielectric constant and at low pH, conditions that, in principle, simulate those in the proximity of the inner mitochondrial membrane.[116,119,120] Two alcohol-induced conformational transitions have been detected at intermediate and high alcohol concentrations. The former leads to a compact species which retains most of the secondary structure of the native state but lacks most features of the tertiary structure (molten globular state), whereas the latter is a more helical state that differs from the extensively unfolded state obtained at high concentrations of strong denaturants such as guanidine hydrochloride or urea.[116] Much less is known about the effects of other organic solvents, or about the redox properties of cytochrome *c* in not purely aqueous media in general. We have investigated the redox behavior of bovine heart cytc in mixed water/dimethyl sulfoxide (DMSO) solutions containing up to 40% (v/v) DMSO. We used cyclic voltammetry, under varying conditions of temperature and pH, and we also monitored the effects on protein conformation by means of ^{1}H NMR and circular dichroism.[29] The main observations can be summarized as follows. The redox behavior of cytc in solutions containing less than 10% DMSO is very similar to that in water. An increase in the proportion of DMSO induces a number of effects, which are different for the native and alkaline forms. Regarding the native form, at constant temperature, its $E^{\circ\prime}$ decreases to a similar extent as that determined in aqueous solution following an increase in temperature reproducing the same dielectric constant as that of the mixed solvent. This indicates that the effect of DMSO on $E^{\circ\prime}$ is mainly determined by the decrease in the average dielectric constant of the solvent. It would appear that DMSO does not significantly alter the protein conformation, as indicated by the absence of appreciable DMSO-induced changes in the hyperfine-shifted ^{1}H NMR resonances and CD spectra of native cytc. Regarding

