ELSEVIER

FEBS Letters 342 (1994) 193–196

FEBS 13865

FEBS LETTERS

Achievement of renaturation of subtilisin BPN′ by a novel procedure using organic salts and a digestible mutant of *Streptomyces* subtilisin inhibitor

Mamoru Matsubara[a], Eiji Kurimoto[a], Shuichi Kojima[b], Kin-ichiro Miura[b], Tomoya Sakai[a,*]

[a]*Department of Chemical Reaction Engineering, Faculty of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467, Japan*

[b]*Institute for Biomolecular Science, Gakushuin University, Mejiro, Toshima-ku, Tokyo 171, Japan*

Received 14 February 1994

Abstract

The pro-sequences of proteases have been considered to be required for the refolding of denatured proteases. However, here we report achievement of almost complete restoration of enzymatic activity of subtilisin BPN′ in the absence of its pro-sequence. The presence of 2 M potassium acetate in the folding medium enhanced the refolding efficiency of guanidine hydrochloride (GdnHCl)-denatured subtilisin BPN′ by up to 28%, and other organic salts were also found to be useful, suggesting that general contribution of the bulky hydrophobic moieties of the salts to the formation of a favorable environment required for folding. This finding will provide new insights into the folding mechanisms not only of proteases but also of various other proteins. Almost complete restoration of enzymatic activity of denatured subtilisin in the organic salt solution was accomplished by further addition of mutated *Streptomyces* subtilisin inhibitor (SSI), which had been converted to a digestible temporary inhibitor by removal of the disulfide bridge near the reactive site.

Key words: Protein folding; Subtilisin BPN′; Serine protease; *Streptomyces* subtilisin inhibitor; Pro-sequence, Site-directed mutagenesis

1. Introduction

Some proteins refold spontaneously in vitro to their native states, some proteins do not once they have been fully denatured. Subtilisin, α-lytic protease and carboxypeptidase Y are the notorious examples of the latter case [1–4]. In vivo folding of subtilisin BPN′, a 275 amino acid serine protease from *Bacillus amyloliquefaciens*, depends on a 77 amino acid pro-sequence, succeeded by the processing to delete the pro-sequence and to prepare the active mature subtilisin BPN′ [1,5,6]. Recently, Eder et al. made it possible to express an inactive pro-subtilisin variant (pro-subtilisinS221A mutant) in *Escherichia coli* and demonstrated that the pro-subtilisin variant unfolds and refolds reversibly [7]. Further, they reported that, in the absence of the pro-sequence, subtilisin could fold up to the metastable intermediate state and that only addition of the pro-sequence led the intermediate to fold to the final native state [8]. Recent reports from Inouye's groups [9–13] demonstrated that a denatured subtilisin in 6 M guanidine hydrochloride (GdnHCl) was impossible to refold to a biologically active enzyme in the absence of its pro-sequence. Thus, it is believed at present that the fully denatured subtilisin can never be refolded in the absence of the pro-sequence.

We have been examining the effects of various inorganic or organic salts on the folding efficiency of GdnHCl-denatured subtilisin BPN′ in the absence of pro-sequence, and found a striking evidence that the fully denatured subtilisin BPN′ can renature quantitatively even in the absence of the pro-sequence [14]. First, we observed that, when a denatured subtilisin in 6 M GdnHCl was allowed to refold by dilution in 2 M potassium acetate solutions, nearly 30% of the activity was recovered. Second, we also demonstrated that almost all the denatured molecules of subtilisin were stabilized as tightly bound complexes with *Streptomyces* subtilisin inhibitor (SSI), a proteinaceous strong inhibitor of subtilisin [15], presumably after refolding in the presence of potassium acetate. A quantitative refolding of subtilisin possibly achieved in this medium, however, was unable to be confirmed by enzyme activity measurement. In this report, we examined the generality of organic salts for enhancement of subtilisin BPN′ folding in the absence of the pro-sequence. Further, in an effort to attain the complete renaturation of subtilisin BPN′ as an active form, we introduced a novel renaturation strategy by applying a temporary inhibitor which is gradually degraded by the renatured protease. Ultimately, we found that a supple-

* Corresponding author. Fax: (81) (52) 834 9309.

SSDI 0014-5793(94)00250-Y

mentation of an excess amount of the digestible mutant of SSI in the renaturation solution such as 2 M potassium acetate led to the recovery of proteolytic activity of subtilisin to almost 100%.

