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Proteases of Enhanced Stability: Characterization of a Thermostable Variant of Subtilisin

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ABSTRACT **A procedure has been developed for the isolation and identification of mutants in the bacterial serine protease subtilisin that exhibit enhanced thermal stability. The cloned subtilisin BPN′ gene from *Bacillus amyloliquefaciens* was treated with bisulfite, a chemical mutagen that deaminates cytosine to uracil in single-stranded DNA. Strains containing the cloned, mutagenized subtilisin gene which produced subtilisin with enhanced thermal stability were selected by a simple plate assay procedure which screens for esterase activity on nitrocellulose filters after preincubation at elevated temperatures. One thermostable subtilisin variant, designated 7150, has been fully characterized and found to differ from wild-type subtilisin by a single substitution of Ser for Asn at position 218. The 7150 enzyme was found to undergo thermal inactivation at one-fourth the rate of the wild-type enzyme when incubated at elevated temperatures. Moreover, the midpoint in the thermally induced transition from the folded to unfolded state was found to be 2.4-3.9°C higher for 7150 as determined by differential scanning calorimetry under a variety of conditions. The refined, 1.8-Å crystal structures of the wild-type and 7150 subtilisin have been compared in detail, leading to the conclusion that slight improvements in hydrogen bond parameters in the vicinity of position 218 result in the enhanced thermal stability of 7150.**

Key words: thermal stability, protein engineering, mutagenesis, plate assay, thermophilic enzymes

INTRODUCTION

The coupling of advances in recombinant DNA technology with detailed biophysical and structural characterization of proteins promises to lead to a greater understanding of the complexity of forces underlying protein folding and to the design of improved and novel biological catalysts. The ability to create localized mutations in proteins of known structure and then to correlate the resulting alterations in structure with changes in activity and stability provides a vital experimental approach to these fundamental problems. Once structure-function correlations have been made, site-directed mutagenesis can provide a means for redesigning proteins with altered properties in predictable and desirable ways.

One important parameter of potential commercial utility that should be alterable by protein engineering is the thermal stability of proteins. In one approach taken by several different laboratories,[1-4] site-directed mutagenesis has been employed for the introduction of disulfide bonds as a means of increasing stability. While the introduction of disulfide bonds with favorable conformations is one possible route to a more thermostable protein, we also have been interested in identifying other strategies to stabilize the folded state. Comparison of the same enzyme from mesophilic and thermophlic organisms has generated some ideas as to why a thermophilic enzyme has enhanced thermal stability over its mesophilic counterpart.[5-7] Nevertheless, it has not been possible in such studies to prove that single amino acid changes or even isolated regions of a protein are responsible for the altered thermal properties. Although information about single-site mutations which alter the thermal stability of a protein is now beginning to accumulate,[8-16] no variant protein with increased thermal stability has been characterized crystallographically until now. Overall the ability to predict the effects of specific mutations on protein thermal stability is poor at the present time.

To increase the number of known mutations resulting in increased thermal stability, we have used the bacterial serine protease subtilisin BPN′ as a model. We have found it efficient to introduce random mutations into the subtilisin gene and then to identify those variants with significantly enhanced resistance to thermal inactivation by using a rapid screening procedure. The method we have devised has allowed us to identify several thermostable subtilisins by screening 5×10^4 random variants. One of these variants is considerably more resistant to thermal inactivation than wild-type subtilisin and was chosen for detailed physical characterization. Here we report the identity of this single-site variant and its chemical and physical properties, including a comparison of its refined x-ray structure with that of wild-type subtilisin.

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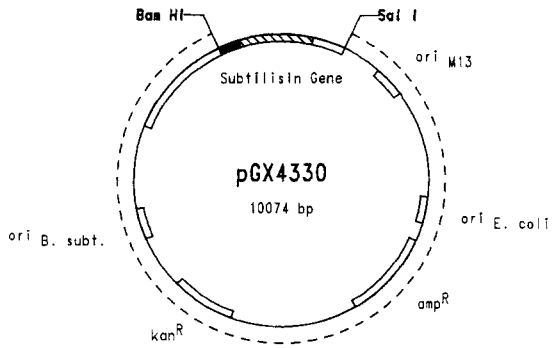

Fig. 1. For mutagenesis and expression, the subtilisin gene from *Bacillus amyloliquefaciens* including its natural promoter sequences was cloned into a vector containing the β-lactamase gene and replicon from pBR322 for growth in *E. coli*; the kanamycin nucleotidyl transferase gene and replicon from pUB110 for growth in *B. subtilis*; and the M13 origin of replication for production of single-stranded plasmid DNA upon superinfection with helper phage IR1. The resulting plasmid is designated pGX4330. Before mutagenesis with sodium bisulfite, single-stranded pGX4330 DNA was annealed with denatured *Bam*HI-*Sal*I fragment of pGX4330. This results in gapped duplex molecules in which the coding region of the subtilisin gene remains single stranded. Typical conditions for mutagenesis were 2.5 mg/ml gapped duplex DNA with 4.0 M bisulfite for 5.0 min at 37°C and pH 6.5.

