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 respect 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) 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 bridges 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](4-Dithio-iot-thiolethol (DIT) and N-(3-[2-furyl]acyloyl)-Gly-Leu amide were purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany), and urea was from ICN Biomedicals GmbH)
activity of a 0.1 M solution of purified enzyme in 20 mM sodium acetate, pH 5.3, 5 mM CaCl$_2$, 0.5% (v/v) isopropanol, 62.5 mM NaCl, 0.03% (w/v) sodium azide, and 0.01% (v/v) Triton X-100 was retracted after 30 min. Initial and residual enzyme activities were determined using a casein assay at 37 °C as described previously (51). The assay was calibrated using the specific activity of the wild type enzyme. The time course of thermal inactivation was followed using the same conditions as described above. The enzymes were incubated at defined temperatures, and the aliquots removed after different time intervals were assayed for activity toward casein at 37 °C.

The kinetic parameter $k_{cat}/K_m$ for N-(3-[2-furyl]acryloyl)-Gly-Leu amide was determined according to the method of Feder (53) using a buffer containing 10 mM MOPS, pH 7.0, 5 mM CaCl$_2$, 0.02% (v/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 M$^{-1}$ cm$^{-1}$. The decrease in absorption was recorded at 27 °C, using a thermostat-ted and a Perkin-Elmer Lambda 11 spectrophotometer (Perkin-Elmer Corp.).

Free thiols were determined according to Ellman (54) under denaturing conditions (6 x 1 µl); the presence of free thiol groups in the G8C/N60C mutant 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 sulphhydryl 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, G8C, and G8C/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 ± 5.2 units/mg, 82.4 ± 6.2 units/mg protein for purified wild type, G8C, N60C, and double mutant enzymes, respectively). The $k_{cat}/K_m$ 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 × 10$^{-3}$ and 30.0 ± 4.4 × 10$^{-3}$ m$^{-1}$ s$^{-1}$, respectively).
the expected disulfide bond was formed 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 ± 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.

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.
Engineered disulfide bridges is at least partly due to the fact that these bridges were introduced in regions of the protease molecule that do not play a role in stability-determining local unfolding processes. Designed disulfide bridges have been extremely successful in cases where the stability measurement was based on monitoring global (as opposed to local) unfolding (24, 35). In these cases, the success of the bridge is more exclusively determined by the success of the design, and much less (if at all) by the location of the bridge in the molecule. Unfortunately, the phenomenon of autolysis prevents analysis of reversible unfolding in TLPs (e.g. Ref. 13). In cases where stability is determined by autolysis or other irreversible mechanisms of inactivation that do not depend on complete unfolding, mutational effects are at least partly determined by the stability-determining local unfolding processes. Designed disulfide bridges have been extremely successful in cases where the stability measurement was based on monitoring global (as opposed to local) unfolding (24, 35). In these cases, the success of the bridge is more exclusively determined by the success of the design, and much less (if at all) by the location of the bridge in the molecule.

The half-life of the enzyme at 92.5 °C was increased more than 120-fold, from less than 0.3 to 36 min. Remarkably, even the stability of the most stable naturally occurring TLP, thermolysin, was exceeded considerably (Table II).

**DISCUSSION**

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 N-terminal domain (important mutations have been found between positions 1 and 70). It remains to be elucidated where the stability determining local unfolding processes take place and what extent of local unfolding is needed for those to occur. The present study shows, however, that highly successful rational design of stabilizing mutations in TLP-ste is possible on the basis of the information that is currently available.