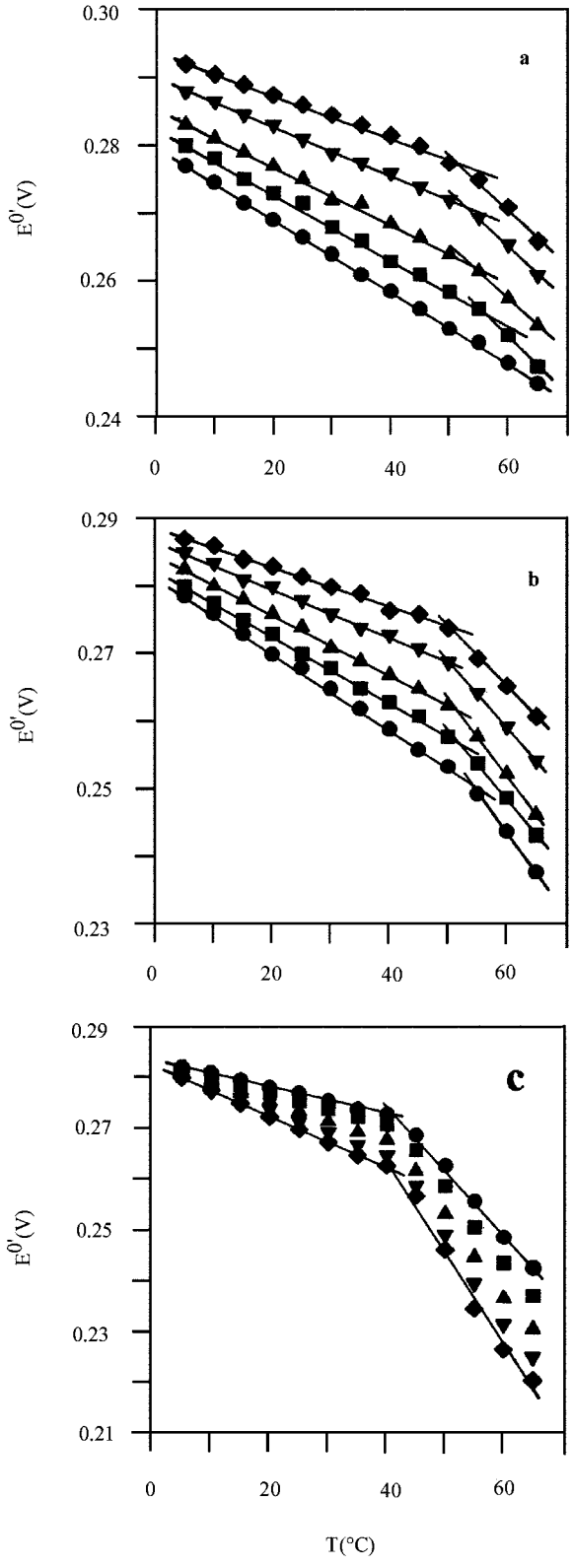

Figure 10. Temperature dependence of the reduction potential of beef heart cytochrome *c* at various ionic strengths (sodium acetate); I = 0.01 M (◆), 0.02 M (▼), 0.05 M (▲), 0.1 M (■), and 0.2 M (●); (a) pH = 7, (b) pH = 8, (c) pH = 9; solid lines are least-squares fits to the data points [those for intermediate ionic strength in (c) are omitted for clarity] (reproduced by permission from G. Battistuzzi et al., *J. Biol. Inorg. Chem.* **1999**, *4*, 601−607; copyright Springer, 1999)

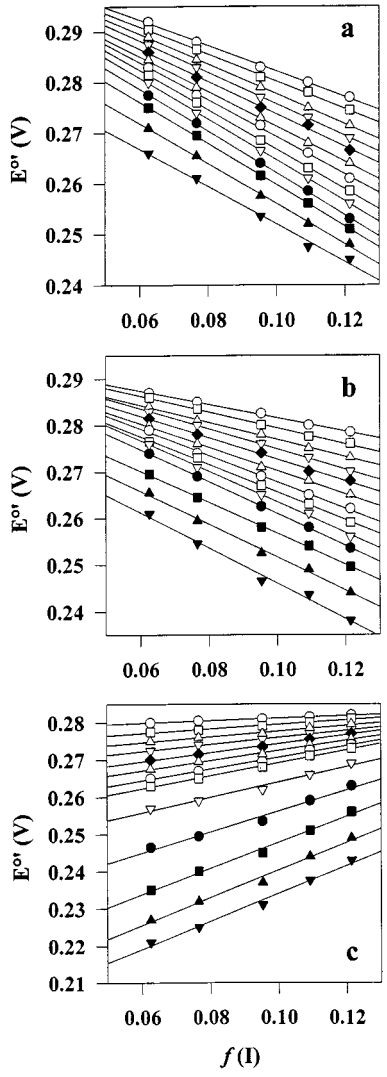

Figure 11. Reduction potential of beef heart cytochrome *c* as a function of $f(I) = \sqrt{I}/(1 + 6\sqrt{I})$ at various temperatures; T [°C] = 5 (○), 10 (□), 15 (Δ), 20 (▽), 25 (◆), 30 (Δ), 35 (○), 40 (□), 45 (▽), 50 (●), 55 (■), 60 (▲), 65 (▼); (a) pH = 7, (b) pH = 8, (c) pH = 9; solid lines are least-squares fits to the data points (reproduced by permission from G. Battistuzzi et al., *J. Biol. Inorg. Chem.* **1999**, *4*, 601−607; copyright Springer, 1999)

the alkaline form(s), at constant pH, the electrochemical wave corresponding to the alkaline conformer(s) of cytc (wave II) increases in intensity to the detriment of wave I (Figure 12). This is paralleled by a decrease in the apparent pK_a value for the transition of native cytc to the alkaline form, estimated from the pH dependence of the peak currents (not shown). Moreover, the electrochemical reversibility of wave II increases (Figure 12) and detectable changes are observed in the ^{1}H NMR and CD spectra of the alkaline form. These data indicate that, in contrast to the native form, DMSO affects to some extent the conformation of alkaline cytc and stabilizes both redox states of this form to the detriment of the native form. The latter effect is not unexpected since the alkaline species is less positively charged and therefore should be preferentially stabilized over the neutral species by a decrease in solvent polarity.

