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Functional Interaction Among Catalytic Residues in Subtilisin BPN′

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ABSTRACT **Variants of the serine protease, subtilisin BPN′, in which the catalytic triad residues (Ser-221, His-64, and Asp-32) are replaced singly or in combination by alanine retain activities with the substrate *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (sAAPF-*p*na) that are at least 10^3 to 10^4 above the nonenzymatic rate [Carter, P., Wells, J.A. Nature (London) 322:564–568, 1988]. A possible source of the residual activity was the hydrogen bond with the $N^{\delta 2}$ of Asn-155 that helps to stabilize the oxyanion generated in the tetrahedral transition state during amide bond hydrolysis by the wild-type enzyme. Replacing Asn-155 by Gly (N155G) lowers the turnover number (k_{cat}) for sAAPF-*p*na by 150-fold with virtually no change in the Michaelis constant (K_M). However, upon combining the N155G and S221A mutations to give N155G:S221A, k_{cat} is actually 5-fold *greater* than for the S221A enzyme. Thus, the catalytic role of Asn-155 is dependent upon the presence of Ser-221. The residual activity of the N155G:S221A enzyme (~10^4-fold above the uncatalyzed rate) is not an artifact because it can be completely inhibited by the third domain of the turkey ovomucoid inhibitor (OMTKY3), which forms a strong 1:1 complex with the active site. The mutations N155G and S221A individually weaken the interaction between subtilisin and OMTKY3 by 1.8 and 2.0 kcal/mol, respectively, and in combination by 2.1 kcal/mol. This is consistent with disruption of stabilizing interactions around the reactive site carbonyl of the OMTKY3 inhibitor. These data suggest that Ser-221 functions together with Asn-155 to accelerate amide bond hydrolysis and that other transition state stabilizing interactions account for the residual rate enhancement of 10^3- to 10^4-fold. More generally, these studies illustrate the limitations of using site-directed mutagenesis to probe the energetic importance of a single catalytic group whose function is dependent upon the interaction with others.**

Key words: serine protease, catalytic triad, oxyanion binding site, site-directed mutagenesis, protease inhibitor

INTRODUCTION

Subtilisin BPN′ is a serine-class endoprotease secreted by *Bacillus amyloliquefaciens* (reviewed in ref. 1 and 2). The catalytic machinery of this enzyme (Fig. 1) comprises a catalytic triad (Ser-221, His-64, and Asp-32), an oxyanion binding site ($N^{\delta 2}$ of Asn-155 and amido nitrogen of Ser-221), and additional binding determinants that help to stabilize the tetrahedral transition state during amide bond hydrolysis.[3] Subtilisin employs these catalytic elements to enhance the hydrolysis of the favorable *p*-nitroanilide substrate, sAAPF-*p*na, by a factor of ~4 × 10^9 over the nonenzymatic rate.[4] Understanding the partitioning of this huge rate enhancement among these catalytic groups is of fundamental importance to enzyme function and thereby to the design of new enzymes and catalytic antibodies.

Site-directed mutagenesis has been used to probe the importance of various active site residues in subtilisin BPN′ to specificity and catalysis (reviewed in ref. 5). Replacement of Asn-155 in the oxyanion binding site by a number of other residues[6,7] results in large reductions in k_{cat} (150- to 830-fold) and small changes in K_M with the substrate, sAAPF-*p*na. Replacement of the catalytic serine by alanine (S221A) lowers k_{cat} by 2 × 10^6-fold, to a level which is 3000-fold above the nonenzymatic hydroly-

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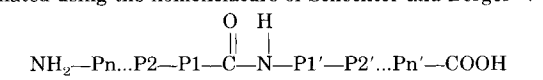
Abbreviations used: sAAPF-*p*na, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide; sAAPF-sbz; *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-thiobenzyl ester; OMTKY3, third domain of the turkey ovomucoid inhibitor; Tris, tris(hydroxymethyl)aminomethane; Me_2SO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PMSF, phenylmethanesulfonyl fluoride; DTT, D,L-dithiothreitol; EDTA, ethylenediaminetetra-acetic acid. Mutants are designated by the single letter code for the wild-type residue, followed by the codon number and then the replacement residue. Multiple mutants are identified by listing the single substitutions separated by colons; for example, the double variant containing the S24C and S221A mutations is designated S24C:S221A. Protease substrate residues are designated using the nomenclature of Schechter and Berger[14]:



where the scissile peptide bond is between the P1 and P1′ residues.

