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KINETIC CHARACTERIZATION OF A PEPTIDE INHIBITOR OF TRYPSIN ISOLATED FROM A SYNTHETIC PEPTIDE COMBINATORIAL LIBRARY

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Abstract

Recently Eichler and Houghten[1] have described the use of a combinatorial peptide library to optimize small trypsin inhibitors. Our kinetic analysis of the most inhibitory peptide, YYGAKIYRPDKM, reveals that it is an inefficiently cleaved substrate for trypsin due to a low k_{cat}, possesses a relatively low K_M, and may exhibit nonproductive binding.

Introduction

The ability to rapidly synthesize and screen vast numbers of peptides represents a powerful tool for analysis of interactions between macromolecules and their ligands or substrates. Such studies may be of value in development of lead compounds of therapeutic interest. Recently Houghten and Eichler carried out a screen for short peptides capable of inhibiting bovine trypsin, and observed that the sequence YYGAKIYRPDKM (I) inhibited trypsin with an IC_{50} of 10 μM.[1] Another group has shown that their technique can be extended to screen compounds other than linear peptides,[2] thus greatly broadening its number of useful applications and emphasizing a need for a quantitative evaluation of the technique's effectiveness. Knowledge of structural and kinetic origins of inhibition by YYGAKIYRPDKM will help to provide this quantitative assessment of the power and sensitivity of Houghten and Eichler's method, facilitating greater understanding of factors underlying development of successful peptide screens for protease inhibitors. This knowledge would allow a rational approach to manipulation of screening parameters to achieve a desired effect. Insights into the mechanism of inhibition by seemingly labile peptides may also contribute to resolution of the paradox of how seemingly protease-sensitive sequences can be integral components of the reactive centers of proteinaceous protease inhibitors. Our interest in effects of subsite interactions on serine protease activity led us to quantify the interaction of (I) with trypsin as both an inhibitor and a substrate. We find that, as with proteinaceous protease inhibitors, the observed inhibition is due to a combination of relatively high affinity binding and relatively low turnover. We also show that the key residues for efficient inhibition are the P1 lysine and the P1' isoleucine. This information may aid in the structural interpretation of the inhibitory properties of (I) and should facilitate rational development of peptide-based serine protease inhibitors.

Results and Discussion

We examined the inhibition by (I) of trypsin-mediated Tyr-Gly-Arg-p-nitroaniline hydrolysis by plotting 1/v versus 1/s in the presence of three different concentrations of (I), and observed a K_I of 9.4 ± 2.0 μM. This value is in close agreement with the reported IC_{50} of 10 μM. The inhibition seen is almost entirely an effect on K_M, indicating that inhibition is competitive. Trypsin is selective for cleavage at

lysine and arginine, each of which is present in (I). The presence of proline after arginine in (I) would prevent hydrolysis after that residue, but it was unclear to what extent cleavage could occur after

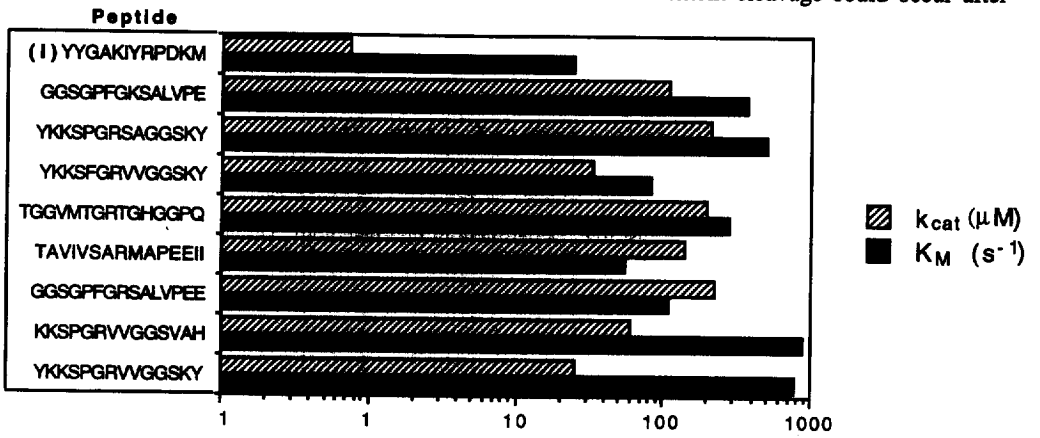

Figure 1. Kinetics of peptide (I) in comparison to previously analyzed peptides. Errors range from 5 to 20%. (G. S. Coombs, E. L. Madison, unpublished results)

