Differences in the Unfolding of Procerain Induced by pH, Guanidine Hydrochloride, Urea, and Temperature†

Vikash Kumar Dubey and M. V. Jagannadham*

Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, India

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ABSTRACT: The structural and functional aspects along with equilibrium unfolding of procerain, a cysteine protease from *Calotropis procera*, were studied in solution. The energetic parameters and conformational stability of procerain in different states were also estimated and interpreted. Procerain belongs to the $\alpha + \beta$ class of proteins. At pH 2.0, procerain exists in a partially unfolded state with characteristics of a molten globule-like state, and the protein is predominantly a β-sheet conformation and exhibits strong ANS binding. GuHCl and temperature denaturation of procerain in the molten globule-like state is noncooperative, contrary to the cooperativity seen with the native protein, suggesting the presence of two parts in the molecular structure of procerain, possibly domains, with different stability that unfolds in steps. Moreover, tryptophan quenching studies suggested the exposure of aromatic residues to solvent in this state. At lower pH, procerain unfolds to the acid-unfolded state, and a further decrease in the pH drives the protein to the A state. The presence of 0.5 M salt in the solvent composition directs the transition to the A state while bypassing the acid-unfolded state. GuHCl-induced unfolding of procerain at pH 3.0 seen by various methods is cooperative, but the transitions are noncoincidental. Besides, a strong ANS binding to the protein is observed at low concentrations of GuHCl, indicating the presence of an intermediate in the unfolding pathway. On the other hand, even in the presence of urea (8 M), procerain retains all the activity as well as structural parameters at neutral pH. However, the protein is susceptible to unfolding by urea at lower pH, and the transitions are cooperative and coincidental. Further, the properties of the molten globule-like state and the intermediate state are different, but both states have the same conformational stability. This indicates that these intermediates may be located on parallel folding routes of procerain.

Understanding the structure−function relationships of an enzyme under different conditions is fundamentally important for both theoretical and applicative aspects. Such studies may provide insight into the molecular basis of the stability of the enzyme, which in turn can be used to design protocols and/or a protein with special properties for biotechnological applications.

The stability of a native protein is a function of external variables such as pH, temperature, ionic strength, and solvent composition as they disrupt the different kinds of bonds that are responsible for the intrinsic stability. Therefore, a quantitative analysis of the role of such variables in the formation of the structure of the protein is a prerequisite in describing the forces that are responsible for the conformational stability. A simple method for such studies involves the monitoring of conformational changes due to perturbation of a protein molecule by various agents such as acid, GuHCl,[1] urea, and temperature.

Unfolding of many proteins that have been studied can be described in terms of a two-state model (*1*), where only folded and unfolded states of the protein exist in rapid equilibrium with no observable intermediates. However, the development of a wide range of sensitive techniques has led to the detection as well as characterization of intermediates in the folding of several proteins, which were previously believed to obey the two-state model. It has been also shown in the case of many proteins that folding involves a discrete pathway with the formation of intermediate states between native and denatured states (*2*−*4*). Such intermediates are found to possess special characteristics, which is evident from the spectroscopic and hydrodynamic measurements. Identification and characterization of intermediate states populated during the folding process of many proteins have received considerable attention, and much effort is being spent on this objective. An understanding of the structural and thermodynamic properties of such intermediate states may provide insight into the factors involved in guiding the pathway of folding.

The results of kinetic experiments with some of the proteins indicate the rapid formation of intermediates with properties consistent with those of the molten globule state, supporting the idea that the molten globule state may be an obligatory intermediate on the folding pathway (*5*−*8*). The role of the molten globule state as a functional entity in protein folding is hypothesized, and further evidence is also shown that such state is involved in several biological or pathological processes such as membrane insertion, transmembrane trafficking, and chaperone-assisted refolding that

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* To whom correspondence should be addressed. E-mail: jaganmv@satyam.net.in or jvm@banaras.ernet.in. Phone: 91-542-2367936. Fax: 91-542-2367568.

[1] Abbreviations: GuHCl, guanidine hydrochloride; CD, circular dichroism; ANS, 8-anilino-1-naphthalenesulfonic acid; UV, ultraviolet.

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require the protein to become partially unfolded (*9*). Further, point mutation may convert the native protein into the molten globule-like state, and some of these mutations are correlated with the genetic predisposition for human diseases (*10*−*12*). Thus, exploring the structure and dynamics of the molten globule state of a protein is necessary not only to understand the mechanism of the protein folding but also to shed light on many natural or disease-related processes. The exact role of molten globule states is still controversial; consequently, it is important to study this aspect with several proteins to determine the generality of such states and their role in protein folding.

Very little information about the general folding aspects of plant cysteine proteases is available. Extensive studies on the folding of papain as well as other related proteins have been carried out in our laboratory (*13*−*16*). Two other cysteine proteases, ervatamin C and B, isolated in our laboratory, have also been used as model systems (*17*−*19*) in understanding the folding of cysteine proteases. Folding studies on similar proteins from different sources and proteins within a family with individual variations would certainly help in generalizing the folding behavior of cysteine proteases as well as complementing the mechanisms proposed thereafter. With this view, conformational studies in solution have been initiated with procerain, a novel plant cysteine protease isolated in our laboratory from *Calotropis procera* (*20*). Procerain is a monomeric protein with a molecular mass of 28.8 kDa, containing eight tryptophans and twenty tyrosines. The protein molecule has seven cysteine residues, six forming three disulfide bridges and one remaining free.

From our initial studies on the folding of procerain, a stable intermediate state exhibiting characteristics similar to those of the "molten globule" state is detected at pH 2.0. Unfolding of procerain under this condition is profoundly different from unfolding of the protein at neutral pH with different stability toward denaturants, and the transitions are biphasic in nature, suggesting sequential unfolding of the molecule. Moreover, we report evidence of another intermediate in the GuHCl-induced unfolding pathway of procerain at pH 3.0. It was important and necessary to carry out further studies to characterize these intermediate states in detail to visualize the spectroscopic and hydrodynamic nature of the states to gain insight into the folding of cysteine protease in general and of procerain specifically.

Further, the molecular structure of procerain is probably made of two domains, as indicated by the noncooperativity in the GuHCl- and temperature-induced unfolding of procerain in the molten globule state. Not much information about the molten globule state in multidomain proteins and its role in the folding of the protein is available. It has been proposed that domains in the native molecule are independent folding units that assemble and produce native molecules (*21*). These structural regions are expected to fold independently whether they are isolated or together. However, further studies in this respect are necessary as such studies of the different conformational states in the unfolding and refolding of multidomain proteins are important in understanding the principles governing folding of multidomain proteins (*22*).

This investigation describes the biophysical characterization of procerain, as an endeavor of understanding the structure−function relationship, the basis and rationale of its distinct physicochemical properties, and the folding−unfolding mechanism. Evidence of the presence of a partially structured intermediate with molten globule-like properties at pH 2.0, as well as another intermediate state in the GuHCl-induced unfolding pathway of procerain at pH 3.0, is also presented.

EXPERIMENTAL PROCEDURES

Materials

Procerain was purified from fresh latex of *C. procera* using the method of Dubey and Jagannadham (*20*). Sodium tetrathionate was used throughout the purification procedure of the protein to prevent any complications due to autodigestion of the enzyme, and such a tetrathionate-inactivated enzyme was used for all the physicochemical studies reported here. All the physical properties of the procerain are the same in both active and inactive states. The concentration of the enzyme was determined spectrophotometrically using an extinction coefficient ($\epsilon^{1\%}_{1cm}$) of 24.9 (*20*). ANS, CsCl, urea, and GuHCl were purchased from Sigma Chemicals. Concentrations of urea and GuHCl solutions were determined from the refractive index of the solution (*23*). All other reagents that were used were analytical grade, and solutions were prepared in doubly distilled water. Samples for spectroscopic measurements were centrifuged and filtered through 0.45 μm filters, and the exact concentration of the protein and pH were determined before the measurements.

Methods

Absorbance Spectroscopy. Absorbance measurements were carried out on a Beckman DU-640B spectrophotometer equipped with a constant-temperature cell holder. The protein concentration for all absorbance measurements was between 9 and 10 μM. Absorbance spectra were recorded between 240 and 320 nm.

