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Specificity of Thrombin: Evidence for Selectivity in Acylation Rather Than Binding for *p*-Nitrophenyl α-Amino-*p*-toluate†

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ABSTRACT: The lysyl ester analogue *p*-nitrophenyl α-amino-*p*-toluate hydrobromide was synthesized, and its reactions with thrombin, trypsin, and plasmin were studied by stopped-flow and conventional methods. Kinetic parameters were compared with those determined for the arginyl ester analogue, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride, with these enzymes. By following nitrophenol release or proflavin absorption changes in the stopped-flow spectrophotometer, the constants K_s (enzyme-substrate binding), k_2 (acylation), and k_3 (deacylation) were determined. The major findings were: (1) K_s values were similar regardless of the substrate or the enzyme; (2) k_3 was approximately the same for the reaction of the lysyl ester analogue with any enzyme; (3) k_2 for the lysyl ester analogue was 1100 times greater with trypsin than with thrombin; and (4) k_2 with thrombin was 60 times greater for the arginyl than for the lysyl ester analogue. The results suggest that the limited cleavage of lysyl bonds by thrombin is due in part to restricted acylation rather than substrate binding. The active site of thrombin, compared with that of trypsin, appears to have a more stringent requirement for the spatial relationship between the cationic group and the bond cleaved in substrates.

Crucial to thrombin's key role in blood coagulation is its restricted proteolytic specificity. Thrombin hydrolyzes only four arginyl-glycine bonds in its main physiological substrate, fibrinogen. It cleaves only two such bonds in activating factor XIII (Schwartz et al., 1973; Takagi and Doolittle, 1974). Thrombin also shows a marked preference for cleavage of arginyl rather than lysyl small-molecule substrates. In contrast, plasmin, a homologous protease, preferentially cleaves lysyl substrates, while trypsin hydrolyzes both arginyl and lysyl bonds with little discrimination (Weinstein and Doolittle, 1972).

Studies with synthetic peptide substrates and defined fibrinogen fragments (Hageman and Scheraga, 1974) have indicated that the high specificity of thrombin for protein substrates resides both in the active site and in secondary binding sites outside, but close to, the active center. The structural requirements for active site binding and reaction can be determined by investigations into the kinetic behavior of small-molecule substrate analogues of arginine and lysine.

In a previous study (Fasco and Fenton, 1973), the effect of N_β substituents on the hydrolysis of *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride (NPGB)[1] by trypsin, thrombin, and plasmin was examined. The results of that

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[1] Abbreviations used are: PMS-trypsin, phenylmethylsulfonyl-trypsin; CU, Michigan Department of Health casein unit; NIH unit, National Institutes of Health clotting unit; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride; NPMT, *p*-nitrophenyl α-amino-*p*-toluate hydrobromide; K_s, enzyme-substrate binding constant; k_2, acylation constant; k_3, deacylation constant.

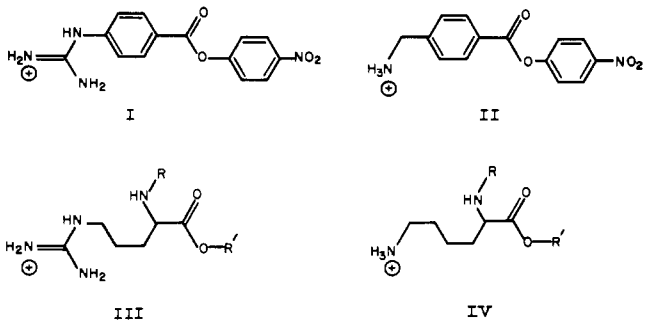

FIGURE 1: Structures of *p*-nitrophenyl *p*-guanidinobenzoate (I) and *p*-nitrophenyl α-amino-*p*-toluate (II) depicting their resemblance to possible conformations of arginyl (III) and lysyl (IV) esters.

work suggested that, relative to trypsin and plasmin, thrombin is less able to accommodate bulky groups in this region of the active site.

