Thermodynamics of the Alkaline Transition of Cytochrome *c*[†]

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ABSTRACT: The apparent equilibrium constant (K_{app}) of the alkaline transition (AT) of beef heart cytochrome *c*, obtained from pH titrations of the current intensities in cyclic voltammetry experiments, has been measured as a function of the temperature from 5 to 65 °C, at different ionic strength ($I = 0.01-0.2$ M). The temperature profile of the pK_{app} values is biphasic and yields two distinct sets of $\Delta H^{\circ\prime}_{AT}$ and $\Delta S^{\circ\prime}_{AT}$ values below and above approximately 40 °C. In the low-temperature range, the process is endothermic and is accompanied by a small positive entropy change, while at higher temperatures it becomes less endothermic and involves a pronounced entropy loss. The temperature dependence of the transition thermodynamics is most likely the result of the thermal transition of native ferricytochrome *c* from a low-T to an high-T conformer which occurs at alkaline pH values at a temperature comparable with above (Ikeshoji, T., Taniguchi, I., and Hawkridge, F. M. (1989) *J. Electroanal. Chem. 270*, 297−308; Battistuzzi, G., Borsari, M., Sola, M., and Francia, F. (1997) *Biochemistry 36*, 16247−16258). Thus, it is apparent that the transitions of the two native conformers to the corresponding alkaline form(s) are thermodynamically distinct processes. It is suggested that this difference arises from either peculiar transition-induced changes in the hydration sphere of the protein or to the preferential binding of different lysines to the heme iron in the two temperature ranges. Extrapolation of the K_{app} values at null ionic strength allowed the determination of the thermodynamic equilibrium constants (K_a) at each temperature, 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. Finally, it is found that the Debye−Hückel theory fits the ionic strength dependence of the pK_{app} values, at least qualitatively, as observed previously for the ionic strength dependence of the reduction potential of this protein class. It is apparent that the increase in the pK_{app} values with increasing ionic strength is for the most part an entropic effect.

Axial ligation to the heme iron in oxidized cytochromes *c* is sensitive to changes in pH, temperature, ionic strength, and solvent composition. In particular, the moderate affinity of the methionine sulfur for the ferric ion in the native form (state III) makes this residue susceptible to substitution by an endogenous ligand at high pH or when the protein is moderately heated or subjected to a chemically induced partial denaturation (*1, 2*). This transition, leading to the "alkaline" isomer (state IV) in which the ferric ion retains a low-spin state, is accompanied by spectroscopic changes such as the loss of the 695 nm band in the electronic spectra, the appearance of new set(s) of hyperfine-shifted resonances in the ^{1}H NMR spectra, and changes in the vibrational modes of the heme pocket in resonance Raman spectra (*3*−*26*). These changes have been exploited to determine the equilibrium and kinetic properties of this isomerization and to identify the nature of the substituting ligands and the role of residues neighboring the heme in the relative stabilization of the two conformers. At present, the overall picture which is generally accepted is that of a two-step mechanism in which a fast residue deprotonation with a pK of approximately 11 triggers a slow and thermodynamically favored conformational change (p$K = -2$) to a new low-spin Fe(III) state (Scheme 1) (*27*−*30*). Consistently, the apparent pK_a for the neutral to alkaline transition measured at equilibrium from electronic and NMR spectra and electrochemical measurements is approximately 9 (*1, 2, 20*). The ionizing residue has not been unambiguously identified yet. The possible candidates include a surface lysine, the iron-binding His18, the heme propionate-6, and a buried water molecule (*13, 31, 32*). A wealth of experimental evidence indicates that the conformational change involves replacement of Met80 with a surface lysine in axial heme ligation (*1*−*9, 11*−*14, 16, 27*−*29*). The existence of two alkaline isomers in a pH-dependent ratio for all mitochondrial cyt[1] *c* suggested that at least two Lys residues can substitute for the methionine at high pH (*7, 12*). Recent studies on site-

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[1] Abbreviations: cyt, cytochrome; SCE, saturated calomel electrode; SHE, standard hydrogen electrode; N, native cytochrome *c*; N_1, low-temperature conformer of native cytochrome *c*; N_2, high-temperature conformer of native cytochrome *c*; AT, alkaline transition of cytochrome *c*; A, alkaline conformer of cytochrome *c*.

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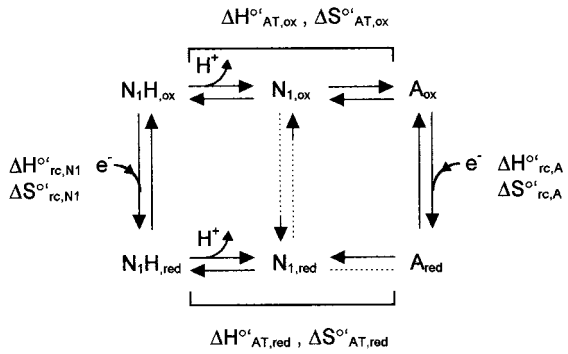
Scheme 1: Minimal Reaction Scheme for the Alkaline Isomerization of Cytochrome *c* (*27*−*29*)



Reference is made to the low-T neutral conformer in its resting state, N_1H, in the deprotonated state, N_1, and in the lysine-ligated alkaline forms, A. Subscript rc stands for "redox couple" and indicates the thermodynamics of reduction determined from non-isothermal variable temperature experiments (*30*, *74*). Subscript AT stands for alkaline transition.

directed mutants of yeast iso-1 cyt *c* indicated Lys79 and Lys73 as the iron ligands (*3*, *4*, *7*, *33*). The most likely candidates in horse heart cyt *c* are Lys79 and Lys72 (*3*, *4*, *6*, *9*). An increase in temperature at neutral or mild alkaline pH was shown to cause the same residue substitution in axial heme ligation (*5*, *8*), most likely as a consequence of the T-induced decrease of the overall pK_a of the alkaline transition. Evidence has been provided for the involvement of a third alkaline isomer at high T in horse heart cyt *c* (*8*). Additional conformers have been detected in strong alkaline solutions (state V), in which an hydroxide ion is likely to act as the sixth axial ligand (*4*). Although much effort has been devoted to the characterization of the III → IV transition through a variety of experimental approaches, still no detailed information is available on the structural features of the alkaline conformers.

