pH-Induced Conformation Changes and Equilibrium Unfolding in Yeast Iso-2 Cytochrome *c*†

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Received January 10, 1985

ABSTRACT: The relationship between pH-induced conformational changes in iso-2 cytochrome *c* from *Saccharomyces cerevisiae* and the guanidine hydrochloride induced unfolding transition has been investigated. Comparison of equilibrium unfolding transitions at acid, neutral, and alkaline pH shows that stability toward guanidine hydrochloride denaturation is decreased at low pH but increased at high pH. In the acid range the decrease in stability of the folded protein is correlated with changes in the visible spectrum, which indicate conversion to a high-spin heme state—probably involving the loss of heme ligands. The increase in stability at high pH is correlated with a pH-induced conformational change with an apparent p*K* near 8. As in the case of homologous cytochromes *c*, this transition involves the loss of the 695-nm absorbance band with only minor changes in other optical parameters. For the unfolded protein, optical spectroscopy and ^{1}H NMR spectroscopy are consistent with a random coil unfolded state in which amino acid side chains serve as (low-spin) heme ligands at both neutral and alkaline pH. However, the paramagnetic region of the proton NMR spectrum of unfolded iso-2 cytochrome *c* indicates a change in the (low-spin) heme–ligand complex at high pH. Apparently, the folded and unfolded states of the (inactive) alkaline form differ from the corresponding states of the less stable native protein.

We have undertaken a systematic study of the folding–unfolding properties of yeast cytochromes *c* in order to relate the process and product of protein folding reactions to specific changes in amino acid sequence (Nall & Landers, 1981; Nall, 1983; Zuniga & Nall, 1983). Iso-1 and iso-2 cytochromes *c* from yeast provide a particularly attractive experimental system for such studies since they belong to what is, next to the globins, the largest family of homologous proteins with known amino acid sequences (Dickerson & Geis, 1983). In addition, genetic investigations of the regulation, biosynthesis, and maturation of the yeast cytochromes *c* have provided an almost unparalleled amount of information on the in vivo function and regulation of these proteins (Sherman & Stewart, 1978). These studies have also resulted in a number of characterized mutant forms of yeast cytochromes *c*. Thus, the yeast cytochrome *c* system is unique in that detailed information is available on which residues are most important (and thus conserved in the homologous series) and in having mutant forms available with known substitutions at or near these same critical sites.

A detailed understanding of the available conformational states and folding–unfolding properties of the native protein is a prerequisite to deciphering the mechanism by which differences in amino acid sequence give rise to changes in these same phenomena in mutant proteins. In addition, the properties of yeast iso-2 cytochrome *c* may be compared to the more thoroughly investigated cytochrome *c* from horse as a test of the degree of conservation of the conformational and folding properties for two distantly related homologous proteins. These two proteins differ by about 46% in amino acid sequence but undoubtedly share the same tertiary structure.

Here we report investigations of the relationship between three conformationally distinct forms of folded iso-2 cytochrome *c* and changes in the overall stability of the tertiary structure toward disruption by guanidine hydrochloride.[1]

Materials and Methods

Procedures for growing yeast and purification of iso-2 have been described (Nall & Landers, 1981). Previously, protein was stored at −5 °C in 0.1 M sodium phosphate buffer. In the present case, the protein was dialyzed into 0.032 M NH_4HCO_3 buffer, pH 7.8, lyophilized, resuspended in water, and lyophilized (twice) before storage at −20 °C.

pH Titrations and Equilibrium Unfolding. Procedures for measurement of the guanidine hydrochloride induced equilibrium unfolding transition by relative fluorescence have been described (Zuniga & Nall, 1983). In the present case, 0.1 M sodium phosphate was used as a buffer with the pH adjusted to 3.0, 5.0, 7.2, 9.0, or 10.0 with sodium hydroxide or phosphoric acid. At pH 10.0, it was not possible to increase Gdn·HCl concentrations above about 3.5 M without precipitation of the guanidine. Absorbance measurements were made on a Hewlett-Packard 8450A UV–visible spectrophotometer as described (Nall & Landers, 1981).