CONSTRUCTION AND IDENTIFICATION OF THERMOSTABLE VARIANTS

The technique for generating and screening subtilisin variants involves three steps: 1) mutagenesis of the cloned subtilisin gene; 2) expression of mutated genes in *Bacillus subtilis*; and 3) screening for altered stability.

Random mutations were introduced into the cloned subtilisin gene by using sodium bisulfite. Sodium bisulfite mutagenesis results primarily in the conversion of cytosine to uracil in single-stranded DNA and can be limited to particular regions of a plasmid by using variations of the methods of Shortle and Nathans.[17] Single-stranded plasmid DNA containing the cloned subtilisin gene was annealed with a double-stranded DNA fragment containing all the vector sequences except for the subtilisin coding region. When the resulting gapped duplex molecule (Fig. 1) was subjected to sodium bisulfite mutagenesis, mutations were restricted to the single-stranded region containing the subtilisin gene. Reaction time and bisulfite concentration were varied in different experiments such that from one to five mutations on the average were created per subtilisin gene. After mutagenesis, the gapped molecules were treated in vitro with the Klenow fragment of DNA polymerase I to regenerate complete double-stranded molecules and fix the mutations. Competent *E. coli* cells then were transformed with the mutagenized DNA to produce an amplified library of mutated subtilisin genes. The variant library then was transformed into a *B. subtilis* strain carrying chromosomal deletions in the alkaline and neutral protease genes but capable of expressing and secreting plasmid-encoded subtilisin.[18]

The transformed *B. subtilis* cells were screened for variant protease activity by using tryptose blood agar plates containing 10 μg/ml kanamycin prepared as follows. Before plating the transformed cells, a nitrocellulose filter was placed directly on the agar plate and a cellulose acetate filter was placed on top of the nitrocellulose filter. The colonies thus grew on top of the cellulose acetate filter while the secreted subtilisin passed through the permeable cellulose acetate and bound to the nitrocellulose filter at positions corresponding to individual colonies. In this manner, subtilisin from hundreds of colonies was bound to a single filter, thus permitting the screening of thousands of variants by processing multiple filters. To detect filter-bound protease, the filters were soaked in a solution containing the subtilisin substrate, Tosyl-L-Arg methyl ester (TAME), and a pH indicator, phenol red, titrated to pH 9.0. As the TAME is hydrolyzed, the pH drops, and the phenol red changes color in localized areas on the filter containing esterase

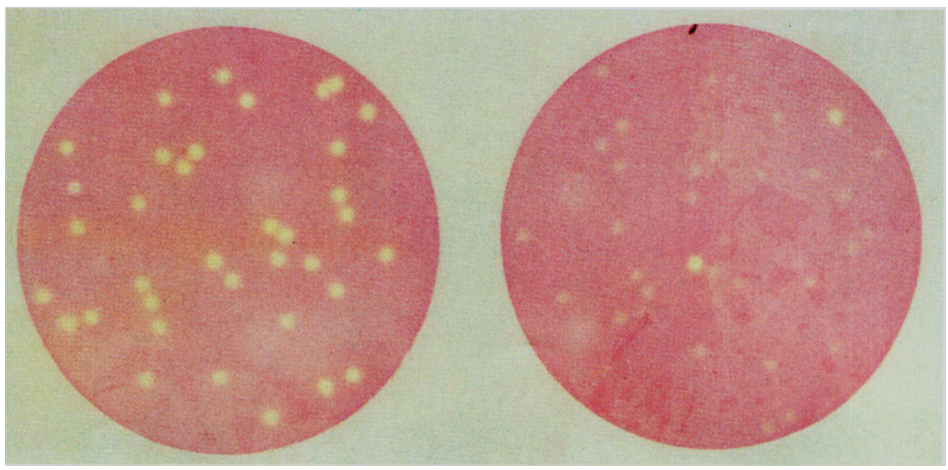

Fig. 2. Filter-bound esterase activity is shown before (left) and after (right) heat treatment. For the plates shown, *B. subtilis* was transformed with pGX4330 spiked with 5% of pGX4350 carrying the Asn 218 to Ser mutation and grown on cellulose acetate as described in the text. After heat treatment, the right filter has only two zones persisting in activity corresponding to colonies bearing pGX4350.