We have recently focused on the thermodynamic parameters of the electron exchange for cytochromes *c* and other electron transport metalloproteins, determined through variable temperature direct electrochemistry experiments (*34*−*37*). The enthalpy and entropy changes accompanying protein reduction proved to be a valuable tool to interpret differences in $E^{\circ\prime}$ among the members of the same protein family, as well as temperature- and pH-induced changes in $E^{\circ\prime}$ for a given species. It is known that at alkaline pH values native ferricytochrome *c* undergoes a reversible temperature-induced conformational transition (above 35−50 °C, depending on the species) with no changes in axial heme ligation (*34*, *38*−*42*). The reduction thermodynamics of the low-T and high-T native isomers (N_1 and N_2, respectively) were found to differ sensibly, indicating that the thermal transition affects to some extent the heme−protein interface and/or the dynamic and solvation properties of the polypeptide chain (*34*). This prompted us to check whether this T-induced conformational change has an effect on the thermodynamic parameters of the alkaline transition. Here, we show that this is the case. In particular, we describe the results of the determination of the apparent equilibrium constant for the alkaline isomerization of beef heart cytochrome *c*, carried out through direct electrochemistry over a wide temperature range (5−65 °C), from which two distinct sets of transition thermodynamics were obtained, that are clearly attributable to the low-T and high-T native isomers. In addition, we have measured the ionic strength dependence of the apparent equilibrium constant at different temperatures. This allowed determination of the thermodynamic equilibrium constants (at null ionic strength) and of the "true" thermodynamic parameters of the transition. Overall, this work provides a detailed picture of the contribution of the enthalpic and entropic terms to the free-energy change of the alkaline isomerization of cytochrome *c* (Scheme 1), and of how this process is affected by the ionic composition of the medium (*43*). These data complement the as yet poor structural information in gaining a deeper understanding of this transition that for some aspects has been suggested to possess a functional relevance (*21*, *22*, *28*, *44*−*46*).

EXPERIMENTAL PROCEDURES

Materials. Beef heart cytochrome *c* was purchased from Sigma and further purified by cation-exchange chromatography (SP-Sepharose HP). Removal of bound anions was achieved following the procedure described elsewhere (*47*). All chemicals were reagent grade. Nanopure water was used throughout.

Electrochemical Measurements. Cyclic voltammetry experiments were carried out with a Potentiostat/Galvanostat PAR mod. 273A at different scan rates (0.02−1 V s^{-1}) using a cell for small volume samples (V = 0.5 mL) under argon. A 2 mm diameter gold disk was used as working electrode, and a Pt sheet and a saturated calomel electrode (SCE) as counter and reference electrode, respectively. The electric contact between the SCE and the working solution was obtained with a Vycor set. Potentials were calibrated against the MV^{2+}/MV^{+} couple (MV = methyl viologen) (*48*). All of the redox potentials reported here referred to the standard hydrogen electrode (SHE). The working electrode was cleaned by first dipping it in ethanol for 10 min and then polishing it with an alumina (BDH, particle size of about 0.015 mm) water slurry on cotton wool; finally the electrode was treated in an ultrasonic pool for about 10 min. To minimize residual adsorbed impurities, the electrode was first set at +1 V (vs SCE) for 180 s and then subjected to 10 voltammetric cycles between +0.7 and −0.6 V at 0.1 V s^{-1}. Modification of the electrode surface was performed by dipping the polished electrode into a 1 mM solution of 4-mercaptopyridine for 30 s and then rinsing it with Nanopure water. Sodium acetate in different concentration was used as the base electrolyte. Protein solutions were freshly prepared before use and their concentration, varying over 0.1 to 0.3 mM, was checked spectrophotometrically. The pH was changed by adding small amounts of concentrated NaOH or acetic acid under fast stirring. The ionic strength (I) was adjusted with sodium acetate and the real I value was calculated taking into account also the concentration of the cytochrome. The experiments were performed several times, and the reduction potentials were found to be reproducible within ± 2 mV. In the pH titrations at different temperatures the reference electrode was kept at constant temperature (21 ± 0.1 °C), while the half-cell containing the working electrode and the Vycor junction to the reference electrode were under thermostatic control and the temperature was varied from 5 to 65 °C.

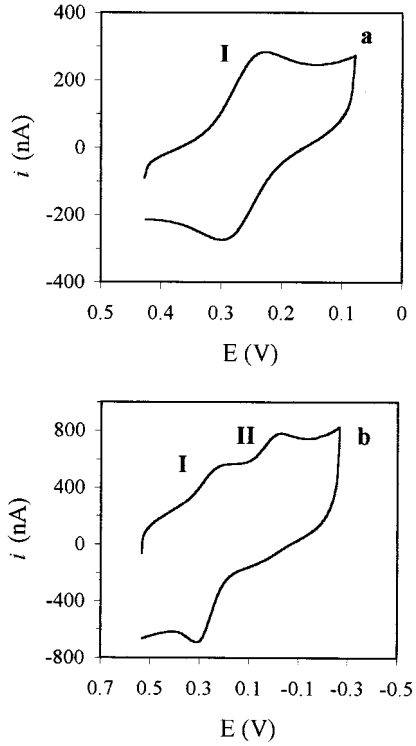


FIGURE 1: Cyclic voltammograms of beef heart cytochrome *c* at a 4-mercaptopyridine surface-modified gold disk electrode; (a) pH 6.95, $T = 293$ K, sweep rate, 50 mV s^{-1}, protein concentration, 0.17 mM; (b) pH 9.13, $T = 293$ K, sweep rate, 50 mV s^{-1}, protein concentration, 0.25 mM. I and II refer to the waves of the native and alkaline conformer, respectively: base electrolyte, 0.1 M sodium acetate.