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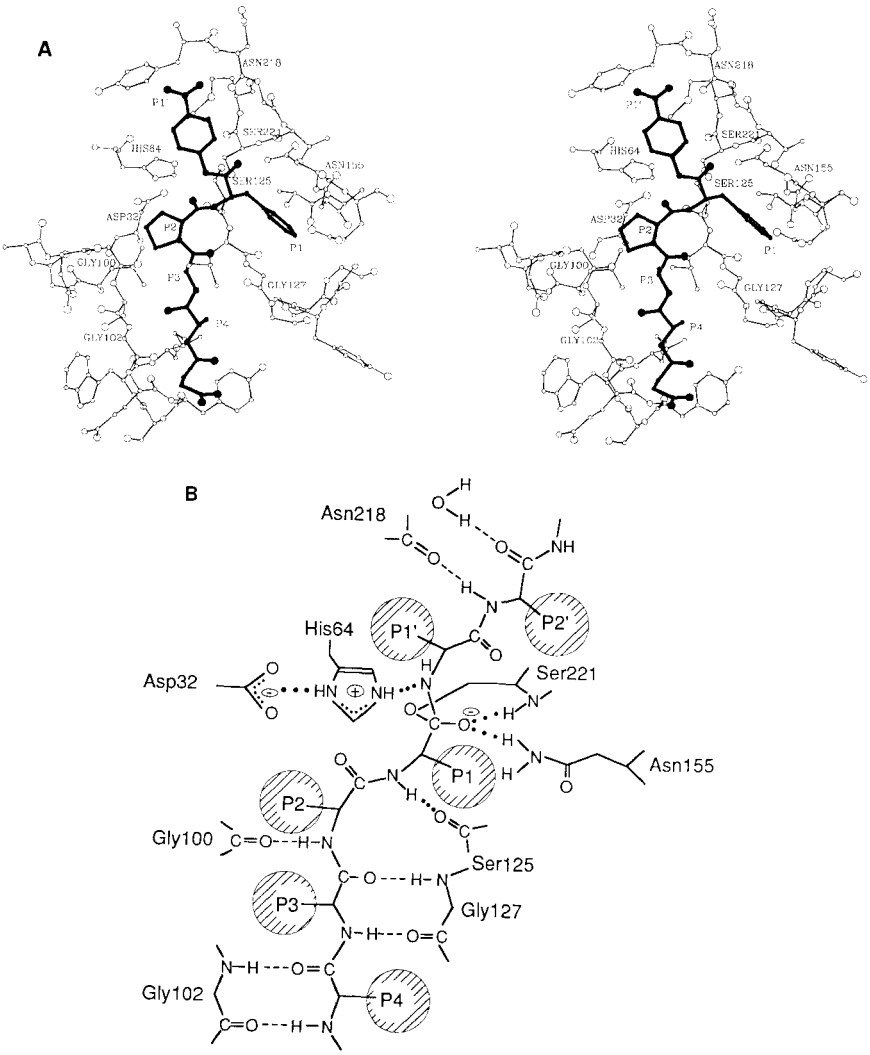


Fig. 1. **(A)** Stereoview of a model containing the substrate sAAPF-*p*NA (bold lines) bound to the active site of *B. amyloliquefaciens* subtilisin (adapted from ref. 4). Important residues in the enzyme and substrate are labeled according to standard nomenclature.[14] This enzyme–substrate model is based upon a 2.0 Å X-ray structure of a product bound to subtilisin and the succinyl and *p*-nitroanilide groups were introduced by modelling (R. Bott and M. Ultsch, unpublished data). **(B)** Diagram (adapted from ref. 8) showing the structure and contacts between subtilisin and the first tetrahedral intermediate (kinetically rate-limiting) for a polypeptide substrate extended from P4 to P2′. Hydrogen bonds formed between the backbone of the substrate and the main chain of the enzyme are shown by dashed lines, and those believed to form additionally in the tetrahedral intermediate[2,9] are represented by dotted lines. Catalysis of amide bond hydrolysis by subtilisin proceeds via an acyl enzyme intermediate following nucleophilic attack of Ser-221 O_γ on the scissile bond (for review see refs. 10–13). His-64 acts as a general acid and general base catalyst in proton transfer steps and Asp-32 stabilizes the protonated form of His-64. In addition to covalent and general acid–base catalysis by the catalytic triad (Ser-221, His-64, and Asp-32), transition state stabilization also is proposed to contribute to catalysis by subtilisin.[3,9]

