Proton-Linked Protein Conformational Switching: Definition of the Alkaline Conformational Transition of Yeast Iso-1-ferricytochrome *c*[‡]

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Abstract: The alkaline conformation (state IV) of yeast iso-1-ferricytochrome *c* and variants in which selected lysyl residues were replaced with alanyl residues has been studied by ^{1}H NMR spectroscopy, electronic spectroscopy, EPR spectroscopy, direct electrochemistry, pH-jump kinetics, and temperature-dependent circular dichroism spectroscopy. On the basis of the NMR studies, Lys73 and Lys79 are shown to replace Met80 as the axial ligand in the two conformers of state IV that were detected in previous studies (Hong, X. L.; Dixon, D. W. *FEBS Lett.* **1989,** *246*, 105−108; Ferrer, J. C.; Guillemette, J. G.; Bogumil, R.; Inglis, S. C.; Smith, M.; Mauk, A. G. *J. Am. Chem. Soc.* **1993,** *115*, 7507−7508). The pK_a for the conformational equilibrium between state III (native conformation) and state IV of the wild-type protein (8.70(2)) is found to be intermediate between that of the Lys73 bound conformer (8.44(1)) and that of the Lys79 bound conformer (8.82(2)) (0.1 M NaCl, 25 °C) as are the kinetic parameters for the conversion of native protein to each of the two alkaline conformers and the midpoint reduction potentials of the two alkaline forms. The EPR spectra of the Lys73Ala and Lys79Ala variants permit interpretation of the corresponding spectrum of the wild-type protein as the sum of two component conformers. The Lys79Ala variant is slightly more susceptible to thermal denaturation at pH 6.15, but the Lys73Ala variant is less thermally stable than the wild-type cytochrome or the Lys79Ala variant at alkaline pH. The Lys73Ala/Lys79Ala double variant retains the spectroscopic characteristics of the native cytochrome at moderately high pH and appears to undergo a change of axial ligation only under more alkaline conditions (pK_a ∼ 10.5). This observation suggests that the coordination of the amine ligands is a significant contribution toward the driving force for formation of the state IV conformers. These results establish the axial ligation of yeast iso-1-ferricytochrome *c* state IV, characterize the kinetics with which state III converts to state IV, and establish the electrochemical properties and thermal stabilities of the two conformers that constitute state IV. The results of this work are discussed with reference to pH-dependent structural behavior of other proteins, the mechanism by which these conformers of the ferricytochrome are formed, and the relationship of the present results to those reported previously for the formation of state IV from state III.

The influence of pH on the electronic spectrum of ferricytochrome *c* has been recognized for 60 years.[1] As described in this early work,[1c] five distinct spectroscopic forms, states I−V, of this protein can be observed between pH 1 and 12. State III dominates near neutral pH and is regarded as the native conformation of the protein.[2] Despite the extended time elapsed, elucidation of the structural changes that the cytochrome undergoes as a function of pH has been surprisingly elusive. In the past 25 years, particular attention has been directed toward the characterization of the conformational change that occurs upon titration of mitochondrial forms of ferricytochrome *c* from neutral (state III) to alkaline (state IV) pH (pK_a ∼ 8.5−9.5, depending on the species from which the cytochrome originates and the solution conditions[3]). From spectroscopic studies, it was established that conversion of native ferricytochrome *c* to the alkaline conformational state results in the disruption of the Met80−Fe bond[4] with the concomitant introduction of a new axial ligand to the heme iron that is probably provided by one of the many lysyl residues present on the surface of the protein[5] (Figure 1). Nevertheless, chemical modification studies (Table 1) failed to provide unambiguous identification of the residue

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[‡] Abbreviations: MES, 2-(*N*-morpholino)ethane sulfonic acid; CAPS, 3-(cyclohexylamine)-1-propane sulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethane sulfonic acid; TAPS, *N*-tris(hydroxymethyl)methyl-3-aminopropane sulfonic acid; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

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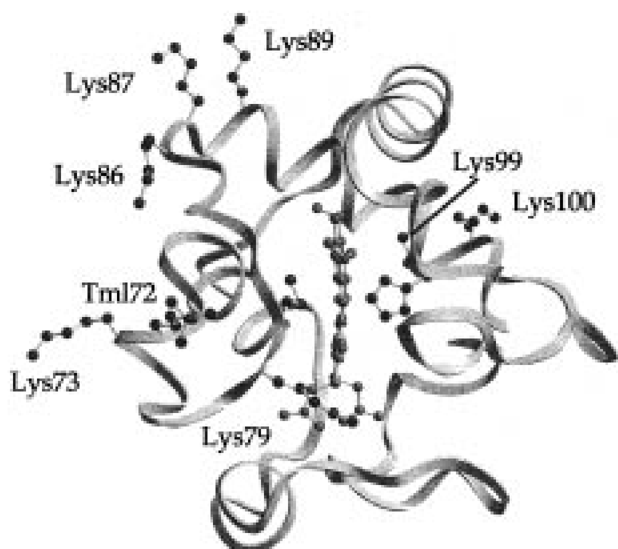

Figure 1. Structure of yeast iso-1-ferricytochrome *c* as determined by X-ray crystallographic analysis.[6] The eight lysyl residues located toward the carboxyl terminus from residue 66 onward are shown, including trimethyllysine-72 (Tml72).

involved and in some cases led to the suggestion that Met80 is replaced by a residue other than lysine.[7,9,10]

Alkaline cytochrome *c* has been known for some time to be far more difficult to reduce than the native protein,[20] yet it was not until recently that work from this laboratory[21] resulted in determination of the reduction potential for this form of the protein and demonstration that it is nearly 0.5 V lower than that of the native cytochrome. This dramatic effect of pH on the fundamental functional property of this small and relatively simple protein makes a structure-based understanding of the origin of this effect of particular interest. Presumably, modified versions of this mechanism could also explain related behavior of bacterial forms of cytochrome *c*.[22]

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With the demonstration by Hong and Dixon[23] that horse heart ferricytochrome *c* exhibits two conformers at alkaline pH and that these conformers can be differentiated by ^{1}H NMR spectroscopy, it became apparent that the limitation in previous attempts to identify the specific lysyl residue providing the sixth heme iron ligand in alkaline ferricytochrome *c* was the method used to detect conversion to the alkaline form. On the basis of this knowledge, we have previously studied the alkaline conformation of the wild-type yeast iso-1-ferricytochrome *c* and the Lys79Ala variant of this protein by ^{1}H NMR and EPR spectroscopy[24] and established that Lys79 replaces Met80 in one of the two alkaline conformers of this cytochrome. While the proximity of Lys79 to Met80 made it a logical choice for investigation in this previous study, the presence of 14 other lysyl residues in yeast iso-1-cytochrome *c* (a 15th lysyl residue, Lys72, is trimethylated[25]) makes selection of the lysyl residue most likely to be involved in formation of the second alkaline conformer more challenging (Figure 1). We now report complementary studies of additional cytochrome *c* variants that identify the lysyl residue responsible for the second alkaline conformer and that have allowed us to initiate a systematic characterization of the molecular mechanism involved in the pH linkage of ferricytochrome *c* tertiary structure. As part of this work, we have also undertaken an extended characterization of the effect of alkaline pH on these two alkaline conformers by resonance Raman spectroscopy.[26]

Experimental Procedures

Protein Preparation and Purification. The yeast iso-1-cytochrome *c* gene (*CYC*1) in the vector *p*ING-4 was mutated[27] to encode the following variants: Lys73Ala, Lys79Ala, Lys73Ala/Lys79Ala, Lys86Ala, and Lys87Ala. These variants were expressed and isolated as described previously.[28] All of the cytochromes used in this work also possessed the Cys102Thr substitution to eliminate intermolecular disulfide bond formation and to prevent autoreduction of the ferricytochromes involving Cys102.[29] For this work, therefore, the cytochrome possessing only the Cys102Thr substitution is referred to as the wild-type protein. Purified cytochromes exhibited $A_{409.5}/A_{280} \geq 4.5$ (sodium phosphate buffer, pH 7.2). Cytochrome solutions were concentrated by centrifugal ultrafiltration (Centricon-10 and Centriprep-10, Amicon, Lexington, MA) and stored at −70 °C. Concentrations of cytochrome solutions were determined on the basis of the extinction coefficient for horse heart cytochrome *c* ($\epsilon_{410} = 106.1$ mM^{-1} cm^{-1}, pH 7.0).[30] All chemicals were of reagent grade or better. A Radiometer Model 84 pH meter fitted with a Radiometer Model 2321C or pHC4406 combination electrode or an Aldrich microcombination electrode (no. Z 11,343-3) was used for determining solution pH. For NMR solutions prepared in D_2O, pH readings were not corrected for the deuterium isotope effect and are, therefore, denoted as pH*.

