Classification of Acid Denaturation of Proteins: Intermediates and Unfolded States†

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ABSTRACT: A systematic investigation of the effect of acid on the denaturation of some 20 monomeric proteins indicates that several different types of conformational behavior occur, depending on the protein, the acid, the presence of salts or denaturant, and the temperature. Three major types of effects were observed. Type I proteins, when titrated with HCl in the absence of salts, show two transitions, initially unfolding in the vicinity of pH 3–4 and then refolding to a molten globule-like conformation, the A state, at lower pH. Two variations in this behavior were noted: some type I proteins, when titrated with HCl in the absence of salts, show only partial unfolding at pH 2 before the transition to the molten globule state; others of this class form an A state that is a less compact form of the molten globule state. In the presence of salts, these proteins transform directly from the native state to the molten globule conformation. Type II proteins, upon acid titration, do not fully unfold but directly transform to the molten globule state, typically in the vicinity of pH 3. Type III proteins show no significant unfolding to pH as low as 1, but may be caused to behave similarly to type I in the presence of urea. Thus, the exact behavior of a given protein at low pH is a complex interplay between a variety of stabilizing and destabilizing forces, some of which are very sensitive to the environment. In particular, the protein conformation is quite sensitive to salts (anions) that affect the electrostatic interactions, denaturants, and temperature, which cause additional global destabilization. The specific behavior of a particular system is determined by the underlying conformational phase diagram. A general model to account for these observations is proposed.

Recently, there has been growing recognition of the importance of the compact denatured states of proteins and of the fact that under many unfolding conditions, the denatured protein may retain substantial structure. In acid denaturation, intramolecular charge repulsion is the driving force for unfolding. The acid denaturation of proteins has been known to result in denatured states that are often less unfolded than those obtained with high concentrations of Gdn·HCl or urea (Aune et al., 1967; Tanford et al., 1967; Tanford, 1970). This may be attributed to electrostatic repulsion failing to overcome the interactions favoring folding, such as hydrophobic forces, disulfide bonds, salt bridges, and metal ion–protein interactions. However, detailed studies of acid-induced denaturation, and especially the role of salts, have not been performed.

Recently we noted that both acid and base denaturation of β-lactamase resulted in unfolded or compact denatured states, depending on the presence of salt (Goto & Fink, 1989). This prompted us to undertake a more detailed examination of the effects of acids and salts on denaturation. Preliminary results on three small monomeric proteins demonstrated that it is the presence of the anion that is critical in determining the extent of unfolding at acidic pH (Goto et al., 1990a). A detailed investigation of the effect of anion structure revealed that the key factor is the affinity of the anion for the ammonium ions of the protein and that the effectiveness of different anions in promoting structure in the protein varies dramatically (K_d's varying from micromolar to molar) and follows the electroselectivity series, rather than the Hofmeister series (Goto et al., 1990b). Probably the earliest reported observation of a salt-induced effect in acid denaturation is that of Tanford and co-workers (1955).

In order to investigate the generality of these effects, we have carried out a systematic investigation of the acid-induced unfolding of several monomeric proteins as a function of ionic strength by monitoring their spectral and hydrodynamic properties. The results show that acids induce a wide range of effects on proteins, ranging from initial complete unfolding followed by refolding to a compact molten globule as the acid concentration is further increased to essentially no effect to pH values as low as 0.5. That these effects are due to the anions is evident from the fact that the addition of neutral salts to the acid-unfolded state will bring about similar conformational transitions.

EXPERIMENTAL PROCEDURES

Materials. The following proteins were obtained from Sigma: bovine carbonic anhydrase, *Bacillus* α-amylase, chymotrypsinogen, concanavalin A, α-lactalbumin (types I and III), β-lactoglobulin, chicken lysozyme, protein A, papain, subtilisin BPN′, subtilisin Carlsberg, horse myoglobin, ferricytochrome *c*, soybean trypsin inhibitor, ubiquitin, and bovine serum albumin. Bovine ribonuclease A was of chromatographically pure grade from Calbiochem. β-Lactamase I from *Bacillus cereus* was prepared as previously described (Goto & Fink, 1989). T4 lysozyme was a generous gift from R. Wetzel and B. Matthews. Staphylococcal nuclease (SNase[1])

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[1] Abbreviations: Gdn·HCl, guanidinium chloride; Gdn·SCN, guanidinium thiocyanate; CD, circular dichroism; PAGE, polyacrylamide gel electrophoresis; SNase, staphylococcal nuclease; ANS, 1-anilino-8-naphthalenesulfonic acid.

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Table 1: Classification of the Acid Denaturation of Various Proteins (See Text)

classification type	proteins
IA	β-lactamase (*B. licheniformis*, *B. cereus*), cytochrome *c*, apomyoglobin[a]
IB	apomyoglobin,[b,c] α-amylase, myoglobin, subtilisin BPN′, subtilisin Carlsberg, β-lactamase PC1 (*Staph. aureus*), staph. nuclease
IC	papain, parvalbumin, ribonuclease A
II	α-lactalbumin, carbonic anhydrase
III	T4 lysozyme, ubiquitin, chicken lysozyme, chymotrypsinogen, protein A, β-lactoglobulin, concanavalin A

[a] In the presence of trichloroacetate. [b] In the presence of chloride. [c] The situation for apomyoglobin is more complicated since, in the presence of low concentrations of KCl, the HCl titration results in an initial transition from native state to A state prior to unfolding: i.e., N → A → U → A (Goto & Fink, 1990).

was grown and purified from a cloned gene kindly supplied by D. Shortle. Frog parvalbumin was generously provided by R. Roche. The homogeneity of the proteins was checked by SDS–PAGE using the Phastsystem (Pharmacia). Protein solutions were desalted by extensive dialysis and then concentrated, using Centricon filters. Protein concentrations were determined from published molar extinction coefficients. 8-Anilino-1-naphthalenesulfonate (ANS) was purified by recrystallization and gel filtration on Sephadex LH-20 using water as the eluant (York et al., 1978).

