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Engineering Subtilisin and Its Substrates for Efficient Ligation of Peptide Bonds in Aqueous Solution

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ABSTRACT: Protein engineering techniques were used to construct a derivative of the serine protease subtilisin that ligates peptides efficiently in water. The subtilisin double mutant in which the catalytic Ser221 was converted to Cys (S221C) and Pro225 converted to Ala (P225A) has 10-fold higher peptide ligase activity and at least 100-fold lower amidase activity than the singly mutated thiolsubtilisin (S221C) that was previously shown to have some peptide ligase activity [Nakatsuka, T., Sasaki, T., & Kaiser, E. T. (1987) *J. Am. Chem. Soc. 109*, 3808-3810]. A 1.5-Å X-ray crystal structure of an oxidized derivative of the double mutant (S221C/P225A) supports the protein design strategy in showing that the P225A mutation partly relieves the steric crowding expected from the S221C substitution, thus accounting for its improved catalytic efficiency. Stable and synthetically reasonable alkyl ester peptide substrates were prepared that rapidly acylate the S221C/P225A enzyme, and aminolysis of the resulting thioacyl-enzyme intermediate by various peptides is strongly preferred over hydrolysis. The efficiency of aminolysis is relatively insensitive to the sequence of the first two residues in the acyl acceptor peptide whose α-amino group attacks the thioacyl-enzyme. To obtain greater flexibility in the choice of coupling sites, a set of three additional peptide ligases were engineered by introducing mutations into the parent ligase (S221C/P225A) that were previously shown to change the specificity of subtilisin for the residue nearest the acyl bond (the P_1 residue). The specificity properties of the parent ligase and derivatives of it paralleled those of wild type and corresponding specificity variants. The set of specific peptide ligases should be useful for blockwise synthesis or semisynthesis of proteins in aqueous solution.

Chemical approaches for synthesis and engineering of proteins offer many advantages to recombinant methods in that one can incorporate nonnatural or selectively labeled amino acids. However, peptide synthesis is practically limited to small proteins (typically <50 residues) due to the accumulation of side products and racemization that complicate product purification and decrease yields [for recent reviews see Kaiser (1989) and Offord (1987)].

Proteolytic enzymes, in particular serine proteases, have been used as alternatives to synthetic peptide chemistry because of their stereoselective properties and mild reaction conditions [for reviews see Kullmann (1987) and Chaiken (1981)]. Such enzymes have also been used to complement chemical coupling methods and allow larger proteins to be synthesized by blockwise enzymatic coupling of synthetic fragments [Inouye et al., 1979; for review see Chaiken (1981)]. However, the narrow substrate specificities and hydrolytic activities of serine proteases have limited their use in peptide synthesis.

A central problem in the use of serine proteases for peptide synthesis is that hydrolysis of the acyl-enzyme intermediate is strongly favored over aminolysis (Figure 1). The partitioning may be effectively shifted to favor aminolysis by catalyzing the reaction in mixed or pure organic solvents (Coletti-Previero et al., 1969; Barbas et al., 1988). However, enzymes are insoluble and often less stable in organic solvents (Wong et al., 1990), and kinetic activation barriers are higher for the charged transition states involved. An alternative approach was presented by Kaiser and co-workers (Nakatsuka et al., 1987) who showed that thiolsubtilisin, a derivative of the bacterial serine protease in which the active-site Ser221 was chemically converted to a Cys (S221C), shifted the preference for aminolysis relative to hydrolysis by >1000-fold. This is likely due to the inherently greater kinetic lability of thioesters toward amines as opposed to water (Chu & Mautner, 1966). On the basis of similar principles, Wu and Hilvert (1989) showed that selenolsubtilisin had a 14000-fold shift in preference for aminolysis over hydrolysis. However, the catalytic efficiencies by either thiol- or selenolsubtilisin for aminolysis of chemically activated esters are about 10^2-10^4-fold below the esterase activity of wild-type subtilisin. Thus, to promote acylation of thiolsubtilisin a chemically active *p*-chlorophenyl ester was used to activate an octapeptide for ligation with a tetrapeptide in 50% DMF. However, such activated esters present synthetic difficulties as well as creating substrates prone to uncatalyzed hydrolysis in aqueous solvents (Nakatsuka et al., 1987).

Here we describe the design and high-resolution structure of a variant of thiolsubtilisin whose ligase activity is enhanced

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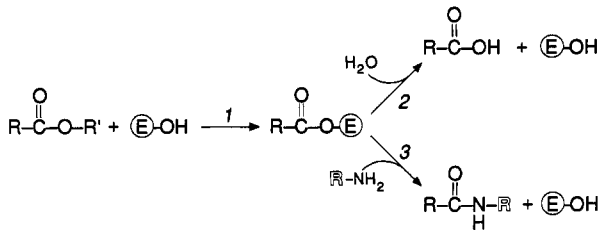


FIGURE 1: Diagram showing the production of the acyl-enzyme intermediate (step 1) from the free enzyme (Ⓔ–OH) and ester. This intermediate is partitioned either by hydrolysis (step 2) or aminolysis (step 3) to regenerate the free enzyme and products. Amide hydrolysis is the reverse of step 3 followed by step 2.

10-fold and amidase activity reduced more than 100-fold compared to S221C. Alkyl ester substrates are presented that are chemically easy to synthesize and rapidly acylate the variant of thiolsubtilisin. This enzyme, and three specificity derivatives of it, rapidly ligate peptides in aqueous solution and should be useful for blockwise synthesis or semisynthesis of proteins.

Materials and Methods

Materials. Enzymes for DNA manipulations were from New England Biolabs or Bethesda Research Labs. Oligonucleotides were synthesized by the DNA synthesis group at Genentech. All peptides contain L-amino acids unless otherwise indicated and were synthesized by standard methods (Barany & Merrifield, 1980). DL-Dithiothreitol (DTT),[1] DTNB, HEPES, 2-mercaptoethanol, MES, NEM, TFA, Tween 80, Tricine, DMSO, DMA, and the substrates s-Ala-Ala-Pro-Phe-*p*NA and s-Ala-Ala-Pro-Phe-Sbz were from Sigma. Ethanol and acetonitrile were from J. T. Baker Inc., and ammonium sulfate from ICN Biochemicals Inc. 2-Methyl-2,4-pentanediol was from Aldrich Chemical Company, Inc. The boronic acid inhibitors were provided by Dr. Charles Kettner, E. I. du Pont de Nemours and Co., Inc. Dipeptides Ala-Phe-amide, Arg-Gly-amide, Arg-Phe-amide, Gly-Ala-amide, Gly-Leu-amide, Gly-Phe-amide, Leu-Phe-amide, and Phe-Gly-amide and the tripeptide Ala-Phe-Ala-amide were obtained from BACHEM Feinchemikalien AG. Activated thiol Sepharose as well as G-25 and G-75 Sepharose were obtained from Pharmacia LKB Technology AB.

