

REVIEW

The role of calcium ions in the stability and instability of a thermolysin-like protease

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Abstract: Thermolysin and other secreted broad-specificity proteases, such as subtilisin or alpha-lytic protease, are produced as pre-pro-proteins that stay at least partially unfolded while in the cytosol. After secretion, the pro-proteases fold to their active conformations in a process that includes the autolytic removal of the pro-peptide. We review the life cycle of the thermolysin-like protease from *Bacillus stearothermophilus* in light of the calcium dependent stability and instability of the N-terminal domain. The protease binds calcium ions in the regions that are involved in the autolytic maturation process. It is generally assumed that the calcium ions contribute to the extreme stability of the protease, but experimental evidence for TLP-ste indicates that at least one of the calcium ions plays a regulatory role. We hypothesize that this calcium ion plays an important role as a switch that modulates the protease between stable and unstable states as appropriate to the biological need.

Keywords: calcium and instability; folding; protease; thermolysin like proteases; unfolding

Introduction to TLPs

Bacteria of the genus *Bacillus* are known to secrete proteins with a wide variety of functions. Many of these proteins are involved in cell wall biogenesis and integrity, but extracellular proteins have been detected with roles in processes as diverse as amino acid metabolism, mobility, sporulation, and nutrient scavenging. An important class of secreted proteins are the enzymes involved in detoxification and metabolism such as DNAses, RNAses, lipases, and a series of proteases. Some secreted enzymes are dan-

gerous for the producer cell. Therefore mechanisms have evolved to ensure that such enzymes only become active after secretion from the cell. The best-known examples of such enzymes in the bacilli are secreted broad-specificity proteases such as the alkaline proteases often referred to as subtilisins and the metallo-proteases or 'neutral proteases' referred to in this review as thermolysin-like proteases (TLPs).

The secreted proteases are produced as pre-pro-proteins. The pre-peptide is cleaved-off during Sec-controlled secretion, and the active protease emerges outside the cell after folding of the pro-protein and autolytic removal of the pro-peptide. The autolytic processing step and the role of the pro-peptide have been described in considerable detail for several proteases, including TLPs.^{1–12} The pro-peptide acts as an intra-molecular chaperone, helping the protease to obtain its correct structure.

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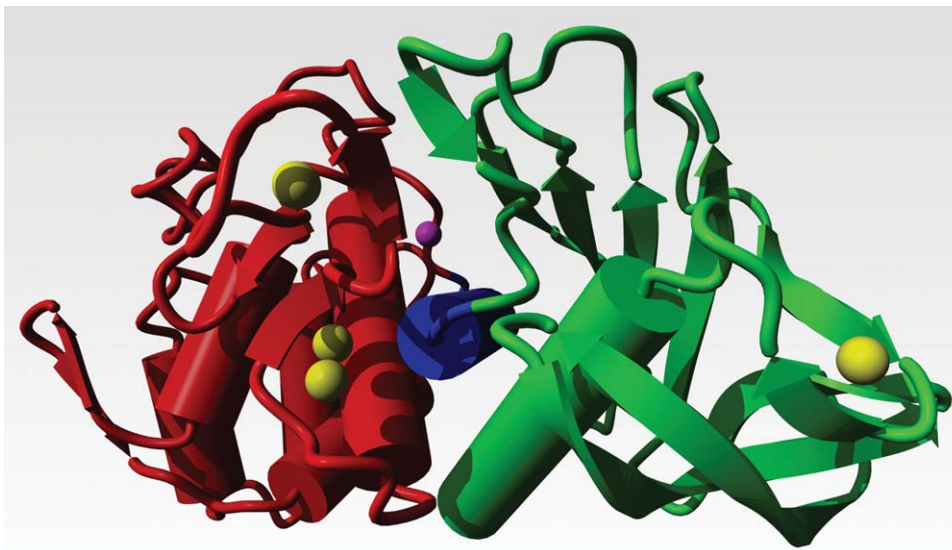


Figure 1. TLPs have a two domain structure. The N-terminal domain that typically comprises residues 1-135 is shown in green. The central helix connecting the two domains is shown in blue. This helix holds several active site residues. The C-terminal domain is in red. The active site Zn is shown in magenta, and the calcium ions are in yellow. Ca3 is at the far right; Ca4 at the top left; Ca1 is located just above and behind Ca2. This picture is based on a homology model of the TLP from *B. stearothermophilus* that shares 85% sequence identity with thermolysin.