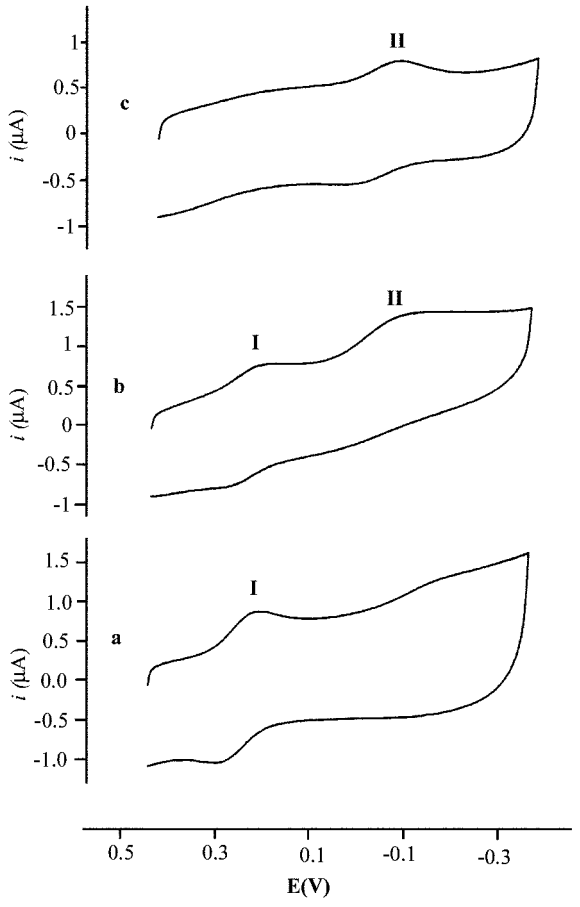

Figure 12. Cyclic voltammograms for bovine heart cytc at a 4-mercaptopyridine surface-modified gold electrode in (a) water, pH = 6.7; (b) 30% DMSO, pH* = 6.1; (c) 40% DMSO, pH* = 6.3; scan rate 50 mVs^{-1}; waves I and II are due to native and alkaline cytc, respectively; protein concentration 0.2 mM; T = 55 °C (this fairly high temperature allows the effects of DMSO on the current and reversibility of wave II to be better visualized) (reproduced by permission from G. Battistuzzi et al., *Inorg. Chim. Acta* **1998**, *272*, 168−175; copyright Elsevier Science S.A., 1998)

The highly homologous horse heart cytc was previously found to behave in a similar manner in water/methanol mixtures at pH = 6.[121]

[1] J. A. Cowan, *Inorganic Biochemistry: An Introduction*, Wiley-VCH, New York, **1996**.
[2] H. B. Gray, W. R. Ellis, in *Bioinorganic Chemistry* (Eds.: I. Bertini, H. B. Gray, S. J. Lippard, J. S. Valentine), University Science Books, Mill Valley, CA, **1994**, chapter 6, p. 315−363.
[3] L. Banci, I. Bertini, A. Rosato, G. Varani, *J. Biol. Inorg. Chem.* **1999**, *4*, 824−837.
[4] G. R. Moore, G. W. Pettigrew, *Cytochromes c: Evolutionary, Structural and Physicochemical aspects*, Springer-Verlag, Berlin, **1990**.
[5] R. Scott, A. G. Mauk, *Cytochrome c: A Multidisciplinary Approach*, University Science Books, Sausalito, CA, **1996.**
[6] G. W. Pettigrew, G. R. Moore, *Cytochromes c: Biological Aspects*, Springer-Verlag, Berlin, **1987**.
[7] F. A. Armstrong, *Struct. Bonding* **1990**, *72*, 137−222.
[8] F. M. Hawkridge, I. Taniguchi, *Comments Inorg. Chem.* **1995**, *17*, 163−187.
[9] M. J. Eddowes, H. A. O. Hill, *J. Am. Chem. Soc.* **1979**, *101*, 4461−4464.
[10] H. A. O. Hill, N. I. Hunt, *Methods Enzymol.* **1993**, *227*, 501−522.
[11] A. M. Bond, *Inorg. Chim. Acta* **1994**, *226*, 293−340.
[12] G. Battistuzzi, M. Borsari, M. Sola, F. Francia, *Biochemistry* **1997**, *36*, 16247−16258.
