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Refolding of the non-specific neutral protease from *Bacillus*
stearothermophilus proceeds via an autoproteolytically sensitive
intermediate

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ABSTRACT

A very thermostable variant of the thermolysin-like protease from *Bacillus stearothermophilus* (G8C/N60C) was previously created by introduction of a disulfide bond into the cysteine-free pseudo-wild type variant (pWT) and thus fixing the unfolding region 56-69. In the present paper, we show that G8C/N60C and pWT can be reactivated from the completely unfolded states, accessible at ≥ 7.5 M guanidine hydrochloride, and analyze the kinetics of folding, autoproteolytic degradation and aggregation. From changes in the fluorescence spectra with time of renaturation, it can be concluded that a folding intermediate with native-like structure, but which is still inactive and sensitive to autoproteolysis, is rapidly formed after renaturation has initiated. The critical region 56-69 of pWT is involved in the autoproteolytic sensitivity of the intermediate as we conclude from the differences in the chevron plots of the first order rate constants of reactivation and the fragmentation patterns in SDS-PAGE of pWT and G8C/N60C.

Keywords

Autoproteolysis, disulfide bond, folding intermediate, neutral protease, reactivation, refolding

Abbreviations:

Abz-AGLA-Nba, 2-aminobenzoyl-Ala-Gly-Leu-Ala-4-nitrobenzylamide; GdnHCl, guanidine hydrochloride; k_{obs} , observed rate constant; pWT, pseudo-wild type; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; $\Delta G^{\#}$, Gibbs free energy of activation; ΔG^0 , Gibbs free energy.

1. Introduction

Proteolytic enzymes differ from other enzymes by their capability of autoproteolysis. Generally, they are not susceptible to autoproteolysis under native conditions but become susceptible to autoproteolysis when their native structure is impaired. For this reason proteases are irreversibly inactivated if they are exposed to denaturing influences such as high temperatures or chemical denaturants. During protein biosynthesis, proteases are liable to be degraded as long as their native folded structure has not been attained. In nature several strategies have evolved to protect proteases from autoproteolytic degradation. Many proteases are produced as inactive proenzymes, which attain their active conformation only after a specific proteolytic cleavage and release of the propeptide [1, 2]. Thus, most digestive proteases as well as bacterial extracellular proteases are secreted as inactive zymogens, e.g. elastase as proelastase or subtilisin as prosubtilisin. Alternatively, many proteases have specific inhibitors that prevent activity. Thus, serpins are a well-known class of structurally similar compounds with particular importance as inhibitors of serine proteinases [3].

Due to phenomenon of autoproteolysis, it is generally not possible to follow the unfolding of proteases without concomitant protein degradation [4-6]. Similarly, folding (refolding) processes cannot be observed by biophysical methods. Therefore, the thermodynamic stability (ΔG°) of proteases is also mostly inaccessible to biochemical investigation.

Many proteases also need a propeptide for correct folding [7]. From extensive studies on the α -lytic protease from *Lysobacter enzymogenes*, it was concluded that mature proteases are thermodynamically unstable but kinetically trapped by a high energetic barrier to unfolding, whereas folding is catalyzed by the propeptide [8]. These constraints also seemed to be valid for the unspecific proteases of the thermolysin type [9]. Recently, however, we could show that the

neutral protease from *Bacillus stearothermophilus* can be expressed in *E. coli* with an N-terminal His tag without the help of the propeptide [10]. The enzyme accumulated as inclusion bodies and, unexpectedly, it could be renatured in high yields after solubilisation of the protein aggregates in guanidine hydrochloride (GdnHCl). Moreover, we could show in a study comparing this protease to its extremely stable disulfide-engineered variant G8C/N60C in GdnHCl-induced denaturation [11, 12] that these enzymes can be unfolded without significant autoproteolysis in the presence of very high concentrations of GdnHCl. These findings motivated us to investigate the reactivation of this protease and its thermostable variant G8C/N60C, although there are only a few related studies in the literature, probably because of the low renaturation yields and the complex interplay of folding and irreversible processes, such as autoproteolysis and aggregation. For example, however, almost complete restoration of enzymatic activity was reported for subtilisin BPN' in the presence of organic salts and a temporary inhibitor [13]. Moreover, high yields of renaturation could also be achieved with immobilized thermolysin in the presence of salts of organic acids [14].

The neutral protease from *B. stearothermophilus* as one of the thermolysin-like members of the M4 family (with 85 % sequence identity to thermolysin) consists of 319 amino acid residues and belongs to the large family of the zinc-dependent metalloendopeptidases. Because of its phylogenetic relationship with the human matrix metalloproteinases and other physiologically important mammalian proteases such as endothelin- and angiotensin-converting enzymes, neprilysin or neurolysin, it has also importance as a model system for these proteases. Based on the resolved X-ray structures of its homologous bacterial relatives from *B. thermoproteolyticus* (known as thermolysin) and *B. cereus*, a 3D model of this enzyme is available [15]. Fig. 1 shows this model for the disulfide-containing variant G8C/N60C. The enzyme is organized in two

domains with the active site between, containing the catalytic zinc ion and four binding sites for calcium ions with stabilizing function.