It is cleaved-off through intra-molecular proteolysis upon completion of the folding process.

Thermolysin was among the first enzymes to have its crystal structure determined.¹³ The thermolysin structure was the first structure of a thermostable enzyme. The T_{50} of thermolysin, i.e. the temperature at which it loses 50% of its activity under standard conditions after 30 minutes of incubation, is 86.9°C.¹⁴ Unexpectedly at that time, but confirmed by many subsequent crystallographic studies on natural and engineered thermostable enzymes, the structure of thermolysin did not show obvious features that could be linked to its stability. It was concluded that “the enhanced stability of thermostable proteins relative to thermolabile ones cannot be attributed to a common determinant, such as metal ions or hydrophobic stabilization, but in a given instance may be due to rather subtle differences in hydrophobic character, metal binding, hydrogen binding, ionic interactions, or a combination of all of these.”¹⁵ Many subsequent studies have supported this idea. Today we indeed know that the stability of a protein can never be attributed to one single factor and there are many alternative ways to reach extreme protein stability.^{16–18} Calcium ions have long been known to contribute to the stability of thermolysin and are also important for TLP stability,^{19,20} albeit to different extents as was shown by Veltman *et al.*^{21,22}

TLP structure

Structurally, TLPs consist of two domains (Fig. 1) that are connected by a central helix containing several catalytically crucial residues. It has been shown

that substrate-binding involves a hinge-bending motion between the two domains.^{23,24} The C-terminal domain is rich in alpha-helices and the final 62 residues form a four-helix bundle that displays considerable stability on its own.²⁵ The N-terminal domain contains a series of β -strands that cradle one long helix. The N-terminal 13 residues form the middle β -strand of a three-stranded β -sheet (Fig. 2).

TLP Stability

TLPs have been the subject of many site-directed mutagenesis studies aimed at manipulating their thermal stability, in particular the TLP from *Bacillus stearothermophilus* (TLP-ste; $T_{50} = 73.4^\circ\text{C}$) and the TLP from *Bacillus subtilis* (TLP-sub; $T_{50} = 58.6^\circ\text{C}$; see De Kreij *et al.*²⁶ for comparative stability data). TLP-ste is a close relative of thermolysin; the two proteins share 85% sequence identity and have all important residues in common (including all residues involved in catalysis and ion-binding). Thermal inactivation of TLPs is a consequence of inter-molecular proteolysis and the rate-limiting step is the local unfolding that precedes this process.^{17,27,28} In this review, we refer to inter-molecular proteolysis as cannibalism to distinguish this process from the autolytic step during protease maturation. It is important to note that the first order rate constant of thermal inactivation does not reflect the rate of the actual proteolytic cleavage nor of a bi-molecular association needed for cannibalism to occur. Instead, it reflects the rate of an unfolding process that makes the protein proteolytically susceptible. All data suggest that the rate-limiting unfolding processes have

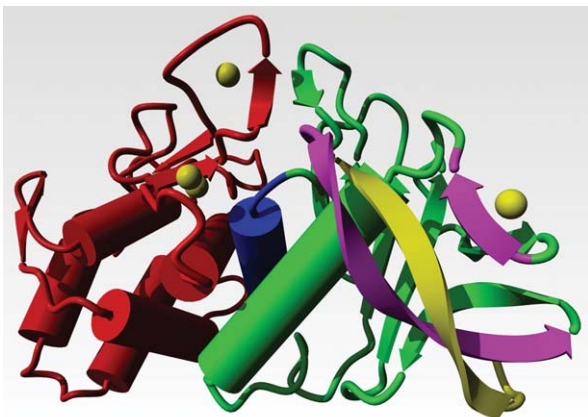


Figure 2. As Figure 1, but slightly rotated to better show the N-terminal β -strands. The N-terminal β -strand of 13 amino acids, shown in yellow, is sandwiched between the β -strands 14-26 and 60-63 that are shown in purple. The structural integrity of the β -strand 60-63 depends on the presence of calcium ion Ca3 (at the top right). Note that Gln61 in the strand 60-63 has a double role. Its side chain interacts with Phe63, while its backbone carbonyl is one of the Ca3 ligands (see also Figure 3).

a local character. While stability data on the mesophilic TLP-sub have not revealed a specific stability determining area,²⁹⁻³¹ studies on TLP-ste have shown that stability is governed by local unfolding processes in the N-terminal domain,^{14,28,32-34} involving the three β -strands and Ca3 (Figs. 2, 3).