^{1}H NMR Spectroscopy. ^{1}H NMR spectra were recorded with a Bruker MSL-200 spectrometer operating in the quadrature detection mode at 200 MHz and equipped with a Bruker VT-1000 temperature controller. Protein samples (2 mM) were exchanged into deuterated sodium phosphate buffer (50 mM). Spectra were obtained by an 8 K

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Table 1. Effect of Chemical Modification of Surface Lysyl Residues on the Alkaline Transition of Horse Heart Cytochrome *c*

modification	protein[a]	pK_a [μ (M)]	method[b]	spin state[c]	conclusions/comments	ref
tryptic digest/complexation	1−38/39−104 T	7.1 [0.05]	EAS	low		7
	1−65/66−104 T	8.9 [0.05]	EAS	low		8
acetimidylation[d]						
complete	holoprotein	9.2−9.9	EAS			9
partial	1−38*/39−104	7.0	EAS			7
	1−38/39−104*	8.3	EAS	low	Lys is not an axial ligand in alkaline ferricytochrome *c*	7
	1−38*/39−104*	8.15	EAS			7
	1−65*/66−104	9.98	EAS			7
	1−65/66−104*	9.67	EAS			7
	1−65*/66−104*	9.18	EAS			7
selective	holoprotein vs 66−80	−	differential chemical modification	−	no difference detected in the susceptibility of Lys72, 73, or 79 to acetic anhydride	10
maleylation	holoprotein	>9 [0.15]	EAS	low	not 100% modified, denaturation occurs with reaction of >15 Lys/cyt *c*	9
(trifluoro)phenylcarbamylation						
selective	Lys13	8.9 [0.05]	EAS	low	Lys79 and possibly Lys72 may be axial ligands of alkaline ferricytochrome *c*	11
	Lys72	9.3 [0.05]	EAS	equil		11
	Lys79	8.9 [0.05]	EAS	low		11
trifluoroacetylation						
complete	holoprotein	9.9 [0.2]−10.3 [0.5]	EAS, FTIR EAS	equil	unstable OH^- bound at pH > 11	12−15
selective	Lys72	9.6 [0.05]	EAS	low		11
	Lys79	8.9 [0.05]		low		11
guanidination						
complete	holoprotein	8.8−9.4	EAS EPR, RR	equil		13, 14, 16−18
selective	1−65*/66−104	8.9 [0.05]		low		8
	1−65/66−104*	8.8 [0.05]		equil		8
acetylation						
selective	holoprotein vs 66−80	−	differential chemical modification	−	Lys72, 73, and 79 are equally modified by acetic anhydride	10
β-thiopropionylation						
selective	Lys72		EAS, EPR, NMR, and ESEEM	equil	non-native ligation	19
	Lys73			low	dimerizes easily, otherwise native characteristics	19
	Lys79			low	similar to Lys72 modified HH but slowly dimerizes like the Lys73-modified derivative	19

[a] Sequence numbers refer to peptides derived from tryptic hydrolysis or CNBr treatment of cytochrome *c* that form cytochrome-like complexes when mixed with complementary peptides (interacting peptides are separated by "/"). The asterisk denotes chemical modification of a peptide by the method indicated. [b] Abbreviations: EAS, electronic absorption spectroscopy; ESEEM, electron spin−echo envelope modulation; RR, resonance Raman spectroscopy. [c] None of the cytochrome derivatives referred to here occurs in a purely high-spin ferric form; therefore, "equil" refers to a spin equilibrium. [d] Also referred to as amidination in the older literature.

points transform of 38.5-kHz spectral width after 2000−3000 pulses with a superWEFT pulse sequence[31] and a recycle delay of 220 ms. Spectroscopic resolution was enhanced by multiplication of the free induction decay with a phase-shifted sine bell. Chemical shifts are referenced with respect to DSS through use of the residual HOD resonance. Magnetization transfer measurements were performed at 50 °C with a presaturation time of 0.5 s.

Electronic Absorption Spectroscopy. Electronic spectra were obtained with a Cary Model 219 spectrophotometer that was interfaced to a microcomputer (OLIS, Bogart, GA) and fitted with a jacketed cuvette holder that was maintained at 25 °C with a thermostated, circulating water bath (Lauda Model RMS6). The variation of the electronic spectrum (500−725 nm) of the wild-type and variant cytochromes (0.2 mM in 0.1 M NaCl) with pH was determined by titration with base (0.1 M, NaOH) starting at pH 6.0. The resulting change in absorbance at 695 nm was used to derive the equilibrium pK_a of the transition by a nonlinear least-squares fit of the data to the Hendersson−Hasselbach equation (MINSQ version 4.02, MicroMath, Salt Lake City, UT) for the ionization of a single titrating group.

The ionic strength dependence of the alkaline pK_a was studied in KCl solutions of varying concentration and in the absence of buffer (25 °C). The results were analyzed by a weighted least-squares fit of the data to the Gouy−Chapmann relationship as implemented by Koutalos and co-workers:[32]

$$\sinh[0.5 \ln 10\,(pK_{true} - pK_{apparent})] = A\sigma C^{-1/2} \quad (1)$$

In this equation, $A = 8\epsilon\epsilon_0 N_A kT^{1/2} = 76.3\ M^{1/2}\ Å^2$ (assuming that T = 25 °C and that ϵ, the dielectric constant for yeast cytochrome *c* (25 °C), is 25 ± 10 °C[33]). This analysis yields the parameter σ, which in principle represents the surface charge density in the vicinity of the deprotonating group that titrates with the intrinsic pK_{true}.

EPR Spectroscopy. EPR spectra (10 K) were obtained at X-band frequency (∼9.5 GHz) with a Bruker ESP Model 300E spectrometer equipped with an Oxford Instruments Model 900 liquid helium cryostat, an Oxford Instruments ITC4 temperature controller, and a Hewlett-Packard Model 5352B frequency counter. To obtain spectra of the native cytochromes, protein solutions were exchanged into potassium phosphate buffer (20 mM, pH 6.6), concentrated to 2 mM by centrifugal ultrafiltration, and diluted with an equal volume of glycerol. For measurements at alkaline pH, protein solutions (3 mM protein in 10 mM NaCl) were mixed with buffer (CAPS, 100 mM, pH 10.5) and glycerol in a 1:1:2 ratio. Solution pH was adjusted to 10.5 by addition of sodium hydroxide solution, and the solution was frozen immediately in liquid nitrogen. The experimental conditions were as follows: microwave power 0.63−2 mW, microwave frequency 9.45 GHz, modulation frequency 100 kHz, and modulation amplitude 1 mT.

Direct Electrochemistry. Midpoint reduction potentials of the native cytochromes (state III; 0.2 mM, pH 6.0) were determined from

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cyclic voltammograms[28,34] collected between 0 and 500 mV vs SHE at a gold electrode (3 mm diameter) modified with 4,4′-dipyridyl disulfide (Aldrich) and with a sweep rate of 20 mV s^{-1}. Cyclic voltammetry of the Lys73Ala and the Lys79Ala variants was also studied as a function of pH (25 °C) through use of faster sweep rates and a carbon electrode as described previously for wild-type cytochrome.[21] For these latter experiments, the variants (320 μM) were studied at scan rates of 2 V s^{-1} with a 2 × 3 mm edge-oriented pyrolytic graphite electrode that was polished prior to each measurement with 0.3-μm alumina (Buehler, Lake Bluff, IL) slurries. The buffer used in previous work[21] was replaced with NaCl solution (μ = 0.1 M) to achieve the same solution conditions as used in the spectrophotometric titrations of these proteins.

Solution potentials were measured against a saturated calomel reference electrode (Radiometer Model K401) that was standardized against quinhydrone. Measured potentials were converted to the standard hydrogen electrode (SHE).[35] Electrode potentials and sweep rates were controlled with an Ursar Electronics (Oxford, U.K.) potentiostat. Current output was either recorded with a Kipp and Zonen BD 90 X−Y recorder or, for sweep rates of 0.5 V s^{-1} or greater, digitized with a digital storage oscilloscope (Hitachi Model VC-6050; 12 bit, 20-kHz A/D conversion) and then downloaded to the plotter. In more recent experiments, current output was digitized with a microcomputer that was interfaced to the potentiostat (Blue Moon Technical Services, Vancouver) with LabWindows software (National Instruments).

pH-Jump Kinetics. Unbuffered solutions of the wild-type cytochrome and the Lys73Ala and Lys79Ala variants (9 μM) were prepared in 0.1 M NaCl and adjusted to pH 6.0 by addition of HCl solution (0.1 M). These protein solutions were then mixed in the stopped-flow apparatus with the following buffers: MES (Sigma) (40 mM, pH 6.07), sodium phosphate (BDH, Fisher) (pHs 7.07, 7.50, and 7.57), TAPS (Sigma) (pHs 8.09, 8.19, 8.43, 8.76, and 8.94), CHES (Sigma) (pHs 9.05, 9.54, and 9.80), NaH_2BO_3 (Baker) (pHs 9.01, 9.28, 9.50, 9.70, and 9.89), and CAPS (Sigma) (pH 10.17). Each of these buffers was prepared with sufficient NaCl to achieve a final ionic strength of 0.1 M.

