Mechanism of Acid-Induced Folding of Proteins†

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ABSTRACT: We have previously shown [Goto, Y., Calciano, L. J., & Fink, A. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 573-577] that β-lactamase, cytochrome *c*, and apomyoglobin are maximally unfolded at pH 2 under conditions of low ionic strength, but a further decrease in pH, by increasing the concentration of HCl, refolds the proteins to the A state with properties similar to those of a molten globule state. To understand the mechanism of acid-induced refolding of protein structure, we studied the effects of various strong acids and their neutral salts on the acid-unfolded states of ferricytochrome *c* and apomyoglobin. The conformational transition of cytochrome *c* was monitored at 20 °C by using changes in the far-UV CD and in the Soret absorption at 394 nm, and that of apomyoglobin was monitored by changes in the far-UV CD. Various strong acids (i.e., sulfuric acid, perchloric acid, nitric acid, trichloroacetic acid, and trifluoroacetic acid) refolded the acid-unfolded cytochrome *c* and apomyoglobin to the A states as was the case with HCl. For both proteins neutral salts of these acids caused similar conformational transitions, confirming that the anions are responsible for bringing about the transition. The order of effectiveness of anions was shown to be ferricyanide > ferrocyanide > sulfate > trichloroacetate > thiocyanate > perchlorate > iodide > nitrate > trifluoroacetate > bromide > chloride. This series is similar to the electroselectivity series of anions toward the anion-exchange resins [Gregor, H. P., Belle, J., & Marcus, R. A. (1955) *J. Am. Chem. Soc. 77*, 2713-2719; Gjerde, D. T., Schmuchler, G., & Fritz, J. S. (1980) *J. Chromatogr. 187*, 35-45], showing that preferential binding of anions to the A states causes the conformational transitions.

Acid-induced unfolding of proteins is often incomplete, and the acid-unfolded proteins assume conformations that are different from the fully unfolded ones observed in the presence of high concentration of Gdn-HCl[1] (Tanford, 1968). A molten globule conformation under acidic conditions has been reported for several proteins, and the role of such conformation in the mechanism and pathway of protein folding has been discussed (Ohgushi & Wada, 1983; Kuwajima, 1989; Ptitsyn, 1987; Goto & Fink, 1989). However, the mechanism of acid-induced unfolding and the generality of the molten globule conformation under such conditions are unknown.

We have recently shown (Goto et al., 1990) that β-lactamase, cytochrome *c*, and apomyoglobin are unfolded extensively by HCl at around pH 2 under conditions of low ionic strength (the U_A state) and that further decrease in pH due to increasing HCl concentration induces the refolding of the proteins to a structure (the A state) with properties similar to those of the molten globule conformation reported for other proteins. The addition of KCl at pH 2 to the U_A states of these proteins induced similar conformational transitions to the HCl-induced transition, indicating that the chloride anion plays a key role in the acid- or salt-induced transitions. Characteristic properties of the A states are a high content of secondary structure, a compact structure compared to that of the unfolded state, buried tryptophan residues, and largely disordered tertiary structure. In the case of β-lactamase, ANS binding specific to the A state was observed, indicating partially exposed hydrophobic regions (Goto & Fink, 1989).

Because all pK_a values of ionizable groups of the unfolded proteins are above 3 (Tanford, 1968), these groups are already maximally protonated in the U_A state at pH 2. In principle, therefore, a further decrease in pH by the addition of acid will have no effect on the ionization state. On the other hand, the increase in the concentration of the anion introduces three effects which may affect the protein conformation. Provided that the electrostatic repulsion of the positive charges on the protein molecule is the major force of unfolding at pH 2 under the conditions of no salt, anions reduce the repulsion by (1) the Debye-Hückel screening effect and (2) the interaction with positive charges by ion-pair formation (or anion binding). These may result in the manifestation of the intrinsic hydrophobic (or solvophobic) interactions of proteins which favor folding. (3) In addition to these electrostatic effects, anions are known to affect the water structure, and this consequently results in an increase or decrease in the hydrophobic interactions of proteins.

If the first factor (Debye-Hückel screening) has the major contribution, the effects of various ions will be determined by the ionic strength of the solution. If the third factor (the effect on water structure) is important, the effectiveness of various ions should follow the Hofmeister series (Collins & Washabaugh, 1985). The importance of the second factor (anion binding) can be determined by comparing the effectiveness of various anions with the electroselectivity series of the anions toward anion-exchange resins (Gregor et al., 1955; Gjerde et al., 1980). The electroselectivity series is different from the Hofmeister series.

We examined the effects of various acids and salts on the conformational transitions of cytochrome *c* and apomyoglobin. In the case of cytochrome *c*, we could examine the effects of more than 10 anions by using the spectral change in the Soret absorption around 400 nm. The results clearly show the im-

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[1] Abbreviations: ANS, 1-anilino-8-naphthalenesulfonate; CD, circular dichroism; Gdn-HCl, guanidine hydrochloride; TCAH, trichloroacetic acid; TCANa, sodium trichloroacetate; TFAH, trifluoroacetic acid; TFANa, sodium trifluoroacetate.

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portance of the anion binding.

Materials and Methods

Materials. Horse cytochrome *c* (type VI) was purchased from Sigma. The cytochrome *c* was more than 98% in the ferri form. If this protein sample was treated with potassium ferricyanide to oxidize the heme completely, we were unable to obtain the maximal unfolding of cytochrome *c* at pH 2 in the absence of salt even if the protein solution had been dialyzed extensively against water to remove the reagent. We found later that the ferricyanide interacts very strongly with acid-unfolded cytochrome *c* at micromolar concentrations. Therefore, to avoid any contamination of ferricyanide, which prevents the formation of U_A state, we used the cytochrome *c* as purchased without the treatment with ferricyanide.

Horse myoglobin (type I) was purchased from Sigma. Apomyoglobin was prepared by 2-butanone extraction of the heme (Hapner et al., 1968). The content of the holoprotein was less than 1% judging from a ratio of absorption at 280 and 410 nm.

Other chemicals were of reagent grade and were used without further purification.

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

CD measurements were carried out with a Jasco spectropolarimeter, Model J-500A, equipped with a DP-501 data processor, or an AVIV spectropolarimeter, Model 60DS. The instruments were calibrated with *d*-10-camphorsulfonic acid. The results are expressed as mean residue ellipticity $[\theta]$, which is defined as $[\theta] = 100\theta_{obsd}/lc$, where θ_{obsd} 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 CD spectra were measured at protein concentrations of 5 μM with a 1 mm path length cell from 250 to 195 nm.

Absorption Measurements. Soret absorption spectra of cytochrome *c* were measured with a Jasco spectrophotometer, Model Ubest-50, at protein concentrations of 5 μM with 1 or 0.5 cm path length cells.