Calciums

Thermally stable TLPs such as thermolysin and TLP-ste bind four calcium ions numbered Ca1-Ca4 (Fig. 1), whereas less stable TLPs seem to bind only two (Ca1 and Ca2). Ca1 and Ca2 bind in a double calcium site close to the interface between the two domains. Ca4 binds near the Ca1-2 site. All ligands for these three calciums are located in the C-terminal domain. Ca3 binding to the N-terminal domain is known to be an important stability determinant of TLPs. The calcium affinity of the four sites in thermolysin has been studied in early work by Dahlquist, Voordouw, Roche and co-workers.^{19,20} On the basis of structural inspections it seems safe to assume that the TLPs cannot be folded without calciums bound to the double calcium binding site and most experimental studies on calcium binding concluded that Ca1 binds with the highest affinity. The ranking of the other three affinities is not straightforward as has been discussed extensively.^{22,35} Veltman *et al.*²² were able to show that the dependency of the thermal stability of TLP-ste on calcium in the 0.1–10 mM concentration range involves the Ca3 site (Figs. 3, 4).

Figure 3 shows a detailed picture of the Ca3 site. Ca3 is liganded by seven atoms, three of which are water oxygens. It is important to note that integrity of the Ca3 site is crucial for the integrity of the β -strand 60-63 that in the mature, folded TLP-ste forms a β -sheet with the two N-terminal β -strands (Fig. 2). Most residues that are known from

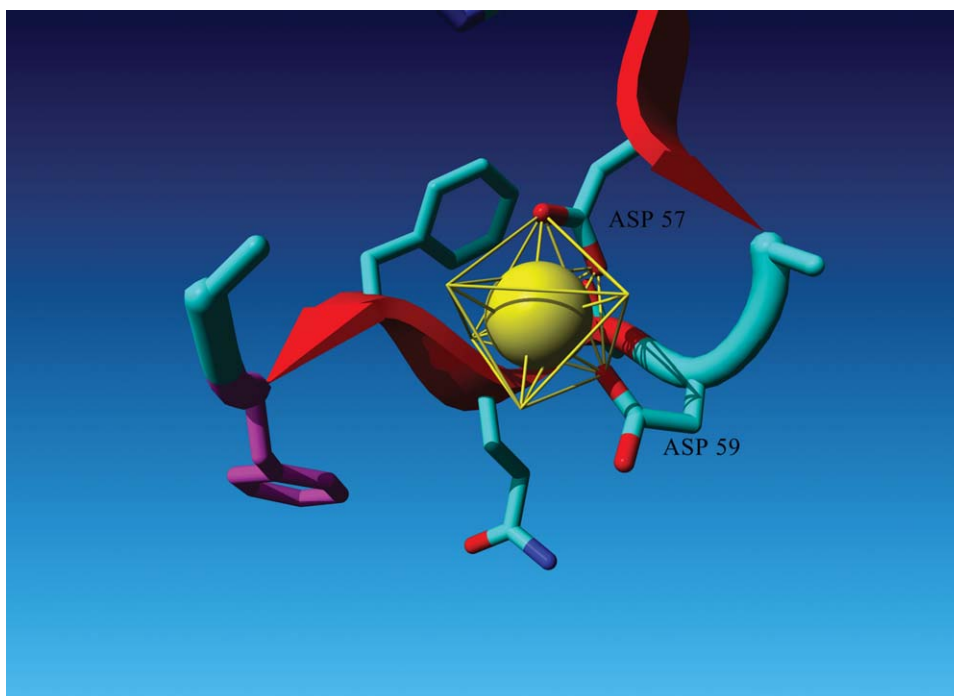


Figure 3. Left. The area around Ca3 in thermolysin. The small yellow sphere is Ca3, the yellow lines connect the seven ligands for this calcium (2 O_{δ} atoms of Asp57, 1 O_{δ} atom of Asp59, the backbone carbonyl of Gln61, and 3 water molecules. The three water molecules are not shown).

