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Acid-induced folding of proteins

(acid denaturation/molten globule/anion binding/protein folding)

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ABSTRACT **The addition of HCl, at low ionic strength, to the native state of apomyoglobin, β-lactamase, and cytochrome *c* caused these proteins to adopt an essentially fully unfolded conformation in the vicinity of pH 2. However, contrary to expectation, the addition of further acid resulted in refolding to a compact conformation with the properties of a molten globule. The major factor responsible for the refolding is believed to be the binding of the anion, which minimizes the intramolecular charge repulsion that initially brought about the unfolding.**

One of the oldest known methods of denaturing proteins is by the addition of acids. Reports on the conformational state of the denatured protein have varied from apparently fully unfolded to substantial remaining structure. We have recently reported that β-lactamase I from *Bacillus cereus* adopts a relatively fully unfolded conformational state at pH 2 at low ionic strength but on the addition of salts converts to a compact, molten globule-like state (1). We now show for several proteins that, if one starts with a native protein at low ionic strength and lowers the pH by adding HCl, the protein will initially change to a relatively fully unfolded conformation, typically in the vicinity of pH 2, and then, as the acid concentration increases, it will change into a compact conformation containing substantial secondary structure.

We use the following experimental criteria to define the protein conformational states expected in the low pH region. (*i*) The native state is characterized by its near-UV and far-UV circular dichroism (CD) spectra as determined at pH 7, a tryptophan fluorescence emission spectrum with λ_{max} as determined at pH 7 (usually in the vicinity of 335 nm), and the Stokes radius as determined at pH 7 using dynamic light scattering or gel-exclusion chromatography. (*ii*) The acid-unfolded state, U_A, has a CD spectrum similar to that of the protein unfolded in 5 or 6 M guanidine hydrochloride (Gdn·HCl) at neutral pH, a tryptophan fluorescence λ_{max} around 350 nm, and a Stokes radius ≥2 times that of the native state (for proteins without disulfide bonds) (1). (*iii*) State A is a compact state (as determined from its hydrodynamic radius) with substantial secondary structure (as determined by far-UV CD) but little or no native-like tertiary structure (as determined by near-UV CD), and tryptophan fluorescence emission with a λ_{max} similar to that of the native state. State A has the properties ascribed to a compact state that has been called a molten globule (1–3). We believe that this species, as described above, may be considered to consist of an overall fold similar to that of the native state in which structural elements, typically secondary structural units, have been pulled apart somewhat, leading to some solvent penetration and enhanced side-chain mobility. In some cases (e.g., apomyoglobin, discussed below), part of the molecule (e.g., the C-terminal region) may be fully unfolded.

MATERIALS AND METHODS

Materials. β-Lactamase I from *B. cereus* was prepared as described (1). Apomyoglobin was prepared from horse myoglobin (Sigma) by 2-butanone extraction of the heme (4). The content of the holoprotein remaining was <1%. Horse cytochrome *c* (Sigma) was >98% in the oxidized form. Protein concentrations were determined from molar extinction coefficients.

Methods. CD spectra were recorded on an Aviv model 60DS instrument at 20°C. Either 1.0- or 0.2-mm pathlength cells were used for far-UV measurements, and a 10.0-mm path length was used for the near-UV region. Tryptophan fluorescence was measured at 20°C with a Perkin–Elmer MPF 4 instrument with excitation at 280 nm. pH measurements were made with a microcombination glass electrode (Microelectrodes, Londonderry, NH; model MI-410). Hydrodynamic radii were determined by molecular exclusion chromatography on Superose 12 with a Pharmacia FPLC apparatus. The Stokes radius was determined by the procedure of Corbett and Roche (5). Protein sample concentrations were 2 μM. No difference in elution volume for the A state was detected over the concentration range 0.5–2 μM when apomyoglobin was used.

