Mapping the Active Sites of Bovine Thrombin, Factor IX_a, Factor X_a, Factor XI_a, Factor XII_a, Plasma Kallikrein, and Trypsin with Amino Acid and Peptide Thioesters: Development of New Sensitive Substrates†

Brian J. McRae, Kotoku Kurachi,‡ Ronald L. Heimark, Kazuo Fujikawa, Earl W. Davie, and James C. Powers*

ABSTRACT: The subsite specificities of bovine factor IX_a, factor X_a, factor XI_a, factor XII_a, thrombin, plasma kallikrein, and trypsin were mapped with amino acid, dipeptide, and longer peptide thioester substrates. Each substrate contained a P_1 Arg residue. The P_1' residues included thiol residues which are analogues of valine, leucine, and isoleucine, respectively, and the P_2 residue included 12 representative amino acid residues. Longer substrates with the sequence at the antithrombin III reactive site and at the zymogen activation site of various coagulation factors were also studied. The enzymatic hydrolysis of the thioesters was measured in the presence of 4,4′-dithiodipyridine which provides a very sensitive assay for the free thiol. The thioesters were excellent substrates for the coagulation factors studied, and the k_{cat}/K_m values for the best thioester substrates were higher than those previously reported for most of these enzymes. Thrombin and plasma kallikrein were the most active of the coagulation factors toward the thioester substrates. The best substrate for thrombin was Z-Gly-Arg-$SCH_2C_6H_5$, although substrates containing proline in the P_2 position were also quite effective. Some of the better substrates for plasma kallikrein had a P_2 Phe or Trp residue. Factor IX_a was the least reactive of the coagulation factors and hydrolyzed only four of the dipeptide thioesters. Substrates with bulky hydrophobic groups such as Phe or Trp in the P_2 position were the most reactive with factor IX_a. Factor X_a hydrolyzed all the thioester substrates tested, the most reactive being Z-Gly-Arg-$SCH_2C_6H_5$. This is consistent with the fact that glycine and arginine are present in the P_2 and P_1 positions, respectively, of the factor X_a sensitive bonds in prothrombin which is the physiological substrate for factor X_a. Bovine factor XI_a showed the least amount of specificity of the various coagulation factors and was quite reactive toward all of the thioester substrates. The most sensitive substrate for this enzyme was also Z-Gly-Arg-$SCH_2C_6H_5$. Factor XII_a preferred the dipeptide with a P_2 Phe, although the simpler thioester Z-Arg-$SCH_2CH(CH_3)_2$ was more reactive. Trypsin hydrolyzed all of the thioester substrates at a high rate and showed little substrate specificity. With all enzymes studied, extension of the thioester substrate beyond P_2 or the P_1' thiol leaving group did not lead to an improvement in hydrolysis. Due to their high k_{cat}/K_m values and the ease of detecting the thiol leaving group, thioester substrates should be extremely useful for future studies of coagulation proteases.

A substantial number of the plasma proteins are zymogens of serine proteases. These proteins are involved in various physiological processes, such as blood coagulation, fibrinolysis, kinin generation, and complement activation. Most of the activated plasma proteases are highly specific when compared with the digestive enzymes, such as pancreatic trypsin or chymotrypsin. In most cases, the plasma proteases cleave only one or two peptide bonds in their natural substrates. This high enzyme specificity apparently is due to the recognition of a specific amino acid sequence near the sensitive bond or a specific conformation of these (or other) amino acids near the sensitive bond or a combination of both. The N-terminal sequences formed during the activation of the various coagulation factors are known (Titani et al., 1972; Fujikawa et al., 1974, 1977; Kisiel et al., 1977a). Also, the C-terminal sequences generated during the activation reaction have been determined for most of the coagulation factors (Magnusson et al., 1975; Titani et al., 1975; Lundblad et al., 1976; Katayama et al., 1979).

A number of studies of the effect of the various blood coagulation factors on synthetic substrates have appeared (Adams & Elmore, 1971; Svedsen et al., 1972; Wuepper, 1972; Aurell et al., 1977; Mattler & Bang, 1977; Zur & Nemerson, 1978). Substrates containing 4-nitroaniline and amides of 7-amino-4-methylcoumarin have been particularly useful. None of these substrates, however, are completely specific, and few have been characterized with respect to their k_{cat} and K_m values. Thus far, most of the kinetic work with synthetic substrates has been centered on factor X_a and thrombin, and little is known about factor IX_a, factor XI_a, and factor XII_a.[1]

A major problem with many of the synthetic substrates is their low rate of enzymatic hydrolysis. Accordingly, large amounts of enzyme are usually required for the assay. Amino acid and peptide thioesters have recently been utilized as substrates for chymotrypsin (Farmer & Hageman, 1975), elastase (Castillo et al., 1979), and trypsin (Green & Shaw, 1979). Cleavage of the thioester bond by these enzymes yields a thiol which can be determined continuously in the presence of 4,4′-dithiodipyridine.

In this paper, we report the hydrolysis of a number of thioester substrates by homogeneous samples of bovine thrombin, factor IX_a, factor X_a, factor XI_a, factor XII_a, plasma kallikrein, and trypsin. The S_2, S_1', and S_2' subsites[2] for these enzymes were studied with a series of single amino acid, di-

† From the School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332 (B.J.M. and J.C.P.), and the Department of Biochemistry, University of Washington, Seattle, Washington 98195 (K.K., R.L.H., K.F., and E.W.D.). *Received May 26, 1981.* This research was supported in part by grants to the Georgia Institute of Technology (HL 22530) and the University of Washington (HL 16919) from the National Institutes of Health.

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[1] The nomenclature for the various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959).

[2] The nomenclature for the individual amino acid residues (P_2, P_1, P_1', etc.) of a substrate and for the subsites (S_2, S_1, S_1', etc.) of the enzyme is that of Schechter & Berger (1967).

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peptide, and tripeptide substrates. Longer peptide thioesters, with sequences corresponding to the sequence found at the reactive site of antithrombin III, were also investigated. A nitroanilide and an aminomethylcoumarin substrate for factor IX_a were also synthesized. These studies represent the first step toward designing specific and sensitive substrates for the enzymes involved in blood coagulation. In addition, this study represents the first time that pure samples of the coagulation factors have been systematically investigated with the same set of substrates under identical conditions.

Materials and Methods

Bovine trypsin (2× crystallized) was purchased from Sigma Chemical Co., St. Louis, MO. Bovine thrombin, factor X_a, and factor XI_a were prepared as described by Kisiel et al. (1977b), Kurachi et al. (1976a), and Kurachi et al. (1980), respectively. Bovine factor IX_a was prepared by activating bovine factor IX with factor XI_a in a manner similar to the activation of human factor IX as described by DiScipio et al. (1978). Bovine factor XII_a was prepared as described by Fujikawa et al. (1977) and plasma kallikrein as described by Heimark et al. (1980). All the enzymes were shown to be homogeneous by gel electrophoresis in sodium dodecyl sulfate ($NaDodSO_4$).[3] The 4,4′-dithiodipyridine (Aldrithiol-4) was purchased from Aldrich Chemical Co., Milwaukee, WI. All common chemicals and solvents were reagent grade.

The active site concentrations of all enzymes were determined by titration with *p*-nitrophenyl *p*-guanidinobenzoate, purchased from Sigma Chemical Co. The procedure of Chase & Shaw (1967) was slightly modified, and the titrations were carried out in 0.10 M Hepes, pH 7.5, and 10 mM $CaCl_2$ at 25 °C. Smith (1973) reports that titration of factor X_a by the original procedure gave results that were 8–10% low. Based on the factor X_a extinction, we obtained activity measurements of 100, 100, and 97% on three separate titrations. These results were also checked by use of a tritium-labeled irreversible inhibitor for factor X_a.

Synthesis. *N*-Carbobenzyloxyphenylalanine and *N*-carbobenzyloxyarginine hydrochloride were synthesized by using standard procedures. *N*-Carbobenzyloxyalanine and tryptophan were purchased from Eastman Organic Chemicals, Rochester, NY. *N*-Carbobenzyloxyglycine and asparagine were purchased from Sigma Chemical Co., St. Louis, MO, and *N*-carbobenzyloxythreonine was from Vega Biochemicals, Tucson, AZ. All other *N*-carbobenzyloxy amino acids and *tert*-butyloxycarbonyl amino acids were obtained from Bachem Inc., Torrance, CA. Benzyl mercaptan, 3-methyl-1-butanethiol, 2-methyl-1-propanethiol, and pentachlorophenol were obtained from Aldrich Chemical Co., and 2-methyl-1-butanethiol was from Fairfield Chemical Co., Blythewood, SC. 4-Nitroaniline and imidazole were purchased from Eastman Organic Chemicals, and 7-amino-4-methylcoumarin was a generous gift from Dr. Morris Zimmerman at the Merck Institute of Therapeutic Research, Rahway, NJ. The EM LiChroprep Si60 silica gel chromatography columns were obtained from Curtin-Matheson Scientific, Inc., Atlanta, GA. All NMR spectra were obtained on a Varian T60A instrument and were consistent with the assigned structures. Elemental analyses were performed by Atlantic Microlabs of Atlanta, GA. TLC of the peptides was carried out on silica gel plates, which were developed in 2:1 chloroform–methanol (system 1) or 10:1 chloroform–methanol (system 2).

