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# Extensive Comparison of the Substrate Preferences of Two Subtilisins As Determined with Peptide Substrates Which Are Based on the Principle of Intramolecular Quenching†

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ABSTRACT: Subtilisins are serine endopeptidases with an extended binding cleft comprising at least eight binding subsites. Interestingly, subsites distant from the scissile bond play a dominant role in determining the specificity of the enzymes. The development of internally quenched fluorogenic substrates, which allow polypeptides of more than 11 amino acids to be inserted between the donor and the acceptor, has rendered it possible to perform a highly systematic mapping of the individual subsites of the active sites of subtilisin BPN′ from *Bacillus amyloliquefaciens* and Savinase from *Bacillus lentus*. For each enzyme, the eight positions $S_5$–$S'_3$ were characterized by determination of $k_{cat}/K_M$ values for the hydrolysis of substrates in which the amino acids were systematically varied. The results emphasize that in both subtilisin BPN′ and Savinase interactions between substrate and $S_4$ and $S_1$ are very important. However, it is apparent that interactions between other subsites and the substrate exert a significant influence on the substrate preference. The results are rationalized on the basis of the structural data available for the two enzymes.

The active site of a proteolytic enzyme is occasionally divided into a catalytic site and a binding site. The catalytic site consists of the limited number of amino acid residues which are directly involved in the breakage of the peptide bond, e.g., by functioning in proton transfer or binding of the oxyanion of the tetrahedral intermediate. The binding site is composed of a fairly large number of amino acid residues which secure the proper alignment of the substrate prior to catalysis. In addition, binding energy is used to transform the substrate into the transition state (Jencks, 1975), and thus, it is not entirely meaningful to distinguish between catalytic site and binding site.

The binding site as defined by Schechter and Berger (1967) may be divided into a number of subsites, each by multiple interactions securing the binding of a single amino acid residue from the substrate. The properties of the amino acid residues which constitute a given binding subsite determine which amino acid residue(s) of the substrate may bind, and thus, they provide the basis of subsite specificity or preference in the case of less restrictive subsites. The nature of these interactions may be studied by site-directed mutagenesis and chemical modifications, but it is a prerequisite that the individual subsites are carefully mapped. Although X-ray crystallography and NMR studies can provide information about which amino acids are involved in substrate binding, the side-chain preference of each subsite is best determined by systematic variations of substrate structures. Such studies have been performed over the past 20 years (Svendsen, 1976), but they were restricted by the means to follow hydrolysis and the availability of substrates (Meldal & Breddam, 1991). The recent development of highly efficient donor/acceptor pairs for substrates based on intramolecular fluorescence quenching, allowing the use of long peptide substrates spanning the entire binding site (Meldal & Breddam, 1991); Matayoshi et al., 1990), represents a significant improvement in this context. We here describe the complete subsite mapping of two enzymes belonging to the subtilisins which are known to have extensive binding sites and, yet, are considered to be unspecific but with a well-defined substrate preference. The known three-dimensional structures of these enzymes form the basis for the interpretation of the results.

## Materials and Methods

*Materials.* Sephadex CM and Sepharose CL 6B were from Pharmacia LKB Biotechnology, Sweden, Bio-Gel P-4 was from Bio-Rad, Richmond, CA, and bacitracin–Sepharose was prepared as previously described (Mortensen et al., 1989). Macrosorp SPR-250 was purchased from Sterling Organics.

Solvents were distilled at the appropriate pressure in a packed column of Raschig rings. DMF[1] was analyzed by

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mixing with Dhbt-OH prior to use. THF and dioxane were passed over a short column of active alumina for purification and drying. Active Dhbt esters and Fmoc-Tyr($NO_2$) were prepared as previously described (Atherton et al., 1988; Meldal & Breddam, 1991). The purity of the Dhbt esters was checked by HPLC and NMR and IR spectroscopy.

Subtilisin BPN′, a gift from Sven Branner, Novo Nordic A/S, was kept at −18 °C in 0.01 M 3,3-dimethylglutaric acid, 0.2 M boric acid, 2 mM $CaCl_2$, 0–0.1 M NaCl, pH 6.5.

*Multiple Column Synthesis of Internally Quenched Substrates.* The fluorescent peptide substates were of the general structure ABz-(Xaa)$_n$-Tyr($NO_2$)-Asp-OH, where ABz is a fluorescent group, Tyr($NO_2$) is a quencher, and $n$ = 4–7. They were prepared either by multiple column synthesis on a 96-column synthesizer (Holm & Meldal, 1989) as described by Meldal and Breddam (1991) or in the following way: Fmoc-Asp(tBu)-OH (514 mg, 1.25 mmol) was lyophilized and dissolved in dichloromethane (20 mL) and tetrahydrofuran (2 mL). *N*-methylimidazole (74 μL, 0.94 mmol) and MSNT (367 mg, 1.24 mmol) were added. Lyophilized Macrosorp SPR-250 resin (2.5 g, 0.63 mmol) was derivatized with ethylenediamine and then (hydroxymethyl)benzoylated. The suspension was shaken for 1 h, and the resin was washed with dichloromethane and DMF. The degree of incorporation was 92% as determined by quantitative amino acid analysis. The Fmoc group was cleaved with piperidine, and the resin was washed with DMF. Fmoc-Tyr($NO_2$) (840 mg, 1.88 mmol) and TBTU (602 mg, 1.88 mmol) were added and dissolved in DMF (15 mL). Diisopropylethylamine (0.25 mL) was added, and the mixture was shaken for 2 h. All reagent was removed by washing with DMF which subsequently was removed with diethyl ether. The resin was dried and distributed with a measuring spoon (25 mg) into the 96 wells of the multiple-column peptide synthesizer.

The synthesis cycle consisted of one wash with 20% piperidine in DMF (40 mL for 10 min), four washings with DMF (45 mL), addition of Fmoc-amino acid Dhbt ester solutions (3 equivalents) with a multipipet, and gentle shaking for at least 4 h followed by three washings with DMF (45 mL). The last residue added was Boc-ABz-O-Dhbt (2.5 equivalents), and after a wash with DMF and diethyl ether the resins were dried by an air flow. The protection groups were cleaved off in the synthesizer with aqueous TFA (95%, 1 mL), and the resins were washed with 95% acetic acid, DMF, 5% triethylamine in DMF, DMF, and finally diethyl ether. The resins were dried by air flow, and the peptides were cleaved off with 0.1 M NaOH (0.2 mL in each column) for 1 h and washed out with water. The solutions were neutralized with 0.1 M HCl (0.2 mL) and lyophilized to yield 3–6 mg of each peptide and 1.2 mg of sodium chloride. The peptides were dissolved in DMF, and sodium chloride was removed by filtration.

The purity of all peptide substrates was better than 97% as determined by reverse-phase HPLC. HPLC was carried out on a Waters Delta Prep 3000 equipped with a Waters 991 photodiode array detector. Analytical chromatograms were obtained using a Waters RCM 8 10 module with a Waters 8 NV $C_{18}$ 4-μm column. Buffer A was 0.1% aqueous TFA; buffer B was 90% acetonitrile, 9.9% water, and 0.1% TFA. All the crude peptides were analyzed using a linear gradient of 0–100% in 30 min followed by isocratic elution with 100% B at a flow rate of 1.3 mL/min. Some of the substrates were further checked by amino acid analysis, and in all cases the amino acid composition was as expected.