Methods. Circular dichroism spectra were recorded on an AVIV Model 60DS instrument at 25 °C unless otherwise specified. Either a 1.0 or 0.2 mm path length cell was used for the far-UV measurements (protein concentrations were in the 15–20 μM range), and a 10.0 mm path length cell was used for the near-UV region (protein concentrations were in the 50 μM range). 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 micro combination glass electrode (Microelectrodes Inc., Model MI-410). Hydrodynamic radii were determined using molecular exclusion chromatography with a Superose 12 column with a Pharmacia FPLC system or with a Bio-Rad Bio-Sil-250 column using a Beckman HPLC system. Protein sample concentrations were 2 μM. No difference in elution volume for the compact intermediate state was detected over the concentration range 0.5–2 μM using apomyoglobin. The Stokes radius was determined by the procedure of Corbett and Roche (1984). ANS binding was measured by fluorescence emission with excitation at 400 nm. Typically, ANS concentrations were in the region of 50 μM, and protein concentrations were in the vicinity of 0.3 mg/mL. HCl was used to adjust the pH for pH titrations at low-salt conditions. Salt titrations at low pH (usually pH 1.8–2.0) were carried out by making a series of solutions of the desired salt (KCl) concentration and adjusting the pH with HCl. Urea and guanidinium concentrations were determined from the refractive index of the solution (Pace et al., 1989).

RESULTS

The acid denaturation of some 20 small monomeric proteins was investigated with several structural probes as a function of pH and anion concentration. Three major types of conformational states were observed under acid-denaturation conditions: (1) a native-like state, N′, that is similar to the native state; (2) the acid-unfolded state, U_A, which may retain somewhat more secondary structure than the corresponding Gdn·HCl-denatured protein; and (3) an acid-denatured state, the A state, that is relatively compact and exhibits a high level of secondary structure and minimal tertiary structure. The degree of compactness, as well as the extent of folding (i.e., the amount of structure), varies substantially among different A states, as will be subsequently discussed. The more compact A states have the properties of molten globules (Ohgushi & Wada, 1983; Ptitsyn, 1987; Kuwajima, 1989; Christensen & Pain, 1991; Baldwin, 1991).

The following experiments were performed: (1) A pH titration was carried out on the salt-free protein from pH 7 to 0.5 using HCl in the absence of salt. (2) In the cases of proteins that showed incomplete unfolding in the above titration, the effect of urea and temperature on the conformation in the vicinity of pH 2 was investigated; where justified, the HCl titration was repeated in the presence of urea. (3) A salt titration with KCl was carried out, usually over the 10–500 mM range, at the pH of maximum unfolding (around pH 2). (4) In some cases urea titrations were carried out in the vicinity of pH 2, either in the presence or absence of salt. (5) In some cases thermal unfolding experiments were undertaken at low pH.

To detect and characterize the protein structure, measurements of near-UV CD, intrinsic tryptophan fluorescence, and ANS binding were used to monitor tertiary structure. Far-UV CD was used to quantify secondary structure, and size exclusion chromatography was employed for hydrodynamic studies. At moderate protein concentrations, many of the A states tended to aggregate, thus precluding the use of dynamic light-scattering measurements for size measurement. In addition, for a number of proteins the A states interacted with the FPLC column support, frequently leading to later elution times than those of the native state. This occurred for aldolase, BSA, ovalbumin, chymotrypsinogen, RNase A, T4 lysozyme, carbonic anhydrase, and α-lactalbumin. In several cases the differential binding of ANS by the molten globule state (Goto & Fink, 1989; Semisotnov et al., 1991), compared to that by the native or denatured states, was used to confirm the presence of molten globule-like states.

On the basis of the results of these studies, five different classes, representing three major types, of behavior were observed. The key features of each category will be described by representative examples. Table 1 lists the class to which each of the proteins examined belongs. Upon HCl titration of a salt-free solution of the protein starting at neutral pH, proteins in the type I class first unfold in the vicinity of pH 3–4. They are thus substantially unfolded at pH 2. The addition of more acid results in the collapse to a compact intermediate or molten globule state, the A state, below pH 2. Furthermore, the addition of salt at pH 2 also leads to collapse to the A state. Type II proteins transform directly from the native to the molten globule state, and type III proteins remain essentially native-like to pH values ≤1. There are three subclasses of type I behavior that reflect varying degrees of unfolding in the first transition, as well as different amounts of secondary structure and compactness in the A state. We previously demonstrated that the very low pH values used in these experiments had negligible effects on the covalent structure on a time scale of a few tens of minutes (Goto & Fink, 1989).