The rapid mixing experiments were performed with a Dionex Model S-100 stopped-flow spectrophotometer (2-cm path length) that was interfaced to a microcomputer and maintained at constant temperature (25.0 ± 0.2 °C) with a thermostated, circulating water bath (Haake Model F3). Absorbance changes were monitored at 390 nm; absorption changes at this wavelength have been shown to reflect the same event as that recorded by monitoring the 695-nm band.[3,36]

The resulting kinetics data were fitted to a first-order exponential function, and the rate constants (k_{obsd}) determined in this manner were fitted to the minimal model described by Davis et al.[37] This model postulates a two-step mechanism in which the protonation state of a titratable (or "trigger") group is functionally linked to the interconversion of states III and IV of ferricytochrome *c*. For this model, the observed rate constant can be described by eq 2:

$$k_{obsd} = k_b + k_f K_H (K_H + [H^+])^{-1} \quad (2)$$

where k_f and k_b are the forward and reverse rate constants, respectively, K_H is the dissociation constant of the titrating group, and $[H^+]$ is the proton concentration determined by the pH of the buffer. In this analysis, the rate and dissociation constants were constrained to agree with the equilibrium pK_a of the alkaline transition determined independently by monitoring the change in absorbance at 695 nm as a function of pH. This constraint obeys eq 3:

$$pK_a = -\log_{10}(k_f K_H / k_b) = pK_c + pK_H \quad (3)$$

Data fitting was performed by a nonlinear least-squares method with the program SCIENTIST (vers. 2.0, MicroMath, Salt Lake City, UT).

Analysis of Thermal Stability. The stability of selected ferricytochromes to thermal denaturation was studied with a Jasco Model J-720 spectropolarimeter that was equipped with a Neslab Model RT 110 circulating water bath. Protein samples [10−15 μM; sodium phosphate buffer (pH 6.15, μ = 0.01 M) or sodium borate buffer (pH 9.58, μ = 0.01 M)] were placed in a water-jacketed, cylindrical quartz cuvette (0.1-cm path length), and the cuvette temperature was measured with a Neslab RS-2 remote sensor interfaced to a computerized data acquisition system. Thermal denaturation was studied by monitoring ellipticity at 222 nm as the temperature was increased from 55 to 85 °C at 50 °C/h. For each protein studied, three thermal denaturation curves were added and then smoothed with the function provided by the Jasco software; the first derivative of each such curve was then calculated to determine T_m of the thermal denaturation curve. If thermal denaturation is assumed to be a two-state process, the T_m is the temperature at which 50% of the sample was denatured. The reversibility of thermal denaturation was evaluated by reversing the direction of the thermal ramp soon after protein denaturation was completed. Reversibility determined in this manner is expressed in terms of the fraction of α-helical structure restored upon cooling. We estimate the uncertainty of T_m determined by this procedure to be ∼±0.5 °C.

Results

^{1}H NMR Spectroscopy. The ^{1}H NMR spectroscopy of the ferricytochrome *c* variants at neutral pH* indicates that no major perturbations in the heme pocket result from the amino acid substitutions employed in this study. In the high-field region of the spectra, all of the variant cytochromes display the characteristic γ-CH_2 and ϵ-CH_3 resonances of the Met80 axial ligand (Table 2). The Fischer nomenclature used to identify structural elements of the heme prosthetic group in this work is defined in Figure 2. The small differences observed in the chemical shifts of equivalent heme methyl groups reveal that the unpaired electron spin distribution in the heme of most of the variants is not significantly different from that observed for the wild-type cytochrome. A representative downfield spectrum of the wild-type protein recorded at neutral pH* and 20 °C is provided in Figure 3A.

The ^{1}H NMR spectra of the Lys79Ala and Lys73Ala/Lys79Ala variants exhibit a number of similarities to each other and differ more than the other variants from the spectrum of the wild-type protein. The substitution for Lys79 affects not only the resonances of the heme substituents but also those of residues that are not bound directly to the heme iron. Notably, Pro30 undergoes a downfield paramagnetic shift of 1.1 ppm that is solely dipolar in nature in the spectra of variants in which Lys79 has been replaced with an alanyl residue (Table 2). Although this is not the greatest shift observed, it indicates that replacement of the lysyl residue at position 79 by an alanyl residue influences the magnetic anisotropy axes of the protein.

The downfield ^{1}H NMR spectra of the variants at alkaline pH* are compared to that of the wild-type protein in Figure 3B-G. The resonances corresponding to the heme methyl group protons of both the native (>30 ppm; labeled III and with the corresponding position on the porphyrin ring) and the alkaline (25−5 ppm; labeled IV) conformations are most informative in this region. These spectra reveal that the wild-type protein, the Lys86Ala variant, and the Lys87Ala variant can assume two conformational states at alkaline pH as previously observed for the horse[23] and yeast[24] wild-type proteins. At high pH, the signals of each heme methyl group appears as a pair of

(34) (a) Taniguchi, I.; Toyosawa, K.; Yamaguchi, H.; Yasukouchi, K. *J. Chem. Soc., Chem. Commun.* **1982**, 1032. (b) Taniguchi, I.; Toyosawa, K.; Yamaguchi, H.; Yasukouchi, K. *J. Electroanal. Chem.* **1982**, *140*, 187−193. (c) Allen, P. M.; Hill, H. A. O.; Walton, N. J. *J. Electroanal. Chem.* **1984**, *178*, 69−86.

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Table 2. ^{1}H NMR Parameters of Native Wild-Type and Variant Yeast Iso-1-cytochromes *c*[a]

group	chemical shift (ppm)					
	WT	Lys73Ala	Lys79Ala	Lys73Ala/ Lys79Ala	Lys86Ala	Lys87Ala
8-CH_3	35.2	32.5	34.5	34.6	35.0	34.6
3-CH_3	32.1	32.0	32.5	32.5	32.0	31.6
His18, δ-CH	24.9	25.0	23.8	23.7	25.0	24.2
5-CH_3	10.4	10.3	11.9	11.8	10.3	10.5
Pro30, δ-CH_2	−6.0	−6.0	−4.9	−4.9	−6.0	−5.7
Met80, ϵ-CH_3	−23.7	−23.8	−22.2	−22.3	−23.7	−23.2
His18, ϵ-CH	−26.9	−26.9	−24.8	−24.8	−26.9	−26.3
Met80, γ-CH_3	−31.1	−31.3	−31.0	−31.2	−31.2	−30.6

[a] Deuterated sodium phosphate buffer (20 °C, pH* 7.0).

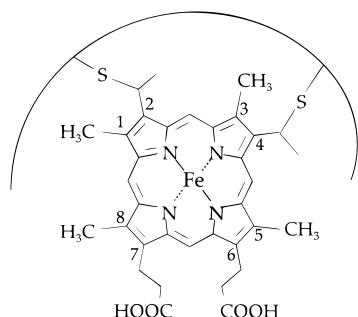

Figure 2. Diagram of the heme prosthetic group of cytochrome *c* and the Fischer nomenclature used in the text. The carbon atoms of the propionate substituents at positions 6 and 7 are labeled α and β. The His18 ligand lies below and the Met80 ligand lies above the plane of the heme as drawn in this diagram.

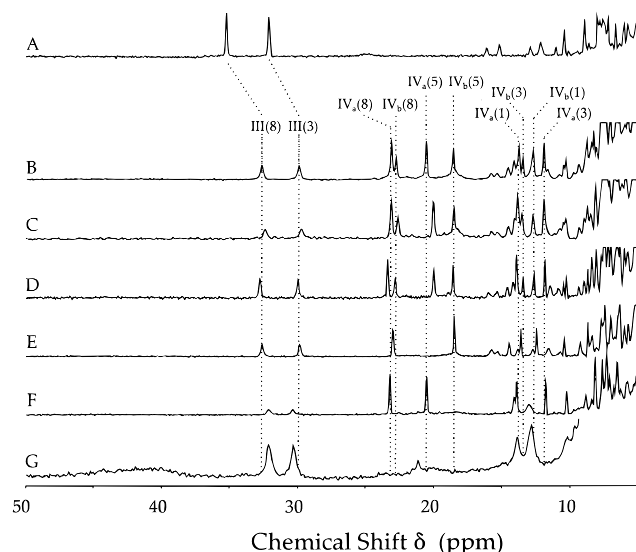

Figure 3. Downfield region of the 200-MHz ^{1}H NMR spectra of yeast iso-1-ferricytochromes *c* (50 mM deuterated sodium phosphate buffer). Native ferricytochrome is denoted as state III, the alkaline conformer with Lys73 coordinated is denoted as state IV_a, and the alkaline conformer with Lys79 coordinated is denoted as state IV_b. The number of the heme methyl group (Figure 2) to which each resonance is assigned is indicated in parentheses. (A) Native wild-type ferricytochrome *c* (pH* 7.0, 20 °C). Corresponding spectra of the alkaline cytochromes (pH* 9.3, 45 °C) of the (B) wild-type, (C) Lys86Ala, (D) Lys87Ala, (E) Lys73Ala, and (F) Lys79Ala proteins. Spectrum G is the spectrum of the Lys73Ala/Lys79Ala variant recorded at pH* 10.0 and 45 °C.

resonances that correspond to the two alkaline conformers. In contrast, no such degeneracy is observed in the spectra of the Lys73Ala or Lys79Ala variants (Figure 3E,F). This latter observation is consistent with the formation of a single alkaline conformational state by these two variants. Significantly, the alkaline protein conformer exhibited in the spectrum of the Lys73Ala variant is absent from the spectrum of the Lys79Ala variant and vice versa. Furthermore, addition of the spectra of these two variants produces a spectrum that is equivalent to that observed for the wild-type protein under similar conditions. From these results, we conclude that the two alkaline conformers of ferricytochrome *c* result from replacement of Met80 with either Lys79 or Lys73.