**REFERENCES**

1. Titani, K., Hermodson, M. A., Ericsson, L. H., Walsh, K. A., and Neurath, H. (1972) *Nature* **238**, 35–37.
2. Stidler, W., Niederer, E., Suter, F., and Zuber, H. (1986) *Bioll. Chem. Hoppe-Seyler* **367**, 643–657.
3. Takagi, M., Imanaka, T., and Aiba, S. (1986) *J. Bacteriol.* **163**, 824–831.
4. Vasantha, N., Thompson, L. D., Rhodes, C., Banner, C., Nagle, J., and Filpula, D. (1984) *J. Bacteriol.* **159**, 811–819.
5. van den Burg, B., Enequist, H. G., van der Haar, M. E., Eijsink, V. G. H., Stulp, B. K., and Venema, G. (1991) *J. Bacteriol.* **171**, 4107–4115.
6. Yang, M. Y., Ferrari, E., and Henner, D. J. (1984) *J. Bacteriol.* **160**, 15–21.
7. Matthews, B. W., Jansonius, J. N., Colman, P. M., Schoenborn, B. P., and Dupouy, D. (1972) *Nature* **238**, 37–41.
8. Holmer, M. A., and Matthews, B. W. (1982) *J. Mol. Biol.* **160**, 623–639.

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**Table I**

| Mutant | $T_{50}$ | $\Delta T_{50}$ |
|--------|---------|----------------|
|        | -DTT    | +DTT           |
|        | -DTT    | +DTT           |

Half-lifes of TLP-ste variants at 60 and 92.5 °C

| Mutant | Half-life |
|--------|-----------|
|        | at 60 °C  | at 92.5 °C |
| Wild type | 813 ± 31 | <0.3 |
| G8C    | 170 ± 7  |
| N60C   | 35.2 ± 1.9 |
| G8C/N60C | 35.9 ± 1.5 |
| G8C/N60C + 10 mM DTT | 15.8 ± 0.8 |
| Thermolysin | 9.5 ± 0.5 |

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

**TABLE I**

*Thermal stabilities of TLP-ste mutants*

| Mutant | $T_{50}$ (°C) | $\Delta T_{50}$ (°C) |
|--------|---------------|----------------------|
| Wild type | 75.4          | 0.9                  |
| G8C    | 64.4          | -11.0                |
| N60C   | 59.2          | -16.2                |
| G8C/N60C | 92.1          | +16.7                |

