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Engineering an enzyme to resist boiling

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ABSTRACT **In recent years, many efforts have been made to isolate enzymes from extremophilic organisms in the hope to unravel the structural basis for hyperstability and to obtain hyperstable biocatalysts. Here we show how a moderately stable enzyme (a thermolysin-like protease from *Bacillus stearothermophilus*, TLP-ste) can be made hyperstable by a limited number of mutations. The mutational strategy included replacing residues in TLP-ste by residues found at equivalent positions in naturally occurring, more thermostable variants, as well as rationally designed mutations. Thus, an extremely stable 8-fold mutant enzyme was obtained that was able to function at 100°C and in the presence of denaturing agents. This 8-fold mutant contained a relatively large number of mutations whose stabilizing effect is generally considered to result from a reduction of the entropy of the unfolded state ("rigidifying" mutations such as Gly → Ala, Ala → Pro, and the introduction of a disulfide bridge). Remarkably, whereas hyperstable enzymes isolated from natural sources often have reduced activity at low temperatures, the 8-fold mutant displayed wild-type-like activity at 37°C.**

Denaturation of proteins at elevated temperatures is usually the result of unfolding, which is followed by an irreversible process, most often aggregation (1). The notion that the unfolding processes involved in irreversible denaturation often have a partial (as opposed to global) character has been confirmed experimentally in several cases (2–4). We have studied the thermal stability and denaturation of a broad-specificity metalloprotease produced by *Bacillus stearothermophilus* CU21 (ref. 5; called TLP-ste; EC 3.4.24.4) that shares 85% sequence identity with its more stable and better known counterpart thermolysin (ref. 6; Table 1). Thermal denaturation of thermolysin-like proteases (TLPs) also depends on partial unfolding processes that, however, are not followed by aggregation but by autolytic degradation starting at unknown sites in the partially unfolded molecule (7–9). An extensive mutation study in which residues in TLP-ste were replaced by the corresponding amino acid in thermolysin showed that only a few of the 43 substitutions between the two enzymes are important for stability (10). All important substitutions are clustered in the N-terminal domain of the protein, in particular in the 55–69 surface loop (refs. 10–12; Fig. 1). Remarkably, combination of only a few of the stabilizing substitutions identified in the 55–59 region resulted in a TLP-ste variant that was more stable than thermolysin itself (11).

Based on the observation that the difference in stability between thermolysin and TLP-ste is mainly determined by mutations in the 55–69 area, we set out to search for several additional stabilizing mutations in this same area. Indeed, several stabilizing mutations involving residues in the 55–69 region have been identified. These include Xaa → Pro mutations (13, 14), the introduction of a salt bridge (15), and the introduction of a disulfide bridge (16). These latter mutations were not based on comparison of the sequences and structures of naturally occurring TLPs, but on rational design.

In the present report we describe the construction of a TLP-ste variant in which five TLP-ste → thermolysin mutations (A4T, T56A, G58A, T63F, A69P) were combined with S65P and a disulfide bridge between residue 60 and 8 in the underlying β-hairpin (Fig. 1). It is shown that this engineered enzyme resembles proteins isolated from thermophilic Archaea and Eubacteria in terms of its resistance to high temperatures and denaturing agents. It is also shown that conferring extreme stability to TLP-ste did not result in major changes in catalytic properties. These findings are discussed in light of the ongoing search for the principles of protein hyperstability.

MATERIALS AND METHODS

Modeling, Mutant Design, and Site-Directed Mutagenesis. The model for TLP-ste was constructed on the basis of homology with thermolysin (ref. 6; 85% sequence identity) by using WHAT IF (17), as described previously. Compared with thermolysin, TLP-ste contains three extra amino acids near position 27 that could not be modeled satisfactorily and therefore were omitted from the model. Considering the high sequence homology between thermolysin and TLP-ste, the model was expected to be sufficiently reliable to predict and analyze the effect of the amino acid substitutions (8, 18, 19). Details of the modeling procedures used to design and analyze mutations have been described elsewhere (8, 10). Details of the design and construction of the disulfide bridge, as well as an extensive analysis of the effects of introducing this bridge in TLP-ste have been described elsewhere (16). The 8-60 bridge was, in geometrical terms, the most promising disulfide bridge that was found in a search for possibly stabilizing disulfide bridges affecting the 55–69 region in TLP-ste. Site-directed mutagenesis, production, and purification of TLPs were performed essentially as described previously (10).

