Active-Site Mapping of Bovine and Human Blood Coagulation Serine Proteases Using Synthetic Peptide 4-Nitroanilide and Thio Ester Substrates†

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ABSTRACT: A series of 14 tripeptide 4-nitroanilide substrates of the type Z-AA-Gly-Arg-NA and Z-AA-Phe-Arg-NA where AA = Ala, Asn, Glu, Lys, Phe, Pro, or Ser were used to map the S_3 subsite of several serine proteases involved in blood coagulation. The enzymes studied included bovine thrombin, factor IX_a, factor X_a, factor XI_a, human β-factor XII_a (factor XII_a fragment), and activated bovine and human protein C. Kinetic constants (k_{cat}, K_M, and k_{cat}/K_M) for the enzymatic hydrolysis of the substrates by each enzyme were determined and used to compare the relative reactivities of the individual enzymes. Most of the enzymes reacted with all the substrates, although a few showed considerable specificity. Human β-factor XII_a showed the highest reactivity of all the coagulation proteases studied and was also very substrate specific (k_{cat}/K_M ranged over 470-fold). The best substrate was Z-Lys-Phe-Arg-NA with k_{cat}/K_M = 140 000 M^{-1} s^{-1}. Activated bovine protein C (best substrate = Z-Ser-Phe-Arg-NA), factor X_a (best substrate = Z-Glu-Gly-Arg-NA), and thrombin (best substrate = Z-Lys-Gly-Arg-NA) were the group of enzymes that showed next highest reactivity toward the substrates. Activated bovine protein C, thrombin, and factor X_a displayed relatively little substrate specificity. Activated human protein C (best substrate = Z-Ser-Phe-Arg-NA) and factor XI_a (best substrate = Z-Glu-Gly-Arg-NA) are moderately reactive enzymes. Activated human protein C is an extremely specific enzyme since it has such a large range of k_{cat}/K_M values. Factor IX_a, although very slow, hydrolyzed 10 of the 14 substrates with the best substrate having k_{cat}/K_M = 503 M^{-1} s^{-1}. Human β-factor XII_a (best substrate = Z-Phe-Arg-SBu-*i*) and both bovine (best substrate = Z-Arg-SBzl) and activated human protein C (Z-Phe-Arg-SBu-*i*) were studied with a set of amino acid and dipeptide thio ester substrates. The best thio ester substrates were 10–359-fold better substrates than the best 4-nitroanilide substrates. Comparison of bovine α-factor XII_a with human β-factor XII_a and activated bovine with human protein C showed substantial differences both in reactivity and in subsite preferences. There were also significant similarities. The results reported in this paper should be useful for the future development of sensitive and specific assays for coagulation enzymes.

Blood coagulation involves the concerted reaction of a series of serine proteases with specificity toward arginine peptide bonds. Most of the enzymes exhibit considerable subsite specificity and will cleave only one or a small number of peptide bonds in their natural substrates. In contrast, trypsin has very little subsite specificity and cleaves most arginine and lysine peptide bonds. Assays of blood coagulation enzymes and their natural inhibitors are widely used in the diagnosis of disease states.

Synthetic peptide substrates for coagulation enzymes have been known for over 20 years and are increasingly used clinically in preference to clotting assays. The assays use specific peptide sequences to which is attached a chromogenic or fluorogenic leaving group. The most widely used chromogenic substrates are 4-nitroanilides. Fluorogenic substrates which are derivatives of 7-amino-4-methylcoumarin or other fluorophores have also gained widespread acceptance [for recent reviews, see Lottenberg et al. (1981), Claeson & Aurell (1981), Iwanaga et al. (1978), and Huseby & Smith (1980)]. More recently, peptide thio esters (Green & Shaw, 1979; McRae et al., 1981a) and fluorescent quench substrates (Castillo et al., 1983) have been studied with many coagulation factors including some of the less reactive enzymes.

Subsite mapping studies have been carried out on many of the coagulation factors, particularly the more abundant enzymes such as thrombin and factor X_a. Peptide 4-nitroanilides have been the most widely studied, and a number of these substrates are now commercially available for coagulation assays (Lottenburg et al., 1981). However, relatively few studies have been carried out with some of the less reactive coagulation factors such as factors IX_a, XI_a, and XII_a, and few comparisons have been made on the specificity of each substrate toward the various coagulation factors.

In our recent studies of peptide thio esters with coagulation factors, we discovered that most of the enzymes had a preference for substrates with either a P_2 Gly or a Phe residue.[1] Therefore, we decided to synthesize 14 tripeptide substrates with the sequences Z-AA-Phe-Arg-NA and Z-AA-Gly-Arg-NA where AA is 7 representative amino acid residues.[2] In this paper, we report the use of these substrates to map the S_3 subsite of bovine thrombin, factor IX_a, factor X_a, factor XI_a, human β-factor XII_a, and activated protein C. We have also extended our earlier subsite mapping studies with amino acid and dipeptide thio ester substrates to activated protein C and report on the comparative reactivity of bovine α-factor XII_a, human β-factor XII_a, and human and activated bovine protein C with the various substrates.[3]

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[1] The nomenclature for the individual amino acid residues (P_3, P_2, and P_1) of a substrate and for the subsites (S_3, S_2, and S_1) of the enzyme is that of Schechter & Berger (1967).

[2] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Me_2SO, dimethyl sulfoxide; Z, benzyloxycarbonyl; NA, 4-nitroanilide; SBzl, $SCH_2C_6H_5$; SBu-*i*, $SCH_2CH(CH_3)_2$; AMC, 7-amino-4-methylcoumarin; Abz, 2-aminobenzoyl; Nba, 4-nitrobenzylamide; Boc, *tert*-butyloxycarbonyl.