The protein concentration was determined spectrophotometrically. Extinction coefficients used to calculate the concentration of native ferricytochrome *c* at 410 nm, native myoglobin at 409 nm, and native apomyoglobin at 280 nm were 1.06×10^5 (Babul & Stellwagen, 1972), 1.6×10^5 (Crumpton & Polson, 1965), and 1.43×10^4 M^{-1} cm^{-1} (Crumpton & Polson, 1965), respectively. The numbers of amino acid residues in cytochrome *c* and apomyoglobin are 104 and 153, respectively.

Conformational Transitions. Cytochrome *c* or apomyoglobin, dialyzed against deionized water, was mixed with the acid or salt solutions. The acid or salt solutions were prepared before the measurements and used as soon as possible. Acid- or salt-induced conformational transitions below pH 2 were rapid (complete within a few minutes), and the measurements were carried out soon after the preparation of the solution. In the measurements of apomyoglobin with TCAH, TCANa, $K_3Fe(CN)_6$, and $K_4Fe(CN)_6$ at high concentrations of acid or salt, where the cooperative transitions are almost over, the CD intensity sometimes decreased gradually with time during the measurements (10–20 min). In such cases, we extrapolated the signal to time zero to estimate the initial value. At much higher concentrations of such acids and salts, solutions often became turbid with time, indicating that the decreases in signal are due to aggregation of the proteins.

Since we used protein solutions containing high concentrations of acid, up to 0.9 M HCl, the stability of the covalent structure of the proteins was checked by HPLC with a C18 reverse-phase column, in which the proteins were eluted with a gradient of acetonitrile in 0.1% TFAH. Cytochrome *c* was intact for at least 24 h under the conditions of 0.9 M HCl at 20 °C. Apomyoglobin was degraded slowly, with a half-life of 40 h in 0.9 M HCl at 20 °C. This shows that the degradation during the spectroscopic measurements was negligible.

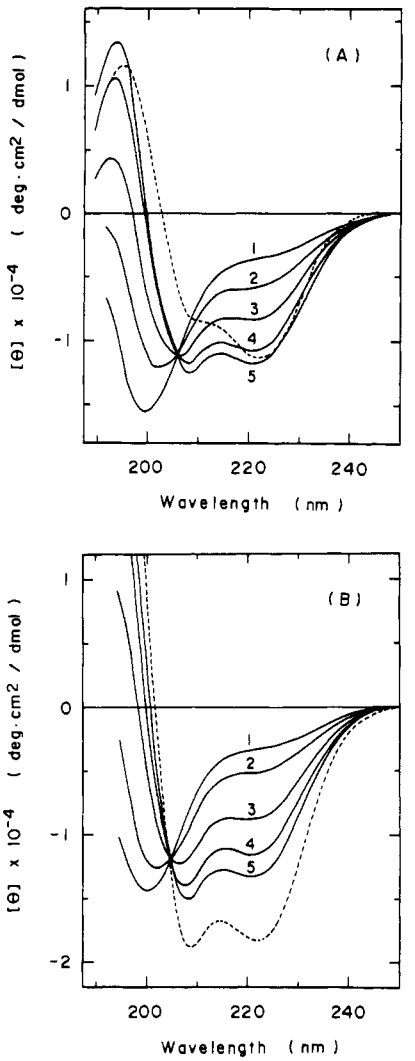


FIGURE 1: Far-UV CD spectra of cytochrome *c* (A) and apomyoglobin (B) as a function of perchloric acid concentration at 20 °C. The solutions of cytochrome *c* and apomyoglobin contained 18 and 9 mM HCl, respectively, to induce the acid-unfolded state. $HClO_4$ concentration and pH: (A) 1, 0 mM, pH 1.82; 2, 4.5 mM, pH 1.70; 3, 9 mM, pH 1.61; 4, 18 mM, pH 1.49; 5, 45 mM, pH 1.24: (B) 1, 0 mM, pH 2.11; 2, 6.75 mM, pH 1.86; 3, 13.5 mM, pH 1.69; 4, 22.5 mM, pH 1.54; 5, 90 mM, pH 1.05. The spectra of the native state (dotted lines) in 25 mM phosphate buffer at pH 7.0 are shown for comparison. Protein concentrations were 5 μM.

pH Measurements. pH was measured with a Radiometer PHM26c at 25 °C.

Results

CD Spectra. Panels A and B of Figure 1 show the effect on the far-UV CD of adding perchloric acid to the HCl-unfolded cytochrome *c* and apomyoglobin, respectively. To examine the effects of strong acids other than HCl, it was necessary to add HCl to induce the U_A state before the perchloric acid was added. When the HCl was not added, the unfolding by 10–20 mM perchloric acid (pH 1.7–2.0) was incomplete for both proteins, suggesting a partial formation of the A state because of the high tendency of perchlorate, compared to chloride, to induce the A state.

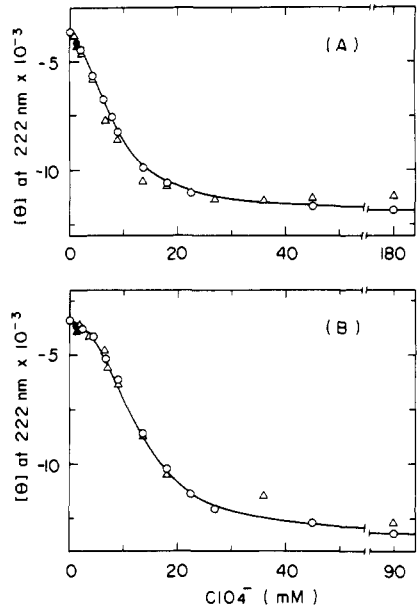


FIGURE 2: Dependence on perchlorate anion concentration of the ellipticity at 222 nm of cytochrome *c* (A) and apomyoglobin (B) at 20 °C. The transitions were induced by perchloric acid (circles) or sodium perchlorate (triangles). The solutions of cytochrome *c* and apomyoglobin contained 18 and 9 mM HCl, respectively. The sodium perchlorate induced transition of cytochrome *c* was measured at pH 1.7–1.8 and that of apomyoglobin was measured at pH 2.0–2.1. Solid symbols are for the solutions in which the perchlorate concentration was first set to 50 mM, kept for 1 h, and then diluted to the final concentration. The lines are calculated curves of the perchloric acid induced transitions with eq 10 and the parameters in Tables I and II.

