

[Chem. Pharm. Bull.]
34(2) 913—916 (1986)

Comparative Studies on the Stability of Acyl Enzyme Intermediates: An Approach to the Design of Temporary Inhibitors for Trypsin-like Enzymes

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(Received July 1, 1985)

The deacylation rates of hydrolysis of inverse substrates (*p*-amidinophenyl esters) catalyzed by trypsin, thrombin and plasmin were studied from the standpoint of designing inhibitors which can discriminate between trypsin-like enzymes. It was shown that plasmin and thrombin behave quite differently towards these substrates; plasmin affords more stable acyl enzymes with aromatic acyl groups than with aliphatic acyl groups, while thrombin shows the opposite behavior. The half-life time of *p*-methoxybenzoyl plasmin is as long as 15 h while that of *p*-methoxybenzoyl thrombin is 35 min. This approach seems promising for the design of selective inhibitors of trypsin-like enzymes.

Keywords—trypsin; thrombin; plasmin; acyl enzyme; inhibitor; deacylation rate constant; inverse substrate; drug design

Many enzymes which have key roles in hematologic phenomena such as coagulation, fibrinolysis and blood pressure regulation exhibit trypsin-like specificity. Thus, the development of compounds which can discriminate between a variety of trypsin-like enzymes would be clinically significant. It is generally considered, however, that the design of such compounds is a rather difficult task since trypsin-like enzymes display only very subtle differences in specificity.

In a previous report,¹⁾ it has been shown that esters of *p*-amidinophenol are specifically hydrolyzed by trypsin and trypsin-like enzymes. In these esters the site-specific group for the enzyme, a charged amidinium group, is liberated during acylation to produce an acyl enzyme intermediate. Kinetic analyses showed that the specificity of binding and the efficiency of acylation of these esters are comparable to those for normal-type substrates, and so a new term, "inverse substrates," was proposed for these esters. This discovery enabled the preparation of a wide range of acyl enzyme derivatives for trypsin and trypsin-like enzymes which differ in their ease of hydrolysis.²⁾

In this respect, inverse substrates are expected to be applicable for the design of compounds which can discriminate between trypsin-like enzymes through the differences in their susceptibilities at the deacylation stage. For this purpose, it is necessary to search for acyl groups which display vastly different deacylation rates among trypsin-like enzymes.

In the present study, the deacylation rates of a series of acylated bovine tryptins, human thrombins and human plasmins are compared. Both aliphatic and aromatic acid residues are represented in the acyl groups.

Experimental

Materials—Bovine trypsin was purchased from Worthington Biochemical Co. (code TRL). Human plasmin was prepared following the reported procedure.³⁾ Human thrombin was prepared from Cohn fraction III by the method of Fenton *et al.*⁴⁾

Synthesis of *p*-Amidinophenyl Esters—Preparations of *p*-amidinophenyl *n*-heptanoate and *p*-amidinophenyl cyclopentanoate *p*-toluenesulfonates were carried out by the method reported previously.¹⁾ *p*-Amidinophenyl *n*-heptanoate *p*-toluenesulfonate: Colorless needles from ethanol-ether, mp 184.5–185°C. *Anal.* Calcd for C₂₂H₃₀N₂O₅S: C, 60.81; H, 6.96; N, 6.42; S, 7.38. Found: C, 60.97; H, 7.07; N, 6.42; S, 7.30. *p*-Amidinophenyl cyclopentanoate *p*-toluenesulfonate: Colorless prisms from ethanol, mp 234–236°C. *Anal.* Calcd for C₂₀H₂₄N₂O₅S: C, 59.42; H, 5.93; N, 6.92; S, 7.92. Found: C, 59.17; H, 5.98; N, 6.72; S, 7.77. *p*-Amidinophenyl isovalerate *p*-toluenesulfonate: Colorless prisms from ethanol-ether, mp 220–222°C. *Anal.* Calcd for C₁₉H₂₄N₂O₅S: C, 58.14; H, 6.16; N, 7.15. Found: C, 58.12; H, 6.14; N, 7.28. Other esters were prepared as reported previously.^{1,5,6)}

