pH-Linked Conformational Regulation of a Metalloprotein Oxidation–Reduction Equilibrium: Electrochemical Analysis of the Alkaline Form of Cytochrome *c*

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Abstract: The direct electrochemistry of a variety of cytochromes *c* at pyrolytic graphite surfaces has been studied by cyclic voltammetry over a wide range of sweep rate and over a range of solution pH in which ferricytochrome *c* can exist in either the native or alkaline conformations. The electrochemistry observed is entirely consistent with both conformers of cytochrome *c* being fully electroactive at this electrode surface. Experiments at sweep rates of 1 V s^{-1} and above show that both cytochrome conformers behave as simple one-electron-transfer proteins. This finding has provided the first measurement of the midpoint reduction potential for the alkaline form of cytochrome *c* and thereby allows a complete thermodynamic analysis of the alkaline conformational cycle involving both oxidation states of the two conformers of the protein. Central to the evidence provided for this analysis are the results obtained from site-specific variants of yeast iso-1-cytochrome *c* which have altered alkaline pK_as. The reduction potential of the alkaline form of wild-type yeast iso-1-cytochrome *c* at pH 8.45 is −205 mV vs SHE. The same value is obtained for the equivalent conformer of the horse heart protein at pH 10.0. Experiments at lower sweep rates are consistent with the rearrangement of the reduced alkaline conformer to the reduced native conformer, and the rates of this interconversion have been estimated from the sweep rate dependence for all the cytochromes studied here. This study highlights the role for dynamic electrochemical analysis in the study of cytochrome *c* and its variants which undergo significant oxidation state linked conformational changes.

Introduction

Mitochondrial cytochromes *c* are the most extensively studied electron-transfer proteins[1] and as such have served as a model for many other less well characterized proteins of this type. At moderately high pH, ferricytochrome *c* undergoes a concomitant ligand replacement and conformational change to produce the so-called alkaline form of cytochrome *c*. While some effort has gone into understanding the nature of the axial ligation in this alkaline form and the kinetics of the conformational conversion, many properties of this protein remain poorly defined. In particular, the midpoint potential of alkaline cytochrome *c* has not been reported, and its electron-transfer properties have been studied in only a preliminary fashion. In this report, we use a dynamic electrochemical analysis to provide the missing thermodynamic information required for the characterization of the electron-transfer properties of alkaline cytochrome *c*.

Cytochrome *c* can exist in at least 5 pH-dependent forms,[2] but most effort has been directed at understanding the structural and functional properties of the form of the protein that exists at neutral pH (i.e. the native form). Greenwood and Palmer[3] and Wilson and Greenwood[4] were the first to show the existence of two *functionally* distinct forms of ferricytochrome *c* at alkaline pH. Their studies established that the alkaline form of cytochrome *c* is reducible by dithionite but not by ascorbate. The rate of cytochrome *c* reduction by hydrated electrons was also shown to be different at neutral and high pH.[5] In a thorough investigation of ferricytochrome *c* reduction by dithionite at alkaline pH, Palmer and colleagues[6] detected a transient species that they proposed was the reduced, ferrous, alkaline form. This intermediate subsequently underwent a pH-dependent, first-order ligand rearrangement to yield native ferrocytochrome *c*. The decrease in midpoint potential measured for many cytochromes *c* with increasing pH above their alkaline pK_a[1,7] can clearly be attributed to the presence of the alkaline form of the oxidized protein, and the pH dependence of cytochrome *c* midpoint potential can be satisfactorily described by equations involving such a pK_a.[8] However, all previous measurements of cytochrome *c* potential at high pH have used equilibrium techniques that cannot measure the true thermodynamic potential of individual species in systems involving interconverting species. Instead, such experiments measure the thermodynamic potential of the *whole system*. Consequently the midpoint potential of the alkaline form has not been reported although an upper limit of +90 mV vs SHE has been proposed.[7]

It is generally accepted that in the alkaline form of ferricytochrome *c*, one axial ligand in the native conformer, the thioether of methionine-80, is replaced by an as yet unidentified nitrogenous ligand.[1] Many have speculated that the new ligand is the deprotonated ε-amino group of a surface lysine residue,[9,10] but structural evidence for this remains elusive. It is also well-known that the trigger for the conversion is the deprotonation of the same or another residue, the pK_a of which is lowered by the subsequent conformational change[11,12] and the equilibrium constant of which favors the alkaline conformation. A minimal scheme describing the proton and conformational equilibria involved in the alkaline conversion in both ferri- and ferrocytochrome *c* is shown in Figure 1. This scheme is essentially that put forward previously[6] except that it includes the possibilities of the existence of a pH-linked alkaline conformational change for ferrocytochrome *c* and the reoxidation of the alkaline form of ferrocytochrome *c*, neither of which has so far been demonstrated conclusively.

