Intermediate Conformational States of Apocytochrome *c*†

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ABSTRACT: Horse apocytochrome *c* has been assumed to be a typical unfolded protein. At low ionic strength, the far- and near-UV circular dichroism spectra are typical of an unfolded protein at all pH values between 2 and 9. On the other hand, in the presence of high concentrations of salt, substantial secondary structure is present at both neutral and acidic pH. At low pH, perchlorate anion, either from salt or from acid, stabilizes an intermediate state (the A state) with secondary structure similar to that previously observed in the molten globule state of holocytochrome *c*. To further characterize the conformational states of apocytochrome *c* as a function of pH and salt, a fluorescence-labeled derivative was prepared, in which the two cysteine residues were labeled with *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS). The conformational transitions of the fluorescence-labeled apocytochrome *c* measured by circular dichroism were similar to those of unmodified apocytochrome *c*, indicating that the effects of the modification on the conformation and stability are small. Fluorescence energy transfer from tryptophan to the fluorescence label revealed several salt- and pH-dependent transitions. At very low ionic strength, apocytochrome *c* became compact as the pH was increased with a transition midpoint at pH 4.5. At acidic pH, increasing concentration of perchlorate induced a more compact state with a transition midpoint similar to that observed by circular dichroism. In contrast, at neutral pH, increasing perchlorate concentration had little effect on the compactness, as determined by a lack of change in the energy-transfer efficiency (but did increase the amount of secondary structure). A phase diagram of the conformational states was constructed from the pH- and perchlorate-dependent conformational transitions. The phase diagram of apocytochrome *c* was notably different from that of holocytochrome *c*, and from those reported for other proteins, in that a compact denatured state was present at neutral pH which was less structured than the A state at acidic pH.

Removal of the covalently bound heme group from cytochrome *c* produces apocytochrome *c*. The conformation of apocytochrome *c* has been studied extensively by several groups (Fisher et al., 1973; Damaschun et al., 1991; de Jongh et al., 1992); it is unfolded under aqueous conditions of neutral or acidic pH, but assumes an α-helical structure in the presence of lipids. Although horse cytochrome *c* and several other proteins are largely unfolded in the absence of salt in the vicinity of pH 2, they adopt a molten globule state in the presence of anions either from salt or from acid (Ohgushi & Wada, 1983; Goto et al., 1990a,b). Goto, Fink, and co-workers (Goto et al., 1990a,b; Goto & Aimoto, 1991; Goto & Nishikiori, 1991) have shown that the conformation of acid-denatured proteins is determined by a subtle balance between unfolding and folding forces. Acid-denatured cytochrome *c* is unfolded under conditions of low salt by repulsion between the positively-charged groups. Added anions bind to the charged groups, shielding the Coulombic charge repulsion, so that the intrinsic forces, including hydrophobic interactions, are able to stabilize the molten globule state.

The molten globule was proposed as a compact denatured state with significant secondary structure but largely disordered tertiary structure and has been suggested to be a major intermediate in protein folding (Ohgushi & Wada, 1983; Ptitsyn, 1987, 1992; Kuwajima, 1989, 1992; Kim & Baldwin, 1990; Christensen & Pain, 1991; Baldwin, 1991; Dobson, 1992). Examination of various proteins revealed that the amount of secondary structure and the compactness of intermediate conformational states are not necessarily close to those of the native protein, but vary significantly depending on the protein species (Kuwajima, 1989). This suggests the presence of various intermediate states from one close to the fully unfolded state to one close to the native state, depending on the protein species and experimental conditions. A theoretical study based on the statistical mechanics of polyelectrolytes also predicts the various conformational states, depending on the amino acid composition and environmental conditions (Stigter et al., 1991; Alonso et al., 1991). Therefore, it is uncertain whether the molten globule state, as originally defined, is a unique intermediate of protein folding. In addition, confusion has arisen from use of the term "molten globule" for intermediate species which are distinct from the original definition (Kim & Baldwin, 1990; Baldwin, 1991; Ptitsyn, 1992). In the present paper, we use the term "molten globule" for a compact denatured state with specific secondary structure.

Horse cytochrome *c* is a basic protein with p*I* = 10.1. Our previous results suggest that the addition of anions to the acid-unfolded state, either from salt or from acid, or an increase in pH should decrease the repulsive forces which arise from the protein net charge and, as a consequence, increase the folding forces. Comparison of the intermediate conformations of holo- and apoproteins may reveal the role of heme in stabilizing the folded and molten globule states. In addition,

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it may shed light on the controversy about the definition and structure of the molten globule.

In the present study, we measured the conformation of apocytochrome *c* and a fluorescence-labeled derivative under various conditions of pH and salts. The results show that, as predicted, apocytochrome *c* adopts an intermediate state (the A state), resembling the molten globule of the holoprotein, at acidic pH in the presence of anions. However, contrary to prediction, the A state was significantly destabilized with increasing pH. The results indicated that, although the decrease in net charge favors a compact denatured state, this does not necessarily result in the stabilization of the molten globule state. The results indicate that apocytochrome *c* can assume distinct compact denatured conformations depending on the environmental conditions.

MATERIALS AND METHODS

Materials. Horse cytochrome *c* (type IV) was purchased from Sigma. The heme group linked to the two cysteine residues by thioether bonds was removed by reaction with silver sulfate (Fisher et al., 1973). Apocytochrome *c* was passed through a column of Sephadex G-25 equilibrated with 5 mM sodium acetate buffer at pH 4 containing 0.1 mM ethylenediaminetetraacetic acid (EDTA)[1] and stored at −20 °C. The thiol content of apocytochrome *c* was determined to be 2 equiv on the basis of titration with DTNB.