Figure 1

Extensive mutational studies had revealed that mutations in the loop region 56-69 (Fig. 1) are critical for the thermal stability of the protease whereas mutations in other regions have only marginal effects [16]. In the engineered enzyme variant G8C/N60C [17] a disulfide bond connects this region with the underlying β -sheet structure (Fig. 1), which causes a dramatic stabilization against thermal inactivation. The variant was produced by site-directed mutagenesis starting from the cysteine-free variant of the protease (C288L), which does not significantly differ from the wild-type enzyme either in activity or in stability [11, 18]. This enzyme, called pseudo-wild type (pWT), is used as reference enzyme in studies on G8C/N60C. The introduction of the disulfide bond produced a shift in the half-life at 92.5°C from < 0.3 min to 35.9 min and increased the temperature after which half of the initial activity is lost within 30 min (T_{50}) by 16.7°C [17]. In GdnHCl-induced denaturation of pWT and G8C/N60C, unfolding and autoproteolysis could be separated because the proteolytic activity is strongly inhibited by GdnHCl even in the presence of low denaturant concentrations [11, 12]. The results suggested that the disulfide bond stabilizes the protease against autoproteolysis more than against global unfolding [11]. Obviously, unfolding of pWT proceeds via local unfolding of the critical region which is sensitive to autoproteolysis. The disulfide bond fixes this region and protects therefore against autoproteolysis. A similar effect is obtained at high Ca^{2+} concentrations (100 mM) by saturation of the low affinity calcium binding site III being located in the critical loop region between amino acids 56-69 [12].

In the present paper we show that pWT and G8C/N60C can undergo substantial reactivation starting from the completely GdnHCl-unfolded states. Comparison of the reactivation kinetics with the kinetics of refolding by fluorescence spectroscopy and autoproteolytic degradation by SDS-PAGE reveals that refolding proceeds via a native-like intermediate which is susceptible to autoproteolysis. The combination of the kinetic refolding data with the results of unfolding [12] allows an estimate of the standard Gibbs free energies of the reversible transition from the native state to the intermediate and also allows a comparison of their kinetic and thermodynamic stabilities, which has been difficult to achieve with proteases until now.

2. Materials and Methods

2.1 Chemicals

Casein was purchased from Merck (Darmstadt, Germany), 2-aminobenzoyl-Ala-Gly-Leu-Ala-4-nitrobenzylamide (Abz-AGLA-Nba) from Bachem (Heidelberg, Germany), GdnHCl from ICN Biomedicals GmbH (Eschwege, Germany), sodium deoxycholate from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany), and tris(hydroxymethyl)aminomethane (Tris) from Amersham Biosciences (Uppsala, Sweden). All other reagents were of the purest quality available.

2.2 Enzymes

pWT and G8C/N60C were produced as described previously [17, 18]. Immediately before use, the enzyme solution was dialyzed against 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl₂, incubated at 65°C for 8 min and dialyzed again. After this procedure the enzyme solutions were homogeneous as judged by SDS-PAGE. The absence of low molecular weight fragments was checked by size-exclusion chromatography using a Superdex 75 (10/30) column (Pharmacia,

Uppsala, Sweden) with 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl₂ and 20% (v/v) isopropanol as eluent.

For the preparation of solutions containing denatured enzymes at protein concentrations of 5 to 20 µg mL⁻¹ (for reactivation experiments) the native enzymes were adjusted to a protein concentration of 0.1 to 0.4 mg mL⁻¹, diluted 1:20 into 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl₂ and 8.4 M GdnHCl and incubated for at least 3 hours. For the preparation of solutions containing denatured enzymes at protein concentrations higher than 20 µg mL⁻¹ (for fluorescence spectroscopy, SDS-PAGE analysis and aggregation experiments) the native enzymes were first precipitated by TCA in the presence of sodium deoxycholate, washed with acetone and ethanol, and dried under vacuum as described in [11]. Then they were dissolved in 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl₂ and 8.4 M GdnHCl to the protein concentration needed for the subsequent experiments (typically 5 – 10 mg mL⁻¹).

2.3 Protein concentration

The protein concentration was determined using the BCA protein assay (Pierce, Germany) with bovine serum albumin as standard.

2.4 Activity toward casein

The activity was determined at 37°C by 30 min incubation in 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl₂ and 0.5% (w/v) casein as described previously [19, 20]. To determine the reactivation of pWT and G8C/N60C, the denatured enzymes (in GdnHCl-containing solution) were diluted into 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl₂. Samples were taken from this renaturation mixture at different times and assayed for activity.

2.5 Activity toward Abz-AGLA-Nba

The activity was measured at a substrate concentration of 20 μM in 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl_2 at 25°C. The increase in fluorescence emission at 415 nm after excitation at 340 nm was recorded over at least 10 min [21]. To determine the reactivation of pWT and G8C/N60C with half life > 5 min, the denatured enzymes (in GdnHCl containing solution) were diluted into 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl_2 . After several periods of time samples were taken from this renaturation mixture and assayed for activity. The progress curves (change of activity vs. renaturation time) were fitted to a single exponential function yielding the rate constants k_{obs} .

Figure 2

For monitoring fast reactivation kinetics (half life < 5 min) the substrate was directly added to the renaturation mixture and the change in the fluorescence signal was followed as function of time as exemplified in Fig. 2. The linear section of the curve representing the final recovered activity was fitted using the function $y = m \cdot x + n$, where y is the fluorescence signal and x is time. Subtraction of the corresponding y -values from the fluorescence data in the non-linear initial phase yielded exponential curves (Fig. 2, insert) from which the rate constants of reactivation (k_{obs}) were calculated.

2.6 Autoproteolysis

SDS-PAGE was used to follow autoproteolysis of the enzymes at a protein concentration $\geq 20 \mu\text{g mL}^{-1}$. To concentrate the protein solution and to remove GdnHCl, the samples taken from the renaturation mixture were treated with sodium deoxycholate [11]. The precipitates were dried

under vacuum, dissolved in SDS-sample buffer and separated on 15 % polyacrylamide gels according to Laemmli [22]. The proteins were stained with Coomassie Brilliant Blue G-250 for quantitative evaluation of the bands of intact enzyme by scanning with a CD 60 densitometer (Desaga, Darmstadt, Germany), or with silver [23] for evaluation of the fragmentation pattern.

2.7 Fluorescence measurements

Fluorescence spectroscopy was carried out in 1 cm quartz cuvettes at 25°C with a FluoroMax-2 spectrofluorometer (Yvon-Spex, Grasbrunn, Germany) with excitation at 278 nm. The slit width was 5 nm for both excitation and emission. Protein spectra were corrected by blank spectra.

