Selective Inhibition, Separation, and Purification of Serine Proteases: A Strategy Based on a Photoremovable Inhibitor

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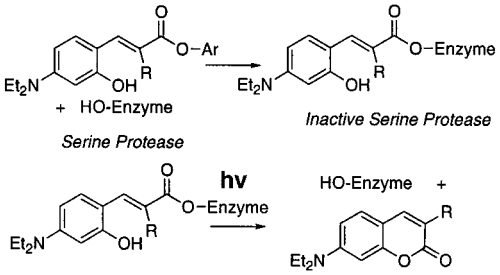
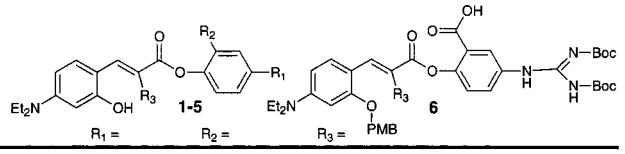
Received March 12, 1999
Revised Manuscript Received May 5, 1999

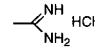
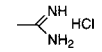
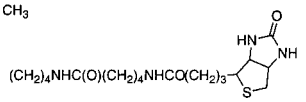
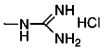
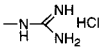
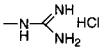
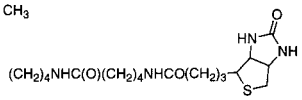
Controlling biological activity with light has become an important strategy for the study of processes as diverse as the activity of neurotransmitters[1] and the coagulation of blood plasma.[2] In the past decade, our own efforts have involved the successful use of a strategy to modify the catalytic residue of serine proteases with a photolabile moiety,[3] effectively modifying the enzyme with a photoremovable "cage".[4] Caged enzymes generated from proteases of the coagulation cascade can be used to initiate plasma coagulation in vitro and in vivo.[5] The potential



for therapeutic and diagnostic applications of such strategies has, however, been largely untapped as has its utility in approaching more general chemical and biochemical problems. We report here a strategy that permits the selective inhibition, separation, and reactivation of two of the coagulation enzymes, thrombin and factor Xa. These studies demonstrate that specific proteases in a mixture of enzymes can be targeted, isolated, and regenerated in their fully active form by means of generating caged enzymes selectively.

A series of compounds based upon the parent *o*-hydroxycinnamate structure **1**−**5** were prepared by a combination of solution- and solid-phase techniques. The cinnamates **2** and **5** were prepared with a biotin sidearm attached to the α carbon of the cinnamate by a synthetic route that is described in detail in Supporting Information. The parent compound **1** has been shown to be an excellent inhibitor for a number of serine proteases such as thrombin, factor Xa, trypsin, and tissue plasminogen activator (tPA) that hydrolyze substrate peptide bonds adjacent to arginine residues. The cinnamate acyl enzyme generated from **1** and the protease can be isolated and purified and shows no enzymatic activity. The acyl enzymes can be efficiently reactivated by light.[6,7]



1		H	CH_3
2		H	
3		-C(O)NHP$_2$	CH_3
4		-C(O)NHP$_2$-P$_3$-NH$_2$	CH_3
5		-C(O)NHP$_2$-P$_3$-NH$_2$	

Preliminary experiments were carried out with the compound **3** having only one amino acid residue at the guanidiniophenyl carboxylate, P_2 in **3**. These experiments demonstrated that substitution at this position can affect the utility of these compounds as thrombin inhibitors.[8] An Ala methyl ester at P_2 gave an inhibitor that eliminated thrombin activity completely (k_i = 1.7 M^{-1} s^{-1}). Enzyme activity for factor Xa was also eliminated by this inhibitor (k_i = 0.9 M^{-1} s^{-1}). Both inhibited enzymes could be reactivated to 100% activity with UV light. An inhibitor with P_2 in **3** = D-PheOH failed to inhibit thrombin. These results suggest that a proper choice of amino acid residues attached to the salicylate moiety might lead to more potent and/or selective thrombin inhibitors, vide infra.

A library of compounds was prepared having the structures **4** with different peptide residues in the positions P_2 and P_3. This library was constructed by standard solid-phase synthesis.[9] The synthesis of the library was completed on the solid phase by reaction of the protected peptide with the compound **6**, a protected version of the cinnamate photoremovable group. Using trifluoroacetic acid−5% triethylsilane deprotected the cinnamate and the peptide in the same step that the peptide was removed from the solid support. The details for the library preparation and assay are presented in Supporting Information.