The two SH groups of apocytochrome *c* were modified with IAEDANS. Apocytochrome *c* (5 mg/mL) was reacted with 5 molar excess of IAEDANS in 20 mM Tris-HCl buffer at pH 8.6 for 1 h at room temperature. The excess reagents were removed by passing through a column of Sephadex G-25 equilibrated with 0.1 M NaCl. The amount of AEDANS groups attached to the protein was determined to be 1.8–2.0 from the absorption spectra assuming the additivity of the spectra of intact apocytochrome *c* and the reagent reacted with cysteine (see Figure 3). The molar absorption coefficient of AEDANS at 337 nm was assumed to be 6100 M^{-1} cm^{-1} (Hudson & Weber, 1973).

Methods. All measurements in this work were carried out at 20 °C with thermostatically controlled cell holders. Usually, 0.1 mL of protein solution, dissolved in deionized water, was mixed with 0.9 mL of salt solutions. Titrations of the acid-, salt-, or pH-dependent conformational transition under various conditions were repeated at least twice, and the typical results were presented. The buffers used were glycine-hydrochloride (pH 2–3), sodium acetate (pH 3–6), sodium phosphate (pH 6–8), and Tris-HCl (pH 8–9). The buffer concentrations were 5–10 mM, and the ionic strength was kept to be 0.01 with NaCl. Solutions below pH 2 were prepared using the appropriate concentration of HCl. The pH was measured using a Radiometer PHM83 at 20 °C.

Circular dichroism 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 *d*-camphorsulfonate. 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. The protein concentration was, except where otherwise specified, 0.1 mg/mL, and a cell of 2-mm light path was used.

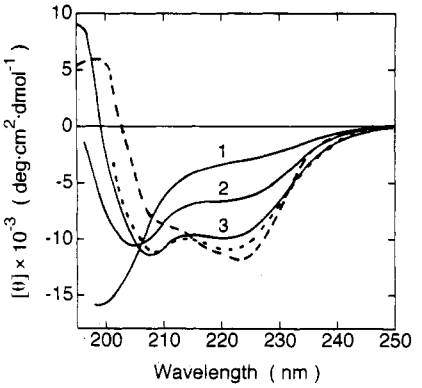

FIGURE 1: Far-UV CD spectra of apocytochrome *c* and holocytochrome *c* at 20 °C. Apocytochrome *c* in 18 mM HCl at pH 1.8 (1), in 62.5 mM perchloric acid at pH 1.3 (2), or in 450 mM perchloric acid at pH 0.4 (3); holocytochrome *c* in 6 mM sodium phosphate buffer at pH 7 (dashed line); holocytochrome *c* in 18 mM HCl and 0.4 M NaCl at pH 1.8 (dotted line).

Fluorescence spectra were measured with a Hitachi-Perkin Elmer fluorescence spectrophotometer, Model MPF-4. Tryptophan fluorescence was measured with excitation at 285 nm. The protein concentration was 0.02 mg/mL.

SAXS measurements were performed with the solution scattering station installed at BL-10C, the Photon Factory, Tsukuba, Japan (Ueki et al., 1985; Kataoka et al., 1991), under approval from the Program Advisory Committee of the Photon Factory (proposal no.92-067). The sample cell was 50 μL in volume and 1-mm X-ray pathlength. Protein concentrations were varied within the range of 5–25 mg/mL, and correction for the concentration dependence of the scattered intensity was made to obtain the scattering curves at infinite dilution (Kataoka et al., 1989).

The concentration of apocytochrome *c* was determined spectrophotometrically using $E^{1cm}_{1\%} = 10.1$ at 276 nm (Damaschun et al., 1991). The concentration of AEDANS-apocytochrome *c* was determined from the absorption at 276 nm after subtraction of the contribution of AEDANS groups.

RESULTS

Conformation of Apocytochrome c. We first confirmed that apocytochrome *c* is unfolded by measuring the CD as a function of pH at low ionic strength (Figures 1 and 2A,C). The spectrum of apocytochrome *c* at pH 2 in the absence of salt showed a minimum at 200 nm ($[\theta] = -15\,000$), indicating that the protein is largely unfolded. The spectrum at pH 7 in the absence of salt (data not shown) was similar to that at pH 2, although the minimum intensity ($[\theta] = -12\,000$) at 200 nm was slightly smaller than that at pH 2. No significant near-UV CD spectral signal was observed for apocytochrome *c* at low ionic strength at either acidic or neutral pH, also consistent with an unfolded conformation.

Figure 1 shows the far-UV CD spectra of apocytochrome *c* in the presence of various concentrations of perchloric acid; for comparison, the spectra of the native and molten globule states of cytochrome *c* are also shown. At low pH, with increasing concentrations of perchloric acid, the CD spectra showed the formation of secondary structure, especially α-helix. The spectra at different concentrations of perchloric acid formed an isodichroic point, consistent with a two-state transition from the acid-unfolded state (U_A state) to the state containing secondary structure (A state). The spectrum of

[1] Abbreviations: AEDANS-apocytochrome *c*, apocytochrome *c* in which two SH groups are labeled with AEDANS; CD, circular dichroism; D, deuterium; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; Gdn-HCl, guanidine hydrochloride; IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; NMR, nuclear magnetic resonance; R_g, radius of gyration; SAXS, small-angle X-ray scattering.

