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THE ESTEROLYTIC SPECIFICITY OF BOVINE THROMBIN AND FACTOR Xa

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Summary

Steady state kinetics are compared for the hydrolysis of *t*-butoxycarbonyl-L-lysine methyl ester and several peptidyl lysine methyl esters catalysed by bovine thrombin and Factor Xa. Thrombin-catalysed reactions have lower K_m values and higher k_{cat}/K_m values than do reactions catalysed by Factor Xa. Values of k_{cat} are comparable and do not show any particular trend. The best substrate in the present series was *t*-butoxycarbonylglycylglycyl-L-lysine methyl ester. Thrombin and Factor Xa may possess a hydrophobic region near the P_2 binding site which is unfavourable for either asparagine or D-alanine but which readily accommodates glycine, L-alanine or L-phenylalanine. The major improvement in Factor Xa hydrolysis occurred with the occupation of the P_2 site by an amino residue while for thrombin the major improvement occurred with the occupation of the P_3 site.

Introduction

Thrombin (EC 3.4.21.5) and Factor Xa (EC 3.4.21.6) are enzymes of critical importance in the blood-clotting process. Both are two-chain molecules with their catalytic sites located on the heavy chains [1—3]. While the catalytic activity of thrombin has been the subject of much study [4—7], considerably less is known about the activity of Factor Xa. Johnson and Smith [8] have compared the primary specificity sites of trypsin and Factor Xa by using inhibitory aromatic amidino and guanidino compounds and conclude that, since the two enzymes differ very little in their inhibition, the large differences

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normally observed between the two enzymes reflect major structural differences at the secondary binding sites. However, Adams and Elmore [9] have shown that Factor Xa has a much more restricted primary site than either trypsin or thrombin since it is less able to accommodate in its primary binding site substrates of different type or length. Nevertheless, thrombin and Factor Xa are also clearly dependent upon secondary interactions for the full expression of their catalytic activity [4—7,10—12]. By varying the amino acid composition at P_2 or P_3 (notation of Schechter and Berger [13]) of Factor Xa and thrombin substrates and by observing the changes in kinetic constants, we have compared the secondary binding sites of the two enzymes.

Materials and Methods

β-Trypsin was prepared by the method of Schroeder and Shaw [14]. Bovine thrombin was prepared by the method of Magnusson [15] and purified by chromatography on CM-Sephadex using 20 mM Tris-HCl buffer (pH 7.5), 0.15 M NaCl as loading buffer. Elution was achieved with a linear gradient of NaCl in the same buffer and the peak of thrombin appeared at 0.27 M NaCl. Peak fractions were pooled and concentrated by ultrafiltration at 2°C and subjected to gel filtration on Sephadex G-25 in 0.2 mM sodium phosphate buffer (pH 7.5) and freeze-dried. The purified thrombin had an activity of 2000—2400 N.I.H. units/mg when assayed by the method of Johnson and Seegers [16]. Bovine Factor X was prepared from fresh blood by the method of Esnouf et al. [17]. The combined pools of Factor X_1 and X_2 were activated by the coagulant fraction of Russell's viper venom [18] and purified on DEAE-Sephadex [17]. The activation of the Factor X was followed by observing the increase in the rate of hydrolysing α*N*-benzoyl-L-arginine ethyl ester. The purified Factor Xa (15—20 mg from 40 l blood) was stored at −20°C in 1-ml aliquots (1 mg/ml). The activity was in the range 590—1050 units/mg. One unit of Factor Xa is defined as that amount produced by the full activation of the Factor X present in 1 ml of normal oxalated plasma. The method of Denton [19] was used to assay Factors X and Xa, the latter with the omission of Russell's viper venom.

Kinetic and computational techniques. The rates of ester hydrolyses were followed on a pH-stat comprising a Radiometer titrator (TTTla) connected to an Activion glass micro-electrode (M27DR) and to a titrigraph. The titrigraph recorded the volume of alkali added from a micrometer syringe as a function of time. The electrode was standardized daily with 8.665 mM KH_2PO_4/30.32 mM Na_2HPO_4 buffer (pH 7.413) [20]. Solutions were prepared from water which had been three times glass-distilled and flushed with CO_2-free nitrogen until the resistance on an Elgastat meter was 1 MΩ. The water and standard alkali solutions were stored in polypropylene containers fitted with $Na_2O \cdot CaO$ guard tubes. CO_2-free NaOH was standardized against Analar-grade benzoic acid. Solutions of thrombin (0.5 mg/ml) were prepared daily in 0.1 M NaCl and kept in an ice-bath throughout the day.