RESULTS

Apparent Equilibrium Constant of the Alkaline Isomerization. A typical cyclic voltammogram of the neutral form (N) of beef heart cyt *c*, obtained on a gold electrode surface modified with 4-mercaptopyridine, is shown in Figure 1a. The electrochemically reversible, one-electron and diffusion-controlled voltammetric signal (wave I) at a potential ($E°'$) of +0.263 V at pH 7 and 25 °C is due to the Fe^{3+}/Fe^{2+} equilibrium of the heme iron (*34*, *49*−*51*). If the pH is increased above 8 at room temperature (25 °C), a new diffusion-controlled chemically irreversible signal (wave II) is observed at negative potentials ($E_{pc} = -0.040$ V vs SHE) (Figure 1b), which is due to the lysine-ligated alkaline conformer(s) (A) (*29*, *34*, *52*, *53*). The intensity of the anodic counterpart increases with increasing sweep rate and becomes comparable to that of the cathodic peak using scan rates higher than 0.6 V s^{-1}. The electrochemical behavior of wave II improves with increasing temperature, as observed elsewhere for other class I cytochromes *c* (*29*, *34*, *54*). 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 wave I, while the sum of the two currents remains constant. Moreover, at each pH value, the cathodic peak currents of both waves (and their E°′ values as well) are independent of the potential sweep rate in the range 0.02−1 V s^{-1}. This holds for all ionic strengths and temperatures considered in this work. Individual peak intensities simply depend on the root square of the scan rate, as expected for diffusion-controlled processes (Figure 2). Thus, at variance with the case of the Phe82His variant of yeast iso-1-cyt *c* (*55*), waves

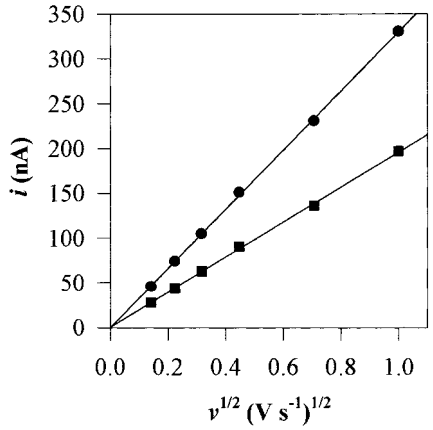


FIGURE 2: Current intensity of the cathodic peak of wave I (●) and II (■), corresponding to the native and alkaline isomers, respectively, as a function of the root square of the potential sweep rate: pH = 9.13; base electrolyte, 0.1 M sodium acetate; $T = 293$ K.

I and II are not "kinetic waves" (in the range of potential sweep rate used), and at each pH value, their currents are those determined by the chemical equilibrium between the native and alkaline forms. In fact, the absence of sweep rate-dependent changes of the cathodic current intensity of the two waves indicates that, at variance with the above mutant, the k_f and k_b values for the overall process of ligand exchange are much smaller than the rate of the process of electron uptake in the range of potential sweep rates used in this work; hence, the reduction process for wave I (wave II) is invariably over before significant A → N (N → A) conversion can take place (*56*). As a consequence, the concentrations of the neutral and alkaline forms at each pH at the solution/electrode interface are not perturbed appreciably with respect to those at equilibrium in the bulk of the solution; therefore the measured currents can be used to determine the apparent equilibrium constant. The current intensity (*i*) of the cathodic peak of wave I was measured as a function of pH at 5, 10, 20, 30, 40, 50, 55, 60, and 65 °C. The fit of the sigmoidal decrease of *i* with increasing pH to a one-proton equilibrium equation yields the apparent p*K* value (pK_{app}) for the alkaline transition at a given temperature (Figure 3). In the pH range investigated, the anodic and cathodic peak potentials of both waves decrease by about 15−25 mV, depending on the temperature and ionic strength.

Equilibrium Constants and Thermodynamic Parameters at Different Ionic Strength. Measurement of the pK_{app} values at different ionic strength allows evaluation of the effects of the screening of the protein charge by the ionic atmosphere on the relative stability of the two protein forms and determination of the thermodynamic equilibrium constant of the transition (through extrapolation of the pK_{app} values at $I = 0$). The pK_{app} values at each temperature were measured as described above at different ionic strength ($I = 0.01-0.2$ M) obtained with sodium acetate. The acetate ion was chosen because it was shown previously not to interact with beef (and horse) heart cyt *c* at specific sites up to 0.2 M concentration (*49*). Hence acetate simply exerts a general ionic strength effect. The only influence of *I* on the voltammetric response was a cathodic shift of E°′ due to the selective stabilization of the more positively charged oxidized state of the protein by the surrounding ionic

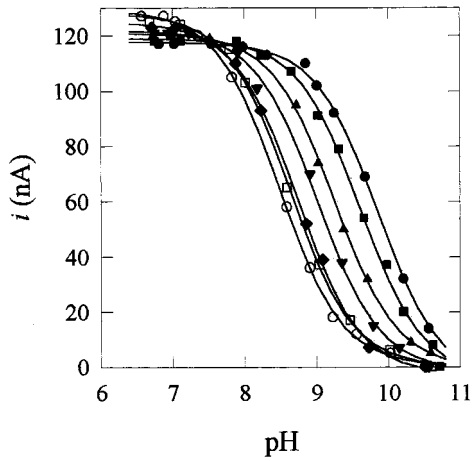


FIGURE 3: pH dependence of the current intensity of the cathodic peak (*i*) of wave I for beef heart cytochrome *c* at different temperatures in sodium acetate at $I = 0.1$ M: $T = 5$ (●), 10 (■), 20 (▲), 30 (▼), 40 (◆), 50 (□), and 60 (○) °C; sweep rate, 50 mVs^{-1}. The pH values were corrected for the temperature (*73*). Solid lines are fits to a conventional one-proton equilibrium equation.

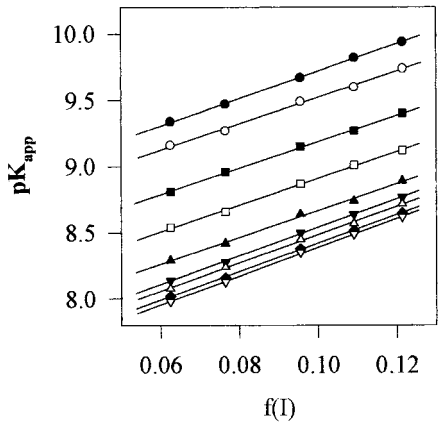


FIGURE 4: Apparent p*K* values for the alkaline transition of beef heart cytochrome *c* as a function of $f(I) = \sqrt{I}/(1 + 6\sqrt{I})$ at different temperatures: T (°C) = 5 (●), 10 (○), 20 (■), 30 (□), 40 (▲), 50 (▼), 55 (△), 60 (◆), 65 (▽). The temperature dependence of f(*I*), due to the parameter *B* (*63*), was taken into account. Solid lines are least-squares fits to the data points.