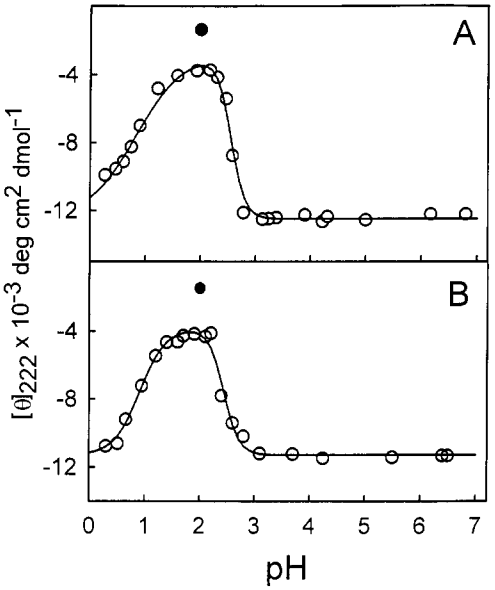

FIGURE 1: Effect of HCl on the conformation of horse cytochrome *c* (panel A) and *Bacillus licheniformis* β-lactamase (panel B), as monitored by far-UV circular dichroism. The filled circle shows the ellipticity value corresponding to the protein unfolded by 6 M Gdn·HCl at pH 2. The wavelength was 222 nm, and the protein concentration was 20 μM. At neutral pH the protein is in the native state. Between pH 3 and 2 it unfolds to the acid-unfolded state (U_A), and below pH 2 it refolds to the A state. The solid lines were drawn according to the following equation: $\theta_{obs} = [\theta_{UA}/[1 + [X^-]^n/K_1 + K_2/[H^+]^m]] + \theta_N$, where θ_{UA} represents the ellipticity for the U_A state and θ_N is that for the native state, $[X^-]$ is the concentration of added anion, and $[H^+]$ is the hydrogen ion concentration. The equation is based on the assumptions that the unfolding transition corresponds to the protonation of *m* carboxyl groups, approximated by a single ionization, K_2', and that the refolding corresponds to the binding of *n* anions to positively charged residues (ammonium groups), again approximated by a single binding (dissociation) constant, K_1'. For panel A the values for *n* and *m* were 1.05 and 3.4, respectively, and the values of the intrinsic dissociation constants for anion and proton, K_1' and K_2', were 0.15×10^{-3} and 3.0×10^{-3} M, respectively. For panel B the values of *n* and *m* were 1.9 and 3.1, and the values of K_1' and K_2' were 0.13×10^{-3} and 3.6×10^{-3} M, respectively.

Type IA Proteins. This class of protein exhibits the following behavior when titrated with HCl in the absence of salts and may be considered to represent the archetypal behavior. A cooperative transition from the native to the unfolded state[2] occurs in the vicinity of pH 2–4, usually with a midpoint around pH 3. Typical data for cytochrome *c* and *Bacillus licheniformis* β-lactamase are shown in Figure 1. A cooperative transition from the native to the unfolded state as monitored by far-UV CD occurs in the vicinity of pH 4–2, reflecting loss of secondary structure. In the aromatic CD region (data not shown), a similar transition is also observed between pH 4 and 2, reflecting the loss of the native-like tertiary structure, which is not regained at lower pH values, and it is similar to that found in the presence of 6 M Gdn·HCl, pH 2. This loss of secondary as well as tertiary structure yields the acid-unfolded state, U_A. Typically 2–4 protons are involved in the unfolding transition, i.e., the protonation of 2–4 key carboxyl groups leads to sufficient net positive charge in the protein to trigger the electrostatically driven unfolding transition (see the legend to Figure 1).

Further addition of HCl leads to a second transition manifested as an *increase* in secondary structure, leading to the A state, with the properties of a molten globule, e.g., an amount of secondary structure comparable to that in the native state (Figure 1), but no native-like tertiary structure (Goto et al., 1990a,b). Typically 1–3 anions are involved in triggering this transition. The addition of anions, as salts (e.g., KCl), to U_A also results in a similar transition to the A state. In some cases a transition is also observed by fluorescence from the U_A state to the A state (Goto & Fink, 1989). The Stokes radius, R_s, of these type IA proteins in the A state is expanded by about 10% compared to the native state, as shown in Table 2. Examples of this class of protein include cytochrome *c* and class A β-lactamases from *B. cereus* and *B. licheniformis*. In the presence of certain acids, e.g., trifluoroacetic acid, apomyoglobin also behaves as a type IA protein; however, in the presence of HCl, a less compact A state, with less secondary structure, was observed.

Table 2: Stokes Radii of A States (Calculated from Size Exclusion Chromatography)[a]

protein	Stokes radius (Å) native	unfolded	A state
β-lactamase (*B. cereus*)	21	52	23
β-lactamase (*B. licheniformis*)[b]	23	51	25
cytochrome *c*	17	34	19
staph. nuclease	18	40	25
apomyoglobin	19	43	35

[a] Unless otherwise noted, a Superose 12 column was used in a Pharmacia FPLC system. The native proteins were eluted in pH 7.0, 0.5 M KCl buffer, the unfolded proteins with 6 M Gdn·HCl, pH 2, buffer, and the A states with pH 2.0, 0.15–0.5 M KCl, buffer. [b] A Bio-Sil 250 (Bio-Rad) column (60 cm) was used in a Beckman HPLC system under conditions similar to those in footnote a.

