Effect of urea at lower concentration on the structure of papain — Formation of a stable molten globule and its characterization

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Effect of lower concentrations of urea on papain was monitored by optical spectroscopy, calorimetry and partial specific volume measurements. At lower concentrations of urea, papain exhibits a different structure and showed an increase in the intensity of circular dichroic (CD) spectra as compared to the native molecule. At lower concentrations (0.2-1.5 M) of urea, binding of 8-anilino-naphthalene sulfonic acid (ANS) to the papain molecule was higher; at 0.5 M, there was about 50% increase in ANS binding. Both calorimetric and spectroscopic studies indicated an increased thermal stability of the molecule at lower concentrations. At 0.5 M urea concentration, the apparent thermal denaturation temperature increased from a control value of 83 ± 1°C to 86 ± 1°C. At isopotential conditions, the partial specific volume of papain was found to be higher in presence of lower concentrations of urea, than the native protein or unfolded molecule. The preferential interaction parameter (δg3/8g2 h) showed a negative value in the presence of lower concentrations of urea (0.2-2 M), which was maximum at 1 M urea with a value of -0.019 g/°. Above 3 M urea, the preferential interaction parameter was positive.

Unfolding of small compact proteins is defined well by a simple 2-state transition, i.e., in these proteins the probability of all the intermediate states between native and unfolded state is very low and they appear as a single cooperative unit. But, it is now generally accepted that unfolding/refolding of a number of proteins involves different steps, i.e., under certain conditions different states have been observed that do not appear to be native or completely unfolded. Equilibrium unfolding studies of bovine and human α-lactalbumins demonstrated the existence of equilibrium intermediates between native and unfolded states. Similar type of intermediates was observed in the case of carbonic anhydrase B and α-lactalbumin. These states were termed as molten globule states by Ogahushi and Wada. These molten globule states exhibit substantial secondary structure and lacking tertiary structure.

Other types of intermediates include those where a domain folds independently and adopts both native secondary and tertiary structure within the folding units. Multistate behaviour has been detected in equilibrium studies of the unfolding of many proteins like α subunit of tryptophan synthase, organophosphorus hydrolase and α subunit of bacterial luciferase. Characterization of these intermediate states of proteins, wherein only certain fraction of the native folding interactions may be present is of importance to understand the nature of protein folding.

Papain, a cysteine protease is a monomeric protein having 212 amino acid residues with a molecular weight of 23400. The molecule is folded into two domains forming cleft at the surface of the enzyme and the active site residues viz., Cys-25, His-159 and Asn-175 are present in this cleft. The left domain which comprise residues 10-111 and 208-212 is mainly made up of α helical structure, while the right domain consists of residues 1-9 and 112-207 is mainly made up of anti parallel β structure. Earlier studies have shown that both the domains in papain will unfold independently. Sathish and Prakash have also demonstrated that two domains unfold independently.
independently during the unfolding process. Thus, the two domains could be effectively treated as two separate polypeptide in terms of denaturation profile. Further, Edwin and Jagannadhams have shown the formation of molten globule state under acidic conditions in papain.

However, the detailed information on the behaviour of papain in presence of lower concentrations of denaturants is not available. It is assumed that partial specific volume in case of molten globule state would be much greater than that of native or unfolded state. Even though molten globule state has been observed in large number of proteins, none of the reports have established the mechanism of interaction of urea at these concentrations with protein. The transition between preferential hydration and preferential interaction is reported for the first time in the case of ribonuclease by Timasheff. Thus, in order to understand the unfolding pathway of papain, studies were carried out at lower as well as higher urea concentrations. We report here the results of spectroscopic, calorimetric and densitometric studies.

Materials and Methods
Papain (two-times crystallized, lot # P 4762), urea, benzoyl-L-arg-p-nitroanilide, dimethyl sulphoxide, and cysteine hydrochloride were procured from Sigma Chemical Company, St. Louis, Missouri, USA. All other chemicals were of analytical grade. Dialysis membrane of 23 mm flat width and 6000-8000 molecular weight cut-off was obtained from Spectrum Inc., Houston, Texas, USA.

Unfolding experiments
Unfolding of papain was studied by incubating or dialyzing papain ($2.12 \times 10^{-6}$ M) with various concentrations of denaturants for 24 hr in 0.02 M acetate buffer, pH 5.6. The extent of unfolding was monitored either by measurement of enzyme activity, change in the emission maximum or change in the ellipticity values at 222 nm.

Refolding experiments
Refolding was performed using papain that had been completely unfolded by a 24-hr incubation with 8 M urea. The completely unfolded papain was either diluted 1:40 times into the appropriate concentration of urea, and the mixture was incubated for 24 hr or alternatively the completely unfolded protein was dialyzed against desired concentration of urea for 24 hr with at least three changes of the dialysate.

