Acid-Induced Unfolding and Refolding Transitions of Cytochrome *c*: A Three-State Mechanism in H_2O and D_2O†

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ABSTRACT: Whereas the salt-dependent conformational transition of acid-denatured horse ferricytochrome *c* at pH 2 is approximated by a two-state mechanism from the acid-unfolded state to the molten globule state [Kataoka, M., Hagihara, Y., Mihara, K., & Goto, Y. (1993) *J. Mol. Biol. 229,* 591–596], the corresponding transition in D_2O has been proposed to involve a noncompact, α-helical intermediate state (the pre-molten globule state)[Jeng, M.-F., & Englander, S. W. (1991) *J. Mol. Biol. 221,* 1045–1061]. To examine the proposed difference in the conformational transitions, we carried out the HCl and DCl titrations of cytochrome *c* in H_2O and D_2O, respectively, measured by far-UV circular dichroism, tryptophan fluorescence, and Soret absorption. In both D_2O and H_2O, unfolding from the native state to the acid-unfolded state and subsequent refolding to the molten globule state were observed. In either solvent, the conformational transitions were well approximated by a minimal three-state mechanism consisting of the native, molten globule, and acid-unfolded states. Thus, our results did not substantiate the presence of a pre-molten globule state in D_2O. Acetylation of amino groups of cytochrome *c* is known to stabilize the molten globule state at pH 2. On the basis of the three-state mechanism, we constructed a conformational phase diagram for the effect of pH and the degree of acetylation. This phase diagram was similar to that of the pH- and salt-dependent conformational transition of cytochrome *c*, suggesting that the effects of acetylation on the conformational states are similar to those of salt.

The molten globule state has been proposed, initially with cytochrome *c* (Ohgushi & Wada, 1983) and α-lactalbumin (Ptitsyn, 1987, 1992; Kuwajima, 1989, 1992), to be a compact denatured state with a significant amount of secondary structure but largely disordered tertiary structure, and it is now recognized for various proteins (Kim & Baldwin, 1990; Baldwin, 1991; Christensen & Pain, 1991; Barrick & Baldwin, 1993). The molecular structure of the molten globule state of several proteins has been examined by two-dimensional NMR[1] (Baum et al., 1989; Jeng et al., 1990; Hughson et al., 1990; Harding et al., 1991; Dobson, 1992; Alexandrescu et al., 1993; Chyan et al., 1993). Kinetic results suggest that the rapidly formed intermediate during protein refolding is analogous to the equilibrium molten globule state (Elöve & Roder, 1991; Elöve et al., 1992; Ptitsyn, 1992; Radford et al., 1992).

Although much is still unknown about the details and generality, the molten globule state is assumed to be a major intermediate of protein folding. There is considerable interest in whether a new type of intermediate state, which might be located between the fully unfolded and molten globule states or the molten globule and native states, exits. Characterization of the transition state of protein folding, which is believed to be located between the molten globule and native states, is also an important topic of protein folding (Kuwajima, 1989; Bycroft et al., 1990; Serrano et al., 1992).

Horse ferricytochrome *c* is substantially unfolded under conditions of low salt at pH 2; the addition of anion, from either salt or acid, stabilizes the molten globule state (Ohgushi & Wada, 1983; Goto et al., 1990a,b; Kuroda et al., 1992). Jeng et al. (1990) examined the structure of the molten globule state by two-dimensional NMR using the H–D exchange of amide protons and demonstrated that the major helices and their common hydrophobic domains are largely preserved, while loop regions of the native structure are flexible and partly disordered.

Goto, Fink, and co-workers have shown that the anion-dependent conformational transition is common to several acid-denatured proteins (Goto et al., 1990a,b). They proposed that the conformation of acid-denatured proteins is determined by a balance of various forces stabilizing or destabilizing the molten globule state. Acid-denatured cytochrome *c* is unfolded under conditions of low salt at pH 2 by charge repulsion between the positive groups. Added anions bind to the positive groups, shielding the charge repulsion, and this results in the manifestation of the intrinsic forces stabilizing the molten globule state. The conformational change is explained by a cooperative two-state transition between the unfolded and molten globule states.

To confirm this mechanism, Goto and Nishikiori (1991) prepared a series of acetylated cytochrome *c* species which have various degrees of charge repulsion at pH 2. They examined by CD the conformation of modified cytochrome *c* species at pH 2 in the absence of salt, where the unmodified protein is unfolded. With the progress of acetylation and, as a result, with the decrease in charge repulsion at pH 2, the acid-unfolded cytochrome *c* was transformed cooperatively to the molten globule state. This further supported the two-state mechanism.

On the other hand, Jeng and Englander (1991) examined the NaCl-induced stabilization of the molten globule state of cytochrome *c* in D_2O at pD_{app} 2.2 (pH meter reading). They

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[1] Abbreviations: CD, circular dichroism; D, deuterium; NMR, nuclear magnetic resonance; SAXS, small-angle X-ray scattering; Lys_n-acetylated cytochrome *c*, cytochrome *c* in which *n* lysyl amino groups are acetylated.

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used various methods, including NMR, intrinsic viscosity, tryptophan fluorescence, CD, and H–D exchange rate of amide protons. On the basis of the non-coincidence of the transitions measured by these methods, they proposed a sequential folding mechanism with a noncompact intermediate state (the pre-molten globule state) having significant nativelike helical segments. However, the results are contradictory to our two-state approximation in H_2O.

