Chapter 3

Mutational analysis of a critical surface area that is critical for the thermal stability of thermolysin-like proteases.


ABBREVIATIONS.

TLP, thermolysin-like protease; TLPs, thermolysin-like proteases, TLP-ste, TLP of B. stearothermophilus, TLP-cer, TLP of B. cereus; TLP-sub, TLP of B. subtilis; TLP-amy, TLP of B. amyloliquefaciens; TLP-cal, TLP of B. caldolyticus.

$T_{50}$, the temperature that causes a 50% loss of activity in 30 minutes.

$\delta T_{50}$, the difference in $T_{50}$ between wild type TLP-ste and variants thereof.

RMSD, Root mean square deviation.

ENZYMES.

thermolysin (EC 3.4.24.27)

KEYWORDS:

Thermal stability, Thermolysin, Autolysis, Calcium Binding, Unfolding pathway.
Summary

Site-directed mutagenesis was used to assess the contribution of individual residues and a bound calcium in the 55-69 region of the thermolysin-like protease of Bacillus stearothermophilus (TLP-ste) to thermal stability. The importance of the 55-69 region was reflected by the fact that almost all mutations had drastic effects on stability. These effects (both stabilising and destabilising) were obtained by mutations affecting main chain flexibility, as well as by mutations affecting the interaction between the 55-69 region and the rest of the protease molecule. The calcium-dependency of stability could be largely abolished by mutating one of its ligands (Asp57 or Asp59). In the case of the Asp57→Ser mutation, the accompanying loss in stability was modest as compared to the effects of other destabilising mutations or the effects of (combinations of) stabilising mutations. The detailed knowledge of the stability-determining region of TLP-ste permits effective rational design of stabilising mutations, which, presumably, are also useful for related TLPs such as thermolysin. This is demonstrated by the successful design of a stabilising salt bridge involving residues 65 and 11.

Introduction

Bacillus thermolysin-like proteases (TLPs) are metallo-endopeptidases consisting of 300-319 residues. These enzymes contain one zinc ion that is essential for catalysis and they bind a varying number of calcium ions for which it is known that they contribute to stability (Dahlquist et al., 1976; Roche & Voordouw, 1978). The amino acid sequences of many TLPs are known and the crystal structures of thermolysin (the TLP of B. thermoproteolyticus; Matthews et al., 1972; Holmes & Matthews, 1982) and TLP-cer (the TLP of B. cereus; Stark et al., 1992) have been determined. TLPs consist of an N-terminal domain (residues 1-154; thermolysin numbering) characterised by a predominance of β-pleated sheet and a C-terminal domain (residues 155-316) that is mainly α-helical (Holmes & Matthews, 1982; Stark et al., 1992). TLPs differ in thermal stability (Vriend & Eijsink, 1993) and the structural determinants of these differences have been analysed by several site-directed mutagenesis studies (e.g. Van den Burg et al., 1991; Imanaka et al., 1986; Eijsink et al., 1995; Frigerio et al., 1996; Veltman et al., 1996).
At elevated temperatures TLPs are irreversibly inactivated as a result of autolysis. Considering the broad specificity of TLPs (Heinrikson; 1977; Stoeva & Kleinschmid; 1989), conformational features rather than sequence characteristics of the TLP molecule are expected to dictate the sites of proteolytic attack (Fontana, 1988). It has been shown that local unfolding processes that render the protein susceptible towards autolysis are the rate-limiting steps in thermal inactivation (Vriend & Eijsink, 1993; Braxton & Wells, 1992; Kidokoro, 1995). It has been proposed that these thermally induced local unfolding processes mainly involve surface located regions of the protein (Fontana, 1988; Vriend & Eijsink, 1993; Eijsink et al., 1995). Accordingly, it has been shown that the changes in the stability of TLP-ste is most easily effected by mutation of surface-located residues that are clustered in one particular region (residues 55-69) of the protein (Eijsink et al., 1995; Veltman et al., 1996; and see Figures 1 and 2).