*Preparation of Savinase.* Savinase was purified from 10 L of fermentation broth. After centrifugation of the fermentation broth, the supernatant was concentrated and diafiltrated with 10 mM Mes, 100 mM boric acid, 2 mM $CaCl_2$, pH 7.0, on a Millipore Pellicon Cassette Ultrafiltration System, equipped with a PTGC 000 05 membrane. The sample was adsorbed on a 300-mL bacitracin affinity column equilibrated with 10 mM Mes, 100 mM boric acid, 2 mM $CaCl_2$, pH 7.0. The protease was eluted from the column with 10 mM Mes, 100 mM boric acid, 2 mM $CaCl_2$, 1 M NaCl, 25% 2-propanol, pH 7.0. The eluate was applied to a Sephadex G50 column (10 × 85 cm), equilibrated with 10 mM Mes, 200 mM boric acid, 2 mM $CaCl_2$, pH 6.5, to remove the 2-propanol. The fractions with protease activity were applied to a CM Sepharose CL 6B cation-exchange column (2.6 × 10 cm), equilibrated with the previous buffer. The enzyme was eluted using a linear gradient of 0–0.1 M NaCl in the same buffer (10 times the column volume). After concentration in an Amicon ultrafiltration cell, equipped with a YM10 filter (Amicon), the enzyme was desalted on a Bio-Gel P-4 column, equilibrated with 5 mM Mes, 2 mM $CaCl_2$, pH 6.5, and stored frozen in the buffer at −18 °C.

*Characterization of the Enzyme Preparations.* Savinase was checked for purity and possible autolysis by SDS–PAGE on a 0.45-mm-thick 20% homogeneous gel using an SDS–Tris–acetate continuous buffer system on a Pharmacia PhastSystem. To avoid autodigestion during SDS treatment, the enzyme had previously been denatured in 0.1 M HCl for 30 min and then lyophilized. The concentration of Savinase was determined spectrophotometrically, $\epsilon_{280}$ = 23 $mM^{-1}$ (Grøn et al., 1990).

The purity of subtilisin BPN′ was checked by FPLC. It was not possible to exceed a purity of more than 90–95% due to autolytic digestion in accordance with previous findings (Ottesen & Svendsen, 1970). The concentration of subtilisin BPN′ was determined spectrophotometrically using $\epsilon_{280}$ = 32 $mM^{-1}$ $cm^{-1}$, determined from the amino acid composition, and the percentage of active subtilisin BPN′ was determined by active site titration with *N-trans*-cinnamoylimidazole (Schonbaum et al., 1961). The concentration of active enzyme was used to determine the kinetic parameters.

*Determination of Enzyme Activity and Kinetic Constants.* The enzymatic hydrolysis of the peptide substrates was followed on a Perkin Elmer luminescence spectrometer LS 50. The substrates were dissolved in DMF at concentrations of 2.5–25 μM. A total of 50 μL of substrate solution was added to 2400 μL of 50 mM Bicine, 2 mM $CaCl_2$, 0.1 M KCl, pH 8.5, to give initial substrate concentrations in the cuvette, $s_0$, of 0.05–1.0 μM. The initial fluorescence, $E_{initial}$, was determined, and 50 μL of an enzyme solution, appropriately diluted into the assay buffer, was added (final enzyme concentrations, $e_0$, of 0.1–100 nM). To prevent loss of enzyme due to adsorption on surfaces, dilution of enzyme was carried out in polypropylene micro test tubes which had been filled with 0.1% poly(ethylene glycol) 20 000, drained, and oven dried to coat the surface. The cleavage of substrate was followed with time by monitoring the emission at 420 nm (10-nm slit) upon excitation at 320 nm (10-nm slit), at 25 °C. At each substrate

[1] Abbreviations: ABz, *o*-aminobenzoyl; DMF, dimethylformamide; Dhbt, 3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl; THF, tetrahydrofuran; Fmoc, fluoren-9-ylmethyloxycarbonyl; Ph, phenyl; Suc, succinyl; NH-Ph-$NO_2$, *p*-nitroanilide; tBu, *tert*-butyl; MSNT, 1-(2,4,6-mesitylenesulfonyl)-3-nitro-1,2,4-triazole; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium tetrafluoroborate; Boc, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid; DMSO-$d_6$, perdeutero dimethyl sulfoxide; Tyr($NO_2$), 3-nitrotyrosine; Y′, Tyr($NO_2$); Xaa, unspecified amino acid; Yaa, unspecified amino acid; Savinase, subtilisin 309 from *Bacillus lentus*; *h*, Planck constant; $k_B$, Boltzmann constant; ↓, the scissile bond.

concentration, $s_0$, the initial velocity for the cleavage of substrate, $v_0$, was determined from the slope of the initial part of the progress curve (emission versus time) (Figure 1). The substrate concentration could be determined from the final fluorescence, $E_{final}$, using ABz-Gly-$NH_2$ as a standard. In the case of slow reactions, total hydrolysis was accomplished by the addition of 5 μL of concentrated enzyme solution.

The $k_{cat}/K_M$ values were determined from initial velocities using the relation

$$v_0 = (k_{cat}/K_M)e_0s_0$$

which is valid at low substrate concentrations ($s_0 \ll K_M$) for systems that obey Michaelis–Menten kinetics. For each substrate, the validity of this equation was ascertained by using a minimum of three substrate concentrations. For $k_{cat}/K_M$ values in the range $1–10^2$ $min^{-1}$ $\mu M^{-1}$, the determination was ±2%, in the range $10^{-2}–1$ $min^{-1}$ $\mu M^{-1}$, it was ±5%, and in the range $10^{-4}–10^{-2}$ $min^{-1}$ $\mu M^{-1}$ it was ±10%.

In the transition-state theory, the activation energy for the enzymatically catalyzed transition of a substrate into product(s), i.e., the free-energy difference between the free enzyme and substrate and the transition-state complex, $\Delta G_T^*$, is given by the sum of $\Delta G_S$ and $\Delta G^*$, where $\Delta G_S$ is the binding energy released upon binding of substrate to enzyme in the most stable complex, and $\Delta G^*$ is the activation energy of the chemical steps associated with bringing the enzyme–substrate complex from the ground state to the transition state (Fersht, 1985; Jencks, 1975):

$$\Delta G_T^* = \Delta G_S + \Delta G^* = RT \ln (k_BT/h) - RT \ln (k_{cat}/K_M)$$

For each subsite investigation, a substrate series was synthesized with 9–11 different amino acids systematically substituted into the investigated position. The discrimination of an enzyme between two different substrates was evaluated from the differences in $\Delta G_T^*$ values, $\Delta\Delta G_T^*$, as this scales the data in dimensions of free energy. Discrimination between two substrates is determined by the relative binding of their transition states to the enzyme. With an optimal complementarity between the enzyme and the transition state of the substrate, $\Delta G_T^*$ is lowered to a minimum. For each subsite the preference for different amino acids relative to Gly, $\Delta\Delta G_T^*$(Xaa → Gly), was determined from the $k_{cat}/K_M$ values for the hydrolysis of each substrate:

$$\Delta\Delta G_T^*(\text{Xaa} \rightarrow \text{Gly}) = \Delta G_T^*(\text{Gly}) - \Delta G_T^*(\text{Xaa}) = RT \ln \frac{k_{cat}/K_M(\text{Xaa})}{k_{cat}/K_M(\text{Gly})}$$

