Probing the Mechanism and Improving the Rate of Substrate-Assisted Catalysis in Subtilisin BPN'

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ABSTRACT: A mutant of the serine protease, subtilisin BPN', in which the catalytic His64 is replaced by Ala (H64A), is very specific for substrates containing a histidine, presumably by the substrate-bound histidine assisting in catalysis [Carter, P., & Wells, J. A. (1987) *Science (Washington, D.C.) 237*, 394-399]. Here we probe the catalytic mechanism of H64A subtilisin for cleaving His and non-His substrates. We show that the ratio of aminolysis to hydrolysis is the same for ester and amide substrates as catalyzed by the H64A subtilisin. This is consistent with formation of a common acyl-enzyme intermediate for H64A subtilisin, analogous to the mechanism of the wild-type enzyme. However, the catalytic efficiencies (k_{cat}/K_M) for amidase and esterase activities with His-containing substrates are reduced by 5000-fold and 14-fold, respectively, relative to wild-type subtilisin BPN, suggesting that acylation is more compromised than deacylation in the H64A mutant. High concentrations of imidazole are much less effective than His substrates in promoting hydrolysis by the H64A variant, suggesting that the His residue on the bound (not free) substrate is involved in catalysis. The reduction in catalytic efficiency k_{cat}/K_M for hydrolysis of the amide substrate upon replacement of the oxyanion stabilizing asparagine (N155G) is only 7-fold greater for wild-type than H64A subtilisin. In contrast, the reductions in k_{cat}/K_M upon replacement of the catalytic serine (S221A) or aspartate (D32A) are about 3000-fold greater for wild-type than H64A subtilisin, suggesting that the functional interactions between the Asp32 and Ser221 with the substrate histidine are more compromised in substrate-assisted catalysis. Thus, while the mechanism of substrate-assisted catalysis by H64A is qualitatively similar to the wild-type mechanism, there are important quantitative differences. In an attempt to improve the performance of Asp32, the conservative mutations I31L and S33T were installed into a variant of H64A subtilisin BPN'[Carter, P., Nilsson, B., Burnier, J. P., Burdick, D., & Wells, J. A. (1989) *Proteins 6*, 240-248]. Together these mutations increased k_{cat}/K_M for hydrolysis of the amide substrate an additional 4-fold. These studies provide a functional insight into the mechanism and catalytic efficiency of substrate-assisted catalysis.

Our goal over the last few years has been to engineer the broadly specific serine-class endoprotease from *Bacillus amyloliquefaciens* subtilisin BPN' into a site-specific protease. In the mechanism of wild-type subtilisin, His64 accepts a proton from Ser221 and subsequently donates it to the leaving group amine to promote acylation (Figure 1). In deacylation, His64 is thought to act as a general base by removing a proton from water leading to nucleophilic attack upon the acyl-enzyme and finally to reprotonation of Ser221. A mutant in which the histidine in the catalytic triad is replaced with alanine (H64A)[1] was designed to be specific for certain histidine-containing substrates (Figure 2), by the P2 substrate histidine substituting for the missing catalytic group—"substrate-assisted catalysis" (Carter & Wells, 1987). However, the catalytic efficiency, k_{cat}/K_M, of this prototype site-specific protease with the original histidine-containing substrate, sFAHF-*p*na, is about 5000-fold below wild-type subtilisin.

In this paper, we evaluate the mechanism of substrate-assisted catalysis by comparing the catalytic properties of the H64A mutant to wild-type subtilisin in the cleavage of both ester and amide substrates. The basis of the low catalytic efficiency of the H64A enzyme is further probed, by examining the contribution of the remaining catalytic residues (Ser221, Asp32, and Asn155) to substrate-assisted catalysis, and compared with their role in the wild-type enzyme. Our data suggest that Asp32 and Ser221 function inefficiently in substrate-assisted catalysis. This led to a successful improvement in the performance of the H64A enzyme by replacing residues flanking Asp32.

Materials and Methods

Materials

Oligonucleotides and *p*-nitroanilide substrates were synthesized and purified by the DNA synthesis group and the Bioorganic Chemistry Department at Genentech, respectively. Thiobenzyl ester substrates and AF-NH_2 were purchased from Bachem Bioscience Inc. Enzymes for DNA manipulations were obtained from New England Biolabs or Bethesda Research Laboratories and used according to suppliers recom-

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[1] Abbreviations: Mutant enzymes are designated by the single letter code for the wild-type amino acid followed by the residue number and then the amino acid replacement. Multiple mutants are identified by listing the single mutant components separated by a slash. For example, the double mutant in which Ser24 is converted to Cys and His64 to Ala is designated S24C/H64A. Protease substrate residues are designated according to 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. Substrates are represented as s-$P_4P_3P_2P_1$-P_1' where s is a succinyl group, X_4 to X_1 are L-amino acids, and P_1' is the leaving group. For *p*-nitroanilide substrates, the leaving group is designated *p*na, and for thiobenzyl ester substrates, Sbz; AF-NH_2, L-Ala-L-Phe-NH_2; DMA, *N*,*N*-dimethylacetamide; DMSO, dimethyl sulfoxide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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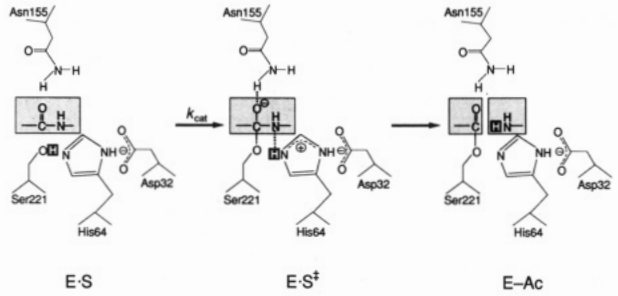

FIGURE 1: Diagram of the mechanism for rate-limiting acylation of subtilisin by an amide substrate. Deacylation is the reverse of this reaction sequence where water occupies the position of the leaving group amine. Important catalytic residues are labeled, and the scissile peptide bond is shown in the shaded box. Adapted from Carter and Wells (1988).

mendations. Reagents for chromatography were purchased from Pharmacia Biotechnology. Centriprep-10 and Centricon-10 microconcentrators were obtained from Amicon.

