Effect of pH on Formation of a Nativelike Intermediate on the Unfolding Pathway of a Lys 73 → His Variant of Yeast Iso-1-cytochrome *c*†

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ABSTRACT: Previous work on a Lys 73 → His (H73) variant of iso-1-cytochrome *c* at pH 7.5 [Godbole et al. (1997) *Biochemistry 36*, 119−126] showed that this variant unfolds through a nativelike intermediate that has properties consistent with replacement of the Met 80 heme ligand by His 73. Here, the pH dependence of the equilibrium unfolding of the wild type (WT) and H73 proteins have been investigated, since a characteristic pH dependence is expected for the stability of an intermediate stabilized by histidine−heme ligation. Stability has been evaluated using guanidine hydrochloride and pH denaturation methods. Above pH 5, the *m*-values from guanidine hydrochloride denaturation of the WT and H73 variants remain significantly different, consistent with continued population of this intermediate. At pH 4.5 the *m*-values for the two proteins are within error the same. To assess stability at lower pH, acid denaturation was carried out. The midpoint is about 3.3 for both proteins but the transition is broader for the H73 protein, suggestive of intermediates again being populated during the unfolding of the H73 protein at this lower pH. Heme ligation by Met 80 was monitored (695 nm absorbance) during gdnHCl (pH 4.5 and 5.0) and acid denaturation, confirming, respectively, the absence and presence of intermediates. A thermodynamic analysis demonstrates that this complex pH dependence for the presence of histidine ligation induced intermediates is expected and implicates a titratable group with a pK_a of ∼6.6. The analysis also demonstrates when the pH dependences of global stability and stability of an intermediate differ significantly, population of folding intermediates as a function of pH will show novel behavior.

Small globular proteins have generally been assumed to have unfolding equilibria which can be described as highly cooperative two state processes (*1*). In recent years, a number of experimental results have caused this assumption to be questioned. Chief among these results has been the observation that single-site mutations to some proteins can substantially alter the magnitude of the unfolding *m*-value, the rate of change of free energy as a function of denaturant concentration, $\delta\Delta G/\delta$[denaturant] (*2*). In some instances there is substantial evidence that changes in denatured state compactness play an important role in these mutation-induced changes in *m*-values (*3*−*8*). However, decreases in *m*-values, in particular, can be caused by the presence of intermediates on the unfolding pathway (*9, 10*). Thus, these results have renewed interest in the role of stable intermediates in the folding equilibria of small globular proteins (*11*). A number of recent results appear to support a connection between decreased *m*-values and the presence of intermediates on the folding pathway of a protein (*12*−*15*).

Hydrogen-exchange NMR experiments have shown that cytochrome *c* has a number of partially unfolded states that are in thermodynamic equilibrium with the native state of the protein (*16*−*18*). The heme coordination properties of cytochrome *c* make it likely that some of these partially unfolded intermediates could be stabilized. In particular, the Met 80 ligand is relatively easily displaced in the Fe(III) oxidation state of the heme by exogenous ligands such as cyanide and imidazole (*19*−*22*). In fact, in a Phe 82 → His variant of iso-1-cytochrome *c*, the Met 80 ligand is replaced by histidine in the native state of the oxidized protein (*23, 24*). This site is thus a weak point in the native state structure of the oxidized protein. We have recently demonstrated that an iso-1-cytochrome *c* variant which introduces a potential heme ligand onto the surface of the protein can stabilize a nativelike intermediate on the equilibrium unfolding pathway of this protein (*15*). The histidine introduced at position 73 in this variant is located in the least stable surface loop of the protein (residues 70−85 or Ω-loop D; *17*) and appears to stabilize an intermediate involving loss of this loop structure (See Figure 1).

It is well-known that cytochrome *c* maintains a low-spin heme under denaturing conditions (*25*) which can be titrated to a high-spin state at low pH. For horse heart cytochrome *c*, in 8 M urea, the spin state transition occurs with an apparent pK_a of 5.2, consistent with a histidine ligand. NMR (*26*) and folding kinetics data (*27*) have confirmed this assignment. Data on the His 73 iso-1-cytochrome *c* variant show that the histidine−heme ligation titrates with an apparent pK_a of ∼4.7 (*15*) in 3 M guanidine hydrochloride (gdnHCl).[1] A pH dependence for formation of a histidine−heme stabilized partially unfolded intermediate stabilized by the H73 variant of iso-1-cytochrome *c* is similarly expected. As the pH is decreased, the availability of deprotonated histidine

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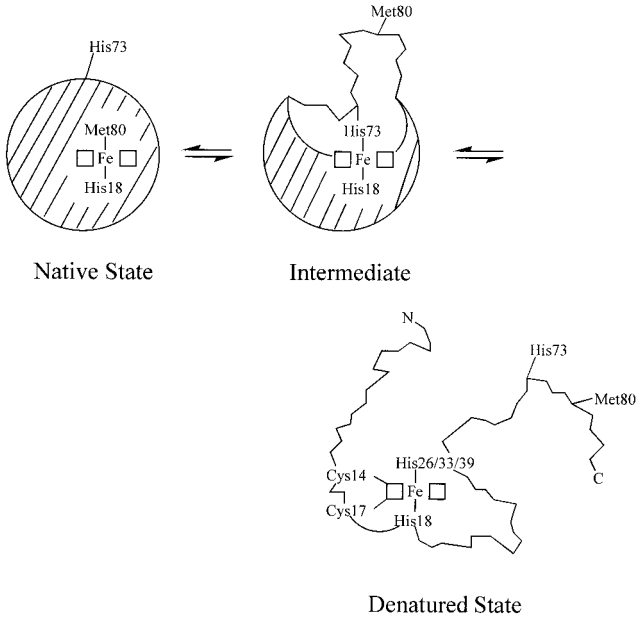

FIGURE 1: Schematic representation of the proposed unfolding mechanism of the H73 variant of iso-1-cytochrome *c*. At low [gdnHCl], Met 80 → His 73 ligand exchange stabilizes unfolding of the least stable substructure of cytochrome *c* (Ω-loop D; residues 70−85). At higher [gdnHCl], the protein unfolds completely and His 26, 33 and 39 dominate heme ligation due to the shorter loop sizes generated by these histidines in the fully unfolded protein (see ref 26).

to displace the Met 80 heme ligand should decrease and a decrease in the ability to form an intermediate state is expected. The presence of the nativelike intermediate should show properties characteristic of a histidine and thus provide confirmatory evidence for the participation of a histidine in the formation of this intermediate. To explore this process, we have investigated the pH dependence of gdnHCl-induced unfolding of WT and H73 iso-1-cytochromes *c* as monitored by CD spectroscopy. The acid unfolding has also been studied to allow assessment of the presence of His 73 induced intermediates in a pH range not accessible to gdnHCl denaturation methods at 25 °C. Heme−Met 80 ligation has been monitored during unfolding in a few instances of particular interest to allow assessment of the presence of heme ligation intermediates during unfolding. The observed pH dependence of the presence of the intermediate stabilized by the H73 variant is more complex than would be expected for simple protonation of a histidine. Thermodynamic analysis indicates that the strength of histidine−heme ligation relative to overall protein stability controls the population of the intermediate. Since histidine−heme ligation is a one proton process and the pH unfolding of iso-1-cytochrome *c* is an ~2.5 proton process, the intermediate and the native state of the H73 variant have similar stabilities in two distinct pH ranges. This work is thus a novel demonstration of how differing pH dependencies for the stabilities of the native state and an intermediate state of a protein can lead to unusual behavior for the population of an equilibrium folding intermediate.

