Articles

Mutational Replacements of the Amino Acid Residues Forming the Hydrophobic S_4 Binding Pocket of Subtilisin 309 from *Bacillus lentus*

Steen Bech Sørensen, Lene M. Bech, Morten Meldal, and Klaus Breddam*

Carlsberg Laboratory, Department of Chemistry, Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby, Denmark

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ABSTRACT: The amino acid side chains of Ile107, Leu126, and Leu135 participate in the formation of the important hydrophobic S_4 binding pocket of the subtilisin Savinase. Ile107 and Leu126, located on each side of the pocket, point toward each other, and Leu135 is situated at the bottom of the pocket. These amino acid residues have been substituted for other hydrophobic amino acid residues by site-directed mutagenesis, and the resulting enzymes have been characterized with respect to their P_4 substrate preferences. The Leu126 → Ala or Phe substitutions reduce k_{cat}/K_M for the hydrolysis of all substrates to around 5% without altering the substrate preference. It is concluded that Leu126 is an essential structural part of the pocket which cannot be replaced without seriously affecting catalysis, consistent with the fact that Leu126 is conserved among all subtilisins. In contrast, the Ile107 → Gly, Ala, Val, Leu, or Phe and Leu135 → Ala, Val, or Phe substitutions strongly influence the P_4 substrate preference, and some of the mutants exhibit large specificity changes for particular substrates when compared to wild-type Savinase. The results can be rationalized on the basis of Ile107 and Leu135 being responsible for steric repulsion of branched aliphatic and aromatic P_4 side chains, respectively. Leu135 exclusively interacts with aromatic P_4 side chains, and its replacement with less bulky amino acid residues alleviates steric repulsion such that the activity toward this type of substrates is enhanced. Conversely, the introduction of a more bulky amino acid residue at position 135 produces more steric repulsion and reduces the activity toward substrates with aromatic P_4 side chains. Ile107 provides important van der Waals contacts with aromatic as well as aliphatic side chains, but some steric repulsion in the case of branched aliphatic P_4 side chains, in particular with Leu, complicates the picture. Some of this strain is alleviated by the Ile107 → Gly, Ala, or Val substitution. All the mutants, including those with the most sterically restricted S_4 sites, hydrolyze an optimized substrate (very high k_{cat}/K_M) with a Phe at the P_4 position at rates varying only 2–3-fold as compared to 84-fold for a nonoptimized substrate (low k_{cat}/K_M). These results suggest that the availability of sufficient overall binding energy can overcome the steric repulsion at a single subsite.

The interactions between amino acid side chains of the substrate and particular regions of the active site cleft of proteolytic enzymes provide the basis for substrate preference. A number of binding subsites are poorly defined such as the S_3 and S'_1 sites[1] of the subtilisins, with the consequence that they discriminate very little between different amino acid residues and contribute insignificantly to transition-state stabilization (Grøn et al., 1992). On the other extreme, some binding subsites are well-defined pockets in the active site cleft containing charged groups or groups capable of participating in hydrogen bond formation, e.g., the S_1 site in trypsin. Such subsites provide efficient, specific, and highly defined binding of charged amino acid side chains, and often it is a prerequisite for cleavage that a specific amino acid side chain bind to this site. A third type of binding pocket is well-defined but largely hydrophobic, e.g., the S_1 and S_4 sites of the subtilisins. Such sites efficiently bind a group of hydrophobic amino acid side chains, but it is not a prerequisite for cleavage of a peptide substrate that this preference is met.

In a recent study it was shown that the hydrophobic S_4 binding pocket of the subtilisin Savinase[2] was only used when a favorable interaction could be achieved (Bech et al., 1993). It was found that blocking the access to the S_4 binding pocket by incorporation of bulky groups at positions 102 and 128[3] (situated at the rim of the S_4 subsite) seriously affected the activity toward substrates with hydrophobic P_4 side chains while that toward substrates with hydrophilic P_4 side chains was much less affected. This suggests that hydrophilic amino acid side chains do not employ the hydrophobic S_4 binding pocket in the wild-type enzyme and that the high activity toward substrates with hydrophobic side chains is due to beneficial interactions within the binding pocket. Thus, the S_4 binding pocket is rather "intolerant", but in spite of this it accommodates a range of widely different "preferred" hydrophobic side chains. The question is how this is possible. Studies on the S_1 binding pockets of the subtilisins and α-lytic protease have previously shown that movements of structural elements of the enzyme in response to the properties of the bound side chain are involved (Takeuchi et al., 1991a; Bone

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

[2] Abbreviations: ABz, *o*-aminobenzoyl; CI-2, chymotrypsin inhibitor 2; mut., mutant; pNA, *p*-nitroanilide; Savinase, subtilisin 309 from *B. lentus*; Suc, succinyl; Tyr(NO_2), 3-nitrotyrosine; w.t., wild-type; Y′, Tyr(NO_2); ↓, the scissile bond; X126Z, Savinase where residue X at position 126 has been replaced by residue Z.