Methods

Mutant Construction. A hexamutant, S24C/D32A/H64A/E156S/G169A/Y217L, of the cloned *B. amyloliquefaciens* subtilisin gene (Wells et al., 1983) was constructed by ligating three fragments: 0.5 kb *Eco*RI–*Cla*I from S24C/D32A (Carter & Wells, 1988), 1.0 kb *Cla*I–*Bam*HI from S24C/H64A/E156S/G169A/Y217L (Carter et al., 1989), and 6.0 kg *Eco*RI–*Bam*HI from the phagemid pSS5 (B. Cunningham, D. Powers, and J.A.W., unpublished results). Further mutations were then introduced into this hexamutant by site-directed mutagenesis using restriction–selection (Wells et al., 1986) of the unique *Nhe*I site at codon 32 to isolate mutant clones (Carter, 1990) by using the oligonucleotides

5' AAAGTAGCGGTICTAGACAGCGGTATCGATTC 3': (CODES FOR I31L/A32D)
XbaI

5' GTAGCGGTTATCGACACCGGGATCGATTCTTC 3': (CODES FOR A32D/S33T)
NciI

5' AAAGTAGCGGTTCTCGACACCGGGATCGATTCTTCTCAT 3' (CODES FOR I31L/A32D/S33T)

The asterisks indicate mismatches, and restriction sites introduced are underlined. The N155G mutation was introduced into the S24C/H64A mutant (Carter & Wells, 1987) as previously described (Carter & Wells, 1990). Mutant phagemids were verified by dideoxynucleotide sequencing (Sanger et al., 1977) and then transformed into a protease deficient strain of *B. subtilis*, BG2036 (Yang et al., 1984). Other subtilisin variants have been described: S24C (Wells & Powers, 1986); S24C/S221A, S24C/D32A/H64A, and S24C/H64A/S221A (Carter & Wells, 1988).

Expression and Purification of Subtilisin. Separate *B. subtilis* BG2036 cultures containing a plasmid encoding a catalytic triad mutant subtilisin or the functionally silent A48E mutant (Carter & Wells, 1987; J.A.W., unpublished data) were grown in 2× TY media (Miller, 1972) containing 2 mM $CaCl_2$ and 12.5 μg/mL chloramphenicol at 37 °C for 14–18 h. Cocultures were inoculated by diluting the active-site mutant (also containing the S24C mutation) 100-fold (v/v) and the A48E "helper" mutant 10^5-fold (v/v) in 2× TY media containing 2 mM $CaCl_2$ and 12.5 μg/mL chloramphenicol and grown at 37 °C for 18–22 h with vigorous aeration.

Cocultures were centrifuged at 6000*g* and 4 °C for 15 min, and the supernatant was adjusted to 20 mM $CaCl_2$, 0.1% (v/v) 2-mercaptoethanol, 1 mM PMSF, and 50% (v/v) ethanol (prechilled to −20 °C). After centrifugation at 13000*g* and 4 °C for 15 min, the supernatant was adjusted to 75% (v/v) ethanol (prechilled to −20 °C) and then chilled (≥2h, −20 °C). After centrifugation at 6000*g* and 4 °C for 15 min, the pellet was resuspended in 50 mM Tris-HCl (pH 8.0), 5 mM $CaCl_2$, 1 mM PMSF, and 10 mM DTT and then dialyzed overnight at 4 °C against 4 L of 10 mM MES (pH 5.5), 5 mM $CaCl_2$, 0.1 mM PMSF, and 0.1% (v/v) 2-mercaptoethanol (SP buffer). Protein was precipitated with ammonium sulfate (0.65 g per millilter of sample) and then recovered by centrifugation at 38000*g* and 4 °C for 30 min. The pellet was resuspended in SP buffer and desalted by gel filtration against the same buffer with a G-25 Sepharose column. The sample was loaded on to a column of SP-Trisacryl equilibrated with SP buffer. After the column was washed with SP buffer (without 2-mercaptoethanol), subtilisin was eluted with degassed MCNE buffer: 10 mM MES (pH 5.5), 5 mM $CaCl_2$, 150 mM NaCl, and 1 mM EDTA. The eluted protein was dialyzed overnight against 2 L of degassed MCNE buffer. One-tenth volume of 1 M Tris-HCl (pH 7.5) was added to the dialyzed sample immediately before it was loaded onto an activated thiopropyl Sepharose column equilibrated with degassed TCNE buffer: 100 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, 200 mM NaCl, and 1 mM EDTA. The S24C-containing mutant subtilisin was eluted with TCNE buffer containing 10 mM DTT and 0.1 mM PMSF. The enzyme preparation was concentrated by ultrafiltration with microconcentrators and then chromatographed on a G-50 Sephadex column (50 cm × 2.5 cm) equilibrated with 10 mM MES (pH 5.5), 5 mM $CaCl_2$, 0.1 mM PMSF, 10 mM DTT, and 100 mM NaCl. The major peak containing subtilisin was reduced in volume by ultrafiltration.

The enzyme concentration was estimated spectrophotometrically ($\epsilon_{280}^{0.1\%} = 1.17$; Matsubara et al., 1965). Purified enzyme samples were flash frozen in aliquots and stored at −70 °C. The surface S24C substitution does not affect enzyme activity when introduced into active-site variants. This mutation enables affinity purification of weakly active mutants free of contaminating proteases, as shown by numerous control experiments (Carter & Wells, 1987, 1988, 1990; Carter et al., 1989).