[1] Abbreviations: gdnHCl, guanidine hydrochloride; CD, circular dichroism; $\Delta G°_u$, free energy of unfolding; $\Delta G°_u{}^{H_2O}$, free energy of unfolding in the absence of denaturant; *m*, denaturant dependence of $\Delta G°_u$; C_m, midpoint of gdn HCl denaturation; $pH_{1/2}$, midpoint of pH denaturation; *n*, number of protons taken up during pH denaturation.

MATERIALS AND METHODS

Isolation and Purification of Proteins. The Lys 73 → His mutation was introduced using site-directed mutagenesis as previously described (*28*). The WT and H73 proteins were isolated and purified as described previously (*29*) from the *Saccharomyces cerevisiae* GM-3C-2 cell line carrying the pRS425 multicopy vector (*30*) with the iso-1-cytochrome *c* gene cloned into it (*28*). In both the WT and the H73 proteins, the Cys 102 has been replaced with a serine residue to avoid any possible dimerization of protein molecules resulting from intermolecular disulfide cross-links. Prior to all unfolding experiments, proteins were oxidized with K_3-$Fe(CN)_6$ to produce ferricytochrome *c* and separated from the oxidizing agent by G-25 chromatography using resin equilibrated to the appropriate buffer.

GdnHCl Denaturations as a Function of pH Monitored by CD Spectroscopy. Oxidized WT and H73 proteins at a final concentration of 2 μM were used in all experiments. Experiments at pH values of 7.5 and 7.2 were carried out in 20 mM Tris buffer with 40 mM NaCl. At pH 6.5, 6.12, and 5.8 experiments were done with 20 mM MES buffer with 40 mM NaCl, whereas 20 mM acetate buffer (pH adjusted with HCl) containing 40 mM NaCl was used for studies at the lower pH values of 4.0, 4.5, and 5.0. Stock solutions of 6 M gdnHCl were prepared in the respective buffers appropriate to the pH being studied. Concentration of gdnHCl stock solutions was assessed by refractive index measurements (*31*). Preliminary experiments carried out to see the effect of gdnHCl on the pH of each of these buffers showed minimal changes in pH up to 3 M gdnHCl (data not shown). Denaturations were carried out at 25 ± 0.1 °C by incrementally increasing the gdnHCl concentration as previously described (*32*) and monitoring the change in ellipticity at 220 nm using a Jasco 500-C spectropolarimeter.

Analysis of the denaturation data to obtain the free energy of unfolding and the *m*-value of the transition was done assuming a linear dependence of free energy on gdnHCl concentration (*31, 33*). The ellipticity versus gdnHCl data was fit directly to eq 1 using a nonlinear least-squares fitting

$$\theta = [(\theta°_N + m_N[\text{gdnHCl}]) + \{(\theta°_D + m_D[\text{gdnHCl}])\exp((m[\text{gdnHCl}] - \Delta G°_u{}^{H_2O})/\text{RT})\}]/1 + \exp\{(m[\text{gdnHCl}] - \Delta G°_u{}^{H_2O})/\text{RT}\} \quad (1)$$

procedure, where θ is the ellipticity of the sample, $\theta°_N$ and $\theta°_D$ are the intercepts for the native and the denatured state baselines respectively at 0 M gdnHCl, and m_N and m_D are the [gdnHCl] dependences of the native and the denatured state baselines, respectively. At pH 4.0 and 4.5 for the H73 protein and pH 4.0 for the WT protein, the pretransition baseline was held constant because there was not adequate data to reliably fit the m_N parameter (*34*). A minimum of three trials was done for each protein at every pH. The reported values of $\Delta G°_u{}^{H_2O}$ and *m* are averages of the fits from the individual trials. The reported error is the standard deviation of the parameters at each pH.

pH Denaturations Monitored by Soret Absorbance. Protein concentration was kept constant at 3.0 μM in a total volume of 3 mL in 5 mM Tris and 10 mM NaCl buffer at 25 °C. A 60 μL sample was removed to obtain the spectrum at each pH using a Beckman DU 640 spectrometer and a 50 μL

volume microcell. The volume of the main sample was restored by adding 30 μL of 2×-concentrated protein and 30 μL of an HCl solution of appropriate concentration so as to bring about the desired change in pH. $A_{398\ nm}$ was plotted against the pH and the data fit to eq 2, where A_N is the

$$A_{398\ nm} = (A_N + A_D 10^{n(pH_{1/2}-pH)})/(1 + 10^{n(pH_{1/2}-pH)}) \quad (2)$$

absorbance at 398 nm of the native protein and A_D is the absorbance of the denatured protein at 398 nm, n is the number of protons involved in the transition, and $pH_{1/2}$ is the midpoint pH of the proton induced conformational transition. Fitting was done using a nonlinear least squares routine. The magnitude of $\Delta G°_u$ in the transition region was calculated using eq 3, where R is the universal gas constant,

$$\Delta G°_u = -2.303nRT(pH_{1/2} - pH) \quad (3)$$

T is 298 K, and n and $pH_{1/2}$ are the parameters obtained from the nonlinear least-squares fit to eq 2. For each protein, the reported errors in n and $pH_{1/2}$ are the standard deviation of two experiments.

pH Denaturations Monitored at 695 nm. The experimental set up was exactly as described for measurement at 398 nm except that the protein concentration was kept constant at 100 μM. Absorbance at 695 nm was measured at every pH, and the extinction coefficients were plotted against pH. The data were fit to eq 2, modified to account for the observed pH dependence of ϵ_{695} in the pretransition baseline ($A_N = A_N° + m_N$pH), when necessary. For each protein, the reported errors in n and $pH_{1/2}$ are the standard deviation of two experiments.

GdnHCl Denaturations at pH 4.5 and 5.0 Monitored by 695 nm Absorbance. Oxidized protein at a final concentration of 100 μM in 20 mM sodium acetate, 40 mM NaCl buffer at pH 4.5 or 5.0 was used for these experiments. A 6 M gdnHCl stock solution at pH 4.5 or 5.0 was prepared in the same buffer. Denaturations were carried out at 25 ± 0.1 °C in a constant volume of 0.5−1.5 mL by incrementally increasing the gdnHCl concentration from 0 to 1.8 M. The absorbance at 695 nm at each gdnHCl concentration was measured using a Beckman DU 640 spectrophotometer. Extinction coefficients at 695 nm for the WT and H73 protein were plotted against [gdnHCl] and fit to eq 1. Since the pre- and posttransition values of ϵ_{695} showed no clear [gdnHCl] dependence, their values were held constant during nonlinear least-squares fitting. The errors in the reported parameters are the standard deviation of two experiments at each pH. Values of $\Delta G°_u{}^{H_2O}$ and m for data at pH 7.5 also were obtained in this way using data reported previously (*15*).

