A Single Calcium Binding Site Is Crucial for the Calcium-Dependent Thermal Stability of Thermolysin-like Proteases†

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ABSTRACT: Thermostable thermolysin-like proteases (TLPs), such as the TLP of *Bacillus stearothermophilus* CU-21 (TLP-ste), bind calcium in one double (Ca1,2) and two single (Ca3, Ca4) calcium binding sites. The single sites are absent in thermolabile TLPs, suggesting that they are determinants of (variation in) TLP stability. Mutations in the Ca3 and Ca4 sites of TLP-ste indeed reduced thermal stability, but only mutations in the Ca3 site affected the calcium-dependence of stability. The predominant effect of the Ca3 site results from the fact that the Ca3 site is part of a region of TLP-ste, which unfolding is crucial for thermal inactivation. Thermal inactivation is not caused by the absence of calcium from the Ca3 site per se, but rather by unfolding of a region of TLP-ste for which stability depends on the occupancy of the Ca3 site. In accordance with this concept is the observation that the effects of mutations in the Ca3 site could be compensated by stabilizing mutations near this site. In addition, it was observed that the contribution of calcium binding to the Ca3 was substantially reduced in extremely stable TLP-ste variants containing multiple stabilizing mutations in the Ca3 region. Apparently, in these latter variants, unfolding of the Ca3 region contributes little to the overall process of thermal inactivation.

Thermolysin-like proteases (TLPs)[1] are a group of metalloendopeptidases with maximum activity at neutral pH. They contain one catalytic zinc atom (*1*), and two to four calcium ions that were shown to be important for stability (e.g., *2*). The amino acid sequences of the TLPs of several *Bacillus* species have been determined (e.g., *3*−*6*), and the X-ray structures of thermolysin (*7, 8*) and the TLP of *Bacillus cereus* (TLP-cer) (*9, 10*) have been elucidated. These structures revealed the locations of the catalytic zinc and four calcium ions. Calcium ions 1 and 2 (Ca1,2) are found in the so-called double calcium binding site close to the active site zinc. Calcium 3 (Ca3) is located at the surface in the N-terminal domain, and calcium 4 (Ca4) is bound by a surface-located ω-type loop (*11*) in the C-terminal domain. The locations and the atomic details of the calcium binding sites in thermolysin are shown in Figure 1.

A sequence alignment of various TLPs (Figure 2) shows that those residues known to be involved in coordinating calcium ions in thermolysin are conserved in the more stable TLPs. In the least stable TLPs, residues making up the Ca1,2 double site are conserved, but residues coordinating Ca3 and Ca4 are not. This sequence alignment, as well as a three-dimensional model of the TLP of *Bacillus subtilis* (*12, 13*), shows that the Ca3 and Ca4 sites are absent in the least stable TLPs. This suggests that binding of Ca3 and/or Ca4 is a determinant of the observed differences in stability between members of the TLP family.

The importance of calcium for the stability of thermolysin has been studied experimentally, and it has been shown that removal of calcium by chelators such as EDTA results in rapid autolytic degradation (*14*−*16*). Removal of calcium presumably results in a partially unfolded, flexible molecule which is prone to autolysis (*12, 14, 17*). There is considerable confusion in the literature about the binding affinities of the four individual calcium sites in thermolysin and their importance for stability. Weaver et al. (*18*) performed crystal soaking studies and ranked the calcium binding affinities Ca1 $\gg$ Ca3 > Ca4 $\geq$ Ca2. Roche and Voordouw (*15*) ranked the affinities Ca3 = Ca4 > Ca1,Ca2 and proposed that there was a cooperative release of the ions from the double calcium binding site (*19*). Finally, Tajima et al. (*20*) observed similar affinities for all calcium binding sites (Ca1 = Ca2 = Ca3 = Ca4). In terms of the effect of calcium binding on stability, the disagreement between the various studies concerns the question whether Ca1 and/or Ca2 are bound in the autolytically susceptible state. Most authors agree, however, that autolysis of thermolysin, as induced by removal of calcium ions, is correlated with the release of

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[1] Abbreviations: TLP, thermolysin-like protease; TLP-ste, thermolysin-like protease of *Bacillus stearothermophilus*; TLP-cer, thermolysin-like protease of *Bacillus cereus*.