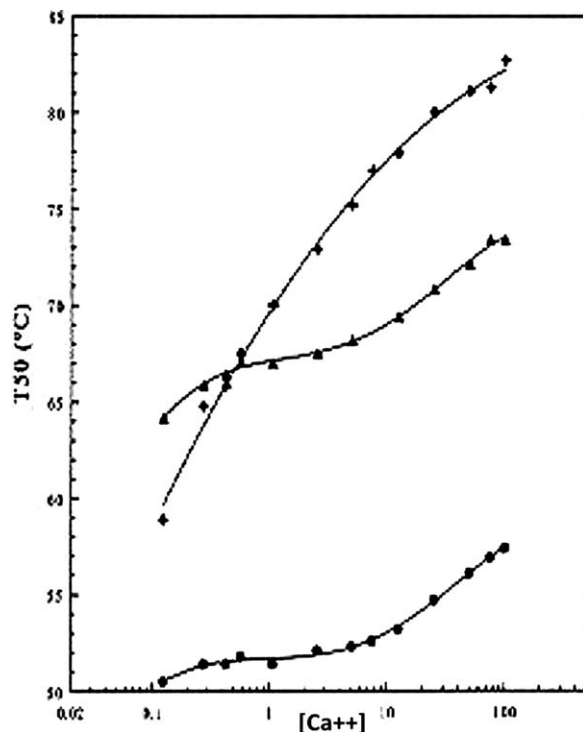


Figure 4. Stability of TLP-ste as a function of the Ca^{2+} concentration. Top curve: wild-type; middle curve Asp57Ala mutant; bottom curve Asp59Ala mutant. TLP-ste variants with mutations in the Ca3 site show reduced stability and an almost abolished calcium-dependency of this stability in the 0.1 – 10 mM concentration range. Asp 57 is buried in the molecule. If no Ca3 is bound, as at low $[\text{Ca}^{2+}]$ or in the case of the Asp59Ala mutant, the buried Asp57 residue will be very detrimental to stability. In the Asp57Ala mutant, also, relatively little calcium is bound, but in this case the remaining aspartic acid, Asp59, is located at the surface, limiting the detrimental effect of the D57A mutation relative to D59A. (Figure copied with permission from O.R. Veltman, dissertation; 1997; see also Veltman *et al.*²²).

comparisons of naturally occurring enzymes³² and from site-directed mutagenesis experiments to be crucial for TLP-ste stability are in the vicinity of Ca3.

Figure 4 shows that the protease is highly unstable at low Ca^{2+} concentrations, indicating that local unfolding of the region around Ca3 and subsequent cannibalistic degradation are promoted as the Ca3 site becomes less populated. The lowest Ca^{2+} concentration that can be achieved in these stability assays is $\sim 100 \mu\text{M}$. The Ca^{2+} concentration in the cytosol is a factor of 1000 lower.³⁶

The Hypothesis

We hypothesize that in order to carry out its full range of biological functions the stability of TLP-ste is variable. Under some circumstances it is stable and fully folded, whereas it can also be partially unfolded and susceptible to cannibalism. We also propose that this change in stability is regulated by

calcium ion binding. Other secreted broad specificity proteases such as subtilisins and alpha-lytic proteases are likely to need similar regulatory mechanisms and it may be noted that the regulation of the stability and instability of such enzymes often involves calcium binding.

The 5 Steps in the Life of TLP-ste

In this section, we summarize the life of TLP-ste starting at the ribosome. Five steps are identified which will be described briefly followed by a more detailed discussion. In Step 1 TLP-ste is synthesized as a pre-pro-protein. While in the cytosol, the pre-pro-protein remains sufficiently unfolded not to be active and to be secretion competent. All healthy living cells, eukaryotes and prokaryotes alike, have an intracellular Ca^{2+} concentration that is on the order of 100 nM.^{36,37} This low Ca^{2+} concentration plays a series of roles in signalling³⁸ but for the purpose of this study, it is important to note that it will contribute to keeping TLP-ste in an at least partially unfolded state because one or more of the calcium sites is not sufficiently occupied. In Step 2, the pre-pro-protein is secreted via the Sec-system and the pre-peptide is removed. In Step 3, outside the cell, the pro-protein folds and the pro-peptide is autolytically cleaved off and degraded.⁷ The relatively high Ca^{2+} concentration outside the cell is an essential aspect of the folding and maturation of the protease. In Step 4, the protease does its work. This normally will be defence or food scavenging. At some point the activity of the protease is, for a series of reasons that we can at best speculate on, no longer needed and in Step 5 the enzyme is degraded. The same Ca3-containing region that is needed to stabilize the β -sheet involving the N-terminal β -strand (Fig. 2) is now the weak link, permitting digestion of the protease, either cannibalistic, or perhaps by members from other protease families.

