# Incorporation of a Stabilizing $Ca^{2+}$-Binding Loop into Subtilisin BPN′ †

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ABSTRACT: A rational approach was taken to improve the stability of subtilisin BPN′ to autoproteolysis. Two sites of autoproteolysis were identified by isolation of early autolysis products and amino-terminal sequence analysis. These studies showed that subtilisin rapidly cleaves Ala48–Ser49 and Ser163–Thr164 peptide bonds at elevated temperatures. These two sites appear in regions of high mobility as estimated from crystallographic *B*-factors and are in extended surface loops. To improve the resistance to thermal-induced autolysis, we replaced sequences around these two sites with sequences derived from a thermophilic homologue of subtilisin, thermitase. Thermitase contains a $Ca^{2+}$-binding site in the region surrounding Ser49. When the $Ca^{2+}$-binding segment of thermitase corresponding to residues 45–63 of subtilisin BPN′ was installed into subtilisin BPN′, the chimeric protein gained the ability to bind another $Ca^{2+}$ with moderate affinity ($K_d \sim 100\ \mu M$). This enzyme had the same $k_{cat}$ as wild-type, had a $K_M$ value 8-fold larger than wild-type, and was slightly less stable to thermal inactivation in EDTA. However, in 10 mM $CaCl_2$, the mutant subtilisin BPN′ was 10-fold more stable to irreversible inactivation at 60 °C than wild-type subtilisin BPN′ as measured by residual activity against the substrate sAAPF-*p*na. Next, mutations and deletions derived from thermitase were introduced near the second autolysis loop in subtilisin BPN′ (residues 158–165). However, all of these mutants were less stable than wild-type subtilisin. Thus, some (but not all) mutations derived from a thermophilic homologue near sites of autolysis can be stabilizing to a mesophilic protease.

Structure-based protein design has been extensively applied to enhance the thermal stability of proteins [for a review, see Alber (1989)]. These design strategies are often based on the thermodynamic assumption that if the protein of interest undergoes reversible folding transitions it can be stabilized by lowering the free energy of the folded state relative to that of the unfolded state. Thus, the introduction of disulfide bonds (Perry & Wetzel, 1984; Villafranca et al., 1987; Matsumura et al., 1989), charge interactions (Alber et al., 1987), or mutations geared to stabilize $\alpha$-helices (Horovitz et al., 1990; Nicholson et al., 1991) or remove hydrophilic groups from the protein interior (Eijsink et al., 1991) can stabilize proteins to reversible unfolding.

Subtilisin is an enzyme of considerable practical as well as theoretical interest [for a review, see Wells and Estell (1988)]. Structure-based approaches to stabilize subtilisin against autolysis by protein engineering of disulfide bonds (Wells & Powers, 1986; Pantoliano et al., 1987; Mitchinson & Wells, 1989) or salt bridges (Erwin et al., 1990) have met with little success. These methods, which rely on affecting the equilibrium between unfolded (inactive) and folded (active) protein, may not apply in cases where inactivation is an irreversible process. These studies and others (Voordouw et al., 1976) suggest that inactivation of subtilisin is not simply controlled by reversible unfolding.

Fontana and co-workers (Fontana et al., 1986) have correlated sites of partial proteolysis and autolysis in thermolysin with exposed sites of high segmental mobility. We reasoned that it may be possible to slow the rate of autolysis in subtilisin BPN′ by identifying sites of autolysis and mutating them to correspond to those of a more stable subtilisin variant, thermitase. Indeed, we show here that grafting a stabilizing $Ca^{2+}$-binding site from thermitase into an autolysis site of subtilisin BPN′ improved its autolytic stability more than 10-fold in the presence of calcium.

## MATERIALS AND METHODS

*Construction and Purification of Subtilisin Variants.* Mutants 1 and 3–6 (Figure 1) were produced by site-directed mutagenesis (Zoller & Smith, 1987) of the *Bacillus amyloliquefaciens* subtilisin gene (Wells et al., 1983) that was cloned into the phagemid vector pSS5 (Carter & Wells, 1988). Mutant 2 was made using mutant 1 as a template. Mutagenesis employed a single-stranded pSS5 uracil-containing template that was prepared in CJ236 cells (Kunkel et al., 1988) with the oligonucleotides shown in Figure 1. The mutated plasmids were verified by dideoxy sequencing (Sanger et al., 1977) and transformed into a protease-deficient strain of *Bacillus subtilis* (BG2036) (Yang et al., 1984). Subtilisin variants were purified by ethanol precipitation (50–75% cut) followed by passage over fast-flow DEAE onto SP Trisacryl M in 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES),[1] pH 5.5. Subtilisin was eluted with a linear gradient of NaCl (0–150 mM) in 10 mM MES, pH 5.5. Enzymes were stored flash-frozen at −70 °C.