sis rate[4] (k_{uncat}). The residual activity of the S221A enzyme is not reduced further by alanine replacements of one or both of the remaining triad members (His-64 and Asp-32). A plausible source of this residual activity for the S221A enzyme was transition state binding contacts in the oxyanion binding site (Fig. 1).

Here, we report the effect of mutating Asn-155 to Gly in the context of the S221A enzyme to examine the functional interaction between these residues both in catalysis and in binding of the inhibitor, OMTKY3.

MATERIALS AND METHODS

Construction and Purification of Subtilisin Variants

The N155G mutation was introduced into the wild-type *Bacillus amyloliquefaciens* subtilisin gene[15] cloned into the phagemid vector pSS5 (B. Cunningham, D. Powers, and J.A.W., unpublished) by site-directed mutagenesis[16] using a 38-mer oligonucleotide (5′ GGCAGCCGGTGGCGAAGGCACT-TCCGGGAGCTCAAGCA 3′; asterisk indicates a mismatch) which also introduces a unique *Sac*I site

(underlined). The N155G mutation was introduced into the S24C:S221A variant[4] using the same 38-mer primer and the corresponding single-stranded pSS5 template. The S24C:S221G variant was constructed using a template corresponding to the S24C:S221A variant and a 36-mer oligonucleotide (5′ TACAACGGTAC*G*GGAATGGC*T*AG*C*CCGCAC-GTTGCC 3′; asterisk indicates a mismatch), which also introduces a unique *Nhe*I site (underlined) that was utilized in isolating the mutant.[6] Variant phagemids were verified by dideoxy sequencing[17] and then transformed into a protease-deficient strain of *B. subtilis*, BG2036 (ref. 18). Subtilisin variants were expressed and purified as described.[4,19] In the case of weakly active subtilisin variants affinity purification was performed by reversible attachment to an activated thiol support using the engineered single thiol S24C (ref. 4).

Kinetic Procedures

Enzymes were assayed with the substrate sAAPF-*p*na[20] against corresponding blanks in 1 ml 100 mM Tris-HCl, pH 8.60, 4% (v/v) dimethyl sulfoxide (Me_2SO) at (25 ± 0.2)°C as previously described.[4] Enzyme concentrations were determined spectrophotometrically ($\epsilon_{280}^{0.1\%}$ = 1.17; see ref. 21). The enzyme concentrations in the assays were 23 nM for the wild-type and S24C enzymes, and 1–3 μM for the N155G, S24C:N155G:S221A, S24C:S221A, and S24C:S221G enzymes. Substrate concentrations were in the range of 0.1 to 7 times K_M, and were determined spectrophotometrically after total hydrolysis (ϵ_{410} = 8480 cm^{-1} M^{-1}; see ref. 20).

