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A Lysine 73 → Histidine Variant of Yeast Iso-1-cytochrome *c*: Evidence for a Native-like Intermediate in the Unfolding Pathway and Implications for *m* Value Effects†

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ABSTRACT: In this paper we report thermodynamic studies on a variant of yeast iso-1-cytochrome *c* in which a surface lysine residue at position 73 has been replaced with a histidine (H73). Guanidine hydrochloride denaturation studies monitored by circular dichroism spectroscopy indicated decreased thermodynamic stability (a lower $\Delta G^\circ_{u^{H_2O}}$) and a smaller *m* value for the H73 protein as compared to the wild type (WT) protein. Further investigations to probe the causes for the thermodynamic stability differences between the two proteins involved guanidine hydrochloride and urea denaturations monitored by tryptophan fluorescence. The stability of heme ligation in the denatured state in the presence of either guanidine hydrochloride or urea was monitored by the spin-state transition of the heme iron induced by pH. None of these studies supported the hypothesis that the decreased *m* value was due to heme−His73 ligation in the denatured state. Guanidine hydrochloride denaturations monitored by the change in the extinction coefficient at 695 nm, which is sensitive to the presence of heme−Met80 ligation, revealed a native-like intermediate for the H73 protein, probably caused by displacement of the Met80 heme ligand by histidine 73 at guanidine hydrochloride concentrations much lower than required for full cooperative unfolding. Presence of the native-like intermediate is most likely the cause of the smaller *m* value and decreased thermodynamic stability for the CD-monitored H73 protein unfolding as compared to the unfolding of the WT protein. Guanidine hydrochloride denaturations in the presence of 200 mM imidazole provide further evidence in support of the proposed mechanism.

How polypeptides fold into specific tertiary or quaternary conformations to achieve highly selective and specific functions has been of immense interest to researchers. Anfinsen (1973) concluded that the unique amino acid sequence of any protein, by itself, has all the information for spontaneous folding of the protein to a definite three-dimensional structure rendering its specific functionality. The discovery in the last decade of molecular chaperone proteins, and their roles in assisting folding and targeting of some proteins in cells, revealed the complex nature of interactions involved in *in vivo* protein folding. The presence of ionic and nonionic solutes, co- and post-translational events, macromolecular crowding, and the relatively low concentrations of specific proteins under study in the cellular environment make *in vivo* folding studies extremely complicated (see Freedman, 1992). Another approach has been *in vitro* kinetic and equilibrium studies where the folding of a purified protein is studied under a controlled environment to avoid interference from extraneous factors. Although the data obtained from such *in vitro* studies might not yield exact information about protein folding within cells, it has added a wealth of information about protein folding and structure−function relationships for a number of proteins.

Site-directed mutagenesis provides a tool to study the effects of individual amino acid replacements, at a specific site in a protein, on the structure and thermodynamic stability of the protein. It has been extensively used to study variants of staphylococcal nuclease (Shortle, 1995), T4 lysozyme (Matthews, 1993; Elwell & Schellman, 1979), barnase (Matouschek & Fersht, 1991), apomyoglobin (Hughson & Baldwin, 1989; Barrick et al., 1994), cytochrome *c* (Betz & Pielak, 1992; Bowler et al., 1993), and other proteins.

The conformational stability of the native state plays a very important role in deciding the N ↔ D equilibrium, but contributions from the occurrence of stable intermediates and residual structure in the denatured state can be quite significant. In the past few years, there has been increasing recognition of the fact that the denatured states of certain proteins retain some residual structure and play an active role in determining the equilibrium between the native and the denatured state. Residual structure has been reported in the 434 repressor protein denatured in 7 M urea (Neri et al., 1992), acid and heat denatured states of α-lactalbumin (Griko et al., 1994), and the thermally-denatured state of barnase (Oliveberg et al., 1994), among other examples. NMR studies on tryptophan synthase have shown some residual structure near His92 in 5 M urea where the unfolding transition monitored by absorbance or far UV CD is essentially complete (Saab-Rincon et al., 1993, 1996).

The degree of exposure of hydrophobic residues to the solvent upon denaturation is related to the slope of the denaturation curve (Shortle, 1995; Schellman, 1978). Occurrence of intermediates along the unfolding pathway can

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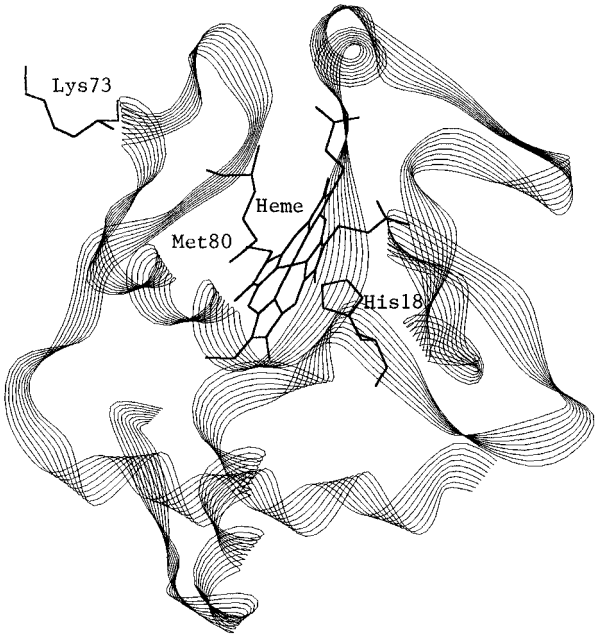

FIGURE 1: Structure of yeast iso-1-cytochrome *c* (Louie & Brayer, 1990; Berghuis & Brayer, 1992) taken from the Brookhaven Databank, file pdb1ycc.ent (Bernstein et al., 1977). The position of Lys73 with respect to the heme is shown, as are the Met80 and His18 heme ligands.

result in altered thermodynamics of the system and smaller *m* values for data evaluated using a two-state model (Pace, 1975). Intermediates usually observed in kinetic studies are thermodynamically unstable and often do not accumulate in sufficient stoichiometric amounts for structural studies. Detection of stable intermediates is difficult in equilibrium unfolding studies resulting in apparent two-state mechanisms. Urea induced unfolding of reduced phosphorylase *b* monitored by CD has demonstrated the existence of equilibrium intermediates (Chignell et al., 1972), as has pH denaturation of apomyoglobin (Hughson et al., 1990).