Wild-type subtilisin and mutants containing an intact catalytic triad were prepared by growing *B. subtilis* BG2036 harboring corresponding subtilisin phagemids in 2× TY containing 2 mM $CaCl_2$ and 12.5 μg/mL chloramphenicol at 37 °C for 24 h. Subtilisin was purified as described above, except that no PMSF was added and 2-mercaptoethanol and DTT were added only for the S24C enzyme. Subtilisin was eluted from SP-Trisacryl with a gradient of 0–100 mM NaCl in 10 mM MES (pH 5.5) and 5 mM $CaCl_2$. The major subtilisin-containing peak was immediately flash frozen and stored in aliquots at −70 °C.

Kinetic Procedures. Subtilisin variants were assayed with *p*-nitroanilide substrates in 1 mL of 100 mM Tris-HCl (pH 8.60) and 4% (v/v) DMSO at 25 ± 0.2 °C with a Kontron Uvikon 860 spectrophotometer by the method of initial rates as applied by Carter and Wells (1988). For thiobenzyl ester substrates, initial hydrolysis rates were determined by including 0.4 mM DTNB in the reaction mixture and following the increase in absorbance at 412 nm upon formation of the thionitrobenzoate anion ($\epsilon_{412} = 13\,600\ cm^{-1}\ M^{-1}$) as previously described (Carter & Wells, 1990). Although DTNB will react with the free thiol in S24C-containing enzymes to form the thionitrobenzoate derivative, the kinetic properties of the S24C/H64A enzyme with sFAHF-*p*na were not detectably changed in the presence of DTNB. DTT was removed from the enzyme preparations by extensive dialysis prior to assays with thiobenzyl ester substrates.

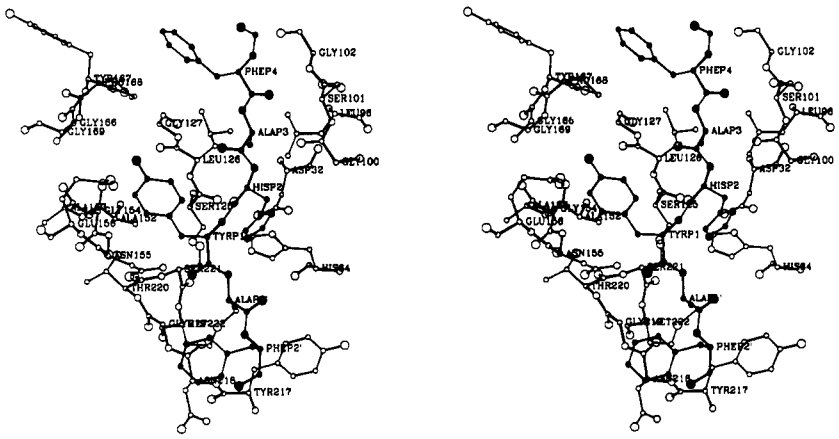

FIGURE 2: Stereoview of a substrate model (filled atoms) L-Phe-L-Ala-L-His-L-Tyr-L-Ala-L-Phe bound to the active site of subtilisin BPN' (open atoms). This model shows the superposition of the catalytic histidine (His64) with the HisP2 substrate [taken from Wells et al. (1987b)]. This model is based upon a 2.0-Å X-ray crystallographic study of the product complex bound to subtilisin (NH_2-L-Phe-L-Ala-L-Ala-L-Leu-OH). (R. Bott and M. Ultsch, unpublished data).

Determination of Product Ratios. Subtilisin variants were assayed with 0.73 mM sFAHF-*p*na or 0.75 mM sFAHF-Sbz in 1.0 mL containing 90 mM Tricine (pH 8.0), 0.005% (v/v) Tween 80, 2% (v/v) DMA, and 3.5 mM AF-NH_2 as described (Abrahmsén et al., 1991). At various times, an aliquot of the reaction mixture was applied to a C18 reverse-phase HPLC column (Waters) and eluted with a gradient of acetonitrile in 0.1% (v/v) TFA. Elution of the substrates, hydrolysis product (sFAHF-OH) and aminolysis product (sFAHFAF-NH_2) were monitored at 214 nm. Peaks were identified and quantified by peak integration, amino acid composition analysis, and mass spectroscopy. The ratio of aminolysis to hydrolysis was estimated from the initial rates of formation in several experiments each involving four to six successive time points.

Results

Evidence for Formation of an Acyl–Enzyme Intermediate. The acyl–enzyme intermediate that is formed in all serine proteases (Figure 1) normally undergoes hydrolysis but can also undergo aminolysis when in the presence of an amine nucleophile such as a dipeptide. The partitioning of the acyl–enzyme intermediate into hydrolysis or aminolysis products is *independent* of the substrates used for acylation (Fastrez & Fersht, 1973). Thus, if H64A subtilisin forms an acyl–enzyme intermediate, then the product ratios for aminolysis to hydrolysis should not depend upon whether a *p*-nitroanilide (sFAHF-*p*na) or thiobenzyl ester substrate (sFAHF-Sbz) is used in the reaction. Indeed, the ratios of aminolysis to hydrolysis for the wild-type (S24C) or the H64A subtilisin (S24C/H64A) were independent of the His-containing substrate used (Table I). This strongly suggests but does not formally prove (Fersht, 1985) that both enzymes exhibit a two-step mechanism: first acylation, followed by aminolysis or hydrolysis. The ratio of aminolysis to hydrolysis for the H64A derivative was nearly 30-fold lower than that for the wild-type, suggesting there are quantitative differences in the catalytic mechanisms.