Although the compactness of a protein molecule is an essential property characterizing the degree of protein folding (Dill, 1990; Alonso et al., 1991), direct measurement of compactness during the process of protein folding is not easy. At present, small-angle X-ray scattering (SAXS) is probably the best technique with which we can carry out such measurements (Kataoka et al., 1989, 1991; Flanagan et al., 1992). To clarify the cooperativity of the salt-induced stabilization of the molten globule of cytochrome c, Kataoka et al. (1993) examined the transition from the unfolded state to the molten globule state in H_2O at pH 2, induced by NaCl or acetylation of amino groups, by using several methods, including SAXS. Both salt- and acetylation-induced stabilization of the molten globule in H_2O at pH 2.0 was consistent with the two-state mechanism.

It has been reported for some proteins that D_2O stabilizes the folded state (Henderson et al., 1970; Gomez-Puyou et al., 1978; Masson & Laurentie, 1988; Antonino et al., 1991). Because Jeng and Englander (1991) carried out their experiments in D_2O, whereas ours were in H_2O, we cannot exclude the possibility that D_2O changes the mechanism of protein folding. If D_2O stabilizes a pre-molten globule state which is unstable in H_2O, it is of much interest to characterize the difference between folding in H_2O and D_2O.

Goto and Fink [1993; see also Goto et al. (1990a,b)] established that acid titration of proteins to pH regions as low as pH = 0 is a simple and useful method to characterize mechanisms of conformational transition. We utilize this method in the present study to examine the difference between protein folding in H_2O and D_2O. The results show that conformational transitions in D_2O are very similar to those in H_2O, suggesting that no significant accumulation of a pre-molten globule state occurs in D_2O. On the basis of a minimal three-state mechanism consisting of the native, molten globule, and unfolded states, we constructed a phase diagram for the pH and acetylation-dependent conformational transitions.

MATERIALS AND METHODS

Materials. Horse cytochrome c (type IV), being essentially in the oxidized form, was purchased from Sigma. Acetylated cytochrome c species with different degrees of acetylation were prepared as described (Goto & Nishikiori, 1991). We did not identify the sites of acetylation, and each species may be a mixture of cytochromes with different sites of modification. Protein concentrations were determined spectrophotometrically (Goto et al., 1990b; Goto & Nishikiori, 1991). DCl (37% in D_2O) was purchased from Merck. D_2O (>99.8%) was a product of the Commissariat a L'energie Atomique (CEA), France.

Methods. All measurements in this work were carried out at 20 °C with thermostatically controlled cell holders.

HCl or DCl titration of cytochrome c was carried out as described (Goto et al., 1990a; Goto & Fink, 1993). A specific point of the present experiments is that, to minimize the artifacts arising from the sample preparation, a single sample solution for respective conditions was prepared and divided for the CD, fluorescence, and absorption measurements. Typically, 0.15 mL of cytochrome c stock solution (1 mg/mL), which had been dialyzed against deionized water, was mixed with 1.35 mL of HCl or DCl solution of appropriate acid concentration. Final protein concentration was 0.1 mg/mL. Samples of 0.3, 0.6, and 0.6 mL were used for the far-UV CD, tryptophan fluorescence, and Soret absorption measurements, respectively.

CD measurements were carried out with a Jasco spectropolarimeter, Model J-500A, equipped with an interface and a personal computer. The instrument was calibrated with ammonium camphorsulfonate-*d*. The results are expressed as the mean residue ellipticity, $[\theta]$, which is defined as $[\theta] = 100\theta_{obs}/lc$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue moles per liter, and l is the length of the light path in centimeters. A cell with a 2-mm light path was used. Fluorescence spectra were measured with a Hitachi-Perkin-Elmer fluorescence spectrophotometer, Model MPF-4. Tryptophan fluorescence was measured with excitation at 295 nm. A microcell with a 5-mm light path was used. The Soret absorption of the heme group was monitored with a Jasco spectrophotometer, Model Ubest, with a cell with a 2-mm light path.

pH titration of acetylated cytochrome c species was carried out at ionic strength $I = 0.02$ at a protein concentration of 0.1 mg/mL. The change in the Soret absorption spectrum was monitored with a cell with a 5-mm light path. The buffers used were glycine hydrochloride (pH 2–3), sodium acetate (pH 3–6), and sodium phosphate (pH 6–7). The ionic strength was controlled with NaCl.

The pH or pD was determined after respective measurements using a Radiometer PHM83 at 20 °C. pD is a true pD value corrected from the apparent pH meter reading, pD_{app}, by using the equation $pD = pD_{app} + 0.4$ (Glasoe & Long, 1960).

RESULTS

HCl-Induced Unfolding and Refolding Transitions in H_2O. We first measured the HCl-induced unfolding and refolding transitions of cytochrome c in H_2O by CD at 222 nm (supplementary material). Cytochrome c has an α-helical content of about 30%. The CD intensity at 222 nm, largely reflecting the helical structure, showed a highly cooperative unfolding transition with a midpoint at pH 2.5. The results are consistent with the pH titration under the conditions of low ionic strength ($I = 0.02$) measured by the Soret absorption (Dyson & Beattie, 1982). The subsequent refolding transition, arising from the increase in the concentration of chloride anion, is also cooperative and has a midpoint at pH 1.0. It has been proposed (Goto et al., 1990a,b) that, whereas the initial unfolding is a transition from the native state to the acid-unfolded state, the subsequent refolding is a transition from the unfolded state to the molten globule state.

