Engineering specificity and activity of thermolysin-like proteases from Bacillus

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Probing catalytic hinge-bending motions in Thermolysin-Like Proteases by Gly → Ala mutations.

Abstract

The active site of thermolysin-like proteases (TLPs) is located at the bottom of a cleft between the N-terminal and C-terminal domain. Crystallographic studies have shown that the active-site cleft is more closed in ligand-binding TLPs than in ligand-free TLPs. Accordingly, it has been proposed that TLPs undergo a hinge-bending motion during catalysis resulting in "closure" and "opening" of the active-site cleft. Two hinge regions have been proposed. One is located around a conserved glycine 78; the second involves residues 135 and 136. The importance of conserved glycine residues in these hinge regions was studied experimentally by analyzing the effects of Gly→Ala mutations on catalytic activity. Eight such mutations were made in the TLP of *Bacillus stearothermophilus* (TLP-ste) and their effects on activity towards casein and various peptide substrates were determined. Only the Gly78Ala, Gly136Ala, and Gly135Ala + Gly136Ala mutants decreased catalytic activity significantly. These mutants displayed a reduction in k_{cat}/K_m for 3-(2-furylacryloyl)-L-glycyl-L-leucine amide of 73%, 62%, and 96% respectively. Comparisons of effects on k_{cat}/K_m for various substrates with effects on the K_i for phosphoramidon, suggested that the mutation at position 78 primarily had an effect on substrate binding, whereas the mutations at positions 135 and 136 primarily influence k_{cat}. The apparent importance of conserved glycine residues in proposed hinge-bending regions for TLP activity supports the idea that hinge-bending is an essential part of catalysis.

Introduction

Thermolysin-like proteases (TLPs) are a group of homologous metalloendopeptidases from *Bacillus* with similar enzymatic characteristics. The amino acid sequences of several TLPs have been determined [see (122) or the Merops data base (http://www.merops.co .uk/merops/famcards/m4.htm)] and the three-dimensional structures of thermolysin of *Bacillus thermoproteolyticus* (52), the TLP from *Bacillus cereus* (53), aureolysin from *Staphylococcus aureus* (56) and elastase from *Pseudomonas aeruginos*a (55) have been solved by X-ray crystallography. All TLPs have a similar fold, consisting of an α-helical C-terminal domain and an N-terminal domain that consists mainly of β-strands. The domains are connected by a central α-helix (residues 137-150). This helix is located at the bottom of the active-site cleft and contains several of the catalytically important residues [(93); Fig. 3.1].

Holland *et al.* (86) superposed the crystal structures of thermolysin, TLP-cer and elastase [the homologous TLP from *Pseudomonas aeruginos*a; (55)]. They observed a hinge-bending displacement between the N- and C-terminal domains. The hinge-bending angle between the two domains was larger (meaning a more open active-site cleft) in TLP-cer than in thermolysin. A comparison of the 3D structures of elastase crystallized with and without inhibitors bound to the active-site (55, 86), revealed that the structure of this remote TLP family member was more closed when a ligand is bound. Further refinement of the thermolysin electron density map revealed that the active-site contained a dipeptide (valine-