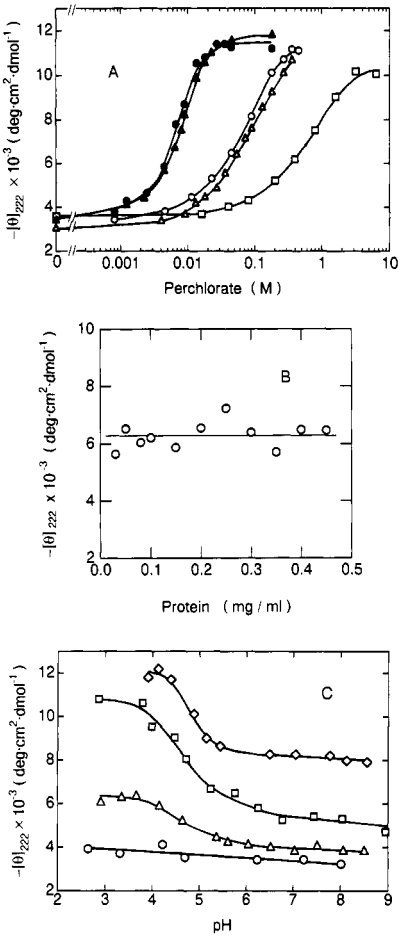

FIGURE 2: Dependence of the ellipticity of apocytochrome c on perchlorate concentration (A), protein concentration (B), or pH (C) at 20 °C. Panel A: (○) $HClO_4$; (△) $NaClO_4$ in 18 mM HCl at pH 2; (□) $NaClO_4$ in 6 mM phosphate buffer at pH 7. Closed circles and closed triangles show the ellipticities of holocytochrome c at various concentrations of $HClO_4$ and $NaClO_4$ in 18 mM HCl at pH 2, respectively, taken from Goto et al. (1990b). Panel B: in 60 mM $NaClO_4$ and 18 mM HCl at pH 2. Panel C: (○) in the absence of salt; (△) 0.05 M $NaClO_4$; (□) 0.2 M $NaClO_4$; (◇) 2 M $NaClO_4$.

apocytochrome c at pH 2 in the presence of 0.45 M perchloric acid was similar to that of cytochrome c in 0.4 M NaCl at pH 2, where the molten globule state is stable. A similar change in the far-UV CD spectrum was also induced by increasing sodium perchlorate concentration at pH 2 (data not shown).

Figure 2A shows the conformational transition of apocytochrome c at a protein concentration of 0.1 mg/mL from the U_A state to the A state as a function of perchloric acid or sodium perchlorate concentration as detected by the ellipticity at 222 nm. Assuming the $[\theta]_{222}$ values for the U_A and A states to be −3000 and −11 500, respectively, the midpoint concentration of the transition (C_m) induced by perchloric acid was 60 mM. The effects of sodium perchlorate at pH 2 were comparable to those of perchloric acid (Table I). Figure 2A also shows the sodium perchlorate or perchloric acid-induced transitions of cytochrome c taken from Goto et al. (1990b). Whereas apocytochrome c and holocytochrome c exhibit similar perchlorate-induced conformational transitions, C_m values of the former were 10 times larger than those of the latter.

Apocytochrome c showed a tendency to aggregate after the transition was over. High tendency to aggregate is one of the properties of the molten globule states (Goto & Fink, 1989; Goto et al., 1990b). To examine the role of intermolecular interactions in the conformational transition, we measured at

Table I: Midpoint Perchlorate Concentration of Conformational Transitions of Horse Apocytochrome c and AEDANS-Apocytochrome c Induced by Perchloric Acid or Sodium Perchlorate at 20 °C

pH	C_m (mM) apocytochrome c	C_m (mM) AEDANS-apocytochrome c	method[a]
	$HClO_4$-Induced U_A to A Transition		
<2	60	60	CD
	$NaClO_4$-Induced U_A to A Transition		
1.8	61	30	CD
3.0	ND[b]	30	fluorescence
4.0	65	50	CD
	$NaClO_4$-Induced C to A Transition		
4.9	200	200	CD
7.0	700	1000	CD

[a] See text for details. [b] Not determined.

C_m (60 mM sodium perchlorate at pH 1.8) the dependence on protein concentration of the ellipticity at 222 nm. As shown in Figure 2B, the ellipticity was independent of the protein concentration in a range of 0.02–0.45 mg/mL. This indicates that the intermolecular interactions are not responsible for the conformational transition.

We measured the near-UV CD spectra of apocytochrome c in the presence of sodium perchlorate. The molten globule state of the holocytochrome c is characterized by the absence of the sharp peaks in the vicinity of 280 nm observed with the native state [see Figure 2 of Goto and Nishikiori (1991)]. Apocytochrome c showed no significant CD intensity even under conditions where the far-UV CD spectrum indicates the formation of significant secondary structure (data not shown). These results suggest that the A state of apocytochrome c is similar to the molten globule state of holocytochrome c.

Although HCl showed a tendency to induce a similar conformational transition, its effect was much less than that of perchloric acid (data not shown). We could not observe the entire refolding transition induced by HCl because of the acid hydrolysis which occurred above 2 M HCl. The C_m value was estimated to be 1.0 M on the basis of the above limiting values. Although the concentration range was much higher, these results were similar to those for the anion-induced stabilization of the molten globule of holocytochrome c (Goto et al., 1990a, b), suggesting that anion binding is responsible for the conformational transition.

