Significance of Hydrophobic S_4–P_4 Interactions in Subtilisin 309 from *Bacillus lentus*

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ABSTRACT: The subtilisins have an extended substrate binding cleft comprising at least 8 subsites. Two pockets at the S_1 and S_4 sites are particularly conspicuous, and the interactions between substrate and these two pockets are very important for the substrate specificity. Phe residues have mutationally been introduced at one of positions 102, 128, 130, and 132 of the subtilisin Savinase from *Bacillus lentus* to investigate the effects of introducing bulky groups along the rim of the S_4 binding pocket. It is shown that the marked P_4 preference of wild-type Savinase for aromatic groups is eliminated by the Gly102 → Phe and Ser128 → Phe mutations, indicating that bulky groups at positions 102 and 128 block the S_4 binding site. In contrast, the activity toward hydrophilic P_4 residues is not nearly as affected by these mutations, suggesting that the binding mode of the P_4 side chain is dependent on its properties. Introduction of a bulky -CH_2-S-CH_2-CH_2-pyridyl group at position 128, by mutational incorporation of Cys followed by chemical modification with 2-vinylpyridine, has essentially the same effect. The Ser130 → Phe mutation hardly affects the activity of the enzyme while the Ser → Phe mutation at position 132 renders the preference for hydrophobic groups in P_4 even more pronounced. This mutation furthermore affects the size of the S_4 pocket. An analysis of double mutants at positions 132 and 104 suggests that the S_4 region is flexible and is adjusted upon binding of substrates.

The three-dimensional structures of subtilisins in complex with various proteinaceous inhibitors (Bode et al., 1987; McPhalen et al., 1985; McPhalen & James, 1988; Takeuchi et al., 1991a,b) suggest that two types of interactions between such enzymes and substrate are important: (a) hydrogen bonds between the main chains producing an antiparallel β-sheet and (b) hydrophobic interactions between the side chains at the P_1 and P_4 positions[1] and the corresponding binding pockets at the S_1 and S_4 subsites, respectively. The significance of the P_4–S_4 interactions, remote from the catalytic event, for rapid hydrolysis (Grøn et al., 1992; Grøn & Breddam, 1992; Svendsen, 1977) is of particular interest, but the features within the S_4 binding pocket influencing the catalytic efficiency are not well-established. The structures of the various enzyme–inhibitor complexes suggest that the amino acid residues 107, 126, and 135,[2] all located at the bottom of the pocket (Heinz et al., 1991; McPhalen & James, 1988), determine its hydrophobic nature, and the residue at position 104 appears to function as an adjustable lid on the pocket (Bech et al., 1992b; McPhalen et al., 1985). However, along the rim of the pocket a number of generally conserved, nonbulky amino acid residues are conspicuous (Figure 1) with residues Gly100-Ser101-Gly102, Ser125-Leu126-Gly127, Ser130, and Ser132 being invariant. Gly102 is involved in main chain to main chain interaction with the P_4 amino acid residue (McPhalen & James 1988). The backbone of the peptide segment 128–130 participates in formation of the S_4 binding pocket (Bode et al., 1987; Hirono et al., 1984; McPhalen & James, 1988), and a study of a number of mutants produced by random mutagenesis within this region implied that position 128, when occupied by a hydrophobic residue, is fixed at the S_4 binding pocket (Teplyakov et al., 1992). Residue 132 is also located on the rim but in the most remote position relative to the P_4 position of the substrate. However, this residue is of interest since it is suspected to change position upon binding of inhibitors (McPhalen & James, 1988) in concert with the movement of the side chain of Tyr104 (Bech et al., 1992b; Kraut, 1977; Takeuchi et al., 1991a).

The significance of these nonbulky amino acid residues on the rim of the P_4 pocket is here investigated by mutational introduction of bulky side chains at positions 102, 128, 130, and 132 of the subtilisin 309, Savinase,[3] from *Bacillus lentus*, followed by an extensive kinetic characterization of mutant enzymes. The mutants G102F and S128F which lack the marked P_4 preference of wild-type Savinase for aromatic groups are used to elucidate to which extent the favorable S_4–P_4 interactions are employed to stabilization of the transition state. Double mutants at positions 132 and 104 are constructed to investigate the interdependence of the effects of substitutions at these positions.

MATERIALS AND METHODS

Materials. A *Bacillus subtilis* strain (Δapr, Δnpr) was kindly provided by Sven Hastrup, Novo Nordisk A/S, Denmark. The *Escherichia coli*/*B. subtilis* shuttle vector containing the structural gene for Savinase (Bech et al., 1992b), the *E. coli* strain DH5α (Hanahan, 1985), and the M13mp18 subclone of the Savinase gene (Bech et al., 1992b) were from house collections. The synthetic oligonucleotides were synthesized on an Applied Biosystems 380 DNA synthesizer, and the oligonucleotide-directed in vitro mutagenesis system version 2 kit was from Amersham. Restriction endonucleases,

[1] The binding site notation is that of Schechter and Berger (1967). Accordingly, P_n denotes a substrate position, S_n denotes the corresponding enzyme binding subsite.

[2] The amino acid numbering is that of subtilisin BPN′ (Markland & Smith, 1967).

[3] Abbreviations: ABz, *o*-aminobenzoyl; -EP, ethylpyridine; pNA, *para*-nitroanilide; Pyr, pyridine; Savinase, subtilisin 309 from *B. lentus*; Suc, succinyl; Tyr(NO_2), 3-nitrotyrosine; Y′, Tyr(NO_2); ↓, the scissile bond; X102Z, Savinase where residue X at position 102 has been replaced by residue Z; S128C-EP, Savinase where the mutationally introduced Cys at position 128 has been alkylated with 2-vinylpyridine.