The ^{1}H NMR spectra of the Lys79Ala and Lys73Ala variants at alkaline pH confirm that no high-spin or mixed-spin iron species are formed under the conditions studied and, therefore, that upon raising the pH, elimination of either Lys73 or Lys79 does not lead to the formation of alternative types of ligation to the heme iron. In contrast, the Lys73Ala/Lys79Ala variant possesses neither of the ϵ-amino groups that are capable of coordinating to the heme iron. As a result, Met80 is retained as an axial ligand to the heme iron of the double variant at pH* 10 as indicated by the characteristic resonances in the high-field region of the spectrum (Figure 3G) that are attributed to protons of the Met80 ligand (Table 2) and by retention of the downfield resonances of heme methyl groups 3 and 8 that are characteristic of the native protein (Figure 3A).

At higher pH*, a high-spin iron species (state V[1]) dominates. Formation of this species is correlated with the appearance of a broad resonance downfield in the ^{1}H NMR spectrum of the double variant (~40 ppm) (Figure 3G). Increasing the pH* to 10.7 results in abolition of the resonances characteristic of the native protein, retention of the resonance at 40 ppm, and concurrent appearance of three new resonances between 10 and 25 ppm. These latter resonances are tentatively assigned to heme methyl groups of low-spin heme iron complexes in which hydroxide has probably replaced Met80 as an axial ligand (cf. ref 26).

The temperature dependence of the equilibrium of the native (state III) conformation of the Lys73Ala variant and its alkaline conformer (state IVa/b) is depicted by the spectra shown in Figure 4. Similar data have been reported previously for the wild-type protein and for the Lys79Ala variant.[24] These spectra confirm that the alkaline form of the protein is favored as temperature is increased.[38] Equilibrium constants (K_{eq}) were determined from these spectra by integration of the heme methyl peaks of both forms and analyzed with van't Hoff plots (Figure 5). For the binding of Lys79 (Lys73Ala variant), $\Delta H^\circ = 6.4(3)$ kcal/mol and $\Delta S^\circ = 20.8(8)$ eu, while for the binding of Lys73 (Lys79Ala variant), $\Delta H^\circ = 13.2(4)$ kcal/mol and $\Delta S^\circ = 43(1)$ eu. Both the entropic and enthalpic changes associated with the alkaline conformational change of the Lys79Ala variant are twice those determined for the corresponding conformational change of the Lys73Ala variant and wild-type[24] horse heart ferricytochrome *c*. Coordination of Lys79 to the heme iron

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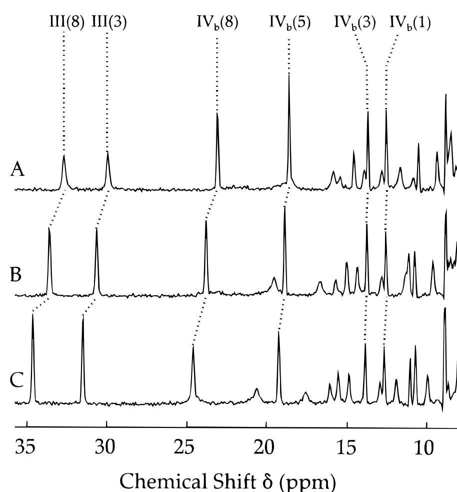

Figure 4. Representative downfield 200-MHz ^{1}H NMR spectra of iso-1-ferricytochrome *c* Lys73Ala/Cys102Thr variant as a function of temperature in deuterated sodium phosphate buffer (50 mM, pH* 9.3). The heme methyl resonances are labeled as in Figure 2, and the convention for labeling of resonances is the same as in Figure 3: A, 45 °C; B, 35 °C; C, 25 °C.

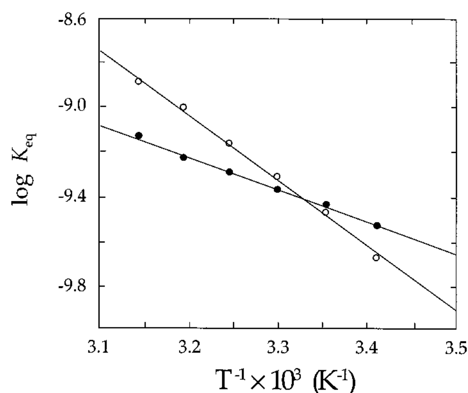

Figure 5. van't Hoff plot of the neutral/alkaline equilibria of the Lys73Ala and Lys79Ala variants of yeast iso-1-ferricytochrome *c* at pH* 9.3 (solution conditions are the same as for Figure 3). K_{eq} values were determined from the relative areas of the heme 8-methyl resonances in the alkaline and native conformations: (●) Lys73Ala, (○) Lys79Ala.

requires the disruption of hydrogen bonds formed by this residue with Ser47, Tyr46, and heme 6-propionate followed by movement of the polypeptide chain to bring Lys79 into the coordination sphere of the heme iron. On the other hand, nearly the entire left face of the protein (as viewed in Figure 1) must rearrange to move the ε-amino group of Lys73 ∼18 Å to permit coordination by this residue.

With identification of Lys73 and Lys79 as the residues that replace Met80 as an axial ligand in states IV of ferricytochrome *c*, all subsequent studies have been limited to the variants in which these two residues were replaced with alanine.

Electronic Absorption Spectroscopy. The electronic spectra of the reduced and oxidized forms of the variants used in this study are essentially identical to those of wild-type yeast iso-1-cytochrome *c* at neutral pH where the native conformation (state III) of the protein dominates. Notably, all six ferricytochromes display the weak absorption maximum centered at 695 nm that is characteristic of methionine−Fe(III) ligation.[3,39]

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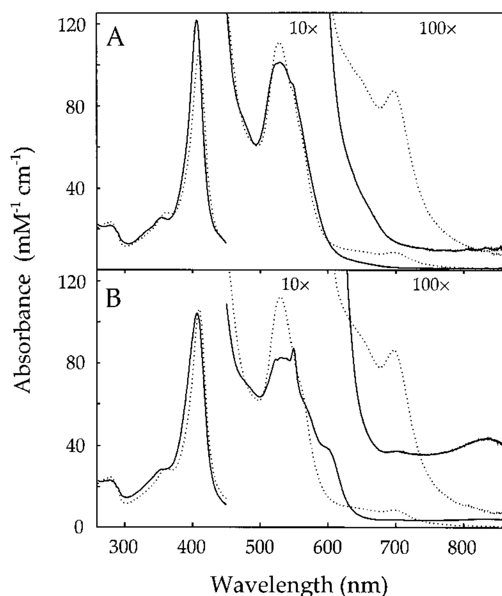

Figure 6. Electronic absorption spectra of the native (dotted lines) and alkaline (solid lines) forms of (A) wild-type iso-1-ferricytochrome *c* and (B) the Lys73Ala/Lys79Ala variant. Native proteins were prepared in sodium phosphate (100 mM)/CAPS buffer (20 mM), pH 6.8, 25.0 ± 0.2 °C. The alkaline proteins were prepared from these same samples by titration to pH ∼ 10.5 with NaOH solution (0.1 M).

At alkaline pH, the electronic spectra of the variants other than the Lys73Ala/Lys79Ala variant are nearly indistinguishable from that of the wild-type ferricytochrome *c* at high pH and 25 °C (Figure 6A). The Soret maxima of these variants are shifted (∼3.3 nm) to higher energy and exhibit increased intensity (∼14%). In the visible region, the intensities of the α and β bands decrease (∼9%), broaden, and shift (∼0.8 nm) to higher energy. As expected, the intensity of the weak band centered at 695 nm decreases at alkaline pH for all of the cytochromes used in this study as the Fe(III)−Met80 bond is disrupted by the pH-induced change in protein structure.

In contrast, weak absorption maxima at ∼600 and 840 nm are observed in the spectrum of the Lys73Ala/Lys79Ala variant at pH 10.5 (Figure 6B) that are not present in the corresponding spectra of the other proteins studied here. These maxima suggest the presence of a high-spin heme iron species when this variant is exposed to this pH. Such species may also be formed by the single variant and the wild-type proteins under these conditions but to an extent that is not sufficient to be detected by electronic spectroscopy. At elevated pH, the Soret band of the double variant is shifted to higher energy as observed for the wild-type protein, and the intensities of the α and β bands decrease (∼24%) and resolve into three maxima at 534.5, 523, and 550 nm. The maxima at 523 and 550 nm presumably result from partial autoreduction of the protein.