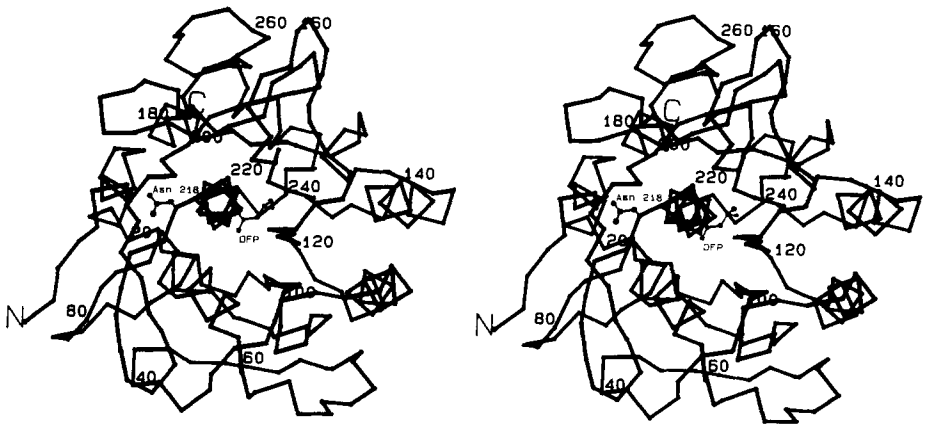

Fig. 3. Alpha carbon backbone model of wild-type subtilisin BPN' showing position of Asn 218. Also shown is the DFP inhibitor covalently attached to the catalytic Ser 221.

activity. Active subtilisin produces a distinct zone of yellow against a red background (Fig. 2).

To screen for thermostable variants, filters containing bound subtilisin were incubated at 70°C for 30 minutes before assaying for TAME hydrolytic activity. Because these conditions inactivate nearly all wild-type subtilisin, this procedure has allowed us to isolate several variants significantly more stable than the wild-type enzyme. Once thermostable variants were identified from the nitrocellulose filters, the corresponding colonies from the cellulose acetate filters were grown in liquid cultures (2% yeast extract, 10 μg/ml kanamycin) for 18 hours. Culture supernatants then were tested for the presence of thermostable subtilisin. To confirm that a thermostable subtilisin phenotype had resulted from a plasmid-borne mutation, plasmid DNAs from positive colonies were purified and used to retransform *B. subtilis*. In nearly all cases, the transformed *B. subtilis* strain produced a variant subtilisin with a phenotype identical to the original.

Although several thermostable subtilisins have been identified by the above procedure, one of the most interesting is a single-site variant, designated 7150, which is significantly more stable than wild type (see below). DNA sequencing of the 7150 gene revealed a single base-pair change resulting in the substitution of Asn 218 with Ser. The location of Asn 218 in the α-carbon backbone of subtilisin is highlighted in the model shown in Figure 3. In order to verify that the enhanced thermal stability of 7150 was due exclusively to the Asn 218 to Ser change, site-directed mutagenesis[19] was used to insert the same base-pair change into the wild-type subtilisin gene. The resulting variant subtilisin behaves identically in all respects to the original random variant.

CHARACTERIZATION OF THE SUBTILISIN VARIANT, 7150

Both wild-type subtilisin and the variant enzyme, 7150, were purified essentially as described by Bryan et al.[20] In all cases, purified protein appeared as a single major band upon sodium dodecyl sulfate–polyacryamide gel electrophoresis and isoelectric focusing. Subtilisin activity was assayed by monitoring the hydrolysis of 1.0 mM solutions of the peptide substrate succinyl-(L)-Ala-(L)-Ala-(L)-Pro-(L)-Phe-p-nitroanilide (SAAPF-pNA) at 25°C as described by DelMar et al.[21] The 7150 enzyme is somewhat more active against the synthetic peptide substrate (kcat/Km = $5.30\pm0.12\times10^5$ M^{-1} s^{-1}) than the wild-type enzyme (kcat/Km = $2.63\pm0.1\times10^5$ M^{-1} s^{-1}). The relative proteinase activity against azocasein of the 7150 enzyme is about 75% that of wild type.

The rates of thermal inactivation of 7150 and wild-type subtilisin at 65°C, pH 8.0, and in the presence of 10 mM $CaCl_2$ are shown in figure 4. Under these conditions the rate of thermal inactivation of wild-type subtilisin appears to obey first-order kinetics and exhibits a half-life ($t_{1/2}$) of 59 ± 3 min in agreement

