Inverse Substrates for Trypsin and Trypsin-like Enzymes

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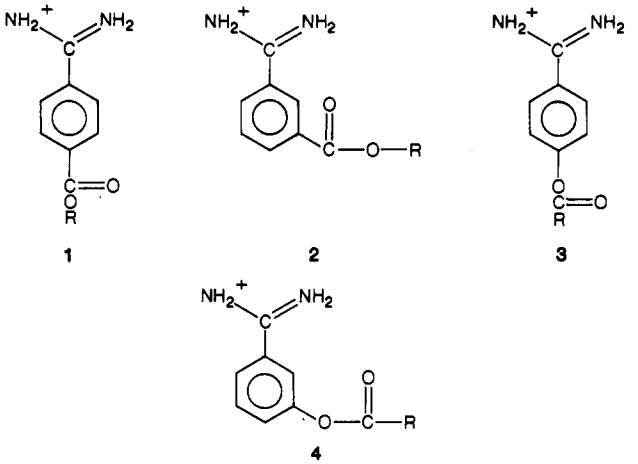
Enzymes responsible for the degradation of biologic macromolecules have remarkable substrate specificity. These enzymes recognize both the particular unit residue in the sequential chain of the substrate and the direction of the chain. For example, ribonuclease cleaves 3′-phosphates of pyrimidine nucleotide residues but not 5′-phosphates, and trypsin hydrolyzes peptide bonds which involve the carboxyl group but not the amino group of arginine or lysine residues (Figure 1). Similar specificity occurs in the hydrolysis of a variety of low-molecular-weight synthetic substrates and quasi-substrates. These are ester or amide derivatives in which the active site specific group containing a positive charge is located adjacent to the carbonyl group at which cleavage occurs.

Because this type of natural specificity was exhibited by trypsin with both proteins and synthetic substrates, it was assumed that any modification in the fundamental molecular structure of the substrate would cause a loss of susceptibility. Some workers doubted, however, whether enzymes could be always "perfect" in their recognition of a variety of synthetic (artificial) substances.

Development of Trypsin Substrates of a New Type: Inverse Substrates

Our early work showed that esters of *p*-amidinobenzoic acid (**1**) behave as specific substrates of trypsin and that meta isomers (**2**) are very inefficient as substrates.[1] These esters conform to the natural specificity of trypsin, but their structures are simplified by lack of an amino function. In an extension of this research we designed esters of an inverted structure, namely, acyl derivatives of *p*-amidinophenol (**3**).



1 **2** **3** **4**

Spectrometric analysis of the interaction between trypsin and compound **3** (R = CH_3) showed that compounds of this type are good substrates for the enzyme. After rapid mixing of enzyme and substrate, fast acylation occurs and slow deacylation follows. The reaction is monitored by amidinophenol liberation under conditions of substrate excess. The reaction follows Michaelis–Menten kinetics with strong binding affinity, efficient acylation, and rate-determining slow deacylation, exactly the same as with normal substrates. As a result, an acyl enzyme intermediate (EA) accumulates in the course of the steady-state hydrolysis:

$$E + S \overset{K_s}{\rightleftharpoons} ES \overset{k_2}{\underset{P_1}{\rightleftharpoons}} EA \xrightarrow{k_3} E + P_2 \qquad (1)$$

Since the site-specific group for the enzyme in the esters (**3**), an amidinium ion, is included not in the acyl moiety but in the leaving group, these esters were termed "inverse substrates".[2] The kinetic parameters for some inverse substrates of type **3** are listed in Table I together with those for the meta isomers (**4**) and for a normal type (**1**). *p*-Amidinophenyl acetate (**3**: R = CH_3), for example, has a binding constant of about 10^{-5} M, efficient acylation with a rate constant of 17 s^{-1}, and a slow deacylation ($k_3 = 9.3 \times 10^{-3}$ s^{-1}). In constrast, the meta isomers (**4**) are very poor substrates, probably because of an unfavorable positioning of the carbonyl carbon, as shown by the small acylation rate constant. The situation is analogous to that of **2**. The acylation rate constants of inverse substrates are nearly comparable to those of normal substrates. The binding constants of inverse substrates (ca. 10^{-5} M) are small