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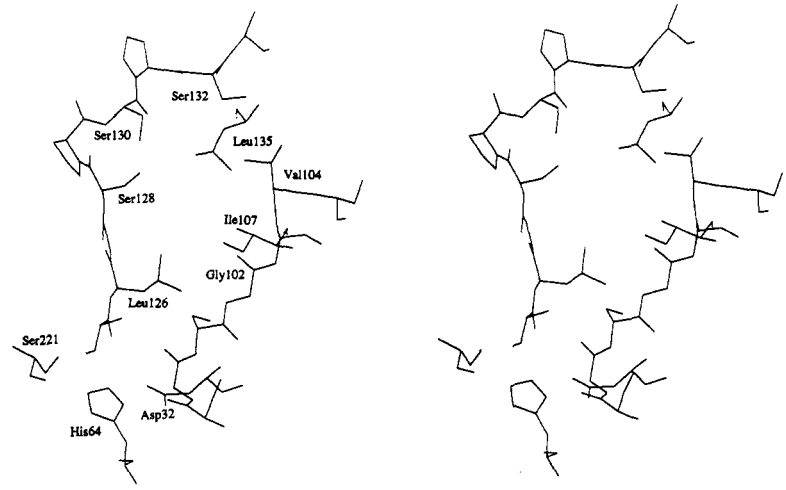

FIGURE 1: Stereoview of the S_4 binding site of Savinase (Betzel et al., 1992). The P_4 amino acid residue is involved in main chain to main chain interactions with Gly102 (McPhalen & James, 1988) and the P_4 side chain protrudes into the S_4 binding pocket bounded by Ser128, Ser130, and Ser132. Val104 functions as an adjustable lid on the pocket.

T4 polynucleotide kinase, and adenosine triphosphate were from Boehringer Mannheim, FRG. DNA sequencing was performed using the Taq Dye–Deoxy terminator cycle sequencing kit and the Model 373A DNA sequencing system from Applied Biosystems. Prep-A-Gene was from Bio-Rad Laboratories. Mes, Hepes, Bicine, and bacitracin were from Sigma, and bacitracin–Sepharose was prepared as previously described (Stepanov & Rudenskaya, 1983). Sephadex G50 was from Pharmacia LKB Biotechnology, Sweden. Suc-Ala-Ala-Pro-Phe-↓pNA was purchased from Bachem, Switzerland, and the fluorogenic substrates were synthesized as previously described (Grøn et al., 1992; Meldal & Breddam, 1991).

Production of Savinase Derivatives. In vitro mutagenesis was performed using a M13mp18 subclone, containing a 415-bp *Eco*RI–*Pvu*II fragment of the Savinase gene. High frequency of mutants was obtained using the oligonucleotide-directed in vitro mutagenesis system version 2 kit from Amersham, on the basis of the methods of Eckstein and coworkers (Nakamaye et al., 1986; Sayers et al., 1988; Taylor et al., 1988a,b), and the mutated sequences were reintroduced into an *E. coli*/*B. subtilis* shuttle vector as previously described (Bech et al., 1992b). Mutant enzymes were purified from a 1-L culture grown at 37 °C for $2^1/_2$ days with vigorous shaking in LB-medium containing chloramphenicol (6 mg/L). The enzyme purifications were performed as described (Bech et al., 1992b) and followed by assay against 0.35 mM Suc-Ala-Ala-Pro-Phe-↓pNA in 50 mM Bicine, 2 mM $CaCl_2$, 0.1 M KCl, 5% dimethylformamide, pH 8.5. The enzyme preparations were stored frozen in buffer at −18 °C. The enzymes were stable under these conditions.

Reduction of S128C with 100 mM β-mercaptoethanol and a subsequent alkylation with 94 mM 2-vinylpyridine was performed in 0.05 M Hepes, 2 mM $CaCl_2$, pH 7.5, using an enzyme concentration of 46 μM. The reaction was followed with assays against Suc-Ala-Ala-Pro-Phe-↓pNA until the activity remained constant and addition of another aliquot (94 mM) of 2-vinylpyridine had no further effect on activity.

Characterization of Mutant Savinase. The purity of the mutant enzymes was ascertained by SDS–polyacrylamide gel electrophoresis on 20% homogeneous gels using the Phast-System from Pharmacia LKB Biotechnology. The enzymes had previously been denatured by incubation in 0.1 M HCl for 30 min and then lyophilized in order to avoid autolysis on boiling in sample buffer containing SDS. The concentration of Savinase mutants was determined spectrophotometrically using a $\epsilon_{280} = 23\ mM^{-1}\ cm^{-1}$ or $27\ mM^{-1}\ min^{-1}$ (S128C-EP). These values were determined by amino acid analysis of a solution of enzyme with known absorbance, performed after acid hydrolysis for 24 h at 110 °C using a Pharmacia LKB α plus amino acid analyzer. N-Terminal sequencing was performed on an Applied Biosystems Model 470 A gas-phase sequencer using the program provided by the company.

The specific activity toward Suc-Ala-Ala-Pro-Phe-↓pNa (0.35 mM) was determined by assay in 50 mM Bicine, 0.1 M KCl, 2 mM $CaCl_2$, 5% dimethylformamide, pH 8.5, at 25 °C and 410 nm using a Perkin Elmer λ7 spectrophotometer. The kinetic constants for hydrolysis of ABz-Asp-Phe-Arg-Leu-Phe-↓Ala-Phe-Tyr(NO_2)-Asp-OH[3] were determined using the nonlinear regression data analysis program Enzfitter (Leatherbarrow, 1987). Assays were performed in 50 mM Bicine, 2 mM $CaCl_2$, 0.1 M KCl, 2% dimethylformamide, pH 8.5, and hydrolysis rates were determined by monitoring the fluorescence emission at 420 nm after excitation at 320 nm using a Perkin Elmer LS50 luminescence spectrometer.

The k_{cat}/K_M values for hydrolysis of the fluorogenic peptide substrates were determined from initial rates using the following relation: $k_{cat}/K_M = v_0/S_0E_0$ which is valid at $S_0 \ll K_M$ for systems that obey Michaelis–Menten kinetics. The validity of this equation was ascertained by performing determinations at a minimum of three substrate concentrations, and the values listed are the mean of these determinations. The initial rates were measured as described above. The cleavage site for typical representatives of the fluorogenic substrates has been determined as previously described (Grøn et al., 1992).

With Ac-Phe-↓OMe, the same approach was applied but the initial rates were determined at 25 °C using a pH-stat. The assays were performed at pH 8.5, and pH was kept constant by titration with 0.02 M NaOH. The reaction mixture contained 0.1 M KCl and 2 mM $CaCl_2$.