Peptide Ester Synthesis. The peptides were synthesized manually via solid-phase methodology by using *t*-Boc chemistry (Barany & Merrifield, 1980). The lactic acid was coupled as its sodium salt by using the BOP reagent [benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate]. The glycolic acid was incorporated as the *tert*-butyl ether. Removal of the *tert*-butyl ether was accomplished with 50% trifluoroacetic acid in methylene chloride. Coupling of the subsequent amino acid to form the ester bond required the catalytic addition of 5 mol % (dimethylamino)pyridine to the coupling media. The peptides were cleaved from the resin with hydrogen fluoride. Crude peptides were cleaved from the resin with hydrogen fluoride. Crude peptides were purified by HPLC and analyzed by mass spectrometry.

Mutant Design and Construction. Molecular modeling was performed on an Evans and Sutherland PS300 using the program FRODO (Jones, 1978) and coordinates from a 1.8-Å resolution structure of subtilisin BPN′ *Bacillus amyloliquefaciens* (Bott et al., 1988). The S221C mutation was introduced (Carter et al., 1986) into the wild-type *B. amyloliquefaciens* subtilisin gene (Wells et al., 1983) by using the oligonucleotide 5′-ACAACGGTACC*TG*C*ATGGCA-TCTCC-3′ (asterisks indicate altered nucleotides and underlined is a unique *Kpn*I site). The S221C/P225A mutations were introduced into the S221C template by using the oligonucleotide 5′-GCGTACAACGGTACT*TGCATGGCA-TCTG*CGCACGTTGCC-3′ (asterisks indicate altered nucleotides and underlined is a new *Fsp*I site) by restriction–selection (Wells et al., 1986) against the *Kpn*I site. The construction of the mutants G166E and E156Q/G166K was described by Wells et al. (1987a) and that of the mutant G166I by Estell et al. (1986). Combinations of the mutations around the active site (positions 221 and 225) and around the P_1 binding pocket (156 and 166) were obtained by ligation of mutated restriction fragments split by the enzyme *Ppu*MI. All mutants were verified by dideoxy sequencing (Sanger et al., 1977). The mutated gene encoding the P225A mutant was provided by T. Graycar (Genencor, South San Francisco, CA).

Expression and Purification of Variant Subtilisins. The subtilisin gene in the M13–*Escherichia coli*–*Bacillus subtilis* shuttle plasmid pSS5 (Carter & Wells, 1987) was expressed in a *B. subtilis* host strain (BG2036) that is lacking its endogenous subtilisin and neutral protease genes (Yang et al., 1984). Since maturation of subtilisin involves autoproteolytic removal of the prosequence (Power et al., 1986), the mutants with reduced protease activity had to be expressed in the presence of active subtilisin. This was done either by adding a small amount of purified subtilisin (to a final concentration of 500 μg/L) late in the logarithmic growth phase or by coculturing from an inoculum of BG2036 containing 0.1% wild-type subtilisin expressing cells (Carter & Wells, 1987).

The purification of inactive subtilisin mutants was essentially as described (Carter & Wells, 1987) except that an equal amount of cold ethanol was added to the supernatant to precipitate impurities prior to the precipitation of subtilisin by the addition of two additional volumes of ethanol and the CM-Trisacryl was substituted by SP-Trisacryl M in the ion-exchange chromatography step. For the S221C mutants, the active-site cysteine was utilized for purification on activated thiol-Sepharose. This latter step is essential in order to separate the mutant proteins from any traces of wild-type "helper" subtilisin. The equivalent step in the original procedure used a cysteine residue introduced on the surface of the protein that results in efficient removal of wild-type activity (Carter & Wells, 1987, 1988). The mutant P225A is capable of autoproteolytic processing and therefore was cultured without helper subtilisin and purified by standard procedures (Estell et al., 1985). Aliquots of purified enzymes were flash-frozen and stored at −70 °C until analyzed.

[1] Abbreviations: DMA, dimethylacetamide; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, DL-dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate, s-Ala-Ala-Pro-Phe-*p*NA, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide; s-Ala-Ala-Pro-Phe-Sbz, the thiobenzyl ester of the same succinylated peptide; TFA, trifluoroacetic acid; Tricine, *N*-[tris(hydroxymethyl)-methyl]glycine; Tris, tris(hydroxymethyl)aminomethane; E-Ac, acyl- or thioacyl-enzyme intermediate; glc, glycolate ester; lac, L-lactate ester; Sbz, thiobenzyl ester; MeO-Suc, methoxysuccinyl; F_o, observed reflection amplitude; F_c, calculated reflection amplitude; ϕ_c, calculated phase. Mutant proteins are designated by the wild-type residue (single-letter amino acid code) followed by their position and the mutant residue. Multiple mutants are separated by a slash. For example, S221C/P225A indicates that the serine at position 221 and the proline at position 225 have been replaced by cysteine and alanine, respectively. Protease substrate residues are designated by using the nomenclature of Schechter and Berger (1967), NH_2–P_n ... P_2–P_1–C(=O)–NH–P_1'–P_2' ... P_n'–COOH, where the scissile peptide bond is between the P_1 and P_1' residues.

Table I: Data Collection and Refinement Statistics

crystal dimensions (mm)	0.25 × 0.25 × 0.25
total no. of observations ($I > 0$)	127954
no. of observations accepted	118946
no. of unique reflections accepted	32538
completeness (%)	90
merging R factor[a]	0.069
refinement resolution (Å)	8.0–1.5
no. of unique data points used in model refinement ($F_o > 2\sigma$)	31934
total no. of atoms in model	2113
no. of solvent molecules in model	169
rms deviation from ideality	
bond distance (Å)	0.012
bond angle (degrees)	2.454
B factor bond distance (Å^2)	2.35
B factor bond angle (Å^2)	3.55
crystallographic R factor	0.176

[a] $R_{merge} = (\sum|I - \langle I\rangle|)/\sum|I|$.

Crystallization. To prevent possible autolysis during crystallization the protein was incubated overnight at 4 °C with the active-site-directed inhibitor MeO-Suc-Ala-Ala-Pro-boro-Phe-OH (Kettner & Shenvi, 1984). Crystals were grown by vapor diffusion. A solution of 4 mg/mL protein in 16% saturated $(NH_4)_2SO_4$, 1% 2-methyl-2,4-pentanediol, 4 mM $CaCl_2$, and 40 mM Tris at pH 8.0 was equilibrated with a reservoir containing 50% saturated $(NH_4)_2SO_4$, 1% 2-methyl-2,4-pentanediol, 5 mM $CaCl_2$, and 50 mM Tris at pH 8.0. Crystals with dimensions 0.25 × 0.25 × 0.25 mm were grown in 3 weeks. The space group of the crystals was $P2(1)2(1)2(1)$ with cell dimensions of a = 39.30 Å, b = 72.99 Å, and c = 76.50 Å. These crystals are not isomorphous with wild-type subtilisin BPN′, which has a 1.3 Å shorter c dimension (75.20 Å) (Bott et al., 1988).

