Interdependency of the Binding Subsites in Subtilisin†

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ABSTRACT: Subtilisins are endopeptidases with an extended binding cleft comprising at least eight subsites, and kinetic studies have revealed that subsites distant from the scissile bond are important in determining the substrate preference of the enzymes. With the subtilisin enzyme Savinase, the interdependency of the individual S_n–P_n interactions has been investigated. It was found that the contributions from each subsite interaction to k_{cat}/K_M are not always additive. Such interdependency was also observed between subsites which are remote from each other. With a series of substrates covering S_6 to S'_4 of Savinase, it was observed that favorable amino acids in P_1 or, more significantly, P_4 of the substrate 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. Furthermore, with substrates in which all positions had been optimized, an upper limit of k_{cat}/K_M ($\sim 2 \times 10^9$ min^{-1} M^{-1}) was seen, both for a substrate with a high k_{cat} and for one with a low K_M. These results emphasize that the design of optimal substrates or substrate-derived inhibitors for endopeptidases preferably should be based on subsite mappings where interdependent substrate–subsite interactions have been eliminated.

Proteases participate in many processes of physiological importance, and to control their action it is of interest to synthesize selective and metabolically stable inhibitors [reviewed by Barrett and Salveson (1986) and Scharpe et al. (1991)]. One approach in the search for appropriate inhibitors, which exhibit low K_i values due to favorable interactions with the target enzyme, is to apply the transition-state analogue concept in which the region around the scissile bond of an optimal peptide substrate is replaced by a nonhydrolyzable analogue (Wolfenden, 1976; Dreyer et al., 1989). In order to obtain substrate-derived inhibitors for the HIV[1] protease, significant effort has been directed toward determining the importance of the binding subsites, using synthetic peptides and protein substrates (Poorman et al., 1991; Moore et al., 1989, Kay & Dunn, 1990). However, caution must be exerted when such data are interpreted for the purpose of inhibitor design. It is possible that enzyme–substrate interactions important for the creation of specific inhibitors are not revealed due to inappropriate substrate design. In addition, it is often assumed that the catalytic efficiency toward a substrate results from the cumulative effect of independent subsite–substrate interactions, an assumption that is not always valid.

A number of proteases have been investigated by the method of free energy perturbation scan in an attempt to predict how genetic modification of enzymes might affect the catalytic efficiency as evaluated with a fixed substrate (Wells, 1990; Warshel et al., 1988; Rao et al., 1987). In the majority of cases, the differences in free energy between substrate cleavage by wild-type enzyme and enzyme mutated at two or more amino acid residues approached the sum of the free energy changes derived from enzymes mutated at a single amino acid residue. However, deviation from additivity was observed, not always accountable on the basis of existing structural data.

With the subtilisin enzyme Savinase, the eight subsites S_5–S'_3 have been characterized on the basis of k_{cat}/K_M values obtained with series of peptide substrates in which each position was systematically varied (Grøn et al., 1992). On this basis it is possible to design a substrate with optimal interactions with Savinase by substituting the most favorable amino acid residues into each of the positions P_5 to P'_3, such that the highest possible k_{cat}/K_M value is achieved. However, the results presented here illustrate that subsite–substrate interactions do not affect the overall transition-state binding energy in an additive manner.

MATERIALS AND METHODS

Savinase and BL-GSE were isolated as previously described (Grøn et al., 1992; Svendsen & Breddam, 1992). The substrates were synthesized as previously described (Grøn et al., 1992; Meldal & Breddam, 1991). The enzymatic hydrolysis of the peptide substrates was followed on a Perkin-Elmer Luminescence Spectrometer LS 50 as previously described (Grøn et al., 1992). Assays were carried out in 50 mM Bicine, 2 mM $CaCl_2$, and 0.1 M KCl, pH 8.5. k_{cat}/K_M values were determined from at least three initial velocities using the relation $v = (k_{cat}/K_M)e_0s_0$, which is valid at low substrate concentrations ($s_0 \ll K_M$). With one substrate, Savinase exhibited a K_M value too low to allow the use of this relation, and k_{cat}/K_M was determined from 20 initial velocities measured at substrate concentrations covering the range 0.1–3 times K_M, using the program ENZFITTER (Leatherbarrow, 1987). The cleavage sige for the substrates was determined as previously described (Grøn et al., 1992).