*Kinetic Characterization.* Enzymes were assayed with the substrate sAAPF-*p*na[1] in 1 mL of 100 mM Tris·HCl (pH 8.60)/4% (v/v) DMSO at 25 ± 0.2 °C as previously described (Carter & Wells, 1988). Wild-type enzyme concentrations were determined spectrophotometrically ($\epsilon_{280nm}^{0.1\%} = 1.17$; Matsubara et al., 1965). The extinction coefficients of the mutant

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[1] Abbreviations: sAAPF-*p*na, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide; PMSF, phenylmethanesulfonyl fluoride; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide.

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A. Calcium binding site sequences

| | 45 | | | | | 50 | | | | | 55 | | | | 60 | | | | | | 65 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WT | V | A | G | G | A | S | M | V | P | S | E | T | N | P | F | Q | D | N | N | S | H | G | T |
| Mut 1 | V | A | G | G | W | D | M | V | D | S | D | T | N | P | – | Q | D | N | N | S | H | G | T |
| Mut 2 | V | A | G | G | W | D | F | V | D | N | D | T | N | P | – | Q | D | G | N | G | H | G | T |
| Thermitase | V | V | G | G | W | D | F | V | D | N | D | S | T | P | – | Q | D | G | N | G | H | G | T |

Oligo 1 AAG GTA GCA GGC GGA TGG GAC ATG GTC GAC TCT GAT ACA AAT CCT – CAA GAC AAC AAC TCT CAC; SalI
Oligo 2 GGC GGA TGG GAC TTT GTA GAC AAT GAT ACA AAT CCT – CAA GAC GGC ACA GGT CAC GGA ACT

B. Flexible loop sequences

| | 155 | | | | | | | 160 | | | | 165 | | | | | | | 170 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WT | A | G | N | E | G | T | S | G | S | S | S | T | V | G | Y | P | G | K | | |
| Mut 3 | A | G | N | E | G | N | T | – | – | – | – | A | P | G | Y | P | G | K | | |
| Mut 4 | A | G | N | E | G | N | – | – | – | – | C | T | V | G | Y | P | G | K | | |
| Mut 5 | A | G | N | E | G | T | S | – | – | – | S | T | V | G | Y | P | G | K | | |
| Mut 6 | A | G | N | E | G | T | – | – | – | – | S | T | V | G | Y | P | G | K | | |
| Thermitase | A | G | N | A | G | N | T | – | – | – | – | A | P | N | Y | P | A | Y | | |

Oligo 3 GCC GGT AAC GAA GGC AAT ACG – – – – GCG CCG GGG TAC CCT GGC AAA; NarI
Oligo 4 GCC GGT AAC GAA GGC AAC – – – – TGC ACA GTG GGG TAC CCT; BspMI
Oligo 5 GGT AAC GAA GGC ACG ACG – – – TCC ACA GTG GGC TAC; AatII
Oligo 6 GGT AAC GAA GGC ACG – – – – TCG ACA GTG GGG TAC; SalI

FIGURE 1: Protein sequence comparison between subtilisin and thermitase spanning a $Ca^{2+}$-binding site (A) and a flexible loop region in subtilisin (residues 158–164) (B). Mutants 1 and 2 were designed to confer $Ca^{2+}$ binding to subtilisin in a region susceptible to autolysis. Residues involved in coordination of calcium are Asp49, Asp52, Asp54, Thr56, and Gln59. Mutants 3–6 were designed to alter a flexible loop surrounding position 164 that is susceptible to autolysis. The boxed sequences indicate sequence identity, and the open letters indicate sites of mutation. The oligonucleotides used in the mutagenesis are shown, and the underlined segments indicate new restriction sites.