*Determination of Cleavage Sites.* To ascertain that the design of the substrate series secured the predicted specific cleavage, the scissile peptide bond was determined for 22 typical representatives of the 78 substrates used for the subsite mapping. Lyophilized substrate (~250 nmol) was dissolved in 5 μL of DMF, and 195 μL of assay buffer (50 mM Bicine, 2 mM $CaCl_2$, 0.1 M KCl, pH 8.5) was added. Enzyme was added to the substrate medium, and when the degree of hydrolysis was optimal (40–70%), as determined by HPLC, the reaction products were separated by semipreparative HPLC using a Waters HPLC system equipped with a $C_{18}$ Vydac column. A linear gradient from 20% to 90% acetonitrile in 0.1% aqueous TFA (flow rate 1 mL/min) was adequate in most cases. The peptide products were collected and lyophilized. The amino acid content was determined after acid hydrolysis for 24 h in vacuo, using a Pharmacia LKB·Alpha Plus amino acid analyzer.

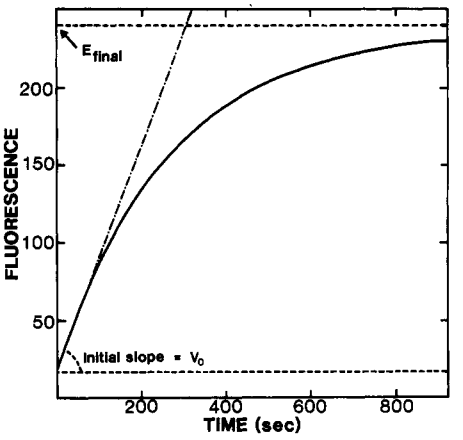


FIGURE 1: Progress curve for the hydrolysis of ABz-Phe-Gly-Pro-Phe-↓Tyr($NO_2$)-Asp-OH by Savinase in 50 mM Bicine, 2 mM $CaCl_2$, 0.1 M KCl, pH 8.5, 25 °C. $v_0$ is the initial velocity for the hydrolysis of the substrate and $E_{final}$ is the fluorescence after total hydrolysis.

## Results and Discussion

In the present work, we have characterized the active sites of two subtilisins, i.e., Savinase from *Bacillus lentus* and subtilisin BPN′ from *Bacillus amyloliquefaciens*, with respect to the eight subsites $S_5$ to $S'_3$.[2] The amino acid side-chain preference of each subsite was determined by means of long and very sensitive fluorogenic substrates. The substrates, ABz-$(Xaa)_n$-Tyr($NO_2$)-Asp-OH, consisted of a polypeptide, $(Xaa)_n$, with 4–7 amino acid residues, a fluorogenic group, ABz, linked to the N-terminus, and a quenching group, Tyr($NO_2$), linked to the C-terminus followed by an Asp to increase the solubility of the substrates. Upon cleavage of any of the peptide bonds between ABz and Tyr($NO_2$), the fluorescence of ABz is released (Figure 1). Eight substrate series were synthesized, and in each series only a single position between ABz and Tyr($NO_2$)-Asp-OH was varied by substituting 9–11 different amino acids, representing different properties of side chains, into the peptide. To secure the correct cleavage point, the sequences of amino acids were designed on the basis of the existing structural and kinetic data for subtilisin BPN′ and subtilisin Carlsberg from *Bacillus licheniformis*: (1) a preference exists for an aromatic amino acid residue in $P_1$ and $P_4$, as well as a nonbulky residue in $P'_1$; (2) Pro is very poorly accepted in $S_1$ and $S_3$ but is accepted in $S_2$ and $S_4$; (3) the rate of hydrolysis is beneficially influenced by an increase in the chain length of the substrate (Brömme et al., 1986; Phillip & Bender, 1983; Morihara & Oka, 1977; Morihara et al., 1970). On the basis of the structural similarities between Savinase and subtilisin BPN′ (Outtrup & Boyce, 1990; Wright et al., 1969; Betzel et al., 1992) and preliminary studies with Savinase using smaller chromogenic substrates, it was deemed probable that both enzymes could be studied with the same series of substrates. With seven out of eight substrate series, the cleavage point was directed by a Pro in $P_2$ and a Phe in $P_4$ and/or $P_1$ (Table I; A1, B1, B2, B3, D1, D2, and D3). Preliminary studies with Savinase demonstrated that ABz is very favorable in $P_4$ (data not shown), and this was utilized to direct the cleavage points for substrates used to determine the $P_2$ preference; the cleavage point was directed by the aromatic groups ABz and Phe in $P_4$ and $P_1$, respectively, and the nonbulky Gly in $P'_1$ (Table

[2] In accordance with Schechter and Berger (1967), the enzyme binding sites are denoted $S_1$, $S_2$, ..., $S_i$ and $S'_1$, $S'_2$, ..., $S'_i$ away from the scissile bond toward the N- and the C-terminus, respectively. Amino acid residues in the substrates are referred to as $P_1$, $P_2$, ..., $P_i$ and $P'_1$, $P'_2$, ..., $P'_i$ in correspondence with the binding site.

Table I: Substrates Used in the Subsite Mapping of Subtilisin BPN′ and Savinase[a]

| | $P_6$ | $P_5$ | $P_4$ | $P_3$ | $P_2$ | $P_1$ | ↓ | $P'_1$ | $P'_2$ | $P'_3$ | $P'_4$ | $P'_5$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 | ABz | Xaa | Phe | Gly | Pro | Phe | | Tyr($NO_2$) | Asp | | | |
| B1 | | ABz | Xaa | Gly | Pro | Phe | | Tyr($NO_2$) | Asp | | | |
| B2 | | ABz | Phe | Xaa | Pro | Phe | | Tyr($NO_2$) | Asp | | | |
| B3 | | ABz | Phe | Gly | Pro | Xaa | | Tyr($NO_2$) | Asp | | | |
| C1 | | | ABz | Asp | Xaa | Phe | | Gly | Tyr($NO_2$) | Asp | | |
| D1 | | ABz | Phe | Ala | Pro | Phe | | Xaa | Gly | Gly | Tyr($NO_2$) | Asp |
| D2 | | ABz | Phe | Ala | Pro | Phe | | Gly | Xaa | Gly | Tyr($NO_2$) | Asp |
| D3 | | ABz | Phe | Ala | Pro | Phe | | Gly | Gly | Xaa | Tyr($NO_2$) | Asp |
| D4 | | ABz | Xaa | Ala | Pro | Phe | | Gly | Gly | Gly | Tyr($NO_2$) | Asp |
| D5 | | ABz | Phe | Xaa | Pro | Phe | | Gly | Gly | Gly | Tyr($NO_2$) | Asp |
| D6 | | ABz | Phe | Ala | Pro | Xaa | | Gly | Gly | Gly | Tyr($NO_2$) | Asp |

[a] The arrow (↓) indicates the scissile bond.