The unfolding and refolding transitions were also monitored by tryptophan fluorescence and Soret absorption (supplementary material). Horse cytochrome c has one tryptophan residue at position 59. Tryptophan fluorescence of the acid-unfolded cytochrome c at pH 2 has a maximum at 345 nm, indicating that the residue is largely exposed to the solvent (Ohgushi & Wada, 1983; Kataoka et al., 1993). In the native state, the fluorescence is quenched completely by energy transfer to the heme group attached covalently to cysteines at positions 14 and 17. Similar quenching occurs in the molten globule state. The HCl titration of cytochrome c monitored by the fluorescence at 345 nm showed cooperative changes corresponding to the unfolding and refolding transitions.

The Soret absorption reflects the spin state of iron and, as a result, depends on the conformational state of the protein (Stellwagen & Babul, 1975; Dyson & Beattie, 1982). While the native state (low-spin state) has a maximum at 410 nm with $\epsilon = 1.06 \times 10^5$ M^{-1} cm^{-1}, the acid-unfolded state (high-spin state) has a maximum at 394 nm ($\epsilon = 1.90 \times 10^5$ M^{-1} cm^{-1}). The molten globule state has a mixed-spin state with a maximum at 397 nm ($\epsilon = 1.20 \times 10^5$ M^{-1} cm^{-1}). Whereas the unfolding of the native state causes a decrease in absorption at 410 nm, the refolding transition from the acid-unfolded state to the molten globule state is accompanied by a large decrease in absorption at 394 nm (Goto et al., 1990b). The HCl titration of cytochrome *c* monitored by the absorption at 394 nm showed cooperative changes corresponding to the unfolding and refolding transitions (supplementary material). Similar two-step transitions with opposite directions were observed at 410 nm (data not shown).

The similarity of the transition curves measured by the three methods suggested that both transitions are explained by a two-state mechanism, i.e., the former transition, between the native and unfolded states and the latter transition, between the unfolded and molten globule states. To validate a two-state mechanism, the coincidence of the transition curves measured by various properties can be used. Assuming the appropriate base-line values (supplementary material), the fraction of unfolded species (f_U) was calculated by

$$f_U = (A - A_F)/(A_U - A_F) \quad (1)$$

where A, A_F and A_U are the observed signal intensity, the signal intensity of the folded state (native or molten globule), and the signal intensity of the unfolded state, respectively. In the case of the Soret absorption at 394 nm, the extinction coefficient for the native state is different from that of the molten globule state. Therefore, we assumed that, above pH 2, the transition is from native to unfolded and, below pH 2, it is from unfolded to molten globule. Figure 1A shows the normalized transition curves measured by CD, fluorescence, and Soret absorption. The HCl-induced unfolding and refolding transitions agreed with each other very well.

DCl-Induced Unfolding and Refolding Transitions in D_2O. The DCl-induced transitions of cytochrome *c* in D_2O were measured by CD at 222 nm, by tryptophan fluorescence at 345 nm, and by absorption at 394 nm (supplementary material). The acid-induced unfolding and refolding transitions were very similar to those in H_2O. For the three methods used here, there was no significant difference in the spectra in D_2O of the native, unfolded, and molten globule states from those in H_2O.

We assumed that the CD, fluorescence, and absorption values for the native, unfolded, and molten globule states in D_2O are the same as those in H_2O. With the base line values indicated in the supplementary material, the fraction of unfolded species was calculated with eq 1. Figure 1B shows the pD dependence of the fraction of unfolded species in D_2O. It is evident that, even in D_2O, the unfolding and refolding transitions measured by various methods agree with each other very well.

In Figure 1B, a common transition curve in H_2O is also shown. It is a theoretical curve calculated on the basis of a minimal three-state model as described in the Discussion. The common transition curve in H_2O was close to that in D_2O in both the unfolding and refolding regions; the transitions in D_2O were shifted to higher pD regions only by 0.1 pH unit. These results indicate that the acid-induced conformational transitions in D_2O are very similar to those in H_2O.

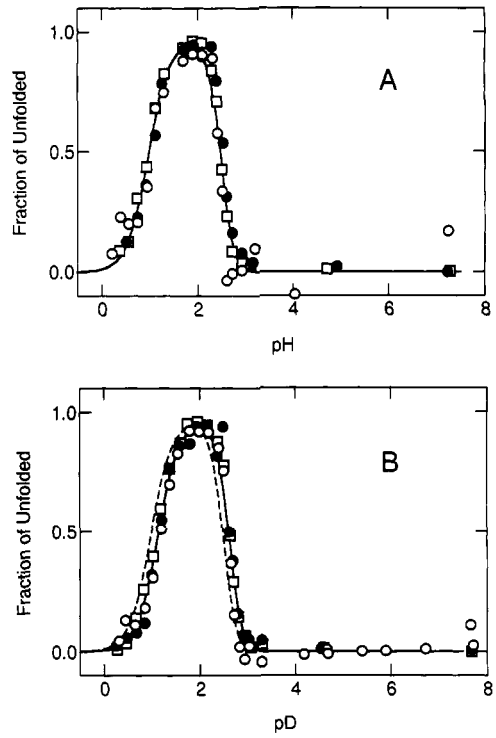

FIGURE 1: Normalized HCl (A) and DCl titration curves (B) of horse ferricytochrome *c* at 20 °C measured by ellipticity at 222 nm (○), tryptophan fluorescence at 345 nm (●), or Soret absorption at 394 nm (□). The solid line in both panels indicates a theoretical curve constructed on the basis of eqs 2, 5, and 6 and the parameters of Table I. The dotted line in panel B is the theoretical curve in H_2O.

