pH Dependence of Formation of a Partially Unfolded State of a Lys 73 → His Variant of Iso-1-cytochrome *c*: Implications for the Alkaline Conformational Transition of Cytochrome *c*†

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ABSTRACT: The alkaline conformational transition of a lysine 73 → histidine variant of iso-1-cytochrome *c* has been studied. The transition has been monitored at 695 nm, a band sensitive to the presence of the heme−methionine 80 bond, at the heme Soret band which is sensitive to the nature of the heme ligand, and by NMR methods. The guanidine hydrochloride dependence of the alkaline conformational transition has also been monitored. The histidine 73 protein has an unusual biphasic alkaline conformational transition at both 695 nm and the heme Soret band, consistent with a three-state process. The conformational transition is fully reversible. An equilibrium model has been developed to account for this behavior. With this model, it has been possible to obtain the acid constant for the trigger group, pK_H, of the low-pH phase from the equilibrium data. A pK_H value of 6.6 ± 0.1 in H_2O was obtained, consistent with a histidine acting as the trigger group. The NMR data for the low-pH phase of the alkaline conformational transition are consistent with an imidazole ligand replacing Met 80. For the high-pH phase of the biphasic alkaline transition, the NMR data are consistent with lysine 79 being the heme ligand. Guanidine hydrochloride *m* values of 1.67 ± 0.08 and 1.1 ± 0.2 kcal mol^{-1} M^{-1} were obtained for the low- and high-pH phases of the biphasic alkaline transition of the histidine 73 protein, respectively, consistent with a greater structural disruption for the low-pH phase of the transition.

Understanding how proteins efficiently fold from a disordered state to a unique and fully functional native state is an area of intense interest (*1*). A number of models have been developed to address this issue (*1*−*5*). Protein folding intermediates have been studied intensely as they can provide a means of promoting efficient protein folding (*6*, *7*). Equilibrium intermediates have been widely used to provide insight into the possible structures of kinetic folding intermediates, since structural characterization of equilibrium species is more straightforward. In a number of instances, combined kinetic and equilibrium data have provided validation of this approach (*8*). The molten globule state (*7*, *9*) which is normally stabilized at equilibrium by low pH and high salt (*10*) has been widely studied by both structural (*11*) and thermodynamic methods (*7*) as a model for protein folding intermediates. This state can have a wide range of residual structure and is commonly characterized by the presence of significant secondary structure but limited tertiary structure. The molten globules of apomyoblobin (*12*−*17*), cytochrome *c* (*18*−*20*), α-lactalbumin (*21*), and lysozyme (*22*, *23*) have been particularly well-studied.

Cytochrome *c* undergoes a base-induced conformational transition, commonly termed the alkaline transition (state III to state IV; for a recent review, see ref *24*). This transition is characterized by loss of the heme−Met 80 ligation of the native state of the protein (*24*). Since the enthalpy of this transition is small (7−16 kcal/mol) (*24*−*27*) relative to the enthalpy for the full unfolding of the protein (∼80 kcal/mol at pH 5; see refs *28* and *29*), the structural perturbation is also expected to be small. Thus, the partial unfolding of cytochrome *c* coupled to this transition likely maintains the majority of the structure of the fully folded protein. Hence, the alkaline conformation could represent a model for a late folding intermediate of cytochrome *c*.

This contention is supported by a number of experimental results. In yeast iso-1-cytochrome *c*, the ligands which replace Met 80, in state IV, are known to be lysines 73 and 79 (*27*, *30*). The lowest-energy partially unfolded form (PUF) of cytochrome *c*, as detected by equilibrium hydrogen−exchange experiments, is known to be surface Ω-loop D (*31*) encompassing residues 70−85 (*8*, *32*−*34*), which includes lysines 73 and 79. Thus, replacement of Met 80 as a heme ligand with either of these amino acids would be expected to disrupt Ω-loop D. Furthermore, previous studies on the Lys 73 → His (K73H)[1] iso-1-cytochrome *c* variant (*35*, *36*), which is the topic of this work, have shown that it unfolds in the presence of guanidine hydrochloride (gdnHCl) through an intermediate involving the loss of heme−Met 80 ligation. The gdnHCl *m* value for formation of this intermediate is ∼1.8 kcal mol^{-1} M^{-1} (*35*), very close to the gdnHCl *m* value for the lowest-energy PUF of cytochrome *c* derived from

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[1] Abbreviations: gdnHCl, guanidine hydrochloride; K73H, lysine 73 to histidine mutation; WT, wild type.

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native state hydrogen exchange experiments (1.6 kcal mol^{-1} M^{-1}; see ref *32*). The K73H variant of iso-1-cytochrome *c* replaces one of the lysine side chains implicated in heme ligation in the alkaline conformer of cytochrome *c* with another high-affinity heme ligand, histidine. These observations suggest that the alkaline conformer of cytochrome *c* might be a good model for the lowest-energy PUF of cytochrome *c* and that the K73H variant of iso-1-cytochrome *c* stabilizes this conformer in a pH regime that is lower than the regime that is typical for the alkaline conformational transition.

To investigate this possibility, we have studied the properties of the alkaline conformational transition of K73H iso-1-cytochrome *c* using both the 695 nm absorbance band associated with heme−Met 80 ligation and the heme Soret absorbance which is sensitive to the nature of the incoming ligand. NMR studies of the paramagnetically shifted heme methyl groups are also used to characterize the transition. An unusual three-state alkaline conformational transition is observed. We have developed an equilibrium model to fit the data which has allowed extraction of the pK_a of the group triggering formation of the intermediate state in this three-state process and determination of the populations of each of the three states as a function of pH. *m* values derived from the gdnHCl dependence of this unusual three-state alkaline transition have allowed evaluation of the relative degree of structural disruption for the two phases of this transition.

MATERIALS AND METHODS

Isolation and Purification of Proteins. The K73H mutation was introduced using site-directed mutagenesis as previously described (*37*). The wild type (WT) and K73H proteins were isolated and purified as described previously (*38*, *39*) from the *Saccharomyces cerevisiae* GM-3C-2 cell line carrying the pRS425 multicopy vector (*40*) with the iso-1-cytochrome *c* gene cloned into it (*37*). In both the WT and the K73H proteins, the Cys 102 has been replaced with a serine residue to avoid dimerization of protein molecules resulting from intermolecular disulfide bonds.

Alkaline Transition Monitored by Absorbance Spectroscopy at 695 nm. Approximately 5 mg of protein was oxidized with $K_3Fe(CN)_6$ for 1 h at 4 °C. The protein was then run down a G-25 column using 100 mM NaCl as the eluant to separate the oxidizing agent from the protein. The protein was collected and exchanged into 200 mM NaCl with a centriprep-10 concentrator (Waters). The final volume was adjusted to 1.5 mL, and the concentration was determined spectrophotometrically as described previously (*38*). The sample was diluted, by mixing 1 mL of the 1.5 mL (2×) protein stock with 1 mL of deionized water in a disposable polystyrene cuvette. The final concentration was between 180 and 190 μM in 100 mM NaCl. After the concentration was determined, the Beckman DU 640 spectrophotometer was set up to read absorbances at 695 and 750 nm. The 695 nm band measures the loss of the heme−Met 80 band, and the 750 nm measurement was used as background. The pH was measured with a Corning model 220 pH meter and a Corning Semimicro Combination pH electrode. The initial spectrum was obtained at pH 4.5. Aliquots of different concentrations of NaOH solution were added to the protein sample to titrate the sample up to pH 10.5. The same volume of a 2× protein stock (200 mM NaCl) was added. The protein sample was mixed with a 1000 μL pipetman, and then the pH and spectrum were recorded. All pH experiments were carried out at 25 ± 0.1 °C using a thermostated cuvette holder attached to a circulating water bath. When the pH was at 10.5, the protein solution was back-titrated with various concentrations of HCl solution and equal volumes of the 2× protein stock as described above. After the titration, 2 mL of protein was removed from the sample and transferred to a fresh cuvette. To add gdnHCl into the sample, 2*x* μL of the sample was removed, and replaced with 1*x* μL of a 6 M gdnHCl stock and 1*x* μL of the 2× protein stock. The gdnHCl-containing sample was then subjected to pH titration as described above, except 1 μL of 3× guanidine-HCl was added in addition to 0.5 μL of NaOH (appropriate concentration) and 1.5 μL of 2× protein. The sample was then back-titrated by substituting HCl solutions with the appropriate concentrations. Typically, this procedure was carried out so that pH titrations at 0, 0.1, 0.2, and 0.3 M gdnHCl were obtained in sequence. An experiment was also performed on the protein in D_2O (no gdnHCl), using similar methodology. No correction was made for the isotope effect on the pH electrode reading (*41*).