$T_{50}$ values represent the difference between the wild type and the mutant enzymes with respect to the temperature at which, after 30 min of incubation, 50% of the initial activity remains. The concentration of DTT used was 10 mM. The $T_{50}$ values are the averages of at least three independent assays. Standard deviations were below 0.5 °C in all cases.
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9. Paupitz, R. A., Karlsson, R., Picot, D., Jenkins, J. A., Niklaus-Reimer, A. S., and Jansonius, J. N. (1988) J. Mol. Biol. 199, 525–537
10. Stark, W., Paupitz, R. A., Wilson, K. S., and Jansonius, J. N. (1992) Eur. J. Biochem. 207, 781–791
11. Vriend, G., and Eijsink, V. G. H. (1993) J. Comput. Aided Mol. Des. 7, 367–396
12. Eijsink, V. G. H., Veltman, O. R., Aukema, W., Vriend, G., and Venema, G. (1995) Nat. Struct. Biol. 2, 374–379
13. Dahlquist, F. W., Long, J. W., and Bigbee, W. L. (1976) Biochemistry 15, 1103–1111
14. Van den Burg, B., Eijsink, V. G. H., Stulp, B. K., and Venema, G. (1990) Biochem. J. 272, 93–97
15. Braxton, S., and Wells, J. A. (1992) Biochemistry 31, 7796–7801
16. Heinrichsen, R. L. (1977) Methods Enzymol. 47, 175–189
17. Fontana, A. (1988) Biophys. Chem. 29, 181–193
18. Kidokoro, S., Miki, Y., Endo, K., Wada, A., Nagao, H., Miyake, T., Aoyama, A., Yoneya, T., Kii, K., and Ose, S. (1995) FEBS Lett. 367, 73–76
19. Eijsink, V. G. H., Vriend, G., Van der Vinne, B., Hazes, B., Van den Burg, B., and Venema, G. (1992) Proteins 14, 224–236
20. Abraham, L. D., and Breuil, C. (1995) Biochim. Biophys. Acta 1245, 76–84
21. Matsudochi, A., Kellis, J. T., Serrano, L., and Fersht, A. (1989) Nature 340, 122–126
22. Jackson, S. E., and Fersht, A. R. (1991) Biochemistry 30, 10436–10443
23. Van den Burg, B., Dijkstra, B. W., Vriend, G., Van der Vinne, B., Venema, G., and Eijsink, V. G. H. (1984) Eur. J. Biochem. 220, 981–985
24. Wetzel, R. (1987) Trends Biochem. Sci. 12, 478–482
25. Cooper, A., Eyres, S. J., Radford, S. E., and Dobson, C. M. (1992) J. Mol. Biol. 225, 939–943
26. Thornton, J. M. (1981) J. Mol. Biol. 151, 261–287
27. Pace, C. N., Grimsley, G. R., Thomson, J. A., and Barnett, B. J. (1988) J. Biol. Chem. 263, 653–658
28. Flory, P. J. (1956) J. Am. Chem. Soc. 78, 5222–5235
29. Zhang, T., Bertelsen, E., and Alber, T. (1994) Nat. Struct. Biol. 1, 434–438
30. Villafranca, J. E., Howell, E. E., Oatley, S. J., Xuong, N., and Kraut, J. (1987) Biocchemistry 26, 2077–2083
31. Eijsink, V. G. H., and Powers, D. B. (1986) J. Mol. Biol. 261, 6564–6570
32. Katz, B., and Kossiakoff, A. A. (1986) J. Mol. Biol. 210, 15480–15485
33. Pantoliano, M. W., Ladner, R. C., Bryan, P. N., Rollence, M. L., Wood, J. F., and Poulos, T. L. (1987) Biochemistry 26, 2077–2082
34. Mitchinson, C., and Wells, J. A. (1989) Biochemistry 28, 4807–4815
35. Matsumura, M., Signor, G., and Matthews, B. W. (1989) Nature 342, 291–293
36. Takagi, H., Takahashi, T., Momose, H., Inouye, Y., Mueda, Y., Matsuawa, H., and Ohta, T. (1990) J. Biol. Chem. 265, 6874–6878
37. Kanaya, S., Katsuda, C., Kimura, S., Nakai, T., Kitakuni, E., Nakamura, H., Katayanagi, K., Morikawa, K., and Ikehara, M. (1991) J. Biol. Chem. 266, 6038–6044
38. Van den Burg, B., Dijkstra, B. W., van der Vinne, B., Stulp, B. K., Eijsink, V. G. H., and Venema, G. (1993) Protein Eng. 6, 521–527
39. Wakarchuk, W. W., Sung, W. L., Campbell, R. L., Cunningham, A., Watson, D. C., and Yaguchi, M. (1994) Protein Eng. 7, 1379–1386
40. Vriend, G. (1999) J. Mol. Graphics 8, 52–56
41. Hazes, B., and Dijkstra, B. (1998) Protein Eng. 2, 119–125
42. Eijsink, V. G. H., Dijkstra, B. W., Vriend, G., van der Zee, J. R., Veltman, O. R., van der Vinne, B., van den Burg, B., Kempe, S., and Venema, G. (1992) Protein Eng. 5, 421–426
43. Eijsink, V. G. H., Vriend, G., van den Burg, B., Venema, G., and Stulp, B. K. (1990) Protein Eng. 4, 99–104
44. Zell, R., and Fritz, H.-J. (1987) EMBO J. 6, 1809
45. Barik, S., and Galinski, M. (1991) BioTechniques 10, 489–490
46. Stanssens, P., Opsomer, C., McKeon, Y. M., Kramer, W., Zabeau, M., and Fritz, H.-J. (1989) Nucleic Acids Res. 17, 4441–4445
47. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 643–657
48. Van den Burg, B., Eijsink, V. G. H., Stulp, B. K., and Venema, G. (1989) J. Biochem. Biophys. Methods 18, 209–220
49. Laemmli, U. K. (1970) Nature 227, 680–685
50. Kunitz, M. (1947) J. Gen. Physiol. 30, 74–85
51. Fujii, M., Takagi, M., Imanaka, T., and Aiba, S. (1983) J. Bacteriol. 154, 831–837
52. Kunitz, M. (1947) J. Gen. Physiol. 30, 74–85
53. Barik, S., and Galinski, M. (1991) BioTechniques 10, 489–490
54. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 643–657
55. Perry, L. J., and Wetzel, R. (1987) Protein Eng. 1, 101–105