RESULTS

Along the rim of the S_4 binding pocket of the subtilisins, a number of well-conserved and nonbulky amino acid residues are found (Betzel et al., 1992; Bode et al., 1987; Hirono et al., 1984; McPhalen & James, 1988). To investigate the significance of this for substrate specificity, bulky residues (Phe) were introduced at positions 102, 128, 130, and 132 in the subtilisin Savinase. Ser128 was also exchanged with a cysteine

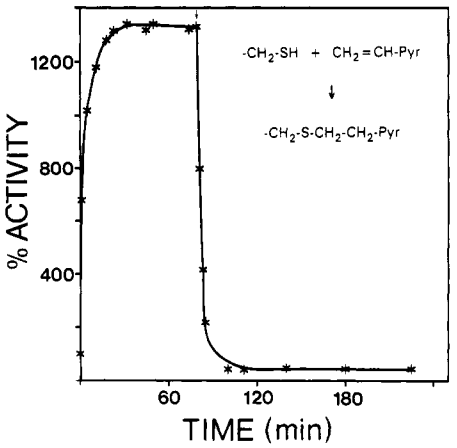

FIGURE 2: Reduction of S128C with β-mercaptoethanol followed by modification with vinylpyridine. An arrow indicates the addition of 2-vinylpyridine. The activity of the enzyme toward Suc-Ala-Ala-Pro-Phe-pNA was measured during the reaction.

Table I: k_{cat}/K_M for Hydrolysis of Fluorogenic Substrates of Varying Chain Length Catalyzed by Wild-Type Savinase, G102F, S128F, S130F, and S132F

	k_{cat}/K_M (μM^{-1} min^{-1})				
substrate[a]	wild type	G102F	S128F	S130F	S132F
ABz-P-F-↓G-Y'-D*	0.030	0.023	0.038	0.030	0.032
ABz-G-P-F-↓G-Y'-D*	265	2.9	3.4	204	490
ABz-F-G-P-F-↓G-Y'-D*	226	2.4	1.4	242	710
ABz-G-F-G-P-F-↓G-Y'-D*	530	6.6	2.1	436	860

[a] The asterisk indicates that the cleavage point has been checked. The standard deviations for k_{cat}/K_M values were <±5%.

residue, thus allowing chemical introduction of a bulky group with a higher conformational flexibility at this position, and two double mutants V104A;S132F and V104F;S132F were also constructed. The mutations were all accomplished in the structural gene for Savinase by standard procedures, and the presence of the mutations was confirmed by DNA sequencing. After reintroduction of the mutated sequences into an *E. coli*/*B. subtilis* shuttle vector (Bech et al., 1992b), the mutated enzymes were isolated and characterized. SDS–PAGE of the purified enzyme preparations demonstrated their homogeneity and correct molecular weight. The N-terminal sequences of S128F and G102F were identical to that of wild-type Savinase. Reduction of S128C with β-mercaptoethanol increased the activity toward Suc-Ala-Ala-Pro-Phe-↓pNA to 1500%, due to the removal of groups blocking the Cys, and subsequent treatment with 2-vinylpyridine reduced the activity to 50% of that of the original preparation (Figure 2). The identity of the blocking groups has not been determined, but it has previously been demonstrated that free Cys can be blocked by glutathione upon expression of recombinant barley α-amylase in yeast (Søgaard et al., 1991). An amino acid analysis confirmed the stoichiometry of the reaction; i.e., the preparation contained 0.8 mol of (2-pyridylethyl)cysteine/mol of enzyme. The specific activity toward Suc-Ala-Ala-Pro-Phe-↓pNA for G102F, S128F, S128C-EP, S130F, S132F, V104A;S132F, and V104F;S132F was 0.37, 5.4, 1.4, 37, 84, 17 and 2.8 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively. The corresponding value for wild-type Savinase was 48 $\mu mol \cdot min^{-1} \cdot mg^{-1}$.

For wild-type Savinase and the four mutated enzymes, G102F, S128F, S130F and S132F, a kinetic investigation was carried out using a series of fluorogenic substrates of increasing chain lengths (Table I). Wild-type Savinase hydrolyzed the short substrate ABz-Pro-Phe-↓Gly-Tyr(NO_2)-Asp-OH (spanning P_3–P'_3) with a low k_{cat}/K_M value. Elongation of the substrate with a single amino acid residue (Gly), hence positioning the hydrophobic ABz-group in the P_4 position, increased k_{cat}/K_M dramatically. Addition of another amino acid residue displaced the ABz group to P_5, but the hydrophobic nature of the new P_4 residue (Phe) secured that k_{cat}/K_M was maintained at the same level. With the longest substrate, the ABz group was located in the P_6 position and k_{cat}/K_M was increased further. With G102F and S128F, the activity toward the short substrate (ABz-Pro-Phe-↓Gly-Tyr(NO_2)-Asp-OH) was at the level of the wild-type enzyme, but with the substrates of increasing chain lengths the activity was drastically reduced. With S130F the k_{cat}/K_M values for hydrolysis of all four substrates were only slightly affected compared to those of the wild-type enzyme, but with S132F elevated values were observed with the longer substrates. For wild-type Savinase as well as for the mutant enzymes, the scissile peptide bond was determined (marked with an asterisk in Table I) and in all cases the cleavage point was as expected.

Table II: Substrates Used for Kinetic Characterization of Savinase Mutants

series	P_6	P_5	P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3
I		ABz	Phe	Gly	Pro	Xaa	Tyr(NO_2)	Asp	
II			ABz	Asp	Xaa	Phe	Gly	Tyr(NO_2)	Asp
III		ABz	Phe	Xaa	Pro	Phe	Tyr(NO_2)	Asp	
IV		ABz	Xaa	Gly	Pro	Phe	Tyr(NO_2)	Asp	
V	ABz	Gly	Xaa	Gly	Pro	Phe	Tyr(NO_2)	Asp	
VI	ABz	Xaa	Phe	Gly	Pro	Phe	Tyr(NO_2)	Asp	

The effect of the mutations on hydrolysis of a very short substrate was tested using the ester substrate Ac-Phe-↓OMe. With wild-type enzyme k_{cat}/K_M was 2000 mM^{-1} min^{-1} while the corresponding values for G102F, S128F, S130F, and S132F were 1500, 2700, 2100, and 3100 mM^{-1} min^{-1}, respectively.