Alkaline Transition Monitored by Absorbance Spectroscopy between 350 and 450 nm. Studies were carried out with ferricytochrome *c* at a concentration of approximately 5 μM in 100 mM NaCl and 5 mM sodium phosphate. The spectrum was scanned from 350 to 450 nm at each pH step. This experiment was carried out at 25 ± 0.1 °C. The titration method was as described above for titrations at 695 nm.

Alkaline Transition Monitored by NMR. Studies were carried out with ferricytochrome *c* at concentrations of approximately 2.5 mM in 100 mM NaCl in D_2O. The pH was adjusted with 1−2 μL of 1 M NaOD or 1 M DCl, as appropriate. The pH* was measured before and after acquiring each spectrum, and no correction was made for the isotope effect on the pH meter reading (*41*). The spectra were obtained at 25 ± 0.1 °C with a 500 MHz Varian Innova NMR spectrometer at the NMR Center at the University of Colorado Health Sciences Center. The one-dimensional spectra were obtained with 512 scans, 11 712 points per scan, and a sweep width of 40 000 Hz using presaturation water suppression. The two-dimensional NOESY spectrum was acquired over a 38 h time span at pH* 7.5, with a mixing time of 100 ms, 256 t_1 increments, 160 scans per increment, and 8192 points per scan. A sweep width of 40 000 Hz was used. Residual H_2O was suppressed by presaturating at the frequency of the HOD signal.

Data Analysis of Monophasic Alkaline Conformational Transitions. To analyze the data from pH titrations of the WT protein, we use the model developed by Davis et al. (*25*) to describe the alkaline conformational transition. First, consider the equilibrium for a single ionizable trigger group, T. Ionization of this trigger group then leads to replacement of Met 80 with an alternate ligand, L.

$$(\text{Fe}-\text{Met80}|\text{TH}^+) \overset{K_H}{\rightleftharpoons} (\text{Fe}-\text{Met80}|\text{T}) + \text{H}^+ \overset{K_C}{\rightleftharpoons} (\text{Fe}-\text{L}|\text{Met80})$$

For historical consistency (*25*), we use the symbol K_H to represent the triggering deprotonation equilibrium and K_C

to represent the conformational equilibrium associated with replacement of Met 80 with an alternative heme ligand, L. For yeast iso-1-cytochrome *c*, it has been definitively demonstrated that lysines 73 and 79 both act as L in the alkaline state (*27*, *30*). It is possible that T and L are the same chemical group, but this is a matter of considerable debate (*24*, *27*).

The equilibrium constant expressions for K_H and K_C are given in eqs 1 and 2.

$$K_H = \frac{[(Fe-Met80|T)][H^+]}{[(Fe-Met80|TH^+)]} \quad (1)$$

$$K_C = \frac{[(Fe-L|Met80)]}{[(Fe-Met80|T)]} \quad (2)$$

If the alkaline conformational transition occurs as a concerted two-state process, an apparent acid equilibrium expression, K_H(app), in the terminology of Davis et al. (*25*), can be written (eq 3).

$$K_H(app) = \frac{[(Fe-L|Met80)][H^+]}{[(Fe-Met80|TH^+)]} = K_H K_C \quad (3)$$

Typically, the *K*(obs) that can be measured is simply the ratio of species with or without Met 80 bound. In terms of spectroscopically observable quantities, *K*(obs) may be written as in eq 4

$$K(obs) = (A_N - A_{695})/(A_{695} - A_{alk}) \quad (4)$$

where A_{695} is the observed absorbance at 695 nm during the alkaline transition, A_N is the absorbance at 695 nm of the Met 80-bound native state, and A_{alk} is the absorbance at 695 nm of the alkaline state where either lysine 73 or lysine 79 (for iso-1-cytochrome *c*) is bound to the heme.

In the most general case, the expression for *K*(obs) must account for the presence of Met 80-bound species with both a protonated and a deprotonated trigger group, T, as in eq 5.

$$K(obs) = \frac{[(Fe-L|Met80)]}{[(Fe-Met80|TH^+)] + [(Fe-Met80|T)]} \quad (5)$$

Taking a ratio of eq 5 to eq 2, we obtain eq 6

$$\frac{K(obs)}{K_C} = \frac{[(Fe-Met80|T)]}{[(Fe-Met80|TH^+)] + [(Fe-Met80|T)]} \quad (6)$$

Using eq 1 to substitute for [(Fe−Met80|TH$^+$)] in eq 6 and rearranging, we obtain eq 7.

$$K(obs) = \frac{K_C}{\left(1 + \frac{[H^+]}{K_H}\right)} = \frac{10^{-pK_C}}{1 + 10^{pK_H - pH}} \quad (7)$$

For the alkaline transition of cytochrome *c*, normally [H$^+$] $\gg K_H$, in the region where *K*(obs) can be evaluated and eq 7 reduces to $K(obs) = (K_C K_H)/[H^+]$. Under these circumstances, the alkaline transition occurs as the concerted two-state process described by eq 3, because the population of the species (Fe−Met80|T) in eq 5 is negligible. Since $K_H(app) = K(obs)[H^+]$ under these conditions, eqs 8a and 8b are rigorously true, where pK_H(app), pK_C, and pK_H are the p*K* values of the equilibrium constants K_H(app), K_C, and K_H, respectively.

$$K_H(app) = K_H K_C \quad (8a)$$

$$pK_H(app) = pK_C + pK_H \quad (8b)$$

Also, where $K_H(app) = K(obs)[H^+]$, the *K*(obs) data as a function of pH can be fit to the Henderson−Hasselbalch equation (eq 9) to obtain pK_H(app)

$$pH = pK_H(app) + \log[(A_N - A_{695})/(A_{695} - A_{alk})] \quad (9)$$

where the expression in eq 4 has been substituted for *K*(obs). This is the expression used by Davis et al. (*25*) to fit their equilibrium data for the alkaline transition of cytochrome *c*.

