Biochimica et Biophysica Acta, 445 (1976) 182—193

BBA 67883

DENATURATION OF SUBTILISIN BPN′ AND ITS DERIVATIVES IN AQUEOUS GUANIDINE HYDROCHLORIDE SOLUTIONS

ATSUSHI IKAI

Dept. of Biophysics and Biochemistry, Faculty of Science, The University of Tokyo, Hongo, Tokyo 113 (Japan)

(Received February 19th, 1976)

Summary

The denaturation of subtilisin BPN′ (EC 3.4.21.14) in guanidine hydrochloride was studied in order to find possible reasons for the exceptional stability of this enzyme against the action of denaturing agents including guanidine hydrochloride. Chemically modified subtilisins, i.e., phenylmethanesulfonyl-subtilisin and thiol-subtilisin, were completely denatured in 2 M guanidine hydrochloride at pH 7 without autolysis but they were stable in 0.5 M guanidine hydrochloride for at least 60 h. On the other hand, once completely denatured, the subtilisins remained inactive and in highly unfolded conformations for 60 h or longer after transfer into 0.5 M guanidine solution at pH 7 or 9. No enzymatic activity was regained when the guanidine concentration was lowered to almost zero.

We concluded from these and other results described in this paper that this enzyme was thermodynamically unstable in 2 M guanidine hydrochloride at 20°C and at pH 7. We wish to point out the possibility that the denaturation of this enzyme could indeed be irreversible.

Introduction

The thermodynamic explanation of the stability of globular proteins has been one of the major problems in biochemistry for some time. Recently some serine proteases have attracted the attention of several workers because of their exceptional stability against the action of denaturing agents such as guanidine hydrochloride and urea [1—5]. This property has already been propitiously exploited in the purification of these enzymes [1] and in the use of proteolytic activity in some biochemical work [6,7]. Subtilisin BPN′ (EC 3.4.21.14) and subtilisin Carlsberg are well documented examples and it has been claimed that they are stable and active even in 6 M guanidine hydrochloride [2,3]. Subtilisin BPN′ is made of 275 amino acid residues with no disulfide or sulfhydryl

groups. It requires no prosthetic groups or metal ions for its activity. It is a very good example for study of the folding mechanism of polypeptide chains except that it is a proteolytic enzyme and self-digestion could be expected to interfere severely with such a denaturation study. We, therefore, made extensive use of modified enzymes in this paper. One of them is phenylmethanesulfonyl-subtilisin in which the active site serine (serine 221) is blocked by the phenylmethanesulfonyl group. The other is thiol-subtilisin in which serine 221 is chemically replaced by cysteine. The reference for the properties and the preparation of these modified enzymes will be given in the following section.

We have been interested in the folding and unfolding mechanism of this protein and in our investigation of this we found that the apparent stability of this protein was very probably kinetic and not thermodynamic in nature. As a result of this investigation we found it important to distinguish between kinetic and thermodynamic stability in the study of protein denaturation.

Materials and Methods

Subtilisin BPN′ was purchased from Sigma Co. as Type VII Alkaline Protease and purified further by the carboxymethyl cellulose column chromatography according to the method of Ottesen and Svendsen [8]. Phenylmethanesulfonyl-subtilisin and thiol-subtilisin were prepared and purified according to the method of Polgar and Bender [9]. The esterase activity of the modified and the unmodified enzymes was measured by their method with *p*-nitrophenylacetate as substrate [9]. Phenylmethanesulfonyl fluoride and *p*-nitrophenylacetate were purchased from Sigma Co. and Wako Pure Chemical Industries, Ltd. respectively and used without further purification. Specially Prepared Reagent (Lot V5M8160) guanidine hydrochloride was purchased from Nakarai Chemicals, Ltd. and used in most studies. Guanidine hydrochloride of somewhat inferior quality purchased from Wako Pure Chemical Industries, Ltd. was used in column chromatogrpahy when the concentration of guanidine hydrochloride in the eluting solvent was higher than 1 M. Sephadex G-200 was the product of Pharmacia Co. All other reagents were of reagent grade.

Polyacrylamide gel electrophoresis in the medium containing 0.1% sodium dodecyl sulfate was performed according to the method of Weber and Osborne [10]. Sedimentation equilibrium experiments were performed on a Beckman Spinco Model E analytical ultracentrifuge with Rayleigh interference optics. Fluorescence spectrum was recorded with a Hitachi 204 spectrofluorometer, and optical rotatory dispersion was recorded on a Jasco ORD/UV-5 Spectropolarimeter. The optical density of the sample solution used in fluorescence experiment was always less than 0.10 at the excitation wavelength. Emission spectra were corrected for the wavelength dependence of photomultiplier sensitivity.

