Process Biochemistry 46 (2011) 458–464

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Comparison of activity and conformational changes of ficin during denaturation by urea and guanidine hydrochloride

K.B. Devaraj, Parigi Ramesh Kumar, V. Prakash*

Department of Protein Chemistry and Technology, Central Food Technological Research Institute (A Constituent Laboratory of CSIR), Mysore 570020, India

ARTICLE INFO

Article history:
Received 17 July 2010
Received in revised form 6 September 2010
Accepted 21 September 2010

Keywords:
Cysteine protease
Ficin
Guanidine hydrochloride
Protein denaturation
Stability
Urea
Unfolding

ABSTRACT

The activity and conformational changes of ficin (EC 3.4.22.3), a cysteine protease from *Ficus carica* have been investigated during the denaturation by urea and guanidine hydrochloride (GuHCl). The denaturation of ficin was followed by activity measurements, fluorescence and circular dichroism (CD) spectroscopic studies. The enzyme activity decreased significantly at low concentration of both urea and GuHCl before unfolding of the enzyme molecule. The enzyme molecule was resistant for unfolding by urea under neutral conditions even at higher concentrations. However, the protein is susceptible to unfolding by urea at lower pH and transition follows a cooperative two-state rule with increasing concentration of urea. On the other hand, ficin molecule loses its complete structure in presence of 4 M GuHCl under neutral conditions. The GuHCl-induced unfolding occurs in a simple two-state cooperative process. These results indicate the differential structural stability and fragility of active site of the enzyme towards denaturation by urea and GuHCl.

1. Introduction

The stability of proteins in solutions is a major concern of biologists and pharmacologists. Although many mechanisms are in vogue, the protein folding from a denatured state to a biologically active form is not very well understood. The denatured state of proteins is equally important as much as the native state in determining the stability and folding pathway of proteins. Therefore more in depth knowledge of protein denaturation and unfolding/refolding process are central to understanding the protein stability. Further, studying of the denaturation process of proteins provides a basis for the protein designing and helps to establish a molecular description of protein folding. It is well known that urea and GuHCl are the most frequently used classical denaturants to study the protein stability and folding pathways. In comparison to either acid or thermal unfolding, chemical agents such as urea and GuHCl are more effective in disturbing the non-covalent interactions. The extent of unfolding is generally greater than that of any other means of denaturation [1–3]. Despite their widespread use, the mode of action of these agents on protein conformation is not clearly known. They may exert their effect directly, by binding to the protein molecule, or indirectly, by altering the solvent environment [4,5]. It has been shown in several studies that the inactivation of many enzymes occurs before protein molecule undergoes significant conformational changes which can be detected during the process of denaturation by urea, GuHCl and temperature [6–8].

Ficin (EC 3.4.22.3) is one of the commercially important plant cysteine protease isolated from the latex of *Ficus* species. The available information indicates that ficin apparently shares many common properties with papain with respect to substrate specificity, esterase activity, transpeptidase reactions, and activation by reducing agents [9]. Very few references are available on the structural aspects of ficin till date as compared to papain [10,11] as well as other related cysteine proteases [12–15]. In our initial studies, we have reported the purification and the properties of ficin from *F. carica*. It is a single polypeptide chain with a molecular mass of 23.1 kDa. The enzyme was active at neutral pH and complete inactivation of the enzyme occurs below pH 3.0 [16]. The characterized enzymatic properties of this ficin shared many similarities with many other plant cysteine proteases. The results of our earlier studies demonstrated that the pH-induced denaturation of ficin leads to a partially unfolded state at acidic pH. The partial unfolded structure of ficin at low pH showed the characteristics of molten globule like intermediate state as studied by various biophysical techniques [17]. In the present study we primarily focus on the effect of chemical denaturants such as urea and GuHCl on the structure and stability aspects of ficin. These studies help to understand the mechanism of denaturation and stability of ficin.

Abbreviations: ANS, 8-anilino-1-naphthalene-sulfonic acid; CD, circular dichrosism; GuHCl, guanidine hydrochloride; F_u, fraction unfolded; K_{sv}, Stern–Volmer constant.

