Extreme Stabilization of a Thermolysin-like Protease by an Engineered Disulfide Bond*

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The thermal inactivation of broad specificity proteases such as thermolysin and subtilisin is initiated by partial unfolding processes that render the enzyme susceptible to autolysis. Previous studies have revealed that a surface-located region in the N-terminal domain of the thermolysin-like protease produced by Bacillus stearothermophilus is crucial for thermal stability. In this region a disulfide bridge between residues 8 and 60 was designed by molecular modelling, and the corresponding single and double cysteine mutants were constructed. The disulfide bridge was spontaneously formed in vivo and resulted in a drastic stabilization of the enzyme. This stabilization presents one of the very few examples of successful stabilization of a broad specificity protease by a designed disulfide bond. We propose that the success of the present stabilization strategy is the result of the localization and mutation of an area of the molecule involved in the partial unfolding processes that determine thermal stability.

Several members of the bacterial genus Bacillus are known to produce extracellular neutral proteases (1-6) that resemble thermolysin, the extremely stable protease from Bacillus thermoproteolyticus. These so-called thermolysin-like proteases (TLPs)1 consist of 300–319 residues and share similar structural and functional characteristics. The three-dimensional structures of thermolysin (7, 8) and the TLP produced by Bacillus cereus (9, 10) have been solved by x-ray crystallography. On the basis of these structures, reasonably accurate models of other TLPs have been built (11). Naturally occurring TLPs exhibit large differences in thermal stability (11) and the structural features causing these differences have been the subject of several site-directed mutagenesis studies (11, 12).

At elevated temperatures TLPs as well as subtilisins are irreversibly inactivated as a result of autolysis (13-15). Because of the broad specificity of TLPs (16), conformational features rather than sequence characteristics determine the sites of autolytic attack (17), and it has been shown that the rate of thermal inactivation is determined by the rate of local unfolding processes that render the protease susceptible to autolysis (11-13, 15, 18, 19). Previous studies on autolysis of broad specificity proteases (13, 15, 17, 20) together with observations concerning the structural changes during protein unfolding (21, 22) suggest that the local unfolding processes that lead to autolysis involve solvent-exposed regions (17, 19, 20). Accordingly, it has recently been shown that the difference in stability between TLP of Bacillus stearothermophilus (TLP-ste) and the more stable thermolysin is determined mainly by amino acid differences at the surface (12). Furthermore, it turned out that the important mutations were clustered in a limited part of the N-terminal domain (especially residues 56–69) of the protein, illustrating the localized nature of the stability-determining unfolding processes (11, 12). One mutant that stabilized TLP-ste rather strongly was T63F (23) (see Fig. 1A). Based on these observations we decided to try to stabilize TLP-ste by introducing a disulfide bond in this critical area (preferably close to position 63), the rationale being that, in principle, a disulfide bond can reduce local mobility and unfolding more than any other type of mutation.

Disulfide bonds can make considerable contributions to the stability of proteins (24–26), an effect mainly attributed to the decrease of conformational chain entropy of the denatured protein (26–29). Many attempts have been made to increase protein stability by introduction of novel disulfide bonds (24, 27, 30–39). Some studies turned out to be successful (35–37, 39), whereas others did not give the expected results (30, 31, 34, 38). Disappointing results have been mainly attributed to side effects of the individual Xaa → Cys mutations (31, 34, 35, 38) and/or to the introduction of strain resulting from suboptimal geometry of the disulfide bridge (30, 32). In the case of industrially important broad specificity proteases such as subtilisin (31, 33, 34) and TLPs (38), most attempts to stabilize these enzymes by the introduction of disulfide bonds have been unsuccessful. Only for one engineered disulfide bridge in subtilisin E has a considerable increase in thermal stability been reported (36). However, this disulfide bridge was not designed de novo but was designed on the basis of a disulfide bridge encountered in a naturally occurring, more thermostable subtilisin variant.

In the present study we show how TLP-ste can be stabilized dramatically by introducing a de novo designed disulfide bridge. Furthermore, we provide an explanation for the lack of success in earlier attempts to stabilize broad specificity proteases by engineered disulfide bridges.

MATERIALS AND METHODS

Reagents—1,4-Dithiothreitol (DTT) and N-(3-[2-furyl]acryloyl)-Gly-Leu amide were purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany), and urea was from ICN Biomedicals GmbH

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1 The abbreviations used are: TLP, thermolysin-like protease; TLP-ste, TLP of B. stearothermophilus; DTT, 1,4-dithiothreitol; PAGE, polyacrylamide gel electrophoresis; MOPS, 3-(N-morpholino)propanesulfonic acid.
Design of Disulfide Mutants—A three-dimensional model of TLP-ste was built exploiting the sequence homology with thermolysin (7, 8) as described previously (11). Most model building procedures were done using the program WHAT IF (40). Residues are numbered throughout this paper according to the sequence of thermolysin (1).

