order dimer systems, it is the binding of the second ligand which is responsible for increasing the affinity between subunits. Additionally, the second mole of ligand cannot be responsible, as those authors stated, for increasing the affinity of its own binding. In cooperative dimeric protein systems in which 2 mol can bind, the binding of the first mole of ligand changes the protein's affinity for the second mole of ligand (Lehninger, 1975).

We hope to have clarified the application of Weber's theory of the order of free energy couplings between ligand binding and oligomerization as well as the frictional resistance to rotation and critical amplitude. These types of analyses, if properly understood, can be important tools in the study of the structural and dynamic changes that may occur in oligomeric proteins upon ligation.

ACKNOWLEDGMENTS

We thank Dr. Gregorio Weber for his support, advice, and encouragement in all aspects of this work.

Registry No. Cys(*S*-methyl)-Tyr-Ile-NH_2, 82668-39-1; Cys(*S*-methyl)-Phe-Ile-NH_2, 37637-14-2.

REFERENCES

Breslow, E. (1979) *Annu. Rev. Biochem. 48*, 251–274.
Breslow, E., Aanning, H. L., Abrash, L., & Schmir, M. (1971) *J. Biol. Chem. 246*, 5179–5188.
Hope, D. B., Walti, M., & Winsor, D. J. (1975) *Biochem. J. 147*, 377–379.
Jameson, D. M., Spencer, R. D., & Weber, G. (1976) *Rev. Sci. Instrum. 47*, 1034–1038.
Lehninger, A. L. (1975) *Biochemistry*, p 236, Worth, New York.
Miner, M., & Dalton, N. (1953) *Glycerol*, pp 246–286, Reinhold, New York.
Nicolas, P., Camier, M., Dessen, P., & Cohen, P. (1976) *J. Biol. Chem. 251*, 3951–3975.
Nicolas, P., Dessen, P., Camier, M., & Cohen, P. (1978) *FEBS Lett. 86*, 182–192.
Pearlmutter, A. F., & McMains, C. (1977) *Biochemistry 16*, 628–633.
Pearlmutter, A. F., & Dalton, E. J. (1980) *Biochemistry 19*, 3550–3556.
Rholam, M., & Nicolas, P. (1985) *Biochemistry 24*, 1928–1933.
Rholam, M., Scarlata, S., & Weber, G. (1984) *Biochemistry 23*, 6793–6796.
Royer, C. A. (1985) Doctoral Thesis, University of Illinois, Urbana, IL.
Scarlata, S. F. (1984) Doctoral Thesis, University of Illinois, Urbana, IL.
Scarlata, S., Rholam, M., & Weber, G. (1984) *Biochemistry 23*, 6789–6792.
Spencer, R. D., & Weber, G. (1969) *Ann. N.Y. Acad. Sci. 158*, 361–367.
Sur, S. S., Rabbani, L. D., Libman, L., & Breslow, E. (1979) *Biochemistry 18*, 1026–1036.
Tellman, R., & Winsor, D. J. (1980) *Arch. Biochem. Biophys. 201*, 20–24.
Weber, G. (1972) *Biochemistry 11*, 864–875.
Weber, G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7098–7102.
Weber, G., Scarlata, S., & Rholam, M. (1984) *Biochemistry 23*, 6785–6788.

p-Amidino Esters as Irreversible Inhibitors of Factors IXa and Xa and Thrombin†

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Received February 10, 1986; Revised Manuscript Received April 29, 1986

ABSTRACT: A number of inhibitors of thrombin and factor Xa have been described; however, only one inhibitor of factor IXa has been reported. This compound, dansyl-Glu-Gly-Arg chloromethyl ketone (DEGER), inhibits porcine factor IXa with a second-order rate constant of 2.2×10^4 M^{-1} min^{-1}. We now describe the synthesis and characterization of three *p*-amidinophenyl esters that inhibit human factor IXa with second-order rate constants comparable to those observed with human and bovine factor Xa and α-thrombin. These rate constants of inhibition, moreover, are 2–30-fold greater than observed when DEGER is employed to inhibit porcine factor IXa. Additional advantages of these derivatives include their ease of synthesis and low degree of toxicity. The *p*-amidinophenyl ester of benzoic acid was employed to inhibit human factor IXa, and the plasma clearance of the protein was studied in mice. These experiments demonstrate for the first time that the endothelial binding previously reported with factor IXa is independent of the active site, a finding similar to the behavior observed with factor Xa and α-thrombin in this and previous reports.

The vitamin K dependent proteinases play a crucial role in normal and aberrant hemostasis. Factor IX can be activated by both the intrinsic and extrinsic pathways (Davie & Hanahan, 1977). In the presence of factor VIII it catalyzes the activation of factor X. Factor Xa functions as the common end point of both the intrinsic and extrinsic mechanisms of coagulation, and its generation results in a rapid conversion of prothrombin to thrombin (Davie & Hanahan, 1977; Jackson & Nemerson, 1980). The in vivo regulation of these factors is complex, involving several of the major plasma proteinase inhibitors as well as a number of endothelial binding proteins

† This work was supported by Grants HL-24066, HL-31932, HL-06350, HL-07255, and HL-17921 from the National Institutes of Health.

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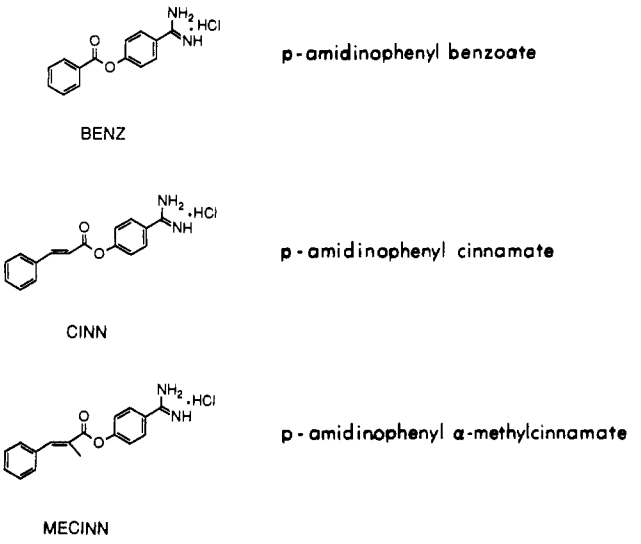

FIGURE 1: *p*-Amidinophenyl esters of benzoic, cinnamic, and α-methylcinnamic acids.