Figure 2A also shows the transition induced by sodium perchlorate at pH 7. The transition at neutral pH required a much higher concentration of salt than that at pH 1.8 (Table I). The situation is opposite that of apomyoglobin (Goto & Fink, 1990), the basic amphiphilic peptide (Goto & Aimoto, 1991), or melittin (Goto & Hagihara, 1992), where an increase in pH results in a decrease in the salt concentration necessary to stabilize the helical (folded) state.

To clarify the effects of pH on conformation, we measured the pH-dependent conformational transition under fixed concentrations of perchlorate ion (Figure 2C). Apocytochrome c had no significant secondary structure in the absence of salt at pH between 2 and 9. In the presence of 0.2 M perchlorate, the helical state was stabilized at acidic pH regions. The pH titration in the presence of various concentration of perchlorate showed a cooperative unfolding transition with an apparent midpoint at pH 4.5–5.0, suggesting that the deprotonation of carboxyl groups destabilizes the helical structure.

Fluorescence-Labeled Apocytochrome c. Apocytochrome c has two SH groups at positions 14 and 17 which had linked

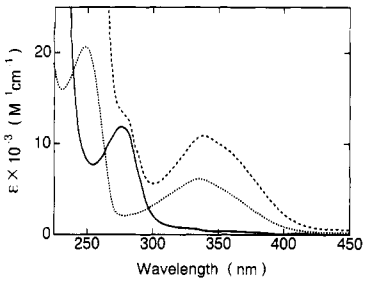

FIGURE 3: Absorption spectra of apocytochrome *c* (solid line), apocytochrome *c* reacted with IAEDANS (AEDANS-apocytochrome *c*) (dashed line), and IAEDANS reacted with cysteine (dotted line).

the heme group to the protein. IAEDANS is a hydrophobic fluorescent reagent with an absorption maximum at 337 nm (Figure 3). We modified apocytochrome *c* with 2 mol of IAEDANS, assuming that the AEDANS groups could be used as a probe to monitor conformational changes by taking advantage of the fluorescence energy transfer from the tryptophan at position 59. We were also interested in determining if the introduction of the hydrophobic reagent stabilizes the helical state of apocytochrome *c*.

We first measured the far-UV CD spectra of AEDANS-apocytochrome *c* in the presence of various concentrations of sodium perchlorate (data not shown). In the absence of salt either at pH 2 or at pH 7, the spectra were similar to those of apocytochrome *c*, showing that the modification does not stabilize the helical state. With increasing concentration of sodium perchlorate at pH 1.8, the spectrum changed to indicate significant helical structure as was the case of unmodified apocytochrome *c*. We then measured the conformational transition of AEDANS-apocytochrome *c* as a function of perchloric acid or sodium perchlorate concentration, as measured by the ellipticity at 222 nm. We also measured the pH-dependent transition under fixed concentrations of perchlorate ion. The maximal ellipticity ($[\theta]$ = −9000) at 222 nm induced by perchlorate was slightly smaller than that of apocytochrome *c*. However, the perchlorate- or pH-induced transitions were similar to those of apocytochrome *c* (Table I). This shows that the AEDANS groups do not affect the conformational transition of apocytochrome *c* significantly, whereas they cause a small decrease in the amount of secondary structure in the A state. Thus, the conformational transitions of the modified protein are similar to those of unmodified apocytochrome *c*.

Figure 4A shows the fluorescence spectra of AEDANS-apocytochrome *c* excited at 285 nm. As a reference state, we measured the spectrum of the unfolded state in the presence of high concentrations of Gdn-HCl (U_{GdnHCl}). The spectrum in 6.5 M Gdn-HCl at pH 3 had two maxima at 350 and 500 nm, the intensity of the former being twice that of the latter. Figure 4A also shows the spectrum of apocytochrome *c* at pH 3. The fluorescence intensity at 350 nm of AEDANS-apocytochrome *c* in Gdn-HCl was 90% of that of apocytochrome *c*, indicating that the quenching by the AEDANS groups is small in 6.5 M Gdn-HCl. At pH 3 in the absence of salt, the fluorescence intensity of AEDANS-apocytochrome *c* at 350 nm decreased and that at 500 nm increased by 10% and 25%, respectively, compared to those in 6.5 M Gdn-HCl. These results suggested that the unfolded state (U_A) at pH