2. Materials and methods

2.1. Materials

Subtilisin BPN′ was purchased from Nagase Biochemicals. The mutated SSI, in which the disulfide bridge (Cys^{71}–Cys^{101}) had been removed by replacing these cysteine residues with serine residues, was expressed in *Streptomyces lividans* and purified as described before [16,17]. The inhibitory activity of mutant SSI toward subtilisin decreased gradually with increasing incubation time after mixing with subtilisin due to proteolytic degradation of SSI (a temporary inhibitor) [17]. Synthetic substrate, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide, was purchased from Sigma. All other chemicals were of analytical grade.

2.2. Protein denaturation and renaturation

Subtilisin BPN′ (0.1 mg/ml) was denatured in 6 M GdnHCl solution at pH 2.0 for 2 h at room temperature. We adopted a low pH level for unfolding in order to suppress the otherwise proceeding autoproteolytic digestion during unfolding. Refolding was initiated by mixing the denatured enzyme solution at 100-fold dilution with the refolding solution containing various salts at several concentration levels, raising the resultant pH level to 6.5. Protein concentrations in the renaturation solutions were around 1 μg/ml. These low protein concentrations were adopted to minimize protein aggregation in the renaturation solution. To study the effectiveness of the presence of SSI on the renaturation, certain excess amounts of the mutated SSI were added to the renaturation mixture consisting of, e.g. 2 M potassium acetate. After incubation at either 4°C or 15°C for indicated periods, the enzyme activity was assayed spectrophotometrically by use of the substrate *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide [18]. The recovered activity of subtilisin was calculated on the basis of enzyme activity as a percentage relative to that of native subtilisin at an equimolar concentration. All experiments were performed at least in triplicate and their averages are presented.

2.3. HPLC analyses

Involvement of autoproteolytic digestion of subtilisin during unfolding or refolding was monitored by the reverse-phase high-performance liquid chromatography (HPLC) on a Vydac C_4 column (5.4 mm × 21 cm) eluted by 0.1% trifluoroacetic acid with a linear gradient from 0 to 50% acetonitrile for 40 min, followed by a 50–80% acetonitrile gradient for 10 min. The flow rate was 1.0 ml/min. Both subtilisin and autoproteolytic fragments were detected by the absorbance at 220 nm.

Table 1
Effects of various salts on the recovery of denatured subtilisin.

Salt	Concentration (M)	Recovered activity (%)
LiCl	2	0.0
NaCl	2	6.8
KCl	2	7.2
RbCl	2	2.0
$MgCl_2$	2	0.0
$CaCl_2$	2	0.0
Na_2SO_4	2	0.0
CH_3COOK	2	28.0
CH_3COONa	2	23.5
$(CH_3COO)_2Mg$	2	27.9
$(CH_3COO)_2Ca$	1.6	35.1
HCOOK	2	16.3
CH_3CH_2COOK	2	46.0
CH_2COOK CH_2COOK	1	15.2

The concentration of subtilisin in renaturation solutions (pH 6.5) was 1.0 μg/ml. The samples were incubated at 4°C for 24 h and then assayed for subtilisin activity. Details are presented in section 2.

3. Results and discussion

3.1. Effectiveness of organic salt solutions for renaturation of subtilisin without pro-sequence

The folding of denatured subtilisin has been extensively studied by trapping folding intermediates