With the four mutated enzymes, G102F, S128F, S130F, and S132F the k_{cat}/K_M values were determined using the substrate series I, II, III, IV, and VI in which one amino acid residue (Xaa) at positions P_1, P_2, P_3, P_4, and P_5 was systematically varied (Table II). In each substrate series Xaa = Arg, Asn, Ala, Leu, and Phe (i.e., a charged, an uncharged/polar, and three hydrophobic residues of increasing size) was tested. With the substrates of the series I, II, III, and VI varying at the P_1, P_2, P_3, and P_5 position, respectively, the preferences of the four mutants were very similar to those observed with wild-type enzyme (Figure 3). S128F and G102F hydrolyzed all substrates of these series with very low k_{cat}/K_M values while with S130F and S132F the k_{cat}/K_M values were as high as or higher than those of wild-type enzyme. In contrast, the P_4 preference of Savinase seemed to be influenced by the presence of bulky groups on the rim of the S_4 binding subsite, and therefore a more elaborate investigation of the P_4 preferences was carried out.

The mutations Gly102 → Phe and Ser128 → Phe strongly affected the P_4 substrate preference (Table III). With these two enzymes the substrates with bulky and hydrophobic Xaa (i.e., Val, Leu, Phe, Trp, and Pro) were hydrolyzed with dramatically reduced k_{cat}/K_M values compared to wild-type enzyme (<2%). The activity toward the substrate with the nonbulky and hydrophobic Ala was less affected (4–8%) and toward Xaa = Gly it was only reduced to 45–73%. The activities toward substrates with hydrophilic Xaa (Asp, Asn, and Arg) were between 7% and 140% of the values obtained with the wild-type enzyme.

This substrate-dependent effect of a bulky Phe at positions 102 or 128 was further investigated with two longer substrates, ABz-Asp-Xaa-Gly-Pro-Phe-↓Gly-Gly-Tyr(NO_2)-Asp-OH

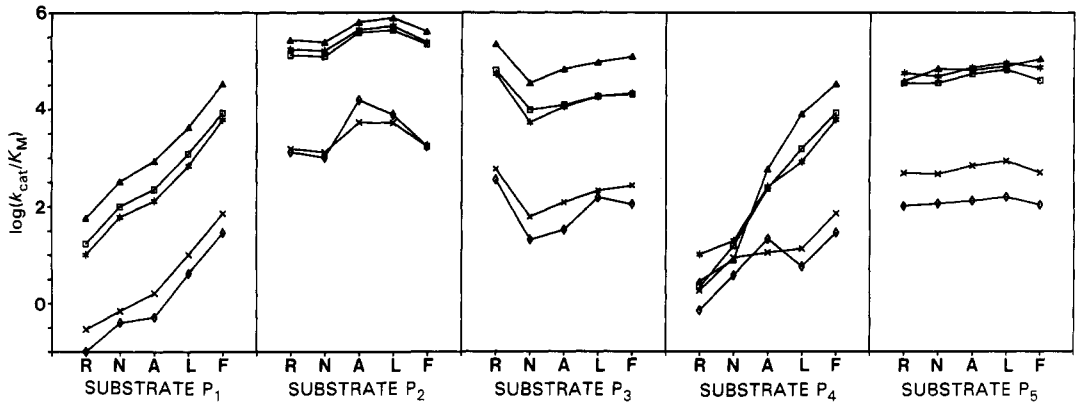

FIGURE 3: P_1, P_2, P_3, P_4, and P_5 preferences of wild-type Savinase (*), G102F (×), S128F (◇), S130F (□), and S132F (▲). The preferences were determined using the substrate series I, II, III, IV, and VI, respectively. For each subsite Xaa = Arg, Asn, Ala, Leu, and Phe is listed in order of increasing hydrophobicity.

Table III: k_{cat}/K_M for Hydrolysis of Fluorogenic Peptide Substrates Varying at the P_4 Position, Catalyzed by Wild-Type Savinase, G102F, S128F, S128C-EP, S130F, S132F, V104A;S132F, and V104F;S132F

	k_{cat}/K_M (mM^{-1} min^{-1})							
substrate	wild type[a]	G102F	S128F	S128C-EP	S130F	S132F	V104A;S132F	V104F;S132F
ABz-D-G-P-F-↓Y′-D	2.1	3.0	0.36	0.33	4.2	2.7	1.4	0.39
ABz-N-G-P-F-↓Y′-D	19	8.6	3.7	2.6	15	7.7	4.6	16
ABz-R-G-P-F-↓Y′-D*	10	1.8	0.7	0.67	2.1	2.8	2.0	1.4
ABz-G-G-P-F-↓Y′-D	22	16	10	4.4	24	42	28	20
ABz-A-G-P-F-↓Y′-D*	250	11	21	20	230	580	520	510
ABz-V-G-P-F-↓Y′-D	2100	24	25	112	2400	5600	4700	4000
ABz-L-G-P-F-↓Y′-D	820	13	5.6	65	1500	8000	5700	1700
ABz-F-G-P-F-↓Y′-D*	6000	71	28	320	8400	33000	53500	6000
ABz-W-G-P-F-↓Y′-D	2600	41	14	84	4300	1400	1500	120
ABz-P-G-P-F-↓Y′-D	690	7.7	2.3	9.2	180	230	380	570

[a] Data are taken from Grøn et al. (1992). For k_{cat}/K_M values in the range 10^2–10^5 mM^{-1} min^{-1}, the standard deviations were less than ±5%; in the range 10–10^2 mM^{-1} min^{-1}, they were less than ±10%, and for k_{cat}/K_m < 10 mM^{-1} min^{-1}, they were less than ±15%. The asterisk denotes that the cleavage point has been determined.

Table IV: k_{cat}/K_M Values for Hydrolysis of Substrates of Varying Amino Acid Sequence Catalyzed by Wild-Type Savinase, G102F, and S128F

		k_{cat}/K_M (μM^{-1} min^{-1})		
	substrate	wild type[a]	G102F	S128F
A	ABz-D-F-R-L-F-↓A-F-Y′-D	1700[b]	39[c]	52[d]
B	ABz-D-F-G-P-F-↓G-G-Y′-D	520	4.9	1.3
C	ABz-D-G-G-P-F-↓G-G-Y′-D	1.1	0.21	0.34

[a] Data are taken from Grøn and Breddam (1992). [b] k_{cat} = 360 min^{-1}, K_M = 0.2 μM. [c] k_{cat} > 200 min^{-1}, K_M > 5 μM. [d] k_{cat} > 520 min^{-1}, K_M > 10 μM. The standard deviations for the k_{cat}/K_M values are <±5%.