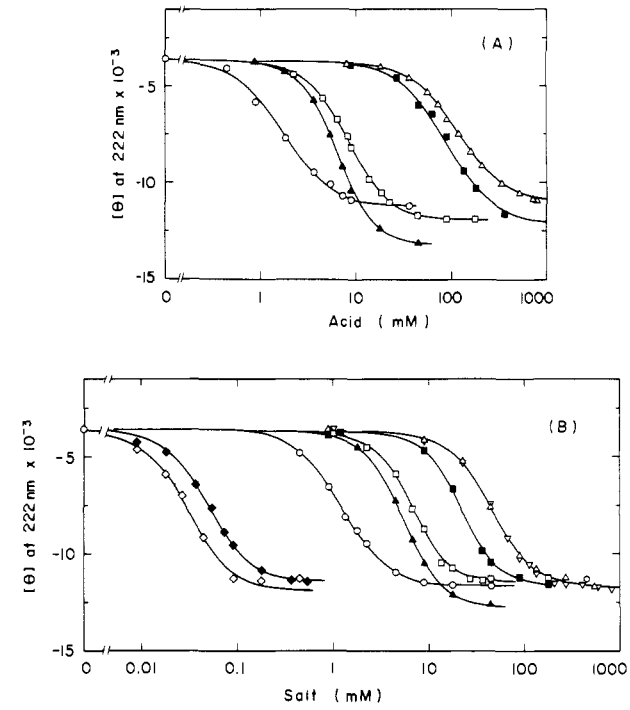


FIGURE 3: Acid-induced (A) or salt-induced (B) conformational transitions of cytochrome *c* in the presence of 18 mM HCl measured by the ellipticity at 222 nm at 20 °C. Acids used were HCl (△), TFAH (■), perchloric acid (□), TCAH (▲), and sulfuric acid (○). Salts were NaCl (△), KCl (▽), TFANa (■), sodium perchlorate (□), TCANa (▲), sodium sulfate (○), potassium ferrocyanide (◆), and potassium ferricyanide (◇). The HCl-induced transition was plotted after subtraction of the 18 mM HCl component. Salt-induced transitions were measured at pH 1.7–1.9. The lines are calculated with eq 10 and the parameters in Table I. The calculated line of the KCl-induced transition is not shown.

As was the case with HCl, perchloric acid induced the refolding of the cytochrome *c* and apomyoglobin. For both proteins, the addition of perchloric acid changed the CD spectra of the U_A state, which has a minimum at 200 nm, to the spectra of the A state, with minima at 208 and 222 nm, representing the formation of α-helical conformation. The CD spectra at different concentrations of perchloric acid show an isosbestic point at 206 nm for cytochrome *c* and at 205 nm for apomyoglobin. The minimum at 222 nm of the A state of cytochrome *c* induced by perchloric acid has an ellipticity value similar to the minimum of the native protein at pH 7, but the minimum at 208 nm has a more negative value than that of the native protein. In the case of apomyoglobin, the values of ellipticity at 222 and 208 nm of the A state are smaller than those of the native protein at pH 7 (Goto et al., 1990). The helical contents of the U_A state, the A state induced by perchloric acid, and the native state of cytochrome *c* are 4, 31, and 30%, respectively, on the basis of the ellipticity values at 222 nm and the method of Chen et al. (1972). Those of apomyoglobin are 3, 35, and 53%, respectively. While the helical content of the A state of cytochrome *c* formed by perchloric acid was similar to that of the A state formed by HCl, that of apomyoglobin was a little larger than the helical content of the A state formed by HCl (see below; Figures 3 and 6).

Panels A and B of Figure 2 show the perchloric acid induced U_A to A transitions of cytochrome *c* and apomyoglobin, respectively, measured by the ellipticity at 222 nm. The perchlorate-induced transitions were reversible provided the concentration of perchlorate to induce the A state was not sufficiently high so as to cause precipitation. The transitions were cooperative in both cases.

Figure 2 also shows that the conformational transitions of cytochrome *c* and apomyoglobin, induced by sodium perchlorate at pH 2, coincide with the transitions induced by perchloric acid. For both proteins, the CD spectra of the A states induced by sodium perchlorate were similar to the corresponding spectra induced by perchloric acid, although, in the case of cytochrome *c*, the ellipticity at 208 nm of the A state induced by acid was about 10% larger than that induced by salt, indicating some difference between the two conformations. A corresponding small difference was also observed between the A state of cytochrome *c* induced by HCl and the A state induced by KCl at pH 1.8 (Goto et al., 1990). These results show that it is the perchlorate anion which is responsible for the U_A to A state transitions.

Effects of Various Anions on Acid-Unfolded Cytochrome c. Panels A and B of Figure 3 show the effects of various acids and salts, respectively, on the U_A state of cytochrome *c* measured by the ellipticity at 222 nm. The acids examined were HCl, TFAH, perchloric acid, TCAH, and sulfuric acid. The salts examined were the corresponding sodium salts of the acids as well as KCl, potassium ferricyanide, and potassium ferrocyanide. As shown in Figure 3B, we found very strong effects of ferricyanide and ferrocyanide. These anions were also very effective in inducing the U_A- to A-state transition of apomyoglobin (see below), indicating that the effects are not specific to cytochrome *c*.

As can be seen from Figure 3, all of the acids and salts induced cooperative transitions to the U_A state. The concentration range of acid or salt required to bring about the transition, however, varied greatly among different acids and salts. The transition induced by NaCl is the same as the transition induced by KCl. The values of ellipticity at high concentrations of acids or salts were similar, indicating that

Table I: Acid- or Salt-Induced U_A- to A-State Transition of Cytochrome *c* at 20 °C[a]

acid or salt	C_m[b] (mM)	Δn[c]	K_b[d]
acid			
H_2SO_4	1.7 (1.6)	1.8 (1.9)	1.3×10^4 (1.1×10^4)
TCAH	6.3 (6.6)	2.4 (2.6)	1.4×10^3 (1.2×10^3)
$HClO_4$	8.1 (7.3)	2.1 (2.1)	1.8×10^3 (1.8×10^3)
HNO_3	nd (29)	nd (2.1)	nd (4.6×10^2)
TFAH	84 (90)	1.8 (2.1)	2.6×10^2 (1.6×10^2)
HCl	114 (138)	1.8 (1.8)	1.9×10^2 (1.5×10^2)
salt			
$K_3Fe(CN)_6$	0.030 (0.028)	1.9 (2.2)	6.0×10^5 (4.4×10^5)
$K_4Fe(CN)_6$	0.048 (0.041)	2.0 (1.8)	3.2×10^5 (5.3×10^5)
Na_2SO_4	1.2 (1.7)	1.9 (1.9)	1.6×10^4 (1.2×10^4)
TCANa	5.5 (4.9)	2.2 (2.7)	2.2×10^3 (1.4×10^3)
NaSCN	nd (5.6)	nd (2.1)	nd (2.4×10^3)
$NaClO_4$	6.6 (6.5)	2.4 (2.3)	1.4×10^3 (1.7×10^3)
NaI	nd (13)	nd (2.3)	nd (8.9×10^2)
$NaNO_3$	nd (22)	nd (2.3)	nd (4.5×10^2)
TFANa	22 (25)	2.5 (2.0)	4.0×10^2 (6.2×10^2)
NaBr	nd (31)	nd (2.3)	nd (3.5×10^2)
NaCl	45 (45)	2.1 (2.0)	3.3×10^2 (3.5×10^2)
KCl	47 (48)	2.0 (2.1)	3.3×10^2 (3.0×10^2)