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Materials and Methods

4,4′-Dithiodipyridine (Aldrithiol-4) was purchased from the Aldrich Chemical Co., Milwaukee, WI. All common chemicals and solvents were reagent grade. The synthesis of the amino acid and dipeptide thio ester substrates is described in McRae et al. (1981). The synthesis of the tripeptide 4-nitroanilides is described in Tanaka et al. (1983).

Bovine thrombin, factor X_a, and factor XI_a were prepared as described by Kisiel et al. (1977), Kurachi et al. (1976), and Kurachi et al. (1980), respectively. Bovine factor IX_a was prepared by activating factor IX with bovine factor XI_a in a similar manner to the activation of human factor IX as described by Discipio et al. (1978). Bovine factor IX_a was separated from factor XI_a by DEAE-Sephadex column chromatography. Human β-factor XII_a was prepared as described by Fujikawa & McMullen (1983). Activated bovine protein C and activated human protein C were prepared according to the procedures of Kisiel et al. (1977) and Kisiel (1979), respectively. All enzymes were shown to be homogeneous by gel electrophoresis in sodium dodecyl sulfate.

The active-site concentrations of thrombin, factor IX_a, and factor X_a 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 titration was carried out in 0.1 Tris-HCl buffer, pH 8.3, and 5 mM $CaCl_2$ at 25 °C. The concentration of factor XI_a was determined with Z-Trp-Arg-SBu-*i*, by applying kinetic constants based on active-site titration of the enzyme (McRae et al., 1981). β-Factor XII_a was titrated with the benzyl ester of 4-guanidinothiobenzoic acid (Cook & Powers, 1983). Activated bovine protein C was titrated by benzyl *p*-guanidinothiobenzoate; however, activated human protein C could not be titrated by either benzyl *p*-guanidinothiobenzoate or *p*-nitrophenyl *p*-guanidinobenzoate. Thus, the concentration of the human enzyme was determined by A_{280} measurement, assuming a molecular weight of 59 000.

4-Nitroanilide Kinetics. The rates of enzymatic hydrolysis of 4-nitroanilide substrates were followed spectrophotometrically at 410 nm with a Beckman Model 25 spectrometer. The kinetic assays of factors X_a and β-factor XII_a, thrombin, and activated protein C were conducted by adding 50 μL of enzyme solutions to polystyrene cuvettes containing 2.0 mL of substrate solutions. For factors IX_a and XI_a, quartz microcuvettes were employed (total volume of 0.550 mL of assay mixture). All glassware was silylized by treatment with 5% trimethylsilyl chloride in toluene in order to prevent adhesion of the proteins to the surface of the glass. Kinetic constants were determined from the initial rates of hydrolysis by using Lineweaver–Burk plots. An extinction coefficient of 8800 M^{-1} cm^{-1} at 410 nm was used for the hydrolysis product, 4-nitroaniline (Erlanger et al., 1961). Correlation coefficients were better than 0.99 in all cases.

The rate assays of factor IX_a, factor X_a, factor XI_a, and β-factor XII_a were conducted at 25 °C in 0.10 M Tris buffer at pH 7.5 containing 5 mM $CaCl_2$ and 10% Me_2SO. The hydrolysis rate by thrombin was measured under the same condition, but no $CaCl_2$ was present in the assay mixture since the enzyme solution contained sodium phosphate. The rate assay of activated bovine protein C was conducted at 25 °C in 0.10 M Tris buffer at pH 8.0 containing 50 mM KCl, 1 mM $CaCl_2$, and 10% Me_2SO. The rate assay of activated human protein C was carried out at 25 °C in 0.05 M Tris buffer at pH 8.0 containing 0.10 M NaCl, 1 mM $CaCl_2$, 0.10 M CsCl, and 10% Me_2SO.

Thio Ester Kinetics. Hydrolysis of the thio ester substrates was measured at 324 nm in the presence of 4,4′-dithiodipyridine by using ϵ = 19 800 M^{-1} cm^{-1} for the product thiopyridone (Grassetti & Murray, 1967; Castillo et al., 1979; McRae et al., 1981). The rate assays with activated bovine protein C were performed at 25 °C in 0.10 M Tris-HCl buffer at pH 7.5 containing 0.10 M NaCl and 10% Me_2SO. The hydrolysis rates of activated human protein C and human β-factor XII_a were measured by using the same buffer solution as described for the 4-nitroanilide kinetics.

The reaction mixture was prepared by adding 50 μL of a 10 mM 4,4′-dithiodipyridine solution to a cuvette containing 2.0 mL of buffer followed by the addition of 20 μL of enzyme and, finally, varying amounts of a stock substrate solution in Me_2SO to reach the proper substrate concentration. The Me_2SO concentration was kept constant throughout by adding appropriate additional amounts of Me_2SO to the cuvettes. The concentrations of the substrates were measured by complete trypsin hydrolysis which was brought about within a few seconds by addition of a concentrated trypsin solution to the cuvette. For activated human protein C and β-factor XII_a, the reference cell contained the same assay mixture as the sample cell with the exception of enzyme. This was necessary since at the pH employed (pH 8.0), the thio ester substrates have nonnegligible spontaneous hydrolysis rates. The kinetic constants were calculated from Lineweaver–Burk plots by taking initial slopes which were converted into molar rates. Enzyme concentrations were identical with those used for the 4-nitroanilide substrates.

Results

The kinetic parameters for the hydrolysis of the 14 tripeptide nitroanilide substrates by bovine thrombin, factor IX_a, factor X_a, factor XI_a, and human β-factor XII_a are reported in Table I. The results with activated bovine protein C and activated human protein C are reported in Table II.