The functional significance of the alkaline form, which is normally observed only at a pH greater than about 9 (depending on the species of cytochrome), is relevant to several recent lines of investigation. First there are suggestions that cytochrome *c*

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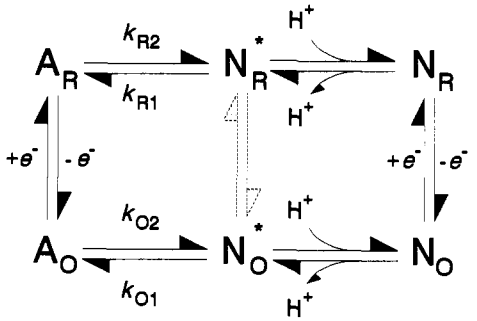

Figure 1. A minimal scheme describing the conformational equilibria involved in the alkaline transition of cytochromes *c*. N and A refer to the native (Met/His ligation) and alkaline (putative Lys/His ligation) conformations of the protein, respectively. Subscripts O and R refer to oxidized and reduced iron. Superscript asterisk denotes a singly deprotonated state with respect to the native structure, and the rates k_{O1}, k_{O2}, k_{R1}, and k_{R2} describe conformational changes. This scheme omits the changes in protonation accompanying the change of redox state in each conformational state.

may undergo some related conformational changes upon binding to its redox partners.[13] Second, site-specific variants of cytochromes *c* have been described that are drastically altered in their conformational stability.[14] In fact amino acid substitution at certain positions yields proteins in which the alkaline form is the most thermodynamically stable form of ferricytochrome *c* at neutral pH.[14b,15] Third, amino acid substitutions have been made to alter the ligands to the heme iron,[16] in various species of cytochrome *c*, and the resultant proteins exhibit multiple conformers. Finally, an understanding of the structure and properties of the alkaline conformer of cytochrome *c* will greatly enhance our ability to design novel heme-containing proteins rationally.

The present investigation assesses the oxidation–reduction properties of the alkaline form of cytochrome *c* through the use of direct electrochemical methods.[17] The achievement of direct electrochemistry at functional electrode surfaces allows the use of dynamic electrochemical techniques that are ideally suited to the identification and electrochemical characterization of interconverting species such as those depicted in Figure 1. Results presented for the wild-type and variant forms of yeast iso-1-cytochrome *c* and for horse heart cytochrome *c* are interpreted to provide a thermodynamic and kinetic characterization of the pH linkage of the conformational state and reduction potential of cytochrome *c*. The variants included in this study possess substitutions at position 82 that have been shown to lower the pK_a for the alkaline transition in ferricytochrome *c*. Significant concentrations of the alkaline form of these proteins are, therefore, accessible at pH values closer to neutrality than required for the wild-type protein.

Experimental Procedures

Wild-type, Ser-82 and Ile-82 yeast iso-1-cytochromes *c* in which the cysteine at amino acid position 102 is replaced with threonine[18] were isolated as described previously.[19] Protein concentrations were determined spectrophotometrically at pH 6.0 based on $\epsilon_{409.5}$ = 106 100 M^{-1} cm^{-1} for ferricytochrome *c*.[20] The presence of some alkaline form in the Ile-82 mutant even at pH 6.0 required determination of its concentration at 410.6 nm (an isosbestic point for native and alkaline forms) with ϵ = 105 300 M^{-1} cm^{-1}.[21] Horse heart cytochrome *c* (Sigma Type VI) was purified by cation exchange chromatography (Mono-S HR10/10, Pharmacia) as described previously.[19]

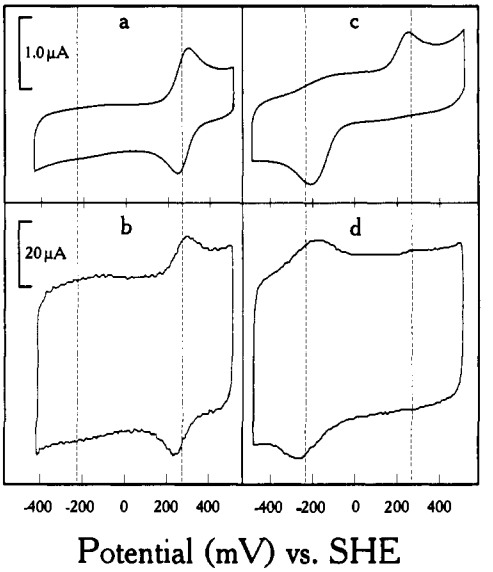

Figure 2. Cyclic voltammograms (first scans) of yeast iso-1-cytochrome *c* at a pyrolytic graphite electrode: pH 7.1 (a and b) and pH 10.3 (c and d). Sweep rates were 20 mV s^{-1} (a and c) and 2 V s^{-1} (b and d). Vertical dashed lines indicate the midpoint potential at pH 7.1 of the high potential reversible wave and of the midpoint potential at pH 10.3 of the low potential wave.