[a] Transitions were measured in the presence of 18 mM HCl by using the change in ellipticity at 222 nm. The values in parentheses were obtained by using change in absorption at 394 nm. The HCl-induced transition was analyzed after subtraction of 18 mM HCl. nd, not determined. [b] Midpoint concentration of transition. [c] Preferential binding parameter of anion obtained by eq 7. [d] Binding constant of anions obtained by eq 10 using the values of Δn in this table and the value K_1 = 0.0035.

the nature of the A state does not depend much on the acid or salt species. As can be seen from Table I, the values of the midpoint concentration (C_m) for the transitions induced by sulfuric acid, TCAH, and perchloric acid are similar to the values of C_m of the corresponding salt-induced transitions. On the other hand, the values of the C_m for the transitions induced by TFAH and HCl are a little larger than the values of C_m of the corresponding salt-induced transitions.

The heme absorption of cytochrome *c* in the vicinity of 400 nm reflects the spin state of the iron and, as a result, depends on the conformational state of the protein (Figure 4). While the native protein (low spin state) has a maximum at 410 nm with an extinction coefficient of 1.06×10^5 M^{-1} cm^{-1}, the U_A state (high spin state) has a maximum at 394 nm with an extinction coefficient of 1.90×10^5 M^{-1} cm^{-1}. As shown in Figure 4A, on going from the U_A state to the A state by the addition of HCl, the maximal wavelength increased with an accompanying decrease in intensity. The spectra at different concentrations of acids showed an isosbestic point at 400 nm. The A state induced by HCl had a maximum at 397 nm with extinction coefficient of 1.2×10^5 M^{-1} cm^{-1}. The change in the absorption spectrum observed in the transition induced by KCl at pH 1.8 is shown in Figure 4B. The A state induced by KCl at pH 1.8 showed a spectrum with a maximum at 400 nm and an extinction coefficient of 1.2×10^5 M^{-1} cm^{-1}. The different absorption maximum also suggests some difference between the A state induced by acid and that induced by salt at pH 1.8.

Because the absorption coefficients of most of the acids and salts are negligible or small at 400 nm, we can study the effects of acids and salts that were difficult to use in the far-UV CD measurements owing to their high absorption.

Panels A and B of Figure 5 show the conformational transitions from U_A to A for cytochrome *c* induced by acids and salts, respectively, measured by the change in absorption at 394 nm. In the absorption measurements, the effects of bromide, nitrate, iodide, and thiocyanate ions could be examined in addition to the effects of the ions used for the CD measurements. The transitions measured by the absorption change at 394 nm were consistent with the transitions measured by CD at 222 nm (Table I).

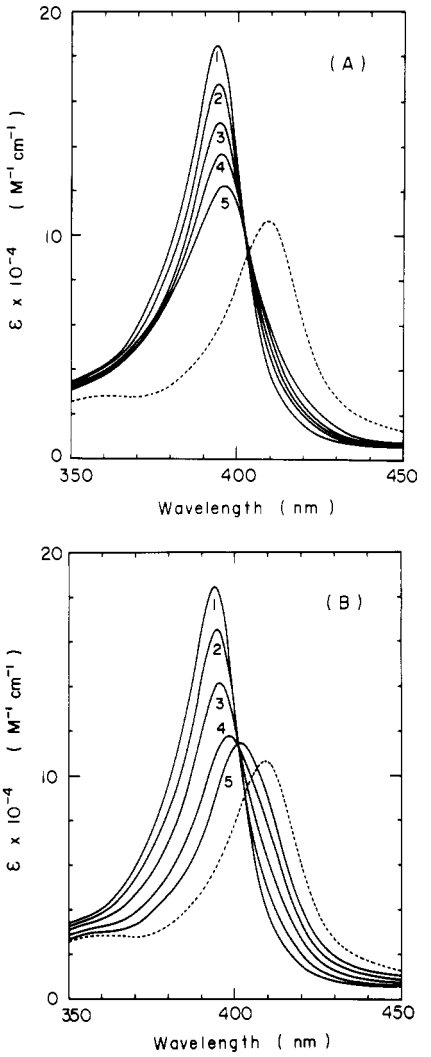


FIGURE 4: Soret absorption spectra of cytochrome *c* as a function of HCl concentration (A) or KCl concentration in the presence of 18 mM HCl at pH 1.8 (B), at 20 °C. (A) HCl concentration and pH: 1, 18 mM, pH 1.85; 2, 135 mM, pH 0.95; 3, 180 mM, pH 0.86; 4, 270 mM, pH 0.67; 5, 540 mM, pH 0.36. (B) KCl concentration: 1, 0 mM; 2, 22.5 mM; 3, 45 mM; 4, 90 mM; 5, 540 mM. Dotted lines show the spectrum of the native state in 25 mM phosphate buffer at pH 7. Protein concentrations were 5 μM.

From these results, the effectiveness of various ions to stabilize the A state of cytochrome *c* at acidic pH follows the series

ferricyanide > ferrocyanide > sulfate > trichloroacetate > thiocyanate > perchlorate > iodide > nitrate > trifluoroacetate > bromide > chloride (1)

Effects of Various Ions on Acid-Unfolded Apomyoglobin. Panels A and B of Figure 6 show the refolding transitions of apomyoglobin induced by various acids and salts, respectively, measured by the change in ellipticity at 222 nm. As can be seen, the transitions were cooperative, and the values of C_m of the acid-induced transitions agreed well with those of the corresponding salt-induced transitions (Table II). In contrast with cytochrome *c*, where the difference between the CD spectra of the A states induced by various anions was small, the values of ellipticity at 222 nm of the A states of apomyoglobin induced by trichloroacetate, trifluoroacetate, and