The substrate binding constants (K_M) and the turnover number (k_{cat}) were determined for bovine thrombin and factor X_a without any difficulty. For other enzymes, only some k_{cat} and K_M values could be determined. With factor IX_a, the Lineweaver–Burk plots went through the origin for all substrates tested, and only the specificity constant k_{cat}/K_M could be measured. Michaelis–Menten kinetics require some substrate concentrations to be in the range of K_M, but for factor IX_a and for the other cases in which k_{cat} and K_M could not be determined, the substrate concentrations were probably much lower than the K_M, and the Lineweaver–Burk plots went through the origin. The substrate concentrations could not be increased due to their poor solubility.

Assay conditions were varied slightly for some enzymes in order to detect significant hydrolysis rates. These variations were necessary to increase reactivities of the enzymes. The addition of 50 mM KCl and a pH increase to 8.0 were utilized to increase the reactivity of activated bovine protein C. Activated human protein C was practically inactive under the normal assay conditions, and the addition of CsCl and an increase in pH to 8.0 were necessary. Thus, these differences in assay conditions must be taken into account when comparing the reactivities of these enzymes to the other enzymes in this paper.

[3] α-Factor XII_a refers to the enzymatically active factor XII molecule composed of a heavy chain (M_r 52 000) and a light chain (M_r 30 000) held together by disulfide bonds. The bovine XII_a preparation which we studied with peptide thio esters (McRae et al., 1981a) was α-factor XII_a. β-Factor XII_a is composed of a light chain (9 amino acid residues) and a heavy chain (243 amino acid residues) held by one disulfide bond (Fujikawa & McMullen, 1983). This molecule is present only in the human species and has previously been called factor XII_a fragment.

Table I: Kinetic Constants for the Hydrolysis of Tripeptide 4-Nitroanilide Substrates by Bovine Thrombin, Factor IX_a, Factor X_a, Factor XI_a, and Human β-Factor XII_a

substrate		enzyme				
P_4 P_3 P_2 P_1	constants[a]	thrombin[b]	factor IX_a[c]	factor X_a[c]	factor XI_a[c]	factor XII_a[c]
Z-Ala-Gly-Arg-NA	k_{cat}	0.92		95		25
	K_M	0.24		5.2		3.9
	k_{cat}/K_M	3.8×10^3	15[d]	1.8×10^4	1.4×10^3[d]	6.5×10^3
Z-Asn-Gly-Arg-NA	k_{cat}	0.41		95	21	18
	K_M	0.22		3.4	14	2.7
	k_{cat}/K_M	1.9×10^3	11[d]	2.8×10^4	1.5×10^3	6.6×10^3
Z-Glu-Gly-Arg-NA	k_{cat}	1.3		433	12	
	K_M	0.52		11	2.9	
	k_{cat}/K_M	2.5×10^3	5.03×10^2[d]	3.9×10^4	4.1×10^3	5.9×10^3[d]
Z-Lys-Gly-Arg-NA	k_{cat}	2.3		78	21	23
	K_M	0.22		6.2	12	0.70
	k_{cat}/K_M	1.0×10^4	6.4[d]	1.3×10^4	1.8×10^3	3.2×10^4
Z-Phe-Gly-Arg-NA	k_{cat}	3.5		152		14
	K_M	0.49		4.5		8.2
	k_{cat}/K_M	7.1×10^3	nh[f]	3.4×10^4	3.8×10^3[d]	1.7×10^3
Z-Pro-Gly-Arg-NA	k_{cat}	0.91		121	3.3	9.2
	K_M	0.16		16	8.4	6.7
	k_{cat}/K_M	5.7×10^3	0.070[d]	7.8×10^3	3.9×10^2	1.4×10^3
Z-Ser-Gly-Arg-NA	k_{cat}	2.3			7.1	
	K_M	3.8			2.4	
	k_{cat}/K_M	6.1×10^3	1.7[d]	2.9×10^4[d]	3.0×10^3	5.1×10^3[d]
Z-Ala-Phe-Arg-NA	k_{cat}	0.32		1.8		34.6
	K_M	0.51		0.24		1.5
	k_{cat}/K_M	6.3×10^2	nh[f]	7.5×10^3	1.6×10^3[d]	2.3×10^4
Z-Asn-Phe-Arg-NA	k_{cat}	0.70		20	2.0	23
	K_M	5.7		2.1	0.71	0.66
	k_{cat}/K_M	8.0×10^3	7.9[d]	9.5×10^3	2.8×10^3	3.5×10^4
Z-Glu-Phe-Arg-NA	k_{cat}	0.18		1.0	3.8	
	K_M	0.33		0.17	3.8	
	k_{cat}/K_M	1.8×10^3	1.9[d]	5.9×10^3	1.0×10^3	1.2×10^4[d]
Z-Lys-Phe-Arg-NA	k_{cat}	0.18		0.58	2.5	23
	K_M	1.3		0.24	2.7	0.16
	k_{cat}/K_M	7.2×10^3	3.7[d]	2.4×10^3	9.3×10^2	1.4×10^5
Z-Phe-Phe-Arg-NA	k_{cat}	0.15		0.43	12	
	K_M	0.026		0.038	15	
	k_{cat}/K_M	5.8×10^3	nh[e]	1.1×10^4	8.1×10^2	1.9×10^4[d]
Z-Pro-Phe-Arg-NA	k_{cat}			1.1		
	K_M			0.99		
	k_{cat}/K_M	nh[f]	nh[f]	1.1×10^3	2.9×10^2[d]	3.0×10^2[d]
Z-Ser-Phe-Arg-NA	k_{cat}	1.2		7.5	4.5	28
	K_M	0.18		0.94	1.2	0.96
	k_{cat}/K_M	6.7×10^3	0.85[d]	8.0×10^3	3.8×10^3	2.9×10^4