It is known that tryptophan fluorescence is highly sensitive to the solvent conditions (Cowgill, 1963). To examine the intrinsic tryptophan fluorescence under the experimental conditions, the effects of HCl or DCl on the fluorescence of *N*-acetyl-L-tryptophanamide were measured (data not shown). *N*-Acetyl-L-tryptophanamide was titrated with HCl in H_2O or with DCl in D_2O. In both solvents, tryptophan fluorescence with an emission maximum at 355 nm showed a significant decrease in intensity below pH (or pD) 2, probably due to the increased collisions of protons with the excited chromophore. Fluorescence intensity at pH 1 and 0.5 was 40% and 20%, respectively, of that at pH 6. This suggested that the HCl-induced refolding transition measured by fluorescence could be affected by the intrinsic quenching caused by HCl or DCl. However, the refolding transition measured by fluorescence coincided well with those measured by absorption and CD (Figure 1), suggesting that the fluorescence transition corresponds to the conformational transition. Kataoka et al. (1993) examined the stabilization of the molten globule state of cytochrome *c* by NaCl at pH 2, where NaCl does not quench the intrinsic fluorescence of tryptophan. They found that the transition measured by fluorescence agrees well with those measured by other properties, supporting the two-state mechanism. Therefore, we believe that the uncorrected transition curves shown in Figure 1 represent the conformational transition. Probably, the tryptophan fluorescence of cytochrome *c* is quenched by HCl or DCl less effectively, especially in the folded state, than is that of *N*-acetyl-L-tryptophanamide.

Acid-Induced Transition of Acetylated Cytochrome c. A phase diagram of conformational states is particularly useful in considering the mechanism of the transition between the different conformational states (Goto & Fink, 1989, 1990; Goto & Aimoto, 1991; Stigter et al., 1991; Alonso et al.,

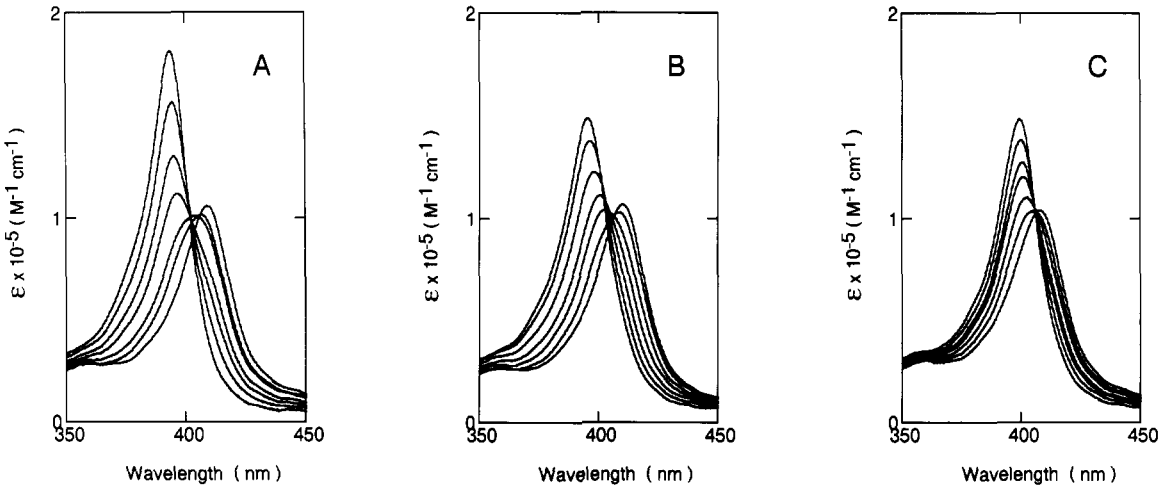

FIGURE 2: Soret absorption spectra of the unmodified (A) and the Lys_3- (B) and Lys_{12}-acetylated cytochrome *c* species (C) as a function of pH at 20 °C and an ionic strength of 0.02. pH values of the spectra from lowest to highest intensity at 394 nm: (A) 5.0, 2.6, 2.5, 2.4, 2.3, 2.2, 1.8; (B) 5.0, 3.2, 2.6, 2.4, 2.2, 2.0, 1.8; (C) 5.0, 4.2, 4.0, 3.6, 3.4, 3.2, 1.8

1991; Goto & Hagihara, 1992). The results obtained so far with unmodified cytochrome *c* are consistent with cooperative conformational transitions between the native, unfolded, and molten globule states. Goto and Nishikiori (1991) showed that the acetylation of amino groups stabilizes the molten globule state. Kataoka et al. (1993) indicated that the acetylation-induced stabilization of the molten globule state at pH 2 in the absence of salt, where the intact protein is unfolded, is consistent with a two-state mechanism. However, the effects of acetylation on the acid-unfolding transition of cytochrome *c* are unknown. Thus, we measured the acid unfolding of acetylated cytochrome *c* to obtain a phase diagram dependent on pH and extent of acetylation.

Of the three methods employed here, the Soret absorption clearly distinguishes the three states, whereas the far-UV CD and fluorescence spectra do not distinguish the native and molten globule states. Panels A, B, and C of Figure 2 show the pH-dependent change in the Soret absorption spectra of the intact cytochrome *c*, and the Lys_3- and Lys_{12}-acetylated species, respectively. The measurements were carried out between pH 6 and 2 under conditions of constant ionic strength ($I = 0.02$) controlled by NaCl. Whereas the unmodified and Lys_3-acetylated species showed two isosbestic points, suggesting that three states are involved, the Lys_{12}-acetylated cytochrome *c* showed an isosbestic point at 405 nm, suggesting a two-state transition.