OMTKY3 Inhibition of Subtilisin Variants

The concentration of OMTKY3 was determined by amino acid composition analysis after total acid hydrolysis. Fixed amounts of subtilisin variants (1.8 pmol wild-type, 13 pmol N155G, or 21 pmol S24C:N155G:S221A) were incubated for 45–60 minutes at (25 ± 0.2)°C in 1.00 ml containing 100 mM Tris-HCl, pH 8.60, 5 mM $CaCl_2$, and varying amounts of OMTKY3. Under these conditions the half-time for binding of wild-type subtilisin to OMTKY3 is <4 minutes. Once the binding reaction had equilibrated, residual enzyme activity was determined by adding sAAPF-*p*na to 0.7 mM and measuring the initial change in A_{410} upon release of *p*-nitroaniline. The residual activity did not vary significantly over the few minutes that were required for measurement, which is consistent with an equilibrium having been reached and not detectably perturbed by the addition of substrate. The residual activity of the S24C:N155G:S221A enzyme after binding of OMTKY3 was measured using the substrate sAAPF-sbz which is hydrolyzed ~2000-fold more rapidly than sAAPF-*p*na (P. Carter, unpublished data). The initial rate of hydrolysis of sAAPF-sbz (0.3 mM final) was determined in the presence of 0.4 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) from the increase in absorbance at 412 nm upon formation of the thionitrobenzoate anion (ϵ_{412} = 13,600 cm^{-1} M^{-1}; see ref. 22). Although DTNB will also react with the free thiol in the S24C variant enzymes to form the thionitrobenzoate derivative, the kinetic properties of the S24C:N155G:S221A enzyme with sAAPF-*p*na were not detectably changed in the presence of 0.4 mM DTNB. For the S24C:S221A and S24C:N155G:S221A enzymes, the values of K_d for OMTKY3 were determined indirectly by incubating fixed amounts of wild-type subtilisin (1.8 pmol) and OMTKY3 (13 pmol) with varying amounts of S24C:S221A (or S24C:N155G:S221A) and following the residual activity of the wild-type enzyme with sAAPF-*p*na as described above. In this case, the incubation time prior to assay was increased to 2 hours to ensure that equilibrium had been reached. Values of K_d were calculated by directly fitting the residual rate data or by Scatchard analysis as described below.

Hydrolysis of the P1–P1′ peptide bond in OMTKY3 by subtilisin is extremely slow and the system behaves as if it were a simple equilibrium between enzyme (E) and free OMTKY3 (I_F) and the complex (EI)[23]:

$$E + I \underset{}{\overset{K_d^E}{\Leftrightarrow}} EI \qquad (1)$$

The measured residual activity of the inhibited enzyme (a_{res}) is related to the activity of the uninhibited enzyme (a_{max}), the total concentrations of enzyme ($[E_T]$) and inhibitor ($[I_T]$), and the apparent dissociation constant (K_d^E):

$$a_{res} = a_{max}\left(1 - \frac{([E_T]+[I_T]+K_d^E - \sqrt{(([E_T]+[I_T]+K_d^E)^2 - 4[E_T][I_T])})}{2[E_T]}\right) \qquad (2)$$

K_d^E values were estimated by either directly fitting the a_{res} data using Eq. (2), or by Scatchard analysis using an adaptation of the Ligand program.[24] The concentrations of bound ([EI]) and free ($[E_F]$) enzymes were calculated using Eqs. (3) and (4), respectively:

$$[EI] = [E_T](1 - a_{res}/a_{max}) \qquad (3)$$

$$[E_F] = [E_T]\, a_{res}/a_{max} \qquad (4)$$

Competition between a highly active (E) and a weakly active subtilisin mutant (M) for OMTKY3 (I) may be represented:

$$\begin{array}{ccc} & \overset{K_d^E}{} & \\ E + M + I & \Leftrightarrow & EI \\ \Updownarrow & K_d^M & \\ MI & & \end{array} \qquad (5)$$

The concentration of inhibitor bound to the weakly

TABLE I. Kinetic Parameters for Hydrolysis of sAAPF-*p*na by Subtilisin Variants*

Enzyme	Ser-221	Asn-155	$k_{cat}(s^{-1})$	K_M (μM)	k_{cat}/K_M (s^{-1} M^{-1})	$\frac{k_{cat}(\text{mutant})}{k_{cat}(\text{wild-type})}$
Wild-type†	+	+	$(4.4 \pm 0.1) \times 10^{1}$	180 ± 10	$(2.5 \pm 0.1) \times 10^{5}$	(1)
N155G	+	−	$(3.0 \pm 0.1) \times 10^{-1}$	210 ± 10	$(1.4 \pm 0.1) \times 10^{3}$	6.8×10^{-3}
S24C†	+	+	$(5.9 \pm 0.2) \times 10^{1}$	220 ± 20	$(2.7 \pm 0.2) \times 10^{5}$	1.3
S24C:S221G	−	+	$(2.2 \pm 0.2) \times 10^{-5}$	1400 ± 200	$(1.5 \pm 0.2) \times 10^{-2}$	5.0×10^{-7}
S24C:S221A†	−	+	$(3.4 \pm 0.1) \times 10^{-5}$	420 ± 40	$(8.2 \pm 0.6) \times 10^{-2}$	7.7×10^{-7}
S24C:N155G:S221A	−	−	$(1.8 \pm 0.1) \times 10^{-4}$	290 ± 30	$(6.4 \pm 0.5) \times 10^{-1}$	4.1×10^{-6}