[a] The units of k_{cat}, K_M, and k_{cat}/K_M are s^{-1}, mM, and M^{-1} s^{-1}, respectively. [b] Tris buffer (0.1 M), pH 7.5, and 10% Me_2SO at 25 °C. [c] Tris buffer (0.1 M), pH 7.5, 5 mM $CaCl_2$, and 10% Me_2SO at 25 °C. [d] The Lineweaver–Burk plot went through the origin, and only k_{cat}/K_M could be obtained. [e] No hydrolysis observed at 20% Me_2SO. At 10% Me_2SO, the substrate precipitated at high concentrations. At low substrate concentrations, no hydrolysis was observed. [f] No hydrolysis.

Table II: Kinetic Constants for the Hydrolysis of Tripeptide 4-Nitroanilide Substrates by Activated Bovine Protein C and Activated Human Protein C[a]

substrate	activated bovine protein C[a]			activated human protein C[b]		
P_4 P_3 P_2 P_1	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})
Z-Ala-Gly-Arg-NA			3400[c]			29[c]
Z-Asn-Gly-Arg-NA	5.0	0.94	5200	0.18	0.90	148
Z-Glu-Gly-Arg-NA			2400[c]			42[c]
Z-Lys-Gly-Arg-NA	3.2	0.72	4500	1.3	9.9	100
Z-Phe-Gly-Arg-NA	3.8	3.3	1100			nh[d]
Z-Pro-Gly-Arg-NA			1200[c]			nh[d]
Z-Ser-Gly-Arg-NA			5400[c]	0.16	1.6	73
Z-Ala-Phe-Arg-NA	11	0.86	13000			1300[c]
Z-Asn-Phe-Arg-NA			22000[c]			2400[c]
Z-Glu-Phe-Arg-NA			8000[c]	1.2	0.59	1600
Z-Lys-Phe-Arg-NA	5.5	0.37	15000	7.9	1.8	3200
Z-Phe-Phe-Arg-NA			8900[c]			600[c]
Z-Pro-Phe-Arg-NA			1800[c]			74[c]
Z-Ser-Phe-Arg-NA			65000[c]			3900[c]

[a] Tris (0.1 M), pH 8.0, 50 mM KCl, 1 mM $CaCl_2$, and 10% Me_2SO at 25 °C. [b] Tris (0.05 M), pH 8.0, 0.1 M NaCl, 1 mM $CaCl_2$, 0.1 M CsCl, and 10% Me_2SO at 25 °C. [c] The Lineweaver–Burk plot went through the origin, and only k_{cat}/K_M could be obtained. [d] No hydrolysis.

Table III: Kinetic Constants for the Hydrolysis of Amino Acid and Dipeptide Thio Ester Substrates by Activated Bovine and Human Protein C and Human Factor XII_a

	activated bovine protein C[a]			activated human protein C[b]				human β-factor XII_a[c]			
substrate	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})	relative[e] k_{cat}/K_M	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})	relative[f] k_{cat}/K_M
Boc-Arg-SBu-*i*	41	48	8.5×10^5			ND[g]		9.2	95	6.1×10^5	0.016
Boc-Arg-SBzl	94	120	7.8×10^5			7.4×10^4[d]	11	29	180	1.6×10^5	0.26
Z-Arg-SBzl	39	36	1.1×10^6			ND[g]				ND[g]	
Z-Arg-SBu-*i*			ND[g]			5.4×10^4[d]		22	103	2.1×10^5	0.37
Z-Gly-Arg-SBu-*i*	30	30	1.0×10^6			4.8×10^4[d]	21	43	36	1.2×10^6	0.018
Z-Ala-Arg-SBu-*i*	47	52	9.0×10^5			3.6×10^4[d]	25	14	34	4.1×10^5	0.046
Z-Val-Arg-SBu-*i*	25	44	5.7×10^5			5.3×10^4[d]	11	17	46	3.7×10^5	0.0086
Z-Met-Arg-SBu-*i*	23	27	8.5×10^5	4	790	5.6×10^3	150	2.5	19	1.3×10^5	
Z-Phe-Arg-SBu-*i*	81	11	7.4×10^5	4	32	1.2×10^5	6.2	8.3	6	1.4×10^6	0.032
Z-Pro-Arg-SBu-*i*	41	70	5.8×10^5	3	100	2.8×10^4	21	8.3	36	2.3×10^5	0.065
Z-Ser-Arg-SBu-*i*	30	83	3.6×10^5			1.3×10^4[d]	28	13	56	2.3×10^5	0.012
Z-Thr-Arg-SBu-*i*	47	72	6.5×10^5	4	100	4.4×10^4	15	11	54	2.1×10^5	0.0095
Z-Glu-Arg-SBu-*i*	14	53	2.6×10^5		nh[h]			9.2	150	6.1×10^4	0.0092
Z-Lys-Arg-SBu-*i*	140	150	9.3×10^5			5.0×10^4[d]	19	15	54	2.8×10^5	0.079
Z-Ala-Arg-SBzl	112	120	9.3×10^5	18	180	9.8×10^4	9.5	15	12	1.3×10^6	

[a] Tris-HCl (0.10 M), pH 7.5, 0.10 M NaCl, and 10% Me_2SO at 25 °C. [b] Tris-HCl (0.05 M), pH 8.0, 0.10 M NaCl, 1 mM $CaCl_2$, 100 mM CsCl, and 10% Me_2SO at 25 °C. [c] Tris-HCl (0.10 M), pH 7.5, 5 mM $CaCl_2$, and 10% Me_2SO at 25 °C. [d] The Lineweaver-Burk plot went through the origin, and only k_{cat}/K_M could be determined. [e] Relative k_{cat}/K_M refers to bovine protein C/human protein C. [f] Relative k_{cat}/K_M refers to bovine factor XII_a/human factor XII_a. [g] ND indicates not determined. [h] nh indicates no hydrolysis.