Nuclear Magnetic Resonance Spectroscopy. Samples were prepared in 99.7% deuterium oxide (Cambridge Isotopes). Sodium phosphate and Gdn·HCl were deuterated by dissolving

† Supported by grants from the National Institute of General Medical Sciences (GM 32980) and the Robert A. Welch Foundation (AU-838). This work was done during the tenure of an Established Investigatorship of the American Heart Association (B.T.N.) and with funds contributed in part from the Texas Affiliate. J.J.O. is a Robert A. Welch Foundation postdoctoral fellow.

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[1] Abbreviations: Gdn·HCl, guanidine hydrochloride; Gdn·DCl, guanidine deuteriochloride; D_2O, deuterium hydroxide; HOD, a water molecule containing one hydrogen atom and one deuterium atom; NMR, nuclear magnetic resonance; iso-2, iso-2 cytochrome *c* from *Saccharomyces cerevisiae*; [$^2H_4$]TSP, sodium 3-(trimethylsilyl)tetradeuteriopropionate; ppm, parts per million; NATA, *N*-acetyltryptophanamide.

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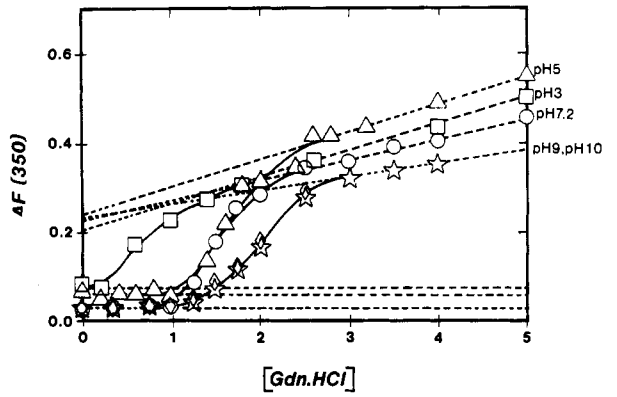

FIGURE 1: Guanidine hydrochloride induced unfolding transition of iso-2 cytochrome *c*. As the Gdn·HCl concentration is increased, the fluorescence of solutions of iso-2 cytochrome *c* is monitored relative to that of an equal molar amount of *N*-acetyltryptophanamide: (□) pH 3.0, (△) pH 5.0, (○) pH 7.2, (☆) pH 9.0, and (◇) pH 10.0. Measurements are at 20 °C in the presence of 0.1 M sodium phosphate with an excitation wavelength of 287 nm and an observation wavelength of 350 nm. The dashed lines (– –) indicate base-line fluorescence for the folded and unfolded species. The base lines for the folded protein were assumed to be independent of Gdn·HCl concentration while those for the unfolded protein are least-squares fits to the data at 3.0 M Gdn·HCl and above (2.5 M and above for the pH 3.0 transition).

them in D_2O and then removing the solvent by lyophilization or rotoevaporation. All samples contained 0.1 M sodium phosphate with the apparent pH (meter readings with no correction for isotope effects) being adjusted with deuterium chloride or sodium deuteroxide. Gdn·DCl (guanidine deuteriochloride) concentrations were determined from refractive index differences between protein solutions with and without Gdn·DCl. It was assumed that the refractive index relations (Nozaki, 1972) for aqueous solutions of Gdn·HCl hold for Gdn·DCl solutions. Protein concentrations were in the range of 1.0–2.0 mM.

Proton NMR (nuclear magnetic resonance) spectra were obtained on a JEOL GX-270WB NMR spectrometer by Fourier transformation of signal-averaged free induction decays. Typically, 32K data points were collected with quadrature detection and a sampling rate sufficient to give a 30-kHz frequency bandwidth. At high Gdn·DCl concentrations, residual protons from guanidinium ions interfered with the aromatic region of the spectrum. This interference was almost completely eliminated by cross-saturation with a selective 100-ms presaturating pulse at the resonance frequency of HOD. In the absence of Gdn·DCl, the HOD resonance was attenuated by a similar 100-ms presaturating pulse. The 90° pulse length was 18 μs with a 5-mm $^1H/^{13}C$ probe. Pulse delays of 3 s were used between acquisitions. Each spectrum is an average of 512–1024 transients. Chemical shifts are expressed relative to an internal [$^2H_4$]TSP standard added to the solutions at a molar ratio of 0.05 of that of the protein.