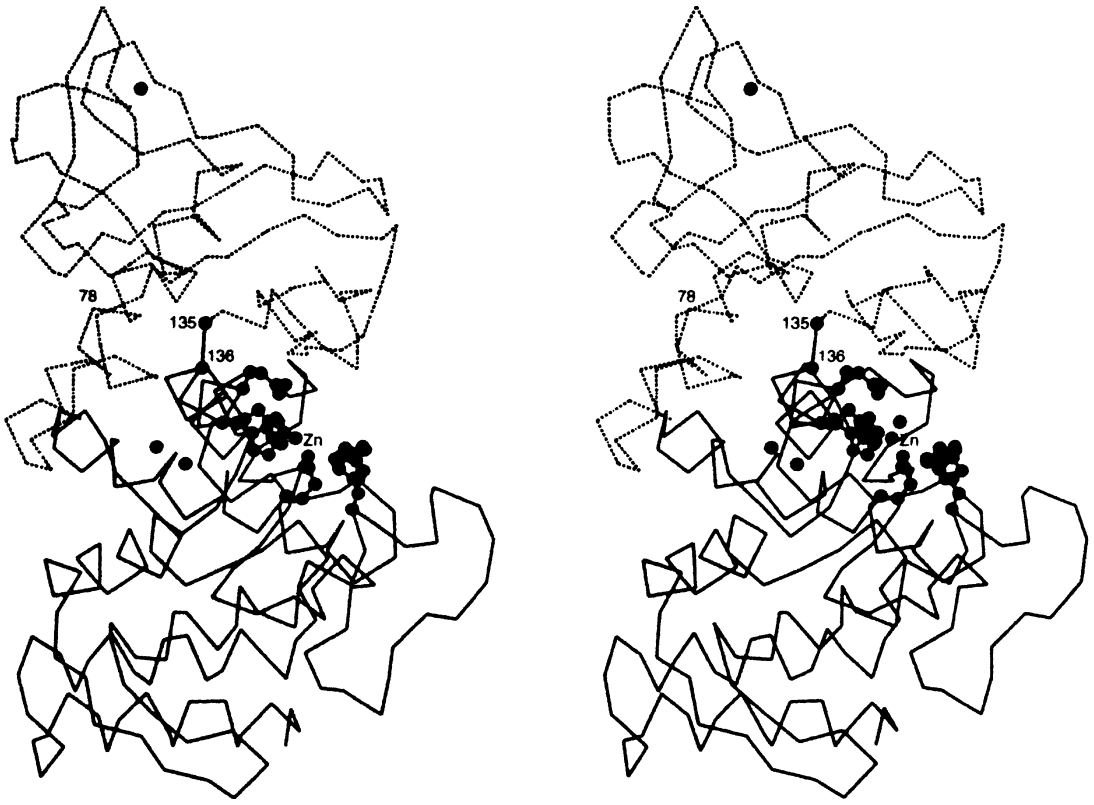
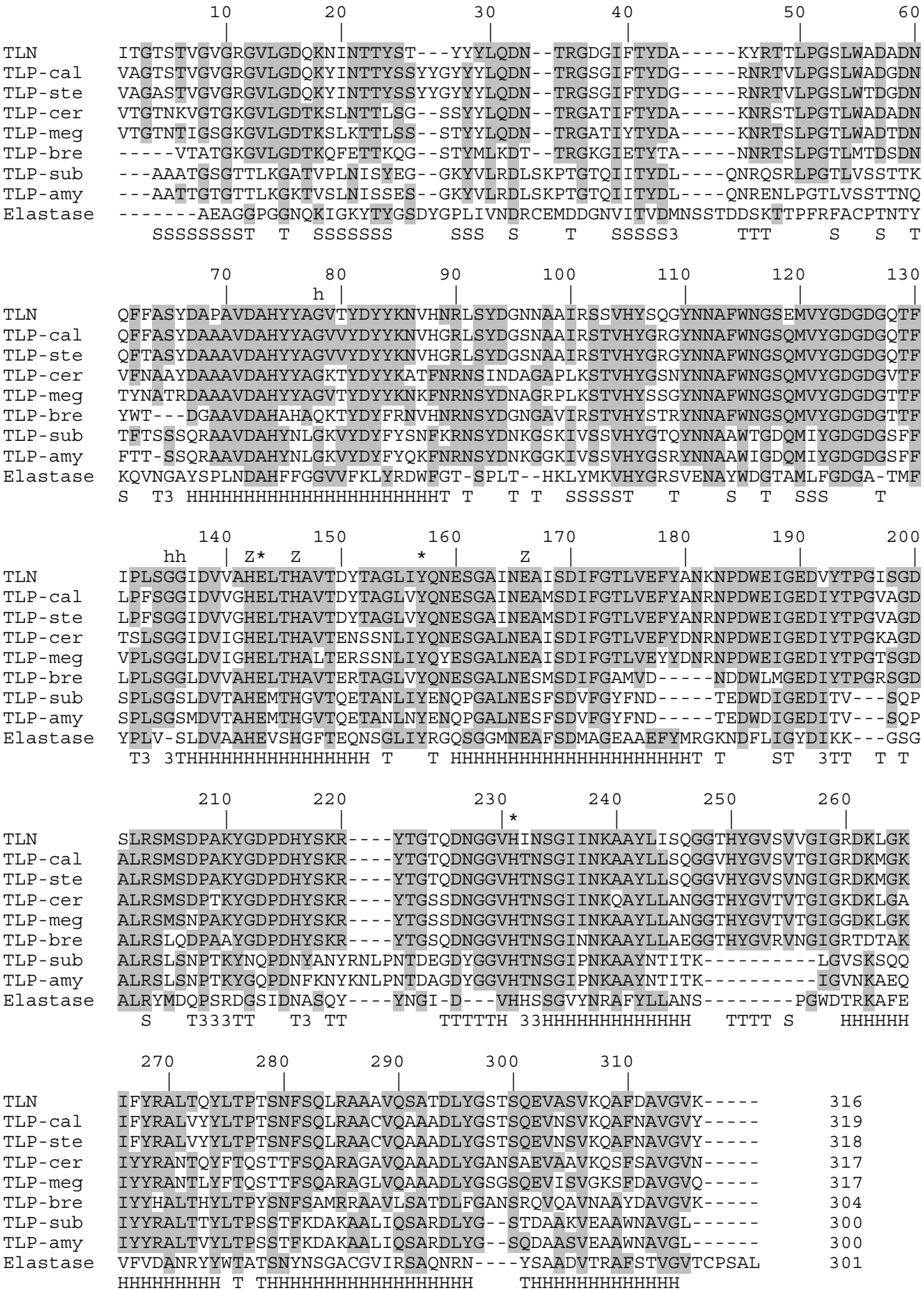


Figure 3.1. Stereo C-α trace of thermolysin. Residues 1-135 are shown with dotted lines, and the α-carbons of Gly135 and Gly136 are marked with small spheres. The active site residues that are involved in zinc binding (His142, His146, and Glu166) and catalysis (Glu143, Tyr157, and His231) are added in ball-and-stick representation. The five single spheres are the zinc and the four calciums; the zinc is labeled. The structure given is that of a closed TLP. The axis describing the hinge-bending displacement is observed by comparing TLP structures in the vicinity of residues 78, 135, and 136 (see (86) for details). Opening of the active-site cleft is associated with a change in hinge-bending angle of approximately 6° (86), as well as a small change in the dihedral angles of Gly135 and Gly136 (54).

lysine), explaining why thermolysin appeared to be more closed than TLP-cer (86, 150).