In the present studies, *p*-nitrophenyl α-amino-*p*-toluate hydrobromide (NPMT) was synthesized and its detailed kinetic properties were compared with those of NPGB. This analogue substrate is of particular interest, not only because of its different cationic group and shorter length, but because it closely resembles a possible configuration of a lysyl ester in the same way that NPGB resembles a possible arginyl configuration (Figure 1).

The kinetic parameters normally reported for serine protease-catalyzed hydrolysis are based on the acyl enzyme hypothesis (reviewed by Bender and Kezdy, 1965) shown in eq 1:

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

The enzyme (E) forms an ES complex with the substrate (ester, amide, peptide) with binding constant K_s. Formation of the covalent acyl enzyme intermediate (ES′) and release of the leaving group (P_1) follow with rate constant k_2 (acylation). Hydrolysis of this acyl enzyme (deacylation) is characterized by k_3. Under conditions of conventional Michaelis–Menten kinetics (low enzyme concentration), the apparent constants K_m(app) and k_{cat} are measured (eq 2):

$$E + S \overset{K_m(app)}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P_1 + P_2 \qquad (2)$$

The relationships between the true molecular constants and the steady-state constants are given in eq 3 and 4:

$$K_m(app) = k_3K_s/(k_2 + k_3) \qquad (3)$$

$$k_{cat} = k_2k_3/(k_2 + k_3) \qquad (4)$$

Under conditions of excess enzyme in a stopped-flow rapid-mixing device, the molecular constants K_s, k_2, and k_3 can be determined. Release of nitrophenol during acylation by nitrophenyl esters is conveniently monitored in the visible absorption range. By concomitant use of the proflavin binding technique (Bernhard et al., 1966; Li et al., 1974; Koehler and Magnusson, 1974), the various parameters can be determined. Thus the effects of structural modifications in substrates can be studied with respect to individual chemical steps.

Materials and Methods

Enzymes. Bovine trypsin (purchased twice crystallized from Worthington Biochemicals Corp.) was used as purchased or was separated into its α and β forms by the method of Schroeder and Shaw (1968) as modified by Luthy et al. (1973). Phenylmethylsulfonyltrypsin (PMS) was prepared according to Fahrney and Gold (1963) and purified on benzamidine-agarose (Schmer, 1972; Martin et al., 1975). A molecular weight of 25 000 was used for calculations.

Human thrombin was prepared from Cohn fraction III paste essentially by the method outlined by Fasco and Fenton (1973). The thrombin had a specific clotting activity of 2350 NIH units/mg and was 90% active by NPGB titration, assuming a molecular weight of 38 000.

Human plasminogen was isolated from Cohn fraction III paste by a modification of the procedure of Liu and Mertz (1971). This material had caseinolytic activities of 20–25 CU/mg. The plasminogen was activated to plasmin by passing a solution through a column of urokinase immobilized on Sepharose 4B (Pharmacia Fine Chemicals, Inc.) (Wiman and Wallen, 1973). The plasmin was 60–70% active by NPGB titration for a molecular weight of 81 000. Gel electrophoresis of reduced samples in sodium dodecyl sulfate–10% polyacrylamide gels (Weber et al., 1972) indicated that the major contaminant was unactivated plasminogen.

Human Cohn fraction III pastes were obtained as gifts through the generosity of Drs. Joseph D. Fisher and Robert M. Silverstein of Armour Pharmaceutical Co. Human urokinase (Winkase, Winthrop Laboratories) was supplied through the courtesy of Dr. Joseph Fratantoni, National Heart and Lung Institute, Bethesda, Md. Standard plasmin was generously provided by Dr. James Sgouris, Michigan Department of Health, Lansing, Mich. Reference human thrombin was supplied by Dr. David L. Aronson of the Bureau of Biologics, Food and Drug Administration, Bethesda, Md.

Chemicals. *p*-Nitrophenyl *p*-guanidinobenzoate hydrochloride was purchased from Nutritional Biochemicals Corp. Proflavin sulfate (Schwarz/Mann) was recrystallized from ethanol in the dark. Other chemicals were reagent grade and were used without purification.