atmosphere (*1*, *49*, *57*−*62*). Peak separations (ΔE_p) in wave I were of 60 ± 2 mV for acetate concentrations lower than 0.2 M. At higher salt concentrations, ΔE_p increased up to 70 mV, indicating a slight decrease in electrochemical reversibility, possibly due to either partial anion-induced disruption of the conduction layer formed by the promoter bound to the metal surface and/or alteration of the promoter−cytochrome interaction which facilitates electron transfer. K_{app} is related to the thermodynamic equilibrium constant (K_a) by the following relationship:

$$pK_{app} = pK_a + \log \frac{\gamma_{\pm cyt\text{-}}}{\gamma_{\pm cytH}} \quad (1)$$

The mean ionic activity coefficient $\gamma_\pm$ of a solvated ion at a given ionic strength can be calculated by the extended Debye−Hückel equation within the finite-ion-size model (*63*). In particular,

$$-\log \gamma_\pm = \frac{Az^2\sqrt{I}}{1 + Ba\sqrt{I}} \quad (2)$$

where z is the charge of the ion and a is the "ion-size parameter", defined as the mean distance of closest approach between the ion and one of opposite charge belonging to the ionic atmosphere. The value of a is comprised between the sum of the crystallographic radii and the sum of the solvated radii. A and B are dimensionless constants related to solvent properties such as density and permittivity, which are dependent on $T^{-3/2}$ and $T^{-1/2}$, respectively. In water at 25 °C, $A = 0.51$ and $B = 0.33 \times 10^8$ (*63*). In the temperature range considered in this work the influence of the temperature on A and B is very small. It has been previously shown that the activity coefficient of a cytochrome *c* (which is positively charged up to pH values slightly above 10) can be successfully expressed within this theoretical framework under the assumption that the protein behaves like a low-dielectric cavity with the charge uniformly distributed on the surface, embedded in an ionic atmosphere of equal and opposite net charge (*1*, *59*, *60*, *62*). In particular

$$-\log \gamma_\pm = \frac{0.5z^2\sqrt{I}}{1 + 0.33(18)\sqrt{I}} \quad (3)$$

where z is the net protein charge and a is given the value of 18 Å (*47*, *62*) by assuming an average radius of 15 Å for bovine ferricytochrome *c*. Substitution of eq 3 into eq 1 yields the following:

$$pK_{app} = pK_a + 0.5(z^2_{cytH} - z^2_{cyt\text{-}})\frac{\sqrt{I}}{1 + 6\sqrt{I}} = pK_a + 0.5(z^2_{cytH} - z^2_{cyt\text{-}})f(I) \quad (4)$$

where z_{cytH} and z_{cyt-} are the net charges of the neutral and alkaline form, respectively (*1*, *47*). Hence, the intercept of the pK_{app} vs f(*I*) plot yields the thermodynamic equilibrium constant for the conversion of the neutral to the alkaline conformer. The plots of pK_{app} vs f(*I*) at different temperatures are shown in Figure 4. The pK_{app} values increase linearly with increasing f(*I*), according, at least qualitatively, to eq 4. The transition thermodynamics ($\Delta H^{\circ\prime}_{AT}$ and $\Delta S^{\circ\prime}_{AT}$, where AT stands for alkaline transition) can then be evaluated from the integrated van't Hoff equation:

$$pK_{app} = \frac{\Delta H^\circ_{AT}}{2.3RT} - \frac{\Delta S^\circ_{AT}}{2.3R} \quad (5)$$

namely from the plot of pK_{app} vs $1/T$. These plots for the pK_{app} values at each f(*I*) and the pK_a values extrapolated at f(*I*) = 0 are reported in Figure 5. They invariably consist of two linear segments which intersect at approximately 40 °C. The enthalpy and entropy changes for the alkaline transition in the two temperature ranges, obtained according to eq 5 at different ionic strengths, are listed in Table 1. These biphasic behaviors closely parallel that of the temperature profile of $E^{\circ\prime}$ for the native form of beef (and horse) heart cyt *c* at pH values above 7, in which an analogous break point is observed at approximately the same temperature (*34*, *38*−

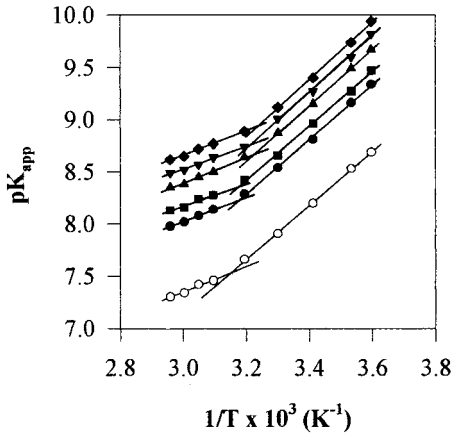


FIGURE 5: Apparent pK values for the alkaline transition of beef heart cytochrome *c* as a function of 1/T at different ionic strength (sodium acetate): $I = 0.01$ M (●); 0.02 M (■), 0.05 M (▲), 0.1 M (▼), and 0.2 M (◆). The pK values extrapolated at $I = 0$ (○) are true thermodynamic equilibrium constants. Solid lines are least-squares fits to the data points.

Table 1: Thermodynamic Parameters for the Alkaline Transition (AT) of Beef Heart Ferricytochrome *c* at Different Ionic Strengths[a] Obtained from the pK_{App} vs 1/T Plots of Figure 5[b]

		$\Delta H^{\circ\prime}_{AT}$ (kJ mol^{-1})		$\Delta S^{\circ\prime}_{AT}$ (J mol^{-1} K^{-1})	
I (M)	f(I)	low-T[c]	high-T	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 on $\Delta H^{\circ\prime}_{AT}$ 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- and high-T range 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 different temperatures (see Figure 3). These values are true standard thermodynamic parameters.

42). The ΔH°_{AT} and ΔS°_{AT} values obtained from the linear fits of the pK_a values (extrapolated at $I = 0$), are true standard thermodynamic parameters (Table 1). From these data, a pK_a value of 8.0 at 25 °C is calculated.