**Figure 1.** Ribbon drawing of TLP-ste. The active site zinc (dark sphere) is located at the bottom of the active site cleft, between the β-pleated N-terminal domain (right) and the largely α-helical C-terminal domain (left). The 55-69 area, which binds calcium-3, is shown in black. Bound calcium ions are indicated by the light grey spheres. The picture was obtained using the program Ribbons (Carson, 1987).
Figure 2. Alignment of the 50-75 region from six TLPs from different Bacillus species. For the determination of T<sub>50</sub> values, the various TLPs were purified using the expression systems and the standard purification protocol which are described under Materials and Methods. The listed enzymes are from B. thermoproteolyticus (thermolysin), B. caldolyticus (TLP-cal), B. stearothermophilus (TLP-ste), B. cereus (TLP-cer), <sup>1</sup>B. megaterium (TLP-meg; Kühn & Fortnagel, 1993), B. subtilis (TLP-sub) and B. amyloliquefaciens (TLP-amy). Residues presumably interacting with the calcium ion in binding site 3 (Ca<sub>3</sub>) in the thermally stable variants are indicated by grey boxes; see text. <sup>2</sup>The T<sub>50</sub> of TLP-amy is not known; studies by other workers indicate that the stability of TLP-amy is similar to that of TLP-sub (Chumakov et al., 1995).

The difference in stability between thermolysin (T<sub>50</sub> = 86.9 °C; see below for definition of T<sub>50</sub>) and the TLP of B. stearothermophilus CU21 (TLP-ste; T<sub>50</sub> = 73.4 °C) is primarily determined by mutations in the 55-69 region, in particular at positions 63 and 69. Upon replacing Thr63 and Ala69 in TLP-ste by the amino acids present at the corresponding positions in thermolysin (Phe and Pro, respectively), large increases in thermal stability were obtained (Van den Burg et al., 1991; Hardy et al., 1993). All other ‘TLP-ste to thermolysin’ mutations with large effects on stability were located in the direct environment of residues 63 and 69 (Veltman et al., 1996). The combination of multiple mutations in this region has yielded extremely stable TLP-ste variants (Eijsink et al., 1995).

Here we describe structural details of the 55-69 region, using the crystal structures of thermolysin and TLP-cer (73 % sequence identity with thermolysin), as well as a model of TLP-ste (86 % sequence identity with thermolysin). We give a short summary of previously published mutations in the 55-69 region of TLP-ste and we describe a series of new mutations aimed at assessing the contribution of individual residues to stability in detail. Mutations were designed to investigate (1) the intrinsic stability of the 55-69 region, (2) the importance of contacts with other parts of the molecule, and (3) the role of the calcium ion that is coordinated by Asp57 and Asp59. The results show how residues in the 55-69 region can contribute to stability by different mechanisms and they permit assessment of the importance of calcium-binding.
Mutational analysis of a critical surface area

Materials and Methods

Genetics

The gene encoding TLP-ste was originally cloned and sequenced by Fujii et al. (1983) and Takagi et al. (1985) and sub-cloned as described previously (Eijsink et al., 1992d). Plasmid pCO3, a Bacillus vector containing the gene of *Bacillus cereus* encoding TLP-cer, was a gift of Dr. R. Roche, Calgary, Canada (Wetmore et al., 1992). The gene encoding the TLP of *Bacillus subtilis* (TLP-sub; Yang et al., 1984) was a gift of Gist-Brocades N.V., Delft, The Netherlands, and subcloned as described elsewhere (Eijsink et al., 1992a). Cloning, sequencing and expression of the gene encoding TLP of *Bacillus caldolyticus* (TLP-cal) have been described by Van den Burg et al. (1991). All genetic procedures, including site-directed mutagenesis were performed as described earlier (Eijsink et al., 1992d).

Production, purification and characterisation of TLP-ste

All procedures used for production, purification and SDS-PAGE analysis of wild type TLPs and mutants thereof have been described previously (Eijsink et al., 1992d). Partially pure thermolysin was obtained from Boehringer Mannheim, Germany and purified further using the standard protocol. Thermal stability was determined by incubating aliquots of diluted purified enzyme (0.1 μM in 20 mM sodium acetate, pH 5.3, 5 mM CaCl₂, 0.5 % (v/v) isopropanol, 62.5 mM NaCl and 0.01 % Triton-X100) at various temperatures during 30 minutes. Subsequently, residual protease activity was determined using casein as a substrate (Fujii et al., 1983) and expressed as percentage of the initial activity. Thermal stability was defined by T₅₀, being the temperature at which 50 % activity was preserved after 30 minutes of incubation. Triton X-100 was included to prevent unspecific binding of the protease to the surface of the reaction vessels. Wild-type TLP-ste was included in every assay and the stabilities of mutant enzymes were expressed as δT₅₀, being the difference in T₅₀ between the mutant and the wild-type enzyme. The δT₅₀ values presented are average values derived from at least three independent assays. Errors in the δT₅₀ values were less than 10 % of the value in all cases, with a maximum of 0.6 °C.

