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THE HYDROLYSIS OF CHEMICALLY MODIFIED PROTEINS BY FACTOR Xa AND THROMBIN

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Summary

Bovine thrombin and Factor Xa were shown to hydrolyse slowly several chemically modified proteins. Both enzymes hydrolyse the proteins at trypsin-susceptible bonds, with arginine, lysine or the synthetically generated *S*-(β-aminoethyl)cysteine at the P_1 position. Both enzymes, however, cleave at far fewer sites than trypsin. The presence of highly polar groups in the P_2 position appears to hinder hydrolysis by Factor Xa or thrombin. The presence of hydrophobic or neutral amino acids around this site may make the site more susceptible to hydrolysis. Differences in the hydrolysis patterns between thrombin and Factor Xa are observed.

Introduction

Bovine thrombin (EC 3.4.21.5) and Factor Xa (EC 3.4.21.6) resemble trypsin in their ability to hydrolyse synthetic esters such as *N*-toluene-*p*-sulphonyl-L-arginine methyl ester, *N*-benzoyl-L-arginine ethyl ester and several peptide lysine methyl esters [1—6]. The homology of their primary structures to trypsin [7,8] and their irreversible inhibition by aryl esters of *p*-guanidinobenzoic acid [9—11], diisopropyl phosphorofluoridate [12] and phenylmethylsulphonyl fluoride [13] demonstrate that these enzymes are trypsin-like serine proteinases. The specificity of thrombin and Factor Xa, however, is much more restricted than that of trypsin. Thrombin cleaves only four bonds in fibrinogen out of a total of approximately 250 arginyl and lysyl bonds [14]. It cleaves only one arginyl bond in prothrombin out of a total of 41 arginyl and 34 lysyl bonds [15]; only one arginyl bond is cleaved in Factor XIII [6] and protein C [17]. Factor Xa cleaves two arginyl bonds in Factor X [18] out of a total of 25 arginyl and 23 lysyl bonds and it also cleaves two arginyl bonds in prothrombin

[15]. The proclivity of both enzymes to cleave arginyl in preference to lysyl bonds in natural protein substrates is confirmed by kinetic studies on ester substrates [5,19].

Several methods have been used to map the subsites of thrombin and Factor Xa. Peptidyl chloromethyl ketones have been developed as differential irreversible inhibitors of thrombin [20]. The kinetics of hydrolysis of peptide esters containing a C-terminal Lys residue by thrombin and Factor Xa have been determined [6]. These methods assess the importance of only those residues on the N-terminal side of the scissile bond. In the case of thrombin, the kinetics of hydrolysis of synthetic or degradative fragments of fibrinogen have been determined [21–24]. These substrates might have been expected to contain enough amino acid residues to occupy all the subsites in the active centre of thrombin. Nevertheless, most of these peptides were poor substrates by comparison with the natural substrates of thrombin. A number of native proteins, which are probably not natural substrates of thrombin, have nevertheless been found to undergo limited proteolysis. It has been suggested that Glu, Gln, Asp or Asn at the P'_6, P'_7, P'_8 or P'_9 positions favour hydrolysis by thrombin [25]. The present study, of which a brief communication has been presented [19], utilises chemically modified proteins as potential substrates for thrombin and Factor Xa. By comparing and contrasting the amino acids surrounding the various points of cleavage, a fuller understanding of the restricted specificity of thrombin and Factor Xa may be obtained. The possibility of using these enzymes to effect limited proteolytic cleavage in sequencing studies is also discussed.