RESULTS

GdnHCl Denaturation as a Function of pH. To assess the affect of pH on the stability of WT and H73 iso-1-cytochromes *c*, gdnHCl denaturation experiments were carried out at eight pH values ranging from pH 7.5 to pH 4.0. A typical gdnHCl denaturation monitored by CD spectroscopy at 220 nm is shown in Figure 2, and the values of $\Delta G°_u{}^{H_2O}$, m, and C_m (the gdnHCl concentration at the midpoint of unfolding) for all pH values are presented in Table 1. The $\Delta G°_u{}^{H_2O}$ and m-values were calculated using nonlinear least-squares fits to the CD data assuming a two-state transition (see Materials and Methods). The trends for the values of $\Delta G°_u{}^{H_2O}$ and m as a function of pH are shown graphically in Figure 3A,B, respectively.

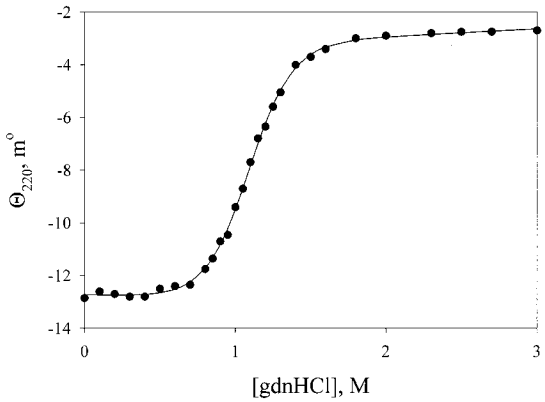

FIGURE 2: GdnHCl induced unfolding of wild-type iso-1-cytochrome *c* at pH 7.2 in 20 mM Tris, 40 mM NaCl buffer. Ellipticity at 220 nm is plotted against [gdnHCl]. The curve represents a nonlinear least-squares fit to the data as described in Materials and Methods. $\Delta G°_u{}^{H_2O}$, m-values and C_m obtained from nonlinear least-squares fits at all pH values studied are collected in Table 1.

At all values of pH, $\Delta G°_u{}^{H_2O}$ is greater in magnitude for the WT protein relative to the H73 variant. Both proteins have relatively constant values of $\Delta G°_u{}^{H_2O}$ above pH 5.0, but below pH 5.0 stability drops sharply. The denaturation midpoint, C_m (see Table 1), reflects this loss in stability at lower pH, as well. At higher pH values, in particular, C_m values for the WT and H73 proteins are within error the same. Thus, the lower apparent values of $\Delta G°_u{}^{H_2O}$ for H73 in the upper pH regime certainly reflect fitting data that deviates from two-state behavior to an equation for two-state unfolding. At lower pH, similar drops in stability are observed for the gdnHCl denaturation of both the horse protein (*35*) and the iso-2 variant of yeast cytochrome *c* (*36*). Studies on the thermal denaturation of wild-type iso-1-cytochrome *c* (*37*) show that the stability drops abruptly as the pH decreases from 5 to 3. Values of ΔG_u(298 K) of 5.3 ± 0.3 kcal/mol at pH 5.0, 3.7 ± 0.2 kcal/mol at pH 4.5, and 2.9 ± 0.2 kcal/mol at pH 4.0 obtained from thermal denaturation data for the wild-type protein (*37*) agree reasonably well with our gdnHCl denaturation data at these pH values.

At higher values of pH, the m-value for the WT protein is significantly larger than the m-value for the H73 protein. At pH 4.5, the m-values for the two proteins are within error of each other, and at pH 4.0, the m-values may diverge again; however, this divergence at pH 4.0 is uncertain because of the error in the measured m-values. The m-values for the WT and H73 proteins move toward each other at pH 4.5, the m-value for WT dropping somewhat and that of the H73 protein increasing somewhat.

Previous studies on the properties of the wild type versus the H73 protein at pH 7.5 indicated that at this pH the lower m-value for the H73 protein could be assigned to the presence of a nativelike intermediate (*15*). The nativelike intermediate was attributed to displacement of the native-state Met 80 heme ligand by histidine 73. From the simplest perspective, as pH is lowered the ability of His 73 to replace Met 80 should diminish and the role of the intermediate observed at pH 7.5 for the H73 variant in the equilibrium folding

Table 1: Thermodynamic Parameters Obtained from gdnHCl Denaturations of the WT and H73 Proteins at Various pH Values

	$\Delta G°_u{}^{H_2O}$ (kcal/ mol)		*m* (kcal/(mol M))		C_m (M)	
pH	WT	H73	WT	H73	WT	H73
7.5[a]	5.66 ± 0.48	3.68 ± 0.19	4.92 ± 0.41	3.20 ± 0.18	1.15 ± 0.01	1.15 ± 0.01
7.2	4.68 ± 0.35	3.41 ± 0.52	4.31 ± 0.20	2.97 ± 0.37	1.09 ± 0.04	1.15 ± 0.05
6.5	5.44 ± 0.14	3.52 ± 0.32	4.45 ± 0.26	3.14 ± 0.19	1.23 ± 0.07	1.12 ± 0.03
6.12	5.66 ± 0.19	3.60 ± 0.20	4.86 ± 0.24	3.31 ± 0.21	1.16 ± 0.02	1.09 ± 0.01
5.8	5.28 ± 0.47	3.70 ± 0.20	4.84 ± 0.37	3.54 ± 0.20	1.09 ± 0.04	1.05 ± 0.02
5.0	4.77 ± 0.25	3.60 ± 0.01	4.42 ± 0.21	3.67 ± 0.08	1.08 ± 0.01	0.98 ± 0.02
4.5	4.11 ± 0.16	3.10 ± 0.31	4.28 ± 0.25	3.75 ± 0.29	0.96 ± 0.03	0.83 ± 0.05
4.0	2.72 ± 0.37	1.71 ± 0.10	4.10 ± 0.37	3.33 ± 0.17	0.66 ± 0.04	0.51 ± 0.01

[a] Data from ref 15, refit using the nonlinear least-squares method described in Materials and Methods.

Table 2: Data from Acid Denaturation of the WT and H73 Proteins

protein	parameter	A_{398} data	ϵ_{695} data
WT	$pH_{1/2}$	3.33 ± 0.07	3.45 ± 0.02
	n	2.6 ± 0.1	3.8 ± 0.7
H73	$pH_{1/2}$	3.31 ± 0.04	3.63 ± 0.09
	n	1.9 ± 0.1	2.2 ± 0.2

pathway should decrease. If the role of this intermediate is less, the *m*-value for the H73 variant should increase toward that of the WT protein (*10*). The behavior is clearly more complex than this scenario, since the two appear to converge near pH 4.5 and may begin to diverge as the pH is lowered further.