* Corresponding author. Tel.: +91 821 2517760; fax: +91 821 2516308.
E-mail address: prakash@cftri.com (V. Prakash).

1359-5113/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2010.09.016

2. Materials and methods

2.1. Materials

Ficin (*F. carica*) Lot #031K76652, 8-anilino-1-naphthalene-sulfonic acid (ANS), urea, guanidine hydrochloride (GuHCl) and acrylamide were purchased from Sigma–Aldrich Chemical Co., St. Louis, Mo, USA. Sodium acetate, acetic acid, hydrochloric acid, monosodium and disodium salts of phosphate, glycine, and hydrochloric acid were purchased from E-Merck, Mumbai, India. All the above chemicals used were of analytical grade. Quartz triple distilled water was used in all experiments.

2.2. Purification of ficin

Ficin was purified from the commercial crude preparation (Sigma) according to the method described in our earlier report [16]. The homogeneity of the purified ficin was evaluated by SDS-PAGE to confirm the purity. Concentration of the enzyme was determined by measuring absorbance at 280 nm using an absorption coefficient of $E^{1\%}_{280}$ = 20.9 on Shimadzu spectrophotometer [16] or alternatively by Lowry's method [18].

2.3. Equilibrium denaturation experiments

Denaturation of ficin was induced by incubating or dialyzing the enzyme with various concentrations of denaturants till the equilibrium was attained. Refolding was performed using the ficin that had been completely denatured by 8 M urea or 6 M GuHCl. The denatured ficin was either diluted with appropriate concentration of urea or GuHCl and the mixture was incubated till the reaction reached to the equilibrium or alternatively the denatured protein was dialyzed against desired concentration of GuHCl/urea with several changes. The extent of unfolding and refolding of ficin was measured either by changes in the emission maximum or ellipticity values at 222 nm by fluorescence or circular dichroic measurements, respectively. The data are expressed in terms of the fraction unfolded (F_u) calculated from the standard equation [19,20]:

$$F_u = \frac{F_{obs} - F_n}{F_u - F_n} \quad (1)$$

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. From these measurements, values of ΔG_{N-U} for a two-state process were determined using the relation:

$$\Delta G_{N-U} = -RT \ \ln \frac{F_{obs} - F_n}{F_u - F_n} \quad (2)$$

If a standard two-state model is assumed, the GuHCl and urea transitions are fitted to the equation

$$\Delta G_{N-U} = \Delta G(H_2O) - m[D] \quad (3)$$

where $\Delta G(H_2O)$ and ΔG_{N-U} are the free energy of the folding in water and at a denaturation concentration D, respectively. m is the slop of the ΔG_{N-U} vs [denaturant] plot, and D is the denaturant concentration [20].

2.4. Ficin assay

Enzyme activity of ficin was quantified by measuring its ability to cleave an amide bond in small-molecular-weight synthetic substrate benzoyl-D,L-arginine *p*-nitroanilide hydrochloride (BAPNA) as described in our earlier studies using 0.05 M sodium phosphate buffer containing 5 mM cysteine hydrochloride [16]. The extent of hydrolysis was determined by measuring the product (*p*-nitroaniline) formed at 410 nm (ε = 8800 for *p*-nitroaniline). All the enzyme activity was measured in both the presence of different concentrations of urea or GuHCl in 0.05 M sodium phosphate buffer containing 5 mM cysteine hydrochloride at 55 °C [16]. The enzyme activity in the absence of the denaturants served as the control, and the percentage residual activity was calculated on the basis of its original activity.

2.5. Fluorescence measurements

Fluorescence measurements of ficin under different conditions were carried out on Shimadzu (Model RF 5000) spectrofluorimeter. All the measurements were made at 25 °C using appropriate blanks for baseline correction of fluorescence intensity. The intrinsic fluorescence was recorded in the wavelength raging from 300 to 400 nm and the excitation of protein solution was set at 280 nm. The slit width for both excitation and emission was set at 5 nm. For binding studies of ANS, ficin samples at different conditions were incubated with 100-fold molar excess of ANS for 30 min at 25 °C in dark. The fluorescence of ANS was excited at 380 nm and emission was collected between 400 and 600 nm. In order to give correction for the unbound ANS fluorescence emission intensities, assays were performed with ANS and buffer only.