Type IB Proteins. This class is similar to type IA proteins in that they undergo an unfolding transition in the vicinity of pH 4–2 upon HCl titration of a salt-free solution, leading to the acid-unfolded state, and then undergo a refolding transition to the A state at lower pH. However, these proteins differ in that the A state is considerably less compact (typically about a 50% greater hydrodynamic radius than that for the native state; Table 2), has correspondingly less secondary structure than the native state, and has the near-UV CD spectrum of the unfolded state. For some proteins, the addition of KCl to the unfolded protein at pH 2 induced more secondary structure than the corresponding concentration of HCl. This class of proteins thus forms an A state that is considerably more expanded than a molten globule. The possibility of a mixture of species (native/unfolded or unfolded/molten globule) was ruled out by FPLC gel filtration. In the case of apomyoglobin, under A state conditions (pH 2, 0.5 M KCl), the protein appeared as a single species, with an R_s value corresponding to an approximately 50% increase over that of the native state, as shown in Table 2. Kinetic measurements indicated that interconversion between states was very slow, so that rapid exchange could be ruled out. These data were confirmed by dynamic light-scattering experiments, which also showed only a single species. It should be noted that apomyoglobin appears to be an unusual case in that the amount of secondary structure and the degree of compactness vary, depending on the anion present. Thus, in the presence of trichloroacetate, the A state has almost as much secondary structure as is found in the native state and has a compactness only slightly expanded over that of the native state. A more detailed characterization of the apomyoglobin A states will be published subsequently.

[2] The ellipticity at 222 nm was used in ascertaining the degree of unfolding. For type IA proteins, the acid-unfolded state typically had a θ_{222} value corresponding to at least 80% unfolded, based on the difference in ellipticity for the protein unfolded in 6 M Gdn·HCl at pH 2, relative to the native state at neutral pH.

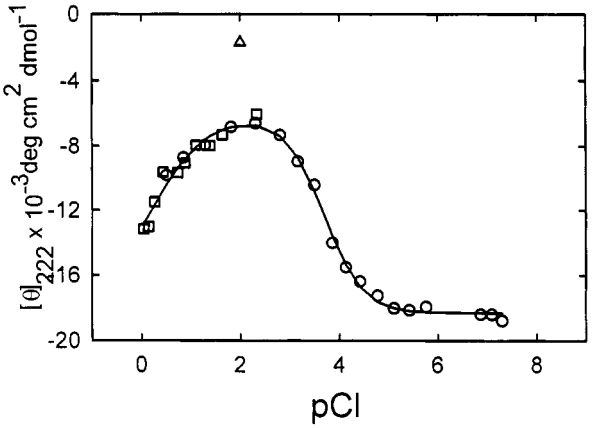

FIGURE 2: Effect of chloride on frog parvalbumin, monitored by ellipticity at 222 nm. The HCl titration data are shown by circles. The triangle shows the value for the protein unfolded in 6 M Gdn·HCl at pH 2. The effect of chloride ion (from KCl) on the acid-unfolded state at pH 2 is shown by squares. The latter data are plotted in the form of pCl (–log [Cl$^-$]) for direct comparison with the effect of Cl$^-$ from HCl in the HCl titration. Below pCl 2, the curves from the HCl and KCl titrations are effectively the same.

Examples of type IB proteins include α-amylase, apomyoglobin and myoglobin in the presence of most anions, subtilisin BPN′, subtilisin Carlsberg, β-lactamase from *Staphylococcus aureus* PC1, and SNase. In the case of subtilisin BPN′, the salt-induced molten globule state at pH 2.0 showed slightly more tertiary structure (as determined by near-UV CD) than the acid or Gdn·HCl-unfolded states. The unfolding of subtilisin is not reversible due to posttranslational processing of the initially folded proenzyme. The detailed results for SNase, which is representative of this class of behavior, have been reported separately (Fink et al., 1993).

Type IC Proteins. This class differs from types IA and IB in that only partial unfolding occurs in the vicinity of pH 2 as monitored by CD. This type of acid-denaturation behavior also was rather common: examples are papain, parvalbumin, and ribonuclease A. Typical data for the HCl titration and for the KCl titration at pH 2 are shown for frog parvalbumin (Figure 2). The addition of HCl to these proteins at pH 7 induces an unfolding transition, as detected by far-UV CD at 222 nm, with transition midpoints at pH 2 for RNase A and papain and pH 3 for parvalbumin. The magnitude of the CD signal at the completion of this transition indicated that unfolding was incomplete: 63% unfolded for parvalbumin, 57% for papain, and 33% for RNase A. These calculations are based on the difference in ellipticity at 222 nm between the native and denatured states (6 M Gdn·HCl, pH 2) of 100%. Thus, the acid-induced unfolding transition for this class of proteins appears to involve conversion from the native state to a somewhat collapsed unfolded state with significant residual secondary structure. Further addition of HCl to the acid-unfolded protein resulted in a second transition, corresponding to the formation of additional secondary structure, i.e., the formation of the A state. At very low pH (0.5), the amount of secondary structure was approximately 70% of that in the native state for all of the type IC proteins. The near-UV CD spectra, obtained at various pH values, demonstrated that these species possess little or no tertiary structure, as is shown in Figure 3 for papain.

The addition of KCl to the acid-unfolded type IC proteins induced a cooperative transition to a compact species with secondary structure similar to that produced by the corresponding concentration of HCl (Figure 2). For most of these proteins it was possible to achieve a higher concentration of KCl than HCl, resulting in an increase in the final amount of secondary structure, as is also seen in type IB proteins. However, aggregation or precipitation (salting out) usually was observed before the amount of secondary structure approximated that present in the native state.

As observed previously with β-lactamase (Goto & Fink, 1989) and apomyoglobin (Goto & Fink, 1990), HCl titration of type I proteins in the presence of salts can lead directly from the native state to the A state, without the intermediacy of the U_A state, as illustrated in Figure 4 for papain. No difference was observed in the pH titration of papain whether DTT was present or not. The presence of the KCl resulted in a shift in the position of the transition from the native to denatured state to somewhat higher pH.