(Xaa = Phe or Gly) (Table IV). With Xaa = Phe the k_{cat}/K_M was reduced to 0.94% and 0.25% with G102F and S128F, respectively, while with Xaa = Gly the activities were reduced to 19% and 31%, respectively. Thus, the same pattern was observed with long substrates.

To ascertain whether the effects of Phe at position 102 and 128 on k_{cat}/K_M were due to reduced k_{cat} values or increased K_M values, the hydrolysis of the substrate ABz-Asp-Phe-Arg-Leu-Phe-↓Ala-Phe-Tyr(NO_2)-Asp-OH was investigated. With wild-type Savinase, this substrate was hydrolyzed with a k_{cat} value of 360 min^{-1} (Table IV) and the values with G102F and S128F were similar to this. In contrast, the Gly102 → Phe and Ser128 → Phe mutations caused a more than 25-fold increase in K_M, from 0.2 μM (wild type) to values exceeding 5 μM.

The P_4 preference of S128C-EP was determined using the substrate series IV. Incorporation of the -CH_2-S-CH_2-CH_2-Pyr group at Cys128 affected the activity in almost the same manner as the Ser → Phe mutation, but the dramatic loss of activity observed with Xaa = Val, Leu, Phe, and Trp was less pronounced than with S128F (Table III).

Introduction of a Phe at position 130 had only a slight effect on the activity toward the substrates with hydrophobic Xaa which were hydrolyzed with k_{cat}/K_M values between 92% and 180% of those measured with wild-type Savinase (Table III). With Xaa = Asp the activity was increased 2-fold while with Xaa = Arg or Pro k_{cat}/K_M was reduced to about 25%. The activity toward substrates with Xaa = Asn or Gly was essentially unchanged.

Replacement of Ser132 with a bulky Phe residue significantly affected the P_4 preference. With Xaa = Ala, Val, Leu, or Phe k_{cat}/K_M was significantly increased and the degree of activation was dependent on the size of Xaa (highest with Xaa = Leu). With Xaa = Gly or Asp, a significantly smaller activation was observed and substrates with Xaa = Trp, Arg, Asn, and Pro were hydrolyzed with reduced k_{cat}/K_M values.

To ascertain that the effects of the introduction of a Phe at position 132 were not due to choice of the substrate series, the P_4 preference was determined with the substrate series V for both wild type and S132F (data not shown). Comparison of the kinetic data for wild-type enzyme with the two series showed that the preference for an aromatic residue was equally high; i.e., $\Delta\Delta G_T^{\ddagger}$(Phe → Gly)[4] was 13.6 ± 0.13 kJ/mol and 16.4 ± 0.10 kJ/mol within series IV and V, respectively. The higher activities previously observed with S132F toward the substrates with hydrophobic P_4 residues were also found within the longer substrate series, although the beneficial effect of the Phe at position 132 was slightly smaller.

[4] $\Delta\Delta G_T^{\ddagger}$(observed(A → B)) = $-RT\ln((k_{cat}/K_M(B))/(k_{cat}/K_M(A)))$.

Two double mutants, V104A;S132F and V104F;S132F, were also characterized using the substrate series IV (Table III). These mutations at positions 104 and 132 reduced k_{cat}/K_M for the hydrolysis of substrates with hydrophilic Xaa, i.e., Arg, Asp, Asn, or Gly, to 15–85% of those found with wild-type Savinase. Within the hydrophobic series of substrates, the activities were also significantly affected: relative to wild-type enzyme, V104A;S132F hydrolyzed the substrates with Xaa = Ala, Val, Leu, and Phe with increased k_{cat}/K_M values compared to those of wild type, the increase being most pronounced with Xaa = Phe. With Xaa = Trp or Pro, the activity was reduced. V104F;S132F hydrolyzed substrates with Xaa = Ala, Val, or Leu with elevated k_{cat}/K_M (2-fold) compared to those of wild type. With Xaa = Phe, k_{cat}/K_M was unchanged, and with Xaa = Trp, k_{cat}/K_M was reduced to 5%.

For the substrates with Xaa = Arg, Ala, and Phe, i.e., substrates hydrolyzed with low, medium, and high k_{cat}/K_M values, the cleavage point was determined (marked with an asterisk in Table III), and no shift in the site of cleavage was observed with any of the mutants.

DISCUSSION

The binding site of the subtilisins can be divided into at least eight subsites of which S_1 and S_4 are the most important (Grøn et al., 1992). Favorable S_4–P_4 interactions distant from the seat of reaction at the scissile bond seem to have profound effects on the catalytic activity as reflected by the response of the catalytic efficiency to increasing substrate chain length. The increase in k_{cat}/K_M of wild-type Savinase on going from the short substrate ABz-Pro-Phe-↓Gly-Tyr(NO_2)-Asp-OH (spanning P_3–P'_3) to ABz-Gly-Pro-Phe-↓Gly-Tyr(NO_2)-Asp-OH (with favorable interactions between the hydrophobic ABz in P_4 and the S_4 binding pocket) is 8800-fold (Table I). Mutations at positions 102, 128, and 132 along the rim of the S_4 binding pocket seem to affect the S_4–P_4 interactions. With S132F, the beneficial effect of including the S_4–P_4 interactions has increased to 15 000-fold whereas with G102F and S128F it has been reduced to 100-fold, suggesting that the S_4–P_4 interactions with these enzymes are severely impaired. However, the low activities of G102F and S128F toward the longer substrates are not due to adverse effects of the bulky groups on the catalytic apparatus, since the two short substrates ABz-Pro-Phe-↓Gly-Tyr(NO_2)-Asp-OH and Ac-Phe-↓OMe are hydrolyzed with k_{cat}/K_M values comparable to those of wild-type enzyme.

For wild-type Savinase, a marked preference for aromatic groups in the P_4 position has been reported (Grøn et al., 1992), and it seems that hydrophobic S_4–P_4 interactions are predominant. However, the marked preference for aromatic groups observed with wild-type enzyme is invalidated by the Gly102 → Phe and Ser128 → Phe mutations, confirming the adverse effect of bulky groups at positions 102 and 128 on the S_4–P_4 interactions. The kinetic parameters k_{cat} and K_M for the hydrolysis of a substrate with Phe in P_4 show that the effect is almost exclusively due to impaired binding in the Michaelis complex (Table IV).