We are studying yeast iso-1-cytochrome *c* and its surface variants constructed by site-directed mutagenesis. Cytochrome *c* is a low molecular weight, soluble, monomeric protein that can be easily isolated and purified. Being ideally suited for denaturation studies, it has been the subject of both chemical and thermal denaturation experiments (Myer, 1968; Ikai et al., 1973; Tsong, 1973). Lysine 73, which is an evolutionally conserved highly solvent-exposed site of iso-1-cytochrome *c*, has been the focus of initial studies in our laboratory (see Figure 1). Mutations at this site would be expected to cause minimal perturbation to the native state structure as opposed to mutations at more buried sites in the protein core. Previous studies with aromatic (Bowler et al., 1993) and aliphatic (Herrmann et al., 1995) amino acid replacements at site 73 of yeast iso-1-cytochrome *c* showed a strong negative correlation of both $\Delta G^\circ_{u}{}^{H_2O}$ and the *m* value to the free energies of transfer, ΔG_{tr} (1-octanol to water transfer; Fauchere & Pliska, 1983), of the respective amino acids. This reverse hydrophobic effect is indicative of a denatured state effect on the folding equilibrium (Pakula & Sauer, 1990). For the histidine 73 (H73)[1] variant, however, the change in the $\Delta G^\circ_{u}{}^{H_2O}$ and the *m* value with respect to the wild type (WT) were much more dramatic than would be expected solely based upon the ΔG_{tr} of histidine. This observation implied other factors were affecting the unfolding equilibrium in this case. These effects might be acting on the native state, the denatured state, or possibly intermediate states of the H73 protein and prompted further investigations.

It has been shown that, in urea or guanidine hydrochloride (gdnHCl) denatured cytochrome *c*, one of the native histidine residues ligates to the heme, displacing the methionine 80 ligand of the native state (Babul & Stellwagen, 1971; Muthukrishnan & Nall, 1991). A number of kinetic studies also suggest the participation of histidine instead of methionine 80 in non-native states of cytochrome *c* (Elove et al., 1994; Sosnick et al., 1994). For our histidine mutant, there could be a possibility of this non-native His73 ligating to the heme in the denatured state and changing the structure of the denatured state and thus possibly its energetics. To specifically monitor the heme environment in the denatured state, pH titrations of the denatured proteins in 3 and 1.5 M gdnHCl and in 5 M urea were undertaken using the heme Soret absorption as a spectroscopic probe of loss of histidine ligation to the heme iron. The pK_a values determined for these titrations were used to ascertain whether a direct thermodynamic stabilization of heme ligation in the denatured state could be demonstrated.

Hawkins et al. (1994) observed that a Phe82His variant of iso-1-cytochrome *c* resulted in His82 replacing the Met80 ligand of the native ferricytochrome *c*. Ligation of Met80 to ferriheme iron can be monitored by a weak absorption band at 695 nm arising from sulfur−iron ligation. To see if His73 was interfering with Met80 ligation, gdnHCl denaturations were monitored by absorbance at 695 nm.

We have used multiple spectroscopic probes (tryptophan fluorescence, Soret absorption, circular dichroism, iron−ligand charge transfer band at 695 nm) that monitor different structural aspects of cytochrome *c* in combination with various denaturants to obtain maximum insight into the unfolding events of the H73 variant versus the WT protein. These data are consistent with the main cause of the anomalous decreases in the $\Delta G^\circ_{u}{}^{H_2O}$ and *m* values of the H73 variant being due to a native-like intermediate as opposed to a denatured or native state effect.

EXPERIMENTAL PROCEDURES

Isolation and Purification of Proteins. The H73 variant was constructed using site-directed mutagenesis as previously described (Herrmann et al., 1996). The WT and H73 proteins were isolated and purified as described previously (Bowler et al., 1993) from the *Saccharomyces cerevisiae* GM-3C-2 cell line carrying the pRS425 multicopy vector (Christianson et al., 1992) with the iso-1-cytochrome *c* gene cloned into it (Herrmann et al., 1996). In both the WT and the H73 proteins, the Cys102 has been replaced with a serine residue to avoid any possible dimerization of protein molecules resulting from intermolecular disulfide bond formation. This mutation does not affect the physical or functional properties of the protein (Mayo, 1988). Amide I FTIR spectra for the WT and the H73 proteins were obtained as described previously (Bowler et al., 1994).