*At (25 ± 0.2)°C in the presence of 100 mM Tris-HCl at pH 8.60 and 4% (v/v) Me_2SO (see Materials and Methods). The presence (+) or absence (−) of Ser-221 or Asn-155 side chains is indicated. Data are presented ± standard errors in the measurements. The non-enzymatic hydrolysis of rate of sAAPF-*p*na under these conditions (k_{uncat}) is $(1.1 \pm 0.1) \times 10^{-8}$ s^{-1} (see ref. 4).
†From ref. 4.

active variant ([MI]) is related to the known parameters by

$$[MI] = [I_T] - [E_T](1 - a_{res}/a_{max}) - K_d^E(a_{max}/a_{res} - 1) \quad (6)$$

The free concentration of the inactive mutant ($[M_F]$) is given by

$$[M_F] = [M_T] - [MI] \quad (7)$$

where $[M_T]$ is the total concentration.

RESULTS

Activity of Subtilisin Variants With sAAPF-*p*na

The N155G mutation causes a 150-fold reduction in k_{cat} for amide bond hydrolysis and almost no change in K_M (Table I). Replacement of the catalytic serine by either alanine (S221A) or glycine (S221G) lowers k_{cat} by ~10^6-fold, whereas K_M is increased by 2- and 6-fold, respectively, relative to the S24C parent enzyme. Introduction of the N155G mutation into the S24C:S221A enzyme increases k_{cat} by more than 5-fold with only a slight reduction in K_M. A surface accessible thiol mutation (S24C) was introduced into the most weakly active subtilisin variants to enable affinity purification away from detectable contaminating protease activity, including the E48A subtilisin variant ("helper") used to process proenzymes containing the S221A or S221G mutations to their mature forms.[19] The activity of the S24C mutant has been shown to be virtually identical to wild-type subtilisin.[4]

Binding of OMTKY3 Inhibitor to Subtilisin Variants

The N155G and S221A mutations increase the K_d of subtilisin for OMTKY3 by 19-fold and 29-fold, respectively, and together (S24C:N155G:S221A) they increase K_d by 34-fold (Table II). Inhibition of the wild-type and N155G subtilisin enzymes with OMTKY3 was followed by directly measuring their residual activities with sAAPF-*p*na. The residual activity of the S24C:N155G:S221A variant after binding OMTKY3 was followed using sAAPF-sbz (Fig. 2A), where k_{cat}/K_M is 7300-fold greater than for the corresponding *p*-nitroanilide substrate (P. Carter, unpublished results). A reliable estimate of K_d could not be obtained by direct activity measurement for the weakly active S24C:S221A variant, but it could be measured indirectly by competition with the wild-type enzyme for OMTKY3 (Fig. 2B). The concentration of OMTKY3 estimated by Scatchard analysis [$(1.5 \pm 0.02) \times 10^{-8}$ M] was in good agreement with that expected from total acid hydrolysis of OMTKY3 [$(1.25 \pm 0.10) \times 10^{-8}$ M]. Thus, complete inhibition occurs upon forming a 1:1 complex between the active site mutant and the OMTKY3 inhibitor. The K_d values for binding of OMTKY3 by the S24C:N155G:S221A enzyme, estimated by direct and indirect methods, are essentially the same (Table II).

The residual activity of S221A variant enzymes is resistant to inhibition by PMSF (not shown). These enzymes and N155G containing enzymes show small but significant changes in K_M for *p*-nitroanilide substrates and K_d for OMTKY3. These and numerous other control experiments[4,19] show that the activity measured for these weakly active enzymes is not the result of protease contamination (including the E48A helper subtilisin), a mistranslated "revertant" with serine at position 221, assay artifact, or nonspecific catalysis away from the mutated active site.