Figure 3 shows the Soret absorption spectra of the native and unfolded states and the molten globule states induced by salt at pH 2 and by acetylation. The molten globule state is stabilized by eight acetylations at pH 2 and 20 °C (Goto & Nishikiori, 1991). The spectra of the molten globule state at pH 2 were essentially the same for the various species with more than eight acetylations (data not shown). This indicated that the spectrum of the molten globule state is independent of the extent of acetylation. It should be noted, however, that the spectrum of the molten globule state induced by acetylation is notably different from that induced by salt, suggesting that the heme environment of the acetylation-induced molten globule state is more perturbed than that of the salt-induced state. A similar difference was also suggested by the analysis of the small-angle X-ray scattering (M. Kataoka and Y. Goto, unpublished results). We assumed that the respective spectra of Figure 2 consist of only the contributions of native, unfolded, and molten globule states and that the spectra of these three states are independent of pH and the extent of acetylation.

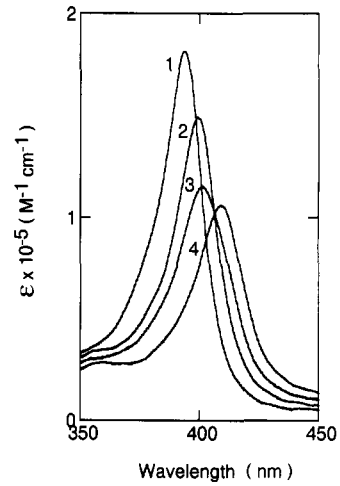

FIGURE 3: Soret absorption spectra of the native state (4), the acid-unfolded state (1), and the molten globule states induced by salt at pH 2 (3) and by acetylation (2). Conditions of the measurements: native, sodium phosphate buffer ($I = 0.02$) at pH 7; acid-unfolded, 20 mM HCl at pH 1.8; molten globule induced by NaCl, 20 mM HCl and 0.5 M NaCl; molten globule induced by acetylation, Lys_{15}-acetylated cytochrome *c* in 20 mM HCl at pH 1.8.

Then, we analyzed the apparent spectra to obtain the contribution of the three states. The absorption spectra at various pH values (Figure 2) were fitted by a combination of the three spectra, as shown in Figure 3, using a least-squares analysis program. Panels A, B, and C of Figures 4 show the pH dependence of the fractions of the three states for the intact and the Lys_3- and Lys_{12}-acetylated species, respectively. In the transition of unmodified cytochrome *c*, a small amount (25% at maximum) of the molten globule state accumulated transiently. With an increase in the extent of acetylation, the amount of the molten globule state increased. For the Lys_{12}-acetylated species, the transition became two-state from the native state to the molten globule state without the accumulation of the unfolded state.

The crossing point of the two species in Figure 4 provides a boundary in the phase diagram. Figure 5A shows the acetylation- and pH-dependent phase diagram of cytochrome *c*. With an increase in the extent of acetylation, the molten globule state was stabilized. On the other hand, acetylation destabilized the native state relative to the molten globule state. For comparison, Figure 5B shows the salt- and pH-dependent phase diagram of cytochrome *c* redrawn with the

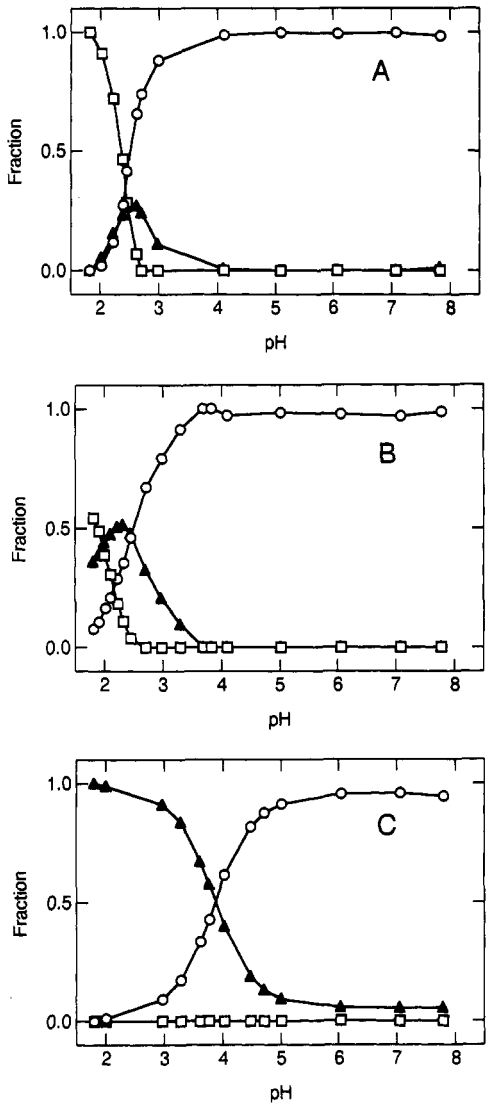

FIGURE 4: Dependence on pH of the fractions of the native (○), acid-unfolded (□), and molten globule states (▲) of the unmodified (A) and the Lys_3- (B), and Lys_{12}-acetylated cytochrome *c* species (C) as a function of pH at $I = 0.02$ and 20 °C. Errors in estimation of the fractions are ±5%.

data of Ohgushi and Wada (1983) and our data. The effects of acetylation were similar to those of salt, as represented by the similarity of the phase diagrams.