Substrate inhibition was observed with factor IX_a at high concentrations (≥1 mM) of Z-Glu-Gly-Arg-NA. The k_{cat}/K_M value was calculated from the lower concentrations where no substrate inhibition occurred. Slight curvature of the rate lines often resulted from a decrease in the hydrolysis rate with time. Usually, this was most evident at low substrate concentrations, and in such cases, only initial slopes were taken as the hydrolysis rates.

The kinetic parameters for the hydrolysis of amino acid and dipeptide thio ester substrates by activated bovine protein C, activated human protein C, and human β-factor XII_a are reported in Table III. Significant curvature was observed with the activated bovine protein C hydrolysis of Z-Phe-Arg-SBu-*i* and somewhat less curvature with Z-Ala-Arg-SBu-*i*, Z-Ala-Arg-SBzl, and Z-Ser-Arg-SBu-*i*. Curvature was also observed with the activated human protein C and human β-factor XII_a hydrolysis of Z-Phe-Arg-SBu-*i* and with activated human protein C hydrolysis of Z-Ala-Arg-SBzl and Z-Gly-Arg-SBu-*i*. In each case, only initial hydrolysis rates were used.

Discussion

In choosing a set of substrates to map the S_3 subsite of blood coagulation serine proteases, we had to compromise in our choice of a leaving group. Peptide thio ester substrates are the most reactive and allow the determination of kinetic constants by using small amounts of enzyme (McRae et al., 1981a). However, they are not very specific, and each substrate will usually react with a number of coagulation proteases, although at widely varying rates. In addition, thio esters seem to exhibit relatively little increase in reactivity when extended beyond dipeptides. The most specific substrates are those where cleavage involves a real peptide bond such as the fluorescent-quench substrates which we have recently studied with factor IX_a, factor X_a, and factor XI_a (Castillo et al., 1983). These substrates, however, have low k_{cat} values, generally involve greater difficulty in synthesis, and are usually not as sensitive as thio ester substrates. Peptide 4-nitroanilides and substrates containing other activated amine leaving groups such as 7-amino-4-methylcoumarin, 6-aminoquinoline, or 4-methoxy-2-naphthylamine are intermediate in reactivity and specificity between thio esters and peptides. Since peptide 4-nitroanilides are readily synthesizable, we decided to compromise and use them for our studies of the S_3 subsite of blood coagulation enzymes. In our studies of the S_2 subsite of bovine thrombin, factor IX_a, factor X_a, factor XI_a, α-factor XII_a, and plasma kallikrein, we observed that substrates with either a P_2 Phe or a Gly residue were among the most reactive for each enzyme (McRae et al., 1981a). Thus, we elected to synthesize the tripeptide sequences Z-AA-Phe-Arg-NA and Z-AA-Gly-Arg-NA where AA = Ala, Ser, Pro, Asn, Phe, Glu, or Lys. These particular amino acid residues were chosen to give a fairly representative cross section of the 20 standard amino acids found in proteins and yet still give a manageable synthetic problem.

Bovine Thrombin. Thrombin showed considerable selectivity toward the nitroanilide substrates with k_{cat}/K_M values ranging from 10 000 M^{-1} s^{-1} to no hydrolysis. As expected from our earlier work with dipeptide thio esters, the series of nitroanilide substrates with a P_2 Gly residue was more reactive than the series with a P_2 Phe. The positively charged polar residue Lys was favored in both sets of tripeptide nitroanilide substrates. The order of reactivity in the Gly series is Lys > Phe > Ser > Pro > Ala > Glu > Asn and in the P_2 Phe series Asn > Lys > Ser > Phe > Glu > Ala while the Pro derivative was not hydrolyzed. Consistent with Morita's observation that Z-Pro-Phe-Arg-AMC is cleaved 8-fold more slowly than Z-Pro-Gly-Arg-AMC (Morita et al., 1977; Iwanaga et al., 1978), we observed that Z-Pro-Phe-Arg-NA was not hydrolyzed while Z-Pro-Gly-Arg-NA is a reasonably good substrate. Z-Phe-Phe-Arg-NA had a K_M (0.026 mM) which was an order of magnitude lower than any of the other K_M values. Most of the best commercially available thrombin substrates such as Tos-Gly-Pro-Arg-NA and D-Phe-Pip-Arg-NA also have a hydrophobic group (tosyl) or amino acid residue (D-Phe) in the P_4-P_3 position (Lottenberg et al., 1981, 1982; Pozsgay et al., 1981).

Subsite specificity appears to be dependent on a combination of the residues in P_2 and in P_3. Perhaps enzymatic hydrolysis rates are partially determined by the conformations of the various peptides in solution which would be highly dependent on the sequences. Alternatively, the enzyme may be interacting with certain combinations of amino acid residues at two or more subsites in a concerted manner. Although thrombin has been widely studied with 4-nitroanilide (Claeson & Aurell,

1981; Pozsgay et al., 1981) and other types of peptide substrates (Iwanaga et al., 1978; Lobo et al., 1976; Liem & Scheraga, 1973), much remains to be learned about the nature of the individual subsites in thrombin and how they interact.