For WT iso-1-cytochrome *c*, the data can be fit to the Henderson−Hasselbalch equation. To fit A_{695} versus pH data directly using nonlinear least-squares methods, eq 9 is rearranged to give eq 10. The equation has also been modified slightly to allow the number of protons, *n*, in the process to be varied during fitting to see how closely the data fit a one-proton process.

$$A_{695} = \{A_N + A_{alk} \times 10^{n[pK_H(app) - pH]}\}/\{1 + 10^{n[pK_H(app) - pH]}\} \quad (10)$$

The more general expression in eq 7 for *K*(obs) can also be used to fit the alkaline conformational transition of the WT iso-1-cytochrome *c*. The bindings of the separate ligands, lysines 73 and 79, are not distinguishable for WT iso-1-cytochrome *c* (*27*) when monitored through the heme absorbance bands. Thus, a single averaged value must be assumed for the K_H of the trigger group, and the conformational equilibrium, K_C. Rearranging eq 4 in terms of A_{695} gives eq 11.

$$A_{695} = \frac{A_N + A_{alk} K(obs)}{1 + K(obs)} \quad (11)$$

Substituting eq 7 into eq 11 gives eq 12.

$$A_{695} = \frac{A_N + A_{alk}\left[\frac{10^{-pK_C}}{(1 + 10^{pK_H - pH})}\right]}{1 + \frac{10^{-pK_C}}{1 + 10^{pK_H - pH}}} \quad (12)$$

Since eq 8b holds for WT iso-1-cytochrome *c*, pK_C and pK_H are not uniquely determined. When using eq 12 to fit data, literature values for pK_H of WT iso-1-cytochrome *c*, determined by kinetic methods (*27*, *42*), will be used so that pK_C may be determined. We note that eq 12, unlike eq 10, assumes a strict one-proton process. A_{695} versus pH data were fit to eq 12 using nonlinear least-squares methods (SigmaPlot, version 4.0, SSPS, Inc.).

RESULTS

In Figure 1, data from pH titrations of WT (□) and K73H (○) iso-1-cytochromes *c* at 695 nm are shown. This band is viewed as characteristic of the presence of the bond between Met 80 and the heme iron (*43*), and thus monitors loss of

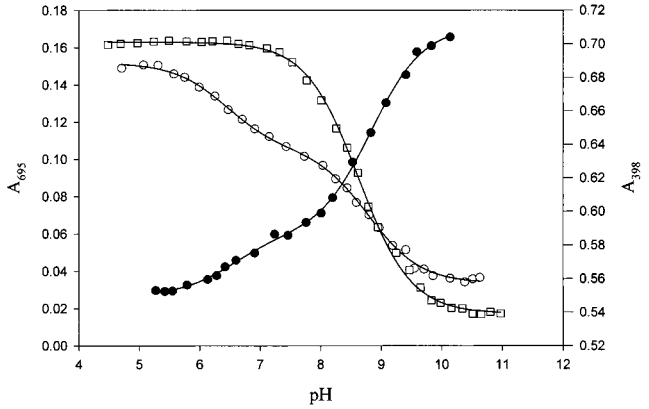


FIGURE 1: Plots of A_{695} vs pH for WT (□) and K73H (○) iso-1-cytochrome *c* and of A_{398} vs pH for K73H (●) iso-1-cytochrome *c*. Data were collected in 0.1 M NaCl at 25 °C with protein concentrations near 200 μM for data at 695 nm and in 0.1 M NaCl and 5 mM sodium phosphate at 25 °C with protein concentrations near 5 μM for data at 398 nm. Titrations were carried out as described in Materials and Methods. The solid curve for the K73H data at 695 nm is a fit to eq 17 in the Discussion, where $pK_{H2} = 10.8$, the value obtained from kinetic experiments for the Lys 79 alkaline state (*27*). The solid curve for the WT data at 695 nm is a fit to eq 12. The value of pK_H was also set to 10.8 for convenience of comparison with the second phase of the transition for the K73H protein. Reported pK_H values for WT iso-1-cytochrome *c* range from 11.0 to 11.7, and thus, the pK_H value for this protein has some uncertainty (*27*, *42*). The solid curve for the data at 398 nm is simply meant to aid the eye.

this bond which is associated with the alkaline conformational transition (*24*). The data for the WT protein show a typical monophasic pH titration curve and can readily be fit to the Henderson−Hasselbalch equation as a one-proton process ($n = 0.92 \pm 0.01$ in fits of the data to eq 10), as is typical for the alkaline transition of cytochrome *c* (*24*). The pK_H(app) for the WT protein is 8.62 ± 0.02, typical for WT iso-1-cytochrome *c* (*27*). The K73H variant, where one of the lysines implicated in the alkaline transition of iso-1-cytochrome *c* (*27*) has been replaced with histidine, has a dramatically different alkaline conformational transition. It is biphasic, which is highly unusual behavior for this conformational transition. The low-pH phase of the transition does not completely eliminate the 695 nm absorbance characteristic of heme−Met 80 ligation. In the higher-pH phase of the transition, the 695 nm band is completely lost. Qualitatively, these observations indicate that $K_C \sim 1$ (conformational equilibrium constant; see eq 2 in Materials and Methods) for the low-pH phase and that $K_C \gg 1$ for the high-pH phase.

This process can also be monitored at the intense heme Soret band near 400 nm and also demonstrates the biphasic behavior for the K73H variant (Figure 1, ●). The data at 695 nm and the data at the heme Soret band track with each other, indicating that the same process is being monitored at both wavelengths. The evidence for a distinct intermediate state in the transition is further strengthened by the presence of two separate isosbestic points in the Soret absorption band (Figure 2), one in the low-pH regime (414 nm) and another in the high-pH regime (409 nm) of the transition.

The paramagnetically shifted heme methyl substituents were also used to monitor the alkaline conformational transition of the WT versus the K73H protein (Figures 3 and 4). For the WT protein, the profile observed as a function

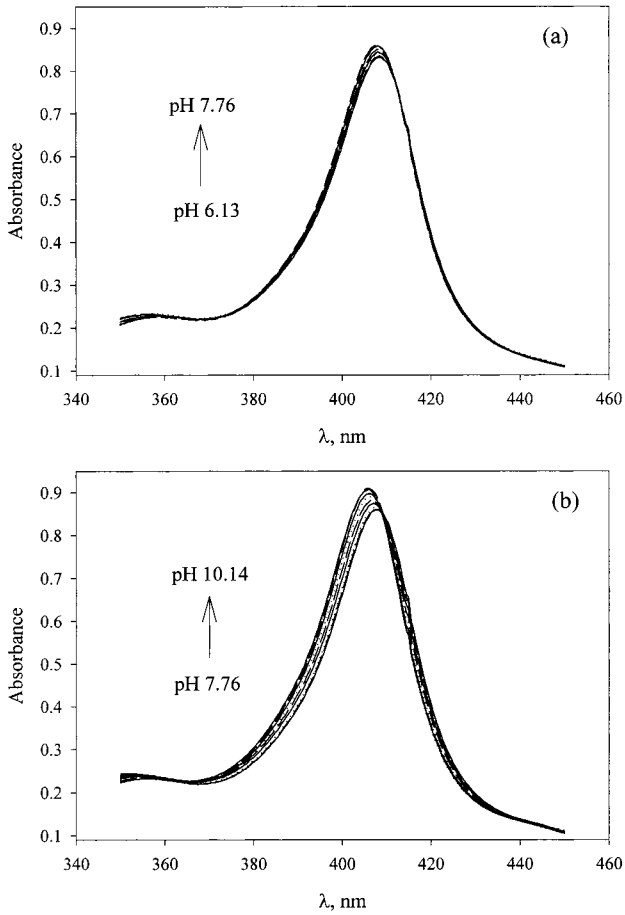


FIGURE 2: Absorbance spectra as a function of pH in the Soret region for K73H iso-1-cytochrome *c*. (a) Low-pH phase of the transition: pH 6.13, 6.60, and 7.46 (—), pH 6.28, 6.91, and 7.76 (− − −), and pH 6.42 and 7.24 (···). The isosbestic point for this phase is 414 nm. (b) High-pH phase of the transition: pH 7.76, 8.53, 9.41, and 10.14 (—), pH 8.01, 8.83, and 9.59 (− − −), and pH 8.20, 9.08, and 9.83 (···). The isosbestic point for this phase is 409 nm.