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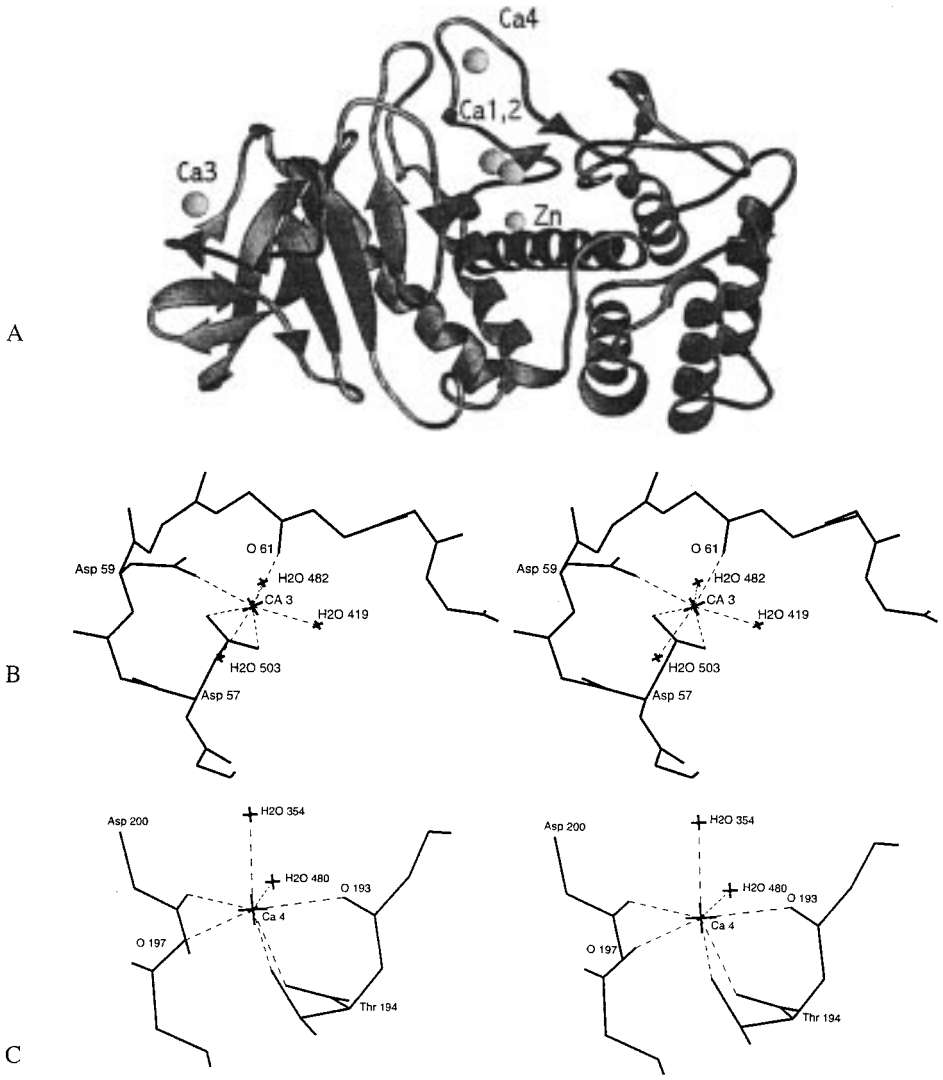

FIGURE 1: (A) Ribbon representation of the 3D structure of thermolysin. The N-terminal domain (containing Ca3) consists mainly of β-strands, whereas the C-terminal domain (containing Ca1,2 and Ca4) is mainly α-helical. The catalytic zinc is positioned in the active site cleft (small sphere). The calcium ions important for stability are indicated by the larger spheres. The catalytic zinc is positioned at the bottom of the active site cleft. (B) Line drawing of the structure of calcium binding site 3 (Ca3). (C) Line drawing of the structure of calcium binding site 4 (Ca4).

either Ca3 or Ca4 (e.g., *14*, *17*, *21*, *22*). Dahlquist et al. (*14*) and Roche and Voordouw (*15*) each concluded that the critical step in thermal inactivation of thermolysin involves the release of only one calcium ion.

Due to the broad specificity of TLPs, conformational features rather than primary sequence are thought to determine the sites of proteolytic attack (*16*, *22*). At elevated temperatures, TLPs are irreversibly inactivated as a result of autolysis (*23*). Accordingly, it has been shown that the rate-limiting step in thermal inactivation of TLPs is a partial unfolding process that renders the protease susceptible to autolysis (*12*, *14*, *23*−*25*; see also ref *26*). Dahlquist et al. (*14*) were the first to show that under most assay conditions only minute amounts of active protease are necessary for immediate and complete autolysis of TLP molecules that reach a sufficiently unfolded state (see also refs *12*, *23*, *27*). The early unfolding areas (weak links) are likely to be found at the surface of the protein, since early steps in protein unfolding are thought to involve mainly surface-located structure elements (*28*−*30*).

The local nature of the stability-determining unfolding processes has been confirmed by the observation that the effects of site-directed mutations on the stability of the TLP from *Bacillus stearothermophilus* CU21 (TLP-ste; 86% sequence identity with thermolysin) are highly dependent on the location of the mutation. Large mutational effects were primarily observed for mutations in one surface-located region (residues 56−69; close to Ca3) which, thus, seems to be involved in unfolding processes that trigger autolysis (*31*−*33*). Likewise, calcium binding will only be related to thermal stability if the calcium ion in question stabilizes a part of the TLP that is involved in the stability-determining unfolding processes. In other words, the contribution of a bound calcium ion to stability is determined by the location of the binding site, not primarily by the affinity of this site. In much of the literature on the effects of calcium binding on thermolysin stability and autolysis, the connection between calcium binding constants and thermal stability is inferred without adequate reference to the peculiarities of the mechanism for thermal inactivation of TLPs.