Bovine Factor IX_a. Factor IX_a was the least reactive coagulation enzyme studied under the assay conditions. Of the 14 substrates, 10 were hydrolyzed, but at minimal hydrolysis rates. Z-Glu-Gly-Arg-NA was the most reactive substrate with a k_{cat}/K_M of 503 M^{-1} s^{-1}. However, substrate inhibition was observed at the highest concentration (1 mM) investigated. The specificity at P_2 is clearly defined for the small aliphatic amino acid residue Gly, as the enzyme demonstrated a clear preference for the P_2 Gly series over Phe.

It is puzzling but interesting that factor IX_a has failed to hydrolyze any peptides bearing the factor X activation site sequence -Val-Val-Arg-. This includes peptide thio esters (McRae et al., 1981a), the 4-nitroanilide substrate Bz-Ser-Gln-Val-Val-Arg-NA (Suomela et al., 1977), and fluorogenic peptides bearing activation site sequences from P_4 to P_3' (Castillo et al., 1983). Instead, substrates that do not bear the natural substrate activation site sequence, such as Abz-Phe-Ser-Arg-Val-Val-Gly-Nba and Z-Trp-Arg-SBzl, are hydrolyzed. Incidentally, the Glu-Gly-Arg sequence of the best 4-nitroanilide substrate is the activation site sequence of prothrombin, the natural substrate of factor X_a. In the future, it would be useful to test nitroanilide substrates in the presence of both phospholipids and factor VIII, and to investigate substrates containing Gla residues.

Bovine Factor X_a. Factor X_a showed high reactivity toward the peptide 4-nitroanilide substrates but relatively little specificity. The k_{cat}/K_M values for all substrates were within a 35-fold range. The enzyme exhibited a preference for P_2 Gly over Phe, and the best substrate was found to be Z-Glu-Gly-Arg-NA. This is the activation site sequence of prothrombin, the natural substrate for factor X_a. Model building of the factor X_a–prothrombin complex indicates that the P_3 residue of a substrate probably forms a salt link with Arg-143 and a hydrogen bond with Gln-192 of the enzyme (Greer, 1981). This interaction accounts for the high reactivity of substrates with P_3 Glu residues. With our series of substrates, the effect seems to be totally in k_{cat}. Factor X_a has been widely studied with synthetic substrates (Iwanaga et al., 1978; Lottenberg et al., 1981; Lonsdale-Eccles et al., 1980; Svendsen et al., 1972), and some subsite mapping has been carried out at P_3 (Aurell et al., 1976, 1977, 1978). Many of these studies reported only relative reactivities without kinetic constants and did not examine a wide variety of P_3 amino acid residues.

Bovine Factor XI_a. Factor XI_a demonstrated much lower reactivity toward the 4-nitroanilide substrates than factor X_a. We have previously observed the same difference with a dipeptide 4-nitroanilide and a dipeptide aminomethylcoumarin (McRae et al., 1981a). This is in contrast to dipeptide thio ester substrates (McRae et al., 1981a) and fluorogenic peptide substrates (Castillo et al., 1983) where factor XI_a is more reactive. At present, we do not understand the reason for this inversion in the order of reactivity with activated amide substrates.

The subsite specificity of factor XI_a is not well-defined although Gly was slightly preferred over Phe in S_2. The specificity of the S_3 subsite is not pronounced enough to draw any conclusion with the exception of Pro which resulted in the least favorable interaction. The second best substrate Z-Phe-Gly-Arg-NA has considerable resemblance to the Phe-Ser-Arg- sequence in bovine factor IX which is cleaved by factor XI_a.

Human β-Factor XII_a. β-Factor XII_a is unlike the other coagulation factors discussed so far in that it was much more specific for Phe at the P_2 residue than Gly. Although the amino acid sequence of the P residues at the activation site of factor XI (factor XII_a's natural substrate) is not known, one of the best 4-nitroanilide substrates (D-Pro-Phe-Arg-NA) for human α-factor XII_a and β-factor XII_a also has a P_2 Phe (Hojima et al., 1980; Silverberg et al., 1980). β-Factor XII_a was one of the most specific enzymes as measured by the variation in reactivity toward the substrates. The k_{cat}/K_M values varied by 460-fold. The enzyme not only is very specific but also is the most reactive coagulation factor. The best substrate, Z-Lys-Phe-Arg-NA, is more reactive than any of the amino acid and dipeptide thio esters that we investigated with bovine α-factor XII_a. This substrate also has a higher k_{cat} than D-Pro-Phe-Arg-NA (14–15 s^{-1}) and should make a more sensitive assay substrate.

Specificity requirements at S_3 appeared to be quite stringent. The enzyme liked the positively charged polar side chain of Lys but disliked the hydrophobic side chain of Phe and the negatively charged polar residue Glu. Again, Pro was the most unfavored.

Activated Bovine Protein C. Activated protein C has been demonstrated to be an anticoagulant (Kisiel et al., 1977). The enzyme is a vitamin K dependent glycoprotein activated by the cleavage of an Arg–Ile bond in the heavy chain of protein C, and its apparent function is to prolong the coagulation time by inactivating factor V_a and factor $VIII_a$.