Kinetic measurements were started by dilution of the denatured enzymes (in GdnHCl-containing solutions) into 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl₂. If necessary, GdnHCl was added to adjust the final GdnHCl concentration. The progress curves (change of signal vs. renaturation time) were fitted to a single exponential function yielding the rate constants k_{obs} .

2.8 Light scattering

Light scattering to monitor aggregation was measured in 1 cm quartz cuvettes at 25°C with a FluoroMax-2 spectrofluorometer (Yvon-Spex, Grasbrunn, Germany) and with excitation and emission at 360 nm. The slit width was 1 nm for both excitation and emission. First, the baseline was recorded by measuring the renaturation buffer for 5 minutes with gentle stirring. Then the denatured enzyme (in GdnHCl-containing solutions) was added and the change in the signal was recorded for 15 minutes while gently stirring the solution. The amplitude of the increase in the signal was used for quantifying the extent of aggregation. The calibration of the signal was performed by measuring the light-scattering of the protein completely precipitated by TCA (2.5

%, w/w) and setting the amplitude of this signal as 100 % aggregation. The completeness of precipitation was checked by SDS-PAGE analysis.

3. Results

Despite the strong tendency of being degraded by autoprolysis, the variants of the neutral protease from *B. stearothermophilus* pWT and G8C/N60C can be obtained in the intact, unfolded state at high concentrations (≥ 7.5 M) of GdnHCl [11, 12]. This finding enabled us to investigate refolding of these proteases. Other denaturants such as urea or high temperature were not appropriate for observing refolding because they were not able to yield completely unfolded protein without autoprolytic fragmentation. The transfer of the denatured enzymes ($[\text{GdnHCl}] \geq 7.5$ M) to native-like conditions ($[\text{GdnHCl}] \leq 1.0$ M) was investigated by following enzyme activity, intrinsic fluorescence, autoprolysis, and aggregation. Native pWT and G8C/N60C are stable toward autoprolysis and keep their structural integrity for several weeks [12].

In all the studies, Ca^{2+} ions (5 mM) were added to stabilize the protein. Zn^{2+} ions, although essential cofactors and exhibiting extremely high affinity, were without effect on the stability and were not added exogenously. For technical reasons, different protein concentrations had to be chosen in the various experiments, which must be considered in the interpretation of the results.

3.1 Reactivation

Figure 3

Fig. 3 demonstrates by two independent assays that protease activity can be significantly regained if the completely unfolded enzymes are transferred from 8 M GdnHCl into native-like conditions (0.4 M GdnHCl) by dilution with buffer. As the standard casein assay is a discontinuous method, in which the reactivation reaction is continued during the incubation with casein, it is not appropriate to determine the rate constants of reactivation. Moreover, it is not sensitive enough to follow reactivation at protein concentrations of $< 0.2 \mu\text{g mL}^{-1}$. Therefore, the synthetic fluorogenic substrate 2-aminobenzoyl-Ala-Gly-Leu-Ala-4-nitrobenzylamide (Abz-AGLA-Nba), which enables the emerging activity to be followed directly (section 2.5) at protein concentrations of $0.1 \mu\text{g mL}^{-1}$, was used to analyze the kinetics of the reactivation process at varying residual GdnHCl and protein concentrations. In all cases the reactivation kinetics could be fitted to a single exponential function. The resulting values for the observed rate constant (k_{obs}) were plotted semi-logarithmically versus the final GdnHCl concentration in the renaturation assay (chevron plot). Straight lines were obtained for both pWT and G8C/N60C (Fig. 4) but with different slopes. The curves intersected at 0.4 M GdnHCl. The observed rate constants were independent of the protease concentrations as checked at 0.2 M GdnHCl for the concentration range of $0.1 - 5 \mu\text{g mL}^{-1}$ (Fig. S-1).

Figure 4

Reactivation did not reach completion as concluded from the comparison of the recovered activity with the activity of the native enzyme determined under the same reaction conditions. The maximum reactivation yields were dependent on the final GdnHCl and protein concentrations. At $0.1 \mu\text{g mL}^{-1}$ protein they were maximal (50 % for pWT and 70 % for G8C/N60C) when the GdnHCl concentration was 0.4 M (Fig. S-2). No significant reactivation was observed at $> 1 \text{ M}$

GdnHCl. As concluded from light-scattering studies and SDS-PAGE analyses at higher enzyme concentrations (see below), aggregation and autoproteolysis strongly compete with reactivation and are probably the reason for incomplete reactivation even at low protein concentrations.

3.2 Spectroscopic characterization of refolding

For spectroscopic measurements of the refolding process higher enzyme concentrations ($\geq 5 \mu\text{g mL}^{-1}$) were necessary than for the determination of reactivation ($0.1 \mu\text{g mL}^{-1}$). To study refolding, fluorescence spectra of pWT and G8C/N60C were recorded in the protein concentration range of $5 - 30 \mu\text{g mL}^{-1}$. Immediately after transferring the unfolded enzymes to native-like conditions ($\leq 1 \text{ M GdnHCl}$) native-like fluorescence spectra were observed (Fig. 5). The characteristic separation of the tyrosine and tryptophan fluorescence, which is characteristic for the denatured state of the enzymes (Fig. 5) and has been studied in detail previously [11], was abolished.

Figure 5

These spectral changes occur within 10 – 15 seconds, which is a time-scale where the reactivation is not yet completed. Therefore, an inactive intermediate with an at least partly folded structure, allowing resonance energy transfer from the tyrosine to the tryptophan residues, must be formed within the first seconds after the initiation of refolding. The fluorescence spectra of the refolded enzymes showed lower intensities than those of the native enzymes under the same conditions which may be attributed to concomitant aggregation (section 3.4). The maximum fluorescence of refolded G8C/N60C was somewhat larger than that of pWT, reflecting a higher refolding yield as

was also found in the reactivation study (section 3.1). Examination of the shape of the spectra reveals, however, that there were only minor deviations between the two enzyme variants.