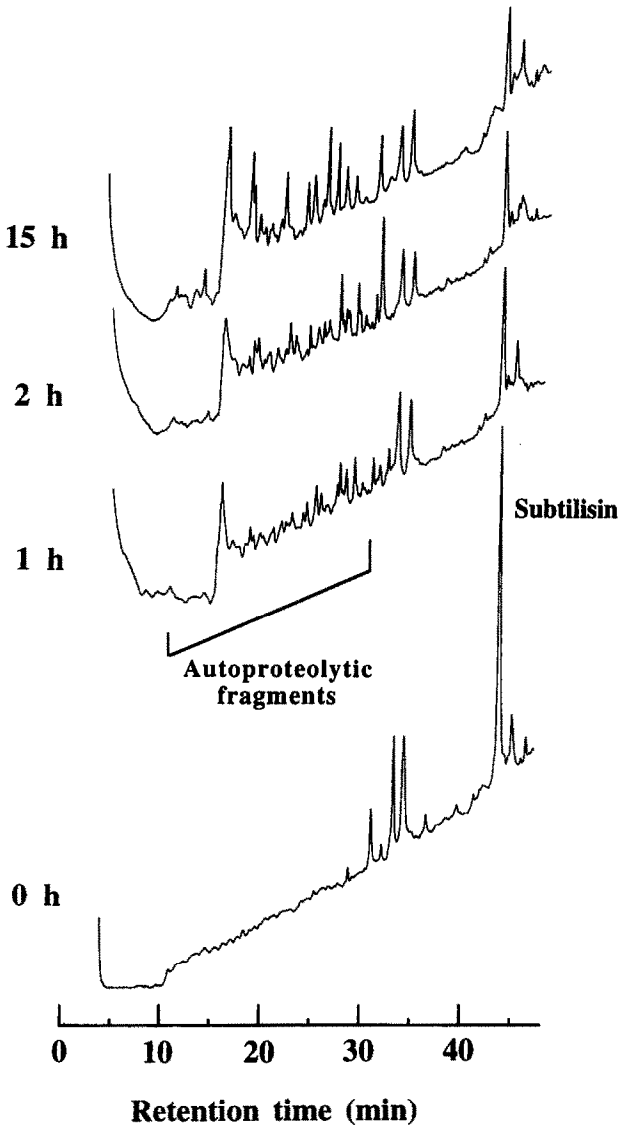

Fig. 1. Time course of the reverse-phase HPLC pattern of the subtilisin and autoproteolytic fragments during refolding in the presence of 2 M potassium acetate. Denatured subtilisin in 6 M GdnHCl was diluted 50-fold with 2 M potassium acetate at a resultant pH of 7.5 and at 4°C. The concentration of subtilisin in renaturation solution was 2.0 μg/ml. Aliquotes of the renaturation solution were subjected to reverse-phase HPLC analysis at the indicated incubation times (0, 1, 2 and 15 h).

[4,7,8,12] or by generation of several mutants of subtilisin BPN′ [6,19,20], combined with structural analyses by CD and NMR. Up to now, it has been considered that a success, if any, in refolding of GdnHCl-denatured subtilisin requires the presence of pro-sequence of subtilisin [7,8,10,12,13]. We have examined the effects of various inorganic or organic salts on the folding efficiency of GdnHCl-denatured subtilisin BPN′ in the absence of the pro-sequence, and reported recently that a high concentration of potassium acetate was remarkably effective [14]. To verify the generality of organic salts for the enhancement of subtilisin folding, we examined the effects of several organic salts and inorganic salts on renaturation of the denatured subtilisin. Table 1 summarizes the recovered activity of GdnHCl-denatured subtilisin BPN′ after 24-h incubation at 4°C in the solution containing various salts at high concentrations. In addition to potassium or sodium acetate, which had been shown to be effective as folding mediators, various salts of organic acids were also found to be useful. Propionate was found to be more effective than acetate or formate. Detailed mechanisms of the contribution of the concentrated salt, especially those of organic salts with bulky hydrophobic moiety, are not yet clear. However, since efficient refolding requires a high concentration of organic salt and propionate is more effective than acetate, it seems reasonable that the bulky organic anion may exert an interaction with the cationic residues in the refolding subtilisin molecule, thereby inhibiting the formation of unfavorable ionic bondings and, at the same time, facilitating the formation of a hydrophobic core in the molecule driven by intrinsic strong hydrophobic interactions. It is likely that kinetically trapped folding intermediates of proteases detected in the absence of their prosequences [4,7,8] are misfolded species resulting from unfavorable ion-pairing of charged groups in the molecule before a hydrophobic core can form. Our results indicate that contribution of organic anions to the prevention of such unfavorable interactions is more appropriate than that of inorganic anions, maybe due to the presence of bulky hydrophobic moiety. The notable enhancement of the refolding efficiency of subtilisin in the presence of organic salts may thus be due to such specific interactions of subtilisin and organic salt. We have also observed refolding acceleration by organic salts in another protein, thermolysin (unpublished data), and thus the new concept described here will provide new insights into the mechanisms of protein folding.

3.2. *Achievement of renaturation of subtilisin using a mutant SSI (temporary inhibitor)*

A low refolding yield of subtilisin BPN′ even in the presence of organic salts seems to be caused mostly by autolysis of subtilisin itself, that is, proteolytic attack by the folded 'active' molecule of subtilisin on the refolding molecule, which is very sensitive to attack by protease, since HPLC analysis of the reaction mixture has indicated that the peak intensity of subtilisin decreases with increasing incubation time, and many peaks of proteolytic fragments appear (Fig. 1). Therefore, inhibition of proteolysis of refolding subtilisin appears to enhance the refolding efficiency. We found previously that almost all the molecule of denatured subtilisin formed tightly bound complexes with SSI, presumably after refolding in the presence of potassium acetate [14]. However, in this case, an 'active' form of subtilisin could not be isolated, due to complex formation with SSI. In these situation, a temporary inhibitor is considered to be suitable for isolation of an 'active' form of subtilisin refolded in the presence of organic salts, because it is an inhibitor which is initially potent but is gradually degraded by protease, as represented schematically in Fig. 2. Mutant SSI, in which the disulfide bridge near the reactive site had been removed [17], was used as a digestible temporary inhibitor. Fig. 3 shows the renaturation profiles of subtilisin at 15°C in the presence of 1.5 M or 2 M potassium acetate with a 24-fold molar excess of mutant SSI. The recovered activity of GdnHCl-denatured subtilisin