The library consisted of 112 compounds having the structure **4**.[10] It was found that variations at P_3 have less effect on inhibitory activity than substitution at P_2, where residues of Ala, Arg, Lys, and Pro constitute most of the potent inhibitors. However, seven of eight inhibitors with P_2 = Pro showed a different inhibitory pattern than other inhibitors in the library. Enzyme activity returned rapidly in the absence of light for these compounds. This suggests that acylation of thrombin active-site serine did not occur with the inhibitors having P_2 = Pro but rather that another mechanism was operable for these compounds. All of the other inhibitors in the library behaved as expected in studies with

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(6) The photochemistry involves a *trans-cis* photoisomerization followed by lactonization that occurs on the ms time scale, see refs 3a and 4.

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(8) Fujioka, T.; Tanizawa, K.; Kanaoka, Y. *Chem. Pharm. Bull.* **1980**, *28*, 1899−1905.

(9) Details of the library synthesis and assay are described in Supporting Information.

(10) Substitution at P_2 in **4** was Ala, Arg, Asp, ChA, Glu, Gly, Ile, Lys, Orn, Phe, Phg, Pro, Trp, and Tyr. Substitution at P_3 was Asp, Gly, Ile, Lys, Phe, Phg, Tyr, and Val.

10.1021/ja990802y CCC: $18.00
Published on Web 08/05/1999

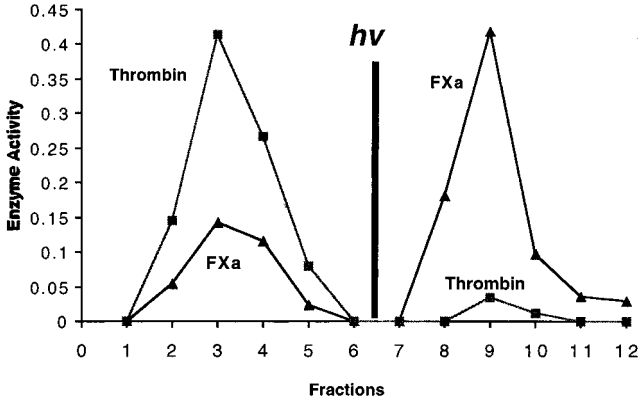

Figure 1. Factor Xa and thrombin activity eluting from an avidin column after inhibition of a 1:1 mixture of the enzymes with 3 and 2 (see text). Enzyme activity in fractions 1−6 is measured after photolyis of the collected fractions. Fractions 6−12 are collected after photolysis of the avidin column. Factor Xa is shown as solid trangles, thrombin as solid squares. Enzyme activity is reported as nmol/ml.

thrombin; they reduced enzyme activity over time, and photolysis regenerated full enzyme activity.

The library of **4** was also tested as an inhibitor of human factor Xa. On the basis of a comparison of the results of these studies and the survey described above of the same library as thrombin inhibitors, selected compounds from the library were resynthesized by solid-phase techniques, fully characterized, and studied for selective enzyme inhibition. A 1 μM:1 μM mixture of thrombin and factor Xa with 40 μM of **4**, P_2 = Lys and P_3= Tyr-NH_2 (**4-KY**)[11] gave, after 3 h, a solution having 10% residual thrombin activity (thrombin $k_i = 1.9\ M^{-1}\ s^{-1}$) and 80% residual factor Xa activity (Xa $k_i = 0.2\ M^{-1}\ s^{-1}$). Photolysis of this mixture returned both thrombin and Factor Xa to their preinhibition levels.

Screens of the library **4** for selective inhibitors of thrombin and factor Xa were intended to provide leads for examination of the inhibitor **5** that is substituted with a biotin sidearm at R_3. It was therefore surprising that **5-KY** was completely unselective as a thrombin/factor Xa inhibitor. Inhibition of a 1:1 mixture of the two enzymes by this derivative of **4** led to comparable loss of activity for both thrombin ($k_i = 0.6\ M^{-1}\ s^{-1}$) and Factor Xa ($k_i = 0.5\ M^{-1}\ s^{-1}$) throughout the course of inhibition. While the inhibitor **4-KY** is a thrombin selective inhibitor, the biotin sidearm apparently negates this selectivity for the analogous inhibitor **5-KY**. Indeed, **2** having the biotin sidearm and no substitution at R_2 is Factor Xa selective. This inhibitor (40 μM) decreases 1 μM factor Xa to 10% activity in 2 h. ($k_i = 7.0\ M^{-1}\ s^{-1}$) at the same time that 1 μM thrombin activity is reduced to 80% ($k_i = 0.5\ M^{-1}\ s^{-1}$).