Figure 6

With increasing time, the native-like spectra changed and the fluorescence signals of tyrosine and tryptophan split again (Fig. 6A and B). This slow process becomes measurable at a higher residual GdnHCl concentration in the renaturation assay (e.g. 0.64 M) as demonstrated in Fig. 6. A kinetic evaluation of the fluorescence signals revealed first order kinetics (Fig. 6C and D) with rate constants in the same range for pWT ($2.6 \cdot 10^{-4} \text{ s}^{-1}$) and G8C/N60C ($3.0 \cdot 10^{-4} \text{ s}^{-1}$).

3.3 Autoproteolysis

The decrease in fluorescence intensity as described above was accompanied by autoproteolysis as revealed by SDS-PAGE analysis in the protein concentration range of 5 – 50 $\mu\text{g mL}^{-1}$. Obviously, the intermediate state occurring immediately after the transfer of denatured enzyme from high to low GdnHCl concentrations (section 3.2.) was susceptible to autoproteolysis. In contrast, both native pWT and G8C/N60C were very stable and did not undergo autoproteolysis. Interestingly, distinct fragments accumulated during this autoproteolysis. The optimal conditions to visualize fragments were found to be at a residual GdnHCl concentration of 0.42 M and a final protein concentration of 50 $\mu\text{g mL}^{-1}$ (Fig. 7). There were some slight differences in the fragmentation pattern between pWT and G8C/N60C (Fig. 7A and B), which was probably caused by differences in the accessibility of the region around the amino acids 8 and/or 60.

Figure 7

Under the conditions shown in Fig. 6, the kinetics of autoproteolysis determined from the decreasing band intensities of the intact proteases in SDS-PAGE analysis after Coomassie staining could be described by a first-order reaction (Fig. 6C and D). The resulting rate constants ($1.6 \cdot 10^{-4} \text{ s}^{-1}$ for pWT and $1.9 \cdot 10^{-4} \text{ s}^{-1}$ for G8C/N60C) were very similar to the rate constants as determined from the decrease of the fluorescence signals (section 3.2). In both cases, the degradation of the autoproteolytically sensitive folding intermediate was observed.

3.4 Aggregation

Aggregations occurring during renaturation of $5 - 60 \mu\text{g mL}^{-1}$ pWT and G8C/N60C were detected by light scattering at 360 nm (Fig. 8). In relation to the light-scattering of solutions containing the native enzymes precipitated by trifluoroacetic acid, the aggregation of pWT and G8C/N60C amounted to 12 and 22 %, respectively, at a final GdnHCl concentration of $< 100 \text{ mM}$.

Figure 8

Solutions of native pWT and G8C/N60C did not show any light-scattering. Analytical ultracentrifugation confirmed that the enzymes remained monomeric up to protein concentrations of at least 0.5 mg mL^{-1} (data not shown).

4. Discussion

4.1 Reactivation competes with autoprolysis and aggregation

In contrast to the general view on the folding of proteases, pWT and G8C/N60C can be renatured (Fig. 3), after denaturation in highly concentrated GdnHCl solutions (where no significant autoprolysis occurs [11]) followed by subsequent dilution with buffer to native-like conditions. The reactivation competes with autoprolysis and aggregation processes, which can be suppressed at low protein concentrations. At protease concentrations of $0.1 \mu\text{g mL}^{-1}$ maximal reactivation yields of 50 % (pWT) and 70 % (G8C/N60C) could be obtained, indicating that the proteases can attain the correctly folded tertiary structure without the help of folding helpers such as the natural propeptide or chaperones.

Based on the first-order kinetics of the reactivation reaction (Fig. 4), it can be concluded that the observed rate constants reflect the folding step in which the enzymes obtain their native state. In autoprolysis and aggregation reactions, where two or more molecules are involved, rate constants are of second or higher order.

The reactivation rate constants give linear branches in the chevron plot (Fig. 4). From fluorescence experiments we learned that refolding proceeds via a rapidly formed intermediate (sections 3.2. and 4.2.). Therefore, the first-order reaction observed in activity measurements is suggested to reflect the second, slower step in which the active state is formed from the intermediate. The derived Gibbs free activation energies of this folding step (ΔG^\ddagger) and the slopes of the straight lines (m) in the plots of Fig. 4 are summarized in Table 1. The smaller slope of the refolding branch of G8C/N60C compared to that of pWT reflects the more rigid structure of the disulfide variant. Moreover, the differences in the slopes of the straight lines indicate that the disulfide bond in G8C/N60C influences the observed refolding.

Table 1*4.2 Refolding proceeds via intermediates sensitive to autoproteolysis*

As fluorescence measurements in the protein concentration range of 5 – 30 $\mu\text{g mL}^{-1}$ showed, refolding of pWT and G8C/N60C proceeds via intermediates with native-like structure. Upon dilution of the enzymes previously unfolded using 8 M GdnHCl, these intermediates are formed within 10 to 15 seconds and are spectroscopically very similar to the native states (Fig. 5). The finding that the total fluorescence intensities of the corresponding amounts of native enzymes are not reached can be attributed to accompanying aggregation of the proteases, as indicated by results of light-scattering experiments (Fig. 8). The folding intermediates are slowly degraded as concluded from the changes in the fluorescence spectra with time (Fig. 6) and the generation of peptide fragments as monitored by SDS-PAGE (Fig. 7). Obviously, traces of the proteases, that have already attained their native, autoproteolytically-resistant state, are sufficient to attack and degrade the remaining molecules in the intermediate state. Only at very low protein concentrations as used in the reactivation experiments (0.1 - 5 $\mu\text{g mL}^{-1}$), does a major percentage of the molecules have the chance to fold to the native structure.