Activities of Subtilisin Variants with Thiobenzyl Ester Substrates. The role of the catalytic His64 is different in acylation (Figure 1) as compared to deacylation. To compare the role of the catalytic His in the wild-type enzyme with that of a HisP2 substrate in the H64A variant in deacylation, we determined the kinetics of hydrolysis for thiobenzyl ester substrates (Table II). The k_{cat}/K_M ratio for hydrolysis of the sFAHF-Sbz substrate was only 14-fold lower for the H64A variant than for the wild type subtilisin. The wild-type enzyme hydrolyzes the HisP2 and GlnP2 substrates with virtually identical efficiency. In sharp contrast, the k_{cat}/K_M ratios for hydrolysis of the sFAQF-Sbz substrate by the H64A variant is reduced by about 2×10^4-fold compared to the sFAHF-Sbz substrate. This enormous difference derives from a reduction in k_{cat} (~700-fold) and increase in K_M (~25-fold). These data suggest that a HisP2 residue in the substrate is critical for the H64A variant and that it can promote deacylation almost as well as His64 in the wild-type in the enzyme.

Table I: Aminolysis/Hydrolysis Product Ratios for Subtilisin Variants[a]

enzyme	substrate	aminolysis/hydrolysis[b]
S24C	sFAHF-*p*na	2.2×10^{-1}
	sFAHF-Sbz	2.4×10^{-1}
S24C/H64A	sFAHF-*p*na	8.2×10^{-3}
	sFAHF-Sbz	8.0×10^{-3}

[a] Assays were performed at 25 ± 0.2 °C in the presence of 3.5 mM AF-NH_2, 90 mM Tricine (pH 8.0), 2% (v/v) DMA, and 0.005 % (v/v) Tween 80 (see Materials and Methods for further details). [b] Standard errors in the data are ≤±10%.

Substrate-Bound Imidazole is More Effective than Free Imidazole for Amide Hydrolysis by H64A Subtilisin. It was possible that the HisP2 substrate is cleaved 400-fold more rapidly than GlnP2 substrate (from k_{cat} ratios) by H64A subtilisin because a free (not bound) imidazole on the substrate participates in catalysis. We investigated this possibility by measuring the rate of hydrolysis of a non-His substrate (sFAAF-*p*na) by H64A subtilisin in the presence of increasing concentrations of imidazole (Figure 3). At concentrations of imidazole that are nearly 300-fold higher than the K_M for the amide substrate (100 mM), there is only a 6-fold increase in k_{cat}. Further addition of imidazole (up to 750 mM) causes an increase in k_{cat} of up to about 20-fold. The effect does not appear to saturate, suggesting imidazole is binding very weakly or not at all. Control studies show that imidazole does not increase the k_{cat} for hydrolysis of amide substrates by wild-type enzyme (not shown). Imidazole causes increases in K_M for H64A (Figure 3) and wild-type subtilisin that are comparable (not shown). Other studies on wild-type subtilisin indicate that imidazole is a weak competitive inhibitor for substrate binding (not shown).

These studies indicate that imidazole can participate in the hydrolysis of amide substrates once His64 is replaced by Ala. However, it binds extremely weakly and is not nearly as effective as a HisP2 substrate. This suggests that the HisP2 in

Table II: Activity of Subtilisin Variants with Thiobenzyl Ester Substrates[a]

enzyme	substrate	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} M^{-1})
S24C	sFAHF-Sbz	$(1.0 \pm 0.1) \times 10^2$	3.8 ± 0.7	$(2.7 \pm 0.5) \times 10^7$
	sFAQF-Sbz	$(1.8 \pm 0.1) \times 10^2$	6.3 ± 1.0	$(2.9 \pm 0.4) \times 10^7$
S24C/H64A	sFAHF-Sbz	7.1 ± 0.2	3.5 ± 0.3	$(2.0 \pm 0.1) \times 10^6$
	sFAQF-Sbz	$(1.0 \pm 0.1) \times 10^{-2}$	89 ± 10	$(1.1 \pm 0.1) \times 10^2$

[a] Assays were performed at 25 ± 0.2 °C in the presence of 100 mM Tris-HCl (pH 8.6), 0.4 mM DTNB, and 4% (v/v) DMSO (see Material and Methods). Data are presented plus or minus standard errors. The nonenzymatic hydrolysis rates (k_{uncat}) for sFAQF-Sbz and sFAHF-Sbz under these conditions are $(1.2 \pm 0.1) \times 10^{-5}$ s^{-1} and $(9.0 \pm 0.2) \times 10^{-6}$ s^{-1}, respectively.

Table III: Activity of Subtilisin Variants with sFAHF-*pna*[a]

	catalytic triad[b]			oxyanion hole[b]				$k_{cat(mutant)}$
enzyme	Ser	His	Asp	Asn	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} M^{-1})	$k_{cat(S24C)}$
WT[c]	+	+	+	+	4.4 ± 0.5	17 ± 2	$(2.6 \pm 0.2) \times 10^5$	$(9.5 \pm 0.5) \times 10^{-1}$
N155G	+	+	+	–	$(1.0 \pm 0.1) \times 10^{-2}$	11 ± 1	$(9.0 \pm 0.4) \times 10^2$	$(2.2 \pm 0.1) \times 10^{-3}$
S24C	+	+	+	+	4.6 ± 0.1	13 ± 1	$(3.6 \pm 0.1) \times 10^5$	(1)
S24C/S221A	–	+	+	+	$(4.9 \pm 0.5) \times 10^{-5}$	470 ± 80	$(1.1 \pm 0.2) \times 10^{-1}$	$(1.1 \pm 0.1) \times 10^{-5}$
S24C/D32A	+	+	–	+	$(1.2 \pm 0.1) \times 10^{-3}$	120 ± 10	$(1.1 \pm 0.1) \times 10^1$	$(2.7 \pm .1) \times 10^{-4}$
S24C/H64A[c]	+	–	+	+	$(2.1 \pm 0.1) \times 10^{-2}$	340 ± 30	$(6.2 \pm 0.4) \times 10^1$	$(4.5 \pm 0.1) \times 10^{-3}$
S24C/H64A/N155G	+	–	+	–	$(1.5 \pm 0.1) \times 10^{-4}$	95 ± 13	1.6 ± 0.2	$(3.3 \pm 0.1) \times 10^{-5}$
S24C/H64A/S221A	–	–	+	+	$(1.4 \pm 0.1) \times 10^{-5}$	220 ± 20	$(6.3 \pm 0.3) \times 10^{-2}$	$(3.1 \pm 0.1) \times 10^{-6}$
S24C/D32A/H64A	+	–	–	+	$(1.4 \pm 0.1) \times 10^{-3}$	160 ± 20	9.0 ± 1.0	$(3.0 \pm 0.1) \times 10^{-4}$