Fluorescence Spectroscopy. Fluorescence measurements were carried out on a Perkin-Elmer LS-50B spectrofluorimeter equipped with a constant-temperature cell holder. The temperature was controlled using a Julabo F 25 water bath. The protein concentration was 1 μM for all fluorescence measurements. Tryptophan was selectively excited at 292 nm, whereas for both the tryptophan and tyrosine fluorescence of procerain, the excitation wavelength was 278 nm. The emission was recorded from 300 to 400 nm with 10 and 5 nm slit widths for excitation and emission, respectively.

Spectropolarimetric Studies. CD measurements were taken on a JASCO J-500A spectropolarimeter equipped with a 500N data processor and equipped with a constant-temperature cell holder. The instrument was calibrated using ammonium (+)-10-camphorsulfonate. The temperature of the cell holder was controlled using a Julabo F 25 water bath. Conformational changes in the secondary structure of the protein were monitored in the region between 200 and 260 nm with a protein concentration of 3.5 μM in a cuvette with a path length of 1 mm, while changes in the tertiary structure were observed in a cuvette with a path length of 10 mm in the region between 260 and 320 nm at a protein concentration of 25 μM. After appropriate blanks had been subtracted, mean residue ellipticities were calculated using the formula $[\theta] = \theta_{obs}\mathrm{MRW}/10cl$, where θ_{obs} is the observed ellipticity

in degrees, MRW is the mean residue weight, c is the concentration of the protein (grams per milliliter), and l is the path length in centimeters (*24*). A mean residue molecular weight of 110 was used. Sensitivities of 1 and 2 m°/cm (millidegree per centimeter) were used for far-UV and near-UV measurements, respectively.

Assay for Enzyme Activity. The hydrolyzing activity of procerain under various conditions of pH or in the presence of a chemical denaturant was monitored using the denatured natural substrate azoalbumin following the procedure described previously (*20*). The enzyme was incubated overnight in denaturants before assays were carried out.

Acid Denaturation of Procerain in the Presence and Absence of Salt. Acid denaturation of procerain was carried out as a function of pH using KCl-HCl (pH 0.5−1.5), Gly-HCl (pH 2.0−3.5), sodium acetate (pH 4.0−5.5), sodium phosphate (pH 6.0−8.0), Tris-HCl (pH 8.5−10.5), and Gly-NaOH (pH 11.0−12.5). Concentrations of all buffers were 50 mM. A stock solution of the protein was added to the appropriate buffer and the mixture was incubated for 24 h at 25 °C. The final pH and concentration of the protein in each sample were measured again. Acid denaturation of procerain was also performed at various pH values in the presence of increasing concentrations of KCl.

ANS Binding Assay. The extent of exposure of hydrophobic surfaces in the enzyme was measured by its ability to bind to the fluorescent dye ANS (*25*). A stock solution of ANS was prepared in methanol, and the dye concentration was determined using an extinction coefficient ϵ of 5000 M^{-1} cm^{-1} at 350 nm (*26*). The protein was incubated with a 100-fold molar excess of ANS for more than 30 min at room temperature in the dark, and the ANS fluorescence was measured. The protein concentration was 1 μM. The excitation wavelength was 380 nm, and emission spectra were collected between 400 and 600 nm. Slit widths of excitation and emission were 10 and 5 nm, respectively.

Quenching of Tryptophan Fluorescence. The quenching of tryptophan fluorescence of procerain with increasing concentrations of anionic (I^-) and cationic (Cs^+) quencher was assessed to estimate the extent of exposure of the buried aromatic amino acids. The samples of the protein with quencher were incubated at 25 °C in the dark for 30 min before fluorescence measurements were taken. An excitation wavelength of 292 nm was used to ensure selective excitation of tryptophan residues. The absorbance of the sample at 292 nm was always kept below 0.06, so no correction of an inner filter effect was necessary. The intensity of the fluorescence at the emission maximum was monitored as a function of the increasing concentration of the quencher. The quenching data were analyzed using the modified Stern−Volmer equation (*27*)

$$F_o/(F_o - F) = 1/K_{sv}[Q]f_a + 1/f_a \quad (1)$$

where F_o and F are the fluorescence intensities of the protein in the absence and presence, respectively, of a given concentration of quencher [Q], K_{sv} is the Stern−Volmer quenching constant, and f_a refers to the fraction of tryptophans accessible to the quencher.

Guanidine Hydrochloride- and Urea-Induced Unfolding. Chemical-induced denaturation of the enzyme, at a given pH, was performed with increasing concentrations of the denaturant. The protein sample was incubated at a desired denaturant concentration for approximately 24 h at 25 °C to attain equilibrium. The final concentrations of the protein and denaturant, in each sample, were determined by spectrophotometry and refractive index measurements, respectively. Data are expressed in terms of the fraction unfolded (F_u) calculated from the equation

$$F_u = (F_{obs} - F_n)/(F_u - F_n) \quad (2)$$

where F_{obs} is the observed value of the signal at a given denaturant concentration and F_n and F_u are the values of native and unfolded protein, respectively. If a standard two-state model is assumed, the GuHCl and urea transitions were fitted to the equation

$$\Delta G_d = \Delta G_w - m_{D-N}D \quad (3)$$

where ΔG_w and ΔG_d are the free energy of the folding in water and at a denaturant concentration D, respectively, m_{D-N} is the slope of the transition (proportional to the increase in solvent-accessible surface area upon the transition from the native to denatured state), and D is the denaturant concentration (*23*).

Thermal Unfolding. Temperature-induced denaturation of the enzyme, under given conditions, was performed as a function of increasing temperature. Protein samples were incubated at the desired temperature for 15 min before the measurements were taken. The actual temperature of the sample in the cuvette was obtained with a thermocouple using a digital multimeter. Occasionally, samples were also checked for any possible aggregation due to heat, by light scattering measurements.

RESULTS

The structure−function relationship and folding behavior of procerain are studied using different spectroscopic and activity measurements. The absorbance spectrum of procerain under native and unfolded conditions shows no significant differences in either the shape or magnitude of intensity (data not shown). Therefore, all the investigations are carried out using circular dichroism (far- and near-UV), fluorescence, and activity as measures of changes.

CD Spectra. The circular dichroism spectra of procerain, in both near- and far-UV wavelength regions, are shown in Figure 1. Near-UV CD spectra provide a measure of the level of interactions of side chain aromatic rings with other groups such as side chain amide carboxylate groups and main chain peptide bonds and are applicable in assessing the tertiary structure of proteins (*28*). In the aromatic region, the CD spectrum of native procerain exhibits a positive peak centered at 276−278 nm (Figure 1A) and a negative band centered at 297−299 nm. At the latter wavelength, tyrosine and phenylalanine residues do not contribute to the CD spectra of proteins (*29*, *30*) and the negative band probably originates from tryptophan residues located in an asymmetrical environment (*31*). The intensity of the negative band at 298 nm is much lower than that of the positive peak at 276−278 nm. This fine stricture suggests that many of the Phe, Tyr, and Try side chains are ordered in the native structure. However, the near-UV CD spectrum of procerain at pH 2.0 as well as in the presence of 6 M GuHCl is featureless,

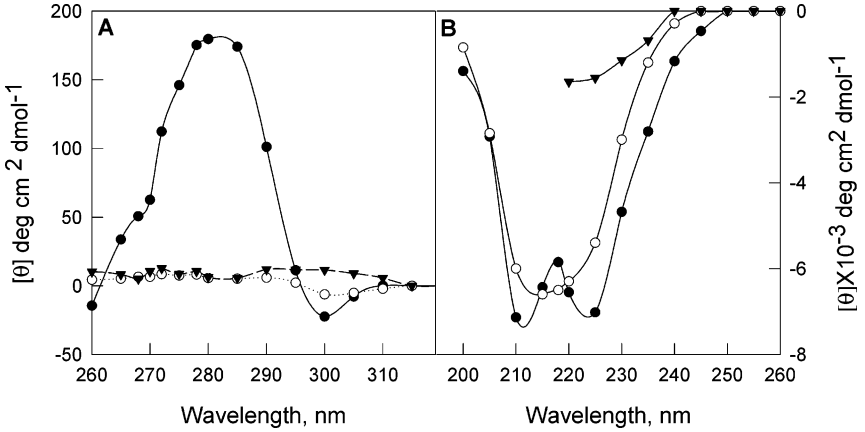


FIGURE 1: (A) Near-UV and (B) far-UV spectra of procerain. The filled circle, empty circle, and filled triangle represent CD spectra of procerain at pH 7.0, at pH 2.0, and in the presence of 6 M GuHCl, respectively. The samples were incubated under the given condition for 24 h at 25 °C before the measurements were taken. The protein concentration was 25 μM for the near-UV and 3.5 μM for the far-UV measurements.

indicating that the aromatic side chains are disordered as expected when the protein unfolds.

