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Applying the increase in rate constants of cooperative proteolysis to the determination of transition curves of protein denaturation

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Abstract

The transition curves of soybean glycinin egg lysozyme, bovine serum albumin (BSA) denaturation under the action of increasing urea concentration were monitored by determination of the increase in rate constants of cooperative enzymatic proteolysis. The results were compared with those obtained using intrinsic fluorescence and the number of accessible tyrosine. It was shown that the determination of the changes of proteolysis rate permits to disclose conformation changes not detected by other methods.

Keywords: Denaturation; Proteolysis rate; Urea; Glycinin; Lysozyme; Bovine serum albumin

1. Introduction

The usefulness of studies of protein denaturation for the understanding the process of protein folding is well known [1,2]. The folding–unfolding transition of proteins may proceed directly from the folded to fully unfolded structure or some intermediate structures may be formed. In fact, the latter are always formed. However, if their equilibrium concentration is very low, only two states of protein, the native and the denatured one, may be considered. In such case, the study of the thermodynamics of the denaturation process is simplified greatly.

The main criterion for discerning the two ways of denaturation is comparing the denaturation curves obtained by different methods [1]. In principle, any protein

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property that changes during the transition from native to denatured state may be used. A comprehensive review of modern methods used for determining the folding/unfolding transitions was published recently [3]. Denaturation curves based on different properties may reflect different structural changes. Their coincidence points to one-step unfolding of protein, whereas if they are at different values of denaturating agents (temperature, concentration, pH, etc), relatively stable partially unfolded intermediate structures exist.

The increase of proteolysis rate during denaturation was recognized long ago [4]. Its use in the unfolding–refolding studies was mainly concerned with the detecting the intermediates formed by the appearance or disappearance of new sites susceptible to proteolytic attack. These works were reviewed by Rupley [5] and later by Price and Johnson [6]. Afterwards, they were applied to the study of ribonuclease A denaturation in guanidine chloride [7] and of its thermal denaturation [8]. The determination of initial proteolysis velocity was used for comparing the stability of lysozyme and its several derivatives [9].

About 30 years ago, it was found that the peptic proteolysis of lysozyme proceeds according the pseudo-first order reaction [10]. More recently, the pseudo-first order kinetics of proteolysis was established for a number of other proteins (see Ref. [11] and articles cited therein). It was shown that such kinetics is characteristic of cooperative proteolysis [11,12]. In this case, the first order rate constants can be determined, and their changes may be used for quantitative following the course of denaturation. In the first attempt [10], the rate constants of peptic hydrolysis of lysozyme at pH 2 were determined and used for following its thermal denaturation. Only a part of the denaturation curve was obtained due to the inactivation of pepsin at high temperatures.

As far as we know, no other attempts to construct denaturation curves by the method based on changes of rate constants of cooperative proteolysis (PRC method) have been made till now. In this work, we have applied this method to urea denaturation of glycinin (storage protein from soybean seeds), lysozyme and bovine serum albumin (BSA). The results were compared with those obtained by determining the changes in fluorescence. Changes in the number of accessible tyrosine during the denaturation of glycinin and those of lysozyme activity were also determined.

2. Materials and methods

2.1. Materials

Three times crystallized chicken egg white lysozyme, research grade BSA (both from Serva, Heidelberg, RFG) and glycinin isolated by isoelectric precipitation according to Thanh and Shibasaki [13] were used as substrates. Twice crystallized papain suspended in acetate buffer pH 4.5 was also from Serva, while lyophilized cells of *Micrococcus lysodeicticus* were from Sigma (St. Louis, MO, USA). Urea for biochemistry was from Merck (Darmstadt, RFG) and Bz-Phe-Val-Arg-*p*NA·HCl from Bachem (Bubendorf, Switzerland).