Results

Denaturation of modified and unmodified subtilisin

The equilibrium effect of varying concentration of guanidine hydrochloride on the conformation of subtilisin BPN′ could not be studied due to the inter-

ference of autolysis. Since autolysis was particularly extensive in the transition region, where the slow rate of denaturation and large accumulation of the denatured protein culminated to almost complete self-digestion, we used modified enzymes to study the denaturation profile. In Fig. 1 we show the change in fluorescence intensity of phenylmethanesulfonyl-subtilisin and thiol-subtilisin against the change in guanidine hydrochloride concentration at pH 7.0. Even with these modified enzymes (twice purified by CM-cellulose column chromatography after modification in order to separate from the unmodified contaminant) autolysis became significant after 60 h in approx. 1—1.5 M guanidine hydrochloride. The exact transition midpoint could not, therefore, be determined, but two things were immediately clear from this study.

First, denaturation in 2—5 M guanidine hydrochloride proceeded to completion without self-digestion (as confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and therefore the native conformations of the modified enzymes are thermodynamically unstable in 2 M guanidine hydrochloride. This observation was duplicated with the unmodified enzyme when the protein concentration was less than 0.01 mg/ml. Secondly, the two modififed enzymes showed remarkably similar denaturation profiles although they bore quite different chemical groups at the active site. This result suggests that the unmodified enzyme would probably show the same denaturation profile were it not for the extensive autolysis.

Denaturation in acidic solutions

At pH 1, the unmodified and thiol-subtilisin enzymes were inactivated and unfolded as the fluorescence and optical rotation at 233 nm indicated (Fig. 2).

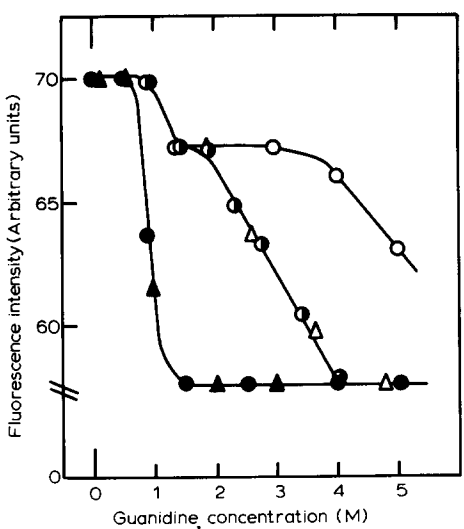


Fig. 1. Denaturation profile of modified subtilisin BPN′ varieties. Phenylmethanesulfonyl-subtilisin and thiol-subtilisin were denatured in guanidine hydrochloride solution containing 0.05 M phosphate buffer at pH 7.0. The change in fluorescence peak intensity is plotted against the concentration of guanidine hydrochloride at different time intervals. Protein concentration: 0.04 mg/ml. Excitation wavelength: 280 nm. Symbols are: ○, phenylmethanesulfonyl-subtilisin after 1 min; ◑, phenylmethanesulfonyl-subtilisin after 2 h; ●, phenylmethanesulfonyl-subtilisin after 30 and 60 h; △, thiol-subtilisin after 2 h; ▲, thiol-subtilisin after 30 and 60 h.

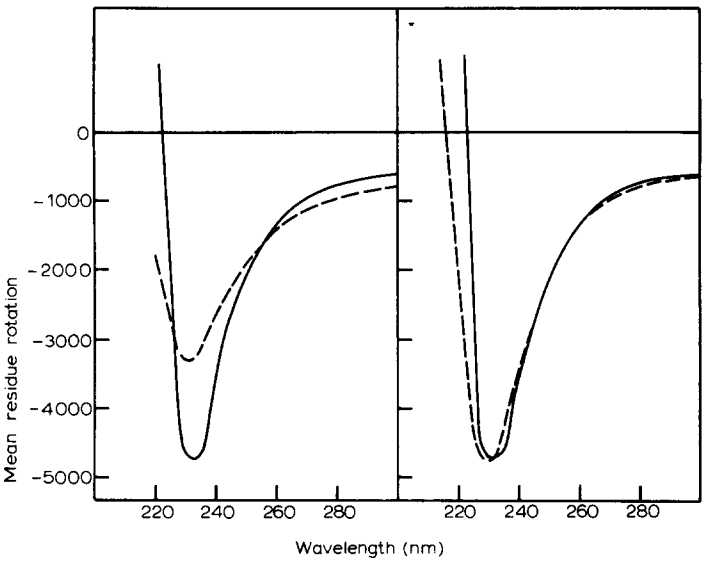
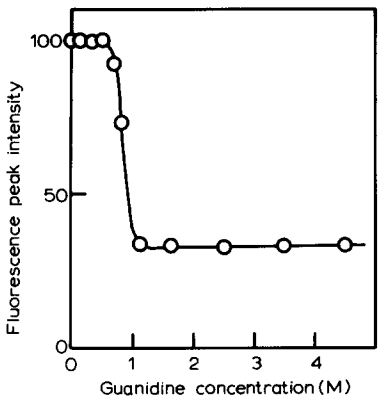