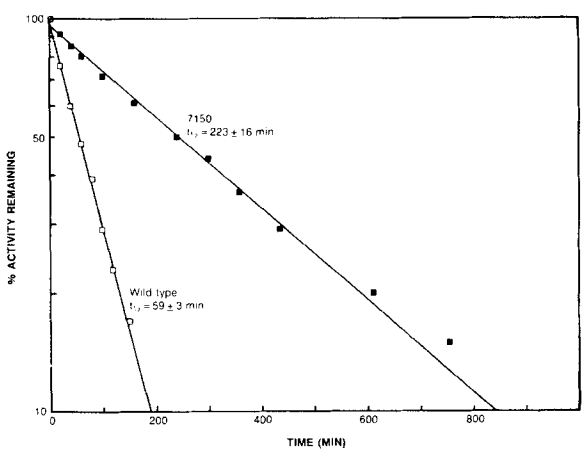

Fig. 4. Kinetics of thermal inactivation of wild-type (□) and variant 7150 (■) subtilisin at 65°C and pH 8.0 (50 mM Tris-HCl, 50 mM KCl) in the presence of 10 mM $CaCl_2$. The enzyme concentration in each case was 50 μg/ml and the samples were immersed in a thermostatted circulating water bath equilibrated at 65°C. At various time intervals, 10-μl aliquots were removed and diluted into 1.0 ml of peptidase assay solution equilibrated at 25°C. The straight lines are the least-squares fit with regression coefficients 0.9987 (wild type) and 0.9975 (7150).

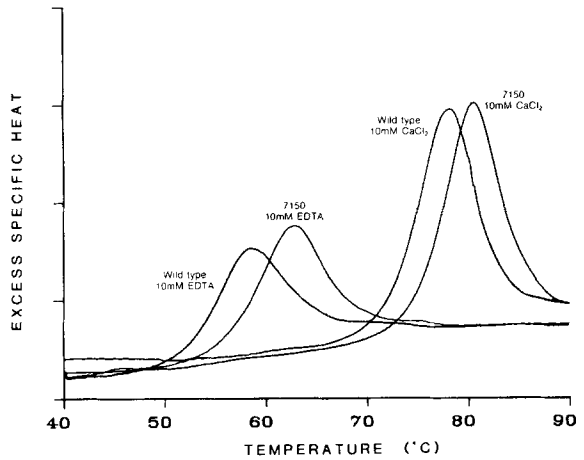

Fig. 5. Differential scanning calorimetry data for wild-type and 7150 subtilisin. These results were obtained for both enzymes under two extremes of free-calcium ion concentration: 10 mM EDTA and 10 mM $CaCl_2$ (as labeled). The excess specific heat as a function of temperature was measured with a Hart Scientific microcalorimeter interfaced to an IMB personal computer (XT). The temperature was increased at a rate of 1°C/min from 20°C to 90°C. The protein concentration for all samples was 3.0 ± 0.1 mg/ml, and the sample size was 0.70 ml. All solutions were buffered with 50 mM Tris-HC1, 50 mM KC1, pH 8.0, and were made 2 mM in N-dansyl-3-aminobenzene-boronic acid, a competitive inhibitor of subtilisin (see text).

with previously reported values.[22] The rate of thermal inactivation of the 7150 enzyme also appeared to be first order, but the half-life was found to be 223 ± 16 min, or approximately four fold greater. In the presence of the divalent cation chelating agent EDTA (1 mM), which has been previously shown to eliminate the well-known stabilizing effect of calcium ions,[22] the half-life for thermal inactivation of 7150 was still approximately threefold greater than that of the wild-type enzyme. In this case, however, the rate of inactivation must be determined at a lower temperature (e.g., 45°C) to obtain comparable values, and the kinetics are not simply first order. The enhanced stability of the 7150 enzyme was also demonstrated in thermal inactivation experiments conducted under a variety of other conditions including pH 10.5 (1 mM EDTA, or 10 mM $CaCl_2$), and with no added $CaCl_2$ or EDTA at pH 8.0 and 10.5.

To obtain an estimate of the relative thermodynamic stabilities of 7150 and the wild-type enzyme, we have employed differential scanning calorimetry (DSC), which measures the amount of excess heat absorbed by a protein sample as the temperature is increased through a transition from the folded to the unfolded state.[23] The thermal unfolding of subtilisin was found to be an irreversible process, as previously reported.[24] Since differential scanning calorimetry measures the heat capacity change with temperature, accurate estimates of the ΔH and other thermodynamic parameters of unfolding are often possible. With subtilisin, however, the autolysis that accompanies the unfolding process precludes the precise determination of many of these parameters.[24] In the studies reported here, this problem was circumvented to some extent by addition of the competitive inhibitor, N-dansyl-3-aminobenzeneboronic acid, which has Ki = 2 μM at pH 8.0.[25] Despite these precautions, however, the small amount of autolytic activity that remained prevented an accurate determination of ΔH. Even in the presence of the inhibitor, no heat can be recovered by reversing the temperature scan after the melting transition has occurred. Nevertheless, the midpoint for the thermal unfolding transition, Tm, where the concentration of native and unfolded states is approximately equal (i.e., $\Delta G \doteq 0$), was precisely determined form the positions of the thermal transition peaks. The excess specific heat absorbed by 7150 and wild-type subtilisin as a function of temperature is shown in Figure 5. The Tm of each enzyme was found to be highly dependent upon the free-calcium ion concentration. Wild-type subtilisin was found to have a Tm of 78.3 ± 0.1°C in the presence of 10mM $CaCl_2$ or 58.9 ± 0.2°C in the presence of 10 mM EDTA. These results are consistent with the calcium ion effect on the kinetics of thermal inactivation reported here and elsewhere.[22] The calorimetry results for 7150 are instructive since they illustrate a clear shift in the Tm to higher temperatures for both extremes of free-calcium ion concentration; the Tm is increased by 3.9° to 62.8 ± 0.1°C in the presence of 10 mM EDTA, and by 2.4° to 80.7 ± 0.1°C in the presence of 10 mM $CaCl_2$. Thus, the single change of Asn 218 to Ser results in a thermodynamically more stable protein molecule.