Stark *et al.* (54) also noticed the above-mentioned difference between TLP-cer and thermolysin. These authors proposed the hinge region to reside near two (rather conserved) glycine residues at positions 135 and 136 (thermolysin numbering; Fig. 3.1 and 3.2). On the basis of inhibitor studies, Thayer *et al*., (55) proposed the presence of a hinge in the corresponding location in elastase. A similar conclusion was reached by Van Aalten *et al.* (89), who studied the dynamics of thermolysin by essential dynamics analyses of molecular dynamics simulations (151). On the basis of detailed crystallographic studies of various TLPs, Holland *et al.* (86, 150) suggested that also the conserved glycine 78 could be important for hinge-bending (Fig. 3.1 and 3.2). Gly78 is located in the middle of the long

Figure 3.2. Multiple alignment of the mature part of thermolysin-like proteases. The enzymes listed are from *B. thermoproteolyticus* (TLN, thermolysin; (35)), *B. caldolyticus* (TLP-cal; (199)), *B. stearothermophilus* CU21 (TLP-ste; (137)), *B. cereus* (TLP-cer; (125)), *B. megaterium* (TLP-meg; (200)), *B. brevis* (TLP-bre; (201)), *B. subtilis* (TLP-sub, (30)), *B. amyloliquefaciens* (TLP-amy; (202)), and elastase from *P. aeruginosa* ((203)). h indicates the glycine residues in the proposed hinge regions. * indicates residues involved in catalysis, Z indicates residues which coordinate the Zn^{2+}. are indicated by light grey boxes. Black boxes indicate residues involved in catalysis. The secondary structure assignment given is that for thermolysin (S, β-strand; T, turn; H, helix; 3, 3_{10} helix).