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Table I.
Kinetic Parameters for Trypsin-Catalyzed Hydrolysis of "Inverse Substrates" and Normal-Type Substrates at pH 8.0 and 25 °C

substrate	K_s, μM	k_2, s^{-1}	k_3, $s^{-1} \times 10^2$	k_2/K_s,[a] $s^{-1} \cdot \mu M^{-1}$
3				
RCO = CH_3CO	38.7 ± 2.9	17.0 ± 3.4	0.926 ± 0.097	0.44
RCO = CH_2CH_3CO	26.7 ± 3.7	9.40 ± 0.66	1.30 ± 0.08	0.35
RCO = Ac-Gly	29.3 ± 2.3	19.2 ± 0.6	61.1 ± 0.7	0.66
RCO = Ac-Gly-Gly	27.4 ± 2.2	20.9 ± 2.8	126 ± 2	0.76
RCO = Ac-Gly-Gly-Gly	46.5 ± 2.0	14.9 ± 0.2	146 ± 3	0.32
RCO = Ac-L-Ala	45.9 ± 10.0	4.82 ± 0.39	208 ± 3	0.11
RCO = Ac-D-Ala	30.2 ± 3.9	6.99 ± 0.45	1.21 ± 0.06	0.22
RCO = Ac-L-Ala-Gly	36.4 ± 10.1	9.55 ± 0.84	183 ± 4	0.26
RCO = Ac-D-Ala-Gly	19.2 ± 2.3	6.56 ± 0.12	27.6 ± 0.3	0.34
4				
RCO = CH_3CO	30.3 ± 7.8	0.0301 ± 0.0038	0.498 ± 0.050	0.00099
RCO = CH_2CH_3CO	28.6 ± 4.3	0.0677 ± 0.0052	0.889 ± 0.028	0.0024
1				
R = p-NO_2-C_6H_4	5.03 ± 0.43	30.4 ± 0.6	6.53 ± 0.13	6.04
p-nitrophenyl acetate	21000	1.5	1.30	0.000071

[a] The value is equal to k_{cat}/K_m(app).

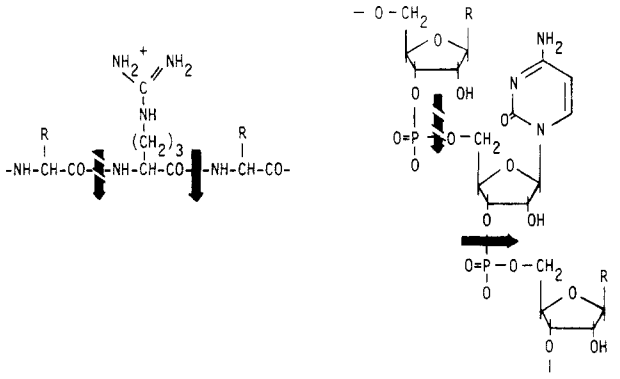


Figure 1. Enzyme specificity on sequential chain substrates.

enough for specific binding, though they are substantially larger than that of **1**. The nonspecific p-nitrophenyl acetate exhibits both very poor binding and a slow acylation rate with trypsin.

The catalytic process with an inverse substrate is compared to that with normal-type substrates in Figure 2. With an inverse substrate, after specific binding and efficient acylation, the site-specific amidinophenyl moiety departs as the leaving group to give acyl enzyme in a very specific manner.

Applications of Inverse Substrates

Inverse substrates are useful for specific introduction of a wide variety of acyl groups, including reporter groups, into the trypsin active site. Development of various new acyl enzymes is expected to reveal novel features of enzymatic reaction mechanisms and of the environment of the active sites. Extending the "inverse" concept to trypsin-like enzymes is especially important, since inverse substrates of these biologically important enzymes have potential for clinical applications.

Application to Studies on Structure and Function of Trypsin

Deacylation Rates in Tryptic Hydrolysis of Nonspecific Substrates. Kinetic parameters for inverse esters derived from acetyl amino acids and acetyl