of pH* is analogous to that reported previously (*27*). A single set of heme methyl resonances is transformed into two sets of heme methyl resonances, one for Lys 73 ligation and the other for Lys 79 ligation. In the work reported here, the 8-methyl resonances, (8)a and (8)b for Lys 73 and Lys 79 ligation, respectively, are not resolved. The 3-methyl resonance for Lys 79, (3)b, and the 1-methyl resonance for Lys 73, (1)a, do not appear to be resolved either. Both these sets of resonances were resolved in previously reported work (*27*). It is likely that this difference results from the lower temperature used in the experiments reported here. For the K73H protein, changes in the spectrum begin to occur at much lower pH*. Specifically, a broad resonance near 22 ppm can be seen in the spectrum at low pH*. At high pH*, the spectrum resembles that of the K73A variant reported previously, at 25 °C (*27*), with heme ligation being dominated by resonances due to Lys 79 ligation. A NOESY spectrum of the K73H protein was run at pH* 7.5 to detect exchange correlations between the native heme methyl resonances and the heme methyl resonances in the intermediate alkaline state (see ref *26*). In Figure 5, the paramagnetically shifted native state heme methyl resonances clearly correlate to broad resonances in the 16−22 ppm range. Table 1 shows the chemical shifts of the heme methyl groups in

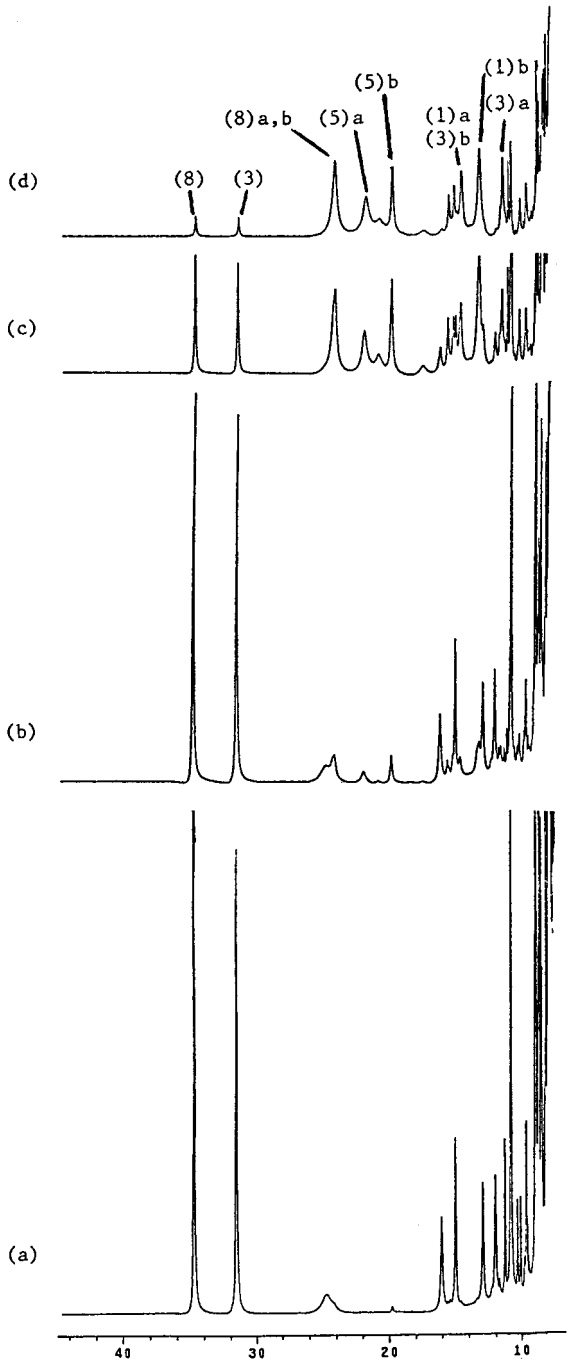


FIGURE 3: NMR spectra in a 0.1 M NaCl, D_2O solution as a function of pH* for WT iso-1-cytochrome *c* at 25 °C. The protein concentration was approximately 2.5 mM. Spectra from bottom to top are at pH* (a) 7.51, (b) 8.39, (c) 9.40, and (d) 10.05. The number for the heme methyl group is given above its ^{1}H resonance. The ^{1}H resonances for the alkaline conformer with Lys 73 coordinated to the heme are indicated with an "a" next to the number for the heme methyl group. The ^{1}H resonances for the alkaline conformer with Lys 79 coordinated to the heme are indicated with a "b" next to the number for the heme methyl group. Assignments are based on the literature values (*27*).

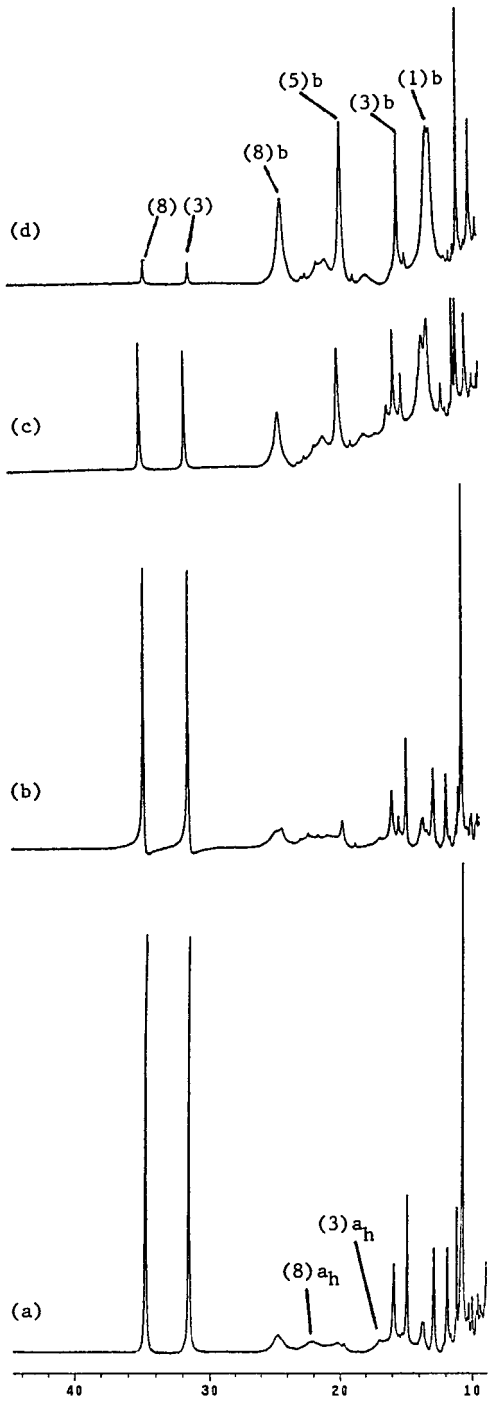


FIGURE 4: NMR spectra in a 0.1 M NaCl, D_2O solution as a function of pH* for K73H iso-1-cytochrome *c* at 25 °C. The protein concentration was approximately 2.5 mM. Spectra from bottom to top are at pH* (a) 7.61, (b) 8.48, (c) 9.52, and (d) 10.51. The number for the heme methyl group is given above its ^{1}H resonance. The ^{1}H resonances for the alkaline conformer formed at low pH, due to heme−His 73 ligation (see Discussion), are indicated with an "a_h" next to the number for the heme methyl group. The ^{1}H resonances for the high-pH alkaline conformer with Lys 79 coordinated to the heme are indicated with a "b" next to the number for the heme methyl group.

the native and intermediate alkaline states, derived from this experiment. Chemical shifts for the heme group with Lys 79 ligation in the K73H protein at high pH* and for the WT protein in the native and alkaline states derived from the one-dimensional spectra in Figures 3 and 4 are also included in Table 1. Clearly, the chemical shifts observed for specific heme methyl substituents in the intermediate alkaline state of the K73H variants are very different from those observed for lysine 73 or 79 ligation. In particular, the resonance for the 3-methyl (3-Me) protons is downfield of the resonance for the 5-methyl (5-Me) protons, which is the reverse of the ordering of the chemical shifts for these protons when a lysine is bound (see Table 1). Thus, a completely different

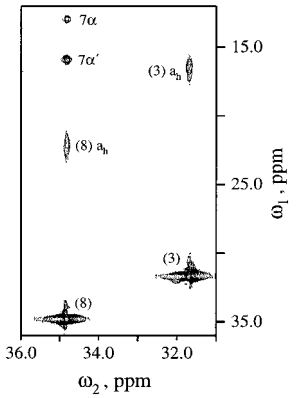


FIGURE 5: Downfield region of the two-dimensional NOESY spectrum of oxidized K73H iso-1-cytochrome *c* at 25 °C and pH* 7.5 in a 0.1 M NaCl, D_2O solution. The diagonal peaks for the native state 8-Me, (8), and 3-Me, (3), are shown. Exchange cross-peaks between the 8-Me and 3-Me native state ^1H resonances and the intermediate state alkaline conformer, $(8)a_h$ and $(3)a_h$, respectively, are shown. The cross-peaks due to dipolar interaction between the 8-Me and the 7α′ and 7α protons of the heme proprionate are also labeled.