10 20 30 40 50 60
TLN ITGTSTVGVGRGVLGDQKNINTTYST---YYYLQDN--TRGDGIFTYDA-----KYRTTLPGSLWADADN
TLP-cal VAGTSTVGVGRGVLGDQKYINTTYSSYYGYYYLQDN--TRGSGIFTYDG-----RNRTVLPGSLWADGDN
TLP-ste VAGASTVGVGRGVLGDQKYINTTYSSYYGYYYLQDN--TRGSGIFTYDG-----RNRTVLPGSLWTDGDN
TLP-cer VTGTNKVGTGKGVLGDTKSLNTTLSG--SSYYLQDN--TRGATIFTYDA-----KNRSTLPGTLWADADN
TLP-meg VTGTNTIGSGKGVLGDTKSLKTTLSS--STYYLQDN--TRGATIYTYDA-----KNRTSLPGTLWADTDN
TLP-bre -----VTATGKGVLGDTKQFETTKQG--STYMLKDT--TRGKGIETYTA-----NNRTSLPGTLMTDSDN
TLP-sub ---AAATGSGTTLKGATVPLNISYEG--GKYVLRDLSKPTGTQIITYDL----QNRQSRLPGTLVSSTTK
TLP-amy ---AATTGTGTTLKGKTVSLNISSES--GKYVLRDLSKPTGTQIITYDL----QNRENLPGTLVSSTTNQ
Elastase -------AEAGGPGGNQKIGKYTYGSDYGPLIVNDRCEMDDGNVITVDMNSSTDDSKTTPFRFACPTNTY
SSSSSSSST T SSSSSSS SSS S T SSSSS3 TTT S S T
70 80 90 100 110 120 130
h
TLN QFFASYDAPAVDAHYYAGVTYDYYKNVHNRLSYDGNNAAIRSSVHYSQGYNNAFWNGSEMVYGDGDGQTF
TLP-cal QFFASYDAAAVDAHYYAGVVYDYYKNVHGRLSYDGSNAAIRSTVHYGRGYNNAFWNGSQMVYGDGDGQTF
TLP-ste QFTASYDAAAVDAHYYAGVVYDYYKNVHGRLSYDGSNAAIRSTVHYGRGYNNAFWNGSQMVYGDGDGQTF
TLP-cer VFNAAYDAAAVDAHYYAGKTYDYYKATFNRNSINDAGAPLKSTVHYGSNYNNAFWNGSQMVYGDGDGVTF
TLP-meg TYNATRDAAAVDAHYYAGVTYDYYKNKFNRNSYDNAGRPLKSTVHYSSGYNNAFWNGSQMVYGDGDGTTF
TLP-bre YWT---DGAAVDAHAHAQKTYDYFRNVHNRNSYDGNGAVIRSTVHYSTRYNNAFWNGSQMVYGDGDGTTF
TLP-sub TFTSSSQRAAVDAHYNLGKVYDYFYSNFKRNSYDNKGSKIVSSVHYGTQYNNAAWTGDQMIYGDGDGSFF
TLP-amy FTT-SSQRAAVDAHYNLGKVYDYFYQKFNRNSYDNKGGKIVSSVHYGSRYNNAAWIGDQMIYGDGDGSFF
Elastase KQVNGAYSPLNDAHFFGGVVFKLYRDWFGT-SPLT--HKLYMKVHYGRSVENAYWDGTAMLFGDGA-TMF
S T3 HHHHHHHHHHHHHHHHHHHHHT T T T SSSSST T S T SSS T
140 150 160 170 180 190 200
hh Z* Z * Z
TLN IPLSGGIDVVAHELTHAVTDYTAGLIYQNESGAINEAISDIFGTLVEFYANKNPDWEIGEDVYTPGISGD
TLP-cal LPFSGGIDVVGHELTHAVTDYTAGLVYQNESGAINEAMSDIFGTLVEFYANRNPDWEIGEDIYTPGVAGD
TLP-ste LPFSGGIDVVGHELTHAVTDYTAGLVYQNESGAINEAMSDIFGTLVEFYANRNPDWEIGEDIYTPGVAGD
TLP-cer TSLSGGIDVIGHELTHAVTENSSNLIYQNESGALNEAISDIFGTLVEFYDNRNPDWEIGEDIYTPGKAGD
TLP-meg VPLSGGLDVIGHELTHALTERSSNLIYQYESGALNEAISDIFGTLVEYYDNRNPDWEIGEDIYTPGTSGD
TLP-bre LPLSGGLDVVAHELTHAVTERTAGLVYQNESGALNESMSDIFGAMVD-----NDDWLMGEDIYTPGRSGD
TLP-sub SPLSGSLDVTAHEMTHGVTQETANLIYENQPGALNESFSDVFGYFND-----TEDWDIGEDITV---SQP
TLP-amy SPLSGSMDVTAHEMTHGVTQETANLNYENQPGALNESFSDVFGYFND-----TEDWDIGEDITV---SQP
Elastase YPLV-SLDVAAHEVSHGFTEQNSGLIYRGQSGGMNEAFSDMAGEAAEFYMRGKNDFLIGYDIKK---GSG
T3 3THHHHHHHHHHHHHHHH T T HHHHHHHHHHHHHHHHHHHHT T ST 3TT T T
210 220 230 240 250 260
*
TLN SLRSMSDPAKYGDPDHYSKR----YTGTQDNGGVHINSGIINKAAYLISQGGTHYGVSVVGIGRDKLGK
TLP-cal ALRSMSDPAKYGDPDHYSKR----YTGTQDNGGVHTNSGIINKAAYLLSQGGVHYGVSVTGIGRDKMGK
TLP-ste ALRSMSDPAKYGDPDHYSKR----YTGTQDNGGVHTNSGIINKAAYLLSQGGVHYGVSVNGIGRDKMGK
TLP-cer ALRSMSDPTKYGDPDHYSKR----YTGSSDNGGVHTNSGIINKQAYLLANGGTHYGVTVTGIGKDKLGA
TLP-meg ALRSMSNPAKYGDPDHYSKR----YTGSSDNGGVHTNSGIINKAAYLLANGGTHYGVTVTGIGGDKLGK
TLP-bre ALRSLQDPAAYGDPDHYSKR----YTGSQDNGGVHTNSGINNKAAYLLAEGGTHYGVRVNGIGRTDTAK
TLP-sub ALRSLSNPTKYNQPDNYANYRNLPNTDEGDYGGVHTNSGIPNKAAYNTITK----------LGVSKSQQ
TLP-amy ALRSLSNPTKYGQPDNFKNYKNLPNTDAGDYGGVHTNSGIPNKAAYNTITK----------IGVNKAEQ
Elastase ALRYMDQPSRDGSIDNASQY----YNGI-D---VHHSSGVYNRAFYLLANS--------PGWDTRKAFE
S T333TT T3 TT TTTTTH 33HHHHHHHHHHHH TTTT S HHHHHH
270 280 290 300 310
TLN IFYRALTQYLTPTSNFSQLRAAAVQSATDLYGSTSQEVASVKQAFDAVGVK----- 316
TLP-cal IFYRALVYYLTPTSNFSQLRAACVQAAADLYGSTSQEVNSVKQAFNAVGVY----- 319
TLP-ste IFYRALVYYLTPTSNFSQLRAACVQAAADLYGSTSQEVNSVKQAFNAVGVY----- 318
TLP-cer IYYRANTQYFTQSTTFSQARAGAVQAAADLYGANSAEVAAVKQSFSAVGVN----- 317
TLP-meg IYYRANTLYFTQSTTFSQARAGLVQAAADLYGSGSQEVISVGKSFDAVGVQ----- 317
TLP-bre IYYHALTHYLTPYSNFSAMRRAAVLSATDLFGANSRQVQAVNAAYDAVGVK----- 304
TLP-sub IYYRALTTYLTPSSTFKDAKAALIQSARDLYG--STDAAKVEAAWNAVGL------ 300
TLP-amy IYYRALTVYLTPSSTFKDAKAALIQSARDLYG--SQDAASVEAAWNAVGL------ 300
Elastase VFVDANRYYWTATSNYNSGACGVIRSAQNRN----YSAADVTRAFSTVGVTCPSAL 301
HHHHHHHHH T THHHHHHHHHHHHHHHHHHH THHHHHHHHHHHHHH

α-helix (residues 68-87) that crosses the entire N-terminal domain. When studying the structural effects of zinc substitutions in the active-site of thermolysin, Holland *et al*. (150), observed global structural changes that resembled the previously observed hinge-bending displacement at glycine 78 (86), which was taken to confirm the importance of bending in the 68-88 helix for the overall hinge-bending motion.

The studies described above suggest that conserved glycine residues provide the flexibility required for a catalytic hinge-bending motion. In the present study we experimentally validated the importance of these glycine residues by studying the effects of Gly → Ala mutations at various positions in the TLP of *B. stearothermophilus* CU21 [TLP-ste (152); 86 % overall sequence identity with thermolysin]. Five other Gly → Ala mutations were analyzed as controls. Modelling studies were performed to ensure that all alanines could be accommodated by TLP-ste without the introduction of atomic clashes or strained backbone torsion angles. The effects of mutations on catalytic activity were evaluated by determining the specific activities towards casein and k_{cat}/K_m values for three furylacryloylated synthetic peptides. Mutational effects on ligand binding were assessed by determining K_i values for the transition-state inhibitor phosphoramidon. The results provide experimental evidence for the hypothesis that conserved glycine residues, in particular Gly78 and the Gly135-Gly136 combination, contribute to catalytically important hinge-bending motions in TLPs.