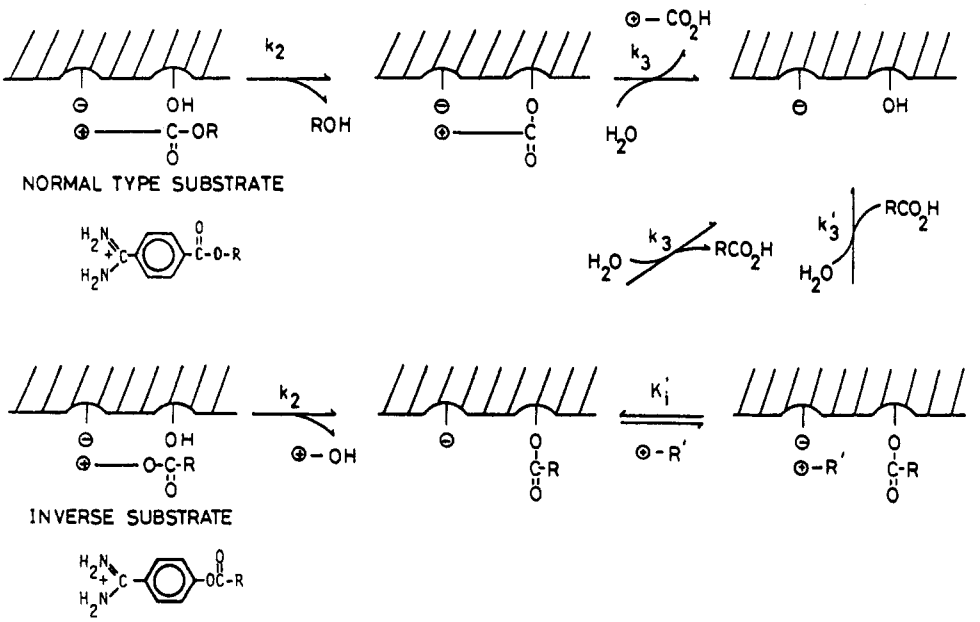


Figure 2. Reaction sequences of trypsin with normal and inverse substrates. Hydroxy function and negative charge represent the catalytic residue Ser-195 and the binding residue Asp-189 at the active site, respectively. The process to the formation of acyl trypsin–ligand complex (below, right) is only for the case where a cationic compound is present.

peptides are shown in Table I. Even in the case of D amino acid derivatives, catalysis yielded acyl enzyme intermediates in a very specific manner. Since inverse substrates are artificial substances designed to afford acyl enzymes in a specific manner, their K_s and k_2 values have no significant meaning in a physiologic sense.

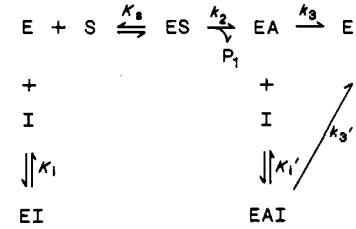
The hydrolytic behaviors of these aminoacyl or peptidyl enzymes (k_3), however, are pertinent to the mechanism of enzymatic action because trypsin is a proteolytic enzyme which cleaves peptides via acyl enzyme intermediates. The k_3 values for some D amino acid derivatives, which are difficult to obtain for trypsin with conventional substrates because of their extremely slow acylation rates, have now been determined.[3] The acetyl-D-alanine residue undergoes deacylation, but the rate is quite slow, 172 times slower than for the L enantiomer. This result shows that enantiomeric discrimination is exhibited both in the acylation step and in the deacylation step. The enantiomeric preference of trypsin was much less marked between a pair of acetylalanylglycine derivatives. These observations can be analyzed in terms of the chiral requirements of subsite interactions consistent with the crystallographic analysis of the trypsin–pancreatic trypsin inhibitor complex.[4] The effect of the peptide chain length with glycine residues was also determined. The rate of cleavage of the derivative of acetyltriglycine is slightly faster than that of the acetyldiglycine one. Both rates are about twice that of the acetylglycine derivative.

Enhancement of the Efficiency of Tryptic Hydrolysis. An important characteristic of inverse substrates is that the acyl enzyme formed from the substrate lacks a site-specific cationic group which can interact with the binding site (Figure 2). Therefore, the acyl enzyme can accept an external charged molecule at this vacant site to form an acyl enzyme–charged molecule complex, provided that the vacant cavity is large enough for both the acyl residue and the charged molecule. The dissociation constant of this complex is denoted as K_i' in Figure 2. The deacylation rate constant for the complex, k_3', is expected to be different from that of the simple acyl enzyme. The presence of a cationic compound accelerated the overall catalytic rate for *p*-amidinophenyl acetate. This acceleration must occur at the rate-determining deacylation stage. Cationic compounds which are competitive inhibitors for trypsin generally have a rate-accelerating effect, the extent of which depends on the ligand concentration.[5]

Our observation of rate acceleration is similar to the findings of Inagami and Murachi that tryptic catalysis of ethyl acetylglycinate was accelerated by cationic compounds.[6] Ethylamine enhanced the catalysis, but butylamine caused inhibition. They concluded that a specific ligand can interact with the vacant binding site if the ligand is small and that the interaction induces a conformational transition of the enzyme structure to promote catalytic efficiency. However, some evidence which cannot be fully explained by this mechanism has since been reported.[7,8] Some alkylamines which are much larger than butylamine, such as *n*-hexyl- and *n*-octylamines, were found to accelerate the trypsin-catalyzed hydrolysis of ethyl- or *p*-nitrophenyl acetylglycinates. Therefore, the effect of cationic ligands on tryptic efficiency remained ambiguous.