To ensure that the kinetic efficiency observed with Savinase was not limited by diffusion control, k_{cat}/K_M was determined in media of different viscosity. Viscous buffers containing Ficoll [0%, 3%, 6%, and 8% (w/w)] were prepared by adding 50 mM Bicine, 2 mM $CaCl_2$, and 0.1 M KCl, pH 8.5, to Ficoll (0, 7.5, 15, and 20 g) to a final weight of 250 g. The relative viscosities of the buffers, η_{rel}, were taken from the literature (Brouwer & Kirsch, 1982) (η_{rel} = 1, 1.4, 1.8,

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[1] Abbreviations: HIV, human immunodeficiency virus; Savinase, subtilisin 309 from *B. lentus*; BL-GSE, glutamic acid specific endopeptidase from *B. licheniformis*, ABz, *o*-aminobenzoyl (anthraniloyl); Y', Tyr(NO_2), 3-nitrotyrosine. The binding site notation is that of Schechter and Berger (1967), i.e., P_n denotes a substrate position, and S_n denotes an enzyme binding subsite. A vertical arrow (↓) indicates the scissile bond in a peptide substrates.

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Table I: BL-GSE Catalyzed Hydrolysis of Substrates with Variations in P'_2: The Influence of Substrate Construction on the $\Delta\Delta G_T^{\ddagger}$(Xaa→Arg) Values

substrate series I	k_{cat}/K_M[a] (min^{-1} μM^{-1})	$\Delta\Delta G_T^{\ddagger}$ (kJ/mol)	substrate series II	k_{cat}/K_M (min^{-1} μM^{-1})	$\Delta\Delta G_T^{\ddagger}$ (kJ/mol)
ABzAAE↓AVY'D	12 ± 0.5	2.2	ABzAFAFE↓VVY'D	320 ± 10	1.7
ABzAAE↓AFY'D	380 ± 11	11	ABzAFAFE↓VFY'D	1200 ± 35	5.0
ABzAAE↓ASY'D	58 ± 2	6.1	ABzAFAFE↓VSY'D	650 ● 20	3.5
ABzAAE↓ADY'D	38 ± 1	5.1	ABzAFAFE↓VDY'D	510 ± 15	2.9
ABzAAE↓ARY'D	4.9 ± 0.3	0	ABzAFAFE↓VRY'D	160 ● 5	0

[a] Data taken from Breddam and Meldal (1992).

Table II: Savinase-Catalyzed Hydrolysis of Substrates with Variations in P'_1: The Influence of Substrate Construction on the $\Delta\Delta G_T^{\ddagger}$(Xaa→Gly) Values

substrate series III	k_{cat}/K_M (min^{-1} μM^{-1})	$\Delta\Delta G_T^{\ddagger}$ (kJ/mol)	substrate series IV	k_{cat}/K_M[a] (min^{-1} μM^{-1})	$\Delta\Delta G_T^{\ddagger}$ (kJ/mol)
ABzAAF↓GY'D	580 ± 70	0	ABzFAPF↓GGGY'D	89. ± 2	0
ABzAAF↓AY'D	630 ● 30	0.2	ABzFAPF↓AGGY'D	97. ± 4	0.2
ABzAAF↓VY'D	560 ± 40	0.09	ABzFAPF↓VGGY'D	25. ± 1	−3.1
ABzAAF↓LY'D	420 ± 20	−0.8	ABzFAPF↓LGGY'D	7.8 ± 0.4	−6.0
ABzAAF↓FY'D	680 ± 70	0.4	ABzFAPF↓FGGY'D	22. ± 1	−3.5
ABzAAF↓NY'D	590 ± 20	0.04	ABzFAPF↓NGGY'D	13.6 ± 0.3	−4.7
ABzAAF↓DY'D	490 ± 20	−0.4	ABzFAPF↓DGGY'D	6.8 ± 0.1	−6.4
ABzAAF↓RY'D	530 ± 30	−0.2	ABzFAPF↓RGGY'D	24. ± 2	−3.2

[a] Data taken from Grøn et al. (1992).

and 2.2, respectively). The ionic strength was adjusted to I = 14 mS by addition of KCl; pH was not affected by Ficoll.