X-ray Data Collection. The data were collected with the Enraf Nonius FAST Area Sensitive TV detector and a Rigaku rotating anode generator (Cu Kα radiation, 45 kV, and 110 mA). The MADNES data processing software (Messerschmidt & Pflugrath, 1987) was used in data collection and data reduction. PROCOR software was used for profile fitting and internal scaling (Kbasch, 1988).

The crystal was mounted on the goniostat at $\chi = 0°$ with b^* parallel to the rotation axis. The crystal to detector distance was 40.0 mm. The detector swing angle was −32° to give a maximum resolution of 1.5 Å. A total of 155° of data was collected at 120 s/0.10° frame. To complete the sphere of data, 60° of data were collected at 120 s/0.10° frame with the crystal oriented at $\chi = 90°$. Limitations in the analog-to-digital converter caused ~10% of the reflections to 3-Å resolution to saturate the detector. For this reason, 108° of data were recollected for 30 s/0.10° frame with X-ray generator settings of 40 kV and 30 mA (detector swing angle = −8°, $\chi = 0°$). The data were scaled with an overall scale factor and B factor. A total of 118946 reflections were merged to give 32538 unique data (90% of theoretical) with a merging $R(I)$ factor = 0.069. Data collection and refinement statistics are summarized in Table I.

Refinement. The starting model used for refinement was that of a wild-type subtilisin–boronic acid inhibitor (MeO-Suc-Ala-Ala-Pro-boro-Val-OH) complex (K. Butcher and A. Kossiakoff, unpublished results). This complex crystallized in a cell (a = 39.21 Å, b = 72.95 Å, and c = 77.23 Å) that was more isomorphous with the S221C/P225A mutant than uncomplexed wild-type subtilisin BPN′. The model of the complex had been partially refined (1.6 Å, R = 0.21) from the subtilisin BPN′ coordinates (Bott et al., 1988). No solvent molecules were included in the model and there was no evidence in the electron density maps for the presence of a bound boronic acid inhibitor. The only significant changes in this model and the wild-type subtilisin BPN′ model were at crystal contact points and the conformations of several side chains.

All refinement was done with the X-PLOR package (Brünger, 1990), which combines energy minimization or molecular dynamics with X-ray diffraction data during the model refinement. Because the crystals were not completely isomorphous with the starting structure, the model was reoriented with rigid body refinement in X-PLOR (Δx = −0.06 Å, Δy = −0.90 Å, and Δz = 0.17 Å). Refitting of the structure was done by using the FRODO graphics program (Jones, 1978) on an Evans and Sutherland PS390. Simulated annealing in X-PLOR was used to allow the structure to move out of local energy minima if required. With 10.0–2.0-Å data, the structure was allowed to adjust for 0.5 fs/step for 50 steps in each loop of dynamics from 4000 to 300 K. Twelve rounds of positional and B-factor refinement followed by model building were performed at 8.0–1.5-Å resolution. Minimization was considered complete when the total energy gradient norm was less than 0.0001 kcal/mol. The final crystallographic R factor for the protein model and one SO_4^{2-}, one Ca^{2+}, and 169 water molecules, was 0.176.

Kinetic Assays. The esterase and amidase activities were obtained from initial rate measurements on a Kontron Uvikon 860 spectrophotometer. The assay for esterase activity utilized the substrate s-Ala-Ala-Pro-Phe-Sbz at (25 ± 0.2 °C) in 100 mM Tris-HCl (pH 8.60), 4% (v/v) dimethyl sulfoxide, and 0.005% (v/v) Tween 80. For non-cysteine-containing proteases, DTNB (Ellman, 1959) was added to a concentration of 37.5 μM to visualize the release of benzylthiol upon hydrolysis of the substrate. For the S221C variant proteases, we could not use DTNB because it reacts with the active-site thiol. Therefore, the difference in absorbance at 250 nm between the Sbz-substrate and the hydrolyzed products (tetrapeptide and benzylthiol) was used to monitor the reaction directly. The amidase activities were measured under identical conditions by following the increase in absorbance at 412 nm upon release of p-nitroaniline from s-Ala-Ala-Pro-Phe-pNA.

Enzymatic ligations of peptides were performed at 25 ± 0.2 °C in 90 mM Tricine (pH 8.0), 2% (v/v) dimethylacetamide, and 0.005% (v/v) Tween 80. The steady-state kinetic parameters for substrates with different leaving groups were obtained from initial rate measurements for ligation with variable substrate concentrations (50 μM–2 mM) and a fixed concentration of the dipeptide Ala-Phe-amide (3.1 mM). For substrates with high values of K_M, the errors in K_M and k_{cat} are larger than for other substrates but the k_{cat}/K_M values are of equivalent accuracy. Ligation reactions comparing the efficiency of ligation by using the P_1-specificity mutants with alternative substrates (s-Ala-Ala-Pro-Xaa)-glc-Phe-amide) with the nucleophile (Ala-Phe-amide) at 1.5 mM were performed at low substrate concentrations (52-69 μM) under the same conditions as above.

Aminolysis with di- and tripeptides were performed at low concentrations of the nucleophilic peptides. The aminolysis rate (v) = $k_{aminolysis}$ [N][E-Ac]/K_N, where [N] is the nucleophile concentration, [E-Ac] is the concentration of acyl-enzyme intermediate, and K_N is the dissociation constant for the binding of the nucleophile to the acyl-enzyme intermediate (Riechmann & Kasche, 1984, 1985). Since K_N = [N][E-Ac]/[N·E-Ac], a change in [N] will result in a change in [E-Ac]. At low [N], where [E-Ac] ≫ [N·E-Ac], v/[E-Ac] = $k_{aminolysis}/K_N$ (the apparent second-order rate constant for the reaction between each nucleophile and the acyl-enzyme

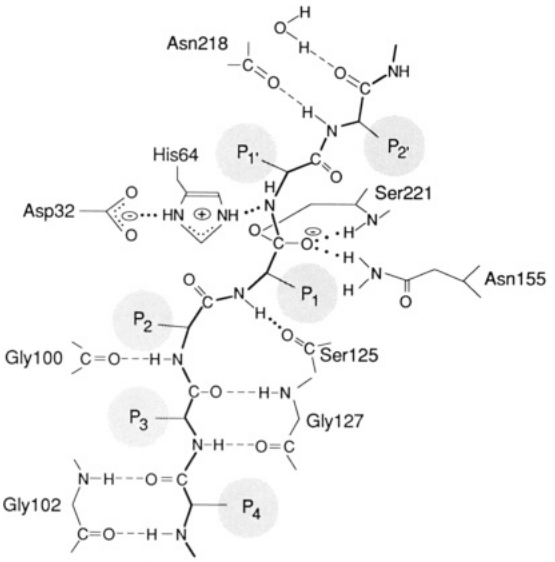


FIGURE 2: Structural diagram showing a model peptide substrate (bold lines) in a transition-state complex with subtilisin [adapted from McPhalen and James (1988)]. The substrate from P_4 to P_2' (Schechter & Berger, 1967) binds in an extended conformation in which most of its main-chain amides form hydrogen bonds with corresponding main-chain amides on the enzyme (positions labeled). The catalytic triad (Asp32, His64, and Ser221) and the oxyanion binding site (Asn155 and Ser221) are shown. Hydrogen bonds thought to form in the transition-state complex are indicated by dotted lines.