Enzymatic Characterization. Specific activities of TLP-ste and the 8-fold mutant toward casein were determined at 37°C, in 50 mM Tris·HCl, pH 7.5/5 mM $CaCl_2$/0.8% (wt/vol) casein. The k_{cat}/K_m values for furylacryloylated dipeptides of the enzymes were determined at 37°C, in a thermostatted Perkin–Elmer Lambda 11 spectrophotometer. The reaction mixture (1 ml) contained 50 mM MOPS [4-morpholino-ethanesulfonic acid] (pH 7.0), 5 mM $CaCl_2$, 5% DMSO, 0.5% isopropanol, 0.01% Triton X-100, 50 mM NaCl, and 100 μM of substrate, and the reaction was followed by measuring the decrease in absorption at 345 nm ($\in_{345} = -317\ M^{-1}\cdot cm^{-1}$). Stock solutions of the furylacryloylated dipeptides (Sigma) were prepared by dissolving 3-(-2-furylacryloyl)-L-glycyl-L-leucine

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Abbreviation: TLP-ste, thermolysin-like protease from *Bacillus stearothermophilus.*

A commentary on this article begins on page 2035.

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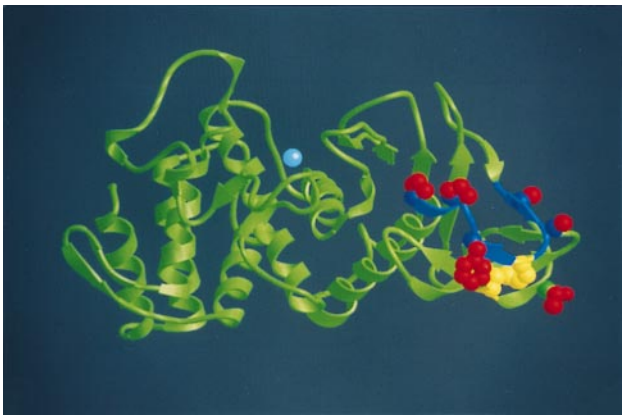

FIG. 1. Ribbon drawing of the three-dimensional model of the 8-fold mutated TLP-ste variant. The 55–69 region is shown in blue. The side chains for the amino acids that were mutated in this study (A4T, T56A, G58A, T63F, S65P, A69P) are shown in red and the disulfide bridge cross-linking residue 60 (N60C) in the critical region with residue 8 (G8C) in the underlying β-hairpin is shown in yellow. The light-blue sphere indicates the catalytic zinc ion.

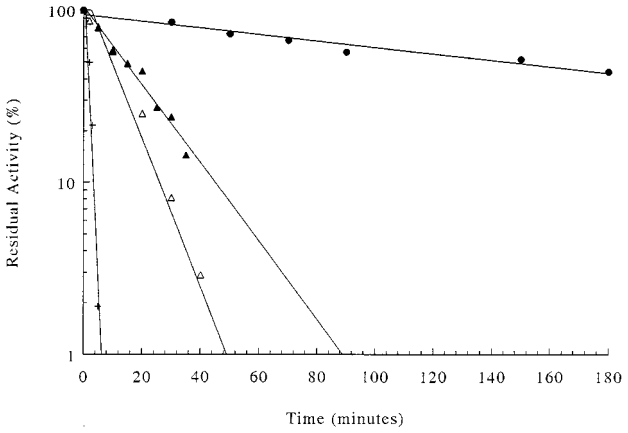

FIG. 2. First-order inactivation of TLPs. Stabilities of TLPs were determined at different temperatures. Shown are the first-order inactivation curves for TLP-ste incubated at 80°C (▲) and 90°C (+), thermolysin at 90°C (△), and the 8-fold mutant at 100°C (●).