Structural analysis

For molecular modelling and database searches, the program WHAT IF was used (Vriend, 1990). The modelling procedures have been described in detail elsewhere (Vriend & Eijsink, 1993). A three-dimensional model of TLP-ste was built on the basis of the crystal structure of thermolysin (Holmes & Matthews, 1982). Considering the high sequence similarity between these two enzymes (86 %), the model of TLP-ste was expected to be sufficiently reliable to predict and analyse the effects of site-directed mutations (Vriend & Eijsink, 1993; Mosimann et al., 1995). The model is expected to be highly reliable around the 55-69 loop because the similarity is even higher in this region. Comparisons
with the crystal structure of TLP-cer (Stark et al., 1992) strengthened this idea. The latter enzyme differs from thermolysin to a larger extent (73 % sequence identity) than TLP-ste, yet it has the same fold as thermolysin in the 55-69 region (Holmes & Matthews, 1982; Stark et al., 1992). Residues are numbered throughout this paper according to the sequence of thermolysin. Compared to thermolysin, TLP-ste contains three additional residues (Tyr-Tyr-Gly), inserted between residues 26 and 27. These residues were omitted from the TLP-ste model because they could not be modelled satisfactorily. The insertion point is far away from calcium atom 3 (app. 13 Å), and the side chains of Asp57 and Asp59 that coordinate this calcium point away from the insertion site. The distances between the insertion point and most residues mutated and/or discussed in this study are even larger (in the 16 - 23 Å range, for e.g. residues 11, 63, 65, 69). Thus, it was assumed that omitting the insertion from the model would not result in general misdesign of mutations and misinterpretations of mutational effects. As far as residues 56 and 58 are concerned, some caution is in order, however, since the side chains of these residues point into the direction of the insertion site and may have contacts with the inserted amino acids (see below).

The side chains of residues introduced by mutation were modelled by searching the structure database for position-specific rotamers (De Filippis et al., 1994; Chinea et al., 1995). The use of position-specific rotamers was previously shown to be valuable for prediction of local structural changes that result from the introduction of a point mutation (De Filippis, 1994). Side chains were positioned in the most preferred rotamer that was attainable without the introduction of unfavourable Van der Waals overlaps. Subsequently, a short energy minimisation was performed using GROMOS (Van Gunsteren & Berendsen, 1987).

**Results and Discussion**

**Structure of the 55-69 region**

In Figure 1 the position of the 55-69 region and the location of the four calcium binding sites are shown in the three-dimensional structure of TLP-ste. Figure 2 shows an alignment of the 50-75 regions in six TLPs that exhibit large differences in thermal stability. The most stable TLPs (TLP-ste, TLP-cal and thermolysin) have highly conserved sequences and in the 50-75 region only at four positions (all in the 55-69 stretch) differences occur. There are many more differences between the three stable TLPs and the less stable ones, and none of the fifteen residues in the 55-69 stretch is fully conserved. Interestingly, residues whose side chains are thought to contribute to the binding of calcium-3 (residues 55, 57, 59 and possibly 67; see Figures 2, 3 and below for details) vary in a correlated manner. Thermolysin, TLP-cer and supposedly TLP-ste and TLP-meg bind calcium-3 and contain a conserved Trp at position 55.
and three conserved Asp's at positions 57, 59 and 67. At all these four positions the less stable TLPs contain other residues, which are not likely to contribute to calcium-binding (e.g. Val55, Ser57, Thr59 and Gln67 in both TLP-sub and TLP-amy). The 55-69 stretch contains all residues coordinating Ca3 (Figure 3), as well as most of the residues that were previously shown to be important for thermal stability (Eijsink et al., 1995; Veltman et al., 1996).