The enzyme was quite reactive toward the nitroanilide substrates, and its specificity for the P_2 Phe series over the P_2 Gly series was pronounced. Z-Ser-Phe-Arg-NA was the best substrate for activated protein C, and the importance of the P_3 Ser was reiterated in that Z-Ser-Gly-Arg-NA was the best substrate from the P_2 Gly series. The negatively charged polar residue Glu, the hydrophobic residue Phe, and Pro were unfavorable residues at P_3. Ohno et al. (1981) have shown that Boc-Leu-Ser-Thr-Arg-AMC is the best substrate out of 25 fluorogenic substrates surveyed and had k_{cat}/K_M = 25 000 M^{-1} s^{-1}. The k_{cat}/K_M value of Z-Ser-Phe-Arg-NA, our best substrate, is 65 000 M^{-1} s^{-1}.

Activated Human Protein C. Activated human protein C is one of the most substrate-specific enzymes involved in the coagulation–anticoagulation system. The specificity constant k_{cat}/K_M ranged from 3900 M^{-1} s^{-1} to no hydrolysis for the nitroanilide substrates. As in the case of the activated bovine protein C, the P_2 Phe series was much preferred over the Gly series. The S_3 subsite specificity was practically identical with the bovine enzyme, and Z-Ser-Phe-Arg-NA was again the best substrate. The reactivity of activated human protein C was extremely low under the same assay conditions as the bovine protein. However, the reactivity of the human protein was increased many fold by adding 100 mM CsCl (Steiner et al., 1980) and raising the pH to 8.0. Although activated human protein C remained much lower in reactivity toward synthetic substrates than activated bovine protein C under the assay conditions, it is reported to be a strong anticoagulant when employed in human plasma (Kisiel, 1979).

Reaction of Human β-Factor XII_a with Thio Ester Substrates and Comparison with Bovine α-Factor XII_a. Previously, we reported the kinetic constants for the hydrolysis of amino acid and dipeptide thio ester substrates by bovine α-factor XII_a (McRae et al., 1981a). We have now extended these studies to the human enzyme (Table III). Human β-factor XII_a was highly reactive, and the most reactive substrates (Z-Phe-Arg-SBu-*i*, Z-Ala-Arg-SBzl, and Z-Gly-Arg-

Table IV: Relative k_{cat}/K_M Values for the Hydrolysis of the Synthetic 4-Nitroanilide Substrates[a]

	bovine enzyme					human enzyme		
substrate	thrombin	factor IX_a	factor X_a	factor XI_a	activated protein C	factor XII_a	activated protein C	bovine trypsin[b]
Z-Ala-Gly-Arg-NA	0.211	0.00083	1.0	0.078	0.19	0.36	0.0022	23
Z-Asn-Gly-Arg-NA	0.11	0.00061	1.6	0.083	0.29	0.37	0.011	9.4
Z-Glu-Gly-Arg-NA	0.14	0.028	2.2	0.23	0.13	0.33	0.0032	40
Z-Lys-Gly-Arg-NA	0.56	0.00036	0.72	0.10	0.25	1.8	0.0078	18
Z-Phe-Gly-Arg-NA	0.39	0	1.9	0.21	0.061	0.094	0	20
Z-Pro-Gly-Arg-NA	0.32	0.0000039	0.43	0.022	0.067	0.078	0	7.2
Z-Ser-Gly-Arg-NA	0.34	0.000094	1.6	0.17	0.30	0.28	0.0054	33
Z-Ala-Phe-Arg-NA	0.035	0	0.42	0.089	0.72	1.3	0.10	8.9
Z-Asn-Phe-Arg-NA	0.44	0.00044	0.53	0.16	1.2	1.9	0.18	15
Z-Glu-Phe-Arg-NA	0.10	0.00011	0.33	0.056	0.44	0.67	0.12	23
Z-Lys-Phe-Arg-NA	0.40	0.00021	0.13	0.052	0.83	7.8	0.24	7.2
Z-Phe-Phe-Arg-NA	0.32	0	0.61	0.045	0.49	1.1	0.046	13
Z-Pro-Phe-Arg-NA	0	0	0.061	0.016	0.10	0.017	0.0056	3.6
Z-Ser-Phe-Arg-NA	0.37	0.000047	0.44	0.21	3.6	1.6	0.29	23

[a] The k_{cat}/K_M value for the hydrolysis of Z-Ala-Gly-Arg-NA by bovine factor X_a was set at 1.0. The best substrate for each enzyme is underlined. [b] The values for bovine trypsin were taken from the data reported by Tanaka et al. (1983).

SBu-*i*) had k_{cat}/K_M values of over 10^6 M^{-1} s^{-1} which should be compared with 140 000 M^{-1} s^{-1} for the best 4-nitroanilide substrate. The S_1' subsite of the enzyme showed a preference for a P_1' SBu-*i* over SBzl.

Human β-factor XII_a was much more reactive toward the same thio ester substrates than the bovine enzyme. The ratios of the k_{cat}/K_M values for the bovine/human enzymes are given in Table III. The k_{cat}/K_M values ranged over 213-fold for the human enzyme and 80-fold for the bovine enzyme which indicates that the human enzyme may be slightly more substrate selective. In only two cases, Boc-Arg-SBzl and Z-Arg-SBzl, were the reactivities of the two enzymes within 3–4-fold of each other. With the rest of the substrates, the differences were 15–115-fold. The kinetic constants for the human enzyme and the bovine enzyme were measured at different times but in each case were based on titrated enzyme. There were also considerable similarities between the two enzymes. Both enzymes preferred the aromatic amino acid residue Phe and the small amino acid residues Gly and Ala at the S_2 subsite. The best dipeptide isobutyl thio ester substrate for both enzymes was Z-Phe-Arg-SBu-*i*, and the least reactive substrate was Z-Glu-Arg-SBu-*i*.