In the far-UV region, CD spectra of proteins are particularly sensitive to protein secondary structure. Procerain revealed two well-resolved negative peaks around 220−222 nm and at 208 nm with the signal pronounced little in magnitude at the latter wavelength (Figure 1B). Strong negative ellipticities at 208, 215, and 222 nm suggest that procerain, in the native state, is composed of α-helix and β-sheet-rich regions and seems to belong to the $\alpha + \beta$ class of proteins (*32*). The mean residue ellipticity at 222 nm was $(7.0 \pm 0.25) \times 10^3$ deg cm^2 dmol^{-1}, and this value can be used to determine the α-helicity of a protein using the simple calculation (*33*)

$$\% \text{ α-helicity} = (\theta_{222} - \theta_{min}/\theta_{max} - \theta_{min}) \times 100$$

where θ_{222} is the molar ellipticity of the protein, θ_{min} (2340) is the minimum value of the molar ellipticity at 222 nm calculated for the "unordered" fraction of five proteins, and θ_{max} (30 300) is the maximum value for the ellipticity at 222 nm as measured for the helical fraction of five proteins (*33*). The estimated α-helicity of procerain at neutral pH is approximately 16.6%.

On the other hand, procerain exhibits a negative peak centered at 215−217 nm observed at pH 2.0, indicating the presence of a β-sheet structure with a molar ellipticity value at 222 nm of $(5.5 \pm 0.25) \times 10^3$ deg cm^2 dmol^{-1}. The calculated α-helicity at pH 2.0 was 11.3%, which is significantly lower than that of the native protein.

Secondary and tertiary structural features of the enzyme are completely lost with the disappearance of all the prominent peaks in 6 M GuHCl.

Fluorescence Spectra. Fluorescence spectra provide a sensitive means of characterizing proteins and their conformation. The spectrum is determined chiefly by the polarity of the environment of the tryptophan and tyrosine residues and by their specific interactions. Basically, the fluorescence emission maximum suffers a red shift when chromophores become more exposed to solvent, and the quantum yield of fluorescence decreases when the chromophores interact with quenching agents either in a solvent or in the protein itself (*34*−*37*).

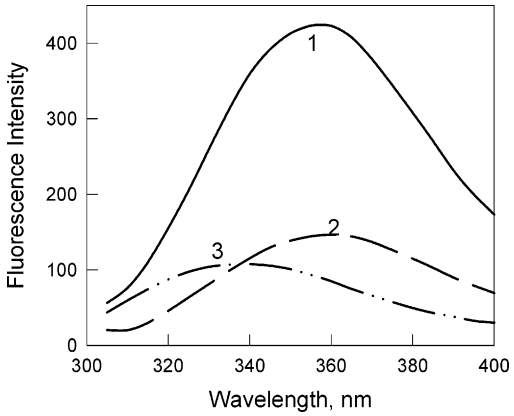


FIGURE 2: Intrinsic fluorescence spectra of procerain. The numbers 1−3 denote spectra at pH 7.0, at pH 2.0, and in the presence of 6 M GuHCl, respectively. The samples were incubated for 24 h at 25 °C before the measurements were taken. The protein concentration was 1 μM.

Intrinsic fluorescence properties of procerain are identical when excited at either 278 or 292 nm (data not shown). Intrinsic tryptophan fluorescence spectra of procerain under neutral conditions, at pH 2.0, and in the completely unfolded state are shown in Figure 2. The intensity of the fluorescence decreases by 75−80% along with a blue shift of 16 nm in the emission maximum from 352 to 336 nm, as the pH is lowered from 7.0 to 2.0, indicating a nonpolar environment of tryptophan residues. Whereas the fluorescence spectrum of completely unfolded procerain in 6 M GuHCl remains similar in shape, the emission maximum suffers a red shift from 352 to 358 nm along with a decrease in fluorescence intensity of 65−70%. This red shift in the wavelength maximum indicates that more tryptophan residues of the protein are exposed to a polar environment which is characteristic of unfolding. Besides, the decrease in fluorescence intensities may be the result of a decreased distance between tryptophan and specific quenching groups, such as protonated carboxyl, protonated imidazole, deprotonated ϵ-amino groups, and tyrosinate, which consequently resulted in quenching of tryptophan fluorescence (*37*).

Acid-Induced Unfolding. The structural and functional changes in procerain upon acid unfolding were followed by far- and near-UV CD, fluorescence, and activity measure-

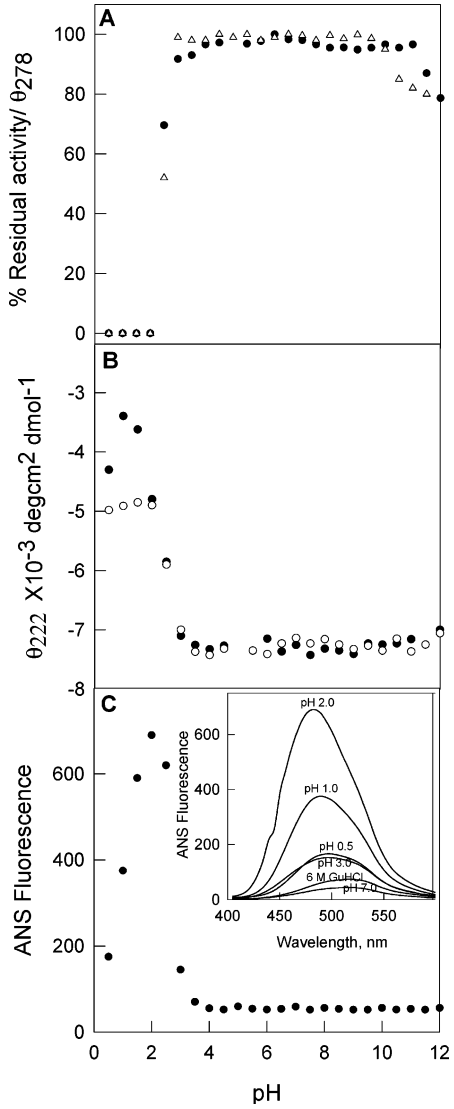


FIGURE 3: pH-induced conformational changes in procerain. (A) The residual proteolytic activity and ellipticity at 278 nm of procerain are denoted with empty triangles and filled circles, respectively. (B) Ellipticity at 222 nm. The filled and empty circles denote the change in ellipticity in the absence and presence of 0.5 M salt, respectively. (C) ANS binding to procerain as a function of pH. The samples were incubated for 24 h at 25 °C before the measurements were taken (see Materials and Methods for details).

ments over a wide pH range (Figure 3). The enzyme retains proteolytic activity in the pH range of 3.0−11.0 and drops only on either side of this pH range. The effect of pH on the proteolytic activity of the enzyme as well on ellipticity at 278 nm is shown in Figure 3B. The changes in ellipticity at 278 nm versus pH follow a bell-shaped curve with a loss of structure beyond pH 3.0 and 11.5. The spectral characteristics are typical of the native state until pH 3.0 with a positive peak at 276−278 nm and a negative band at 297−299 nm. At pH ≤2.0, all the prominent peaks are lost and the spectrum is identical to that in 6 M GuHCl, indicating that the enzyme has lost its tertiary structure (Figure 1A).