Kinetic runs with Factor Xa and thrombin were performed at pH 8.0 and pH 8.4, respectively, and at 25.0°C. CO_2 was excluded from the reaction vessel by nitrogen saturated at 25°C with water. Mixing was accomplished by a magnetic

stirrer. The operational molarities of thrombin solutions were determined spectrofluorimetrically [21]. Factor Xa solutions were standardized by the steady-state assay with Bz-Arg-OEt [21].

The kinetic parameters were calculated using an ICL-1907 computer and programs written in FORTRAN IV based on the methods described by Elmore et al. [22], and Roberts [23].

Results and Discussion

Thrombin and Factor Xa are known to hydrolyse arginine esters such as tosyl arginine methyl ester and α-*N*-benzoyl arginine ethyl ester [9,24—29]. The present work extends such studies to lysine esters. With the exception of Boc-D-Ala-Lys-OMe all the lysine esters were susceptible to hydrolysis by both Factor Xa and thrombin (Tables I and II). The K_m values were consistently lower and the specificity constants (k_{cat}/K_m) consistently higher with thrombin. For Factor Xa the K_m with Boc-Lys-OMe (approx. 1.2 M) is approx. 1000-fold greater than for thrombin and since the k_{cat} values for the two enzymes with Boc-Lys-OMe are similar, this difference is probably due to less efficient binding by Factor Xa. Changing the substrate from Boc-Lys-OMe to Boc-Gly-Lys-OMe or Boc-Ala-Lys-OMe results in a large drop in K_m with Factor Xa. Thrombin, on the other hand, shows only a small decrease in K_m with this change. In contrast, when the substrate peptide chain is further increased (Boc-Gly-Gly-Lys-OMe), the K_m for Factor Xa hydrolysis is only slightly decreased, while the K_m for the thrombin-catalysed reaction is substantially reduced. Surprisingly, k_{cat} for the hydrolysis of Boc-Lys-OMe is higher than for either Boc-Gly-Lys-OMe or Boc-Ala-Lys-OMe. Moreover, using 1,4-butanediol as a competing nucleophile [30] in thrombin-catalysed reactions, it was found that $k_{cat} = k_3$ for Boc-Ala-Lys-OMe, but both acylation and deacylation are partly rate-determining for the hydrolysis of Boc-Lys-OMe; k_2 and k_3 were calculated to be approx. 300 and 100 s^{-1}, respectively.

The chiral stringency of the P_2 binding site of Factor Xa and thrombin is clearly demonstrated with Boc-D-Ala-Lys-OMe. Factor Xa failed to hydrolyse this substrate at all and thrombin did so only very slowly. Furthermore, compared with the other dipeptides, the occupation of P_2 by asparagine

TABLE I

KINETIC PARAMETERS FOR THE THROMBIN-CATALYSED HYDROLYSIS OF LYSINE DERIVATIVES

The hydrolyses were performed in 0.1 M NaCl at pH 8.4, 25°C.

Substrate	k_{cat} (s^{-1})	10^3 K_m (M)	$10^3 k_{cat}/K_m$ ($m^{-1} \cdot s^{-1}$)
Boc-Lys-OMe	74.43 ± 1.13	1.54 ± 0.05	48.3 ± 0.9
Boc-Gly-Lys-OMe	46.31 ± 1.53	0.63 ± 0.04	72.8 ± 2.6
Boc-Ala-Lys-OMe	30.65 ± 0.49	0.73 ± 0.03	42.0 ± 1.2
Boc-D-Ala-Lys-OMe	0.94 ± 0.02	16.84 ± 0.64	0.06 ± 0.003
Boc-Phe-Lys-OMe	23.11 ± 0.44	0.35 ± 0.02	66.5 ± 1.3
Boc-Asn-Lys-OMe	4.25 ± 0.31	13.44 ± 0.13	0.32 ± 0.01
Boc-Gly-Gly-Lys-OMe	72.17 ± 0.49	0.0462 ± 0.011	1561.0 ± 30.0

TABLE II

KINETIC PARAMETERS FOR THE FACTOR Xa CATALYSED HYDROLYSIS OF LYSINE ESTERS

The hydrolyses were performed in 0.1 M NaCl at pH 8.0, 25°C.

Substrate	k_{cat} (s^{-1})	10^3 K_m (M)	$10^3 k_{cat}/Km$ ($m^{-1} \cdot s^{-1}$)
Boc-Lys-OMe	99.9 ± 21.1	1180.0 ± 270.0	0.085 ± 0.015
Boc-Gly-Lys-OMe	35.49 ± 0.09	4.57 ± 0.23	7.77 ± 0.22
Boc-Ala-Lys-OMe	10.92 ± 0.04	13.21 ± 0.09	8.27 ± 0.03
Boc-D-Ala-Lys-OMe *	—	—	—
Boc-Phe-Lys-OMe	12.70 ± 0.47	1.59 ± 0.15	7.97 ± 0.49
Boc-Asn-Lys-OMe	3.57 ± 0.19	56.9 ± 5.2	0.063 ± 0.003
Boc-Gly-Gly-Lys-OMe	136.7 ± 5.3	3.81 ± 0.30	35.9 ± 1.7

* No hydrolysis was observed.

adversely affects catalysis by both enzymes as expressed by both higher K_m and lower k_{cat} values. The adverse effect of polar residues at P_2 was also observed by Lonsdale-Eccles et al. [10] in studies utilising proteins as potential substrates.