[for review, see Fuchs and Pizzo (1983) and Fuchs et al. (1984)].

A problem in the in vivo and in vitro study of the vitamin K dependent proteinases is the availability of suitable inhibitors. This is particularly the case with factor IXa, which is resistant to most small inhibitors. This enzyme is poorly, if at all, inactivated by such agents as phenylmethanesulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP), or *p*-nitrophenyl *p*-guanidinobenzoate (pNpGB) (Di Scipio et al., 1978; Byrne et al., 1980; Fuchs et al., 1984; Lollar & Fass, 1984). Recently, Lollar and Fass (1984) described an inhibitor of porcine factor IXa, dansyl-Glu-Gly-Arg chloromethyl ketone (DEGER), whose second-order rate constant for inhibition is 2.2×10^4 M^{-1} min^{-1}. This agent, like other chloromethyl ketones, is too toxic for in vivo or tissue culture studies.

Synthetic inhibitors have been employed to establish that the active or inhibited forms of α-thrombin and factor Xa bind to endothelial cells with comparable affinities (Lollar & Owen, 1980; Shifman & Pizzo, 1983; Heimark & Schwartz, 1983; Stern et al., 1983; Fuchs & Pizzo, 1983; Fuchs et al., 1984). Similar studies have not been reported with factor IXa since DEGER was unavailable when the endothelial binding of factor IXa was studied (Heimark & Schwartz, 1983; Stern et al., 1983; Fuchs et al., 1984).

We now report the synthesis and chemical and biochemical characterization of three inhibitors of the vitamin K dependent proteinases (for structures, see Figure 1). These agents are the *p*-amidinophenyl esters of benzoic (BENZ), cinnamic (CINN), and α-methylcinnamic (MECINN) acids. In kinetic studies with these compounds, the second-order rate constants for inhibition of human and bovine factor Xa and α-thrombin and human factor IXa are comparable to each other. Moreover, the magnitudes of the rate constants were 2–30-fold greater than that observed for the inhibition of porcine factor IXa by DEGER. Of these inhibitors, BENZ was employed to study the clearance behavior of inhibited factor IXa for the first time. The ease of synthesis of these compounds and the low degree of toxicity generally observed with such esters make these agents ideally suited for development as inhibitors of the vitamin K dependent proteinases.

Materials and Methods

Reagents. Diisopropyl fluorophosphate (DFP), Russell's viper venom (RVV), *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride (pNpGB), bovine serum albumin (BSA), Sepharose 4B-CL, and *p*-aminobenzamidine (pABAm) were purchased from Sigma Chemical Co., St. Louis, MO. [^{125}I]Iodine, carrier free, and iodo-beads were obtained from New England Nuclear, Boston, MA, and Pierce Chemical Co., Rockford, IL., respectively. Sephadex G-25 was purchased from Pharmacia Fine Chemicals, Piscataway, NJ. The factor Xa substrate, *N*-benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide hydrochloride (and its methyl ester), S-2222, and the thrombin substrate H-D-Phe-L-pipecolyl-Arg-*p*-nitroanilide dihydrochloride, S-2238, were purchased from Helena Laboratories, Beaumont, TX. Cinnamoyl chloride, α-methylcinnamic acid, and *p*-cyanophenol were obtained from Aldrich Chemical Co., Milwaukee, WI. Benzoyl chloride was purchased from Kodak Co., Rochester, NY. Long-fiber cellulose (Whatman Co.) was purchased from H. Reeve Angel and Co., London. 1,4-Dioxane was passed through neutral alumina and distilled prior to use. All other reagents were of the best commercial grade available.

Proteins. Human α-thrombin and factors IX and X were purified and characterized as detailed elsewhere (Fuchs & Pizzo, 1983; Fuchs et al., 1984). Human thrombin was greater than 95% in the α form. For kinetics studies, human and bovine α-thrombins were the kind gift of Dr. Frank Church and Herbert C. Whinna. Bovine factor X (Stuart) was purchased from Sigma Chemical Co., St. Louis, MO. This preparation appeared homogeneous as determined by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (data not shown). Factors IXa and Xa, diisopropylphosphoryl (DIP)–thrombin, and DIP–factor Xa were prepared as previously described (Fuchs & Pizzo, 1983; Fuchs et al., 1984).

Protein Concentrations. The concentrations of purified proteins were determined by using the extinction coefficients and molecular weights as previously detailed (Fuchs & Pizzo, 1983; Fuchs et al., 1984). The activities of factor Xa (bovine and human) and human thrombin were determined by active site titration with pNpGB (Chase & Shaw, 1967).

Protein Radiolabeling. Human α-thrombin and factor IXa and bovine factor Xa were radiolabeled with ^{125}I by using Iodo-beads as previously described (Fuchs & Pizzo, 1983; Fuchs et al., 1984). Radioactivity was measured in a γ counter (LKB-Wallac Clini Gamma, Model 1272). Proteins were labeled to specific radioactivities of 1000–2000 cpm/ng.

Clearance Studies. Clearance studies were performed in duplicate as previously described (Imber et al., 1980). In general, inhibition of human α-thrombin and factor IXa and bovine Xa was achieved by reacting the enzymes at room temperature with a 20-fold excess of the synthetic inhibitor to be tested just prior to injection.