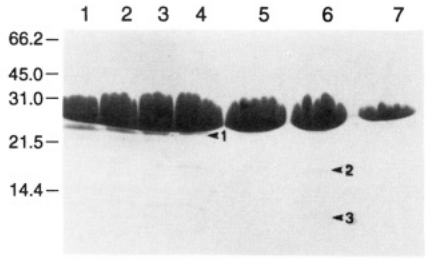


FIGURE 2: Identification sites for autolysis. Twenty microliters of PMSF-inhibited wild-type subtilisin (6.8 mg/mL) was preincubated at 65 °C in 100 mM Tris (pH 8.6) and 30 mM $CaCl_2$ for 1 min. Ten microliters of uninhibited subtilisin was added to final concentrations of 0.375, 1.50, 2.63, and 3.75 μM (lanes 1–4) for 30 s and 7.50 and 15 μM (lanes 5 and 6) for 15 s. Autolysis was quenched by addition of 2.5 μL of 100 mM PMSF followed by freezing on dry ice. Lane 7 is PMSF-inhibited subtilisin heated to 60 °C without addition of active subtilisin. SDS–PAGE was run according to the method of Laemmli (1972) using 20% acrylamide. See Results for identity of bands 1–3 indicated by arrows.

enzymes were adjusted for any changes in tryptophan and tyrosine content (Wetlaufer, 1962). Final enzyme concentrations in assays were between 10 and 30 nM. Initial rates of hydrolysis were measured at substrate concentrations in the range of 0.2–5 times $K_M$.

*Determination of Autolysis Sites.* Wild-type subtilisin was inhibited with PMSF and dialyzed against 50 mM Tris (pH 8.0) and 30 mM $CaCl_2$. Twenty microliters of a 6.8 mg/mL subtilisin solution was preincubated at 65 °C for 1 min followed by addition of 10 μL of uninhibited subtilisin at various concentrations for 15 or 30 s (Figure 2). Autolysis was quenched by the addition of 2.5 μL of 100 mM PMSF followed by freezing samples on dry ice. Autolysis products were visualized by electroblotting onto ProBlot (Applied Biosystems) after SDS–PAGE. Protein bands were excised, and amino-terminal sequencing was done on each.

*Determination of Rates of Inactivation.* Protein solutions were passed through a Sephadex PD-10 column to exchange buffer solution to 10 mM Tris (pH 8.6) or 10 mM MES (pH 5.5) or 50 mM sodium acetate (pH 3.5), all containing 1 mM $CaCl_2$. The protein concentrations were adjusted to 0.1 mg/mL. Enzyme samples (99 μL) were diluted with 1 μL of 0.25 M EDTA, 1 μL of $H_2O$, or 1 μL of 1 M $CaCl_2$ and incubated at 60 °C. To stop autolysis, 10-μL samples were added to 0.950 mL of 100 mM Tris(pH 8.6) at 25 °C. Activity measurements were initiated by the addition of 40 μL of 25 mg/mL sAAPF-*p*na in DMSO.

*Determination of Calcium-Binding Stoichiometry.* Wild-type or mutant subtilisin was dialyzed against 1000 volumes of 10 mM MES, pH 5.5 at 4 °C, with three changes of buffer over 16–24 h. A 2-fold calcium dilution series (from 0.01 to 1.2 mM) was added to one side of a microdialysis cell with subtilisin at about 45 μM. To the other side was added a $^{45}CaCl_2$ solution in pH 5.5 dialysate. After equilibration by slow rotation for 14 h at 4 °C, $^{45}Ca^{2+}$ concentrations were determined on each side of the dialysis cell by scintillation counting. Calcium-binding studies were also performed in 50 mM Tris (pH 8.0) with PMSF-inhibited subtilisin (18 μM).

*Differential Scanning Calorimetry.* Enzyme solutions were inactivated with PMSF and dialyzed against 50 mM Tris (pH 8.3) with either 10 or 100 mM $CaCl_2$. Subtilisin solutions were adjusted with dialysate to 1.0 mg/mL and scanned using a MicroCal MC-2 calorimeter (MicroCal, Inc.).