RESULTS AND DISCUSSION

The binding site of proteases may be divided into a number of subsites each, by multiple interactions, securing the binding of a single amino acid residue from peptide substrates (Schechter & Berger, 1967). The properties of the amino acid residues which constitute a given binding subsite determine which amino acid residue(s) of a 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 (Bech & Breddam, 1988; Grøn et al., 1990), 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. To examine the interactions between an enzyme and a given substrate position, P_n, it is normal to compare the catalytic efficiency, k_{cat}/K_M, toward substrates which are only substituted in P_n. For two substrates, A and B, the difference in transition-state stabilization energy can be determined from the k_{cat}/K_M values (Fersht, 1985):

$$\Delta\Delta G_T^{\ddagger}(A\rightarrow B) = \Delta H_T^{\ddagger}(B) - \Delta H_T^{\ddagger}(A) - T[\Delta S_T^{\ddagger}(B) - \Delta S_T^{\ddagger}(A)] = -RT \ln [k_{cat}/K_M(B)/k_{cat}/K_M(A)]$$

$\Delta\Delta G_T^{\ddagger}$(A→B) is the difference in specificity energy observed with substrates of a given structure. The entropy term in the expression for $\Delta\Delta G_T^{\ddagger}$ accounts for the difference in loss of entropy upon formation of the transition-state intermediate (loss of translational and rotational motion). If A is a better substrate than B, $\Delta\Delta G_T^{\ddagger}$(A→B) will be positive.

Optimally, a substrate series used for a subsite mapping should be designed such that with two substrates, differing only at a single position, P_n, $\Delta\Delta G_T^{\ddagger}$(A→B) approaches $\Delta\Delta H_T^{\ddagger}$(A→B), thus reflecting the changes in S_n–P_n interactions. This is achieved when $\Delta S_T^{\ddagger}(B) \approx \Delta S_T^{\ddagger}(A)$. The loss of entropy upon binding of a substrate to an enzyme cannot exceed the amount of entropy available in the system, and, therefore, substrate series with maximal loss of entropy can be constructed such that additional favorable interactions have no further effects on $\Delta S_T^{\ddagger}$, i.e., the decrease in entropy upon binding is similar for all substrates (Jencks, 1975). If the substrates in a series are not sufficiently favorable, additional beneficial interactions would increase $\Delta S_T^{\ddagger}$, and for such series $\Delta\Delta G_T^{\ddagger}$ is only an upper limit for $\Delta\Delta H_T^{\ddagger}$. This has been observed with the protease papain (Berti et al., 1991). With pairs of substrates differing only at a single position, $\Delta\Delta G_T^{\ddagger}$ was larger with the substrates characterized by high k_{cat}/K_M values, i.e., more favorable enzyme–substrate interactions, versus those with lower k_{cat}/K_M values (Berti et al., 1991).

During a kinetic study of the glutamic acid specific endopeptidase isolated from *Bacillus licheniformis*, BL-GSE, the opposite of an entropic effect was observed (Table I). With a series of long substrates, i.e., ABz-Ala-Phe-Ala-Phe-Glu-↓-Val-Xaa-Tyr(NO_2)-Asp-OH, with favorable interactions introduced in five out of six positions the beneficial effects of introducing an additional favorable interaction at P'_2 were less pronounced than observed with a series of shorter, less optimal substrates, i.e., ABz-Ala-Ala-Glu-↓-Ala-Xaa-Tyr(NO_2)-Asp-OH. The difference in $\Delta G_T^{\ddagger}$ between the substrate with the least favorable (Xaa = Arg) and the most favorable (Xaa = Phe) P'_2 substituent was 5 kJ/mol for the substrate series exhibiting high k_{cat}/K_M values (160–1200 min^{-1} μM^{-1}) as compared with 11 kJ/mol for the substrate series exhibiting lower k_{cat}/K_M values (4.9–380 min^{-1} μM^{-1}). Thus, in this case $\Delta\Delta G_T^{\ddagger}$ was smaller with the better substrates than with the poorer substrates.