In Vitro Studies. Human α-thrombin and bovine factor Xa were diluted in 0.05M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.2. Up to 50 μL of BENZ, CINN, or MECINN (dissolved in methanol) was added to the enzyme solution (final volume, 1 mL), and the absorbance of the solution was determined at 405 nm by using the chromogenic substrates S-2238 and S-2222, respectively. The times, temperatures, and *x*-fold molar excesses for the reaction are given in the text. All measurements are the average of triplicate determinations.

To follow reactivation of deactivated enzyme in the absence of inhibitor, radiolabeled bovine factor Xa was inactivated at room temperature by a large excess of inhibitor. The inhibitied enzyme was then purified at 4 °C on BSA-treated Sephadex G-25 and subsequently monitored with S-2222. Parallel gel filtration columns were run simultaneously on buffer-treated enzymes for control studies. The radioactivity of the fractions

was used to determine recovery of enzyme.

Fluorescence Studies. *p*-Aminobenzamidine has been shown to bind to the active site of serine proteinases (Evans et al., 1982; Monroe et al., 1985). Second-order rate constants were determined by employing the mathematical analysis of Leytus et al. (1984). Fluorescence measurements were made on a Perkin-Elmer LS-5 fluorescence spectrophotometer. In order to reduce inner filter effects with pABAm, the samples were excited at 335 nm instead of at the excitation maximum of 295 nm. A 3 × 10 mm cuvette was oriented so that the excitation beam entered the short axis, and a low concentration of pABAm was used (150 μM). Serial dilutions of a concentrated methanol stock solution of each inhibitor were made so that constant volumes (usually 20 μL) of the inhibitor solution were added for each inhibitor/enzyme concentration ratio to be tested. In every case, final methanol concentrations were maintained under 10%, and the total titration volume added was less than 3% of the final solution volume so that no corrections for dilution effects were necessary.

For fluorescence titrations, the initial fluorescence values were obtained by adding 9 μL of a 16.67 mM pABAm stock solution to 1 mL of a 1 μM enzyme solution in the fluorescence cuvette. The inhibitor solution was mixed with the enzyme solution manually for approximately 30 s before measuring. In general, the fluorescence signal was recorded every 6 s for the first 10 min through an HP Model 3478A analog to digital converter to an HP85 computer. Simultaneously, the decay in fluorescence was recorded for up to 60 min by a Perkin-Elmer Model R-100A chart recorder.

Synthesis of p-Amidinophenol. *p*-Amidinophenol, mp 222 °C, was prepared by a published two-step procedure (Wagner et al., 1973) in an overall yield from *p*-cyanophenol of 96%: IR (KBr) 3000–3400 (H-bonded OH, amidine NH, aromatic CH), 1680 (amidine C=N), 1610, 1600 cm^{-1} (amidine NH); 300-MHz ^1H NMR (CD_3OD) δ 7.75 (d, 2 H, J = 7.8 Hz), 6.95 (d, 2 H, J = 7.8 Hz); ^{13}C NMR (CD_3OD) δ 167.3, 164.4 (phenolic C and aromatic amidine C), 119.0 (amidine C), 131.1, 117.1; MS (chemical ionization) 137 (100% M – HCl), 120 (21.21%, M – HCl – OH); UV λ_{max} (H_2O) 305, 237 nm.

General Procedure for Coupling of Acid Chlorides and p-Amidinophenol. One gram of the acid chloride and 1 equiv of *p*-amidinophenol were coupled neat under argon at 180 °C for 2 h (Wagner et al., 1973). The resulting ester was purified by elution through approximately 140 g of long-fiber cellulose with 95:5 dioxane/1 N HCl. The solid thus obtained was further purified by recrystallization from 95:5 dioxane/1 N HCl.

Analytical Data for p-Amidinophenyl Benzoate (BENZ). The overall yield after chromatography was 81%: sintering point, 160 °C; IR (KBr) 3000–3400 (amidine NH_2 and NH, aromatic CH), 1740 (ester C=O), 1680 (amidine C=N), 1600 (amidine NH), 1590, 1480 cm^{-1} (aromatic C=C); 300-MHz ^1H NMR (CD_3OD) δ 8.2 (d, 2 H, J = 7.2 Hz), 7.9 (*d*, 2 H, J = 3.8 Hz), 7.73 (t, 1 H, J = 7.65 Hz), 7.55 (m, 4 H); ^{13}C NMR (CD_3OD) δ 167.7, 165.9 (carbonyl and aromatic amidine C), 156.8 (phenolic C), 135.4, 131.2, 130.7, 130.1, 130.0, 127.1, 124.1; MS (electron ionization) 241 (100%, M – HCl), 105 (95%); UV λ_{max} (H_2O) 240 nm.

Analytical Data for p-Amidinophenyl Cinnamate (CINN). The overall yield after chromatography was 53%: sintering point, 192 °C; IR (KBr) 3000–3400 (amidine NH_2 and NH, aromatic CH), 1735 (ester C=O), 1680 (amidine C=N), 1620, 1600 (amidine NH), 1580, 1480 cm^{-1} (aromatic C=C); 300-MHz ^1H NMR (CD_3OD) δ 7.9 (m, 3 H), 7.7 (m, 2 H), 7.45 (m, 4 H), 6.95 (d, 1 H, J = 8.2 Hz), 6.8 (d, 1 H, J = 15.8 Hz); ^{13}C NMR (CD_3OD) δ 168.0, 166.3 (carbonyl and aromatic amidine C), 156.8 (phenolic C), 148.8 (HC=CH-CO), 135.3, 132.1, 131.1, 130.7, 130.1, 129.6, 123.9, 117.4; MS (electron ionization) 266 (26.95%, M – HCl), 131 (100.0%), 120 (71.46%); UV λ_{max} (H_2O) 286 nm.