amide (FaGLa) and 3-(-2-furylacryloyl)-L-alanyl-L-phenylalanine amide (FaAFa) in DMSO. The k_{cat}/K_m ratios of the enzymes were determined by varying the enzyme concentrations (over a 50-fold range) under pseudo-first-order conditions and measuring the initial activity, essentially according to the method described by Feder (20). The K_i for phosphoramidon (*N*-[α-L-rhamnopyranosyl-oxyhydroxyphosphinyl]-L-leucyl-L-tryptophan) was determined by a 30-min preincubation of a 100-pM protease solution with varying concentrations of the inhibitor (10^{-8} to 10^{-3} M), in 50 mM MOPS, pH 7.0/5 mM $CaCl_2$/50 mM NaCl prior to the addition of the furylacryloylated substrate (FaAFa). Because K_m values of TLPs for FaAFa are higher than the attainable substrate concentrations, IC_{50} values were taken to be equal to K_i values (21). The optimum temperature for activity was determined by incubating proteases with casein (0.8%) in 50 mM Tris·HCl, pH 7.5/5 mM $CaCl_2$ at different temperatures for 30 min, after which the amount of released soluble peptides was determined as described previously (22).

Stability Measurements. For the determination of their half-lifes, the proteases were incubated in 20 mM NaAc, pH 5.3/15 mM $CaCl_2$/62.5 mM NaCl/0.5% isopropanol/0.01% Triton X-100 at 80, 90, or 100°C. Samples, taken at the various time points, were quickly cooled on ice (15 s) and transferred to 50°C to prevent cold denaturation. Residual activity was determined by using casein (0.8%) as a substrate in 50 mM Tris·HCl, pH 7.5/5 mM $CaCl_2$ as described previously (11). Protease activities in the presence of denaturing agents were determined in 50 mM Mops, pH 7.0/5 mM $CaCl_2$/50 mM NaCl/0.01% Triton X-100/0.5% isopropanol using 100 μM FaGLa (3-(-2-furylacryloyl)-L-glycyl-L-leucine amide) as a substrate at 50°C. Enzymes and denaturing agents were preincubated in the reaction mixture at 50°C for 15 min before the addition of the substrate. The reaction was followed by measuring the change in absorbance at 345 nm. The change in absorbance was linear during the time of measurement (30–60 min), indicating that the proteases were active in this time interval and that substrate depletion was negligible.

Proteolytic Properties. β-Casein (1 mg/ml) was incubated with TLP-ste or the 8-fold mutant at a molar ratio of 1,000:1 for 1 hr at 60°C and 100°C, respectively. At both these temperatures, β-casein behaves as a noncompact and largely flexible structure (23). The peptides resulting from hydrolysis were derivatized with dansyl-chloride. The proteolytic products were separated by loading a sample corresponding to 50 μg β-casein on a reversed-phase column (RP-304, Bio-Rad). The mobile phase used was 50 mM NaAc, pH 5.2. Peptides were eluted with a linear gradient from 0 to 60% acetonitrile in 30 min at a flow rate of 1 ml/min. Absorption of the eluting peptides was monitored at 254 nm.

Bacillus licheniformis α-amylase (1 mg/ml) in 50 mM Mops, pH 7.0/5 mM $CaCl_2$/0.01% Triton X-100 was incubated with purified TLP-ste (1 μg/ml), the 8-fold mutant, or without protease for 60 min at different temperatures. The reaction volume was 500 μl. After incubation the samples were cooled on ice, which resulted in aggregation of the substrate in the samples that had been incubated at 100°C. Precipitates (only observed in the 100°C samples) were collected by centrifugation and redissolved in 500 μl 6 M urea. Both supernatants and redissolved precipitates were subjected to standard SDS/PAGE, including pretreatment with sample loading buffer (5 min at 100°C). The samples were identical in size (20 μl supernatant and 20 μl dissolved precipitate). Gels were stained with Coomassie brilliant blue.