intermediate). Thus the relative catalytic efficiencies ($k_{aminolysis}/K_N$) can be approximated for the different nucleophiles from ratios of aminolysis rates measured at low nucleophile concentrations and high ester concentration. The concentrations of the peptide nucleophiles and the calibration of the absorbance data for the different ligation products was obtained from amino acid compositional analysis. The rates of peptide ligation were measured at four or five different concentrations for each nucleophile.

Ligation reactions were analyzed by taking aliquots at different times and analyzing the peptide products by C-18 reversed-phase HPLC. Peptides were eluted in a gradient of acetonitrile/0.1% TFA in water, and the absorbance at 214 nm was monitored. Amino acid composition was used to confirm both the hydrolysis and ligation products and to calibrate the absorbance values. The chemical compositions of the hydrolysis and ligation products (with the dipeptide Ala-Phe-amide as a nucleophile) were confirmed by mass spectrometric analysis.

RESULTS

Protein Design. The lower activity of thiolsubtilisin may result from steric crowding in the active site (Figure 2) because the covalent radius of sulfur is significantly larger than that of oxygen (1.03 Å versus 0.65 Å, respectively; Pauling, 1960). One way to make more room for the substrate would be to alter the position of the γ-thiol. A mutation (P225A) in a highly conserved proline residue in the α-helix that supports Ser221 is known to move the γ-hydroxyl of Ser221 away from the oxyanion hole and the catalytic histidine by 0.5–1.0 Å (Caldwell et al., 1989). We therefore prepared the S221C/P225A double mutant and determined its X-ray crystal structure.

1.5-Å Resolution Structure of S221C/P225A Subtilisin BPN′. A $F_{o(mutant)} - F_{c(wt)}$, $\phi_{c(wt)}$ difference map indicated two principal areas of change in the S221C/P225A variant structure compared to the wild-type subtilisin model (BPN′). The first region comprises residues 16–22, which are at a crystal contact point and presumably arise from observed packing differences in the two cells. The second region is found in the segment of α-helix that includes the P225A mutation site. Two major peaks (of magnitude 7σ) in the difference map were found 1.5 Å from the Sγ of Cys221. These peaks were ascribed to two oxygens indicating the presence of the sulfone of Cys221 in the crystal structure. Mass spectral analysis confirmed that the crystalline enzyme had an additional mass equal to two oxygens. However, the enzyme used for the kinetics analysis was shown by mass spectral analysis not to be modified. The oxidation of Cys221 in the crystals reflects the fact that it required 3 weeks to grow crystals and

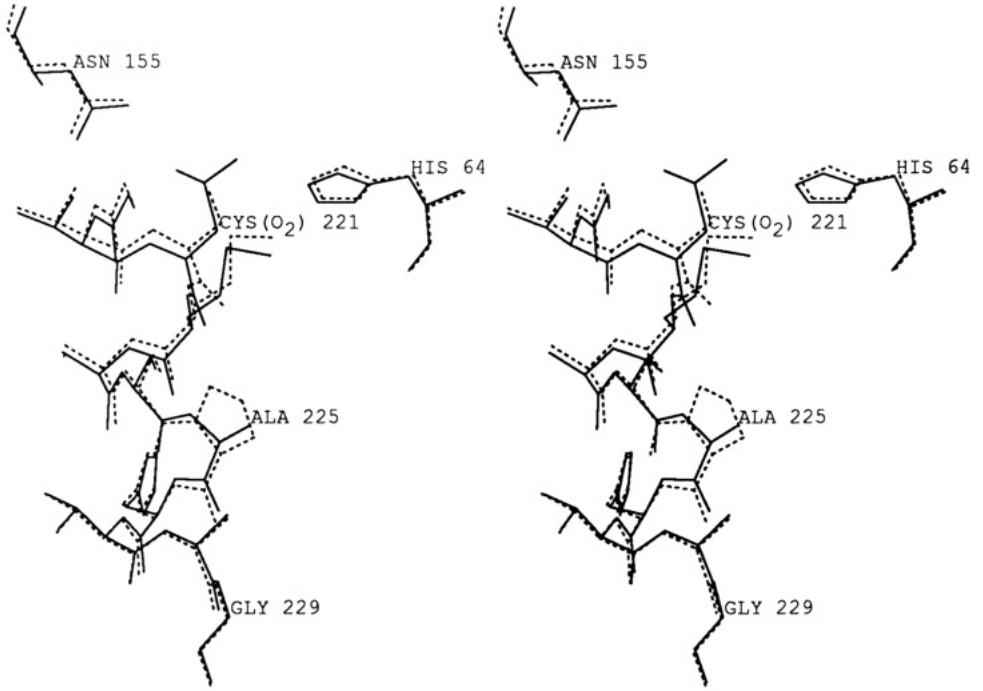


FIGURE 3: Stereographic view of wild-type subtilisin BPN′ (dashed lines) and the S221C/P225A mutant (solid lines) superimposed in the region of the active site and the α-helix that supports residue 221. The sulfur in Cys221 is shown in the oxidized form as observed in the crystal structure.

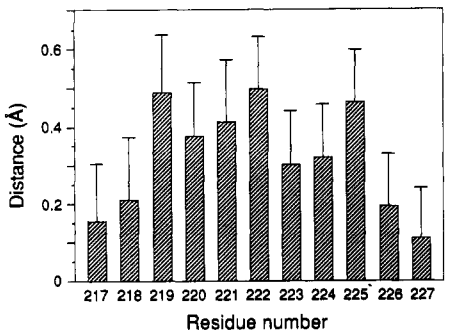


FIGURE 4: Histogram showing the distances and standard deviations between the Cα positions of residues 217–227 of wild-type subtilisin BPN′ and the S221C/P225A mutant. Standard deviations were calculated as in Perry et al. (1990).