DISCUSSION

The voltammetric wave II which appears above pH 7 with increasing pH and/or temperature (Figure 1b) is due to the alkaline conformers (A) (*29*, *34*, *52*, *53*). The harder base character of the ϵ-amino group of the lysine ligand as compared to the thioether sulfur of the native methionine appears to be the main determinant of the dramatic decrease in $E^{\circ\prime}$ of the A form(s) as compared to the native conformer, which was shown indeed to be largely due to an enthalpic stabilization of the ferriheme (*34*). Since only one wave of the alkaline form is observed, the redox behavior of the various lysine-ligated conformers turns out to be the same, at least within the resolution of the technique: the shape of the voltammetric signal sets the upper value for the difference in reduction potentials, if any, at about 0.015 V. Since the electrochemical measurements were carried out on species at chemical equilibrium, the pK values and thermodynamic parameters determined here refer to the overall process of alkaline isomerization and must be considered average values for the various alkaline conformers. The observation of the anodic counterpart of wave II only for high scan rates is due to the fact that the reduced alkaline form is unstable and transforms rapidly in the corresponding neutral form (*29*, *34*).

Thermodynamics of the Alkaline Transition. The overall transition from the neutral to the alkaline conformer of cytochromes *c* turns out to be an endothermic process involving a small positive entropy change at low temperature and a moderate entropy loss at higher temperatures (Table 1). The $\Delta H^{\circ\prime}_{AT}$ values have coordinative and noncoordinative sources. Due to the higher affinity of the lysine nitrogen for the ferric ion as compared to the methionine sulfur, ligand substitution should not contribute to the endothermicity of the process. Hence, the determinants of the positive $\Delta H^{\circ\prime}_{AT}$ values must include the residue ionization and other noncoordinative factors such as transition-induced changes in the hydrogen bonding 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. Several contributions also concur to determine the transition entropy, $\Delta S^{\circ\prime}_{AT}$. The transition involves a net release of a proton, and it is known to originate multiple conformers. Moreover, it has been argued elsewhere that it may involve opening of the closed heme crevice structure through disruption of two key stabilizing interactions on the protein surface between Lys13 and Glu90 and between Lys79 and Ser/Thr47 (*20*). Furthermore, molecular graphics observations indicate that the Met80 side chain, once detached from the iron, would cause the displacement of the internal water molecule with the consequent disruption of the hydrogen bond network that connects it with the side chains of Tyr67, Asn52, and Thr78 (*23*). All of these processes would increase the degrees of freedom of the system; hence they would result in an increase in entropy. The fact that the ΔS°_{AT} values are either slightly positive or negative must be ascribed to the presence of opposing contributions. The rearrangement of the solvation sphere of the protein may play a significant role in this effect. In particular, we suggest two alternative hypotheses. First, there might be an increase in solvent exposition of the heme crevice in the alkaline form: for example, water molecules could originate internally hydrogen-bonded ordered molecular arrays within the hydrophobic crevice. The fact that the alkaline conformers in D_2O were found to experience greater isotope effects on the vibrational modes of the heme pocket as compared to the native species (*4*) may be consistent with this hypothesis. Second, the transition may induce a net release of water molecules from the hydration sphere of the protein to the bulk solvent, which is a solvation effect that was shown to result in an entropy loss (*64*−*67*). We note that also a depression of vibrational, rotational, and torsional motions in the A form as compared to the N form, and specific anion binding to the alkaline forms (as mentioned below) would cause a decrease in entropy. Nevertheless, at this stage clear definition of the molecular details of the processes underlying the entropy change is not at hand.

The biphasic profile of the pK_{app} vs 1/T plots (Figure 5) shows that, independently of the ionic strength, the thermodynamics of the alkaline transition of cytochrome *c* undergo a sizable modification above approximately 40 °C. The most reasonable explanation of this effect relies on the well-known

existence of two neutral conformers of cytochrome *c* [N_1 (low-T) and N_2 (high-T)] which are stable below and above approximately the same temperature (*34*, *38*−*42*), and would imply that the alkaline transitions involving the two conformers are thermodynamically distinct processes. Table 1 clearly shows that the positive free-energy change for the $N_1 \rightarrow A$ transition is entirely enthalpic in origin, because the entropy change is very small and positive. Both $\Delta H^{\circ\prime}_{AT}$ and $\Delta S^{\circ\prime}_{AT}$ decrease for the $N_2 \rightarrow A$ transition: now the isomerization is less enthalpically disfavored but involves a negative entropy change. The two effects are compensative; hence the pK_{app} values do not change dramatically in the two temperature ranges. A detailed discussion on the molecular bases of the differences between the two sets of transition thermodynamics is hampered by the absence of structural information on both the high-T native and the alkaline conformers. However, we note that the average values of $\Delta\Delta H^{\circ\prime}_{AT}$ [$= (\Delta H^{\circ\prime}_{AT})_{N1} - (\Delta H^{\circ\prime}_{AT})_{N2} = +29$ kJ mol^{-1}] and $\Delta\Delta S^{\circ\prime}_{AT}$ [$= (\Delta S^{\circ\prime}_{AT})_{N1} - (\Delta S^{\circ\prime}_{AT})_{N2} = +93$ J mol^{-1} K^{-1}] calculated from Table 1 could be indicative of differences between N_1 and N_2 in the number of water molecules released in the transition to the alkaline form. In fact, a loss of a water molecule from the hydration sphere of the protein may be responsible for a (compensating) decrease in entropy and enthalpy up to 29 J mol^{-1} K^{-1} and 8.7 kJ mol^{-1}, respectively (*64*−*67*), and the above $\Delta\Delta S^{\circ\prime}_{AT}$ and $\Delta\Delta H^{\circ\prime}_{AT}$ values would be consistent with the transition-induced release of three more water molecules from the hydration sphere of N_2 as compared to N_1. The two native isomers were already proposed to differ to some extent in terms of protein conformation around the heme and/or in solvation properties (*34*, *38*, *42*, *68*). Nevertheless, a contribution to the observed effect from peculiarities of the alkaline isomer(s) formed in the two temperature ranges cannot be excluded. In fact, $\Delta\Delta H^{\circ\prime}_{AT}$ and $\Delta\Delta S^{\circ\prime}_{AT}$ values comparable with those above (approximately +28 kJ mol^{-1} and +90 J mol^{-1} K^{-1}, respectively) have been recently determined for two different mutants of yeast iso-1 cyt *c* differing in the binding lysine (Lys 79 or Lys73) (*3*). Thus, at least part of the difference in the transition thermodynamics could originate from a temperature-induced change in the molar ratio of the two alkaline conformers possessing a different liganding lysine (detected indeed in ^{1}H NMR experiments (*54*)), or to the possible formation of a third alkaline isomer (*8*).