Guanidine Hydrochloride Denaturations Monitored by CD Spectroscopy. Iso-1-cytochrome *c* oxidized with $K_3Fe(CN)_6$

[1] Abbreviations: gdnHCl, guanidine hydrochloride; FTIR, Fourier transform infrared; CD, circular dichroism; ΔG°_u, free energy of unfolding; $\Delta G^\circ_{u}{}^{H_2O}$ free energy of unfolding in the absence of denaturant; WT, wild type protein; H73, histidine 73 variant protein.

was used in all studies. Oxidized protein was separated from the oxidizing agent using Sephadex G-25 chromatography. A buffer containing 20 mM Tris and 40 mM NaCl was used for studies at pH 7.5. A 6 M gdnHCl stock solution was made using the same buffer. Denaturations were carried out at 25 ± 0.1 °C using a modification of Shortle and Meeker's method (1986) of incrementally increasing the gdnHCl concentration as previously described by Herrmann et al. (1995). A Jasco 500-C spectropolarimeter was used to monitor the changes in the α-helical structure at 220 nm. Analysis of the denaturation data to obtain the free energy of unfolding and the *m* value of the transition was done as described previously (Bowler et al., 1993).

GdnHCl and Urea Denaturations Monitored by Tryptophan Fluorescence. Studies were carried out with ferricytochrome *c* at a concentration of 5 μM for gdnHCl and 4 μM for urea in 20 mM Tris/40 mM NaCl buffer at pH 7.5. The denaturations were done in exactly the same way as those monitored by CD spectroscopy. Protein samples were excited at 287 nm, and the fluorescence of the Trp59 residue was measured at an emission wavelength of 350 nm. A Spex Fluorolog 2 spectrofluorimeter was used with excitation and emission slit widths of 4 mm. The background fluorescence for the buffer plus gdnHCl blank at each [gdnHCl] was subtracted from the respective fluorescence values obtained for each protein sample. Experiments with the buffer blank, the WT, and the H73 protein were conducted simultaneously to minimize possible errors due to variations in the lamp intensity.

The data were analyzed for $\Delta G^{\circ}_{u}{}^{H_2O}$ and *m* values using nonlinear least-squares fits on the basis of a two-state model with a linear dependence of ΔG_u on the denaturant concentration using the equation:

$$I = \{(I^{\circ}_N + m_N[\text{denaturant}]) + (I^{\circ}_D + m_D[\text{denaturant}])\exp[(m[\text{denaturant}] - \Delta G^{\circ}_{u}{}^{H_2O})/RT]\}/\{1 + \exp[(m[\text{denaturant}] - \Delta G^{\circ}_{u}{}^{H_2O})/RT]\}$$

In this equation, *I* is the fluorescence intensity at a given [gdnHCl], I°_N and I°_D are the intercepts for the native and the denatured state baselines, respectively, at zero molar denaturant, and m_N and m_D are the [denaturant] dependencies of the native and the denatured state baselines, respectively. Four trials were done for each sample and the data processed to obtain the averages and the standard deviations for $\Delta G^{\circ}_{u}{}^{H_2O}$ and *m*.

GdnHCl Denaturations in the Presence of Imidazole. GdnHCl denaturations were carried out in a similar way as described above with 20 mM Tris/40 mM NaCl buffer and 5 μM protein concentration in the presence of 200 mM imidazole at pH 7.5. The buffer blanks also contained 200 mM imidazole. Denaturations were monitored by tryptophan fluorescence and the data analyzed by nonlinear least-squares fitting as described above.

pH Titrations of Proteins in the Presence of GdnHCl and Urea. Ferricytochrome *c* (3 μM) in 5 mM Tris/10 mM NaCl buffer at pH 7.5 was used for these experiments, in the presence of either 3 or 1.5 M gdnHCl or 5 M urea. The pH of the protein solution was gradually lowered by addition of HCl while keeping the protein and gdnHCl or urea concentrations constant in a total sample volume of 3 mL.

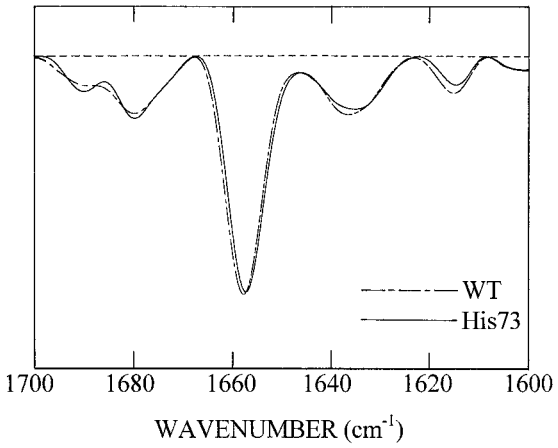

FIGURE 2: The second derivative amide I FTIR spectra for the WT and H73 proteins.

For every data point, a 60 μL sample was removed and used to obtain the spectrum in a microcell of 50 μL volume. The volume of the main solution was remade up to 3 mL by addition of 20 μL of the protein stock solution of 3× (9 μM) concentration, 30 μL of 2× denaturant (6 and 3 M gdnHCl for experiments with 3 and 1.5 M gdnHCl, respectively, or 10 M urea), and 10 μL of an HCl solution of appropriate molarity (0.001−0.05 M), depending upon the pH range being studied. The sample holder was equipped with a magnetic stirrer to ensure thorough mixing after every addition. The pH of each sample was measured by directly dipping the pH electrode into the sample. The effect of increasing H^+ ion concentration on the spin state of the heme iron was followed with a Beckman DU 640 spectrometer by measuring the absorbance at 398 nm (A_{398nm}). The A_{398nm} values were plotted against pH to obtain curves that were fitted to the equation given below:

$$A_{398nm} = \{A_{LS} + A_{HS}[10^{n(pH_{1/2}-pH)}]\}/\{1 + 10^{n(pH_{1/2}-pH)}\}$$

In this equation, A_{LS} is the absorbance at 398 nm of the low-spin denatured protein, A_{HS} is the absorbance of the high-spin denatured protein at 398 nm, *n* is the number of protons involved in the transition, and the midpoint pH, $pH_{1/2}$, corresponds to the pK_a for the proton induced spin-state transition concomitant with loss of histidine ligation.