Materials and Methods

Genetics.

The *npr*T gene encoding the TLP of *B. stearothermophilus* CU21 [TLP-ste, (152)] was cloned, subcloned, and expressed as described previously (80). Site-directed mutagenesis was performed using the pMa/c gapped duplex method as described before (80) or (for G78A and G135A+G136A) by the PCR-based mega-primer method, essentially as described by Sarkar and Sommer (139).

Production and characterization of mutant enzymes.

Production, purification, and subsequent characterization of the enzymes were performed as described earlier (80), with the exception of the G78A mutant, for which a different purification protocol was used (82). For the determination of thermal stability 0.1 μM solutions of purified protease (in 20 mM sodium acetate, pH 5.3, 5 mM $CaCl_2$, 0.01% Triton X-100, 0.5% 2-propanol, and 62.5 mM NaCl) were incubated at various temperatures for 30 min, after which the residual proteolytic activity was determined using casein as a substrate (136). Thermal stability was quantified by T_{50}, being the temperature giving 50% residual protease activity after a 30 min period of incubation. Wild-type TLP-ste (T_{50} = 73.4 °C) was included in every assay and thermal stabilities of mutants are presented by the change in T_{50} compared to wild-type TLP-ste (δT_{50}).

The specific activity of the TLPs on casein was determined according to a method adapted from Fujii *et al.* (136): approximately 0.5 μg of protease was incubated in 1 ml of 50 mM Tris-HCl, pH 7.5, containing 0.8 % (wt/vol) casein and 5 mM $CaCl_2$ at 37 °C for 1 h. The reaction was quenched by adding 1 ml of a solution containing 100 mM TCA, pH 3.5. The specific activity was calculated as the average from at least three independent assays. One unit of activity is defined as the amount of enzyme activity needed to liberate a quantity of acid-soluble peptide corresponding to an increase in A_{275nm} of 0.001 per min.

The catalytic performance (k_{cat}/K_m) for three furylacryloylated peptides at 37 °C was determined essentially as described by Feder (114) using a thermostatted Perkin-Elmer Lambda 11 spectrophotometer. The reaction mixture (1 ml) contained 10 mM MOPS-NaOH, pH 7.0, 5 mM $CaCl_2$, 5 % Me_2SO (v/v), 1 % 2-propanol (v/v), 0.02% Triton-X100 (v/v), 125 mM NaCl, 100 μM substrate, and varying amounts of enzyme. Activities were derived by measuring the decrease in absorption at 345 nm using a Δε of -317 $M^{-1}cm^{-1}$ as described by Feder (114).

The furylacryloylated peptides 3-(2-furylacryloyl)-L-glycyl-L-leucine amide (FaGLa), 3-(2-furylacryloyl)-L-alanyl-L-phenylalanine amide (FaAFa) and 3-(2-furylacryloyl)-L-glycyl-L-leucinyl-L-alanine (FaGLA) were obtained from Bachem Feinchemikalien AG, Bubendorf, Switzerland.

The K_i for phosphoramidon (N-[α-L-rhamnopyranosyl-(oxyhydroxyphosphinyl)]-L-leucyl-L-tryptophan; Boehringer Mannheim, Germany) was determined by a 30 min preincubation of 100 pM protease with varying concentrations of the inhibitor (10^{-8} to 10^{-5}), in a buffer consisting of 50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 5% Me_2SO (v/v), 1% 2-propanol (v/v), and 125 mM NaCl, after which FaAFa (final concentration 100 μM) was added as substrate. K_i s were calculated by the method described by Hunter & Downs (145).

Structural analysis.

A three-dimensional model of TLP-ste was built on the basis of the crystal structure of thermolysin, using the molecular modelling program WHAT IF (140). The modelling procedures have been described in detail elsewhere (78). Because of the 86 % sequence identity between the template and the model, the TLP-ste model was expected to be sufficiently reliable to predict and analyze the effects of site-directed mutations (78, 141). This idea was corroborated by the successful *de novo* design of many stabilizing mutations (*e.g.*, (43, 78, 82)). In this report all TLPs are numbered following the corresponding residues in thermolysin. All glycine residues mutated in this study have Φ, Ψ angles that are favorable for alanine (see http://swift.EMBLHeidelberg.DE/neutpep/ for structural details). The Φ, Ψ angles of Gly89 and Gly109 are in the left-handed α-helix region. The feasibility of mutating these was, however, apparent from previous studies (153) and from the fact that residues other than glycine do occur at these positions in TLPs from other bacilli.

Results

Selection of mutations and production of mutant proteins.

In addition to TLP-ste variants with Gly→Ala mutations at positions 78, 135, and 136 (and the 135/136 double mutant), five other TLP-ste variants, each containing one Gly→Ala mutation were included in this study. Four of these 'control' mutations were in parts of the protein (positions 58, 89, 109, and 264) that are unrelated to catalysis or hinge-bending (Fig. 3.1). The fifth (at position 141) is located next to His142, which is part of the HExxH motif common to all zinc metalloproteases [Fig 3.1 and 3.2; see also (154)]. The two histidines in this motif are ligands of the active-site zinc and the glutamate is directly involved in the catalytic mechanism (92, 155). The C-β of Ala141 points away from the active-site and is highly unlikely to influence the binding of the ligand.