The possible presence of a hydrophobic P_2 binding site in both thrombin and Factor Xa as reported in the literature [10,31,32] is only weakly supported by the present data. The K_m values for Boc-Phe-Lys-OMe are only slightly lower than for the other dipeptides. Furthermore, the diminished k_{cat} values suggest that the apparent improvement in binding is at the expense of catalysis. A similar effect is observed when the Factor Xa hydrolysis of α-*N*-acetyl arginine methyl ester is compared with the hydrolysis of Tos-Arg-OMe and Bz-Arg-OEt [9]. The benzoyl and tosyl derivatives have significantly lower K_m values, but lower k_{cat} values than the acetyl derivative. These reduced k_{cat} values suggest that substrates which bind at this hydrophobic site may do so in a slightly distorted manner.

Both thrombin and Factor Xa have increased specificity constants (k_{cat}/K_m) with Boc-Gly-Gly-Lys-OMe compared to Boc-Gly-Lys-OMe. For Factor Xa the major improvement occurs in the rate constant (k_{cat} = 137 s^{-1}), a value which exceeds that of trypsin (106 s^{-1}) with tosyl arginine methyl ester [33]. In this latter case the full catalytic apparatus, which consists of the charge relay system and oxyanion hole [34], may be presumed to be operative. By inference one may assume that the same system is also fully operative in the hydrolysis of Boc-Gly-Gly-Lys-OMe by Factor Xa. Inhibition by 1,4-butanediol [30] of the Factor Xa hydrolysis of Boc-Gly-Gly-Lys-OMe permitted the calculation of the binding constant K_s ($6.7 \cdot 10^{-3}$ M), as well as the individual rate constants k_2 (270 s^{-1}) and k_3 (286 s^{-1}), and clearly shows that the binding of this substrate by Factor Xa is very weak. The replacement of lysine by arginine at P_1 would probably partially rectify this lesion since kinetic studies of the hydrolysis of Bz-Arg-OEt and Bz-Lys-OEt by Factor Xa revealed that the value of k_{cat}/K_m for the arginine derivative is 334 times greater than for the lysine derivative [35]. With thrombin such a substitution would probably be less effective since it only shows a 40—60-fold preference for arginine over lysine [9,35], a preference which Ryan et al. [36] suggest is due to restricted acylation rather than binding. Indeed Takasaki et al. [37] showed that the

TABLE III

KINETIC PARAMETERS FOR THE TRYPSIN CATALYSED HYDROLYSIS OF LYSINE ESTER DERIVATIVES

The hydrolyses were performed in 0.1 M NaCl at pH 8.0, 25°C.

Substrate	k_{cat} (s^{-1})	10^5 K_m (M)	$10^5 k_{cat}/K_m$ ($M^{-1} \cdot s^{-1}$)
Boc-Lys-OMe	132.7 ± 3.3	9.76 ± 0.69	13.59 ± 0.07
Boc-Ala-Lys-OMe	53.3 ± 0.8	4.37 ± 0.15	12.21 ± 0.03
Boc-D-Ala-Lys-OMe	11.9 ± 0.1	17.42 ± 0.26	0.68 ± 0.01

principal difference between thrombin and trypsin in their action on synthetic substrates was in the efficiency of their acylation steps.

Kinetic constants for the hydrolysis of three of the substrates by β-trypsin are given (Table III) for comparison. The lower K_m values for reactions catalysed by this enzyme give rise to considerably larger specificity constants. As with thrombin, occupancy of the P_2 site by an Ala residue in place of a butoxycarbonyl-group only marginally affects the kinetics of hydrolysis. Of the three enzymes, trypsin is least sensitive to the chirality of the residue at the P_2 site.

In summary, the present study has shown that despite the weak binding, the easily synthesized peptidyl lysine esters can be used to probe the active sites of Factor Xa and thrombin. While the kinetic parameters of both enzymes vary in similar manners with the various amino acid substitutions, they nevertheless show many differences. Factor Xa is more sensitive than thrombin to the occupation and variation of P_2 amino acids, while thrombin is more sensitive than Factor Xa to the occupation of P_3. Thrombin is the more efficient catalyst for the hydrolysis of these substrates.

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