The kinetic properties of an inverse substrate, *p*-amidinophenyl acetylglycinate, toward trypsin showed that it behaved as a specific substrate with stronger binding and a much more efficient acylation rate constant than *p*-nitrophenyl and ethyl esters.[9] These kinetic characteristics allowed the acyl enzyme intermediate to be produced quantitatively and very specifically. Nonspecific ethyl and *p*-nitrophenyl acetylglycinates give the same acyl enzyme, acetylglycyltrypsin, but the yield is much lower due to their kinetic characteristics.

When an amidine or amine was added during the steady state, hydrolysis of the *p*-amidinophenyl esters was accelerated. Indeed, all cationic compounds tested had an acceleration effect. The parameters for the acceleration process, k_3'/k_3 and K_i', were determined from



(2)

where k_3'/k_3 is the ratio of rates from the acyl enzyme–ligand complex (EAI) and acyl enzyme (EA) and K_i' is the dissociation constant of EAI. Every k_3'/k_3 value obtained from *p*-amidinophenyl esters was >1, indicating that the inhibitory effect of *n*-propyl- and *n*-butylamines seen in previous work with conventional substrates is apparently due to the somewhat nonspecific character of these substrates.[9]

Spectrometric Analysis of Acyl Trypsins Labeled with Reporter Groups. *p*-Amidinophenyl esters carrying a fluorophore,[10] an optically active chromophore,[11] or a stable free radical[12] were synthesized (Figure 3). Each of these esters showed strong binding affinity and efficient acylation of trypsin. The dinitrophenyl-D-alanine derivative yielded the acyl trypsin in a very specific manner. Acyl trypsins were successfully isolated by the following general procedure: The enzyme was treated with excess substrate at pH 8.0 for several minutes. The pH was then lowered to 2, and the protein was separated from free substrate by gel filtration and lyophilized. Isolated acyl trypsins were completely inactive. Reactivation as a result of deacylation was observed during incubation at pH 8.0. One mole of acyl group per mole of enzyme was determined by spectrometric analysis of the acyl trypsin sample.

Information about the microenvironment of the trypsin active site was obtained by spectrometric

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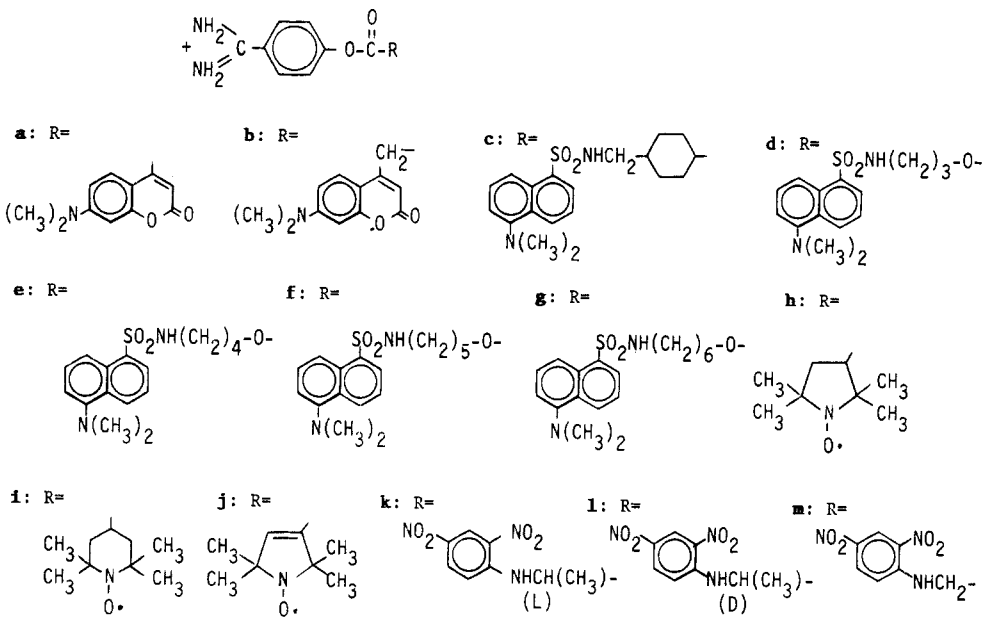

Figure 3. Inverse substrates carrying reporter groups.

analysis of these acyl trypsin preparations. Fluorescence spectra of acyl trypsins derived from compounds a–g (Figure 3) were analyzed. Each dansyl group in the acyl trypsins derived from compounds d–g may be located in a different region near the active site. Polarities of these regions were estimated from the emission wavelengths of the spectra by using Kosower's Z value.[13] The polarities of each environment containing the dansyl group derived from compounds d–f are almost identical with $Z = 90$, whereas the environment occupied by that from compound g is less polar , $Z = 85$.[14]