These results indicate that bulky groups at positions 102 and 128 block the S_4 binding pocket such that favorable interactions between a hydrophobic residue at the P_4 position of the substrate and the S_4 binding pocket are no longer possible. Consistent with this, the hydrolysis of a substrate with Gly at P_4 is only slightly affected by these mutations (Table IV). It is remarkable that the change in activity toward substrates with hydrophilic groups in P_4 is not nearly as pronounced as observed with the hydrophobic P_4 residues; these substrates which possess fairly bulky groups at the P_4 position fit better into the pattern of the substrate with Gly at the P_4 position. This indicates that within the S_4 site of the wild-type enzyme the orientations of hydrophobic side chains differ from those of the hydrophilic. The reduced activity of G102F and S128F toward substrates with bulky/hydrophobic P_4 residues may be due to the bulky groups at positions 102 and 128 preventing the proper orientation and optimal binding of hydrophobic P_4 residues such that they are forced into the less optimal binding mode of the hydrophilic substrates. This is consistent with the fact that G102F and S128F hydrolyze substrates with widely different P_4 groups essentially equally well.

The higher activity toward substrates with hydrophobic residues at the P_4 position observed with S128C-EP compared to S128F indicates that the aliphatic chain of the -CH_2-S-CH_2-CH_2-Pyr group renders the blocking of the S_4 pocket less efficient, presumably due to a higher degree of conformational flexibility or to a partial displacement of the bulky pyridyl group away from the S_4 pocket.

Recently, a study introducing random mutations at positions 128–130 (BPN′ numbering) of a subtilisin from *Bacillus alcalophilus* (Teplyakov et al., 1992) has implied that hydrophobic residues at position 128 affect the binding of substrates since K_M for hydrolysis of Suc-Ala-Ala-Pro-Phe-↓pNA is significantly increased. Structural studies have positioned the bulky groups at the S_4 binding pocket (Teplyakov et al., 1992). Although these results are not simple to interpret, since the mutants at position 128 appear in various combinations and not as single mutants, they seem to be consistent with the results on S128F obtained here.

On the basis of the results in Table III, the difference in transition-state stabilization between substrates of the general formula ABz-Xaa-Gly-Pro-Phe-↓Tyr(NO_2)-Asp-OH with Phe and Gly in P_4, i.e., $\Delta\Delta G_T^{\ddagger}$(Phe → Gly),[4] may be calculated. With the wild-type enzyme, G102F and S128F values of 13.9 ± 0.13, 3.4 ± 0.35, and 2.6 ± 0.30 kJ/mol, respectively, are obtained, signifying the loss of the P_4 substrate preference of the two mutants. These $\Delta\Delta G_T^{\ddagger}$ values may be higher than observed with more optimized substrate pairs containing the Phe → Gly substitutions. This is due to an interdependence of the individual S_n–P_n interactions with the consequence that the k_{cat}/K_M value cannot be rationalized on the basis of simple additive contributions from each subsite–substrate interaction (Grøn & Breddam, 1992). In such cases, $\Delta\Delta G_T^{\ddagger}$(observed) deviates significantly from $\sum_{P_n}\Delta\Delta G_T^{\ddagger}(P_n)$.[5] It is suggested that the strong interactions between the S_4 binding pocket and hydrophobic P_4 residues contribute significantly to this deviation (Grøn & Breddam, 1992).

The extent to which such interdependence is affected by the blocking of the S_4 binding pocket was investigated with the two substrates ABz-Asp-Phe-Arg-Leu-Phe-↓Ala-Phe-Tyr-(NO_2)-Asp-OH (A) and ABz-Asp-Gly-Gly-Pro-Phe-↓Gly-Gly-Tyr(NO_2)-Asp-OH (C), containing four invariant and five variant positions and among these the Phe → Gly substitution in P_4. Wild-type Savinase hydrolyzes these substrates with widely different k_{cat}/K_M values (see Table IV), corresponding to a $\Delta\Delta G_T^{\ddagger}$(observed) of 18.2 ± 0.03 kJ/mol as compared with a $\sum_{P_n}\Delta\Delta G_T^{\ddagger}(P_n)$ of 27.2 ± 0.17 kJ/mol (Table V). This difference is due to the binding energy at the different S_n–P_n interactions being only partially utilized for

[5] $\sum_{P_n}\Delta\Delta G_T^{\ddagger}(P_n)$ is the sum of the $\Delta\Delta G_T^{\ddagger}$(Xaa → Yaa) values for the substituted amino acids in the substrate pairs. The $\Delta\Delta G_T^{\ddagger}$(Xaa → Yaa) values are obtained from substrate pairs in which only one position is substituted.

Table V: Contributions from Each Substrate–Subsite Interaction to the Catalytic Efficiency, Comparing the Substrates Listed in Table IV[a]

substrate pair	ABzDFRLFAFY'D A → B || || ABzDFGPFGGY'D			ABzDFRLFAFY'D A → C ||| || ABzDGGPFGGY'D		
enzyme	wt	G102F	S128F	wt	G102F	S128F
P_4 $\Delta\Delta G_T^{\ddagger}$ (kJ/mol)	0	0	0	13.9 ± 0.13	3.4 ± 0.35	2.6 ± 0.30
P_3 $\Delta\Delta G_T^{\ddagger}$ (kJ/mol)	5.4 ± 0.06	5.4 ± 0.06	5.4 ± 0.06	5.4 ± 0.06	5.4 ± 0.06	5.4 ± 0.06
P_2 $\Delta\Delta G_T^{\ddagger}$ (kJ/mol)	4.2 ± 0.05	4.2 ± 0.05	4.2 ± 0.05	4.2 ± 0.05	4.2 ± 0.05	4.2 ± 0.05
P_1 $\Delta\Delta G_T^{\ddagger}$ (kJ/mol)	0	0	0	0	0	0
P'_1 $\Delta\Delta G_T^{\ddagger}$ (kJ/mol)	0.2 ± 0.06	0.2 ± 0.06	0.2 ± 0.06	0.2 ± 0.06	0.2 ± 0.06	0.2 ± 0.06
P'_2 $\Delta\Delta G_T^{\ddagger}$ (kJ/mol)	3.5 ± 0.03	3.5 ± 0.03	3.5 ± 0.03	3.5 ± 0.03	3.5 ± 0.03	3.5 ± 0.03
$\sum_{P_n}\Delta\Delta G_T^{\ddagger}(P_n)$ (kJ/mol)	13.3 ± 0.10	13.3 ± 0.10	13.3 ± 0.10	27.2 ± 0.17	16.7 ± 0.36	15.9 ± 0.32
$\Delta\Delta G_T^{\ddagger}$ (obsd) (kJ/mol)	2.9 ± 0.02	5.1 ± 0.04	9.1 ± 0.05	18.2 ± 0.03	13.0 ± 0.04	12.1 ± 0.05

