Probing Catalytic Hinge Bending Motions in Thermolysin-like Proteases by Glycine → Alanine Mutations

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ABSTRACT: The active site of thermolysin-like proteases (TLPs) is located at the bottom of a cleft between the N- and C-terminal domains. 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 toward 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.

Thermolysin-like proteases (TLPs)[1] are a group of homologous metalloendopeptidases from *Bacillus* with similar enzymatic characteristics. The amino acid sequences of several TLPs have been determined (e.g., *1*−*5*) and the three-dimensional structures of the TLPs of *Bacillus thermoproteolyticus* (thermolysin) and of *Bacillus cereus* (TLP-cer) have been solved by X-ray crystallography (*6, 7*). All TLPs have a similar fold, consisting of a 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 (*8*; Figure 1).

Holland et al. (*9*) superposed the crystal structures of thermolysin, TLP-cer, and elastase (the homologous TLP from *Pseudomonas aeruginosa*; *10*). 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 (*9, 10*) 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-lysine), explaining why thermolysin appeared to be more closed than TLP-cer (*9, 11*).

Stark et al. (*7*) 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; Figures 1 and 2). On the basis of inhibitor studies, Thayer et al. (*10*) proposed the presence of a hinge in the corresponding location in elastase. A similar conclusion was reached by Van Aalten et al. (*12*), who studied the dynamics of thermolysin by essential dynamics analyses (*13*) of molecular dynamics simulations. On the basis of detailed crystallographic studies of various TLPs, Holland et al. (*9, 11*) suggested that also the conserved glycine 78 could be important for hinge-bending (Figures 1 and 2). Gly78 is located in the middle of the long 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. (*11*) observed global structural changes that resembled the previously observed hinge

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[1] Abbreviations: TLP, thermolysin-like protease; TLP-ste, thermolysin-like protease of *Bacillus stearothermophilus*; TLP-cer, thermolysin-like protease of *Bacillus cereus*; FaGLa, 3-(2-furylacryloyl)-L-glycyl-L-leucine amide; FaAFa, 3-(2-furylacryloyl)-L-alanyl-L-phenylalanine amide; FaGLA-OH, 3-(2-furylacryloyl)-L-glycyl-L-leucinyl-L-alanine.

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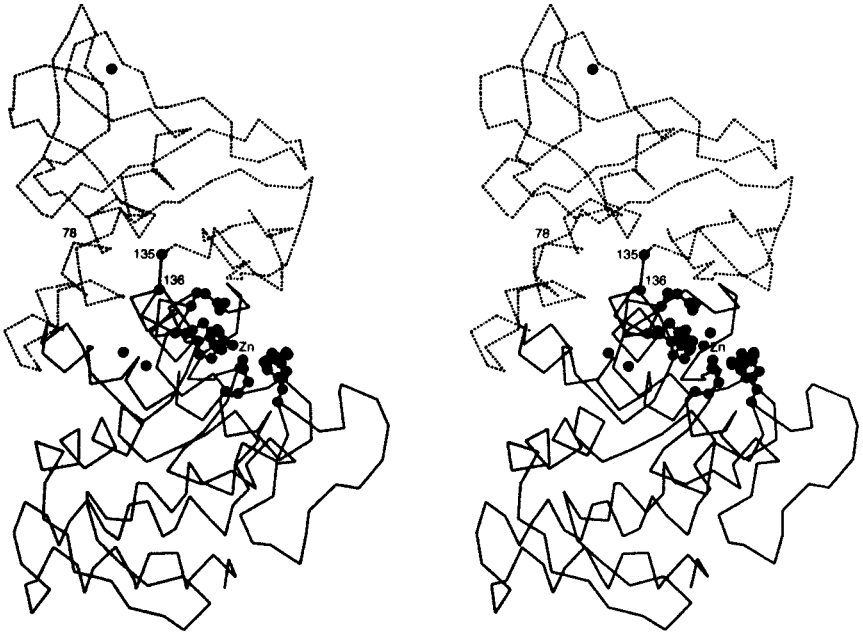

FIGURE 1: Stereo C-α trace of thermolysin. Residues 1−135 are shown with dotted lines, and the α carbons of Gly135 and Gly136 are marked with small spheres. The active-site residues that are involved in zinc binding (His142, His146, Glu166) and catalysis (Glu143, Tyr157, His231) are added in a ball-and-stick 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 ref *9* for details). Opening of the active-site cleft is associated with a change in hinge bending angle of approximately 6° (*9*), as well as a small change in the dihedral angles of Gly135 and Gly136 (see ref *7*).

bending displacement at glycine 78 (*9*), 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 *Bacillus stearothermophilus* CU21 (TLP-ste, ref *3*; 86% overall sequence identity with thermolysin). Five other Gly → Ala mutations were analyzed as controls. Modeling 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 toward 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.