Fluorescence emission spectrum
Protein solution of concentration $2.12 \times 10^{-6}$ M treated with different concentrations (0.2-8 M) of urea was used for fluorescence measurements. Fluorescence emission spectrum was recorded with a Shimadzu RF-5000 automatic recording spectrofluorophotometer equipped with a thermostated cuvette holder. The protein was excited at 280 nm and the emission was recorded at 300-400 nm.

ANS binding
Papain was treated with different concentrations (0.2-8 M) of urea for 24 hr, thus equilibrated with different concentration of denaturants and then treated with freshly prepared ANS. The mixture was incubated at 37°C for 3 hr with constant stirring in a Queue orbital shaker. A protein concentration of $2.12 \times 10^{-6}$ M and ANS concentration in the final mixture was $2.18 \times 10^{-3}$, such that there is a large excess of ANS in solution. The binding of the hydrophobic dye ANS to protein was monitored by measuring the fluorescence intensity at 470 nm after exciting at 375 nm.

Enzyme activity measurements
Enzyme activity of papain was quantified by measuring its ability to cleave an amide bond in a small molecular weight synthetic substrate, benzoyl-L-arg-p-nitroanilide. The assay was based on the method adopted by Arnon. All the assays were performed in 0.02 M acetate buffer, pH 5.6 after incubating for 4 hr with desired concentrations (0.01-1 M) of urea.

Thermal denaturation studies
The effect of different concentration of urea (0.2-2 M) on the thermal denaturation profile of papain was studied using a Gilford Response-II UV-visible spectrophotometer equipped with six-position thermostated cuvette manifold. The thermal unfolding of papain was monitored by recording absorbance at 287 nm as a function of temperature in the range of 30-95°C. Data were stored and analyzed using specific software supplied with the instrument. From the thermal denaturation profile, fraction of papain unfolded was calculated using standard equation.

Circular dichroic (CD) spectrum
Far-ultraviolet circular dichroic studies were performed from 200 to 260 nm using a Jasco J-20C Spectropolarimeter calibrated with d-10-camphorsulfonic acid. To measure the CD spectrum
of papain, samples were scanned from 200-260 nm. The blank spectra without enzyme was run and appropriate corrections incorporated in the final spectra. Protein concentration of $7 \times 10^{-6}$ M was dialyzed exhaustively for 24 hr versus different concentrations (0.2-5 M) of urea. The solution was centrifuged at 6000xg for 30 min. and the supernatant was used for CD spectral measurements. The mean residue ellipticities were calculated using a mean residue weight of 110.80 for papain from the amino acid sequence data.\textsuperscript{21}

**Differential scanning calorimetric (DSC) measurements**

All the DSC experiments were performed on a MicroCal MC-2 Ultrasensitive Differential Scanning Calorimeter. Protein solutions were exhaustively dialyzed against different concentrations (0.2-4 M) of urea for a minimum of 24 hr. The final dialysate was used for reference cell. For all the experiments, a protein concentration of $2 \times 10^{-4}$ M was used. Concentrations of protein samples were calculated from the absorbance at 278 nm by using an $E^\text{m}$ value of 25±0.1 for papain.\textsuperscript{21} All the protein solutions and buffer were degassed with gentle stirring under vacuum before being loaded into the calorimeter to ensure no bubbles during heating process. Experiments were performed over a range of 30-100°C at a scan rate of 1.5°C per min. Normalized heat capacity (Cp) data were corrected for buffer baseline. Raw data from the DSC run were curve fitted using Origin\textsuperscript{TM} scientific plotting software which uses Levenberg-Marquardt non-linear least square method.

**Partial specific volume measurements**

The partial specific volume of papain was measured using Anton paar DMA 55 or DMA 58 densitometer at 20.00±0.005°C according to the standard procedure. The densities of the solvent and of the protein solution were measured and the partial specific volume was calculated using the equation.\textsuperscript{22,23} The data were analyzed for thermodynamical parameters using water, protein and denaturant as components 1, 2 and 3 respectively, following the standard notations.\textsuperscript{24,25}

**Results**

The unfolding of papain was carried out by CD spectral studies. Far UV CD and near UV CD studies provide information about changes in the secondary and tertiary structure respectively. Fig. 1 shows the effect on the far UV CD spectra of papain at various concentrations of urea. The spectra of native papain have a trough at 208 and 222 nm, which are characteristics of $\alpha$ helical proteins in general. In the presence of lower concentrations (0.2-2 M) of urea, there was a significant change in the CD spectra as compared to native protein. These changes in rotations at 222 nm at lower concentrations of urea, are shown in Fig. 1 (Inset). Further, increase in the denaturant concentration leads to an expected loss of secondary structure. In the case of near UV-CD, there was a reduction in the CD spectra even in the presence of lower concentrations of urea, as compared to native protein molecule, indicating the changes in the environment of the aromatic amino acids. Enzyme activity measurement in presence of different concentrations of urea shows that the enzyme loses nearly 50% of its activity at 0.06 M urea and is completely inactivated at above 0.25 M urea.