The nativelike intermediate due to displacement of Met 80 can be detected readily by monitoring the unfolding of cytochrome *c* at 695 nm (*15*). To investigate the role of the nativelike intermediate at pH 4.5, where the *m*-values appear to converge, gdnHCl unfolding was monitored at 695 nm at this pH for both proteins. Figure 4A,B compares the unfolding data at 695 nm to CD data for the H73 and WT proteins, respectively. Several observations are noteworthy. At 0 M gdnHCl, the extinction coefficient at 695 nm for the H73 protein is very close to that of the WT protein. At pH 7.5, the 0 M gdnHCl extinction coefficient is ~65% that of the wild-type protein (LaConte and Bowler, unpublished results). At pH 7.5 and 0 M gdnHCl, some loss of Met 80 ligation appears to have occurred for the H73 variant, whereas at pH 4.5 much less has occurred even though overall the protein is less stable at this pH. The apparent *m*-value at pH 7.5 was ~1.8 kcal/(mol M) for denaturation monitored at 695 nm. At pH 4.5 the *m*-value for the 695 nm data is clearly similar to that for the CD data (see Figure 4A). A value of ~3.9 kcal/(mol M) is obtained from nonlinear least-squares fits to the 695 nm data. Similar behavior is also observed at pH 5.0 (data not shown) for denaturation monitored at 695 nm (m = ~3.8 kcal/(mol M)). At pH 7.5 the difference in C_m between the CD and the 695 data, ΔC_m, for the H73 protein is ~0.93 M (*15*). At pH 4.5, ΔC_m is 0.35 ± 0.06 M and, at pH 5, ΔC_m is 0.28 ± 0.04 for the H73 protein. For the WT protein, ΔC_m is 0.21 ± 0.02 at pH 7.5, 0.28 ± 0.08 at pH 4.5, and 0.16 ± 0.03 at pH 5.0. A slight deviation from two-state behavior occurs for the WT protein at all pH values. It is evident at pH 4.5 and 5.0 that the behavior of the H73 protein is quite similar to the WT protein, with regard to the presence of a nativelike intermediate involving displacement of Met 80, suggesting minimal involvement of His 73 ligation at these pH values.

pH Denaturation of WT and H73 Proteins. Below pH 4.0, it is not possible to obtain reliable gdnHCl denaturation data

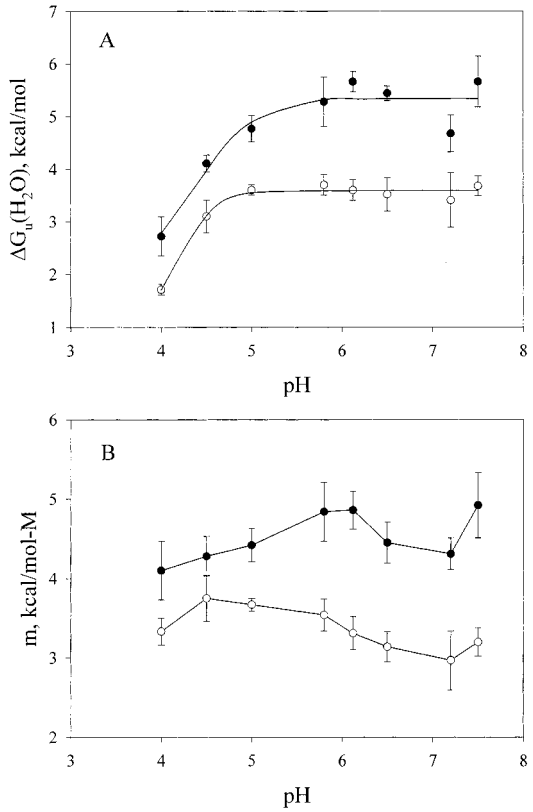

FIGURE 3: (A) Plot of $\Delta G°_u{}^{H_2O}$ against pH for the WT (solid circles) and H73 (open circles) proteins. The error bars indicate one standard deviation. (B) Plot of the *m*-values for the WT (solid circles) and H73 (open circles) against pH. The error bars represent one standard deviation. The pH 7.5 data in parts A and B are from ref 15.

for both the H73 and WT proteins. Also, at pH 4.0, due to the errors in *m*-values, it is uncertain if the *m*-values of the WT and H73 proteins are truly diverging. To further investigate the effect of pH on the ability of the H73 variant to form a nativelike intermediate during equilibrium denaturation, we have carried out pH unfolding studies of the WT and H73 proteins. Figure 5 compares the pH denaturations for the WT and H73 proteins monitored by changes in Soret absorption band intensity measured at 398 nm. Changes in the extinction coefficient and maximum of the Soret band monitor spin-state changes that occur when a strong field ligand such as methionine or histidine is replaced by a weak field ligand such as water (*38*). It can be seen in Figure 5 that the spin-state transition induced at low pH is broader for the H73 protein than for the WT protein. Differences in the breadth of pH transitions reflect apparent differences in the number of protons involved in the process. In Table 2, it can be seen that the midpoints of the two spin-

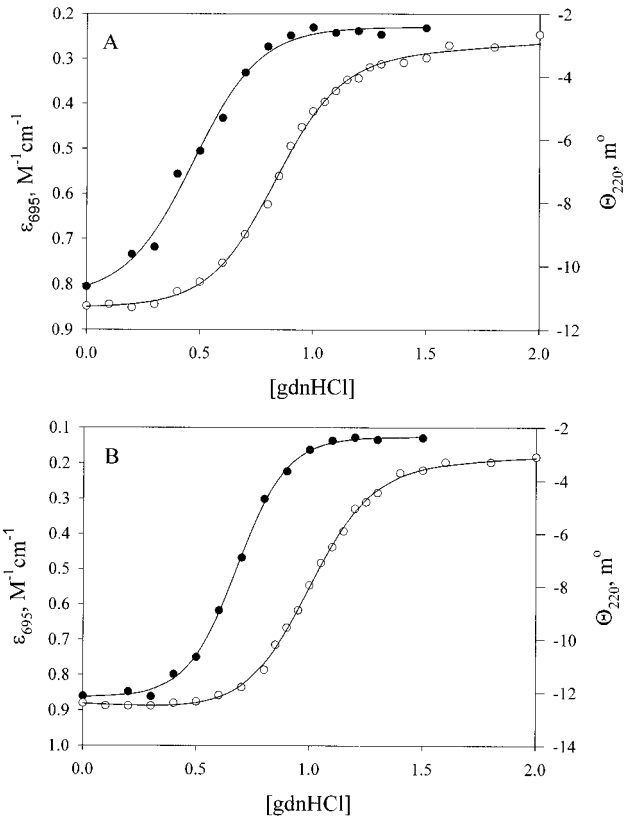

FIGURE 4: (A) Data at pH 4.5 for the extinction coefficient at 695 nm (solid circles) versus gdnHCl concentration for the H73 protein. CD data (open circles) from 0 to 2 M gdnHCl at 220 nm are included for comparison. (B) Data at pH 4.5 for the extinction coefficient at 695 nm (solid circles) versus gdnHCl concentration for the WT protein. CD data (open circles) from 0 to 2 M gdnHCl at 220 nm are included for comparison. The solid curves in parts A and B represent nonlinear least-squares fits to eq 1 as described in Materials and Methods.