Step 1: The partially unfolded cytosolic phase

Bacteria have developed several ways to accomplish the complex process of protein secretion.³⁹ The challenge for a bacterium is to coordinate translation, transport to the secretion machinery, translocation through the membrane, and folding. The most ubiquitous export system, used for TLPs, is the so-called Sec-system.^{39–41} Studies on protein secretion of mainly the mesophile *B. subtilis* have been instrumental for generating insight into the functioning of the Sec machinery in bacilli. In the current model for Sec-driven secretion in bacilli, summarized by Harwood and Cranenburgh,⁴¹ a nascent TLP would start its life as a pre-pro-protein that remains at least partially unfolded. The so-called signal recognition particle (SRP) recognizes the N-terminal signal sequence (the 'pre' part). The SRP directs the protein to the translocase complex in the cell membrane while its

chaperone function keeps the nascent protein in a 'secretion competent', partially unfolded state.

There are several good reasons for the *Bacillus* to keep the pre-pro-protein in the secretion competent and at least partially unfolded state. Obviously, it would be energetically inefficient to fold the protease in the cytosol, to unfold it again during translocation, and to refold it again outside the cell. In the particular case of broad specificity proteases such as subtilisins or TLPs, there is the additional problem that these proteases potentially can digest many cytosolic proteins. The bacilli combine several mechanisms to keep TLPs inactive: first, both the secretion machinery and the low intracellular calcium concentration will promote (partial) unfolding. Second, in the unlikely case that a pre-pro-protein occasionally would populate an active conformation, the pro-protein would act as an inhibitor.^{10,42,43}

Step 2: Translocation

The protein is translocated through the membrane at the expense of ATP and the pre-peptide is cleaved off by a Type I signal peptidase. Once outside, the still unfolded pro-protein must fold rapidly and correctly. Failure to do so may damage the producer cell (e.g. by aggregate formation in the cell wall) or may lead to the protein being degraded by quality-control proteases. The pro-part plays two roles in this process: it facilitates folding by acting as an intra-molecular chaperone and it inhibits protease activity of the folded pro-enzyme.¹⁰

Step 3: Folding, maturation, and stability

Once at the trans-side of the membrane and without folding restraints conferred by the secretion machinery, the protein folds in an environment that is rich in bivalent metal ions (including Ca^{2+}), possibly assisted by foldases such as peptidyl prolyl cis-trans isomerases and in some cases also disulphide isomerases (most TLPs, though, neither contain cis-peptide bonds nor cysteine bridges and thus seem not to rely on these enzymes). Many secreted *Bacillus* proteins are metallo-proteins and there is experimental data supporting the idea that bivalent cations such as Ca^{2+} act as folding catalysts.^{41,44} Looking at what is known about calcium binding in TLP-ste and thermolysin and considering intra- and extra-cellular calcium concentrations, it can only be concluded that calcium ions indeed play a crucial role in the maturation process of TLP-ste. The calcium titrations by Veltman *et al.*²² (see also Fig. 4) show that the intracellular calcium concentration of a few hundred nanomolar is so low that in the cytosol not all calcium sites can be occupied in TLP-ste, which would contribute to keeping it at least partly unfolded and thus inactive. Once outside the cell, i.e. in or near the Gram-positive cell wall, the calcium concentration typically is in the millimolar

range due to the ability of the *B. subtilis* cell wall to concentrate calcium ions present in the surrounding medium.^{44,45} Consequently, all calcium binding sites will become occupied, leading to structural stabilization and protease activation.

