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Hydrolysis of small peptide substrates parallels binding of chymotrypsin inhibitor 2 for mutants of subtilisin BPN′

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Variants of subtilisin BPN′ that possess improved specificity towards isoleucine compared with alanine at the P_4 position of small peptide substrates, were analysed for their ability to bind chymotrypsin inhibitor 2. The binding of the inhibitor with isoleucine (wild-type) and with alanine as the P_4 residue parallels the hydrolysis of tetrapeptide substrates. There is a linear relationship between the free energy of binding of the transition state of the substrate and the free energy of binding of the inhibitor with a slope of 2.0. The data suggest that the inhibitor uses predominantly ground state rather than transition state binding energy.

Subtilisin; Chymotrypsin inhibitor 2; S_4 pocket; P_4 substrate; Linear free-energy relationship

1. INTRODUCTION

Subtilisin BPN′ is a serine-class endoprotease from *Bacillus amyloliquefaciens* [1], which binds substrates with a rather broad specificity. The enzyme has two binding pockets, S_1 and S_4, and its substrate specificity is determined mainly by the interaction of the P_1 and P_4 amino acid side chains of the substrate with these pockets (nomenclature of the binding sites as defined by Schechter and Berger+ [2]). The specificity towards both the P_1 and P_4 residues of small oligopeptide substrates can be substantially increased by altering amino acid side chains in the S_1 and S_4 pocket, respectively [3–6], e.g. increasing the size of the S_4 pocket by shortening amino acid residues can improve the specificity of subtilisin towards tetrapeptides with large hydrophobic P_4 residues by up to 200-fold. However, subtilisin has a total of at least eight binding subsites [7,8] and it is still speculation as to whether the binding of longer peptides or whole proteins is similarly affected by these mutations. Inspection of the high-resolution crystal structure of the complex between subtilisin BPN′ and chymotrypsin inhibitor 2, a potent polypeptide inhibitor of chymotrypsin and subtilisin, reveals that the P_1 and P_4 residues together account for only 48%, and the P_1,P_2,P_3 and P_4 residues for 65%, of all < 4 Å contacts to subtilisin [7]. In fact, it has been reported that the catalytic efficiency of subtilisin BPN′ for the hydrolysis of small peptide substrates is sensitive to the bulkiness of the P_4 side chain, while binding of *Streptomyces* subtilisin inhibitor is not [9]. In this work, we analyse wild-type and four variants of subtilisin BPN′ that carry different amino acid replacements in the S_4 pocket, for their capacity to bind wild-type CI2 (isoleucine at the P_4 position) and a mutant CI2 in which I56 has been replaced by alanine (I56A CI2; alanine at the P_4 position). The corresponding binding constants are compared with the catalytic constants for the hydrolysis of tetrapeptide substrates.

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Abbreviations: CI2, chymotrypsin inhibitor 2; SSI; *Streptomyces* subtilisin inhibitor. Mutants are designated by the single letter code of the wild type amino acid followed by the residue number and the amino acid replacement.

+Enzyme binding sites S_1,S_2,... and S_1',S_2',... are away from the scissile bond to the N- and C-terminus, respectively; amino acid residues in the substrate are P_1,P_2,... and P_1',P_2',... in correspondence with their binding sites.

2. MATERIALS AND METHODS

Restriction enzymes and other DNA-modifying enzymes were purchased from Boehringer Mannheim Corporation, Pharmacia or New England Biolabs. Oligonucleotides were synthesised on an Applied Biosystems ABI 380B oligonucleotide synthesiser. All other reagents were of analytical grade.

The vector pCI2, used for the mutagenesis and expression of the CI2 gene, was constructed by subcloning the 328 bp *BglII*/*Hin*dIII fragment from the plasmid pPO1 (F. Poulsen, pers. comm.), carrying a T7 RNA polymerase ϕ10 promoter, a ribosome binding site and the CI2 gene, into the multiple cloning site of pTZ18U [10]. In the resulting pCI2 plasmid, the codon for isoleucine 56 was replaced by that of alanine in an inverse polymerase chain reaction [11] using the primers 5′-CATGGTACAGCTGTCCCCAC-3′ and 5′-GAATATCGGATCG-

ACCGCGTCCGCCTCTTT-3'. The constructs were verified by sequencing [12] of the entire coding sequence.

Wild-type and the I56A mutant CI2 were expressed in, and purified from *Escherichia coli* strain TG2 harbouring the appropriate pCI2 plasmid. Cells were ruptured by sonication and the nucleic acids were precipitated from the resulting homogenate by addition of 1% (w/v) polyethylenimine. Following centrifugation at 18,000 × *g* for 10 min, the CI2 protein was precipitated from the supernatant by addition of 65% ammonium sulphate and collected by centrifugation at 18,000 × *g* for 30 min. The pellet was dissolved in 50 mM Tris-HCl at pH 8.6 and dialysed against the same buffer. Since CI2 does not bind to anion-exchange resin under these conditions, a partial purification was effected by binding contaminating proteins to DEAE-Trisacryl (1 ml resin/20 ml solution). The solution was centrifuged at 4,000 × *g* for 10 min and the supernatant applied to a HiLoad 26/60 Superdex G75 column (Pharmacia) equilibrated with a buffer of 50 mM Tris-HCl at pH 8.6. CI2 eluted after 200 ml at a flow rate of 2.6 ml · min^{-1}. Fractions containing pure protein were pooled, dialysed against water, lyophilised and stored at −70°C. A molar extinction coefficient of 6965 $M^{-1} \cdot cm^{-1}$ (S. Jackson, personal communication) was used for the determination of wild-type and I56A CI2 concentrations. The construction, expression, and purification of the different subtilisin variants have been reported earlier [5,6].