Fig. 2. ORD spectra of subtilisins at pH 1.0. The left figure is for subtilisin and thiol-subtilisin at pH 7.0 (———) and pH 1.0 (- - - - - -). The right figure is for phenylmethanesulfonyl-subtilisin at pH 7.0 (———) and pH 1.0 (- - - - - -).

phenylmethanesulfonyl-subtilisin, on the contrary, retained its tertiary structure in 0.1 M NaCl at pH 1. The optical rotatory dispersion spectrum of phenylmethanesulfonyl-subtilisin at pH 1 is shown in Fig. 2 with that of the unmodified enzyme at pH 7. There is a shift of the 233 nm trough to shorter wavelengths by 3—4 nm, but the depth of the trough is about the same as that of the unmodified enzyme at pH 7. In 2 M guanidine hydrochloride the trough was substantially destroyed. In Fig. 3 we show the change in fluorescence intensity of phenylmethanesulfonyl-subtilisin at pH 1 as a function of guanidine hydrochloride concentration. The curve was constructed 30 h after the addition of guanidine hydrochloride. It actually became constant within 10 h. Dilution of the denaturant concentration or dialysis of the solution to 0.1 M NaCl at pH 1 could almost restore the native flueorescence but not the ORD spectrum, primarily because of aggregate formation after reduction of guanidine hydrochloride concentration. It was also confirmed that autolysis was non-existent in all of the samples after the 30-h denaturation experiment. When we compare the results at pH 1 with those at pH 7, we notice that the apparent stability of phenylmethanesulfonyl-subtilisin after 30-h incubation in guanidine hydrochloride solution was, to our surprise, about the same at pH 1 as at pH 6.

Irreversible denaturation

When modified and unmodified subtilisins were quickly denatured at acidic pH or in very concentrated guanidine hydrochloride at pH 7 the denaturation was irreversible involving little autolysis. In Fig. 4 we show how much of the esterase activity was recovered after transferring the unmodified enzyme from 4 and 6 M guanidine hydrochloride at 6 different pH values to a strongly buffered solution of *p*-nitrophenylacetate at pH 7. The final concentration of guanidine hydrochloride was always less than 0.3 M. The proteolytic activity was



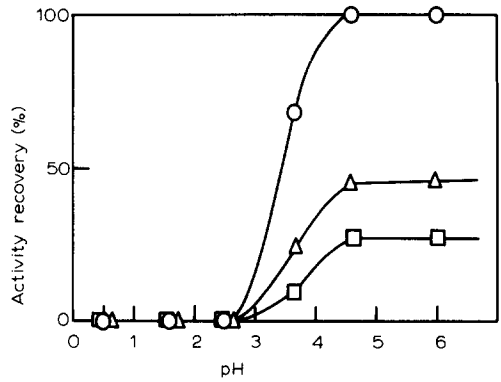


Fig. 3. Denaturation profile of phenylmethanesulfonyl-subtilisin at pH 1.0. The fluorescence peak intensity measured 30 h after the onset of denaturation was plotted against guanidine concentration.

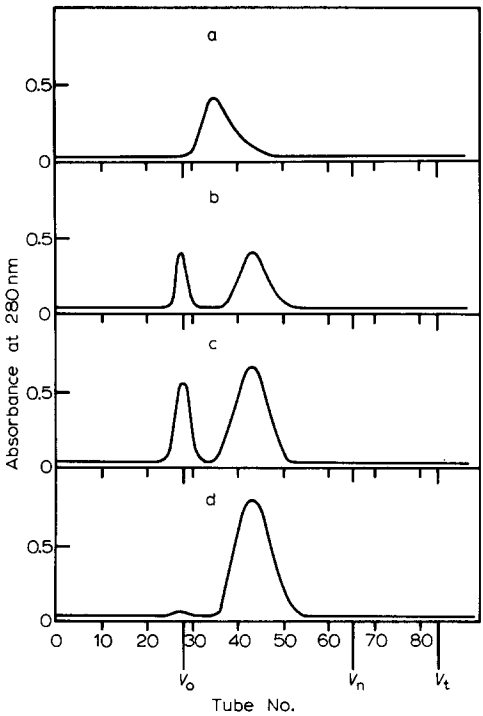
Fig. 4. Irreversible inactivation of subtilisin BPN' in acidic media. The recovery of esterase activity of the unmodified enzyme was measured at pH 7.0 after its brief exposure to various pH values between 0.5 and 6.0 with 4 and 6 M guanidine hydrochloride. Esterase activity was usually measured within 2 h of dilution of the enzyme solution. The zero activity recovery below pH 3.0 was not improved at all even after 24- and 48-h incubation in the renaturing medium. ○, 5 minutes exposure with 4 M guanidine concentration: △, 60 minutes exposure with 4 M guanidine concentration; □, 5 minutes exposure with 6 M guanine concentration. Protein concentration in denaturing media was 1 mg/ml; that in activity was 0.05 mg/ml.