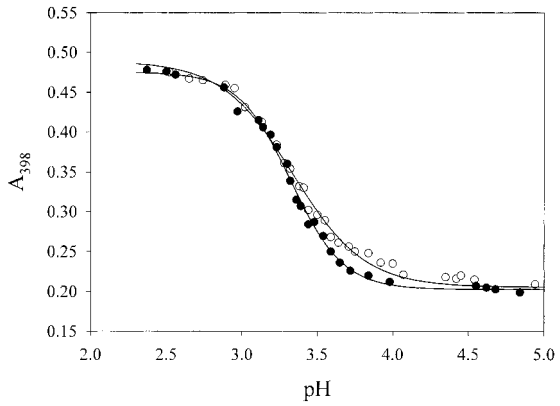

FIGURE 5: Acid denaturation of the WT (solid circles) and H73 (open circles) iso-1-cytochromes *c* monitored by absorbance at 398 nm. The solid curves represent nonlinear least-squares fits to eq 2 as described in Materials and Methods. The values of $pH_{1/2}$ and *n* obtained from these fits are collected in Table 2.

state transitions are about the same. The number of protons involved in the spin-state transition is 0.7 ± 0.2 less for the H73 protein.

To investigate whether intermediate displacement of the Met80 ligand by histidine 73 plays a role in the broader transition observed for the H73 protein at 398 nm, the pH unfolding was also monitored at 695 nm. The 695 and 398 nm data are compared for the H73 and WT proteins in Figure 6A,B, respectively. The 695 and 398 nm monitored conformational transitions are nearly coincident for the WT protein ($\Delta pH_{1/2} = 0.12 \pm 0.09$). For the H73 protein, the 695 nm band is lost somewhat in advance of the spin-state transition ($\Delta pH_{1/2} = 0.32 \pm 0.13$). The pH unfolding is essentially two state for the WT protein, yet clearly deviates from two-state behavior for the H73 protein. The Met 80 ligand is lost prior to the low-spin to high-spin transition during pH denaturation for the H73 protein. It appears to be transiently replaced by another strong field ligand, presumably His 73.

At pH 7.5, the nativelike intermediate induced by the presence of His 73 is highly populated during equilibrium unfolding. At pH 4.5 and 5.0, changes in Met 80 ligation during gdnHCl unfolding are similar for both proteins. At the lower values of pH required to denature iso-1-cytochrome *c* in water, loss of Met 80 ligation is again induced preferentially by the presence of His 73. Thus, the ability of histidine 73 to induce a nativelike intermediate has complex behavior as a function of pH.

DISCUSSION

A number of factors in our previous study of the equilibrium unfolding of H73 iso-1-cytochrome *c* implicated histidine 73 displacement of Met 80 as the cause of formation of an unfolding intermediate (*15*). Met 80 ligation was lost substantially in advance of the CD monitored unfolding. His 73 is part of the least stable loop of cytochrome *c* which encompasses residues 70−85 (*17*), and the imidazole ring of histidine is a strong heme ligand (*38*). The *m*-value for formation of this intermediate as monitored at 695 nm at pH 7.5 (∼1.8 kcal/(mol M)) is nearly identical to the *m*-value (1.6 kcal/(mol M)) reported for the stability of the 70−85 loop structure as a function of gdnHCl concentration in NMR-detected H/D exchange experiments (*17*).

The ability of His 73 to induce intermediate formation, presumably by disrupting the loop 70−85 structure, should depend on both the strength of this ligation as a function of pH and on the stability of the structure disrupted by ligation as a function of pH. Therefore, a full understanding of the effects of pH on the population of the intermediate for the H73 variant of iso-1-cytochrome *c* requires careful consideration of the pH dependence of both the global stability of iso-1-cytochrome *c* and the pH dependence of the stability gain achieved by replacing the Met 80 heme ligand with His 73 in the H73 variant.

pH Dependence of Iso-1-cytochrome c Stability and of the Strength of Histidine-Heme Ligation. To analyze the data, it will be assumed that a surface histidine at the solvent-exposed position 73 of iso-1-cytochrome *c* minimally affects the energy of the native state. Therefore, the pH dependence of the WT protein stability will be used to model the native state energy of the H73 variant. The $\Delta G^{\circ}_{u}{}^{H_2O}$ values of the H73 protein are consistently lower than those for WT protein (Table 1, Figure 3A). This difference, at higher pH, results mainly from the lower *m*-value due to deviations from two-state behavior. The similarity of the denaturation midpoints, C_m, in the high-pH range (see Table 1), suggests that the assumption of similar global stability for the two proteins is reasonable. It is possible to map out the stability of the wild type protein over the pH range 3−7.5 using a combination of the gdnHCl denaturation data and pH denaturation data

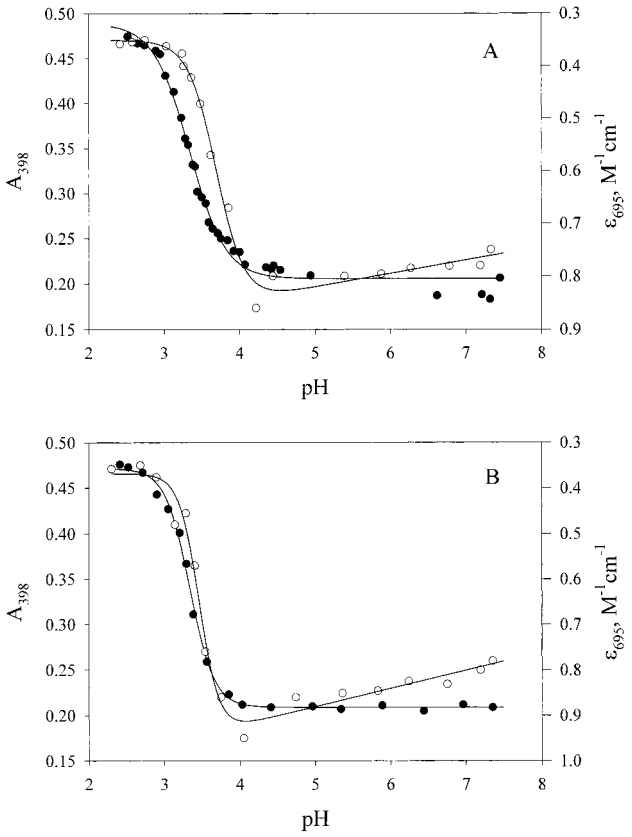

FIGURE 6: (A) Extinction coefficient at 695 nm (open circles) and absorbance at 398 nm (solid circles) plotted against pH for the H73 protein. (B) Extinction coefficient at 695 nm (open circles) and absorbance at 398 nm (solid circles) plotted against pH for the WT protein. The solid lines represent fits to eq 2 as described in Materials and Methods. The values of $pH_{1/2}$ and n obtained from these fits are collected in Table 2.

presented here, along with thermal denaturation data for the WT protein reported previously (*37*). In Figure 7, it can be seen that the stability of WT iso-1-cytochrome *c* is essentially constant at a value of ~5.3 kcal/mol at pH 5 and above. Below pH 5, the stability drops off, rapidly reaching a value of 0 kcal/mol by pH 3.3 (the midpoint of the pH denaturation). The three methods used to monitor stability appear to be monitoring the same process since there is good agreement between stability values where the different methods overlap.