RESULTS AND DISCUSSION

Acid Denaturation Monitored by CD. Fig. 1*A* shows the effect on the far-UV CD of adding HCl (in the absence of other ions) to β-lactamase. At relatively low concentrations of acid (corresponding to pH 1.5–2) the spectrum resembles that of the Gdn·HCl unfolded state and indicates the presence of the acid unfolded state U_A. As the acid concentration increases, and the pH decreases, the spectrum changes, becoming more and more similar to that of the native state. The CD spectrum in the aromatic region (Fig. 2*A*) at high concentrations of acid was essentially featureless, indicating the absence of native-like tertiary structure. Both the far- and near-UV CD spectra at high concentrations of HCl resemble those of the A state, the molten globule-like state previously obtained at low pH and high salt concentration (1). Although the far-UV CD spectra for native and A state β-lactamase are very similar, this does not mean that the secondary structures are identical, only that there are equivalent amounts of secondary structure.

Similar results were obtained with horse apomyoglobin (Fig. 1*B*) and horse cytochrome *c* (Fig. 1*C*). Both proteins were maximally unfolded by HCl at pH 2 in the absence of salt, although some differences between the CD spectra of the U_A states and the Gdn·HCl unfolded states suggest the possibility of some residual structure in the U_A states. The addition of higher HCl concentrations induced refolding to the corresponding A states. Similar conformations were induced by the addition of KCl at pH 2 (Fig. 1). In the case

Abbreviation: Gdn·HCl, guanidine hydrochloride.

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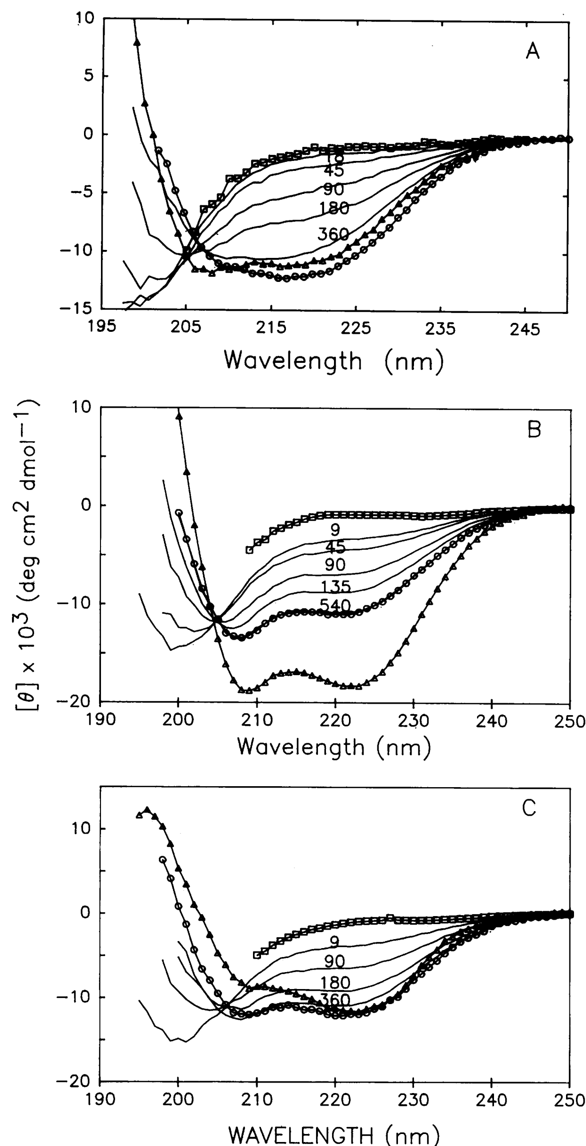

FIG. 1. Far-UV CD spectra of β-lactamase (*A*), apomyoglobin (*B*), and ferricytochrome *c* (*C*) as a function of HCl concentration. Protein concentrations were 10 μM. The numbers refer to the HCl concentration in millimolar units. The spectra of the native state (△), the A state induced by KCl (○) (10–20 mM HCl/0.45 M KCl, pH ≈2), and the Gdn·HCl-unfolded state (□) (4–5 M Gdn·HCl/25 mM sodium phosphate buffer, pH 7.0) are shown for comparison. Measurements were made soon after preparation of the solutions. The solutions at high [HCl] (0.5–0.9 M) were stable for at least 2 hr.