The occurrence of peptides originating from the degradation of the intermediates during refolding as visualized by SDS-PAGE (Fig. 7), is in contrast to the unfolding experiments where no conditions could be found in which fragment accumulation due to autoproteolysis [12] occurred. It is likely that fragments can accumulate during refolding because the recovered activity is very low compared to the conditions during unfolding where the produced susceptible molecules are rapidly degraded by the large amount of still active species. Differences in the fragment patterns

of pWT and G8C/N60C (Fig. 7) indicate that the folding intermediates of the two enzymes are differentially accessible to autoproteolysis.

For GdnHCl-induced denaturation it has been shown recently [11, 12] that unfolding of pWT also proceeds via a native-like intermediate, which is sensitive to autoproteolysis and that autoproteolysis apparently promotes unfolding. In contrast, during the denaturation of G8C/N60C this intermediate does not seem to play any role in autoproteolysis and unfolding. The present results suggest that the same intermediates are formed during folding and unfolding. For pWT and G8C/N60C, these intermediates differ in their sensitivities to degradation. In the intermediate of pWT the region 56-69 is highly flexible and prone to autoproteolysis, whereas in G8C/N60C this region is fixed and insensitive to autodegradation. While we assumed in our earlier studies of unfolding of G8C/N60C that autoproteolysis starts from the completely unfolded enzyme [12], the present results of folding show that autoproteolysis also starts from an intermediate with native-like tertiary structure (Fig. 6B). Therefore, we assume now that this intermediate is also formed but not populated during unfolding of G8C/N60C, and instead only accumulates during refolding.

4.3 Combination of the refolding and unfolding data allows the thermodynamic characterization of the intermediate - native state equilibrium

Unfortunately, no conditions could be found to characterize the transition of the intermediate to the native state in equilibrium. With some caution, however, the present and previous kinetic data can be used to estimate the Gibbs free energy, ΔG^0 , of this equilibrium. Assuming that the first-order rate constants of reactivation reflect the folding from the intermediate to the native state, these data can be combined with the corresponding data of unfolding [12], which have been inserted in Fig. 4 and Table 1. Disregarding the incomplete reactivation and making the

assumption that the intermediate is the same during folding and unfolding, this allows an energetic characterization of the intermediate-native state equilibrium to be undertaken (Table 1). The midpoint of the transition $[\text{GdnHCl}]_{0.5}$ obtained from the intersection of the corresponding refolding and unfolding branches is higher by 1.5 M for G8C/N60C than for pWT. Using Eyring's equation [24, 25] the Gibbs free energy of activation under native conditions ΔG^\ddagger can be obtained from the rate constants extrapolated to 0 M GdnHCl. Comparison of ΔG^\ddagger with the Gibbs free energy ΔG^0 for both enzyme variants shows that the high stability of these proteins mainly is due to a high kinetic barrier. From the difference of the ΔG^0 values it can be concluded that the contribution of the disulfide bond to the stabilization is 8.1 kJ mol^{-1} .

4.4 Concluding remarks

For the first time, the interplay of folding and autodegradation processes of an unspecific protease have been analyzed quantitatively. The results of refolding show that in a first very fast folding step a native-like structure arises. At low protein concentrations ($0.1 - 5 \mu\text{g mL}^{-1}$) this intermediate structure can slowly fold to the native active protease, whereas it is degraded at higher protein concentrations by autoproteolysis. Moreover, this study is the first attempt to obtain thermodynamic parameters for the stability of a neutral protease and the influence of an engineered disulfide bond.

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Figure Legends

Figure 1. Model of the three-dimensional structure of the neutral protease of *B. stearrowthermophilus*, including the disulfide bond in variant G8C/N60C.

The model was created on the basis of the X-ray structure of thermolysin using homology modeling with the program WHAT IF [15]. The orange and violet spheres indicate the catalytic zinc ion and four bound calcium ions with numbering of the binding sites. The sensitive loop region between amino acids 56-69 is drawn in red and the disulfide bond connecting positions 8 and 60 is drawn in yellow.

Figure 2. Progress curve of pWT reactivation.

Refolding of pWT denatured in 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl₂ and 8.0 M GdnHCl was initiated by 1:50 dilution with the same buffer without GdnHCl, containing the fluorogenic substrate Abz-AGLA-Nba and the reaction was followed by measuring the fluorescence at 415 nm (excitation at 340 nm). The final enzyme concentration was 0.1 µg mL⁻¹. The section of constant fluorescence increase (90-600 s) was fitted to a linear function. The insert shows the fluorescence data reduced by the values of this function, representing the reactivation curve.

Figure 3. Reactivation of pWT (●) and G8C/N60C (○) followed by the casein assay.

The denatured enzymes ($20 \mu\text{g mL}^{-1}$ in 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl_2 and 8 M GdnHCl) were diluted 1:20 into the same buffer but without GdnHCl. After several periods of time samples were removed and assayed for activity using the casein assay as described in section 2.4. Each value is the mean of three independent measurements. The error bars represent the standard deviation. The regained activity was related to the activity of the native enzyme at the same GdnHCl concentration, which was used as control.

Figure 4. Rate constants of reactivation of pWT (●) and G8C/N60C (○) as a function of the final GdnHCl concentration.

The denatured enzymes ($5 \mu\text{g mL}^{-1}$ in 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl_2 and 8 M GdnHCl) were diluted to the indicated residual GdnHCl concentration by 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl_2 and the fluorogenic substrate Abz-AGLA-Nba. The enzyme concentration during reactivation was adjusted to $0.1 \mu\text{g mL}^{-1}$. The error bars indicate the error of the fitted rate constants.

The insert shows the data in combination with the rate constants of unfolding for pWT (▲) and G8C/N60C (Δ) as a function of the GdnHCl concentration (data taken from [12]).