## RESULTS

*Identification of Autolytic Cleavage Products.* Isolation of large amounts of autolyzed subtilisin is complicated by the fact that single cleaved products of autolysis are far less stable than subtilisin, and thus do not accumulate. To generate these unstable products, a large excess of PMSF-inhibited

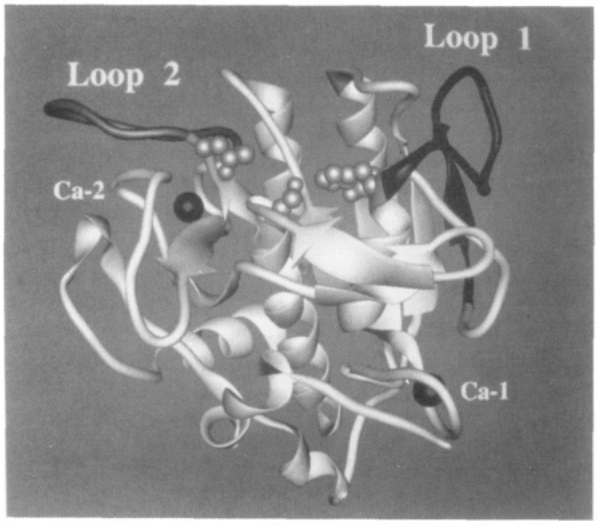


FIGURE 3: Ribbon diagram of subtilisin showing regions that were mutated. The black loop on the right is from codon 44 to 63 and the gray loop on the left is from codons 156 to 168. The active-site residues Asn155, Ser221, His64, and Asp32 are shown in gray. The strong-binding calcium (Ca-1) and weak-binding calcium (Ca-2) are shown in black. Coordinates are from the Protein Data Bank (Bernstein et al., 1977) entry pdb2sni (McPhalen & James, 1988).

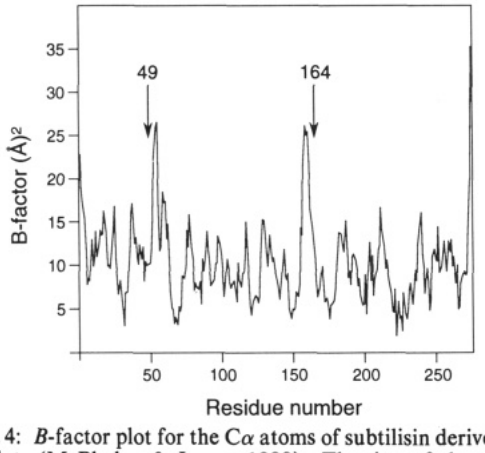


FIGURE 4: *B*-factor plot for the Cα atoms of subtilisin derived from X-ray data (McPhalen & James, 1988). The sites of cleavage are shown with arrows. The amino-terminal residue generated by proteolysis at these sites is Ser49 and Thr164.

subtilisin was digested briefly at 60 °C with small amounts of wild-type subtilisin. Reactions were quenched on ice with additional PMSF, and the products were separated by SDS–PAGE (Figure 2). Three products appeared (bands 1–3, Figure 2) plus a number of minor products. Under these conditions, less than 5% of the PMSF-inhibited subtilisin was digested. Longer incubations resulted in greater loss of full-length subtilisin. The autolysis products were not present in PMSF-inhibited subtilisin heated to 60 °C without the addition of active subtilisin (Figure 2, lane 7). The products were isolated by electroblotting onto ProBlot. N-Terminal sequencing showed that band 2 contained the amino terminus of subtilisin, band 1 started at Ser49, and band 3 began with Thr164. Characterization of the order at which these products appear is complicated by the fact that they are very unstable and thus the steady-state levels are very low.

Inspection of an X-ray structure of subtilisin (Bott et al., 1988; McPhalen & James, 1988) shows that Ser49 and Thr164 are located in separate accessible loop structures near the active site (Figure 3). Furthermore, a plot of crystallographic *B*-factor versus sequence position (Figure 4) shows that these loops are at sites of maximal *B*-factor and presumably regions of high segmental mobility.

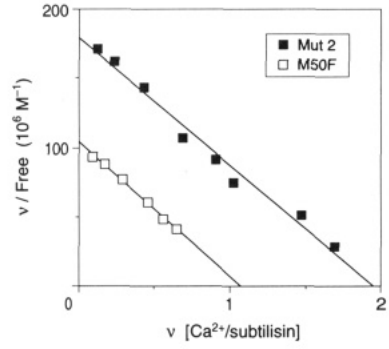


FIGURE 5: Scatchard analysis of $^{45}Ca^{2+}$ binding to M50F subtilisin and mutant 2 at pH 5.5. The ordinate is bound moles of $^{45}Ca^{2+}$ per mole of enzyme divided by micromoles of free $^{45}Ca^{2+}$. The abscissa is moles of $^{45}Ca^{2+}$ bound per mole enzyme. See Materials and Methods for further details.