2.2. Unfolding and refolding

Proteins were dissolved in urea solutions containing 0.05 M tris–HCl buffer, pH 8.2 (standard buffer). Urea concentrations varied from 0 to 9.5 M (lysozyme) or to 8 M (glycinin and BSA). The solutions were kept 20 h at room temperature (20–22 °C) to guarantee the completeness of unfolding. For refolding, the protein was dissolved in 8 M urea and after 20 h dialyzed against urea solution of lower concentration. The final urea concentration was calculated on the basis of known volumes and concentrations of both solutions.

The denaturation curves were expressed as percents of the maximum change of measured values. Except that of accessible tyrosine, they were the result of three independent determinations.

Free energy changes (ΔG_U) of the transitions detected in urea solutions by PRC method were calculated as described by Shirley [14]. They were back extrapolated to zero concentration of urea for estimating ΔG_{H_2O}. A linear extrapolation was used based on the assumption that the linear dependence of ΔG_U on urea concentration in the transition region continued to zero concentration of urea [14]. The concentrations of urea at $\Delta G_U = 0$ were taken for the midpoints of transition curves. The confidence limits of ΔG_{H_2O} were calculated according to Snedecor [15].

2.3. Proteolysis

Proteolysis was performed by papain. Hydrolysis of a synthetic substrate showed that papain retained its activity in all range of urea concentrations (Fig. 1). The increase of urea concentration brought about even a slight but significant rise of its activity.

The incubation mixture contained 0.5% protein. Papain was activated by β-mercaptoethanol (5 μl/ml) and added to the incubation mixture. The enzyme/substrate ratio was

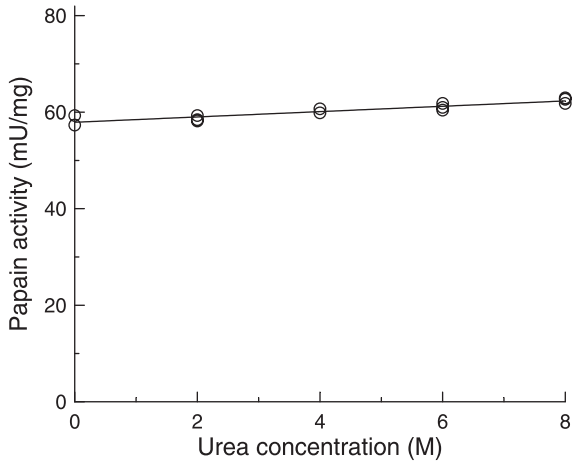

Fig. 1. Papain activity in dependence on urea concentration. Bz-Phe-Val-Arg-*p*NA·HCl as substrate.

1:1000, 1:50 and 1:300 for glycinin, lysozyme and BSA, respectively. Proteolysis was performed at 37 °C. Its duration varied from 7 to 9 h for native proteins to 15–30 min when unfolded protein was hydrolyzed. The residual (TCA-insoluble) protein was determined in samples (8 μl) taken from the incubation mixture at specified intervals. Three parallel samples were taken each time. The dependence of the logarithm of residual protein on proteolysis time was plotted. The rate constants were equal to the slope of the plot.

2.4. Protein determination

Protein was determined by a dye-binding method [16]. The samples taken from the incubation mixture were applied on chromatographic paper, washed with 5% TCA and dyed with 0.05% solution of bromophenol blue in 5% TCA. The excess of dye was removed by washing with 5% TCA, and the paper was dried at room temperature. The bound dye was eluted by 1.5 ml of 0.01 M NaOH, and the absorbance at 591 nm was measured. The standard deviation does not exceed 0.01 absorbance units.

2.5. Protein fluorescence

Fluorescence was measured by MPFA spectrofluorimeter (Hitachi, Japan). Protein concentration was 0.02 mg/ml, excitation at 297 nm and emission at 345 nm. Lysozyme fluorescence was determined also in 6 M guanidine chloride solution. The results of measurements were corrected for the fluorescence of denaturant.