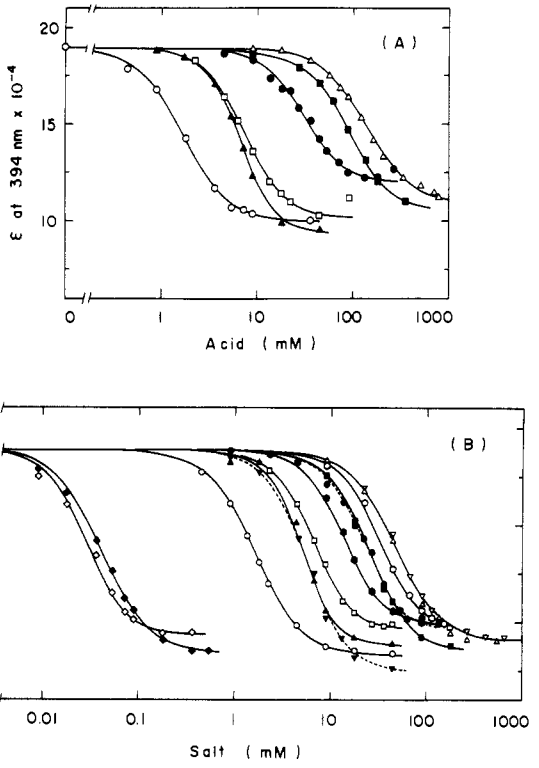


FIGURE 5: Anion-induced transitions of cytochrome *c* measured by change in absorption at 394 nm at 20 °C. The transitions were induced by various acids (A) or salts (B) in the presence of 18 mM HCl. In addition to the acids and salts shown in Figure 3, the effects of HNO_3 (●), NaBr (○), $NaNO_3$ (●), NaI (●), and NaSCN (▼) were examined. The HCl-induced transition was plotted after subtraction of the 18 mM HCl component. Salt-induced transitions were measured at pH 1.7–1.9. The lines are calculated with eq 10 and the parameters in Table I.

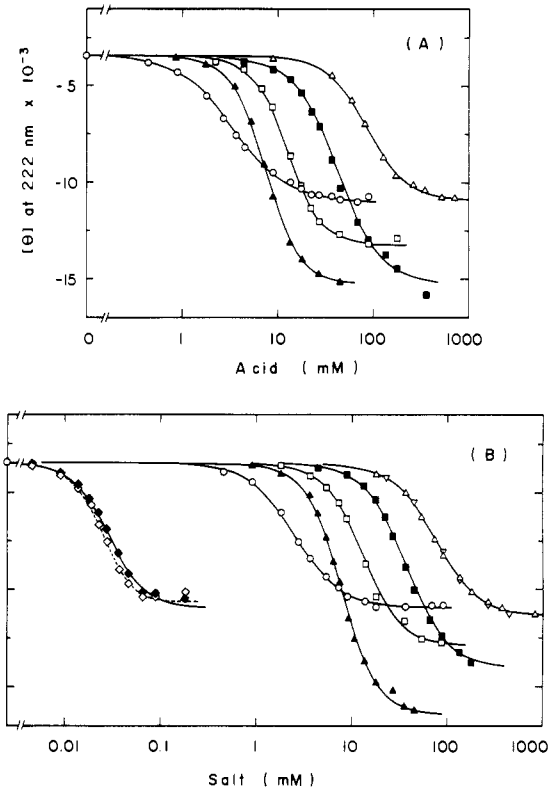


FIGURE 6: Anion-induced transitions of apomyoglobin measured by change in ellipticity at 222 nm at 20 °C. The transitions were induced by acids (A) and salts (B) in the presence of 9 mM HCl. The symbols are the same as shown in Figure 3. Salt-induced transitions were measured at pH 2.0–2.2. The lines were calculated with eq 10 and the parameters in Table II.

Table II: Acid- or Salt-Induced U_A- to A-State Transition of Apomyoglobin at 20 °C[a]

acid or salt	C_m[b] (mM)	Δn[c]	K_b[d]
acid			
H_2SO_4	3.5	1.6	1.7×10^4
TCAH	7.3	2.5	1.5×10^3
$HClO_4$	12	2.4	1.0×10^3
TFAH	41	2.0	5.7×10^2
HCl	85	2.2	2.1×10^2
salt			
$K_3Fe(CN)_6$	0.025	2.6	4.1×10^5
$K_4Fe(CN)_6$	0.029	2.4	4.5×10^5
Na_2SO_4	2.5	1.8	1.3×10^4
TCANa	7.7	2.5	1.5×10^3
$NaClO_4$	13	2.3	1.2×10^3
TFANa	38	2.1	5.7×10^2
NaCl	73	2.0	3.3×10^2
KCl	75	2.0	3.0×10^2

[a] Transitions were measured in the presence of 9 mM HCl by using the change in ellipticity at 222 nm. The HCl-induced transition was analyzed after subtraction of 9 mM HCl. [b] Midpoint concentration of transition. [c] Preferential binding parameter of anion obtained by eq 7. [d] Binding constant of anions obtained by eq 10 using the values of Δn in this table and the value $K_1 = 0.0017$.

perchlorate were larger than those of the A states induced by other anions. The order of effectiveness of various anions to induce the A state of apomyoglobin is, however, consistent with the series observed with cytochrome *c* (eq 1).

Discussion

Role of Anions in the Conformational Transitions. We considered three factors that might be responsible for the anion-induced transitions: (1) the Debye–Hückel screening effect of anions, (2) anion binding to the proteins, and (3) the effect of anions of water structure. The results show that the transitions induced by monovalent anions are dependent on the anion species. This is inconsistent with the role of the Debye–Hückel screening effect of anions as a major factor.

It is known that the effects of anions under neutral conditions in stabilizing protein structure follow the Hofmeister series (von Hippel & Schleich, 1969). The representative series is

sulfate > phosphate > fluoride > chloride > bromide > iodide > perchlorate > thiocyanate (2)

The anions to the left of chloride in eq 2 are called kosmotropic and stabilize the native structure of proteins (Collins & Washabaugh, 1985). The anions located to the right of chloride are called chaotropic or lyotropic and destabilize the native structure. The exact mechanism of stabilization or destabilization of protein native structure by these salts is unknown, but the results are consistent with the idea that these salts affect the water structure (Collins & Washabaugh, 1985; Washabaugh & Collins, 1986). While a kosmotropic ion (water structure maker) interacts with surrounding water molecules more strongly than would bulk water in its place, a chaotropic ion (water structure breaker) interacts with water molecules less strongly than would bulk water in its place. The former causes the preferential exclusion of the kosmotropes from protein surface (or preferential hydration of protein) (Arakawa & Timasheff, 1982), and the latter causes the preferential interaction of the chaotropes with proteins. While the former is unfavorable and, consequently, the protein minimizes its surface area by folding, the latter is favorable and proteins maximize their surface by unfolding. If the anion-induced stabilization of the A state results from the

changes in water structure, the effects of various anions should follow the Hofmeister series.

Both eqs 1 and 2 represent the relative effectiveness of anions in stabilizing folded structure of proteins. The series in eq 1 is, however, different from the Hofmeister series shown in eq 2. Only the strong effect of sulfate is consistent between the two series, and the order of the monovalent anions in inducing the U_A to A transition is, in fact, opposite to the order of the monovalent anions in the Hofmeister series.