Pro-enzymes secreted and folded in this manner will not become active before the pro-peptide is cleaved off and removed. This process has been studied in varying detail for different secreted proteases. It is well established that in most cases, including TLP-ste, the necessary cleavage is intra-molecular,^{6,7,46} but in trans processing is possible.⁴⁷ Intra-molecular processing seems to be the default and biologically most relevant situation. Once separated from the mature protease, the pro-parts have low stability^{48,49} and are readily degraded by the mature protease or by other, exogenous proteases.^{7,50,51}

This folding and maturation mechanism has important implications for the conformational flexibility and stability of the N-terminus of the mature proteins. During autolytic maturation, a stretch of amino acids comprising the pro-peptide/N-terminus boundary needs to insert productively into the catalytic centre, a process that obviously requires conformational flexibility. Structural studies on the isolated pro-domain from alpha lytic protease showed a disordered C-terminus that became ordered in the complex of the protease with its pro-domain.⁵⁰ During maturation, the N-termini of the mature proteases need to relocate from a position in the catalytic centre to their final position in the mature enzyme. Structural studies on the intact or processed precursor complexes of subtilisin,^{9,52} alpha-lytic protease,⁵⁰ a TLP with a long, 'thermolysin-like' pro sequence,¹² and a TLP with a short pro-sequence,¹¹ have shown that these relocations are considerable.

Figure 5 shows the result of a short (unrealistic) molecular dynamics simulation on the thermolysin structure. In this simulation, a strong force was applied manually (interactively) to pull the N-terminal amino acid into the active site pocket (such that Val-1 occupies the S1' pocket). Obviously, this simulation is highly unrealistic, but it shows that the N-terminal residue can be located in the active site without the need for any large rearrangements other than the location of the N-terminal 13 amino acids. Recent structural, mutagenesis and modelling studies of the autolytic processing of a TLP from *Pseudomonas* led to similar conclusions.¹² Interestingly, this study showed that the reorganization of the N-terminal beta-strand (amounting to a displacement of 33 Å for the N-terminal amino acid) takes place prior to release of the pro-part, i.e. in a complex of the pro-part and the mature enzyme.

The N-termini of all the aforementioned proteases have extended structures and tend to be a

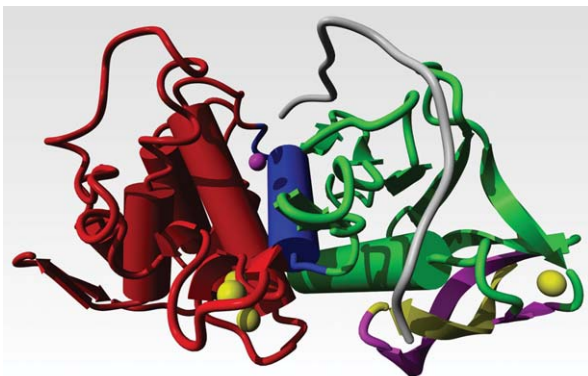


Figure 5. Thermolysin shown in the same colouring scheme as figure 2. The gray loop shows a possible conformation of the residues 1–13 that has the N-terminal amino acid in the active site of the protease. The gray loop was interactively brought into the conformation shown by pulling the N-terminal residue along a wide curve towards the active site Zn while continuously energy minimizing the structure with the YASARA software.

part of β -sheets. Mature, fully folded TLPs have an N-terminal β -hairpin that is detailed in Figures 1, 2, and 5. Figure 6 shows the result of a molecular dynamics simulation on subtilisin E, performed similar to the one described above for thermolysin. In subtilisin, only 10 N-terminal residues need to be relocated to place the N-terminal residue in front of the active site serine. Also in this case, relocation of the N-terminus takes place prior to release of the pro-part, as observed in the crystal structure of a complex of the pro-part and the mature enzyme.⁵²

It seems crucial for both proteases that relocation of the intrinsically flexible N-terminus is

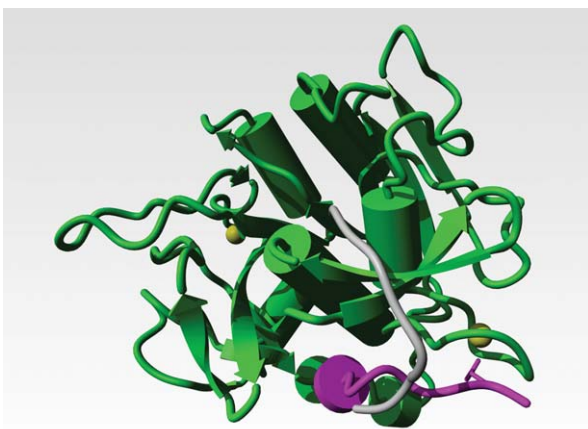


Figure 6. Subtilisin E from *Bacillus subtilis*; PDB file 1scj⁵². The two calcium ions are shown as yellow balls. Residues 1–10 in the mature protease are shown in magenta. Residues 1–10 after the energy minimisation-supported manual operation that brought the N-terminal residue close to the serine in the active site cleft are shown in gray. The side chain of Gln-2, which interacts strongly with one of the calcium ions, is shown as a magenta stick-model.