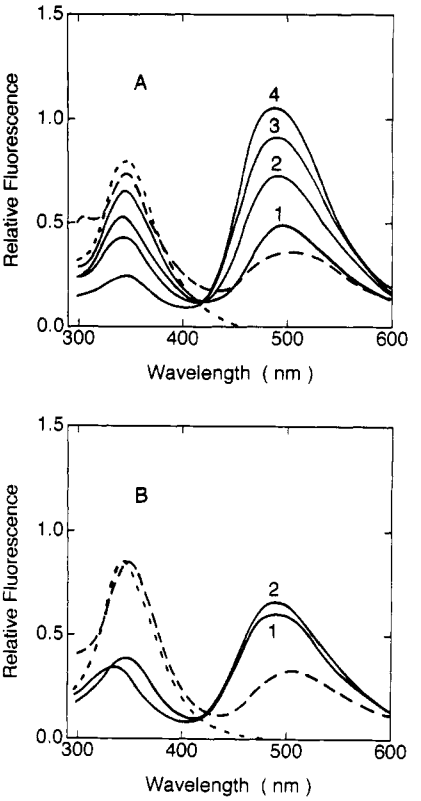

FIGURE 4: Fluorescence spectra of AEDANS-apocytochrome *c* in 9 mM glycine hydrochloride buffer at pH 3 (A) and in 6 mM sodium phosphate buffer (I = 0.01) at pH 7 (B) at 20 °C in the presence of various concentrations of $NaClO_4$. Panel A: $NaClO_4$ concentrations are 0 (1), 20 (2), 40 (3), and 200 mM (4). Panel B: $NaClO_4$ concentrations are 0 (1) and 4 M (2). For comparison, the fluorescence spectrum of AEDANS-apocytochrome *c* in the presence of 6.5 M Gdn-HCl (dashed line) and that of apocytochrome *c* in the absence of salt (dotted line) under the same buffer conditions are shown. The ordinate shows the relative fluorescence intensity to that of *N*-acetyltryptophanamide at 345 nm. Excitation was at 285 nm.

3, even in the absence of salt, is somewhat compact compared to that (U_{GdnHCl}) in 6.5 M Gdn-HCl.

With increase in perchlorate concentration at pH 3, the tryptophan fluorescence of AEDANS-apocytochrome *c* at 350 nm decreased and that of the AEDANS group increased (Figures 4A and 5A). This indicates fluorescence energy transfer between the chromophores, suggesting that the distance between the tryptophan and AEDANS groups becomes closer with the increase in perchlorate concentration. The increase in AEDANS fluorescence was accompanied by a blue shift of the maximum emission wavelength from 500 to 480 nm, suggesting that the AEDANS groups are buried in a hydrophobic environment. As shown in Table I, the midpoint of the transition at pH 3 measured by the change in fluorescence at 350 nm was 30 mM, consistent with that measured by CD at 222 nm at pH 2. The fluorescence measurements were done at a protein concentration of 0.02 mg/mL whereas the CD measurements were at 0.1 mg/mL. The agreement of C_m values again indicates that the intermolecular interactions are not responsible for the conformational transition.

Figure 4B shows the fluorescence spectra of AEDANS-apocytochrome *c* at neutral pH. The spectrum in the absence of salt indicates that, whereas the tryptophan emission intensity relative to that of apocytochrome *c* is smaller by 40% than that at pH 3 in the absence of salt, the emission intensity from the AEDANS groups is larger by about 35% (see Figure 5A,B). This indicates that the molecule is more compact than that

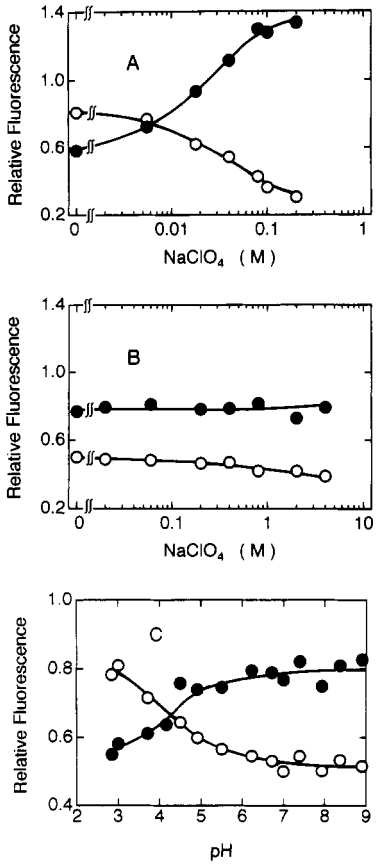

FIGURE 5: Dependence on $NaClO_4$ concentration (A, B) and pH (C) of the fluorescence of AEDANS-apocytochrome c at 350 nm (open symbols) and at 500 nm (closed symbols) at 20 °C. Panel A: [$NaClO_4$] dependence in 10 mM glycine hydrochloride buffer at pH 3. Panel B: [$NaClO_4$] dependence in 6 mM sodium phosphate buffer at pH 7. Panel C: pH dependence in the absence of salt. The ordinate shows the relative fluorescence intensity to that of apocytochrome c at 350 nm.

at the acidic pH. With increase in salt concentration, a slight decrease in fluorescence intensity at 350 nm and an increase at 500 nm were observed under conditions where the CD spectrum showed formation of secondary structure.

Figure 5C shows the dependence on pH of the fluorescence intensities at 350 and 500 nm of AEDANS-apocytochrome c under conditions of no salt (I = 0.01). The change in fluorescence energy transfer shows that the molecule becomes compact in a transition with an apparent midpoint of pH 4.5. The transition measured by the increase in intensity at 500 nm agreed well with the transition measured at 350 nm.

Small-Angle X-ray Light Scattering. To determine the compactness of the different conformational states of apocytochrome c, we tried to measure the radius of gyration (R_g) by SAXS. However, the measurements could not be performed at pH above 4 or in the presence of salt, because the high protein concentration (more than 5 mg/mL) necessary for the SAXS measurements induced aggregation. Measurements in the presence of high concentrations of Gdn-HCl were possible at both acidic and neutral pH regions.

X-ray scattering intensities at the small angle region are given as $I(Q) = I(0) \exp(-R_g^2Q^2/3)$, where Q and $I(0)$ are momentum transfer and intensity at 0 scattering angle, respectively (Glatter & Kratky, 1982). Q is defined by $Q = 4\pi \sin\theta/\lambda$, where 2θ and λ are the scattering angle and the wavelength of X-ray, respectively. Therefore, a Guinier plot, $\ln[I(Q)]$ vs Q^2, should be approximated by a straight line, and the slope gives R_g.