also lost completely after acid denaturation. The denaturation in 8 M guanidine hydrochloride at pH 6 was also irreversible. The sodium dodecyl sulfate polyacrylamide gel electrophoresis of the denatured protein revealed some autolysis products in the case of unmodified enzyme, but the protein mainly remained undigested in these quick denaturation experiments, particularly when the concentration of the protein was less than 1 mg/ml.

In order to clarify the true cause of irreversibility of subtilisin denaturation, combined studies of sedimentation equilibrium, the sodium dodecyl sulfate polyacrylamide gel electrophoresis and Sephadex G-200 gel chromatography were performed on the denatured enzyme under several defferent conditions. Experiments at pH 7 were performed in parallel for the native and phenylmethanesulfonyl-subtilisin to avoid the interference of autolysis in the interpretation of the results.

First we show the result of gel chromatography. Phenylmethanesulfonyl-subtilisin and unmodified subtilisin that were solubilized in unbuffered water were first inactivated by bringing the pH of the solution to 1.0 for ten minutes. Crystalline guanidine hydrochloride was then added to make the solution 6 M, with the denaturant. The solution was then neutralized either by adding crystalline Tris-(hydroxymethyl)-aminomethane or dialyzing the solution against 2 M guanidine hydrochloride buffered at pH 7 or 9. About 1.5 ml of the protein solution was applied to a column (90 × 1.5 cm) equilibrated with an appropriate buffer.

The elution profiles of denatured and renatured subtilisins under several different conditions are shown in Figs. 5 and 6. In Fig. 5, we show the elution profiles of denatured phenylmethanesulfonyl-subtilisin in 2, 1, 0.5 M and zero guanidine hydrochloride solution with 0.05 M phosphate buffer at pH 7. The



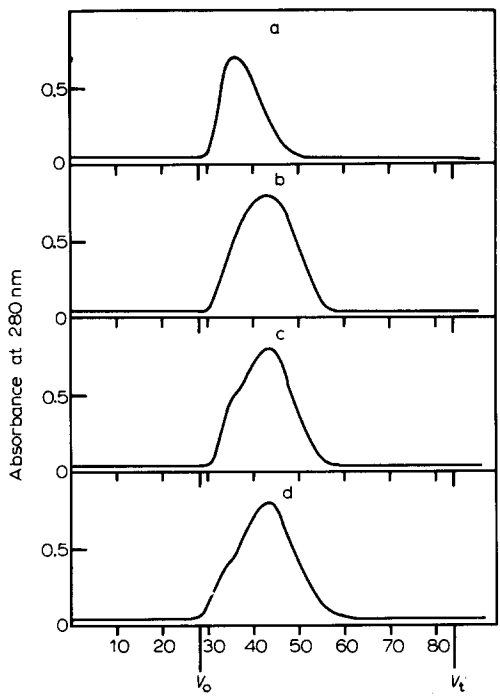


Fig. 5. Elution profiles of denatured phenylmethanesulfonyl-subtilisin in sephadex G-200 column chromatography at pH 7.0. Phenylmethanesulfonyl-subtilisin was first denatured in acidic medium and chromatographed in phosphate buffers containing 4 different concentrations of guanidine hydrochloride. V_0 and V_t represent the void volume and the total volume of the column. V_e is the elution volume of the native enzyme. a: no guanidine hydrochloride, 0.05 M phosphate. b: 0.5 M guanidine hydrochloride, 0.05 M phosphate. c: 1.0 M guanidine hydrochloride, 0.05 M phosphate. d: 2.0 M guanidine hydrochloride, 0.05 M phosphate.