The direct electrochemistry of the cytochromes *c* was studied at a pyrolytic graphite surface with the *ab* (edge) plane of the graphite disc oriented parallel to the electrode surface.[22,23] Pyrolytic graphite (Le Carbone, U.K.) was the generous gift of Dr. H. A. O. Hill, University of Oxford. The electrode (0.12 cm^2) was cleaned by polishing with alumina (0.3 μm, Beuhler)/water slurry on a Mastertex polishing cloth (Beuhler) followed by sonication and copious washing with water. Cyclic voltammetry was carried out in three-electrode, two-compartment glass cells. One cell design allows the simultaneous measurement of solution pH by a combination electrode (Radiometer 2226C) introduced through a side arm. The calomel reference electrode (Radiometer K401) was maintained at 25 °C and connected to the sample compartment by a Luggin capillary (0.1 mm). The sample temperature was maintained at 25 °C by immersing the cell in a thermostated water-jacketed vessel. Oxygen was removed from solution by continuous flushing of the electrochemical cell with humidified oxygen-free argon. All experiments reported were carried out in a mixed buffer system consisting of MES[24] (Sigma) (5 mM), MOPS (Calbiochem) (5 mM), TAPS (Sigma) (5 mM), and NaCl (BDH) (95 mM). The pH was adjusted with NaOH (BDH) so the ionic strength of this solution therefore varies from 95 mM at low pH to 115 mM at high pH. Protein concentrations used for experiments varied from 0.1 to 0.4 mM depending on the pH. pH titration experiments were performed at 0.3–0.4 mM.

The electrode potential and sweep rate (ν) were controlled by an Ursar Electronics (Oxford, U.K.) potentiostat. The current output was either recorded directly on a Kipp and Zonen BD 90 X-Y recorder or, for sweep rates of 0.5 V s^{-1} and above, digitized (12 bit, 20 kHz A/D conversion) using a digital storage oscilloscope (Hitachi VC-6050) before output to the same chart recorder as an analog signal. The maximum sweep rate that could be handled with this system was 10 V s^{-1}.

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(24) Abbreviations used: MES, 2-[*N*-morpholino]ethanesulfonic acid; MOPS, 3-[*N*-morpholino]propanesulfonic acid; TAPS, *N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid.

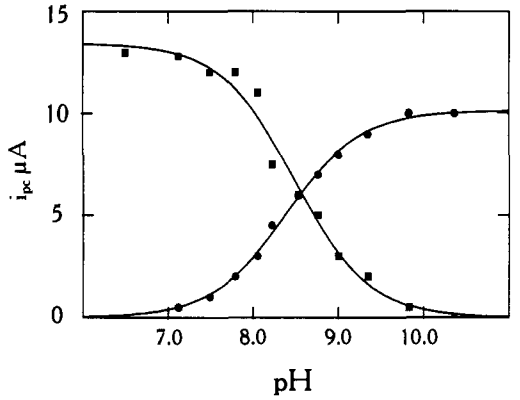

Figure 3. Cathodic peak currents at 2 V s^{-1} (second scan) for reversible electrochemistries observed at ~+270 mV (■) and at ~−200 mV (●) as a function of pH for WT yeast iso-1-cytochrome *c* (320 μM). The solid lines represent fits to an equation describing a single pK_a.

Results

The electrochemistry of yeast iso-1-cytochrome *c* at an edge oriented pyrolytic graphite electrode at neutral and alkaline pH and at two sweep rates is shown in Figure 2. The response observed at neutral pH is due to the direct electrochemistry of the native conformation of cytochrome *c* and as such this species (for the horse heart protein[22a]) has been extensively studied at a variety of electrode surfaces in many laboratories. The midpoint potential of the yeast protein (+275 mV vs. SHE) is consistent with that reported previously.[25] Estimates[26] of the diffusion coefficient (5×10^{-11} m^2 s^{-1}) and heterogeneous electron transfer rate constant (8×10^{-3} cm s^{-1}) for this electrochemical reaction are also similar to those obtained for the horse protein at this surface. Indeed, the electrochemistry is seen to be essentially reversible even at sweep rates of 2 V s^{-1} (Figure 2b) and above.[27]

At a relatively low sweep rate, the electrochemistry at pH 10.3 (at which yeast iso-1-ferricytochrome *c* is predominantly in the alkaline form[14a]) is similar (Figure 2c) to that already published for the horse protein at a modified gold surface[28] or at a glassy carbon electrode.[29] The electrochemistry consists of two waves, neither of which is reversible. On the first cathodic sweep no wave corresponding to the reduction of the native form is observed, but a reductive wave occurs at a much lower potential. On the anodic, return sweep, no reoxidation is seen at this lower potential, but a wave is observed close to that for the oxidation of the native form of the protein. This response, however, is dramatically dependent on sweep rate as highlighted in Figure 2d. At 2 V s^{-1} the response at pH 10.3 is essentially a single reversible wave with a midpoint potential of −230 mV vs SHE with no electrochemistry observed at the formal potential of the native form of the protein. The response at pH 10.3 at the sweep rates below 200 mV s^{-1} is also dependent on the scan number (data not shown). On the second and subsequent scans, a cathodic (reducing) wave is observed at the higher potential of the native protein (~230 mV). The peak current of this wave increases over several scans but never equals that measured for the associated anodic wave.