The effect of varying pH on the secondary structure of procerain, in the presence and absence of salt, was also studied and is shown in Figure 3B. The far-UV CD spectrum of procerain remains unchanged in the pH range of 3.0−11.5, and the spectra reveal two distinct peaks, one at 222 nm and the other at 208 nm. The unfolding of the enzyme, in the absence of added salt, followed by ellipticity at 222 nm, is noncooperative. A cooperative transition from the native state to an acid-unfolded state occurs in the vicinity of pH 3.0−1.5, and a second transition occurs on further lowering the pH from 1.5 to 0.5. The unfolded state at lower pH exhibiting a reduced secondary structure, loss of tertiary structure, and activity represents the acid-unfolded state of the enzyme (*39*), indicating partial unfolding of the protein molecule. Thus, procerain at pH 1.5−1.0 exists in an acid-unfolded state. Further, addition of acid leads to a second transition between pH 1.5 and 0.5, manifested with an increase in secondary structure, leading to the A state (*39*). Interestingly, a negative peak centered at 215−217 nm was observed in the CD spectra of procerain at pH 2.0, indicating the presence of predominant β-sheet structure.

Besides, in the presence of 0.5 M KCl, pH-induced unfolding of procerain is cooperative as manifested by a single transition (Figure 3B), in which the enzyme passes from the native state to the A state directly without passing through the acid-unfolded state. The secondary structural content of such a salt-induced A state is more ordered than that seen at pH 0.5 in the absence of added salt. The CD spectra of the protein at pH 2.0−0.5, either in the presence or in the absence of 0.5 M KCl, exhibit predominantly β-sheet secondary structure. Some aggregation or salting out of the protein was observed at higher concentrations of KCl (>0.6 M) under similar conditions.

The exposure of any hydrophobic regions, buried inside the enzyme in the native state, on acid unfolding, was also monitored by ANS binding to the protein. The level of ANS binding to procerain at different pH values is shown in Figure 3C. ANS binding to the enzyme is maximal at pH 2.0 in comparison to that in the native and completely unfolded states. The ANS fluorescence intensity increases approximately 12-fold at this pH, and the emission maximum shifts to shorter wavelengths from 515 to 482 nm. However, at a lower pH, a decrease in ANS fluorescence is also observed.

The pH dependence of the intrinsic fluorescence properties of procerain is also monitored. Changes in the fluorescence intensity and emission maximum of the enzyme at various pH values are shown in Figure 4. The pH-induced transition is noncooperative and exhibits a biphasic nature with one transition between pH 4.0 and 6.0 with a midpoint of approximately pH 4.9 and the latter between pH 1.0 and 3.0 with a midpoint of pH 2.2. The shape of the spectrum remains the same at all pH values, but a blue shift of 17 nm in emission maximum along with decrease in fluorescence intensity is observed when pH was reduced from 6.0 to 4.0. In the second transition, a red shift in the wavelength maximum and an increase in fluorescence intensity are observed that may be attributed to unfolding of the protein to the acid-unfolded state. At pH 2.0, the fluorescence spectrum of procerain exhibited a 75−80% decrease in intensity with a blue shift of 16 nm from 352 to 336 nm compared to the native protein at pH 7.0.

Quenching of Tryptophan Fluorescence. Measurement of the extent of quenching of tryptophan fluorescence by an external quencher is very useful in sensing the exposure of tryptophan residues in the molten globule state of a protein (*40*). In the study presented here, such quenching of tryptophan fluorescence by anionic (I^-) and cationic (Cs^+) quenchers is carried out. Generally, a nonionic quencher is

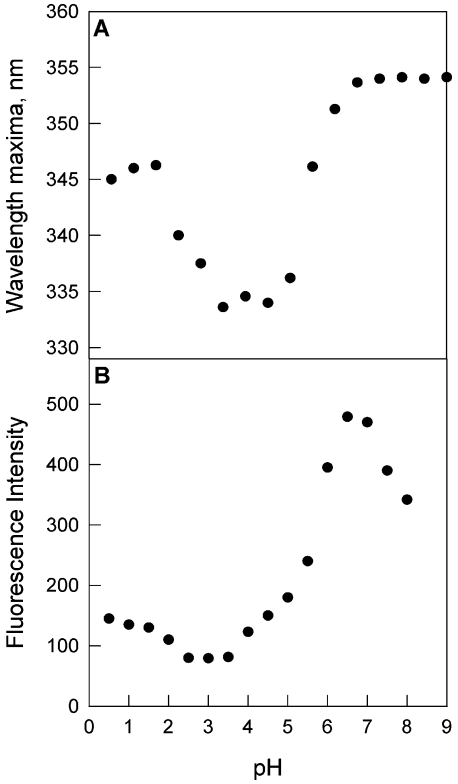


FIGURE 4: Effect of pH on the intrinsic fluorescence of procerain. The wavelength maximum (A) and fluorescence intensity (B) at different pH values are plotted. The protein concentration was 1 μM. The excitation wavelength was 292 nm with slit widths of 10 and 5 nm for excitation and emission, respectively.

used to obtain information about the exposure of the hydrophobic surfaces as a charged quencher does not readily penetrate the hydrophobic interior if the same charged amino acids surround it. On the other hand, if the fluorescence quenching is followed in the presence of an anionic quencher (I^-) or cationic quencher (Cs^+) quencher, independently, at least one, with opposite charge, should penetrate the hydrophobic interior. This approach will provide information about the extent of the exposure of the hydrophobic interior but also the nature of the charge surrounding it. The Stern−Volmer plot of I^- quenching is represented in Figure 5. There are more quenchable fractions in procerain at pH 2.0 than in the native protein under neutral conditions as well as in the presence of 6 M GuHCl. Interestingly, Cs^+, in the experimental range (0−0.2 M), failed to quench the tryptophan fluorescence of procerain either at pH 2.0 or in the presence of 6 M GuHCl. However, at neutral pH, Cs^+ causes 15% quenching (data not shown). No distinguishable changes in either the shape or emission maximum of fluorescence spectra of the protein are observed, with both quenchers, in the concentration range of the quencher that was used, indicating that no structural changes occurred in the protein molecule.

GuHCl-Induced Unfolding. GuHCl-induced changes in procerain under different pH conditions were followed by CD, fluorescence, and activity measurements, and the unfolding was found to be irreversible. Under neutral conditions, GuHCl-induced unfolding, when monitored by changes in the secondary or tertiary structural content of procerain, is cooperative and the transition curves are sigmoidal (Figure 6A). A red shift of 6 nm from 352 to 358

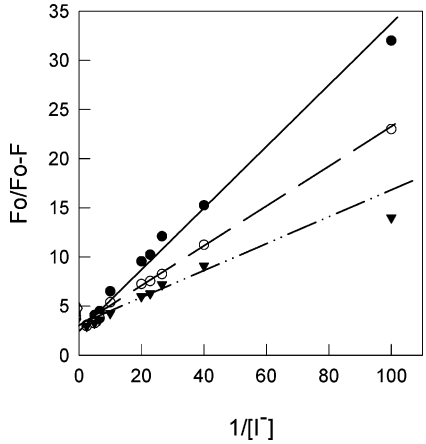


FIGURE 5: Stern−Volmer plot of tryptophan fluorescence quenching by iodide (I^-). Quenching of tryptophan fluorescence intensity of procerain at pH 7.0 (●), at pH 2.0 (○), and in 6 M GuHCl (▼) was carried out with 0.0−0.2 M KI at 25 °C. KCl was added to keep the ionic strength constant. The reciprocal of the *y*-intercept indicates quenchable fractions of fluorescence intensity.

nm in the wavelength emission maximum of intrinsic fluorescence is seen upon chemical-induced unfolding from the native to denatured state. The loss in proteolytic activity upon unfolding coincides with the loss in tertiary and secondary structure reflecting a good correlation between the activity and structural integrity of the molecule. Data that were obtained were normalized and analyzed according to eqs 2 and 3, respectively, as described in Materials and Methods, and the respective thermodynamic parameters along with corresponding transition midpoints were summarized in Table 1.

As the enzyme is structurally stable with no loss in proteolytic activity even after prolonged exposure in the pH range of 3.0−11.5, it was important and interesting to check the structural integrity of the molecule at low pH. Any subtle changes in the structure of the protein should reflect in its behavior toward denaturants. At pH 3.0, GuHCl-induced unfolding of procerain was cooperative but the transition curves were noncoincidental (Figure 6B). The proteolytic activity and tertiary structure are lost initially, followed by the loss of fluorescence and secondary structure. Such noncoincidental transitions indicate the probable existence of intermediate states in unfolding (*41*). Further, the existence of an intermediate in the unfolding pathway of procerain is substantiated by strong ANS binding at 1.6 M GuHCl (inset of Figure 6B). The data obtained by various methods were normalized and analyzed as described above and are summarized in Table 1. The conformational stability of the protein by proteolytic activity and tertiary structure measurement was -3.3 ± 0.2 kcal/mol. Besides, ΔG_{U-N} was found to be -4.7 ± 0.2 kcal/mol when far-UV CD and fluorescence were used as probes.