DISCUSSION

Energetics of Interactions Between OMTKY3 and Subtilisin BPN

The relative reduction in binding affinity with OMTKY3 caused by the S221A mutation in subtilisin BPN′ is virtually identical to that observed for anhydrotrypsin relative to trypsin for binding ovomucoid inhibitor, soybean trypsin inhibitor, or lima inhibitor.[25] Although a crystal structure is not available for OMTKY3 with subtilisin BPN′, high resolution structures have been determined for OMTKY3 complexed to both *Streptomyces griseus* protease B and α-chymotrypsin,[26–28] and for subtilisin BPN′ complexed with *Streptomyces* subtilisin inhibitor,[29] and α-chymotrypsin inhibitor 2 from

TABLE II. Dissociation Constants (K_d) and Change in Free Energies of Binding ($\Delta\Delta G$) for Subtilisin Variants With OMTKY3*

Enzyme	K_d (M)	$\Delta\Delta G$ (kcal/mol)†
Wild-type	$(3.5 \pm 0.2) \times 10^{-9}$‡	(0)
S24C:S221A	$(1.0 \pm 0.1) \times 10^{-7}$¶	2.0
N155G	$(6.6 \pm 0.6) \times 10^{-8}$‡	1.8
S24C:N155G:S221A	$(1.2 \pm 0.1) \times 10^{-7}$§	2.1
	$(1.1 \pm 0.5) \times 10^{-7}$¶	2.0

*At (25 ± 0.2)°C in the presence of 100 mM Tris-HCl at pH 8.60, 5 mM $CaCl_2$, and 4% (v/v) Me_2SO (see Materials and Methods). Data are presented ± standard errors in the measurements.

†$\Delta\Delta G = -RT \ln[(K_d)_{mutant}/(K_d)_{wild\text{-}type}]$; where the gas constant (R) is 1.99 cal/K/mol and the temperature (T) is 298 K.

‡Residual activity was followed with sAAPF-*p*na.

¶K_d was determined indirectly by competition with wild-type subtilisin for OMTKY3 and measuring the residual activity of the wild-type enzyme with sAAPF-*p*na.

§Residual activity was followed with sAAPF-sbz.

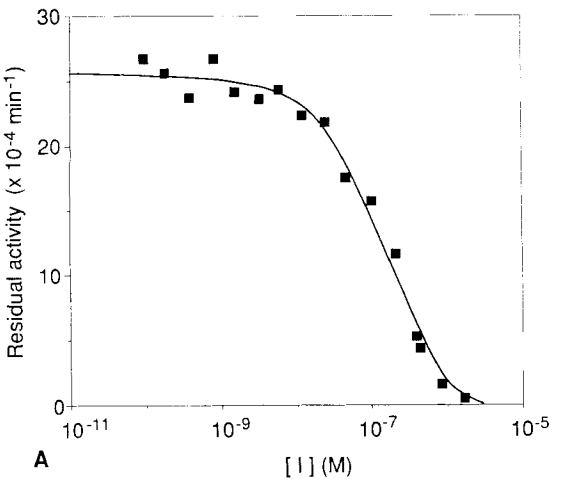
barley seed.[8,30] The conformations of the reactive site loops between OMTKY3 and CI-2 inhibitors are very similar[30] as they are for a homolog of OMTKY3 from Japanese quail and SSI.[29] In all of these structures the reactive site carbonyl of the inhibitor remains closely trigonal and no cleavage of the peptide bond is observed (for review see ref. 23). However, the distance between the O^{γ} of the catalytic serine and the carbonyl carbon of the inhibitor (2.5–2.8 Å)[8,27,30] is shorter than the van der Waals contact distance of (~3.6 Å) but much longer than the typical O–C covalent bond distance (1.7 Å).

Another common structural feature in complexes between the protease inhibitors and subtilisin is hydrogen bonds between the carbonyl oxygen of the reactive site peptide bond from the inhibitor and $N^{\delta 2}$ of Asn-155 and also the amide nitrogen of Ser-221. The interaction with Asn-155 would be disrupted in the N155G mutant, and we find that this causes a reduction in binding affinity of 1.8 kcal/mol. Fersht and co-workers[31] found that disruption of neutral hydrogen bond pairs causes a reduction in binding free energy of 0.5–1.5 kcal/mol, whereas disruption of a hydrogen bond in which one of the donors or acceptors on the substrate is charged causes a 3–5 kcal/mol reduction in binding energy. Accordingly, a larger reduction is observed for N155G in catalysis (3.2 kcal/mol) where Asn-155 contacts the oxyanion in the transition state as compared to binding the neutral carbonyl oxygen in the protease inhibitor (1.8 kcal/mol).