Figure 3 charts the current observed at each of the cathodic peak potentials of the two waves, at a sweep rate of 2 V s^{-1} and measured on the 2nd cathodic sweep, as the pH of the solution is increased from its initial value of 6.5. The appearance (pK = 8.4 ± 0.1) of the reversible wave at around −200 mV vs SHE directly mirrors the disappearance (pK = 8.5 ± 0.1) of the wave at around +270 mV, which corresponds to the electrochemistry

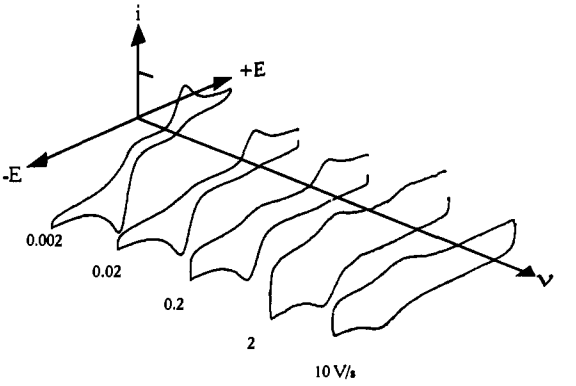

Figure 4. Representative voltammograms describing the electrochemistry of isoleucine-82 yeast iso-1-cytochrome *c* at pH 8.4 (sweep rates indicated). Potential limits are +520 and −600 mV vs SHE. The current scale bar is provided for qualitative comparison only and has *approximately* the following values for the different voltammograms: 0.002 V/s, 0.5 μA; 0.020 V/s, 2 μA; 0.20 V/s, 10 μA; 2.0 V/s, 25 μA; 10.0 V/s, 100 μA.

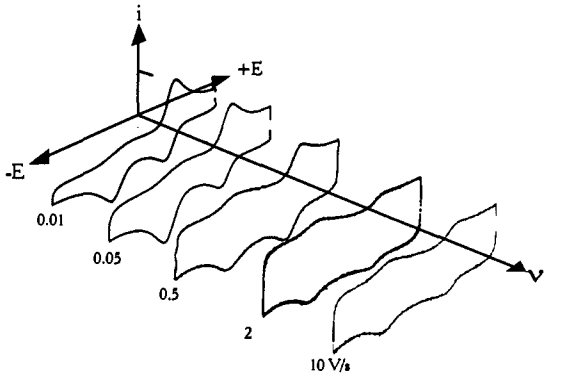

Figure 5. Representative voltammograms describing the electrochemistry of serine-82 yeast iso-1-cytochrome *c* at pH 7.7 at the indicated sweep rates. Potential limits are +520 and −600 mV vs SHE. The current scale bar is provided for qualitative comparison only and has *approximately* the following values for the different voltammograms: 0.010 V/s, 2.5 μA; 0.050 V/s, 5 μA; 0.50 V/s, 10 μA; 2.0 V/s, 25 μA; 10.0 V/s, 100 μA.

of the native form of cytochrome *c*. (The exact potentials for each process are pH dependent.) These pK values compare well with the alkaline pK_a previously reported for this cytochrome *c*.[14a] The peak currents observed for the low-potential waves are about 75% of those measured for the high-potential process at the same protein concentration. As a result, the diffusion coefficient (4×10^{-11} m^2 s^{-1}), calculated from the sweep rate dependence of the peak current, is lower than that for the high-potential response. The peak shapes also appear different in that the low-potential wave is broader, but the integrated currents of low- and high-potential waves are roughly equal. Limited analysis[26] of the sweep rate dependence of the peak separation at these high sweep rates[27] indicates that the heterogeneous electron transfer rate constant for this electrochemistry is of the same order as that of the native protein.[30]

Further correlation between the ratio of the two electrochemical waves and the pH of the ferricytochrome *c* solution has been obtained for the single-site variants Ile-82 and Ser-82. These variants have previously been shown to exhibit alkaline pK_a values of 7.2 and 7.7, respectively.[14a] Both variants exhibit electro-

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Table I. Midpoint Potentials for the Native and Alkaline Forms of the Cytochromes *c* Studied and the Observed First-Order Rate of Disappearance of the Reduced Alkaline Form[a]