lysine. To determine this, we examined (I) as a substrate for trypsin. Partial digests of the peptide were analyzed by reverse phase HPLC for product formation,[3] and the cleavage site was confirmed to be between lysine and isoleucine by mass spectral analysis. We observed k_{cat} and K_M values of 0.73 s^{-1} and 24.5 μM respectively. To put these numbers in perspective, we have previously obtained kinetic constants for 31 different peptide substrates for trypsin of 12 to 15 amino acids and have observed values for k_{cat} ranging from 25 to 370 s^{-1} and values for K_M ranging from 54 to 1500 μM (see figure 1). High k_{cat} values correlated with low K_M values for these peptides. Apparently, the screen carried out by Houghten and Eichler was able to select for both reduced k_{cat} and reduced K_M. However, the reduction in k_{cat} is much larger than that of K_M. It is interesting to note that the value of K_M is 2.6 times higher than the K_I or the reported IC_{50}. This discrepancy suggests that a substantial degree of nonproductive binding is occuring.

Most protein protease inhibitors are cleaved at their reactive site peptide bond by their cognate proteases with a k_{cat}/K_M which is two to three orders of magnitude higher than that of the same proteases' physiological substrate(s).[4] This value is partitioned into very low k_{cat} and K_M values, i. e. soybean trypsin inhibitor (STI) is processed by bovine trypsin with a k_{cat} of ~$3x(10)^{-6}$ s^{-1}, a K_M of ~$1x(10)^{-12}$ M and a k_{cat}/K_M of ~$3x(10)^6$.[5] By contrast, (I) possesses only moderately reduced k_{cat} and K_M, and k_{cat}/K_M is approximately two orders of magnitude smaller than those of many substrate peptides.

At least three mechanisms can be envisioned for inhibition by proteinaceous protease inhibitors which contain a susceptible P1 arginine or lysine: 1) exclusion of water, thus preventing hydrolysis of the acyl enzyme intermediate, 2) binding of the peptide with a scissile bond orientation or conformation that is incompatible with efficient cleavage, and 3) distortion or shifting of catalytically important trypsin residues by complex formation. Mechanism one requires a rigid 3-dimensional structure and is probably inconsistent with inhibition by a short flexible peptide. Mechanisms two and three, however, might apply

to peptides; the character of subsite occupancy might either lead to suboptimal orientation of the scissile bond or interference with the catalytic machinery of the enzyme. Our initial modeling of the inhibitory peptide with trypsin based on the bovine trypsin-BPTI complex suggested that the P2' tyrosine and/or the P3' arginine might produce a conformational shift of either histidine 57, a member of the catalytic triad, or glycine 193, which helps form the oxyanion hole. Peptides containing alanine substitutions at each of these positions were synthesized and kinetically analyzed to test this hypothesis. In each case, k_{cat} was virtually unchanged while K_M increased 3.5 (P2'-ala) or 4.7 (P3'-ala) fold relative to peptide (I), suggesting that these residues do not play a primary role in maintaining the inhibitor-like properties of (I). They do contribute subtly to enhancement of binding however, illustrating the sensitivity of the screening method.

It is also sterically possible for isoleucine at P1' to interact with histidine 57; however, its means of interaction are reduced because its side chain lacks the ability to hydrogen bond. It has previously been noted that the presence of residues with large side chains at both P1' and P3' in a substrate may produce a steric interaction which can interfere with efficient catalysis.[6] For these reasons we also kinetically analyzed a variant of (I) with alanine at P1'. This single substitution increased both k_{cat} (119 fold) and K_M (8.8 fold) producing a 13 fold increase in overall efficiency. The concurrent increase in k_{cat} and decrease in K_M upon replacement of the isoleucine by alanine suggests that the manner of isoleucine binding at P1' is detrimental to the positioning of the scissile bond, and supports mechanism two as the means of inhibition.