Arginine-containing products were visualized by using the Sakaguchi reaction (0.05% α-naphthol and 12.5% urea in 47.5% ethanol followed by 5% aqueous NaOBr). It was observed that *tert*-butyloxycarbonylarginine hydrochloride gave a very weak positive Sakaguchi test, but all final products gave a strong pink color when sprayed with the reagents. The plates were then heated on a hot plate to visualize all organic components.

The syntheses of some representative thioesters are given below. The syntheses of all other new compounds are described under Supplementary Material.

tert-Butyloxycarbonylarginine Isopentyl Thioester Hydrochloride. *tert*-Butyloxycarbonylarginine hydrochloride hydrate (3.29 g, 10.0 mmol) and 1-hydroxybenzotriazole (HOBt) (0.14 g, 1.0 mmol) were dissolved in DMF and cooled to 0 °C with stirring. To this was added 3-methyl-1-butanethiol (2.5 mL, 20 mmol) followed by dicyclohexylcarbodiimide (DCC) (2.46 g, 12.0 mmol). After the solution was stirred overnight at 0 °C, 4 mmol of dicyclohexylcarbodiimide was added, and the reaction proceeded for another 24 h at 0 °C. The dicyclohexylurea (DCU) was filtered, and the solvent and excess thiol were removed in vacuo. The yellow oil was chromatographed on a silica gel column equilibrated with $CHCl_3$ and eluted with increasing percentages of methanol. The product eluted with 7.5% methanol–chloroform, and the solvent was removed in vacuo. The residual material was recrystallized from chloroform–ether to yield a very hygroscopic white solid (1.22 g, 30.7%); single spot on TLC, R_f^1 = 0.86. Anal. Calcd for $C_{16}H_{33}N_4O_3SCl\cdot0.5H_2O$: C, 47.34; H, 8.44; N, 13.80. Found: C, 47.21; H, 8.47; N, 13.79.

tert-Butyloxycarbonylarginine sec-Pentyl Thioester Hydrochloride. This compound was prepared from *tert*-butyloxycarbonylarginine hydrochloride and 2-methyl-1-butanethiol by using the DCC–HOBt procedure as described above. Silica gel chromatography was used to purify the product, but it would not crystallize. The product was isolated and used as a white foam (19%); single spot on TLC, R_f^1 = 0.86. Anal. Calcd for $C_{16}H_{33}N_4O_3SCl\cdot1.5H_2O$: C, 45.32; H, 8.56; N, 13.21. Found: C, 45.32; H, 8.57; N, 13.21.

tert-Butyloxycarbonylarginine Isobutyl Thioester Hydrochloride. *tert*-Butyloxycarbonylarginine hydrochloride hydrate (6.58 g, 20 mmol) was dissolved in DMF and dried overnight over $MgSO_4$. The solution was filtered to remove the $MgSO_4$ and was stirred at room temperature, protected by a $CaCl_2$ drying tube. 1,1′-Carbonyldiimidazole (3.24 g, 20 mmol) was added, and the solution was stirred for 1 h. 2-Methyl-1-propanethiol (2.38 mL, 22 mmol) was then added, and the reaction was stirred for 3 h at room temperature. The DMF was removed, and the product was purified on an EM silica gel 60 column (size B) with 7% methanol–chloroform. The tubes containing only product were combined, and the solvent was removed to yield a light yellow foam (3.17 g, 40.8%). This was then rechromatographed on the same column with 6% methanol–chloroform; single spot on TLC, R_f^1 = 0.86. Anal. Calcd for $C_{15}H_{31}N_4O_3SCl\cdot2.5H_2O$: C, 42.10; H, 8.48; N, 13.09. Found: C, 42.01; H, 8.50; N, 13.07.

tert-Butyloxycarbonylarginine Benzyl Thioester Hydrochloride. *tert*-Butyloxycarbonylarginine hydrochloride hydrate and benzyl mercaptan were coupled by using the DCC–HOBt

[3] Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Me_2SO, dimethyl sulfoxide, Boc, *tert*-butyloxycarbonyl; Z, carbobenzyloxy; Tos, 4-toluenesulfonyl; Pe-*i*, isopentyl; Pe-*s*, *sec*-pentyl; Bzl, benzyl; H-SVal-OH, $HSCH[CH(CH_3)_2]CO_2H$; H-SIle-OH, $HSCH[CH(CH_3)CH_2CH_3]CO_2H$; Ac, acetyl; H-AMC, 7-amino-4-methylcoumarin; TLC, thin-layer chromatography; HOBt, *N*-hydroxybenzotriazole; DMF, dimethylformamide; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCU, *N*,*N*′-dicyclohexylurea; NA, 4-nitroanilide; $NaDodSO_4$, sodium dodecyl sulfate.

procedure as described above and chromatographed on an EM silica gel 60 column (size C) with 5% methanol–chloroform. A white foam was isolated after the solvent was removed (49.3%); single spot on TLC, $R_f^1 = 0.86$. Anal. Calcd for $C_{18}H_{29}N_4O_3SCl \cdot 0.5H_2O$: C, 50.22; H, 7.14; N, 13.02. Found: C, 50.33; H, 7.15; N, 13.34.

Carbobenzyloxyglycylarginine Benzyl Thioester Hydrochloride. tert-Butyloxycarbonylarginine benzyl thioester hydrochloride (156 mg, 0.375 mmol) was deblocked by using 2.5 N HCl–dioxane (3 mL). After 3 h, the solvent was removed, and the resulting foam was dried for 3 h over P_2O_5 and NaOH. To this were added carbobenzyloxyglycine pentachlorophenyl ester (189 mg, 0.412 mmol) and imidazole (25.5 mg, 0.375 mmol), followed by 7 mL of DMF. After dissolution of the components, the reaction sat at room temperature overnight. The DMF was removed, and the residue was purified by liquid chromatography (LC) on an EM silica gel 60 column (size A) with 5.5% methanol–chloroform. The product was then isolated as a white foam (68%); single spot on TLC, $R_f^1 = 0.85$. Anal. Calcd for $C_{23}H_{30}N_5O_4SCl \cdot H_2O$: C, 52.51; H, 6.13; N, 13.35. Found: C, 52.67; H, 5.90; N, 13.66.

Carbobenzyloxytryptophanylarginine Benzyl Thioester Hydrochloride. Carbobenzyloxytryptophan pentachlorophenyl ester and deblocked *tert*-butyloxycarbonylarginine benzyl thioester hydrochloride were coupled in DMF as described above. Purification was by LC on a size B EM silica gel 60 column, and the product was isolated as a light yellow foam (50%); single spot on TLC, $R_f^1 = 0.85$. Anal. Calcd for $C_{32}H_{37}N_6O_4SCl \cdot H_2O$: C, 58.64; H, 5.99; N, 12.83. Found: C, 58.32; H, 5.83; N, 12.94.

Kinetic Measurements. The enzymatic hydrolysis of the thioesters was measured with 4,4′-dithiodipyridine (Grassetti & Murray, 1967; Castillo et al., 1979). Assays were conducted at 30 °C in 0.10 M Hepes buffer, pH 7.5, containing 0.01 M $CaCl_2$ and 9.8% v/v Me_2SO. Stock solutions of the substrates were prepared in Me_2SO, as all exhibited slow hydrolysis in aqueous buffers. Aliquots of the stock solution were diluted with buffer immediately before use. To a cuvette containing 2.0 mL of the substrate solution was added 150 μL of a 2.0 mM solution of 4,4′-dithiodipyridine in the same buffer and Me_2SO concentration. The instrument was zeroed, and the initial rate of hydrolysis was measured spectrophotometrically at 324 nm after adding 50 μL of enzyme. After the initial rates were obtained, 20 μL of a 10^{-5} M (or greater) trypsin solution was added in order to complete the reaction within several minutes. The final absorbance was read, and the substrate concentration was calculated from the A_∞ reading ($\epsilon_{324} = 19800\ M^{-1}\ cm^{-1}$). The background hydrolysis rate was negligible for all enzymes except bovine factor IX_a, factor XI_a, and factor XII_a where enzymatic rates were slow. For these enzymes, a reference cell containing substrate and 4,4′-dithiodipyridine was used for the kinetic measurements. All initial slopes were converted into molar rates, and K_m and k_{cat} values were obtained by using Lineweaver–Burk plots. Most correlation coefficients were 0.999 or greater, and none were lower than 0.97.