Fig. 6. Elution profiles of denatured subtilisin BPN′ (unmodified) in sephadex G-200 column chromatography at pH 9.0. a: no guanidine hydrochloride, 0.1 M Tris. b: 0.25 M gunaidine hydrochloride, 0.1 M Tris. c: 0.50 M guanidine hydrochloride, 0.1 M Tris. d: 1.0 M guanidine hydrochloride, 0.1 M Tris.

elution position of the peak with the largest elution volume had the value of $V_e/V_0 = 1.50 \pm 0.03$ in 2, 1 and 0.5 M guanidine hydrochloride solution, where V_e and V_0 are the elution volumes of the sample and that of blue dextran. In a similar experiment bovine carbonic anhydrase with a molecular weight of 30000 was eluted with $V_e/V_0 = 1.45$ in 6 M guanidine hydrochloride at pH 7, where the protein is known to be in a randomly coiled state [11]. When the denaturant concentrations were 1.0 and 0.5 M there was another peak at the void volume apparently due to the extensive aggregation. The elution profile obtained in the absence of the denaturant shows the aggregated material with $V_e/V_0 = 1.35$. The aggregates in this case had smaller Stokes radii than those in the presence of 0.5 and 1.0 M guanidine hydrochloride. The proof that the peak with $V_e/V_0 = 1.35$ was aggregate came from the sedimentation study. At 30000 rev./min, all of the solute precipitated to the bottom.

The elution profiles of the denatured subtilisin (unmodified) in 2.0, 1.0, 0.5 M and no guanidine hydrochloride at pH 7 were virtually the same as those in

Fig. 5 except that the amount of aggregated material in lower guanidine concentrations was much larger in this case.

In Fig. 6, we show the elution profile of the denatured subtilisin (unmodified) in 1.0, 0.5, 0.25 M and zero guanidine hydrochloride with 0.1 M Tris buffer at pH 9.0. In 1.0, 0.5 and 0.25 M guanidine hydrochloride the protein eluted with $V_e/V_0 = 1.52 \pm 0.05$ with a slight shoulder on the left. In the absence of the denaturant, it came out with $V_e/V_0 = 1.30$. The presence of aggregates was less pronounced at this pH.

Summarizing the results from gel chromatography we conclude that, at least in 0.5 M guanidine hydrochloride and at pH between 7 and 9, the protein species with the largest elution volume had the value of $V_e/V_0 = 1.5$.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was used to see the extent of autolysis in samples recovered from gel chromatography. The dominant band in all cases was the one that corresponded to the unfragmented subtilisin. The peak that came out at the void volume of the column was entirely composed of unfragmented protein. Phenylmethanesulfonyl-subtilisin, as was expected of course, showed a negligible amoutn of autolysis product either in the fraction with $V_e/V_0 = 1.5$ or in the fraction at void volume.

Meniscus depletion-type sedimentation equilibrium studies [12] were carried out on fractions in the peak with $V_e/V_0 = 1.5$ that were shown to contain little fragmented protein in order to see whether subtilisins in these fractions were aggregated or not. Typical experimental results of such runs are shown in Fig. 7 in the form of the natural logarithm of the fringe displacement versus r^2 type plots. The molecular weight of the solute was calculated from the slope of such plots as described by Yphantis [12]. As is summarized in Table I, proteins in the peak with $V_e/V_0 = 1.5$ were found to have molecular weights around 29000 as long as the guanidine hydrochloride concentration was higher than 0.5 M. The partial specific volume of subtilisins was calculated to be 0.73 from the amino acid composition given in Ref. 8 according to the method of Cohn and Edsall [13] and assumed to be independent of the change in solvent composition. When the concentration of guanidine hydrochloride was lower than 0.25 M, subtilisins invariably showed aggregation even at the lowest protein concentration observable in sedimentation studies using Rayleigh interference optics, i.e. about 0.1—0.2 mg/ml. The results of sedimentation experiments were little affected by storing the sample solution either at 4° C for 24 h or at 20° C for 24 h.

These studies established that subtilisins in the peak with $V_e/V_0 = 1.5$ were minimally fragmented, not aggregated and had Stokes radii close to those of random coils of similar molecular weights as long as the concentration of guanidine hydrochloride was higher than 0.5 M.