To investigate the generality of the different observations with papain and BL-GSE, a series of experiments was set up with the subtilisin enzyme Savinase from *Bacillus lentus*. This enzyme has been thoroughly investigated with respect to the substrate preferences of the eight subsites S_5–S'_3 (Grøn et al., 1992), and thus it provides a kinetically well-characterized system. A series of substrates, ABz-Ala-Ala-Phe-↓-Xaa-Tyr(NO_2)-Asp-OH, with multiple favorable interactions and variations in P'_1 was constructed. The cleavage point was directed by very favorable residues (ABz and Phe) in the two important substrate positions, P_4 and P_1, respectively (Table II, substrate series III) (Grøn et al., 1992). With Xaa = Phe, Leu, Val, Ala, Gly, Asn, Asp, or Arg, Savinase cleaved the

Table III: k_{cat}/K_M Values for the Savinase-Catalyzed Hydrolysis of Substrates of Varying Amino Acid Sequence

	substrate series V	k_{cat}/K_M (min^{-1} μM^{-1})
1	ABzDFRLF↓AFY'D	1700 ± 150
2	ABzDFGPF↓GGY'D	520 ± 15
3	ABzDFRPG↓AFY'D	17 ± 0.8
4	ABzDGGPF↓GGY'D	1.1 ± 0.06
5	ABzDGGPG↓GGY'D	0.0002 ± 0.00005
6	ABzFRAF↓AFFY'D	1290 ± 30

peptide bond between Phe and Xaa, as verified by amino acid analysis of the isolated cleavage products. The k_{cat}/K_M values varied from 420 to 680 min^{-1} μM^{-1}, which corresponds to a difference in transition-state stabilization energy, $\Delta\Delta G_T^{\ddagger}$, between the most and the least favorable substrate of only 1.2 kJ/mol. Thus, with this substrate series, characterized by high k_{cat}/K_M values, Savinase discriminated very little between different amino acid residues in P'_1. With an analogous series of substrates, ABz-Phe-Ala-Pro-Phe-↓-Xaa-Gly-Gly-Tyr(NO_2)-Asp-OH, Savinase exhibited a much larger variation in k_{cat}/K_M (Table II, substrate series IV). With Xaa corresponding to the same eight amino acid residues, Savinase cleaved at the Phe–Xaa peptide bond with k_{cat}/K_M values ranging from 6.8 (Xaa = Asp) to 97 min^{-1} μM^{-1} (Xaa = Ala), corresponding to a transition-state stabilization of 6.6 kJ/mol in response to the Asp→Ala substitution in P'_1. Thus, a selectivity with respect to P'_1 was observed with this substrate series which is hydrolyzed with k_{cat}/K_M values 10–100-fold lower than those observed with the previous series. The lower k_{cat}/K_M values may probably be accounted for by the disruption of the S_4–P_4 interactions and the S_1–P_1 interactions due to an ABz in P_5 and a Pro in P_2, respectively. The discrimination between different P'_1 amino acid residues observed with the poorer substrate series is in agreement with the pronounced preference of other subtilisins for certain P'_1 amino acid residues (Bratovanova & Petkov, 1987). Furthermore, the crystal structure of the complex between subtilisin BPN′ and the *Streptomyces* subtilisin inhibitor (SSI) indicates that the P'_1 side chain points toward the enzyme (Hirono et al., 1984), suggesting an influence of the P'_1 side chain of substrates as well.