Guanidine Hydrochloride Denaturations Monitored by the Change in the Extinction Coefficient at 695 nm. Denaturations were carried out with ferricytochrome *c* at 100 μM concentration in a total volume of 1.5 mL in 20 mM Tris/40 mM NaCl, pH 7.5 buffer using a 6 M gdnHCl stock solution and techniques similar to those described above for CD and fluorescence monitored denaturations. The absorbance at 695 nm was measured for each sample at gdnHCl concentrations ranging from 0.0 to 1.8 M using a Beckman DU 640 spectrometer and a 50 μL capacity microcuvette. The extinction coefficients at 695 nm were then plotted against the gdnHCl concentration.

RESULTS

The FTIR amide I spectra for the WT and the H73 mutant are shown in Figure 2. There are no major changes in the α-helix content (strong band near 1656 cm^{-1}) of the two proteins and only small changes in other regions of the

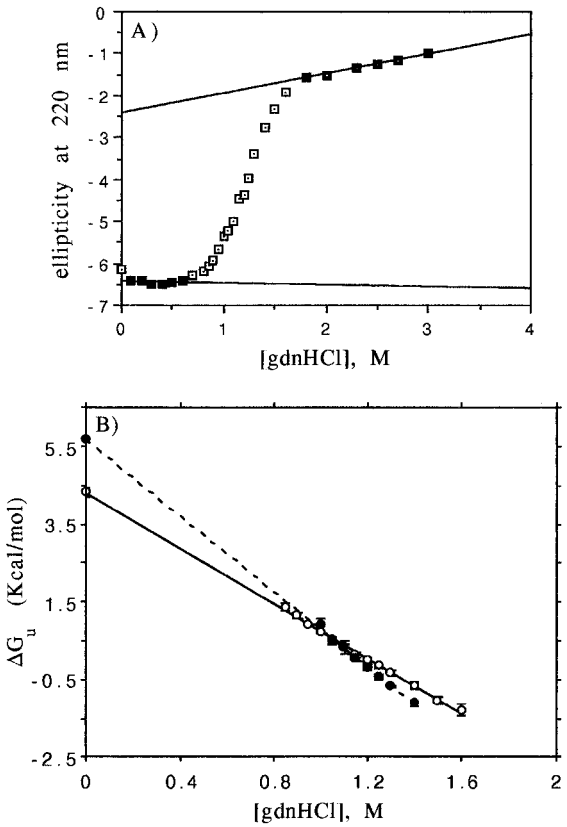

FIGURE 3: (A) A typical gdnHCl denaturation curve monitored by measuring the change in ellipticity at 220 nm. The data shown are for the H73 protein at pH 7.5. The native and the denatured state baselines used to calculate K_u are shown. (B) A plot of ΔG_u versus [gdnHCl] for the WT and H73 mutants at pH 7.5. Open and filled circles represent data for the H73 and WT proteins, respectively. The lines shown are linear fits to the equation $\Delta G_u = \Delta G^\circ_u{}^{H_2O} - m$[gdnHCl]. The error bars are one standard deviation. The intercept of each line shown on the *y*-axis is not an actual data point.

spectrum, indicating structural similarity of the native states of the two proteins.

A typical sigmoidal curve for the gdnHCl induced unfolding of cytochrome *c* monitored by change in ellipticity at 220 nm is shown in Figure 3A. The ΔG°_u at each denaturant concentration was calculated for the transition region using the equation:

$$\Delta G_u = -RT \ln K_u$$

where K_u is the equilibrium constant at a given gdnHCl concentration. Figure 3B shows the graph of ΔG_u versus [gdnHCl] for the WT and H73 proteins. The data is fitted using the equation:

$$\Delta G_u = \Delta G^\circ_u{}^{H_2O} - m[\text{gdnHCl}]$$

The intercept of the curve gives the ΔG_u in the absence of denaturant ($\Delta G^\circ_u{}^{H_2O}$), whereas the slope of the line is the *m* value of the transition. According to Schellman (1978), the slope *m* is proportional to the difference (ΔA) between the solvent exposed surface areas of the native (A_n) and the denatured (A_d) states of the protein:

$$\Delta A = A_d - A_n$$

The *m* value is thus considered to give an estimate of the compactness or the degree of residual structure in the denatured state (Dill & Shortle, 1991; Shortle, 1995). It is evident from Figure 3B and Table 1 that the WT and H73 proteins differ significantly with respect to the in $\Delta G^\circ_u{}^{H_2O}$ and *m* values at pH 7.5.

Tryptophan fluorescence data, where the fluorescence intensity is plotted against [gdnHCl] and [urea], are shown in Figure 4A, and the thermodynamic parameters are given in Table 1. For the native ferricytochrome *c* at pH 7.5, the fluorescence of the single Trp59 residue is almost completely quenched by the heme. As the protein unfolds, the Trp59 moves further away from the heme, resulting in the observed increase in its fluorescence intensity. The curves in Figure 4A for the H73 protein show a considerably less steep transition as compared to the WT. Also, the denatured state fluorescence intensity obtained for the H73 protein is lower than that for the WT. The observed difference is about 4−5% for 3 M gdnHCl and about 3% for 6 M urea, after correcting for slight differences in sample concentrations. This effect was fully reproducible for 4 independent trials. The difference in the fluorescence intensity of denatured WT versus H73 is somewhat more pronounced at 2 M gdnHCl and 4 M urea where the cooperative unfolding transition is just complete.