Modelling studies indicated that the mutations at positions 78, 135, and 136 would not affect substrate binding by direct contacts between the introduced alanine C-β and the

Table III.I. Properties of *B.stearothermophilus* thermolysin-like protease (TLP-ste) variants.

	k_{cat}/K_m			FaAFa	FaGLA	K_i Phosphoramidon	specific activity
	FaGLa	FaAFa	FaGLA	FaGLa	FaGLa		for casein
		$s^{-1} \cdot M^{-1} \times 10^{-4}$		ratio k_{cat}/K_m	ratio k_{cat}/K_m	nM $\times 10^{-2}$	U $\times 10^{-3}$
TLP-ste	21 ± 1	43± 3	99± 4	20	48	0.8 ± 0.2	30 ± 5
G58A	14 ± 1.4	27± 3	95± 3	19	68	1.0 ± 0.1	16 ± 3
G89A	29 ± 2	47± 3	110± 12	16	38	0.7 ± 0.2	33 ± 7
G109A	27 ± 8	43± 5	132± 6	16	49	0.7 ± 0.2	35 ± 7
G141A	13 ± 0.6	23± 5	63± 8	18	48	0.9 ± 0.3	35 ± 6
G264A	33 ± 5	56± 4	200± 10	17	60	0.6 ± 0.1	40 ± 7
G78A	5.5 ± 1.1	9± 1	18± 2	16	33	2.7 ± 0.9	1.7 ± 0.3
G135A	23 ± 3	48± 6	115± 12	21	50	0.7 ± 0.2	27 ± 5
G136A	7.9 ± 1.3	8.4± 0.3	45± 2	11	57	0.8 ± 0.3	30 ± 6
G135A+G136A	0.9 ± 0.1	1.1± 0.1	12± 2	12	133	2.2 ± 0.5	17 ± 3

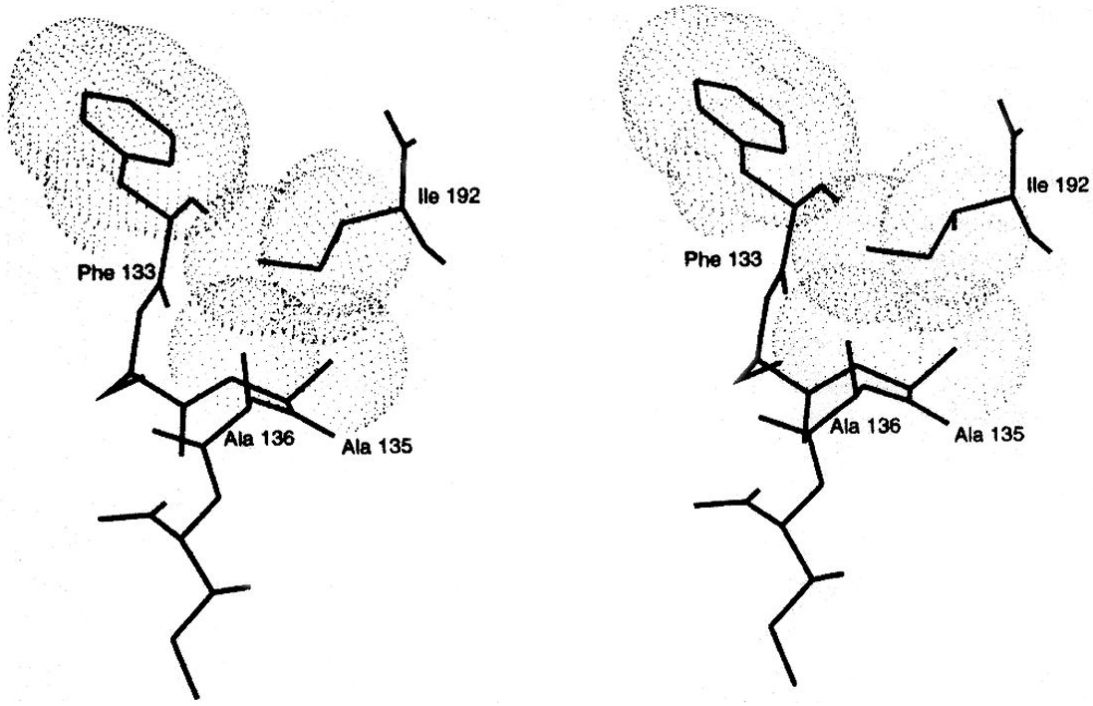


Figure 3.3. **Local environment of in the hinge area of the G135A+G136A mutant of TLP-ste.** There is clearly some van der Waals overlap between the C-β of Gly136Ala and the C-δ of Ile192, which again could affect Ph133 at the bottom of the S_1' subsite.

bound ligand (nor would the other mutations). The model indicated a minor Van der Waals clash between the C-β of Ala136 and the C-δ of Ile192, which interacts with the side chain of Phe133 at the bottom of the S_1' pocket (Fig. 3.3). The G136A mutation could, therefore, result in a small displacement of the Ile192 and Phe133 side chains, which, in turn could disturb binding of substrates with large side chains (*e.g.* phenylalanine) in the P_1' position (Fig. 3.3). However, modelling studies and inspection of the crystal structure of the phosphoramidon-thermolysin complex (52) indicated that the C-β of Ala136 would not affect binding of phosphoramidon. All mutant proteins could be produced in standard amounts, with the exception of the G78A mutant and the G135A+G136A double mutant, that yielded 5–8-fold less protease upon fermentation. With the exception of the G78A mutant, all mutants were purified using the standard protocol based on affinity chromatography with bacitracin-silica (80, 144). The G78A mutant did not bind efficiently to the bacitracin-silica column and a different purification method (82), was therefore employed to purify this mutant.