Spectroscopic experiments

The fluorescence spectra of the native, denatured and renatured forms of Pms-subtilisin are shown in Fig. 8. Although there was little difference between the results obtained for the unmodified and phenylmethanesulfonyl-subtilisins, there were clear differences between the three spectra of the same protein in different states. We are particularly interested in the difference between the spectra of the native and "renatured" proteins. The fluorescence spectrum of

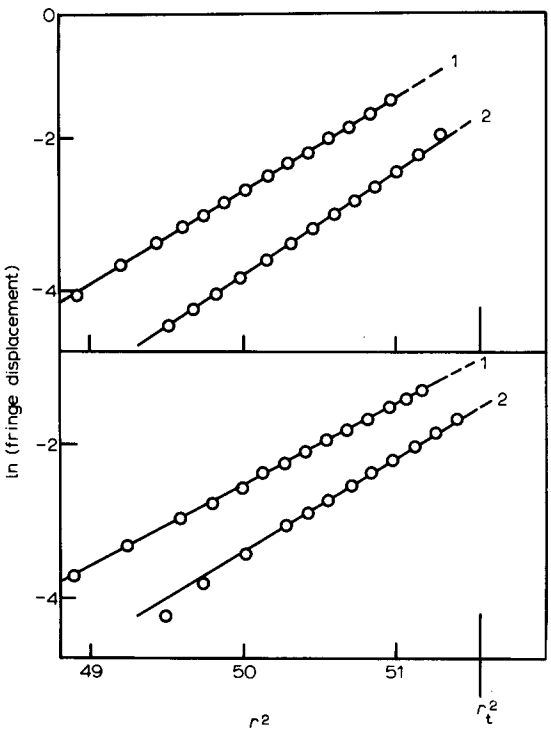


Fig. 7. Meniscus depletion sedimentation equilibrium of denatured subtilisins. The upper figure is for phenylmethanesulfonyl-subtilisin in 2 M guanidine hydrochloride at pH 7.0 (Line 1) and in 0.5 M guanidine hydrochloride at pH 7.0 (Line 2). The lower figure is for unmodified subtilisin in 2 M guanidine hydrochloride at pH 9.0 (Line 1) and in 0.5 M guanidine hydrochloride at pH 9.0 (Line 2). r is the distance from the rotor center and r_b is the distance of the cell bottom from the rotor center.

TABLE I

MOLECULAR WEIGHT OF DENATURED SUBTILISINS

The weight average molecular weight, M_r, was calculated by using the following expression given in ref. 12:

$$M_r = \frac{2RT \cdot \frac{d \ln y}{d r^2}}{(1 - \bar{\nu}\rho)\omega^2}$$

where $(d \ln y/dr^2)$ is the slope of a plot such as the ones given in Fig. 7, $\bar{\nu}$ is the partial specific volume of subtilisin (= 0.73), ρ is the solvent density, ω is the angular velocity, R is the gas constant and T is the temperature in Kelvin.

Guanidine concentration (M)	Phenylmethanesulfonyl-subtilisin pH 7.0	Unmodified subtilisin pH 7.0	Unmodified subtilisin pH 9.0
0.5	28 700	27 500	28 100
1.0	29 600	29 500	28 700
2.0	27 700	—	27 500

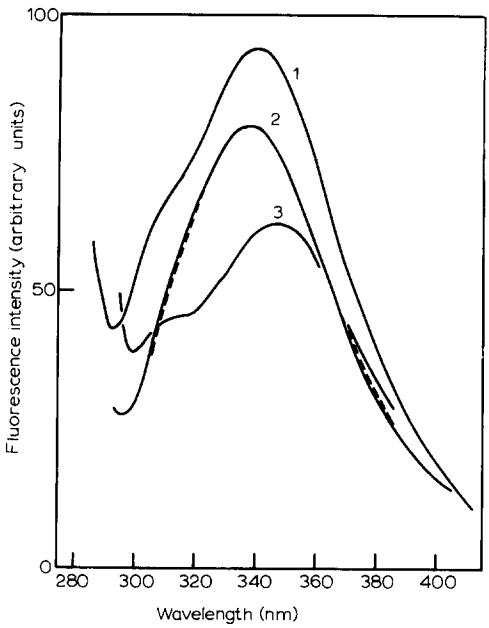


Fig. 8. Fluorescence spectra of native, denatured and renatured phenylmethanesulfonyl-subtilisin at pH 7.0 and at 20°C. Curve 1: native in 0.05 M phosphate buffer. Curve 2: renatured in 0.5 M guanidine solution at pH 7.0 from 6 M guanidine solution at pH 1.0, and from 6 M guanidine solution at pH 7.0 (- - - - -). Curve 3: Denatured in 6 M guanidine solution. Excitation wavelength: 280 nm. Protein concentration: 0.01 mg/ml.

the renatured form had a peak at 337 nm, that is, 5 nm and 10 nm lower than those of the native and the denatured forms respectively. Its peak intensity was lower than that of the native but higher than that of the denatured protein. Moreover the shoulder at around 310 nm was lost. This spectrum was obtained when the guanidine concentration was equal to or less than 1.0 M. A similar spectrum was obtained for the inactively renatured enzyme that had been denatured by acetic acid and 2-chloroethanol.