Inhibition of a mixture of thrombin and factor Xa by the thrombin selective inhibitor **4-KY** followed by **2** provides a means for separating the two enzymes. This experiment started with a mixture of 1.7 μM factor Xa and 1.6 μM thrombin, which was inhibited for 1 h with 60 μM **4-KY**. At this time thrombin activity was 16% and factor Xa activity was 78% of the original. After removal of the excess **4-KY** by a size exclusion column, the second inhibitor **2** was added (30 μM). After incubation and another size exclusion column, the mixture was applied to an avidin column and eluted with Tris buffer. The results of this experiment are shown in Figure 1. The first six fractions taken from the avidin column consisted of a small amount of thrombin and factor Xa that are uninhibited. The major component of these fractions was thrombin that was inhibited with **4-KY** and which could be reactivated with photolysis by UV light from a hand-held 5-W mercury lamp. Figure 1 shows the total thrombin and factor Xa activity in each fraction, respectively, after collection of the fraction and subsequent photolysis.

(11) The residue at P_2 and at P_3 is identified by the single letter symbol.

After the collection of six fractions, no additional enzyme activity eluted from the column until the column itself was photolyzed with the 5-W mercury lamp (1 min per side). Thus, the enzyme eluting from the avidin column in fractions 7−12 (Figure 1) resulted from enzyme photoactivation on the avidin column. Pooling fractions 7−12 gave a mixture containing 46% of the original factor Xa used in the experiment. Thrombin in the first six fractions accounted for 55% of the original enzyme, while 1.5% of this enzyme elutes in fractions 7−12. The ratio of factor Xa/thrombin collected in the pooled fractions 7−12 (post-photolysis of the avidin column) was 30/1, while the pooled thrombin-rich fractions (1−6) contained an enzyme ratio of 1/2.8.[12]

In a separate experiment, a mixture of thrombin and factor Xa was incubated for 2 h at room temperature with 60 μM of the inhibitor **2.** The resulting mixture was applied to an avidin column and eluted with Tris buffer. The first six fractions taken from the avidin column consisted of factor Xa and thrombin that are fully active. These pooled fractions contained 53% of the original thrombin and 3.6% of the factor Xa giving a factor Xa/thrombin ratio of 1/15. Fractions 7−12 taken after photolysis of the avidin column, contained 83% of the original factor Xa and 21% of thrombin, giving an enzyme ratio in these pooled fractions of 4/1.

A judicious choice of inhibition strategy permits the isolation of either enzyme in a purified state from the original mixture. Thus, the experiments reported here show that use of **2** alone can give a purified thrombin while the sequence **4-KY** and then **2** gives factor Xa that is highly purified. Retreatment of the enzyme should further improve the enzyme purity. Comment should also be made on the selectivity patterns for the inhibitors **1**−**5**. These compounds, being inverse inhibitors,[13] still should reflect the P recognition sites of the proteases. Attachment of the biotin sidearm to the substructure, however, leads to a factor Xa selective inhibitor. This result may reflect the fact that the "open" active site of factor Xa is more accessible to the biotin-substituted inhibitor than is the narrow active-site channel of thrombin.[14]

The experiments described here demonstrate the potential of the enzyme photoactivation strategy for use in the isolation and separation of proteases. While the enzymes of the coagulation cascade are reasonably well understood, new and important classes of serine[15] and cysteine proteases[16] are the subject of intense current investigation. Enzyme photoactivation coupled with the parallel synthesis approach used to prepare the library of compounds described by **4** and **5** may provide a strategy for the identification, isolation, and purification of new proteases.

Acknowledgment. Support of this research from an NIH MERIT Award (H.L. 17921) is gratefully acknowledged. J.-W.T. acknowledges the Niels Stensen Foundation (The Netherlands) for receipt of a postdoctoral fellowship.

Supporting Information Available: Experimental procedures and characterization data for compounds, preparation of libraries **4** and **5** and enzyme assays are included (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA990802Y

(12) The enzyme recoveries reported here are lower limits since a considerable amount of enzyme was consumed in assays of fractions.

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