2.6. Accessible tyrosine

For spectrophotometric titration of accessible tyrosine, 5 ml samples of protein solutions (2 mg/ml) were brought by 0.5 N NaOH to the desired values of pH (between 8.5 and 12.3). Their volume was adjusted to 10 ml using urea solutions of corresponding concentrations and pH. After 20 h, their absorbance at 294 nm was measured. The protein solutions at pH 8.25 and of the same concentration of urea were used as control solution. The amount of accessible tyrosine residues was calculated from the increase in tyrosine absorbance at pH 10 where the ionization of accessible tyrosine is complete. The molar absorbance of each tyrosine residue in these conditions is 2300 [17].

2.7. Enzymes activity

Papain activity at different urea concentrations was determined using Bz-Phe-Val-Arg-*p*NA·HCl, a good papain substrate. Papain suspension (25 μl) was added to urea solution (1 ml) in standard buffer containing β-mercaptoethanol (2 μl/ml). After 30 min standing for papain activation, 25 μl of 0.5 mM substrate were added. The increase of absorbance of the liberated *p*-nitroaniline at 405 nm at 37 °C was determined by Spekol 220 spectrophotometer. Papain activity was calculated using the built-in kinetic program.

The decrease of the turbidity of lyophilized cells of *M. lysodeicticus* suspension was used for the activity determination of lysozyme. It was performed according the protocol described by Epstein and Goldberger [18].

3. Results

Papain hydrolysis of lysozyme followed the pseudo-first order kinetics throughout the range of urea concentration used. In case of glycinin, in accordance with the previous results [12], the cooperative type of proteolysis superseded the noncooperative one shortly after the onset of the hydrolysis. The same proteolysis course was observed during the action of papain on BSA. Representative curves of proteolysis kinetics are shown in Fig. 2.

3.1. Glycinin

The changes in proteolysis rate constants and fluorescence of glycinin in urea solutions of increasing concentration are shown in Fig. 3A. We also succeeded in determining the changes in the number of accessible tyrosine shown on the same figure.

The rate constants of glycinin hydrolysis increased slowly with the increase of urea concentration to 3 M. In the range from 3 to 4 M, a sharp increase took place. The maximum value attained showed a 14-fold increase in rate constants. Then the rate constants remained nearly unchanged during further increase in urea concentration, while in 8 M urea the proteolysis rate decreased markedly.

The determination of fluorescence led to a practically identical curve. Midpoints ($\Delta G = 0$) of the denaturation curves obtained by the two methods were at 3.19 and 3.28 M urea concentration.

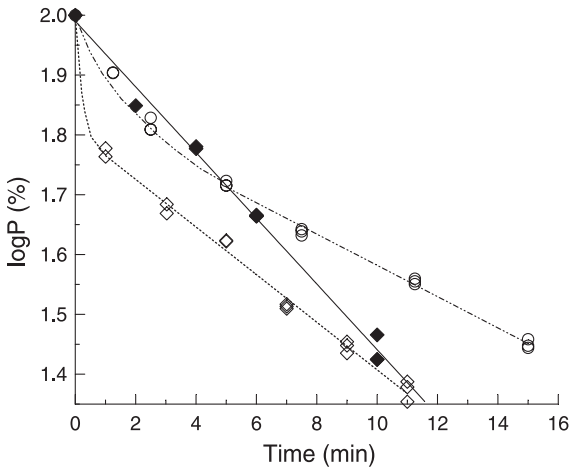

Fig. 2. Representative pseudo-first order kinetic curves of proteolysis. Concentration of residual protein (*P*) is expressed as percent of the initial concentration. (○) glycinin, 6.0 M urea; (◆) lysozyme, 8.0 M urea; (◇) BSA, 4.2 M urea. For glycinin, the time labels should be multiplied by four.