RESULTS

Unfolding and pH. On addition of Gdn·HCl to a solution of iso-2 at pH 9–10, there is a cooperative increase in fluorescence. A similar transition is observed at a slightly lower Gdn·HCl concentration near neutral pH and at an even lower Gdn·HCl concentration in the acid range (Figure 1). The limiting value of the relative fluorescence above the transition zone is 5–15% greater for the transition monitored at acid or neutral pH compared to the transition at high pH. Nevertheless, least-squares fits to the data at 3.0 M Gdn·HCl and above (2.5 M and above for pH 3.0) extrapolate to nearly the same value at 0.0 M Gdn·HCl: 0.210–0.235 of the fluorescence of an equal molar amount of NATA. This could indicate that there are pH-dependent differences in the interaction of Gdn·HCl with the unfolded protein, that unfolding transitions do not always produce fully unfolded proteins as products, or, merely, that ionizable groups provide more efficient fluorescence quenching at high pH. By analogy to the more thoroughly investigated unfolding transition of horse cytochrome *c* (Ikai et al., 1973; Tsong, 1976), we have previously interpreted the transition at pH 7.2 as an unfolding transition to an unstructured random coil (Nall & Landers, 1981).

NMR Analysis of the Product of Unfolding. (A) Aromatic and Aliphatic Regions. In order to check for differences in residual structure in unfolded protein at high pH and neutral pH, proton NMR spectra of the oxidized form of the native protein (Figure 2A) are compared to spectra of the unfolded protein in 3.0 M Gdn·DCl at pH 7.2 (Figure 2B) and pH 9.1 (Figure 2C). The spectrum of the folded protein is similar to those of homologous cytochromes *c* (Cookson et al., 1978; McDonald & Phillips, 1973; Wuthrich, 1969; Keller & Wuthrich, 1978) and apparently identical with that previously reported for yeast iso-2 cytochrome *c* (Senn et al., 1983). Many of the resonances are well resolved, with the resonance position of any given proton resulting from some or all of the following conformation-dependent factors: paramagnetic contact shifts and pseudo contact shifts, aromatic side chain and heme ring current shifts, and environment-dependent diamagnetic shifts. Most of these contributions to the chemical shifts are expected to be removed if unfolding converts the protein to a structureless form. Thus, the resulting spectrum of a protein unfolded to a state approximating a "random coil" is predicted to be close to that of the constituent amino acids. The neutral pH and alkaline pH spectra of the unfolded protein (Figure 2B,C) are almost identical and indicate largely structureless polypeptides as products of the Gdn·DCl-induced transitions. The major differences in these spectra near 4.7 ppm are due to spectral distortion resulting from preirradiation of the HOD resonance. Minor differences at 7.8 and 8.0 ppm appear to result from a titratable side chain. In Figure 2D a schematic diagram of the aromatic proton region is presented showing the expected spectrum for a random coil polypeptide with the amino acid composition of iso-2 cytochrome *c*. Superimposed on this diagram is the observed aromatic proton region from Figure 2B. By comparing parts A–D of Figure 2, it is clear that (1) at both pH 7.2 and pH 9.1 the product of unfolding closely approximates a random coil polypeptide and (2) unfolding is complete by 3.0 M Gdn·DCl.

Spectra have also been obtained for unfolded protein at pH 5.0, 3.0, and 2.0 (data not shown). Although these spectra are consistent with a structureless unfolded state, they provide a poor test for the presence of structure for two reasons. First, at acid pH, residual amide proton resonances from Gdn·HCl cannot be removed by cross-saturation from HOD, because of a decrease in the amide proton exchange rate in the pH 2.0–5.0 region. This seriously distorts the aromatic spectral region, making a comparison as in Figure 2D difficult. Second, the heme converts to a high-spin state below pH 5.0. The slower electron relaxation time for the high-spin heme will selectively broaden nearby protons, leading to ambiguity in the interpretation of spectral differences between high-spin and low-spin unfolded proteins. For example, selectively broadened resonances might be due to residual structure, proximity to the heme, or both.