2.6. Fluorescence quenching experiments

Acrylamide quenching studies of the intrinsic fluorescence were performed by adding aliquots from the stock solution of the quencher (5 M acrylamide) into a cuvette containing protein solution. The intrinsic fluorescence was excited at 295 nm, and the emission was recorded between 300 and 400 nm. Final fluorescence intensities were corrected for dilution effects. The decrease in fluorescence intensity at λ_{max} was analyzed by the Stern–Volmer equation [21]

$$\frac{F_o}{F} = 1 + K_{sv}[Q] \quad (4)$$

where F_o and F are the fluorescence intensities at an appropriate wavelength in the absence and presence of quencher (acrylamide), respectively; K_{sv} is the Stern–Volmer constant and [Q] is the concentration of the quencher.

2.7. Circular dichroic measurements

Circular dichroic (CD) spectral measurements of ficin under different conditions were carried out using an automatic recording Jasco J-810 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) fitted with a xenon lamp and calibrated with +D-10-camphor sulfonic acid. All the measurements were performed at 25 °C on a thermostatically controlled cell holder attached to a water bath. Far-UV CD measurements were recorded in the range of 190–260 nm using protein concentration of 10 μM with 1 mm path length cell. The scan speed was 10 nm/min using a bandwidth of 1 nm and the spectra were taken as an average of three scans. The results were expressed as the mean residual ellipticity $[\theta]_{MRW}$ obtained from the relation

$$[\theta] = \frac{100 \times \theta_{obs}}{l \times c} \quad (5)$$

where θ_{obs} is the observed ellipticity in degrees. The $[\theta]_{MRW}$ was calculated using a value of 115 for mean residue mass of the protease, c is the concentration in g/l and l is the length of the light path in cm. The values obtained were normalized by subtracting the base line recorded for the buffer under similar conditions. The analysis of the data for the secondary structure elements was done according to computer program of Yang et al. [22].

3. Results and discussion

3.1. Effect of urea on the conformation of ficin under neutral condition

The effect of urea on structural and functional properties of ficin was studied by equilibrating the enzyme at neutral conditions (pH 7.0) in the presence of different concentrations of urea. The enzyme was equilibrated for 36 h before taking all spectral measurements. Changes in the fluorescence emission spectra of ficin after equilibration in presence of increasing concentrations of urea are shown in the Fig. 1A. As seen from the spectra, a slight increment in the fluorescence intensity was observed with increasing concentration of urea without any shift in the emission maximum. This increase in the intensity is probably due to the loss of specific interaction of tryptophan residues with vicinal quenching groups, which consequently resulted in the increment in the fluorescence intensity [23]. These results suggest that ficin molecule under neutral conditions resist to unfold even in the presence of high concentration of urea. The stability or conformational rigidity of ficin at neutral conditions was further evaluated by far-UV CD spectral studies.

Far-UV CD spectra of ficin in presence of different concentrations of urea are shown in Fig. 1B. The spectra of ficin in presence of different concentrations of urea remained unaffected except for marginal changes in the ellipticity values at 222 nm. The mean residual ellipticity value at 222 nm for native enzyme is −5150 deg cm^2 dmol^{-1} and in presence of 8 M urea is −4504 deg cm^2 dmol^{-1}. These results clearly indicate the structural stability of ficin molecule towards urea denaturation. The structural stability of ficin, isolated from *Ficus glabrata* towards urea denaturation has been reported earlier by Englund et al. [24]. Similar to the results indicated here stem bromelain and procerain, a cysteine proteases from *Calotropis procera* also showed unusual structural stability against urea in neutral conditions [15,25]. α-Amylase from malted sorghum (*Sorghum bicolor*) and lectin from *Erythrina indica* are also stable towards denaturation by urea under neutral conditions [26,27].