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

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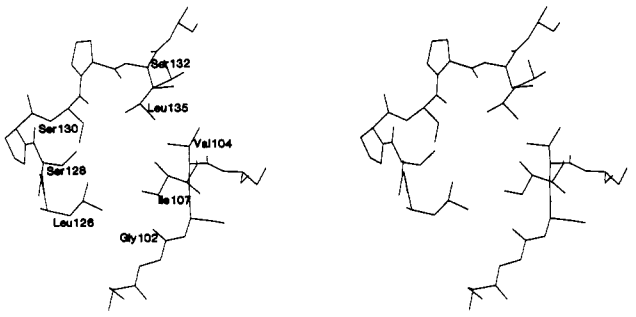

FIGURE 1: Stereoview of the S_4 binding site of Savinase (Betzel et al., 1992). The P_4 amino acid residue is involved in main-chain to main-chain interactions with Gly102 (McPhalen & James, 1988), and the P_4 side chain protrudes into the S_4 binding pocket formed by Ile107, Leu126, and Leu135 while Val104, Ser128, Ser130, and Ser132 are situated at the rim of the pocket.

et al., 1991). However, flexibility of the substrate, i.e., multiple binding modes, appears also to contribute to securing a wide specificity of a particular binding pocket.

The S_4 binding pocket is very important for the substrate preference of the subtilisins (Grøn et al., 1992). It has been shown that the mobility of the amino acid residues located on the rim of the S_4 pocket to some extent influences the P_4 preference of the subtilisin Savinase (Bech et al., 1992b, 1993). However, although Rheinnecker et al. (1993) by performing the mutations Ile107 → Gly, Ala, or Val found specificity changes for subtilisin BPN′, the contribution of the amino acid residues forming the S_4 pocket to the side-chain preference of the subtilisins has not been thoroughly delineated. We here describe the mutational replacement in Savinase of the amino acid residues Ile107, Leu126, and Leu135, which form the hydrophobic S_4 pocket (Figure 1), and it is shown that van der Waals interactions in combination with a complex pattern of steric repulsions determine the P_4 side-chain preference.

MATERIALS AND METHODS

Materials. A *Bacillus subtilis* strain (Δ *apr*, Δ*npr*) 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 in-house collections. The synthetic oligonucleotides were synthesized on an Applied Biosystem 380 DNA synthesizer, and the "oligonucleotide directed *in vitro* mutagenesis system version 2" kit was from Amersham, England. Restriction endonucleases, 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, USA. Prep-A-Gene was from Bio-Rad Laboratories, USA. Mes and Bicine were from Sigma, USA. 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 415bp *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, based on the methods of Eckstein and co-workers (Nakamaye et al., 1986; Sayers et al., 1988; Taylor et al. 1985a,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, and 5% dimethylformamide, pH 8.5. The enzyme preparations were stored frozen in buffer at −18 °C. The enzymes were stable under these conditions.

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, Sweden. 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 $\epsilon_{280} = 23\ mM^{-1}\ cm^{-1}$ (Bech et al., 1992a).

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. Assays were performed in 50 mM Bicine, 2 mM $CaCl_2$, 0.1 M KCl, and 5% dimethylformamide, pH 8.5, and hydrolysis rates at 25 °C were determined by monitoring the fluorescence emission at 420 nm after excitation at 320 nm using a Perkin Elmer LS50 luminescence spectrometer. The standard deviation for the k_{cat}/K_M values was ±8% unless otherwise indicated. Cleavage sites in the fluorogenic substrates have previously been determined with the wild-type enzyme (Grøn et al., 1992), and these cleavage sites were not shifted by drastic modifications at the S_4 pocket, e.g., by incorporating Phe at positions 102 or 128 hence blocking the access of the P_4 side chain to the S_4 pocket (Bech et al., 1993). It was therefore extremely unlikely that the much smaller changes in enzymatic properties described here would cause shifts in the cleavage points.