I; C1). In each substrate series, the cleavage point with Savinase was determined for three substrates, hydrolyzed with $k_{cat}/K_M$ values spanning the entire range, i.e., a substrate hydrolyzed with a high, a medium, and a low $k_{cat}/K_M$ value. In the substrate series A1, B1, B2, B3, C1, D2, and D3, all cleavage points were as predicted (indicated by an arrow in Table II). Thus, it is inconceivable that substrates belonging to these series, exhibiting $k_{cat}/K_M$ values ranking between those tested, would be cleaved at positions other than the predicted. Both Savinase and subtilisin BPN′ exhibited extremely low $k_{cat}/K_M$ values (<0.004 $\mu M^{-1}$ $min^{-1}$) with a Pro in $P'_1$ (series D1, Xaa = Pro), and with this substrate an unexpected cleavage site was found. The substrate was cleaved between two glycines and not as expected between Phe and Pro, thus placing the two prolines in $S_2$ and $S_4$. It is explicable that this cleavage point is favored, as prolines are well accepted in both $S_2$ and $S_4$ (Brömme et al., 1986; Morihara & Oka, 1977; Table II). A similar undesirable shift of prolyl residues to favorable positions is not possible with the other substrate series. With the rest of the substrates in series D1, the $k_{cat}/K_M$ values varied only 14-fold, ranging from 6.8 $\mu M^{-1}$ $min^{-1}$ (Xaa = Asp) to 97.0 $\mu M^{-1}$ $min^{-1}$ (Xaa = Ala), and within this range the expected cleavage point was confirmed. The similarity in substrate preferences of Savinase and subtilisin BPN′ would predict that the cleavage points in all substrates are identical, and this was confirmed with one or two substrates from each of the eight substrate series (indicated by an asterisk (*) in Table II).

For each subsite investigation, i.e., within each substrate series, the preference for a given amino acid, Xaa, given in dimensions of free energy, was evaluated relative to Gly, by calculating the difference in transition-state binding energies, $\Delta\Delta G_T^{\ddagger}$(Xaa → Gly) (Table II), hence bypassing problems in calculating the numeric values of $\Delta G_T^{\ddagger}$. A negative $\Delta\Delta G_T^{\ddagger}$(Xaa → Gly) value indicates that Xaa is less favorable than Gly at the given substrate position; a positive value indicates that Xaa is more favorable. The more selective the subsite, the larger $\Delta\Delta G_T^{\ddagger}$ between the substrate with the most preferred and the least preferred amino acid residue, i.e., $\Delta\Delta G_T^{\ddagger}$(max). In Table III, $\Delta\Delta G_T^{\ddagger}$(max) is calculated for each subsite with both enzymes. Since a Pro may influence the conformation of the enzyme-bound peptide substrate, the results with Pro are not included unless specifically stated.

The individual subsites need not necessarily be independent of each other, preventing comparisons of $\Delta\Delta G_T^{\ddagger}$(max) values from different substrate series. This was investigated by comparing $\Delta\Delta G_T^{\ddagger}$(Xaa → Gly) obtained with two series of different structures but with the same variations in each of the three positions $P_4$ (Table I, series B1 and D4), $P_3$ (Table I, series B2 and D5), and $P_1$ (Table I, series B3 and D6). The $\Delta\Delta G_T^{\ddagger}$(Xaa → Gly) values were essentially independent of the substrate series (Table II), and thus, it appears that comparisons of results obtained with different series are permissible. With subtilisin BPN′, the $\Delta\Delta G_T^{\ddagger}$(max) values provide the following selectivity rating of the subsites: $S_1 \geq S_4 > S_2 > S'_1 \geq S_3 > S'_2 > S_5 = S'_3$. With Savinase, the rating is similar: $S_1 > S_4 > S_2 = S_3 > S'_1 \geq S'_2 > S_5 > S'_3$ (Table III). This rating supports the predictions from X-ray crystallographic studies of subtilisin BPN′ in complex with different inhibitors. On the basis of the number of close contacts (<4 Å) at each subsite between enzyme and inhibitors, it is assumed that $S_4$ and $S_1$ dominate the substrate preference (McPhalen et al., 1985; Heinz et al., 1991; Mitsui, 1985) (Table III). In comparison, $S_2$, $S_3$, $S'_1$, $S'_2$, and, in particular, $S_5$ and $S'_3$ have fewer close contacts with the inhibitors (Table III).

Each subsite was characterized by its substrate preference as determined by the $k_{cat}/K_M$ values.

$S_5$: Both enzymes were very flexible with respect to accommodation of different $P_5$ amino acid residues in substrates of the general formula ABz-Xaa-Phe-Gly-Pro-Phe-↓Tyr-($NO_2$)-Asp-OH. $\Delta\Delta G_T^{\ddagger}$(max) was 3.9 kJ/mol with Savinase and 3.4 kJ/mol with subtilisin BPN′. With Savinase, the following decreasing order of $k_{cat}/K_M$ was observed: V > L ≥ D ≥ F = A > R > N > P > G. With subtilisin BPN′, the following was observed: V ≥ D > L > F ≥ P ≥ A > N > R = G.

$S_4$: Savinase and subtilisin BPN′ both exhibited marked preferences for an aromatic group in $P_4$, and pronounced changes in the $k_{cat}/K_M$ values were brought about by relatively small variations in substrates of the general formula ABz-Xaa-Gly-Pro-Phe-↓Tyr($NO_2$)-Asp-OH. $\Delta\Delta G_T^{\ddagger}$(max) was 20.7 kJ/mol with Savinase and 20.9 kJ/mol with subtilisin BPN′. With Savinase, the following decreasing order of $k_{cat}/K_M$ was observed: F > W > V > L > P > A > G ≥ N > R > D. With subtilisin BPN′, the following was observed: F > L > V > W ≥ A > P > G > N > R > D. It is well established that subtilisin BPN′ has an $S_4$ binding crevice which can accommodate an aromatic side chain from $P_4$ (Robertus et al., 1972a,b). It has been suggested that the preference of subtilisin BPN′ for aromatic $P_4$ residues is directed by Tyr104[3] aligning its aromatic ring parallel to an aromatic ring in the substrate, e.g., Phe (Kraut, 1977). However, as described here, Savinase with a Val occupying position 104 also exhibits a high preference for aromatic groups, i.e., ABz and Phe. A comparison of the structures of the subtilisin Carlsberg/eglin-c complex ($P_4$ = Pro) and the subtilisin BPN′/CI-2 complex ($P_4$ = Ile) reveals that the $S_4$ pockets differ considerably in size and shape in spite of the

[3] The numbering of amino acids is that of subtilisin BPN′ (Wells et al., 1983); an amino acid followed by a number refers to the corresponding amino acid residue positioned in the enzyme according to this numbering; e.g., Tyr104 is the tyrosyl residue in position 104.