Reactivity of Activated Bovine and Human Protein C with Thio Ester Substrates. Activated bovine protein C displayed high reactivity toward the thio ester substrates. The best substrate for this enzyme was Z-Arg-SBzl whose k_{cat}/K_M was 17-fold times greater than that of Z-Ser-Phe-Arg-NA, the best 4-nitroanilide substrate for the enzyme.

In contrast, activated human protein C was much more specific toward the thio ester substrates than the bovine enzyme since we observed a 17-fold variation in k_{cat}/K_M values (compared to 4-fold for the bovine enzyme). Z-Glu-Arg-SBu-*i* was not hydrolyed by the human enzyme whereas the bovine enzyme hydrolyzed the substrate quite rapidly (k_{cat}/K_M = 260 000 M^{-1} s^{-1}). The best substrate was Z-Phe-Arg-SBu-*i* whose k_{cat}/K_M value was 359 times higher than that of the best 4-nitroanilide substrate (Z-Ser-Phe-Arg-NA).

The human enzyme was 6–150 times less reactive than the bovine enzyme toward thio ester substrates and 5–117 times less reactive toward the 4-nitroanilide substrates. Since we were unable to titrate the human enzyme, it is possible that some of this difference may be due to an unreliable active enzyme concentration which would affect all the k_{cat} and k_{cat}/K_M values. However, there are also variations in K_M values, and it is clear that there are subsite differences between the two enzymes.

Relative Reactivities of Coagulation Proteases. Relative k_{cat}/K_M values for seven coagulation serine proteases and bovine trypsin are given in Table IV. This table should be useful in choosing a specific 4-nitroanilide substrate for a particular enzyme. For example, it is clear from the data that Z-Lys-Phe-Arg-NA could be used as a specific substrate for factor XII_a in the presence of the other coagulation enzymes. Likewise, Z-Ser-Phe-Arg-NA could be used for activated bovine protein C, and Z-Glu-Gly-Arg-NA could be used to assay factor X_a. It would be much more difficult to assay for factor IX_a or factor XI_a in the presence of the other coagulation proteases using tripeptide 4-nitroanilide substrates. However, we have previously reported a dipeptide thio ester substrate (Z-Trp-Arg-SBzl) which can be used with factor IX_a (McRae et al., 1981a) and the fluorogenic substrate Abz-Glu-Phe-Ser-Arg-Val-Val-Nba (Castillo et al., 1983) which can be used with factor XI_a. For thrombin, a substrate such as the commercially available Tos-Gly-Pro-Arg-NA, D-Phe-Pip-Arg-NA, or D-Phe-Pro-Arg-NA would probably be most useful (Lottenberg et al., 1981, 1982). We would like to emphasize that very few substrates are completely specific for one protease, and although substrates are often claimed in the literature to be highly specific, most have never been tested with many of the other coagulation enzymes. Even then, only relative reactivities with fairly common enzymes such as thrombin, factor X_a, kallikrein, and plasmin are reported.

Conclusions. In conclusion, we have used tripeptide 4-nitroanilide substrates to map the S_3 subsite of bovine thrombin, factor IX_a, factor X_a, factor XI_a, activated bovine protein C, human β-factor XII_a, and activated human protein C. Dipeptide and amino acid thio ester substrates have been used to map the S_2 subsite of protein C and human β-factor XII_a. We have discovered a number of new and sensitive substrates for most of the enzymes studied and have reported significant species differences between bovine α-factor XII_a and human β-factor XII_a and activated human and bovine protein C. The substrates reported in this paper should be useful for the future development of sensitive and specific assays for blood coagulation enzymes.

Registry No. Z-Ala-Gly-Arg-NA, 88253-73-0; Z-Asn-Gly-Arg-NA, 88253-74-1; Z-Glu-Gly-Arg-NA, 88253-75-2; Z-Lys-Gly-Arg-NA, 88253-76-3; Z-Phe-Gly-Arg-NA, 88253-77-4; Z-Pro-Gly-Arg-NA, 70217-80-0; Z-Ser-Gly-Arg-NA, 88253-78-5; Z-Ala-Phe-Arg-NA, 88253-79-6; Z-Asn-Phe-Arg-NA, 88253-80-9; Z-Glu-Phe-Arg-NA, 88253-81-0; Z-Lys-Phe-Arg-NA, 88253-82-1; Z-Phe-Phe-Arg-NA, 88253-83-2; Z-Pro-Phe-Arg-NA, 88253-84-3; Z-Ser-

Phe-Arg-NA, 88253-85-4; Boc-Arg-SBu-*i*, 79684-03-0; Boc-Arg-SBzl, 79684-06-3; Z-Arg-SBzl, 88253-86-5; Z-Arg-SBu-*i*, 79684-07-4; Z-Gly-Arg-SBu-*i*, 79684-08-5; Z-Ala-Arg-SBu-*i*, 79684-09-6; Z-Val-Arg-SBu-*i*, 79684-10-9; Z-Met-Arg-SBu-*i*, 88253-87-6; Z-Phe-Arg-SBu-*i*, 79684-12-1; Z-Pro-Arg-SBu-*i*, 79684-14-3; Z-Ser-Arg-SBu-*i*, 79684-15-4; Z-Thr-Arg-SBu-*i*, 79684-16-5; Z-Glu-Arg-SBu-*i*, 79684-18-7; Z-Lys-Arg-SBu-*i*, 79684-19-8; Z-Ala-Arg-SBzl, 88253-88-7; thrombin, 9002-04-4; factor IX_a, 37316-87-3; factor X_a, 9002-05-5; factor XI_a, 37203-61-5; factor XII_a, 37203-62-6; activated protein C, 42617-41-4; β-factor XII_a, 75216-42-1.

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