RESULTS AND DISCUSSION

Studies of subtilisins in complex with proteinaceous inhibitors such as the *Streptomyces* subtilisin inhibitor, Eglin c, or CI-2 (Bode et al., 1987; McPhalen et al., 1985; McPhalen & James, 1988; Takeuchi et al., 1991a,b) suggest that the hydrophobic nature of the S_4 binding pocket is determined by the side chains of amino acid residue 135 (situated at the bottom of the pocket) as well as 107 and 126 (on each side of the pocket) (Figure 1). Among the subtilisins, residue 107 is either Ile or Val, 126 is consistently Leu, and 135 is either Leu or Met (Betzel et al., 1992). These amino acid side chains provide a very hydrophobic environment within the S_4 binding pocket. To understand the contribution of each of these amino acid side chains to the hydrophobicity and volume of the S_4 site, other hydrophobic amino acid residues were introduced at these positions in the subtilisin Savinase. The mutations were all accomplished in the structural gene for Savinase by standard procdures, 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.

In the event that the interaction between the P_4 side chain and the pocket is purely hydrophobic in nature a plot of log-

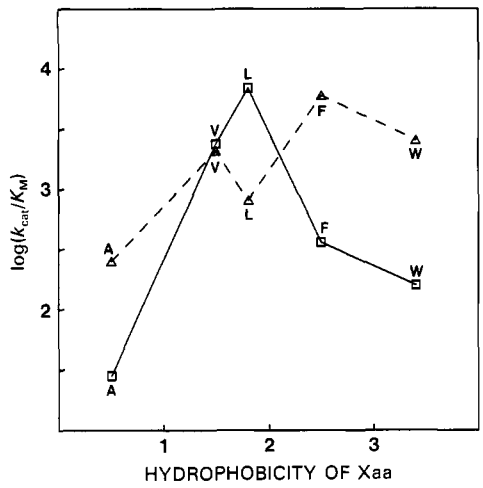

FIGURE 2: Effect of hydrophobicity (Chotia, 1984) of Xaa on log-(k_{cat}/K_M) for the hydrolysis of ABz-Xaa-Gly-Pro-Phe-↓Tyr(NO_2)-Asp-OH catalyzed by wild-type Savinase (△) and I107A (□).

(k_{cat}/K_M) versus the hydrophobicity of the P_4 amino acid residue would be linear. With the substrate series ABz-Xaa-Gly-Pro-Phe-↓Tyr(NO_2)-Asp-OH, where Xaa = Trp, Phe, Leu, Val, or Ala, it was previously shown that this is not the case with the subtilisin Savinase (Bech et al., 1993; Grøn et al., 1992). The k_{cat}/K_M values increase in the following order of Xaa: Ala < Leu < Val < Trp < Phe, as opposed to the hydrophobicity scale: Ala < Val < Leu < Phe < Trp (Chothia, 1984) (see Figure 2). In this treatment hydrophilic P_4 side chains are excluded since they seem to bind in a different way at the S_4 site (Bech et al., 1993). The fact that the k_{cat}/K_M value for the hydrolysis of the substrate with Xaa = Leu is lower than that with Xaa = Val suggests the existence of significant steric repulsion with the more bulky Leu side chain. If so, it is surprising that with Xaa = Phe this repulsion has at least partially vanished and then reappears with the even larger Trp. This could be due to aliphatic side chains binding in a mode which differs from that of the aromatic side chains or more likely, a very narrow S_4 pocket matching the flat aromatic structure of a Phe side chain but not the branched and bulky side chains of, e.g., Leu.

To investigate the structural features determining the P_4 preference of Savinase, Leu135, located at the bottom of the pocket, was replaced with Ala, Val, and Phe by site-directed mutagenesis. The P_4 preference of the resulting enzymes was determined using the substrate series ABz-Xaa-Gly-Pro-Phe-↓Tyr(NO_2)-Asp-OH, where Xaa = Gly, Ala, Val, Leu, Phe, Trp, Pro, Asp, Asn, and Arg (Table I). For the mutant enzymes L135A, L135V, and L135F the k_{cat}/K_M values toward substrates with Xaa = Gly, Ala, Val, or Leu were 60–170% of the values obtained with the wild-type enzyme. With L135A, L135V, and L135F and Xaa = Phe the corresponding values were 320%, 350%, and 7%, respectively, and for the same mutants but with Xaa = Trp the values were 970%, 970%, and 15%, respectively. The dramatic increase in k_{cat}/K_M for the hydrolysis of the substrate with Xaa = Trp when the volume of the amino acid residue at position 135 is reduced, i.e., L135A and L135V, suggests that some steric repulsion has been removed. This is further supported by the fact that the two mutants hydrolyze the substrate with Xaa = Phe at lower rates than that with Xaa = Trp consistent with their relative hydrophobicities. It may therefore be concluded that aromatic P_4 side chains are in contact with Leu135 at the bottom of the S4 pocket of Savinase (Figure 3), but the observation that substitution of Leu135 has no effect or a much smaller effect on the hydrolysis of substrates with