EXPERIMENTAL PROCEDURES

Genetics. The *npr*T gene encoding the TLP of *B. stearothermophilus* CU21 (*3*) was cloned, subcloned, and expressed as described previously (*14*). Site-directed mutagenesis was performed using the pMa/c gapped duplex method as described before (*14*) or (for G78A and G135A + G136A) by the PCR-based mega-primer method, essentially as described by Sarkar and Sommer (*15*).

Production and Characterization of Mutant Enzymes. Production, purification, and subsequent characterization of the enzymes were performed as described earlier (*14*), with the exception of the G78A mutant, for which a different purification protocol was used (*16*). 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 with casein as a substrate (*17*). Thermal stability was quantified by T_{50}, 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. (*17*): approximately 0.5 μg of protease was incubated in 1 mL of 50 mM Tris-HCl, pH 7.5, containing 0.8% 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, 1.9% acetic acid, and 17 mM sodium acetate (pH 3.5). The specific activity was calculated as the average from at least three independent assays. One unit was defined as the amount of acid-soluble peptide that gives an increase in A_{275} of 0.001 per minute per milligram of TLP.

The catalytic performance (k_{cat}/K_m) for three furylacryloylated peptides at 37 °C was determined essentially as described by Feder (*18*) using a thermostated Perkin-Elmer Lambda 11 spectrophotometer. The reaction mixture (1 mL) contained 10 mM MOPS-NaOH, pH 7.0, 5 mM $CaCl_2$, 1% DMSO (v/v), 1% 2-propanol (v/v), 0.02% Triton X-100 (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 $\Delta\epsilon$ of -317 M^{-1} cm^{-1} as described by Feder (*18*).