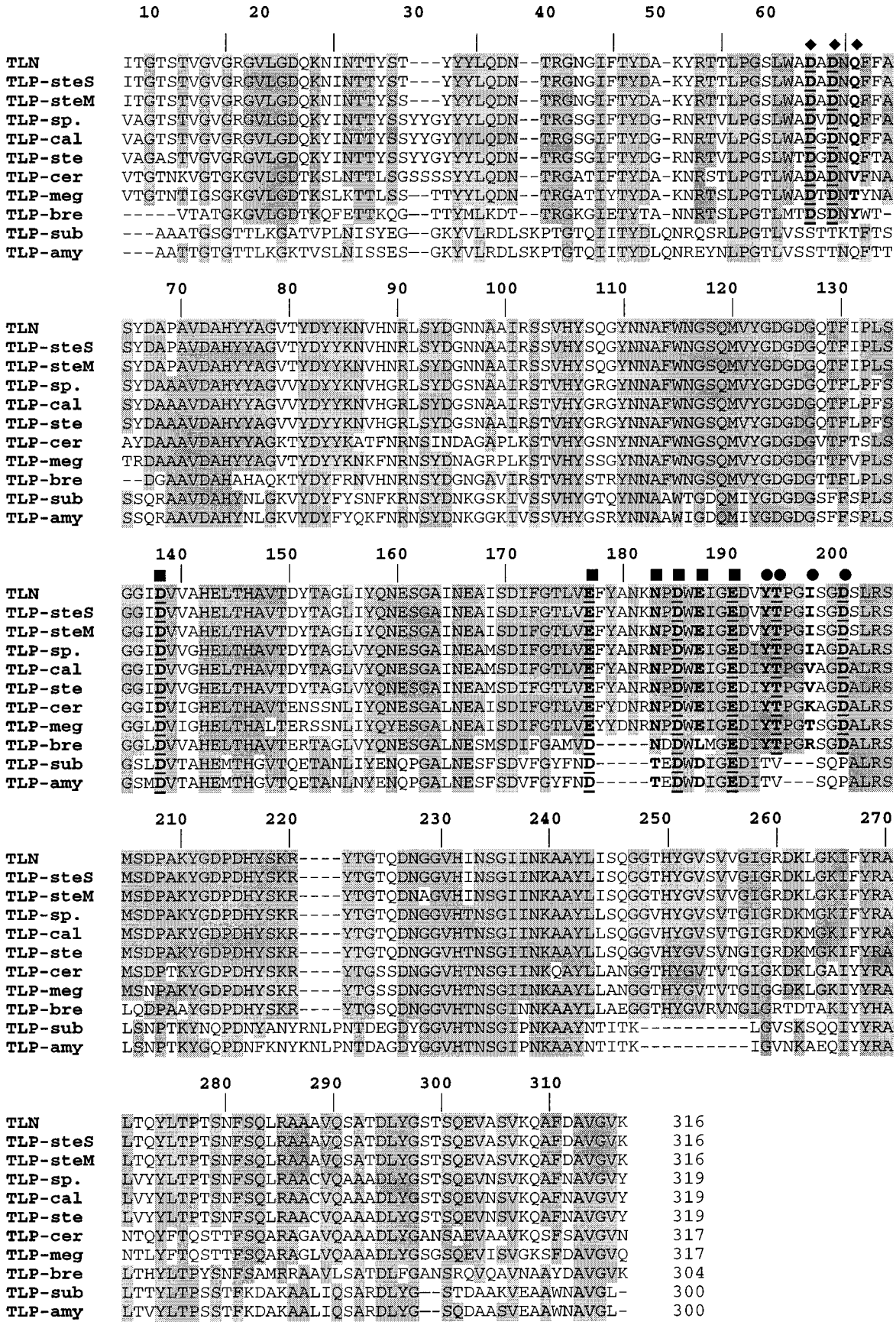

FIGURE 2: Alignment of thermostable and thermolabile thermolysin-like proteases. Residues that are ligands of the conserved double calcium binding site Ca1,2 (■), and the single Ca3 (◆) and Ca4 (●) binding sites are shown in bold. Residues that are liganding via their side chain are underlined.

Site-directed mutagenesis is an obvious approach to the study of the contribution of bound calcium ions to TLP stability. For example, on the basis of the presence of a clear weakest link in TLP-ste near the binding site of Ca3,

one may suggest that Ca3 is crucial for stability, even without knowledge concerning its binding constant (*32*). In the present study, we have further exploited site-directed mutagenesis to study the contribution of calcium binding to TLP-ste stability. We describe the influence of the calcium concentration on the thermal stability for a series of TLP-ste variants, including variants with deteriorated calcium binding sites. The results reveal the relative binding constants of Ca3 and Ca4, and they show that the calcium-dependence of TLP-ste stability in the 1−100 mM [Ca^{2+}] range reflects binding to site 3.