Table I: The P_4 Substrate Preference[a] of Savinase Mutants with Replacements at Position 135

substrate	L135A	L135V	L135L (w.t.)	L135F
ABz-G-G-P-F-↓Y′-D	13	20	22	14
ABz-A-G-P-F-↓Y′-D	200	150	250	400
ABz-V-G-P-F-↓Y′-D	2200	1200[b]	2100	3600
ABz-L-G-P-F-↓Y′-D	720	870	820	670
ABz-F-G-P-F-↓Y′-D	19000	21000	6000	450
ABz-W-G-P-F-↓Y′-D	25000	25000	2600	380
ABz-P-G-P-F-↓Y′-D	80	150	690	30
ABz-D-G-P-F-↓Y′-D	10	8.1	2.1	6.8
ABz-N-G-P-F-↓Y′-D	35	18	19	32
ABz-R-G-P-F-↓Y′-D	4.0	2.6[b]	10	1.4

[a] k_{cat}/K_M ($mM^{-1}\cdot min^{-1}$) values are listed. The assays were performed at pH 8.5 as described in Materials and Methods. The values for the wild-type enzyme are from Grøn et al. (1992). The underlined amino acid residues are those situated in the P_4 position of the substrates. The standard deviations for the k_{cat}/K_M values were less than ±8% unless otherwise indicated. [b] Standard deviations from ±8% to ±15%.

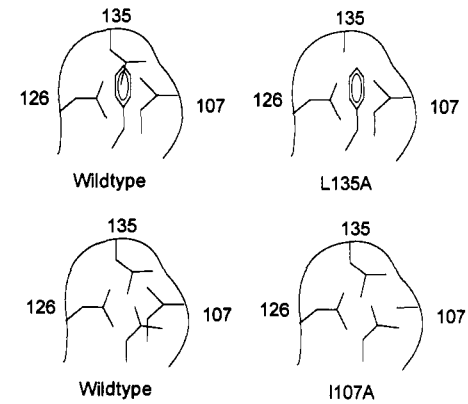

FIGURE 3: Schematic drawing of the steric repulsion of the P_4 side chain of Phe and Leu in the S_4 binding pocket of wild-type Savinase, and the better accommodation of the same two side chains in the S_4 pocket of the mutants L135A and I107A.

aliphatic P_4 amino acid residues suggests that these do not have this contact. This may be explained by the P_4 side chains of, e.g., Leu and Phe binding in identical modes but such that the shorter side chain of Leu does not reach Leu135. One might expect that a Leu at position 135 and a Phe at P_4 should be equivalent to a Phe at position 135 and a Leu at P_4 in terms of their combined side-chain volumes, and therefore one might expect similar k_{cat}/K_M values in these two cases. However, the wild-type enzyme (Leu at position 135) hydrolyzes the substrate with Xaa = Phe with 9 times the k_{cat}/K_M obtained for the hydrolysis of the substrate with Xaa = Leu and the mutant enzyme L135F. This is presumably due to the fact that the steric repulsion with Xaa = Leu observed with the wild-type enzyme (Figure 3) is maintained in L135F as well as the other mutants substituted at position 135.

The replacement of Leu135 with Ala or Phe has beneficial as well as adverse effects on k_{cat}/K_M for the hydrolysis of ABz-Xaa-Gly-Pro-Phe-↓Tyr(NO_2)-Asp-OH, where Xaa = hydrophilic amino acid residues (Table I). However, the k_{cat}/K_M values are low, and the effects do not seem to be correlated to the structure of the amino acid residue occupying position 135 but may instead be due to minor local conformational changes. This supports previous results which indicate that hydrophilic P_4 side chains do not employ the S_4 binding pocket (Bech et al., 1993). In this context it is interesting that the substrate with Xaa = Pro, which is strongly hydrophobic, falls into this category as well, possibly due to its influence on the conformation of the peptide, preventing optimal interaction with the S_4 pocket.