X-RAY CRYSTAL STRUCTURES

In order to analyze the structural basis for the enhanced thermal stability of the 7150 enzyme, high-resolution crystal structures were determined for both 7150 and wild-type subtilisin. Purified samples of both enzymes were first inhibited with diisopropylflurophosphate (DFP) to prevent autolysis and then di-

TABLE I. Summary of Least Squares Refinement at 1.8 Å

	Data collected	Data used $I > \sigma(I)$	r.m.s. Deviation of Final Models: Through bonds	Through bond angles	Through dihedral angles	R-factor*
Wild-type	15,033	14,189	0.019 Å	0.030 Å	0.040 Å	0.132
7150	17,346	15,166	0.017 Å	0.027 Å	0.034 Å	0.144

*The R-factor = $\Sigma|F_o - F_c|/\Sigma F_o$ and the r.m.s. deviation represents the root-mean-square deviation between atom pairs in the final models from distances expected from small molecule crystal structures.

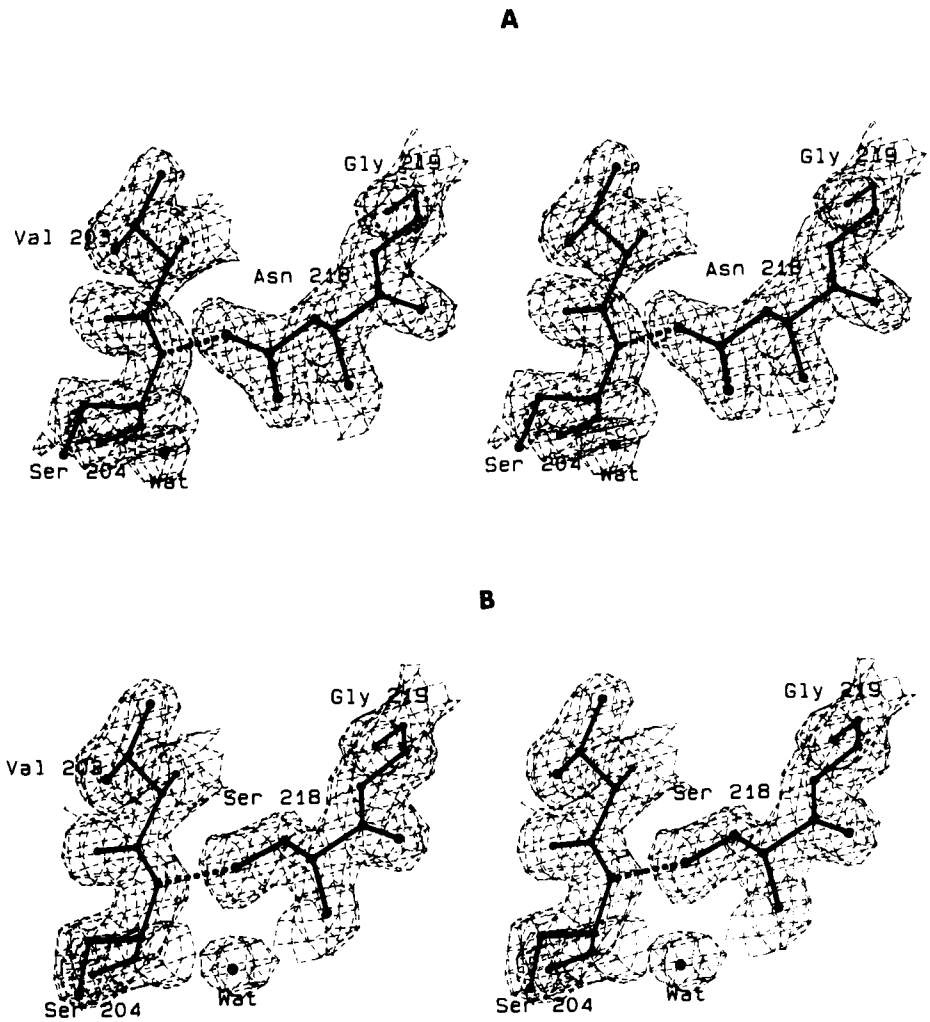

Fig. 6. Final 2Fo-Fc electron density maps for wild-type (**A**) and 7150 (**B**) subtilisin in the vicinity of the single amino acid substitution at position 218. Electron density (displayed on a 0.6-Å grid) clearly illustrates the position of side-chains in both molecules. The least-squares residual or R-factor $\Sigma|F_o-F_c|/\Sigma F_o$ for the wild-type and 7150 structures are, respectively, 0.13 and 0.14 for data from 10 Å to 1.8 Å. Atomic model geometry (bond distances, angles, and nonbonded contacts) has been tightly restrained to values found in small molecule crystal structures. The overall r.m.s. deviation from ideal bond distances is 0.019 Å for the wild-type model and 0.017 Å for 7150. (These numbers should provide a means of evaluating the quality for the molecular models.)

alyzed against 50 mM glycine-NaOH, pH 9.0. Crystals were grown by vapor diffusion against 55% acetone in hanging drops[26] and large single crystals of both 7150 and wild-type subtilisin were obtained within two to seven days. The crystals were isomorphous with the monoclinic crystal form reported by Drenth and Hol,[27] belonging to space group $P2_1$ with cell dimensions a = 41.6Å, b = 79.5 Å, c = 37.3 Å, and $\beta = 114.5°$.

Diffraction intensities were measured with a Xentronics imaging proportional counter mounted on a modified Supper oscillation camera utilizing an Elliott GX-21 x-ray source.[28] Single crystals were used to collect each data set to 1.8-Å resolution. For the wild-type enzyme, 37,209 observations of 15,033 unique reflections to 1.8 Å scaled to give Rmerge= 0.038 where Rmerge= $\Sigma((I-<I>)/\sigma(I))^2/\Sigma(I/\sigma(I))^2$. For 7150, 37,860 observations of 17,346 unique reflections scaled with Rmerge= 0.040.