(B) Paramagnetically Shifted Resonances. In the unfolded protein, the heme and heme ligand resonances are subject to

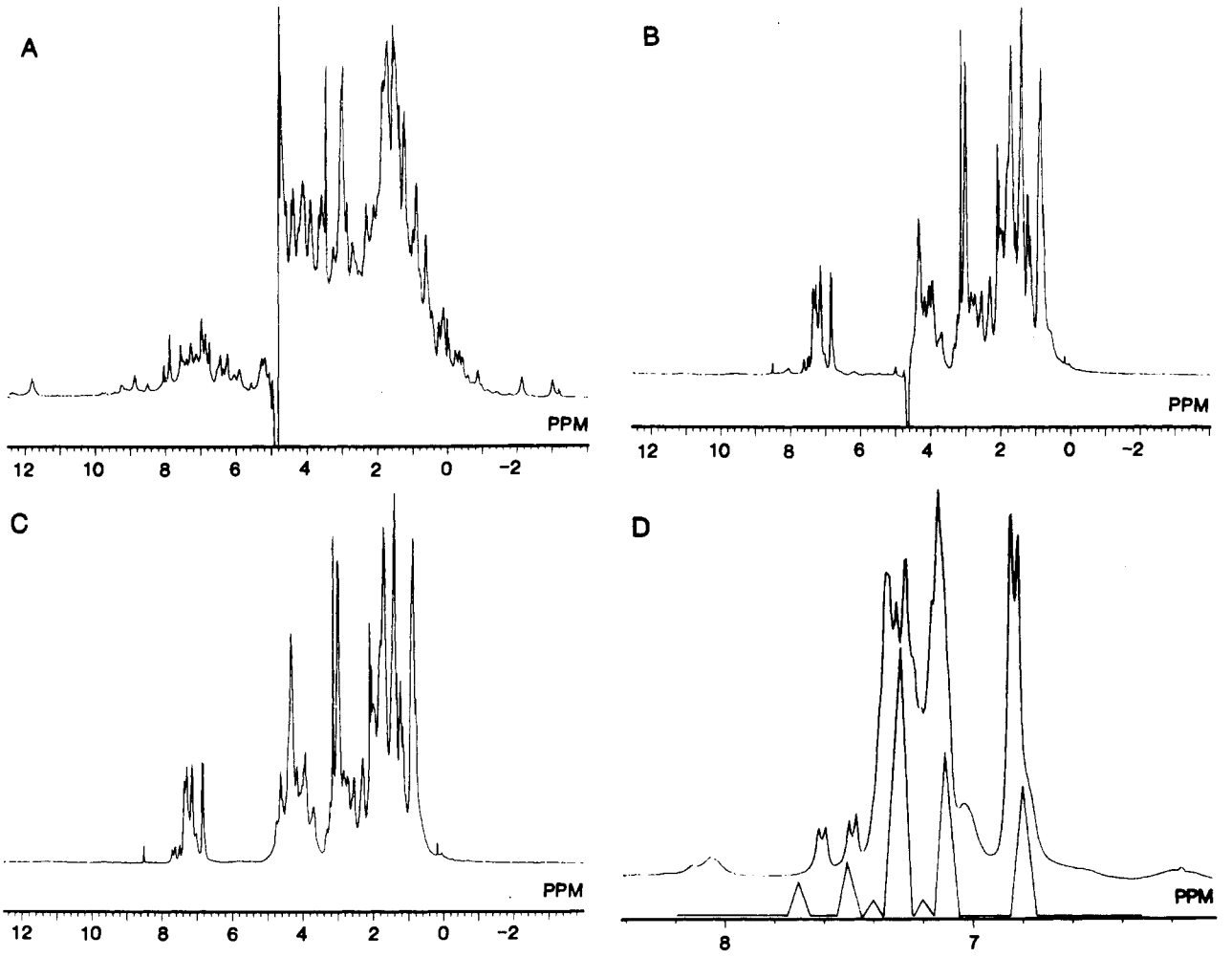

FIGURE 2: ^1H NMR spectra of the diamagnetic region of iso-2 cytochrome *c*. The aromatic and aliphatic proton regions are shown for (A) the folded protein at pH 7.2, (B) the unfolded protein in the presence of 3.0 M Gdn·HCl, pH 7.2, and (C) the unfolded protein in the presence of 3.0 M Gdn·HCl, pH 9.1. In (D) the aromatic proton region of the unfolded protein at pH 7.2 is compared to a schematic diagram of the expected spectrum for a structureless polypeptide with the same amino acid composition as iso-2. The chemical shift parameters for the schematic spectrum are taken from Wuthrich (1976). All spectra were taken in D_2O at 21 °C in the presence of 0.1 M sodium phosphate, with chemical shifts expressed relative to internal [$^2H_4$]TSP. All pH values are apparent values—meter readings of D_2O solutions without correction for isotope effects. The residual HOD resonance has been attenuated with a selective 100-ms saturating pulse applied immediately before a 90° sampling pulse. The saturating pulse is also effective in eliminating residual guanidinium protons by cross-saturation from HOD.