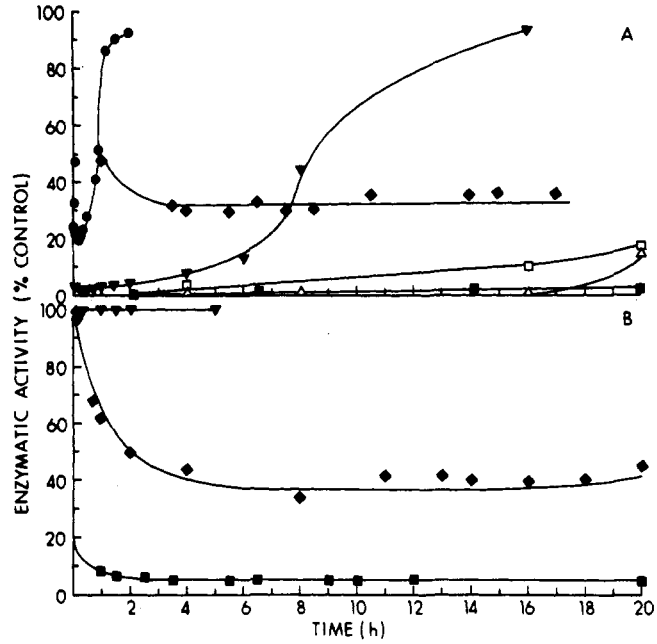

FIGURE 2: Time courses for the inhibition of human thrombin and bovine factor Xa with the *p*-amidinophenyl esters of benzoic, cinnamic, and α-methylcinnamic acids using the chromogenic substrates S-2238 and S-2222, respectively. Studies were done in triplicate at 15, 23, and 37 °C with inhibitors present in a 2–4-fold excess. (Panel A) Bovine factor Xa: (●) CINN–Xa, 37 °C; (▼) CINN–Xa, 23 °C; (△) CINN–Xa, 15 °C; (□) BENZ–Xa, 37 °C; (■) BENZ–Xa, 23 °C; (◆) MECINN–Xa, 23 °C. (Panel B) Human thrombin: (■) BENZ–T, 23 °C; (▼) CINN–T, 23 °C; (◆) MECINN–T, 23 °C.

Analytical Data for p-Amidinophenyl α-Methylcinnamate (MECINN). α-Methylcinnamoyl chloride was prepared from the corresponding acid by using thionyl chloride. The resulting acid chloride was coupled as described above in an overall yield after chromatography of 47.6%: sintering point, 204 °C; IR (KBr) 3450 (amidine NH), 3000–3250 (amidine NH_2, aromatic CH), 1725 (ester C=O), 1680 (amidine C=N), 1610 (amidine NH), 1590, 1480 (amidine NH), 1465 cm^{-1} (CH_3); 300-MHz ^1H NMR (CD_3OD) δ 7.9 (m, 3 H), 7.45 (m, 7 H), 2.2 (d, 3 H, J = 2 Hz); ^{13}C NMR (CD_3OD), δ 167.7, 167.5 (carbonyl and aromatic amidine C), 157.0 (phenolic C), 142.6 ($HC=CCH_3$), 136.5, 131.0, 130.7, 130.1, 129.6, 128.2, 126.6, 124.0, 14.4 (methyl); MS (electron ionization) 163 (18.2%), 145 (100%), 117 (75.86%); UV λ_{max} (H_2O) 278 nm. Anal. Calcd for $C_{17}H_{17}N_2O_2Cl$: C, 64.46; H, 5.42. Found: C, 64.95; H, 5.58. Infrared spectra were determined on a Perkin-Elmer Model 297 spectrophotometer. Ultraviolet spectra were obtained on a Shimadza UV–vis recording spectrophotometer, Model UV-240. MS data were determined at 70 eV on a Finnigan 4000 spectrometer with INCOS system. Carbon-13 spectra were obtained on a Varian XL300 series instrument.

RESULTS

Reaction of Human Thrombin and Bovine Xa with p-Amidinophenyl Esters of Benzoic, Cinnamic, and α-Methylcinnamic Acids (Figure 2). BENZ in a 2–4-fold excess was found to be a good inhibitor of human thrombin at 23 °C as previously reported (Wagner et al., 1973). Triplicate time courses of the reaction using the chromogenic substrate S-2238 showed thrombin activity in the presence of the inhibitor

Table I: Inhibition of Factor Xa by the *p*-Amidino Esters of BENZ, CINN, and MECINN

time (h)	% inhibition[a] BENZ	CINN	MECINN
	Bovine Factor Xa[b]		
0.5	100	97	40
1	100	96	48
4	99	93	72
8	99	59	72
16	97	3	72
	Human Thrombin[c]		
0.5	19	0	23
1	92	0	35
4	95	0	59
8	95		63
16	95		63

[a] Experiments performed at 23 °C as described in Figure 2. [b] Determined by using S-2222. [c] Determined by using S-2238.

(BENZ–T) to be less than 10% (relative to a control) within 1 h (Figure 2; Table I). Maximal inhibition of approximately 95%, reached within the second hour, was maintained for over 24 h. Thrombin activity was still less than 20% after 53 h. BENZ was also found to be an excellent inhibitor of bovine factor Xa under the same conditions as described above for α-thrombin. The percent activity of BENZ–Xa was not measurable in the initial hours of study and remained under 7% even beyond 32 h. At 37 °C under otherwise similar conditions, comparable inhibition was achieved initially, although at later time points factor Xa activity was more than double that obtained at ambient temperature. Chromogenic assays performed on the 1:1 BENZ–Xa complex resulted in complete reactivation after more than 16 h at ambient temperature.

CINN in a 2–4-fold excess at 23 °C did not diminish thrombin activity relative to a control even after 5 h. However, in sharp contrast to its behavior with human α-thrombin, CINN achieved greater than 95% inhibition of bovine factor Xa under identical conditions within the first hour. Reactivation of the CINN–Xa complex was more rapid than that of BENZ–T, with factor Xa activity above 40% after 8 h and greater than 95% after 16 h. At 15 °C the inhibitory capacity of CINN–Xa was roughly that of BENZ–Xa at 37 °C, while at 37 °C the CINN–Xa mixture regained full activity in approximately 2 h. Chromogenic assays performed on the 1:1 CINN–Xa adduct resulted in complete reactivation after 4 h at 4 °C.