To determine the ability of a histidine to displace a methionine, it is necessary to estimate the difference in stability between heme−histidine and heme−methionine ligation. Estimates of these relative stabilities come from direct measurements of the binding of imidazole and *N*-acetyl methionine to a heme octapeptide fragment obtained by proteolytic digest of horse heart cytochrome *c* (*39*). The fragment encompasses residues 14−20 and thus maintains His 18 ligation and both thioether linkages to the heme (through cysteines 14 and 17). The intrinsic binding affinities, log K_{heme}, are 4.45 (*39, 40*) and 0.58 (*39*) for imidazole and *N*-acetylmethionine at 25 °C, respectively. The log K_{heme} value for imidazole is extrapolated to $[H^+] = 0$. Thus, the equilibrium constant for imidazole binding to heme can be as much as 4 orders of magnitude larger than for methionine. The stability of methionine binding is expected to be pH independent; however, histidine binding will be pH-dependent because of the protonation equilibrium of the nitrogen

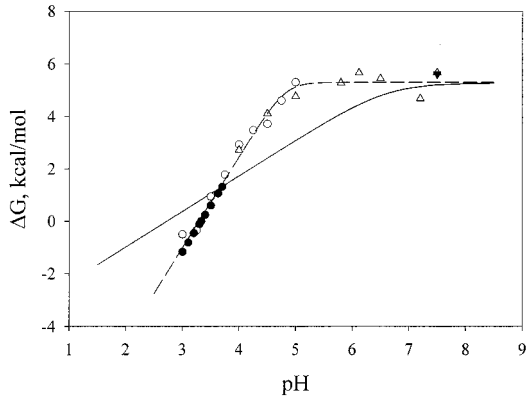

FIGURE 7: Plots of $\Delta G°_u$ for the WT protein and the negative of the free energy of exchanging methionine for histidine ligation to the heme, $-\Delta\Delta G_{His/Met}$ (solid line), versus pH. The pH dependence of $-\Delta\Delta G_{His/Met}$ is calculated according to eq 7 which is parametrized as described in the text. $\Delta G°_u$ data for the WT protein is from three sources, gdnHCl denaturation studies (open triangles), thermal denaturation data (open circles, taken from ref 37), and pH denaturation data (solid circles). The dashed line is a fit of the data to an equation similar in form to eq 7 but which allows for a multiple proton process. The fit implicates titratable groups with an average pK_a of 4.82, indicates a 2.56 proton process (as in Table 2), and gives a value of 5.30 kcal/mol for the asymptotic protein stability above pH 5. The upside-down solid triangle at pH 7.5 indicates the free energy required to produce the partially unfolded state involving loss of the 70−85 loop. This value was obtained by adding $-\Delta\Delta G_{His/Met}$ to $\Delta G°_u{}^{H_2O}$ for production of the intermediate at pH 7.5. The $\Delta G°_u{}^{H_2O}$ value for formation of the intermediate at pH 7.5, ~0.4 kcal/mol, was obtained from a nonlinear least-squares fit of ϵ_{695} versus [gdnHCl] to eq 1 as described in Materials and Methods.

which ligates to the heme. The effective binding constant as a function of pH, K_{heme}(pH) will vary due to the change in the proportion of the imidazole ring available in the deprotonated form as a function of pH. It is straightforward to show that this dependence has the form given in eq 4 (*39*),

$$K_{heme}(H^+) = K_{heme}(0)/(1 + ([H^+]/K_a)) \quad (4)$$

where $K_{heme}(0)$ is the stability at $[H^+] = 0$ and K_a is the acid dissociation constant of the heme ligand. Writing this equation in terms of pH and pK_a, we obtain eq 5, where

$$K_{heme}(pH) = K_{heme}(\infty)/(1 + 10^{(pK_a - pH)}) \quad (5)$$

$K_{heme}(\infty)$ is the binding constant at infinite pH (ie. $[H^+] = 0$). Rewriting this equation in terms of free energy, we obtain eq 6, where $\Delta G_{heme}(\infty)$ is the free energy of heme binding

$$\Delta G_{heme}(pH) = \Delta G_{heme}(\infty) + 2.3RT \log(1 + 10^{(pK_a - pH)}) \quad (6)$$

at infinite pH. Since methionine binding is pH independent, the difference in stability when histidine replaces methionine, $\Delta\Delta G_{His/Met}$, is given by eq 7, where $\Delta G_{heme-His}(\infty)$ is the free

$$\Delta\Delta G_{His/Met}(pH) = \Delta G_{heme-His}(\infty) + 2.3RT \log(1 + 10^{(pK_a(His) - pH)}) - \Delta G_{heme-Met} \quad (7)$$

energy of heme−histidine binding at infinite pH, $\Delta G_{heme-Met}$ is the free energy of methionine−heme binding, and pK_a(His) is the pK_a of histidine. The difference $\Delta G_{heme-His}(\infty)$

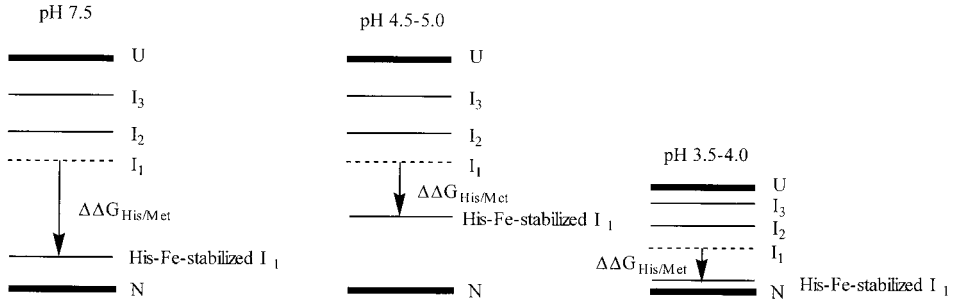

FIGURE 8: Schematic representation of the interplay between $\Delta\Delta G_{His/Met}$ and the overall stability of cytochrome *c* and its substructures. The vertical scale is free energy. This interplay controls the formation of the nativelike intermediate resulting from the Lys 73 → His mutation. At pH 7.5 the value of $\Delta\Delta G_{His/Met}$ is large, allowing the ligand exchange reaction to significantly lower the energy of the lowest energy partially unfolded state, I_1 (Ω-loop D, residues 70−85), of cytochrome *c*. At pH 4.5−5.0 the stability of the protein has not decreased significantly but the magnitude of $\Delta\Delta G_{His/Met}$ has. I_1 is no longer adequately stabilized to allow the intermediate to be observed during equilibrium unfolding. Below pH 5, the stability of the protein drops more rapidly than $\Delta\Delta G_{His/Met}$. I_1 again is lowered adequately in energy relative to the native state, N, to be observed as a folding intermediate. U is the fully unfolded state of the protein and I_1, I_2, and I_3 are the partially unfolded states of cytochrome *c* defined previously (*17*).