EXPERIMENTAL PROCEDURES

Production and Characterization of Mutants. The *nprT* gene encoding TLP-ste from *B. stearothermophilus* CU21 was originally cloned and sequenced by Aiba and co-workers (*4*, *34*) and subcloned as described previously (*35*). Site-directed mutagenesis was performed using the pMa/c gapped duplex method as described earlier (*35*) or (for Thr194Val and Asp200Ala) by the PCR-based megaprimer method essentially as described by Sarkar and Sommer (*36*). The expression, production, purification, and subsequent characterization of wild-type and mutant TLPs were performed as described earlier (*35*, *37*).

In the standard thermal stability assay, 0.1 μM solutions of purified protease were incubated in 5 mM $CaCl_2$, 20 mM sodium acetate, pH 5.3, 0.01% Triton X-100, 0.5% 2-propanol, and 62.5 mM NaCl at various temperatures for 30 min. After the incubation, the residual proteolytic activity was determined using casein as a substrate (*34*). The influence of calcium on thermal stability was determined by varying the calcium concentration between 0.125 and 100 mM in the assay buffer described above. Thermal stability was quantified as T_{50} being the temperature at which 50% of the activity remains after a 30 min incubation. When testing various calcium concentrations, a sample of protease at the standard 5 mM calcium concentration was included in each assay as a control. The T_{50} values are averages of at least three independent assays with an error margin of approximately 0.3 °C in all cases.

Structural Analysis. A three-dimensional model of TLP-ste was built on the basis of the crystal structure of thermolysin (*7*, *8*) using WHAT IF (*38*), as described in detail previously (*12*). Considering the high sequence identity (86%) between the template thermolysin and TLP-ste, the model of TLP-ste is expected to be sufficiently reliable to predict and analyze the effects of site-directed mutations and the influence of the calcium atoms on their environment (*39*, *40*). This assumption is corroborated by the fact that the model has been used successfully for de novo design of stabilizing mutations (*12*, *33*, *41*, *42*).

Calcium Binding Studies. Thermally induced autolysis of TLPs is a first-order process (*14*, *23*): $dE/dt = k_i E$ in which E is the enzyme concentration and k_i the (first order) rate contact of the autolysis. At each calcium site, there exists a binding equilibrium ECa ⇌ E + Ca that is described by K_d = [E][Ca]/[ECa] in which ECa is the complex between the enzyme and the calcium in question, and E and Ca are the enzyme without the calcium in question and free calcium, respectively. Square brackets denote concentrations. K_d is the calcium dissociation constant. The strong dependence of T_{50} on the calcium concentration indicates that E (with one or more unoccupied calcium sites) is susceptible to autolysis, but ECa [with, at least, the stability-determining calcium site(s) occupied] is not. In the present analysis, it is assumed that, within the range of calcium concentrations and temperatures used in the stability assays, calcium-dependence of thermal stability reflects calcium binding to only one critical site (*14*, *15*, *21*).

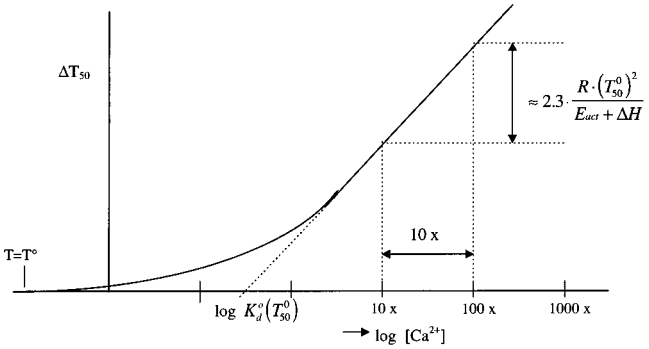

FIGURE 3: Hypothetical plot of T_{50} vs calcium concentration assuming only one critical calcium site. See text for details. The K_d for calcium binding in the critical calcium site can be determined by extrapolating to $\Delta T_{50}{}^0 = 0$.

Using the above-mentioned equations and assumptions (*14*), and denoting the hypothetical $T_{50}{}^0$ at zero calcium concentration by T_{50}, the T_{50} for any given [Ca^{2+}] can be computed from the following implicit relation:

$$1 + ([Ca^{2+}]/K_d{}^0)\exp\left\{\frac{\Delta H}{R}\left(\frac{1}{T_{50}} - \frac{1}{T_{50}{}^0}\right)\right\} = \exp\left\{-\frac{E_{act}}{R}\left(\frac{1}{T_{50}} - \frac{1}{T_{50}{}^0}\right)\right\}$$

Here $K_d{}^0$ is the calcium dissociation constant at $T_{50}{}^0$, ΔH is the calcium binding enthalpy, and E_{act} is the activation enthalpy for the local unfolding process. The solution of this equation is sketched in Figure 3. We note that at calcium concentrations well above $K_d{}^0$ the solution can be approximated by

$$\Delta T_{50} = T_{50} - T_{50}{}^0 \approx (R(T_{50}{}^0)^2/(E_{act} + \Delta H))\ln([Ca^{2+}]/K_d)$$

which represents a linear dependence of ΔT_{50} on the logarithm of the calcium concentration.