Materials and Methods

Materials

Chemicals and biochemicals were obtained as follows: Bovine trypsin (Type I), bovine trypsinogen (Type I), bovine chymotrypsinogen A (Type II), ribonuclease A (Type XI A), diisopropylphosphorofluoridate-treated carboxypeptidase B, Seitz-filtered Factor VII and X deficient plasma and rabbit brain cephalin from Sigma London Chemical Co. Ltd.; crystalline egg white lysozyme, Cheng-Chin polyamide layers, dansyl amino acid standards and anhydrous trifluoroacetic acid from BDH Ltd., Poole, Dorset, U.K.; dansyl chloride from Fluka AG, Basel, Switzerland; bovine fibrinogen (grade B1) from Kabi AB, Sweden; 'Stypven' from Burroughs-Wellcome Ltd., U.K.; phenylisothiocyanate from Koch-Light Laboratories Ltd., U.K.; performic acid and hydrogen peroxide from Hopkin and Williams, Chadwell Heath, Essex, U.K.; silica gel Kieselgel G nach Stahl, Type 60, from Merck Laboratory Chemicals; crystalline sperm whale myoglobin from Miles-Servac Pty, Ltd., U.K. Porcine glucagon was a gift from Dr. K.N. Holohan. All common reagents were Analar grade and solutions were prepared in water which had been distilled three times in glass including one distillation over potassium permanganate. Benzoyl arginine ethyl ester was prepared by the method of Bergmann et al. [26]. *N*-Dansyl-L-cysteic acid was prepared by treatment of bisdanyl-L-cystine (1 mg) with a mixture of 99% formic acid/30% hydrogen peroxide (19 : 1, v/v) at −5°C for 2.5 h followed by removal of volatile reactants under vacuum. *N*-Dansyl-*S*-(β-dansyl-

aminoethyl)-L-cysteine was prepared by treatment of *S*-(β-benzyloxycarbonyl-aminoethyl)-L-cysteine (1 mg) [27] with HBr/acetic acid (50% v/v, 0.1 ml) for 45 min at room temperature. The mixture was evaporated to dryness under vacuum, the residue dissolved in 2 ml 0.2 M sodium bicarbonate solution and 5 mg dansyl chloride in 2 ml acetone was added. After 1 h at 37°C, the solution was evaporated to dryness. Both dansyl derivatives were dissolved in 50% (v/v) aqueous pyridine to provide a reference solution for chromatography.

β-Trypsin, thrombin and Factor Xa were prepared and assayed as described by Lonsdale-Eccles et al. [6].

Protein modifications

Trypsinogen, chymotrypsinogen A, and ribonuclease A were oxidised with performic acid by the method of Hirs [28]. Trypsinogen, prior to oxidation, was desalted on a column of Sephadex G-25 in 0.1 M acetic acid and freeze-dried. Lysozyme was either reduced and carboxymethylated [29] or reduced and *S*-aminoethylated [30]. Apomyoglobin was prepared according to Edmundson and Hirs [31]. Since this product retained some of its original red colour, the remaining haem was removed by the methods of Teale [32] and Breslow [33]. Maleylation of proteins was performed with 1 M maleic anhydride in redistilled dioxan as described by Butler et al. [34]. In the case of chymotripsinogen A, it was necessary to incorporate 8 M urea to solubilise the protein (10 mg/ml). Maleylated proteins failed to undergo dansylation [35], suggesting that maleylation of amino groups was complete. Glucagon was used without modification.

The quantitative conversion of cystine into the respective reduced or oxidised residue was demonstrated by amino acid analysis. The samples were hydrolysed at 110°C for 20, 48 and 76 h in constant boiling HCl in sealed and evacuated tubes. For tryptophan analysis the hydrolyses were performed in *p*-toluenesulphonic acid [36] using tryptamine as a scavenger.

Enzymatic hydrolysis of proteins

The enzymatic hydrolyses were performed at 37°C in either NH_4HCO_3 buffer (0.2 M, pH 8.0) or in a pH-stat from which estimates of the number of bonds cleaved could be calculated from the amount of alkali uptake. In the latter case a Radiometer Titrator (TTT1a) and Titrigraph (Type SBR 2c) were used in conjunction with an Activion glass electrode. The protein solutions (1—3%, w/v) in 0.1 or 0.15 M NaCl were maintained at pH 8.0 by the addition of 0.1 M NaOH. Factor Xa, thrombin and trypsin concentrations were 0.02—0.04, 0.015—0.02 and 0.01% (w/v), respectively. An inert atmosphere of CO_2-free nitrogen saturated the reaction vessel. The reaction was usually terminated after 8—9 h by the addition of 0.1 M HCl to pH 3.0, and the mixture was freeze-dried.