[a] Assays were performed at 25 ± 0.2 °C in the presence of 100 mM Tris-HCl (pH 8.6) and 4% (v/v) DMSO (see Materials and Methods). Data are presented plus or minus standard errors. The nonenzymatic hydrolysis rate of sFAHF-*pna* under these conditions (k_{uncat}) is $(4.1 \pm 0.1) \times 10^{-8}$ s^{-1}. [b] Catalytic triad (Ser221, His64, and Asp32) and oxyanion hole (N155) residues are represented by (+) and Ala and Gly replacements by (–). [c] Data taken from Carter et al. (1989).

Table IV: Activity of Subtilisin Variants with sFAQF-*pna*[a]

	catalytic triad[b]			oxyanion hole[b]				$k_{cat(mutant)}$
enzyme	Ser	His	Asp	Asn	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} M^{-1})	$k_{cat(S24C)}$
WT	+	+	+	+	$(1.0 \pm 0.1) \times 10^1$	60 ± 4	$(1.7 \pm 0.1) \times 10^5$	1.0 ± 0.1
N155G	+	+	+	–	$(2.7 \pm 0.1) \times 10^{-2}$	34 ± 2	$(8.0 \pm 0.3) \times 10^2$	$(2.7 \pm 0.1) \times 10^{-3}$
S24C	+	+	+	+	$(1.0 \pm 0.1) \times 10^1$	68 ± 4	$(1.5 \pm 0.1) \times 10^5$	(1)
S24C/S221A	–	+	+	+	$(2.3 \pm 0.4) \times 10^{-5}$	520 ± 130	$(4.5 \pm 1.6) \times 10^{-2}$	$(2.3 \pm 0.2) \times 10^{-6}$
S24C/D32A	+	+	–	+	$(9.3 \pm 0.3) \times 10^{-4}$	120 ± 10	7.9 ± 0.6	$(9.1 \pm 0.2) \times 10^{-5}$
S24C/H64A	+	–	+	+	$(5.1 \pm 0.3) \times 10^{-5}$	190 ± 20	$(2.7 \pm 0.2) \times 10^{-1}$	$(5.0 \pm 0.2) \times 10^{-6}$
S24C/H64A/N155G	+	–	+	–	$(8.0 \pm 0.6) \times 10^{-5}$	70 ± 20	1.2 ± 0.6	$(8.0 \pm 0.3) \times 10^{-6}$
S24C/H64A/S221A	–	–	+	+	$(2.2 \pm 0.2) \times 10^{-5}$	260 ± 50	$(8.4 \pm 1.7) \times 10^{-2}$	$(1.7 \pm 0.1) \times 10^{-6}$
S24C/D32A/H64A	+	–	–	+	$(9.1 \pm 0.2) \times 10^{-4}$	150 ± 10	6.2 ± 0.3	$(8.9 \pm 0.2) \times 10^{-5}$

[a] The assay conditions and other details are as described in Table III. The nonenzymatic hydrolysis rate for sFAQF-*pna* under these conditions (k_{uncat}) is $(5.1 \pm 0.3) \times 10^{-8}$ s^{-1}. [b] Catalytic triad (Ser221, His64, and Asp32) and oxyanion hole (N155) residues are represented by (+) and Ala and Gly replacements by (–).

the E·S complex (not free substrate) participates in catalysis by the H64A variant subtilisin.

Functional Interaction of HisP2 with Other Catalytic Residues in H64A Subtilisin. To compare the catalytic contributions of Asn155, Ser221, and Asp32 in the wild-type and H64A variants, we introduced alanine or glycine substitutions at these sites into the parent enzymes. We then examined the effects of mutations upon hydrolysis of HisP2 (Table III) or GlnP2 (Table IV) substrates.

The reduction in the k_{cat}/K_M ratio for both wild-type and S24C/H64A subtilisin with sFAHF-*pna* upon mutating S221A, N155G, and D32A is dominated by reductions in k_{cat} (Table III). Mutating the oxyanion-stabilizing asparagine residue (N155G) lowers k_{cat} for wild-type and S24C/H64A subtilisins with sFAHF-*pna* by comparable amounts (440-fold and 140-fold, respectively). In contrast, replacing the catalytic aspartate with alanine (D32A) in the S24C parent enzyme gives a much greater reduction in k_{cat} (3800-fold) than for the S24C/H64A mutant (only 15-fold). Likewise, mutating the catalytic serine to alanine (S221A) reduces k_{cat} with sFAHF-*pna* by 94 000-fold and 1500-fold for the S24C enzyme and S24C/H64A enzymes, respectively. Thus, the catalytic function of Asp32 and Ser221 for hydrolysis of the HisP2 substrate by the H64A variant appears to be much more compromised than the function of Asn155.