This implies that at high calcium concentrations there will be little enzyme lacking the stability-determining calcium ions and being sufficiently unfolded to be susceptible to autolysis. At very low calcium concentrations, the critical calcium site is calcium-free and the T_{50} is independent of the calcium concentration. It should be noted that the assumption that stability depends on only one critical calcium site is only valid within a certain calcium concentration range. Outside this range, calcium binding to other sites plays a role which will cause deviations of the idealized curve depicted in Figure 3.

The major experimental problem is that K_d can vary over many decades, but experimentally accessible calcium concentrations vary roughly from 0.1 to 100 mM. Therefore, a

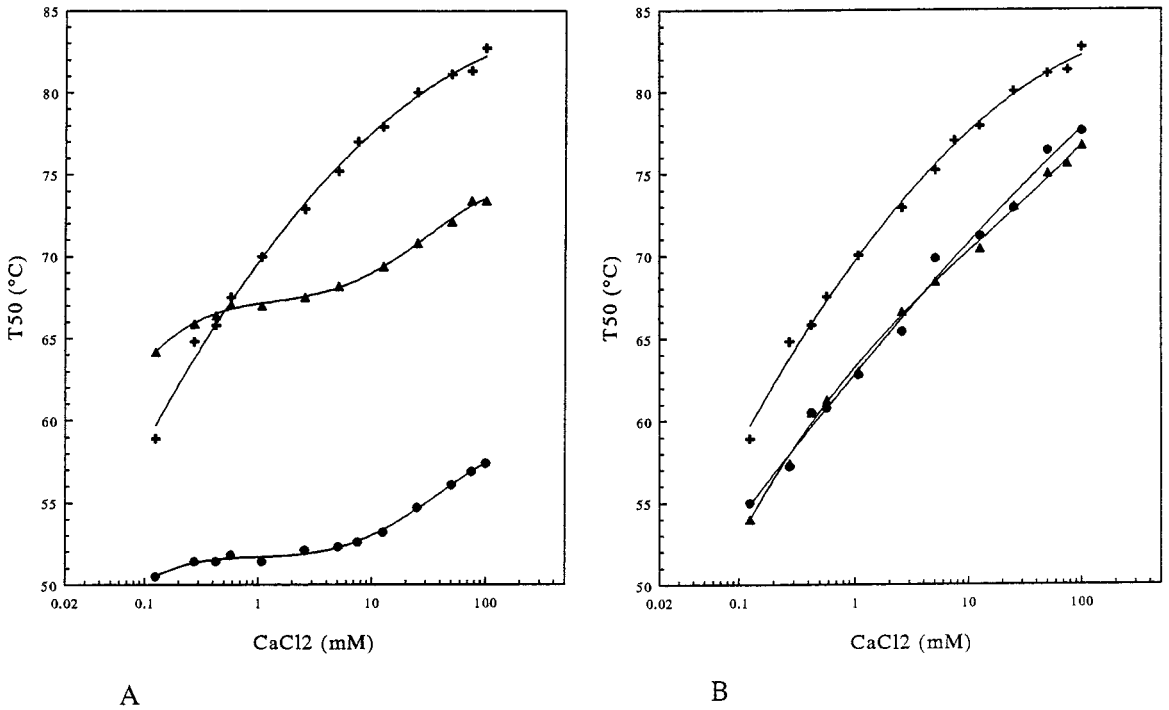

FIGURE 4: Calcium-dependence of the T_{50} for TLP-ste mutants with deteriorated calcium binding sites. (A) TLP-ste (+), and the Asp57Ser (▲) and Asp59Ala (●) mutants in calcium binding site 3. (B) TLP-ste (+), and the Thr194Val (▼) and Asp200Ala (■) mutants in calcium binding site 4.

K_d value can only be measured if it falls roughly between 0.1 and 100 mM.

RESULTS AND DISCUSSION

In Figure 1A, the X-ray structure of thermolysin is shown with the location of the four calcium binding sites indicated. As shown in Figure 1B, Ca3 is bound by the side chains of Asp57 (Oδ1 and Oδ2) and Asp59 (Oδ1). In addition, the backbone carbonyl of residue 61 and three water molecules interact with Ca3. Ca4 (Figure 1C) is bound by the side chains of Thr194 (Oγ1) and Asp200 (Oδ1), and it interacts further with the backbone carbonyl groups of residues 193, 194, and 197 and two water molecules. Obviously, only the roles of residues that interact with calcium through their side chains can be assessed by site-directed mutagenesis.