Diffusion control occurs when an enzyme/substrate system is optimized such that substrate association and/or product diffusion processes are slower than the enzymatically catalyzed chemical steps involved in the conversion of substrate (Cleland, 1975). At $k_{cat}/K_M \approx 10^4$–10^5 min^{-1} μM^{-1}, enzymatic catalysis approaches diffusion control (Davis et al., 1991). To investigate whether the k_{cat}/K_M values obtained with the better substrate series ($k_{cat}/K_M \approx 500$ min^{-1} μM^{-1}) were limited by diffusion, two substrates, which, on the basis of a previously performed subsite mapping of Savinase (Grøn et al., 1992), were believed to exhibit even higher k_{cat}/K_M values were synthesized: ABz-Asp-Phe-Arg-Leu-Phe-↓-Ala-Phe-Tyr(NO_2)-Asp-OH and ABz-Phe-Arg-Ala-Phe-↓-Ala-Phe-Phe-Tyr(NO_2)-Asp-OH (Table III, 1 and 6). As expected, these substrates exhibited similar k_{cat}/K_M values, 1700 min^{-1} μM^{-1} (K_M = 0.2 μM, k_{cat} = 360 min^{-1}) and 1290 min^{-1} μM^{-1} (K_M = 2.5 μM, k_{cat} = 4 × 10^3 min^{-1}), respectively. Thus, with these substrates k_{cat}/K_M was significantly increased. To ensure that, even with the best substrates, hydrolysis was not diffusion limited, k_{cat}/K_M was determined in media of different viscosity, obtained by different concentrations of Ficoll, a nonionic polymer of sucrose. In cases of diffusion dependency, k_{cat}/K_M should be sensitive to viscosity. With ABz-Phe-Arg-Ala-Phe-↓-Ala-Phe-Phe-Tyr(NO_2)-Asp-OH, k_{cat}/K_M decreased from 1190 to 1040 min^{-1} μM^{-1} (13%) upon a change in η_{rel} from 1.0 to 2.2. With a poorer substrate, ABz-Phe-Ala-Pro-Phe-↓-Gly-Gly-Gly-Tyr(NO_2)-Asp-OH, the same increase in viscosity decreased k_{cat}/K_M similarly, from 21.6 to 20.3 min^{-1} μM^{-1} (9%). Thus, within experimental error the effects of variations in viscosity are very similar for the two substrates which exhibit a 10^2-fold difference in k_{cat}/K_M. It is therefore inconceivable that k_{cat}/K_M is limited by diffusion control, even with the best substrates employed in this investigation.

The results obtained with Savinase (and BL-GSE) suggest that some enzyme interactions are less important with a good substrate as compared with a poor substrate, and this is in contrast to the entropic effect observed with papain. Furthermore, even with the poorer substrate series, ABz-Phe-Ala-Pro-Phe-↓-Gly-Gly-Gly-Tyr(NO_2)-Asp-OH, the loss of entropy apparently is maximized since substrates of this structure with variations in either P_4, P_3, or P_1 exhibit $\Delta\Delta G_T^{\ddagger}$ (Gly→Xaa) values which are identical to those observed with similar series of shorter and slower hydrolyzed substrates (Grøn et al., 1992). The effects observed with Savinase and BL-GSE could be due to some degree of interdependency of subsite–substrate interactions as opposed to previous observations with chymotrypsin where it was shown that the k_{cat}/K_M values for the hydrolysis of peptide substrates could be rationalized on the basis of simple additive contributions from each subsite–substrate interaction (Schellenberger et al., 1991). To investigate the validity of this in the case of Savinase, the k_{cat}/K_M values were determined for the hydrolysis of a series of substrates with substitutions in multiple positions (Table III). The difference in $\Delta G_T^{\ddagger}$ [$\Delta\Delta G_T^{\ddagger}$(observed)] was calculated for pairs in which 1, 2, 4, 5, or 6 positions were substituted (Table IV). Provided the subsites were independent of each other, $\Delta\Delta G_T^{\ddagger}$(observed) should equal $\sum_{P_n}\Delta\Delta G_T^{\ddagger}(P_n)$, where $\Delta\Delta G_T^{\ddagger}(P_n)$ is the value obtained with substrates in which a single position is systematically varied (Table IV). As expected (Grøn et al., 1992), this relation was valid for pairs of substrates where only a single position was substituted (Table IV, 2→4 and 4→5). However, with two or more replacements $\Delta\Delta G_T^{\ddagger}$(observed) deviated significantly from $\sum_{P_n}\Delta\Delta G_T^{\ddagger}(P_n)$. The most pronounced case of breakdown of additivity was observed when both the S_4–P_4 and the S_1–P_1 interactions were optimized, i.e., with a Phe in P_4 and P_1. With the substrate pair 1→2 (Table IV), $\sum_{P_n}\Delta\Delta G_T^{\ddagger}(P_n)$ = 13.3 kJ/mol as compared with $\Delta\Delta G_T^{\ddagger}$(observed) = 2.9 kJ/mol. Thus, it appears that for substrates with Phe in P_4 and P_1 the nature of the amino acid residues in P_3, P_2, P'_1, and P'_2 is of only minor importance. With pairs of substrates in which either the S_4–P_4 or the S_1–P_1 interactions were optimized, i.e., with Phe in either P_4 or P_1 (Table IV, 1→3, 2→3, and 1→4), a similar observation was made: $\sum_{P_n}\Delta\Delta G_T^{\ddagger}(P_n)$ = 28.8, 15.5, and 27.2 kJ/mol, respectively, as compared with $\Delta\Delta G_T^{\ddagger}$(observed) = 11.4, 8.5, and 18.2 kJ/mol. The difference was, however, not as pronounced as with both positions optimized. Only with one pair of substrates did $\Delta\Delta G_T^{\ddagger}$(observed) exceed $\sum_{P_n}\Delta\Delta G_T^{\ddagger}(P_n)$ (Table IV, 3→4). This pair involved a Phe→Gly substitution at P_4 and a Gly→Phe substitution P_1. Apparently the favorable interactions introduced at P_1 could not fully compensate for the transition-state stabilization lost upon the unfavorable substitution Phe→Gly in P_4 such that $\sum_{P_n}\Delta\Delta G_T^{\ddagger}(P_n)$ was 8.4 kJ/mol lower than $\Delta\Delta G_T^{\ddagger}$(observed). In pairs of substrates where P_4 was substituted Phe→Gly (Table IV, 2→5, 3→5, and 1→5), the consequent transition-state destabilizations, $\Delta\Delta G_T^{\ddagger}$(observed) = 26.6, 11.0, and 39.6 kJ/mol, respectively, were not as large as expected from $\sum_{P_n}\Delta\Delta G_T^{\ddagger}(P_n)$ = 38.5, 23.0, and 51.8 kJ/mol, respectively.