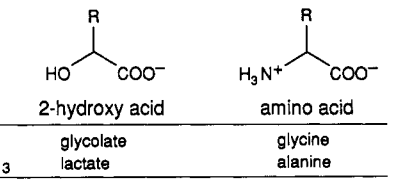
Table II: Comparison of the Distances between the Main-Chain Nitrogens (Donors of Hydrogen Bonds) and the Main-Chain Carbonyl Oxygens (Acceptors of Hydrogen Bonds) of the α-Helix That Supports Residue 221

acceptor residue no.	donor residue no.	distance in wild type (Å)	distance in mutant (Å)
224	228	2.99	2.96
223	227	3.64	3.38
222	226	4.35	3.40
221	225	4.37	3.29
220	224	3.12	2.84
219	223	3.47	3.36

crystallization was done in the absence of reductants.

Figure 3 shows the superposed models of the wild-type subtilisin BPN′ and the P225A structures (rms deviation = 0.186 Å for least-squares superposition of α-carbons). The most significant differences in position are in the segment of chain between residues 218 and 226 and have a maximal shift in Cα (0.49 Å) at residue 222 (Figure 4). The Asn155 side chain is also seen to move, keeping its H-bonding interaction with the peptide amide of Thr220 (Asn155 Oδ1–N220 distance, 2.76 Å in wild-type subtilisin BPN′ and 2.85 Å in S221C/P225A). This movement results in the Nδ2 of Asn155, which is part of the oxyanion binding site, being closer (3.93 Å) to Sγ of Cys221 than to the Oγ of Ser221 (4.92 Å).

The direction of the displacements of the residues preceding the P225A mutation clearly indicates a concerted shift of the N-terminal end of the helix. This concerted shift has the effect of pushing potential helix H-bonding pairs closer together (by up to 1.08 Å for N221–O225), although most of these distances remain longer than those normally regarded as good H-bonding distances (Table II). The ordered water structure near this α-helix does not change. At the active site there is seen a deletion of two waters from the wild-type subtilisin BPN′ structure whose van der Waals radii would overlap the oxygen peaks of the Sγ of Cys221. Thus, although the Cys221 Sγ is oxidized, we do not expect this to alter the position of its supporting α-helix. In fact, the two oxygens overlay those in typical boronic acid complexes in which one is H-bonded in the oxyanion binding site while the other is H-bonded to the Nε2 of His64.



	2-hydroxy acid	amino acid
R = H	glycolate	glycine
R = CH_3	lactate	alanine

FIGURE 5: Structures of 2-hydroxy acids (and corresponding amino acids) used to construct alkyl ester donor substrates.

Kinetic Analyses of Mutant Subtilisins. The individual P225A and S221C mutations lead to large reductions in amidase activity and smaller reductions in esterase activity as evidenced by their catalytic efficiencies (k_{cat}/K_M) against *p*-nitroanilide and thiobenzyl ester substrates, respectively (Table III). The combination of these two mutants (S221C/P225A) further reduces the amidase activity below our level of accurate detection (>10^7-fold reduction from wild type). However, the double mutant exhibits a 10-fold *increase* in esterase activity compared to S221C subtilisin. The S221C/P225A enzyme retains an aminolysis/hydrolysis ratio that is 500-fold improved over that of wild-type subtilisin BPN′. Even though this ratio is 10-fold below the S221C parent, it is sufficient to give over 95% aminolysis and without hydrolysis of the peptide adduct (see below). The k_{cat} values presented in Table III are minimal estimates of turnover number because active-site titrations were not determined for the S221C variant enzymes. However, the mass spectral analysis of the purified S221C/P225A enzyme indicates little or no oxidation of the Cys221. Moreover, the S221C-containing enzymes were purified by binding to an activated thiol-Sepharose column and elution with DTT (Carter & Wells, 1987).

Optimizing the Donor Ester Substrate. Although activated aryl ester and thioaryl ester substrates are more efficient than corresponding alkyl esters in acylating subtilisin, aryl esters are more difficult to synthesize and inherently less stable. We therefore prepared a series of alkyl ester substrates (Figure 5B) to improve upon their catalytic efficiencies as donor substrates. Peptide substrates bind to subtilisin in an extended antiparallel β-sheet conformation from residues P_4 to P_3' (Figure 2). Although the P_4 and P_1 residues dominate the substrate specificity of the enzyme [for review see Philipp and Bender (1983) and Estell et al. (1986)], the catalytic efficiency

Table III: Kinetic Constants for the Hydrolysis of an Amide Substrate (s-Ala-Ala-Pro-Phe-*p*NA) or an Activated Ester Substrate (s-Ala-Ala-Pro-Phe-Sbz)[a]

	s-Ala-Ala-Pro-Phe-*p*NA			s-Ala-Ala-Pro-Phe-Sbz				
enzyme	k_{cat}	K_M	k_{cat}/K_M	k_{cat}	K_M	k_{cat}/K_M	esterase/amidase[b]	aminolysis/hydrolysis
wild type[b]	(4.4 ± 0.01) × 10^1	(1.8 ± 0.1) × 10^{-4}	(2.5 ± 0.1) × 10^5	(2.3 ± 0.1) × 10^3	(1.9 × 0.1) × 10^{-4}	(1.2 ± 0.1) × 10^7	4.8 × 10^1	5.6 × 10^{-2}
P225A	4.1 ± 0.04	(7.8 ± 0.2) × 10^{-4}	(5.2 ± 0.1) × 10^3	(2.3 ± 0.03) × 10^3	(3.8 ± 0.1) × 10^{-4}	(6.2 ± 0.1) × 10^6	1.2 × 10^3	1.6 × 10^{-1}
S221C	(1.3 ± 0.04) × 10^{-3}	(4.9 ± 0.4) × 10^{-4}	2.7 ± 0.1	1.4 ± 0.1	(5.5 ± 0.8) × 10^{-5}	(2.5 ± 0.3) × 10^4	9.3 × 10^3	3.0 × 10^2
S221C/P225A	<3 × 10^{-5}	nd	nd	(4.1 ± 0.2) × 10^1	(1.9 ± 0.2) × 10^{-4}	(2.1 ± 0.2) × 10^5	nd	2.5 × 10^1

[a] The ratio of esterase to amidase activities is calculated from the ratio of the apparent second-order rate constants (k_{cat}/K_M). The aminolysis/hydrolysis ratio was determined by using the thiobenzyl ester substrate with 3.6 mM of the dipeptide Ala-Phe-amide as the nucleophile. See Materials and Methods for furthr details. [b] Data for the *p*NA substrate and for the Sbz substrate are from Carter and Wells (1988) and Wells et al. (1986), respectively.

Table IV: Substrate Leaving Group Comparison[a]

leaving group	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} M^{-1})
-glc-amide	17 ± 3	4.2 ± 0.9	(4.0 ± 1.1) × 10^3
-glc-Phe-amide	16 ± 1	0.89 ± 0.15	(1.8 ± 0.3) × 10^4
-glc-Phe-Gly-amide	21 ± 1	0.62 ● 0.05	(3.4 ± 0.2) × 10^4
-lac-amide	2.4 ± 0.2	2.3 ± 0.2	(1.0 ± 0.1) × 10^3
-lac-Leu-amide	1.9 ± 0.4	1.2 ± 0.4	(1.5 ± 1.0) × 10^3
-lac-Phe-amide	3.8 ± 0.2	1.1 ± 0.1	(3.6 ± 0.3) × 10^3

[a] The acyl donor (s-Ala-Ala-Pro-Phe-) is linked to alternative leaving groups. The initial rate of ligation of each ester with the nucleophilic dipeptide NH_2-Ala-Phe-amide (3.1 mM final) was measured.