DISCUSSION

Conformational Transition in D_2O. To examine the proposed difference in the conformational transitions of cytochrome *c* (Jeng & Englander, 1991; Kataoka et al., 1993), we carried out the HCl and DCl titration of cytochrome *c* in H_2O and D_2O. We have shown that, after the pD value correction ($pD = pD_{app} + 0.4$; Glasoe and Long, 1960), the DCl titration of cytochrome *c* in D_2O is very similar to the HCl titration in H_2O. For some proteins, it is reported that D_2O increases the stability of the protein conformational states probably by strengthening the hydrogen bonds (Henderson et al., 1970; Gomez-Puyou et al., 1978; Masson & Laurentie, 1988; Antonino et al., 1991). Such effects can be expected to be dramatic for an intermediate conformational state where the contribution of hydrogen bonds to stability is larger than that of other forces. However, no significant stabilization of the native or intermediate states of cytochrome *c* by D_2O was observed. In addition, the coincidence of the transition curves

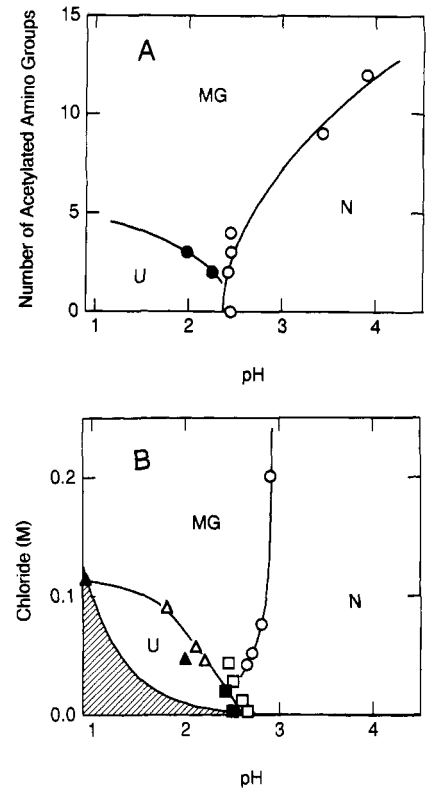

FIGURE 5: Phase diagram for the conformational states of cytochrome *c* dependent on the pH and the extent of acetylation at 20 °C (A) and that dependent on the pH and the chloride concentration at 20 °C (B): N, native state; U, acid-unfolded state; MG, molten globule state. Chloride concentration was controlled by NaCl or KCl. In panel B, open symbols are taken from Ohgushi and Wada (1983) and closed symbols are from our previous reports (Goto et al., 1990a,b). The hatched area in panel B is prohibited owing to the increase in the minimal chloride concentration with a decrease in pH ($[Cl^-] = 10^{-pH}$).

monitored by CD, fluorescence, and absorption indicated that no unique intermediate state which is unstable in H_2O was stabilized in D_2O.

Jeng and Englander (1991) proposed accumulation of the pre-molten globule state at pD = 2.6 under low-salt conditions. The present results did not confirm such an intermediate state. The conformational transitions in both H_2O and D_2O are apparently consistent with the sequential transition from the native (N) state to the unfolded (U) state and then to the molten globule (M) state.

$$N \rightarrow U \rightarrow M \qquad \text{(mechanism 1)}$$

However, this does not mean that the molten globule state is an off-pathway intermediate. The molten globule state is located between the native and unfolded states (Kuwajima, 1989; Ptitsyn, 1992; Barrick & Baldwin, 1993). Thus, the relation of the three states on the folding pathway is:

$$N \leftrightarrow M \leftrightarrow U \qquad \text{(mechanism 2)}$$

The molten globule state should be involved in the initial unfolding process of mechanism 1, but it does not accumulate because of the high cooperativity of the transition.

Dyson and Beattie (1982) analyzed the acid-induced unfolding transition of horse cytochrome *c* under various salt conditions. The molten globule state, which had not been recognized in those days, corresponds to state II in their terminology [see also Tsong (1975) and Stellwagen and Babul (1975)]. Under low ionic strength conditions ($I = 0.01$, controlled by NaCl), the N to M transition coincided with the

M to U transition. As a result, the equilibrium unfolding was approximated by a two-state mechanism from the native state to the unfolded state. An increase in salt concentration separates the two transitions by slightly decreasing the stability of the native state and significantly increasing the stability of the molten globule state. This results in the two-step unfolding transition involving the molten globule intermediate state. These effects of salt on the unfolding transition are evident from the pH- and salt-dependent phase diagram (Figure 5B). It was proposed that the replacement of the methionine ligation to the heme iron by a carboxyl group is responsible for the N to M transition, and protonation of His 18, which coordinates to the iron, is involved in the M to U transition (Dyson & Beattie, 1982).

On the other hand, the HCl- or DCl-induced refolding transition arises from the increase in chloride concentration. Chloride binds to the positively charged protein, thus shielding the charge repulsion and resulting in the stabilization of the molten globule state by the intrinsic forces, including hydrophobic interactions. Irrespective of the interpretation of the acid-induced unfolding transition, a pre-molten globule state might be expected to accumulate during the acid-induced refolding transition since it is a transition between the fully unfolded and molten globule states. However, in both H_2O and D_2O, the transition was consistent with a two-state mechanism between the unfolded and molten globule states. Kataoka et al. (1993) examined the NaCl-dependent stabilization of the molten globule of cytochrome c at pH 2 by various methods, including SAXS, and showed that the transition is consistent with the two-state approximation. Because the salt- and acid-induced transitions both arise from the chloride effects, the cooperative refolding transition observed here is as expected from the NaCl-induced refolding transition at pH 2. It is interesting to note that, because unprotonated His 18 coordinates to the iron in the molten globule state, the acid-induced refolding accompanies deprotonation of His 18 with a decrease in pH. In other words, the increase in chloride concentration causes the drastic decrease in the pK_a of His 18.