It has previously been demonstrated that trypsin cleaves substrates containing arginine at P1 about 6 times more efficiently than those containing lysine.[7] It therefore seemed that the P1 lysine in (I) must contribute to its low rate of catalysis. To quantitate this contribution we synthesized and kinetically analyzed a peptide containing this substitution. We found that this substitution had little effect on binding (K_M = 18 μM) but produced a 41 fold increase in k_{cat}. This improvement in the rate of catalysis is significantly larger than has been observed for the same substitution in other peptide substrates and suggests that lysine, with its shorter side chain and single amino group, is less rigidly positioned within the S1 binding pocket of trypsin than is arginine. This malleability allows subsite interactions to exert a greater influence on the position or conformation of peptide binding. In this case, that effect is a substantial degree of nonproductive binding.

Table 1. Kinetic data for catalysis of peptides based on selected inhibitor sequence.

Peptide	Sequence	$k_{cat}(s^{-1})$	K_M(mM)	$K_{cat}/K_M(M^{-1},s^{-1})$	ratio*
I	YYGAKIYRPDKM	0.73	24	$3.0x(10)^4$	1
II	YYGARIYRPDKM	30.0	18	$1.7x(10)^6$	57
III	YYGAKAYRPDKM	87.0	210	$4.1x(10)^5$	14
IV	YYGAKIARPDKM	1.3	86	$1.5x(10)^4$	0.5
V	YYGAKIYAPDKM	0.60	115	$5.2x(10)^3$	0.17

* *Efficiency of peptide hydrolysis relative to* (I).

Kinetic analysis of the peptide isolated by Houghten and Eichler's screening technique indicates that it does inhibit trypsin activity with affinity similar to the originally published IC_{50}. The most likely mechanism of action appears to be substrate binding in a nonproductive conformation, probably induced chiefly by interactions between trypsin and the P1' isoleucine. Nonproductive binding appears to be more prevalent for peptides containing a P1 lysine than for those containing arginine at this position, and is enhanced by selection for relatively tight binding residues at the remaining subsites. As the screen utilized an iterative process to maximize inhibitory ability at each individual subsite in the peptide, it is likely that this peptide represents the best trypsin inhibitor that can be produced by a linear dodecapeptide. Our quantitation of the kinetic parameters for this peptide may therefore be taken as an estimate of the power of this screening technique. While the technique possesses substantial sensitivity, the use of short peptides containing only naturally occuring amino acids sets definite limits to its power. The five hundred fold difference between the IC_{50}s of the peptide and of BPTI[1] illustrates the superiority of large folded structures over small flexible peptides in specific recognition and binding of other macromolecules. The next logical step may be to focus on reducing K_M, perhaps by developing methods to induce folding of the peptides in the library. Some possible methods for this include single or multiple disulfide bond formation and/or induction of coiled-coil formation.[8] Techniques of this nature could enhance binding affinity by providing added rigidity and increased solvent exclusion and may allow characterization of minimal size and structural requirements for acheiving protein proteinase inhibitor-like protease inhibition.

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References and Notes

1. Eichler, J.; Houghten, R.A. *Biochemistry* **1993**, *32*, 11035.
2. Carell, T.; Wintner, E. A.; Rebek,J. *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 2061.
3. Corey, D.R.; Craik, C. S. *J. Am. Chem. Soc.* **1992**, *114*, 1784.
4. Laskowski, M.; Ikunoshin, K. *Ann. Rev. Biochem.* **1980**, *49*, 593.
5. Finkenstadt, W. R.; Hamid, M. A.; Mattis, J. A.; Schrode, J.; Sealock, R. W.; Wang, D.; Laskowski, M. *Bayer Symposium V "Proteinase Inhibitors"*; Fritz H. et. al.,Ed.; Springer-Verlag: New York, 1974; pp.389-411.
6. Schellenberger, V.; Turck, C. W.; Rutter, W. J. *Biochemistry* **1994**, *33*, 4251.
7. Evnin, L. B.; Vasquez, J. R.; Craik, C. S. *Proc. Natl. Acad. Sci.* **1990**, *87*, 6659.
8. Myszka, D. G.; Chaiken I. M. *Biochemistry* **1994**, *33*, 2363.

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