Table II: The P_4 Substrate Preference[a] of Savinase Mutants with Replacements at Position 126

substrate	L126L (w.t.)	L126A	L126F
ABz-G-G-P-F-↓Y′-D	22	1.7	2.9
ABz-A-G-P-F-↓Y′-D	250	26[b]	14
ABz-V-G-P-F-↓Y′-D	2100	190[b]	92
ABz-L-G-P-F-↓Y′-D	820	60	53
ABz-F-G-P-F-↓Y′-D	6000	280	620
ABz-W-G-P-F-↓Y′-D	2600	81	140
ABz-P-G-P-F-↓Y′-D	690	13	7.7
ABz-D-G-P-F-↓Y′-D	2.1	0.37	0.31
ABz-N-G-P-F-↓Y′-D	19	1.0	2.1
ABz-R-G-P-F-↓Y′-D	10	0.25	0.26

[a] k_{cat}/K_M ($mM^{-1}\cdot min^{-1}$) values are listed. The assays were performed at pH 8.5 as described in Materials and Methods. The values for the wild-type enzyme are from Grøn et al. (1992). The underlined amino acid residues are those situated in the P_4 position of the substrates. The standard deviations for the k_{cat}/K_M values were less than ±8% unless otherwise indicated. [b] Standard deviations from ±8% to ±15%.

The origin of steric repulsion with Xaa = Leu at the P_4 position could be the side chains of either Leu126 or Ile107 each forming a side of the S_4 pocket as defined by the inhibitor studies (see above). This was investigated by mutational replacement of these amino acid residues. The Leu residue at position 126 was replaced with an Ala or a Phe, and the P_4 substrate preferences of these enzymes were determined using the substrate series described above. Replacement with an Ala resulted in k_{cat}/K_M values of around 5% of the values obtained with the wild-type enzyme without any apparent dependence on the nature of Xaa, i.e., identical P_4 preference (Table II). Even with Xaa = Gly, in which case the S_4 pocket is not being used, low k_{cat}/K_M values were observed. Since similar results were obtained with the mutant enzyme with a Phe at position 126, the change in activity appears to be due to the removal of Leu rather than to the nature of the side chain introduced at this position. The amino acid residues at positions 125–127 (Ser-Leu-Gly) are conserved among the subtilisins (Betzel et al., 1992), and this, together with the present results, suggests that Leu126 is an important structural element of the active site cleft, possibly participating in orienting the loop consisting of residues 125–127 which is involved in backbone–backbone interactions with the substrate. This renders it difficult to study the contribution of the side chain of Leu126 to the properties of the S_4 pocket. However, the fact that significant variation in the bulkiness of the side chain at position 126 has no influence on the P_4 preference suggests that Leu126 is not responsible for the steric repulsion observed with Xaa = Leu.

Replacement of Ile107 with Gly, Ala, Val, Leu, and Phe had a pronounced influence on the P_4 side-chain preference of Savinase as also observed by Rheinnecker et al. (1993) for the subtilisin BPN′ mutants I107G and I107A. In Table III the k_{cat}/K_M values for the hydrolysis of ABz-Xaa-Gly-Pro-Phe-↓Tyr(NO_2)-Asp-OH with the different mutants are listed according to increasing size of the amino acid residue at position 107. With Xaa = Gly, Ala, Phe, Trp, and Pro the highest activities were obtained with I107V or I107I (wild-type enzyme), i.e., when the amino acid side chain at position 107 was medium sized. Widening the S_4 pocket by decreasing the volume of the group at position 107, i.e., I107G and I107A, would be predicted to reduce the number of van der Waals interactions between P_4 and S_4, and this may explain the lower activity. The lower activity with I107L and in particular I107F, on the other hand, is presumably due to increased steric repulsion. Inspection of the three-dimensional structure of Savinase (Figure 1) supports that the introduction of a Phe would have such effects, but the effect of the Ile107 → Leu substitution, moving one methyl group, could not be predicted. With Xaa = Val the same pattern was observed, with the exception that the enzyme with Ile at position 107 is less active than that with Leu. This was also the case with Xaa = Leu, but in addition, the k_{cat}/K_M values obtained with I107G and I107A are comparatively high. These results may be interpreted in terms of the S_4 site of the wild-type enzyme being narrow and deep, accommodating the side chains of Ala, Pro, and Phe (on its narrow edge) between Leu126 and Ile107. The branched and bulky side chains of Val and Leu, on the other hand, clash with Ile107 (Figure 3). This steric repulsion is reduced by the Ile107 → Val substitution, and as a consequence, a substantial increase in k_{cat}/K_M is seen with Xaa = Val and Leu. A further reduction in the volume of the side chain at position 107, i.e., I107A and I107G, has an adverse effect on k_{cat}/K_M for the hydrolysis of the substrate with Xaa = Val, presumably due to loss of van der Waals interactions. However, with Xaa = Leu the activity is maintained at essentially the same level, signifying that the loss of van der Waals interactions is counteracted by the beneficial effects of alleviating strain. This is supported by the fact that the activity with Xaa = Leu is much higher than with Xaa = Val, consistent with their relative hydrophobicities. As a consequence, the decline in k_{cat}/K_M with Xaa = Leu characteristic of the wild-type enzyme has disappeared for I107A (Figure 2). The lower activity with I107A compared to wild-type enzyme with Xaa = Ala, Phe, and Trp (Figure 2) is due to reduced van der Waals interactions as explained above.