Table 1: Chemical Shifts of the Heme Methyl Groups of WT and K73H Iso-1-ferricytochromes *c*

	chemical shift of heme methyl groups (ppm)			
protein	1-Me	3-Me	5-Me	8-Me
K73H, native[a]	7.88	31.69	10.82	34.79
K73H, alkaline, low-pH phase[a]	11.50	16.60	14.50	22.26
K73H, alkaline, high-pH phase[b]	13.28	15.49	19.70	24.31
WT, native[c]	(8.01)[d]	31.72	10.81	34.86
WT, alkaline, Lys 73[e]	14.64	11.52	21.84	24.21
WT, alkaline, Lys 79[e]	13.27	14.64	19.84	24.21

[a] Chemical shifts are with respect to DSS, referenced at the residual HOD resonance. Chemical shifts are taken from the cross-peaks of the NOESY spectrum in D_2O at 25 °C and pH* 7.5. [b] Data are from the one-dimensional spectra at pH* 10.51. Assignments are based on previously reported data for WT and mutant iso-1-cytochromes *c* (see refs *27* and *44*). [c] Data are from the one-dimensional spectrum at pH* 7.51. Assignments are based on previously reported data for WT and mutant iso-1-cytochromes *c* (see refs *27* and *44*). [d] Chemical shift taken from ref *47*. [e] Data are from the one-dimensional spectrum at pH* 10.05. Assignments are based on previously reported data for WT and mutant iso-1-cytochromes *c* (see refs *27* and *44*).

ligand environment is indicated for the intermediate alkaline state of the K73H variant. The chemical shifts in the intermediate state of the alkaline transition are similar to those observed when exogenous imidazole derivatives bind to the heme of cytochrome *c* displacing Met 80 (*45*, *46*). For example, when imidazole binds to horse cytochrome *c*, the chemical shifts of the heme methyl groups are as follows: 1-methyl (1-Me), 10.69 ppm; 3-Me, 16.69 ppm; 5-Me, 14.05 ppm; and 8-methyl (8-Me), 24.20 ppm (*45*). Thus, the chemical shift pattern for the intermediate state in the biphasic transition of the K73H protein is consistent with an imidazole ligand displacing Met 80 and binding to the heme iron. The broad resonances near 16.5 and 22 ppm at pH* 7.5 which correlate with the native heme 3-Me and 8-Me groups disappear at high pH* (Figure 4). Thus, the lysine 79 alkaline state appears to replace the intermediate state at high pH. This is consistent with the qualitative interpretation of the spectroscopic data in Figure 1. It is also noteworthy that the chemical shifts for the heme methyl

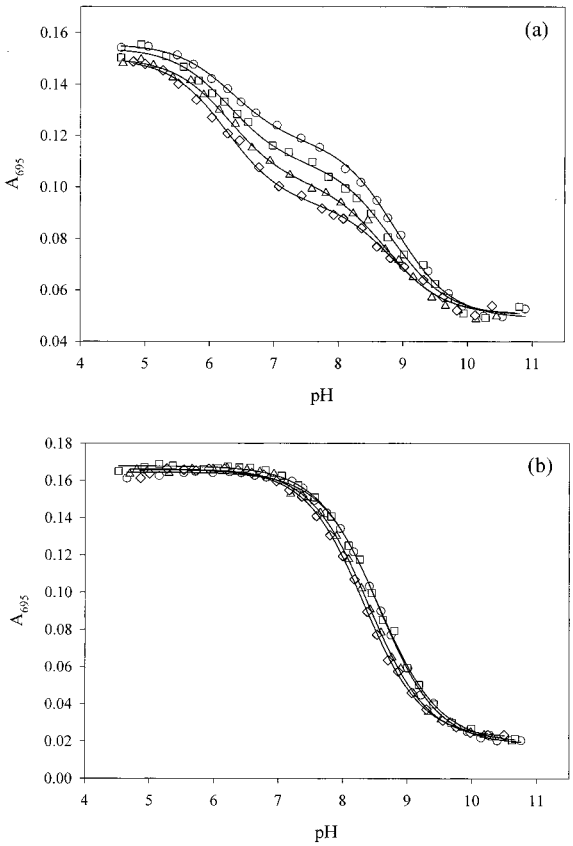


FIGURE 6: Plots of A_{695} vs pH for K73H (a) and WT (b) iso-1-cytochromes *c* at different concentrations of gdnHCl. Data were collected in 0.1 M NaCl at 25 °C at protein concentrations near 200 μM, with 0 (○), 0.1 (□), 0.2 (△), or 0.3 M (◇) gdnHCl added. Titrations were carried out as described in Materials and Methods. The solid curves for the K73H data are fits to eq 17 in the Discussion, where $pK_{H2} = 10.8$, the value obtained from kinetic experiments for the Lys 79 alkaline state (*27*). The solid curves for the WT data are fits to eq 12. The value of pK_H was also set to 10.8 as discussed in the legend of Figure 1. Parameters obtained from these fits are collected in Table 2.

groups of the native states of the WT and K73H proteins are almost identical, indicating that the K73H mutation does not significantly perturb the native state heme environment of iso-1-cytochrome *c*. The chemical shifts of the native state heme methyl groups also agree well with those reported previously for iso-1-cytochrome *c* (*47*, *48*). NMR data for the previously reported K73A and K79A variants of iso-1-cytochrome *c* indicate more perturbation to the native state heme environment than is observed for the K73H variant (*27*).

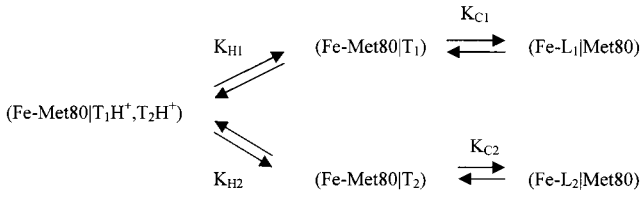
The alkaline transition was also monitored as a function of gdnHCl concentration at the 695 nm band for the K73H and WT proteins (Figure 6). The sensitivity of the WT protein to gdnHCl concentration is small. The first phase of the transition of the K73H protein is obviously sensitive to gdnHCl concentration. It clearly goes further to completion as the gdnHCl concentration increases. The alkaline transition was also monitored at 695 nm in D_2O for both proteins (data not shown). For the WT protein, the transition remained an approximately one-proton process ($n = 0.89 \pm 0.02$), but the pK_H(app) was raised significantly to 8.93 ± 0.02. For the K73H protein, the alkaline transition remains biphasic but less of the absorbance at 695 nm is lost in the low-pH phase of the alkaline transition, indicating that the low-pH

phase of the transition is less favorable than in H_2O. The stabilizing effect of D_2O toward partial protein unfolding, apparent here for the alkaline conformational transition, has been observed previously for the full unfolding of proteins (*49*, *50*).