Table II: $k_{cat}/K_M$ Values for the Cleavage of Fluorogenic Peptide Substrates by Subtilisin BPN′ and Savinase and the Associated $\Delta\Delta G_T^{\ddagger}$(Xaa → Gly) Values[a]

| | Savinase | | subtilisin BPN′ | |
|---|---|---|---|---|
| | $k_{cat}/K_M$ ($min^{-1}$ $\mu M^{-1}$) | $\Delta\Delta G_T^{\ddagger}$ (Xaa → Gly) (kJ $mol^{-1}$) | $k_{cat}/K_M$ ($min^{-1}$ $\mu M^{-1}$) | $\Delta\Delta G_T^{\ddagger}$ (Xaa → Gly) (kJ $mol^{-1}$) |
| **$P_5$, Series A1** | | | | |
| ABz-GFGPF-Y′D | 35.6 | 0 | 5.0 | 0 |
| ABz-AFGPF-Y′D | 70.0 | 1.7 | 8.5 | 1.3 |
| ABz-VFGPF-Y′D | 170.0 | 3.9 | 20.0 | 3.4 |
| ABz-LFGPF-Y′D | 90.0 | 2.3 | 13.5 | 2.5 |
| ABz-FFGPF-↓Y′D* | 70.0 | 1.7 | 9.5 | 1.6 |
| ABz-NFGPF-Y′D | 47.0 | 0.7 | 6.8 | 0.8 |
| ABz-PFGPF-Y′D | 37.1 | 0.1 | 9.0 | 1.5 |
| ABz-DFGPF-Y′D | 87.5 | 2.2 | 19.5 | 3.4 |
| ABz-RFGPF-Y′D | 55.0 | 1.1 | 5.0 | 0.0 |
| **$P_4$, Series B1** | | | | |
| ABz-GGPF-Y′D | 0.022 | 0 | 0.028 | 0 |
| ABz-AGPF-↓Y′D* | 0.25 | 6.0 | 0.59 | 7.6 |
| ABz-VGPF-Y′D | 2.1 | 11.3 | 1.1 | 9.1 |
| ABz-LGPF-Y′D | 0.82 | 9.0 | 2.1 | 10.7 |
| ABz-FGPF-↓Y′D* | 6.0 | 13.9 | 3.6 | 12.1 |
| ABz-WGPF-Y′D | 2.6 | 11.8 | 0.60 | 7.6 |
| ABz-NGPF-Y′D | 0.019 | −0.4 | 0.005 | −4.4 |
| ABz-PGPF-Y′D | 0.69 | 8.5 | 0.075 | 2.5 |
| ABz-DGPF-Y′D | 0.0014 | −6.8 | 0.0008 | −8.8 |
| ABz-RGPF-↓Y′D | 0.010 | −2.0 | 0.0027 | −5.8 |
| **$P_4$, Series D4** | | | | |
| ABz-GAPF-GGGY′D | 0.31 | 0 | nd[b] | nd[b] |
| ABz-LAPF-GGGY′D | 8.5 | 8.2 | nd[b] | nd[b] |
| ABz-FAPF-↓GGGY′D* | 89.0 | 14.0 | nd[b] | nd[b] |
| **$P_3$, Series B2** | | | | |
| ABz-FGPF-↓Y′D* | 6.0 | 0 | 3.6 | 0 |
| ABz-FAPF-Y′D | 10.7 | 1.4 | 5.7 | 1.1 |
| ABz-FLPF-Y′D | 17.6 | 2.7 | 9.8 | 2.5 |
| ABz-FFPF-Y′D | 20.8 | 3.1 | 18.1 | 4.0 |
| ABz-FWPF-Y′D | 21.0 | 3.1 | 14.0 | 3.4 |
| ABz-FNPF-Y′D | 5.2 | −0.4 | 3.4 | −0.1 |
| ABz-FPPF-↓Y′D | 0.003 | −19.0 | 0.00001 | −31.7 |
| ABz-FDPF-Y′D | 1.3 | −3.8 | 0.32 | −6.0 |
| ABz-FRPF-↓Y′D* | 53.0 | 5.4 | 77.0 | 7.6 |
| **$P_3$, Series D5** | | | | |
| ABz-FGPF-GGGY′D | 61.0 | 0 | nd[b] | nd[b] |
| ABz-FAPF-↓GGGY′D* | 89.0 | 0.9 | nd[b] | nd[b] |
| ABz-FRPF-GGGY′D | 150.0 | 2.2 | nd[b] | nd[b] |
| **$P_2$, Series C1** | | | | |
| ABz-DGF-↓GY′D | 125 | 0 | 0.29 | 0 |
| ABz-DAF-↓GY′D* | 440 | 3.1 | 16.0 | 10.0 |
| ABz-DVF-GY′D | 400 | 2.9 | 0.38 | 0.6 |
| ABz-DLF-GY′D | 525 | 3.6 | 2.4 | 5.2 |
| ABz-DFF-GY′D | 242 | 1.6 | 0.40 | 0.8 |
| ABz-DWF-GY′D | 21.0 | 3.1 | 14.0 | 3.4 |
| ABz-DNF-GY′D | 155 | 0.5 | 0.47 | 0.5 |
| ABz-DPF-GY′D | 100 | −0.6 | 0.93 | 2.9 |
| ABz-DDF-↓GY′D | 13.0 | −5.6 | 0.020 | −6.6 |
| ABz-DRF-GY′D | 165 | 0.7 | 2.7 | 5.5 |
| **$P_1$, Series B3** | | | | |
| ABz-FGPG-↓Y′D | 0.0003 | 0 | 0.0029 | 0 |
| ABz-FGPA-Y′D | 0.13 | 15.1 | 1.1 | 14.7 |
| ABz-FGPV-Y′D | 0.0008 | 2.4 | 0.042 | 6.6 |
| ABz-FGPL-↓Y′D* | 0.68 | 19.2 | 10.7 | 20.4 |
| ABz-FGPI-Y′D | 0.00001 | −8.0 | 0.0015 | −1.6 |
| ABz-FGPF-↓Y′D* | 6.0 | 24.6 | 3.6 | 17.7 |
| ABz-FGPW-Y′D | 0.13 | 15.05 | 0.38 | 12.1 |
| ABz-FGPN-Y′D | 0.060 | 13.1 | 1.2 | 14.9 |
| ABz-FGPP-Y′D | $\approx 5 \times 10^{-7}$ | <−16 | $\approx 5 \times 10^{-6}$ | <−16 |
| ABz-FGPD-↓Y′D | 0.0006 | 1.7 | 0.0069 | 2.1 |
| ABz-FGPR-Y′D | 0.010 | 8.7 | 2.26 | 16.5 |
| **$P_1$, Series D6** | | | | |
| ABz-FAPG-↓GGGY′D | 0.01 | 0 | nd[b] | nd[b] |
| ABz-FAPA-GGGY′D | 4.3 | 15.0 | nd[b] | nd[b] |
| ABz-FAPF-↓GGGY′D* | 89.0 | 22.5 | nd[b] | nd[b] |
| **$P'_1$, Series D1** | | | | |
| ABz-FAPF-↓GGGY′D* | 89.0 | 0 | 75.0 | 0 |
| ABz-FAPF-AGGY′D | 97.0 | 0.2 | 9.5 | −2.1 |
| ABz-FAPF-VGGY′D | 25.4 | −3.1 | 4.9 | −6.7 |
| ABz-FAPF-LGGY′D | 7.8 | −6.0 | 3.25 | −7.8 |
| ABz-FAPF-FGGY′D | 22.0 | −3.5 | 31.0 | −2.2 |
| ABz-FAPF-WGGY′D | 32.0 | −2.5 | 133 | 1.4 |
| ABz-FAPF-NGGY′D | 13.6 | −4.7 | 2.35 | −8.6 |
| ABz-FAPFPG-↓GY′D* | <0.004[c] | <−25 | <0.0008[c] | <−28 |
| ABz-FAPF-↓DGGY′D | 6.8 | −6.4 | 0.4 | −13.0 |
| ABz-FAPF-RGGY′D | 24.0 | −3.2 | 38.0 | −1.7 |
| **$P'_2$, Series D2** | | | | |
| ABz-FAPF-↓GGGY′D* | 89.0 | 0 | 75.0 | 0 |
| ABz-FAPF-GAGY′D | 140 | 1.1 | 175 | 2.1 |
| ABz-FAPF-GVGY′D | 231 | 2.4 | 255 | 3.0 |
| ABz-FAPF-GLGY′D | 277 | 2.8 | 250 | 3.0 |
| ABz-FAPF-↓GFGY′D* | 358 | 3.5 | 410 | 4.2 |
| ABz-FAPF-GWGY′D | 268 | 2.7 | 322 | 3.6 |
| ABz-FAPF-GNGY′D | 76.0 | −0.4 | 46.0 | −1.2 |
| ABz-FAPF-↓GPGY′D | 5.4 | −6.9 | 1.2 | −10.3 |
| ABz-FAPF-GDGY′D | 44.4 | −1.7 | 36.0 | −1.8 |
| ABz-FAPF-GRGY′D | 92.6 | 0.1 | 180 | 2.2 |
| **$P'_3$, Series D3** | | | | |
| ABz-FAPF-↓GGGY′D* | 80.0 | 0 | 78.0 | 0 |
| ABz-FAPF-GGAY′D | 159 | 1.7 | 120 | 1.1 |
| ABz-FAPF-GGVY′D | 180 | 2.0 | 80.0 | 0.1 |
| ABz-FAPF-GGLY′D | 149 | 1.5 | 80.0 | 0.1 |
| ABz-FAPF-↓GGFY′D | 173 | 1.9 | 100 | 0.6 |
| ABz-FAPF-GGNY′D | 79.0 | −0.0 | 55.0 | −0.9 |
| ABz-FAPF-GGPY′D | 83.0 | 0.1 | 165 | 1.9 |
| ABz-FAPF-GGDY′D | 58.0 | −0.8 | 42.0 | −1.5 |
| ABz-FAPF-GGRY′D | 100 | 0.6 | 110 | 0.9 |