A further reduction of the pH to 2.0 disrupts the tertiary structure of the protein completely, as seen from near-UV CD, with the complete loss of proteolytic activity. At pH 2.0, the GuHCl-induced denaturation of procerain was noncooperative as shown in Figure 6C. The transition midpoints by far-UV CD and fluorescence wavelength maximum were summarized in Table 1. Moreover, a strong ANS binding to the protein was also observed in this state, which concomitantly decreases with the first transition (inset

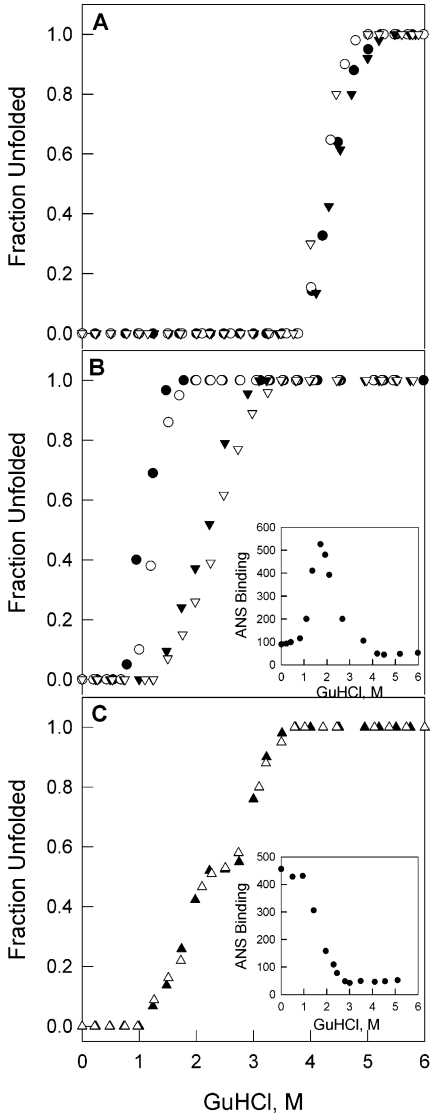


FIGURE 6: Equilibrium GuHCl denaturation of procerain. GuHCl-induced unfolding of procerain at pH 7.0 (A), 3.0 (B), and 2.0 (C) was carried out. The data that were obtained were normalized and analyzed according to eqs 2 and 3, respectively (see Materials and Methods). The filled circles, empty circles, filled triangles, and empty circles denote the activity, near-UV CD, far-UV CD, and fluorescence, respectively. The insets show ANS binding as a function of GuHCl concentration.

of Figure 7C), indicating that most of the hydrophobic surfaces are in the domain that unfolds first.

Urea-Induced Unfolding. Under neutral conditions, urea did not cause any structural perturbations and the enzyme suffers no loss in proteolytic activity. The enzyme is susceptible to urea unfolding at lower pH, and the changes were irreversible. Urea-induced unfolding of procerain at pH 3.0, as followed by near-UV activity, far-UV activity, and fluorescence is shown in Figure 7A. Unfolding transitions, monitored by various methods, are cooperative and coincidental. The data that were obtained were normalized and analyzed according to eqs 2 and 3, and the respective thermodynamic parameters as well as transition midpoints are summarized in Table 1.

Since at pH 2.0 there is no tertiary structure and proteolytic activity, urea-induced changes in the protein are followed by far-UV CD and fluorescence only. The conformational stability of the protein was found to be -1.6 ± 0.1 kcal/mol. The unfolding transitions by different measures are overlapping and cooperative (Figure 7B), and all changes occur between 1.25 and 3.5 M urea with a transition midpoint of 2.2 ± 0.1 M. There was no significant increase in the level of ANS binding in the urea-induced unfolding pathway. However, a marginal ANS binding at 2.0−2.5 M urea (inset of Figure 7B) indicates little exposure of hydrophobic patches. It is important to note that the ANS binding to procerain in the molten globule state or intermediate state is several times higher. Thus, urea-induced unfolding of procerain can be described by a two-state model, which is profoundly different from the unfolding in the presence of GuHCl.

Thermal Unfolding. Temperature-induced unfolding of procerain was incomplete and completely reversible under neutral conditions. At least 30−35% of the native structure of the protein is retained even at a higher temperature (87 °C) as monitored by near-UV CD, far-UV CD, and fluorescence measurements. The reversibility of thermal unfolding of the protein was supported by the fact that the protein regains the native structure after cooling and follows the same unfolding transition if reheated (data not shown). At pH 2.0, the observed properties of procerain agree formally with the definition of the molten globule-like state, while the temperature-induced transition was irreversible and noncooperative (Figure 8) with two transition midpoints at 36 ± 0.5 and 58 ± 0.5 °C.

DISCUSSION

The physicochemical characteristics of procerain have been elucidated to gain insight into the structure−function relationship of the enzyme in view of its stability toward pH and denaturants. Circular dichroism, intrinsic fluorescence, and activity measurements were used to study the solution conformation of procerain as well as its unfolding by various denaturants. Procerain belongs to the $\alpha + \beta$ class of proteins as seen by CD, and members of this class present separate α-helix and β-sheet-rich regions in the molecular structure (*32*). This is also actually known to be the case in papain, whose molecular structure consists of an all α-helix domain and an antiparallel β-sheet domain (*42*). Thus, from the CD data of procerain, it can be concluded that it probably belongs to the papain superfamily. However, CD spectra and intrinsic fluorescence properties are distinguishable from those of other members of the same family and behave differently when protein unfolding is followed. The equilibrium unfolding of procerain by various denaturants reveals the high stability of the enzyme. Procerain retains activity and other structural parameters in the presence of 8 M urea as well as in the presence of 3.75 M GuHCl, indicating the high rigidity of the molecule. These observations make it unique among other reported plant cysteine proteases, probably suggesting a different folding pattern and structural integrity for procerain.

The fluorescence emission spectrum of procerain is identical when it is excited at 278 and 295 nm. Thus, it appears that the fluorescence of tyrosine is effectively quenched by tryptophan. The fluorescence emission maximum of $\sim 352 \pm 1$ nm, in the native state, indicates that the excitable chromophore(s) is in a substantially hydrophilic environment in a rigid conformation as in the case of some

Table 1: Unfolding Parameters of Procerain[a]

condition	denaturant	method	transition midpoint (C_m) (M)	ΔG_{U-N} (kcal/mol)	m_{U-N} (kcal mol^{-1} M^{-1})
pH 7.0, 25 °C	GuHCl	CD $[\theta]_{222}$	4.4 ± 0.1	−12.7 ± 0.5	−2.7 ± 0.1
		CD $[\theta]_{278}$	4.3 ± 0.1	−13.0 ± 0.5	−2.6 ± 0.1
		fluorescence	4.4 ± 0.1	−12.8 ± 0.5	−2.7 ± 0.1
		activity	4.3 ± 0.1	−12.9 ± 0.5	−2.9 ± 0.1
pH 3.0, 25 °C	urea	CD $[\theta]_{222}$	2.3 ± 0.1	−4.3 ± 0.2	−1.5 ± 0.1
		CD $[\theta]_{278}$	2.2 ± 0.1	−4.5 ± 0.2	−1.4 ± 0.1
		fluorescence	2.3 ± 0.1	−4.4 ± 0.2	−1.4 ± 0.1
		activity	2.3 ± 0.1	−4.6 ± 0.1	−1.5 ± 0.1
	GuHCl	CD $[\theta]_{222}$	2.2 ± 0.1	−4.7 ± 0.2	−1.7 ± 0.1
		CD $[\theta]_{278}$	1.0 ± 0.1	−3.2 ± 0.2	−3.2 ± 0.1
		fluorescence	2.3 ± 0.1	−4.6 ± 0.2	−1.6 ± 0.1
		activity	1.2 ± 0.1	−3.4 ± 0.2	−3.3 ± 0.1
pH 2.0, 25 °C	urea	CD $[\theta]_{222}$	2.1 ± 0.1	−1.5 ± 0.1	−1.9 ± 0.2
		fluorescence	2.2 ± 0.1	−1.7 ± 0.1	−1.9 ± 0.2
	GuHCl	CD $[\theta]_{222}$	1.7 ± 0.1 (C_{m1})		
			3.0 ± 0.1 (C_{m2})	not measured as unfolding	
		fluorescence	1.6 ± 0.1 (C_{m2})	is a three-state process	
			3.0 ± 0.1 (C_{m2})		

[a] The calculated thermodynamic parameters are approximate values, as it does not take into account some interactions present in native procerain that are irreversibly lost upon unfolding.