*Substitution of Sequences Derived from Thermitase.* The sequence of the thermophilic homologue thermitase (Meloun et al., 1985) was aligned with that of subtilisin and found to contain a number of substitutions around the two autolysis sites (Figure 1). The region between the conserved Gly47 and the catalytic His64 contains 10 substitutions and a single deletion. Thermitase contains a calcium-binding site in which Asp49, Asp52, Asp54, Thr56, and Gln59 coordinate calcium (Gros et al., 1991). Mutant 1 (Figure 1A) was designed with minimal changes to introduce the thermitase $Ca^{2+}$-binding sequence into subtilisin. However, expression was so poor that we could not purify enough enzyme for analysis. We added four additional substitutions in this region from thermitase (M50F, S53N, N61G, and S63G) to produce mutant 2 which could be expressed at high levels in *B. subtilis*.

The region around Thr164 of thermitase contains a deletion of 4 residues (160–163) plus substitutions at 158, 159, 164, and 165 (Figure 1B). A series of 4 mutants (mutants 3–6) were produced that deleted 3 or 4 residues plus flanking substitutions between residues 158 and 165. Each could be expressed at levels suitable for study.

*Analysis of the First Autolysis Loop Mutant.* Equilibrium dialysis with $^{45}Ca^{2+}$ was carried out to determine the change in $Ca^{2+}$ binding for mutant 2 as a result of installing the thermitase $Ca^{2+}$-binding loop (Figure 5). To minimize autolysis, the experiment was conducted at 4 °C at pH 5.5 where protease activity is greatly reduced. To further reduce autolysis for the wild-type enzyme, we used a slightly more stable variant, M50F (Cunningham & Wells, 1987), as our wild-type control for these studies. Under these conditions, M50F subtilisin contains one exchangeable $Ca^{2+}$-binding site with a $K_d$ of ~100 μM whereas mutant 2 contains two $Ca^{2+}$ sites of comparable affinity ($K_d$ ~100 μM).

Wild-type subtilisin actually binds two calciums; however, at pH 5.5, the tighter site ($K_d$ ~$10^{-8}$ M) does not undergo exchange (Pantoliano et al., 1988). When the equilibrium dialysis experiments were conducted at pH 8.0 using PMSF-inhibited enzymes, the stoichiometries of $Ca^{2+}$ binding for the M50F subtilisin and mutant 2 were about 2 and 3, respectively (data not shown). Thus, under either pH condition, mutant 2 contains an additional $Ca^{2+}$-binding site of affinity comparable to the weaker $Ca^{2+}$-binding site in subtilisin.

To measure thermal-induced autolysis rates, wild-type or mutant 2 subtilisin was incubated at 60 °C in 10 mM Tris (pH 8.6) for various times, and sAAPF-*p*na was used as the substrate to measure the residual enzyme activity (Figure 6). A plot of the log activity versus time is linear for these