The enzymatic hydrolysis of Z-Trp-Arg-NA was followed spectrophotometrically at 410 nm. All assays were conducted at 30 °C in 0.10 M Tris buffer, pH 7.5, containing 5 mM $CaCl_2$ and 9.8% v/v Me_2SO. For thrombin, factor IX_a, factor XI_a, and factor XII_a, absorbance readings were taken over a period of 19–20 h. No significant background hydrolysis was observed in a control lacking enzyme. Molar rates were obtained from initial slopes ($\epsilon_{410} = 8800\ M^{-1}\ cm^{-1}$), and K_m and k_{cat} values were calculated.

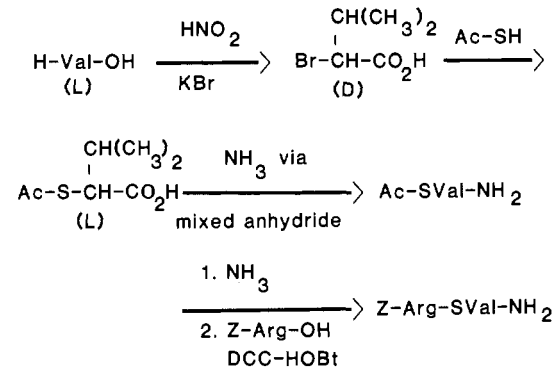

FIGURE 1: Scheme used in the synthesis of Z-Arg-SVal-NH_2.

The hydrolysis of Z-Trp-Arg-AMC was followed spectrofluorometrically at excitation and emission wavelengths of 370 and 460 nm, respectively (Castillo et al., 1979). Assays were conducted at 25 °C in Tris buffer, pH 7.5, containing 5 mM $CaCl_2$ and 9.5% v/v (factor XI_a) or 9.8% v/v (other enzymes) Me_2SO. For thrombin, factor IX_a, and factor XII_a, measurements were made over a period of 3 h, recording initial and final fluorescence values. Initial slopes were converted into molar rates by using a standard curve obtained by measuring the fluorescence of known amounts of H-AMC under identical conditions. For all fluorescence measurements, the instrument was standardized by setting a 1.25 μM solution of quinine sulfate in 0.1 N H_2SO_4 equal to 1.0 fluorescence unit. With the aminomethylcoumarin substrate as well, no background hydrolysis was observed, and K_m and k_{cat} values were calculated as described above.

All ultraviolet and visible measurements were performed with a Beckman Model 35 spectrophotometer and recorder, and fluorescence measurements were obtained with a Perkin-Elmer Model 204-S fluorescence spectrophotometer and recorder.

Results

Synthesis. The synthesis of a number of simple blocked arginine thioesters was accomplished by coupling a blocked arginine derivative with a thiol by using the HOBt–DCC or the 1,1′-carbonyldiimidazole methods. Di- and tripeptide thiol esters were prepared by coupling blocked amino acids or peptides with H-Arg-SR (usually prepared by deblocking Boc-Arg-SR with HCl in dioxane). Coupling was usually accomplished with the pentachlorophenyl ester method to minimize racemization, and products were purified to homogeneity by liquid chromatography on silica gel. The preparation of some thioesters of Tos-Arg-OH has been described (Plattner et al., 1979; Herrin et al., 1980), but no other arginine thioester derivatives have yet been reported. The acid chloride method utilized for the preparation of Tos-Arg-SR would not be applicable to most other arginine thioesters.

The key to the synthesis of the P′-extended thioester substrates was the conversion of an α-amino group to an α-thiol, with an overall retention of configuration. Figure 1 shows the synthetic scheme for the substrate Z-Arg-SVal-NH_2. It has been reported that the diazotization of valine followed by nucleophilic attack leads to an inversion of configuration (Levene et al., 1927). The same source reported inversion of the same center upon displacement of a Br with KSH. The optical rotations ($[\alpha]_D$) of the compounds L-Val-OH (+28.3°), L-Ile-OH (+39.5°), D-BrVal-OH (−14°), D-BrIle-OH (−21°), L-Ac-SVal-OH (+43°), and L-Ac-SIle-OH (+22°) seem to support a double inversion in each case, with overall retention of configuration in the final product. Although 100% optical purity cannot be proven, there is strong evidence that the

Table I: Kinetic Constants for the Hydrolysis of Amino Acid and Dipeptide Thioester Substrates by Bovine Thrombin, Factor IX_a, Factor X_a, Factor XI_a, Factor XII_a, Plasma Kallikrein, and Trypsin[a]

substrate	kinetic constants[b]	enzyme						
		thrombin	factor IX_a	factor X_a	factor XI_a	factor XII_a	plasma kallikrein	trypsin
Boc-Arg-SBu-*i*	k_{cat}	18	no hydrolysis	78	47		160	39
	K_m	12		2600	350		180	18
	k_{cat}/K_m	1.5×10^6		3.0×10^4	1.4×10^5	1.0×10^4 [c]	8.6×10^5	2.1×10^6
Boc-Arg-SPe-*i*	k_{cat}	21	no hydrolysis	94	150	7.7	230	81
	K_m	11		3600	2400	270	850	20
	k_{cat}/K_m	1.9×10^6		2.6×10^4	6.2×10^4	2.8×10^4	2.7×10^5	4.1×10^6
Boc-Arg-SPe-*s*	k_{cat}	17	no hydrolysis	40	63	21	710	50
	K_m	7.1		1900	760	1900	3300	18
	k_{cat}/K_m	2.4×10^6		2.2×10^4	8.4×10^4	1.1×10^4	2.1×10^5	2.8×10^6
Boc-Arg-SBzl	k_{cat}	8.3	no hydrolysis	120	39	7.8	340	100
	K_m	5.2		1700	190	190	820	15
	k_{cat}/K_m	1.6×10^6		7.1×10^4	2.0×10^5	4.1×10^4	4.1×10^5	6.7×10^6
Z-Arg-SBu-*i*	k_{cat}	8.5	no hydrolysis	110	50	6.2	330	64
	K_m	9.3		1400	300	80	480	9.6
	k_{cat}/K_m	9.2×10^5		7.9×10^4	1.7×10^5	7.7×10^4	6.9×10^5	6.7×10^6
Z-Gly-Arg-SBu-*i*	k_{cat}	17	no hydrolysis	100	110	7.1	240	95
	K_m	30		750	960	320	290	21
	k_{cat}/K_m	5.6×10^5		1.3×10^5	1.2×10^5	2.2×10^4	8.2×10^5	4.5×10^6
Z-Ala-Arg-SBu-*i*	k_{cat}	27	no hydrolysis	46	61	0.86	190	42
	K_m	40		760	630	46	490	15
	k_{cat}/K_m	6.9×10^5		6.1×10^4	9.7×10^4	1.9×10^4	3.9×10^5	2.8×10^6
Z-Val-Arg-SBu-*i*	k_{cat}	7.6	no hydrolysis	11	78		290	49
	K_m	7.7		540	1300		810	12
	k_{cat}/K_m	9.9×10^5		2.0×10^4	6.1×10^4	3.2×10^3 [c]	3.6×10^5	4.0×10^6
Boc-Met-Arg-SBu-*i*	k_{cat}	2.8	no hydrolysis	18	31	0.76	41	13
	K_m	13		1500	440	900	100	5.6
	k_{cat}/K_m	2.1×10^5		1.2×10^4	7.0×10^4	8.5×10^2	4.0×10^5	2.4×10^6
Z-Phe-Arg-SBu-*i*	k_{cat}	12		24	52	9.8	140	57
	K_m	14		630	450	220	63	8.4
	k_{cat}/K_m	9.6×10^5	2.3×10^4 [c]	3.8×10^4	1.2×10^5	4.5×10^4	2.2×10^6	6.7×10^6
Z-Trp-Arg-SBu-*i*	k_{cat}	3.0		24	110	1.1	82	110
	K_m	950		750	990	57	37	17
	k_{cat}/K_m	3.1×10^3	9.2×10^4 [c]	3.1×10^4	1.1×10^5	1.9×10^4	2.2×10^6	6.3×10^6
Z-Trp-Arg-SBzl	k_{cat}	not determined		not determined	not determined	not determined	not determined	not determined
	K_m							
	k_{cat}/K_m		3.4×10^5 [c]					
Boc-Pro-Arg-SBu-*i*	k_{cat}	11	no hydrolysis	26	32	not determined		36
	K_m	5.4		3500	1800			17
	k_{cat}/K_m	2.0×10^6		7.3×10^3	1.8×10^4		2.2×10^4 [c]	2.1×10^6
Z-Pro-Arg-SBu-*i*	k_{cat}	22	no hydrolysis	46	30	4.8	26	61
	K_m	15		1300	820	330	310	63
	k_{cat}/K_m	1.5×10^6		3.7×10^4	3.6×10^4	1.5×10^4	8.4×10^4	9.6×10^5
Z-Ser-Arg-SBu-*i*	k_{cat}	4.6	no hydrolysis	18	54		100	89
	K_m	33		530	510		280	19
	k_{cat}/K_m	1.4×10^5		3.3×10^4	1.1×10^5	2.8×10^3 [c]	3.7×10^5	4.7×10^6
Z-Thr-Arg-SBu-*i*	k_{cat}	2.7	no hydrolysis	6.6	54		120	29
	K_m	18		630	710		640	4.6
	k_{cat}/K_m	1.4×10^5		1.1×10^4	7.6×10^4	2.0×10^3 [c]	2.0×10^5	6.3×10^6
Z-Asn-Arg-SBu-*i*	k_{cat}	3.8	no hydrolysis	8.6	20	no hydrolysis	15	54
	K_m	40		620	210		250	60
	k_{cat}/K_m	9.4×10^4		1.4×10^4	9.6×10^4		5.9×10^4	9.0×10^5
Z-Glu-Arg-SBu-*i*	k_{cat}	5.1	no hydrolysis	7.7	23			14
	K_m	450		2300	190			27
	k_{cat}/K_m	1.2×10^4		3.4×10^3	1.2×10^5	5.6×10^2 [c]	8.2×10^3 [c]	5.0×10^5
Z-Lys-Arg-SBu-*i*	k_{cat}	7.5	no hydrolysis	19	13	1.7	73	18
	K_m	270		1500	130	81	90	7.1
	k_{cat}/K_m	2.8×10^4		1.3×10^4	1.0×10^5	2.2×10^4	8.1×10^5	2.5×10^6