[a] The $k_{cat}/K_M$ values for the hydrolysis of the fluorogenic peptide substrates were determined using steady-state enzyme kinetics, the $\Delta\Delta G_T^{\ddagger}$(Xaa → Gly) = $RT$ ln [($k_{cat}/K_M$(Xaa))/($k_{cat}/K_M$(Gly))] values were calculated and the cleavage point (−) was checked with Savinase for the substrates indicated with an arrow (↓). An asterisk (*) indicates that the cleavage point is checked with subtilisin BPN′. The underlined amino acids are the ones which were varied and Y′ is Tyr($NO_2$). [b] Not determined. [c] This substrate was cleaved between the two glycines and not as expected between Phe and Pro.

fact that the amino acid residues forming the pockets are identical. With Pro bound, the pocket is shorter and broader than with Ile bound (McPhalen & James, 1988). It has been suggested that the side chain of Tyr104 is flexible such that it can adjust the size of the $S_4$ pocket (Takeuchi et al., 1991a). This can explain why $k_{cat}/K_M$ within the hydrophobic series Ala, Val, Leu, and Phe only increased by a factor of less than 6 with subtilisin BPN′, whereas with Savinase (without a flexible Tyr at position 104) a 24-fold increase was observed. It seems that hydrophobic forces are predominant in both enzymes in determining the side-chain preference with respect to $P_4$. However, steric effects are also important since the substrate with the most hydrophobic amino acid residue in $P_4$, i.e., Trp, apparently due to its bulkiness, was hydrolyzed with a lower $k_{cat}/K_M$ than one with Phe in $P_4$. The steric effects appear to be more pronounced in subtilisin BPN′ than in Savinase, most likely due to the difference in bulkiness of the group at residue 104 in the two enzymes. With the hydrophilic Asp, Arg, Asn, and Gly, $k_{cat}/K_M$ decreased to less than 1% relative to Phe with both enzymes.

$S_3$: Substrates used in the $S_3$ subsite mapping were of the general formula ABz-Phe-Xaa-Pro-Phe-↓Tyr($NO_2$)-Asp-OH.

Table III: Contributions of the Individual Subsite–Substrate Interactions to the Substrate Preferences of Savinase and Subtilisin BPN′[a]

| | Savinase $\Delta\Delta G_T^{\ddagger}$(max) (kJ mol$^{-1}$) | subtilisin BPN′ $\Delta\Delta G_T^{\ddagger}$(max) (kJ mol$^{-1}$) | CI-2[b,c] contacts <4 Å | eglin-c[b,d] contacts <4 Å | SSI[b,e] contacts <4 Å |
|---|---|---|---|---|---|
| $S_5$ | 3.9 | 3.4 | 8 (Thr55) | 0 (Ser41) | 9 (Val69) |
| $S_4$ | 20.7 | 20.9 | 31 (Ile56) | 20 (Pro42) | 24 (Met70) |
| $S_3$ | 9.2 | 13.6 | 12 (Val57) | 13 (Val43) | 7 (Cys71) |
| $S_2$ | 9.2 | 16.6 | 16 (Thr58) | 16 (Thr44) | 13 (Pro72) |
| $S_1$ | 32.6 | 22.0 | 45 (Met59) | 29 (Leu45) | 32 (Met73) |
| $S'_1$ | 6.6 | 14.4 | 11 (Glu60) | 13 (Asp46) | 14 (Val74) |
| $S'_2$ | 5.2 | 6.0 | 11 (Tyr61) | 5 (Leu47) | 17 (Tyr74) |
| $S'_3$ | 2.8 | 3.4 | 2 (Arg62) | 7 (Asp48) | |

[a] The significance of each subsite–substrate interaction for determining the overall substrate preference exhibited by subtilisin BPN′ and Savinase can be evaluated on the basis of $\Delta\Delta G_T^{\ddagger}$(max) = $\Delta\Delta G_T^{\ddagger}$(Xaa → Gly) − $\Delta\Delta G_T^{\ddagger}$(Yaa → Gly), where Xaa is the most favorable amino acid and Yaa is the least favorable amino acid to be substituted in the actual position. [b] X-ray crystallographic data provide the number of close intermolecular contacts (<4.0 Å) between subtilisin BPN′ and the following inhibitors: (*c*) CI-2 (McPhalen et al., 1985), (*d*) eglin-c (Heinz et al., 1991), and (*e*) SSI (Takeuchi et al., 1991b) (the amino acid occupying the P position in the inhibitor is given in parentheses).