Synthesis of NPMT. α-Amino-*p*-toluic acid was prepared by hydrogenation of *p*-cyanobenzoic acid (Eastman Kodak Co.) by the method of Levine and Sedlecky (1959) except that Raney nickel (Ventron Corp.) was substituted for Raney cobalt.

N-Benzyloxycarbonyl-α-amino-*p*-toluic acid was prepared by dissolving α-amino-*p*-toluic acid (2 g, 13 mmol) in 1.25 N sodium hydroxide (50 ml, 62.5 mmol) at 4 °C. Benzyl chloroformate (3.5 ml, 20 mmol, Aldrich Chemical Co.) was added with stirring, and the stirring was continued overnight at 4 °C. A white solid precipitate was collected by filtration, triturated with 10% hydrochloric acid solution, and dried under vacuum. This yielded 2 g, 7 mmol (54%), mp 184–189 °C.

p-Nitrophenyl *N*-benzyloxycarbonyl-α-amino-*p*-toluate was prepared by addition with stirring of *p*-nitrophenol (1 g, 7.2 mmol) and dicyclohexylcarbodiimide (1.7 g, 8.2 mmol) to a solution of *N*-benzyloxycarbonyl-α-amino-*p*-toluic acid (2 g, 7 mmol) in pyridine. The mixture was allowed to stand at room temperature for 5 days. The precipitate of dicyclohexylurea was removed by filtration; the filtrate was evaporated to dryness under high vacuum (1 Torr); and the residue was dissolved in methylene chloride (50 ml). The solution was filtered, and the addition of pe-

troleum ether (bp 30–60 °C) precipitated an off-white solid, which was redissolved in methylene chloride (40 ml). The solution was decolorized with Darco G-60 (Fisher Scientific Co.) and reprecipitated by addition of petroleum ether. The precipitate was collected by filtration and dried under vacuum to yield 1.25 g, 3.0 mmol (43%), of a white solid, mp 142–144 °C.

p-Nitrophenyl α-amino-*p*-toluate hydrobromide was synthesized by dissolving *p*-nitrophenyl *N*-benzyloxycarbonyl-α-amino-*p*-toluate (1.25 g, 3.0 mmol) in 20 ml of acetic acid saturated with hydrogen bromide. The solution was heated to 70–80 °C with stirring for 1 h and then poured into 200 ml of ethyl ether. The precipitate was collected by filtration and recrystallized from ethanol–ether to yield 500 mg, 1.4 mmol (20%), of a white solid, mp 222–224 °C. Two further recrystallizations from ethanol–ether followed by drying overnight at 80 °C under vacuum (1 Torr) gave a product with mp 230–231 °C: ir (KBr) 3400 cm^{-1} (NH), 2980, 1733 (ester), 1615, 1597, 1523, 1493, 1467, 1425, 1355, 1315, 1267, 1219, 1188, 1165, 1113, 1068, 1020, 963, 890, 868, 838, 763. Anal. Calcd for $C_{14}H_{13}N_2O_4Br$: C, 47.61; H, 3.71; N, 7.93. Found: C, 47.32; H, 3.98, N, 7.62 (average of three determinations). Elemental analyses were performed by Instranal Laboratory, Inc., Rensselaer, N.Y., and Galbraith Laboratories, Inc., Knoxville, Tenn. This compound released 100% of the theoretical amount of *p*-nitrophenol as determined spectroscopically after hydrolysis in 0.1 N sodium hydroxide.

Kinetic Methods. Kinetic experiments were carried out at 25 °C in pH 7.8 buffer: 0.1 M Tris-HCl, 0.05 M NaCl, 0.01 M $CaCl_2$. Spectrophotometric measurements for slow reactions were made on a Beckman Acta III. Rapid kinetics were determined on an Aminco-Morrow stopped-flow apparatus with a measured dead time of 3–5 ms, using a Philbrick Model 1531 logarithmic amplifier and a Tektronix storage oscilloscope. The kinetic analysis was based on eq 1 and 2.