DISCUSSION

Analysis of the Biphasic Alkaline Conformational Transition. The alkaline conformational transition of K73H iso-1-cytochrome *c* is unique in its biphasic nature. The spectroscopic data at both 695 nm and in the Soret region demonstrate the presence of two separable equilibria involved in the replacement of the Met 80 heme ligand. The equilibria are clearly reversible since data from back-titrations (pH going down) overlay the data for forward titrations (pH going up). It is evident that the low-pH phase does not go to completion because it does not result in the full loss of heme−Met 80 ligation. This is clear from the NMR data, as well. At pH* 7.5, where the population of the intermediate state begins to plateau (see Figure 1), the native state heme methyl groups are strongly evident (Figure 4). In other words, $K_C \sim 1$ for this first phase ($pK_C \sim 0$). The NMR data are also consistent with very different ligand environments for the heme for the low-pH phase and the high-pH phase of the alkaline transition of the K73H variant. The chemical shifts of the heme methyl groups in the intermediate state at the end of the low-pH phase are not consistent with either Lys 73 or Lys 79 ligation, but are consistent with an imidazole ligand (*45*, *46*) such as histidine. The high-pH phase of the alkaline transition is dominated by Lys 79 ligation by comparison with literature NMR data (*27*).

For the biphasic alkaline conformational transition of the K73H variant of iso-1-cytochrome *c*, the data are consistent with two trigger groups with different pK_H values, and two different chemical groups replacing Met 80 as the heme ligand. The following partitioned equilibrium is consistent with the spectroscopic data.



We note that the trigger groups, T_1 and T_2, may be the same chemical groups as ligands L_1 and L_2, respectively. We will use T_1 and L_1 and thus K_{H1} and K_{C1} to refer to the low-pH phase of the alkaline conformational transition of the K73H variant. T_2 and L_2, and K_{H2} and K_{C2}, will refer to the high-pH phase of the alkaline conformational transition of the K73H variant. This analysis assumes that the replacement of Met 80 by either branch of the equilibrium is a strict one-proton process. We also note that we cannot rule out direct interconversion of the L_1 and L_2 liganded states. However, in equilibrium measurements, only the relative favorability, of the states which can be observed, can be assessed. Thus, no mechanistic information about how species interconvert is available. For simplicity, we use the least complicated equilibrium consistent with the data and thus have not included equilibrium arrows between (Fe−L_1|Met80) and (Fe−L_2|Met80).

Since this is a partitioned equilibrium, the observed equilibrium constant for loss of Met 80 with increasing pH can be written in terms of the populations, *P*, of the states involved in the equilibrium as given in eq 13. P_{Fe-L1} is the population of the state with ligand L_1 bound to the heme; P_{Fe-L2} is the population of the state with ligand L_2 bound to the heme, and $P_{Fe-Met80}$ is the population of the state with Met 80 bound to the heme.

$$K_{1,2}(\text{obs}) = \frac{P_{Fe-L1} + P_{Fe-L2}}{P_{Fe-Met80}} \tag{13}$$

The population of each state is readily obtained from partition function analysis using the Met-bound state as the reference state as in eqs 14a−c, where K_1(obs) and K_2(obs) have the same form as in eq 5 (Materials and Methods), and refer to the observed equilibria involving T_1 and L_1, and T_2 and L_2, respectively.

$$P_{Fe-Met} = 1/[1 + K_1(\text{obs}) + K_2(\text{obs})] \tag{14a}$$

$$P_{Fe-L1} = K_1(\text{obs})/[1 + K_1(\text{obs}) + K_2(\text{obs})] \tag{14b}$$

$$P_{Fe-L2} = K_2(\text{obs})/[1 + K_1(\text{obs}) + K_2(\text{obs})] \tag{14c}$$

Substituting eqs 14 into eq 13 yields the trivial result given in eq 15.

$$K_{1,2}(\text{obs}) = K_1(\text{obs}) + K_2(\text{obs}) \tag{15}$$

Using the result for the pH dependence of *K*(obs) for a single ionizable ligand displacing Met 80 in eq 7 (Materials and Methods), we obtain eq 16.

$$K_{1,2}(\text{obs}) = \frac{K_{C1}}{1 + \dfrac{[H^+]}{K_{H1}}} + \frac{K_{C2}}{1 + \dfrac{[H^+]}{K_{H2}}} = \frac{10^{-pK_{C1}}}{1 + 10^{pK_{H1} - pH}} + \frac{10^{-pK_{C2}}}{1 + 10^{pK_{H2} - pH}} \tag{16}$$

The properties of eq 16 bear some discussion with regard to which parameters can be uniquely determined. First consider the high-pH phase of the transition, denoted by K_{C2} and K_{H2}. Our experimental data indicate that the high-pH phase of the alkaline transition of K73H iso-1-cytochrome *c* goes to completion and that the ligand involved is Lys 79. Previously reported data on a K73A variant (*27*) of iso-1-cytochrome *c* (with only the Lys 79 ligand) also indicate that lysine 79 drives the alkaline transition to completion. Thus, $pK_{C2} < -1$, and the alkaline transition occurs at a pH [pK_H(app)] that is significantly lower than the pK_{H2} of the trigger group. In this case, eq 8b (Materials and Methods) holds and pK_{C2} and pK_{H2} cannot be determined independently with eq 16. Thus, one or the other must be known when using eq 16. For lysine 79, pK_H has been reported to be 10.8 from kinetic data (*27*). We will use this value. On the other hand, our data indicate that $pK_C \sim 0$ for the low-pH phase of the alkaline transition of K73H iso-1-cytochrome *c*. Examination of eq 8b (Materials and Methods) indicates that $pK_H(\text{app}) \cong pK_H$ under such a circumstance, which means the simplifying assumption $[H^+] \gg K_H$ (i.e., $pH \ll pK_H$) during the transition does not hold and eqs 8a and 8b no longer apply. Inspection

of the two *K*(obs) terms in eq 16 shows that if the transition occurs at a pH much lower that the pK_H of the trigger group, the value of *K*(obs) changes by a factor of 10 for each unit change in pH {i.e., the slope of a plot of $-\log[K(\text{obs})]$ vs pH is 1; see eq 9 in Materials and Methods}. This rate of change of *K*(obs) can be obtained with multiple combinations of K_C and K_H, and thus, these parameters are not uniquely determined. However, when the conformational transition occurs at a pH near the pK_H of the trigger group, the rate of change of *K*(obs) with pH is no longer constant. On a log scale, the rate of change of $-\log[K(\text{obs})]$ versus pH goes from a slope of 1 to a slope of 0 and asymptotically approaches the value of pK_C, which will be near 0 as indicated above. Thus, for the low-pH phase of the alkaline transition of K73H iso-1-cytochrome *c*, pK_{C1} can be determined uniquely from the leveling off of the curve, near the end of the first phase of the transition. Thus, pK_{C1} and pK_{H1} for the trigger group, T_1, can be deconvoluted from fitting the data to eq 16, since pK_{C1} is near 0.

For the purposes of nonlinear least-squares fitting of the data in Figures 1 and 6, eq 16 is substituted into eq 11 (Materials and Methods), giving eq 17.

$$A_{695} = \frac{A_N + A_{alk}\left(\dfrac{10^{-pK_{C1}}}{1+10^{pK_{H1}-pH}} + \dfrac{10^{-pK_{C2}}}{1+10^{pK_{H2}-pH}}\right)}{1 + \dfrac{10^{-pK_{C1}}}{1+10^{pK_{H1}-pH}} + \dfrac{10^{-pK_{C2}}}{1+10^{pK_{H2}-pH}}} \quad (17)$$

When data are fit to this equation, it is assumed that the end points, A_N and A_{alk}, are the same for both the low- and high-pH phases of the transition. Since the 695 nm band is due to the presence of Met 80 ligation and is expected to be absent when other ligands bind to the heme, irrespective of the nature of the incoming ligand, this is a reasonable assumption. This same assumption is not tenable for fitting data to the Soret band near 400 nm as is evident from the spectra in Figure 2. Thus, we restrict extraction of the parameters pK_{C1}, pK_{H1}, and pK_{C2} to curve fitting analysis of data obtained at 695 nm.