The solvent accessibility of the active site vicinity was estimated from the effect of deuterium oxide on the fluorescence spectrum of the acyl trypsin derived from compound c. Further structural information was obtained by measuring the energy-transfer efficiency in the fluorescence spectra between intrinsic tryptophan residues and the extrinsic fluorophore.[10] The emission maxima of the spectra of acyl trypsin from compound c shifted to a longer wavelength (12 nm) when cationic ligands were added. As mentioned previously, these ligands increased tryptic efficiency. The spectral changes caused by addition of cationic ligands may accompany conformational changes related to the acceleration phenomena[10] and probably reflect an alteration of the active site vicinity, caused by the more polar environment. Electron spin resonance spectra of acyl trypsins from compounds h–j revealed freedom of motion of the active site.[12]

Inverse Substrates for Trypsin-like Enzymes

Many enzymes which have key roles in coagulation and fibrinolysis exhibit trypsin-like specificity and should also react with inverse substrates for trypsin. Kinetic analysis of the reactions of trypsin-like enzymes with *p*-amidinophenyl esters revealed that the "inverse" concept is also applicable to thrombin, plasmin, urokinase, kallikrein, and trypsins from various sources.[15]

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	57	189 195
Bovine Trypsin	-Ala-Ala-His-Cys-Tyr-Lys-	-Asp-Ser-Cys-Gln-Gly-Asp-Ser-Gly-Gly-Pro-
Hog Trypsin	-Ala-Ala-His-Cys-Tyr-Lys-	-Asp-Ser-Cys-Gln-Gly-Asp-Ser-Gly-Gly-Pro-
SG Trypsin	-Ala-Ala-His-Cys-Val-Ser-	-Asp-Thr-Cys-Gln-Gly-Asp-Ser-Gly-Gly-Pro-
Human Plasmin	-Ala-Ala-His-Cys-Leu-Glu-	-Asp-Ser-Cys-Gln-Gly-Asp-Ser-Gly-Gly-Pro-
Human Thrombin	-Ala-Ala-His-Cys-Leu-Leu-	-Asp-Ala-Cys-Glu-Gly-Asp-Ser-Gly-Gly-Pro-
Hog Kallikrein	-Ala-Ala-His-Cys-Lys-Asn-	-Asp-Thr-Cys-Met-Gly-Asp-Ser-Gly-Gly-Pro-

	215
Bovine Trypsin	-Gly-Ile-Val-Ser-Trp-Gly-Ser-Gly-Cys-Ala-
Hog Trypsin	-Gly-Ile-Val-Ser-Trp-Gly-Ser-Gly-Cys-Ala-
SG Trypsin	-Gly-Ile-Val-Ser-Trp-Gly-Tyr-Gly-Cys-Ala-
Human Plasmin	-Gly-Val-Thr-Ser-Trp-Gly-Leu-Gly-Cys-Ala-
Human Thrombin	-Gly-Ile-Val-Ser-Trp-Gly-Glu-Gly-Cys-Asp-
Hog Kallikrein	-Gly-Ile-Thr-Ser-Trp-Gly-His-Thr Pro-Cys-Gly-

Figure 4. Comparisons of amino acid sequences of the "binding holes". On the basis of a molecular model of bovine trypsin, three peptide fragments which constitute the "binding hole" are shown. Covalent structures of the corresponding peptide fragments of other enzymes are compared. Different residues from those of bovine trypsin are framed. The numbering is that of chymotrypsinogen A.

These enzymes are similar to bovine trypsin in their binding constants and acylation rate constants with inverse substrates. Their deacylation rate constants, however, show considerable variability.

Comparative Studies on the Active Site Structures of Trypsin-like Enzymes. The active site structures of trypsin-like enzymes are assumed to be very similar to that of bovine trypsin, but much less is known about them. Elucidation of these structures is important for the purpose of designing physiologically active substances. With a view to comparing the spatial requirements of the active sites of these enzymes, the dissociation constants of the acyl enzyme–ligand complex, K_i', were determined. The kinetic reactivity of hog trypsin was quite similar to that of bovine trypsin. Kallikrein and urokinase showed strongly restricted spatial requirements at the active site.[16]

A molecular model of bovine trypsin constructed from the X-ray data[17] revealed that the "binding hole" is