With Savinase, the following decreasing order of $k_{cat}/K_m$ was observed: R > F ≥ L > A > G ≥ N > D >> P. With subtilisin BPN′, the following was observed: R > F > L > A > G ≥ N > D >> P. With Pro in $P_3$, $k_{cat}/K_M$ decreased dramatically, possibly due to the importance of the $S_4$–$P_4$ interactions which could be disturbed by the presence of a Pro in $P_3$. X-ray crystallographic studies of the interactions between subtilisin Carlsberg and eglin-c show that the $P_3$ side chain of the inhibitor (Val) does not bind at any specific site of the enzyme but is involved only in hydrophobic interactions with the backbone of the inhibitor itself (McPhalen et al., 1988). Consistent with this, both enzymes discriminate only little among the uncharged amino acid residues (Gly, Ala, Leu, Phe, Trp, and Asn) with $\Delta\Delta G_T^{\ddagger}$ < 3.5 kJ/mol between the most preferred and the least preferred. However, an interesting difference was found with the positively charged Arg and the negatively charged Asp ($\Delta\Delta G_T^{\ddagger}$(Arg → Asp) = 9.2 kJ/mol with Savinase and 13.6 kJ/mol with subtilisin BPN′). Both enzymes exhibited a significant preference for Arg but only low activity toward Asp in $P_3$. It is conceivable that charged $P_3$ residues interact with the enzyme, presumably with the negative charge of Asp60 which is conserved in both enzymes.

$S_2$: With a substrate series of the general formula ABz-Asp-Xaa-Phe-↓Gly-Tyr($NO_2$)-Asp-OH the following decreasing order of $k_{cat}/K_M$ was observed with Savinase: L > A ≥ V > F > R ≥ N > G > P > W > D. With subtilisin BPN′, the following was observed: A > R ≥ L > P > N > F ≥ V > G > W > D. $\Delta\Delta G_T^{\ddagger}$(max) was 9.2 kJ/mol with Savinase and 16.6 kJ/mol with subtilisin BPN′, suggesting that Savinase discriminates less strongly between different $P_2$ residues than does subtilisin BPN′. Both enzymes exhibited a preference for aliphatic amino acid residues in $P_2$ (Ala and Leu) whereas the negatively charged Asp was very poorly accepted. This is in accordance with structural data that propose the $S_2$ binding pocket in subtilisin Carlsberg to be composed of Thr33, the His64 side chain, Leu96, and Gly100 into which Thr58 of eglin-c fits (McPhalen et al., 1988). These amino acids are all conserved in Savinase, and in subtilisin BPN′ only position 33 differs (it is occupied by a Ser). Pro is rather well accepted by both enzymes in this subsite.

$S_1$: Pronounced changes in the $k_{cat}/K_M$ values were brought about by relatively small changes in the $P_1$ amino acids in substrates of the general formula ABz-Phe-Gly-Pro-Xaa-↓Tyr($NO_2$)-Asp-OH. With Savinase the following decreasing order of $k_{cat}/K_M$ was observed: F > L > A = W > N > R > V ≥ D ≥ G > I >>> P. With subtilisin BPN′, the following order was observed: L > F > R > N ≥ A > W > V > D ≥ G ≥ I >>> P. With Savinase, $\Delta\Delta G_T^{\ddagger}$(max) was 32.6 kJ/mol, and with subtilisin BPN′ it was 22 kJ/mol. For both enzymes, this was the subsite with the highest $\Delta\Delta G_T^{\ddagger}$(max) indicating that it is the most restrictive subsite. Both enzymes exhibited a marked preference for hydrophobic groups whereas a Pro in $P_1$ led to very low $k_{cat}/K_M$ values presumably by preventing proper alignment of the scissile bond. Studies of the interactions between subtilisin BPN′ and different mutants of *Streptomyces* subtilisin inhibitor (SSI) support our results that in $P_1$ Phe, Arg, and Asn are favored over Asp, Gly, and Pro (Kojima et al., 1991; Takeuchi et al., 1991a). As observed by Morihara and Tsuzuki (1969), substrates with the β-branched amino acids Val and Ile in $P_1$ are very poor. This inefficiency in cleaving substrates with a β-branched $P_1$ residue is common to many proteolytic enzymes (Pettit et al., 1991; Breddam, 1986; Breddam et al., 1987). In the case of subtilisin BPN′, it has been suggested that this is due to the $S_1$ pocket being "sock-shaped" with a too narrow entrance to accommodate the β-branched amino acids (Takeuchi et al., 1991a). The $P_1$ specificity for subtilisin BPN′ as determined in the present investigation is in good agreement with that obtained by a series of Suc-Ala-Ala-Pro-Xaa-NH-Ph-$NO_2$ substrates although the preference for Leu over Phe observed here (Table II) is reversed in the earlier studies (Wells et al., 1987). Subtilisin BPN′ accommodates the positively charged Arg, the polar Asn, and the bulky Val and Ile more easily than Savinase. The $P_1$ preference of subtilisin BPN′ and subtilisin Carlsberg also differ, and this has been ascribed to the amino acid residues occupying positions 156 (Glu in BPN′/Ser in Carlsberg), 169 (Gly in BPN′/Ala in Carlsberg), and 217 (Tyr in BPN′/Leu in Carlsberg) (Wells et al., 1987). Since Savinase at these positions is identical to subtilisin Carlsberg, some of its differences in substrate preference relative to that of BPN′ may be explained by these substitutions. However, since the substrate preference of Savinase also differs from that of subtilisin Carlsberg, an additional effect of the smaller $S_1$ binding pocket of Savinase, due to the deletion of amino acids 164, 165, and 166 and/or the presence of Ser at position 128 (Gly in subtilisin BPN′) (Betzel et al., 1992), is conceivable. Furthermore, the crystallographic studies on subtilisin BPN′/eglin-c mutant complexes have indicated that the contacts between residues Gly127/Gly128 of subtilisin BPN′ and the $P_1$ position of eglin-c are dependent on the nature of the amino acid residue occupying this position (Leu or Arg) (Heinz et al., 1991) which emphasizes the potential influence of Ser128 in Savinase on the $P_1$ preference.

The geometries of the $S'_1$, $S'_2$, and $S'_3$ subsites are rather poorly defined due to the fact that the X-ray crystallographic data have been obtained with inhibitors that do not bind in a well-defined mode on the C-terminal side of the scissile bond. Furthermore, kinetic data have been difficult to obtain due to the limited availability of suitable chromogenic or fluorogenic substrates.