The bicarbonate-buffered digests contained the substrate protein (5 mg/ml) and either Factor Xa (200 μg/ml) or thrombin (50 μg/ml). After 2 h, an additional 200 μg Factor Xa or 50 μg thrombin were added and the reaction was allowed to proceed for a total of 8—9 h. The digest was then divided into 100-μl aliquots and freeze-dried under high vacuum using a mercury diffusion pump in conjunction with a rotary vacuum pump to ensure complete removal

of NH_4HCO_3. The freeze-dried samples were stored at −20°C prior to further analysis. Protein hydrolyses by β-trypsin (25 μg/ml) were terminated after 4 h.

Identification of bonds cleaved

Identification of the bonds cleaved by the enzymes was generally performed by a combination of dansyl-Edman analysis and carboxypeptidase digestion on unseparated peptide mixtures. Since the sequence of the proteins was known and the number of points of cleavage limited, two cycles of the dansyl-Edman procedure were generally sufficient to identify the bonds cleaved.

Manual Edman degradation and dansylation were performed as described by Gray [35,37]. The dansylated proteins were hydrolysed for 18 h in vacuo at 110°C using constant boiling HCl. For N-terminal tryptophan analysis the dansylated protein was hydrolysed with *p*-toluenesulphonic acid [36] and the hydrolysate (0.5 ml) was applied to a column (0.8 × 10 cm) of Sephadex G-10, equilibrated with glass-distilled water. Dansyl tryptophan remained bound to the top of the column while the other reaction products were eluted with distilled water. When no more fluorescent material was eluted from the column, the eluant was changed to 50% (v/v) aqueous pyridine. The fluorescent fractions were collected and concentrated to a volume suitable for analysis. All samples were applied to thin-layer chromatograms in 50% (v/v) aqueous pyridine.

Thin-layer chromatography on polyamide layers was performed using the solvent systems of Woods and Wang [38] and Hartley [39] on plates cut to 7.5 × 7.5 cm. The two-dimensional thin-layer chromatography of the dansyl amino acids on silica gel (20 × 20 cm × 250 μm) utilised the solvent systems of Cole et al. [40]. Prior to sample application the plates were activated at 110°C for 30 min then cooled to room temperature. Unambiguous identification was obtained by replicate analyses including appropriate internal standards.

Carboxypeptidase hydrolyses were performed as described by Ambler [41]. The samples were then dansylated and separated by chromatography without acid hydrolysis.

Results

Several chemically modified proteins are susceptible to proteolytic cleavage by Factor Xa or thrombin as shown in Table I. Thrombin and Factor Xa cleaved at the same site in maleylated apomyoglobin and at one common site in maleylated oxidised chymotrypsinogen A. In maleylated oxidised trypsinogen and chymotrypsinogen A, Factor Xa and thrombin cleaved at different sites. While thrombin hydrolysed oxidised ribonuclease in three positions, Factor X had no action on this substrate. Experiments with derivatives of lysozyme, however, were less satisfactory. Reduced and *S*-carboxymethylated lysozyme proved to be insoluble at pH 8.0 and was not hydrolysed by Factor Xa and only slightly by thrombin, although it was rapidly solubilised by trypsin. The maleylated derivative was both soluble and susceptible to hydrolysis by Factor Xa (Table I). The alkali uptake suggested that 3.5 bonds were split by Factor Xa. Aspartic acid was identified as a new N-terminal amino acid following Factor Xa digestion of the maleylated and *S*-carboxymethylated lysozyme. This