Slow binding kinetics were measured as described previously [13] using a range of inhibitor concentrations (1–25 nM) and an enzyme concentration of approximately 0.25 nM. Data from each curve at different inhibitor concentrations were fitted to Equation 1

$$A = v_s t + (v_o - v_s)(1 - \exp(-k_{obs} t))/k_{obs} + A_o \quad (1)$$

where A is the absorbance, A_o is the initital absorbance, v_o is the initial rate, v_s is the final steady-state rate, and k_{obs} is the apparent first-order rate constant for the transition to the steady-state rate. The association rate constant, k_{on}, was determined from a plot of k_{obs} versus the inhibitor concentration. The inhibition constant, K_i, was determined from a plot of $(v_o - v_s)/v_s$ versus the inhibitor concentration, and k_{off}, the dissociation rate constant, was calculated according to $k_{off} = k_{on} \times K_i$ [13].

3. RESULTS AND DISCUSSION

In the crystal structure of the subtilisin-CI2 complex [7,14], close-range intermolecular contacts to the P_4 residue of CI2 are made by residues Y104, I107, and L126 in the S_4 pocket of subtilisin (Fig. 1). Replacement of Y104 by alanine, I107 by glycine, L126 by alanine, and the construction of the double mutant I107G/L126V each increase the size of the S4 pocket, thereby improving the specificity of the protease towards large hydrophobic P_4 residues. The kinetic parameters of wild-type and the mutant subtilisins for the hydrolysis of small peptides have been determined previously [5,6] and are included in Table I to allow comparison with the results found in this work. All four mutant subtilisins possess, to various degrees, increased specificity towards isoleucine compared with alanine at the P_4 position of the tetrapeptides substrates.

Wild-type and I56A CI2 were treated as slow-binding inhibitors, since the attainment of steady-state enzyme activity was observed over a time period of several minutes, and the data fitted to Equation 1, the integrated

Table I

Binding constants of wild-type and I56A CI2 to different subtilisin variants and kinetic constants for the hydrolysis of small peptide substrates[a]

Subtilisin variant	K_i (× 10^{-12} M)	k_{on} (× 10^6 $M^{-1} \cdot s^{-1}$)	k_{off} (× 10^{-6} s^{-1})	K_m[b] (mM)	k_{cat}/K_m[b] (× 10^5 $M^{-1} \cdot s^{-1}$)
		Wild-type CI2/Ile at P_4 site			
Wild-type	1.2	3.5	4.4	0.08	3.75
Y104A	2.2	3.4	7.7	0.07	1.01
I107G	1.1	9.1	9.7	0.04	2.2
L126A	3.4	10.3	35	0.04	0.36
I107G/L126V	6.4	1.4	8.9	0.05	0.07
		I56ACI2/Ala at P_4 site			
Wild-type	1.2	3.1	3.8	0.15	2.33
Y104A	20.6	3.3	67.2	1.62	0.014
I107G	16.2	8.8	140	0.23	0.014
L126A	15.7	19.1	300	0.84	0.013
I107/L126V	49	nd[c]	nd[c]	0.62	0.0013

[a]Slow binding kinetics were measured as described in section 2.
[b]Steady-state kinetic constants for the hydrolysis of the substrate succinyl-X-Ala-Pro-Phe-pNA, where X is the P_4 residue. Data taken from [5,6].
[c]Not determined.

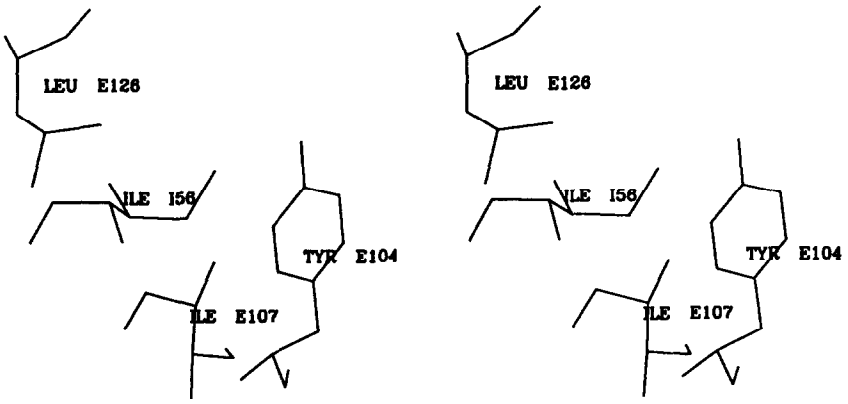

Fig. 1. Stereographic representation of the S_4 binding pocket of subtilisin BPN'. The view is into the pocket. The P_4 residue of CI2 (I56) and the residues Y104, I107, and L126 are shown.