Determination of Deacylation Rates—Kinetic measurements were made on a Hitachi model 200-10 double-beam spectrophotometer. Concentrations of the enzyme stock solutions were determined by the absorbance change at 405 nm for burst titration with *p*-nitrophenyl *p*'-guanidinobenzoate.⁷⁾ Deacylation rates for bovine trypsin-catalyzed and human thrombin-catalyzed reactions were determined by subtracting the velocity of the self-hydrolysis from the velocity of the substrate-enzyme reaction. The velocities were measured at 305 nm, where the liberated product of the reaction, *p*-amidinophenol, has a measured extinction coefficient of 1.48×10^4 . Typically 50 μ l of the substrate stock solution (in dimethylformamide) was added to a thermostated cuvette containing 3.0 ml of 0.05 M Tris buffer (pH 8.0) containing 0.02 M CaCl₂, and the self-hydrolysis velocity was measured. Then 100 μ l of the enzyme stock solution was added and mixed, and the velocity was determined. Values of k_3 were calculated from the apparent net rate, V , and the enzyme concentration, $[E_0]$, following the equation; $V = k_3[E_0]$. This expression could be used since for all substrates $k_2 \gg k_3$ and $[S_0] > K_m$. In these experiments, the concentrations were: enzyme, 8.94×10^{-7} M; substrate, 6.11×10^{-5} – 1.60×10^{-4} M. For determination of slow deacylation rates the concentrations of enzyme and substrate were raised to 1.60×10^{-5} M and 2.50×10^{-4} – 7.51×10^{-4} M, respectively. Human plasmin-catalyzed reactions for which k_3 is greater than 5×10^{-4} s⁻¹ were measured similarly by subtracting the velocity of self-hydrolysis of the substrate from that of the enzyme-substrate in 0.05 M Tris, pH 8.0 containing 0.1 M NaCl and 0.02 M lysine. The concentrations of plasmin and substrate were 2.02×10^{-7} and 3.17×10^{-4} M respectively. Study of the slower rates in the plasmin-catalyzed reaction ($k_3 < 5 \times 10^{-4}$ s⁻¹) necessitated the preparation and isolation of the acyl enzyme derivatives. For the acylation step, 0.4 ml of the enzyme stock solution (6.39×10^{-6} M) was mixed with 1.6 ml of 0.05 M Tris buffer (pH 8.0) containing 0.1 M NaCl and 0.01 M lysine. Then 50 μ l of the substrate stock solution (10^{-2} M in dimethylformamide) was added and the mixture incubated at 25°C for 15 min. Measurements of the residual enzyme activity showed that 15 min was sufficient for complete acylation with the substrates studied. The acyl-enzyme was separated by column chromatography with Sephadex G-25 and eluted with 10^{-3} M HCl. For determination of the deacylation, the lyophilized powder was dissolved in 100 μ l of 10^{-3} M HCl. Then 2 ml of 0.05 M Tris buffer (pH 8.0) containing 0.1 M NaCl and 0.02 M lysine was added and the solution was incubated at 25°C. At various time intervals aliquots of the incubated solution were recovered, and the regenerated enzyme was assayed with ZLysONP (*N*^α-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester) in 3 ml of 0.1 M phosphate buffer (pH 6.0) containing 0.1 M NaCl at 340 nm.⁷⁾

Results and Discussion

As reported previously,¹⁾ hydrolysis of inverse substrates (1) catalyzed by trypsin and

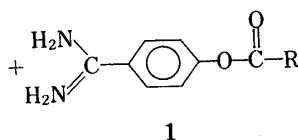


Chart 1

trypsin-like enzymes proceeds by the process given in Eq. 1:

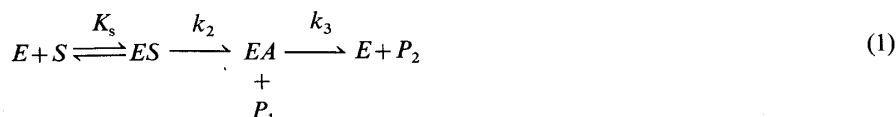


TABLE I. Comparison of Deacylation Rate Constants for Bovine Trypsin-, Human Thrombin- and Human Plasmin-Catalyzed Reactions at pH 8.0, 25°C

Acyl group	k_3 (s ⁻¹)			k_3 (plasmin)
	Bovine trypsin	Human thrombin	Human plasmin	k_3 (thrombin)
CH ₃ CO	$9.26 \pm 0.51 \times 10^{-3}$	$2.23 \pm 0.11 \times 10^{-3}$	$1.06 \pm 0.05 \times 10^{-2}$	4.7
CH ₃ (CH ₂) ₂ CO	$7.50 \pm 0.42 \times 10^{-3}$	$1.19 \pm 0.08 \times 10^{-3}$	$6.45 \pm 0.54 \times 10^{-3}$	5.4
CH ₃ (CH ₂) ₆ CO	$4.07 \pm 0.20 \times 10^{-3}$	$5.21 \pm 0.10 \times 10^{-3}$	$5.42 \pm 0.22 \times 10^{-3}$	1.0
<i>cyclo</i> -C ₅ H ₉ CO	$1.23 \pm 0.09 \times 10^{-2}$	$2.91 \pm 0.34 \times 10^{-3}$	$9.47 \pm 0.77 \times 10^{-3}$	3.3
<i>cyclo</i> -C ₆ H ₁₁ CO	$1.19 \pm 0.10 \times 10^{-2}$	$2.58 \pm 0.21 \times 10^{-3}$	$1.11 \pm 0.08 \times 10^{-2}$	4.3
(CH ₃) ₂ CHCO	$1.24 \pm 0.12 \times 10^{-3}$	$1.23 \pm 0.15 \times 10^{-3}$	$2.35 \pm 0.38 \times 10^{-3}$	1.9
(CH ₃) ₂ CHCH ₂ CO	$1.02 \pm 0.14 \times 10^{-3}$	$4.51 \pm 0.19 \times 10^{-4}$	$2.83 \pm 0.12 \times 10^{-3}$	6.2
(CH ₃) ₃ CCO	$2.59 \pm 0.28 \times 10^{-4}$	$2.15 \pm 0.08 \times 10^{-4}$	$3.53 \pm 0.13 \times 10^{-4}$	1.6
CH ₃ (H)C=C(H)CO	$9.31 \pm 0.52 \times 10^{-4}$	$5.08 \pm 0.15 \times 10^{-4}$	$9.43 \pm 0.57 \times 10^{-4}$	1.8
C ₆ H ₅ CO	$8.09 \pm 0.48 \times 10^{-4}$	$3.32 \pm 0.11 \times 10^{-3}$	$1.34 \pm 0.05 \times 10^{-4}$	0.04
(<i>p</i>)CH ₃ O-C ₆ H ₄ CO	$2.87 \pm 0.11 \times 10^{-4}$	$3.30 \pm 0.21 \times 10^{-4}$	$1.30 \pm 0.05 \times 10^{-5}$	0.04
(<i>p</i>)NO ₂ -C ₆ H ₄ CO	$2.08 \pm 0.12 \times 10^{-2}$	$2.49 \pm 0.15 \times 10^{-3}$	$3.80 \pm 0.36 \times 10^{-3}$	1.52
α -C ₁₀ H ₇ CO	$3.48 \pm 0.20 \times 10^{-4}$	$2.20 \pm 0.08 \times 10^{-4}$	$1.12 \pm 0.03 \times 10^{-5}$	0.05

where P_1 and P_2 are the reaction products (*p*-amidinophenol and acid, respectively), and EA is the acyl enzyme intermediate. For all substrates tested, the reactions with bovine trypsin, human plasmin or human thrombin were all found to be unexceptional *i.e.*, efficient acylation and slow deacylation process were observed. Furthermore, the binding affinity of the enzyme is very strong and the K_s value was analyzed to be around 10^{-5} M in each case. Thus, the production of acyl enzyme is attained in a specific manner for each substrate with these three enzymes and the over-all catalytic rates are determined by their deacylation rates.