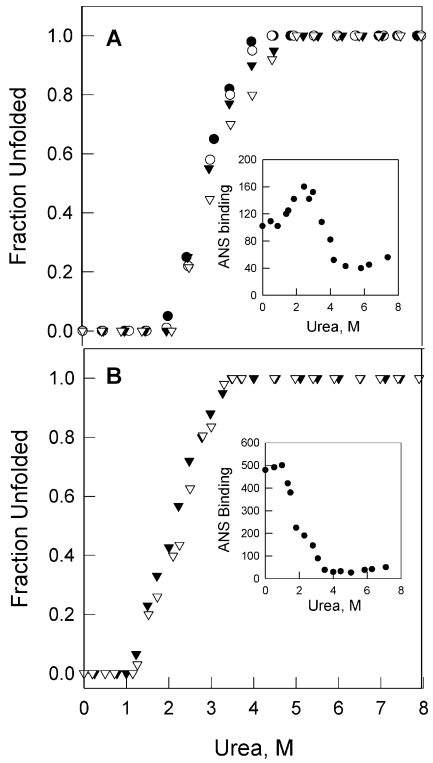


FIGURE 7: Equilibrium urea denaturation of procerain. Urea-induced unfolding of procerain at pH 3.0 (A) and 2.0 (B). The filled circles, empty circles, filled triangles, and empty triangles denote activity, near-UV CD, far-UV CD, and fluorescence wavelength maxima, respectively. The insets show ANS binding to protein as a function of urea concentration. The data that were obtained were normalized and analyzed according to eqs 2 and 3, respectively (see Materials and Methods).

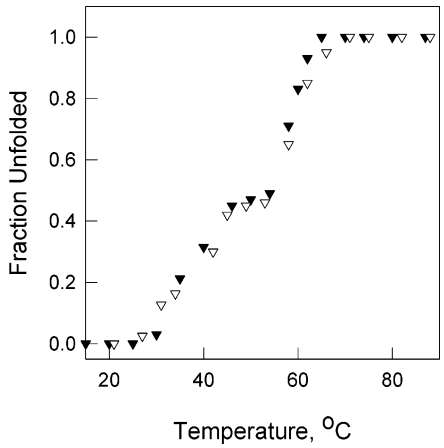


FIGURE 8: Thermal unfolding of procerain in the molten globule state. The filled and empty triangles denote activity, near UV-CD, far-UV CD, and fluorescence wavelength maxima, respectively. The spectra were recorded after incubation for 15 min at each temperature.

other proteins such as TraY (*43*) and ervatamin C (*19*). The fluorescence spectrum of the completely unfolded enzyme in 6 M GuHCl remains similar in shape, but the emission maximum shifts from 352 ± 1 to 358 ± 1 nm with a 65−70% decrease in the fluorescence intensity, indicating that more tryptophan residues of procerain are exposed to a polar environment.

The pH-induced transition of procerain, when followed by intrinsic fluorescence, is noncooperative and exhibits a biphasic nature with one transition between pH 6.0 and 4.0 with a pH midpoint of approximately 4.9 and the latter between pH 3.5 and 1.5 with a midpoint of pH 2.2. The shape of the spectra is same at all pH values. Besides, a blue shift of 15−17 nm and a decrease in fluorescence intensity occur when the pH is lowered from 6.0 to 4.0. Such a blue shift in the wavelength maximum is attributed to a change in the microenvironment around excitable tryptophan(s) without a change in the overall structure of the protein as activity and tertiary structure are preserved in this pH range. The observed decrease in tryptophan fluorescence intensity may be due to the proximity to specific quenching groups formed after a decrease in pH, such as protonated carboxyl, protonated imidazole, deprotonated ϵ-amino groups, and tyrosinate anion (*37*). In the latter transition, a red shift in the wavelength maximum along with an increase in fluorescence intensity in the pH range of 3.5−1.5, which concurrently occurs with a loss of secondary structure, may be attributed to conversion of the protein to the acid-unfolded state.

The acid-induced unfolding transition of procerain as followed by measurement of far-UV CD is also noncoopera-

tive with two transitions. The first transition, in the pH range of 3.0−1.5, appears to involve a conversion of the native-like state to a somewhat collapsed unfolded state (acid-unfolded state) with some residual secondary structure followed by the second transition corresponding to the formation of additional secondary structure at a lower pH which drives the protein into the A state. The molten globule state was populated at pH 2.0, in the transition from the non-native state at pH 3.0 (N′) to the acid-unfolded state at pH 1.0−15. In this state, a very high level of ANS binding and significant secondary structure are observed, whereas the acid-unfolded state seen in the pH range of 1.0−1.5 has less secondary structure and weaker ANS binding than the molten globule state (at pH 2.0) as shown in Figure 3. In the presence of added salt, however, the enzyme switches directly from the native state to the A state. This behavior is typical of the type IC proteins (*39*) like papain and ribonuclease A. Besides, other cysteine proteases studied in our laboratory also exhibited a similar behavior (*17*, *19*). In this class of proteins, addition of a stronger acid adds both protons and anions to the solution. As the protein is already, maximally, protonated in the acid-unfolded state, such addition of more protons has no effect on its ionization. Under the condition of extreme pH, the main force unfolding the protein is the repulsion between the charged groups on the protein molecule. Therefore, the presence of a high concentration of salts will act as counterions. These counterions interact with the charge groups and weaken the repulsions, permitting other forces to favor folding.

Moreover, pH-induced conformational changes were also manifested by ANS binding. ANS binds maximally to procerain at pH 2.0 relative to the native or completely unfolded protein. However, at lower pH values, there is a decrease in the magnitude of ANS binding. A 12-fold increase in ANS fluorescence at pH 2.0 and a shift in the emission maximum to a shorter wavelength (482 nm) compared to that of the native state (515 nm) suggest an increase in the hydrophobicity of the molecule. At this pH, the molecule becomes less compact than the native molecule, and more hydrophobic binding sites are accessible to ANS. The observed negative peak centered on 215−217 nm in far-UV CD spectra indicates predominant β-sheet structure of procerain at this pH. The magnitude of molar ellipticity at 222 nm in this state was $(5.5 \pm 0.25) \times 10^3$ deg cm^2 dmol^{-1}, which is lower than that of the native protein [$(7.0 \pm 0.25) \times 10^3$ deg cm^2 dmol^{-1}] at neutral pH. In terms of α-helical content, at neutral pH procerain has 16.6% α-helix, which is reduced to 11.3% in the molten globule state. The change in the overall ellipticity at pH 2.0 may be due to the partial unfolding of the molecule, especially α-rich regions, as under similar solvent conditions β-rich regions are known to be more stable in the case of papain (*15*, *16*). The conclusion of the relative stability of the α-rich and β-rich region is supported by our observation also as, if the β-rich region would have melted first the CD spectrum in the molten globule-like state (pH 2.0) would have been typical for an α-helix instead of typical for a β-sheet with a decreased ellipticity at 222 nm.