accompanied by the establishment of new stabilizing interactions to fix the N-terminus in its correct position and to confer sufficient stability to the now fully folded and active protease. In thermolysin and in subtilisin E, calcium is crucially involved in stabilizing the N-terminal residues in the mature protease. The structural aspects like packing, location, etc. of the calciums in thermolysin and subtilisin are different, but their functional roles seem highly similar. In both cases, it has been shown that the occupancy of this N-terminal calcium-binding site has a large effect on stability.^{22,53}

One highly successful, and at the time rather surprising stabilizing mutation in TLP-ste concerns the introduction of a phenylalanine at the surface-located position 63. This phenylalanine is naturally present in thermolysin ($T_{50} = 86.9^\circ\text{C}$ versus $T_{50} = 73.4^\circ\text{C}$ for TLP-ste) and stabilizes the N-terminal β -sheet through interactions with the aliphatic parts of the side chains of residues 9, 11, 17 and 61 (see Fig. 7).^{32,54} The introduction of this phenylalanine in TLP-ste adds stabilizing interactions between the three strands in the N-terminal β -sheet and causes a 7° increase in T_{50} .

The relocation of the N-termini needs to happen in an otherwise pre-formed mature enzyme. Thus, it would seem that those parts of the mature enzyme that interact with the N-terminal residues must be among the conformational least restricted and potentially least stable parts of the protein. Interestingly, mutational studies on TLP-ste have clearly shown that the N-terminal domain is considerably less stable than the C-terminal domain. Thus, the instability of the N-terminal domain, which is crucial during maturation of the enzyme, is to some extent maintained in the fully mature protein. Further details are provided in the description of Step 5.

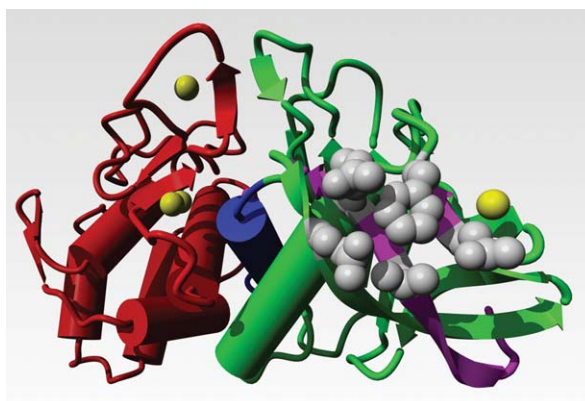


Figure 7. Hydrophobic packing of Phe-63 at the surface. TLP-ste ribbon coloured similar as in Figure 1. Side chains in gray. Phe-63 bridges, Gln-61 (and thus indirectly also Ca3), Val-9, Gln-17, and Arg-11 (shown clockwise around Phe-63).

Step 4: The protease is active

Once folded and without its pro-domain, the protease acts in the extracellular space, its primary functions being to digest food and to detoxify the environment. Broad-specificity proteases including TLP-ste are prone to cannibalistic proteolysis. This process is reduced by the presence of abundant substrate, whereas it is promoted by factors that destabilize the protein such as high temperature or the removal of calcium ions. As the secreted proteases deplete their substrates, the degree of cannibalism will increase, meaning that the proteases will be removed when they are no longer needed. The resulting peptides may potentially be taken up by the bacterium and re-used. It is also possible that protease turnover is accomplished by other secreted broad-specificity proteases (e.g. subtilisin-like) in the environment. Most importantly, as described in detail below (Step 5), the proteolytic degradation of an enzyme, such as TLP-ste, be it by cannibalistic or by the action of another type of protease, requires that the sites of proteolytic attack are sufficiently accessible. This requires some degree of local instability.