TABLE I

PRODUCTS OF DANSYL-EDMAN ANALYSIS

The following abbreviations are used in the table: Mal-Apo-Mb, maleylated apomyoglobin; Mal-Ox-Trg, maleylated performic acid-oxidised trypsinogen; Mal-Ox-Ctg, maleylated performic acid-oxidised chymotrypsinogen; Ox-RNAase, performic acid-oxidised ribonuclease; Mal-CM-Lys, maleylated carboxymethylated lysozyme. With Ox-RNAase the following amino acids were observed after trypsin hydrolysis: Phe, Glu, Asp, Ser, Thr, Tyr, and His. Columns 1 refer to the N-terminal amino acids generated by digestion with the respective enzymes, and columns 2 refer to the amino acids identified by dansylation after the N-terminal had been removed by Edman degradation.

Substrate	Digesting enzyme						Bond cleaved
	Thrombin		Factor Xa		Trypsin		
	1	2	1	2	1	2	
Mal-Apo-Mb	Leu	Phe	Leu	Phe	Leu	Phe	31↓32 33 Arg-Leu-Phe
		Val *		Val *	Phe	Lys	46 46 47 Arg-Phe-Lys
					Lys	Asp	139 140 141 Arg-Lys-Asp
					His	Pro	118 119 120 Arg-His- Pro
						Val *	
Mal-Ox-Trg	Leu	Gly			Leu	Gly	55 56 57 Arg-Leu-Gly
		Val *	Val	Ala	Val	Ala	105 106 107 Arg-Val- Ala
				Val *		Val *	
Mal-Ox-Ctg	Val	Thr	Val	Thr	Val	Thr	230 231 232 Arg-Val- Thr
		Cys *	Ile	Val	Tyr		145 146 147 Arg-Tyr-Thr
			Leu **		Ile	Val	15 16 17 Arg-Ile- Val
				Cys *	Leu	Glu	154 155 156 Arg-Leu-Gln
						Cys *	
Ox-RNAase	Ser	Arg					31 32 33 Lys-Ser- Arg
	Asp		Lys *		(see legend)		37 38 39 Lys-Asp-Arg
	Thr **						98 99 100 Lys-Thr-Thr
	Lys *	Glu					
Mal-CM-Lys			Gly	Cys			125 126 127 Arg-Gly-Cys
				Tyr **			21 22 23 Arg-Gly-Tyr
			Thr **	Pro **			68 69 70 Arg-Thr-Pro
			His **	Gly **			14 15 16 Arg-His- Gly
			Asx	Leu **			73 74 75 Arg-Asn-Leu

* This residue is the original N-terminal amino acid of the undigested protein. In the case of maleylated proteins this residue was not seen in the first cycle of the dansyl-Edman procedure since it was protected by the maleyl group. The acid cleavage step of the Edman degradation deprotects the amino groups and the original N-terminal and the ϵ-amino groups become available for dansylation.

** These residues were obtained in lower yield (<50%) as judged subjectively by less intense fluorescence. In the case of Mal-Ox-Ctg this residue was only faintly visible and it was not possible to determine the following amino acid residue. Consequently, it was placed by homology with the trypsin cleavage.

TABLE II

THE PERCENTAGE OF HYDROPHOBIC OR NEUTRAL AMINO ACIDS ACCOMMODATED AT THE SECONDARY BINDING SITES OF FACTOR Xa

The values are derived from the sequences shown in Discussion where the following amino acids are considered either hydrophobic or neutral: Ala, Val, Leu, Ile, Phe, Tyr, Trp, Gly, Pro and Met. The tryptic cleavage points provide the maximum number of potential cleavage points available for Factor Xa hydrolysis and provides the natural distribution of hydrophobic and neutral amino acids at the subsites.