It has been pointed out[30,32] that many of the contacts between the protease inhibitors and subtilisin mimic contacts made in the transition state of the reaction.[3] These contacts include hydrogen bonds between Asn-155 and Ser-221 amide to the carbonyl oxygen of the P1 residue, the close contact between Ser-221 and the carbonyl carbon of the P1 residue, a long hydrogen bond between the P1 amine nitrogen and the carbonyl oxygen of Ser-125, and others. Our functional data are consistent with this view because mutagenesis of Asn-155 and Ser-221 affects k_{cat} (not K_M) and these residues support each other in binding to OMTKY3. Moreover, once either of the side chains from Ser-221 or Asn-155 is removed, the effect of the other on the binding of OMTKY3 is minimal. Thus for binding of OMTKY3, Asn-155 and Ser-221 are somewhat analogous to a vise in that both sides are needed to support the interaction with the other.

Interaction Between Asn-155 and Ser-221

The fact that Asn-155 is a liability to the S24C:S221A enzyme is most easily explained by a change in enzyme mechanism that must occur upon removal of the catalytic nucleophile, Ser-221. There are plausible mechanisms the S221A enzyme could adopt in which Asn-155 would be a liability. For example, hydrolysis could proceed by direct attack of solvent to generate a single (noncovalent) tetrahedral intermediate that breaks down to products. In this case, it is unlikely that solvent attacks the carbonyl carbon from the same face as Ala-221 (Fig. 1) because expanding the solvent cavity (S221G) does not improve the enzyme, nor does His-64 provide a catalytic advantage (as a general acid–base) to the S221A enzyme.[4] It is more likely that solvent attacks the carbonyl carbon from the face opposite Ala-221. In this situation Asn-155 would be a liability because the oxyanion would develop away from Asn-155, and Asn-155 would sterically interfere with solvent attack. Calculations show that the accessible van der Waals surface (determined using a probe radius of 0.05 Å)[33] at the carbonyl carbon of a *p*-nitroanilide model substrate is 4.8 $Å^2$, and when bound to subtilisin increases from 2.0 to 3.2 $Å^2$ on replacing Asn-155 by glycine. The solvent accessibility of the carbonyl carbon (calculated using a probe radius of 1.4 Å) is zero for wild-type, S221A, and S221G enzymes but is marginally increased to 0.1 $Å^2$ for N155G containing enzymes. It is known from X-ray crystallographic studies that neither S221A nor N155G causes substantial structural changes except for the side chain replacement (R. Bott, unpublished results). These accessibility calculations do not include changes in accessibility resulting from protein motion. Nonetheless, when a mutation causes a gross change in mechanism, the role of other catalytic groups can change to the point where they can even be deleterious to the enzyme. In this way, energetic changes resulting from mutagenesis of catalytically important residues may exaggerate their true catalytic importance.