[13] J. C. Marchon, T. Mashiko, C. A. Reed, in *Electron Transport and Oxygen Utilization* (Ed.: H. Chien), Elsevier, Amsterdam, **1982**, p. 67−72.
[14] F. A. Tezcan, J. R. Winkler, H. B. Gray, *J. Am. Chem. Soc.* **1998**, *120*, 13383−13388.
[15] M. R. Gunner, E. Alexov, E. Torres, S. Lipovaca, *J. Biol. Inorg. Chem.* **1997**, *2*, 126−134.
[16] A. G. Mauk, G. R. Moore, *J. Biol. Inorg. Chem.* **1997**, *2*, 119−125.
[17] P. J. Stephens, D. R. Jollie, A. Warshel, *Chem. Rev.* **1996**, *96*, 2491−2513.
[18] A. Warshel, A. Papazyan, I. Muegge, *J. Biol. Inorg. Chem.* **1997**, *2*, 143−152.
[19] A. K. Churg, A. Warshel, *Biochemistry* **1986**, *25*, 1675−1681.
[20] N. K. Rogers, *Prog. Biophys. Mol. Biol.* **1986**, *48*, 37−66.
[21] G. Battistuzzi, M. Borsari, M. Sola, *Arch. Biochem. Biophys.* **1997**, *339*, 283−290.
[22] G. Battistuzzi, M. Borsari, D. Dallari, S. Ferretti, M. Sola, *Eur. J. Biochem.* **1995**, *233*, 335−339.
[23] G. Battistuzzi, M. Borsari, D. Dallari, I. Lancellotti, M. Sola, *Eur. J. Biochem.* **1996**, *241*, 208−214.
[24] R. L. Cutler, A. M. Davies, S. Creighton, A. Warshel, G. R. Moore, M. Smith, A. G. Mauk, *Biochemistry* **1989**, *28*, 3188−3197.
[25] S. P. Rafferty, L. L. Pearce, P. D. Barker, J. G. Guillemette, C. M. Kay, M. Smith, A. G. Mauk, *Biochemistry* **1990**, *29*, 9365−9369.
[26] G. Battistuzzi, M. Borsari, L. Loschi, F. Righi, M. Sola, *J. Am. Chem. Soc.* **1999**, *121*, 501−506.
[27] G. Battistuzzi, M. Borsari, A. L. Macedo, J. J. G. Moura, P. Rodrigues, M. Sola, *J. Biol. Inorg. Chem.* **2000**, *5*, 748−760.
[28] G. Battistuzzi, M. Borsari, J. A. Cowan, C. Eicken, L. Loschi, M. Sola, *Biochemistry* **1999**, *38*, 5553−5562.
[29] G. Battistuzzi, M. Borsari, G. Rossi, M. Sola, *Inorg. Chim. Acta* **1998**, *272*, 168−175.
[30] G. Battistuzzi, M. Borsari, L. Loschi, M. Sola, *J. Biol. Inorg. Chem.* **1999**, *4*, 601−607.
[31] S. Benini, M. Borsari, S. Ciurli, A. Dikiy, M. Lamborghini, *J. Biol. Inorg. Chem.* **1998**, *3*, 371−382.
[32] P. Bertrand, O. Mbarki, M. Asso, L. Blanchard, F. Guerlesquin, M. Tegoni, *Biochemistry* **1995**, *34*, 11071−11079.
[33] T. Ikeshoji, I. Taniguchi, F. M. Hawkridge, *J. Electroanal. Chem.* **1989**, *270*, 297−308.
[34] K. B. Koller, F. M. Hawkridge, *J. Am. Chem. Soc.* **1985**, *107*, 7412−7417.
[35] K. B. Koller, F. M. Hawkridge, *J. Electroanal. Chem.* **1988**, *239*, 291−306.
[36] I. Taniguchi, T. Funatsu, M. Iseki, H. Yamaguchi, K. Yasukouchi, *J. Electroanal. Chem.* **1985**, *193*, 295−302.
[37] I. Taniguchi, M. Iseki, T. Eto, K. Toyosawa, H. Yamaguchi, K. Yasukouchi, *Bioelectrochem. Bioenerg.* **1984**, *13*, 373−383.