The clear difference between the fluorescence spectrum of the native protein and that of the renatured protein remained intact at the lowest protein concentration we employed for the renaturation, that is, 0.003 mg/ml, and the addition of 1—10 mM $CaCl_2$ did not alter the result. Fluorescence spectra that were taken within 30 s of dilution of guanidine hydrochloride concentration already had characteristic differences from either the native or the denatured subtilisin. In order to eliminate the possibility that the observed irreversibility of subtilisin denaturation was caused by exposure of this enzyme to acidic pH, the fluorescence spectrum of subtilisin that had been first denatured in 6 M guanidine hydrochloride at pH 7 was taken after 12-fold dilution of the protein solution with 0.05 M phosphate buffer. The result is also shown in Fig. 8. The result confirmed that the irreversible denaturation of subtilisin was not due to the alteration of covalent structure of this enzyme under acidic denaturing conditions.

The optical rotatory dispersion spectra of the renatured protein had no trough around 230 nm and revealed that the renatured protein was essentially

in a randomly coiled state in 0.5 M guanidine hydrochloride. When the concentration of guanidine hydrochloride was lower than 0.25 M, the spectrum of the renatured enzyme changed drastically showing a new trough at 227 nm but we think this was due to the aggregated material and of no significance here.

Almost duplicate results were obtained in the spectroscopic studies using unmodified subtilisin instead of phenylmethanesulfonyl-subtilisin. The renatured subtilisin showed a fluorescence spectrum superimposable on Curve 2 in Fig. 8 in 0.5 and 0.1 M guanidine solution at pH 7.0 with the lowest protein concentration of 0.003 mg/ml.

Discussion

In 0.5 M guanidine hydrochloride phenylmethanesulfonyl-subtilisin maintained its native conformation for 60 h or longer. On the other hand, once it had been denatured in acidic guanidine hydrochloride or in concentrated guanidine hydrochloride or in concentrated guanidine solutions at neutral pH values, it remained in highly unfolded states again for 60 h or longer after being transferred to 0.5 M guanidine solution at pH 7. The latter observation was also repeated with the unmodified enzyme. Either the denaturation or the renaturation of this protein must be excessively slow in this solvent compared with other single chain proteins such as cytochrome *c* [14], lysozyme [15] and ribonuclease A [16]. Denaturation of the native subtilisin at higher guanidine concentration was also slower than similar processes for many other proteins, but proceeded to completion at least in 2 M guanidine solution without fragmentation.

These results prompted us to stress that the well documented stability of the native conformation of subtilisin BPN′ against the action of denaturant must be understood in a kinetic sense rather than in a thermodynamic one. It is still beyond our experimental reach to answer which of the two conformations, the native of the random coil, is thermodynamically stable in 0.5 M guanidine solution. We believe it is still very significant that subtilisin was found in highly extended states in 0.5 M guanidine hydrochloride after complete denaturation in acidic guanidine hydrochloride solutions. The folding of this protein in 0.5 M guanidine hydrochloride must be very difficult if at all possible. The result obtained in 2 M guanidine hydrochloride, on the other hand, clearly proved that preferred conformations of phenylmethanesulfonyl-subtilisin in this solvent at pH 7 and those of the unmodified subtilisin in the same solvent at pH 7 and 9 were indeed nonaggregated and randomly coiled ones. We can at least say that the transition point of subtilisin is less than 2 M guanidine hydrochloride at pH 7 and at 20° C, which is not at all significantly different from the transition point of many other proteins under similar external conditions [17].

The fluorescence spectrum of the renatured subtilisin was different from that of the native subtilisin even at a protein concentration of 0.003 mg/ml. Here and in the following discussion the term "renatured subtilisin" will be applied to the subtilisin that was transferred to solutions of less than 1.0 M guanidine concentration after complete denaturation in acidic guanidine and similar solutions, though such proteins were not "renatured" at all in a usual sense. A similar spectrum was obtained for the renatured subtilisin from 2-chloro-

ethanol, a well known helix-forming solvent [18,19]. The difference between denatured conformations has, therefore, little to do with the observed irreversibility of subtilisin denaturation.

Since the refolding process to the native state is a very slow reaction if at all possible, we are forced to think about the efficient process that must be employed in vivo, in order to produce the active subtilisin within a short time. The possibility of proenzyme formation with subsequent activation by proteolysis can not be denied. To the best of our knowledge, however, the proenzyme formation has been observed with multicellular organisms but not, so far, with bacteria. Participation of intracellular materials such as ribosomes can be considered but not easy to establish. We are still making continued efforts to understand the folding mechanisms of this protein.

