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From Poor Substrates to Good Inhibitors: Design of Inhibitors for Serine and Thiol Proteases†

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ABSTRACT: Serine and thiol proteases react with peptide substrates to form an acyl-enzyme. We have synthesized inhibitors which are pseudo-substrates and react with the proteases to generate acyl-enzymes which hydrolyze slowly. This is achieved by incorporating an electron-donating group near the carbonyl group of inhibitors **I** [Ac-Phe−C(O)NH−NH−C(O)X] and **II** [benzyl-O-C(O)-ΨAla-Leu-ArgOMe]. The acyl-enzymes derived from the reaction of **I** with papain and **II** with chymotrypsin hydrolyze with $t_{1/2}$ of 12 and 1 h, respectively. The increased electron density on the carbonyl group of the inhibitor also reduces the rate of acyl-enzyme formation. Components were incorporated into the inhibitor which interact with the leaving group binding site (S′ subsite) and which accelerate the rate of reaction of inhibitor with enzyme. For inhibitor **I**, X = NH(CH_3), $k_{on} < 0.13$ M^{-1} s^{-1} for the reaction with papain, but if X = ΨLeu(CH_3)$_2$, $k_{on} = 10^5$ M^{-1} s^{-1}. Similar results were obtained with **II** and chymotrypsin. Concomitant with acyl-enzyme formation, X is released and a slowly hydrolyzing acyl-enzyme remains.

Serine and thiol proteases are involved in many biological processes, and inhibitors of these enzymes can be of major pharmacological importance (Barrett & McDonald, 1986). We report here a general approach to the design of inhibitors targeted at specific proteases. Our rationale for the design of inhibitors is based upon the mechanism of action of serine and thiol proteases. Cleavage of the peptide and ester bonds catalyzed by these enzymes proceeds in two phases: The enzyme (thiol/serine protease) reacts with the peptide substrate and cleaves a peptide bond (Figure 1), without the intervention of H_2O. This gives rise to an acyl-enzyme derived from the transfer of a substrate acyl group to the −OH group of a serine protease or to the −SH group of a thiol protease. Additionally, a peptide with a free N-terminal amino group called the leaving group is generated. This peptide is released from the enzyme. The leaving group need not necessarily be a peptide. In the second phase H_2O reacts with the acyl-enzyme to regenerate the enzyme and release a peptide with a C-terminal carboxyl group. Nucleophiles other than H_2O can also react with the acyl-enzyme. There is a defined area on the enzyme which binds the acyl group and another which binds the leaving group (Schechter & Berger, 1970). These interactions impart specificity and

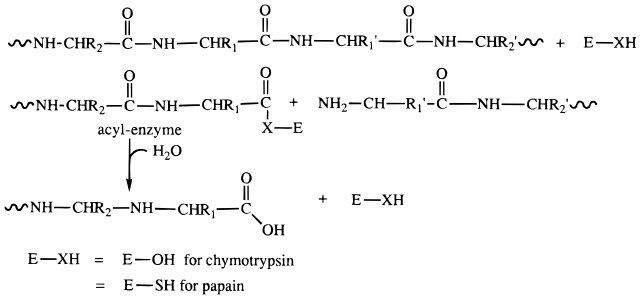

FIGURE 1: Hydrolysis of a peptide by a serine or thiol protease.

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Table 1: Inhibition of Papain by Aza-Peptides[a]

Ac-Phe−C(=O)−NH−NH−C(=O)−X	X	k_{on} (M^{-1} s^{-1})	v_i/v_o	[AX] (mM)
R_1	NHMe	>0.13		
R_2	OMe	13		
YQ5	NH−Gly−OEt	10	0.52	0.25
YQ6	NH−Leu−OMe	770	0.40	0.010
YQ14	NH−Val−OMe	220 ± 15	0.24	0.010
YQ15	NH−Val−PheOMe	550 ± 38	0.15	0.012
YQ16	NH−Val−OCH_2Ph	770 ± 100	0.47	0.0012
YQ4	O−Gly−NMe_2	$<2 \times 10^4$	>0.34	0.0005
YQ7	O−Leu−NMe_2	$\sim 10^5$		

[a] v_o = uninhibited rate; v_i = inhibited rate; AX = azapeptide. Rate of inactivation (k_{on}): The loss of catalytic activity as a function of time was determined. For each time point a separate reaction mixture was used, which consisted of 2 mM KP_i, 5 mM EDTA, 0.22 μM papain, 0.25−1 μM inhibitor, and 2 mM acetyl-L-cysteine, volume 990 μL at 25 °C. At various time intervals BAPNA in 10 μL of DMSO was added to a final concentration of 1.2 mM. The reaction was monitored at 410 nm. For the determination of v_i, the reaction mixture consisted of 50 mM KP_i, pH 7.1, 5 mM EDTA, 5−7 mM L-cysteine, BAPNA, final concentration 5 mM in DMSO (final concentration 5%), and inhibitor added in 10 μL of DMSO, final concentration as indicated in the table, 15 25 °C. The reaction was started by the addition of 0.2 μM enzyme and was monitored at 410 mn. For the determination of v_o, inhibitor was omitted. A plot of $1/(AX_o - E_o) \ln (AX \cdot E)/(E_o \cdot AX)$ vs + was constructed. The slope of the linear portion of this plot gives k_{on}.