Interactions between anions and anion-exchange resins have been examined extensively in various systems (Gregor et al., 1955; Small et al., 1975; Gjerde et al., 1980; Barron & Fritz, 1984). The affinity of a particular anion to the resin relative to a standard anion, which is often chloride, is called selectivity (or electroselectivity). Although the selectivity series of various anions depends critically on the structure of the resin and the solution conditions, the general trend of selectivity can be seen from the following examples of selectivity series:

sulfite > sulfate > perchlorate > thiocyanate > iodide > nitrate > bromide > chloride > acetate = fluoride (Gjerde et al., 1980) (3)

perchlorate > iodide > trichloroacetate = thiocyanate > nitrate > bromide > trifluoroacetate > chloride > acetate > fluoride (Gregor et al., 1955) (4)

Equation 3 represents the retention time of divalent (sulfite, sulfate) and monovalent anions from an anion-exchange column with trimethylamino groups in the presence of 0.1 mM phthalate at pH 6.25 [the data were taken from Table I of Gjerde et al. (1980)]. Equation 4 comes from the selectivity of monovalent anions to a benzyl(hydroxyethyl)dimethylammonium anion-exchange resin (Dowex 2) [the data were taken from Figure 8 of Gregor et al. (1955)].

The selectivity data for ferricyanide and ferrocyanide are not available. As can be seen, however, the series of anions in eq 1 is generally consistent with the series in eq 3 or 4. This suggested that the anion binding to the positively charged groups of proteins is the major factor responsible for the anion-induced formation of the A state. Some anions are effective at very low concentrations; C_m values are about 2 mM for sulfate and 30–40 μM for ferricyanide and ferrocyanide (Tables I and II). This also suggests the importance of effects of anion binding and not effects on water structure, because stabilization of the protein structure by the latter requires moderately high concentration of salt (von Hippel & Schleich, 1969; Goto et al., 1988).

The selectivity of anions to anion-exchange resins is predominantly determined by the valency, or net charge, of the anions. The anion-exchange resins usually prefer the anion with higher valency to the anion with lower valency if other conditions are equal: trivalent > divalent > monovalent. The monovalent acids used in this work are strong acids with pK_a values less than 1, and thus the net charge of the anions at pH 2 is effectively −1.0. The second pK_a of sulfuric acid is 2.0, and the net charge of sulfate ion at pH 2 is −1.5. For both cytochrome *c* and apomyoglobin, the C_m of ferrocyanide is a little larger than the C_m of ferricyanide (Tables I and II). These results at first seemed inconsistent with the valences. However, while all three pK_a values of ferricyanide are less than 1 (Jordan & Ewing, 1962), two pK_a values of ferrocyanide are less than 1 and the others are 2.3 and 4.3 (Hanania et al., 1967). As a result, the net charges of ferricyanide and ferrocyanide at pH 2 are −3 and −2.3, respectively. The position of ferricyanide and ferrocyanide in eq 1 is therefore consistent with the idea that the major determinant of order is the net charge of the anion.

The selectivity constant of monovalent anions varies substantially and is related to the hydrated volume of the anions (Gregor et al., 1955). Because the Hofmeister series reflects the tendency of anions to interact with nearby water molecules (Collins & Washabaugh, 1985), the electroselectivity series of monovalent anions has some relation to the Hofmeister series. Chaotropic anions, which interact less favorably with surrounding water, have higher affinity for positively charged resins because of their smaller effective size. On the other hand, the monovalent kosmotropic anions, which increase the water structure by interacting with nearby water molecules, have lower affinity to the resins because of their larger size. The sulfate anion is heavily hydrated, and its hydrated size is large judging from the elution position in gel chromatography with a Sephadex G-10 column (Washabaugh & Collins, 1986). In the case of the electroselectivity series, however, the net charge of anions is more important than hydration, and the electroselectivity of multivalent anions is generally higher than that of monovalent anions.

Thus, we can conclude from the above considerations that the electrostatic binding of anions to the positively charged sites of proteins is the major factor responsible for the anion-induced conformational transitions.

Conformational Transition and Coordination of Iron of Cytochrome c. The A state of cytochrome *c* induced by acid is the same state as the intermediate state of unfolding observed by others (Babul & Stellwagen, 1972; Stellwagen & Babul, 1975; Dyson & Beattie, 1982; Ohgushi & Wada, 1983). According to Dyson and Beattie (1982), the conformational states of cytochrome *c* are characterized by the coordination and the spin state of the iron in the heme. While the native state (state III in their classification) is low spin with both Met-80 and His-18 coordinated to the iron, the U_A state (their state I) is high spin with neither Met-80 nor His-18 coordinated. The intermediate state (their state II) has His-18 coordinated and, with respect to the coordination of Met-80, is a mixture of two forms, a low-spin form with Met-80 coordinated and a high-spin form with Met-80 not coordinated.

The transition from native to intermediate state accompanies the protonation of an ionizable group with a pK_a of 2.5 in 0.01 M chloride and is relatively insensitive to the salt conditions. In contrast, the second transition from the intermediate to unfolded state, which requires the protonation of His-18, is highly dependent on the salt conditions. While the pK_a of His-18 in 0.01 M KCl is 2.5, the pK_a in 0.1 M chloride is 1.4 (Dyson & Beattie, 1982). Owing to this strong dependence of pK_a of His-18 on salt, the intermediate state is stable even at pH 0.0 in 3.0 M chloride (Stellwagen & Babul, 1975).

Provided that the A state of cytochrome *c* induced by HCl is the same state as the unfolding intermediate reported by others, HCl-induced refolding of the U_A state of cytochrome *c* could be explained on the basis of the very large decrease in pK_a of His-18 due to the addition of chloride. Thus, although the increase in the concentration of protons favors protonation of His-18, the simultaneous increase in the chloride concentration greatly decreases the pK_a of His-18. Consequently, the addition of HCl to U_A, with His-18 already protonated, causes the deprotonation of His-18 and the folding to the A state. This rather unusual phenomenon occurs due to the very strong efficacy of chloride to induce refolding: the effects of chloride, which favor folding, easily compensate for the effects of protons, which favor unfolding.

Stellwagen and Babul (1975) suggested that the chloride ion stabilizes the A state by ligation between iron(III) and a

cationic amino group located close to heme. However, there is no evidence to support this interpretation. Aviram (1973), in a study of the interaction of several monovalent chaotropic anions with acid-unfolded cytochrome *c*, concluded that disordering of the water structure at low pH favored the native-like conformation of cytochrome *c*. Such an interpretation is inconsistent with the very strong effects of sulfate observed here.

An important point of our results is that the acid-induced refolding is not a phenomenon specific to cytochrome *c* but is common to apomyoglobin, β-lactamase (Goto et al., 1990), and several non-heme proteins (L. J. Calciano, A. L. Fink, Y. Goto, and D. Palleros, unpublished results).