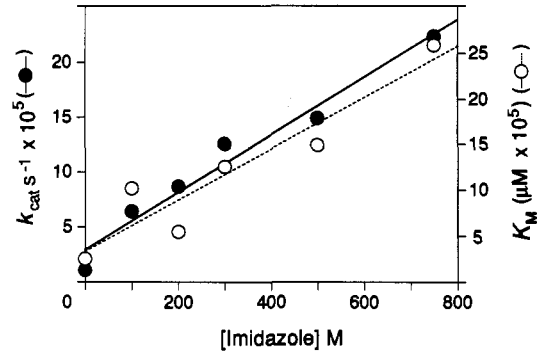

FIGURE 3: Effect of imidazole on the kinetic parameters for hydrolysis of sFAAF-*pna* by S24C/H64A subtilisin. Assays were carried out as described under Materials and Methods and in Table III. KCl was added to assays containing low concentrations of imidazole to maintain a constant ionic strength in all assays (as confirmed by conductivity measurements).

The strong preference of the S24C/H64A enzyme for HisP2 over the GlnP2 substrate (ratio of k_{cat}/K_M terms is 230) is abolished by replacement of any of the remaining catalytic groups (Tables III and IV): in the case of N155G and D32A mutations, k_{cat}/K_M for sFAQF-*pna* is *increased* by 4-fold and

Table V: Activity of S24C and S24C/H64A Derived Subtilisin Variants with *p*-Nitroanilide Substrates[a]

enzyme	substrate	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} M^{-1})
S24C[b]	sFAHF-*p*na	4.6 ± 0.1	13 ± 1	$(3.6 \pm 0.1) \times 10^5$
	sFAQF-*p*na	$(1.0 \pm 0.1) \times 10^1$	68 ± 4	$(1.5 \pm 0.1) \times 10^5$
S24C/H64A[b]	sFAHF-*p*na	$(2.1 \pm 0.1) \times 10^{-2}$	340 ± 30	$(6.2 \pm 0.4) \times 10^1$
	sFAQF-*p*na	$(5.1 \pm 0.3) \times 10^{-5}$	190 ± 20	$(2.7 \pm 0.2) \times 10^{-1}$
S24C/H64A/E156S/G169A/Y217L	sFAHF-*p*na[c]	$(4.5 \pm 0.2) \times 10^{-2}$	200 ± 20	$(2.2 \pm 0.2) \times 10^2$
	sFAQF-*p*na	$(8.7 \pm 0.3) \times 10^{-5}$	79 ± 8	1.1 ± 0.1
S24C/I31L/H64A/E156S/G169A/Y217L	sFAHF-*p*na	$(6.2 \pm 0.1) \times 10^{-2}$	230 ± 11	$(2.7 \pm 0.1) \times 10^2$
	sFAQF-*p*na	$(7.8 \pm 0.5) \times 10^{-5}$	79 ± 12	$(9.9 \pm 1.4) \times 10^{-1}$
S24C/S33T/H64A/E156S/G169A/Y217L	sFAHF-*p*na	$(5.1 \pm 0.1) \times 10^{-2}$	100 ± 10	$(5.0 \pm 0.1) \times 10^2$
	sFAQF-*p*na	$(1.0 \pm 0.1) \times 10^{-4}$	74 ± 5	1.4 ± 0.1
S24C/I31L/S33T/H64A/E156S/G169A/Y217L	sFAHF-*p*na	$(5.7 \pm 0.1) \times 10^{-2}$	63 ± 3	$(9.0 \pm 0.3) \times 10^2$
	sFAQF-*p*na	$(1.5 \pm 0.1) \times 10^{-4}$	60 ± 5	2.5 ± 0.2

[a] The assay conditions and other details are described in Table III. [b] Data taken from Tables III and IV for comparison purposes. [c] Data taken from Carter et al. (1989).

23-fold, respectively, to a level similar to that for the sFAHF-*p*na substrate. These enhancement effects have been attributed to changes in catalytic mechanism that result from replacement of additional catalytic triad residues (Carter & Wells, 1988, 1990). The S221A mutation lowers k_{cat}/K_M with sFAQF-*p*na for the S24C/H64A enzyme by 3-fold to a level similar to that for the sFAHF-*p*na substrate. In contrast to the S24C/H64A variant, S24C and wild-type subtilisins show only a 2-fold preference for sFAHF-*p*na over sFAQF-*p*na, and their activity against these two substrates is similarly affected by any of the S221A, D32A, and N155G mutations.

Enhancing the Activity of H64A Subtilisin. The residues flanking the catalytic Asp32 of H64A subtilisin were mutated to enhance its performance in the hydrolysis of histidine-containing substrates. We chose to introduce substitutions from other natural variants of subtilisin because these seemed most likely to be tolerated in the somewhat buried environment surrounding Asp32. Thus the mutations I31L and S33T from *B. licheniformis* subtilisin were installed singly or together into the highest activity variant of the H64A enzyme previously engineered, namely the pentamutant S24C/H64A/E156S/G169A/Y217L (Carter et al., 1989). Indeed, the mutation S33T increases k_{cat}/K_M by 2-fold for the pentamutant with sFAHF-*p*na, and subsequent incorporation of I31L gives a further 2-fold improvement (Table V). The benefit from the I31L replacement requires the presence of S33T as it does not increase the activity of the pentamutant against sFAHF-*p*na on its own. The increase in activity observed with sFAHF-*p*na is almost entirely due to a reduction in K_M with k_{cat} being almost unchanged. The single replacements I31L and S33T have little effect upon the activity of the pentamutant with the non-histidine substrate sFAQF-*p*na, but together they enhance activity 2-fold by increasing k_{cat}. Thus, the combination of I31L and S33T do not reduce the preference for HisP2 substrates.