Mutations in the Ca3 Site. Figure 4, parts A and B, shows that the dependence of the T_{50} of TLP-ste on log[Ca^{2+}] is reasonably linear over the full experimentally accessible calcium concentration range, indicating that the dissociation constants of the stability-determining calcium ions are less than 0.1 mM. The decrease in the slope of the curve at calcium concentrations above 10 mM can be understood if corrections for the activity coefficients of calcium ions are made. The slope of the curve indicates a value of 250 ± 5 kJ/mol for $E_{act} + \Delta H$. Figure 4A also shows that the Asp57 → Ser and Asp59 → Ala mutations in the Ca3 site decrease stability and also modify the calcium-dependence of stability. The curves show that the K_d for the critical site now is in the 0.1−100 mM range. Using the equation given in Experimental Procedures and the type of extrapolation shown in Figure 3, the K_d values were estimated to be in the order of 5 mM in both mutants. At calcium concentrations significantly above K_d, wild-type and mutant curves run parallel, indicating that the calcium-dependent stability is determined by the same calcium site. At calcium concentrations, below K_d, T_{50} does not significantly depend on calcium concentration. At the lowest calcium concentrations the stability of the two mutants displays a further calcium-dependent reduction of stability. Thus, the two mutant curves reveal that another calcium site is beginning to contribute to the overall autolysis process at these very low calcium concentrations.

When assessing the different stability effects of the two mutations in the Ca3 site, it should be kept in mind that the T_{50} assay does not measure calcium binding, but rather unfolding followed by autolysis. At first sight it seems strange that Asp59 → Ala destabilizes much more than Asp57 → Ser, as the structure of thermolysin suggests that the former interacts more strongly with Ca3 (Figure 1B). In the Asp59 → Ala mutant, however, the release of Ca3 will lead to the very unfavorable situation that the negatively charged oxygens in the side chain of Asp57 are left buried in the molecule without counter charges. This will stimulate local unfolding much more than when Asp59 is left without counter charges, since the latter residue has its side chain solvent-exposed. In the absence of calcium, a Ser at the buried position 57 seems more favorable than an Asp, explaining why the Asp57 → Ser mutant is more stable than wild-type at very low calcium concentrations.

Mutations in the Ca4 Site. Figure 4B shows, besides wild-type TLP-ste, the calcium dependence of the T_{50} of the Thr194 → Val and the Asp200 → Ala, which are both expected to deteriorate calcium binding to the Ca4 site. These two mutants show a reduced stability (by approximately −7.5 °C) but the dependence of their T_{50} on the calcium concentration is very similar to wild-type TLP-ste. An explanation for this observation could be that the change in K_d caused by the mutations cannot be detected within the calcium concentration range tested. In other words, either the K_d has stayed below 0.1 mM or the K_d has become larger than 100