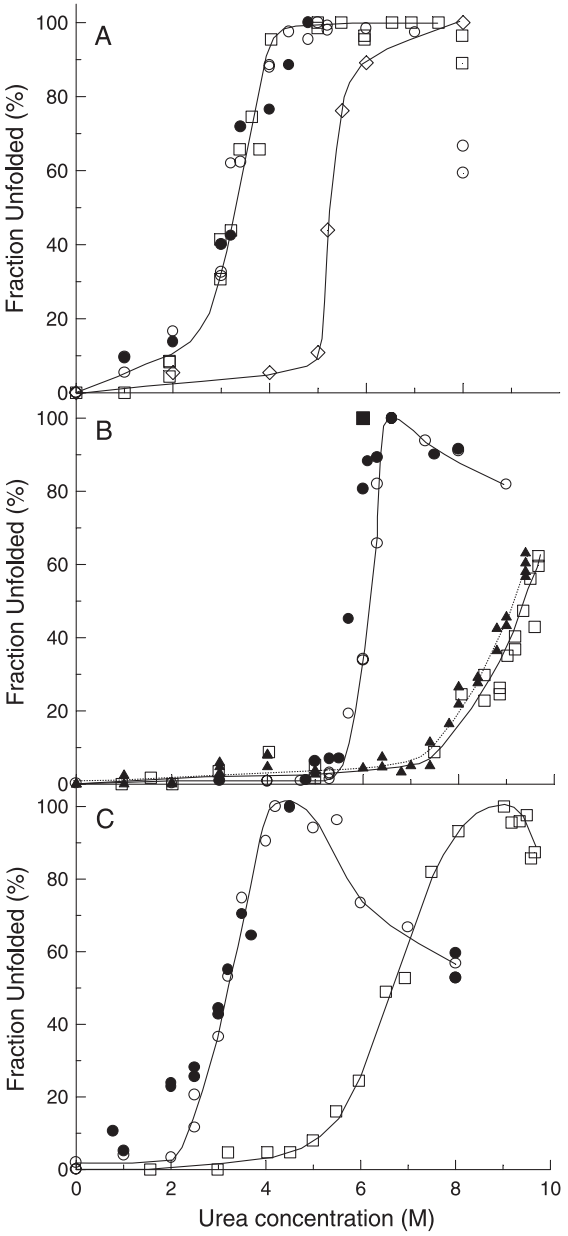

Fig. 3. Transition curves. (A) glycinin; (B) lysozyme; (C) BSA. (○) PRC method, unfolding; (●) PRC method, refolding; (□) fluorescence; (■) fluorescence in 6 M guanidine chloride; (◇) accessible tyrosine; (▲) inactivated lysozyme. Maximum changes of measured values are taken for 100%. In case of lysozyme, the value of fluorescence in 6 M guanidine chloride and zero activity is taken for 100% of fluorescence and inactivation, respectively.

The denaturation curve based on the changes in the number of accessible tyrosine was quite different. A sharp increase from 7 to 18 of accessible tyrosine residues per glycinin molecule occurred between 5 and 6 M concentration of urea. In 8 M urea, it reached 19 residues, which, however, was far from the total number of 71 attained at pH 12.5. The latter was in accordance with the total number of tyrosine residues per glycinin molecule.

3.2. Lysozyme and BSA

The urea-dependent proteolysis rate constants, fluorescence and enzymatic activity of lysozyme are shown in Fig. 3B. We failed to determine the accessible tyrosine.

A very slow hydrolysis occurred in the absence and at low concentrations of urea. Significant increase in proteolysis rate was observed at 5.3 M urea concentration. Further increase in urea concentration brought about a sharp increase in proteolysis rate, which at 6.6 M urea concentration reached its maximum value (midpoint at 6.0 M urea). It was 360 times higher than in the absence of urea. At higher concentrations of urea, decrease of proteolysis rate occurred.

The increase in lysozyme fluorescence started only when urea concentration reached 8 M. However, it did not reach the maximum value even at 9.7 M urea. At this concentration, the increase in fluorescence amounted to only 62% of that observed in 6 M guanidine chloride where the unfolding might be considered complete.