Table IV: Contributions from Each Substrate–Subsite Interaction to the Catalytic Efficiency, Using the Substrates Listed in Table III

	substrate pair	P_4 $\Delta\Delta G_T^*$ (kJ/mol)	P_3 $\Delta\Delta G_T^*$ (kJ/mol)	P_2 $\Delta\Delta G_T^*$ (kJ/mol)	P_1 $\Delta\Delta G_T^*$ (kJ/mol)	P'_1 $\Delta\Delta G_T^*$ (kJ/mol)	P'_2 $\Delta\Delta G_T^*$ (kJ/mol)	$\sum_{P_n}\Delta\Delta G_T^*(P_n)$[a] (kJ/mol)		$\Delta\Delta G_T^*$(observed)[b] (kJ/mol)
2→4	ABzDFGPFGGY'D ABzDGGPFGGY'D	13.9	0	0	0	0	0	13.9	=	15.3
4→5	ABzDGGPFGGY'D ABzDGGPGGGY'D	0	0	0	24.6	0	0	24.6	=	21.3
2→5	ABzDFGPFGGY'D ABzDGGPGGGY'D	13.9	0	0	24.6	0	0	38.5	>	26.6
1→3	ABzDFRLFAFY'D ABzDFRPGAFY'D	0	0	4.2	24.6	0	0	28.8	>	11.4
1→2	ABzDFRLFAFY'D ABzDFGPFGGY'D	0	5.4	4.2	0	0.2	3.5	13.3	>	2.9
2→3	ABzDFGPFGGY'D ABzDFRPGAFY'D	0	−5.4	0	24.6	−0.2	−3.5	15.5	>	8.5
3→5	ABzDFRPGAFY'D ABzDGGPGGGY'D	13.9	5.4	0	0	0.2	3.5	23.0	>	11.0
1→4	ABzDFRLFAFY'D ABzDGGPFGGY'D	13.9	5.4	4.2	0	0.2	3.5	27.2	>	18.2
3→4	ABzDFRPGAFY'D ABzDGGPFGGY'D	13.9	5.4	0	−24.6	0.2	3.5	−1.6	<	6.8
1→5	ABzDFRLFAFY'D ABzDGGPGGGY'D	13.9	5.4	4.2	24.6	0.2	3.5	51.8	>	39.6