An initial F(7150)−F (wild-type) electron density map exhibited significant difference density around position 218 consistent with the Asn to Ser change deduced from the DNA sequence.

The initial models were subjected to extensive crystallographic refinement by using restrained least-squares procedures.[29] All nonhydrogen protein atoms, 184 ordered water molecules, two acetone molecules, and two calcium atoms were included in the refinement.

Each model has been tightly constrained and refined to R<0.15. A summary of the refinement is presented in Table I.

The final, refined electron density maps for the region surrounding position 218 for both enzymes are shown in Figure 6.

Overall, structures of the two enzymes are identical with the exception of the region near the site of mutation. Superimposition of the models gives rise to

r.m.s. differences in C α positions of 0.07 Å and of all atoms in common of 0.10 Å. Differences in atomic positions larger than twice these values are considered significant. Such differences are confined to the immediate vicinity of residue 218 with a mean shift in the 218 backbone atoms of 0.30 Å. The mutation results in movement of Ser 218 and its immediate peptide neighbors toward Ser 204. Ser 204 and Asn (or Ser) 218 are components of an antiparallel β-pair formed by residues 202–219 with residues 204 and 218 directly opposite one another (Fig. 7).

Interestingly, Ser 204 is the site of a local irregularity in the anti-parallel β-pair structure known as a "β-bulge." A β-bulge occurs when an extra residue is inserted into a β-strand, giving rise to a short disruption of the normal antiparallel hydrogen bonding pattern.[30] In the subtilisin structure, either Val 203 or Ser 204 can be considered the "extra" residue that produces the bulge. In the wild-type enzyme, the side-chain carbonyl oxygen atom of Asn 218 is positioned above the plane of the antiparallel β-pair where it accepts a hydrogen bond from the Ser 204 peptide nitrogen, thereby imparting additional stability to the bulge conformation. In the 7150 enzyme, where Asn 218 is replaced by Ser, the Ser 218 side-chain hydroxyl group accepts the hydrogen bond from the peptide nitrogen of Ser 204. Thus, the overall hydrogen bonding pattern of the antiparallel β-pair and the β bulge are maintained in 7150. However, the Ser 218 and Ser 204 hydrogen bond of 7150 may be energetically more favorable because the serine hydroxyl group fits more readily in the plane of the Ser 204 peptide nitrogen atom than does the side-chain carbonyl oxygen atom of Asn 218 (Fig. 6). Moreover, the presence of a shorter side-chain at position 218 permits a closer packing of the two β-strands about the bulge, resulting in a shortening of one hydrogen bond by 0.16 Å and another by 0.10 Å (Fig. 7).