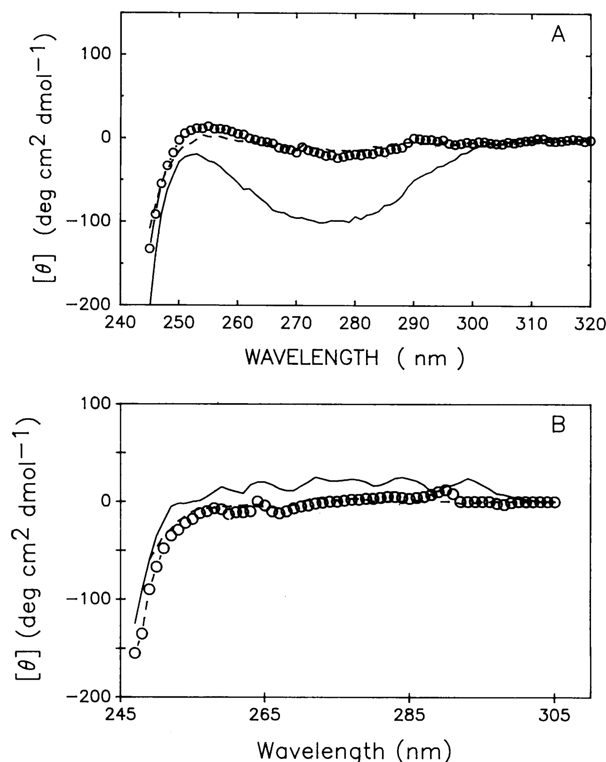

FIG. 2. Near-UV CD spectra of β-lactamase (*A*) and apomyoglobin (*B*) in the native state (solid line), acid-unfolded state (broken line), and A state (○). Conditions are as described in Fig. 1 legend. The Gdn·HCl-unfolded states (not shown) had spectra similar to the acid-unfolded states.

of apomyoglobin the amount of secondary structure (α-helix) in the A state was ≈65% of that in the native state. The A state of cytochrome *c* had comparable amounts of ordered secondary structure to the native state. The aromatic region (260–300 nm) of the CD spectrum of the acid-induced A state for apomyoglobin was similar to that of the corresponding U_A state and indicated the loss of native-like tertiary structure (Fig. 2*B*). In the case of cytochrome *c*, the heme CD contributed significantly to the CD spectrum in the near-UV region for N, U_A, and A states or the unfolded state induced by 4 M Gdn·HCl at pH 2. However, the sharp peaks with minima at 283 and 290 nm from tryptophan and/or tyrosine were observed only for the native state, indicating loss of much of the native-like tertiary structure in the other states.

Anion-Induced Cooperative Transitions Between U_A and A. As shown in Figs. 3 and 4, the transitions between the U_A and A states are cooperative. A comparison of the effect of chloride ion on the U_A to A transition of apomyoglobin and β-lactamase with either HCl or KCl (at pH 2) shows identical results (Fig. 3 *A* and *B*), indicating that it is the chloride ion that is responsible for the transition (the possibility that it is an ionic strength effect was ruled out from investigations with a variety of other ions) (Y.G., N. Takahashi, and A.L.F., unpublished data). In the case of cytochrome *c*, however, the transition induced by KCl at pH 2 is more cooperative than that induced by HCl (Fig. 3*C*). This may reflect specific effects of acid involving the heme iron coordination (see discussion below).

The N → U_A → A transitions for apomyoglobin are illustrated in Fig. 4. As shown in Fig. 4*A*, monitoring the transition by tryptophan fluorescence suggests that the two tryptophan residues are buried in the interior of the protein in the A state, in an environment of comparable hydrophobicity to that in the native state. A similar λ_{max} was observed for the A state induced by KCl at pH 2. In cytochrome *c* the tryptophan fluorescence is quenched in the native state by energy transfer to the heme. The quenching is lost on unfolding to the U_A state but occurs again on going to the A state (formed either by acid or salt), indicating that in the A state the tryptophan and heme are again in close proximity. The fluorescence properties for the A state of β-lactamase are similar to those of apomyoglobin, in that the tryptophan environment is hydrophobic, and the emission intensity is quenched (1).