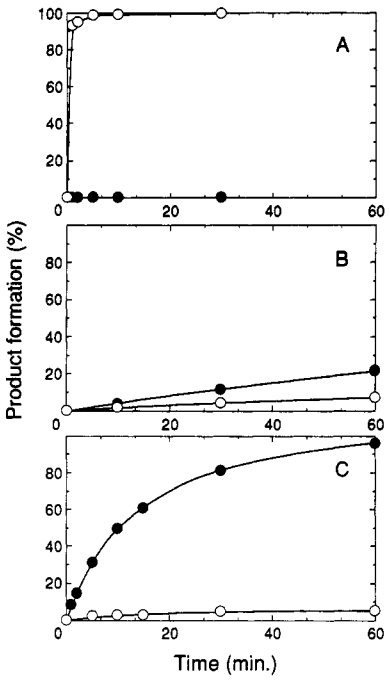


FIGURE 6: Progress curves showing the fraction of substrate converted to either ligation (●) or hydrolysis (○) product as a function of time by either P225A (panel A), S221C (panel B), or S221C/P225A (panel C). The donor substrate s-Ala-Ala-Pro-Phe-glc-Phe-amide (0.35 mM final) and dipeptide Ala-Phe-amide (3.6 mM) were incubated in the presence of indicated enzyme (10 μM final) and ligation buffer as described under Materials and Methods. Products were separated and quantified by reverse-phase HPLC. Their compositions were verified by amino acid composition and mass spectrometry.

for hydrolysis is enhanced significantly when peptide substrates are extended from P_1' to P_3' (Morahara et al., 1970).

We analyzed the efficiency of peptide ligation using a series of glycolate and lactate esters with S221C/P225A subtilisin (Table IV). Indeed, there is a systematic increase in k_{cat}/K_M of about 10-fold in extending esters from -glc-amide through -glc-Phe-Gly-amide. Most of this increase results from lower K_M values. There is a similar progression starting from L-lac-amide that further illustrates the advantage of extending the ester chain length. The k_{cat}/K_M values for the D-lactate ester series varied from 40- to 5000-fold lower than the corresponding L-series substrates (data not shown). This stereochemical effect suggests that efficient hydrolysis of these esters requires proper binding in the P_1' and P_2' binding sites. The lactate ester series is generally 4–5-fold less reactive than the glycolate ester series and contains an additional chiral center. Therefore, because of the improved catalytic efficiency and ease of synthesis of the glycolate esters, we chose to focus on the -glc-Phe-amide ester substrate.

The P225A mutant rapidly and quantitatively hydrolyzes the -glc-Phe-amide ester with very little ligation (Figure 6A). The S221C mutant hydrolyzes roughly one-third of the substrate (Figure 6B). Most of this is from hydrolysis of the ligated peptide product attributed to the residual amidase activity of S221C. In contrast, the S221C/P225A enzyme gives rapid and almost quantitative aminolysis (>90%; Figure 6C). Moreover, as expected from the very low amidase activity of S221C/P225A (Table III), the ligation product was not detectably hydrolyzed as it was for S221C and P225A. On the basis of rapid aminolysis and slow peptide product hydrolysis, S221C/P225A is a more useful enzyme than either of its parent single mutants for ligation of peptides using the -glc-Phe-amide ester donor substrate.

Table V: Relative Catalytic Efficiency for Aminolysis by Different Nucleophilic Di- and Tripeptides with S221C/P225A Subtilisin Acylated by the Donor Peptide s-Ala-Ala-Pro-Phe-glc-Phe-amide[a]

peptide	relative efficiency	peptide	relative efficiency
GF	0.8[b]	GL	0.3
AF	1.0	FG	0.006
LF	0.3	RG	0.04
RF	2.0	AFA	2.0
GA	0.1	LDF	0.3

[a] All nucleophilic peptides were amidated on their carboxyl termini, and sequences are specified by single-letter amino acid codes. [b] A value of 1.0 corresponds to an absolute value of $k_{aminolysis}[E\text{-}Ac]/K_N = 7 \times 10^{-4}$ (s^{-1} M^{-1}) (see Materials and Methods for further details).

Sequence Requirements for the Nucleophilic Acceptor Peptide. We next investigated the ligation efficiency with a series of acceptor dipeptides having the form NH_2-Xaa-Phe-amide, where Xaa corresponds to the amino-terminal residue of the acceptor peptide that would occupy the P_1' binding site during aminolysis of the thioacyl-enzyme intermediate. As the P_1' residue is varied in size or charge (Gly, Ala, Leu, Arg) the apparent second-order rate constant for aminolysis of s-Ala-Ala-Pro-Phe-glc-Phe-amide varies less than 5-fold (Table V). This is consistent with the relatively broad specificity for hydrolyzing various P_1' peptide substrates (Carter et al., 1989).

The P_2' site was probed with a series of dipeptides having the form NH_2-Gly-Xaa-amide (Table V). The rate constant for ligation varies 8-fold in going from GA to GL to GF showing a mild preference for ligating peptides containing a large hydrophobic residue at P_2'. This is consistent with data showing a similar preference for hydrolyzing substrates containing large hydrophobic side chains at P_2' (Morihara et al., 1970). However, for some dipeptide combinations the difference can be much larger; for example, Arg-Phe ligates nearly 100-fold faster than Arg-Gly (showing the importance of the P_2' Phe interaction for favorable rate with Arg P_1'). Moreover, combinations such as a large hydrophobic at P_1' and Gly at P_2' are extremely poor substrates (compare NH_2-Phe-Gly-amide with NH_2-Gly-Phe-amide; Table V). Extending the nucleophilic peptide can enhance the catalytic efficiency of ligation 2–3-fold (compare NH_2-Ala-Phe-amide with NH_2-Ala-Phe-Ala-amide; Table V).

The pH–rate profile (Figure 7) reflecting k_{cat} for the ligation of s-Ala-Ala-Pro-Phe-glc-Phe-amide with Ala-Phe-amide is sigmoidal with a plateau ranging from pH 7.8 to 8.3 and an inflection at about pH 6.8. The acidic limb may reflect the pK_a of His64 in wild-type subtilisin and the pK_a of the α-amino group of the Ala-Phe-amide nucleophile.