The data of Bech et al. (1993) indicate alternate modes of binding for hydrophilic P_4 side chains which may not utilize the S_4 binding pocket of the wild-type enzyme, but instead adopt other binding modes. However, substitution of Ile107, situated at the entrance to the pocket, may change the interaction, and the fact that k_{cat}/K_M is increased 8- and 3-fold with Xaa = Asp by substitution of Ile107 for the widely different Gly and Phe, respectively, provides some evidence for this. Both mutations may increase the number of van der Waals interactions but in different ways. One interpretation is that the Ile107 → Gly mutation allows the Asp side chain to enter the S_4 pocket while the Ile107 → Phe mutation secures contact with the Asp outside the pocket. The results with Xaa = Asn are equally difficult to interpret. With Xaa = Arg the highest k_{cat}/K_M value was obtained with the wild-type enzyme, suggesting that part of the side chain, presumably the β, γ, and δ carbons, interacts with Ile107. However, it is unlikely that the guanidino group enters the S_4 pocket.

As a control of the assignment of the side chains of Ile107, Leu126, and Leu135 to the S_4 pocket, the P_3 and P_5 substrate preferences of the mutants with Ala at positions 107, 126, and 135, i.e., I107A, L126A, and L135A, were investigated. The following substrate series were used: ABz-Phe-Xaa-Pro-Phe-↓Tyr(NO_2)-Asp-OH (P_3 position) and ABz-Xaa-Phe-Gly-Pro-Phe-↓Tyr(NO_2)-Asp-OH (P_5 position), where Xaa = Asn, Ala, Leu, and Phe. For each substrate series the amino acid residues (Xaa) were ranked according to increasing hydrophobicity (Figure 4). The curves obtained by variations at the P_3 or P_5 positions with all three mutants were essentially parallel to that of the wild-type enzyme. Different levels of activity were observed, but these reflect the influence of a Phe in the P_4 position. Thus, the mutations do not appear to significantly influence the properties of the neighboring S_3 and S_5 sites. As a comparison, the curves obtained with the three mutant enzymes by variation at the P_4 position deviated

Table III: The P_4 Substrate Preference[a] of Savinase Mutants with Replacements at Position 107

substrate	I107G	I107A	I107V	I107I (w.t.)	I107L	I107F
ABz-G-G-P-F-↓Y′-D	1.7[b]	1.0	6.2	22	2.7	2.7
ABz-A-G-P-F-↓Y′-D	11	28	720	250	55	15
ABz-V-G-P-F-↓Y′-D	370	2400	18000	2100	8400	360
ABz-L-G-P-F-↓Y′-D	6200	6900	8500	820	4300	750
ABz-F-G-P-F-↓Y′-D	340	360	15000	6000	2500	250
ABz-W-G-P-F-↓Y′-D	140	160	910	2600	220	95
ABz-P-G-P-F-↓Y′-D	12	27	530	690	140	18
ABz-D-G-P-F-↓Y′-D	16	1.8	3.4	2.1	0.7	6.3
ABz-N-G-P-F-↓Y′-D	4.3	19	9.0	19	4.1	7.2
ABz-R-G-P-F-↓Y′-D	3.7	0.4	1.1	10	0.4	0.5

[a] k_{cat}/K_M ($mM^{-1}\cdot min^{-1}$) values are listed. The assays were carried out at pH 8.5 as described in Materials and Methods. The values for the wild-type enzyme are from Grøn et al. (1992). The underlined amino acid residues are those situated in the P_4 position of the substrates. The standard deviations for the k_{cat}/K_M values were less than ±8% unless otherwise indicated. [b] Standard deviations from ±8% to ±15%.