[a] $\sum_{P_n}\Delta\Delta G_T^*(P_n)$ is the sum of the $\Delta\Delta G_T^*$(Xaa→Yaa) values for the substituted amino acids in the substrate pairs. The $\Delta\Delta G_T^*$(Xaa→Yaa) values are obtained from k_{cat}/K_M values for substrate pairs in which only one position is substituted, taken from Grøn et al. (1992). [b] $\Delta\Delta G_T^*$(observed A→B) $= -RT \ln [k_{cat}/K_M(B)/k_{cat}/K_M(A)]$, i.e., the actual difference in transition state stabilization energy of the two substrates A and B. The k_{cat}/K_M values are from Table III.

These data emphasize that the S_n–P_n interactions are strongly interdependent. Apparently Savinase binds substrates productively and favorably when either the P_1 or, more significantly, the P_4 side chain is optimized, and it seems that these favorable interactions can eliminate or reduce effects of less favorable interactions introduced at other positions. It has previously been observed that a Pro in P_3 reduces k_{cat}/K_M drastically (the Arg→Pro substitution in P_3 destabilizes the transition-state intermediate with 24.4 kJ/mol) (Grøn et al., 1992). Since S_3 otherwise tolerates numerous amino acid residues, this effect could be due to an adverse influence of a neighboring Pro on the S_4–P_4 interactions. The significance of the S_4–P_4 interactions for optimal kinetic efficiency has previously been indicated for subtilisin BPN′ (Svendsen, 1976; Morihara, 1974). However, the molecular basis for these interactions which are distant from the scissile bond and still affect catalysis is not clear.

With elastase, interdependent subsite–substrate interactions have been reported (Thompson & Blout, 1973a,b; Thompson, 1974). With two series of oligopeptide inhibitors, it was observed that the influence of the S_1–P_1 interactions on the inhibitory efficiency was lost in inhibitors with favorable S_4–P_4 interactions. It was concluded that favorable interactions between enzyme and P_4 induce a destabilization of the scissile bond large enough to eliminate contributions from S_1–P_1 interactions (Thompson, 1974; Jencks, 1975). On the basis of results with amide substrates of varying length, it was suggested that favorable S_4–P_4 interactions caused a rearrangement of the S_1–S'_1 subsites to allow optimal alignment of the scissile bond (Thompson, 1974). With subtilisin BPN′, X-ray crystallographic studies show that the binding of inhibitors induces a movement in the S_4 area of the enzyme (McPhalen & James, 1988); but, studies with SSI derivatives with different P_4 and P_1 substituents show that these movements are not accompanied by a change in the catalytic geometry around the P_1–P'_1 peptide bond (Takeuchi et al., 1991). Mutants of SSI with different P_4 substituents showed identical K_i values (Kojima et al., 1990) as opposed to the widely different k_{cat}/K_M values obtained in response to replacements at the P_4 position of small substrates [reviewed by Svendsen (1976)]. Takeuchi et al. (1991) have argued that the observations with small substrates are not relevant for "proteinaceous" substrates. However, the data for Savinase obtained with peptide substrates, covering the S_6 to S'_4 subsites, emphasize the importance of the S_4–P_4 interactions even with long peptide substrates. It is thus conceivable that favorable S_4–P_4 interactions can accelerate the catalytic efficiency with protein substrates as well. The results suggest that it is more likely that the inhibitors represent the "unnatural" situation; the rigidity of SSI and other efficient subtilisin inhibitors possibly prevents optimal interactions with the enzyme and consequently conformational changes at S_1–S'_1, thereby impeding hydrolysis.

CONCLUDING REMARKS

With the subtilisin enzyme Savinase, the interactions between substrate and the active site exhibit an interdependency of the individual P_n–S_n interactions. Favorable P_4–S_4 interactions eliminate effects of interactions introduced at other subsites. These data suggest that with this enzyme an upper limit exists to the amount of substrate interaction energy which can be converted into transition-state intermediate stabilization. Thus, the design of substrate-derived inhibitors, e.g., for the HIV protease, should be based on subsite mappings where such "saturation" phenomena are absent.

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