The exposure of any hydrophobic regions at the intermediate state observed at lower concentrations of urea can be studied by monitoring the binding of ANS to the protein molecule. The folding intermediate with loosely packed hydrophobic clusters can be detected using ANS a fluorescent probe. Fig. 2 shows the binding of ANS to the papain molecule at various concentrations of urea. From the Figure, it is clear that binding of ANS to papain is more in the case of lower concentrations (0.2-1.5 M) of urea, as compared to the native or unfolded state. ANS binding was maximum in presence of 0.5 M urea, the increase being almost 50%. Further, increase in the denaturant concentration (2-8 M) resulted in decrease in the binding.

The increase observed in the intensity of the CD bands at far UV region at lower concentrations of urea may have some implications on the thermal stability of the protein molecule. Effect of lower concentrations of urea (0.2-2 M) on the thermal stability of papain was examined by both calorimetric and spectrophotometric studies. In native papain, the two transitions are tightly linked and overlap extensively. The calorimetric scan when deconvoluted using a non-two state fit shows two peaks, peak A at 83±0.2°C and peak B at 89±0.2°C, both of which shift to higher temperature in presence of lower concentrations of urea, maximum being at 0.5 M wherein thermal transition temperature increases to a value of 85±0.2°C and 91.5±0.2°C for transition A and B respectively. The DSC curve of papain in
Fig. 1—Far UV CD spectra of papain in presence of various concentrations of urea in 0.02 M acetate buffer pH 5.6 [Protein concentration of $7 \times 10^{-6} M$ was used in all the experiments. (●), Native; (▲), 0.5 M; (○), 1 M; and (□), 4.5 M urea. Inset: Changes in the CD spectral rotations of papain as a function of lower concentrations of urea at $\theta_{222}$ nm]

Fig. 2—Binding of ANS to papain [Protein concentration was $2.12 \times 10^{-6} M$ and ANS concentration in the final mixture was $2.18 \times 10^{-3} M$ such that there was a large excess of ANS in solution. The binding of ANS to protein was monitored by measuring the fluorescence intensity at 470 nm after exciting at 375 nm]
presence of different concentrations of urea is shown in Fig. 3. Further, increase in the denaturant concentration (2-4 M) results in the decrease of the thermal transition temperature, which is expected.

The increase in the thermal stability of papain in presence of lower concentrations of urea was further supported by spectroscopic studies. Here, the thermal unfolding was followed by measuring the absorbance changes at 287 nm as a function of temperature. Fig. 4 shows the thermal denaturation profile of papain in presence of different concentrations of urea. At 0.5 M urea concentration, the apparent thermal denaturation temperature increases from a control value of 83±1°C to 86±1°C, demonstrating the increased thermal stability of the protein molecule in the presence of lower concentrations of urea.

In order to clearly understand the unfolding mechanism of papain, it is necessary to understand the nature of interaction of urea through the mechanism of preferential interaction. The values of isomolal and isopotential conditions for different concentrations of urea are: 0.5 M (0.724 ml/g, 0.728 ml/g), 1 M (0.723 ml/g, 0.728 ml/g) and 7 M (0.721 ml/g, 0.708 ml/g). The value for native protein in buffer alone is 0.724 ml/g and 0.728 ml/g for isomolal and isopotential conditions respectively. From the value of isomolal and isopotential conditions in the presence of urea, the preferential interaction parameter ($\xi_3$) was calculated for both lower and higher concentrations of urea. Fig. 5A shows the changes in preferential interaction parameter as a function of urea concentration. The preferential interaction parameter showed a negative value in the presence of lower concentrations of urea (0.2-2 M), which is maximum at 1 M urea with a value of -0.019 g/l. Above 3 M concentration, one can see the binding of urea to the protein molecule, wherein the preferential parameter is a positive value. Fig. 5 B shows the changes in the molar volume as a function of urea. At lower concentrations of urea upto 3 M, there is decrease in the $\Delta V$ values, maximum being at 1.5 M urea, wherein the $\Delta V$ value is 84±10 ml/mol. After 3 M concentrations, the $\Delta V$ values change and will have a negative value with a maximum value being at 7 M, urea, where the value is -376±15 ml/mol. After 7 M, the $\Delta V$ value decreases and this decrease in the $\Delta V$ value after reaching a maximum is possibly due to the unfolding of the protein molecule and exposure of number of both