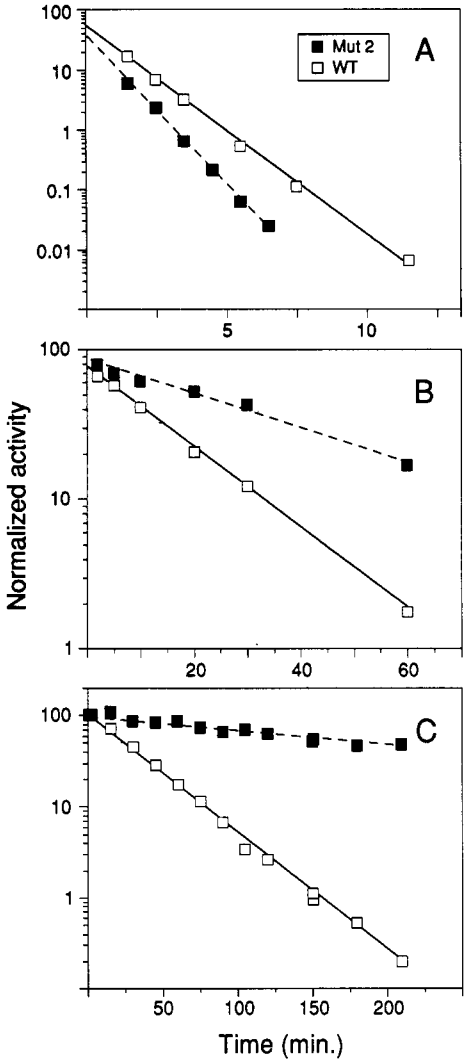


FIGURE 6: Rate of thermal inactivation for $Ca^{2+}$ site mutant 2 relative to wild-type subtilisin. The lines are best fit to the logarithm of remaining activity versus time. All incubations were done at 60 °C in 10 mM Tris (pH 8.6) with the following additions: panel A, 1.0 mM $CaCl_2$ and 2.5 mM EDTA; panel B, 1.0 mM $CaCl_2$; panel C, 10 mM $CaCl_2$. See Materials and Methods for further details.

conditions, indicative of an apparent first-order process. In the presence of EDTA, wild-type is more stable than mutant 2 (Figure 6A), possibly because of the net negative charge repulsion that exists in mutant 2 in the absence of calcium. However, mutant 2 is about 3 times more stable in 1 mM $CaCl_2$ than wild-type (Figure 6B) and 10 times more stable in 10 mM $CaCl_2$ (Figure 6C). The half-life of mutant 2 is increased an additional 2.5–3-fold in 100 mM $CaCl_2$ over the half-life in 10 mM $CaCl_2$ (data not shown). Thus, the half-life at 60 °C increases from less than 1 min in EDTA to 180 min in 10 mM $CaCl_2$ for mutant 2.

The stabilizing effect of calcium is pH-dependent (Table I). At pH 3.5, the wild-type and mutant 2 are not stabilized by calcium and have a half-life of 1 min and 30 s, respectively. As the pH is increased with calcium present, both enzymes become more stable; however, mutant 2 becomes more stable than wild-type. Without the addition of calcium, the half-lives remain unchanged. These data are consistent with the binding of calcium to the new calcium site in mutant 2 via ionized aspartate side chains. Replacement of the loop around Ser49 with a calcium-binding loop from thermitase resulted in a >10-fold reduction in the autolysis rate. Therefore, bond cleavage in this loop appears to be a major route of autolysis at 60 °C.

Table I: Half-Life of Irreversible Inactivation of Wild-Type (WT) or the Calcium-Binding Mutant 2 (Mut) at 60 °C under Various Conditions[a]

| conditions | $t_{1/2}$ (min) WT | Mut |
|---|---|---|
| pH 3.5 | | |
| 1 mM $CaCl_2$ + 2.5 mM EDTA | 0.99 (1.0) | 0.47 (1.0) |
| 1 mM $CaCl_2$ | 0.74 (1.0) | 0.43 (1.0) |
| pH 5.5 | | |
| 1 mM $CaCl_2$ + 2.5 mM EDTA | 1.56 (1.0) | 0.95 (1.0) |
| 1 mM $CaCl_2$ | 10.2 (0.99) | 12.2 (0.99) |
| pH 8.6 | | |
| 1 mM $CaCl_2$ + 2.5 mM EDTA | 0.88 (1.0) | 0.61 (1.0) |
| 1 mM $CaCl_2$ | 11.1 (1.0) | 32.4 (0.98) |
| 10 mM $CaCl_2$ | 22.7 (1.0) | 180 (0.95) |

[a] Buffers were as follows: 50 mM NaOAc (pH 3.5); 10 mM MES (pH 5.5); 10 mM Tris (pH 8.6). All samples were prepared by solvent-exchange over Sephadex PD-10 columns preequilibrated with the appropriate buffer. Half-lives were determined by least-squares linear fit to a plot of log activity versus time (Figure 6). Values in parentheses are the correlation coefficients for the lines fitted to first-order activity decay.

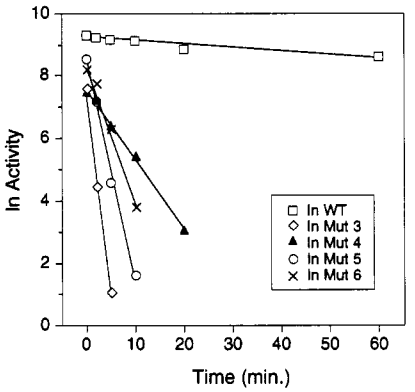


FIGURE 7: Rate of thermal inactivation for flexible loop mutants. The log of remaining activity is plotted versus time at 60 °C. The assay was performed as in Figure 6C.

