Human α- to ζ-Thrombin Cleavage Occurs with Neutrophil Cathepsin G or Chymotrypsin While Fibrinogen Clotting Activity Is Retained†

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Received May 31, 1989; Revised Manuscript Received December 5, 1989

ABSTRACT: Human neutrophil cathepsin G or bovine chymotrypsin proteolytically cleaved human α-thrombin at the B-chain Trp_{148}–Thr_{149} bond generating a new form, ζ-thrombin. While incubation of α-thrombin with cathepsin G at pH 7.4 and 37 °C resulted in a partial loss of fibrinogen clotting activity, 86 ± 13% of the clotting activity and 99 ± 16% of the active sites titratable with *p*-nitrophenyl *p*-guanidinobenzoate were retained upon controlled passage of α-thrombin through chymotrypsin–Sepharose 4B at pH 6.2 or 7.4 and 24 °C (n = 15). Kinetic parameters for H-D-hexahydrotyrosyl-Ala-Arg *p*-nitroanilide were K_m = 1.52 ± 0.60 vs 1.32 ± 0.18 μM and k_{cat} = 51.9 ± 2.9 vs 35.8 ± 6.4 s^{-1} with α-thrombin vs chymotrypsin-prepared ζ-thrombin (n = 4 vs 3), respectively (I = 0.15 M, pH 7.4, and 24 °C). Some 95% of the clotting activity was lost when ζ-thrombin was passed through trypsin–Sepharose 4B under conditions for converting α- to nonclotting β- and subsequently γ-thrombin. The resulting γ-like thrombins eluted bimodally with 260 and 310 mM NaCl when applied to Amberlite CG-50 resin [cross-linked poly(methylacrylic acid)] developed with a linear salt gradient in 50 mM Tris at pH 7.4 and 24 °C. These elution peaks correspond to 240, 330, and 350 mM NaCl for γ-, α-, and ζ-thrombin, respectfully, implying that the anion-binding exosite is partially destroyed in γ-like thrombins but is intact in ζ-thrombin. Unlike α-thrombin, ζ-thrombin more rapidly loses clotting activity when incubated at pH 7.4 and 37 °C, where upon >90% behaves as denatured protein not retained on CG-50 resin. Thus, the ζ-cleavage destabilizes the protein but does not appreciably effect enzymic properties, such as clotting activity requiring both the catalytic site and adjacent regions, as well as the anion-binding exosite.

Procoagulant α-thrombin (EC 3.4.21.5) has central bioregulatory functions in hemostasis, various disease states, and wound healing (Fenton, 1981, 1986, 1988). Other forms, β- and γ-thrombin, which arise from autoproteolytic or limited tryptic cleavage, are of questionable physiological significance. These forms have been exceedingly useful, however, in probing the requirements of various thrombin activities, since these forms essentially lack fibrinogen clotting activity while retaining certain proteolytic capabilities and activities with synthetic substrates. On the other hand, ε-thrombin formed by neutrophil elastase is potentially a physiological form (Brower et al., 1987).

Since activated neutrophils also release the proteinase cathepsin G, we examined the effects of this proteinase obtained as a byproduct of our previous studies (Brower et al., 1987). Since the single cleavage in the thrombin B-chain produced by cathepsin G suggested a chymotrypsin-like specificity, we passed human α-thrombin through chymotrypsin–Sepharose 4B at pH 6.2 and 24 °C and found that it produced the identical cleavage and that the resulting ζ-thrombin retained >85% of the original clotting activity. This technique has enabled the preparation of ζ-thrombin at the 10–100-mg scale and examination of its properties. Cleavage at the Trp_{148}–Thr_{149} bond in the thrombin B-chain polypeptide does not destroy clotting, whereas this cleavage causes protein instability.