[38] E. L. Yee, R. J. Cave, K. L. Guyer, P. D. Tyma, M. J. Weaver, *J. Am. Chem. Soc.* **1979**, *101*, 1131−1137.
[39] E. L. Yee, M. J. Weaver, *Inorg. Chem.* **1980**, *19*, 1077−1079.
[40] V. T. Taniguchi, N. Sailasuta-Scott, F. C. Anson, H. B. Gray, *Pure Appl. Chem.* **1980**, *52*, 2275−2281.
[41] P. Baistrocchi, L. Banci, I. Bertini, P. Turano, K. L. Bren, H. B. Gray, *Biochemistry* **1996**, *35*, 13788−13796.
[42] L. Banci, I. Bertini, H. B. Gray, C. Luchinat, T. Redding, A. Rosato, P. Turano, *Biochemistry* **1997**, *36*, 9867−9877.
[43] L. Banci, I. Bertini, J. G. Huber, G. A. Spyroulias, P. Turano, *J. Biol. Inorg. Chem.* **1999**, *4*, 21−31.

[44] L. Banci, I. Bertini, T. Redding, P. Turano, *Eur. J. Biochem.* **1997**, *256*, 271−278.
[45] L. Banci, G. Gori-Savellini, P. Turano, *Eur. J. Biochem.* **1997**, *249*, 716−723.
[46] A. Soriano, D. Li, S. Bian, A. Agarwal, J. A. Cowan, *Biochemistry* **1996**, *35*, 12479−12486.
[47] H. A. Heering, Y. B. M. Bulsink, W. R. Hagen, T. E. Meyer, *Biochemistry* **1995**, *34*, 14675−14686.
[48] G. Battistuzzi, M. Borsari, L. Loschi, M. Martinelli, M. Sola, *Biochemistry* **1999**, *38*, 7900−7907.
[49] G. Battistuzzi, M. Borsari, S. Ferretti, M. Sola, E. Soliani, *Eur. J. Biochem.* **1995**, *232*, 206−213.
[50] G. R. Moore, D. E. Harris, F. A. Leitch, G. W. Pettigrew, *Biochim. Biophys. Acta* **1984**, *764*, 331−342.
[51] W. Qin, R. Sanishvili, B. Plotkin, A. Schejter, E. Margoliash, *Biochim. Biophys. Acta* **1995**, *1252*, 87−94.
[52] J. S. Cohen, W. R. Fischer, A. N. Schechter, *J. Biol. Chem.* **1974**, *249*, 1113−1118.
[53] H. Ochi, Y. Hata, N. Tanaka, M. Kakudo, T. Sakurai, S. Aihara, Y. Morita, *J. Mol. Biol.* **1983**, *166*, 407−418.
[54] G. W. Pettigrew, R. G. Bartsch, T. E. Meyer, M. D. Kamen, *Biochim. Biophys. Acta* **1978**, *503*, 509−523.
[55] P. D. Barker, A. G. Mauk, *J. Am. Chem. Soc.* **1992**, *114*, 3619−3624.
[56] S. Döpner, P. Hildebrandt, F. I. Rosell, A. G. Mauk, *J. Am. Chem. Soc.* **1998**, *120*, 11246−11255.
[57] J. C. Ferrer, J. G. Guillemette, R. Bogumil, S. C. Inglis, M. Smith, A. G. Mauk, *J. Am. Chem. Soc.* **1993**, *115*, 7507−7508.
[58] X. Hong, D. W. Dixon, *FEBS Lett.* **1989**, *246*, 105−108.
[59] F. I. Rosell, J. C. Ferrer, A. G. Mauk, *J. Am. Chem. Soc.* **1998**, *120*, 11234−11245.
[60] G. Taler, A. Schejter, G. Navon, I. Vig, E. Margoliash, *Biochemistry* **1995**, *34*, 14209−14212.
[61] K. S. Nicholson, I. Shain, *Anal. Chem.* **1964**, *36*, 706−723.
[62] P. J. Spooner, A. Watts, *Biochemistry* **1991**, *30*, 3871−3879.
[63] P. J. Spooner, A. Watts, *Biochemistry* **1992**, *31*, 10129−10138.