[a] Hepes buffer (0.1 M), pH 7.5, 0.01 M $CaCl_2$, and 9.8% v/v Me_2SO at 30 °C. Enzyme concentrations: 2.3–4.6 nM thrombin, 0.59–1.8 nM factor IX_a, 5.5–55 nM factor X_a, 0.57–0.73 nM factor XI_a, 0.53–0.66 nM factor XII_a, 0.11 nM plasma kallikrein, and 0.25–2.7 nM trypsin. [b] The units of k_{cat}, K_m, and k_{cat}/K_m are s^{-1}, μM, and M^{-1} s^{-1}, respectively. [c] The Lineweaver–Burk plot went through the origin, and only k_{cat}/K_m could be determined.

proper optical isomer was synthesized with high optical purity.

Thioester Hydrolysis. The kinetic parameters for the hydrolysis of a number of amino acid, dipeptide, and tripeptide thioesters by bovine thrombin, factor IX_a, factor X_a, factor XI_a, factor XII_a, plasma kallikrein, and trypsin are reported in Tables I and II. When it was observed that Z-Phe-Arg-SBu-*i* and Z-Trp-Arg-SBu-*i* were the only substrates hydrolyzed by factor IX_a and were among the best substrates for factor XI_a, the corresponding 4-nitroanilide (Z-Trp-Arg-NA) and 7-amino-4-methylcoumarin (Z-Trp-Arg-AMC) substrates were also investigated (Table III). Longer peptide thioesters with sequences corresponding to the sequence at the reactive site of antithrombin III are reported in Table IV. Table V lists kinetic constants for the hydrolysis of P′-extended peptide thioester substrates with the various coagulation enzymes studied.

Table II: Kinetic Constants for the Hydrolysis of Tripeptide Thioester Substrates by Bovine Thrombin, Factor IX_a, Factor X_a, Factor XI_a, and Trypsin[a]

substrate P_4 P_3 P_2 P_1	kinetic constants[b]	enzyme: thrombin	factor IX_a	factor X_a	factor XI_a	trypsin
Boc-Phe-Ser-Arg-SBzl	k_{cat}	25	no hydrolysis	700	37	54
	K_m	130		9500	310	9.4
	k_{cat}/K_m	2.0×10^5		7.4×10^4	1.2×10^5	5.8×10^6
Boc-Leu-Ser-Arg-SBzl	k_{cat}	13	no hydrolysis	130	47	65
	K_m	92		2200	570	11
	k_{cat}/K_m	1.4×10^5		5.9×10^4	8.2×10^4	5.8×10^6
Boc-Leu-Thr-Arg-SBzl	k_{cat}	5.1	no hydrolysis	160	25	40
	K_m	22		4000	230	7.0
	k_{cat}/K_m	2.4×10^5		4.1×10^4	1.1×10^5	5.7×10^6
Boc-Val-Val-Arg-SBzl	k_{cat}	27	no hydrolysis	25	65	21
	K_m	130		870	2600	6.6
	k_{cat}/K_m	2.1×10^5		2.8×10^4	2.5×10^4	3.2×10^6
Boc-Val-Phe-Arg-SBzl	k_{cat}	13		140	29	55
	K_m	39		4000	650	12
	k_{cat}/K_m	3.4×10^5	3.7×10^3[c]	3.6×10^4	4.4×10^4	4.6×10^6
Boc-Val-Trp-Arg-SBzl	k_{cat}	12		120	34	34
	K_m	96		3500	850	12
	k_{cat}/K_m	1.2×10^5	1.2×10^4[c]	3.5×10^4	4.0×10^4	2.7×10^6
Boc-Phe-Phe-Arg-SBzl	k_{cat}	13		64	40	32
	K_m	11		1600	760	8.2
	k_{cat}/K_m	1.2×10^6	2.8×10^4[c]	4.1×10^4	5.3×10^4	3.9×10^6
Boc-Phe-Trp-Arg-SBzl	k_{cat}	16		66	28	37
	K_m	17		1500	470	8.6
	k_{cat}/K_m	9.1×10^5	5.7×10^4[c]	4.4×10^4	6.0×10^4	4.3×10^6
Boc-Trp-Phe-Arg-SBzl	k_{cat}	13		49	50	28
	K_m	37		1300	800	8.6
	k_{cat}/K_m	3.5×10^5	7.0×10^4[c]	3.7×10^4	6.3×10^4	3.3×10^6
Boc-Trp-Trp-Arg-SBzl	k_{cat}	8.4		36	31	48
	K_m	31		580	430	9.1
	k_{cat}/K_m	2.7×10^5	7.2×10^4[c]	6.1×10^4	7.3×10^4	5.3×10^6

[a] Hepes buffer (0.1 M), pH 7.5, 0.01 M $CaCl_2$, and 9.8% v/v Me_2SO at 30 °C. [b] The units of k_{cat}, K_m, and k_{cat}/K_m are s^{-1}, μM, and M^{-1} s^{-1}, respectively. [c] The Lineweaver–Burk plot went through the origin, and only k_{cat}/K_m could be determined.

Table III: Kinetic Constants for the Hydrolysis of Z-Trp-Arg-NA and Z-Trp-Arg-AMC by Bovine Thrombin, Factor IX_a, Factor X_a, Factor XI_a, Factor XII_a, Kallikrein, and Trypsin

substrate	enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1}/s^{-1})
Z-Trp-Arg-NA[a]	thrombin	0.15	0.0076	5.0×10^1
	factor IX_a			1.1×10^2[c]
	factor X_a	0.56	6.4	1.1×10^4
	factor XI_a			1.0×10^3[c]
	factor XII_a	0.084	0.00018	2.8
	kallikrein	0.37	8.4	2.2×10^4
	trypsin	0.45	2.9	6.4×10^3
Z-Trp-Arg-AMC[b]	thrombin	0.18	0.053	2.9×10^2
	factor IX_a	0.066	0.0048	7.3×10^1
	factor X_a	0.13	1.4	1.0×10^4
	factor XI_a			5.1×10^3[c]
	factor XII_a	0.065	0.000036	5.5×10^{-1}
	kallikrein	0.32	0.19	5.9×10^2
	trypsin	0.048	6.8	1.4×10^5

[a] Tris buffer (0.1 M), pH 7.5, 5 mM $CaCl_2$, and 9.8% v/v Me_2SO at 30 °C. [b] Tris buffer (0.1 M), pH 7.5, 5 mM $CaCl_2$, and 9.5 or 9.8% v/v Me_2SO at 25 °C. [c] The Lineweaver–Burk plot went through the origin, and only k_{cat}/K_m could be determined.

The rates of thioester hydrolysis were measured by the reaction of the released thiol with 4,4′-dithiodipyridine contained in the assay mixture. The product of this reaction, 4-thiopyridone, has an extremely high extinction coefficient (ϵ_{324} = 19 800 M^{-1} cm^{-1}) which allows the rate to be measured continuously with high sensitivity. In addition, the thioesters are only slowly hydrolyzed in aqueous buffers, and the background rates were negligible except in a few cases of slow enzymatic hydrolysis.