	WT yeast cytochrome *c* (pK_a = 8.5)			Ser-82 yeast cytochrome *c* (pK_a = 7.7)			Ile-82 yeast cytochrome *c* (pK_a = 7.2)		
pH	E_m(Nat)[b]	E_m(Alk)[b]	k_{R2}, s^{-1}	E_m(Nat)[b]	E_m(Alk)[b]	k_{R2}, s^{-1}	E_m(Nat)[b]	E_m(Alk)[b]	k_{R2}, s^{-1}
6.00	+290[c]	−170[d]		+255[c]	−185[d]		+273[c]	−152[d]	
7.27							+265	−160	3.2 (6)
7.65				+240	−200	44 (5)			
8.45	+255	−205	60				+255	−180	5.2 (2)
8.87							+250	−195	13 (2)
9.20				+220[d]	−220	75 (3)	+235[d]	−210	17 (3)
10.0							+225[d]	−215	4.4 (5)
10.4	+230	−230	11 (1)						

[a] These rates were calculated using the shift in cathodic peak potential with sweep rate (from first scan voltammograms) as described for an E_rC_i electrochemical scheme.[31] [b] Versus SHE, ±5 mV, 25 °C, measured at 2 V s^{-1}. [c] Versus SHE, ±2 mV, 25 °C, μ = 0.1 M, measured at 0.02 V s^{-1}.[19] [d] Calculated. See text.

chemistry similar to that observed for the wild-type protein. Except for the midpoint potentials, the electrochemistry of these proteins at pH 6 is indistinguishable from that of wild-type protein.[19] With increasing pH and at high sweep rate, the reversible low-potential wave appears at the expense of the high-potential wave with p*K* values within ±0.1 of the alkaline pK_a values given above (data not shown). Experiments with these variants have the advantage that a less extreme alkaline pH is required to generate the alkaline forms of these proteins.

A detailed sweep rate dependence of the response of the wild-type protein and these two variants was carried out at a variety of pH values. Examples of the results from these experiments are shown in Figures 4 and 5. The electrochemistry of isoleucine-82 cytochrome *c* at pH 8.4 (1.2 pH units above its alkaline pK_a) is shown in Figure 4. The electrochemistry observed at each extreme of sweep rate qualitatively resembles that shown for the wild-type protein in Figure 2, parts c and d. At intermediate sweep rates, both the low- and high-potential waves appear to be partially reversible. At the alkaline pK_a of serine-82 cytochrome *c* (pH 7.7, Figure 5), two reversible electrochemical waves of similar current magnitude are observed at high sweep rates (>2 V s^{-1}). At low sweep rates (<0.05 V s^{-1}), an asymmetric voltammogram is observed. The high-potential wave has peak potentials in a reversible arrangement (ΔE_p = 57 mV), but the anodic peak current is greater than that measured for the cathodic peak. Above 1 V s^{-1}, the peak currents of both low- and high-potential waves are proportional to $\nu^{1/2}$, again suggesting that both processes arise from freely diffusing species rather than surface bound species.

The electrochemistry described above can be explained qualitatively with reference to Figure 1. Knowing that the high-potential wave is due to the one-electron reduction and reoxidation of native (N_O/N_R) cytochrome *c*, we assign the reversible, low-potential wave observed at high pH and high sweep rates to the one-electron reduction and reoxidation of the alkaline form (A_O/A_R). The midpoint potentials of both species are given in Table I. In addition, we have carried out similar experiments with the horse heart protein, for which the alkaline pK_a is 9.1 at 25 °C.[2b] At pH 10.0, the potentials measured for the native and alkaline forms of horse heart cytochrome *c* are +255 and −205 mV vs SHE, respectively.

At pH values above the alkaline pK_a for each cytochrome *c* studied here, the sweep rate dependence of both high- and low-potential waves is characteristic of chemical reactions in homogeneous solution coupled to the electrochemical steps. Both electrochemical reactions are coupled to pH-dependent, conformational reactions that can either precede or succeed the electrochemical reaction. The situation is simplified somewhat by considering only electrochemistry of the oxidized species (A_O and N_O) at bulk equilibrium concentrations, i.e., the results of single sweep experiments. At equilibrium, a solution of ferricytochrome *c* contains a mixture of native (N_O) and alkaline (A_O) forms in a ratio determined by the pH. At a pH where significant concentrations of both forms exist in solution (e.g. at the alkaline pK_a), the reduction of the native protein will alter the alkaline equilibrium, and the electrochemistry of N_O will be affected by the formation of N_O from A_O prior to electron transfer. This situation can be described by Case III (C_rE_r) of Nicholson and Shain's theoretical analysis of electrochemistry coupled to homogeneous kinetics.[31] The interconversion of A_O and N_O has been shown to proceed in a pH-independent, first-order manner. The rate-limiting step of this process is the conformational interchange between A_O and N^*_O which have rate constants of $k_{O1} \sim 10\ s^{-1}$ and $k_{O2} \sim 0.05\ s^{-1}$ for the proteins studied here.[14a] This coupled kinetic electrochemistry could be studied in isolation from the low-potential process by using a cathodic switching potential (e.g. +50 mV) well above potentials at which the alkaline form can be reduced though we have not carried out a detailed analysis of this type. However, we have observed (data not shown) that this electrochemical system does follow the theoretical behavior described for the C_rE_r scheme. In particular, the homogeneous kinetic rates known for these mitochondrial cytochromes *c* are all such[14] that the kinetics will not affect the midpoint potential of the native proteins when it is measured above a sweep rate of 20 mV s^{-1}.[19] At these higher sweep rates, the measured current reflects the concentration of the native species only as governed by the position of the equilibrium which in turn is defined by the pH. The pH dependence of the midpoint potential of native cytochromes *c* can, therefore, be studied without the complication of the alkaline form.[32]