**Figure 3.** Interactions of the 55-69 area with the calcium ion in the Ca3 site of thermolysin. Hydrogen bonds are depicted by thin dashed lines. Interactions of the ligands with the calcium ion are shown with thick dashed lines. The side chains of residues that are different from TLP-ste are shown by small dots. Water molecules are indicated by crosses. More structural details of thermolysin, including details of the hydrogen bonds, can be obtained from HTTP://swift.EMBL-heidelberg.DE/neutpep/.
Figure 3 shows structural details of the 55-69 region and its direct environment in thermolysin. Trp55 is the first residue with a clear role in the binding of Ca3 that is encountered while moving along the chain in N- to C-terminal direction. Residues 55-61 form a common type of calcium binding loop, incorporating all direct contacts between the protein and the calcium ion in Ca3 (Asp57, Asp59 and Asn61). Residues 57 and 59 both form so-called Asx-turns which are regularly encountered in non-helix-loop-helix calcium binding proteins (McPhalen et al., 1991; polar interactions of the residues in the 55-69 region are described in Table 1). At position 59 the backbone changes into a short stretch of $\beta$-structure comprising residues 60-63. This 60-63 $\beta$-strand is part of a $\beta$-sheet comprising stretches 60-63, 8-12 and 15-20. The latter two strands are part of the N-terminal $\beta$-hairpin comprising residues 8-20. Alanine 64 connects the 60-63 $\beta$-strand to the 65-68 $3_{10}$ helix. This $3_{10}$ helix bends away (by ~ 30 degrees) from the axis of a regular, mainly buried $\alpha$-helix that begins at position 68 and extends to residue 87. Despite various sequence differences, including an Ala (TLP-cer) to Pro (thermolysin) substitution at position 69 (Figure 2), the backbone structures of thermolysin and TLP-cer are virtually identical in the 55-69 region (Holmes & Matthews, 1982; Stark et al., 1992). The backbone RMSD (root mean square deviation) after optimal superposition is about 0.3 Å and the largest atomic displacement is about 0.5 Å. Considering the high sequence identity between TLP-ste and thermolysin, structural differences between these two enzymes in the 55-69 region are expected to be similarly small, as confirmed by molecular modelling (Vriend & Eijsink, 1993).

The role of most residues in the 55-69 region was assessed by examining the effects on thermal stability of a series of site-directed mutations in TLP-ste. The results are listed in Table 2 and are discussed below.

The water molecules in the crystal structure are indicated by #xxx.
See also Holmes & Matthews, 1982. More structural details of thermolysin, including details of the hydrogen bonds, can be obtained from HTTP://swift.embl-heidelberg.de/neutpep/. See opposite page
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Table 1  Electrostatic interactions of residues in the 55 to 69 stretch in thermolysin.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Donor</th>
<th>Acceptor(s)</th>
<th>Acceptor</th>
<th>Donor(s)</th>
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<td>O</td>
<td>Leu30-N</td>
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<td></td>
<td>Ne1</td>
<td>#356</td>
<td></td>
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<td>O</td>
<td>#477</td>
</tr>
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<td>N</td>
<td>Tyr28-O</td>
<td>O</td>
<td>Tyr28-N</td>
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<td></td>
<td></td>
<td>Oδ1</td>
<td></td>
<td>Ca3, #356</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oδ2</td>
<td></td>
<td>Ca3, Asp59-N, Asn60-N, Gln61-N</td>
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<td>#508</td>
<td>O</td>
<td>-</td>
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<td></td>
<td></td>
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<td>#482</td>
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<td>Asp59-Oδ1</td>
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</tr>
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<td>N</td>
<td>Val9-O</td>
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<td>Arg11-N</td>
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<td>N</td>
<td>#484</td>
<td>O</td>
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<td>Asp67-Oδ2</td>
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<td></td>
<td></td>
<td>O-γ</td>
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<td>Ala64-O</td>
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<td></td>
<td>Oδ2</td>
<td></td>
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<td>Ala64-O, Ser65-O</td>
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<td>Asp72-N</td>
</tr>
<tr>
<td>Pro69</td>
<td>-</td>
<td></td>
<td>O</td>
<td>Ala73-N, Ser134-Oγ</td>
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Ligands of calcium-3  Distance (Å)

<p>| | |</p>
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</tr>
<tr>
<td>Asp59-Oδ1</td>
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</tr>
<tr>
<td>Gln61-O</td>
<td>2.30</td>
</tr>
<tr>
<td>#419</td>
<td>2.43</td>
</tr>
<tr>
<td>#482</td>
<td>2.30</td>
</tr>
<tr>
<td>#503</td>
<td>2.38</td>
</tr>
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</table>
Mutations in the binding site of calcium 3.

The Ca3 site was described by McPhalen et al. (1991) as a heptacoordinate calcium binding site with a pentagonal bipyramidal geometry, with Gln61 (via its backbone carbonyl oxygen) and water molecule #503 as apical ligands and Asp57 (bidentate, via its Oδ’s), Asp59 (monodentate, via Oδ1) and two water molecules (#419 and #482) as equatorial ligands (see also Figure 3, Table 1 and Holmes & Matthews, 1982). The B-factors of residues 57 and 59 are among the lowest in the protein, whereas the B-factors of water molecules #419 and #482 (15.6 and 21.5 Å²) are much lower than the average value for all water molecules observed in the thermolysin structure (36.9 Å²). This suggests that the conformation around the calcium site as well as the positions of the water molecules are relatively fixed. The Oδ atoms of Asp57 are largely buried whereas Asp59 interacts with the calcium through the fully solvent accessible Oδ1. Water molecule #419 is held in place by H-bonds with the Oδ2 of Asp67 and two other surface located crystal waters (#484 and #628). Water molecule #482 is coordinated by the Oδ2 of Asp59 and the water molecules #484 and #671. Water #503 is coordinated only by #508. This indicates that water molecules are essential components of the calcium ion binding site. It should be noted that the hydrogen bonds mentioned here are part of a larger network, many of them with ideal geometries (see Table 1 and Figure 3). Thus, residues further away from the calcium ion may be important for its binding through interactions via tightly bound water molecules. Interestingly, at the ‘surface side’ the calcium is coordinated by water only, indicating that only marginal modification of the local hydrogen bonding network is required for the calcium to ‘escape’