Preferential Anion Binding to the A State. If the binding of the anion (X) to the protein shifts the equilibrium between the two conformations, the U_A and A states, the observable equilibrium constant K_{app} is expressed in terms of the total concentrations of U_A and A states in all their forms U_A, U_AX, U_AX_2, ..., A, AX, AX_2, ...

$$K_{app} = \text{total A}/\text{total U}_A = \sum_{i=0}^{m_A}(AX_i)/\sum_{i=0}^{m_U}(UX_i) \quad (5)$$

where m_A and m_U represent the maximum number of moles of X that may be bound per mole of protein in the two conformations. If all binding sites of the two states are independent, the K_{app} is

$$K_{app} = K_1\sum_{j=1}^{m_A}(1 + K_{j,A}a_X)/\sum_{j=1}^{m_U}(1 + K_{j,U}a_X) \quad (6)$$

where K_1 is the equilibrium constant in the absence of the ligand, $K_{j,A}$ and $K_{j,U}$ are the intrinsic binding constant at each individual site, and a_X is the activity of the ligand (Tanford, 1970). When anions bind preferentially to the A state, the difference in the number of anions bound between the A and U_A states, Δn, is

$$\Delta n = n_A - n_U = \text{d ln } K_{app}/\text{d ln } (a_x) \quad (7)$$

where n_A and n_U are the number of anions bound to the A and U_A states, respectively (Wyman, 1964; Tanford, 1970; Schellman, 1975; Record et al., 1978).

The values of K_{app} were calculated from

$$K_{app} = (Y - Y_U)/(Y_A - Y) \quad (8)$$

where Y is the observed value of the signal, Y_U and Y_A are the corresponding values for the U_A and A states, respectively. Figure 7 shows plots of log K_{app} against log [anion] for cytochrome *c* and apomyoglobin. It should be noted that in the analysis we used the concentration of anions instead of the activity because it is difficult to obtain the activity of the respective anions. The values presented here are thus the apparent values obtained by using molarity.

The value of Δn varies slightly for the ions used, ranging from 1.8 to 2.7 for cytochrome *c* (Table I) and from 1.6 to 2.6 for apomyoglobin (Table II). There is no significant correlation between the value of Δn and the valency of the anions or the values of C_m. The results indicate that, under the transition regions, for both cytochrome *c* and apomyoglobin, 2–3 mol of anion are preferentially bound to the A state relative to the U_A state.

At pH 2, the net charges of cytochrome *c* and apomyoglobin are +24 and +33, respectively. Because the binding depends on the electrostatic interactions of anions with positively charged proteins, both the U_A and A states should have many binding sites for anions. The analysis based on eq 7 indicates,

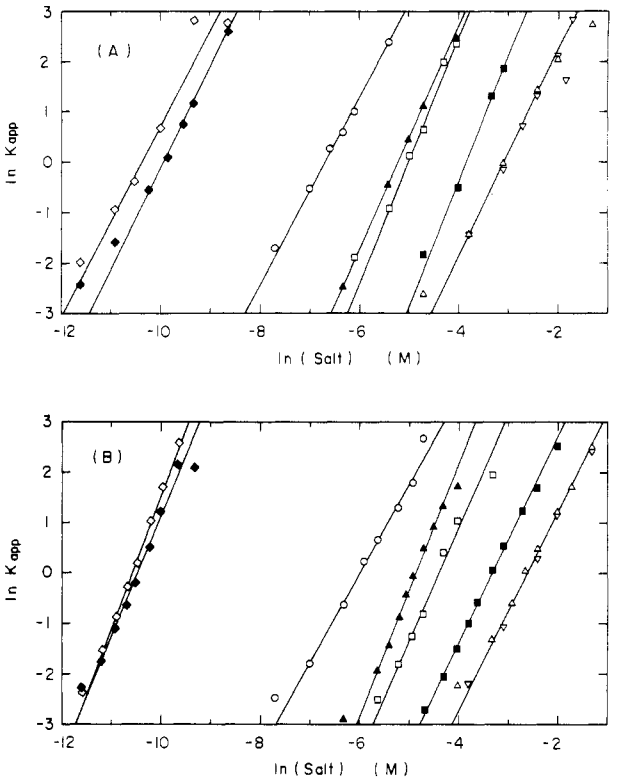
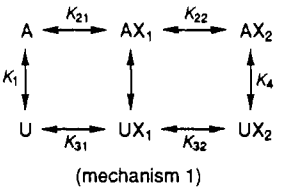


FIGURE 7: Plot of ln K_{app} as a function of ln [anion] according to eq 7 of the conformational transitions of cytochrome *c* (A) and apomyoglobin (B). Transitions were induced by salts and were measured by the ellipticity at 222 nm (Figure 3B for cytochrome *c* and Figure 6B for apomyoglobin). The symbols are the same as shown in Figure 3.

however, the values of Δn are much smaller than the net charges of the proteins, which probably are the maximum values of binding sites (m_A and m_U) for monovalent anions. As a first approximation, we assume the simplest case in which only those Δn sites are responsible for the anion-induced transitions. The mechanism for a case in which $\Delta n = 2$ is represented as



(mechanism 1)

Equation 6 becomes

$$K_{app} = K_1(1 + K_{21}a_X)(1 + K_{22}a_X)/(1 + K_{31}a_X)(1 + K_{32}a_x) \quad (9)$$

The mechanism is analogous to the mechanism of acid denaturation of proteins (Tanford, 1970), in which only a small number of acid-titratable groups of the total many titratable groups are responsible for the unfolding transitions. The acid denaturation is induced by higher affinity of protons to the titratable groups in the unfolded state compared to the affinity of protons to the same group in the native state, i.e., an increase in pK_a upon unfolding. While the proton binding induces the unfolding of protein structure in the acid denaturation, the anion binding to the acid-unfolded proteins in the present case induces the refolding of the protein structure.

In the extreme case where the binding constants of the Δn sites of the A state are the same (K_b) and are much larger than those of the U_A state, the K_{app} is further approximated.

$$K_{app} = K_1(1 + K_b a_X)^{\Delta n} \quad (10)$$

We assumed that K_1 is independent of the anion species and estimated the values of K_b and K_1 which give the fit to all the data from the plot of log K_{app} against log $(1 + K_b[\text{anion}])$ at the constant value of Δn obtained by eq 7. In view of the approximation made, we did not try to get the integral number from Δn. The value of K_1 obtained was 0.0035 for cytochrome *c* and 0.002 for apomyoglobin. These correspond to free energies of 3.3 and 3.6 kcal/mol at 20 °C, respectively. Table I shows the values of K_b for cytochrome *c*, and Table II shows those for apomyoglobin. The order of values of the binding constant is generally consistent with the series of C_m. The lines in Figures 2, 3, 5, and 6 are made by using eq 10 and the parameters shown in Tables I and II.