The scheme in Figure 1 predicts that the electrochemistry of the low-potential form (A_O) should also be complicated by homogeneous reactions. However, at these low potentials the surface concentration of N_O will be very low, so the formation of A_O from N_O will be negligible (as will the second-order electron-transfer cross reaction, $A_R + N_O \rightarrow A_O + N_R$). The relevant reactions affecting this electrochemistry could therefore be the removal of A_O prior to electron transfer ($A_O \rightarrow N^*_O \rightarrow N_O$, the same as given for Case III above) or the removal of A_R subsequent to electron transfer at the electrode ($A_R \rightarrow N^*_R \rightarrow N_R$). Analysis of the low-potential process from single sweep voltammograms shows that the peak potential of the cathodic (reducing) wave shifts to more positive (anodic) potentials with decreasing sweep rates and simultaneously the associated anodic wave disappears, i.e. no reoxidation peak is observed. The data for this process can be analyzed (in isolation from the high-potential process) according to Case VI of the theoretical analysis,[31] which describes a first-order, irreversible chemical reaction succeeding electron transfer (E_rC_i). Thus, the rate of disappearance of the reduced alkaline form (A_O) controls the electrochemistry and can be estimated from the sweep rate dependence of the cathodic peak potential.[33] The rates calculated for the disappearance of A_R (which may or may

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(32) Barker, P. D.; Guillemette, J. G.; Rafferty, S. P.; Mauk, A. G., unpublished results.

(33) This method uses a theoretical working curve to relate the difference between the peak potential of the kinetically perturbed process and that of the reversible, diffusion-controlled process (as observed at high sweep rates) to the kinetic parameter, k_{obs}/a (where $a = nF\nu/RT$ and ν is the sweep rate). k_{obs} is the apparent first-order rate of reaction of the reduced alkaline form. This rate may or may not be equated with k_{R2} in Figure 1. Although the rate constants for the protonation reactions are assumed to be large, the actual rate at low proton concentrations may limit the overall process.

not correspond to the rate of overall conversion of $A_R \rightarrow N^*_R \rightarrow N_R$) at a variety of pH values for all the cytochromes investigated here are given in Table I, together with the midpoint potentials for both native and alkaline proteins (where they exist) as measured at 2 V s^{-1}. The appearance of an anodic wave corresponding to the reoxidation of the native protein after reduction of the alkaline form strongly suggests that the reduced alkaline form (A_R) rearranges to the native conformation as described in Figure 1. This conclusion is consistent with the stopped-flow spectrophotometry observations described by Lambeth et al.[6] However, the observed rate of removal of A_R for the horse heart protein at pH 10 is 33 ± 5 s^{-1}, which is significantly greater than the rates (8.5 s^{-1} at pH 9, 2 s^{-1} at pH 11) observed for the disappearance of the transient-reduced alkaline form following reduction by dithionite.[6]

Discussion

We have demonstrated that the alkaline form of mitochondrial cytochrome *c* can behave as a simple electron-transfer protein that exhibits a drastically altered reduction potential relative to the native protein. However, the reduced, alkaline form is not thermodynamically stable with respect to the reduced, native protein. Under solution conditions in which the alkaline form is the most stable conformation of the ferricytochrome, a conformational rearrangement follows the reduction of the alkaline form. This study provides the first detailed description of redox-linked conformational hysteresis by a metalloprotein.

We have used the direct electrochemistry of cytochrome *c* at an edge-oriented pyrolytic graphite electrode to characterize the voltammetric response of this small, positively charged redox protein over a wide pH range and within a wide potential window. The electrochemistry of yeast iso-1-cytochrome *c* at this surface at neutral pH is fully consistent with the considerable amount of data that have been obtained for the horse protein at graphite surfaces.[22,29] The response we observe at high pH, in which ferricytochrome *c* is present in the alkaline form, is also similar to that already observed for the horse protein.[28,29] However, sweeping the electrode voltage 10 to 100 times faster than in those previous experiments has revealed that a low potential process can be observed to be due to a reversible one-electron process involving a freely diffusing species.[34] We have made the correlation between the presence of this low-potential wave and the presence in solution of the alkaline form of not only the wild-type yeast and horse proteins but also two site-specific mutants that are known to have altered properties with regard to the alkaline transition. Results obtained for these two variants are central to the correlation between the observed electrochemistry and the presence of the alkaline form and also indicate that the residue at position 82 of cytochrome *c* influences the properties of the alkaline conformer of this protein.[14a] The broader peak shapes observed for the electrochemistry of the alkaline cytochrome, compared to that of the native protein, could arise from heterogeneity in the sites of interaction of the protein with the electrode.[23] Alternatively, it has been suggested that more than one non-interconverting alkaline isomer exists.[35] If two or more alkaline conformers are present in our experiments, the results suggest that they have similar reduction potentials.