The results of denaturation in the presence of 200 mM imidazole are shown in Figure 4B and Table 1. The difference in the fluorescence intensities of the denatured proteins disappears in the presence of imidazole. The values for $\Delta G^\circ_u{}^{H_2O}$ and *m* for the WT protein approach those for the H73 protein, indicating that imidazole is mimicking the effect induced by H73. The smaller $\Delta G^\circ_u{}^{H_2O}$ for the WT protein in the presence of imidazole demonstrates the destabilizing effect of imidazole on the protein.

In order to determine if the observed decrease in the *m* value for the H73 protein has a contribution from a denatured state loop due to His73−heme ligation, we measured the pK_a of this linkage in the gdnHCl and urea denatured states. The pH titration data in the presence of 1.5 M gdnHCl are shown in Figure 5 where the absorbance at 398 nm is plotted against pH. As the pH is lowered, one of the histidines (at positions 26, 33, or 39 of yeast cytochrome *c*) ligated to the 6th coordination site of heme in the denatured state is protonated and displaced from the heme by the solvent. This replacement of the strong field histidine ligand with a weak field solvent molecule causes the spin-state transition of the heme iron, resulting in the shift of the Soret absorption maximum from 406 to 397 nm (Pettigrew & Moore, 1990). This shift in λ_{max} is coupled to an increase in absorbance at 398 nm.

The pK_a values obtained for the titrations in 3 and 1.5 M gdnHCl and 5 M urea are given in Table 2. There is no significant difference in the pK_a values for the WT and H73 proteins at both 3 M gdnHCl and 5 M urea. However, the pK_a value for the H73 variant is slightly lower than that of the WT at 1.5 M gdnHCl.

Since the spin-state titration of the heme−histidine ligation in the denatured state gave little evidence for a stabilization of the denatured state for H73 versus WT and since the FTIR data do not indicate large structural perturbations to the native state, we decided to monitor unfolding using the 695 nm absorption band. This absorption band is sensitive to the presence of the Met80 ligand and thus could be useful in detecting the presence of native-like intermediates prior to the major conformational unfolding event. GdnHCl dena-

Table 1: Thermodynamic Parameters for the Denaturant Unfolding of WT and H73 Iso-1-cytochromes *c*

	gdnHCl[a]	gdnHCl[b]	gdnHCl with 200 mM imidazole[b]	urea[b]
$\Delta G^{\circ}_{u}{}^{H_2O}$ (kcal/mol) WT	5.66 ± 0.42	5.43 ± 0.30	4.25 ± 0.18	4.97 ± 0.40
$\Delta G^{\circ}_{u}{}^{H_2O}$ (Kcal/mol) H73	4.32 ± 0.12	4.44 ± 0.30	4.31 ± 0.23	3.63 ± 0.14
m (kcal/(mol·M)) WT	4.87 ± 0.32	4.08 ± 0.40	3.38 ± 0.12	1.65 ± 0.02
m (kcal/(mol·M)) H73	3.59 ± 0.01	3.38 ± 0.28	3.40 ± 0.13	1.23 ± 0.14

[a] Data obtained from CD experiments. [b] Data obtained from Trp fluorescence experiments.

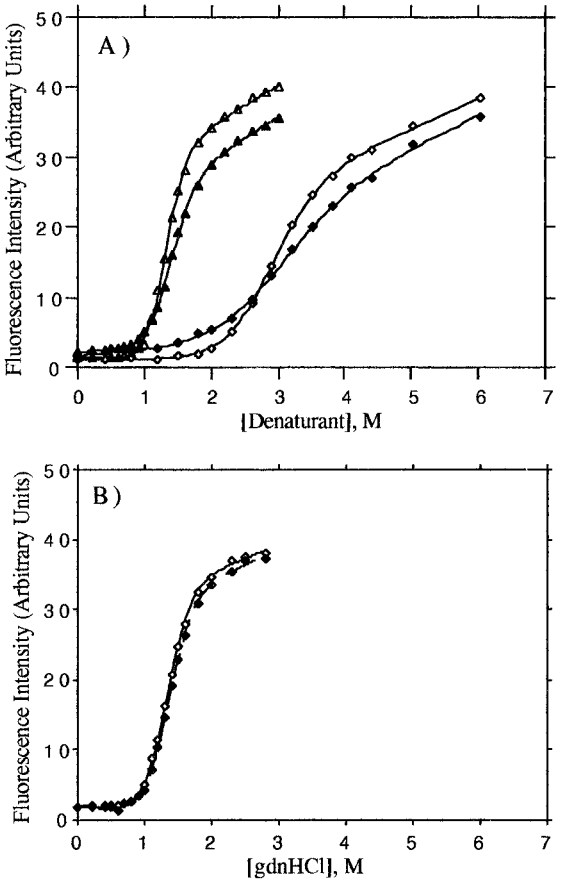

FIGURE 4: The data from the tryptophan fluorescence experiments where the increase in the fluorescence intensity is plotted against the [denaturant] for the WT and H73 proteins. The curves represent nonlinear least-squares fits to the data as described in the Experimental Procedures. (A) Denaturations in the absence of imidazole. The open triangles and diamonds represent data for the WT protein for gdnHCl and urea denaturations, respectively. Filled triangles and diamonds represent data for the H73 protein for gdnHCl and urea denaturations, respectively. (B) Denaturations in the presence of 200 mM imidazole. Open and filled diamonds represent data for the WT and H73 proteins, respectively.

turations monitored at the 695 nm absorption are shown in Figure 6. For the WT protein, the decrease in absorbance for the 695 nm band is very steep and occurs close in concentration to the unfolding transition monitored by CD. For the H73 mutant, however, the transition followed by the 695 nm band occurs at much lower gdnHCl concentrations than the CD-monitored unfolding and is much broader. This result indicates that the iron−sulfur ligation in the mutant protein is destabilized compared to the wild type protein. The His73 side chain is a likely candidate to replace the Met80 ligand upon slight destabilization of the protein conformation due to its structural proximity to the Met80 ligation site (see Figure 1). The fact that such an effect does not occur in the WT protein which does not contain this histidine also supports this assignment.