The ORD of renatured subtilisin showed that the peptide was in randomly coiled states, in agreement with the result from gel chromatography. The fluorescence spectrum of subtilisin under the same condition indicated, however, that the aromatic residues were not in full contact with water below 1 M guanidine hydrochloride. The fluorescence spectrum of tryptophyl residues in full contact with water has been known to display a maximum at around 350 nm [20], whereas the spectrum of subtilisin in less than 1 M guanidine solutions had a maximum at 337 nm. It is therefore most likely that, while the overall configuration of the polypeptide chain was close to a random coil, local interactions between aromatic residues and other hydrophobic residues were preferred to some extent. If such local interactions were preventing the polypeptide chain from directing itself to the correct folding pathway, we may regard them as unwanted interactions leading the protein to incorrectly folded states [14,21] in 0.5 M guanidine solution and to aggregation at lower guanidine concentrations. It is also possible to conjecture that the polypeptide chain of this enzyme fails to form favorable intermediate states that would bridge the transformation of a random coil to the native conformation.

Finally we like to point out the remarkable stability of phenylmethanesulfonyl-subtilisin at pH 1. The inactivation and denaturation of the native subtilisin BPN′ at acidic pH occurred at around pH 3.5. This acidic inactivation was studied by Matsubara et al. [22] and by Gounaris and Ottesen [23] and documented in the Ref. 8. The driving force for the acid denaturation of subtilisin is, according to Gounaris et al., the protonation of masked histidine residues in the interior of the molecule. The stability of phenylmethanesulfonyl-subtilisin at acidic pH indicates that those histidine residues could not be protonated or that their protonation could not be the driving force for unfolding in phenylmethanesulfonyl-enzyme.

Acknowledgement

The author thanks Prof. Haruhiko Noda for his continuous support of this work.

References

1 Awad, W.M., Vosbeck, K.D., Siegel, S. and Ochoa, M.S. (1975) in Isozymes 1. Molecular Structure (Markert, C.L., ed.), p. 823, Academic Press, New York

2 Brown, M.F. and Schleich, T. (1975) Biochemistry 14, 3069—3074
3 Stauffer, C.E. and Sullivan, J.F. (1971) Biochim. Biophys. Acta 251, 407—412
4 Siegel, S., Brady, A.H. and Awad, W.M. (1972) J. Biol. Chem. 247, 4155)4159
5 Fuke, I., Matsubara, H. and Okunuki, K. (1959) J. Biochem. 46, 1513—1520
6 Ikai, A. (1975) J. Biochem. 77, 321—331
7 Ikai, A. (1976) J. Biochem. 179, 679—688
8 Ottesen, M. and Svendsen, I. (1970) in Methods in Enzymology (Perlmann, G.E. and Lorand, L., eds.), Vol. 19, pp. 199—214
9 Polgar, L. and Bender, M.L. (1967) Biochemistry 6, 610—620
10 Weber, K. and Osborne, M. (1969) J. Biol. Chem. 244, 4406—4412
11 Wong, K.P. and Tanford, C. (1973) J. Biol. Chem. 248, 8515—8523
12 Yphantis, D.A. (1964) Biochemistry 3, 297—317
13 Cohn, E.J. and Edsall, J.T. (1950) Proteins, Amino Acids and Peptides, 4th Printing, Chap. 16, p. 370, Reinhold Publishing Co., New York
14 Ikai, A., Fish, W. and Tanford, C. (1973) J. Mol. Biol. 73, 165—184
15 Tanford, C., Aune, K.C. and Ikai, A. (1973) J. Mol. Biol. 73, 185—197
16 Tsong, T.Y. and Baldwin, R.L. (1972) J. Mol. Biol. 69, 149—153
17 Tanford, C. (1968) Adv. Protein Chem. 23, 121—281
18 Weber, R.E. and Tanford, C. (1959) J. Am. Chem. Soc. 81, 3255—3260
19 Ikai, A. and Noda, H. (1968) J. Biol. Chem. 243, 5028—5034
20 Teale, F.W.J. (1957) Biochem. J. 76, 381—388
21 Ikai, A. and Tanford, C. (1971) Nature 230, 100—102
22 Matsubara, H., Hagiwara, B., Nakai, M., Komaki, T., Yonetani, T. and Okunuki, K. (1958) J. Biochem. 45, 251—258
23 Gounaris, A.D. and Ottesen, M. (1965) C. R. Trav. Lab. Carlsberg 35, 37