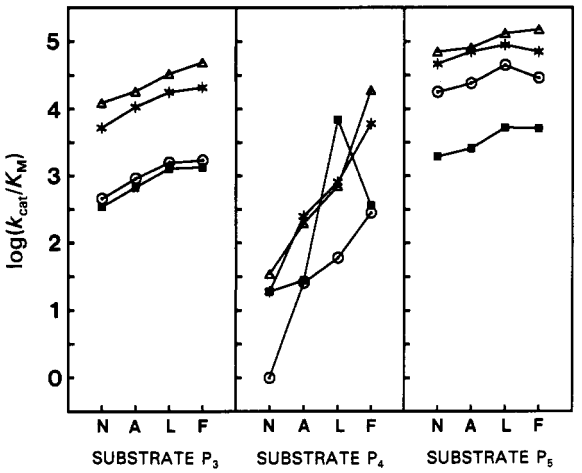

FIGURE 4: P_3, P_4, and P_5 preferences of wild-type Savinase (*), I107A (■), L126A (⊙), and L135A (△). The preferences were determined using substrate series, where for each subsite Xaa = Asn, Ala, Leu, and Phe (see Results and Discussion).

more from the curve for wild-type enzyme; especially I107A.

In a previous study on wild-type Savinase (Grøn & Breddam, 1992) it was shown that the k_{cat}/K_M values for the hydrolysis of peptide substrates, optimized to match the preference of the enzyme, could not be rationalized on the basis of simple additive contributions from each subsite–substrate interaction. With a series of substrates covering S_6 to S'_4 it was observed that favorable amino acids in P_1 and P_4 shield adverse effects of less favorable amino acids at other positions. Thus, an upper limit of k_{cat}/K_M was observed, suggesting a limit on the amount of substrate interaction energy which can be converted into transition-state stabilization. In another study (Bech et al., 1993) it was shown that a significant portion of the unused interaction energy with such optimized substrates can be assigned to the S_4–P_4 interactions. The question then is how k_{cat}/K_M for the hydrolysis of an optimized substrate is influenced by mutations within the S_4 binding pocket. With some of the mutants described in this paper impaired S_4–P_4 interactions are responsible for the low k_{cat}/K_M values for the hydrolysis of the nonoptimized ABz-Phe-Gly-Pro-Phe-↓Tyr(NO_2)-Asp-OH (Phe in P_4). However, with the optimized substrate ABz-Phe-Arg-Ala-Phe-↓Ala-Phe-Phe-Tyr(NO_2)-Asp-OH (Grøn & Breddam, 1992) one could imagine that these mutations would lower the amount of "unused" interaction energy, in which case no change in k_{cat}/K_M should be observed. To investigate this, k_{cat}/K_M values for the hydrolysis of the optimized substrate were determined with the mutants described in Tables I–III (see Table IV). It was found that these values only varied 2–3-fold with the optimized substrate as compared to 84-fold with the nonoptimized substrate; i.e., the variation in $\Delta\Delta G_T^*$(w.t.→mut.)[4] for the optimized substrate was only 2.6 kJ·mol^{-1} as compared to 11 kJ·mol^{-1} for the nonoptimized substrate. Thus, even with the mutants which, due to steric repulsion, exhibit impaired binding of Phe in the S_4 subsite, the remaining interaction energy from binding of the optimized substrate is sufficient to almost attain the very high k_{cat}/K_M values characteristic of the wild-type enzyme. In other words, with the optimized substrate the loss of interaction energy due to the mutations is largely at the expense of the "unused" interaction energy described above. It is remarkable that the substantial steric repulsion introduced into the S_4 site by the Leu135 → Phe and Ile107 → Phe mutations as well as the assumed conformational changes associated with the substitution of Leu126 only slightly affects k_{cat}/K_M for the hydrolysis of the optimized substrate. One explanation may be that the substantial surplus of binding energy is used to induce conformational changes in the vicinity of the S_4 site with the effect that the steric repulsion is alleviated. However, validation of this hypothesis requires structural information not available to us, but it should be mentioned that binding energy previously has been shown to induce conformational changes within the S_1 site of α-lytic protease (Bone et al., 1989).