Mutation of Asp57 and Asp59 resulted in destabilisation of TLP-ste (Table 2). All three mutations that were made largely abolished the calcium-dependency of thermal stability (Figure 4), indicating that in the wild type this dependency primarily reflects binding of calcium in the Ca3 site. The Asp57→Ser mutation (Ser occurs naturally in TLP-sub; Figure 2) had a less drastic effect on stability than the Asp59→Ala and Asp59→Thr mutations (Thr occurs naturally in TLP-sub; Figure 2). The much larger destabilisation obtained after mutations at position 59 is most likely caused by the fact that the remaining carboxyl group of Asp57 is largely buried, which is highly unfavourable in the absence of calcium. Mutations at position 57 do not have this effect since the carboxyl group of residue 59 is fully solvent-exposed.

Previously, it has been shown that two other mutations in the 55-61 calcium-binding site affect stability of TLP-ste in a positive way, namely Thr56→Ala (δT50 = + 1.9 °C) and Gly58→Ala (δT50 = + 3.9 °C; Veltman et al., 1996). Whereas the stabilising effect of Gly58→Ala can be
attributed to the well established rigidifying effect of this type of mutation (Matthews et al., 1987),
the effect of Thr56→ Ala can less readily be explained. Residue 56 is located in a solvent exposed,
extended (β-strand like) structure, meaning that threonine seems more favourable (Minor & Kim,
1994). Steric clashes with a three amino acid insertion in TLP-ste near position 27 (that was
omitted from the model; see Materials and Methods) might underlie the unexpected stabilising effect
of the Thr56→ Ala mutation. To investigate this further, threonine was also mutated into a
cysteine, which has a similar size but has a much less narrow rotamer preference at position 56. Cys
can escape to a less preferred rotamer more easily than Thr and thus avoid possible clashes. The
introduction of Cys stabilised TLP-ste by 0.8 °C strongly suggesting that, indeed, steric hindrance
makes Thr unfavourable.

The mutations at position 56 and 58 affect the stability of the calcium-binding loop without
apparent direct effects on calcium-binding. Indeed the calcium-dependency of stability was the
same for Thr56→ Ala, Gly58→ Ala and wild-type (and all other mutants described in this study,
with the exception of Asp57→ Ser, Asp59→ Ala and Asp59→ Thr; Figure 4). To further assess
the importance of the mutations at positions 56 and 58 they were combined. Remarkably, over-
additivity was observed and the combined effect was as large as + 7.3 °C (Table 2).

Figure 4. Effect of calcium concentration on T50 for wild-type TLP-ste (▲), the Asp57→ Ser mutant (●) and
the Asp59→ Ala mutant (■). Asp59→ Thr gave results similar to those for Asp59→ Ala (same slope). All
other mutants listed in Table 2 gave lines with slopes similar to that observed for the wild type enzyme.
Mutations in the 63-67 region.

Van den Burg et al. (1994) have shown that the stability of TLP-ste is greatly influenced by the extent of hydrophobic interactions between residue 63 and the side chains of Val9, Arg11, Gln17 and Gln61 (all part of the 60-63, 8-12, 15-20 β-sheet). These results suggest the importance of interactions between the environment of residue 63 and the underlying β-sheets. In an attempt to optimise these interactions we focused on mutations of the solvent-exposed Ser65. The stabilisation obtained after the rigidifying Ser65→Pro mutation (δT<sub>50</sub> = + 4.7 °C) had already shown the feasibility of stabilising TLP-ste by mutating this residue (Hardy et al., 1993). Here we examined the possibility to stabilise TLP-ste by engineering a salt bridge between residue 65 and Arg11 in the β-sheet. Rotamer searches indicated that the most favourable side chain conformation for an Asp at position 65 would position the carboxyl group in the direction of the side chain of Arg11. The exact position of the Arg11 side chain was less easy to predict, but in many sterically allowed rotamers its two terminal nitrogen atoms were close to the Asp65.