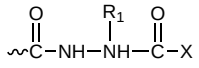
provide energy to facilitate bond cleavage. For our purposes it is important to realize that interaction of the leaving group with its binding sites increases the rate of the catalytic reaction (Fersht, 1984).

A peptide which gives rise to an acyl-enzyme and which hydrolyzes slowly, or possibly not at all, will be an inhibitor of the protease. As long as the binding site is occupied, no additional substrate molecules can be processed. How can slow hydrolysis of the acyl-enzyme be achieved? If the electron density at the carbonyl carbon of the acyl-enzyme is increased, i.e., the carbonyl group is deactivated toward nucleophilic attack, the rate of hydrolysis will be decreased (Caplow & Jencks, 1962). Increased electron density at the carbonyl carbon will be obtained by incorporating electron-donating groups into the inhibitor. Unfortunately, however, the same electron-donating group will also decrease the rate of acyl-enzyme formation, since the chemistry of acyl-enzyme formation (reaction with −OH or −SH on the enzyme) is similar to that involved in the hydrolysis of the acyl-enzyme (Wang & Shaw, 1972). Such a "deactivated carbonyl" compound will fail as a good inhibitor since an effective inhibitor will not be obtained if both the rate of formation and rate of hydrolysis of the acyl-enzyme are decreased.

"Help" is needed, therefore, to overcome the negative effect of the electron-releasing group on the rate of formation of the acyl-enzyme. If the displacement of the leaving group is energetically favorable (good leaving group), the effect of the electron-releasing group on the formation of the acyl-enzyme will be partially or fully neutralized, and formation of the acyl-enzyme will be facilitated. Concomitant with acyl-enzyme formation, the leaving group departs. Slowly hydrolyzing acyl-enzyme will remain and enzyme activity will be inhibited. This approach has been used (Powers et al., 1984) but has serious drawbacks when used in biological systems (Glover & Wang, 1973). The introduction of a good leaving group makes the inhibitor more susceptible to spontaneous hydrolysis and increases the likelihood of nonspecific reaction with nucleophiles. Our strategy circumvents problems by incorporating into the inhibitor an intrinsically unreactive moiety which interacts favorably with the leaving group binding site. Such interactions with leaving group binding sites are known to increase the rate of hydrolysis of the normal substrate (Schechter & Berger, 1970). It is, therefore, likely that these interactions will also increase the rate of formation of the acyl-enzyme.

In summary, an effective inhibitor of serine or thiol proteases requires a compound, frequently a peptide, which delivers a deactivated acyl group to the target enzyme. It also contains a component which interacts with the leaving group binding site of the normal substrate. This interaction facilitates formation of the slowly hydrolyzing acyl-enzyme. Upon formation of the acyl-enzyme the leaving group departs and the slowly hydrolyzing acyl-enzyme remains. Two cases will be described which illustrate the approach outlined above.

Aza-Peptides. Aza-peptides **I** are close analogs of peptides. Reaction of papain with an Aza-peptide (YQ14, Table



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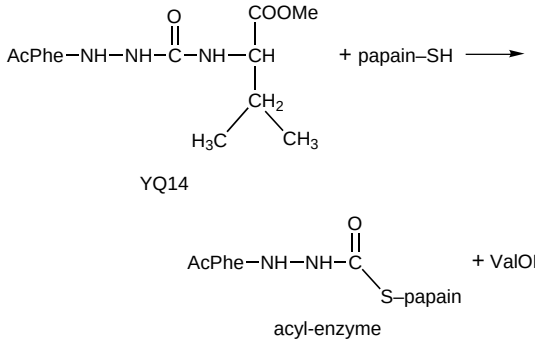
1) results in loss of catalytic activity and the formation of an acyl-enzyme (eq 1) (Magrath & Abeles, 1992). Acyl-enzyme formation was established by NMR and chemical characterization (Wu and Abeles, to be published).



Aza-peptides fulfill all requirements for inhibition of proteases. The carbonyl group of the aza-peptide is deactivated toward nucleophilic attack by the adjacent aza group, and a leaving group (X, Table 1) can be incorporated which interacts with the leaving group binding site. Table 1 summarizes the second-order rate constants for papain inactivation by aza-peptides with varying leaving groups. The rate of inhibition is slow unless a "good" leaving group is

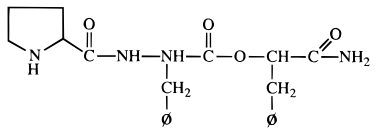


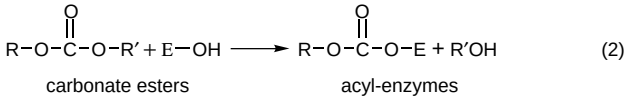
FIGURE 2: Structure of YQ17.