The pK_a for the conformational change that occurs as the pH of the native ferricytochrome is increased can be determined from the dependence of the absorbance at 695 nm on pH. The pK_as determined in this manner for the present variants are shown in Table 3 (last column on the right). Although the standard deviations derived from the nonlinear least-squares fitting process (shown in parentheses in Table 3) are quite small, we estimate that the reproducibility of the value for the Lys73Ala/Lys79Ala double variant may be as poor as ±0.3 pK_a units. The results obtained for the double variant are complicated because the decrease in absorbance at 695 nm that is observed as the pH is increased is followed by an increase in absorbance as the pH is increased further, thereby preventing

Table 3. Equilibrium and Kinetic Parameters for the Alkaline Transition of Yeast Ferricytochromes *c*

protein	k_f (s^{-1})	$k_b \times 10^2$ (s^{-1})	K_c	pK_H	pK_c + pK_H	pK_a[a]
wild-type	48(2)	3.5(1)	13.9(8) × 10^2	11.7(2)	8.6(2)	8.70(2)
wild-type[b]	8.5(3)	3.5(1)	2.4(1) × 10^2	11.0(1)	8.6(2)	8.5
Lys73Ala	1.51(5)	1.6(1)	9.4(7) × 10	10.8(1)	8.8(1)	8.82(2)
Lys79Ala	160(5)	4.0(7)	4.0(7) × 10^3	12.0(3)	8.4(4)	8.44(1)
Lys73Ala/Lys79Ala			nd			10.5
Lys86Ala			nd			8.60(4)
Lys87Ala			nd			8.47(2)

[a] Apparent pK_a determined by nonlinear least-squares analysis of pH titration data measured at 695 nm. [b] Reference 42; in this reference, the parameters for recombinant wild-type cytochrome *c* with Thr at position 102 and for nonrecombinant wild-type cytochrome *c* that possesses Cys102 were inadvertently reversed (i.e., the line labeled "Cys-102 W.T." should bear the label "Thr-102" and vice versa).

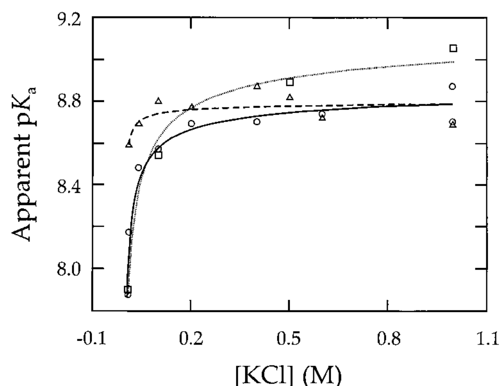

Figure 7. Dependence of the pK_a for the alkaline conformational transition of wild-type and variant forms of yeast iso-1-ferricytochrome *c* on ionic strength (KCl, 25 °C): (○, solid line) wild-type ferricytochrome *c*; (□, dotted line), the Lys79Ala variant; (△, dashed line), the Lys73Ala variant.

precise determination of the end point of the titration. Interestingly, the pK_a values observed for the wild-type protein and for the Lys86Ala and the Lys87Ala variants are intermediate between the values determined for the Lys73Ala and Lys79Ala variants.

Although the sensitivity of the pK_a for the alkaline conformation to ionic strength has been known for some time,[40] this dependence appears not to be widely recognized. As quantification of this dependence is helpful in comparing data from experiments conducted under a range of experimental conditions, the dependence of this pK_a for the wild-type, Lys73Ala, and Lys79Ala variants was determined (Figure 7). Interestingly, the dependence of this pK_a for the Lys79Ala variant on ionic strength is much greater than that observed for the pK_a for the Lys73Ala variant. As a result, the pK_a for the Lys79Ala variant is lower than that of the Lys73Ala variant at lower values of ionic strength and at higher μ this situation is reversed. The wild-type ferricytochrome exhibits behavior that is similar to that of the Lys79Ala variant at low ionic strength and similar to that of the Lys73Ala variant at higher ionic strength.

As with any electrostatic model that might have been used to fit the pK_a vs ionic strength data obtained here, the physical meaning of the parameters derived from use of the Gouy−Chapman formalism is questionable. This uncertainty is particularly noteworthy for applications in which the pK_a does not relate to a simple protonation/deprotonation equilibrium but is a composite of a simple pK_a and a coupled conformational equilibrium as discussed below. For this reason, these values are reported here primarily for the sake of completeness. From this analysis, the resulting pK_{true} values are 8.87 (wild-type cytochrome), 8.82 (Lys73Ala variant), and 9.17 (Lys79Ala variant), while σ values are 0.013 Å^{-2} (wild-type cytochrome), 0.00035 Å^{-2} (Lys73Ala variant), and 0.0028 Å^{-2} (Lys79Ala variant).

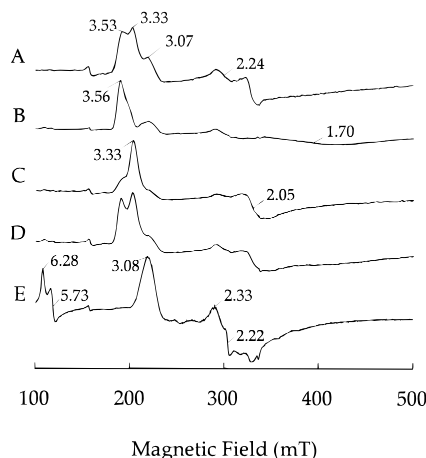

Figure 8. X-band EPR spectra of (A) wild-type, (B) Lys73Ala, (C) Lys79Ala, and (E) Lys73Ala/Lys79Ala yeast iso-1-ferricytochrome *c* (50 mM CAPS buffer, pH 10.5, 50% (v/v) glycerol). Spectrum D is the sum of spectra B and C.

EPR Spectroscopy. The EPR spectra of the native wild-type and variant cytochromes are all indicative of a low-spin ferric heme complex possessing a histidine/methionine coordination environment[5a,d,e] with *g* values of ~3.07, 2.24, and 1.15. On raising the pH, multiple low-spin species are observed in the spectrum of the wild-type protein (Figure 8A), the Lys86Ala and Lys87Ala variants (data not shown), and the Lys73Ala and Lys79Ala variants (Figures 8B and 8C). At pH slightly greater than the pK_a, g_z (3.07) and g_y (2.24) can be assigned to the residual proportion of the sample frozen in the native state. The *g* values of 3.53 and 3.33 have been observed previously for horse heart cytochrome *c*[5a,d,e] and are within the range expected for a heme coordinated axially by a histidine and an amine group as exemplified by cytochrome *f* (g_z = 3.51, g_y ~ 1.70)[41] and the *N*-butylamine adduct of leghemoglobin (g_z = 3.38, g_y ~ 2.05).[5e]

From the EPR spectra of the alkaline forms of the variants, it is clear that the signals with g ~ 3.33 and 3.53 correspond to the two conformational states of the protein that arise from the coordination of Lys79 (g = 3.53) (Figure 8B) or Lys73 (g = 3.33) (Figure 8C) to the heme iron. Gadsby et al.[5e] observed similar *g* values for wild-type horse heart cytochrome *c* under these conditions and, interestingly, reported that the relative intensities of these resonances are reversed by the addition of ethanediol. As in the ^{1}H NMR spectra of the Lys73Ala and Lys79Ala variants described above, the EPR spectra of these proteins lack the spectroscopic features that result from coordination of the Lys residue that is replaced by Ala in each

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(41) Rigby, S. E.; Moore, G. R.; Gray, J. C.; Gadsby, P. M.; George, S. J.; Thomson, A. J. *Biochem. J.* **1988,** *256*, 571−577.

Table 4. Reduction Potentials of the Native and Alkaline Conformers of Yeast Iso-1-cytochrome *c*

protein	reduction potential (mV vs SHE)			
	native[a]	alkaline[b]		
wild-type	290	−170 (6.0)[c]	−205 (8.45)	−230 (∼10.4)
Lys73Ala	287	−160 (∼6.7)	−190 (∼8.9)	−240 (∼10.6)
Lys79Ala	289[d]	−170 (∼6.7)	−190 (∼8.4)	−240 (∼10.4)
Lys73Ala/Lys79Ala	291		nd	

[a] Sodium phosphate buffer (μ = 0.1 M, pH 6.0), at a 4,4′-dithiodipyridine-modified gold electrode. [b] NaCl solution (μ = 0.1 M), at an edge-oriented pyrolytic graphite electrode. The pH is indicated in parentheses. [c] The figure reported at pH 6.0 is calculated value. [d] Reference 9.

variant. Substitution of Lys86 or Lys87 with Ala has little effect on the EPR spectra of these proteins at alkaline pH (data not shown). Addition of the spectra of the Lys73Ala and Lys79Ala variants produces a spectrum (Figure 8D) that is essentially that of the wild-type protein under the same conditions. The spectrum of the double variant at pH 10.5 strongly resembles the spectrum of native cytochrome *c* as expected on the basis of the high pK_a of this variant. However, the spectrum of the double variant exhibits a greater contribution from a high-spin species with $g \sim 6.2$ than observed for wild-type cytochrome.