The observed properties of procerain at pH 2.0 agree formally with the definition of the molten globule state as it contains no tertiary structure and activity but retains substantial non-native secondary structure with strong ANS binding. Though most of the observed molten globule states, in different proteins, exhibit an α-helical conformation, a few proteins exhibit a β-conformation in the molten globule state as in papain (*16*), human carbonic anhydrase B (*44*), and bovine pancreatic ribonuclease (*45*). The fluorescence spectrum of procerain at pH 2.0 exhibited a 75−80% decrease in intensity with a blue shift of 18 nm from 354 to 336 nm compared to that of the native protein at pH 7.0, indicating a nonpolar tryptophan environment. The transition of procerain from the native to molten globule-like state is reversible over a period of 48 h, and all the fluorescence properties of such a renatured protein are regained and are the same as those of the native protein. At higher procerain concentrations (>0.05 mg/mL), aggregation of the protein upon renaturation results. The nonspecific hydrophobic interactions are expected to play a major role in the aggregation phenomenon of proteins, and the same rationale may also be employed for the aggregation of procerain. This aggregation prone nature of procerain further confirms that in the molten globule state hydrophobic surfaces are exposed.

The fluorescence energy transfer studies of the molten globule state of procerain clearly indicate the proximity of some tryptophan(s) with bound ANS. Each molecule of procerain has six strong ANS binding sites with a dissociation constant of 170 μM (data not shown)

Exposure of aromatic groups to the solvent, in the molten globule state of procerain, is also demonstrated by quenching studies as the extent of quencher penetration through the protein core provides information about protein dynamics, and thus provides a probe for monitoring the unfolding and folding of a protein. The quenching data of anionic quencher (I^-) can be fitted to a straight line and confirm that this quencher has accessibility to almost all the fluorescent tryptophan residues of the protein. The Stern−Volmer plot indicates that the tryptophan residues, which were inaccessible to the anionic quencher (I^-) in the native state, become highly accessible in the molten globule state. Since the charged quenchers do not readily penetrate the hydrophobic interior of the protein, the quenching by iodide indicates that the molten globule state of procerain is a loosely packed intermediate, which has largely exposed and hydrated tryptophan residues.

Interestingly, Cs^+ in the experimental concentration range (0−0.2 M) failed to quench any tryptophan fluorescence of the protein in the molten globule state or in the presence of 6 M GuHCl. However, at neutral pH, some quenching (15%) is observed. This indicates that the tryptophan residue is surrounded by amino acids, which are positively charged at pH 2.0, and Cs^+ is being repelled. No distinguishable change in the shapes and wavelength maximum of fluorescence spectra is observed at the concentration of quencher that was used, suggesting that the protein conformation is not being affected.

The temperature- and GuHCl-induced unfolding of procerain in the molten globule-like state to unfolded state is noncooperative, contrary to the cooperative unfolding seen under neutral conditions. The observed cooperativity in unfolding of the protein, at neutral pH, is due to the integrity of the molecule due to side chain packing. This implies that disruption of the tertiary packing is necessary for noncooperative transitions observed in the molten globule-like state. GuHCl- and temperature-induced unfolding of procerain in

the molten globule-like state indicates the existence of two domains in the molecular structure of the protein, which unfold independently, and each transition corresponds to unfolding of one domain. Upon closer examination of the temperature and GuHCl transition of procerain in the molten globule state, at 45−55 °C or 2.25−2.75 M GuHCl, one domain of procerain is unfolded while the other is intact. This interpretation is based on the fact that, under these conditions, the first transition corresponds to the completion of unfolding of one domain while the other is not yet started. Therefore, it can be safely concluded that at 45−55 °C or 2.25−2.75 M GuHCl the protein exists in a state where the α-rich domain is unfolded. Besides, ANS binding to procerain in the molten globule-like state, at pH 2.0, ends parallel with the first transition, suggesting that most of the hydrophobic surfaces exposed are in the domain that unfolds first.

GuHCl-induced unfolding of procerain at neural pH was cooperative, and the conformational stability of the protein (ΔG_{U-N}) and m_{U-N} by various methods were -12.75 ± 0.5 kcal/mol and -2.8 ± 0.2 kcal mol^{-1} M^{-1}, respectively. The enzyme is structurally and functionally stable up to 3.75 M GuHCl, and the transition midpoint by various measures is 4.3 ± 1 M. The loss in proteolytic activity upon unfolding coincides with the loss in tertiary and secondary structure reflecting a good correlation between the activity and structural integrity of the molecule. The observed cooperativity of unfolding, as followed by various methods, is an indication that they are probing structural changes in the same region of the protein molecule or changes occurring concomitantly in all parts. Temperature-induced unfolding of procerain was incomplete under neutral conditions as 30−35% of the native structure is retained even at 87 °C, which is completely reversible. The reversibility of thermal unfolding was supported by the fact that the protein regains its native structure after cooling and follows the same unfolding transition if reheated. However, temperature-induced unfolding of procerain at the lower pH values that were studied (3.0 and 2.0) was irreversible.

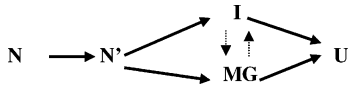
The effect of denaturants was more pronounced at very low pH values. At pH 3.0, temperature-induced unfolding is cooperative and the transitions are coincidental. On the other hand, GuHCl-induced unfolding of the enzyme under similar conditions was cooperative, and the transitions seen by various measures are noncoincidental. The proteolytic activity and tertiary structure are lost first followed by the loss of fluorescence and secondary structure. Besides, the m_{U-N} value determined from near-UV and activity transitions is significantly higher than the value obtained from fluorescence and far-UV curves. This indicates a discrete intermediate state exists in the chemical-induced unfolding pathway between the native state and the denatured state. Further, strong ANS binding to protein at 1.6 M GuHCl substantiates the idea of the existence of an intermediate state in turn, indicating that the state of the molecule at pH 3.0 is different from the native state (we have assigned it as the non-native state or N′ state). It is important to note that in the presence of 1.6 M GuHCl the protein does not show any tertiary structure or activity but exhibits substantial secondary structure. Further, the value of ΔG_{U-N} (ca. −4.8 kcal/mol), determined using far-UV CD and fluorescence transitions, was higher and likely to reflect the conversion of the non-native state at pH 3.0 to the denatured state. Similarly, the conformational stability, obtained by analysis of near-UV CD and activity curves, was lower (ca. −3.3 kcal/mol) and likely to reflect the conversion of the non-native state at pH 3.0 to the intermediate state. Therefore, the conformational stability of the intermediate state was approximately −1.5 kcal/mol, which is the difference between the above two measurements.

On the other hand, procerain retains all the activity as well as structure in the presence of 8 M urea under neutral conditions and is susceptible to urea-induced unfolding at lower pH values. At both pH 3.0 and 2.0, urea-induced unfolding of procerain is cooperative and the transition curves obtained by various measures are coincidental. Since urea-induced unfolding of the molten globule-like state (pH 2.0) is a two-state process, the resultant ΔG_{U-N} (ca. −1.6 kcal/mol) is a measure of the conformational stability of the protein in this state. Overall, observations manifest that the unfolding of procerain by GuHCl and urea is dramatically different with different unfolding mechanisms. Such differences in the unfolding pathway may be attributed to the ionic nature of GuHCl. As GuHCl is an electrolyte with a pK_a of ∼11, which means that at a pH below this the GuHCl molecule will be present in a fully dissociated form, i.e., as Gu^+ and Cl^-. The presence of these ions would influence the stabilizing and destabilizing properties of proteins (*46*), resulting in different unfolding pathways.

Further, under neutral conditions, the conformational stability of procerain (ΔG_{U-N}) as determined by various methods was -12.75 ± 0.5 kcal/mol. It has been shown in chymopapain that a number of salt bridges provide stability for the protein under neutral conditions. At lower pH values, because of an increased level of protonation of acidic groups, many salt bridges may be disrupted as they involve ionizable groups, resulting in the decreased stability of the protein (*47*). The observed decrease in the stability of procerain at pH 3.0 ($\Delta\Delta G = 8.25$ kcal/mol) suggests that the salt bridge may be important in determining the stability of this protein. It is important to note that at pH 3.0 all salt bridges will be broken due to protonation of acidic groups. Thus, it appears that the breakage of electrostatic interactions at pH 3.0 is key in the formation of the intermediate state observed in the GuHCl-induced unfolding pathway. Besides, no significant difference in the value of ΔG_{U-N} is observed upon urea denaturation.