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Table II.
Comparison of Deacylation Rate Constants for Bovine Trypsin, Human Thrombin, and Human Plasmin Catalyzed Reactions at pH 8.0 and 25 °C

acyl group	k_3, $s^{-1} \times 10^3$			k_3(plasmin)/ k_3(thrombin)
	bovine trypsin	human thrombin	human plasmin	
CH_3CO	9.26 ± 0.51	2.23 ± 0.11	10.6 ± 0.50	4.7
$CH_3(CH_2)_2CO$	7.50 ± 0.42	1.19 ± 0.08	6.45 ± 0.54	5.4
$CH_3(CH_2)_6CO$	4.07 ± 0.20	5.21 ± 0.10	5.42 ± 0.22	1.0
c-C_5H_9CO	12.3 ± 0.90	2.91 ± 0.34	9.47 ± 0.77	3.3
c-$C_6H_{11}CO$	11.9 ± 1.10	2.58 ± 0.21	11.1 ± 0.80	4.3
$(CH_3)_2CHCO$	1.24 ± 0.12	1.23 ± 0.15	2.35 ± 0.38	1.9
$(CH_3)_2CHCH_2CO$	1.02 ± 0.14	0.45 ± 0.02	2.83 ± 0.12	6.2
$(CH_3)_3CCO$	0.26 ± 0.03	0.22 ± 0.01	0.35 ± 0.01	1.6
$CH_3(H)C{=}C(H)CO$	0.93 ± 0.05	0.51 ± 0.02	0.94 ± 0.06	1.8
C_6H_5CO	0.81 ± 0.05	3.32 ± 0.11	0.13 ± 0.01	0.04
p-CH_3O–C_6H_4CO	0.29 ± 0.01	0.33 ± 0.02	0.013 ± 0.005	0.04
p-NO_2–C_6H_4CO	20.8 ± 1.20	2.49 ± 0.15	3.80 ± 0.36	1.52
α-$C_{10}H_7CO$	0.35 ± 0.02	0.22 ± 0.01	0.012 ± 0.003	0.05

made up of three peptide fragments.[16] The amino acid sequences of these fragments are shown in Figure 4, together with those expected for hog trypsin,[18] *Streptomyces griseus* (SG) trypsin,[19] and human plasmin,[20] thrombin,[21] and kallikrein.[22] Bovine and hog trypsins are identical in the sequences of these three fragments, while SG trypsin and human thrombin, plasmin, and kallikrein are slightly different from the mammalian trypsins in these sequences. These facts parallel our observations on the spatial requirements of the active site toward acyl enzyme–ligand complexation.

Medicinal Applications. By taking advantage of inverse substrates, which have an unlimited range of acyl components, one can develop stable acyl enzymes. These transient inhibitors for trypsin-like enzymes would be candidates for use as drugs. To investigate this possibility, the deacylation rate constants for plasmin- and thrombin-catalyzed hydrolyses of various esters are being determined. The results obtained thus far are shown in Table II.[23] The *p*-methoxybenzoyl ester, for example, affords a very stable acyl plasmin with a half-life of about 15 h, while that of the acyl thrombin is 35 min. The isovaleryl ester gives an acyl plasmin with a 4-min half-life and an acyl thrombin with a 25-min half-life.[24]

A new approach to thrombosis therapy using acyl plasmins has been reported by Smith et al.[25] Acyl plasmins are catalytically inert and unable to react with plasma inhibitors but can still bind to a fibrin clot. They can therefore circulate without being trapped by inhibitors: When they come in contact with fibrin, deacylation can occur to yield a fibrin–plasmin complex, which undergoes fibrinolysis. Acyl plasmins of appropriate stability were prepared by the general method for application of inverse substrates.[2]

Inverse Substrates: General Considerations

Classification of Protease-Specific Compounds. Compounds which interact specifically with proteases can be classified into five categories:[26]

$$E + S \rightleftharpoons ES \rightarrow (E\text{–}S') \rightarrow E + P \quad (3)$$

$$E + I \rightleftharpoons EI \quad (4)$$

$$E + I' \rightleftharpoons EI' \rightleftharpoons E\text{–}I' \quad (5)$$

$$E + I'' \rightleftharpoons EI'' \rightarrow E\text{–}I'' \quad (6)$$

$$E + I''' \rightleftharpoons EI''' \rightarrow E\text{–}I''' \rightarrow E + P' \quad (7)$$

where S, I, I′, I″ and I‴ are conventional substrates, competitive inhibitors, transition-state inhibitor,[27] specific irreversible inhibitors, and inverse substrates, respectively. The biological effect of inverse substrates is "transient inhibition" because of the formation of a more or less labile covalent bond between the substrate and enzyme (E–I‴). The reaction of inverse substrates is essentially the same as that of conventional ester substrates, proceeding through the same steps to regenerate the original enzyme after a certain "sleeping time", the lifetime of E–I‴, determined by the acyl moiety. This characteristic of inverse substrates is not shared by any other inhibitors, such as the "mechanism-based", or "suicide", and "affinity-labeling" inhibitors classified as I″ and transition-state inhibitor I′.