On the basis of mechanism 1, we can explain the anion-induced U_A- to A-state transition in two ways. Because formation of the A state with bound anions from the U_A state without anions is a thermodynamic process, there are two pathways accounting for it. One consists of the initial folding to the A state and the subsequent anion binding. The alternative pathway consists of the initial binding of 2 mol of anion to the U_A state and the subsequent folding to the A state. The entire equilibrium is independent of the pathway:

$$K_1K_{21}K_{22} = K_{31}K_{32}K_4 \quad (11)$$

Folding of the positively charged U_A state to the A state is an energetically difficult process because of the repulsion of the positive charges. As the protein molecule adopts the A state, the molecule becomes much more compact than the U_A state, and thus the charge density on the molecule becomes higher than that of the U_A state. This results in stronger interactions of anions with the A state compared to those with the U_A state ($K_{21}K_{22} \gg K_{31}K_{32}$). Thus, with the increase in the concentration of anion, the unfolded proteins are transformed into the A states.

From eq 11, the assumption of $K_{21}K_{22} \gg K_{31}K_{32}$ simultaneously assumes $K_4 \gg K_1$. This means that the free energy of folding of the U_A state to the A state decreases due to the anion binding. In other words, the shielding of intramolecular charge–charge repulsive forces in the U_A state by the anion binding manifests the intrinsic forces which favor the formation of the A state.

Salt-Induced Conformational Transitions of Synthetic Polypeptides. There are many reports on the effects of salts on the conformational transitions of synthetic polypeptides (Rifkind, 1969; Conio et al., 1974; Ebert & Ebert, 1977; Ebert et al., 1978; Ebert & Kuroyanagi, 1982a,b). Ebert and Kuroyanagi (1982a) studied the effects of salt on the conformation of statistical copolymers of L-Leu and L-Lys. The copolymers with 50 mol % L-Leu did not form α-helices in water at pH 7 at 20 °C because of the repulsive forces between positive charges on Lys residues. However, sulfate or perchlorate, at concentrations as low as 3 mM, induced α-helix formation with little dependence on cation. The order of effectiveness of several anions was determined to be sulfate > perchlorate > chloride > fluoride. This order is consistent with our results.

Interestingly, sulfate does not stabilize the α-helix of basic poly(amino acids) such as poly(L-Lys) (Ebert & Ebert, 1977; Ebert et al., 1978). The statistical copolymers examined by Ebert and Kuroyanagi contain hydrophobic L-Leu residues. This suggests the involvement of hydrophobic interactions in the salt-induced stabilization of the α-helices.

Ebert and Kuroyanagi (1982b) also examined the salt effects on an alternating copolymer of L-Leu and L-Lys. In contrast to the statistical copolymer of L-Leu and L-Lys, the alternating copolymer of L-Leu and L-Lys undergoes a conformational transition from a coil to an intramolecular β-sheet structure by the addition of salts. The effectiveness of various salts is also consistent with our results.

Conclusion. Salt-dependent conformational transitions under acidic conditions have been observed for several proteins (Arakawa et al., 1987; Goto & Fink, 1989; Goto et al., 1990; Ikeguchi & Sugai, 1989). The present results show that anion binding is responsible for the conformational transitions. Very similar salt dependence has been reported for conformational transitions of synthetic polypeptides consisting of both hydrophobic and hydrophilic amino acid residues, and this suggests that the molten globule state is similar to the salt-induced conformational states of such copolymers. It is possible that such electrostatic interactions and consequent conformational changes may play an important role in the function of some proteins.

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A Dynamic Quaternary Structure of Bovine α-Crystallin As Indicated from Intermolecular Exchange of Subunits†

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ABSTRACT: The structural bovine eye lens protein α-crystallin was dissociated in 7 M urea and its four subunits, A_1, A_2, B_1, and B_2, were separated by means of ion-exchange chromatography. Homopolymeric reaggregates of these subunits were prepared by removal of the denaturant via dialysis. It was found that subunits were exchanged upon incubation of mixtures of two homopolymers under native conditions. New hybrid species were formed within 24 h as demonstrated by isoelectric focusing. Moreover, native α-crystallin molecules also exchanged subunits when incubated with homopolymeric aggregates of B_2 subunits. Subunit exchange between native α-crystallin molecules is postulated, and a "dynamic quaternary structure" is presented that allows the polydisperse protein to adapt to changes in cytoplasmic conditions upon aging of the lens tissue.

Calf lens α-crystallin is a multisubunit protein composed of about 40 A- and B-type 20 000-Da subunits that occur in an average ratio of about 3:1 (Siezen et al., 1978). The primary gene products A_2 and B_2 are nearly 60% identical in primary structure (Van der Ouderaa et al., 1973, 1974), and hence they are likely to have closely similar secondary and tertiary structures. The A_1 and B_1 subunits are formed from A_2 and B_2, respectively, by a phosphorylation step (Spector et al., 1985; Voorter et al., 1986; Chiesa et al., 1987a-c). Many studies have dealt with the quaternary assembly of α-crystallin. A model for the architecture of the polydisperse molecules has been proposed with 14 ± 2, 13 ± 3, and 15 ± 2 subunits in three concentric layers (Bindels et al., 1979; Siezen et al., 1980). However, in the past few years controversy has arisen about the molecular weight of native α-crystallin. Thomson and Augusteyn (1983, 1984) described an α-crystallin of 320 000 Da, isolated at 37 °C. Hydrodynamic studies and symmetry considerations led to dodecameric models of α-crystallin, either as a protein with an intermediate tetrahedral shell organization of its subunits or as a micelle-type aggregate (Thomson, 1985; Augusteyn & Koretz, 1987). On the other hand, others presented further evidence in favor of a molecular mass of about 800 000 Da (Van den Oetelaar et al., 1985; Tardieu et al., 1986). The latter authors presented an extended version of the three-layer model, characterized by tetrahedral symmetry and 12, 24, and 24 sites occupied in the first, second, and third layer, respectively.

The subunits of α-crystallin are very prone to form aggregates. In fact, single subunits only exist in concentrated solutions of denaturing agents, and they aggregate upon dilution of these agents or after their removal by dialysis. The ratio of the subunits is not critical in order to form reaggregates. Not only dissociated native α-crystallin (Bloemendal et al., 1962; Li & Spector, 1973; Siezen & Bindels, 1982; De Block et al., 1986; Tardieu et al., 1986) but also isolated A and B subunits mixed in various ratios could be reassociated (Van Kamp et al., 1974; Bindels, 1982; Thomson & Augusteyn, 1989). Moreover, it was possible to form homopolymers by reassociation of purified subunits (Li & Spector, 1972, 1973, 1974; Bindels, 1982; Thomson, 1985; Thomson & Augusteyn, 1989). Reaggregated α-crystallins and these homopolymers are smaller than the native α-crystallin and generally do not exceed about 20-22 subunits (Bloemendal et al., 1975; Siezen et al., 1978; Siezen & Berger, 1978; Thomson & Augusteyn,

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