large contact and pseudo contact shifts due to the paramagnetic iron atom. As shown in Figure 3, strongly shifted resonances are observed spanning a chemical shift range of about 40 ppm in the unfolded protein. The resonances cannot be assigned unambiguously to specific protons but are almost certainly heme or heme ligand protons in unfolded iso-2. A comparison of the spectrum at neutral pH (Figure 3A) to that at pH 9.1 (Figure 3B) shows numerous differences in chemical shifts, line widths, and line intensities. These results provide a clear indication of pH-induced changes in the heme–ligand complex of unfolded iso-2. The changes could involve one or all of the following: heme ligation, ligand-exchange dynamics, or the distribution of the unpaired electron spin density.

pH Titration of Native Iso-2. To evaluate the extent of pH-induced optical changes in iso-2, titrations of the folded protein have been performed. Absorbance changes (visible and ultraviolet) and fluorescence changes are monitored. The changes in absorbance and fluorescence (Figure 4) are similar to those found for the homologous horse cytochrome *c* (Greenwood & Palmer, 1965; Davis et al., 1974; Dyson & Beattie, 1982; Brems & Stellwagen, 1983). Changes in visible absorbance and fluorescence are observed in the acid pH range where the folded protein undergoes a change in spin state (low spin at neutral pH to high spin in the acid range) involving replacement of one or both heme ligands and possibly some acid-induced unfolding (Stellwagen & Babul, 1975; Robinson et al., 1983). Above neutral pH a major change in absorbance at 696 nm is observed with an apparent p*K* near 8. This is the behavior expected for conversion of native cytochrome *c* to an inactive, but folded, alkaline form (Greenwood & Palmer, 1965; Brandt et al., 1966; Gupta & Koenig, 1971; Davis et al., 1974). Since the presence of an absorbance band at 695 nm has been correlated with Met ligation of the heme (Schechter & Saludjian, 1967), disappearance of this band on conversion to the alkaline form is believed to involve replacement of the Met-80 heme ligand by some other low-spin ligand. The remaining optical properties show only minor changes on titration to high pH.

Titration in Presence of Denaturant. A pH titration has also been performed in the presence of 2.0 M Gdn·HCl (not shown). At this denaturant concentration, the protein is expected to be at the upper edge of the unfolding transition at neutral pH. In the acid region, the optical changes are again consistent with a spin-state transition, but in the presence of denaturant the transition occurs at somewhat higher pH (ΔpK of about 0.7 relative to the folded protein). Since this transition requires replacement of the protein (low-spin) ligands by water, it is reasonable that the transition occurs more readily in the