Burst and Steady-State Kinetics. The initial enzyme concentration $[E]_0$ and turnover number (k_{cat}) were determined by "burst" kinetics (Bender et al., 1966, 1967) by following nitrophenol release at 410 nm. In a typical experiment, the concentrations were 6.67×10^{-5} M NPMT and 1.0×10^{-5} M trypsin in 3 ml of 0.67% dimethyl sulfoxide in buffer. Conventional Michaelis–Menten kinetic studies for the thrombin–NPMT reaction were performed spectrophotometrically. In these experiments, the thrombin concentration was 4.5×10^{-6} M, and substrate was varied up to 6.6×10^{-5} M. For the other substrates, K_m(app) was too low to measure. The constants k_{cat} and K_m(app) were determined by Lineweaver–Burk analysis, using a weighted least-squares program, on a Wang Model 700 calculator.

Transient Kinetics. Under conditions of excess enzyme, the rate of release of nitrophenol is pseudo-first-order in substrate, with rate constant $k_2(\text{obsd}) = k_2[E]_0/(K_s + [E]_0)$. Acylation rates were measured in the stopped-flow apparatus for different enzyme concentrations. The values of k_2 and K_s were determined by Lineweaver–Burk analysis, using the same program as above, for plots of $k_2(\text{obsd})^{-1}$ against $[E]_0^{-1}$ (see Figure 2). For trypsin or thrombin, the final concentrations were: substrate, 5.0×10^{-6} M; enzyme, 1.0×10^{-4} to 1.0×10^{-5} M. For acylation of plasmin, the final concentrations were: substrate, 1.0×10^{-6} M; enzyme, 2.0×10^{-6} to 6.0×10^{-6} M. In the calculation of rate parameters, only experiments where $[E]_0/[S]_0$ was greater than or equal to 3 were used. Analysis of the kinetic data showed that, at lower concentration ratios, the pseudo-first-order approximation is not valid.

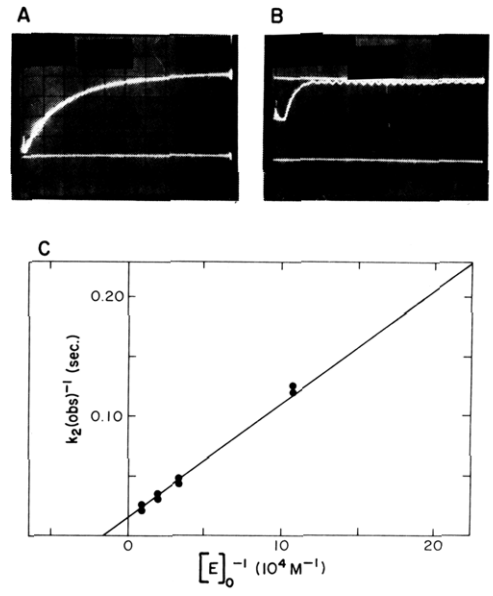

FIGURE 2: (A) Acylation of trypsin (final concentration, 1.55×10^{-5} M) by NPMT (5×10^{-6} M), 0.1 M Tris-HCl buffer (pH 7.8), 410 nm. Vertical axis: 0.02 OD/division. Time: 50 ms/division. (B) Acylation of trypsin (9.3×10^{-5} M) by NPGB (5.0×10^{-6} M), 0.1 M Tris-HCl buffer (pH 7.8), 410 nm. Vertical axis: 0.02 OD/division. Time: 10 ms/division. (C) Dependence of observed acylation rate constant k_2(obsd) on enzyme concentration for trypsin and NPMT. Line is the best fit by weighted least-squares double-reciprocal analysis.

The deacylation constants (k_3) were determined by several methods depending on the enzyme and substrate. For trypsin and thrombin, the proflavin method was used. This acridine dye binds to the active site of serine proteases (Bernhard et al., 1966; Hess, 1971; Li et al., 1974; Koehler and Magnusson, 1974). If $[E]_0 > [S]_0$, where $[S]_0$ is the initial substrate concentration, changes in the absorption spectrum of the enzyme–dye complex (diff λ_{max} 470 nm) can be used to follow displacement of the dye when the substrate binds and to follow rebinding of proflavin as deacylation of the acyl enzyme proceeds. Deacylation rates are measured at 468 nm in the stopped-flow apparatus for high rates or in a conventional spectrophotometer for slow rates. In a representative experiment, the deacylation rate of NPMT and thrombin was measured on a conventional spectrophotometer. The final concentration of proflavin in both sample and reference cuvettes was 1.0×10^{-5} M. The concentration of thrombin in the sample cuvette was 4.0×10^{-5} M. A solution of NPMT in dimethyl sulfoxide was added to the sample cuvette to give a final concentration of 1.67×10^{-5} M.