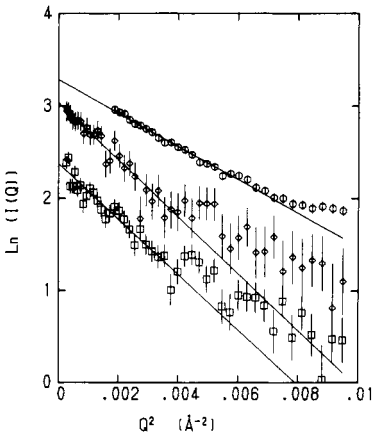

FIGURE 6: Comparison of SAXS profiles of denatured apocytochrome c at infinite dilution in the form of a Guinier plot. (○) Apocytochrome c in 10 mM glycine hydrochloride buffer at pH 3; (◇) apocytochrome c in 10 mM glycine hydrochloride buffer at pH 3 and 4 M Gdn-HCl; (□) apocytochrome c in 10 mM phosphate buffer at pH 6 and 4 M Gdn-HCl. For clarity, the values are shifted on the ln I axis.

Figure 6 shows Guinier plots of scattering curves from AEDANS-apocytochrome c at pH 3 in the presence and absence of 4 M Gdn-HCl, and at pH 6 in 4 M Gdn-HCl. Each curve is obtained by extrapolation to zero protein concentration with the data of five different protein concentrations. The R_g of AEDANS-apocytochrome c in 4 M Gdn-HCl was determined to be 30.4 ± 1.2 and 30.0 ± 0.8 Å at pH 3 and 6, respectively (Table II). On the other hand, the R_g of AEDANS-apocytochrome c in the absence of salt at pH 3 was 22.5 ± 0.2 Å. We previously measured the R_g of holocytochrome c at pH 2 in the absence of salt as 24.2 Å (Kataoka et al., 1993), similar to that of AEDANS-apocytochrome c. These results indicate that the unfolded state of apocytochrome c at pH 3, or that of holocytochrome c at pH 2, is compact compared to that in 4 M Gdn-HCl.

DISCUSSION

Among the significant findings of our study is the observation that apocytochrome c, which has been believed to be a typical example of an unfolded protein, is significantly compact at neutral pH, even though lacking in secondary structure, and adopts compact conformations with significant secondary structure under conditions of high ionic strength at both acidic and neutral pH.

Conformation of the A State. At acidic pH, the addition of perchlorate anion to apocytochrome c induces a specific conformational state, the A state, which has a significant amount of helix (about 30% on the basis of the ellipticity at 222 nm), comparable to that of the molten globule of the holoprotein (Table II). Interestingly, the hydrophobic fluorescent groups introduced in place of the heme did not stabilize the intermediate state, but provided a useful probe to monitor the size of the molecule. The fluorescence energy transfer between the tryptophan and AEDANS groups indicated that apocytochrome c becomes compact with increasing perchlorate concentration at acidic pH.

In the structure of native cytochrome c (Bushnell et al., 1990), the three major α-helices, i.e., the N-helix, the 60's helix, and the C-helix, are grouped around one edge of the heme group. Nonpolar side chains provided from these helices in addition to the heme group constitute a major part of the hydrophobic core of cytochrome c. Jeng et al. (1990) indicated that the helices and the hydrophobic core are also preserved in the acidic molten globule state. This suggests that the

Table II: Conformational Properties of Different States of Horse Apocytochrome *c* and Ferricytochrome *c*

state	condition	$-[\theta]$ at 222 nm (deg cm^2 dmol^{-1})	helix content (%)[a]	R_g[b] (Å)	R[c] (Å)
		Apocytochrome *c*			
U_A	pH 3, no salt	3000	2	22.5	26.9
A	pH 2, 0.2 M $NaClO_4$	11000	29	ND[d]	18.7
C	pH 7, no salt	3000	2	ND[d]	21.9
U_{GdnHCl}	pH 3, 4 M Gdn-HCl	3000	2	30.4, 30.0[f]	30.7[e]
		Holocytochrome *c*			
N	pH 7, no salt	11500	30	13.8	ND[d]
U_A	pH 2, no salt	3000	2	24.2	29.5
MG	pH 2, 0.4 M NaCl	11000	29	16.1	17.0
U_{GdnHCl}	pH 7, 4 M Gdn-HCl	2000	0	32.4	33.1

[a] Calculated from $f_H = -([\theta]_{222} + 2340)/30300$ (Chen et al., 1972). [b] The values of apocytochrome *c* were obtained with AEDANS-apocytochrome *c*, and those of holocytochrome *c* were taken from Kataoka et al. (1993). [c] The average distance between Trp-59 and AEDANS groups attached at Cys-14 and Cys-17 for apocytochrome *c* and that between Trp-59 and the heme group for holocytochrome *c*. [d] Not determined. [e] The value was obtained in 6.5 M Gdn-HCl at pH 3. [f] The value was obtained in 4 M Gdn-HCl at pH 6.

nativelike helix-to-heme contacts are critical for stabilization of the molten globule state. However, the present results suggest that the three helices and hydrophobic core can be formed even in the absence of heme, although the structure is much less stable than that of the holoprotein because of the lack of the contributions from the heme group. In accordance with this suggestion, Kuroda (1993) has reported recently that the synthetic N- and C-terminal peptides corresponding to the N-helix and the C-helix, respectively, when connected to each other by a disulfide bond, assume a conformational state with significant helical content [see Wu et al. (1993) for the opposing results].