Table IV: Comparison of k_{cat}/K_M Values and Differences in Transition-State Stabilization Energies [$\Delta\Delta G_T^*$(w.t.→mut.) Values] between Wild-Type Savinase and Mutant Enzymes in Hydrolysis of ABz-Phe-Arg-Ala-Phe-↓Ala-Phe-Phe-Tyr(NO_2)-Asp-OH or ABz-Phe-Gly-Pro-Phe-↓Tyr(NO_2)-Asp-OH[a]

	ABz-F-R-A-F-↓A-F-F-Y′-D		ABz-F-G-P-F-↓Y′-D	
enzyme	k_{cat}/K_M ($\mu M^{-1}\cdot min^{-1}$)	$\Delta\Delta G_T^*$ (w.t.→mut.) (kJ·mol^{-1})	k_{cat}/K_M ($\mu M^{-1}\cdot min^{-1}$)	$\Delta\Delta G_T^*$ (w.t.→mut.) (kJ·mol^{-1})
wild-type	1300		6	
L135A	760	+1.3	19	−2.9
L135V	1200	+0.2	21	−3.1
L135F	610	+1.9	0.45	+6.4
L126A	970	+0.7	0.28	+7.6
L126F	830	+1.1	0.62	+5.6
I107G	540	+2.2	0.34	+7.1
I107A	660	+1.7	0.36	+7.0
I107V	1500	−0.35	15	−2.3
I107L	840	+1.1	2.5	+2.2
I107F	600	+1.9	0.25	+7.9

[a] The values for ABz-Phe-Gly-Pro-Phe-↓Tyr(NO_2)-Asp-OH are based on the k_{cat}/K_M values from Tables I–III. The values for the wild-type enzyme are from Grøn and Breddam (1992) and Grøn et al. (1992). The standard deviations for the k_{cat}/K_M values were less than ±8%.

[4] $\Delta\Delta G_T^*$(w.t.→mut.) = $-RT\ \ln[(k_{cat}/K_M(\text{mut.}))/(k_{cat}/K_M(\text{w.t.}))]$ (Carter et al., 1984).

CONCLUDING REMARKS

For the subtilisin enzyme Savinase the hydrophobicity of the P_4 side chain, remote from the scissile bond, is important for transition-state stabilization. However, $\log(k_{cat}/K_M)$ does not increase linearly with the hydrophobicity constant, signifying an influence of steric repulsion (Bech et al., 1993; Grøn et al., 1992). Mutational replacements of Ile107, Leu126, and Leu135 which form the hydrophobic S_4 pocket of the subtilisin Savinase have been accomplished. Substitution of Ile107 and Leu135 with other hydrophobic amino acid residues show that the properties of the hydrophobic S_4 binding pocket are strongly influenced by these amino acid residues. Although the hydrophobicity of the amino acid residues incorporated at these positions must influence the hydrophobicity of the pocket, no correlation to the P_4 substrate preference exists. However, we can rationalize the results on the basis of Leu126 and Ile107 forming a narrow binding pocket which provides important van der Waals interactions with the P_4 side chain. The pocket is too narrow to accommodate branched side chains without steric repulsion, and furthermore, due to Leu135, it is not deep enough to be optimal for aromatic side chains (Figure 3). It appears that Leu135 exclusively interacts with aromatic P_4 side chains whereas Ile107 is in contact with all hydrophobic P_4 side chains. The results do not provide evidence for the existence of separate binding modes for the different hydrophobic P_4 side chains.

Two widely different hydrophobic binding pockets have previously been investigated by site-directed mutagenesis. The S_1 site of α-lytic protease represents an extreme case of steric repulsion (Bone et al., 1989, 1991) whereas the S_1 pocket of subtilisin BPN′ provides pure hydrophobic interactions without significant steric repulsion (Estell et al., 1986). The S_4 site of the subtilisin Savinase represents an intermediate type of binding pocket with steric repulsion limiting the hydrophobic interactions. The question is why all subtilisins have a branched amino acid at position 107 when this causes steric repulsion of some P_4 side chains. It may be related to the fact that such an amino acid residue secures that the interaction with the preferred P_4 side chain, i.e., Phe, is optimal for transition-state stabilization. However, in this context it is surprising that the Leu at position 135 of wild-type Savinase is not optimal for Phe in a nonoptimized substrate; the mutant enzymes with Ala135 or Val135 are both superior.

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