[64] R. Jemmerson, J. Liu, D. Hansauer, K.-P. Lam, A. Mondino, R. D. Nelson, *Biochemistry* **1999**, *38*, 3599−3609.
[65] M. T. Wilson, C. Greenwood, *Eur. J. Biochem.* **1971**, *22*, 11−18.
[66] A. Schejter, P. George, *Biochemistry* **1965**, *3*, 1045−1049.
[67] N. Osheroff, D. Borden, W. H. Koppenol, E. Margoliash, *J. Biol. Chem.* **1980**, *255*, 1689−1697.
[68] A. Schejter, T. L. Luntz, T. I. Koshy, E. Margoliash, *Biochemistry* **1992**, *31*, 8336−8343.
[69] D. Gopal, G. S. Wilson, R. A. Earl, M. A. Cusanovich, *J. Biol. Chem.* **1988**, *263*, 11652−11656.
[70] F. Millett, M. A. Miller, L. Green, B. Durham, *J. Bioenerg. Biomembr.* **1995**, *27*, 341−351.
[71] T. Heimburg, D. Marsh, *Biophys. J.* **1995**, *68*, 536−546.
[72] T. Goldkorn, A. Schejter, *Arch. Biochem. Biophys.* **1976**, *177*, 39−45.
[73] R. Margalit, A. Schejter, *Eur. J. Biochem.* **1973**, *32*, 492−499.
[74] I. Aviram, Y. P. Myer, A. Schejter, *J. Biol. Chem.* **1981**, *256*, 5540−5544.
[75] L. Banci, I. Bertini, K. L. Bren, H. B. Gray, P. Sompornpisut, P. Turano, *Biochemistry* **1997**, *36*, 8992−9001.
[76] G. Battistuzzi, M. Borsari, L. Loschi, M. Sola, *J. Biol. Inorg. Chem.* **1997**, *2*, 350−359.
[77] J. M. Guss, E. A. Merritt, R. P. Phizackerley, H. C. Freeman, *J. Mol. Biol.* **1996**, *262*, 686−705.
[78] C. O. Arean, G. R. Moore, G. Williams, R. J. P. Williams, *Eur. J. Biochem.* **1988**, *173*, 607−615.
[79] N. Osheroff, D. L. Brautigan, E. Margoliash, *Proc. Natl. Acad. Sci. USA* **1980**, *77*, 4439−4443.
[80] G. R. Moore, G. C. S. Eley, G. Williams, in *Advances in Inorganic and Bioinorganic Mechanisms* (Ed.: A. G. Sykes), Academic Press, New York, **1984**, chapter 1, p. 1.
[81] D. L. Brautigan, S. Ferguson-Miller, E. Margoliash, *J. Biol. Chem.* **1978**, *253*, 130−139.
[82] D. L. Brautigan, S. Ferguson-Miller, G. E. Tarr, E. Margoliash, *J. Biol. Chem.* **1978**, *253*, 140−148.
[83] S. Ferguson-Miller, D. L. Brautigan, E. Margoliash, *J. Biol. Chem.* **1978**, *253*, 149−159.
[84] E. Stellwagen, R. G. Shulman, *J. Mol. Biol.* **1973**, *75*, 683−695.
[85] J. K. Dethmers, S. Ferguson-Miller, E. Margoliash, *J. Biol. Chem.* **1979**, *254*, 11973−11981.
[86] G. H. Barlow, E. Margoliash, *J. Biol. Chem.* **1966**, *241*, 1473−1477.
[87] S. Sogabe, T. Ezoe, N. Kasai, M. Saeda, A. Uno, M. Miki, K. Miki, *FEBS Lett.* **1994**, *345*, 5−8.
[88] M. M. Benning, G. Wesemberg, M. S. Caffrey, R. G. Bartsch, T. E. Meyer, M. A. Cusanovich, I. Rayment, H. M. Holden, *J. Mol. Biol.* **1991**, *220*, 673−685.
[89] F. R. Salemme, S. T. Freer, Ng. H. Xuong, R. A. Alden, J. Kraut, *J. Biol. Chem.* **1973**, *248*, 3910−3921.