We assumed a two-state folding mechanism between the U_A and A states and a linear dependence of the free energy of unfolding, ΔG_U, of the A state on the logarithm of perchlorate concentration on the basis of the anion binding-induced transition model (Goto et al., 1990b). Then we calculated the free energy contribution of the heme group in stabilizing the A state. At the C_m (6.5 mM) of the sodium perchlorate-induced transition of cytochrome *c* at pH 2, where the ΔG_U of the A state is 0, the ΔG_U of the A state of apocytochrome *c* was estimated to be –6.5 kJ/mol. On the other hand, at the C_m (61 mM) of apocytochrome *c*, the ΔG_U of the A state of holocytochrome *c* was 9.0 kJ/mol. These provide a measure of the heme contributions in stability of the A state.

We estimated the average distance between tryptophan at position 59 and the AEDANS groups attached to cysteines at positions 14 and 17 on the basis of the theory of Förster (Förster, 1965; Fairclough & Cantor, 1978; Cantor & Schimmel, 1980). The relation between the energy-transfer efficiency, E, and the distance between donor and acceptor, R, is given by

$$R = R_0(E^{-1} - 1)^{1/6} \quad (1)$$

$$E = 1 - F_{DA}/F_D \quad (2)$$

where R_0 is the distance at which the efficiency of energy transfer between donor and acceptor is 50% and F_{DA} and F_D are the fluorescence intensities in the presence and absence of acceptor, respectively. R_0 is calculated by the equation:

$$R_0 = (\kappa^2 J_{DA} Q_D n^{-4})^{1/6}(9.79 \times 10^3 \text{ Å}) \quad (3)$$

where n is the refractive index of the medium between the donor and acceptor, Q_D is the quantum yield of the donor, J_{DA} is the spectral overlap integral of the donor fluorescence and acceptor absorption, and κ^2 is the orientation factor and accounts for the relative orientation of the donor emission and acceptor absorption transition dipole. The overlap integral J_{DA} is calculated with the data of Figures 3 and 4 to be 5.06×10^{-15} cm^3 M^{-1}. Q_D was determined to be 0.058 based on the value of 0.13 for *N*-acetyl-L-tryptophanamide (Chen, 1967). A value of 1.4 was used for n, and a value of $^2/_3$ was used for κ^2 assuming the free rotation of donor and acceptors. By using these values, the R_0 value was calculated to be 21.1 Å. The value of E was found to be 0.095, 0.190, and 0.675 for the unfolded state (6.5 M Gdn-HCl), the acid-unfolded state in the absence of salt at pH 3, and the A state induced by 0.2 M sodium perchlorate, respectively. Thus, the R values were calculated to be 30.7, 26.9, and 18.7 Å, respectively (Table II).

The difference in R values for the acid-unfolded state and the unfolded state in 6.5 M Gdn-HCl is consistent with the difference in R_g values determined by SAXS (Table II). Therefore, both SAXS and fluorescence energy transfer indicate that the acid-unfolded state retains intramolecular interactions which result in a relatively more compact structure than in the denaturant-induced unfolded state. The R values for the acid-unfolded and the molten globule states of holocytochrome *c* at pH 2 were calculated by the same procedure to be 29.5 and 17.0 Å, respectively. The value calculated for R for the A state of apocytochrome *c* is comparable to that of the acidic molten globule state of holocytochrome *c*. Although the precise interpretation of the fluorescence energy-transfer data is difficult because of several assumptions, the results suggest that the compactness of the A state of apocytochrome *c* under acidic conditions is comparable to that of the molten globule state of holocytochrome *c*.

Conformation at Neutral pH. At neutral pH, the A state was significantly destabilized; however, at high concentrations of sodium perchlorate, above 1 M, a compact conformational state with significant secondary structure similar to the A state was stabilized (Figure 2).

On the basis of the CD spectrum, apocytochrome *c* in the absence of salt at neutral pH has negligible secondary structure. Surprisingly, however, the fluorescence spectrum of AEDANS-apocytochrome *c* indicated that the molecule at neutral pH is more compact than that at acidic pH. The R value in the absence of salt at pH 7 was calculated by the same procedure as described above to be 21.9 Å, intermediate between those of the A and U_A states at acidic pH (Table II). The R value in 4 M sodium perchlorate at pH 7 was 20.3 Å, comparable to that of the acidic A state. This means that the perchlorate-induced transition at neutral pH (which leads to the formation of secondary structure) involves only a small decrease in compactness.

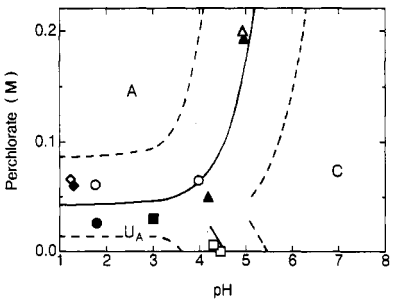

FIGURE 7: pH- and perchlorate-dependent phase diagram for the conformational states of apocytochrome c at 20 °C. The phase diagram consists of the acidic unfolded state (U_A), the acidic intermediate state (A), and the collapsed state at neutral pH (C). The continuous line shows the boundaries between the conformational states determined on the basis of the C_m values of the conformational transitions (Table I). The dashed lines indicate the contour lines corresponding to 80% or 20% progress of the transition. The boundary between the A and the U_A or C states was determined on the basis of CD measurements from the $HClO_4$-induced transition (diamonds), the pH-induced transitions at various concentrations of $NaClO_4$ (triangles), and the $NaClO_4$-induced transitions at various pH values (circles). Open and closed symbols were obtained with apocytochrome c and AEDANS-apocytochrome c, respectively. The boundary between the U_A and C states (open squares) was obtained from the pH-induced transitions of AEDANS-apocytochrome c measured by the fluorescence intensity at 350 nm. The closed square shows the boundary between the U_A and A states obtained from the $NaClO_4$-induced transition of AEDANS-apocytochrome c measured by the fluorescence intensity at 350 nm.