Design of P_1-Specific Peptide Ligases. The variations in substrate specificity for peptide nucleophiles is much smaller (generally about 10-fold) than the range of catalytic efficiencies for hydrolysis of P_1 peptide substrates for wild-type subtilisin (~10^5-fold; Estell et al., 1986). We anticipated that the S221C/P225A mutant, like wild-type subtilisin, would retain a strong preference for large hydrophobic (Estell et al., 1986) or lysine residues (Wells et al., 1987a) in the P_1 position

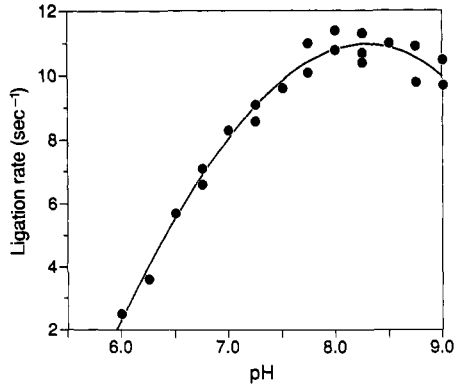


FIGURE 7: pH–rate profile for aminolysis of s-Ala-Ala-Pro-Phe-glc-Phe-amide (1.4 mM) with Ala-Phe-amide (3.6 mM) by S221C/P225A subtilisin (13 nM). Three different buffers were used, MES (pH 6.0–6.75), HEPES (pH 6.75–7.75), and Tricine (pH 7.75–8.75); All were adjusted to the same ion activity by the addition of NaCl.

of the peptide ester substrate. Therefore, to expand the P_1 specificity for ligation, we designed three other variants of S221C/P225A based upon mutations that alter the P_1 specificity of wild-type subtilisin. The subtilisin mutants G166E, E156Q/G166K, and G166I are more efficient than wild-type subtilisin for hydrolysis of Lys or Arg, Glu, and Ala P_1 substrates, respectively (Wells et al., 1987a; Estell et al., 1986). By introducing these mutations into the S221C/P225A enzyme, the peptide ligase specificity was substantially altered as expected (Figure 8). For a small P_1 ester substrate (s-Ala-Ala-Pro-Ala-glc-Phe-amide), the G166I/S221C/P225A enzyme is significantly better than the others (Figure 8A). The E156Q/G166K/S221C/P225A enzyme is the only one that efficiently aminolyzes a Glu P_1 substrate (Figure 8B), and is faster than the other variants in ligating a Phe P_1 substrate (Figure 8C). For a Lys P_1 ester substrate the rates for two of the enzymes (including the complementarily charged G166E/S221C/P225A mutant) are comparable and much more active than for the like-charged mutant E156Q/G166K/S221C/P225A (Figure 8D). The complementary charged enzyme does, however, give a slightly higher ratio of aminolysis to hydrolysis (data not shown). For ligation of a large peptide fragment containing a P_1 Arg ester, preliminary data (not shown) indicate that G166E/S221C/P225A is more active than the parent ligase.

In general, the aminolysis rates for the optimal enzyme–substrate pair were rapid and efficient, indicating it should be possible to efficiently ligate Lys, Ala, Phe, and Glu P_1 substrates with the proper choice of the peptide ligase. Except for the Lys P_1 substrate, at least one of the three other variants was significantly better than the parent peptide ligase. These additional mutant enzymes should provide added flexibility in design of ligation junctions.

Discussion

A protein engineering approach was applied to increase the aminolysis activity of thiolsubtilisin (S221C) and improve its potential use as a peptide ligase. We reasoned that the larger Cys221 side chain would impose significant steric crowding in generating the oxyanion transition state because a C–S bond is substantially longer than a C–O bond (1.8 versus 1.42 Å). This crowding was alleviated in part by moving the main-chain position at 221 by using a second-site mutation (P225A) that is one helical turn away (~4 Å). Structural analysis of the S221C/P225A variant shows that the segment of the α-helix supporting residue 221 is translated away from the active site by about 0.3 Å.

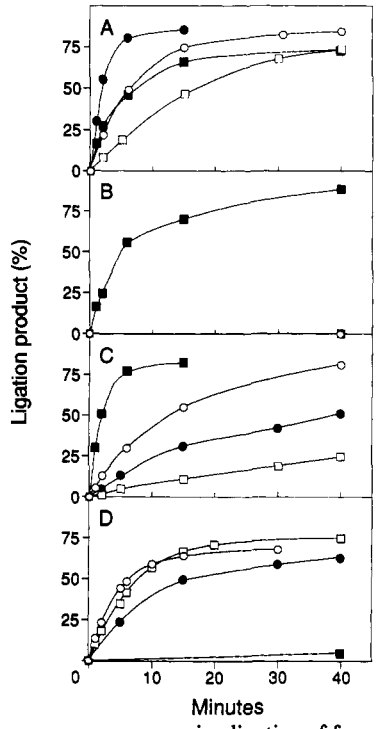


FIGURE 8: Progress curves comparing ligation of four different alkyl ester substrates having the form s-Ala-Ala-Pro-[Xaa]-glc-Phe-amide (where Xaa is the P_1 residue) with the dipeptide amide, Ala-Phe-amide. The alkyl esters had either Ala (panel A), Glu (panel B), Phe (panel C) or Lys (panel D) in their P_1 position. Ligation reactions were catalyzed by either S221C/P225A (○), G166I/S221C/P225A (●), G166E/S221C/P225A (□), or E156Q/G166K/S221C/P225A (■) that exhibit different specificities for ligation of P_1 alkyl esters. Reaction conditions and product analysis is described under Materials and Methods.

To better understand the structural consequences of the two mutations we have superimposed the structure of the S221C/P225A enzyme upon that of a complex between wild-type subtilisin and a peptide boronic acid inhibitor that mimics the deacylation transition-state complex (Figure 9). When the Cys221 side chain in the double mutant structure is rotated so that the Cβ–Sγ bond is parallel to the Cβ–Oγ bond in the wild-type subtilisin–inhibitor complex structure, the Sγ is 1.53 Å from the boron. Although this distance is still about 0.3 Å too close to facilitate an optimum orientation of the substrate in the active site, it is considerably more favorable than the case expected in the absence of the P225A mutation. In this case we estimate the Sγ to boron distance would be about 1.25 Å or 0.6 Å too close.

The kinetic data are consistent with the observed structural interaction between the S221C and P225A mutations. The free energy effects for mutations at structurally independent residues usually exhibit simple additivity [for review see Wells (1990)]. In our case, both single mutants show large reductions in both esterase and amidase activities (Table III). However, the esterase activity of the double mutant is *increased* 10-fold relative to S221C, while its amidase activity is severely reduced. Such nonadditive and differential kinetic behavior is consistent with the hypothesis that a structural perturbation caused by P225A affects the catalytic function of the S221C substitution.