Sites for insertion of disulfide bridges were selected using the program SS-BOND (41) as described previously (38). This program uses the backbone coordinates from the three-dimensional model to select residue pairs on the basis of the calculated Cα-Cα distances. Subsequently, Sα positions with ideal or nearly ideal geometries were generated for the selected pairs. An energy minimization procedure was used to select acceptable conformations. Acceptable residue pairs that were located in the stability limiting region in the N-terminal domain were visually inspected using the thermolysin crystal structure and the TLP-ste model. To minimize the risk of modelling errors, the Xaa → Cys mutations were chosen only in regions where thermolysin and TLP-ste are highly similar. The most promising candidate appeared to be GSC/N60C. This cysteine pair could be modelled with close to ideal geometry and is located close to the aforementioned position 63. The two cysteines, as well as the individual single mutations, were introduced in a cysteine-free variant of TLP-ste (C288L-TLP-ste, called wild type through this article) whose stability is nearly identical to that of unmutated TLP-ste (42).

Plasmids and Strains—Plasmid pGE53042 contains the gene encoding the C288L variant of TLP-ste, which was used as wild type in this study. The protease-deficient strain Bacillus subtilis DB11743 was used as host for this plasmid, and its variants were obtained by site-directed mutagenesis. Cells harboring these plasmids were grown at 37 °C in TY broth containing 5 μg/ml chloramphenicol as antibiotic. The Escherichia coli strains WK6 and WK10 were used as host for this plasmid, and its variants were obtained by site-directed mutagenesis via polymerase chain reaction (megaprimer method (45)).

The enzymes were stored in elution buffer containing 20 mM sodium acetate, pH 5.3, 5 mM CaCl2, 2.5 mM NaCl, 20% (v/v) isopropanol, and 0.03% (w/v) sodium azide.

An alternative purification scheme was applied to samples to be used for CD measurements. After separation of cells from the fermentation broth (10 min at 6000 rpm, Beckman J2-HC), the supernatant was concentrated by ultrafiltration (FILTRON ProVari-o-S) System) using an 8-kDa membrane (Nova series, FILTRON), and proteins were precipitated with ammonium sulfate (85% saturation). The precipitate was collected by centrifugation (20 min at 20000 rpm, Beckman L8–60 M ultracentrifuge), redissolved in 0.05M Tris buffer, pH 7.5, 5 mM CaCl2, and dialyzed with ammonium sulfate (85% saturation). The precipitate was further purified by chromatography on a DEAE-Sephacel (Pharmacia Biotech Inc.) column equilibrated with 0.05M Tris buffer, pH 7.5, 5 mM NaCl, 0.03% (w/v) isopropanol, and 0.03% (w/v) sodium azide, and dialyzed twice against the same buffer. The concentrated enzyme fractions were further purified using gel filtration chromatography (either on Sephacryl S-200, 1% (v/v) isopropanol, and 125 mM NaCl). Activities were derived using the program WHAT IF (40). Residues are numbered throughout this article as described previously (11). Most model building procedures were done using the program WHAT IF (40). Residues are numbered throughout this paper according to the sequence of thermolysin (1).

The kinetic parameter kcat/Km for N-[3-[2-furylacycloxy]-1-Gly-Leu amide was determined according to the method of Feder (53) using a buffer containing 10 mM MOPS, pH 7.0, 0.5 mM CaCl2, 0.02% (w/v) Triton X-100, 1% (v/v) isopropanol, and 125 mM NaCl. Activities were derived from the decrease in absorption at 345 nm, using a Δε of 317 μM cm⁻¹. The decrease in absorption was recorded at 37 °C, using a thermostatted cuvette in a Perkin-Elmer Lambda 11 spectrophotometer (Perkin-Elmer Corp.).

CD spectra were recorded using a JASCO J-710 circular dichroism spectrometer. Measurements were performed at 25 °C using a quartz cell of 1-mm path length. Samples contained 0.08 mg of enzyme/ml in 0.05 M Tris buffer, pH 9.0, 5 mM CaCl2. Protein concentrations were determined with the Micro BCA protein assay reagent (Pierce) using bovine serum albumin as a standard.

Free thiols were determined according to Ellman (54) under denaturing conditions (6 x urea); the presence of free thiols in the GSC/N60C variant was determined without or with previous incubation with reducing agents (0.2 M DTT). Excess of reducing agent was removed via extensive dialysis. The amount of free sulphydryl groups was calculated using an extinction coefficient of 13,600 M⁻¹ cm⁻¹.

SDS-PAGE analysis of purified TLP-ste variants was performed using a method essentially similar to the method described by Laemmli (49). The presence of disulfide bonds was analyzed by comparing mobilities during SDS-PAGE of enzyme samples that had been prepared in the absence or the presence of reducing agent.

RESULTS

Design and Production of the Mutants—Fig. 1 (A and B) shows the 8–60 disulfide mutant as designed in the three-dimensional model of TLP-ste. The disulfide bond connects the N-terminal β-hairpin (residues 1–25) with a region that is crucial for thermal stability (residues 56–69) (11, 12). Inspection of the thermolysin crystal structure and the TLP-ste model indicated that the individual mutations needed for the disulfide bond (G8C and N60C) would not lead to significant clashes or have other negative side effects.