TABLE II. Stability of Subtilisin Variants at Position 218*

Variant	t½ of thermal inactivation at 65°C (min)	Tm (°C)
Ala 218	11 ± 0.5	75.0
Cys 218	35 ± 1.5	—
Asn 218 (wild type)	59 ± 3	78.3 ± 0.1
Asp 218	116 ± 9	—
Ser 218 (7150)	223 ± 16	80.7 ± 0.1

*Experiments carried out in the presence of 10 mM $CaCl_2$, 50 mM KCl, and 50 mM Tris-HCl pH 8.0 as described in Figures 4 and 5.

One final difference concerns the local, ordered water structure. In the Ser 218 mutant, a water molecule (labeled Wat in Fig. 6) is slightly repositioned, presumably to optimize hydrogen bonding with the Ser 218 side-chain.

Since the changes we see in hydrogen bonding distances are comparable to estimates of error in the atomic coordinates, we performed additional comparisons in order to justify our confidence in the significance of these small differences between the subtilisin models. As part of a broader study, a different 1.8-Å data set had been obtained from a single uninhibited 7150 cyrstal and the uninhibited structure had been refined to an R factor of 0.14. The uninhibited and DFP-inhibited 7150 structures are superimposable in the region of the 202–219 antiparallel β-pair to within 0.05 Å and exhibit the same differences when compared to the wild-type structure. Furthermore, the 1.8-Å wild-type structure determined in this study has been compared to a 1.3-Å wild-type structure[31] derived from protein purified under slightly different conditions. These two wild-type structures are also superimposable to within 0.05 Å in the region of the 202–219 antiparallel beta pair. Thus, comparison of

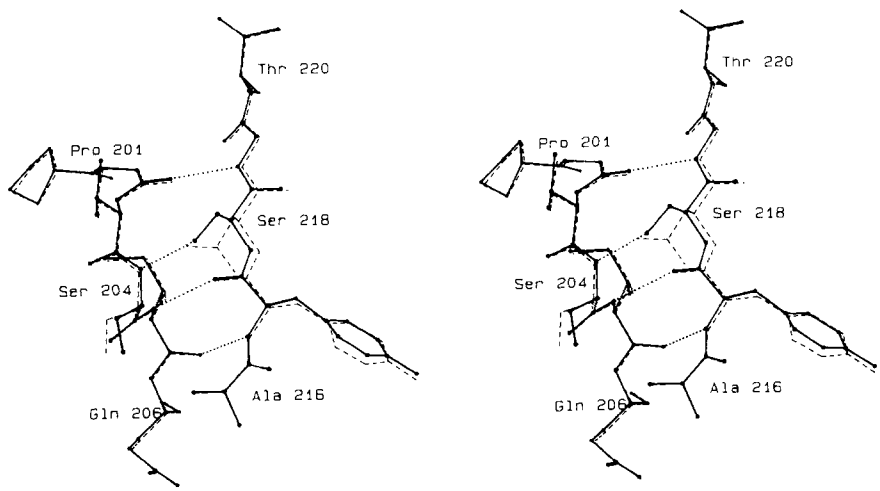

Fig. 7. Comparison of local conformation of wild-type (dashed lines) and 7150 subtilisin (solid lines) in the vicinity of residue 218. Dotted lines designate hydrogen bonds between β-strands of 7150 that are in common with those of the wild-type enzyme. As a result of the mutation, two interstrand hydrogen bonds (0202-N219 and 0217-N205) are shortened slightly (from 2.86 to 2.76 Å and from 2.93 to 2.77 Å, respectively). The hydrogen bond between Ser 204 peptide nitrogen and the side-chain oxygen at position 218 (OD218 or OG218) is longer in 7150 (2.87 vs. 2.77 Å) but exhibits better geometry (see text).

refined models derived from data obtained from different crystals indicates that the small changes in hydrogen bonding distances observed between 7150 and wild-type subtilisin at the site of the 218 mutation are real and significant.

SIGNIFICANCE OF THE ASN 218 TO SER MUTATION

Improvements in hydrogen bonding parameters of the 202–219 β-pair offer the simplest explanation for the enhanced stability of the 7150 subtilisin variant. Replacement of an Asn 218 with Ser, a smaller residue, allows the antiparallel strands to move closer together, thus allowing for slightly shorter and therefore stronger hydrogen bonds. This movement does not disrupt the hydrogen bond between the side-chain of residue 218 and the peptide nitrogen of residue 204. One problem in correlating structural alterations with a change in the free energy of stabilization is our total lack of information about the effect of the mutation on the unfolded form of the enzyme. This is an important consideration since ΔG is a measure of the difference in free energy between the folded and unfolded enzyme. To further investigate this point, we constructed subtilisin mutants with aspartic acid, cysteine, or alanine at position 218 by oligonucleotide-directed mutagenesis and compared their thermal stabilities with the 7150 variant and wild-type subtilisin. The relative stability of these variants based on thermal inactivation experiments and scanning calorimetry (Table II) is as follows: Ser > Asp > Asn > Cys > Ala.