Imperfect Enzymatic Recognition of Substrate Molecules. The kinetic behavior of proteolytic enzymes toward a wide variety of synthetic substrates and inhibitors has been determined, and some apparently irrational enzymatic responses have been observed. These responses suggested that enzymatic recognition of substrates may be imperfect. The reactivity of inverse substrates may be considered to be due to such an imperfection.

In general, peptide bonds adjacent to a D amino acid are not hydrolyzed by proteases, thus avoiding any contamination of optical isomers in subsequent meta-

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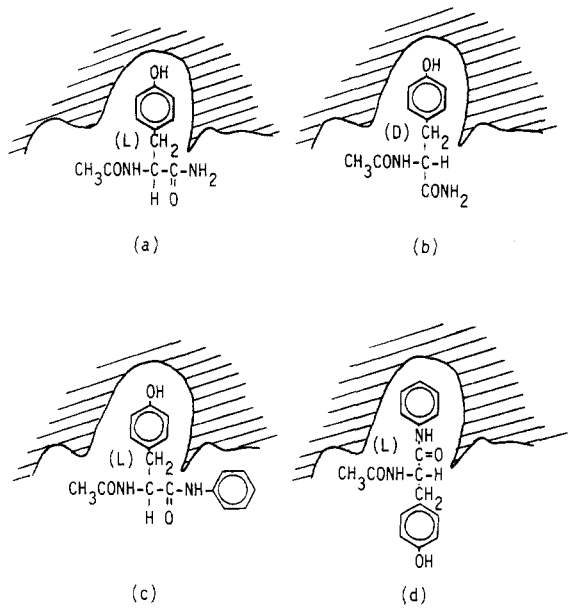

Figure 5. Schematic representation of productive (a, c) and nonproductive (b, d) bindings of tyrosine derivatives to chymotrypsin. Catalytic residues in the active site are illustrated as a sharp edge.

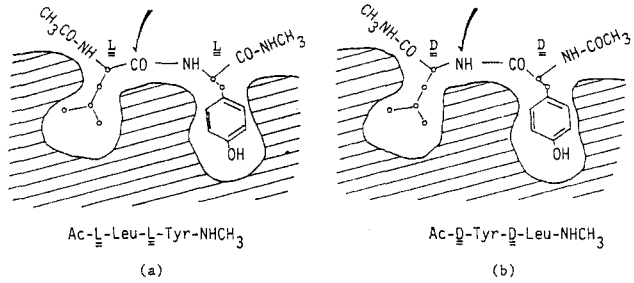

Figure 6. Binding of specific substrate (a) and its "retro-enantiomer" (b) to pepsin. Arrow represents the proximity of the catalytic residue. Reproduced from ref 30.

bolic pathways and other life processes. So-called nonproductive or wrong-way binding must be such a binding, in which the enzyme is prevented from catalyzing reactions of inappropriate substrates. A typical example of such binding is the interaction of chymotrypsin with D amino acid derivatives (Figure 5b). However, nonproductive binding between chymotrypsin and its substrate, acetyl-L-tyrosine anilide, is somewhat different.[28] Chymotrypsin acts specifically on peptides of aromatic amino acids, but in the case shown in Figure 5d it cannot discriminate completely between the aromatic residue of the tyrosine side chain and the anilide moiety, even when the substrate has an L configuration.

The binding constant of the substrate acetyl-L-leucyl-L-tyrosine methylamide to pepsin (K_m) is 2.7 mM, and the binding constant of the inhibitor acetyl-D-tyrosyl-D-leucine methylamide (K_i) is 5.8 mM.[29] The binding model in Figure 6 was proposed to explain why these binding constants are almost identical. This model assumes that the space-filling structure of leucyltyrosine in the L-configuration is similar to that of the reversed sequence, tyrosylleucine, in the D configuration. The term "retro-enantiomer" was proposed to describe the concept.[30] Here again the enzyme may have been misled. The peptide bond in this case (Figure 6b) is resistant to hydrolysis because it does not become properly oriented to the enzyme catalytic residue.