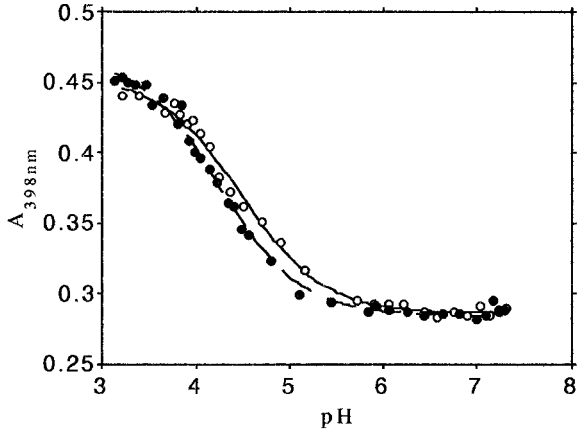

FIGURE 5: Absorbance at 398 nm is plotted against pH in the presence of 1.5 M gdnHCl for the WT and H73 proteins. Open and filled circles represent data for the WT and H73 proteins, respectively. The curves represent nonlinear least-squares fits to the Henderson−Hasselbalch equation as described in the Experimental Procedures.

Table 2: Data from pH-Induced Spin-State Transitions in the Denatured State of WT and H73 Iso-1-cytochromes *c*

exptl conditions	pK_a ($pH_{1/2}$)		*n* (no. of protons)	
	WT	H73	WT	H73
1.5 M gdnHCl	4.45 ± 0.03	4.32 ± 0.01	1.02 ± 0.01	1.21 ± 0.04
3.0 M gdnHCl	4.67 ± 0.03	4.72 ± 0.03	0.95 ± 0.05	1.08 ± 0.09
5.0 M urea	4.81 ± 0.07	4.89 ± 0.06	1.66 ± 0.44	1.70 ± 0.24

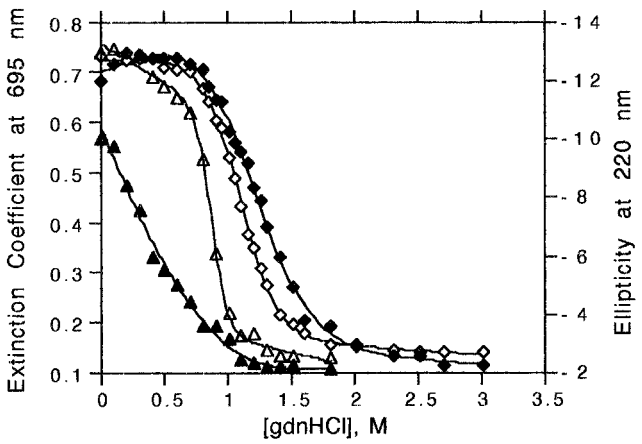

FIGURE 6: The extinction coefficient at 695 nm and ellipticity at 220 nm for the WT and H73 proteins are plotted against [gdnHCl]. Open triangles and diamonds give the data for the WT protein at 695 and 220 nm, respectively. Filled triangles and diamonds represent data for the H73 protein at 695 and 220 nm, respectively. The smooth curves are meant only as visual aids.

DISCUSSION

The spectra of the WT and H73 iso-1-ferricytochrome *c* proteins in the UV−visible region (data not shown) at pH 7.5 were identical with respect to the Soret absorption maximum. The H73 protein showed the presence of a 695

nm absorption which is a charge transfer band arising from the Met80 sulfur ligation to the heme iron (Moore & Pettigrew, 1990). The extinction coefficients for the 695 nm band for the WT and H73 proteins were in the range (0.6−0.7 mM^{-1}) reported in the literature for this charge transfer band (Moore & Pettigrew, 1990); however, the extinction coefficient for the H73 protein was a little lower than for the WT protein. The weak absorbance and broad nature of the band at 695 nm make it difficult to obtain accurate values for the extinction coefficients. There has been a previous report of a mutant histidine at position 82 replacing the methionine 80 ligation in native cytochrome *c* (Hawkins et al., 1994), but the presence of the 695 nm band in the H73 protein ruled out such a possibility in the native state of this protein.

Lys73, being a highly solvent exposed surface residue, has been suggested to be involved in the interaction of cytochrome *c* with its redox partners (Berghuis & Brayer, 1992). Comparable growth rates for the WT and H73 variant on the nonfermentable glycerol medium, which requires functional oxidative metabolism, indicate minimal effects on the functional aspects of the variant protein (Herrmann et al., 1996). The second derivative amide I FTIR spectra (Figure 2) show small but significant differences, suggesting that the WT and the mutant protein have similar secondary structures in the native state. We note that these differences are much less than those observed between oxidized and reduced cytochrome *c* (Bowler et al., 1993; Dong et al., 1992), which have been shown to have essentially identical structures from X-ray crystallographic studies (Louie & Brayer, 1990; Berghuis & Brayer, 1992).