The relation between formation of the secondary structure and collapse of the unfolded molecule is currently an important and controversial topic of protein folding (Baldwin, 1991; Dill, 1990; Alonso et al., 1991; Jeng & Englander, 1991; Kataoka et al., 1993). The present results indicate that whereas, under acidic conditions, formation of the secondary structure of apocytochrome c is coupled with collapse of the molecule, under neutral pH conditions compactness of the molecule proceeds without significant formation of secondary structure, and subsequent formation of secondary structure occurs with relatively little change in compactness.

The equilibrium transition of holocytochrome c from the acid-unfolded state to the molten globule state at pH 2 and 20 °C, induced by either the addition of NaCl or the acetylation of amino groups, is explained by a two-state mechanism (Kataoka et al., 1993), analogous to the case of the perchlorate-induced transition of apocytochrome c at acidic pH.

Phase Diagram of the Conformational Transition. The present results indicate the existence of at least three distinct conformational states of apocytochrome c, i.e., the A state (including the form at high ionic strength, neutral pH), the U_A state, and the relatively compact state without specific secondary structure (the C state) found at neutral pH and low ionic strength. The C state may correspond to a compact (collapsed) denatured state with a hydrophobic core (Baldwin, 1991; Kim & Baldwin, 1990).

Figure 7 shows the pH and perchlorate-dependent phase diagram for the conformational transitions of apocytochrome c. The solid-line boundary between the U_A and C states, and the A state was constructed on the basis of the transitions of apocytochrome c and AEDANS-apocytochrome c, measured by CD at 222 nm. We assumed that the AEDANS groups do not affect the conformational transition, a good assumption since the boundary calculated with AEDANS-apocytochrome c was consistent with that for apocytochrome c. The CD spectrum does not distinguish the U_A and C states. Therefore, the boundary between the U_A and C states was estimated on the basis of the pH-dependent transition of AEDANS-apocytochrome c measured by the fluorescence at 350 nm. As can be seen, the A state of apocytochrome c is stable in the region of low pH and high perchlorate concentration. With increase in pH, the C state is stabilized. In the present phase diagram, however, the relation of the three states around the crossing point (i.e., pH 4 and 0.05 M perchlorate) is not exact.

Goto, Fink and co-workers have reported the phase diagrams of the conformational transition of several acid-denatured proteins and peptides, including β-lactamase (Goto & Fink, 1989), apomyoglobin (Goto & Fink, 1990), basic amphiphilic peptide, consisting of repeats of Lys-Lys-Leu-Leu (Goto & Aimoto, 1991), and melittin (Goto & Hagihara, 1992). For all these cases, the boundary between the unfolded state and the molten globule (or corresponding state) showed a sharp downward curvature with increase in pH, as expected from the decrease in repulsion arising from the protein net charge. Dill and co-workers (Alonso et al., 1991; Stigter et al., 1991) considered the conformation and stability of the denatured state on the basis of the statistical mechanical theory of polyelectrolytes that takes into account the hydrophobic interaction, the chain conformational entropy, and electrostatic effects. Their theory also predicts the downward curvature of the phase boundary between the compact and expanded denatured states, consistent with the previous experimental results.

The stabilization of the relatively compact structure with no specific secondary structure (the C state) with increase in pH means that the collapse to a compact conformation and formation of the secondary structure are independent properties in the case of the conformational transition of apocytochrome c.

The conformation of the denatured protein is determined by a subtle balance of various factors favoring unfolding or folding (Alonso et al., 1991; Goto & Nishikiori, 1991). Although the charge repulsion arising from the protein net charge is an important factor determining the conformation of denatured proteins, the present results indicate that the decrease in net charge does not necessarily stabilize the molten globule state, even though the molecule becomes compact. The cooperative transition at pH around 4 suggests the involvement of carboxyl groups. Deprotonation of carboxyl groups may produce local charge repulsion between negative groups which destabilizes the specific secondary structure. In the case of holocytochrome c, the hydrophobicity of the heme group and the ligation of iron to Met and His residues overcome such unfavorable effects, resulting in the stabilization of the native structure at neutral pH. We are trying to dissect the role of the heme group into contributions from the hydrophobic effect and those arising from the ligation by studying the structure and stability of porphyrin cytochrome c.

Conclusion. We have shown that apocytochrome c is unfolded at low ionic strength at acidic pH, but can assume an intermediate state, similar to the molten globule state of holocytochrome c, in the presence of perchlorate anion. Interestingly, the secondary structure of the intermediate state was substantially less stable at neutral pH relative to acidic pH. At low ionic strength at neutral pH, apocytochrome c exists as a compact state without significant secondary or tertiary structure. This compact state may reflect the hydrophobic collapsed state, and is distinct from the molten globule state, which has specific secondary structure. The results show that the effects of electrostatics on the conformation of the denatured state of apocytochrome c are more complicated than those approximated on the basis of the net charge.

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