Direct Electrochemistry. The midpoint potentials of the native cytochromes were determined with a modified gold electrode (pH 6.0, sweep rate = 20 mV s^{-1}) (Table 4). Under these conditions, all of the variants display essentially reversible electrochemistry with peak separations of ∼59 mV, and the midpoint potentials of the native forms of the variants and of the wild-type protein are identical within the experimental uncertainty (290 ± 2 mV). The cyclic voltammograms of the Lys73Ala and Lys79Ala variants determined with a graphite electrode and faster sweep rates (2 V s^{-1}) at pH < 5 revealed a non-native electroactive species at ∼−60 mV vs SHE. As the pH was increased, the peak currents associated with these waves decreased in intensity and shifted to more negative potentials while the intensity of the peak currents corresponding to the native protein at ∼270 mV increased. At pH > 6, the current peak corresponding to the non-native form of the protein behaved as observed previously for alkaline cytochrome *c*.[21]

The results derived from cyclic voltammetry of the Lys73Ala and Lys79Ala variants performed at fast sweep rates are shown in Figure 9. These results reflect the typical pH dependence of the peak cathodic currents ([protein] ∼ 320 μM) observed in the second scan of these two variants. The pK_a values determined by least-squares analysis of these data have been shown to correspond to the equilibrium pK_a of the alkaline transition of the cytochrome.[21] For the Lys79Ala variant, the pK_a determined from fitting the data in Figure 9A is 8.46(6) and the corresponding value for the Lys73Ala variant is 8.67-(5) (Figure 9B). These values are in good agreement with the corresponding values determined spectroscopically (Table 3). However, the results for these two single variants differ from those reported for the wild-type protein in two ways. First, no low-potential cathodic wave corresponding to the alkaline conformer could be detected in voltammograms obtained for wild-type cytochrome *c* of comparable concentration and pH though a high-potential cathodic wave corresponding to the native conformer exhibited a magnitude of ∼14 μA. On increasing the pH to ∼10.2, the transition of the native protein to its alkaline conformers resulted in a decreased intensity of the high-potential wave and a concomitant increase in the magnitude of the low-potential wave. This low-potential wave reached a maximum of ∼10 μA. In contrast, the pH dependence

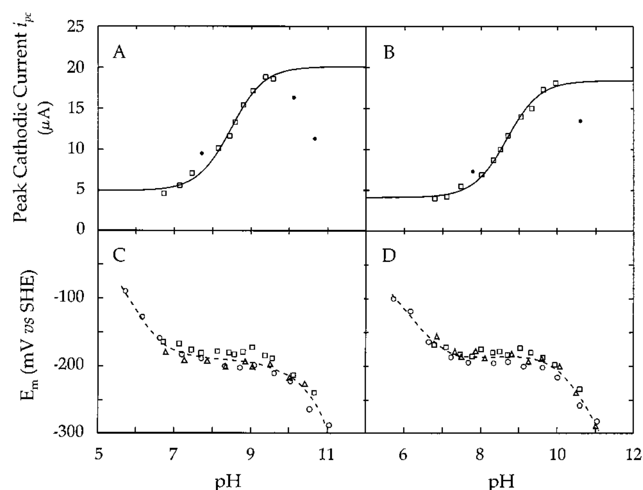

Figure 9. Cyclic voltammetry of the yeast ferricytochrome *c* variants ([protein] ∼ 320 μM, 0.1 M NaCl, 25.0 °C): panels A and C, Lys79Ala; panels B and D, Lys73Ala. The symbols in panels C and D correspond to individual titrations. The points drawn as solid symbols in panels A and B were excluded from these fits.

of the cyclic voltammograms of the alkaline variants are characterized by a low- and a high-potential waves even at pH ∼ 6 (Figure 9). The magnitude of the cathodic peak, which corresponds to the non-native form of the variants, is predicted not to fall below 4 μA despite the fact that at low pH a very small proportion of the protein exists in the alkaline state. Furthermore, the maximum current expected at high pH (between 18 and 20 μA) is greater than that determined for the native cathodic peak of the wild-type protein in the native state.

The dependencies of the midpoint potentials of the alkaline forms of the Lys73Ala and Lys79Ala variants are shown in Figure 9C,D. The experimental uncertainty (±10−15 mV) in the midpoint potentials obtained with the graphite electrode is greater than normally observed (±2 mV) for potentials determined with a dithiodipyridine-modified gold electrode. As a result, the reduction potentials obtained for the alkaline proteins are consistent with those reported previously[21] (Table 4).

Stopped-Flow Kinetics. The mechanism by which Lys73 and Lys79 bind to the heme iron as pH is increased has been analyzed by pH-jump experiments in terms of the model described by Davis et al.[37] (Figure 10). The kinetic parameters derived from analysis of these data are set out in Table 3 along with the parameters reported previously for the wild-type protein[42] and the corresponding pK_a values determined by spectrophotometric titrations. From these results it is apparent that the pK_a for the equilibrium of the wild-type protein is intermediate between the values determined for the two variants. Similarly, the k_f, k_b, and pK_H values obtained from the kinetic analysis decrease in the order Lys79Ala > wild-type > Lys73Ala (cf. Figure 10). Consistent with this analysis, the dependence of k_{obsd} on [H^+] observed for the wild-type protein could be simulated with a suitably weighted combination of the kinetic parameters of the single variants (Figure 10, inset). These results establish that the mechanism by which the Lys79Ala and Lys73Ala variants convert to their respective alkaline conformational states are kinetically similar to each other and to that of the wild-type protein. Therefore, these variants provide reliable model systems through which the pH-linked conformational behavior of the wild-type protein can be investigated.

(42) Pearce, L. L.; Gärtner, A. L.; Smith, M.; Mauk, A. G. *Biochemistry* **1989**, *28*, 3152−3156.

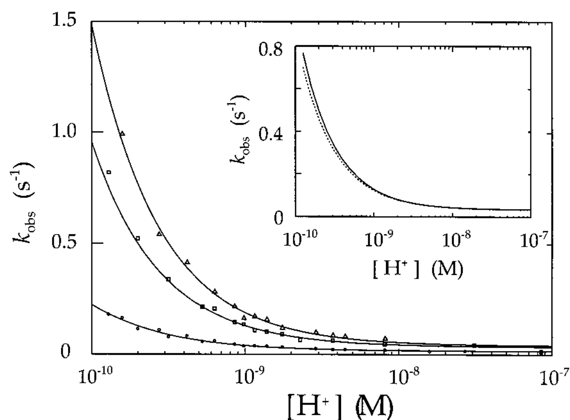

Figure 10. pH-jump kinetics analyses of the rapid mixing reactions of unbuffered 9 μM solutions of (□) wild-type, (O) Lys73Ala, and (△) Lys79Ala yeast iso-1-ferricytochromes *c* in 0.1 M NaCl with a variety of buffers of the same ionic strength (25.0 ± 0.2 °C). The absorbance change at 390 nm was monitored to yield observed first-order rate constants (k_{obsd}) as a function of pH. The lines are the best fits to eq 2. The inset is a comparison of the kinetic response of the wild-type protein (solid line) with a simulation (dotted line) based on the weighted kinetic responses of the Lys73Ala and Lys79Ala variants.

Thermal Stability. The thermal stabilities of the native Lys79Ala and Lys73Ala ferricytochromes were evaluated by CD spectroscopy (sodium phosphate buffer (pH 6.15, $\mu = 0.01$ M) or sodium borate buffer (pH 9.58, $\mu = 0.01$ M)). At pH 6.15, the T_m (°C) values obtained from these measurements are as follows (% reversibility in parentheses): wild-type ferricytochrome *c*, 52.7 (78); Lys73Ala, 53.7 (86); Lys79Ala, 51.4 (89). At pH 9.58, these same values were as follows: wild-type ferricytochrome *c*, 52.0 (68); Lys73Ala variant, 48.2 (47); Lys79Ala, 52.3 (25). These results indicate that the native form of the Lys73Ala variant is slightly more stable to thermal denaturation than is the native form of the Lys79Ala variant. This small difference may arise from the absence of the hydrogen bonds formed by Lys79 with Ser47, Tyr46, and the heme 6-propionate group in the Lys79Ala variant.