Generally we find that the substrate specificity for ligation by S221C/P225A parallels the specificity for amide hydrolysis by wild-type subtilisin. For example, mutations in wild-type subtilisin that improve the catalytic efficiency for hydrolysis of different P_1 substrates produced similar effects for the S221C/P225A mutant in ligation efficiency (Figure 8). Ex-

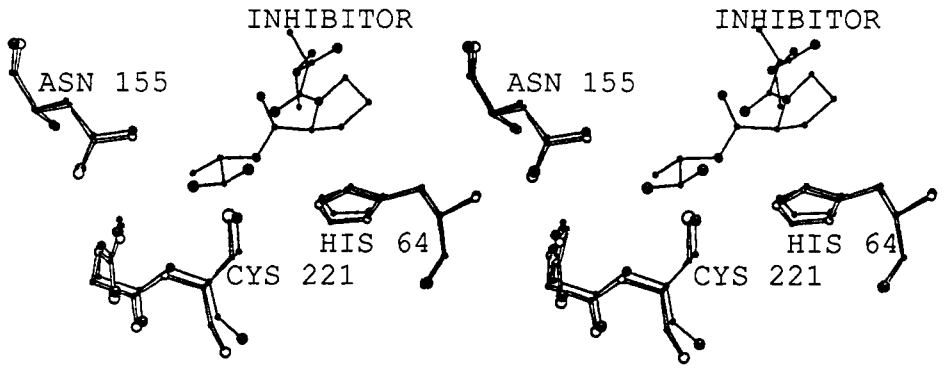


FIGURE 9: Stereographic view of the catalytic triad of the models of either the wild-type subtilisin–boronic acid inhibitor complex (filled bonds and atoms) or the S221C/P225A mutant (unfilled bonds and atoms). The Cys 221 side chain has been rotated so that the Cβ–Sγ bond is parallel to the Cβ–Oγ bond in Ser221 of the complex. The Cys221 Sγ is shown in the reduced form. A model of the boronic acid inhibitor MeO-Suc-Ala-Ala-Pro-boro-Val-OH is included to indicate the optimum position of a bound deacylation transition-state intermediate in the active site.

Table VI: Guideline of Preferred Sequences for Ligating Peptides by Using S221C/P225A Variants of Subtilisin BPN′

	residue						
	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'
avoid				G, P, T, V, I	I, P, D, E[a]	P, G, D, E[a]	
preferred	small or large hydrophobics	flexible	flexible	M, Y, L (1);[b] K, R (3); A (2); F, E (4)	R, C, N, T, K, H, W, Q, Y, A, V, S, G	F, Y, L, M, R, K	flexible
relevant references	*c*, *d*	*d*	*c–e*	*f*	*c*, *g*	*c*, *g*	*d*

[a] The deleterious effects of the acidic P_1' side chains may be minimized in high salt (>1 M; Carter et al., 1989). [b] These residues are preferred with the following variants of thiolsubtilisin: (1) = S221C/P225A; (2) = G166I/S221C/P225A; (3) = G166E/S221C/P225A; (4) = E156Q/G166K/S221C/P225A. [c] Philipp and Bender (1983); Carter et al. (1989). [d] Inferred from inspection of X-ray structures (Robertus et al., 1972; McPhalen & James, 1988) and from Morihara et al. (1970). [e] Carter and Wells (1987). [f] Estell et al. (1986), Wells et al. (1987a,b). [g] Morihara et al. (1970); Morihara and Oka (1977).

tending the chain length of the alkyl ester (Table IV) or the acceptor peptide nucleophile (Table V) enhanced the catalytic efficiency for ligation. A similar trend is observed for extending the peptide substrate from P_1' to P_3' for hydrolysis (Morihara et al., 1970; Morihara & Oka, 1977). Moreover, the specificity determinants for both reactions are similar from P_1' to P_3' [notably a preference for a larger hydrophobic side chain at P_2'; Table V and Morihara et al. (1970)]. Taken together, these data suggest that the substrate-binding determinants in the S221C/P225A mutant for ligation are essentially the same as those in wild-type subtilisin for hydrolysis.

Ligation Strategy. Subtilisin binds peptide substrates from P_4 to P_3' in an extended antiparallel β-sheet conformation (Figure 2; McPhalen & James, 1988). Therefore, the ligation efficiency for peptide fragments will be practically limited by the sequence of the junction from P_4 to P_3' (especially for the P_1 residue of the donor ester) as well as by the ability of the peptide fragments to adjust to the secondary structural features of the active site. Obviously the number of possible sequence combinations is too large to test all substrates (20^7 from P_4 to P_3'). Nonetheless, from specificity studies of the S221C/P225A and variants thereof, plus the large data base on the specificity of wild-type subtilisin, we can make some recommendations about the choice of junction sequences to ligate (Table VI). For example, wild-type subtilisin is good for hydrolyzing the side chains of P_1 residues Tyr, Phe, Met, and Leu (Estell et al., 1986), whereas at least one of the three other specificity variants are much better for Ala, Arg, and Glu. There are a small number of residues at P_1, P_1', and P_2' that are extremely poorly hydrolyzed and should be avoided, especially proline. In addition, difficulties in the synthesis of the alkyl ester substrates may further limit the choice of P_1 residues. Practical experience is sure to reveal more detailed sequence constraints.

We anticipate that in assembling a protein in blocks of 10–20 residues, some junctions may be difficult to ligate because of the sequence constraints. In cases involving poor residues in the P_4–P_1 positions, it may be possible to use more activated esters, such as thiolbenzyl esters. For example, the k_{cat}, K_M, and k_{cat}/K_M values for s-Ala-Ala-Pro-Phe-Sbz ligated with Ala-Phe-amide (3.1 mM) by S221C/P225A subtilisin are 230 s^{-1}, 0.4 mM, and 650 × 10^3 s^{-1} mM^{-1}, respectively. Although Sbz donor peptides are more difficult to synthesize, the rate of ligation is about 20-fold higher than that for the best alkyl ester substrate, suggesting acylation is rate-limiting for ligation with the alkyl ester substrates.

Other peptide sequences, especially longer protein fragments, may be difficult to ligate due to the requirement that about four residues either side of the ligation point need to be exposed and able to assume an extended conformation in the enzyme active site. The stability of subtilisin allows a number of denaturing conditions to be explored [such as higher temperatures and addition of ionic and nonionic detergents; see for example Carter et al. (1989)] to melt the substrate for more efficient ligation. Furthermore, variants of subtilisin have been engineered that have enhanced stability (Pantoliano et al., 1989; Cunningham & Wells, 1987; Bryan et al., 1987) and useful in organic solvents for synthesis (Wong et al., 1990).

We conclude that engineered subtilisins will be practically useful in peptide fragment condensation and/or semisynthesis of proteins in aqueous solution. The flexibility to manipulate the specificity and stability of the enzyme as well as the reaction conditions, ligation junctions, and donor leaving group chemistry makes this system extremely attractive for peptide synthesis in both aqueous solution and organic solvents.

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