Discussion

Mechanism of Substrate-Assisted Catalysis in H64A Subtilisin Is Similar to Wild-Type Subtilisin. The H64A subtilisin variant was designed with the idea (based only on molecular modeling; Figure 2) that, upon removal of the catalytic His64, a substrate bound His would substitute for the catalytic function of His64 in the enzyme mechanism. Here, we provide evidence that the mechanistic features of substrate-assisted catalysis by H64A subtilisin are qualitatively the same as for wild-type subtilisin. First, the partitioning of aminolysis and hydrolysis products for H64A subtilisin is independent of the nature of the leaving group, ester or amide (Table I). This suggests the H64A enzyme undergoes at least a two-step mechanism as would be expected for formation of an acyl–enzyme intermediate (Figure 1). If the H64A subtilisin used a single-step mechanism, then the product partitioning would be expected to change based upon the differential chemical reactivities of the leaving group (Fastrez & Fersht, 1973). Unfortunately, both H64A and wild-type subtilisins hydrolyze sFAHF-Sbz too rapidly to observe burst kinetics by the methods used. Such data would further substantiate the existence of an acyl–enzyme intermediate. Second, substrate-assisted catalysis is reduced substantially upon mutation of Asn155, Ser221, and Asp32 (Table III), and these residues are also important for the mechanism of wild-type subtilisin (Wells et al., 1986; Bryan et al., 1986; Carter & Wells, 1988). Third, once any of these catalytic residues are mutated in the H64A variant, the preference for the HisP2 over GlnP2 substrate collapses (Table IV). This is consistent with studies in wild-type subtilisin showing that the functional importance of His64 depends upon the presence of Ser 221 and Asp32 (Carter & Wells, 1988). Earlier experiments on H64A subtilisin have shown the pH rate profile (P.C. and J.A.W., unpublished results; Carter & Wells, 1987) for substrate-assisted catalysis is similar to wild-type subtilisin but requires a HisP2 substrate. This suggests that a deprotonated HisP2 residue is important in catalysis as is the case for His64. Furthermore, free imidazole is not nearly as effective as the bound HisP2 substrate in promoting amide hydrolysis, suggesting the bound rather than free HisP2 substrate assists in catalysis by H64A subtilisin (Figure 3 versus Table II). Finally, the P_1 subsite specificities are virtually the same for substrate-assisted catalysis by H64A and wild-type subtilisin (Carter et al., 1989). Together, these data strongly suggest that the mode of substrate binding (Figure 2) and mechanism of substrate-assisted catalysis by the H64A enzyme are very similar to those of wild-type subtilisin.

Quantitative Differences in Catalytic Mechanisms. In the kinetic scheme for wild-type subtilisin, acylation is controlled by k_2 and deacylation by k_3

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \xrightarrow{k_2} E\text{–}Pr_2 + Pr_1 \xrightarrow{k_3} E + Pr_2 \quad (1)$$

where E is the enzyme, S is substrate, Pr_1 and Pr_2 are hydrolyzed products, and E–Pr_2 is the acyl–enzyme intermediate. The steady-state rate constant, k_{cat}, for this scheme is (Gutfreund & Sturtevant, 1956)

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \quad (2)$$

For amide hydrolysis by wild-type subtilisin, acylation is rate limiting ($k_2 \ll k_3$) and so $k_{cat} \sim k_2$. For example, the k_{cat} values for hydrolysis of thiobenzyl esters are typically 20–40 times greater than their corresponding *p*-nitroanilides (Wells et al., 1986; Tables II and III), which reflects the shift in rate-limiting step.

The k_{cat} value for hydrolysis of sFAHF-*p*na by the H64A enzyme is reduced 200-fold compared to wild-type subtilisin (Table III), suggesting that the activation barrier for acylation is dramatically increased for substrate-assisted catalysis. In contrast, the k_{cat} for hydrolysis of sFAHF-Sbz is reduced by only 14-fold, suggesting that the activation barrier for deacylation is much less affected. Moreover, we can probe the importance of the HisP2 substrate to deacylation by comparing the k_{cat} values relative to the GlnP2 substrate (Table II). Here, the HisP2 substrate contributes a factor of 700 in k_{cat} for the H64A enzyme and essentially nothing to the wild-type enzyme.

For the two step mechanism, K_M is given by

$$K_M = K_s[k_3/k_2 + k_3] \quad (3)$$

where K_s is the E·S dissociation constant (k_{-1}/k_1). If we assume that K_s for homologous esters and amides against the same enzyme is roughtly the same and that $k_3 \cong k_{cat(esterase)}$ (Table II) and $k_2 \cong k_{cat(amidase)}$ (Tables III and IV), it is possible to calculate k_2 for ester hydrolysis from eq 3. These calculations show $k_{2(esterase\ calculated)}$ is greater than $k_{cat(esterase)}$ for all enzyme–substrate pairs by factors ranging from 10- to 100-fold, except for the H64A enzyme with the GlnP2 ester where $k_{cat(esterase)} \cong k_{2(esterase\ calculated)}$. Thus barring this exception, the assumption made above that $k_2 \gg k_3$ for esters so that $k_{cat(esterase)} \sim k_3$ is consistent with our data. We also note that the K_M values for amidase activity (which is a close approximation of K_s because $k_2 \ll k_3$) are about 10-fold higher for substrate-assisted hydrolysis of the HisP2 substrate by H64A compared to wild-type (Table III). Although we believe the mode of substrate binding is similar, these data indicate substrate binding is weaker and may reflect the alterations in binding at the P_2 binding site.

In summary, our data suggest that the major defect in the H64A enzyme for substrate-assisted hydrolysis of HisP2 containing amides is at the level of substrate binding (~20-fold) and acylation (~200-fold) and not limited by the reduction in deacylation rate (~14-fold).

