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Functional Requirement for Threonine in the P_2 Position of a Cyclic Peptide Mimetic of a Bowman-Birk Proteinase Inhibitor

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We have used the anti-tryptic loop region of the Bowman-Birk proteinase inhibitor; MAI/D4 to create a template assisted combinatorial peptide library to screen for peptides displaying re-directed activity towards chymotrypsin [1]. The inhibitory sequences discovered were found to have both high activity and display a consensus binding motif. One surprising feature was that all active sequences were found to have exclusively threonine in the P_2 position. The reason for this specificity has been probed by maintaining the binding motif but systematically varying the P_2 locus to create a further 24 variants. It is found that the requirements for inhibitory activity at this locus are finely tuned. The results are consistent with a dual requirement for hydrophobic recognition with the enzyme pocket and maintenance of an inhibitory conformation, presumably formed by a hydrogen bond within the peptide loop.

Introduction

Inhibition by serine proteinase inhibitor proteins is often mediated by an exposed loop that is fixed in a characteristic "canonical" conformation, thought to be similar to that of a productively bound substrate [2,3]. The sequence of the loop determines the specificity of the inhibition, which mirrors the specificity of proteinases for their substrates.

The Bowman-Birk family of serine proteinase inhibitors (BBI's) are small proteins (6-9 kD) with a symmetrical structure of two tricyclic domains each containing an independent binding loop [4]. The proteinase binding loop is typically contained within a nonapeptide region joined *via* a disulfide between flanking cysteines. We have used an eleven amino acid monocyclic peptide [5] derived from the anti-tryptic loop domain of BBI as a template for construction of a "one bead, one peptide" library [6] in which three locations considered important for proteinase recognition were varied (P_1, P_2 , and P_2') [1]. When this library was screened for re-directed activity against chymotrypsin, active sequences were found to contain exclusively threonine at the P_2 site; the P_1 site had phenylalanine and tyrosine; and leucine, isoleucine and norleucine

were found at P_2' [1]. Although the preference for aromatic residues at P_1 is consistent with the substrate preference of chymotrypsin, the sequences obtained at the other locations were less predictable, and it was particularly striking that exclusively threonine was found at the P_2 location.

In order to evaluate further what features of the P_2 locus are necessary to the properties of the peptide mimetic, we have undertaken a systematic kinetic analysis of 25 variations of the consensus chymotrypsin binding motif: SCXFSIPPQCY (P_2 position shown in bold).

Materials and Methods

Peptide synthesis and purification.
Peptides for kinetic analysis were synthesized by standard solid phase techniques as described previously [1]. All peptides were characterized by FAB-MS and were >85% by analytical reverse phase HPLC.

Inhibition kinetics.
The inhibition kinetics were determined by competitive binding studies using the chromogenic substrate succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as previously [1].

RESULTS

Effect of P_2 variation on K_i
Using the consensus sequence (SCTFSIPPQCY; P_2 residue in bold) found for re-directed activity of the anti-tryptic loop towards chymotrypsin, a range of P_2 variants was synthesised. The individual K_i values are given in Table 1.

Table 1. *Equilibrium dissociation constants for P_2 variants of the chymotrypsin re-directed anti-tryptic loop of MAI-D4. N.I., no detectable inhibition.*

P_2 residue	K_i (μM)	P_2 residue	K_i (μM)
Thr	0.019	Pro	5.2
Abu	0.13	Gln	7.1
Nva	0.28	Phe	8.7
Ser	0.4	Trp	9
Ala	0.57	Ile	13.4
Val	1.3	Asn	17
His	1.4	Tyr	17.1
Arg	1.8	Glu	84
Met	2.2	Asp	98
Leu	2.4	*allo*-Thr	>100
Hse	2.6	*t*-butylglycine	>100
Lys	2.8	DThr	N.I.
Gly	4.4		

Discussion

P_2 Specificity found in this study

The results confirm a significant influence of the P_2 locus of the peptide. In addition to those residues present in our earlier library, the following amino acids were also examined: Abu, Nva, Hse, *allo*-Thr, D-Thr and *t*-butylglycine. The results confirm that of those variants tested, Thr is optimal. This explains why our earlier library studies [1] returned exclusively Thr at this location.

The requirements for inhibitory activity at this locus are found to be finely tuned. Inversion of asymmetric β-carbon from R to S (*allo*-Thr) results in a very poor inhibitor, as does the replacement of L-Thr with D-Thr. Loss of either the β-hydroxyl or methyl functions of the threonine results in a significant loss of activity. Whilst the loss of the hydroxyl gives a 6.8 fold reduction in activity (Abu), the loss of the methyl (Ser) gives a 21 fold reduction in activity. In the absence of the hydroxyl function, the homologous series Ala, Abu and Nva further demonstrate the requirement for methyl group attached to the β-carbon. When the aliphatic sidechain is reduced to Ala there is an approximate 4 fold loss in activity compared to Abu and increase of the alkyl length to Nva results in a more modest 2 fold loss in activity. Branching of the aliphatic sidechain results in further loss of activity. Hence, valine has a 10 fold higher K_i than Abu. Similarly, in contrast to Ala, *t*-butylglycine is of extremely low activity and branching at the γ-carbon gives an 8.5 fold higher K_i when comparing Leu and Nva. In a similar fashion, the requirement for a β-hydroxyl is demonstrated by comparing Ser and Hse with a γ-hydroxyl resulting in a K_i 6.5 fold higher than the β equivalent. The crystal structure of a BBI-trypsin complex shows a hydrogen bond between P_2 Thr and P_1' Ser, and a hydrophobic contact between the γ-methyl and His-57 [7]. These interactions would explain why both hydroxyl and methyl groups of the threonine residue are important.

Large aliphatic or aromatic side chains result in relatively poor inhibitors, suggesting that they are not well accommodated in the S_2 pocket. Particularly noteworthy is the extremely poor inhibition when a negatively-charged residue is present at P_2 (Asp, Glu). It is possible that these side chains result in an unfavourable electrostatic interactions with Asp-102 of the catalytic triad.

Implications for Library Screening

Our original screening procedure produced exclusively Thr at P_2 [1]. The present results show that the next best sequence that was present at this locus, Ser, had a K_i value that was higher by a factor of 20. This indicates that the selection procedure depends of the K_i value, and that a twenty-fold difference is sufficient to give complete selectivity.

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