RESULTS AND DISCUSSION

The amino acid substitutions A4T, T56A, G58A, T63F, S65P, A69P, G8C, and N60C were combined in TLP-ste, and the

Table 1. Enzymatic and stability properties of wild-type TLP-ste, thermolysin, and the 8-fold mutated variant

	Enzymatic properties at 37°C				Stability			
	Specific activity	k_{cat}/K_m		K_i	T_{opt}	Half-lifes		
Protease	casein, units/μg	FaGLa ($M^{-1}s^{-1}$) $\times 10^{-3}$	FaAFa ($M^{-1}s^{-1}$) $\times 10^{-3}$	Phosphoramidon, nM	Casein, °C	80°C (min.)	90°C (min.)	100°C (min.)
TLP-ste	3.5 ± 0.7	30 ± 8	222 ± 33	50 ± 18	74	17.5	1.5	<0.5
TLN	NA	NA	NA	NA	77	>200	12.5	1
Eightfold mutant	3.9 ± 0.7	30 ± 6	266 ± 23	43 ± 13	95	Stable	Stable	170

NA, not applicable.

Table 2. Effect of denaturing agents on activity toward furylacryloylated peptides at 50°C

GndHCl, M	Relative activity		Urea, M	Relative activity		SDS, % wt/vol	Relative activity	
	TLP-ste	Eightfold mutant		TLP-ste	Eightfold mutant		TLP-ste	Eightfold mutant
0	100	100	0	100	100	0	100	100
1.0	99	95	0.5	96	97	0.25	72	94
2.0	62	83	1.0	87	91	0.5	37	81
3.0	0	68	2.0	75	93	0.75	9.6	57
4.0	0	50	3.0	3	86	1.0	0	40
5.0	0	40	4.0	0	72	1.25	0	29
5.5	0	15	5.0	0	63	1.5	0	22
6.0	0	0	7.5	0	35	2.0	0	0

Activities are expressed as percentage of the activity in the absence of denaturant.

resulting 8-fold mutant (Fig. 1) was produced, purified, and characterized as described in *Materials and Methods*. Production and purification yields were similar to those of the wild-type TLP-ste.

As shown in Table 1 and Fig. 2, the 8-fold mutant displayed a drastically increased thermal stability. Whereas the wild-type enzyme had an undetectable short half-life at 100°C, the 8-fold mutant was quite stable at this temperature, its half-life being 170 min. Table 1 shows that, in terms of the increase in half-life at 100°C, the 8-fold mutant is at least 340 times more stable than the wild-type enzyme. Table 1 also shows that the 8-fold mutant is much more stable than the naturally occurring, more thermostable counterpart of TLP-ste, thermolysin. The extreme stability of the 8-fold mutant is further illustrated by Table 2, which shows that this mutant retains its activity at high concentrations of denaturing agents.

The temperature optimum of the 8-fold mutant was 21°C higher than that of the wild-type. The two enzymes variants were largely identical, however, in terms of their specific activities toward casein and their k_{cat}/K_m values for furylacryloylated dipeptides at 37°C (Table 1). The ratio between the k_{cat}/K_m values for FaGLa and FaAFa was not changed by the mutations, indicating that cleavage specificity had not changed. This is further illustrated by Fig. 3, which shows that identical digestion patterns were obtained on incubating β-casein with both TLP-ste variants at various temperatures.

The potential and stability of the 8-fold mutant, as well as its activity at 100°C, is further illustrated in Fig. 4. This figure shows that, in contrast to the wild-type enzyme, the 8-fold mutant can be used to degrade protease-resistant substrates under extreme conditions. Similar experiments with thermolysin did not result in degradation of the amylase (results not shown).

In summary, the enzymatic properties of the constructed variant resemble those of the wild-type, but its stability resembles that of extremozymes (24) or thermozymes (25) produced by organisms that are capable of surviving in extreme environments such as Archaea and Eubacterial extremophiles (refs. 24, 26, and 27; Table 3).