*Analysis of Second Autolysis Loop Mutants.* Figure 7 shows that the loss in log activity versus time fits a first-order loss in activity for the set of four loop mutants in the region surrounding Thr164. However, none of the mutants were as stable as wild-type.

Three other mutants in this flexible loop region were made by computer-aided design (data not shown). Val165Glu was designed to strengthen calcium binding at site 2 of subtilisin (Figure 3) by providing an additional charge–charge interaction. Ser162Arg was designed to cover a hydrophobic patch and hole present in this flexible loop and to form hydrogen bonds with the Ser163 carbonyl oxygen, Ser195-Oγ, and Glu156-Oε on the other side of the loop. Thr164Asn was designed to alter the autolysis cut site. Of these, only S162R was marginally more stable in the presence of 2.5 mM EDTA than wild-type, but 2-fold less stable in 1 mM $CaCl_2$. Thr164Asn was 10-fold less stable than wild-type, and Val165Glu was expressed at levels too low to determine stability parameters.

The kinetic parameters for each mutant are summarized in Table II. The values are within a factor of 4 of wild-type, except that the $K_M$ of mutant 2 is 8 times higher.

*Mechanism of Autolysis below the $T_M$.* We compared the resistance to thermal inactivation for PMSF-treated wild-type subtilisin or mutant 2 by differential scanning calorimetry. From analysis of these data (not shown), the melting temperature ($T_M$) in 10 mM $CaCl_2$ for wild-type was 73.4 °C

Table II: Summary of Kinetic Parameters for Wild-Type (WT) and Mutants 2–6[a]

| enzyme | $k_{cat}$ ($s^{-1}$) | $K_M$ ($\mu M$) |
|---|---|---|
| WT | 56 ± 0.9 | 162 ± 14 |
| Mut 2 | 50 ± 0.7 | 1460 ± 40 |
| Mut 3 | 16 ± 0.2 | 317 ± 11 |
| Mut 4 | 16 ± 0.2 | 674 ± 28 |
| Mut 5 | 31 ± 0.15 | 247 ± 4.3 |
| Mut 6 | 24 ● 0.2 | 431 ± 13 |

[a] Values are determined from a nonlinear least-squares fit to the Michaelis–Menten equation using initial rates of hydrolysis of sAAPF-*p*na in 100 mM Tris (pH 8.6)/4% DMSO. Mutant 2 gave the same kinetic parameters in the presence or absence of 10 mM $CaCl_2$.

and for mutant 2 was 72.0 °C. Therefore, a difference in the resistance to autolytic inactivation at 60 °C does not appear to be due to a difference in the overall stability of the folded protein since wild-type melts at a higher temperature.

Next we evaluated whether autolysis at 60 °C is prevented by a competitive inhibitor. Addition of casein (5 mg/mL) to a solution containing subtilisin (0.1 mg/mL) prevented autolytic inactivation for 5 min. Moreover, an inactive subtilisin mutant (H64A) whose activity is reduced $10^6$-fold relative to wild-type (Carter & Wells, 1988) resisted any autolytic inactivation for over 3 h at 60 °C (data not shown). Addition of a small amount of active WT subtilisin to an excess of H64A led to the loss of full-length subtilisin, as visualized by SDS–PAGE. These data show loss of activity at elevated temperatures requires the presence of active subtilisin.

The loss of activity at 60 °C is a first-order process (Figures 6 and 7), and is thus independent of subtilisin concentration (Mitchinson & Wells, 1989). There are at least three explanations for this observation: (1) loss of activity follows the irreversible unfolding of one of the loop structures near the active site; (2) loss of activity is a intramolecular event where a molecule of subtilisin digests itself; or (3) loss of activity follows a rate-limiting local, reversible unfolding event, and the locally unfolded region is a good substrate for proteolysis.