Table 2 Effects of site-directed mutations in the 55-69 region on the thermal stability of the thermolysin-like protease of B. stearothermophilus.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>δT&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Mutation</th>
<th>δT&lt;sub&gt;50&lt;/sub&gt;</th>
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<td>Thr56Cys</td>
<td>+ 0.8</td>
<td>Asp67Asn</td>
<td>- 23</td>
</tr>
<tr>
<td>Thr56Ala&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+ 1.9</td>
<td>Asp67Ala</td>
<td>- 5.7</td>
</tr>
<tr>
<td>Asp57Ser&lt;sup&gt;a&lt;/sup&gt;</td>
<td>- 7.0</td>
<td>Ala68Asn</td>
<td>- 9.2</td>
</tr>
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<td>Gly58Ala&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+ 3.9</td>
<td>Ala69Asn</td>
<td>- 16</td>
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<tr>
<td>Asp59Ala</td>
<td>- 23</td>
<td>Ala69Gly</td>
<td>- 6.2</td>
</tr>
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<td>- 19</td>
<td>Ala69Ser</td>
<td>- 3.4</td>
</tr>
<tr>
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</tr>
<tr>
<td>Ser65Asn</td>
<td>- 1.4</td>
<td>T56A-G58A</td>
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<td>Ser65Asp</td>
<td>+ 3.2</td>
<td>T56A-G58A-T63F-A69P&lt;sup&gt;f&lt;/sup&gt;</td>
<td>+ 18.8</td>
</tr>
<tr>
<td>Ser65Pro&lt;sup&gt;e&lt;/sup&gt;</td>
<td>+ 4.7</td>
<td>T56A-G58A-T63F-S65D-A69P</td>
<td>+ 20.2</td>
</tr>
<tr>
<td>Tyr66Pro&lt;sup&gt;e&lt;/sup&gt;</td>
<td>- 18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1<sup>)</sup>Van den Burg et al. (1994) have described twelve different Thr63→Xxx mutations: large hydrophobic residues (Phe, Ile) and the positively charged Lys and Arg were found to have the largest stabilising effects. Some mutations have been described earlier: aVan den Burg et al., 1991; bVeltman et al., 1997a; cImanaka et al., 1986; dVan den Burg et al., 1994; eHardy et al., 1993; fVeltman et al., 1996; gEijsink et al., 1995.
side chain (the shortest $\text{N} \eta - \text{O} \delta$ varied from 3.0 to 8.5 Å, with an average of 5.3 Å). As expected on the basis of these modelling studies, the Ser65$\rightarrow$Asp mutation indeed stabilised the enzyme considerably ($\delta T_{m0} = +3.2 ^\circ \text{C}$).

The side chain of residue 65 is highly exposed to solvent and thus an alternative explanation for the beneficial effect of introducing Asp could simply be an increase in surface hydrophilicity (Pakula & Sauer, 1990). As a control, Ser65 was replaced by Asn, a residue which is similar to Asp in terms of hydrophilicity, but which does not have the charges needed for the salt bridge. The Ser65$\rightarrow$Asn mutation destabilised the enzyme by 1.4 ^\circ \text{C}, indicating that the stabilisation observed for the Ser65$\rightarrow$Asp mutation was indeed a result of the introduction of a negative charge. Database searches for Ser, Asn and Asp residues with the same local backbone conformation as residue 65 in TLP-ste gave many hits for Ser, an intermediate number for Asp, and only very few for Asn. This indicates that Asn is relatively unfavourable (see Vriend, 1990 and Bower et al., 1997 for details) and may explain why the Ser65$\rightarrow$Asn mutation had a destabilising effect.

The stabilising effect of an engineered salt bridge at the protein surface is in apparent contrast with the general notion that such salt bridges have marginal effects on stability (Matthews, 1993; Fersht & Serrano, 1993). This apparent contrast can be explained by the fact that the aliphatic part of the Arg11 side chain is conformationally highly restricted by interactions with other residues. Thus, in the case of the Asp65-Arg11 interaction, the entropic loss associated with salt bridge formation between fully solvent-exposed residues (e.g. Dao-Pin et al., 1991; Sali et al., 1991) is partly absent. Interestingly, the Ser65$\rightarrow$Asp mutation could be used for further stabilisation of extremely stable mutants of TLP-ste that contain various mutations in the 55-69 area (Table 2).