Previous estimates of the thermodynamics of the alkaline transition in cytochrome *c* were made by reference to a simple equilibrium between two protein conformations ["cold" and "hot" (*19*, *20*) or "reducible" and "nonreducible" (by ascorbate) (*18*)], without the involvement of a residue deprotonation. Hence, the previous apparent constants differ from the present "absolute" values by a factor corresponding to the pH at which the measurements were made. The transition enthalpies, which are given by the slope of the pK_{app} vs 1/*T* plot, are in good agreement: at $I = 0.2$ M, $\Delta H^{\circ} = +54.3$ kJ mol^{-1} (*18*), +64.4 kJ mol^{-1} (*20*), and +61.1 kJ mol^{-1} (*19*) vs +53 kJ mol^{-1} of the present work (*69*). However, as a direct consequence of the above scaling factor in the pK_{app}'s, the entropy values, which are obtained from the intercept of the van't Hoff plots, are much higher [at $I = 0.2$ M and 20 °C, $\Delta S^{\circ} = +187$ J mol^{-1} (*18*), +199 J mol^{-1} (*20*), and +180 J mol^{-1} (*19*)] than that determined here (0 J mol^{-1} K^{-1}) and have no real meaning.

The thermodynamics of the neutral to alkaline transition for the reduced cytochrome *c* can be evaluated with the simple thermodynamic cycle shown in Scheme 1 (*29*). We used the enthalpy and entropy values of the low-T neutral ferricytochrome [since the reduced cyt c does not undergo the thermal transition (*38*)] in acetate at $I = 0.1$ M. We obtained $\Delta H^{\circ\prime}_{AT,red} = +81$ kJ mol^{-1} and $\Delta S^{\circ\prime}_{AT,red} = -3$ JK^{-1}mol^{-1}, which correspond to a pK_{app} value of 14.4 at 25 °C. This value somewhat differs from that determined elsewhere for horse heart cyt *c* from direct electrochemistry experiments using the same cycle (p$K_{app} = 16.8$) (*29*). This discrepancy is likely to arise mainly from the different solution conditions used therein (a mixed buffer system plus sodium chloride), as noted elsewhere (*43*). Chloride is known to bind to both redox states of class I cytochromes *c*, inducing changes in E° (*47*, *49*). The alkaline transition for the ferrocytochrome turns out to be disfavored as compared to that of the ferri-form almost exclusively on enthalpic grounds, since the transition entropy is almost the same (Table 1). This is a direct consequence of the fact that the reduction entropies of the N_1 and A species are almost coincident [$\Delta S^{\circ\prime}_{rc} = -43$ and −45 J K^{-1} mol^{-1} in acetate, $I = 0.1$ M, respectively (*54*)]. Thus, the much greater affinity of the methionine sulfur for the Fe(II) ion as compared to the amino nitrogen of the lysine would appear to be the major factor responsible for the oxidation state-induced difference in the p*K* values for the alkaline transition in cytochrome *c*. The stability of the reduced alkaline forms is enhanced in mixed water−DMSO solutions (*43*). In this case it was shown that DMSO causes a structural change of the alkaline conformers, altering the thermodynamics of reduction and particularly the entropic term.

The Ionic Strength Dependence of the Apparent Constant for the Alkaline Transition. The pK_{app} values for the $N \rightarrow A$ transition linearly increase with increasing f(*I*), as expected from eq 4 (Figure 4). The slope of the plots is approximately +10. It decreases slightly with increasing temperature, consistent with the fact that the factor *A* in eq 4 is proportional to $T^{-3/2}$ (*63*). However, this slope is almost twice that expected from the net charge of the native and alkaline forms, namely $0.5 \times (6^2 - 5^2) = +5.5$. This difference is most likely due to the intrinsic limits of the Debye−Hückel theory applied to a complex electrolyte such as a protein (see below). However, we simply note that the observed slope would be exactly that expected in case the transition involved the loss of two positive charges [$0.5 \times (6^2 - 4^2) = +10$]. Since it is well-established that the alkaline isomerization for the mitochondrial species is a one-proton process (*1*, *2*), this result could be due to the specific binding of one acetate ion to a surface site of the alkaline form. In fact, while the acetate ion was shown not to interact specifically with neutral cyt *c*, nothing is known about its interaction with the alkaline conformer. The ability of the finite-ion-version of the Debye−Hückel theory to reproduce, at least on qualitative grounds, the ionic strength dependence of the pK_{app} values for the alkaline transition parallels that shown for the ionic strength dependence of the redox potential of a number of eukaryotic and bacterial cytochromes *c* (*49*, *57*, *58*, *60*−*62*). The fact that in some cases the ion size parameter *a* had to be modified to fit the

experimental data (*60*) is not restricted to proteins, but reflects the main drawback of the theory, which is the nature of *a* as an adjustable parameter which takes into account all of the factors that make the real system deviate from ideality (*63*). For proteins, these include the irregular shape of the molecule, the protein dielectric which can be assumed as low and homogeneous only as a rough approximation, the asymmetry in charge distribution, specific ion binding with the formation of electrically neutral ion pairs, the involvement of short-range non-Coulombic forces such as dispersion forces in the ion−ion interactions, and specific solvent effects besides that of providing the dielectric medium for the Coulombic interactions. We believe that, as long as the *a* parameter possesses a physical meaning, namely, it assumes reasonable positive values in keeping with the geometrical features of the molecule, the contribution of the above factors are such as to not invalidate the basic assumptions of the theory. This appears to be the case of cytochromes *c*. Hence, with a phenomenological reasoning, this model must be considered suitable to describe at least the main physicochemical features of the shielding of the protein charge by the ions in solution. We suggest that at least part of the success of this theory applied to the ionic strength dependence of the redox properties of cytochromes *c* could be due to the fact that these species are roughly spherical, there are only subtle oxidation state-induced structural changes (which fits with an implicit assumption of the model) (*70*−*72*), and the metal center is almost totally embedded in the protein matrix. We observed elsewhere (*35*) 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 hardly 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*.