Fig. 3—Differential scanning calorimetric profile of papain in presence of different concentrations of urea in 0.02 M acetate buffer pH 5.6 [Protein concentration of $2 \times 10^{-4}$ M was used in all experiments. (a), Native; (b), 0.5 M; and (c), 4 M urea. At 1 M urea concentration the DSC profile is same as that of 0.5 M and it reproduces itself on curve b (i.e. 0.5 M)]
Fig. 4—Thermal denaturation profile of papain in presence of various concentrations of urea. [Thermal denaturation was followed by monitoring the changes in the absorbance at 287 nm as a function of temperature. (●), Native; (△), 0.5 M; and (□), 2 M urea: At 1 M urea concentrations, the curve retraces the 0.5 M curve.]

Fig. 5—(A): Changes in the preferential interaction parameters of papain as a function of urea concentrations; (B): Changes in the partial molar volume of papain upon transferring from water to different concentrations of urea.
hydrophobic and hydrophilic sites to bulk solvent leading to the net change in the preferential interaction, which is lower. Similar observations were reported in the case of ribonuclease and human serum albumin\textsuperscript{18,31}.

**Discussion**

Under certain conditions, a number of proteins exist in stable conformation that is neither fully folded nor fully unfolded. These intermediates can be classified into different types. Among them, the molten globules are commonly observed intermediates at low and high pH and in presence of low concentrations of denaturants\textsuperscript{9,20,28}. Considering the wide variety of possible folding intermediates, very careful experimental characterization is required.

Papain, which is a bidomain protein, is known to exhibit the presence of intermediates during the unfolding process\textsuperscript{15,16}, and to exist as a molten globule at low pH\textsuperscript{41}. In the present study on the effect of urea at lower concentrations on the structure of papain, far UV-CD studies showed the presence of pronounced CD spectra at low concentration (0.2-2 M) of urea as compared to the native protein molecule. Similar types of pronounced CD spectra were observed in the far UV region in the case of \textalpha lactalbumins\textsuperscript{34,6} and carbonic anhydrase\textsuperscript{2,27}. This increase in the CD band does not necessarily mean a change in the secondary structure since far UV-CD spectra can also be influenced by aromatic side chains and disulfide bonds\textsuperscript{29}.

Thus, the structure of papain in presence of lower concentrations of urea is different from native or unfolded state. Binding studies of ANS, a charged hydrophobic fluorescent molecule, demonstrated the presence of hydrophobic clusters in these intermediates. The binding of ANS to hydrophobic surface of papain exposed at lower concentrations of urea (0.2-1.5 M) results in increase in the fluorescence intensity, as compared to the native or unfolded state. The above characteristics are typical of a molten globule state observed in the case of many proteins\textsuperscript{22,12,27}. These results indicate that there is sufficient association of hydrophobic residues to constitute ANS binding site in the case of intermediates observed at lower concentrations of urea. Thermal denaturation studies showed that the thermal stability of papain increased at lower concentrations of urea. There have been many reports showing increased thermal stability in presence of lower concentration of denaturants\textsuperscript{30,31}.

Even though it is assumed that in the case of molten globule state, partial specific volume will be greater than that of the native state, there are only limited reports available, wherein careful comparison of the partial specific volume of molten globule, native and the unfolded states has been carried out. The partial specific volume studies showed that at lower concentration of urea the preferential interaction parameter has a negative value indicating that the molecule is preferentially hydrated. Thus, the intermediate state observed in presence of lower concentration of urea is more hydrated as compared to the native papain molecule and this may give more stability to the protein, as indicated by increased thermal denaturation temperature. This type of transition between preferential hydration and preferential interaction has been reported earlier in the case of ribonuclease and human serum albumin\textsuperscript{9,31}.

Thus, from the studies of both far UV and near UV-CD, it appears that papain assumes an intermediate state, which possesses a pronounced secondary structure, but little tertiary structure, characteristic of molten globule state. From the present study it can be concluded that during urea induced reversible unfolding process, papain assumes a molten globule state, in which the far UV-CD bands are more pronounced than the native state, but having a decreased tertiary structure. ANS binding studies showed that in molten globule state, binding of ANS to the molecule was higher than the native, demonstrating that the hydrophobic clusters were exposed. Also, the thermal stability of the protein molecule is higher in this state. The partial specific volume measurements demonstrate that the increased thermal stability observed at lower concentrations of urea is because of preferential hydration of the protein molecule. Thus during interaction of papain with urea at lower concentrations, preferential hydration dominates, but at higher concentrations, normal preferential interaction overrides.

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