$S'_1$: Our data show that the $k_{cat}/K_M$ values were dependent on the $P'_1$ side chain in substrates of the general formula ABz-Phe-Ala-Pro-Phe-↓Xaa-Gly-Gly-Tyr($NO_2$)-Asp-OH. With Savinase, the following decreasing order of $k_{cat}/K_M$ was observed: A ≥ G > W > V ≥ R ≥ F > N > L ≥ D >>> P. With subtilisin BPN′, the following was observed: W > G > R > F > A > V ≥ L ≥ N > D >>> P. $\Delta\Delta G_T^{\ddagger}$(max) is 6.6 kJ/mol with Savinase and 14.4 kJ/mol with subtilisin BPN′. This suggests that $S'_1$ in Savinase is less restrictive than in subtilisin BPN′. As expected from the literature, the small amino acid Gly is very well accepted in $P'_1$ (Bratovanova &

Petkov, 1987). The bulky, hydrophobic Phe and Trp and the positively charged Arg were also well accepted in this position whereas the negatively charged Asp was only poorly accepted. A Pro in $P'_1$ prevents cleavage, most likely by obstructing the proper alignment of the scissile bond. With both enzymes the substrate ABz-Phe-Ala-Pro-Phe-Pro-Gly-Gly-Tyr($NO_2$)-Asp-OH was cleaved between the two glycines shifting the two prolines to $P_2$ and $P_4$. The $k_{cat}/K_M$ values with this substrate were very low reflecting the presence of the unfavorable Gly and Pro in $P_1$ and $P_4$, respectively. The $P'_1$ preference of Savinase and subtilisin BPN′ appears not to be determined by purely steric or hydrophobic effects.

$S'_2$: Substrates of the general formula ABz-Phe-Ala-Pro-Phe-↓Gly-Xaa-Gly-Tyr($NO_2$)-Asp-OH were used in the $S'_2$ subsite investigation. With Savinase, the following decreasing order of $k_{cat}/K_M$ was observed: F > L > W > V > A > R ≥ G > N > D > P. With subtilisin BPN′, the following was observed: F > W > V ≥ L > R ≥ A > G > N > D > P. With Savinase, $\Delta\Delta G_T^{\ddagger}$(max) was 5.2 kJ/mol, and with subtilisin BPN′ it was 6.0 kJ/mol. This suggests that with both enzymes the $S'_2$–$P'_2$ interactions only contribute little to $\Delta G_T^{\ddagger}$. Both enzymes exhibited a preference for aromatic and bulky aliphatic amino acids. Likewise, the positively charged Arg was well accepted by both enzymes whereas Pro in $P'_2$ caused a significant reduction in $k_{cat}/K_M$. Within the hydrophobic series Gly, Ala, Val, Leu, and Phe, $k_{cat}/K_M$ increased, thus indicating that hydrophobic interactions control the $P'_2$ preference of both enzymes. With the very bulky Trp $k_{cat}/K_M$ decreased relative to Phe, probably due to some steric restraints.

$S'_3$: Both enzymes were very flexible with respect to the accommodation of different amino acid residues in this subsite. The substrate preference was investigated with substrates of the general formula ABz-Phe-Ala-Pro-Phe-↓Gly-Gly-Xaa-Tyr($NO_2$)-Asp-OH, and $\Delta\Delta G_T^{\ddagger}$(max) was 2.8 kJ/mol with Savinase and 3.4 kJ/mol with subtilisin BPN′.

*Concluding Remarks.* Our data can be rationalized on the basis of the structural data available for the two enzymes, subtilisin BPN′ and Savinase, suggesting that the structures of proteases and protease/inhibitor complexes to some extent may predict the importance of particular binding sites. Our data emphasize that in both subtilisin BPN′ and Savinase interactions between substrate and $S_4$ and $S_1$ are very important and that changes in $P_4$ and $P_1$ residues can drastically change $\Delta G_T^{\ddagger}$. However, other subsites exert a significant influence on the substrate preference such that changes in substrate structure distant from the scissile bond and in other positions than $P_1$ or $P_4$ can affect the $k_{cat}/K_M$ values by increasing or decreasing $\Delta G_T^{\ddagger}$, even in cases where no specific binding pocket has been identified.

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# Inactivation of Dimeric D-Amino Acid Transaminase by a Normal Substrate through Formation of an Unproductive Coenzyme Adduct in One Subunit†

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ABSTRACT: D-Amino acid transaminase, which contains pyridoxal 5′-phosphate (vitamin $B_6$) as coenzyme, catalyzes the formation of D-alanine and D-glutamate from their corresponding α-keto acids; these D-amino acids are required for bacterial cell wall biosynthesis. Under conditions usually used for *kinetic* assay of enzyme activity, i.e., short incubation times with dilute enzyme concentrations, D-alanine behaves as one of the best substrates. However, the enzyme slowly loses activity over a period of hours when exposed to substrates, intermediates, and products at *equilibrium*. The rate of inactivation is dependent on enzyme concentration but independent of substrate concentration greater than $K_m$ values. Continuous removal of the product pyruvate by enzymic reduction precludes the establishment of equilibrium and prevents inactivation. The formation of small but detectable amounts of a quinonoid intermediate absorbing at 493 nm is proportional to inactivation. Studies with [$^{14}$C]-D-alanine labeled on different carbon atoms indicate that the α-carboxyl group of the substrate is absent in the inactive enzyme; such decarboxylation is not a usual function of this enzyme. The inactive transaminase contains 1.1 mol of [$^{14}$C]-D-alanine-derived adduct per mole of dimeric enzyme; this finding is consistent with the 50% reduction in the fluorescence intensity at 390 nm (due to the PMP form of the coenzyme) for the inactive enzyme. Thus, inactivation of one subunit of the dimeric enzyme renders the entire molecule inactive. Inactivation may occur when a coenzyme intermediate, perhaps the ketimine, is slowly decarboxylated and then undergoes a conformational change from its catalytically competent location. Evidence indicates that acetaldehyde is the adduct attached to the coenzyme and not to the protein in the inactive enzyme. The activity and spectral properties of the native enzyme are restored upon treatment of the inactive enzyme at slightly acidic pH. These findings may have ramifications in terms of a slow decrease in enzyme function under physiological conditions.

In most *kinetic* assays used to measure the activity of enzymes with their normal substrates, the time for analysis with microgram amounts of enzyme is usually short, i.e., of the order of minutes, and reactive intermediates are not readily detectable. In enzymes with cofactors that absorb at higher wavelengths, milligram amounts of enzyme are sometimes used to detect such intermediates and to measure their turnover. Such conditions also present the opportunity to examine the integrity of the enzyme–coenzyme system upon exposure to such intermediates for extended periods. Described herein is the slow substrate-induced inactivation of D-amino acid transminase when it is permitted to remain at *equilibrium* in contact with all of the intermediates in the reaction pathway. This enzyme, which contains pyridoxal 5′-phosphate as coenzyme, catalyzes the reversible interconversion of D-alanine and α-ketoglutarate to form pyruvate and D-glutamate; these two D-amino acids are an integral part of most, if not all, bacterial cell walls. Hence, information on the inactivation of this enzyme by any type of substrate, whether it be a normal or a suicide substrate, may facilitate the development of novel antimicrobial agents that have this protein as their target (Gale et al., 1981; Manning et al., 1974).

## Materials and Methods

D-Alanine, D-glutamate, D-α-aminobutyrate, yeast aldehyde dehydrogenase (EC 1.2.1.5), and bovine liver alkaline phosphatase (EC 3.1.3.1) were purchased from Sigma. The various batches of D-alanine used in this study were judged pure by amino acid analysis and by elemental analysis, which were kindly performed by Mr. Robert Buzolich. Uniformly labeled [U-$^{14}$C]-D-alanine and [1-$^{14}$C]-D-alanine were purchased from Amersham. For accurate determination of their specific ra-

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