Tyr66 has many important interactions with its surroundings (Figure 5). The backbone carbonyl of residue 66 forms a H-bond with the $\text{N} \epsilon$ of the conserved His105. In addition, the Tyr66 side chain stacks nicely with the conserved His105 side chain and its hydroxyl group forms an H-bond with the $\text{O} \delta 1$ of the conserved Asp43, thereby firmly linking the 65-68 $3_{10}$ helix to other parts of the molecule. Finally, the side chain of Tyr66 shields part of the side chains of Trp55 (involved in calcium binding; Figure 3) from the solvent. Thus, Tyr66 seems heavily involved in maintaining the structural integrity of the region and in connecting the 63-67 stretch to the rest of the molecule. The only mutation that has been tested at this position is replacement by a rigidifying proline, which turned out to be highly deleterious for stability ($\delta T_{m0} = -18 ^\circ \text{C}$). In principle, introducing the rigidifying proline residue (Matthews et al., 1987; Hardy et al., 1993) would have been stabilising since it fits perfectly well in the backbone conformation at position 66, and since the amide proton removed by the mutation is solvent-
exposed and not involved in any intra-protein hydrogen bonds in the wild-type enzyme. The fact that a drastic destabilisation was nevertheless obtained indicates that the above-mentioned beneficial interactions of the side chain of Tyr66 are of crucial importance for TLP-ste stability.

The side chain carboxyl group of Asp67 is involved in the large network of hydrogen bonds that also involves Ca3 (Figure 3). The Oδ2 of Asp67 contributes to the intrinsic stability of the 63-67 stretch by hydrogen bonding to the backbone amide of Ala64. Asp67 interacts via two tightly bound crystal waters (#356 and #419) with Ca3 (Figure 3). It would appear that Asp is the ideal residue at position 67 and, indeed, replacement of Asp by Ala or Asn (which is likely to adopt the same rotamer as Asp at this position) had large negative effects on thermal stability of TLP-ste (δT_{50} = -5.6 °C and -23 °C, respectively).

The large

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**Figure 5.** Contacts made by Tyr66 in thermolysin. Hydrogen bonds are indicated by dashed lines; see text for details.
Mutational analysis of a critical surface area
difference between Ala and Asn cannot readily be explained without the availability of crystal
structures of the mutant enzymes. One could speculate that the small alanine side chain allows
a beneficial arrangement of water molecules, leaving intact most of the interactions that were
present in the wild-type enzyme. Such a rearrangement of water molecules can not be
envisaged after replacing Asp by an equally big Asn residue, which would replace one of the
strong H bonds accepting $\delta$ oxygens by a strongly donating NH$_2$ group.

Mutations in the N-terminal turn of the 68 to 87 $\alpha$-helix.

Ala68 and Ala69 are positioned in the N-terminal turn of the 68-87 $\alpha$-helix. This helix end is
somewhat irregular since it is directly preceded by a short stretch of 3$_{10}$ helix comprising residues
65-68. Previously it had been shown that replacing the partly solvent-exposed Ala69 by Pro
drastically stabilised TLP-ste (Table 2; Hardy et al., 1993). No attempts were made to introduce
proline at position 68, because this mutation would result in severe Van der Waals clashes with the
backbone of residue 65. In order to verify whether the effect of the Ala69$\rightarrow$Pro mutation was due
to a beneficial effect on helix stability (e.g. Richardson & Richardson, 1988) or to the more general
effect of main chain rigidification (Matthews et al., 1987) the Ala69$\rightarrow$Gly mutation was made.
Despite the fact that glycine residues in solvent exposed N-terminal caps of $\alpha$-helices can be more
favourable for helix stability than alanine (or at least are not destabilising (Serrano et al., 1992a,b;
Bell et al., 1992; Harpaz et al., 1994), the Ala69$\rightarrow$Gly mutation destabilised TLP-ste by 6.1 °C.
Thus, effects on main chain flexibility seem to predominate the effects of the mutations at position
69.

Molecular modelling studies did not give unambiguous indications for other mutations at
positions 68 and 69 that could be beneficial for stability. Nevertheless, three mutations were made
that could potentially improve hydrogen bonding, but at the expense of introducing a little (Ala69$\rightarrow$
Ser) or considerable (Ala68$\rightarrow$ Asn, Ala69$\rightarrow$ Asn) strain. All three mutations destabilised the
enzyme by amounts (Table 2) that correlated reasonably well with the amount of Van der Waals
clashes (not shown).