The rates of reaction of four different thiol leaving groups with 4,4′-dithiodipyridine were also studied in order to show that this was not the rate-limiting step in the coupled assay. The following rates were obtained for the reaction of the four thiols (thiol concentration in parentheses) with 4,4′-dithiodipyridine (1.8 mM): HSBzl (7.4 μM), 3.9×10^{-4} M s^{-1}; HSBu-*i* (6.4 μM), 3.4×10^{-4} M s^{-1}; HSPe-*i* (6.2 μM), 2.8×10^{-4} M s^{-1}; HSPe-*s* (5.9 μM), 3.1×10^{-4} M s^{-1}. On the other hand, the fastest enzymatic hydrolysis rate obtained with any of the enzymes was 2.6×10^{-6} M s^{-1} observed during the study of the trypsin-catalyzed hydrolysis of Z-Pro-Arg-SBu-*i*. Thus, in all cases, the rate-limiting step is the hydrolysis of the substrate by the enzyme, and not the subsequent reaction of the thiol with 4,4′-dithiodipyridine. Of course, circumstances can be imagined where the thioester would be hydrolyzed faster than the thiol could react with 4,4′-dithiodipyridine. Such conditions might be observed when using extremely high concentrations of the more reactive enzymes such as trypsin. Investigators who utilize thioester substrates under those circumstances should check that the enzymatic hydrolysis rates are much less than the rates of reaction of the thiol with 4,4′-dithiodipyridine.

Since the thioester substrates are so sensitive, some investigators may wish to use them in blood plasma. The high concentration of serum albumin might be expected to interfere with the assays due to the reaction of the thiol group of the albumin with the 4,4′-dithiodipyridine. The hydrolysis of Boc-Arg-SBzl and Z-Trp-Arg-SBu-*i* by bovine trypsin was measured in the presence of 1% bovine serum albumin (BSA) to determine its effect on the assay. With Boc-Arg-SBzl as substrate, 1% BSA increased the K_m from 15 to 26 μM and the k_{cat} from 100 to 110 s^{-1}, thus causing an overall decrease in k_{cat}/K_m from 6.7×10^6 to 4.3×10^6 M^{-1} s^{-1}. With Z-Trp-Arg-SBu-*i*, the K_m was raised from 17 to 39 μM and k_{cat} from 110 to 140 s^{-1}, giving a decrease in k_{cat}/K_m from 6.3 ×

Table IV: Kinetic Constants for the Enzymatic Hydrolysis of Peptide Thioester Substrates with Antithrombin III Reactive Site Sequences[a]

substrate P_5 P_4 P_3 P_2 P_1	kinetic constants[b]	enzyme: thrombin	factor IX_a	factor X_a	factor XI_a	factor XII_a	plasma kallikrein	trypsin
-Val-Ile-Ala-Gly-Arg-Ser[c]								
Z-Gly-Arg-SBzl	k_{cat}	27	5.5	110	4200	5.5	300	140
	K_m	6.6	690	830	9400	19	91	27
	k_{cat}/K_m	4.1×10^6	7.9×10^3	1.4×10^5	4.5×10^5	2.8×10^5	3.2×10^6	4.9×10^6
Z-Ala-Gly-Arg-SBzl	k_{cat}	49	22	150	13000	4.8	280	180
	K_m	30	1200	1600	52000	31	170	40
	k_{cat}/K_m	1.6×10^6	1.8×10^4	9.5×10^4	2.5×10^5	1.5×10^5	1.7×10^6	4.4×10^6
Z-Ile-Ala-Gly-Arg-SBzl	k_{cat}	33	120	210	170	4.5	260	110
	K_m	23	10000	1300	880	18	170	21
	k_{cat}/K_m	1.4×10^6	1.2×10^4	1.6×10^5	1.9×10^5	2.5×10^5	1.6×10^6	5.0×10^6
Z-Val-Ile-Ala-Gly-Arg-SBzl	k_{cat}	27	8.1	100	67	4.8	220	140
	K_m	18	600	800	550	33	200	33
	k_{cat}/K_m	1.5×10^6	1.3×10^4	1.3×10^5	1.2×10^5	1.4×10^5	1.1×10^6	4.2×10^6

[a] Hepes buffer (0.1 M), pH 7.5, 0.01 M $CaCl_2$, and 9.8% v/v Me_2SO at 30 °C. [b] The units of k_{cat}, K_m, and k_{cat}/K_m are s^{-1}, μM, and M^{-1} s^{-1}, respectively. [c] This is the sequence of human antithrombin III at positions 381–386 (Petersen et al., 1979) which is homologous with the reactive site of the α_1-protease inhibitor (α_1-antitrypsin) (Carrell et al., 1980). Bovine antithrombin III is cleaved by thrombin at a site homologous to Arg-385 (Jornvall et al., 1979).

Table V: Kinetic Constants for the Hydrolysis of P′-Extended Thioester Substrates by Bovine Thrombin, Factor IX_a, Factor X_a, Factor XI_a, Factor XII_a, Plasma Kallikrein, and Trypsin[a]

substrate P_1 P_1' P_2'	kinetic constants[b]	enzyme: thrombin	factor IX_a	factor X_a	factor XI_a	factor XII_a	plasma kallikrein	trypsin
Z-Arg-SVal-NH_2	k_{cat}	8.9	no hydrolysis[c]	14				30
	K_m	10		460				11
	k_{cat}/K_m	8.8×10^5		3.0×10^4	3.0×10^4 [d]	7.9×10^3	9.1×10^4	2.9×10^6
Z-Arg-SIle-NH_2	k_{cat}	9.6	no hydrolysis	1.0				29
	K_m	16		22				15
	k_{cat}/K_m	6.1×10^5		4.5×10^4	5.2×10^4	1.7×10^4	4.3×10^4	2.0×10^6
Z-Arg-SVal-Val-NH_2	k_{cat}	51	no hydrolysis		no hydrolysis	no hydrolysis	11	64
	K_m	940					1000	530
	k_{cat}/K_m	5.4×10^4		780			1.1×10^4	1.2×10^5
Z-Arg-SIle-Val-NH_2	k_{cat}	1.5	no hydrolysis	0.68	no hydrolysis	no hydrolysis		13
	K_m	64		740				30
	k_{cat}/K_m	2.4×10^4		920			2.7×10^4	4.3×10^5
Z-Arg-SIle-Ile-NH_2	k_{cat}	0.73	no hydrolysis	no hydrolysis	no hydrolysis	no hydrolysis	7.9	7.8
	K_m	58					1200	110
	k_{cat}/K_m	1.2×10^4					6.5×10^4	7.1×10^4

[a] Hepes buffer (0.1 M), pH 7.5, 0.01 M $CaCl_2$, and 9.8% v/v Me_2SO at 30 °C. [b] The units of k_{cat}, K_m, and k_{cat}/K_m are s^{-1}, μM, and M^{-1} s^{-1}, respectively. [c] No measurable hydrolysis observed at the enzyme concentration used. [d] The Lineweaver–Burk plot went through the origin, and only k_{cat}/K_m could be determined.

10^6 to 3.4×10^6 M^{-1} s^{-1}. In spite of the high initial absorbance caused by the free sulfhydryl group in BSA, no difficulties were encountered in obtaining good initial hydrolysis rates under the conditions used. Thus, the only noticeable effect appears to be a slight modification of the kinetic parameters. Under most conditions, dilution of a plasma sample into the assay buffer would result in a BSA concentration much less than 1% in the assay, and little effect on the hydrolysis rates would be expected.

Kinetics. The kinetic parameters refer to the following well-established scheme for serine proteases:

$$E + S \rightleftarrows E{\cdot}S \xrightarrow{k_2} E{\cdot}S' + P_1 \xrightarrow{k_3} E + P_2$$

where E·S′ is the acyl enzyme. The Michaelis constant, K_m, is then equal to $K_S k_3/(k_2 + k_3)$, k_{cat} is equal to $k_2 k_3/(k_2 + k_3)$, and k_{cat}/K_m (the specificity constant) is equal to k_2/K_s (Bender, 1971). With many esters and thioesters, $k_2 > k_3$, and k_{cat} becomes the rate-limiting deacylation rate k_3 (Hirohara et al., 1977). K_m equals $k_3 K_S/k_2$ and represents a lower limit for K_S (the dissociation constant of E·S). With amides, $k_3 > k_2$, k_{cat} equals the rate-limiting acylation rate k_2, and K_m becomes equal to the true binding constant K_S.

Nonproductive binding of the substrates would result in a decrease in both k_{cat} and K_m (Fersht, 1977). However, k_{cat}/K_m would be unaltered. Thus, the specificity constant $k_{cat}/K_m = k_2/K_S$ represents the most useful parameter for comparing the reactivity of the various thioester and amide substrates.