Relative to the other two compounds tested, MECINN demonstrated both slower inactivation and less maximal inhibition with α-thrombin and factor Xa. At ambient temperature in the presence of a 2–4-fold excess of MECINN, thrombin activity fell to a final value of roughly 30%. Under identical conditions, comparable inhibition of bovine factor Xa was reached in approximately 2 h. Chromogenic assays performed on the purified 1:1 MECINN–Xa complex resulted in no measurable reactivation after more than 48 h at 23 °C.

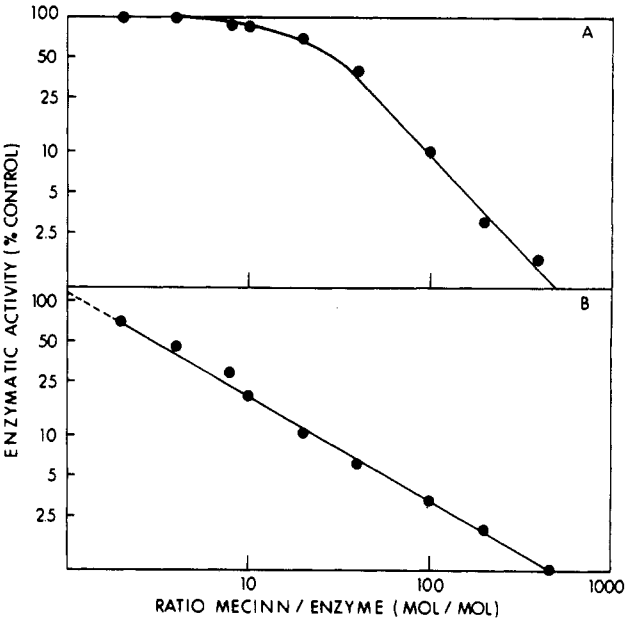

FIGURE 3: Concentration dependence of rate of inactivation of thrombin and factor Xa with the *p*-amidinophenyl ester of α-methylcinnamic acid. The studies were performed at 23 °C, employing S-2238 and S-2222, respectively, to measure enzyme activity. (Panel A) Bovine factor Xa activity after a 5-min incubation with MECINN. (Panel B) Human thrombin activity after a 45-min incubation with MECINN.

At ambient temperature in the presence of a 500-fold excess of MECINN, 5 and 45 min are required for the complete inactivation of bovine Xa and human α-thrombin, respectively. By use of these incubation periods, MECINN concentration vs. percent inhibition curves were generated for both enzymes (Figure 3).

None of the above studies with BENZ, CINN, or MECINN could be performed on factor IXa since no chromogenic substrate is presently available.

Fluorescence Studies. The second-order rate constants for the inhibition of human and bovine factors Xa and α-thrombin and human factor IXa with BENZ, CINN, and MECINN were determined by measuring the rate of displacement of the active site fluorescent probe pABAm (Table II). All these inhibitors were shown to have comparable inhibitory capacities with all three enzymes, the second-order rate constants ranging from 4.8×10^4 to 144×10^4 M^{-1} min^{-1}. These rates were approximately the same order of magnitude as observed for the inhibition of human α-thrombin and were 2–30-fold better than the 2.2×10^4 M^{-1} min^{-1} value obtained for DEGER inhibition of porcine factor IXa (Lollar & Fass, 1984). The second-order rate constant for DEGER inhibition of human factor IXa has not been reported, but preliminary studies in our laboratory indicate a comparable rate constant to that observed with porcine factor IXa. In addition, the conditions of the fluorometric assay used to obtain these rate constants (ambient temperature, pHs in the range of optimal enzyme

Table II: Second-Order Rate Constants for the Inhibition of Human Factors IXa and Xa and Thrombin and Bovine Factor Xa and Thrombin

synthetic inhibitors	second-order rate constants (M^{-1} min^{-1})[a] human factor IXa	human factor Xa	bovine factor Xa	human thrombin	bovine thrombin
BENZ	4.8×10^4	78×10^4	22×10^4	108×10^4	144×10^4
CINN	61×10^4	110×10^4	105×10^4	72×10^4	45×10^4
MECINN	16×10^4	9.5×10^4	3.5×10^4	16×10^4	32×10^4
DEGER[b]	2.2×10^4	2200×10^4		26×10^4	
pAPMSF[c]					

[a] Studies at 23 °C. [b] Taken from Lollar and Fass (1984). The value for factor IXa was determined with porcine material and is shown for comparison. [c] Too unstable for analysis.

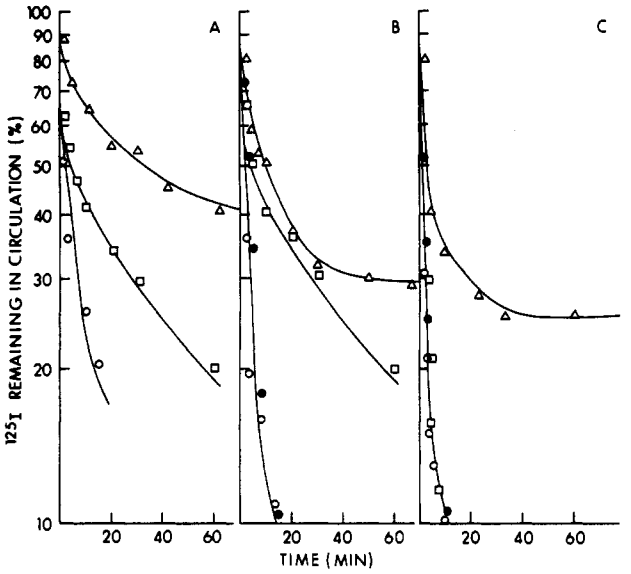

FIGURE 4: Clearance studies of intravenously injected active and inhibited ^{125}I-labeled factors IXa and Xa and thrombin. (Panel A) Human factor IXa: (○) BENZ–^{125}I-IXa; (□) ^{125}I-IXa; (△) 1000-fold molar excess BENZ–T to BENZ–^{125}I-IXa. (Panel B) Human factor Xa: (○) BENZ–^{125}I-Xa; (●) DIP–^{125}I-factor Xa; (□) ^{125}I-factor Xa; (△) 1000-fold molar excess BENZ–T to BENZ–^{125}I-Xa. (Panel C) Human factor IIa: (○) BENZ–^{125}I-T; (●) DIP–^{125}I-thrombin; (□) ^{125}I-thrombin; (△) 1000-fold molar excess BENZ–T to BENZ–^{125}I-T.