Improving the Catalytic Efficiency for Substrate-Assisted Catalysis. The mutational analysis of the catalytic residues, Asn155, Ser221, and Asp32, provides information of their relative importance to the mechanisms of wild-type enzyme and the H64A variant. These experiments show that the effect upon k_{cat} of mutating Asn155 is comparable in both the wild-type and H64A enzymes. In contrast, Ser221 and Asp32 are 60- to 250-fold more important to the wild-type enzyme than H64A. Even larger differences are seen when relative values of k_{cat}/K_M are compared, because S221A and D32A cause increases in K_M of 10- to 40-fold for the wild-type enzyme that are already included in the H64A mutant. The mechanisms of these catalytic triad multiple mutants are likely to be substantially different from the wild-type enzyme, especially when the catalytic nucleophile, Ser221, is altered [for discussion, see Carter and Wells (1990)]. Nonetheless, these studies emphasize that the catalytic inefficiency of H64A in substrate-assisted catalysis is likely the result of a less functional catalytic triad and not the oxyanion hole. In fact, the original molecular modeling studies showed that when the position of the HisP2 substrate side chain was optimized for its interaction with Ser221, the H-bond angle to Asp32 was substantially compromised (Carter & Wells, 1987).

We therefore reasoned that it might be possible to enhance the activity of the H64A enzyme by mutations that cause subtle movements of the polypeptide backbone around Asp32 and/or Ser221 or that perturb the position of the histidine in the substrate. Thus, we decided to mutate the residues immediately flanking Asp32 but faced two major problems. First, it was essential to greatly restrict the number of replacements of residues 31 and 33 from 400 possibilities down to few, to allow characterization. Second, it was not possible to reliably predict the perturbation of Asp32 resulting from replacement of flanking residues, much less the catalytic consequences. Amino acids flanking catalytic residues in subtilisin-like proteases are tightly conserved (Mizuno et al., 1988): only valine, isoleucine, and leucine are found on the N-terminal side of the catalytic aspartate, whereas serine, threonine, and aspartate are found on the C-terminal side. The replacements of I31L and S33T were chosen from subtilisin *B. licheniformis* (Carlsberg), which is more active than the subtilisin BPN′ against a number of substrates (Wells et al., 1987a,b). We were encouraged to do this because the recruitment of three residues from subtilisin Carlsberg into subtilisin BPN′ (viz E156S/G169A/Y217L) enhances the activity of both the wild-type enzyme (Wells et al., 1987a,b) and the S24C/H64A variant (Carter et al., 1989). Furthermore, the mutation I31L has been shown to increase the activity of subtilisin E from the I-168 strain of *B. subtilis*, whereas the I31V mutation lowered activity ~2-fold and replacement by Cys, Thr, Ser, Ala, Gly, and Phe greatly reduced activity (Takagi et al., 1988).

As this study is part of an on-going effort to engineer subtilisin into a site-specific protease, replacements of residues 31 and 33 were installed in the context of the most active H64A variant that we had previously identified, namely the pentamutant S24C/H64A/E156S/G169A/Y217L (Carter et al., 1989). The I31L and S33T mutations together enhance the activity of the pentamutant against sFAHF-*p*na by decreasing K_M and for sFAQF-*p*na by increasing k_{cat}. Interpretation of these data is not straightforward because the γ methyl group of Thr33 in subtilisin Carlsberg forms part of the binding site for the P2 residue of the substrate (McPhalen & James, 1988). Thus, effects of the S33T mutation in the background of the pentamutant may reflect direct interaction between Thr33 and the P2 His in sFAHF-*p*na in addition to any effects that it is has upon Asp32.

The k_{cat}/K_M ratio for the heptamutant enzyme with the most preferred substrate sAAHY-*p*na (Carter et al., 1989) is increased 155-fold over the original enzyme/substrate pair (S24C/H64A, sFAHF-*p*na, P. Carter and L. Abrahmsén, unpublished results). Furthermore, the heptamutant has been used for site-specific proteolysis of fusion proteins and found to cleave the target site more efficiently than the pentamutant (S24C/H64A/E156S/G169A/Y217L) and much more efficiently than the original H64A enzyme (D. Vandlen and K. Miller, unpublished results). These improvements in the catalytic efficiency of substrate-assisted catalysis bring this new enzyme well within the practical range for use as a site-specific protease.

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Differential DNA Binding by Calf Uterine Estrogen and Progesterone Receptors Results from Differences in Oligomeric States†

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ABSTRACT: The studies presented here provided evidence that the calf uterine estrogen and progesterone receptors exhibit different DNA-binding properties in vitro as a result of having different dimerization constants. The affinity of the estrogen and progesterone receptors for DNA was measured by using isocratic elution from DNA-Sepharose. The hormone-free estrogen receptor had a 10-fold higher affinity for DNA than did the hormone-free progesterone receptor when measured at receptor concentrations of 6-12 nM and 180 mM KCl. No effect on DNA binding by binding progesterone to its receptor was detected. This contrasts with the increased affinity for DNA and increased number of ions released upon DNA binding exhibited by the hormone-bound estrogen receptor. Between 2 and 3 ions were released when the progesterone receptor and the diluted estrogen receptor bound DNA. These observations suggested the progesterone receptor was in the monomeric state, whereas the estrogen receptor was in the dimeric state at receptor concentrations of 6-12 nM. When the dimerization constant of the progesterone receptor was measured, the value of ≈7 nM obtained was 20-fold higher than the value of 0.3 nM reported for the estrogen receptor. This makes it likely the two receptors exist in different forms at the same concentration in vitro. It is also suggested the predominant form of the estrogen and progesterone receptors in vivo could differ.

The calf uterine estrogen and progesterone receptors are members of the steroid hormone receptor superfamily of gene-regulatory proteins [for review see Evans (1988)]. The transcription-regulation activity of these proteins in vivo is controlled by steroid binding. The estrogen receptor has the characteristic properties of an allosteric protein: it is a homodimer (Notides et al., 1985); it binds estradiol with a positive cooperative mechanism and with a maximum Hill coefficient of 1.6 (Notides et al., 1981); and hormone binding induces a conformation change in the receptor leading to an increased affinity for DNA (Skafar & Notides, 1985). The calf progesterone receptor, although less well studied, sediments on sucrose gradients as a 4S, monomeric species and a 6S, possibly dimeric species (Theofan & Notides, 1984). It also binds

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