The parameters obtained from fitting the data in Figures 1 and 6 to eq 17 are collected in Table 2. Parameters from fitting the WT alkaline transition data to both eqs 10 and 12 are reported in Table 2, as well. As expected, eq 8b holds for the WT data. The fit to eq 10 is consistent with a one-proton process for the WT protein, as expected. The parameters pK_{C1} and pK_{H1} obtained for the low-pH phase of the alkaline transition of the K73H protein are robust. They are obtained irrespective of the initial parameter set used for curve fitting. The fitting routine also converges smoothly. We also note that if pK_{H1} is constrained to values 1 pH unit above or below the value obtained by curve fitting, it is not possible to obtain a good fit to the data. On the other hand, the value of pK_{H2} can be constrained to a wide range of values, besides the literature value of 10.8 for the transition to Lys 79 ligation (*27*), and a good fit to the data is still obtained.

The value for the pK_{H1} of the trigger group for the low-pH phase of the transition is 6.7 ± 0.1, irrespective of gdnHCl concentration or H_2O versus D_2O as the solvent. This observation is not surprising since the pK_a values of most ionizable amino acid groups are not sensitive to gdnHCl concentration (*51*). We note that this value is the expected value for a solvent-exposed histidine such as His 73 (*52*). The lack of a shift in the value for pK_{H1} in D_2O is consistent with previous observations showing that the difference in the activities of deuterium and hydrogen at a glass electrode is equal to and the opposite of the difference in their activities with respect to the titratable group of histidine (*53*). The value of pK_{C1} becomes progressively more negative (i.e., more favorable) as the gdnHCl concentration increases. This is as expected. GdnHCl should progressively destabilize the substructure of iso-1-cytochrome *c* that is disrupted by the alkaline transition, decreasing the conformational energy that must be overcome for the alkaline transition of cytochrome *c*. The magnitude of pK_{C2} is less than −1, as expected. If a linear free energy relationship is assumed for this transition [$\Delta G_u = \Delta G_u(H_2O) - m[\text{gdnHCl}]$; see ref *54*], *m* values of 1.67 ± 0.08 and 1.1 ± 0.2 kcal mol^{-1} M^{-1} are obtained for the low- and high-pH phases of the alkaline transition of the K73H variant, respectively. Since *m* values appear to correlate with the increase in solvent-exposed surface area of hydrophobic groups (*55*), the *m* values indicate that the structural disruption for the low-pH phase of the transition is greater than for the high-pH phase of the transition. Previously, a larger enthalpy was observed for the Lys 73-driven alkaline transition than for the Lys 79-driven alkaline transition (*27*), which is consistent with a greater structural disruption for the Lys 73-ligated state than for the Lys 79-ligated state. Given the relative structural proximity of these two lysines relative to the heme−Met 80 linkage, much greater structural disruption is expected for a heme ligand at position 73 than for one at position 79 when it displaces Met 80. The *m* value for the alkaline conformational transition of the WT protein is 1.1 ± 0.1 kcal mol^{-1} M^{-1}, which suggests that Lys 79 is the dominant ligand in the alkaline state under the conditions used in our experiments. The relative intensities of (5)a (Lys 73 ligation) and (5)b (Lys 79 ligation) in Figure 3 qualitatively corroborate this conclusion.

In the preceding discussion, a number of factors coincide to implicate His 73 as the ligand in the low-pH phase of the alkaline transition of the K73H protein. First, side chains at

Table 2: Thermodynamic Parameters for the Alkaline Conformational Transitions of WT and K73H Iso-1-cytochromes *c* in 0.1 M NaCl at 25 °C

WT Iso-1-cytochrome *c*			
[gdnHCl] (M)	pK_H(app)[a]	*n*[a]	pK_C[b]
0	8.62 ± 0.02	0.92 ± 0.01	−2.20 ± 0.05
0.1	8.55 ± 0.01	0.94 ± 0.04	−2.26 ± 0.02
0.2	8.47 ± 0.06	0.93 ± 0.05	−2.34 ± 0.06
0.3	8.36 ± 0.04	0.96 ± 0.01	−2.44 ± 0.04
0 (with D_2O)	8.93 ± 0.02	0.89 ± 0.02	−1.88 ± 0.02
K73H Iso-1-cytochrome *c*			
[gdnHCl] (M)	pK_{H1}[c]	pK_{C1}[c]	pK_{C2}[c]
0	6.60 ± 0.06	0.28 ± 0.01	−2.18 ± 0.11
0.1	6.71 ± 0.16	0.14 ± 0.02	−2.24 ± 0.04
0.2	6.70 ± 0.17	0.02 ± 0.08	−2.39 ± 0.14
0.3	6.71 ± 0.11	−0.09 ± 0.05	−2.39 ± 0.10
0 (with D_2O)	6.74 ± 0.09	0.49 ± 0.03	−2.04 ± 0.03

[a] Parameters extracted from fits to eq 10. [b] Parameters obtained from fits of the data to eq 12, where pK_H = 10.8. See the legend of Figure 1. [c] Parameters obtained from fits of the data to eq 17, where pK_{H2} = 10.8 (*27*).

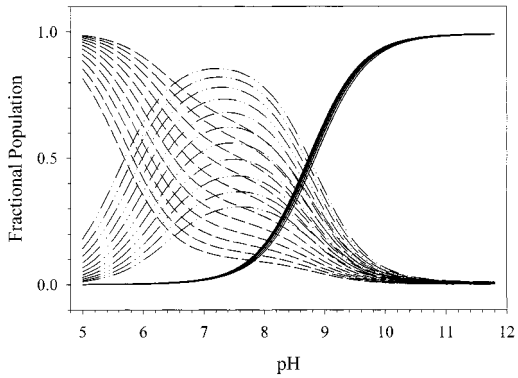


FIGURE 7: Plots of the population of heme−Met 80 (− − −), heme−His 73 (−•−), and heme−Lys 79 (—) states vs pH at different gdnHCl concentrations. The parameters from fitting the data depicted in Figures 1 and 6 to eq 17 along with the *m* values for the low- and high-pH phases reported in the text were used in eqs 14a−c to generate these curves. The gdnHCl concentration is varied from 0 to 1 M in steps of 0.1 M. For heme−Met 80 ligation, the top curve curve is 0 M and the bottom curve is 1.0 M gdnHCl. For heme−His 73 ligation, the bottom curve is 0 M and the top curve is 1.0 M gdnHCl. For heme−Lys 79 ligation, the fractional population at a given pH increases up to ~0.5 M and then decreases again, such that the lowest visible curve is 1.0 M gdnHCl and the second lowest visible curve is 0 M gdnHCl.

position 73 that are good heme ligands are known to cause the alkaline transition (*27*), and histidine is a strong heme ligand (*56*). Replacement of Lys 73 with alanine prevents the alkaline transition due to a protein side chain at this position (*27*). Second, the chemical shifts of the heme methyl groups of the intermediate state at pH* 7.5 are consistent with an imidazole ligand, such as histidine (*45*, *46*). Third, the structural disruption caused by the first phase of the transition is significantly larger than that for the second phase of the transition, consistent with previous data for ligation by lysine from position 73 versus position 79 in the alkaline state (*27*). Finally, the pK_{H1} of ~6.7 is consistent with a histidine. Thus, we assign His 73 as the ligand involved in heme binding in the low-pH phase of the alkaline transition of the K73H protein.