Effects on activity.

The mutant proteins were characterized by determining specific activities towards casein, k_{cat}/K_m values for various furylacryloylated peptides (114), and K_i values for the transition state inhibitor phosphoramidon (52) (Table III.I). Since the low solubilities of the furylacryloylated peptides preclude accurate determination of K_m values (114, 115, 133), the K_i's phosphoramidon were determined to examine whether the mutations had affected ligand binding.

For the control mutations (G58A, G89A, G109A, G141A, and G264A), both increases and decreases in catalytic activity of, at most, a factor of 2 were observed. These small effects were substrate-dependent, being only detectable

for one or two of the substrates tested (Table III.I). Clearly, larger effects were observed for G78A, G136A, and G135A+G136A. These effects were negative in all cases and they were observed for all peptide substrates tested. Depending on the peptide used, G78A, G136A, and G135A+G136A reduced k_{cat}/K_m 4–6-fold, 2–5-fold, and 8–40-fold, respectively. Remarkably, clear effects on specific activity towards casein were only observed for G78A (Table III.I). The G135A+G136A double mutation reduced activity towards casein only 2-fold, which is a reduction in the same order of magnitude as for one of the control mutations.

The G135A single mutant behaved like wild-type enzyme for all substrates tested. Nevertheless, G135A+G136A was much less active than the mutant containing G136A alone. This suggests that the flexibility provided by G135A is not essential for activity as long as there is a glycine present at position 136.

As predicted from the modelling studies described above, G136A affected substrate specificity to some extent. This is shown by a small reduction in the [k_{cat}/K_m (FaAFa)]/[k_{cat}/K_m (FaGLa)] ratio observed for G136A and G135A+G136A (Table III.I). Wild-type TLP-ste and G136A containing mutants gave almost identical digestion patterns when incubated with β-casein as a substrate, indicating that the effects of G136A on cleavage specificity indeed were small [B. van den Burg and O.R. Veltman, unpublished observations; method described in (84)]. Interestingly, the effects of G136A and G135A+G136A were dependent on the length of the substrate used. This is illustrated by the increase in the [k_{cat}/K_m (FaGLA-OH)]/[k_{cat}/K_m (FaGLa)] ratio in the double mutant. For even longer substrates such as casein, hardly any decrease in activity of the G136A and the G135A+G136A mutant enzymes was detectable. The effects of G136A and G135A+G136A on the K_i for phosphoramidon were small in comparison with the effects on k_{cat}/K_m (Table III.I). Since the present study does not involve mutation of residues directly interacting with ligands/susbstrates, it is reasonable to assume that effects on the K_i to some extent reflect effects on binding affinity in general. The modest effects on K_i thus indicate that the larger effects on k_{cat}/K_m are primarily due to effects on k_{cat} and not to effects on K_m. The fact that the G136A and G135A+G136A mutants behaved as the wild-type enzyme during affinity chromatography supports this conclusion.

The effects of G78A were approximately similar for all peptide substrates as reflected by the grossly unchanged ratios given in Table III.I. Thus this mutation did not affect or hardly affected substrate specificity, nor were the observed reductions in activity substantially dependent on substrate-length. Consistent with the latter observation, the effects of G78A are also reflected in reduced activity towards casein. The increase in K_i for phosphoramidon (which is in the same order of magnitude as the decrease in the various k_{cat}/K_m values; Table III.I) and the strong reduction in binding to bacitracin indicate that a significant part of the reduction in catalytic efficiency caused by G78A reflects an increase in K_m and thus a reduced ability to bind substrate.

Effects on stability.

Based on the results of statistical (156, 157), theoretical (158) and experimental analyses [*e.g.* (159-163)] Gly→Ala mutations are expected to be beneficial for protein stability, albeit in a context dependent way (162, 163). In contrast with this notion, most of the Gly→Ala mutations presented in this study had only marginal effects on stability, with the exception of G58A (stabilization by 3.8°C) and G78A (destabilization by 5.5°C) (Table III.II). Interestingly, the rather drastic (in terms of

Table III.II. Thermal stability of glycine→alanine TLP-ste variants.

variant	δT50[a,b]	variant	δT50[a, b]
	°C		°C
TLP-ste	0	G135A	-0.3
G58A	+3.8	G136A	+0.1
G78A	-5.5	G135A+G136A	-0.6
G89A	0	G141A	+0.7
G109A	0	G264A	+0.2

[a] wild-type T_{50}=73.4 °C. Part of the effects on thermal stability have been discussed previously (78, 154).
[b]δT50 values are relative to wild-type; the standard deviations in the δT50 measurements were below ± 0.3°C in all cases.

activity) G135A+G136A double mutation hardly affected protein stability.