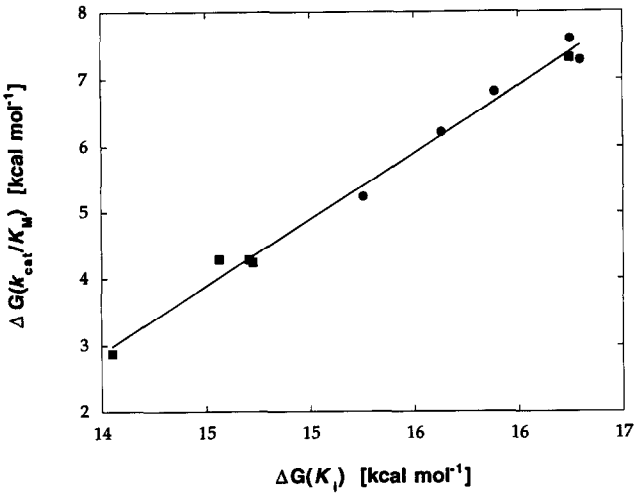

Fig. 2. Comparison of the free energy of binding of the transition state of substrates to subtilisin variants with the binding energy of mutants of CI2 with the same amino acid in the P_4 subsite. The slope of the straight line is 2.0. Squares, ΔG values for alanine at the P_4 position of the substrate or inhibitor; circles, ΔG values for isoleucine at the P_4 position of the substrate or inhibitor.

rate equation describing substrate hydrolysis in the presence of a slow-binding inhibitor [13]. The binding constants for wild-type and I56A CI2 to the various subtilisin variants are summarized in Table I. Wild-type CI2 (having an isoleucine at the P_4 position) binds equally well to wild-type and I107G subtilisin, but possesses somewhat higher K_i values for binding to Y104A, L126A, and the I107G/L126V double-mutant subtilisin. Further, there is an apparent correlation between the K_i values of wild-type CI2 and the k_{cat}/K_m values for the hydrolysis of tetrapeptide substrates: the K_i values increase with decreasing catalytic efficiency of the subtilisin variants. However, no apparent correlation was found between the K_i and K_m or between the K_i and k_{cat} values alone. Introducing an alanine residue at the P_4 position of CI2 has no effect on the binding of the inhibitor to wild-type subtilisin, a similar result has also been reported recently for the binding of *Streptomyces* subtilisin inhibitor [15], but results in substantially increased K_i values for all four mutants of subtilisin. Changes in K_i values are mainly due to increased dissociation rate constants, while k_{on} values remain virtually unaffected. Again, there is an apparent correlation between K_i and k_{cat}/K_m values, but not between K_i and K_m or K_i and k_{cat} values.

The tight binding of CI2 to subtilisin and the apparent correlation between K_i and k_{cat}/K_m values suggest that the inhibitor uses both ground state and transition state binding energy. Free energy values were calculated for the kinetic parameters k_{cat}/K_m and K_i [16] for isoleucine as well as alanine at the P_4 position using the expressions

$$\Delta G(k_{cat}/K_m) = RT \ln(k_{cat}/K_m) \quad (2)$$

and

$$\Delta G(K_i) = -RT \ln(K_i) \quad (3)$$

Changes in energy measured by Equation 2 should be the same as changes in the binding energy of the transition state of the substrate to the enzyme [16] whereas Equation 3 gives the binding energy of the inhibitor. As shown in Fig. 2, there is a linear relationship between $\Delta G(k_{cat}/K_m)$ and $\Delta G(K_i)$ for wild-type and all subtilisin variants with isoleucine and alanine as P_4 residue of the substrate/inhibitor. The slope of the line is 2.0, suggesting that, as far as substrate occupancy is concerned, CI2 uses only part of the available transition state binding energy. Inhibitor binding is, therefore, less sensitive to mutation than is the catalytic efficiency (if the K_i value would be similarly affected as the k_{cat}/K_m value, then the slope of the line would be 1). This result supports earlier findings from crystal structures of protease-inhibitor complexes, which indicate that the inhibited complex is close to a Michaelis complex [9,16,17].

For some mutants of subtilisin, changes in the catalytic efficiency result, to a certain degree, from structural changes on mutation that are transmitted from the S_4 pocket to the active site [5,6]. However, this is not the case for the Y104A mutant subtilisin, and the influence of amino acid replacements in the S_4 pocket on the structure of the S_1 pocket or active site varies for the other subtilisin variants [5,6]. Thus, the binding of inhibitors, such as CI2, and probably also the hydrolysis of long peptide substrates, seem to be affected by changes in the S_4 pocket of subtilisin in a similar manner to the hydrolysis of small tetrapeptide substrates. On the basis of the above data, we conclude that the binding of polypeptide inhibitors to subtilisin BPN′ and also the activity of the enzyme towards long peptide or protein substrates can be remodelled by altering the P_4 residue and/or the structure of the S_4 pocket.

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