Table 1 indicates that the ionic strength does not affect the transition enthalpy of both N_1 and N_2. Thus, the expected selective stabilization of the native conformer (which has one more positive charge than the alkaline form) by the negative ionic atmosphere which surrounds the protein has little or no effect. Of course, the possibility that the invariance of $\Delta H°_{AT}$ is the result of compensating effects arising from opposite contributions cannot be ruled out, although we believe it is rather improbable. The present data thus indicate that the *general* ionic strength effect which determines the increase in the pK_{app} values is almost entirely entropic in origin. This result cannot be easily justified at present, owing to the uncertainty regarding the determinants of the transition entropy (see above). An increase in salt concentration of the solution could (i) influence the solvation effects suggested to be involved in the entropy change; (ii) induce a depression of the flexibility of the polypeptide chain of the alkaline species as compared to the native form; and (iii) determine an increase in the number of acetate anions specifically bound to the alkaline conformers (if any). The increase in pK_a observed with increasing concentration of other ions such as phosphate and chloride (*20*), which bind to the protein at specific sites (*20*, *49*), may involve electrostatic effects that stabilize the neutral form, as suggested elsewhere (*20*).

REFERENCES

1. Moore, G. R., and Pettigrew, G. W. (1990) *Cytochromes c; Evolutionary, Structural and Physicochemical Aspects*, Springer-Verlag, Berlin, Germany.
2. Wilson, M. T., and Greenwod C. (1996) in *Cytochrome c: A Multidisciplinary Approach* (Scott, R. A., and Mauk, A. G., Eds.) Chapter 19, University Science Books, Sausalito, CA.
3. Rosell, F. I., Ferrer, J. C., and Mauk, A. G. (1998) *J. Am. Chem. Soc. 120*, 11234−11245.
4. Döpner, S., Hildebrandt, P., Rosell, F. I., and Mauk, A. G. (1998) *J. Am. Chem. Soc. 120*, 11234−11245.
5. Taler, G., Schejter, A., Navon, G., Vig, I., and Margoliash, E. (1995) *Biochemistry 34*, 14209−14212.
6. Theodorakis, J. L., Garber, E. A. E., McCracken, J., Peisach, J. Schejter, A., and Margoliash, E. (1995) *Biochim. Biophys. Acta 1252*, 103−113.
7. Ferrer, J. C., Guillemette, J. G., Bogumil, R., Inglis, S. C., Smith. M., and Mauk, A. G. (1993) *J. Am. Chem. Soc. 115*, 7507−7508.
8. Banci, L., Bertini, I., Spyroulias, G. A., and Turano, P. (1998) *Eur. J. Inorg. Chem.* 583−591.
9. Smith, H. T., and Millett, F. (1980) *Biochemistry 19*, 1117−1120.
10. Pettigrew, G. W., Aviram, I., and Schejter, A. (1976) *Biochem. Biophys. Res. Commun. 68*, 807−813.
11. Wooten, J. B., Cohen, J. S., Vig, I., and Schejter, A. (1981) *Biochemistry 20*, 5394−5402.
12. Hong, X., and Dixon, D. W. (1989) *FEBS Lett. 246*, 105−108.
13. Gadsby, P. M. A., Peterson, J., Foote, N., Greenwood, C., and Thomson, A. J. (1987) *Biochem. J. 246*, 43−54.
14. Ubbink, M., Campos, A. P., Teixeira, M., Hunt, N. I., Hill, H. A. O., and Canters, G. W. (1994) *Biochemistry 33*, 10051−10059.
15. Gupta, R. K., and Koenig, S. H. (1971) *Biochem. Biophys. Res. Commun. 45*, 1134−1143.
16. Saraiva, L. M., Thomson, A. J., Le Brun, N. E., Liu, M.-Y., Payne, W. J., LeGall, J., and Moura, I. (1994) *Biochem. Biophys. Res. Commun. 204*, 120−128.
17. Greenwood, C., and Wilson, M. T. (1971) *Eur. J. Biochem. 22*, 5−10.
18. Wilson, M. T., and Greenwood, C. (1971) *Eur. J. Biochem. 22*, 11−18.
19. Schejter, A., and George P. (1964) *Biochemistry 3*, 1045−1049.
20. Osheroff, N., Borden, D., Koppenol, W. H., and Margoliash, E. (1980) *J. Biol. Chem. 255*, 1689−1697.
21. Nall, B. T., Zuniga, E. H., White, T. B., Wood, L. C., and Ramdas, L. (1989) *Biochemistry 28*, 9834−9839.
22. Pearce, L. L., Gärtner, A. L., Smith, M., and Mauk, A. G. (1989) *Biochemistry 28*, 3152−3156.
23. Schejter, A., Luntz, T. L., Koshy, T. I., and Margoliash, E. (1992) *Biochemistry 31*, 8336−8343.
24. Angstrom, J., Moore, G. M., and Williams, R. J. P. (1982) *Biochim. Biophys. Acta 703*, 87−94.
25. Schechter, E., and Saludjian, P. (1978) *Biopolymers 5*, 788−790.
26. Brautigan, D. L., Feinberg, B. A., Hoffman, B. M., Margoliash, E., Peisach, J., and Blumberg, W. E. (1977) *J. Biol. Chem. 252*, 574−582.
27. Lambeth, D. O., Campbell, K. L., Zand, R., and Palmer, G. (1973) *J. Biol. Chem. 248*, 8130−8136.
28. Davis, L. A., Schejter, A., and Hess, G. P. (1974) *J. Biol. Chem. 249*, 2624−2632.
29. Barker, P. D., and Mauk, A. G. (1992) *J. Am. Chem. Soc. 114*, 3619−3624.
30. Kihara, H., Saigo, S., Nakatani, H., Hiromi, K., Ikeda-Saito, M., and Iizuka, T. (1976) *Biochim. Biophys. Acta 430*, 225−243.
31. Tonge, P., Wharton, C. W., and Moore, G. R. (1989) *Biochem. J. 258*, 599−605.
32. Takano, T., and Dickerson, R. E. (1981) *J. Mol. Biol. 153*, 95−115.