$-\Delta G_{heme-Met}$ can be derived from the log K_{heme} values given above. It is equal to −5.27 kcal/mol at 25 °C. For H73 iso-1-cytochrome *c*, if His 73 is not bound to the heme, the protein can be assumed to be in the native state. Lysine 73 is highly solvent-exposed and is located at a relatively charge isolated site in native state iso-1-cytochrome *c* (*29*). Therefore, His 73 is expected to behave like an isolated solvent-exposed histidine. From a survey of pK_a values for histidine in unordered peptides and at solvent-exposed sites in proteins, a pK_a value of 6.6 ± 0.1 is expected (*41*). More specifically, in iso-1-cytochrome *c*, values of 6.7 and 6.8 have been reported for His 39 and His 33 respectively (*42*). Thus, His 73 should have a pK_a in the range of 6.6 and this value will be used for pK_a(His) in eq 7.

Figure 7 shows a plot of $-\Delta\Delta G_{His/Met}$ as a solid line; the negative of $\Delta\Delta G_{His/Met}$ is being used for ease of comparison to the magnitude of $\Delta G°_u$. At pH greater than 7, the value of $-\Delta\Delta G_{His/Met}$ approaches 5.27 kcal/mol asymptotically. Below about pH 5, $-\Delta\Delta G_{His/Met}$ decreases linearly at a rate of 1.36 kcal/mol per pH unit. The $\Delta G°_u$ curve crosses the $-\Delta\Delta G_{His/Met}$ curve at pH 3.7 just near the beginning of the pH denaturation transition of WT iso-1-cytochrome *c*. Thus, the amount of free energy that can be supplied to compensate for loss of structure is very close the overall stability of the WT protein above pH 7 and near pH 3.7. At pH values below 7 and above 3.7, the ability of this ligand exchange to compensate for loss of structure is less relative to the overall stability of the protein. The maximal difference between $\Delta G°_u$ and $-\Delta\Delta G_{His/Met}$ occurs at about pH 4.9 with the parameters used. This point of maximal difference between $\Delta G°_u$ and $-\Delta\Delta G_{His/Met}$ results largely from the properties of the pH dependence of global stability. The ability of histidine ligation on the Met 80 side of the heme to stabilize a partially unfolded iso-1-cytochrome *c* intermediate is maximal above pH 7 and near pH 3.7. As the pH is lowered from pH 7 to 5 or raised from pH 3.7 to pH 5, the ability of histidine ligation to stabilize the partially unfolded intermediate state decreases.

This analysis is consistent with the experimental results for the equilibrium unfolding properties of WT versus H73 iso-1-cytochromes *c*. At pH 7.5, the H73 protein clearly unfolds via a well-defined intermediate (*15*) and the *m*-values of the WT and H73 proteins are significantly different. At pH 4.5, the error ranges of the *m*-values overlap and the loss of Met 80 ligation during gdnHCl denaturation is nearly identical relative to the CD-monitored unfolding for the two proteins. At pH 4.5, the difference between $\Delta G°_u$ and $-\Delta\Delta G_{His/Met}$ is significantly larger than at pH 7.5 (∼1.3−1.7 kcal/mol versus ∼0.1−0.4 kcal/mol). Thus, the ability to stabilize a partially folded intermediate state is significantly diminished at pH 4.5. At pH 3.7, the values of $-\Delta\Delta G_{His/Met}$ and $\Delta G°_u$ are equal. Again, there is enough energy in this ligand exchange to stabilize a partially unfolded state of the protein. In the pH unfolding studies, the denaturation curves monitored at 398 and 695 nm are essentially indistinguishable for the WT protein. For the H73 protein, the loss of Met 80 ligation occurs in advance of the spin-state transition at 398 nm which monitors global unfolding. A partially unfolded state is thus being stabilized at this lower pH. Thus, the theoretical analysis of the pH dependence of stabilization of a partially unfolded intermediate by histidine ligation in the H73 variant is consistent with experiment. A schematic representation of the interplay of $\Delta\Delta G_{His/Met}$ and ΔG_u in terms of the energy levels of the partially unfolded states of cytochrome *c* (*17*) is shown in Figure 8.

A few of the parameters in our thermodynamic analysis bear further discussion. The value of $\Delta G_{heme-His}(\infty) - \Delta G_{heme-Met}$ may be questioned since the effective concentrations of Met 80 and His 73 relative to the heme iron may differ somewhat in a looped out region of the protein in a partially unfolded intermediate. Whether the exact values of $\Delta G_{heme-His}(\infty)$ and $\Delta G_{heme-Met}$ obtained in a model system can be extrapolated to the protein system could be questioned as well. However, both of these considerations will only shift the entire $-\Delta\Delta G_{His/Met}$ curve up or down relative to the $\Delta G°_u$ curve. The relative pH dependencies of the $\Delta G°_u$ and $-\Delta\Delta G_{His/Met}$ curves do not change. It is this relative dependence that is important for the interpretation of the pH dependence of the presence of the intermediate during unfolding. In effect, changing the value of $\Delta G_{heme-His}(\infty) - \Delta G_{heme-Met}$ from the value of −5.27 kcal/mol only changes the predicted energy of the partially unfolded state being stabilized by the exchange of methionine ligation for histidine ligation. With the value of −5.27 kcal/mol and the stability of our histidine-stabilized partially unfolded intermediate at pH 7.5 of ∼0.4 kcal/mol (see Materials and Methods), we would predict the stability of the least stable partially unfolded state of iso-1-cytochrome *c* (loop 70−85 from horse

heart cytochrome *c* data, *17*) to be about the same as that for full unfolding of the protein (see Figure 7). Given these considerations, $\Delta G_{heme-His}(\infty) - \Delta G_{heme-Met}$ is probably overestimated in magnitude with the parameters used here. We also assume that the energy of the partially unfolded states tracks with the overall stability of the protein. The observed pH dependence of intermediate formation appears to justify this assumption.