Recently, Kuroda et al. (1992) studied the thermal stability of horse cytochrome c at low pH, between 2.0 and 3.2, and at different KCl concentrations. On the basis of the disagreement of the unfolding curves measured by the ellipticity at 222 nm and that at 282 nm, they proposed the presence of two molten globule states (IIb and IIc). The IIb state is stable at low temperature and high salt, and the IIc state is an intermediate of the thermal unfolding of the IIb state. Whereas the IIb and IIc states are not distinguished from each other by their far-UV CD or tryptophan fluorescence, the IIc state is more disordered than the IIb state in terms of the aromatic CD. As described above, in both D_2O and H_2O, the acid titration of intact cytochrome c at 20 °C was consistent with a minimal three-state model (mechanism 2) involving one molten globule state. Considering the proposed stability of the IIc state, it probably did not accumulate significantly under our experimental conditions.

We tried to simulate the unfolding and refolding transitions by a minimal three-state model. The fraction of the unfolded state (f_U) is defined with the equilibrium constant for the respective processes by

$$f_U = (1 + K_U^{-1} + K_M)^{-1} \quad (2)$$

where $K_U = [U]/[N]$ and $K_M = [M]/[U]$. We assumed that the unfolding transition is cooperative and approximated by a two-state transition from the N to the U state. The acid-induced unfolding can be explained by the protonation of titratable groups (Tanford, 1970). On the other hand, we assumed that the refolding transition is from the U to the M state and is caused by the binding of chloride anion to the protonated groups (Goto et al., 1990a,b). Provided that the binding sites for proton and chloride are independent, the relation of K_U and K_M as a function of proton concentration $[H^+]$ and chloride concentration $[Cl^-]$, respectively, is given by

$$K_U = K_{U0}\prod_{i=1}^{n}(1 + [H^+]/K_{HUi})/(1 + [H^+]/K_{HNi}) \quad (3)$$

and

$$K_M = K_{M0}\prod_{i=1}^{m}(1 + [Cl^-]/K_{CMi})/(1 + [Cl^-]/K_{CUi}) \quad (4)$$

where K_{U0} is the equilibrium constant for the deprotonated species, and K_{M0} is that in the absence of salt. K_{HUi} and K_{HNi} are the dissociation constants of protons of the ith titratable site in the unfolded and folded states, respectively, and K_{CMi} and K_{CUi} are the chloride dissociation constant of the ith binding site in the molten globule and unfolded states, respectively. The number of titratable sites responsible for the unfolding and refolding transitions can be estimated from $d \ln K_U/d \ln [H^+] = \Delta n$ and $d \ln K_M/d \ln [Cl^-] = \Delta m$, respectively. The analysis of the transition curves gave $\Delta n = 4.6$ and $\Delta m = 1.9$ in H_2O (Figure 1A) and $\Delta n = 4.8$ and $\Delta m = 1.6$ in D_2O (Figure 1B). Thus the values in H_2O and D_2O are essentially the same. Assuming that five proton-titratable and two chloride-titratable sites are responsible for the transitions and that sites of the same kind are indistinguishable, eqs 3 and 4 can be reduced to

$$K_U = K_{U0}(1 + [H^+]/K_{HU})^5/(1 + [H^+]/K_{HN})^5 \quad (5)$$

and

$$K_M = K_{M0}(1 + [Cl^-]/K_{CM})^2/(1 + [Cl^-]/K_{CU})^2 \quad (6)$$

respectively. Equations 2, 5, and 6 were used to simulate the unfolding and refolding transitions induced by HCl or DCl. In the analysis, we assumed that $K_{U0} = 4.7 \times 10^{-6}$. This corresponds to a free energy of unfolding of 7.1 kcal/mol at 20 °C (Pace, 1975). We also assumed that $K_{M0} = 3.5 \times 10^{-3}$ from the previous study (Goto et al., 1990b). We estimated the values of K_{HN}, K_{HU}, K_{CM}, and K_{CU} manually so that the calculated curves fit both the unfolding and the refolding transition. Whereas the unfolding transition defines K_{HN} and K_{HU}, the refolding transition defines K_{CM} and K_{CU}. Table I shows the parameters used to draw the theoretical lines in Figure 1, which agree well with the observed transitions.

Table I: Parameters Used to Fit the Unfolding and Refolding Transitions of Cytochrome c at 20 °C[a]

	pK_{HN}	pK_{HU}	K_{CM} (M)	K_{CU} (M)
in H_2O	2.0	3.7	0.5	180
in D_2O	2.1	3.8	0.5	220

[a] The transitions were simulated on the basis of eqs 2, 5, and 6. The values of K_{U0} and K_{M0} were 4.7×10^{-6} and 3.5×10^{-3}, respectively. $pK_{HN} = -\log K_{HN}$, and $pK_{HU} = -\log K_{HU}$.