33. Berghuis, A. M., and Brayer, G. D. (1992) *J. Mol. Biol. 223*, 959−976.
34. Battistuzzi, G., Borsari, M., Sola, M., and Francia, F. (1997) *Biochemistry 36*, 16247−16258.
35. Battistuzzi, G., Borsari, M., Loschi, L., and Sola, M. (1997) *JBIC, J. Biol. Inorg. Chem. 2*, 350−359.
36. Battistuzzi, G., Borsari, M., Loschi, L., and Sola, M. (1998) *J. Inorg. Biochem. 69*, 97−100.
37. Battistuzzi, G., Borsari, M., Loschi, L., Righi, F., and Sola, M. (1999) *J. Am. Chem. Soc. 121*, 501−506.
38. Christen, R. P., Nomikos, S. I., and Smith, E. T. (1996) *JBIC J. Biol. Inorg. Chem. 1*, 515−522.
39. Taniguchi, I., Iseki, M., Takaki, E., Toyosawa, K., Yamaguchi, H., and Yasukouchi, K. (1984) *Bioelectrochem. Bioenerg. 13*, 373−383.
40. Taniguchi, I., Funatsu, T., Iseki, M., Yamaguchi, H., and Yasukouchi, K. (1985) *J. Electroanal. Chem. 193*, 295−302.
41. Koller, K. B., and Hawkridge, F. M. (1988) *J. Electroanal. Chem. 239*, 291−306.
42. Ikeshoji, T., Taniguchi, I., and Hawkridge, F. M. (1989) *J. Electroanal. Chem. 270*, 297−308.
43. Battistuzzi, G., Borsari, M., Rossi, G., and Sola, M. (1998) *Inorg. Chim. Acta 272*, 168−175.
44. Hildebrandt, P., Heimburg, T., Marsh, D., and Powell, G. L. (1990) *Biochemistry 29*, 1661−1668.
45. Weber, C., Michel, B., and Bosshard, H. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6687−6691.
46. Hildebrandt, P., Vanhecke, F., Buse, G., Soulimane, T., and Mauk, A. G. (1993) *Biochemistry 32*, 10912−10922.
47. Gopal, D., Wilson, G. S., Earl, R. A., and Cusanovich, M. A. (1988) *J. Biol. Chem. 263*, 11652−11656.
48. Kuwana, T. (1977) in *Electrochemical studies of biological systems* (Sawyer, D. T., Ed.) ACS Symposium Series, No. 38, American Chemical Society, Washington, D.C.
49. Battistuzzi, G., Borsari, M., Dallari, D., Lancellotti, I., and Sola, M. (1996) *Eur. J. Biochem. 241*, 208−214.
50. Armstrong, F. A., Cox, P. A., Hill, H. A. O., Lowe, V. J., and Oliver, B. N. (1987) *J. Electroanal. Chem. Interfacial Electrochem. 217*, 331−336.
51. Armstrong, F. A., Bond, A. M., Hill, H. A. O., and Oliver, B. N. (1984) *J. Chem. Soc., Chem. Commun.* 976−977.
52. Haladjian, J., Pilard, R., Bianco, P., and Serre, P.-A. (1982) *Bioelectrochem. Bioenerg. 9*, 91−101.
53. Rodrigues, C. G., Farchione, F., Wedd, A. G., and Bond, A. M. (1987) *J. Electroanal. Chem. 218*, 251−264.
54. Unpublished experiments from our laboratory.
55. Feinberg, B. A., Liu, X., Ryan, M. D., Schejter, A., Zhang, C., and Margoliash, E. (1998) *Biochemistry 37*, 13091−13101.
56. Nicholson, K. S., and Shain, I. (1964) *Anal. Chem. 36*, 706−723.
57. Battistuzzi, G., Borsari, M., Dallari, D., Ferretti, S., and Sola, M. (1995) *Eur. J. Biochem. 233*, 335−339.
58. Battistuzzi, G., Borsari, M., and Sola, M. (1997) *Arch. Biochem. Biophys. 339*, 283−290.
59. Tanford, C. (1957) *J. Am. Chem. Soc. 79*, 5340−5347.
60. Goldkorn, T., and Schejter, A. (1976) *Arch. Biochem. Biophys. 177*, 39−45.
61. Aviram, I., Myer, Y. P., and Schejter, A. (1981) *J. Biol. Chem. 256*, 5540−5544.
62. Margalit, R., and Schejter, A. (1973) *Eur. J. Biochem. 32*, 492−499.
63. Bockris, J. O'M., and Reddy, A. K. N. (1970) *Modern Electrochemistry*, Vol. 1, MacDonald, London, U.K.
64. Dunitz, J. D. (1994) *Science 264*, 670.
65. Cohen, D. S., and Pielak, G. (1995) *J. Am. Chem. Soc. 117*, 1675−1677.
66. Benini, S., Borsari, M., Ciurli, S., Dikiy, A., and Lamborghini, M. (1998) *JBIC, J. Biol. Inorg. Chem. 3*, 371−382.
67. Lee, B., and Graziano, G. (1996) *J. Am. Chem. Soc. 118*, 5163−5168.
68. Yuan, X., Hawkridge, F. M., and Chlebowski, J. F. (1993) *J. Electroanal. Chem. 350*, 29−42.
69. In contrast, a much more positive enthalpy value (+96.1 kJ mol^{-1}) was obtained by Davis et al. (*28*) from the apparent constants for the alkaline isomerization obtained from the pH titration of the 695 nm band, measured at three temperatures. In this case we simply note that the fit of the above data is rather poor.
70. Banci, L., Bertini, I., Bren, K. L., Gray, H. B., Sompornpisut, P., and Turano, P. (1997) *Biochemistry 36*, 8992−9001.
71. Banci, L., Bertini, I., Gray, H. B., Luchinat, C., Redding, T., Rosato, A., and Turano, P. (1997) *Biochemistry 36*, 9867−9877.
72. Banci, L., Gori-Savellini, G., and Turano, P. (1997) *Eur. J. Biochem. 249*, 716−723.
73. Bates, R. G. (1973) *Determination of pH, Theory and Practice*, Wiley, New York.
74. Yee, E. L., Cave, R. J., Guyer, K. L., Tyma, P. D., and Weaver, M. J. (1979) *J. Am. Chem. Soc. 101*, 1131−1137.

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