Lys73 is a relatively charge-isolated residue, the only important structural interaction being a hydrogen bond from the side chain of Asn70 to the main chain amide of Lys73 (Louie & Brayer, 1990; Berghuis & Brayer, 1992). The minimal electrostatic interactions of Lys73 with other residues would not seem to contribute significantly to the energy of the native state (see Bowler et al., 1993). Thus, from a combination of spectroscopic, functional, and structural considerations, we can say that the His73 mutation does not significantly perturb the native state conformation of the protein.

The tryptophan fluorescence data (Figure 4A and Table 1) show considerable slope for the denatured baselines as observed previously by Tsong (1974, 1975) and could be due to noncooperative local unfolding in the vicinity of Trp59. The thermodynamic parameters obtained from the fluorescence data are comparable, within error, to those derived from the CD data, except for the *m* value for the WT protein. The lower fluorescence intensity for the H73 protein at 3 M gdnHCl indicates a more compact denatured state (i.e., shorter distance between Trp59 and the heme) or a slightly different local environment for Trp59.

There is a wealth of structural data accumulated over the years regarding the acid, thermal, and chemically denatured states of various eukaryotic cytochromes *c*. It has been shown by optical and NMR spectroscopy that His18, which is one of the heme ligands in the native state of horse heart cytochrome *c*, remains bound to the ferriheme in concentrated gdnHCl or urea solutions at neutral pH (Babul & Stellwagen, 1971, 1972; Tsong, 1975; Muthukrishnan & Nall, 1991). On the other hand, the Met80 ligand of the native protein is replaced by a native histidine (His26 or -33) side chain under typical denaturing conditions at pH 7.0 (Babul & Stellwagen, 1971; Muthukrishnan & Nall, 1991). For the H73 variant, there is thus a possibility of the non-native His73 ligating to the heme in the denatured state, causing a change in the structure and energetics of this state relative to the WT protein.

In denatured horse heart cytochrome *c* in 6 M gdnHCl, the histidine side chain ligated to the heme dissociates from the iron upon acidification with a pK_a of 5.1 (Tsong, 1975). At low pH, the positively charged protonated form of histidine can no longer ligate to the Fe^{3+} of the heme (Moore & Pettigrew, 1990). The change in the Soret absorption maximum is related to the formation of a high spin complex in which the strong field histidine ligands are replaced by weak field solvent molecules (Babul & Stellwagen, 1971). The fact that the pK_a values for the spin-state transitions for WT and H73 in 3 M gdnHCl and 5 M urea (Table 2) are almost identical indicates involvement of predominately the same histidine residues (His26, -33, and -39) in heme ligation in the WT and the H73 variant. Thus, His73 ligation appears to play a minimal role in the energetics of the denatured state of the H73 protein. Interestingly, slightly lower pK_a values are observed for both proteins in 1.5 M gdnHCl. This effect could equally be due to some remaining native state protein at 1.5 M gdnHCl, where the denaturation is only about 90% complete, versus being due to the denatured state being more compact at 1.5 versus 3 M gdnHCl. However, the progressive increase in fluorescence intensity with increasing denaturant concentration in the denatured state of these proteins (see Figure 4A) might support the latter interpretation.

Denaturation experiments in the presence of 200 mM imidazole monitored by Trp59 fluorescence (Figure 4B) indicate that free imidazole mimics the effects induced by His73. It has been shown previously (Schejter & George, 1964) that imidazole binds to oxidized cytochrome *c* in the native state, replacing Met80 as an axial heme ligand. For iso-1-cytochrome *c*, it also has been shown that, under denaturing conditions at neutral pH, imidazole replaces any weakly bound intrinsic ligands such as His26, -33, and -39 (Muthukrishnan & Nall, 1991). Kinetic studies have revealed that the presence of 200 mM imidazole prior to and during refolding eliminates slow phases in the folding kinetics of cytochrome *c* attributable to non-native histidine−heme ligation (Elove et al., 1994).

If His73 were ligating to the heme in the denatured state, it might cause a structural change in the denatured state, producing the observed smaller *m* value. Given this scenario, we would expect the imidazole to displace His73 from the heme in denatured iso-1-cytochrome *c* nullifying the *m* value effect of His73 ligation. The *m* value for the H73 protein should therefore approach that of the WT protein in presence of 200 mM imidazole. Again our data do not support this hypothesis because the *m* values for the H73 protein in the presence and absence of imidazole are identical, indicating that imidazole does not have any effect on the *m* value for denaturation of the H73 variant. Replacement of the Met80 ligand by added imidazole in the native WT protein results in the *m* value for the WT protein approaching that of the H73 protein. These data suggest that His73 might be interacting with the heme replacing the native Met80 ligand under slightly destabilizing conditions, possibly forming a native-like intermediate.

The data obtained from gdnHCl denaturations followed by 695 nm absorbance (Figure 6) strongly support this proposal. In the native H73 protein, in the absence of any denaturant, the 695 nm band is present indicating Met80 ligation. However, slight destabilization by very low concentrations of gdnHCl results in disappearance of the 695 nm band, indicating loss of methionine ligation. Clearly, the His73 residue replaces Met80 without causing the major structural perturbations (detectable by CD or fluorescence spectroscopy) related to full cooperative unfolding (see Figure 6). This formation of a native-like intermediate for the H73 protein might be expected to lead to exposure of buried hydrophobic surface area leading to the smaller *m* value compared to the WT protein for CD-monitored unfolding. For the WT protein, the Met80 ligation of the native protein seems to be much more stable as indicated by loss of the 695 nm band almost concomitant with cooperative unfolding. Apparently, replacement of Met80 by exogenous imidazole prior to the cooperative unfolding leads to a similar exposure of hydrophobic surface as caused by Met80 displacement by His73 (Figure 4B, Table 1). Thus, imidazole appears to lead to the formation of the same native-like intermediate for the WT protein as observed for the H73 variant.