The course of the decrease of lysozyme activity closely followed that of the changes in fluorescence.

The transition curves of BSA denaturation obtained by PRC and fluorescence methods are shown in Fig. 3C. As well as those of lysozyme, they differed significantly (midpoints

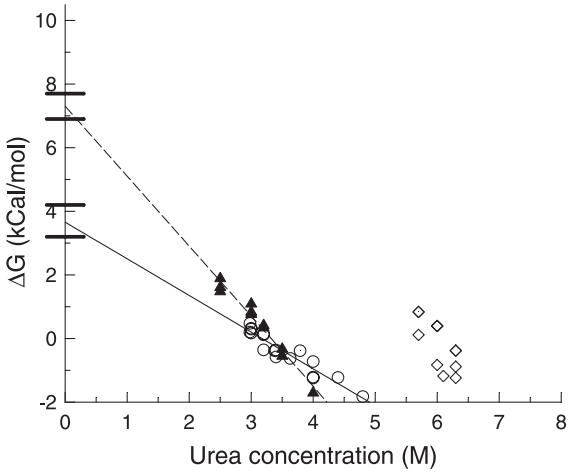

Fig. 4. ΔG_U as a function of urea concentration, (○) glycinin; (◇) lysozyme; (▲) BSA. ΔG_{H_2O} determined as the values of extrapolated regression curves at zero concentration of urea. The bars intersecting the *Y*-axis denote the confidence limits of ΔG_{H_2O}.

at 3.3 and 6.9, respectively). A marked decline of proteolysis rate occurred at high urea concentrations.

3.3. Evaluation of ΔG_{H_2O}

Conformation changes of all three investigated proteins detected by PRC method were found reversible (Fig. 3). The evaluation of ΔG_{H_2O} was, therefore, attempted; the results are shown in Fig. 4. The determination of ΔG_{H_2O} is approximate. The strict constancy of temperature was not maintained during the experiments. Other source of error exists in the case of lysozyme and BSA. These proteins do not attain the plateau of their transition curves due to the decreasing of the proteolysis rate at higher urea concentrations. The lysozyme transition in a very narrow range of urea concentration leads to errors in determination of equilibrium constants of the transition. In addition, the latter occurs at higher urea concentration. The increase of the interval between zero and mean experimental value of the denaturant brings about a considerable broadening of ΔG_{H_2O} confidence limits [15]. In consequence, the confidence limits of lysozyme ΔG_{H_2O} turn out to be too large (about 10 kcal/mol) and the determined value was meaningless. ΔG_{H_2O} of glycinin and BSA were 3.7 and 7.3 kcal/mol, the confidence limits 0.5 and 04 kcal/mol, respectively.

4. Discussion

Glycinin, one of the main reserve proteins of soybean seeds, consists of six nonidentical, but homologous subunits. It dissociates in mild conditions [19]. The transition curve of urea-induced glycinin denaturation determined by PRC method and by fluorescence occurs in the range of urea concentration where the dissociation of glycinin was observed [19]. Therefore, the increase in hydrolysis rate and the change in fluorescence might reflect the dissociation of glycinin. This suggestion is supported by a sharp increase in the rate constants of glycinin proteolysis at low ionic strength [20] where glycinin dissociation occurs as well [19]. The low value of ΔG_{H_2O} is also in compliance with the assumed linkage between dissociation and the increase of glycinin proteolysis rate. The increase in accessible tyrosine observed at higher urea concentration might reflect the unfolding of glycinin subunits that seems to be only partial.