The A State Is Compact. The compact nature of the A states induced by KCl at pH 2 was confirmed by molecular exclusion chromatography. Table 1 shows the hydrodynamic data for the N, U, and A states. The A states for cytochrome *c* and β-lactamase are quite compact relative to the unfolded state but are somewhat expanded compared to the native state. A correlation between the amount of secondary structure and compactness is observed. The hydrodynamic radius of the A

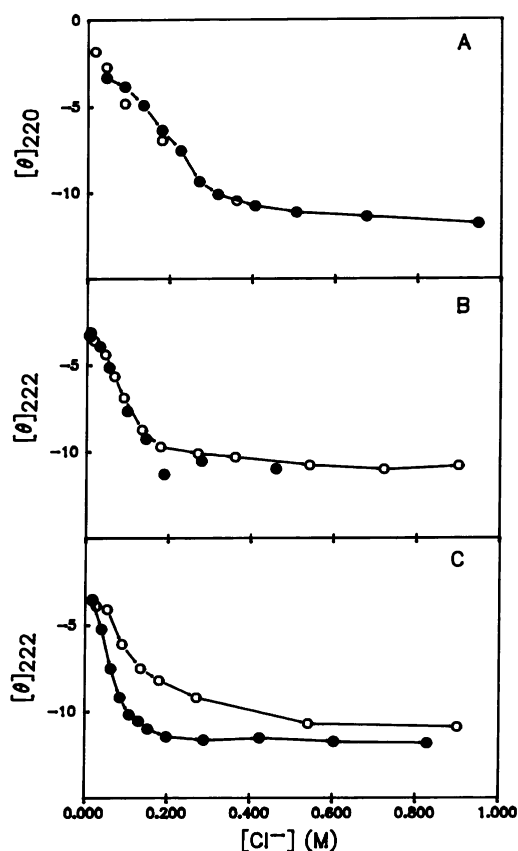

FIG. 3. HCl-induced (○) and KCl-induced (●) transitions of β-lactamase (A), apomyoglobin (B), and cytochrome c (C) between the acid-unfolded state, U_A, and the molten globule-like state, A, as monitored by change in the far-UV CD at 220 nm (A) or 222 nm (B and C). The units for $[\theta]$ are 10^{-3} deg·cm^2·dmol^{-1}. The KCl-induced transitions were measured at pH 1.6 for β-lactamase, and at pH 2.0 for apomyoglobin and cytochrome c. The pH was adjusted with HCl. Conditions for the spectral measurements were as described in Fig. 1.

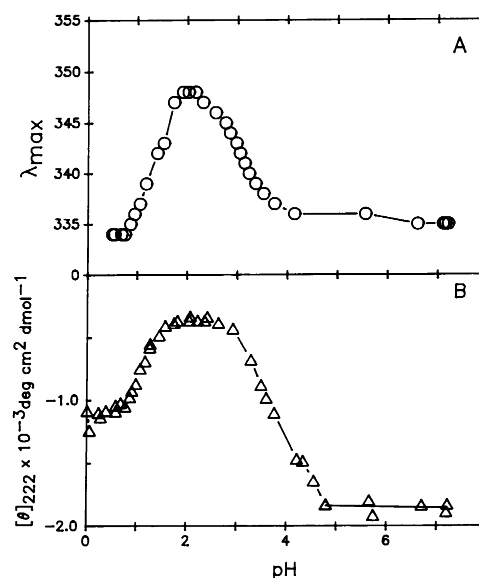

FIG. 4. Effects of increasing concentrations of HCl on protein conformation: the N → U_A → A transition. The transition of apomyoglobin was monitored by tryptophan fluorescence (A) and CD ellipticity at 222 nm (B). Conditions for the CD experiments were as described in Fig. 1.

states is increased by 10–20% for β-lactamase and cytochrome c, and by 60% for apomyoglobin, relative to the native state. The latter figure is consistent with the CD data, indicating that the A state for apomyoglobin has much less native-like character than that for the other A states discussed. We interpret this to mean that the A state for apomyoglobin is a hybrid state in which the core of the molecule is present as a molten globule but either or both of the C and N termini are unfolded.