The other important parameter in eq 7 is the assumed pK_a. It is this pK_a which is characteristic of the titratable group involved in ligating the heme and stabilizing the partially unfolded state of the protein. It is thus useful to explore the effect of changing the pK_a of this group on the ability to populate the intermediate as a function of pH. Lowering the pK_a to 6 or raising it to 7 would not qualitatively change the observation that the intermediate would be populated at pH 7.5 but not at pH 4.5 or 5.0. However, with a pK_a of 6, the solid curve in Figure 7 would cross the global stability curve at pH 4.05 and thus the intermediate would be much more populated during pH denaturation than observed. The opposite would be true for a pK_a of 7 where the solid curve would cross the global stability curve very near the midpoint of the pH denaturation. Thus, within the range of pK_a that might be expected for a histidine, that of a solvent-exposed histidine, 6.6, appears to fit the data the best. What about more extreme pK_a values? Maybe His 73 has simply destabilized Ω-loop D, the least stable substructure of the protein (*17*), allowing other side chains in this loop to displace Met 80. Carboxylate side chains with pK_a values of 4.5−5 are unlikely candidates because the ability of such a side chain to stabilize the intermediate observed at pH 7.5 would change very little as the pH drops to 5. Experimentally, the intermediate is no longer observed at pH 5 for the H73 protein. Other likely candidates are the lysine side chains in Ω-loop D which mediate the alkaline transition of cytochrome *c* (*43*). In this case, loss of the intermediate at pH 4.5−5.0, as observed, would be expected. However, reappearance of the intermediate during pH denaturation would not be expected for the same reasons discussed above for a histidine with a pK_a of 7. On the basis of these considerations, we assign the group responsible for formation of the intermediate produced by the Lys 73 → His mutation to a histidine with a pK_a near 6.6. His 73 is the most likely candidate, since it is the sole sequence difference between the H73 and WT proteins. Also, very little loss of CD signal occurs during formation of this intermediate (*15*), consistent with loss of a surface loop. If histidines 26, 33, or 39 were responsible for the intermediate observed for the H73 protein, much more disruption of ordered secondary structure would be expected.

Observed Changes in m-values. m-values are the rate of change in the free energy of unfolding with respect to denaturant concentration (*2*). Theoretically (*33*) and empirically (*44*), *m*-values are proportional to the change in solvent-exposed surface area upon unfolding a protein. In a system where protein folding remains two state, changes in *m*-values induced by single-site mutation are most simply interpreted in terms of changes in the compactness of the denatured state of the protein (*2, 45*). However, in systems where the protein folding equilibrium changes from two state to three state, changes in *m*-values may reflect changes in the proportional occupation of an intermediate during unfolding (*10, 44*). In the case of the H73 variant of iso-1-cytochrome *c*, this latter possibility appears to be the case (*15*). Since the intermediate stabilized in this case involves histidine−heme ligation, its population should vary with pH and thus the relative *m*-values of the WT and H73 proteins should vary. At pH values above 5, the difference in *m*-values is 1.3−1.7 kcal/(mol M). At pH 4.5 the difference in *m*-values is the same within error (0.5 ± 0.5 kcal/(mol M)). Qualitatively, this is consistent with the behavior expected from the above thermodynamic analysis.

It is perhaps surprising that the *m*-values for both H73 and WT decrease at lower pH. An increase in the *m*-value for the H73 variant, toward that of the WT protein, might have been expected. Previous studies on ribonuclease T1 and ribonuclease A (*47*) have shown that *m*-values nearly doubled at lower pH for urea unfolding, presumably due to electrostatic repulsion. Data in gdnHCl for these two proteins showed a small increase in *m*-value for ribonuclease A and a small decrease in *m*-value for ribonuclease T1 (*47*). Fluorescence studies on unfolded cytochrome *c* indicate a substantially less expanded denatured state for this protein at low pH in 6 M gdnHCl than in 9 M urea (*48*). The decrease in *m*-value observed at lower pH for WT iso-1-cytochrome *c*, thus, appears consistent with previous findings. The decrease in the *m*-value for WT iso-1-cytochrome *c* appears to partially counteract an expected increase in the *m*-value for the H73 variant as the pH is lowered below 7 toward pH 4.5−5.0 where the equilibrium unfolding of the H73 protein becomes more two state in character.

Number of Protons Involved in pH Unfolding. The value of 2.6 ± 0.1 protons for the WT protein compares favorably with the value of 2.2−2.5 protons taken up during thermal unfolding in the pH range of the pH denaturation (*37*). Similar values for proton uptake have been reported in this pH range for thermal denaturation of a Cys 102 → Thr "wild type" form of iso-1-cytochrome *c* (*49*). (Our studies use the Cys 102 → Ser "wild type" iso-1-cytochrome *c*.) Our pH denaturation data monitored at 398 nm for the WT iso-1-cytochrome *c* compares favorably to previously reported data for the wild-type iso-1-cytochrome *c* (Cys 102) obtained at 390 nm (*50*). Values of 3.30 and 2.55 at 25 °C were reported for $pH_{1/2}$ and the number of protons involved in the process, respectively. These authors also reported that the same results were obtained using fluorescence to monitor the pH denaturation. This is consistent with the correlation between CD data from our thermal denaturation work (*37*) and our A_{398} data (Figure 7). The coincidence of data from fluorescence, CD, Soret absorbance (390 nm, 398 nm), and absorbance at 695 nm (Met 80-heme ligation) indicates that pH denaturation is a rigorously two-state process for wild-type iso-1-cytochrome *c*. This contrasts with gdnHCl denaturation which shows noncoincidence of data obtained from CD and absorbance at 695 nm at pH 7.5 (*15*) as well as at pH 4.5 and 5.0 for the WT protein. Thus, gdnHCl denaturation is not rigorously two state.

The number of protons involved in pH denaturation differs significantly for the WT and H73 proteins ($\Delta n = 0.7 \pm 0.2$ for the WT protein versus the H73 protein). An apparently smaller uptake of protons occurs during the pH denaturation of the H73 variant. The change in absorbance at 398 nm monitors the spin-state transition which occurs as cytochrome *c* unfolds. Specifically, the exchange of a strong field ligand

(methionine or histidine) for a weak field ligand (H_2O) is being monitored. The loss of methionine as monitored at 695 nm precedes ($\Delta pH_{1/2} = 0.32 \pm 0.13$, 695 nm versus 398 nm data for the H73 protein) but overlaps the spin-state transition monitored at 398 nm for the H73 protein (Figure 6A). The two conformational transitions are not sufficiently well separated that the breadth of the 398 nm transition can be attributed directly to the proton uptake of the intermediate state. The broadening of the transition, which leads to an apparent decrease in the number of protons taken up, thus likely reflects that pH denaturation is no longer a two-state transition for the H73 protein. Unfortunately, this situation does not allow for structural inferences about the intermediate state from the apparent number of protons involved in pH denaturation, since the native to intermediate state and intermediate to denatured state transitions are not well separated.

CONCLUSIONS

The pH dependence of the formation of a nativelike intermediate during unfolding of cytochrome *c* generated by the mutation of Lys 73 → His has been studied. The intermediate is most prominent at pH 7.5 during gdnHCl denaturation (*15*) and between pH 3.5 and 4.0 during acid denaturation. At intermediate pH values, the H73 variant has unfolding properties very similar to the wild-type protein, indicating lack of any significant population of an intermediate beyond what is observed for the WT protein during unfolding. Analysis of the pH dependence expected for heme ligation of a group with a pK_a of 6.6 relative to the pH dependence of the overall protein stability provides an explanation for this behavior. The congruence of theory and experiment provides strong evidence that the group responsible for the formation of the nativelike intermediate during unfolding is in fact a histidine. Given the lack of similar behavior for the WT protein, the present observations strengthen the conclusion in our earlier work that this intermediate is caused by a heme ligation exchange reaction involving His 73. The data also demonstrate that the ability to form a folding intermediate depends both on the pH dependence of the global stability of the protein and the pH dependence of the stability of the intermediate. If the two pH dependences are different, complex behavior can result.

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