The denaturation of egg white lysozyme has been investigated in detail. The results have been summarized in the review by Pfeil and Privalov [21]. Lysozyme is considered resistant to unfolding by urea in neutral milieu [22–24]. Usually, the studies of lysozyme unfolding were performed in acid milieu [10,21,23,25] where its structure was already partially destabilized. As far as we know, only in an old paper, the transition curves of lysozyme in urea in neutral medium were investigated [26]. The transition point of unfolding was at 8 M urea when viscosity was the criterion, while the changes in optical rotation were incomplete even in 10 M urea and increased significantly in 6 M guanidine chloride. The changes in fluorescence and enzymatic activity observed in the present work are in accordance with the results obtained following the lysozyme denaturation by the

changes in optical rotation [26]. The conformation changes detected by PRC method occur at lower concentration of urea and have no influence on the enzymatic activity of lysozyme.

The overall rate of the cooperative proteolysis is determined by a relatively slow split of the first one or several peptide bonds, which results in the unfolding of protein molecule and its further rapid proteolytic degradation [5]. One may speculate that local conformation changes in vicinity of the first splitting sites take place at low urea concentrations and are the plausible reason of the detected proteolysis rate increase of lysozyme and BSA.

The proteolysis rate constants of all three investigated proteins show a marked decline at high urea concentrations. It is especially high in the case of BSA denaturation. The decrease of proteolysis rate of glycinin at high concentrations of several denaturants including urea was explained by its refolding [27]. It seems probable that it also leads to the decrease of proteolysis rate of lysozyme and BSA. Anomalous optical effects observed in protein solutions in concentrated urea are also due to some structurization of the unfolded polypeptide chains [28].

The sources of errors and the resulting approximate character of ΔG_{H_2O} are stressed in Results. They are especially high for lysozyme and make out of question the determination of its ΔG_{H_2O}. Nonetheless, the confidence limits of glycinin and BSA are reasonable narrow. The low value of ΔG_{H_2O} complies with the assumed linkage between dissociation and the increase of glycinin proteolysis rate.

Two important conditions that restrict the possibilities of PRC method must be fulfilled:

1. The kinetics of proteolysis must follow the pseudo-first order reaction, i.e. the hydrolysis of the protein studied must be of cooperative type.
2. The proteinase used must retain its activity under the action of the denaturating agent at least during the time needed for the determination of the rate constant.

Both requirements are fulfilled in the present work. In accordance with the published data [9], the kinetics of lysozyme proteolysis is of pseudo-first order. The kinetics of glycinin and BSA becomes the same after a short initial period of noncooperative proteolysis.

The published data concerning the papain stability in urea solutions are contradictory. Papain is reported to retain its activity even in 9 M urea solution [29]. According to others, it is already inactivated in 5 M urea [30]. Our results obtained using a low-molecular mass substrate confirm the first conclusion. The linearity of the kinetic curves in all range of urea concentration observed in the present work also indicates constancy of the papain activity at least during the time of proteolysis. The inactivation of papain by urea has been found in the absence of substrate [30]. The presence of substrate may cause its stabilization.

The coincidence of denaturation curves of glycinin determined by PRC method and by fluorescence is an evidence of the applicability of the former. On the other hand, the study of lysozyme and BSA denaturation shows that PRC method is capable of disclosing some subtle conformation changes. These changes have not been detected by the changes in fluorescence, optical rotation and viscosity [26] and in an activity of lysozyme (this study) that occurs at higher values of denaturant. The existence of an intermediate state follows directly from these results.

PRC method determines the equilibrium transition curves and, as the most popular spectroscopic techniques such as fluorescence and circular dichroism, is insensitive to the rate of equilibrium establishment. The high rate of interconversion of folded to unfolded state hampers the use of the methods based on their physical separation (e.g. chromatography or capillary electrophoresis). On the other hand, determining of proteolysis rate is too time-consuming for applying it to the study of denaturation kinetics.

Unlike the other methods of investigation of protein folding/refolding prevalent at present, PRC method does not require sophisticated and expensive equipment. It is, however, more laborious. Nevertheless, the possibility of disclosing conformation changes not detected by other methods may turn out a considerable advantage of PRC method.

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