## DISCUSSION

The data presented here and in previous studies suggest that autolytic inactivation of subtilisin involves a local unfolding event followed by proteolysis. Neither engineered disulfide bonds (Wells & Powers, 1986; Pantoliano et al., 1987; Mitchinson & Wells, 1989) nor salt bridges (Erwin et al., 1990) substantially reduce the rate of irreversible autolytic inactivation, suggesting that the autolysis substrate is somewhat nativelike. Nonetheless, the fact that the rate of inactivation is first-order and exhibits a temperature dependence similar to a melting transition (Wells & Powers, 1986) suggests a rate-limiting unfolding event is involved in autolysis. That the H64A subtilisin, which is greatly reduced in catalytic efficiency, does not inactivate at 60 °C points to a role for proteolysis in irreversible inactivation of subtilisin at elevated temperatures. The fact that specific autolysis products are produced at early times of autolytic digestion suggests that the molecule does not fully unfold. These sites are not cleaved on the basis of primary sequence specificity because the sequences around the cleaved bonds (AGGA$^{48}$–S$^{49}$MVP and GSSS$^{163}$–T$^{164}$VGY) are poor substrates. [Subtilisin prefers large hydrophobic side chains at the P1 or P4 position [for reviews, see Philipp and Bender (1983) and Estell et al. (1986)].] Similarly, the early cleavage products for digestion of thermolysin by subtilisin (Fontana et al., 1986) result from hydrolysis at sites which are not good subtilisin substrate sequences either.

The autolysis sites in subtilisin, and subtilisin digestion sites in thermolysin, are in accessible regions of high segmental mobility (Figure 4; Fontana et al., 1986). Transient local fluctuations in protein structure are well-known from amide proton exchange experiments and protease susceptibility studies [for a review, see Kim and Baldwin, (1990)]. If local unfolding is crucial for autolysis, this would explain how some mutants (notably mutant 2) can be more stable to autolysis yet fully unfold at a lower temperature than wild-type subtilisin (as determined by differential scanning calorimetry). We speculate that local unfolding around the primary sites of autolysis provides extended substrates for cleavage.

We evaluated the possibility of stabilizing subtilisin to autolysis by mutating sequences in regions of autolytic susceptibility so that they more closely match those of a thermophilic homologue, thermitase. Virtually all of the calcium-binding sequence from thermitase surrounding Ser49 was substituted into subtilisin to produce mutant 2. This variant bound one extra calcium and was more than 10-fold more stable to irreversible inactivation in the presence of 10 mM calcium than wild-type subtilisin. In an analogous fashion, Fontana and co-workers (Toma et al., 1991) have elegantly shown that introducing an extra calcium-binding site (derived from thermolysin) into neutral protease improved the stability of neutral protease more than 2-fold to irreversible thermal inactivation.

Subtilisin naturally binds two $Ca^{2+}$ (Pantoliano et al., 1989; Bode et al., 1987). One of these sites is close to the N-terminus and binds $Ca^{2+}$ so tightly ($K_a \sim 10^8$ $M^{-1}$) that it remains bound even in the presence of EDTA. The second site, involving residues 195 and 197, binds $Ca^{2+}$ much more weakly and is not occupied by calcium in other crystallographic studies (Kossiakoff et al., 1991). Nonetheless, EDTA dramatically increases the rate of thermal inactivation more than 100-fold (Voordouw et al., 1976). Addition of $Ca^{2+}$ in the millimolar range decreases the rate of thermal inactivation (Matsubara et al., 1958), suggesting occupation of the weaker calcium site is important for resisting thermal inactivation. Moreover, mutants which enhance the affinity for the weaker $Ca^{2+}$ site require less $Ca^{2+}$ to resist thermal inactivation (Pantoliano et al., 1988b).

Thermitase has a large deletion and a number of substitutions surrounding the autolysis site at Thr164 in subtilisin. A series of mutants designed to replace a flexible loop region of subtilisin around Thr164 with sequences derived from thermitase destabilized the enzyme to autolysis. Mutant 3 (which was very unstable) introduced the thermitase deletion and replaced two residues on either side with thermitase sequence. Mutant 4 was more stable than mutant 3 (but still less stable than wild-type) and was an attempt to "repack" some of the regions exposed by the four-residue thermitase deletion. Attempts to enhance the stability of mutant 3 by mutants 5 and 6 failed. This suggests that autolysis is governed by more than the primary loop sequence in this region.

In summary, we have identified two important sites of autolysis in subtilisin. Attempts to stabilize subtilisin by deleting sequences from an autolysis loop to match thermitase substantially increased the autolysis rate. However, it was possible to graft a functional $Ca^{2+}$-binding loop from thermitase onto subtilisin and stabilize the enzyme to irreversible inactivation by removing a major autolysis site. These and other experiments suggest the mechanism for autolysis involves a rate-limiting local unfolding event followed by rapid proteolysis.

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