[a] $\sum_{P_n}\Delta\Delta G_T^{\ddagger}(P_n)$ is the sum of the $\Delta\Delta G_T^{\ddagger}$(Xaa→Yaa) values for the substituted amino acids in the substrate pairs. The $\Delta\Delta G_T^{\ddagger}$(Xaa→Yaa) values are obtained from substrate pairs in which only one position is substituted. The values are from Grøn et al. (1992) or are calculated from Table III. $\Delta\Delta G_T^{\ddagger}$ (observed (A→B)) = $-RT \ln((k_{cat}/K_M(B))/(k_{cat}/K_M(A)))$. The amino acid substitutions are marked with vertical lines.

transition-state stabilization. It is not clear, however, whether the proportion of unexploited binding energy is similar for every S_n–P_n interaction or if the inability to fully utilize the interaction energy is concentrated at a few sites. With the two mutants G102F and S128F, the $\sum_{P_n}\Delta\Delta G_T^{\ddagger}(P_n)$ is reduced to 16.7 ± 0.3 and 15.9 ± 0.32 kJ/mol, respectively. If the S_4–P_4 interactions were fully utilized for transition-state stabilization in the wild-type enzyme, one would predict that $\Delta\Delta G_T^{\ddagger}$(observed) for these mutants would suffer a similar reduction (12–14 kJ/mol) due to the abolition of the S_4–P_4 interactions. However, the reduction was only 5–6 kJ/mol ($\Delta\Delta G_T^{\ddagger}$(observed) = 13.0 ± 0.04 and 12.1 ± 0.05 kJ/mol with G102F and S128F, respectively), suggesting that only part of the S_4–P_4 binding energy in an optimized substrate is used for transition-state stabilization. Interestingly, $\Delta\Delta G_T^{\ddagger}$(observed) for both mutants equals $\sum_{P_n}\Delta\Delta G_T^{\ddagger}(P_n)$, signifying the contribution of favorable S_4–P_4 interactions to the breakdown of additivity observed for wild-type Savinase.

For the substrate pair A → B in which the P_4 position was not substituted, $\sum_{P_n}\Delta\Delta G_T^{\ddagger}(P_n)$ (13.3 ± 0.1 kJ/mol) was identical for wild-type Savinase, G102F, and S128F, since the preferences of other subsites are not affected by the mutations (Figure 3). $\Delta\Delta G_T^{\ddagger}$(observed) was 2.9 ± 0.02 kJ/mol, 5.1 ± 0.04 kJ/mol, and 9.1 ± 0.05 kJ/mol with wild-type Savinase, G102F, and S128F, respectively; i.e., for the mutants $\Delta\Delta G_T^{\ddagger}$(observed) are higher than for wild-type and for S128F it approaches $\sum_{P_n}\Delta\Delta G_T^{\ddagger}(P_n)$.

X-ray crystallographic studies of complexes of subtilisins with proteinaceous inhibitors do not suggest that the side chains of Ser130 and Ser132 are in direct contact with the side chain of the P_4 residue (Bode et al., 1987; Heinz et al., 1991; McPhalen & James, 1988; Takeuchi et al., 1991b). However, replacement of these residues with bulky Phe residues affected the activities toward the substrates of series IV. With S130F the ratio of activities toward hydrophobic and hydrophilic substrates is essentially constant, but with S132F this ratio is increased (Table III). A plot of log (k_{cat}/K_M) for S132F and wild-type Savinase versus the hydrophobicity of the P_4 residue is shown in Figure 4. With S132F a linear dependency is observed going from Ala to Phe, mainly because the relation between Val and Leu has been reversed. This could be due to an influence of Phe at position 132 on the shape of the S_4 pocket, and this influence is supported by the fact that S132F hydrolyzes the substrates with Xaa = Trp and Pro at a lower rate than the wild-type enzyme. It is possible that the introduction of the bulky and hydrophobic Phe at position 132 causes a shift in the orientation of the P_4 side chain or,

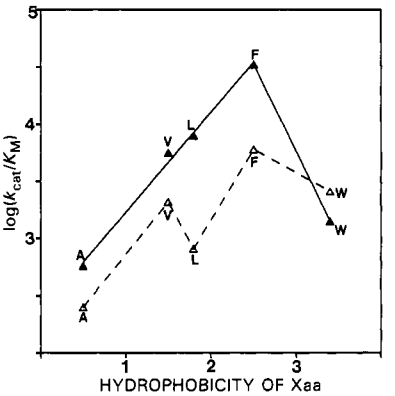

FIGURE 4: Effect of hydrophobicity (Chotia, 1984) of Xaa on the log (k_{cat}/K_M) for the hydrolysis of ABz-Xaa-Gly-Pro-Phe-Tyr(NO_2)-Asp-OH catalyzed by S132F (▲) and wild-type Savinase (△).

alternatively, induces a local conformational change in the enzyme.

The loop including residue 132 has been reported to change position upon binding of a proteinaceous inhibitor (McPhalen & James, 1988) in concert with the apparent mobility of the residue occupying position 104 (Bech et al., 1992b; Kraut, 1977; Takeuchi et al., 1991a). Therefore, it was of interest to combine mutations at these two positions (104 and 132) to investigate the interdependence of the effects of the substitutions.

The mutant V104A has previously been shown to exhibit increased activity toward substrates ABz-Xaa-Gly-Pro-Phe-↓Tyr(NO_2)-Asp-OH with bulky/hydrophobic Xaa (Bech et al., 1992b), and introduction of a Phe at position 132 in V104A increased the activities toward substrates with hydrophobic Xaa even further (Figure 5A). Thus, the Ser132 → Phe mutation had a similar effect as in the wild-type background (Figure 5B). However, the increase in k_{cat}/K_M with Xaa = Phe due to the Ser132 → Phe mutation is less pronounced, and the adverse effect observed with Xaa = Trp is larger with V104A; S132F than with S132F. This indicates that the enhanced complementarity between enzyme (Ala at position 104) and substrate (bulky groups in the P_4 position) is not fully realized in the presence of a bulky group at position 132.