Studies on proteins from extremophiles have revealed that adaptation to extreme environments normally can be attributed to intrinsic properties of these proteins, although in some

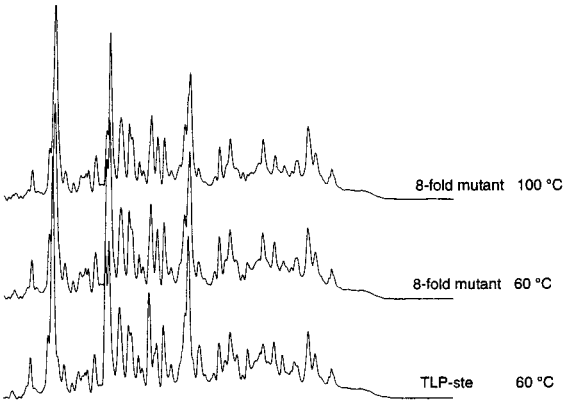

FIG. 3. Proteolytic specificity of TLP-ste and the 8-fold mutant. β-Casein was incubated with TLP-ste or the 8-fold mutant at a molar ratio of 1,000:1 for 1 hr at 60°C and 100°C, respectively. Proteolysis products were analyzed by reversed-phase HPLC.

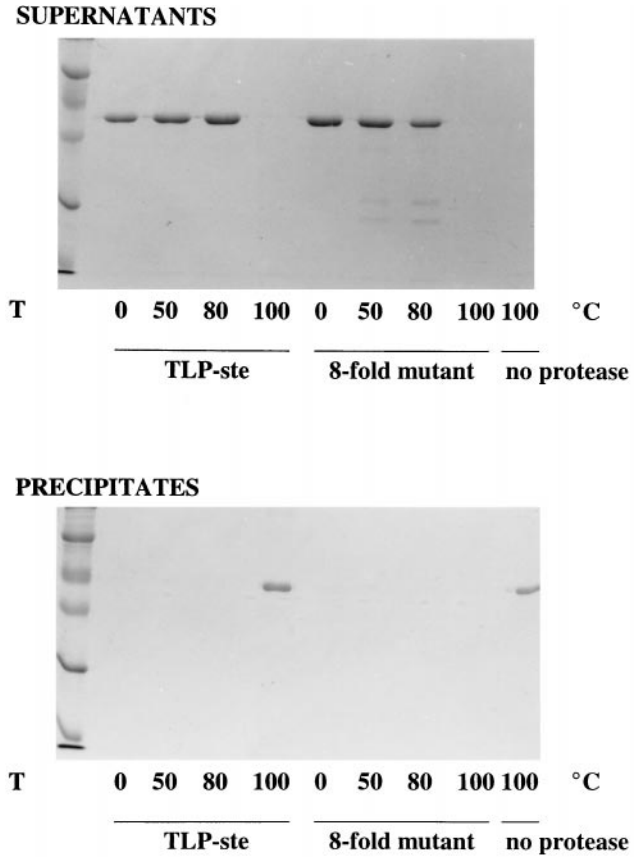

FIG. 4. Hydrolysis of protease-resistant α-amylase from *Bacillus licheniformis* by the 8-fold mutant. *B. licheniformis* α-amylase was incubated with purified TLP-ste, the 8-fold mutant, or without protease for 60 min at the temperature indicated. After incubation the samples were cooled on ice, which resulted in aggregation of the substrate in the samples that had been incubated at 100°C. Precipitates (observed only in the 100°C samples) were collected by centrifugation and redissolved in 6 M urea. Both supernatants (*Upper*) and redissolved precipitates (*Lower*) were subjected to standard SDS/PAGE. No significant degradation of α-amylase occurred at temperatures of 80°C and lower, irrespective of the enzyme used. In cases where the samples were incubated at 100°C without added protease or with TLP-ste the aggregate formed after cooling contained mature α-amylase (*Lower*), indicating that no hydrolysis had occurred. The *B. licheniformis* α-amylase that was incubated with the 8-fold mutant at 100°C was completely hydrolyzed, and no aggregate was formed.

Table 3. Stabilities of the 8-fold TLP-ste mutant and a selection of enzymes from extremophiles