activity) precluded analysis of the (*p*-amidinophenyl)-methanesulfonyl fluoride (pAPMSF)–factor IXa or –factor Xa reaction rates (Laura et al., 1980).

Clearance Studies. The clearance of factors IXa and Xa and thrombin are presented in Figure 4. In each case, the disappearance of the BENZ-inhibited enzyme is essentially first order. This clearance is significantly inhibited by coinjection with a 1000-fold excess of BENZ–T. The clearance of DIP–factor Xa and that of DIP–thrombin are presented for comparison to the clearance of each enzyme inhibited with BENZ (Figure 4B,C). Since factor IXa is not inhibited by DFP (Di Scipio et al., 1978; Fuchs et al., 1984), no comparable data are available for this enzyme.

The clearance behavior of uninhibited factors IXa and Xa is at least second order as previously reported (Fuchs & Pizzo, 1983; Fuchs et al., 1984). This behavior results from the interaction of endothelial-bound enzyme with proteinase inhibitors. On the other hand, such transfer is much slower with thrombin (Figure 4C), and native thrombin demonstrates essentially first-order clearance behavior through 20 min of study.

It should be emphasized that these clearance competition experiments employed approximately 2500 units of BENZ–T. The lethal dose of uninhibited thrombin in the murine model is less than 10 units of active enzyme (data not shown). The animals remained alive 24 h after study.

Discussion

p-Amidinophenyl derivatives are known inhibitors of serine proteinases (Geratz, 1971; Walsmann, 1982). Although most reported amidino compounds have been designed as reversible inhibitors, some esters of *p*- and *m*-amidinophenol have been synthesized as irreversible "inverse substrates". Reactions with trypsin and chymotrypsin as model proteinases have been extensively studied (Tanizawa et al., 1981). In these compounds, the site-specific charged amidino function is associated with the leaving group rather than the carbonyl moieties that form the acyl-enzyme complexes (Figure 5). These amidino esters are also irreversible inhibitors of plasmin and streptokinase–plasmin complex (Smith et al., 1981). Of the compounds synthesized in the present study, only BENZ was previously shown to be a thrombin inhibitor (Markwardt et al., 1972), but there is no published data on its use as an inhibitor of factors IXa and Xa. Similarly, CINN and MECINN derivatives have never been characterized as inhibitors of the vitamin K dependent proteinases. These compounds have a number of advantages as proteinase inhibitors, including the essentially irreversible nature of their inhibition and the low degree of toxicity that they exhibit during in vivo studies (Smith, 1981; Pizzo et al., 1986). In addition, their ease of synthesis makes feasible large-scale rapid production at relatively low cost.

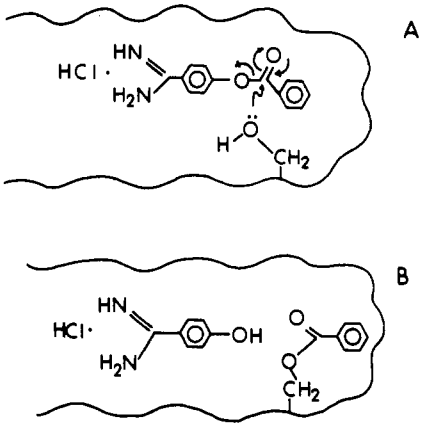

FIGURE 5: Representation of the acylation reaction thought to occur at the active site serine of serine proteinases. (Panel A) BENZ acylation. (Panel B) BENZ–T complex.

These *p*-amidinophenyl esters inhibit serine proteinases by acylation of the active site serine hydroxyl (Bender & Kezdy, 1965). Other serine proteinase inhibitors such as DFP, PMSF, and pAPMSF also react at the active site serine with varying degrees of stability relative to hydrolysis. In contrast, chloromethyl ketones such as DEGER have been shown to react with an active site histidine residue. Of the thiol esters mentioned above, PMSF has been shown to undergo facile O,N-acyl shifts even at pH 7.0 (Gold & Fabrey, 1964), and pAPMSF is reported to be too unstable for study at ambient temperatures and pHs in the range of optimal enzyme activity. The *p*-amidinophenyl esters reported here have been shown to efficiently inhibit α-thrombin and factors IXa and Xa in low molar excesses at 23 °C for up to 53 h.

One can speculate on structure–activity relationships in the BENZ, CINN, and MECINN series (Figure 1). BENZ, the first inhibitor synthesized and tested, contains a benzene ring α to the ester carbonyl. The close proximity to the acylation site of this fairly bulky substituent plays a key role in reactivity, since sterically hindered acylated enzymes are more stable than unhindered serine esters. Separating the aromatic ring from the ester carbonyl by a C=C group (CINN) results in total loss of inhibitory capacity with thrombin. This loss of activity is apparently due to more rapid deacylation of the CINN–serine intermediate. These relationships are depicted in Figure 5.