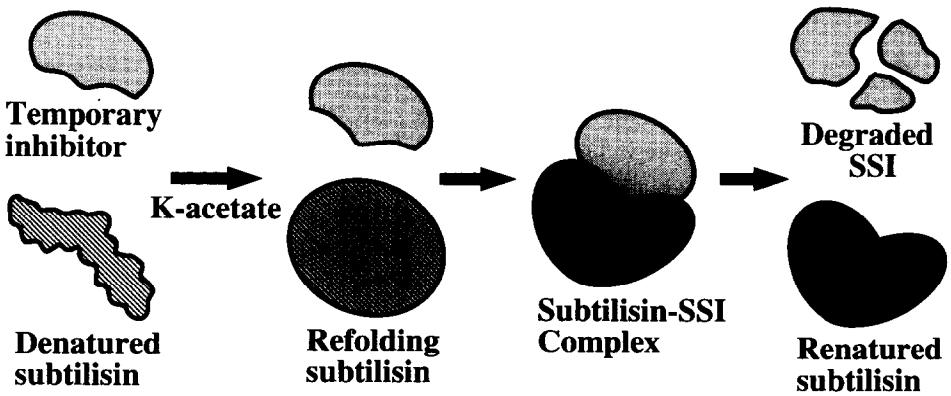

Fig. 2. A schematic representation of the renaturation strategy by use of the digestible mutant SSI. When denatured subtilisin is incubated in the presence of the mutant SSI in 2 M potassium acetate, the mutant SSI binds to refolded or refolding subtilisin to form subtilisin-SSI complexes. The complexes are quite stable initially, but the inhibitor is gradually digested by the complexed renatured subtilisin. Thus, active subtilisin is produced in the renaturation solution. Once the subtilisin renatures and adopts its authentic three-dimensional structure, the rate of autolysis is quite slow.

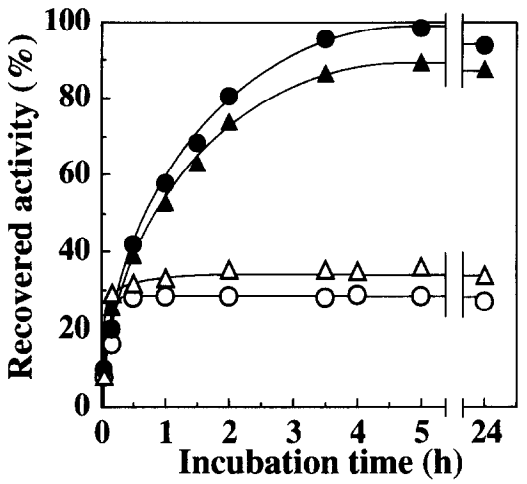

Fig. 3. A remarkable effect of mutant SSI on the renaturation of fully denatured subtilisin in 1.5 M or 2.0 M potassium acetate. Denatured subtilisin in 6 M GdnHCl was diluted 100-fold with 1.5 M or 2.0 M potassium acetate with and without 10.0 μg/ml mutant SSI at a resultant pH of 6.5 and at 15°C. The concentration of subtilisin in renaturation solution was 1.0 μg/ml. The recovered activity of subtilisin was assayed after the indicated incubation time at 15°C. Given experimental conditions: 1.5 M (●) or 2 M (▲) potassium acetate with mutant SSI, and 1.5 M (○) or 2 M (△) potassium acetate without mutant SSI.

in the presence of mutant SSI increased gradually, reaching almost 100% after 5 h of incubation, whereas that in the absence of mutant SSI remained at about 30%.