The model used here is too simple to completely explain the combined effects of protons and anions on the complicated electrostatics of protein molecules. Thus, we regard these values as phenomenological values consistent with the experimental data. However, it is evident from this analysis that the unfolding and refolding transitions can be explained

simply by the minimal three-state model in both H_2O and D_2O. It should be emphasized that we propose the three-state approximation only for the equilibrium transition at 20 °C. As described above, Kuroda et al. (1992) indicated that one additional molten globule state (the IIc state) with more completely disordered tertiary structure than that at 20 °C is necessary to analyze the equilibrium thermal unfolding curves under acidic conditions. Kinetic studies of the refolding of cytochrome *c* (Elöve et al., 1992) indicate the rapid accumulation of a transient intermediate before formation of the relatively stable molten globule state.

In concluding this section, we draw attention to the experimental conditions used by Jeng and Englander (1991), namely, the NaCl-induced titration in D_2O at pD 2.6 (pD_{app} 2.2) at 20 °C. As can be seen from Figure 1B, pD 2.6 is in the transition region between the native and unfolded states. Thus, interpretation of the effects of the change in NaCl concentration at pD 2.6 is complicated, because NaCl affects both the N to U and the U to M transition. It would be difficult to unambiguously detect the accumulation of a pre-molten globule under such conditions. Because of these complications, we did not try to analyze the conformational states present under the conditions used by Jeng and Englander (1991). Although we cannot rule out the possibility of a pre-molten globule at pD 2.6 under the salt conditions used by them (0.01–0.02 M NaCl), the high cooperativity of the DCl-induced refolding transition strongly suggests that if such an intermediate exists, it must be very unstable.

Mechanism of the Stability of the Molten Globule State. The acetylation- and pH-dependent phase diagram indicates that the effects of acetylation on the conformation of cytochrome *c* are similar to those of salt. Both salt and acetylation stabilize the molten globule state relative to the unfolded state. Whereas the salt decreases the charge repulsion by counterion binding to the positively charged groups, acetylation of the amino groups directly removes the charged groups, resulting in the stabilization of the molten globule state by the intrinsic folding forces, including hydrophobic interactions. From a comparison of the two phase diagrams, modification of two acetyl groups corresponds to the addition of 0.1 M NaCl.

On the other hand, acetylation destabilizes the native state relative to the molten globule state, which is also analogous to the effects of NaCl, although the effects of the former are greater than those of the latter. Horse cytochrome *c* is a basic protein with an isoelectric point of 10.1. Because acetylation decreases the net charge of cytochrome *c* even at pH 7, the destabilization cannot be explained by the change in net charge repulsion. The opposing effects of acetylation on the native and molten globule states were also observed in thermal unfolding experiments (our unpublished results). Whereas acetylation stabilizes the molten globule state against thermal unfolding at pH 2 in the absence of salt, it decreases the thermal stability of the native structure at pH 7.

The destabilizing effects of salt on the native structure might be explained by the disruption of the specific salt bridges which are important to maintaining the native structure. Recently, Dill and co-workers (Dill, 1990; Alonso et al., 1991) proposed an alternative explanation, namely, that the salt preferentially stabilizes the denatured state by penetrating into the interior of the unfolded protein molecule. On the other hand, the acetylation of amino groups, which are located on the surface of the molecule, may preferentially increase the hydrophobic surface area of the native state, resulting in the destabilization of the native state. In addition, introduction of the bulky acetyl groups may perturb the rigid packing of the native structure.

At the present stage of our research, we cannot clarify the mechanism of the destabilization of the native state by acetylation. However, these results indicate that the mechanism of the conformational stability of the native state is substantially different from that of the molten globule state. Whereas specific interactions, such as hydrogen bonds, salt bridges, and specific side-chain interactions, are important for the native structure, such interactions are not so important in the molten globule state. Instead, hydrophobicity and global electrostatic properties may be more important. The secondary structural elements of the molten globule state probably interact more weakly than those of the native state, as suggested by Ewbank and Creighton (1991). Baldwin and co-workers (Hughson et al., 1991; Barrick & Baldwin, 1993) studied the stability of the acidic intermediate state of apomyoglobin by site-directed mutagenesis. Mutations were introduced to disrupt the packing of the helices that are identified as native-like in the intermediate state. These mutations reduced the stability of the native state relative to the unfolded state but did not destabilize the intermediate state. They suggested that the intermediate is stabilized by relatively nonspecific hydrophobic interactions, which allow it to adapt easily to mutation.

However, this does not necessarily mean that specific interactions in the molten globule state are unimportant. We have found recently that ligation of the heme iron to the His18 residue is critical in stabilizing the acidic molten globule of cytochrome *c* (our unpublished results). To understand the molten globule state, it is important to dissect quantitatively the contribution of specific and nonspecific interactions contributing to its stability.

Conclusion. We have shown that, in both H_2O and D_2O, the equilibrium conformational transition of cytochrome *c* in the acidic pH region at 20 °C is a cooperative process well approximated by a minimal three-state mechanism. In particular, under conditions of minimal ionic strength, a decrease in pH results in the initial unfolding from the native to the unfolded state and the subsequent refolding to the molten globule state. The effects of acetylation were similar to those of salt both in stabilizing the molten globule state and in destabilizing the native state. The phase diagram suggests that acetylation changes the relative stability of the conformational states but not the cooperativity of the conformational transitions.

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SUPPLEMENTARY MATERIAL AVAILABLE

Raw data of the HCl and DCl titrations of ferricytochrome *c* measured by CD at 222 nm, by tryptophan fluorescence at 345 nm, and by Soret absorption at 394 nm (2 pages). Ordering information is given on any current masthead page.

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