Unfolding and Refolding Can Be Accounted for in Terms of Charge Repulsion and Anion Binding. We interpret our observations as follows. On decreasing the pH from neutrality to approximately pH 2 the protein becomes maximally positively charged, since the pK of most carboxyls is ≥3. The net charge for β-lactamase, apomyoglobin, and cytochrome c will be +36, +33, and +27, respectively. The resulting intramolecular repulsion between the positively charged groups leads to unfolding and a relatively extended conformation, state U_A [in some cases with a greater hydrodynamic radius than in the random coil (1, 6)]. The addition of more strong acid adds both protons and anions to the solution. Because the protein is already maximally protonated, the addition of more protons has, in principle, no effect on its ionization state. In the case of cytochrome c His-18 has a very low pK, and its ionization state will be considered below. However, the addition of more anions leads to electrostatic interactions with the positively charged centers on the protein (binding of the anions), so as to effectively shield the repulsive forces (Y.G., N. Takahashi, and A.L.F., unpublished data). This decreases the internal repulsive forces, which favor unfolding, and consequently the intrinsic hydrophobic (or solvophobic) interactions of proteins in an aqueous environment manifest themselves. These result in the protein folding up to adopt a compact structure with decreased hydrophobic surface area. Furthermore, hydrophobic forces favoring folding may be increased due to the anion's effect on water structure (6). From examination of the effects of various anions, we find that anions with higher electroselectivity (affinity) for anion-exchange resins (e.g., sulfate, perchlorate) are more effective in inducing the U_A → A transition than anions with lower affinity (e.g., chloride, bromide), indicating that anion binding is the major factor determining the conformational transition (Y.G., N. Takahashi, and A.L.F., unpublished data).

The large net positive charge on the protein below pH 3 minimizes both intramolecular interactions that lead to unfolding and intermolecular interactions (aggregation). The abolition of the electrostatic repulsive force by the binding of the anions leads not only to a decrease in the intramolecular repulsion causing folding to a compact structure, but also to minimization of the intermolecular repulsive forces and pro-

Table 1. Hydrodynamic radii for native, unfolded, and molten globule conformational states

Protein	State	K_d	R_s, Å	Relative volume
Apomyoglobin	N	0.50	21.5	1.0
	U	0.27	43	8.0
	A	0.36	35	4.3
Cytochrome c	N	0.54	17	1.0
	U	0.34	34	8.0
	A	0.51	20	1.6
β-Lactamase	N	0.46	24	1.0
	U	0.21	51	9.6
	A	0.48	26.5	1.4

The data were determined by gel-exclusion chromatography on Superose 12 using the experimental protocol and analysis of Corbett and Roche (5), where K_d is the partition coefficient and R_s represents the Stokes radius. The conditions were as follows: for the native state (N), pH 7.0 and 0.15 M KCl; for the unfolded state (U), pH 2.0 and 5 M Gdn·HCl; for the molten globule state (A), pH 2.0 and 0.5 M KCl.

duces the observed propensity for aggregation of the A state at high protein concentrations (typically ≥1 mg/ml).

Conformational State and Heme Coordination of Cytochrome *c*. There have been several previous reports on the conformational state of cytochrome *c* under acidic conditions (e.g., see refs. 3, 7–11). The acid unfolding occurs with an intermediate state (state II in ref. 11), which may coincide to the A state discussed here. The coordination and spin states of the heme are as follows (11): In the native state the iron (III) is low spin, with both Met-80 and His-18 coordinated. The intermediate state (presumably state A) is a mixture of two forms, with His-18 coordinated to the iron in both, and Met-80 coordinated in a low-spin form and not coordinated in a high-spin form. It is generally believed that the dissociation of His-18 from the iron occurs with accompanying protonation of the imidazole of His-18, and that this process is coupled with the global unfolding of cytochrome *c*. The transition between the native and intermediate state requires the partial dissociation of Met-80 from the iron (III) and is relatively insensitive to the ionic conditions (11). On the other hand, the transition from the intermediate state to the unfolded state (U_A), which requires the protonation of His-18, is very dependent on the ionic strength. The pK of His-18 is 2.5 in 0.01 M Cl^- and is 1.4 in 0.1 M Cl^- (11). The potential for anions to induce refolding of cytochrome *c* at low pH has previously been noted (10).