This ranking is exactly as expected based on simple hydrogen-bonding considerations. Ser is the most stabilizing substitution because of the geometric and steric considerations discussed above. Asp at position 218 is presumably more stabilizing than Asn because it is a stronger H-bond acceptor due to the formal charge on the oxygen. The Cys variant is less stable than Asn because the sulfur is a poorer H-bond acceptor than the carbonyl oxygen. Finally, Ala at position 218 is the least stable variant in the group lacking a H-bond accepting group altogether. The difference in rate of thermal inactivation at 65°C between Ser 218 and Ala 218 is about 20-fold and the difference in melting temperature is 5.7°C. Because the stabilities of these variants at position 218 can be rationalized on the basis of hydrogen-bonding considerations, we are somewhat more confident that changes in ΔG are due primarily to improved interactions in the folded enzyme.

The 7150 subtilisin variant clearly has a larger free energy of stabilization, but due to protein autolysis, we have not been able to accurately determine ΔH and the other thermodynamic parameters of unfolding. It is instructive, however, to compare the Tm results reported here with DSC data reported for single-site variants of other proteins. A 1.8°C increase (to 58°C) in the Tm for thermal unfolding of the α subunit of a variant (Gly 211 → Glu) of tryptophan synthase was observed to be due to an increase of 0.6±0.2 kcal/mole in the ΔG of unfolding.[15] Similarly, a 5.9°C increase (to 52°C) in the Tm for unfolding of the amino-terminal domain of phage repressor with a Gln 33→ Tyr mutation was found to be due to an increase in the ΔG of unfolding of 1.3 kcal/mole.[16] By interpolation of these data, we estimate that the 3.9°C increase in Tm observed for the 7150 enzyme relative to wild type in the same temperature range (i.e., near 60°C) is due to an increase of roughly 1 kcal/mole in the ΔG of unfolding of subtilisin (i.e., the free energy of the native form is decreased by about 1 kcal/mole relative to the unfolded form). This increase in stability is significant when one considers that the total ΔG for the unfolding of most proteins in solution is on the order of 5–15 kcal/mole near physiological temperature and pH.[23,32]

In order to further analyze the significance of the Asn 218 to Ser change, we have compared the homologous primary sequences of eight microbial proteases that belong to the subtilisin family. These included five from *Bacillus* strains[33–37] (subtilisin BPN′, subtilisin Carlsberg, subtilisin DY, subtilisin amylosachariticus, and mesenticopeptidase) along with thermitase from *Thermoactinomyces vulgaris*[38] and two fungal proteases, proteinase K[39] from *Tritirachium album* and thermomycolase[40] from the thermophilic fungus, *Malbranchea pulchella*. These enzymes have been shown to be related to subtilisin BPN′, not only through their primary sequences and enzymological properties, but also by comparison of x-ray crystallographic data.[41,42] Thermitase, thermomycolase, and proteinase K have been generally considered more stable than their counterparts from *Bacillus*.[22,43,44] It is therefore intriguing to find that these three enzymes have a Ser at position 218 while all five *Bacillus* enzymes have Asn at this position. Since our current work has shown that the Asn 218 to Ser mutation leads to a more thermostable enzyme, there may have been selective pressure on the fungal and *Thermoactinomyces* enzymes to have Ser at this position due to the requirements for enhanced thermal stability.

In this regard, Shortle and Lin[13] recently provided evidence from studies on staphylococcal nuclease which indicated that a limited number of sites may play a disproportionately large role in the structural stability of a protein. Even so, assignment of a unique role for position 218 in the selection of thermostable subtilisins is not justifiable at this point. Based on our experience thus far, less than 10^3 randomly mutagenized subtilisin variants must be screened to find one with increased thermal stability.[45] Because we have been able to isolate a number of other stabilizing mutations in subtilisin, we would argue against some unique role for the Ser 218 mutation in the evolution of thermostable subtilisins. Rather, it appears more likely that the Asn 218 to Ser change

represents one of a number of possible variations of the subtilisin primary sequence capable of increasing the free energy of unfolding. Of course, our goal in these studies is to learn how to predict where such changes should be made. The occurrence of the mutation at an unusual element of secondary structure, the β-bulge, may prove to be of some predictive value. Appropriate mutations in analogous structural elements of other proteins might also result in enhanced thermostability.

This initial example of a detailed comparison between the x-ray structures of a native protein and a thermostable single-site variant demonstrates how very subtle and highly localized changes in structure can result in a significant change in thermostability. Additionally, our results illustrate the importance of well-refined, high-resolution structures in understanding the differences between wild-type and variant proteins. The structural differences between 7150 and wild-type subtilisin very likely would have gone undetected at lower resolution using less well-refined models. Thus, in this early stage of protein engineering, it is important to select model systems for which it is possible to obtain high-resolution crystal structures.

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