The Savinase mutant with a bulky Phe residue occupying position 104, V104F, exhibited much higher activity toward substrates with nonbulky Xaa as compared with the wild-type enzyme (Bech et al., 1992b). However, with increasing size of Xaa, this beneficial effect was attenuated and substrates with bulky groups at P_4 were hydrolyzed with reduced k_{cat}/

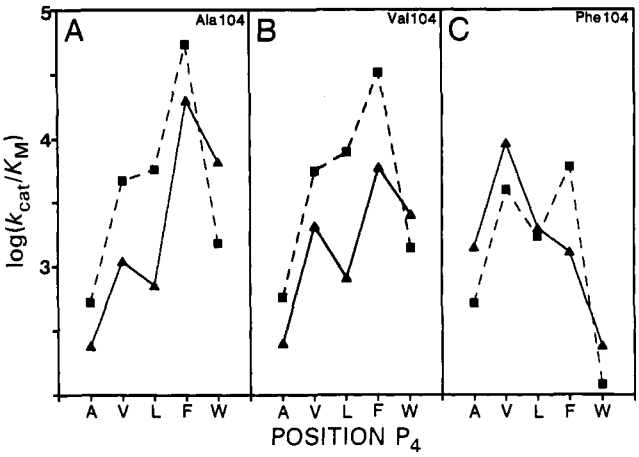

FIGURE 5: Comparison of log (k_{cat}/K_M) for Savinase mutants with Ser (▲) or Phe (■) residues at position 132. The comparison is made with position 104 = Ala (panel A), Val (panel B), and Phe (panel C).

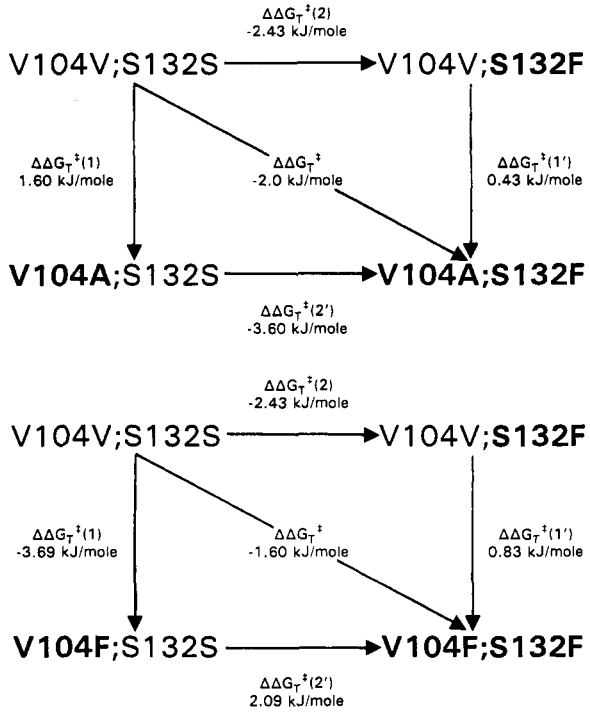

FIGURE 6: The energy of interaction of individual side chains of the enzyme (positions 104 and 132) with the transition state of the substrate, represented by the $\Delta\Delta G_T^‡$ terms. For example, the $\Delta\Delta G_T^‡$-(1) is the difference in transition-state stabilization energy between the mutant V104A, S132S, and wild type (V104V, S132S). $\Delta\Delta G_T^‡ = (-RT\ln((k_{cat}/K_M)_{V104A,S132S}/(k_{cat}/K_M)_{wildtype}))$ (Carter et al., 1984). The standard deviations for the $\Delta\Delta G_T^‡$ values are <0.1.

K_M values. Introduction of a Phe at position 132 abolished the high activity toward substrates with nonbulky Xaa at P_4, while with Xaa = Phe the activity was increased. With Xaa = Trp, the k_{cat}/K_M was further reduced as compared to V104F (Figure 5C).

If replacement of side chains at positions 104 and 132 were independent, the overall change in interaction energy of the transition-state complex in the double mutants would be the sum of the corresponding terms for the single mutants (Carter et al., 1984). In the thermodynamic terms of Figure 6 the following relations should be valid:

$$\Delta\Delta G_T^‡(1) = \Delta\Delta G_T^‡(1') \quad \text{and} \quad \Delta\Delta G_T^‡(2) = \Delta\Delta G_T^‡(2')$$

However, for the $\Delta\Delta G_T^‡$ values listed in Figure 6 (on the basis of the k_{cat}/K_M values for hydrolysis of ABz-Val-Gly-Pro-Phe-↓Tyr(NO_2)-Asp-OH from Table III and Bech et al. (1992b), the relation above is not valid. Likewise, for all substrates in series IV, regardless of the nature of Xaa, the transition-state stabilization energy calculated for the Ser132 → Phe mutation varies depending on the nature of the amino acid residue at position 104, indicating that the amino acid replacement at these two positions is accompanied by structural changes in the enzyme/substrate complex. This supports the results obtained with S132F and is in good agreement with the observation that the loop containing position 132 changes position in concert with position 104 upon binding of proteinaceous inhibitors (McPhalen & James 1988).

The results presented above suggest that the enzyme/substrate interface is rather flexible in the S_4 region. The amino acid residue occupying position 104 is mobile such that it is positioned in the S_4 binding site only when it can interact favorably with the P_4 side chain of the substrate (Bech et al., 1992b), and residue 132 seems to move in concert with residue 104 upon substrate binding. A certain flexibility of the substrate P_4 side chain is also very likely with multiple binding modes, depending on the properties of the side chain, as a result. The significance of the nonbulky amino acid residues at positions 102 and 128 is clearly to reduce the steric hindrance and allow access of bulky and hydrophobic P_4 side chains to the S_4 binding pocket. A bulky Phe at either of these positions appears to function as a lid on the pocket, hence disabling the beneficial hydrophobic interactions. In contrast, it is less clear why nonbulky residues exclusively are found at positions 130 and 132. The enzymes with Phe at these positions appear to function equally well.

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