	P_8	P_7	P_6	P_5	P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4	P'_5	Sample size
Major cleavage	20	40	80	40	80	80	80		100	60	20	80	50	5
Minor cleavage	0	100	75	25	75	50	25		25	100	50	25	25	4
Not cleaved	35	38	45	48	43	57	26		39	39	39	41	29	20--22 *
Tryptic cleavage	27	45	59	44	53	60	34		49	49	37	47	32	30—32 *

* Due to some cleavage occurring near the N- or C-terminal ends of the proteins not all the subsites were occupied in every case.

was tentatively located by the detection of a small yield of dansyl-leucine generated after Edman degradation followed by dansylation and hydrolysis. Because this site could not be identified with certainty, these residues have not been included either as bonds cleaved or as bonds not cleaved in the data presented in Table II. Reduced and *S*-aminoethylated lysozyme was both soluble at pH 8.0 and susceptible to hydrolysis by trypsin, thrombin and Factor Xa. Trypsin cleaved in excess of 12 bonds while thrombin and Factor Xa cleaved approx. 3 and 7 bonds, respectively. Factor Xa differed from the other two enzymes, since it produced a precipitate during the course of the reaction with *S*-aminoethylated lysozyme. Dansylation and amino acid analysis showed that the precipitate was heterogeneous and attempts to separate and purify the different fragments were unsuccessful. The large number of amino acids identified by dansylation and hydrolysis of the peptides in the supernatant (glycine, aspartic acid, lysine, arginine, histidine, serine, alanine, *S*-β-aminoethyl-cysteine, phenylalanine, valine, threonine and leucine or isoleucine in order of decreasing fluorescent intensity) suggests that incomplete cleavages occurred during the hydrolysis and in view of the complexity of the products produced, this substrate was not investigated fully. Nevertheless, it was shown that thrombin and Factor Xa, like trypsin, cleaved at the site of the synthetic amino acid *S*-β-aminoethylcysteine as well as arginine and lysine. Moreover, the generation of an N-terminal valine suggests that the bond Lys_1—Val_2 is susceptible to hydrolysis by thrombin and Factor Xa, although the presence of an unblocked amino group hinders hydrolysis of lysine ethyl ester by thrombin [6].

Glucagon was unaffected by Factor Xa, although it was rapidly hydrolysed by trypsin.

Discussion

The specificity of proteolytic enzymes is modulated by multiple interactions both at the active site and at the secondary binding sites. In this study we have employed chemically modified proteins as potential substrates for thrombin and Factor Xa on the assumption that the modified protein would provide a

random selection of cleavage points at all bonds which fit the respective binding sites. Indeed a tryptic hydrolysis of the proteins suggests that all the potential arginine and/or lysine binding sites were susceptible to cleavage and, therefore, were not buried within the three-dimensional structure of the proteins. Nevertheless, neither thrombin nor Factor Xa cleaved at as many bonds as did trypsin (Table I). The present study shows that the coagulant enzymes cleave at peptide bonds involving the carbonyl group of arginine, lysine and *S*-β-aminoethylcysteine. Since the methyl ester of this latter amino acid is not cleaved by Factor Xa [43] and both thrombin and Factor Xa hydrolyse lysine esters much more slowly than arginine esters [5], the present observations suggests that a major determinant for the restricted specificity of thrombin and Factor Xa must reside outside the active sites of the enzymes.

The general characteristics of the secondary binding site requirements of Factor Xa and thrombin appear to be similar (Tables II and III). The bonds which are susceptible to major cleavage by either enzyme show a high proportion of hydrophobic or neutral amino acids surrounding the cleavage point. While the number of such cleavages is limited, other studies with ester and amide substrates have shown that phenylalanine may be readily accommodated at the P_2 binding site [6,44]. Furthermore, those bonds which are not hydrolysed show a high proportion of hydrophilic residues at P_2. This suggests that the presence of such hydrophilic residues at P_2 may indeed hinder peptide hydrolysis as was shown with ester hydrolysis [6].