Thus, we accomplished the almost complete renaturation of denatured subtilisin BPN′ using organic salts at high concentration and a temporary inhibitor. One possible contribution of a temporary inhibitor to the enhancement of refolding efficiency is inhibition of autolysis of the refolding molecule, as described above. The pro-sequence of protease has also been shown to function as an inhibitor [3,10,21]. However, the functional structure of the pro-sequence was found to be formed only after interaction with protease [7,12,22] and its active center has not yet been specified, although the importance of hydrophobic residues has been suggested [11]. In contrast, mutant SSI lacking the disulfide bridge near the reactive site was shown to inhibit subtilisin initially, as the wild-type SSI does, and thereafter to be gradually degraded by subtilisin via a specific intermediate with a nick at the reactive site [17]. The interaction of mutant SSI and subtilisin is thus specific and strong. Such features would have resulted in almost complete renaturation of subtilisin. Another possible contribution of mutant SSI to the refolding process is a 'template' effect. In this hypothesis, the three-dimensional structure of SSI, particularly that of the contact region, may induce structural formation around the active site of subtilisin, and thus accelerate the refolding. Detailed understanding in this respect is a future problem.

In any event, we have accomplished the almost complete renaturation of GdnHCl-denatured subtilisin BPN′, previously thought to be possible only in the coexistence of the pro-sequence, by a novel procedure using organic salts at high concentrations and a digestible temporary inhibitor in the folding medium. In particular, acceleration of folding by organic salts may provide new insights into the folding mechanisms generally proposed [23,24].

Acknowledgments: This work was supported by a Grant-in-Aid for Developmental Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

[1] Zhu, X., Ohta, Y., Jordan, F. and Inouye, M. (1989) Nature 339, 483–484.
[2] Silen, J.L. and Agard, D.A. (1989) Nature 341, 462–464.
[3] Winter, J.R. and Sørensen, P. (1991) Proc. Natl. Acad. Sci. USA 88, 9330–9334.
[4] Baker, D., Sohl, J.L. and Agard, D.A. (1992) 356, 263–265.
[5] Ikemura, H., Takagi, H. and Inouye, M. (1987) J. Biol. Chem. 262, 7859–7864.
[6] Strausberg, S., Alexander, P., Wang, L., Schwarz, F. and Bryan, P. (1993) Biochemistry 32, 8112–8119.
[7] Eder, J., Rheinnecker, M. and Fersht, A.R. (1993) J. Mol. Biol. 233, 293–304.
[8] Eder, J., Rheinnecker, M. and Fersht, A.R. (1993) Biochemistry 32, 18–26.
[9] Ikemura, H. and Inouye, M. (1988) J. Biol. Chem. 263, 12959–12963.
[10] Ohta, Y., Hojo, H., Aimoto, S., Kobayashi, T., Zhu, X., Jordan, F. and Inouye, M. (1991) Mol. Microbiol. 5, 1507–1510.
[11] Kobayashi, T. and Inouye, M. (1992) J. Mol. Biol. 226, 931–933.
[12] Shinde, U., Li, Y., Chatterjee, S. and Inouye, M. (1993) Proc. Natl. Acad. Sci. USA 90, 6924–6928.
[13] Shinde, U. and Inouye, M. (1993) Trends Biochem. Sci. 18, 442–446.
[14] Matsubara, M., Kurimoto, E., Kojima, S., Miura, K. and Sakai, T. (1993) Chem. Lett. 1783–1786.
[15] Hiromi, K., Akasaka, K., Mitsui, Y., Tonomura, B. and Murao, S. (1985) Protein Protease Inhibitor – The Case of *Streptomyces* Subtilisin Inhibitor (SSI), Elsevier, Amsterdam.
[16] Kojima, S., Nishiyama, Y., Kumagai, I. and Miura, K. (1991) J. Biochem. 109, 377–382.
[17] Kojima, S., Kumagai, I. and Miura, K. (1993) J. Mol. Biol. 230, 395–399.
[18] Carter, P. and Wells, J.A. (1988) Nature 332, 564–568.
[19] Bryan, P., Alexander, P., Strausberg, S., Schwarz, F., Lan, W., Gilliland, G. and Gallagher, T. (1992) Biochemistry 31, 4937–4945.
[20] Strausberg, S., Alexander, P., Wang, L., Gallagher, T., Gilliland, G. and Bryan, P. (1993) Biochemistry 32, 10371–10377.
[21] Baker, D., Silen, J.L. and Agard, D.A. (1992) Proteins: Struct. Funct. Genet. 12, 339–344.
[22] Sørensen, P., Winter, J.R., Kaarsholm, N.C. and Poulsen, F.M. (1993) Biochemistry 32, 12160–12166.
[23] Gething, M.-J. and Sambrook, J. (1992) Nature 355, 33–45.
[24] Kim, P.S. and Baldwin, R.L. (1990) Annu. Rev. Biochem. 59, 631–660.