Assuming that the A state of cytochrome *c* induced by HCl is the same as the previously reported intermediate state, the HCl-induced refolding of the U_A state could be explained on the basis of the large decrease in the pK of His-18 on the addition of chloride. Thus, although the increase in acidity favors protonation of His-18, the simultaneous increase in Cl^- decreases the pK of His-18. Thus, the difference observed between the effect of KCl and HCl on the refolding of the U_A state of cytochrome *c* in Fig. 3 could be accounted for by the addition of HCl to the protonated histidine in U_A leading to deprotonation and refolding to the A state due to the very strong effect of chloride on inducing folding and overcoming the effect of protonation, which favors unfolding.

In spite of the major importance of the effects of chloride ion, the exact mechanism of how the anion stabilizes the A state is unclear. An important point to note is that the HCl-induced refolding is not a phenomenon specific to heme proteins but is common to several quite different proteins. Thus the effect of chloride on unfolded cytochrome *c* is not necessarily related to the heme coordination. Based on the results with the other proteins it is clear that electrostatic interaction of the anion with the positively charged unfolded protein is important in the transition to the molten globule state.

Formation of the A State Is Quite Common. As will be discussed elsewhere, we have also observed the $U_A \rightarrow A$ transition with other acids. In many cases, it was necessary to initially add 5–20 mM HCl to induce the acid-unfolded state prior to adding the acid. When the HCl was not added, the protein went directly from the native state to the A state, with little formation of the U_A state. This is a reflection of the comparative effectiveness of HCl in bringing about the $N \rightarrow U_A$ transition and of other acids in bringing about the $U_A \rightarrow A$ transition.

In preliminary experiments, we have found that several other monomeric globular proteins exhibit the same type of folding behavior at low pH as that for the three proteins discussed here. These include staphylococcal nuclease, parvalbumin, subtilisin (both Carlsberg and BPN′), and myoglobin. The amount of ordered secondary structure in the molten globule-like state varies significantly from one protein to another. A similar salt-dependent conformational transition at acidic pH has been reported recently for γ-interferon (12). For some proteins, especially those that are heavily cross-linked by disulfide bridges, the intramolecular charge–charge repulsion is insufficient to cause complete unfolding in the range of pH 2 in the absence of salt. In these cases, the protein goes directly from the N state to the A state. One such example is bovine α-lactalbumin. In other cases, the situation is a mixture of the above; e.g., for RNase A the protein is only partially unfolded by HCl at pH 2, and further addition of HCl leads to the A state (L.J.C. and A.L.F., unpublished observations).

Several reports over the past 20 years have indicated that acid-denatured proteins possess some residual structure, rather than being fully unfolded as observed in 5 or 6 M Gdn·HCl (13–16). However, the details and widespread generality of the acid-unfolding phenomena have not been well characterized until now. For the three proteins examined here, commonly used conditions for pH-induced unfolding—e.g., pH around 2, ionic strength around 0.1—would yield mixtures of the U_A and A states (cf. Fig. 3) with a substantial amount of the molten globule state and thus have significant amounts of secondary structure and be more compact than the fully unfolded conformation. As shown in Figs. 1 and 4, however, under similar conditions of pH but in the absence of salt, these proteins are much more unfolded—i.e., the U_A state. The nature of the acid used is very important, since other anions are significantly more effective than chloride in inducing the A state (Y.G., N. Takahashi, and A.L.F., unpublished data).

Conclusions. There are a number of important implications from this work. First, to obtain maximal unfolding of a protein by acid denaturation, one should use HCl in the absence of any added salt or buffer and a pH of around 2. Many other acids, including sulfuric, involve anions that are very effective at promoting the $U_A \rightarrow A$ transition and thus lead directly to the A state even at pH 2. Second, as clearly illustrated in Fig. 3, a further decrease in the pH by the addition of more acid will produce a compact, molten globule-like state with significant structure. Third, the presence of low concentrations of salts at acidic pH will prevent formation of the U_A state and lead to a molten globule-like state. Fourth, the absence of native-like tertiary structure in the molten globule states means that caution should be exercised when using probes for monitoring the presence of the unfolded state that are based on the existence of tertiary structure since they cannot distinguish between the formation of a molten globule-like state or a more fully (random coil) unfolded state. Finally, we believe that molten globule-like states, rather than random-coil conformations, may occur under many denaturation conditions.

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