For RNase A the addition of 0.5 M KCl at pH 1.6 led to an amount of secondary structure equivalent to that present in the native state. For RNase A, at the pH of maximum unfolding (pH 1.5), the magnitude of the ellipticity at 275 nm was 58% of that for the Gdn·HCl-unfolded protein. The addition of 0.5 M KCl at this pH led to an increase in the θ_{275} to 83% of that of the native signal. This behavior is apparently atypical and suggests that RNase A may be a special case in which significant tertiary structure is present in the A state. Shearwin and Winzor (1990) have reported results from thermodynamic nonideality studies also indicating that the acid denaturation of RNase A leads to a relatively compact intermediate state.

Confirmation that Cl$^-$ induces a molten globule-like conformation at low pH in this class of protein is illustrated by ANS binding studies. The fluorescence emission of ANS in the presence of native protein is unaltered, indicating the lack of binding between the dye and the protein, as shown in Figure 5 for parvalbumin. In the presence of acid-unfolded parvalbumin a small increase in fluorescence emission is observed, indicating some residual structure in U_A to which ANS binds. However, in the presence of 0.5 M Cl$^-$ a very substantial emission signal is observed, reflecting the preferential binding of the ANS to the A state.

Type II Proteins. The unique characteristic of this class is that the protein undergoes a transition directly from the native state to the molten globule-like state upon salt-free titration with HCl. In contrast to type I proteins, there is no detectable unfolded state at intervening pH values. The behavior is exemplified by α-lactalbumin (with bound Ca^{2+}). Thus, monitoring the far-UV CD shows only a very low-amplitude transition upon HCl titration (Figure 6B), presumably because the amount of secondary structure in the molten globule state is very similar to that in the native state, whereas if the near-UV CD is monitored a pH-induced transition is observed (Figure 6A). Previously described examples include human and bovine α-lactalbumin (Kuwajima et al., 1975; Dolgikh et al., 1985; Gast et al., 1986; Ikeguchi et al., 1986; Baum et al., 1989) and carbonic anhydrase (Wong & Hamlin, 1974; Brazhnikov et al., 1985; Ptitsyn, 1987). In addition, the V_L domains of immunoglobulins appear to belong to this class (A. Talapatra and A. L. Fink, unpublished results).

Type III Proteins. These proteins are resistant to high concentrations of acid. They maintain native-like far- and near-UV spectra over the range from pH 7 to 1 or in some cases lower pH. T4 lysozyme, chicken lysozyme, chymotrypsinogen, ubiquitin, concanavalin A, and protein A belong to this category where minimal alteration in secondary and tertiary structure occurs. For example, in the native state protein A shows a far-UV CD spectrum typical of a helix-rich protein, as seen in Figure 7 (insert). The addition of HCl to

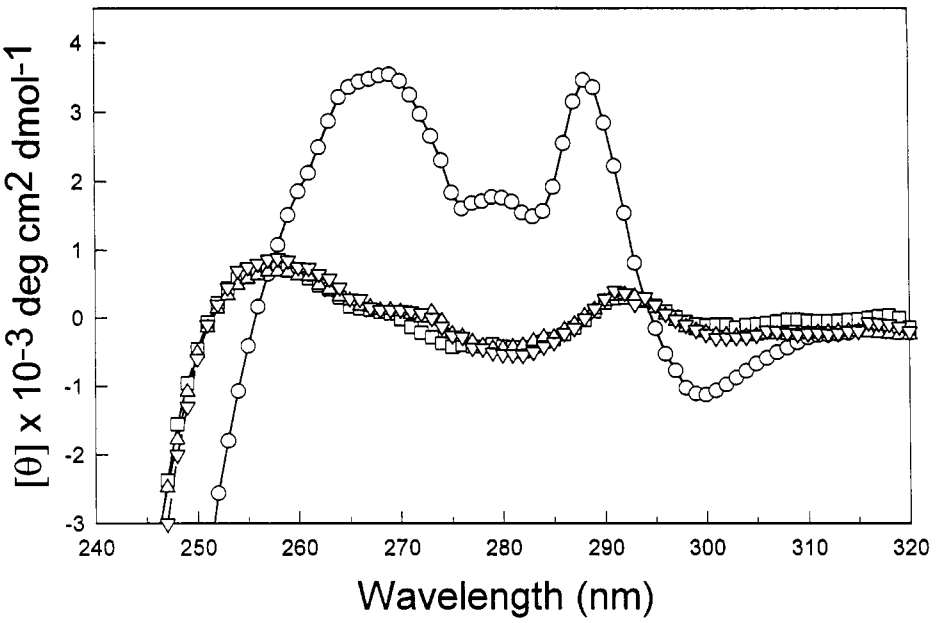

FIGURE 3: Near-UV CD spectrum of papain in the native state (○), acid-unfolded state, pH 1.7 (△), HCl-induced A state, pH 0.77 (□), and Gdn·HCl-denatured state, pH 2 (▽).

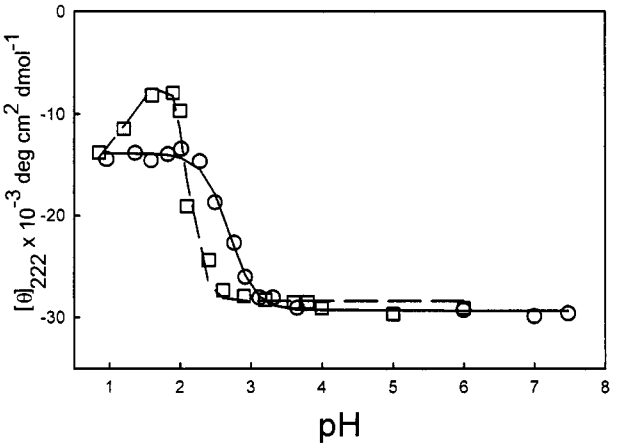

FIGURE 4: Effect of HCl titration on the ellipticity at 222 nm of papain in the presence (○) and absence (□) of 0.25 M KCl. The presence of the 0.25 M Cl^- effectively changes the transition upon HCl titration from N → U_A → A to N → A.