In Figure 7, we show the populations of the His 73 and Lys 79 alkaline conformational states as a function of pH and gdnHCl concentration using the parameters in Table 2 along with eqs 14a−c. The maximal fractional occupation of the His 73-bound state is predicted to be 0.85 and occurs at pH 7.2 and 1.0 M gdnHCl. We do not extend the evaluation of the population of these states as a function of pH beyond 1.0 M gdnHCl, because the fully unfolded state is expected to start to become more stable than the intermediate state above 1 M gdnHCl (*35*, *36*). This situation results because the *m* value for global unfolding (*35*, *36*) is approximately 3 times the magnitude of the *m* value for formation of the His 73 alkaline conformer (see ref *57*). The population of the intermediate His 73 state predicted from data for the alkaline conformational transition of the K73H protein is consistent with the previous data for gdnHCl denaturation of this same protein monitored by CD at 220 nm and at an A_{695} at pH 7.5 (*35*). It is also noteworthy in Figure 7 that as the gdnHCl concentration increases, the pH at which maximal population of the intermediate His 73-ligated state occurs decreases slightly from 7.6 at 0 M gdnHCl to 7.2 at 1.0 M gdnHCl. Also, as the gdnHCl concentration increases, the Lys 79-driven transition initially shifts to lower pH because pK_C becomes more favorable. However, at [gdnHCl] ~ 0.5, the steeper dependence of free energy on [gdnHCl] of the His 73-bound state (larger *m* value) outcompetes Lys 79 ligation at intermediate pH values, pushing the Lys 79-driven transition to higher pH again. It is also clear that as [gdnHCl] increases, even at pH 5, the native state can no longer be fully populated. At pH <5, the global stability of iso-1-cytochrome *c* drops off rapidly (*36*), complicating the analysis presented in Figure 7, and thus, this analysis is not extended below this pH. The partition function analysis in Figure 7 can be extended to the data obtained in D_2O. The stabilizing effect of D_2O limits the maximal fractional population of the intermediate state to ~0.2 (pH* 7.8 at maximum population), significantly below the maximal population of ~0.3 in H_2O. This is consistent with the low intensity observed for the heme methyl proton resonances at pH* 7.6 in Figure 4. Overall, the parameters derived from the data presented here allow the equilibria between the native state and two partially unfolded forms of iso-1-cytochrome *c* to be mapped out as a function of pH and [gdnHCl].

Implications for the Alkaline Transition of Cytochrome c. Significant progress has been made in understanding the nature of the alkaline conformational transition of cytochrome *c*. Although kinetic data are consistent with a fast deprotonation event followed by a simple two-state conformational transition (*25*), NMR (*26*), EPR (*27*, *44*), and Raman (*58*) data demonstrate that more than one alkaline conformer exists. More recently, data on variants of iso-1-cytochrome *c* (*27*) have demonstrated that the two alkaline conformers in this protein result from two different protein ligands replacing Met 80, either Lys 73 or Lys 79. Significant controversy has revolved around the nature of the trigger group for the alkaline conformational transition. The pK_H values obtained from the pH dependence of the kinetics of this transition are consistent with the identity of the trigger group being lysine (*25*, *27*). However, the decreases in pK_H obtained for several mutations at position 82 in yeast iso-1-cytochrome *c* are difficult to reconcile with the trigger group being either of these surface-exposed lysines (*42*). It is equally difficult to understand how the mutations at position 82, which are believed to increase the level of exposure of the heme to water (*59*), would lower the pK_H values of putative trigger groups such as His 18, Tyr 67, or a buried water molecule to values far below the intrinsic pK_a values of these groups in aqueous solution. However, a heme propionate with an unusually high pK_a (*60*), which has been suggested to be the trigger group for the alkaline conformational transition of cytochrome *c* (*24*, *61*), cannot be ruled out on this basis. Mutation of Pro 76 to Gly on the other hand has no effect on the pK_H value (*62*). Possibly, the simple mechanism of Davis et al. (*25*) is no longer adequate for yielding reliable parameters for position 82 variants which have both lysine 73 and 79 competing for heme ligation. As suggested by Mauk and co-workers (*27*), studies on the alkaline conformational transition of iso-1-cytochrome *c* with position 82 mutations where either only lysine 73 or only lysine 79 is present should shed light on the surprising shifts in pK_H for these variants.

In the data presented here, we replaced Lys 73 with histidine. This replacement significantly changes the properties of the alkaline conformational transition, making it

biphasic. It was thus necessary to develop a more complex model to fit the biphasic alkaline transition that is evident in the data. Since the His 73-induced transition does not go to completion, it was possible to evaluate pK_C and thus pK_H for the first phase of the transition by equilibrium methods, a deconvolution not usually possible without resorting to kinetic measurements. The pK_H value obtained is consistent with the pK_a expected for a solvent-exposed histidine in cytochrome *c* (*52*). The fact that replacing Lys 73 with a histidine shifts the pK_H for the alkaline transition involving a ligand at position 73 from 12.0 ± 0.3 (*27*) to 6.60 ± 0.06 in H_2O is very suggestive. In the Lys 73 case, the pK_a is near the intrinsic value for a lysine, and in the His 73 case, the pK_a is near the intrinsic value for a histidine. The chemical shifts of the heme methyl groups for the WT and K73H proteins in the native state are essentially indistinguishable (Table 1), indicating that perturbations to the heme environment in the native state resulting from the K73H mutation are minimal. A significant shift in the pK_a of any of the putative buried trigger groups (His 18, Tyr 67, heme proprionate, or buried water molecule) is highly unlikely if the heme environment in the native state is not perturbed significantly by the K73H mutation. In our view, the simplest explanation of the results presented here is that the alkaline conformational transition is driven by deprotonation of the ligand (Lys 73 or Lys 79 in the WT protein; His 73 or Lys 79 in the K73H protein) which replaces Met 80.

The data presented here are also consistent with the alkaline conformational transition resulting in a relatively small degree of unfolding of the protein. The gdnHCl *m* value for the His 73-driven transition is about $^1/_3$ the value for full unfolding of the protein ($m = 5.11$ kcal mol^{-1} M^{-1}; see ref *63*). The *m* value for the Lys 79-driven transition is even smaller. Solvent denaturation *m* values are proportional to the change in the degree of solvent exposure of buried hydrophobic groups caused by a conformational transition (*54*, *55*). Thus, the disruption of structure is much less than for full unfolding of yeast iso-1-cytochrome *c*.

It has been previously suggested that the alkaline conformation may represent a model for a folding intermediate of iso-1-cyochrome *c* (*62*). Work by Englander and co-workers (*8*, *32*−*34*) has indicated that the lowest-energy partially unfolded form of cytochrome *c* involves unfolding of Ω-loop D (residues 70−85). Englander and co-workers have suggested that formation of this loop may be the last step in folding of this protein (*32*). This segment of the protein includes both sequence positions implicated in the alkaline transition of iso-1-cytochrome *c*. The *m* value obtained here for the His 73-driven alkaline transition is nearly identical to that obtained for unfolding of Ω-loop D by Englander and co-workers (1.6 kcal mol^{-1} M^{-1}; see ref *32*). Thus, the data presented here provide additional support for the notion that the alkaline conformational state of cytochrome *c* is a useful model for a folding intermediate of cytochrome *c*. Recent NMR studies by Bren and co-workers show that lysines replace Met 80 in horse cytochrome *c*, during equilibrium unfolding (*64*). The degree of structural disruption observed for the lysine-liganded state for the horse protein is also consistent with involvement of the alkaline state as an equilibrium folding intermediate (*64*). Thus, further studies on the alkaline state of iso-1-cytochrome *c* are expected to be very fruitful for understanding the folding of this protein.

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