The acyl enzymes formed upon reaction of Boc-Arg-X, where X = SBu-*i*, SPe-*i*, SPe-*s*, and SBzl, with any particular enzyme would be identical. Examination of the data for the hydrolysis of Boc-Arg-X shows that the majority of the k_{cat} values obtained with individual enzymes are similar. This would be expected if k_{cat} represented deacylation rates for identical acyl enzymes. However, a few of the values are much different than the rest. For example, the k_{cat} for the hydrolysis of Boc-Arg-SPe-*i* by trypsin is 2.5 times greater than the average of the other three Boc-Arg-X derivatives, and the k_{cat} for the hydrolysis of Boc-Arg-SBzl by thrombin is less than one-half the value of the average of the other three. This variation could be due either to a change of the rate-limiting step in the case of some thioesters or to nonproductive binding modes for some of the thioesters. Nonproductive binding could explain the one low k_{cat} value obtained with thrombin but not the few higher k_{cat} values obtained with the other enzymes. It is thus more likely that acylation is the rate-limiting step

Table VI: Major Sites of Cleavage of Natural Substrates by Blood Coagulation Factors

enzyme	cleavage sites P_5 P_4 P_3 P_2 P_1 P_1' P_2' P_3' P_4'	substrate	ref
bovine thrombin		bovine fibrinogen	
	Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Leu	α chain	Timpl et al. (1977)
	Gly-Leu-Gly-Ala-Arg-Gly-His-Arg-Pro	β chain	Blomback et al. (1965)
bovine factor XI_a	Asp-Glu-Phe-Ser-Arg-Val-Val-Gly-Gly	bovine factor IX	Katayama et al. (1979)
	Lys-Lys-Leu-Thr-Arg-Ala-Glu-Thr-Ile		
bovine factor IX_a	Ser-Gln-Val-Val-Arg-Ile-Val-Gly-Gly	bovine factor X	Titani et al. (1975)
bovine factor X_a	Tyr-Ile-Glu-Gly-Arg-Ile-Val-Glu-Gly	bovine prothrombin	Magnusson et al. (1975)
	Ala-Ile-Glu-Gly-Arg-Thr-Ser-Glu-Asp		
bovine factor XII_a	Ile-Val-Gly-Gly	bovine factor XI	Kurachi et al. (1980)
bovine plasma kallikrein	Arg-Val-Val-Gly-Gly	bovine factor XII	Fujikawa et al. (1980)
	Phe-Ser-Pro-Phe-Arg-Ser-Val-Gln-Val	high mol wt kininogen[a]	Han et al. (1976)
	His-Tyr-Asp-Trp-Arg-Thr-Pro-Tyr-Leu		

[a] Two other cleavage sites were also reported.

for a few of the thioesters. Such a change in the rate-limiting step has previously been observed in the chymotrypsin-catalyzed hydrolysis of simple alkyl esters of benzoylglycine (Epand & Wilson, 1963).

The kinetics observed with factor IX_a at this stage are difficult to interpret. Since factor IX_a hydrolyzed its substrates much more slowly than the other enzymes, there is considerably more error in the results. In addition, factor IX_a is inactivated by free thiols, the rate of which depends on the structure of the thiol (Lundblad & Kingdon, 1974). We observed experimentally that the hydrolysis rates decreased as the reaction proceeded, and we used only initial rates in our studies. All of the thioester substrates (Tables I and II) and the nitroanilide substrate gave Lineweaver–Burk plots which went through the origin or curved. It appears that the substrates are activating the enzyme at higher substrate concentrations. We have reported k_{cat}/K_m values for factor IX_a for the higher substrate concentrations investigated. The values are 2–4-fold lower at the low substrate concentrations and at this stage can only be considered rough approximations. The anomalous kinetic behavior of factor IX_a could also be due to the absence of essential cofactors such as phospholipids in the reaction mixture. We plan to study in greater depth the effect of cofactors and substrate concentration on the catalytic activity of factor IX_a and will report the results in future papers. The aminomethylcoumarin substrate and the antithrombin III sequence substrates (Table IV) did not show substrate activation and were the only ones tested with measurable K_m values.

Discussion

Although simple synthetic peptide substrates are known for many of the blood clotting enzymes, the subsite specificity of the coagulation factors has not been extensively studied [see Huseby & Smith (1980) for a recent review]. Thrombin has been studied most widely, using peptide substrates (Liem & Scheraga, 1974; Meinwald et al., 1980), peptide esters (Takasaki et al., 1975), peptide 4-nitroanilides (Svedsen et al., 1972; Aurell et al., 1977), peptide β-naphthylamides (Szczeklik et al., 1968), and peptide aminomethylcoumarin derivatives (Morita et al., 1977). Bovine factor X_a, one of the more common coagulation factors, has also been studied with peptide esters (Adams & Elmore, 1971; Lonsdale-Eccles et al., 1980), peptide 4-nitroanilides (Aurell et al., 1977; Suomela et al., 1977), and peptide amides of aminomethylcoumarin (Morita et al., 1977). Plasma kallikrein has been studied extensively with peptide 4-nitroanilides (Claeson et al., 1978) and peptide aminomethylcoumarin derivatives (Morita et al., 1977). Only a few of these papers, however, report K_m and k_{cat} values. Bovine factor XI_a hydrolyzes Tos-Arg-OMe and Bz-Phe-Val-Arg-NA weakly (Kurachi & Davie, 1977), bovine factor IX_a hydrolyzes Bz-Arg-OEt (Byrne & Castellino, 1978; Byrne et al., 1980), and human factor XII_a and factor XII_a fragment hydrolyze 4-nitroanilides such as D-Pro-Phe-Arg-NA and Bz-Ile-Glu-Gly-Arg-NA (Hojima et al., 1980; Silverberg et al., 1980).

One of the goals of this research was to study the subsite specificity of the various blood coagulation enzymes and to compare the reactivities of the various enzymes. We were especially interested in studying factor IX_a, factor XI_a, and factor XII_a since little is known about the specificity of these three blood coagulation enzymes. Peptide esters or 4-nitroanilides were hydrolyzed very slowly or not at all by these enzymes. Accordingly, the use of these substrates requires large amounts of enzyme.

Simple amino acid and peptide thioesters were considered good choices for this investigation, because of the high reactivity of ester bonds as compared with amide bonds. The use of chromogenic thiol reagents such as Ellman's reagent (Ellman, 1959) or 4,4′-dithiodipyridine (Grassetti & Murray, 1967) allows the thiol hydrolysis product to be measured continuously with high sensitivity. For example, the tetrapeptide thioester MeO-Suc-Ala-Ala-Pro-Val-SBzl can be used to detect human leukocyte elastase concentrations as low as 2–6 pM (Castillo et al., 1979), and Z-Lys-SBzl is a sensitive assay substrate for trypsin, thrombin, factor X_a, and other trypsinlike enzymes (Green & Shaw, 1979).

The S_2 subsite of each enzyme was partially mapped by using dipeptide thioesters with various residues in the P_2 site. In the present work, 12 different P_2 residues were chosen so that all classes of amino acids normally found in proteins were represented. The S_1' subsite was investigated by using Boc-Arg-SR with different thiol leaving groups. The thiols HSBu-*i*, HSPe-*i*, and HSPe-*s* were chosen since they have the same structures as the side chain of a P_1' Val, Leu, or Ile, respectively (Figure 2). It was expected that the most reactive substrates would have sequences similar to or identical with those found in the natural protein substrates (Table VI).

Bovine Thrombin. Thrombin showed a wide range of reactivity with k_{cat}/K_m values varying by a factor of 200. The best amino acid peptide substrate was Boc-Arg-SPe-*s*, although the other amino acid peptide substrates were almost as active. The best dipeptide isobutyl thioester substrate for thrombin was Boc-Pro-Arg-SBu-*i* containing a P_2 Pro residue. Thrombin was less active with charged or polar P_2 residues. The P_2 Phe derivative was a good substrate, but the P_2 Trp substrate was much less reactive, indicating that this residue was apparently too bulky. The Boc group in the P_2 position was more reactive than most amino acids in this position. Indeed, Boc-Arg-SPe-*s* has a higher k_{cat}/K_m value than that of any

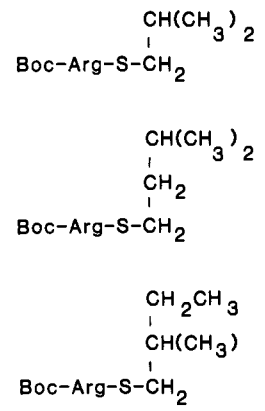

FIGURE 2: Structure of *tert*-butyloxycarbonylarginine isobutyl thioester (Boc-Arg-SBu-*i*), isopentyl thioester (Boc-Arg-SPe-*i*), and *sec*-pentyl thioester (Boc-Arg-SPe-*s*). The three thioesters are truncated analogues of substrates with Val, Leu, and Ile, respectively, in the P_1' position.

previously reported substrate for thrombin. The amide substrates Z-Trp-Arg-NA and Z-Trp-Arg-AMC were very poor substrates for thrombin, with k_{cat}/K_m values of 50 and 300 M^{-1} s^{-1}. This is not unexpected, since the corresponding thioester was also a poor substrate for thrombin.

Bovine Factor IX_a. Factor IX_a was the most specific and least reactive of the blood clotting enzymes. None of the simple arginine derivatives were hydrolyzed. The two dipeptide isobutyl thioesters (Table I) and the tripeptide isobutyl thioesters (Table II) that were hydrolyzed contained a P_2 Phe or Trp. The tripeptide substrate with the sequence Val-Val-Arg was not hydrolyzed. This sequence is present in the amino acid sequence of factor X, which is cleaved during its conversion to factor X_a by factor IX_a. The best tripeptide substrate for factor IX_a was Boc-Trp-Trp-Arg-SBzl. This substrate, however, was less reactive than the dipeptide substrate Z-Trp-Arg-SBu-*i*. Changing the thiol leaving group to benzyl mercaptan resulted in a 3-fold increase in k_{cat}/K_m, and Z-Trp-Arg-SBzl is the best factor IX_a thioester substrate which we prepared. Factor IX_a also hydrolyzed the amide substrates, although they were 200–300 times less reactive than the thioester substrates. These results indicate that the S_2 and S_3 subsites which bind Val residues in the natural substrate prefer hydrophobic or aromatic residues. However, it is apparent that P_3-P_1' residues in a substrate are not sufficient alone to form a good substrate for this enzyme.