The conformational stability and secondary structure content of the intermediate at 1.6 M GuHCl and pH 3.0 are almost equal to those of the molten globule state, whereas both states lack activity and tertiary structure. Thus, one may conclude that these intermediates are the same and populated under different conditions as the idea can be substantiated by the higher level of ANS binding to both states. However, the protein in the molten globule state is a predominantly β-sheet structure, whereas the intermediate state (I) is predominantly α-helical, manifesting a major difference in the structure of the protein in these two states. Therefore, it is not safe to conclude that the two states are the same. Since the conformational stability of the protein in the two intermediates is the same, they may be located on parallel folding routes. On the basis of the above results and the discussion presented above, a model of unfolding of procerain can be presented as given in Scheme 1.

Scheme 1: Proposed Unfolding Pathway of Procerain[a]



[a] N, N′, I, MG, and U represent the native state at neutral pH, the non-native state at pH 3.0, the intermediate at pH 3.0 in the presence of 1.6 M GuHCl, the molten globule-like state, and unfolded state, respectively.

In the study presented here, it is established that procerain unfolds through an intermediate state and the protein unfolds sequentially. Similar unfolding behavior is observed in the case of other cysteine proteases, such as ervatamin B, ervatamin C, and papain, studied in our laboratory as model systems for investigating the folding of proteins that belong to the papain superfamily (*16*−*19*). However, the conditions of the presence of intermediates and their nature vary with each protein. To a first approximation, some generalization for the folding behavior of plant cysteine proteases of the papain superfamily can be drawn. This class of proteins unfolds through intermediates, and the two domains in the molecular structure unfold sequentially. However, the properties and the conditions where intermediates are populated may be different, depending on the stability and structural integrity of the protein molecule.

REFERENCES

1. Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 67−241.
2. Kim, P. S., and Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631−660.
3. Ptitsyn, O. B. (1995) *Adv. Protein Chem. 47*, 83−229.
4. Gast, K., Zirwer, D., Muller-Frohne, M., and Damaschun, G. (1998) *Protein Sci. 7*, 2004−2011.
5. Dolgikh, D. A., Kolomiets, A. P., Bolotina, I. A., and Ptitsyn, O. B. (1984) *FEBS Lett. 165*, 88−92.
6. Dolgikh, D. A., Abaturov, L. V., Bolotina, I. A., Brazhnikhov, E. V., Bychkova, V. E., Gilmanshin, R. A., Lebedev, O. Y., Semisotnov, G. V., Tiktopulo, E. I., and Ptitsyn, O. B. (1985) *Eur. Biophys. J. 13*, 109−121.
7. Ptitsyn, O. B. (1987) *J. Protein Chem. 6*, 273−293.
8. Semisotnov, G. V., Roddionov, N. A., Kutyshenko, E. J., Blank, V. P. B., and Ptitsyn, O. B. (1987) *FEBS Lett. 244*, 9−13.
9. Hartl, F. U., Hlodam, R., and Langer, T. (1994) *Trends Biochem. Sci. 9*, 20−25.
10. Booth, D. R., Sunde, M., Bellotti, V., Robinson, C. V., Hutchinson, M. L., Fraser, P. E., Hokins, P. N., Dobson, C. M., Radford, S. E., Blake, C. C. F., and Pepys, M. B. (1997) *Nature 385*, 787−793.
11. Lim, W. A., Farruggio, D. C., and Sauer, R. T. (1992) *Biochemistry 31*, 4324−4333.
12. Matthews, J. M., Norton, R. S., Hammacher, A., and Simpson, R. J. (2000) *Biochemistry 39*, 1942−1950.
13. Edwin, F., and Jagannadham, M. V. (2000) *Biochim. Biophys. Acta 1492*, 69−82.
14. Edwin, F., and Jagannadham, M. V. (2002) *Biochem. Biophys. Res. Commun. 290*, 1441−1446.
15. Sharma, Y. V., and Jagannadham, M. V. (2003) *Protein Pept. Lett. 10*, 83−90.
16. Edwin, F., and Jagannadham, M. V. (1998) *Biochem. Biophys. Res. Commun. 252*, 654−660.
17. Sundd, M., Kundu, S., and Medicherla, J. V. (2002) *J. Biochem. Mol. Biol. 35*, 143−154.
18. Kundu, S., Sundd, M., and Jagannadham, M. V. (2002) *J. Biochem. Mol. Biol. 35*, 155−164.
19. Kundu, S., Sundd, M., and Jagannadham, M. V. (1999) *Biochem. Biophys. Res. Commun. 264*, 635−642.
20. Dubey, V. K., and Jagannadham, M. V. (2003) *Phytochemistry 62*, 1057−1071.
21. Garel, J. R. (1992) in *Protein Folding* (Creighton, T. E., Ed.) pp 405−454, Freeman, New York.
22. Dobson, C. M. (1992) *Curr. Opin. Struct. Biol. 2*, 6−12.
23. Pace, C. N. (1990) *Trends Biotechnol. 8*, 93−98.
24. Balasubramanian, D., and Kumar, C. (1976) *Appl. Spectrosc. Rev. 11*, 223−286.
25. Semisotnov, G. V., Rodionova, N. A., Razgulyaev, O. I., Uversky, V. N., Gripas, A. F., and Gilmanshin, R. I. (1991) *Biopolymers 31*, 119−128.
26. Khurana, R., and Udgaonkar, J. B. (1994) *Biochemistry 33*, 106−115.
27. Lehrer, S. S. (1972) *Biochemistry 11*, 4120−4131.
28. Sears, D. W., and Beychok, S. (1973) in *Physical properties and techniques of protein chemistry. Part C* (Leach, S. J., Ed.) pp 445−593, Academic Press, New York.
29. Strickland, E. H., Hortwiz, J., and Billups, C. (1969) *Biochemistry 8*, 3205−3213.
30. Strickland, E. H. (1974) *CRC Crit. Rev. Biochem. 2*, 113−175.
31. Solis-Mendiola, S., Arroyo-Reyna, A., and Hernandez-Arana, A. (1992) *Biochim. Biophys. Acta 1118*, 288−292.
32. Manavalan, P., and Johnson, W. C. (1983) *Nature 305*, 831−832.
33. Chen, Y. H., Yang, J. T., and Martinez, H. M. (1972) *Biochemistry 11*, 4120−4131.
34. Ptitsyn, O. B. (1992) in *Protein Folding* (Creighton, T. E., Ed.) pp 243−300, W. H. Freeman, New York.
35. Cairoli, S., Lametti, S., and Bonomi, F. (1994) *J. Protein Chem. 13*, 347−354.
36. Lametti, S., Cairoli, S., De Gregori, B., and Bonomi, F. (1995) *J. Agric. Food Chem. 43*, 53−58.
37. Halfman, C. J., and Nishida, T. (1971) *Biochim. Biophys. Acta 243*, 294−303.
38. Lametti, S., De Gregori, B., Vecchio, G., and Bonomi, F. (1996) *Eur. J. Biochem. 237*, 106−112.
39. Fink, A. L., Calciano, L. J., Goto, Y., Kurotsu, T., and Palleros, D. R. (1994) *Biochemistry 33*, 12504−12511.
40. Eftink, M. R., and Ghiron, C. A. (1981) *Anal. Biochem. 114*, 119−227.
41. Wong, K. P., and Tanford, C. (1973) *J. Biol. Chem. 248*, 8519−8523.
42. Kamphuis, I. G., Kalk, K. H., Swarte, M. B., and Drenth, J. (1984) *J. Mol. Biol. 179*, 233−256.
43. Schildbach, J. F., Robinson, C. R., and Sauer, R. T. (1998) *J. Mol. Biol. 273*, 1329−1333.
44. Jagannadham, M. V., and Balasubramanian, D. (1985) *FEBS Lett. 188*, 326−330.
45. Kumar, T. K. S., Subbiah, V., Ramakrishna, T., and Pandit, M. W. (1994) *J. Biol. Chem. 269*, 12620−12625.
46. Mayr, L. M., and Schmid, F. X. (1993) *Biochemistry 32*, 7994−7998.
47. Solis-Mendiola, S., Gutierrez-Gonzalez, L. H., Arroyo-Reyna, A., Padilla-Zuniga, J., Rojo-Dominguez, A., and Hernandez-Arana, A. (1998) *Biochim. Biophys. Acta 1338*, 363−372.

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