The electrochemistry of these proteins at high pH is qualitatively consistent with an E_rC_i coupled electrochemical reaction. We conclude that the irreversible chemical step following reduction of the alkaline form leads to the production of the reduced native form from the reduced alkaline form. This conclusion is consistent with the observation of a transient, reduced alkaline form of the horse heart protein following chemical reduction by dithionite at pH 10.[6] The chemical step is not truly irreversible but has a large equilibrium constant under these conditions. The analysis using the E_rC_i scheme may break down at low sweep rates under conditions in which significant concentrations of N_O exist (e.g. at the alkaline pK_a) because removal of A_O to N_O becomes significant and the reaction scheme is better described by $C_rE_rC_i$.[36] Our analysis shows that the reduced alkaline form of cytochrome *c* is not stable under the conditions of these experiments and reverts to the native conformation in a pH-dependent first-order manner, with rate constants varying from 3 to 75 s^{-1} depending on the protein and pH.

The measurement of the reduction potential of cytochrome *c* in its alkaline conformation means that ΔG is now known for three sides of the reaction cycle depicted in Figure 1. Therefore, ΔG for the fourth side can be deduced, and the equilibrium constant for the overall conversion of reduced native form to reduced alkaline form can be calculated. The electrochemical experiments were not performed under standard state conditions, and measurement of the midpoint potentials affords $\Delta G'$. Therefore, $\Delta G'$ for the alkaline transition in the ferricytochrome *c* and pertaining to the conditions of the potential measurements must be calculated from $\Delta G°(pK_a)$ and the pH. The sum of $\Delta G'$ for the three known reactions allows $\Delta G'$ and hence $\Delta G°$ and pK_a to be calculated for the fourth. Most data have been collected for the Ile-82 variant. Calculations from the data at pH 7.27, 8.45, and 8.87 (Table I) give pK_a values of 14.4, 14.5, and 14.6, respectively ($\Delta G°$ = 19.6, 19.8, and 20.4 kcal mol^{-1}) for the alkaline transition in this variant of ferrocytochrome *c*. As the pK_a for this transition should be pH independent, we assign it a value of 14.5 ± 0.1. This result then allows us to calculate the midpoint potential of the native protein at high pH where the native ferricytochrome is not present in solution at the start of these experiments and the midpoint potentials have not been measured. These calculated potentials are indicated in Table I.[37]

The calculated pK_as for an alkaline transition in the reduced WT, Ser-82, and horse proteins are 16.3, 15.1, and 16.8, respectively. Clearly, solution conditions cannot be obtained under which the alkaline form of ferrocytochrome *c* is thermodynamically the most stable conformation. If it is reasonable to expect that the pK_a of the titratable group that is involved in the alkaline transition will not vary greatly between oxidized and reduced cytochromes, these alkaline pK_as in the ferrocytochromes suggest that the equilibrium constant for the conformational rearrangement (involving ligand exchange) is 7–9 orders of magnitude different in reduced and oxidized proteins. This difference contrasts with the two orders of magnitude difference between stability constants for methionine ligation to reduced and oxidized hemepeptide fragments of cytochrome *c*[38] and raises the question of whether the difference in potentials of native and alkaline forms (420–460 mV depending on the variant) is solely attributable to a difference in the axial ligation to the heme iron.

The low-reduction potential of the alkaline form is consistent with several proposed properties of the proteins in this form. The potential is within the range of that observed for *c*-type cytochromes containing two nitrogenous ligands.[39] Bis-histidine coordinated horse cytochrome *c* made by semisynthetic methods has a potential of +41 mV vs SHE.[16a] The still lower potential observed here for the alkaline cytochrome *c* may reflect the co-

(34) Previous work[29] has shown that horse heart cytochrome *c* can adsorb strongly to gold and glassy carbon surfaces after prolonged exposure, giving rise to apparently irreversible, low-potential electrochemistry. It was proposed that the adsorbed species was similar to the alkaline form. After a few minutes exposure of the graphite surface to ferricytochrome *c*, we have also observed electrochemistry at low potentials due to surface-bound material. This was not the case when experiments were carried out with ferrocytochrome *c*. As a result, great care was taken to ensure that the surfaces used were thoroughly cleaned between scans, and only data from first or second cycles were analyzed. The close correlation of the currents observed at low potential with the concentration of the alkaline form of the proteins, together with the dependence of peak current of $\nu^{1/2}$ at sweep rates between 1 and 10 V s^{-1}, confirms that the low-potential process is due to the direct electrochemistry of freely diffusing alkaline cytochrome *c*.