In order to explore the relative importance of disubstitution at the carbon α to the carbonyl, the previously unreported MECINN was synthesized. In this compound the α-vinyl hydrogen of CINN has been replaced by a bulkier methyl substituent to better mimic the disubstitution of the aromatic α-carbon in BENZ. It was predicted that this change would result in a derivative more closely simulating the steric bulk of the BENZ α-carbon. When tested, MECINN did prove

to be a thrombin inhibitor. By 4 h of incubation, the relative thrombin inhibitory capacities of CINN, MECINN, and BENZ were 0%, 60%, and 95%, respectively (Table I).

The extent of inhibition depends upon both acylation and hydrolysis rates. The chromogenic assay reflects primarily the acylation reaction since it is performed in the presence of an excess of the inhibitors. Therefore, a study to assess qualitative hydrolysis rates was performed. Factor Xa inhibited at a 1000-fold molar excess of each of the three inhibitors was separated from the free compounds by gel filtration at 4 °C. In this study, by far, the most stable adduct was the MECINN–Xa complex, which showed no hydrolysis even at 48 h and 23 °C. BENZ–Xa demonstrated complete deacylation by 16 h at 23 °C. By contrast, CINN–Xa completely hydrolyzed in less than 4 h even at 4 °C. These studies indicate that while acylation of factor Xa with MECINN is slower than observed with the other two esters, the resultant acyl enzyme is extremely stable to hydrolysis. The acylation reaction can be pushed to completion if a larger molar excess of MECINN is employed for inhibition of factor Xa or thrombin (Figure 3).

In order to obtain data for all three enzymes by a single technique, an approach other than colorimetric assay is necessary. The active site fluorescent probe pABAm was employed to obtain second-order rate constants for the inhibition of these enzymes. The second-order rate constants for inhibition of human and bovine α-thrombin by BENZ were 1.08×10^6 and 1.44×10^6 M^{-1} min^{-1}, respectively. This compares to a second-order rate constant for the inhibition of α-thrombin by antithrombin III which is 3.5×10^5 M^{-1} min^{-1} in the absence of heparin (Church et al., 1985). The CINN and MECINN derivatives also demonstrated better rate constants for inhibition of α-thrombin than observed when antithrombin III is the inhibitor. All three compounds demonstrate comparable inhibitory activity against both human and bovine factors Xa. By this technique, CINN was found to be the best inhibitor of factor IXa. When compared to the published data for DEGER, the only other known inhibitor of factor IXa, the second-order rate constants for the *p*-amidinophenyl esters are 2–30-fold greater. Data from these studies are collected over a period of less than 5 min to obtain initial rates; therefore, they do not reflect the deacylation seen over many hours by colorimetric assay.

In the present report these inhibitors have also been employed for in vivo experiments. These studies were possible because of the relatively low toxicity of the compounds. Previous work demonstrated an endothelial binding protein for thrombin (Lollar & Owen, 1980). This protein, named thrombomodulin, rapidly binds both active and DIP–thrombin. When native thrombin is bound, it subsequently is transferred to antithrombin III by a facilitated mechanism (Lollar & Oven, 1980; Shifman & Pizzo, 1983). The affinity of thrombomodulin for the antithrombin III–thrombin complex appears lower than for thrombin since the complexes reenter the circulation (Lollar & Owen, 1980; Shifman & Pizzo, 1983) and are then removed from the blood by the hepatocyte antithrombin III–proteinase receptor (Shifman & Pizzo, 1982, 1983). Thrombin inhibited with BENZ behaved in a manner identical with that of free thrombin and DIP–thrombin exhibiting the use of such inhibitors in biological studies.

Previous studies also demonstrated the presence of factor Xa and factor IXa binding proteins on endothelial cells (Heimark & Schwartz, 1983; Stern et al., 1983; Fuchs & Pizzo, 1983; Fuchs et al., 1984). The binding of factor Xa to endothelium results in altered specificity for substrates. This is best demonstrated in the transfer of endothelial-bound factor Xa to α_2-macroglobulin (α_2M), which contrasts with the in vitro observation that α_2M is a poor inhibitor of this enzyme. The facilitated transfer of factor Xa to α_2M occurs more rapidly than the transfer of thrombin to antithrombin III, resulting in the higher order clearance behavior of factor Xa in contrast to thrombin (Figure 2) (Fuchs & Pizzo, 1983). When the clearance of DIP–factor Xa is studied, clearance is essentially first order since transfer to α_2M cannot occur with an inhibited enzyme (Fuchs & Pizzo, 1983). The same behavior was observed in the case of factor Xa inhibited with BENZ in that the clearance of DIP–factor Xa and BENZ–factor Xa are identical.

The clearance behavior of factor IXa is more complex since both factor IX and factor IXa bind to endothelial sites (Heimark & Schwartz, 1983; Stern et al., 1983; Fuchs et al., 1984). In vivo studies suggest that the zymogen form may be recognized by a distinct mechanism as compared to factor IXa (Fuchs et al., 1984). The lack of a factor IXa inhibitor at the time this study was performed precluded further analysis of the interaction of factor IXa and endothelium. The clearance of factor IXa is higher order as a result of interaction of endothelial-bound factor IXa with antithrombin III (Fuchs et al., 1984). This reaction occurs at an accelerated rate compared to the in vitro interactions between these proteins (Fuchs et al., 1984). In the present study, it is demonstrated that BENZ–IXa clearance is rapid and nearly first order. These studies suggest that the binding of factor IXa, like that of thrombin and factor Xa is independent of the active site of the enzyme. The slight curvature of the clearance curve may indicate some cross recognition between the factor IXa and factor IX binding proteins as previously suggested (Stern et al., 1983; Fuchs et al., 1984).

The clearance of factors IXa and Xa is inhibited by a large molar excess of DIP–thrombin, suggesting that thrombin binds to multiple classes of binding sites. There is no evidence that thrombomodulin binds proteinases other than thrombin (Fuchs & Pizzo, 1983; Fuchs et al., 1984). These experiments were repeated in the present work, employing BENZ–T in place of DIP–thrombin to compete for the clearance of ^{125}I-BENZ–T, –Xa, and –IXa. The results were equivalent to those previously reported when DIP–thrombin was employed as a competing ligand (Fuchs & Pizzo, 1983; Fuchs et al., 1984).