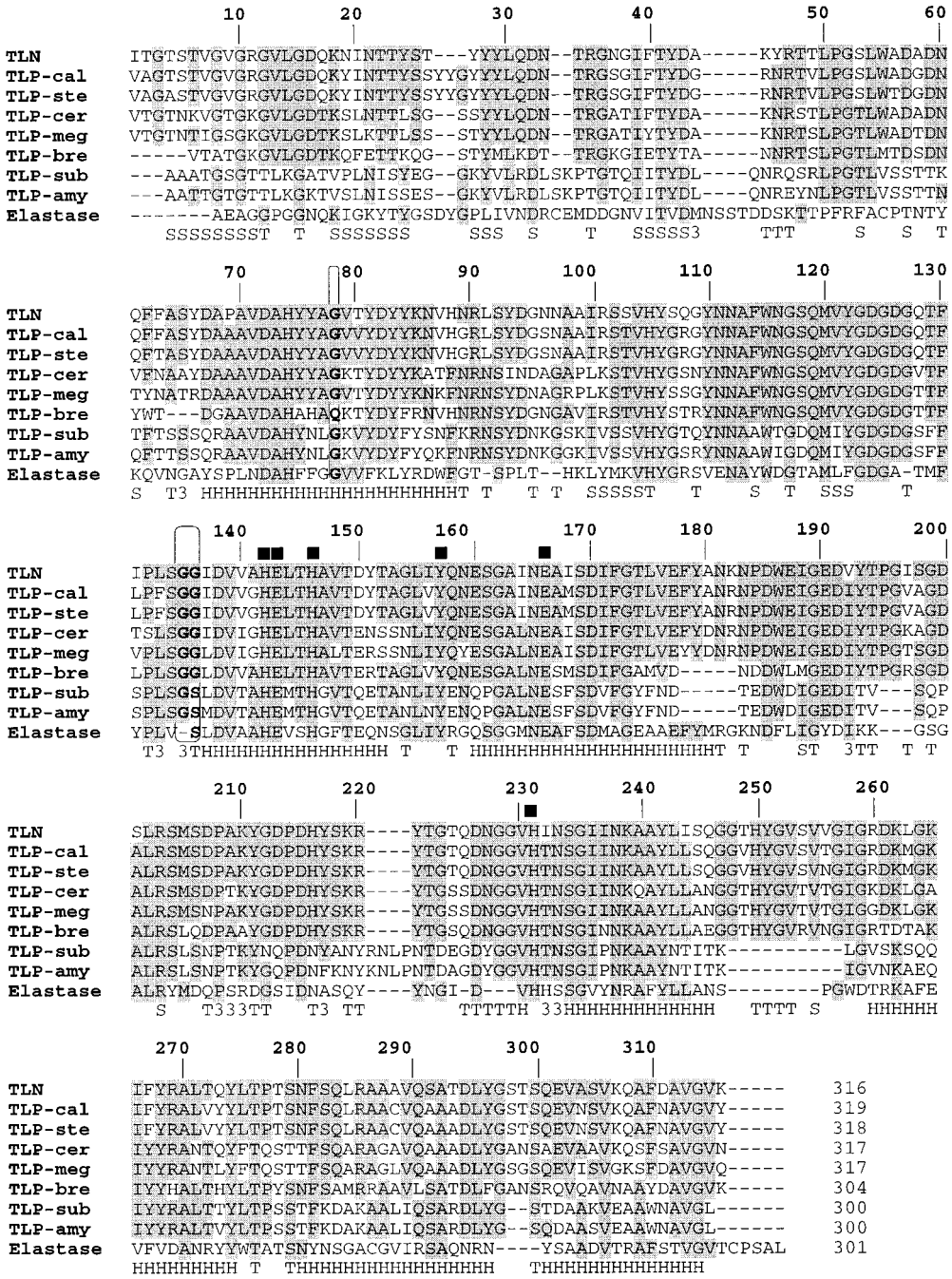

FIGURE 2: Multiple alignment of the mature part of thermolysin-like proteases. The enzymes listed are from *B. thermoproteolyticus* (TLN, thermolysin; *5*), *Bacillus caldolyticus* (TLP-cal; *54*), *B. stearothermophilus* CU21 (TLP-ste; *17*), *B. cereus* (TLP-cer; *4*), *Bacillus megaterium* (TLP-meg; *55*), *Bacillus brevis* (TLP-bre; *56*), *Bacillus subtilis* (TLP-sub, *1*), *Bacillus amyloliquefaciens* (TLP-amy; *2*) and elastase from *P. aeruginosa* (*57*). The glycine residues in the proposed hinge regions are indicated by light gray 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).

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-OH) 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} M), in a buffer consisting of 50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 1% DMSO (v/v), 1% 2-propanol (v/v), and 125 mM NaCl, after which FaAFa (final concentration 100 μM) was added as substrate. K_is were calculated by the method described by Hunter and Downs (*19*).

Structural Analysis. A three-dimensional model of TLP-ste was built on the basis of the crystal structure of thermolysin, using the molecular modeling program WHAT IF (*20*). The modeling procedures have been described in detail elsewhere (*21*). 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 (*21, 22*). This idea was corroborated by the successful *de novo* design of many stabilizing mutations (e.g., *16, 21, 23*). In this report all TLPs are numbered following the corresponding residues in

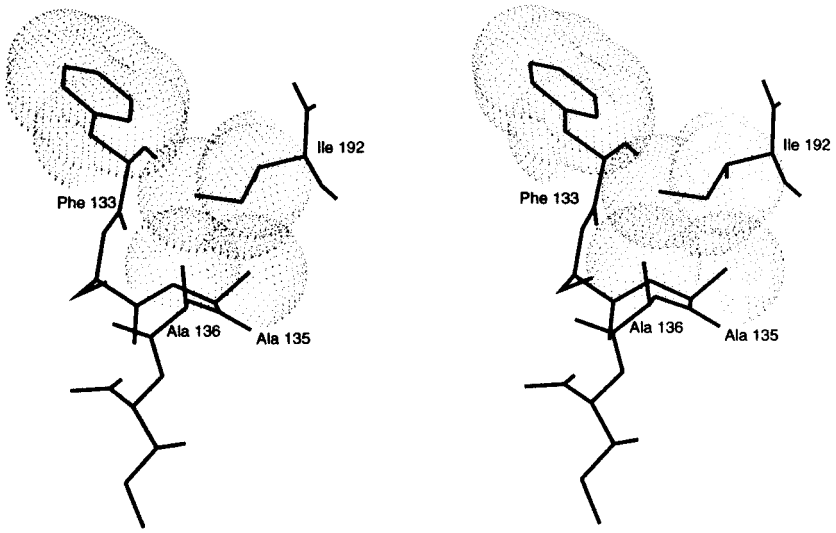

FIGURE 3: Local environment in the hinge area of the G135A + G136A mutant of TLP-ste. There is clearly some van der Waals overlap between the C-β of Gly136 and the C-δ of Ile192, which again could affect Phe133 at the bottom of the S_1' subsite.