Under alkaline conditions, on the other hand, the Lys73Ala variant is clearly less thermally stable than are the other two proteins. In fact, this relative instability of the Lys73Ala alkaline conformer is attenuated in this experiment because the pK_a for the conformational equilibrium of this protein is greater than that of the other two proteins. As a result, a greater fraction of the native conformer of this variant persists at pH 9.58 than is the case for the wild-type or Lys79Ala proteins. Interestingly, the reversibility of thermal denaturation of the three proteins is not only markedly different but the wild-type protein is significantly less susceptible to irreversible changes under these conditions than are either of the two variants. Notably, the reversibility of the Lys79Ala variant to thermal denaturation is the poorest of the three proteins. At present, the mechanistic basis for this difference in behavior cannot be determined from the information available.

Discussion

The demonstration that the two alkaline conformers of yeast ferricytochrome *c* result from coordination of Lys73 or Lys79 to the heme iron defines a critical structural attribute of the pH-linked conformational changes of this protein. Although Lys79 is located in a position near the heme iron in the native conformational state (state III), the side chain of Lys73 is directed in the opposite direction from the heme prosthetic group with its ϵ-amino group fully exposed to solvent on the surface of the protein (Figure 1). As a result the conformational change required to place Lys73 in a position to coordinate to the heme iron must be significantly greater than that required for coordination of Lys79. For this reason, it is surprising that the two conformers exhibit such similarity in terms of their electrochemical properties, their electronic spectra, the pK_a at which they form, and their relative abundance.

While it is tempting to conclude that Lys73 and Lys79 also provide the axial ligands of the alkaline conformers of horse heart and other higher eukaryotic ferricytochromes *c*, this conclusion is premature. Unlike the cytochromes *c* of higher eukaryotes, Lys72 of yeast and fungal cytochromes is trimethylated and, therefore, unable to coordinate to the heme iron. This fact combined with the observation of two alkaline conformers in the ^{1}H NMR spectrum of alkaline horse heart ferricytochrome *c*[23] makes the situation concerning the cytochromes of these higher eukaryotes ambiguous. In our view, if Lys73 and Lys79 are not the axial ligands of alkaline ferricytochrome *c* from horse heart, then Lys72 and Lys79 are the most reasonable alternative ligands. We base this preference in part on our recent observation of three alkaline conformers in the ^{1}H NMR spectrum of the nontrimethylated yeast cytochrome produced by a bacterial expression system.[43] This observation raises two related possibilities. First, trimethylation of the yeast cytochrome may have evolved to prevent the coordination of Lys72 to the heme iron of the alkaline ferricytochrome *c*. Alternatively, structural factors that are currently unapparent may render Lys72 in alkaline ferricytochromes *c* from horse heart and higher eukaryotes unable to coordinate to the heme iron. Clearly, further studies of the type reported here for the yeast cytochrome are also required for the horse heart protein to resolve this issue.

Historically, the principal method used to study the structural origin of the alkaline conformational change of ferricytochrome *c* involved complete or selective chemical modification of the protein followed by spectrophotometric pH titration of the modified derivative in which the change in absorbance at 695 nm is used to indicate the presence of the native conformation. We have attempted to summarize most of these studies and the results obtained from them in Table 1. In general, these studies supported the conclusion that Met80 is replaced by a lysyl residue as solution pH is increased from neutrality. Among these studies, the report by Smith and Millett[11] is unique in considering the possible involvement of more than one lysyl residue. As in the other studies of this type, these authors worked with the horse heart cytochrome, so the two lysyl residues they emphasized in their analysis were Lys79 and Lys72. The use of chemically modified cytochromes, however, introduced the possibility that the modifying group could alter the behavior of the protein in some undetermined fashion, and their reliance on spectrophotometric pH titrations provided limited information concerning the conformational behavior of the modified cytochromes. In retrospect, it is now clear that the common limitation of all such studies was their dependence on spectrophotometric pH titrations of the 695-nm band to monitor the pH-linked conformational equilibrium. In measurements of this type, elimination of one or the other of the two lysyl residues that can replace Met80 as an axial ligand would cause little or no change in the results obtained. Only with the demonstration by Hong and Dixon[23] that alkaline cytochrome *c* is comprised of two conformers and the definition of the conditions by which they could be detected by ^{1}H NMR

(43) Pollock, W. B. R.; Rosell, F. I.; Twitchett, M. B.; Dumont, M. E.; Mauk, A. G. *Biochemistry* **1998**, *37*, 6124−6131.

spectroscopy was it apparent how the ligand exchange process that is central to the alkaline conformational change could be characterized unambiguously.

More recent studies in which cytochrome *c* variants have been investigated demonstrate the complex nature of the structural factors that influence the pH-linked conformational changes of ferricytochrome *c*. For example, Banci et al.[44] have recently reported ^{1}H NMR spectra of the Met80Ala ferricytochrome variant at alkaline pH that demonstrate that this protein forms just one alkaline conformer with a coordinated lysyl residue. On the basis of the results reported here, it appears that the alkaline conformer formed by this axial ligand variant probably is the one in which Lys73 is coordinated to the heme iron. Considering the proximity of Lys79 to the vacant coordination position, it is surprising that replacement of Met80 with the smaller alanyl residue does not promote coordination by the adjacent Lys79 residue. Evidently, the driving force for coordination of Lys73 at alkaline pH is far greater than that for coordination of Lys79, though at present, the structural basis for this observation is unknown. For the wild-type cytochrome or variants that retain the Met80 residue, the dissociation of the Fe(III)−Met80 bond will, of course, also contribute to the overall driving force of the conformational change.

Cytochrome variants substituted at positions that are not directly involved in axial ligation have also been observed to exhibit altered conformational pH linkage. For example, Nall and co-workers[45] have shown that the Pro76Gly substitution in yeast iso-2-cytochrome *c* decreases the pK_a for the alkaline transition from 8.45 to 6.71 by increasing k_f (as defined in eq 2) 70-fold and no change in K_H. Initial studies of a number of other cytochrome variants that involve replacement of Phe82 have also demonstrated that changes at this conserved position perturb both the pK_a for the alkaline conformational change[42] and the nature of the alkaline conformers as detected by ^{1}H NMR spectroscopy (J. C. Ferrer, F. I. Rosell, G. R. Moore, and A. G. Mauk, unpublished work). Through systematic characterization of the properties of these variants and their conformational dynamics, it should be possible to identify the structural factors that are most influential in dictating this fundamental property of cytochrome *c*.

Functional Properties of the Alkaline Conformers. The current results establish that the reduction potentials of the two alkaline conformers of ferricytochrome *c* are quite similar to each other. This observation is fully consistent with the original characterization of the electrochemical properties of alkaline cytochrome *c* which led us to conclude that the potentials of the two conformers could differ by no more than 30 mV.[21] In view of what must be a significant difference in the structure of the two alkaline conformational states, the similarity of their reduction potentials is somewhat surprising. This similarity in potential combined with the identity of the coordination environments in the two conformers makes it likely that the dielectric of the heme environment is also similar for the two forms of the protein. Whether this similarity in dielectric results from similarity in heme exposure to solvent in the two forms of the protein will remain speculative until the structures of the two forms of the protein are determined.

While the thermal stability measurements provided by this work cannot substitute for a rigorous calorimetric analysis, the current results do define the relative stabilities of the two alkaline conformers. Surprisingly, the coordination of Lys79, which presumably requires a relatively small structural change, results in a less stable structure than does the coordination of Lys73, which must involve a significant structural rearrangement. It seems likely that coordination of Lys79 requires some displacement of the polypeptide in which this residue resides that results in relative destabilization of the resulting conformer. Ultimately, the structural basis for this observation and for the significant differences in reversibility of forming the two conformers would be greatly assisted by determination of the structures of the two conformers.

Mechanism of the Alkaline Conformational Change. The present kinetic analysis has emphasized the simple two-step model of Davis et al.[37] which can now be restated as an initial deprotonation event that leads to exchange of the Met80 ligand for either Lys73 or Lys79. As these previous authors and others have recognized, this model is an oversimplification of what must be a more complex sequence of events. One clear limitation of this model is that it does not account for the obligatory transient species in which neither Met80 nor either lysyl residue is bound to the heme iron. This limitation has been considered previously by Kihara and colleagues[36] for the horse heart cytochrome. In time, more detailed stopped-flow and NMR studies of Lys73Ala and Lys79Ala variants of the yeast cytochrome described here should be undertaken to define the subtleties of this mechanism for each of the two alkaline conformers. In recent work, Bowler and colleagues[46] have considered the effects of various substitutions for Lys73 on the stability of yeast iso-1-cytochrome *c* and on the mechanism by which the cytochrome folds. Extension of this work to evaluation of the behavior of these variants in the alkaline conformational transition should also provide useful information.