Discussion

Studies of the crystal structures of enzymes in the ligand-free and ligand-binding state provide accumulating evidence for the notion that larger, concerted motions are an essential part of catalysis in many enzymes (164). A hinge-bending movement between domains that close around an enzyme's active-site is one, relatively simple example of such concerted motions (86, 88). Studies of a large number of wild-type and mutant T4 lysozyme structures (87, 88, 165) strongly indicated that hinge-bending motions resulting in continuous opening and closure of the active-site cleft are an intrinsic property of this enzyme. Interestingly, Mchaourab *et al.* (166) have recently been able to demonstrate the hinge-bending motion in T4 lysozyme in solution by directly measuring inter-residue distances with help of site-directed spin-labeling. Hinge-bending motions have also been detected by molecular dynamics simulations of various proteins (151, 167) including thermolysin (89). These motions were similar to the motions inferred from structure comparisons (86). Since domain closure is supposedly related to entrapment of substrate and, thus, to catalysis, the hinge-bending motion needs to be fast (164). Energy barriers for torsion angle variations thus need to be low, which would be achieved best in the case where the hinge residue is a glycine. The latter is strongly supported by the present results, which show that Gly→Ala mutations in the hinge regions, but not those at five control positions, drastically reduce enzymatic activity.

Inspection of the TLP alignment (Fig. 3.2) shows that, in addition to positions 135 and 136, there are several other positions in and around the central interdomain helix (137-150) where glycines are conserved. The absence of a glycine at position 136 is correlated with the presence of a glycine at position 147, at the other end of the central α-helix. This Gly147 has been changed into an alanine by Margarit *et al*. (161) in an attempt to stabilize the TLP from *B. subtilis* (Gly135, Ser136, Gly147). Interestingly, like the Gly136A mutant in TLP-ste, the Gly147Ala mutant displayed reduced activity towards peptide substrates, whereas the activity towards casein was hardly affected. Elastase lacks both Gly135 and Gly136, but this may be compensated by glycines at positions 147 and 154. Both of these glycines are conserved in almost all more distantly related non-Bacillus TLPs which lack Gly135 and Gly136 (not shown; see http://swift.EMBL-Heidelberg.DE/neutpep/). It should be noted that, in addition to permitting low energy-barrier concerted motions, the various glycines in TLP active-sites (135, 136, 141, 147, 154; see Fig. 3.2) are likely to contribute to the local flexibility in the active-site, that is generally considered to be important for catalysis (106, 168). It has recently been claimed that the active-sites of many enzymes contain recognizable, glycine containing sequence motifs (169).

Some of the control mutations (58, 141, 264) in this study had small but significant

effects on catalytic activity towards the peptide substrates. The negative effect of G141A is not surprising since it may directly affect the conformation and flexibility of crucial parts of the active-site (154, 169). The negative effect of G58A and the positive effect of G264A are less readily explainable. The effects of the control mutations were in all cases much smaller than the effects of G78A or the G135A, G136A double mutation.

As shown in Table III.II, most Gly→Ala mutations had only marginal effects on thermal stability. Previously, it has been shown that thermal inactivation of TLP-ste is determined by (rate-limiting) partial unfolding processes, followed by autolytic degradation (80, 170, 171). Consequently, only mutations in regions that are involved in the these partial unfolding processes are expected to affect stability. In TLP-ste such a region was identified in the N-terminal domain, in particular between residues 4 and 70 (77, 78, 82, 153). Gly58 is an important part of this region and the stabilizing effect of the G58A mutation has been discussed previously (153, 172). Modelling studies have indicated that the destabilizing effect of G78A might be caused by a clash between the alanine C-β and the N-η1 of Arg35, which would disturb a cluster of electrostatic interactions involving several residues in the stability determining region (Arg35, Asp32, Asp85; not shown).

So far, the various types of studies of the (presumed) catalytic hinge-bending motion in TLPs (54, 86, 89, 127, 150) provide a highly consistent picture, showing that, indeed, such a motion occurs. However, although residues 78, 135 and 136 appear to be important for the same catalytic hinge-bending motion, the effects of mutations at these positions differ. G78A reduced activity towards all substrates tested, whereas G135A+G136A reduced activity towards shorter substrates only. Furthermore, the data may be taken to indicate that the k_{cat} component in the mutational effect is relatively small for G78A and relatively large for G135A+G136A. At the moment we can only speculate about the cause of these differences. It is conceivable that mutations at position 78 have a more general effect on mobility in the substrate binding cleft, whereas mutations at 135 and 136 (which are at the beginning of the 'catalytic' inter-domain helix) primarily affect motions directly involved in catalysis. In their detailed structural studies of hinge-bending displacements in thermolysin, Holland *et al.* (150) concluded that closure of the active site may be directly related to optimizing the enzyme for binding of the transition state, thus linking the hinge-bending motion to k_{cat}. For longer substrates, the contribution of binding energy to catalysis (173) may be so large that the negative effect of the G135A+G136A double mutation on the catalytic rate becomes relatively small and hardly detectable. The distortion (kink) in the 68-88 helix that is presumably needed to "open" the active-site of thermolysin [see model presented in (150)] may be less easy to achieve when glycine at 78 has been replaced by alanine. Thus, G78A may have "locked" the enzyme in a more closed state resulting in reduced ligand affinity. In this respect, it is suggestive that the G78A mutant is the only TLP-ste mutant (out of several hundreds made) that did not bind to bacitracin-silica during purification. Insight in the cause of the differences in the mutational effects as well as increased understanding of the hinge-bending motion may be gained by further enzymological and crystallographic analyses of the mutants described above.

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