Figure 5. Fluorescence spectra of native, unfolded and refolded pWT (A) and G8C/N60C (B). The enzymes were incubated at $5 \mu\text{g mL}^{-1}$ in 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl_2 without (solid line) and with 8 M GdnHCl (dotted line) before taking the fluorescence spectra at an excitation wavelength of 278 nm. For refolding the denatured enzymes (5mg mL^{-1} in 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl_2 and 8.4 M GdnHCl) were diluted 1:1000 into the same buffer without GdnHCl and measured immediately (broken line).

Figure 6. Autoproteolysis during the refolding of pWT (A and C) and G8C/N60C (B and D) reflected in the fluorescence spectra and SDS-PAGE.

Enzymes denatured in 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl₂ and 8.4 M GdnHCl were diluted with the same buffer without GdnHCl to a final enzyme concentration of 30 µg mL⁻¹ and a final GdnHCl concentration of 0.64 M. After several periods of time (0.2, 15, 23, 29, 47, 69, 95, 357 min, in the directions of arrows) fluorescence spectra at the excitation wavelength of 278 nm were recorded (A and B).

In C and D, the decrease of the fluorescence signal at 334 nm (●) is shown as a function of time and compared with the relative intensity of the enzyme bands in SDS-PAGE (○).

Figure 7. Fragment patterns monitored by SDS-PAGE during refolding of pWT (A) and G8C/N60C (B).

Refolding of the enzymes denatured in 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl₂ and 8.4 M GdnHCl was initiated by dilution with the same buffer without GdnHCl to the final enzyme concentration of 50 µg mL⁻¹ and the final GdnHCl concentration of 0.42 M. After various periods of time, samples were analysed by SDS-PAGE as described in section 2.6. Lane 1: molecular weight markers; lane 2: native enzymes; lanes 3 to 15: enzymes after refolding for 10, 30 s, and 1, 2, 5, 10, 15, 20, 25, 30, 35, 40, 45 min (A) and 10, 30 s and 1, 2, 5, 10, 20, 40, 60, 80, 100, 120, 140 min (B).

Figure 8. Aggregation during refolding of pWT (●) and G8C/N60C (○).

The enzymes denatured in 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM CaCl₂ and 8.4 M GdnHCl were diluted (1:90 to 1:1000) with the same buffer without GdnHCl to the enzyme concentration indicated. The residual GdnHCl concentration was < 100 mM. Light-scattering was measured as described in section 2.8. The error bars represent the standard deviations of three independent measurements.

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Table 1: Characteristics of the GdnHCl induced unfolding/refolding of pWT and G8C/N60C

The parameters were calculated from the data of Fig. 4 according to Tanford [24, 25] and Eyring [26] using the linear extrapolation model $\ln k_{\text{obs}} = m \cdot [\text{GdnHCl}] + n$, where m represents the slope ($\partial \ln k_{\text{obs}} / \partial [\text{GdnHCl}]$) of the folding or unfolding branch and n is the logarithm of the observed rate constant extrapolated to 0 M GdnHCl ($\ln k_0$). k_{obs} is given in s^{-1} . $[\text{GdnHCl}]_{0.5}$ is the GdnHCl concentration at the point of intersection of the refolding and unfolding branches. ΔG^\ddagger stands for the Gibbs free energy of activation of unfolding or refolding in the absence of GdnHCl. ΔG^0 was calculated from the difference of the ΔG^\ddagger values of unfolding and refolding and represents the Gibbs free energy in the absence of GdnHCl. The data marked by a star were taken from [12].

| Enzyme variant | Reaction | m (M^{-1}) | k_0 (s) | ΔG^\ddagger (kJ mol^{-1}) | $[\text{GdnHCl}]_{0.5}$ (M) | ΔG^0 (kJ mol^{-1}) |
|----------------|--------------------------|----------------------------|-----------------------|---|--------------------------------|--|
| pWT | Reactivation (refolding) | -9.897 | $4.5 \cdot 10^{-1}$ | 75.0 | 1.3 | 35.7 |
| | unfolding | 1.203* | $2.5 \cdot 10^{-7}$ * | 110.7* | | |
| G8C/N60C | Reactivation (refolding) | -4.468 | $5.7 \cdot 10^{-2}$ | 80.1 | 2.8 | 44.0 |
| | unfolding | 1.785* | $1.0 \cdot 10^{-9}$ * | 124.1* | | |