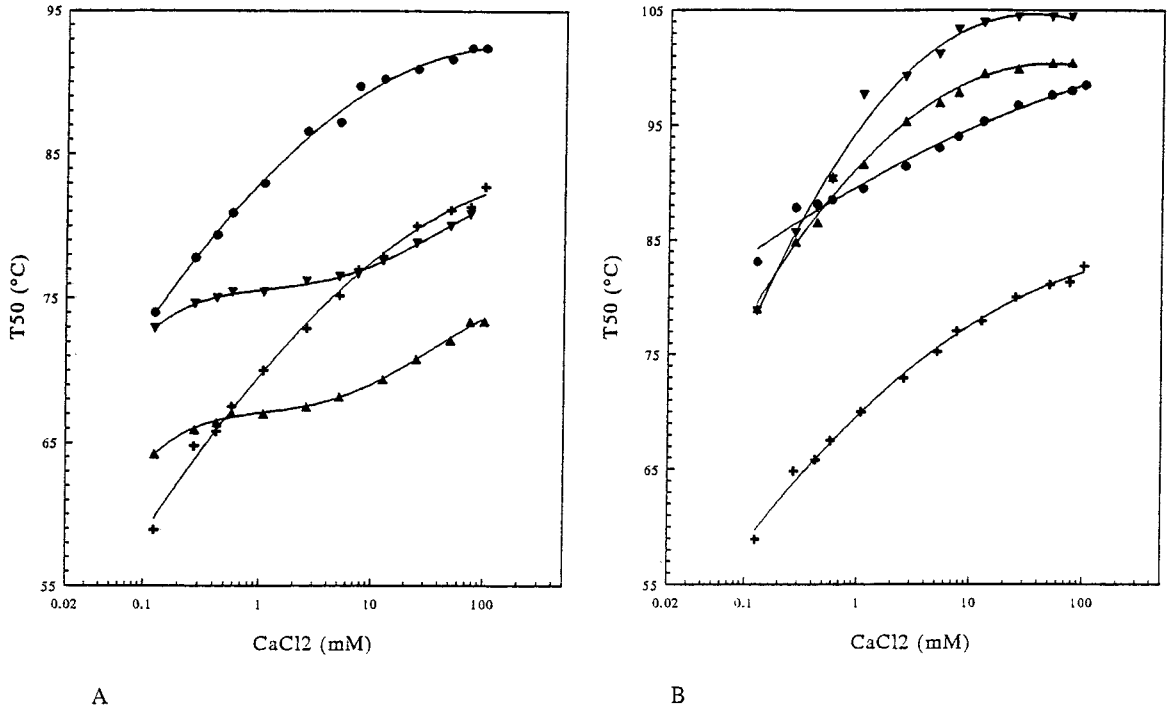

FIGURE 5: Engineering TLP-ste variants whose stability is less dependent on calcium. A) The effect of stabilizing mutations near calcium site 3 on calcium dependence versus T_{50}. Wild-type TLP-ste (+), Asp57Ser (▲), Thr63Phe-Ala69Pro (●), and Asp57Ser-Thr63Phe-Ala69Pro (▼) mutants. (B) The effect of cumulatively stabilizing mutations in the area of calcium site 3 on the calcium-dependence vs T_{50} plot. Wild-type TLP-ste (+), Thr63Phe-Ala69Pro-Ala4Thr-Thr56Ala-Gly58Ala-Ser65Pro(▲), Gly8Cys-Asn60Cys (disulfide bridge) (●); Thr63Phe-Ala69Pro-Ala4Thr-Gly8Cys-Thr56Ala-Gly58Ala-Asn60Cys-Ser65Pro (▼).

mM. The former explanation is highly unlikely since the presence of a valine at position 194 as well as the lack of negative charge at position 200 seems to be incompatible with high-affinity calcium binding (for example, according to our modeling studies a calcium ion would no longer fit in the Ca4 site of the Thr194 → Val mutant). In this respect, it is interesting to note that the destabilizing effects of Thr194 → Val and Asp200 → Ala are almost identical. This may be coincidental, but may also be taken to illustrate that the effect of each of the mutations indeed reflects total loss of calcium-binding in the Ca4 site ($K_d \gg 100$ mM).

The decrease in stability upon the Thr194 → Val and Asp200 → Ala mutations shows that the entirely empty Ca4 site has become a weak link in TLP-ste. The identical slopes for mutant and wild-type enzyme in Figure 4B do however indicate that the Ca4 site does not contribute to the calcium-dependence of stability, either in the wild-type or, obviously, in the mutants. There are two possible explanations for this, one of them being that the Ca4 site has a higher affinity for calcium than the critical site (Ca3) in the wild-type enzyme. This is unlikely, however, since various authors using various experimental approaches have shown that Ca4 binds less tightly to TLPs than Ca3 (*17*, *18*, *22*). Besides, our studies indicate that calcium binding to the Ca4 site can be abolished by one single mutation (in contrast to binding to the Ca3 site). We therefore propose that Ca4 indeed has a lower affinity than Ca3, but that the stability of the wild-type enzyme is not affected by partial depletion of the Ca4 site at the temperatures and calcium concentrations shown in Figure 4B. In other words, for its unfolding to make a noticeable contribution to the overall thermal inactivation process, the Ca4 region needs a higher degree of calcium depletion than the critical (Ca3) region (weakest link). Interestingly, Fontana and co-workers have described experiments indicating that removal of Ca4 from thermolysin at moderate temperatures results in nicking, but not autolytic degradation (*17*).

Reducing the Calcium-Dependence of Stability. The conclusions drawn about the role of Ca3 lead to suggestions for the engineering of TLP-ste variants, for which stability is less dependent on calcium. Thermal inactivation of TLP-ste is not caused by the absence of Ca3 per se, but by the autolysis that takes place after the local unfolding of a region that is stabilized by binding of Ca3. Therefore, every mutant that prevents local unfolding of the region near Ca3 is likely to make the protein more stable. The feasibility of designing TLP-ste variants along the lines of this concept was shown by Veltman et al. (*43*), who compensated for the stability loss caused by the Asp57 → Ser mutation by introducing the stabilizing Thr63 → Phe and Ala69 → Pro mutations. Residues 63 and 69 are near the Ca3 site, without directly affecting it. We have now studied the calcium-dependence of these variants in more detail (Figure 5A), which shows that this dependence is completely identical for the wild-type and the Thr63 → Phe + Ala69 → Pro double mutant on one hand and the Asp57Ser mutant and the triple mutant on the other hand. The net result in the triple mutant is increased stability and decreased calcium-dependent stability at lower calcium concentrations (Figure 5A).