It appears that the loss of the 695 nm band as a function of [gdnHCl] slightly precedes loss of structure as monitored by CD for the WT protein as well (see Figure 6). This observation may indicate that even WT iso-1-cytochrome *c* does not unfold by an entirely two-state mechanism. Replacement of Met80 by lysine or tyrosine side chains located nearby in the sequence (Lys73 or -79 or Tyr74) may be responsible for a deviation from a two-state unfolding mechanism in the case of the WT protein. The much higher pK_a values of these side chains, compared to histidine, should reduce their ability to stabilize the native-like intermediate.

A previous report describes effects of changing the invariant proline 30 of rat and *Drosophila melanogaster* cytochromes *c* to alanine or valine (Koshy et al., 1990). The resulting variants destabilize the iron−Met80 sulfur bond indirectly due to modified hydrogen bonding interactions of residue 30 with His18 or allosteric propagation of the mutation effect along the peptide chain. Temperature titrations of the 695 nm band in a rat cytochrome *c* variant, where His26 is replaced by valine, also revealed weakening of the Fe−Met80 linkage as compared to the wild type protein (Qin et al., 1995). Urea denaturation for this variant showed a decreased thermodynamic stability and a smaller *m* value with respect to the wild type protein. The decreased thermodynamic stability was attributed to loss of hydrogen bonding interactions in the native state of this variant. In contrast to these studies, the H73 protein destabilizes the Met80 bond to the heme iron by direct displacement of Met80 with His73. An intermediate state is stabilized rather than the native state being destabilized.

It is well accepted that *m* values can decrease if an intermediate is formed on the unfolding pathway (Pace, 1975). Calorimetric results suggest that some variants of staphylococcal nuclease with substantially decreased *m* values as compared to the WT protein have a stabilized intermediate on the folding pathway (Carra et al., 1994; Carra & Privalov, 1995). However, high salt and low pH, conditions significantly different from those where the *m* value differences were observed, had to be used to demonstrate the presence of the intermediate. We have been able to demonstrate the presence of an intermediate under the same conditions where the *m* value differences between the WT and H73 protein were observed.

The pK_a values obtained from the spin-state transition data (Table 2) in the presence of denaturant are comparable for the WT and H73 proteins, indicating that in the denatured state the ligation for both proteins is similar (His26, -33, or -39). Since Met80 is apparently replaced by His73 in the native-like intermediate formed by the H73 variant, upon denaturation, His26, -33, or -39 must replace the His73 ligand. Presumably, the unfavorable entropic effect of restricting the unfolded polypeptide into a large loop if His73−heme ligation occurred in the denatured state (see Muthukrishnan & Nall, 1991, for example) favors loss of His73 ligation. In fact, the H73 variant does appear to lead to a small amount of compaction of both the gdnHCl and urea denatured states, as the fluorescence of Trp59 in the denatured state of WT is higher than in the H73 protein (see Figure 4A). This compaction is presumably due to a small amount of His73−heme ligation in the denatured state. Notably, the difference in the fluorescence of the denatured states of the WT and H73 proteins disappears in the presence of 200 mM imidazole, which is known to displace non-native histidines from the 6th coordination site of the heme in the denatured state of iso-1-cytochrome *c* (Muthukrishnan & Nall, 1991). Given the *m* values observed for the WT and the H73 variant in the presence of imidazole, we believe that this small amount of His73−heme ligation in the denatured state does not contribute substantially to the lower *m* value observed for the H73 variant versus the WT.

Finally, it has been noted in HX experiments on horse heart cytochrome *c* carried out as a function of gdnHCl concentration (Bai et al., 1995; Bai & Englander, 1996) that several partially unfolded structures (PUFs) formed by cooperative loss of structure are populated prior to the full cooperative loss of structure. The partially unfolded structure closest in energy to the native state, PUF1, encompasses a surface loop extending from position 70 to 85 in the sequence which includes both His73 and Met80. It is possible that the stabilizing energy of His73 ligation has lowered the energy of PUF1 such that it completely replaces the native state prior to the onset of full cooperative unfolding. Comparing the 695 nm data to the CD data, it appears that, for the H73 variant, formation of the native-like intermediate and full cooperative loss of secondary structure are two nonoverlapping transitions. The *m* value derived from a two-state fit of the 695 nm data for the H73 variant is about 1.8 kcal/(mol·M), which is very close to the *m* value of 1.6 kcal/(mol·M) for PUF1 derived from HX experiments (Bai et al., 1995). The *m* values for H73 from the CD and 695 nm data also approximately add up to the CD-derived *m* value for the WT protein.

CONCLUSIONS

We have reported thermodynamic studies on the unusual effects of a variant of iso-1-cytochrome *c* in which a histidine has been introduced on the surface at position 73. This mutation resulted in minimal changes in the native state as indicated by growth studies and amide I FTIR spectroscopy. Data from denaturation experiments in the presence of 200 mM imidazole and gdnHCl denaturations followed by 695

nm absorbance provide evidence for a native-like intermediate with altered heme ligation. The presence of this unfolding intermediate results in dramatic changes in the *m* value for the H73 protein as compared to the WT protein. These data indicate that changes in *m* values induced by mutation can be important indicators of changes in the equilibrium folding mechanism of a protein.

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