The deacylation rate constants for trypsin, plasmin and thrombin are listed in Table I. It is evident from the data in the table that k_3 for the three enzymes is markedly dependent on the nature of the acyl group. The highest and the lowest values of k_3 for plasmin cover a range of 10^3 . In the case of plasmin, an aromatic acyl group (except *p*-nitrobenzoyl) gives a more stable acyl enzyme than an aliphatic group. In the case of the substituted benzoyl group, the value of k_3 generally parallels the electron-withdrawing ability of the substituent, so that higher k_3 values are obtained as the acyl group more readily withdraws electrons from the carbonyl group, *i.e.*, p -NO₂ > H > *p*-MeO. This behavior is also displayed by trypsin but not by thrombin, with which *p*-nitrobenzoyl is slower than benzoyl. Plots of k_3 for human plasmin and bovine trypsin *versus* the Hammett substituent constant (σ) give a fairly good correlation. Plots for *p*-nitrobenzoyl, benzoyl and *p*-methoxybenzoyl gave a slope of about 2.2 for both trypsin and plasmin. In a previous study of the deacylation rates of a series of *p*-substituted benzoyl trypsins, a ρ value of 2.68 was observed.⁸⁾ In the case of thrombin, the correlation is quite different from those of trypsin and plasmin, though the plots give a good straight line. The ρ value observed is -0.12 . In this case, the deacylation rates are almost independent of the electronic factor of the substituents. The steric effect of the aliphatic acyl group on the deacylation rates was analyzed by a plot of $\log(k_3/k_3(\text{acetyl}))$ *vs.* E_s , the Taft steric effect constants.⁹⁾ This plot did not give a linear relation for the three enzymes. Cyclopentyl and cyclohexyl groups afforded higher values than expected. This result is in marked contrast to the linear correlation reported for the chymotrypsin-catalyzed reaction.^{10,11)} It appears from these studies that the Taft steric effect which is defined from OH⁻-catalyzed ester hydrolysis is not applicable in a straightforward way to the enzymatic processes of bovine trypsin, human plasmin or human thrombin. The deacylation rates for these enzymes are governed by both the steric and electronic nature of the environments of the active sites. In chymotryptic

deacylation, however, the steric factor could be predominant. In the last column of the table, deacylation rates for thrombin and plasmin are compared. *p*-Methoxybenzoyl, for example, affords a very stable acyl plasmin with a half-life of about 15 h, while the half-life of the acyl thrombin is 35 min. However, isovaleryl gives an acyl plasmin with a 4 min half-life and an acyl thrombin with a 25 min half-life.

An approach to the design of selective inhibitors which can discriminate among trypsin-like enzymes by temporarily blocking the catalytic activity by means of acyl enzyme formation was firstly proposed by Glover *et al.*¹²⁾ It has shown that the *p*-guanidinobenzoyl group discriminates effectively between thrombin and plasmin.¹³⁾ In this approach, however, the choice of the acyl groups is limited to those carrying a positively charged substituent. Our approach is characterized by the use of inverse substrates, in which a variety of acyl groups are applicable to the specific blocking of the catalytic activity. Further studies to design more discriminating inverse substrates containing an optically active residue as the acyl group are in progress.

Acknowledgement This work was supported by a Grant-in-Aid for Scientific Research (No. 59570879) from the Ministry of Education, Science and Culture, Japan, by a grant from The Naito Foundation, and by a Grant-in-Aid from the American Heart Association, Finger Lakes Chapter.

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