Concluding remarks.
The present data show how various amino acids and the binding of a calcium-ion in the 55-69
region in TLP-ste contribute to stability. In general, the effects of mutations in this region were
large, indicating its importance for stability. In some cases, mutational effects could be attributed to
local effects on main chain flexibility (e.g. Gly58$\rightarrow$ Ala, Ser65$\rightarrow$ Pro, Ala69$\rightarrow$ Pro, Ala69$\rightarrow$ Gly),
whereas in other cases effects on tertiary interactions (especially those between the 55-69 region and the 8-12, 15-20, 60-63 β-sheet) were clearly predominant (e.g. Thr63 → Phe, Ser65 → Asp, Tyr66 → Pro). Large destabilisations were obtained for all replacements for which negative side effects were predicted, suggested that several residues in the 55-69 region of TLP-ste are optimised for stability (e.g. Asp67 and Ala68). The stabilising effect of the Thr56 → Ala, Gly58 → Ala, Thr63 → Phe and Ala69 → Pro mutations in TLP-ste shows that in thermolysin the 55-69 region is further optimised. Still, improvement of TLP-ste by designed mutations in the 55-69 region appeared possible, as exemplified by the results of mutating Ser65. Another example is a highly stabilising disulphide bridge that recently has been introduced in TLP-ste by replacing the conserved Gly8 and Asn60 by cysteines (Mansfeld et al., 1997).

It is important to note that the accumulating detailed knowledge of the roles of various residues in the stability determining-region in TLP-ste now permits effective stabilisation by site-directed mutagenesis. The clear stabilising effects of the designed mutations at position 65 or the introduction of the disulphide bridge (Mansfeld et al., 1997) contrast strongly with the generally modest effects which were obtained upon introducing rationally designed stabilising mutations at many other positions in TLP-ste (e.g. Veltman et al., 1996) or other aspecific proteases such as subtilisin (e.g. Erwin et al., 1990; Mitchinson & Wells, 1989). Identification of one single region that determines the stability of TLP-ste has been the key to the present successful stabilisation experiments. Detection of such stability-determining regions may be of importance in stabilising any protein against irreversible denaturation, since in the latter process, as in TLP-ste autolysis, partial unfolding may very well be the rate-limiting step (e.g., Mitraki & King, 1989; Chrunyk & Wetzel, 1993; Pepys et al., 1993). Together with previous comparative studies (e.g. Veltman et al., 1996; Serrano et al., 1993) the present study also shows that destabilising and stabilising mutations do not obey ‘traffic rules’ for stability (e.g. Argos et al., 1979; Menéndez-Ariaz & Argos, 1989). A specific analysis of the entire environment seems needed for each mutation. The above-mentioned de novo designed stabilising mutations in the 55-69 region in TLP-ste concern conserved residues. Interestingly, these latter mutations may therefore also be applicable for stabilisation of the industrially important thermolysin (Eijsink & Venema, 1994).

With the exception of mutations at positions 57 and 59, the mutations described in this study did not affect the calcium-dependency of stability. Thus, the observed mutational effects do not reflect modulations of calcium affinity in the Ca-3 site. This, and the fact that the calcium-dependency of stability can be largely abolished by a mutation with a clear but not dramatic destabilising effect (Asp57 → Ser), indicate that calcium-binding is just one of many ways to stabilise the 55-69 region. This is illustrated by the recently described observation that the
destabilising effect of the Asp57→Ser mutation can easily be compensated for by, for example, the combined Thr63→Phe and Ala69→Pro mutation, yielding a stable TLP-ste variant whose stability is largely independent on calcium in the mM range (Veltman et al., 1997a). Finally, the notion that the contribution of calcium-ion binding in the Ca3 site to stability is not predominant is supported by the observation that within the group of TLPs with binding sites for all four calcium ions (thermolysin, TLP-cal, TLP-ste, TLP-cer) large differences in stability occur (Figure 2).

In previous studies on thermal denaturation of thermolysin, it has been suggested that the early steps in the thermally induced (autolytic) denaturation process are accompanied by the release of a calcium ion from one of the single calcium binding sites (Ca3 or Ca4) in the protease molecule (Dahlquist et al., 1976; Roche & Voordouw, 1978). Furthermore, experimental evidence has been presented indicating that the N-terminal domain of thermolysin is less stable than the C-terminal domain (Vita et al., 1989) and that unfolding of thermolysin starts in the former domain (Corbett et al., 1986). The present results are in perfect agreement with these observations, the first calcium to be released being the one bound in the Ca3 site, which, however, as the above results also indicate, is not predominant in determining stability. Further studies of the contribution of calcium ion binding to stability, as well as attempts to use the information gained in the present study to design TLP-variants with characteristics resembling those of enzymes isolated from hyperthermophilic archea (Daniel et al., 1996a) are currently in progress in our laboratory.

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References
References are listed in chapter 9.