Table 1: Properties of *B. stearothermophilus* Thermolysin-like Protease Variants

TLP-ste variant	FaGLa k_{cat}/K_m ($s^{-1}\cdot M^{-1} \times 10^{-3}$)	FaAFa k_{cat}/K_m ($s^{-1}\cdot M^{-1} \times 10^{-4}$)	FaGLA-OH k_{cat}/K_m ($s^{-1}\cdot M^{-1} \times 10^{-4}$)	FaAFa/FaGLa ratio	FaGLA-OH/ FaGLa ratio	K_i (nM $\times 10^{-2}$) phosphoramidon	specific activity for casein (units $\times 10^{-3}$)
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

thermolysin. All glycine residues mutated in this study have φ, ψ angles that are favorable for alanine (see http://swift.EMBL-Heidelberg.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 glycines was, however, apparent from previous studies (*24*) 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 were expected to be unrelated to catalysis or hinge bending (Figure 1). The fifth (at position 141) is located next to His142, which is part of the HExxH motif common to many zinc metalloproteases (Figures 1 and 2; see also ref *25*). The two histidines in this motif are ligands of the active, site zinc, and the glutamate is directly involved in the catalytic mechanism (*6*, *26*). The C-β of Ala141 points away from the active site and is highly unlikely to influence the binding of the ligand.

Modeling 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 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 S1′ pocket (Figure 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 P1′ position (Figure 3). However, modeling studies and inspection of the crystal structure of the phosphoramidon−thermolysin complex (*27*) 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, which 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 (*14*, *28*). The G78A mutant did not bind efficiently to the bacitracin-silica column and a different purification method (*16*) was therefore employed to purify this mutant.

Effects on Activity. The mutant proteins were characterized by determining specific activities toward casein, k_{cat}/K_m values for various furylacryloylated peptides (*18*), and K_i values for the transition-state inhibitor phosphoramidon (*27*, *29*) (Table 1). Since the low solubilities of the

furylacryloylated peptides preclude accurate determination of K_m values (*18, 30, 31*), the K_is for 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 1). 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 toward casein were only observed for G78A (Table 1). The G135A + G136A double mutation reduced activity toward 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 modeling 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 1). 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 ref *32*). 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 caused by 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 1). Since the present study does not involve mutation of residues directly interacting with ligands/substrates, 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 1. 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 toward casein. The increase in K_i for phosphoramidon (which is in the same order of magnitude as decrease in the various k_{cat}/K_m values; Table 1) 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.

Table 2: Thermal Stability of Glycine → Alanine TLP-ste Variants[a]

TLP-ste variant	δT_{50}[b] (°C)	TLP-ste variant	δT_{50}[b] (°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] T_{50} = 73.4 °C. Part of the effects on thermal stability have been discussed previously (*21, 24*). [b] δT_{50} values are relative to wild type; the standard deviations in the δT_{50} measurements were below ± 0.3 °C in all cases.

Effects on Stability. On the basis of the results of statistical (*33, 34*), theoretical (*35*), and experimental analyses (e.g., *35*−*39*), Gly → Ala mutations are expected to be beneficial for protein stability, albeit in a context-dependent way (e.g., *38, 39*). 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 2). Interestingly, the rather drastic (in terms of activity) G135A + G136A double mutation hardly affected protein stability.

DISCUSSION

Studies of the crystal structures of enzymes in the ligand-free and ligand-binding states provide accumulating evidence for the notion that larger, concerted motions are an essential part of catalysis in many enzymes (*40*). A hinge-bending movement between domains that close around an enzyme's active site is one, relatively simple example of such concerted motions (e.g., *9, 41*). Studies of a large number of wild-type and mutant T4 lysozyme structures (*41*−*43*) 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. (*44*) have recently been able to demonstrate the hinge-bending motion in T4 lysozyme in solution by directly measuring interresidue distances with help of site-directed spin-labeling. Hinge-bending motions have also been detected by molecular dynamics simulations of various proteins (e.g., *12, 13, 45, 46*) including TLP (*12*). These motions were similar to the motions inferred from structure comparisons (*9*). Since domain closure is supposedly related to entrapment of substrate and, thus, to catalysis, the hinge-bending motion needs to be fast (*40*). 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 (Figure 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 helix. This Gly147 has been changed into an alanine by Margarit et al. (*37*) 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 toward peptide substrates, whereas the activity toward 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 that 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, and 154; see Figure 2) are likely to contribute to the local flexibility in the active site, which is generally considered to be important for catalysis (*47, 48*). It has recently been claimed that the active sites of many enzymes contain recognizable, glycine-containing sequence motifs (*49*).

Some of the control mutations (58, 141, 264) in this study had small but significant effects on catalytic activity toward 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 (*25, 49*). 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 2, 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 (*14, 21, 50, 51*). Consequently, only mutations in regions that are involved in 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 (*16, 21, 23, 24*). Gly58 is an important part of this region and the stabilizing effect of the G58A mutation has been discussed previously (*24, 52*). Modeling 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 (*7, 9*−*12*; this study) 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 toward all substrates tested, whereas G135A + G136A reduced activity toward 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 interdomain helix) primarily affect motions directly involved in catalysis. In their detailed structural studies of hinge-bending displacements in thermolysin, Holland et al. (*11*) 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 (*53*) 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 by Holland et al., (*11*)] 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 into 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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