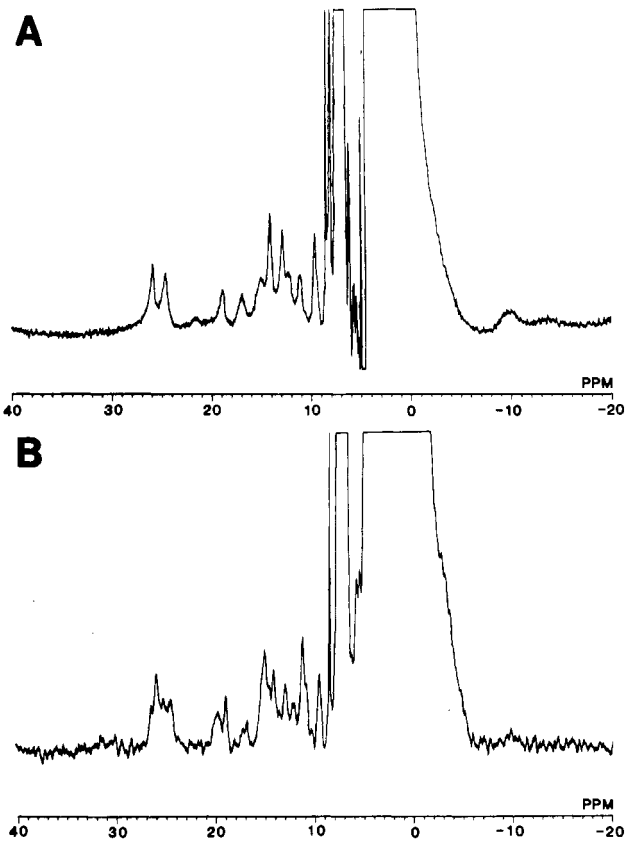

FIGURE 3: ^{1}H NMR spectra of the paramagnetic region of unfolded iso-2 cytochrome *c*. A chemical shift range that includes the paramagnetically shifted resonances is shown for iso-2 in the presence of 3.0 M Gdn·HCl (A) at pH 7.2 and (B) at pH 9.1. All resonances outside the 9 to −5 ppm chemical shift region may be assigned to heme or heme–ligand protons due to their strong paramagnetic shifts. Conditions are the same as in Figure 2B,C.

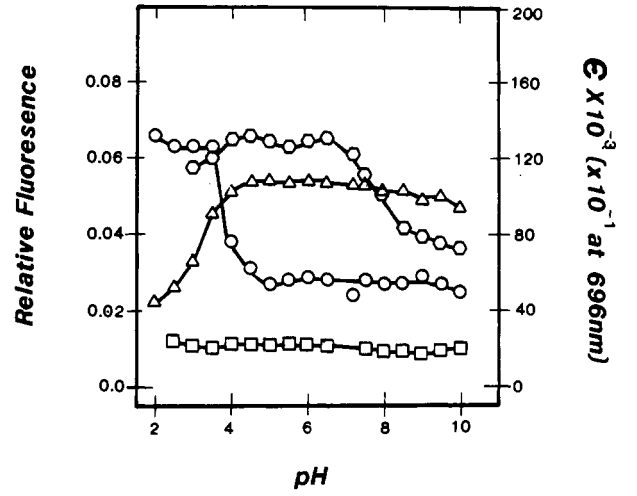

FIGURE 4: Effect of pH on the absorbance and fluorescence properties of iso-2 cytochrome *c* in the presence of 0.4 M Gdn·HCl. Absorbance changes are given at 287 (□), 410 (△), and 696 nm (○). Note that the molar extinction coefficients are multiplied by 10^{-3} except at 696 nm where the factor is 10^{-1}. Fluorescence (○) at 350 nm (excitation at 287 nm) is given relative to an equal molar amount of *N*-acetyltryptophanamide. Conditions are 20 °C, 0.1 M sodium phosphate, and pH 7.2. Protein concentrations are as follows: fluorescence, 5 × 10^{-6} M; absorbance at 287 nm and 410 nm, 30 × 10^{-6} M; absorbance at 696 nm, 10^{-4} M.

absence of the stabilizing effects of an organized folded structure. At high pH, there are only minor changes in all optical parameters other than fluorescence. The magnitude and sign of the observed decrease in relative fluorescence are as expected for partial refolding resulting from the increased stability of the folded form at high pH (Figure 1).

Discussion

Alkaline Form of Iso-2 Is More Stable toward Gdn·HCl Denaturation Than Native Iso-2. The major cooperative changes in fluorescence, believed to represent unfolding, occur at higher Gdn·HCl concentration when the protein is at high pH as opposed to neutral pH (Figure 1). This could be due to pH-induced changes in any or all of the following: Gibbs free energy of the folded or unfolded species, electrostatic free energy, preferential interaction of Gdn·HCl with the folded or unfolded species, or population of folding intermediates. Optical (Figure 4) and NMR (Figure 3) data do show pH-induced changes in the state of both the folded and unfolded species. Corresponding changes in the free energies of these species may be likely but cannot be proven.

Iso-2 Decreases in Stability at Low pH. The transition at pH 5.0 is similar to that at pH 7.2 except that the limiting value of the fluorescence above the transition zone is higher. At pH 3.0 the stability has decreased greatly. By pH 3.0 the folded protein is well into the acid-induced high-spin transition, which is believed to involve displacement of the side-chain ligands (His-18 and Met-80) by extrinsic ligands from the solvent. It is tempting to ascribe the decrease in stability of the folded structure at pH 3 to rupture of the side-chain heme–ligand "cross-links". These bonds are also absent in the the unfolded protein in the acid pH range (the optical changes for the unfolded species show a conversion to a high-spin state implying displacement of the protein ligands by solvent ligands). Thus, in the low-pH limit there is no net change in heme ligation on unfolding. However, an increase in the configurational entropy of unfolding at acid pH is expected, similar to that found for proteins differing in the number of covalent cross-links (Lin et al., 1985).