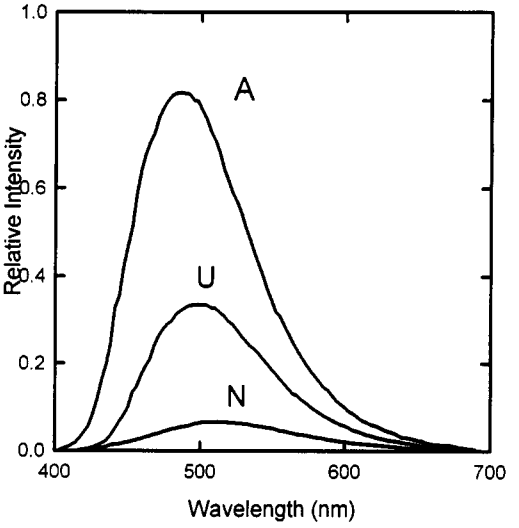

FIGURE 5: Interaction of ANS with frog parvalbumin, monitored by ANS fluorescence. The conditions were 45 mM HCl for the acid-unfolded state, 45 mM HCl, 450 mM KCl for the A state, and pH 7.0 for the N state. The ANS concentration was 50 μM, and the parvalbumin concentration was 0.32 mg/mL.

the salt-free sample had no significant effect on the spectrum to pH 0.6 (Figure 7). The aromatic CD spectra at neutral and low pH's were identical, although the lack of tryptophan results in a very weak near-UV CD signal.

In order to measure the stability of type III proteins at low pH, the effects of high temperature and denaturant were investigated. For example, for protein A at pH 1.4, the ellipticity at 222 nm showed a broad, monotonic increase with increasing temperature, starting around 30 °C and continuing to above 70 °C where the protein was still not fully unfolded. On reversing the temperature gradient the protein refolded (showing some hysteresis), but returning to within 90% of the starting ellipticity. For T4 lysozyme at pH 1.8, increasing the temperature led to a monotonic increase in the ellipticity at 222 nm that leveled off around 40 °C, with a signal corresponding to 20% of the native structure remaining.

On the basis of experiments with T4 lysozyme, it appears that the behavior of some type III proteins can be converted to that of type I by the addition of urea. For example, in the presence of 2 M urea, T4 lysozyme was in the native state at pH 7, but upon HCl titration in the absence of salt, it underwent almost complete unfolding at pH 1.8. The subsequent addition of chloride anion induced a cooperative transition to the A state. In other words, in 2 M urea T4 lysozyme behaves as a typical type IA protein! The behavior of T4 lysozyme at low pH will be described in greater detail elsewhere.

We also examined the effect of urea on several other proteins that exhibited type III behavior, including chymotrypsinogen, chicken lysozyme, β-lactoglobulin, ubiquitin, and concanavalin A; a variety of effects were noted, with the following general trends. In each case, the titration of salt-free protein by HCl showed no significant change in either near- or far-UV CD signals down to pH 1.0. At pH 2, urea titrations showed unfolding transitions with midpoints in the 2–4 M range (monitored by both near- and far-UV CD), except for β-lactoglobulin. The following data for chymotrypsinogen were relatively typical. When the HCl titration was repeated in the presence of 2.5 M urea, an unfolding transition was observed in the vicinity of pH 3–4, leading to loss of the near-UV signal but retention of some residual secondary structure compared to 6 M Gdn·HCl. The addition of KCl to the 2.5

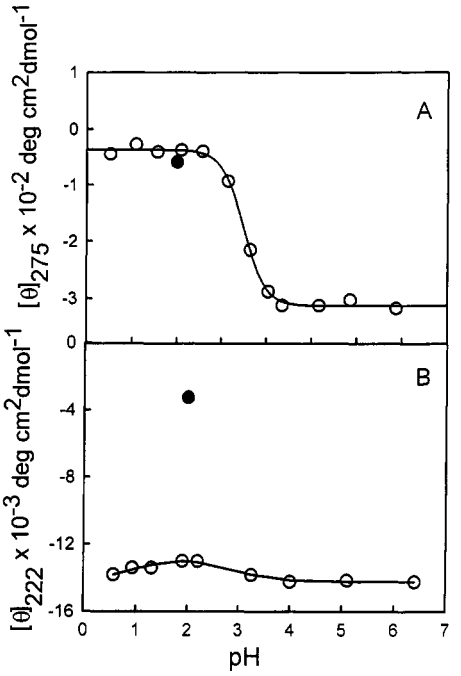

FIGURE 6: Titration of α-lactalbumin by HCl, as monitored by near-UV (A) and far-UV (B) circular dichroism. The filled circle represents the ellipticity for the protein unfolded by 6 M Gdn·HCl, pH 2.