Enzyme	Source	Half-life, hr	T, °C	Ref.
2-Ketoisovalerate-ferredoxin oxidoreductase	*Thermococcus litoralis*	0.8	95	(41)
Carbamoyl-phosphate synthetase	*Pyrococcus abyssi*	3	95	(42)
Sulfide dehydrogenase	*Pyrococcus furiosus*	12	95	(43)
α-Glucosidase	*Thermococcus* AN1	0.6	98	(44)
3-Phosphoglycerate kinase	*Pyrococcus woesei*	0.45	100	(45)
Glyceraldehyde-3-phosphate dehydrogenase	*Pyrococcus woesei*	0.7	100	(46)
DNA-RNA polymerase	*Thermoproteus tenax*	2	100	(26)
Hydrogenase	*Pyrococcus furiosus*	2	100	(47)
Glyceraldehyde-3-phosphate dehydrogenase	*Thermotoga maritima*	>2	100	(48)
Eightfold TLP-ste mutant	*B. stearothermophilus*	2.8	100	(This study)
ADP-dependent glucokinase	*Pyrococcus furiosus*	3.6	100	(49)
Amylase	*Pyrococcus woesei*	6	100	(26)
Glutamate dehydrogenase	*Pyrococcus furiosus*	10	100	(50)
β-Glucosidase	*Pyrococcus furiosus*	85	100	(51)
Cellobiohydrolase	*Thermotoga* sp.	1	108	(26)
α-Amylase	*Pyrococcus furiosus*	2	120	(52)

cases contributions of particular intracellular components, e.g., heat shock proteins and so-called "thermoprotectants," have been demonstrated (28, 29). On the basis of comparative studies of the primary structures of proteins from mesophilic and thermophilic organisms (29, 30), general "rules" for stability have been proposed. Mutational studies (e.g., refs. 10, 31, and 32) have made it increasingly clear, however, that many of these purely statistical rules do not apply and that the effects of site-directed mutations on stability should be evaluated in a context-dependent way. Recently, an expanding number of three-dimensional structures of extremozymes has become available (ref. 25 and references therein, and ref. 33). These structures revealed some conspicuous characteristics, in particular, the presence of a high number of surface-located salt bridges and the presence of metal-binding sites (25). Still, considering that the free energy of stabilization equals no more than, for example, a few hydrogen bonds, it has been proposed that a few structural changes account for observed large differences in stability (26). That this is indeed the case is shown by the present study, where an extremozyme was produced by a limited number of changes. These changes do not involve surface-located salt bridges and they do not affect metal binding (the effect of calcium on stability is largely similar for TLP-ste and the 8-fold mutant; O.R.V. and B.V., unpublished observations). Some of the mutations in the 8-fold mutant seem to be rather TLP-specific (e.g., T63F; ref. 12). It is important to note, however, that a major contribution to stability is made by a more generally applicable type of mutations, namely, those that reduce the entropy of the unfolded state (Gly $\rightarrow$ Ala, Xaa $\rightarrow$ Pro, and the introduction of disulfide bridges). This illustrates the importance of such mutations for stability (13, 34, 35).

Assuming that temperature was the fastest-changing environmental parameter during the early life of microorganisms, easy (and therefore rapid) evolutionary adaptation to reduced temperatures may have been important for survival. The studies with TLPs show that evolutionary processes resulting in molecular adaptation of enzymes to mesophilic conditions may indeed have taken place through a very limited number of mutations. In this view, all other mutations were part of the normal evolutionary process of adaptations to the many additional changes in the environment. Involvement of these other mutations in maintaining an optimal active site conformation has been suggested (36, 37) and has also been used to explain the similar specific activities of enzymes from mesophilic and thermophilic organisms at their respective optimum temperatures (38). Without mutational adaptation, the more stable enzymes would be much more active at their optimal temperatures, as consequence of the temperature dependence of catalysis (which predicts an increase of enzymatic activity by a factor of two for every 10°C raise in temperature; ref. 36). It has been observed that extremozymes are often less active at lower temperatures than their mesophilic counterparts (37, 39, 40). The present results, however, lead to the unexpected but important conclusion that it is possible to engineer extremozymes that retain their full activity at lower temperatures.

The present study shows that boiling-resistant proteins can be obtained by rational design. In this case, the key to success was insight in the (often local) unfolding processes that determine the rate of irreversible thermal inactivation. Rationally designed extremozymes can be valuable biocatalysts, as exemplified by the ability of the 8-fold TLP-ste mutant to hydrolyze stable, protease-resistant substrates (Fig. 4). With respect to applications, it is most important to note that the 8-fold mutant displays adaptation to extreme conditions without forfeiture of enzymatic performance.

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