For plasmin, which does not bind proflavin, deacylation can be measured for NPGB by adding a known amount of the substrate to a slight excess of enzyme in buffer containing 0.01 M benzamidine. Benzamidine is added to the buffer to prevent autolysis of plasmin during the experiment. After acylation is complete, excess enzyme is titrated with NPGB at suitable intervals to determine the amount of acyl enzyme remaining. Plots of $\ln([ES']_t/[ES']_0)$ vs. time have slope $-k_3$. $[ES']_0$ is the initial amount of acyl enzyme formed, and $[ES']_t$ is the amount of acyl enzyme at time t.

The procedure of Chase and Shaw (1969) was used to measure k_3 for NPMT and plasmin and to measure the nonspecific reaction of plasmin and trypsin with substrates.

Table I: Kinetic Constants for NPMT and NPGB Reactions with Trypsin, Plasmin, and Thrombin.

	Trypsin		Plasmin		Thrombin	
Constant	NPGB	NPMT	NPGB	NPMT	NPGB	NPMT
K_s (10^{-5} M)	3.65	5.89	2.27	2.03	11.4	4.48
k_2 (s^{-1})	269[a]	64.25	1.01	0.48	3.4	0.058
k_3 (10^{-5} s^{-1})	4.1[b]	390	0.49	180	239	970
k_{cat} or k_{1st} (10^{-5} s^{-1})	0.9	179	ND[c]	180	105	836
k_{cat}(calcd) (10^{-5} s^{-1})	4.1	390	0.49	180	239	831
K_m(obsd) (M)	(—)[d]	(—)	(—)	(—)	(—)	2.7×10^{-6}
K_m(calcd) (M)	5.6×10^{-12}	3.6×10^{-9}	1.1×10^{-10}	7.6×10^{-8}	8.0×10^{-8}	6.42×10^{-6}

[a] A value of 300 s^{-1} was obtained with α-trypsin and 261 s^{-1} with β-trypsin. [b] Values of 3.3×10^{-5} and 3.4×10^{-5} s^{-1} were reported for α- and β-trypsin by Foucault et al. (1974). [c] ND, not determined. [d] (—) Too low to be measured experimentally.

Post-burst rate constants (rate/burst) are determined at several substrate concentrations. The nonspecific second-order rate constant determined from the slope of a plot of post-burst rate constant vs. substrate concentration is called k_{2nd} and the intercept, $k_{1st} = k_3$.

Results

A typical stopped-flow experiment measuring release of *p*-nitrophenol from NPMT by trypsin is shown in Figure 2A. From the enzyme dependence of the observed acylation rate, K_s and k_2 were determined, as shown in Figure 2C. Values for the deacylation rate constants (k_3) were determined as described in Materials and Methods.

An unusual feature of the reaction of NPGB with trypsin is shown in Figure 2B. At high enzyme concentrations, there is an initial, rapid appearance of *p*-nitrophenol which is too fast to measure. This initial jump may be the early part of normal hydrolysis since at these concentrations the beginning of the reaction falls within the dead time of the stopped-flow instrument. However, PMS-trypsin showed an initial "burst" of *p*-nitrophenol under the same conditions, suggesting that this phenomenon is related to the nonspecific hydrolysis noted by other workers (Chase and Shaw, 1969, 1970).[2] The initial jump was too fast to be due to any trace amounts of active trypsin contaminating the PMS-trypsin. This behavior was not observed in NPMT hydrolysis catalyzed by trypsin, plasmin, or thrombin. This feature of the NPGB kinetics has not been explained and, for the present study, rates were calculated from the reaction after this initial jump. If, in fact, some substrate was consumed by a nonspecific reaction, values for k_2(obsd) at high enzyme concentrations would be too low.