Bovine Factor X_a. The best factor X_a substrates were Z-Gly-Arg-SBu-*i* and Z-Gly-Arg-SBzl which contain a P_2 Gly residue. These substrates were more than twice as reactive as any other dipeptide or tripeptide thioester substrate. Prothrombin, which is the natural substrate for factor X_a, also has a P_2 Gly residue. Factor X_a has a small S_2 subsite since the dipeptides with either a P_2 Gly or an Ala are 2–10 times more reactive than any of the other dipeptides. The k_{cat}/K_m value of Z-Gly-Arg-SBzl is significantly higher than those reported for lysine esters (Lonsdale-Eccles et al., 1980).

Factor X_a preferred the P_1' Val analogue Boc-Arg-SBu-*i* despite the fact that a P_1' Ile occurs in prothrombin. However, it can be seen that the Ile analogue has the lowest K_m of the three. Both amide derivatives were excellent substrates, with k_{cat}/K_m values only 3-fold lower than those for the corresponding thioester. The aminomethylcoumarin amide is probably one of the most sensitive substrates for factor X_a, although it cannot be compared with other aminomethylcoumarin derivatives of peptides until kinetic constants become available (Morita et al., 1977). Factor X_a also differs significantly from the other coagulation factors since it hydrolyzes the amide substrates nearly as fast as the thioesters.

Bovine Factor XI_a. Factor XI_a is much more reactive toward thioesters than either factors IX_a or X_a. Both factors XI_a and X_a are less specific than factor IX_a. The most sensitive thioester substrates for this enzyme were Boc-Arg-SBzl and Z-Gly-Arg-SBzl. Factor XI_a did not demonstrate any clear preference for the two dipeptides with a P_2 Ser or Thr residue. This is a little surprising since these two residues are present in the amino acid sequence of factor IX which is cleaved during its conversion to factor IX_a by factor XI_a. Excluding the P_2 Pro derivative, we determined that k_{cat}/K_m values only varied by a factor of three for the various thioester substrates hydrolyzed by factor XI_a. Specificity was exhibited in the P_1' residues, however, as the P_1' Val analogue (Boc-Arg-SBu-*i*) was significantly better than the Ile or Leu analogues. This was expected since the P_1' residue in factor IX which is cleaved by factor XI_a is Val. The tripeptide substrates were not more reactive than the dipeptide derivatives even though they were designed to match factor XI_a cleavage sites. Thus, it appears that the inclusion of a P_3 residue makes little or no contribution to the reactivity or specificity of factor XI_a. Factor XI_a also hydrolyzes the amide substrates, but the k_{cat}/K_m values were 20–100 times lower than those for the thioester.

Bovine Factor XII_a. Factor XII_a shows only moderate reactivity but a very high degree of specificity. The best dipeptide isobutyl thioester has a P_2 Phe residue. Although the amino acid sequence at the activation site of factor XI (factor XII_a's natural substrate) is not known, one of the best nitroanilide substrates (H-D-Pro-Phe-Arg-NA) for human factor XII_a and factor XII_a fragment also has a P_2 Phe (Hojima et al., 1980; Silverberg et al., 1980). One of the dipeptide thioesters, with a P_2 Asn residue, was not hydrolyzed at all. The other good substrates contain either small, positively charged, or aromatic residues. The thioester containing the P_1' Val analogue was the most reactive, and Val is found in the P_1' position at the activation site of factor XI. Factor XII_a hydrolyzed the amide substrates very poorly, with k_{cat}/K_m values 200–35 000 times lower than those for the corresponding thioester. The thioester Z-Phe-Arg-SBu-*i* is one of the more reactive factor XII_a substrates, although the tripeptide nitroanilide, H-D-Pro-Phe-Arg-NA, has a slightly higher reactivity with human factor XII_a [Silverberg et al. (1980) report k_{cat} = 15 s^{-1}, K_m = 0.190 mM, $k_{cat}/K_m = 7.9 \times 10^4$ M^{-1} s^{-1}].

Bovine Plasma Kallikrein. Kallikrein exhibits very high reactivity and specificity. Its best substrates, Z-Phe-Arg-SBu-*i* and Z-Trp-Arg-SBu-*i*, were only 3 times less reactive than the best trypsin substrate, and k_{cat}/K_m values for the dipeptide thioester substrates varied by a factor of 100. Kallikrein prefers aromatic residues in the P_2 position, and high molecular weight kininogen, one of the kallikrein's natural substrates, has a P_2 Phe or Trp residue. Kallikrein also demonstrated a marked specificity for the P_1' analogue Boc-Arg-SBu-*i*. This was not unexpected, as a Val residue is found in the P_1' position of bovine factor XII, another natural substrate of plasma kallikrein. The two amide substrates were hydrolyzed fairly well by plasma kallikrein, with k_{cat}/K_m values 100–400 times lower than those for the corresponding thioester. Z-Phe-Arg-SBu-*i* is probably one of the most reactive substrates for plasma kallikrein, although it is difficult to compare with data in the literature, since kinetic parameters are not given in most cases.

Bovine Trypsin. As expected, trypsin exhibited little specificity when compared with the coagulation factors and hydrolyzed all the thioester substrates quite well. The range in k_{cat}/K_m values for all the substrates was only 4–5-fold, ex-

Table VII: Relative k_{cat}/K_m Values for the Hydrolysis of the Synthetic Thioester Substrates[a]

substrate	thrombin	factor IX_a	factor X_a	factor XI_a	factor XII_a	plasma kallikrein	trypsin
Boc-Arg-SBu-*i*	50	0	1.0	4.7	0.33	219	70
Boc-Arg-SPe-*i*	63	0	0.87	2.1	0.93	9.0	140
Boc-Arg-SPe-*s*	80	0	0.73	2.8	0.37	7.0	93
Boc-Arg-SBzl	53	0	2.4	6.7	1.4	14	220
Z-Arg-SBu-*i*	31	0	2.6	5.7	2.6	23	220
Z-Gly-Arg-SBu-*i*	19	0	4.3	4.0	0.73	27	150
Z-Gly-Arg-SBzl	140	0.26	4.7	15	9.3	110	160
Z-Ala-Arg-SBu-*i*	23	0	2.0	3.2	0.63	13	93
Z-Val-Arg-SBu-*i*	33	0	0.67	2.0	0.11	12	130
Boc-Met-Arg-SBu-*i*	7.0	0	0.40	2.3	0.03	13	80
Z-Phe-Arg-SBu-*i*	29	0.77	1.3	4.0	1.5	73	220
Z-Trp-Arg-SBu-*i*	0.10	3.1	1.0	3.7	0.63	73	200
Z-Trp-Arg-SBzl	ND[b]	11	ND	ND	ND	ND	ND
Boc-Pro-Arg-SBu-*i*	67	0	0.24	0.60	ND	0.73	70
Z-Pro-Arg-SBu-*i*	50	0	1.2	1.2	0.50	2.8	32
Z-Ser-Arg-SBu-*i*	4.7	0	1.1	3.7	0.09	12	160
Z-Thr-Arg-SBu-*i*	4.7	0	0.37	2.5	0.07	6.7	210
Z-Asn-Arg-SBu-*i*	3.1	0	0.47	3.2	0	2.0	30
Z-Glu-Arg-SBu-*i*	0.40	0	1.1	4.0	0.02	0.27	17
Z-Lys-Arg-SBu-*i*	0.93	0	0.43	3.3	0.73	27	83
Z-Trp-Arg-NA	0.0017	0.0037	0.36	0.033	0	0.73	0.02
Z-Trp-Arg-AMC	0.0097	0.0024	0.33	0.17	0	0.02	3.6

[a] k_{cat}/K_m value for the hydrolysis of Boc-Arg-SBu-*i* by bovine factor X_a set at 1.0. The best substrate for each enzyme is underlined. [b] Not determined.

cluding the P_2 Glu substrate. The best substrates for trypsin contain a hydrophobic P_2 residue such as a carbobenzyloxy or a Phe residue. Trypsin is far less active toward substrates with a negative charge in the P_2 position (Glu). In contrast, the positively charged Lys derivative was quite reactive. The P_1' Leu analogue (Boc-Arg-SPe-*i*) was significantly more reactive than either the P_1' Val or Ile analogues. This effect is in k_{cat} alone. Thus, it is possible that the thioester linkage in Boc-Arg-SPe-*i* is slightly less sterically hindered than those of the other two derivatives. This suggestion is supported by the observation that the background hydrolysis rate (buffer alone) for Boc-Arg-SPe-*i* was noticeably higher than the rates for the other two thioesters.