Cytochrome *c* as a Binary Molecular Switch. As we have noted previously,[21] the pH- and oxidation-state linkage of the conformation of cytochrome *c* constitutes a binary molecular switch in which the reduction potential of the protein is profoundly dependent on pH. This property of the protein has been employed recently by Gray and colleagues[47] as a means of studying photoinitiated induction of protein folding and promises to be of continuing interest as a model for electronic control of protein reactivity. The present studies establish that cytochrome *c*, in fact, possesses two such switching mechanisms that result in similar functional consequences but must result in significantly different structural changes. The possibility of introducing additional structural elements to gain further structural control over this protein is intriguing.[48]

Discussion of cytochrome *c* as a molecular switch necessitates consideration of the identity of the "trigger" group, the protonation state of which is linked to the conformation of the protein. At present, the identity of this group or pair of groups remains unknown though several candidates merit consideration. In evaluating the suitability of a residue for this role, we note that identification of the correct residue will provide an explanation for the known effects of substitutions for Phe82 on the pK_a for this residue as determined by pH-jump experiments.[42] Several of the titratable groups that have been suggested to fulfill this functional role are reviewed briefly below:

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(45) Nall, B. T.; Zuniga, E. H.; White, T. B.; Wood, L. C.; Ramdas, L. *Biochemistry* **1989**, *28*, 9834−9839.

(46) (a) Bowler, B. E.; May, K.; Zaragoza, T.; York, P.; Dong, A.; Caughey, W. S. *Biochemistry* **1993**, *32*, 183−190. (b) Herrmann, L.; Bowler, B. E.; Dong, A.; Caughey, W. S. *Biochemistry* **1995**, *34*, 3040−3047. (c) Herrmann, L.; Flatt, P.; Bowler, B. E. *Inorg. Chim. Acta* **1996**, *242*, 97−103. (d) Godbole, S.; Dong, A.; Garbon, K.; Bowler, B. E. *Biochemistry* **1997**, *35*, 119−126.

(47) (a) Pascher, T.; Chesick, J. P.; Winkler, J. R.; Gray, H. B. *Science* **1996**, *271*, 1558−1560. (b) Mines, G. A.; Pascher, T.; Lee, S. C.; Winkler, J. R.; Gray, H. B. *Chem. Biol.* **1996**, *3*, 491−497.

(48) Ashkenazi, G.; Ripoll, D. R.; Lotan, N.; Scheraga, H. A. *Biosens. Bioelectr.* **1997**, *12*, 85−95.

(i) Lys73/Lys79. The inability of a protonated lysyl ϵ-amino group to coordinate to the heme iron suggests that Lys73 and Lys79 may serve as their own trigger groups. Indeed, interpretation of the pH-jump kinetics data by the model of Davis et al.[37] indicates that the pK_a of the trigger group for coordination of Lys73 is 12.0 while the corresponding pK_a for coordination of Lys79 is 10.8 (Table 3, Figure 9). Moreover, the assignment of pK_a values determined from the pH-jump kinetics to these lysyl residues would be consistent with the pK_a (10.4) for Lys79 reported by Bosshard[10] and with the hydrogen-bonding interactions of Lys79 and the complete exposure of Lys73 to solvent. Alternatively, the replacement of the two lysyl residues with alanine may differentially alter the environment around the "true" trigger group and thereby alter the pK_a at which it titrates. However, it is not clear that this model can explain the effects of position-82 substitutions on the pK for the alkaline conformational transition. While the pK_a of Lys79 might be perturbed by substitutions for Phe82, it seems unlikely that the same substitutions should have a similar influence on Lys73. To test this model further, it would be necessary to perform pH-jump kinetics experiments with Lys79Ala/Phe82Xxx and Lys73Ala/Phe82Xxx double variants to determine the combined effects of these substitutions on the pK_a of the "trigger" group.

(ii) Tyr67. Studies of the cytochrome *c* derivative in which Tyr67 is chemically modified by nitration led to the suggestion[49] that deprotonation of this residue is responsible for initiating the alkaline conformational change of ferricytochrome *c*. In addition, the enthalpy of ionization of the conformationally linked titratable group determined by pH-jump kinetics[37] was consistent with involvement of either a histidyl or tyrosyl residue in this role. Subsequent studies of cytochrome *c* from *Euglena*, which possesses a phenylalanyl residue at position 67, demonstrated that this protein exhibits an apparently normal alkaline conformational change.[22b] On this basis, Tyr67 was argued not to be the "trigger" group. The more recent observation[50] that the Tyr67Phe variant of rat cytochrome *c* retains the native structure to very high pH, on the other hand, could be viewed as support for Tyr67 as the critical residue. Certainly, it is reasonable to expect that substitutions for Phe82 could result in a sufficient perturbation in the environment of Tyr67 to change its pK_a.

(iii) His18. The possibility that deprotonation of His18 provides the conformational "trigger" was first suggested by Margoliash and Schejter[3a] and subsequently reiterated by Pettigrew and co-workers.[9] More recently, Gadsby et al.[5e] also favored this mechanism on the basis of their EPR and MCD analysis of the alkaline conformational equilibrium. As we indicated[42] previously, however, it is not clear how substitutions for Phe82, could perturb the pK_a of this residue as would be required to explain the results of the pH-jump analysis of Phe82 variants.

(iv) Heme 7-Propionate. The first suggestion that a heme propionate could titrate with a pK_a compatible with involvement in the alkaline conformational change resulted from a study by Hartshorn and Moore[51] that provided evidence that one of the heme propionate groups of horse heart ferricytochrome *c* has a $pK_a > 9$. Subsequently, Tonge et al.[15] reported pH-dependent FTIR studies of native tuna and horse ferricytochrome *c* and of the trifluoroacetylated horse ferricytochrome that led them to conclude that one of the heme propionate groups exhibits a pK_a of 9.35. As the authors note, this pK_a does correspond reasonably with the pK_a for the alkaline conformational change. Nevertheless, this pK_a is significantly lower than the pK_a of the "trigger" group as determined by the pH-jump experiments of Davis et al.[37]

Finally, Takano and Dickerson[52] suggested that the ionization state of an internal water molecule located in the heme crevice of cytochrome *c* could be linked to the conformational state of the ferricytochrome. As with the other candidate groups discussed above, the design of an experiment to evaluate this possibility in a definitive fashion is complicated by the likelihood that any potentially relevant chemical or mutagenic modification of the protein will introduce too great a perturbation in the properties of the protein to provide insight into the behavior of the wild-type protein. For this reason, it seems likely that the most compelling evidence for the identification of the "trigger" group of yeast iso-1-ferricytochrome *c* will result from the insightful use of a physical or spectroscopic technique or the development of some new experimental technology to study the pH-linked behavior of the wild-type protein and the Lys79Ala and Lys73Ala variants.

Linkage of Ferricytochrome *c* Binding Interactions and the Alkaline Conformational Change. The role of the ferricytochrome *c* alkaline conformational equilibrium in biological processes remains an issue of considerable debate. The challenge in evaluating this situation is based on the uncertainty of the pH of the microenvironment in which cytochrome *c* occurs and in evaluating the influence of binding of the protein to membrane structures and to other proteins on this equilibrium. Although Wilson and Greenwood[3c] conclude that the physiological role of the alkaline conformational change is limited, a number of studies suggest otherwise. For example, the interaction of ferricytochrome *c* with cardiolipin has been known for some time to induce spectroscopic changes that are consistent with displacement of Met80 as an axial ligand.[53] More recent resonance Raman studies have indicated that the interaction of ferricytochrome *c* with model membrane systems alters the coordination environment of ferricytochrome *c* in a fashion that is consistent with elimination of Met80 as a ligand to the heme iron.[54]

The interaction of ferricytochrome *c* with some other electron-transfer proteins may also induce related structural changes. The first indication that such interactions might induce structural changes in the cytochrome was provided by Weber et al.,[55] in which MCD spectroscopy was used to study the interaction of cytochrome *c* with cytochrome *c* oxidase. Further studies by this group led them to conclude that interaction with cytochrome *c* oxidase in fact stabilizes the native conformation.[56] Subsequent resonance Raman studies of the cytochrome *c*−cytochrome *c* oxidase complex, however, have provided considerable evidence that the binding of the ferricytochrome to the oxidase displaces the Met80 ligand to the heme iron and results in a structure that is related to that of the alkaline cytochrome.[57] Interestingly,

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the interaction of ferricytochrome *c* with cytochrome b_5 does not induce any change in the resonance Raman spectrum of cytochrome *c*.[54a] The possible influence of interaction with yeast flavocytochrome b_2 (lactate dehydrogenase) has not yet been considered in this manner. On the other hand, the interaction of ferricytochrome *c* with polyanions such as heteropolytungstate or heteropolymolybdate does stabilize[58] one or the other or both of the alkaline conformers and may provide a model for surface binding-induced perturbation of the ferricytochrome conformational equilibrium.

In general, proteins are regarded as existing in the native, folded structure in a range of pH that varies from protein to protein and to denature as the solution pH is increased or decreased outside of this range. In recent years, however, examples of proteins that exhibit pH-dependent conformational states are beginning to be recognized.[59] It seems likely that as more proteins are studied in depth under a range of solution conditions that additional examples will be found that exhibit pH-dependent conformational states. As a result, we suggest that, aside from resolving a long-standing question concerning a fundamental property of ferricytochrome *c*, the present work provides the initial characterization of a prototypical protein with this characteristic that can serve as a highly informative model for understanding the basis of this functional property.

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