Theory predicts that if the area around the Ca3 site is stabilized to the extreme, this area will no longer be the weakest link in TLP-ste and, consequently, that the dependence of stability on binding of calcium 3 will diminish. Eijsink et al. (*31*) introduced six stabilizing mutations in the area around Ca3 which resulted in a drastic stabilization of TLP-ste (+ 23.5 °C at 5 mM $CaCl_2$). The last mutation added to a 5-fold mutant of TLP-ste still had a considerable stabilizing effect, indicating that the region around Ca3 still

was a weak link in the 5-fold mutant. As depicted in Figure 5B, the calcium-dependence of stability of this highly stable 6-fold mutant differs slightly from that observed for the wild-type enzyme and for variants that had been stabilized to a lesser degree (e.g. the Thr63 → Phe, Ala69 → Pro double mutant; Figure 5A). We have recently added a stabilizing disulfide bridge (which stabilizes wild-type TLP-ste by 18 °C at 5 mM $CaCl_2$) (*42*) to the 6-fold TLP-ste mutant, which resulted in an enzyme with a T_{50} of 101 °C at 5 mM $CaCl_2$ (*44*). Figure 5B shows that this 8-fold mutant displays a more pronounced change in the calcium-dependence of T_{50}, in the same direction at that observed for the 6-fold mutant. Indeed, calcium-independence of stability was obtained, but only at higher calcium concentrations (from 5 mM and upward). Apparently, at these higher calcium concentrations, the Ca3 region is stabilized to the extent that its unfolding no longer contributes significantly to thermal inactivation. At lower calcium concentrations, however, the stability of the 8-fold mutant displays a strongly increased calcium-dependency. Probably, the contribution of unfolding of a different calcium-binding region becomes noticeable at the high temperatures employed when assaying the 8-fold mutant (see below for further discussion).

Concluding Remarks. In literature there is some agreement about the fact that calcium sites 3 and 4 are more important for thermal stability of TLPs than the double calcium site (*14*, *15*, *22*). It is probably more correct to state that the double calcium site is so important that TLPs cannot exist without this calcium site being occupied. The double calcium site is well conserved in all TLPs, and inspections of TLP crystal structures indicate that it would be impossible for the folded molecule to exist in the absence of at least Ca1 (*14*, *45*). Ca2 may be less important, and it has indeed been concluded by several authors that this calcium easily leaves the molecule under various conditions, without significantly affecting stability (*14*, *15*, *18*, *19*).

The importance of Ca3 and Ca4 for the stability differences is strongly indicated by a sequence comparison (see Figure 2) which shows that all thermophilic TLPs have the conserved calcium binding residues Asp57, Asp59, Asp200, and Thr194, whereas the more labile TLPs have residues at these positions that make calcium binding impossible (Figure 2). The present data clearly show that, indeed, the Ca3 and Ca4 binding sites are beneficial for TLP stability, but that their contributions differ. Within the 0.1−100 mM calcium range, Ca3 determines the calcium-dependence of stability, because binding of calcium to the Ca3 site stabilizes by far the weakest link in TLP-ste. Within this same concentration range, unfolding near the Ca4 site (which is likely to be less occupied by calcium than the Ca3 site) does not contribute to the overall thermal inactivation process.

The fairly straight lines observed for the calcium-dependence of the stability of, for example, wild-type TLP-ste indicate that, indeed, this dependence reflects binding of calcium to only one site. In some cases, however, curves were obtained that show the effects of at least two calcium sites. In the Asp57 → Ser and Asp59 → Ala mutants (Figure 4A), the effect of a second site was detectable at the lowest calcium concentrations tested. This may reflect the fact that the Ca4 site has become depleted to the extent that its unfolding is beginning to contribute to thermal inactivation. In the most stable mutants, the effect of a second site is visible from about 5 mM $CaCl_2$ and downward, indicating that at the high temperatures used in assaying these mutants, higher calcium concentrations are needed to keep the Ca4 region sufficiently stable.

In the present study we have assumed that the calcium binding sites in TLP-ste are almost identical to those in thermolysin. This seems reasonable since the high sequence identity between the two enzymes (86%) is even higher near the calcium binding sites (Figure 2). Another implicit assumption was that the mutations made had no other (structural) effects than a (local) effect on calcium binding. Crystallographic studies of large numbers of mutant proteins have indeed indicated that the effects of most site-directed mutations have a highly local character (*46*−*48*), but, of course, larger changes cannot be completely excluded. However, the fact that stable TLPs could be designed, of which the stability was less dependent on calcium, lends convincing support for the correctness of the model used. The initial success of designing less calcium-dependent hyper-stable TLP-ste variants (Figure 5A,B; ref *43*) opens a promising perspective for design of TLP-ste variants with even further improved properties. Instead of simply removing calcium-binding side chains (as in the highly destabilizing Asp59 → Ala mutant), one might, for example, rebuild calcium sites by introducing stabilizing interactions that replace the beneficial effect of calcium binding. In fact, it would seem that current knowledge about the mechanism of thermal inactivation of TLP-ste and the role of calcium therein provides a basis for designing (hyper-) stable TLP-ste variants in which the Ca3 and Ca4 sites are no longer present.

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