Requirements for the Design of Inverse Substrates. The kinetic behaviors of inverse-type compounds for chymotrypsin and trypsin have been studied by other research groups. Jones et al.[31] prepared esters with alcohol components which imitate tryptophan and phenylalanine residues in the hope of obtaining chymotrypsin substrates. Attempts have been also made by Hartman et al.[32] and Muramatsu et al.,[33] who prepared aminobutyl acetate as a trypsin-specific compound. None of these compounds were hydrolyzed appreciably under the experimental conditions used. Although enzyme function is not always perfect, enzymes are still be able to discriminate against such derivatives. In the design of inverse substrates, some increase in chemical reactivity is needed. For example, phenyl esters are generally much more susceptible to nucleophilic attack than esters of aliphatic alcohols, and the *p*-amidino substituent has an electron-withdrawing character nearly equal to that of the *p*-nitro group.[34] Therefore, the *p*-amidinophenyl group we have used as leaving portion satisfies both conditions: the spatial requirements of the active site of the enzyme and the chemical reactivity.

In the acylation stage, inverse substrates will be distinguished from normal substrates because the leaving oxygen atom is in a different position. In this case, the assistance of the residue which serves as general acid or proton donor to the leaving oxygen is not expected.[35] *p*-Amidinophenol thus compensates chemically for the inherent (steric) disadvantage of the enzymatic process with inverse substrates.[36] These considerations explain why the compounds reported by Jones et al., Hartman et al., and Muramatsu et al. behaved as competitive inhibitors.

It is perhaps worth reconsidering the status of chymotrypsin substrates derived from *p*-nitrophenol in terms of the inverse concept. The well-known *p*-nitrophenyl acetate is an active ester with an aromatic moiety specific for the enzyme in its leaving portion. This compound may be considered to be an inverse substrate. 2-Hydroxy-5-nitro-α-toluenesulfonic acid sultone may be considered a hybrid of normal and inverse substrates.[37]

As early as the 1960s one of us (W.B.L) noticed the important role of the *p*-nitrophenyl group at the binding site of chymotrypsin and utilized it in the design of one of the first affinity labeling agents.[38] However, no one has paid special attention to the specificity of

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the binding of the ester group or thought of these esters as inverted substrates, though many studies have been done with active esters since then.

Concluding Remarks and Further Applicability of Inverse Substrates

The most striking characteristic of inverse substrates is the generation of acyl enzymes with little restriction on the structure of the acyl moiety. As a result, a wide variety of acyl groups can be introduced specifically into an enzyme active site. The new approach to thrombosis therapy reported by Smith et al.[25] succeeded by exploring this characteristic feature of inverse substrates. A further type of application was exemplified recently by the report on photoactivatable acylated thrombin.[39]

Esters derived from *p*-(aminomethyl)phenol and *p*-guanidinophenol have also been effective as substrates for trypsin and trypsin-like enzymes,[15b,40] and (trimethylamino)butanoic acid *p*-nitrophenyl ester is an inverse substrate for butyrylcholinesterase.[41] Application of the inverse concept to thiol enzymes has also been successful: *p*-amidinophenyl esters are substrates for clostripain,[15a] a thiol enzyme with trypsin-like specificity. Although not every hydrolytic enzyme may act on inverse substrates, this concept should be useful with many enzymes for both research and clinical applications.

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Organic Radical Cations in Fluid Solution: Unusual Structures and Rearrangements

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Organic radical ions are important intermediates in a wide variety of chemical processes, and they also command attention because of a multitude of unusual structure types and a variety of interesting reactions.[1-4] Several techniques have been applied to probe various aspects of radical ion chemistry. Fast, time-resolved optical spectroscopy is a useful tool to derive information about the kinetics of formation and decay but usually provides little or no information concerning the identity or structure of the transient.[5-7] Electron spin resonance (ESR) allows insight into the structure of radical ions by probing their unpaired spin density distributions and has proved invaluable for a large variety of radical ions.[8-10] Its application is usually limited to species with lifetimes well above the millisecond range, although selected hydrocarbon cation radicals have been studied on the nanosecond time scale.[10] A large number of radical cations have been studied by matrix isolation optical or EPR spectroscopy.[4] However, the relatively high energy (~1 MeV) of the γ- or X-irradiation employed for the generation of the radical cations combined with the relatively slow dissipation of excess energy in rigid matrices constitutes a potential drawback of this method of study. Since the barriers to radical cation rearrangements are considerably lower than those on the parent energy surfaces, a number of the most interesting radical cations, e.g., those of strained-ring hydrocarbons such as quadricyclane,[11] Dewar benzene,[12] or benzvalene, have eluded this technique because of rapid rearrangements under the conditions of their generation. Clearly, a milder method of generation and a faster method of observation would be useful.[13-15]

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