Trypsin also readily hydrolyzed the amide substrates, although k_{cat}/K_m values were 40–1000-fold lower than those of the best thioester. The aminomethylcoumarin Z-Trp-Arg-AMC is among the most sensitive trypsin amide substrates known, having a k_{cat}/K_m value 30 times that of Z-Arg-AMC (Kanaoka et al., 1977; Zimmerman et al., 1977). One of the best trypsin thioester substrates (Z-Arg-SBu-*i*, $k_{cat}/K_m = 6.7 \times 10^6$ M^{-1} s^{-1}) was slightly better than Z-Lys-SBzl ($k_{cat}/K_m = 1.5 \times 10^6$ M^{-1} s^{-1}; Green & Shaw, 1979).

Peptide Thioester Substrates with Antithrombin III Reactive Site Sequences. The plasma protease inhibitor antithrombin III (heparin cofactor) is a strong inhibitor of thrombin and factor X_a and also inhibits other coagulation factors such as factor IX_a, factor XI_a, and factor XII_a (Kurachi et al., 1976b; Fujikawa et al., 1977; Kurachi & Davie, 1977). The reactive site sequence of antithrombin III has not yet been clearly established but is probably the sequence listed in Table IV (Petersen et al., 1979) which is homologous with the α_1-protease inhibitor (α_1-antitrypsin) reactive site (Carrell et al., 1980). Since we have previously shown that peptides with the α_1-protease reactive site are fairly reactive toward a number of enzymes which are inhibited by α_1-protease inhibitor (McRae et al., 1980), we decided to investigate peptide benzyl thioesters with antithrombin III reactive site sequences (Table IV).

Interestingly, the most reactive substrates for four of the enzymes studied resulted from this study. They were Z-Gly-Arg-SBzl for thrombin, factor XI_a, and factor XII_a and Z-Ile-Ala-Gly-Arg-SBzl for factor X_a. The higher reactivity of these substrates is partially due to the fact that we utilized benzyl thioesters of the antithrombin III sequences. In the majority of cases examined, benzyl thioesters were more reactive than simple alkyl thioesters. None of the substrates listed in Table IV showed substrate activation with factor IX_a in contrast to the other substrates investigated with factor IX_a.

Increasing the length of the peptide chain had relatively little effect on the reactivity of the thioesters. With any one enzyme, a maximum 4-fold change was observed within the series of four peptide thioesters investigated. In general, the longer peptides were slightly poorer substrates.

P'-Extended Thioester Substrates. In order to further test the role of P_1' residues on substrate hydrolysis, we synthesized and tested the P'-extended thioester substrates listed in Table V. The sequences correspond to zymogen activation sites in bovine factor IX, factor X, prothrombin, factor XI, and factor XII (Table VI). These substrates turned out to be more difficult to synthesize and less stable to hydrolysis than alkyl or benzyl thioesters. Disappointingly, in all cases the extended P' substrates had k_{cat}/K_m values which were slightly lower than that for the simple thioester Z-Arg-SBu-*i*. Interestingly, factor X_a, whose natural substrate has a P_1'–P_2' Ile-Val sequence, hydrolyzed the P_1' SIle-NH_2 substrate slightly more effectively than the SVal-NH_2 analogue and hydrolyzed the SIle-Val-NH_2 derivative slightly faster than either SVal-Val-NH_2 or SIle-Ile-NH_2. However, in general, extending the peptide chain in the P' direction resulted in poorer peptide thioester substrates.

Summary. Table VII shows a direct comparison of the relative k_{cat}/K_m values for the blood coagulation enzymes and trypsin with the simpler substrates. Factor IX_a is the least reactive and the most specific, hydrolyzing only four of the dipeptide thioesters. Both factor X_a and factor XII_a showed considerable specificity toward the substrates, but little specificity was observed with factor XI_a. Thrombin and kallikrein were the most reactive of the blood clotting enzymes and were also quite specific. Trypsin is by far the most reactive of the enzymes and as expected does not demonstrate much specificity toward substrates. The tripeptides designed for factors IX_a and XI_a were no more reactive or specific than the di-

peptide substrates. In addition, the two amide derivatives designed for factor IX_a were substrates for most of the enzymes. With the exception of factor X_a, the k_{cat}/K_m values for the amides were 2–3 orders of magnitude lower than those for the thioesters.

The peptide thioesters described in this manuscript should be extremely useful in assaying for the various blood coagulation enzymes. No other substrates for factors IX_a and XI_a have been reported that are anywhere near as reactive as the thioesters. With all the enzymes that were studied, the thioester substrates were the most reactive or among the most reactive substrates that have been reported. High sensitivity assays for the various coagulation factors can be developed by using thioester substrates due to the high turnover rates of these substrates and the sensitivity with which the thiol leaving group can be detected. On the basis of our earlier experience with elastase (Castillo et al., 1979), we would estimate that concentrations as low as 0.5–1.5 pM (thrombin, kallikrein, and trypsin) and 10–30 pM (factor IX_a, factor X_a, factor XI_a, and factor XII_a) can be determined with a standard spectrophotometer. In addition, they could be used in systems such as plasma which contain large amounts of exogenous thiols. On the other hand, thioesters would be less useful in systems where there are a number of different trypsinlike enzymes present. Most of the enzymes studied reacted with most of the substrates. If a limited number of the coagulation enzymes were present in a system under study, the data in Table VII could be used to choose the substrate with the greatest specificity toward the enzyme or enzymes of interest.

The thioester substrates have been very convenient for mapping the extended substrate binding site to the various coagulation enzymes. No other substrates would have been likely to react with many of the enzymes studied. It is apparent that residues on both sides of the sensitive bond in the substrate can affect k_{cat}/K_m values. In particular, both the P_2 residue and the P_1' thiol leaving group significantly affect rates of reaction and the specificity of the substrates. It appears to us that the P residues are more important than the P′ residues. However, with *thioester substrates*, we foresee no significant advantage to extending the substrates beyond the P_2 residue and the P_1' thiol leaving group. It appears that the maximum reactivity with thioester substrates is reached at this point, and further increase in the size of the substrate has relatively little effect on the reactivity. Our results also indicate that use of the amino acid sequence at the cleavage site of the natural substrate may not always lead to the best simple peptide substrates, as in the case of factor IX_a and factor XI_a.

In conclusion, we have reported a series of highly sensitive thioester substrates for most of the coagulation proteases. With two of the substrates, Z-Gly-Arg-SBzl and Z-Trp-Arg-SBzl, sensitive assays for all the coagulation enzymes could be developed. Future studies, using thioester substrates, on the effect of cofactors on the catalytic efficiency of the various enzymes should lead to a better understanding of the blood coagulation mechanism.

Supplementary Material Available

Experimental details for the synthesis of the new compounds reported (10 pages). Ordering information is given on any current masthead page.

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Interaction of α-Dansylated Peptide Inhibitors with Porcine Pepsin: Detection of Complex Formation by Fluorescence Energy Transfer and Chromatography and Evidence for a Two-Step Binding Scheme†

Ben M. Dunn,* Christine Pham, Laurence Raney, Dilip Abayasekara, Wendy Gillespie, and Anna Hsu

ABSTRACT: Peptide inhibitors, specifically labeled at the α-amino terminus by dansylation, have been prepared by utilizing solid-phase peptide synthesis. Changes in fluorescence have been observed upon mixing these peptides with porcine pepsin that can be attributed to the formation of at least two complexes. Energy transfer between tryptophan residues of the protein and the dansyl group of the inhibitors has been detected by the unique excitation spectra generated. The kinetics of formation of the second complex can be correlated with inhibition of the catalytic activity of pepsin. Evidence for complex formation has also been obtained from gel filtration experiments using the fluorescent peptides.

Fragments of the amino-terminal region of pepsinogen are released upon conversion to pepsin. Amino acids from residues 1 to 44 of porcine pepsinogen are released as peptides of varying length depending on the conditions (Dunn et al., 1978). The first fragment released is peptide (1–16) as shown by trapping studies with the use of a specific inhibitor (Dykes & Kay, 1976).

Peptide (1–16), as well as several other fragments, will inhibit the pepsin-catalyzed clotting of a dilute solution of milk (Herriott, 1938, 1939, 1941; Dunn et al., 1978). Inhibition of the cleavage of small, synthetic peptides can also be observed.[1] The molecular details of this inhibition process are unclear at this point. Since preliminary kinetic studies have indicated a complex pathway leading to inhibition, we have sought further information on this process by the use of the fluorescent reporter group method (Stryer, 1978). We have prepared a series of derivatives of the peptide (1–16) sequence

† From the Department of Biochemistry and Molecular Biology, College of Medicine, University of Florida, Gainesville, Florida 32610. *Received June 23, 1981.* Supported by National Institutes of Health Grants AM 18865 and AM 00303.

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