Nevertheless, exceptions to the above observations occur. Polar residues are found at P_2 at the following cleavage sites (for abbreviations see legend Table I):

104 105 ↓ 106
Ser—Arg—Val (Mal-Ox-Trg/Factor Xa or thrombin),

14 15 ↓ 16 153 154 ↓ 155
Ser—Arg—Ile (Mal-Ox-Ctg/Factor Xa), Asp—Arg—Leu (Mal-Ox-Ctg/Factor Xa),

36 37 ↓ 38
Thr—Lys—Asp (Ox-RNase/thrombin),

13 14 ↓ 15 72 73 ↓ 74
Lys—Arg—His (Mal-CM-Lys/Factor Xa) and Ser—Arg—Asn (Mal-CM-Lys/Factor Xa).

In contrast, hydrophobic amino acids are found at P_2 in the following regions which resist hydrolysis:

138 139 140
Phe—Arg—Lys (Mal-Apo-Mb/Factor Xa or thrombin),

54 55 56 6 7 8
Val—Arg—Leu (Mal-Ox-Trg/Factor Xa), Ala—Lys—Phe (Ox-RNase/Factor Xa or thrombin),

30 31 32 87 88 89
Met—Lys—Ser (Ox-RNase/Factor Xa) and Tyr—Lys—Thr (Ox-RNase/Factor Xa).

Some of the latter might have been anticipated to be potentially good sites for cleavage. Consequently, the causes for these discrepancies probably do not reside entirely within an incompatible primary sequence, although the primary

TABLE III

THE PERCENTAGE OF HYDROPHOBIC OR NEUTRAL AMINO ACIDS ACCOMMODATED AT THE SECONDARY BINDING SITES OF THROMBIN

The values are derived from the sequences shown in Discussion where the following amino acids are considered either hydrophobic or neutral: Ala, Val, Leu, Ile, Phe, Tyr, Trp, Gly, Pro and Met. The tryptic cleavage shows the maximum number of potential thrombin cleavage points and also provides the neutral distribution of hydrophobic and neutral amino acids on the subsites.

	P_8	P_7	P_6	P_5	P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4	P'_5	Sample size
Major cleavage	40	40	20	40	60	80	80		60	40	20	40	40	5
Minor cleavage	0	100	100	0	0	100	100		0	0	0	100	0	1
Not cleaved	27	31	50	50	50	56	13		44	50	44	50	25	16
Tryptic cleavage	29	36	45	45	50	64	32		45	45	36	50	27	22

sequence may modulate the reaction rates. A more likely explanation is that the three-dimensional structure of the substrate is incompatible with that of the enzymes. This is rather elegantly demonstrated by the action of thrombin on native and maleylated oxidised by chymotrypsinogen A. While thrombin is reported to activate the native chymotrypsinogen A [45], a process which involves the cleavage of the Arg_{15}—Ile_{15} bond, we have shown that thrombin does not cleavage this bond in the modified protein. Clearly, maleylation and oxidation of chymotrypsinogen A changes the conformation around this part of the molecule so that it does not bind productively with thrombin.

The restricted specificity of thrombin and Factor Xa compared to trypsin makes these enzymes potentially useful for amino acid sequence studies, particularly in situations where trypsin produces too many or too small fragments. The relatively slow and incomplete cleavages by thrombin and Factor Xa, however, tend to lead to complex mixtures which are difficult to purify by simple techniques such as gel filtration as was observed in the digests of lysozyme. This characteristic diminishes their use as a general method for sequence determination. Nevertheless, the enzymes do show differences in their cleavage characteristics which could make them useful for obtaining overlap fragments. For example, while they cleaved at the one common bond in chymotrypsinogen and at the same position in myoglobin, they cleaved at other bonds in chymotrypsinogen A, trypsinogen, and ribonuclease A. Such differences in specificity make the two enzymes useful additions to the tools available for protein sequence studies.

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