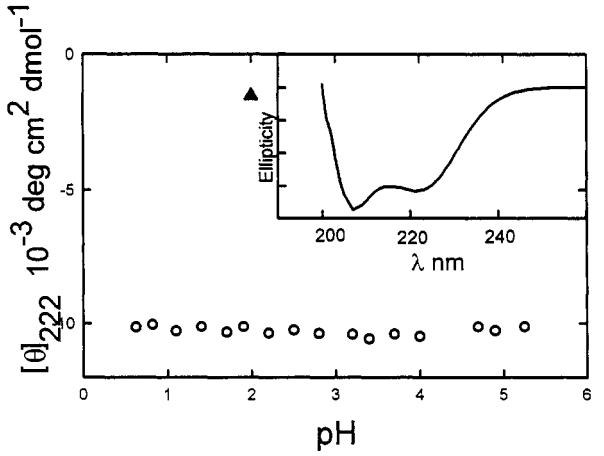

FIGURE 7: Effect of HCl on a salt-free solution of protein A, as monitored by ellipticity at 222 nm. The triangle shows the value for the protein unfolded in 6 M Gdn·HCl. The inset shows the far-UV CD spectrum of the native protein at pH 7.

M urea solution (pH 2) led to some return of secondary structure at 0.1 M KCl and aggregation and precipitation at higher KCl concentrations. It is important to note that at high concentrations of urea at pH 2 or lower a significant amount of chloride ion is present due to the necessity of titrating the *urea* with HCl since its pK is 0.2. In the case of chicken lysozyme, the addition of urea or Gdn·HCl at pH 2 led to broad unfolding transitions, with complete loss of tertiary structure occurring at 6 M Gdn·HCl or 5 M urea. The addition of 1 M KCl to a solution of lysozyme at 5 M urea (pH 2) restored 50% of the near-UV signal of the native state and 70% of the θ_{222} signal of the native state.

DISCUSSION

In a systematic examination of the conformational states of proteins under conditions of acid-induced denaturation, we have made a number of interesting observations. Perhaps the two most unexpected are (1) that some proteins retain their native state to pH's as low as 0.5 and require high concentrations of denaturant for unfolding at low pH and (2) that a number of proteins, as the pH of a salt-free solution is lowered by the addition of hydrochloric acid (HCl), initially unfold in the vicinity of pH 3, as expected due to charge–charge repulsion, but then as the pH is lowered below 2 they begin to refold, in many cases attaining a compact structure with as much secondary structure as the native state! In some cases this compact structure has the properties of a molten globule (Ptitsyn, 1987; Kuwajima, 1989).

The addition of acid has no single effect on the conformation of proteins. Proteins can be classified into three major categories on the basis of their observed behavior upon HCl titration: (1) those proteins that initially unfold (to greater or lesser extent) and then refold into a compact or expanded molten globule-like conformation (A state) (Baldwin, 1991) at lower pH; (2) those that transform directly to the molten globule (A) state; and (3) those that essentially remain native:

$$\text{type I: } N \rightarrow U_A \rightarrow A$$

$$\text{type II: } N \rightarrow A \qquad (1)$$

$$\text{type III: } N \rightarrow N'$$

For type I proteins, the degree of unfolding in the U_A state varies considerably, from essentially completely unfolded in some cases to retention of as much as 50% of the native secondary structure in others. Interestingly, the degree of compactness and the amount of secondary structure in the A states also varied, from very similar to that of the N state to much less. In some cases the addition of denaturant to type III proteins converted their behavior to that of type I proteins.

The results show that the properties of the A states varied significantly among different proteins. In some cases, on the basis of the compactness and amount of secondary structure, the A state strictly adheres to the definition of a molten globule (Ptitsyn, 1987) (i.e., type IA proteins), whereas in other cases both the compactness and the amount of secondary structure are considerably less than expected for a molten globule. Nevertheless, the conformational state is clearly a more compact and structured one than the unfolded state. These A states are best referred to as compact intermediate states or compact denatured states (Dill & Shortle, 1991).

A simple model to account for the observations of A states with varying degrees of structure and compactness is as follows (Palleros et al., 1993; Fink, 1994). The native state is assumed to consist of a number of structural units that are tightly packed together via *tertiary* interactions. These structural units will have significant intrinsic stability due to backbone and side-chain interactions and would minimally consist of a helix or two-stranded β-sheet and maximally of a domain. The difference between the native state and compact intermediate states such as the A state, which experimentally corresponds to the loss of much of the tertiary structure but retention of most of the secondary structure, corresponds to the separation of these structural units, with concomitant solvent penetration, and overall expansion of the molecule. Thus, in the compact A states, there is disruption of the interactions *between* structural units, but little effect on side-chain interactions *within* the structural unit. As more solvent molecules penetrate the space between structural units, the A state expands, and as the structural units become separated, they may become less stable and lose their secondary structure. The loss of secondary structure potentially occurs in a stepwise fashion, correlating with the relative stability of the structural

subunits. This model makes implicit the existence of two kinds of tertiary structure: (1) the majority of such interactions, which occur between structural units and which would be the first to be disrupted by the solvent, and (2) the minority within the structural unit, which would be more protected from interactions with the solvent and hence more stable.

A range of possible conformations for the A states is anticipated, depending on the extent of the loss of side-chain contacts between structural units. When the contacts between units are lost, the molecule becomes a collection of structural units linked by flexible polypeptide chain links. It is likely that these conformations will be quite mobile; however, even with the loss of the building block contacts there may still be transient contacts between them. Depending on the relative stabilizing or destabilizing forces of the external environment, as well as the intrinsic structural properties of the protein, the resulting A states may be quite compact and relatively native-like, as in type IA behavior, or more expanded as in type IB.