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(36) A complete analysis would account for all species and reactions involved in this cyclic process and may be termed a $(CECE)_{cyclic}$. No theoretical analysis exists as yet for this type of electrochemical scheme.

(37) The potentials could be measured if ferrocytochrome *c* were used in the experiments, but this species autoxidizes at high pH unless maintained under strict anaerobic conditions. Such experiments have not been attempted.

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ordination of a more basic ϵ-amino group of lysine although the basicity of ligands can be significantly modified by local protein structure. It may therefore be more enlightening to ask why the potential of the native form of cytochrome *c* is so high. Studies of heme-containing fragments of cytochrome *c*[40] and of noncovalent complexes formed between these fragments and apocytochrome *c*[38] have addressed the effect of methionine coordination and heme exposure to solvent on the potential of the protein. Ligation of free methionine to the iron in hemepeptide fragments leads to reduction potentials about 160 mV more oxidizing when compared to imidazole coordination. The bulk of the rest of the difference between the reduction potentials of the free hemepeptides and the native protein has been attributed to the burial of heme in a low-dielectric medium[41] although specific stereochemical constraints on the ligands within the native protein may also have some effect.[42] We suggest that the exposure of the heme to solvent in the native conformation of cytochrome *c* is significantly less than in the alkaline form.

It is interesting to note that, with only one exception,[43] all currently known modifications and amino acid replacements in mitochondrial cytochrome *c* lower, or have no effect upon, the reduction potential.[44] Carboxymethylation of the ligand, methionine-80, in native horse cytochrome *c* yields a protein that is six coordinate and low spin above pH 8 in both the ferric and ferrous forms.[45] This derivative has been suggested as a model for the alkaline form of the unmodified cytochrome. While the only reported[46] reduction potential for this modified protein is similar (−220 to −250 mV vs SHE, depending on the method used) to that of the alkaline form reported here, it was measured under conditions (pH 7) in which the reduced carboxymethylated cytochrome undergoes a pH-dependent conformational change involving a five- to six-coordination change.[45b]

Cytochrome *f* is the only other heme protein for which spectroscopic evidence suggests His/Lys axial ligation.[47] This protein has a potential of +350 to +365 mV vs SHE at pH 7.[47c,48] The high potential of this particular protein may be very unusual for a bis-nitrogen liganded *c*-type heme. Clearly, a difference of over 500–600 mV between the potentials of two heme proteins with the same putative coordinating amino acids must reflect large differences in the heme environment in each protein. In the absence of structural information for either alkaline cytochrome *c* or cytochrome *f*, we defer further comparison. The reduction potential of the alkaline cytochromes *c* falls significantly with increasing pH. The pH of zero net charge for yeast ferricytochromes *c* is around 9.4,[21] at which pH these proteins are in the alkaline form. The lowering of potentials observed for the alkaline form with increasing pH may therefore reflect the loss of positive charge on the protein that would lead to a net stabilization of the oxidized iron species relative to the reduced species.

The thermodynamics of cytochrome *c* and its variants are such that under certain conditions they exhibit redox-linked conformational hysteresis.[49] For the Ile-82 and Ser-82 variants, this phenomenum occurs at neutral pH. Consequently, measurement of the reduction potentials of these proteins by classical equilibrium techniques (either chemical or electrochemical titrations) does not resolve the mechanistic information provided here by a dynamic, nonequilibrium, electrochemical measurement and would report some average potential for the whole system. This point must be taken into consideration when analyzing the properties of variant cytochromes *c* particularly if mutation leads to destabilization of the conformation of the oxidized, native protein. This conformational hysteresis is the first to be described in such thermodynamic detail for an electron-transfer protein, and the properties are similar to those being engineered into small organometallic molecules.[50] The electrochemistry of Ile-82 cytochrome *c* at pH 8.4 (Figure 4) reveals that this protein behaves as a reasonably fast binary switch. Such behavior is a fundamental requirement in regulation of enzyme activity or in the eventual design of enzymes, the activities of which can be controlled electronically.

Summary

The use of rapid scan cyclic voltammetry and site-specific variants has established that the alkaline form of cytochrome *c* can behave as a perfectly normal one-electron-transfer protein with a midpoint potential of around −200 mV vs SHE. Measurement of this potential means that the thermodynamics of the cycle depicted in Figure 1 can now be described completely for the first time. Such an analysis reveals that the difference in reduction potential between native and alkaline cytochrome *c* is coupled to a difference in conformational energy of the two forms of about 10 kcal mol^{-1}. We ascribe the bulk of this free energy difference to the ligation of the methionine thioether and less solvent exposure of the heme in the native conformation. This study shows once again the additional information that can be obtained by using dynamic electrochemical techniques (only available once direct electrochemistry of proteins has been achieved[17b]) rather than the traditional indirect, equilibrium titrations in the study of metalloproteins. Indeed, care must be taken when interpreting the *macroscopic* equilibrium constants obtained from these latter experiments if applied to a dynamic, multicomponent system such as the one described here.

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