Figure 1

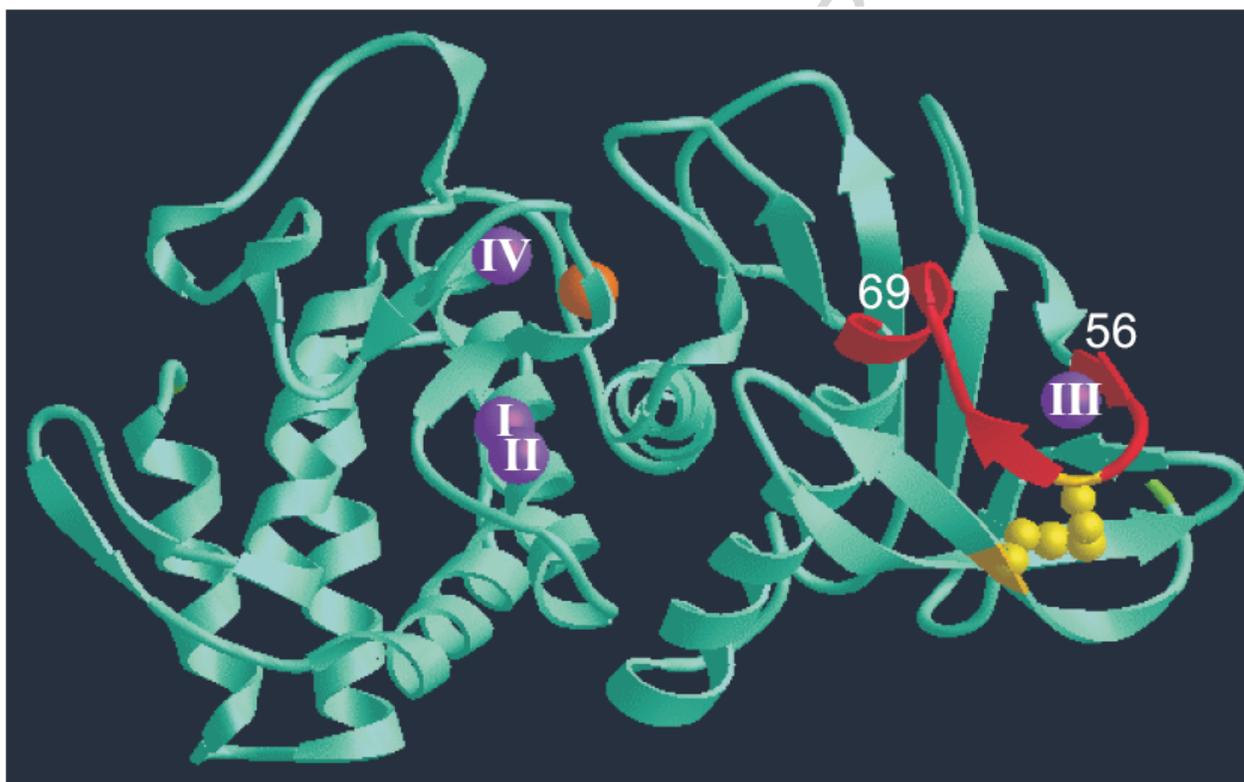


Figure 2

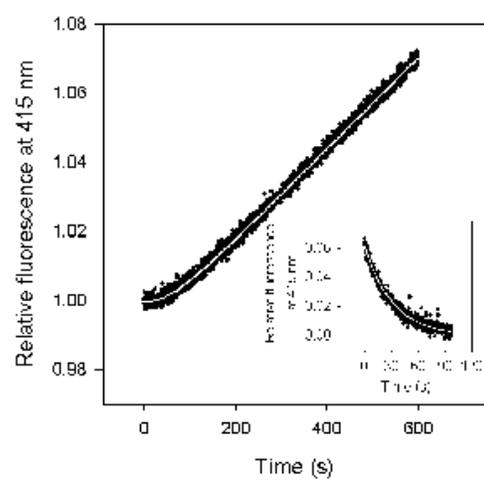


Figure 3

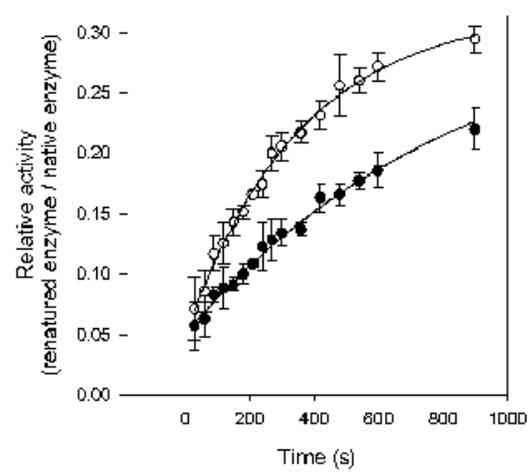


Figure 4

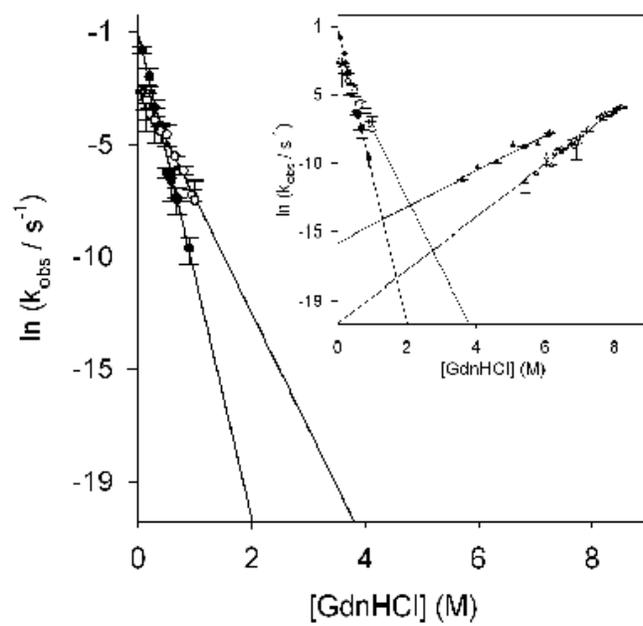