For an acid-unfolded protein, U_A, at a pH in the vicinity of 2, 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 no effect on its ionization state. However, the addition of more anions leads to binding of the anions (Goto et al., 1990) so as to effectively shield the repulsive forces. This decreases the internal repulsive forces, which favor unfolding, and consequently the intrinsic hydrophobic interactions manifest themselves. These result in the protein folding to adopt a compact structure with decreased hydrophobic surface area. Use of a model of Tanford's (1970) for the preferential binding of ligand to the A state compared to the U_A state reveals that the number of anions *preferentially* binding to the A state is typically between 2 and 3 (Goto et al., 1990b; Calciano et al., 1993; Figure 1). Similarly, the number of key protons binding in the transition from native to U_A state is on the order of 2–3. That is, the *difference* in the number of bound anions between the two states (U_A, A) is quite small. The association constants are on the order of 10^2–10^5 M^{-1}. Thus, it is the binding of anions to the ammonium groups of the unfolded state that masks the repulsive positive charge, allowing the intrinsic hydrophobic forces to cause the collapse to a compact state. The presence of secondary structure may be explained by the intrinsic properties of the polypeptide as a consequence of basic polymer physics and conformational entropy [as proposed in models by Dill and co-workers (Chan & Dill, 1989, 1990)].

Upon decreasing the pH from neutrality to approximately pH 2 the protein becomes maximally positively charged, since the pK of most carboxyls is ≥ 3. The resulting intramolecular repulsion between the positively charged groups leads to unfolding and a relatively extended conformation, state U_A. For some proteins the balance between these intramolecular repulsive forces and the hydrophobic interactions (and perhaps electrostatic interactions, disulfide cross-links, and metal–protein interactions) are such that the protein may only partially unfold or, in extreme cases, remain folded. It is the overall small net difference between these forces and the minimization of the intramolecular charge–charge repulsion by anion binding that determine the shape of the phase diagram (or conformational state diagram) for the protein as a function of pH and anion concentration. A typical diagram is shown in Figure 8 for β-lactamase and chloride. Due to differences in structure, which result in a different balance of hydrophobic and electrostatic forces, the positions of the phase boundaries between the native, unfolded, and molten globule states vary

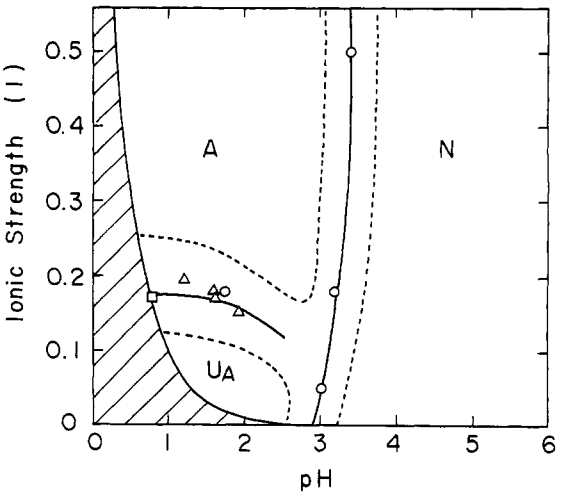

FIGURE 8: Phase diagram for β-lactamase as a function of pH and chloride ion. The solid lines represent the midpoints of the transitions between the native and the A state or the A state and the unfolded state, while the broken lines reflect 20% and 80% completion of the transitions. The shaded area is inaccessible in the sense that its upper boundary reflects the chloride concentration of the HCl solution at the given pH.

significantly from one protein to another. Thus, we find that different proteins appear to respond very differently to the effect of low pH. However, we believe that these differences are really manifestations of relatively minor changes in the position of the boundaries in the conformational phase diagram (Figure 8), which in turn reflect the intrinsic relative stability of the native state. Thus, the difference in behavior of different proteins at acidic pH is really one of degree: for example, protein carboxyl groups with particularly low pK values are predicted to change from type I to type II behavior, if the carboxyl ionization is a key one in the unfolding transition. With the exception of type III proteins, under typical acid-denaturation conditions, with some salt present, most proteins will be present, at least to a significant extent, in compact intermediate or denatured conformations rather than being fully unfolded.

Most other acids are more effective than HCl in bringing about the unfolded to molten globule transition (Goto et al., 1990b). In these cases, upon decreasing the pH, the protein will usually go directly into the molten globule state from the native state. There are several reports in the literature that indicate that other proteins not examined in this study also form compact intermediate states at low pH: examples include interferon (Arakawa et al., 1987), carmin (Rao & Prakash, 1989), the C-terminal fragment of thermolysin (Dalzoppo et al., 1985), interleukin-2 (Dryden & Weir, 1991), interleukin-4 (Redfield et al., 1994), immunoglobulin (Buchner et al., 1991), retinol-binding protein (Bychkova et al., 1992), brain-derived neurotrophic factor (Narhi et al., 1993), transthyretin (Colon & Kelly, 1992), β-globulin (Rajendran & Prakash, 1992), BSA (Tanford et al., 1955), and growth hormone (Holzman et al., 1990). In addition, similar phenomena may be observed at alkaline pH (McPhie, 1982; Goto & Fink, 1989).

We conclude, therefore, that the conformational states induced by acid denaturation are governed by a fine balance between the hydrophobic interaction, i.e., the drive to minimize the exposed hydrophobic surface area, which leads to compact molten globule states, the opposing chain conformational entropy and intramolecular charge–charge repulsion, and the affinity of anions for the positively charged ammonium groups. The unique structure of a given protein, particularly the distribution and number of ammonium groups, and the

presence of disulfide cross-links or metal-binding sites determine how readily the molecule will unfold to a random coil-like structure or molten globule-like structure. This in turn determines the conformational state diagram and, hence, the observed behavior upon acid titration.

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