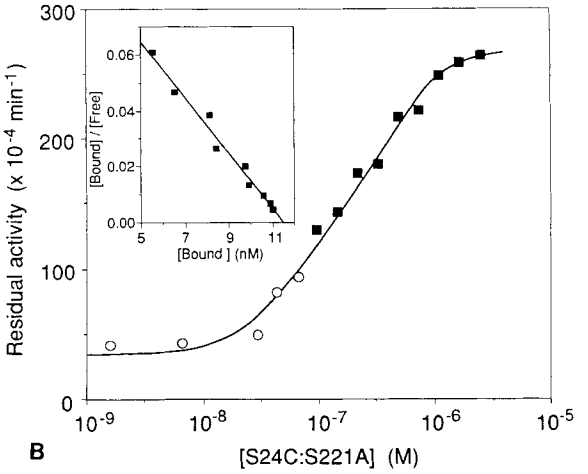


Fig. 2. **(A)** The residual activity ($\Delta A_{412}/\Delta t$) of S24C:N155G:S221A subtilisin with the substrate sAAPF-sbz (0.3 mM) in the presence of 100 mM Tris-HCl, pH 8.60, 5 mM $CaCl_2$, 0.4 mM DTNB, and 1.5 % (v/v) Me_2SO at (25 ± 0.2)°C versus the total concentration of OMTKY3 inhibitor ([I]) (see Materials and Methods). The K_d [(1.2 ± 0.1) × 10^{-7} M] was determined by fitting these data to Eq. (2) (solid line). **(B)** The residual activity ($\Delta A_{410}/\Delta t$) of 1.8 nM wild-type subtilisin with the substrate sAAPF-*p*na (0.7 mM) in the presence of 13 nM OMTKY3, 100 mM Tris-HCl, pH 8.60, 5 mM $CaCl_2$, 1.5% (v/v) Me_2SO, and varying concentrations of S24C:S221A subtilisin at (25 ± 0.2)°C (see Materials and Methods). Scatchard analysis for OMTKY3 binding at high concentrations of S24C:S221A is shown by the insert (■). The data at low concentrations of S24C:S221A (○) were excluded from the Scatchard analysis because of technical difficulties in obtaining precise data points within this region.

Importance of Transition State Stabilization Beyond the Scissile Peptide Bond

The fact that the residual activity for S24C:S221A subtilisin is not reduced by additional mutations in the oxyanion binding site (Table I) or the catalytic triad[4] strongly suggests that other determinants are responsible for the residual activity that is 10^3 to 10^4 above the nonenzymatic rate for hydrolysis of sAAPF-*p*na. It is unlikely that the residual activity results from catalysis from an alternative substrate binding site. First, the values of K_M are essentially the same between the wild-type enzyme and the various mutants (with the exception of S221G subtilisin). OMTKY3 inhibits all of these active site mutants indicating that the residual catalytic activity results at the same site as for the wild-type enzyme. We cannot exclude the possibility that the mode of substrate binding is not subtly changed between the wild-type enzyme and the various mutants. However, X-ray crystallographic studies show that the conformation of a reaction product bound to S221A subtilisin is identical to product bound to the wild-type enzyme (R. Bott, unpublished results). Finally, other studies have shown that the P1 substrate preference is the same between wild-type and another active site mutant of subtilisin, H64A.[34]

Numerous studies have implicated the importance of other transition state binding contacts away from the catalytic triad. Structural analyses of transition state analogs in comparison to bound substrate models for subtilisin BPN′ suggest a number of contacts change outside of the catalytic triad and oxyanion binding site in going from the trigonal ground state to the tetrahedral-like transition state.[3,9,35] For example, a hydrogen bond is formed between the P1 amide nitrogen and the carbonyl oxygen of Ser-125 in the transition state as the aromatic side chain at the P1 residue penetrates ~1 Å further into the P1 binding site of subtilisin.[3,9] Further evidence for additional P1 binding determinants contributing to catalysis comes from analysis of the activity of wild-type subtilisin with a variety of substrates. For example, for the P1 substrates sAAPX-*p*na (where X is Tyr, Phe, Met, Leu, Lys, His, Ala, Gln, Ser, Gly, or Glu), K_M ranges over 300-fold whereas k_{cat} varies over 1700-fold.[36,37] It has been suggested that the positive dipole moment at the N-terminus of the helix extending from 221 to 237 may provide significant electrostatic stabilization to the oxyanion intermediate.[38] In addition, when substrates are extended from P2 to P4, the increase in catalytic efficiency (k_{cat}/K_M) occurs primarily by a systematic increase in the k_{cat} term (nearly 200-fold) with virtually no change in K_M.[39] The importance of transition state binding interactions (for review see ref. 40,41) to catalysis by subtilisin is also suggested by the fact that mutations which increase the activity of subtilisin do not necessarily require an intact catalytic triad to mediate their effects. For example, k_{cat}/K_M increases 10-fold with sAAPF-*p*na by introducing the mutations E156S:G166A:G169A:Y217L into either wild-type subtilisin or the S24C:H64A variant (P. Carter, unpublished data).

Understanding the functional interplay between catalytic elements is fundamental to understanding how enzymes work and evolve. Our view is that the functions of the catalytic triad and oxyanion binding

site are strongly interdependent and functionally distinct from other transition state binding interactions.

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