The selected mutants were constructed and could successfully be expressed in B. subtilis DB117. Wild type TLP-ste, GSC, and GSC/N60C mutants were similar with respect to expression levels and yields of purification. The expression level was approximately three times lower for the N60C mutant. Wild type and mutant proteins had similar specific activities toward casein as substrate at 37 °C, pH 7.5 (18.5 ± 5.3 units/mg, 82.3 ± 5.0 units/mg, 75.0 ± 6.2 units/mg, 82.4 ± 6.2 units/mg protein for purified wild type, GSC, N60C, and double mutant enzymes, respectively). The kcat/Km of the double mutant enzyme for the synthetic dipeptide substrate N-[3-[2-furyl]acryloyl]-Gly-Leu amide (53) was similar to that of the wild type enzyme (27.8 ± 3.5 · 104 and 30.0 ± 4.4 · 104 M⁻¹ s⁻¹, respectively).

Nonreducing SDS-PAGE showed that the double mutant enzyme migrated slightly faster than the wild type enzyme, whereas identical mobilities were observed in the presence of reducing agent (DTT; results not shown). This suggests that...
the expected disulfide bond was formed \textit{in vivo} in the G8C/N60C mutant. No free thiol groups could be detected by thiol titrations (under denaturing conditions) with Ellman’s reagent (54), confirming the spontaneous formation of the disulfide bridge in the double mutant. After treatment with excess of DTT (0.2 M), the number of sulfydryl groups in the double mutant was determined to be \(1.95 \pm 0.15\) /molecule.

The CD spectrum of the double mutant was identical to that of the wild type enzyme (not shown), indicating that the tertiary structure had not changed significantly as a result of the introduced disulfide bond.

\textbf{Thermal Stability}—Purified, electrophoretically homogeneous wild type and mutant enzymes were used for determining \(T_{50}\), as described under “Materials and Methods.” As shown in Table I, the single mutant enzymes were considerably less stable than the wild type enzyme \((\Delta T_{50} = -11.0\) and \(-16.2\) °C, for G8C and N60C, respectively). Reducing agents had a stabilizing effect on the single mutant enzymes but only a small effect on the wild type enzyme. This suggests that the decrease in thermal stability of the single mutants is at least partly due to oxidation of the introduced cysteine residue and, possibly, formation of intermolecular disulfide bonds (55).

Despite the destabilizations observed for the single mutants, the double mutant displayed a drastic increase in \(T_{50}\) \((\Delta T_{50} = +16.7\) °C). DTT reduced the stability of this mutant, but even at 10 mM DTT the mutant was much more stable than the wild type \((\Delta T_{50} = +11.8\) °C). Thus, it seems that the engineered disulfide bridge is rather resistant toward reduction. At higher DTT concentrations (50–100 mM) the stability of the double mutant was further reduced, but stability measurements at such high concentrations could not be performed accurately, because increasing DTT concentrations resulted in considerable decrease of the enzymatic activity in wild type and in all mutant enzymes.

In the temperature range of 80–95 °C for the stable double mutant enzyme and 55–75 °C for the unstable single mutant enzymes, the kinetics of thermal inactivation was measured and compared with those of the wild type TLP-ste. In all cases the inactivation was irreversible and followed a first order kinetics. Thermal inactivation of the double mutant coincided in the usual manner with the disappearance of protein material visible in SDS-PAGE gels (Fig. 2). The results (Table II) confirmed the low stability of the single mutants and the extreme stabilization obtained by introduction of the disulfide bond.
Stabilization by an Engineered Disulfide Bond

Table I

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$T_{50}$</th>
<th>$\Delta T_{50}$</th>
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<tr>
<td></td>
<td>-DTT</td>
<td>+DTT</td>
</tr>
<tr>
<td>Wild type</td>
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<td>76.3</td>
</tr>
<tr>
<td>GSC</td>
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<td>70.7</td>
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<tr>
<td>N60C</td>
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<td>GSC/N60C</td>
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Table II

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<tr>
<th>Mutant</th>
<th>Half-life</th>
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<td></td>
<td>at 60 °C</td>
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<tr>
<td>Wild type</td>
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<tr>
<td>GSC</td>
<td>170 ± 7</td>
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<tr>
<td>N60C</td>
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<tr>
<td>GSC/N60C</td>
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<tr>
<td>GSC/N60C + 10 mM DTT</td>
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<tr>
<td>Thermolysin</td>
<td>9.5 ± 0.5</td>
</tr>
</tbody>
</table>

Fig. 2. Autolysis of TLP-ste variants at elevated temperatures.

In the present study we provide the first example of drastic stabilization of a broad specificity protease by a de novo designed engineered disulfide bridge. In terms of kinetic stability, the disulfide containing mutant of TLP-ste is one of the most stable enzymes ever obtained by protein engineering. The stability of this designed mutant is comparable with the stability of a recently published mutant of TLP-ste in which five amino acids (all in the 1–70 region) had been replaced (12) by the corresponding residues in a naturally occurring more stable TLP variant (thermolysin).

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