One other interpretation for the observation that thrombin competes for the clearance of factors Xa and IXa is that the preparation of α-thrombin is contaminated with traces of these proteinases. This possibility can be eliminated for a number of reasons. Electrophoretic analysis of our preparation of purified human α-thrombin demonstrates that the protein is at least 95% in the α form, with the remainder of the protein consisting primarily of β-thrombin (Shifman & Pizzo, 1982). Even at high protein load, there was no evidence of contamination by factors Xa or IXa. Moreover, when our α-thrombin preparation was assayed for factor Xa with S-2222 as a substrate, there was no evidence of contamination (data not shown). Finally, in numerous previous studies employing the murine clearance model, we have demonstrated that significant in vivo clearance competition does not occur unless the competing ligand is present in at least a 500-fold molar excess [see, for example, Fuchs and Pizzo (1983), Fuchs et al. (1984), Imber and Pizzo (1981), and Shifman and Pizzo (1982, 1983)]. This would require that our α-thrombin preparation be at least 50% factors Xa and/or IXa. Thus it may be concluded that α-thrombin competes for the endothelial

binding of factors Xa and IXa as well as thrombin. It must be emphasized that these competition studies employed about 2700 units (1 mg) of inhibited thrombin. The presence of any free thrombin due to lack of inhibition or regeneration would have resulted in the death of the experimental animals, since 10 units of thrombin is a lethal dose in mice. Moreover, the experimental animals remained alive 24 h after study.

In summary, we report the synthesis and characterization of three *p*-amidinophenyl esters that are potent inhibitors of the vitamin K dependent proteinases. These inhibitors are readily prepared, function at a low molar ratio to proteinase, and can be employed for in vivo studies. Their potential use as antithrombotic agents may warrant further exploration.

Registry No. BENZ, 40761-72-6; CINN, 103499-67-8; MECINN, 103499-68-9; *p*-$HOC_6H_4C(=NH)NH_2$, 15535-98-5; PhCOCl, 98-88-4; PhCH=CHCOCl, 102-92-1; PhCH=C(Me)COCl, 35086-87-4; PhCH=C(Me)CO_2H, 1199-77-5; factor IXa, 37316-87-3; factor Xa, 9002-05-5; thrombin, 9002-04-4.

REFERENCES

Bender, M. L., & Kezdy, F. J. (1965) *Annu. Rev. Biochem. 34*, 49–76.

Byrne, R., Link, R. P., & Castellino, F. J. (1980) *J. Biol. Chem. 255*, 5336–5341.

Chase, T., & Shaw, E. (1967) *Biochem. Biophys. Res. Commun. 29*, 508–514.

Church, F. C., Noyes, C. M., & Griffith, M. J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6431–6434.

Davie, E. W., & Hanahan, D. J. (1977) in *The Plasma Proteins* (Putnam, F. W., Ed.) Vol. III, pp 422–531, Academic Press, New York.

Di Scipio, R. G., Kurachi, K., & Davie, E. W. (1978) *J. Clin. Invest. 61*, 1528–1538.

Evans, S. A., Olson, S. T., & Shore, J. D. (1982) *J. Biol. Chem. 257*, 3014–3017.

Fuchs, H. E., & Pizzo, S. V. (1983) *J. Clin. Invest. 72*, 2041–2049.

Fuchs, H. E., Trapp, H. G., Griffith, M. J., Roberts, H. R., & Pizzo, S. V. (1984) *J. Clin. Invest. 73*, 1696–1703.

Geratz, J. D. (1971) *Thromb. Diath. Haemorrh. 25*, 391–404.

Gold, A. M., & Fabrey, D. (1964) *Biochemistry 3*, 783–791.

Heimark, R. L., & Schwartz, S. M. (1983) *Biochem. Biophys. Res. Commun. 111*, 723–731.

Imber, M. J., & Pizzo, S. V. (1981) *J. Biol. Chem. 256*, 8134–8139.

Jackson, C. M., & Nemerson, Y. (1980) *Annu. Rev. Biochem. 49*, 765–811.

Laura, R., Robinson, D. J., & Bing, D. H. (1980) *Biochemistry 19*, 4859–4864.

Leytus, S. P., Toledo, D. L., & Mangel, W. F. (1984) *Biochim. Biophys. Acta 788*, 74–86.

Lollar, P., & Owen, W. G. (1980) *J. Clin. Invest. 66*, 1222–1230.

Lollar, P., & Fass, D. N. (1984) *Arch. Biochem. Biophys. 233*, 138–446.

Markwardt, F., Wagner, G., Walsmann, P., Horn, H., & Sturzebecker, J. (1972) *Acta Biol. Med. Ger. 28*, K19–K25.

Monroe, D. M., Roberts, H. R., & Griffith, M. J. (1985) *Thromb. Haemostasis 54*, 149 (Abstract 879).

Pizzo, S. V., Turner, A. D., Gonias, S. L., Monroe, D. M., Roberts, H. R., & Porter, N. A. (1986) *Ann. N.Y. Acad. Sci.* (in press).

Shifman, M. A., & Pizzo, S. V. (1982) *J. Biol. Chem. 257*, 3243–3248.

Shifman, M. A., & Pizzo, S. V. (1983) *Biochem. Pharmacol. 32*, 739–741.

Smith, R. A. G. (1981) U.S. Patent 4 285 932.

Stern, D. M., Drillings, M., Nossel, H. L., Hurlet-Jensen, A., LaGamma, K. S., & Owen, J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4119–4123.

Tanizawa, K., Kasaba, Y., & Kanaoka, Y. (1977) *J. Am. Chem. Soc. 99*, 4485–4488.

Wagner, G., & Horn, H. (1973) *Pharmazie 28*, 427–431.

Walsmann, P. (1982) *Folia Haematol. (Leipzig) 109*, 75–82.