The kinetic constants determined for the hydrolysis of NPGB and NPMT are presented in Table I. The enzyme-substrate binding constant (K_s) is approximately the same for each substrate with all three enzymes. This suggests that substrate binding is not a major factor in specificity for these two substrates. The binding in all cases is very tight; these substrates bind tighter than many specific substrates and substantially tighter than the corresponding ethyl esters (Markwardt et al., 1968; Chase and Shaw, 1969). Chase and Shaw (1969) suggest that for NPGB this may be due to nonspecific binding of the *p*-nitrophenyl aromatic nucleus within the active site. Their further suggestion that this would involve alternate orientation at the active site, reflected in a lower reaction rate, seems not to apply here since, at least for trypsin, the acylation rate can be extremely fast.

For the lysine analogue NPMT, the three enzymes show similar deacylation rate constants (k_3) as well as approximately the same K_s. In contrast, the acylation rate (k_2) for trypsin is 134 times greater than that for plasmin and 1100 times greater than that for thrombin, strongly supporting the idea that acylation is the most significant parameter for specificity in these enzymes. For thrombin, the large difference in acylation rates (about 60-fold) is the main difference in its kinetic behavior with the two substrates, again indicating that its active site specificity is expressed in the acylation step.

The rates of acylation appear to be indicative of specificity. The relative increase in rate of the arginine analogue over that of the lysine analogue (k_2^{NPGB}/k_2^{NPMT}) is 60-fold for thrombin, 4-fold for trypsin, and 2-fold for plasmin. This is approximately the order of their preference for substrates containing the corresponding amino acids (Weinstein and Doolittle, 1972).

Discussion

Many factors enter into the determination of enzyme specificity. For protein substrates of proteases, the interactions of the peptide chain of the substrate with secondary binding sites, as well as active-center interactions, are involved in specificity (Hageman and Scheraga, 1974; Berger and Schechter, 1970). The problem is further complicated in the case of enzymes which have multistepped mechanisms. In this work we have examined active site specificity by comparing two small substrates with relatively fixed orientations containing the two different side chains of the natural substrates, arginine and lysine. The fact that the structural difference between the substrates was reflected in acylation rather than binding indicates that specificity is primarily kinetic, at least for the system studied here. This is especially significant because, for protein substrates, acylation would be expected to be the rate-determining step (Bender et al., 1964; Bender and Kezdy, 1965).

Our results suggest that the requirements for orientation of the substrate within the active site may be more stringent for thrombin than for trypsin or plasmin. Positioning of a substrate within the active site is due in large part to inter-

[2] It should be emphasized that these are not "burst" kinetics; enzyme is always in excess, and the observed "burst" is not proportional to enzyme concentration. The initial jump with PMS-trypsin was of the same order of magnitude as that seen with trypsin but was always less than the total enzyme or substrate concentration.

action between the cationic ligand and the active site carboxyl group. Several theories have been proposed to explain the enhanced reactivity due to proper orientation after binding. Geometric accessibility to the active serine, induced conformational changes in the enzyme, and deformation of the substrate to resemble the transition state are all possible mechanisms for the system studied here (see, e.g., Jencks, 1969). In any of these mechanisms the rate of acylation is determined by the geometry of the substrate.

The greatly decreased acylation rate for thrombin with the lysyl-like substrate NPMT is analogous to that reported by Foucault et al. (1974) for trypsin with nonspecific substrates. They proposed that the main function of the aspartyl counteranion in trypsin is to decrease the activation free energy for acylation, rather than for stabilizing the initial enzyme-substrate complex.

The results of the present work show that the specificity of thrombin can be observed even with small substrates such as NPMT and NPGB. The stereochemical requirements of the active site of thrombin appear to be more stringent than those of trypsin and plasmin, and these requirements are expressed most clearly in the acylation step. The preference of thrombin for arginyl-like over lysyl-like substrates is at least partially determined by the spatial relationship between the hydrolyzed bond and the cationic ligand in the substrate.

Acknowledgments

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