Step 5. TLP-ste degradation; N-terminal instability and unfolding

Extensive protein engineering studies of TLP-ste have shown that the N-terminal domain, and in particular the region comprising the N-terminal β -sheet and the Ca3 site are a “weak link” in mature TLP-ste.^{14,27,34} While mutations in the C-terminal domain generally have stability effects on the order of maximally 1° (often close to zero^{24,33,55}), larger mutational effects were obtained when mutating in or around the proposed ‘weak link’.^{14,28,54–57} Although it has not been possible yet to map the first, kinetically critical cannibalistic site in TLP-ste, the studies summarized in Figure 8 show that the N-terminal region of the protein, including the N-terminal three stranded β -sheet and the Ca3 binding site, governs the partial unfolding processes that lead to cannibalistic TLP-ste inactivation. The validity of this model for local instability and unfolding was further illustrated by Vriend *et al.*³⁴ who showed that mutations in the C-terminal part of TLP-ste, which had minimal effects on stability in the wild-type background, had notable effects in extremely stable engineered variants with optimized N-terminal regions. Thus, as the N-terminal ‘weak link’ becomes stabilized, other parts of the protein become relatively vulnerable and their unfolding starts contributing to the overall thermal inactivation process.

The many mutational studies on TLP-ste also showed that binding of Ca3 is merely one of many ways to stabilize the N-terminal region. Using a variety of stabilization strategies, highly stable

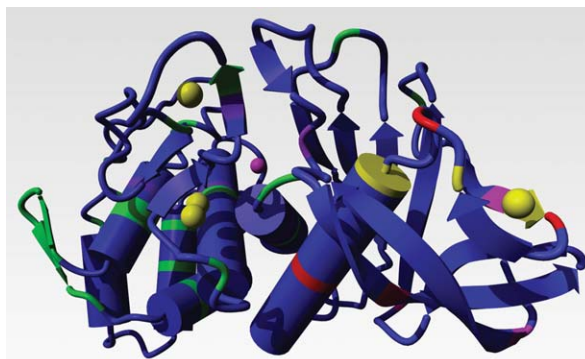


Figure 8. TLP-ste shown as a ribbon, coloured by mutational effect of stabilizing mutations. The relation between colour and mutational effect is: blue = not measured; green = marginal effect; purple = ΔT_{50} up to 2°; magenta = $\Delta T_{50} \sim 3^\circ$; red = $\Delta T_{50} \sim 4^\circ$; yellow = $\Delta T_{50} > 5^\circ$. Calciums shown as yellow balls; zinc as a magenta ball.

variants of TLP-ste could be engineered.^{14,56} One particularly successful example was the introduction of a designed disulfide bridge (through mutations G8C & N60C) that covalently linked the N-terminal β -strand to one of the other strands in the N-terminal three-strand β -sheet.^{28,58} Other mutations in the region were also successful for stabilization of TLP-ste (e.g. positions 4, 56, 58, 65, and 69).^{14,57} Several of these stabilizing mutations were discovered when replacing residues in TLP-ste with the corresponding residue in the more stable thermolysin.^{14,55}

In light of the life cycle of TLP-ste, the relative instability of the N-terminal region and the tendency of this region to be (partially) unfolded can be seen to permit a dual biological function: unfolding of this region is not only critical during maturation (Step 3) but also plays a critical role in cannibalistic turnover (Step 5). Residues such as those at positions 4, 56, 58, 63, and 69, whose mutation to the corresponding residue in thermolysin leads to improved TLP-ste stability, may in fact be crucial to ensure appropriate INstability of TLP-ste. Turnover requires that a certain degree of N-terminal instability is maintained at the temperatures at which the producing organism thrives. A TLP-ste variant containing the stabilizing mutations A4T, T56A, G58A, T63F, and A69P has a T_{50} of 93.3°C¹⁴ and will not show detectable cannibalistic turnover at e.g. the T_{50} of the wild-type enzyme (73.4°C).

The instability of the N-terminal domain, also in the mature protein, places the role of calcium binding to the Ca3 site in another, rather unusual light. The binding affinity of the Ca3 site and the importance of this site for local (un)folded and (in)stability seem to provide an ideal regulatory tool for TLP-ste. Intra-cellular, there is little if any calcium binding to the Ca3 site and this contributes to the protein remaining unfolded. In the calcium rich environment of the Gram-positive cell wall,⁴⁴

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