used or unless a leaving group is used which interacts with the leaving group binding site. For instance, if X (R_1, Table 1) = $-NHCH_3$, no inactivation of papain is detected. When X = OMe (a poor leaving group but better than $-NHCH_3$), inactivation of papain occurs with $k_{on} = 13\ M^{-1}\ s^{-1}$. With a good leaving group such as phenol, $k_{on} = 11 \times 10^3\ M^{-1}\ s^{-1}$. Phenol esters are reactive and tend to hydrolyze spontaneously (Magrath & Abeles, 1992). A more desirable approach is to employ an aza-peptide where X is a peptide or amino acid, which interacts with the leaving group binding site. This will alleviate the use of a reactive compound and will also impart specificity. When X interacts strongly with the leaving group binding site, large acceleration in the rate of inactivation can be achieved (Table 1). For example, when X = ΨLeuN$(CH_3)_2$,[1] $k_{on} > 10^5\ M^{-1}\ s^{-1}$. ΨLeuN-$(CH_3)_2$ is not a good leaving group but interacts strongly with the leaving group binding site.

The rate of hydrolysis of the acyl-enzyme, derived from reaction with YQ14, was determined. Papain was inactivated with an excess of YQ14 and then was passed through a gel-filtration column to separate inactive enzyme from YQ14. The spontaneous rate of hydrolysis of the inactivated enzyme proceeded with $t_{1/2} = 12$ h. An identical experiment was done with YQ4, and its hydrolysis rate was found to be the same as for YQ14. This result shows that the leaving group ($-O-Gly-NMe_2$) is no longer a component of the enzyme−inhibitor complex after inactivation has occurred.

An inhibitor for chymotrypsin was also synthesized. Since the acyl binding site of chymotrypsin binds the phenyl group strongly, we synthesized YQ17 (Figure 2). YQ17 inactivates chymotrypsin with k_{on} 880 = $M^{-1}\ s^{-1}$.

Carbonate Esters. The carbonyl group of a carbonate ester is flanked by two oxygen atoms and will therefore be deactivated toward reaction with nucleophiles. Presumably chymotrypsin reacts with carbonate esters to form an acyl-enzyme (eq 2). Formation of the acyl-enzyme is slow unless



carbonate esters acyl-enzymes (2)

−OR′ is a good leaving group or −OR′ interacts with the leaving group binding site. The chymotrypsin-catalyzed hydrolysis of carbonate esters, in which R′ is a good leaving group, has been investigated (McMahon et al., 1972).

A number of carbonate esters with leaving groups consisting of from zero to three amino acids were synthesized. These amino acids are known to interact with the leaving group binding site of chymotrypsin (Schellenberger et al., 1944). The results are summarized in Table 2. The effect of the leaving group on k_{on} is striking. Changing the leaving group from O-Me (**5**) (Table 2) to ΨAla-Leu-ArgOMe (Table 2) increases k_{on} ~3000-fold. The carbonate−enzyme was isolated as above, and its spontaneous hydrolysis proceeded with $t_{1/2} = 68$ min.

Table 2: Inhibition of Chymotrypsin by Carbonate Esters[a]

carbonate inhibitor	k_{on} ($M^{-1}\ s^{-1}$)	v_i/v_o	AX (mM)
benzyl−OC(O)−O−Ala−Leu−Arg−OMe (1)	>23000	0.058	0.001
benzyl−OC(O)−O−Ala−Leu−OMe (2)	2400	0.29	0.01
benzyl−OC(O)−O−Ala−Leu−NH_2 (3)	2200	0.49	0.01
benzyl−OC(O)−O−Ala−OEt (4)	200	0.39	0.1
benzyl−OC(O)−O−Me (5)	9	0.79	1

[a] For the determination of k_{on} the reaction mixture contained (total volume of 200 μL) 0.4 μM chymotrypsin and 0.1 M KP_i, pH 7.5, at 25 °C. Enzyme activity was assayed periodically by transferring 10 μL aliquots to a 1 mL reaction mixture containing 0.1 M KP_i, pH 7.5, and succinyl-Ala-Pro-Phe-*p*-nitroanilide (0.1 mM). V_i was determined under the conditions described above, except that 0.2 mM substrate was used. v_o is the rate of product formation in the absence of inhibitor.

The mechanism of inhibition by carbonate esters is similar to that for inhibition of papain by aza-peptides. The carbonyl group of the esters shows reduced electrophilicity due to the electron donation by the neighboring oxygen atoms. Incorporation of structures which interact with the leaving group binding site accelerates acyl-enzyme formation, and this neutralizes the retarding effect of the adjacent oxygen atoms. This allows the enzyme to be converted to the inhibitory acyl-enzyme.

The approach described here leads to the synthesis of effective and selective inhibitors of serine and thiol proteases. It seems likely that this approach can be expanded by using methods, other than those described here, to obtain slowly hydrolyzing acyl-enzymes. Furthermore, inhibitors based on the principle described here can also be designed for enzymes other than serine and thiol proteases.

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[1] Abbreviations: Ψ, designates replacement of the α-amino group of an amino acid by oxygen; BAPNA, benzoylarginine *p*-nitroanilide. The synthetic procedures will be furnished upon request.