Product of Unfolding Is a (Cross-Linked) Random Coil at Neutral and Alkaline pHs. Comparison of the diamagnetic region of the ^{1}H NMR spectra for the folded protein with those of the unfolded protein at neutral and alkaline pH indicates major changes in structure (Figure 2). The aromatic region of the spectrum of the folded protein shows much greater dispersion of the over 50 aromatic ring proton resonances than the spectra of the same region for the unfolded protein. This is due to conformation-dependent heme ring current shifts and pseudo contact shifts, which are largely eliminated on disruption of the tertiary structure. For the aromatic region, the spectrum of the unfolded protein is close to that expected for the sum of the constituent amino acids—at both pH 7.2 and pH 9.1 Thus, addition of Gdn·HCl appears to disrupt all organized tertiary structure in the native protein.

An exception is the heme ligation state in unfolded iso-2 at neutral and alkaline pHs. Both optical spectroscopy and NMR spectroscopy (Figure 3) indicate retention of low-spin ligands in the fifth and sixth coordination positions. Since these ligands are likely to be intrinsic to the protein, the picture that emerges is one of a cross-linked random coil where the cross-links result from protein side chains serving as heme ligands.

Titration Indicates Equilibrium Transitions at High and Low pHs. For the folded protein (Figure 4), both fluorescence and heme absorbance changes at 410 nm detect a pH-induced transition with a p*K* near 3.4. Undoubtedly this is due to the low-spin to high-spin transition that has been thoroughly studied in the homologous cytochrome *c* from horse (Tsong, 1975; Andersson et al., 1980; Dyson & Beattie, 1982; Robinson et al., 1983). The changes seen here are similar to the changes

reported for the homologous protein and thus may reflect a combination of spin-state changes and partial unfolding as reported for that protein (Stellwagen & Babul, 1975; Robinson et al., 1983).

On alkaline titration, the major change is in the absorbance at 696 nm, which is sensitive to an alkaline pH induced conformational change of the native protein that may (Hettinger & Harbury, 1964; Morton, 1973; Wilgus & Stellwagen, 1974; Smith & Millett, 1980; Davis et al., 1974) or may not (Pettigrew et al., 1976; Schejter et al., 1970; Bosshard, 1981) involve replacement of the heme ligand, Methionine-80, by a lysine residue. In the presence of 2.0 M Gdn·HCl, titration of the protein to high pH shows large changes in fluorescence while other properties show minimal change (data not shown). Inspection of Figure 1 shows that these fluorescence changes are due to partial refolding resulting from the increased stability of the folded form at high pH.

Conclusions

While changes in stability at extremes of pH are a general property of proteins, it is not often that these changes can be correlated with easily observed states of the folded and unfolded protein. The sensitivity of the optical and NMR properties of heme proteins to conformational changes provides a particularly favorable system. For folded iso-2, distinct conformational states are observed at high pH (alkaline isomerization) and low pH (low-spin to high-spin transition) as with homologous cytochromes *c*. The low-pH transition, believed to involve displacement of the native heme ligands by solvent ligands, leads to a product less stable toward Gdn·HCl denaturation than the native protein. The alkaline transition is thought to involve a change in heme ligation: Met-80 for some unknown but low-spin ligand. Surprisingly, this transition appears to produce a folded structure of increased stability toward Gdn·HCl.

The unfolded states of a protein are as important as the folded states in determining protein stability. In the acid pH region, unfolded iso-2 is high spin, indicating that the fifth and sixth coordination positions are filled by solvent (high spin) ligands. At neutral and alkaline pH, optical spectroscopy indicates that the coordination positions are filled by low-spin ligands, but NMR spectroscopy shows that high pH induces a change (of unknown nature) in the state of the heme–ligand complex.

Acknowledgments

We thank Efrain H. Zuniga for help in purifying and characterizing the protein used in these experiments and for preparing the figures.

Registry No. Cytochrome *c*, 9007-43-6; Gdn·HCl, 50-01-1.

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