[90] M. M. Benning, T. E. Meyer, H. M. Holden, *Arch. Biochem. Biophys.* **1994**, *310*, 460−466.
[91] F. R. Salemme, J. Kraut, M. D. Kamen, *J. Biol. Chem.* **1973**, *248*, 7701−7716.
[92] J. Hall, X. Zha, B. Durham, P. O'Brien, B. Vieira, D. Davis, M. Y. Okamura, F. Millet, *Biochemistry* **1987**, *26*, 4494−4500.
[93] J. E. Long, B. Durham, M. Y. Okamura, F. Millet, *Biochemistry* **1989**, *28*, 6970−6974.
[94] C. Weber, B. Michel, H. R. Bosshard, *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 6687−6691.
[95] H.-X. Zhou, *J. Biol. Inorg. Chem.* **1997**, *2*, 109−113.
[96] I. Bertini, G. Gori-Savellini, C. Luchinat, *J. Biol. Inorg. Chem.* **1997**, *2*, 114−118.
[97] Q. Zeng, E. T. Smith, D. M. Kurtz, R. A. Scott, *Inorg. Chim. Acta* **1996**, *242*, 245−251.
[98] F. A. Armstrong, *J. Biol. Inorg. Chem.* **1997**, *2*, 139−142.
[99] M. Sola, unpublished results.
[100] R. Sanishvili, K. W. Volz, E. M. Westbrook, E. Margoliash, *Structure* **1995**, *3*, 707−716.
[101] D. C. Rees, *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 3082−3085.
[102] M. S. Caffrey, M. A. Cusanovich, *Arch. Biochem. Biophys.* **1991**, *285*, 227−230.
[103] G. Battistuzzi, M. Borsari, A. Ranieri, M. Sola, *Arch. Biochem. Biophys.* **2001**, *386*, 117−122.
[104] Y. Feng, S. W. Englander, *Biochemistry* **1990**, *29*, 3505−3509.
[105] L. Banci, I. Bertini, T. Reddig, P. Turano, *Eur. J. Biochem.* **1998**, *256*, 271−278.
[106] G. Liu, C. A. Grygon, T. E. Spiro, *Biochemistry* **1989**, *28*, 5046−5050.
[107] W. Blokzijl, J. B. N. F. Engberts, *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 1545−1579.
[108] E. Grunwald, *J. Am. Chem. Soc.* **1986**, *108*, 5726−5731.
[109] R. Lumry, S. Rajender, *Biopolymers* **1970**, *9*, 1125−1227.
[110] N. Matubayasi, L. H. Reed, R. M. Levy, *J. Phys. Chem.* **1994**, *98*, 10640−10649.
[111] B. Lee, G. Graziano, *J. Am. Chem. Soc.* **1996**, *118*, 5163−5168.
[112] P. J. Stephens, D. R. Jollie, A. Warshel, *Chem. Rev.* **1996**, *96*, 2491−2513.
[113] A. K. Churg, A. Warshel, *Biochemistry* **1986**, *25*, 1675−1681.
[114] R. Langen, G. M. Jensen, U. Jacob, P. J. Stephens, A. Warshel, *J. Biol. Chem.* **1992**, *267*, 25625−25627.
[115] H.-X. Zhou, *J. Am. Chem. Soc.* **1994**, *116*, 10362−10375.
[116] V. E. Bychkova, A. F. Dujsekina, S. I. Klenin, E. I. Tiktopulo, V. N. Uversky, O. B. Ptitsyn, *Biochemistry* **1996**, *35*, 6058−6063.
[117] F. G. Van der Goot, J. M. Gonzales-Mañas, J. H. Lakey, F. Pattus, *Nature* **1991**, *354*, 408−410.
[118] O. B. Ptitsyn, *Adv. Protein Chem.* **1995**, *47*, 83−229.
[119] P. Fan, C. Bracken, J. Baum, *Biochemistry* **1993**, *32*, 463−479.
[120] K. Shiraki, K. Nishikawa, Y. Goto, *J. Mol. Biol.* **1995**, *245*, 180−192.
[121] H. R. Drew, R. E. Dickerson, *J. Biol. Chem.* **1978**, *253*, 8420−8427.

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