Figure 5

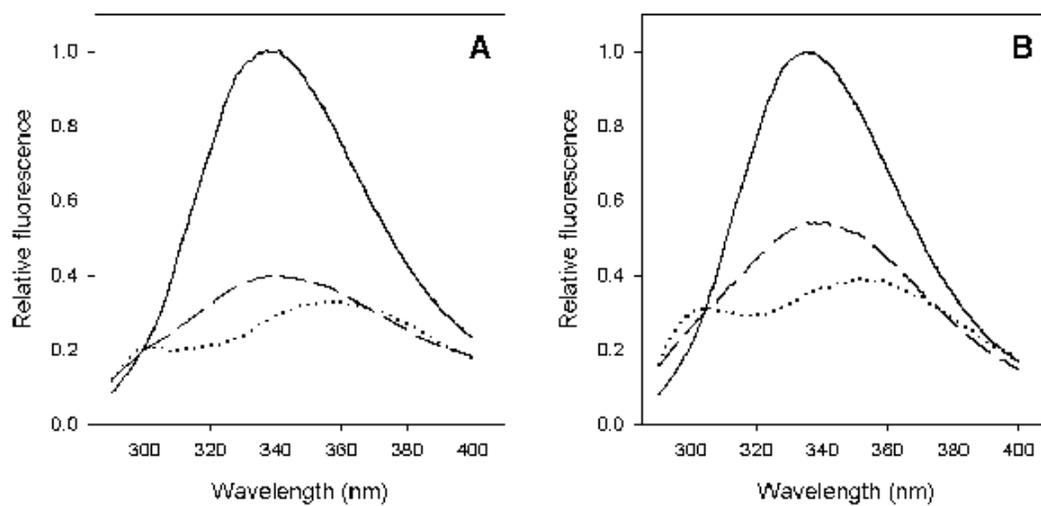


Figure 6

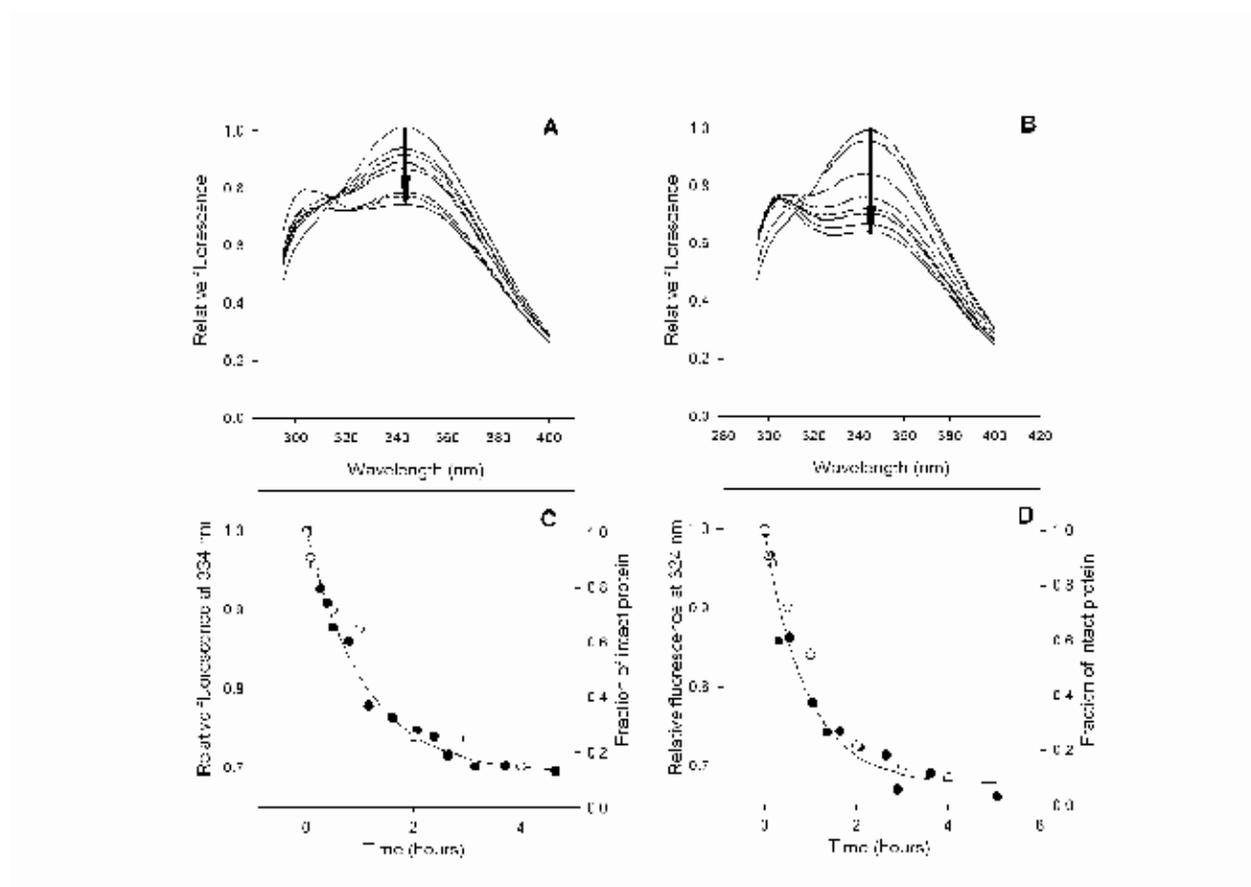
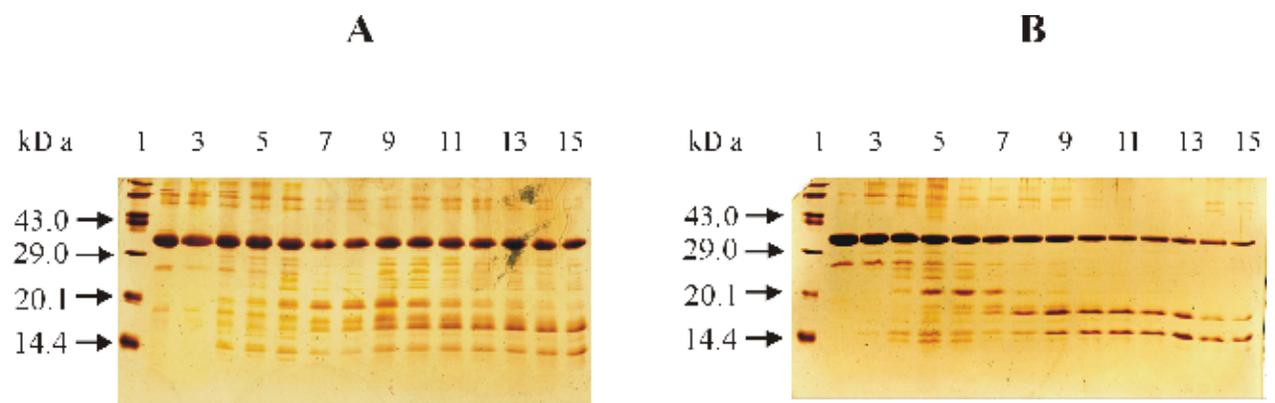


Figure 7



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Figure 8