Experimental Procedures

Materials. The following were gifts: human plasma fraction III from Dr. Henry S. Kingdon (Hyland Therapeutics Division, Travenol Laboratories, Glendale, CA) and Spectrozyme-TH (H-D-HHT-Ala-Arg-pNA)[1] from Dr. Richard Hart (American Diagnostica, New York, NY). Bovine fibrinogen (95% clottable) was purchased from Miles Laboratories (Elkhart, IN), bovine chymotrypsin (twice crystallized) from Calbiochem (San Diego, CA), and soybean trypsin inhibitor from Sigma Chemical Co. (St. Louis, MO). The elastase inhibitor MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl and cathepsin G inhibitor Z-Gly-Leu-Phe-CH_2Cl were from Enzyme Systems Products

† These studies were supported in part by NIH Grants HL-13160 (J.W.F.), HL-27073 (D.A.W.), and HL-32166 (M.S.B.) and Specialized Center of Research in Thrombosis Grant H1-18828 at Cornell Medical Center.

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[1] Abbreviations: *i*-Pr_2P-F, diisopropyl phosphorofluoridate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; K_m, Michaelis–Menten constant; k_{cat}, catalytic constant; k_{cat}/K_m, specificity constant; MES, 2-(*N*-morpholino)ethanesulfonic acid; MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl, methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine chloromethyl ketone; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; PEG, poly(ethylene glycol); PTH, phenylthiohydantoin; SEM, standard error of mean; Spectrozyme-TH, H-D-hexahydrotyrosyl-L-alanyl-L-arginine *p*-nitroanilide; TFA, trifluoroacetic acid; Z-Gly-Leu-Phe-CH_2Cl, carbobenzoxyglycyl-L-leucyl-L-phenylalanyl chloromethyl ketone.

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Table I: Evaluation of ζ-Thrombin (after) Made by Controlled Passage of Human α-Thrombin (before) through Chymotrypsin–Agarose at pH 6.2[a]

prepn no. (source no.)	% thrombin form[b]						enzymic activities					
	before			after			NPGB titration[c]			fibrinogen clotting[d]		
	α	β	γ	α	β	ζ (γ)[e]	before	after	%	before	after	%
1, 2 (307)[f]	98.5	1.3	0.2	0.0	4.0	96.1	0.73	1.01	136	2616	2480	95
3, 4, 7 (312)[f]	97.1	2.9	0.0	4.7	1.9	93.4	1.16	1.14	98	3011	2440	81
5, 10 (311)[f,g]	97.4	2.6	0.0	2.2	0.2	97.7	1.12	1.04	93	2424	2373	98
6 (312)[h]	97.1	2.9	0.0	4.5	0.5	94.9	1.16	0.98	84	3011	2571	85
8 (322)[i]	79.8	19.6	0.7	2.8	1.6	95.7	0.87	0.78	90	1872	1672	89
9 (169)	99.0	1.0	0.0	0.4	6.0	93.6	0.91	1.00	110	2469	2135	77
11 (325)[i]	89.3	10.3	0.4	0.0	0.0	100.0	1.14	0.98	84	3124	2109	68
12 (292)	96.4	3.3	0.4	0.3	3.1	96.6	0.89	1.02	115	3121	2385	76
13 (317)	99.4	0.5	0.1	4.7	1.9	93.5	0.99	0.95	96	2929	3035	104
14 (310)	96.0	4.0	0.0	3.8	1.5	94.7	1.03	0.88	85	3355	2567	77
15 (332)[i]	84.1	14.4	1.5	1.6	0.7	97.7	0.97	1.01	104	2137	1768	83
mean	94.0	5.7	0.3	2.3	1.9	95.8	1.00	0.98	99	2734	2321	86
SEM	±6.6	±6.3	±0.5	±1.9	±1.8	±2.1	±0.14	±0.09	±16	±465	±385	±13

[a] See text for specific details. Two bovine ζ-thrombin preparations made by these methods from the same starting material averaged 3.0, 1.0, and 96.0% α-, β-, and ζ-thrombins, titrating to 1.05 sites/thrombin with 1874 kiloclotting units/g of protein. [b] Determined by labeling preparation with [^{14}C]*i*-Pr$_2$P-F and distribution of radioactivity upon electrophoresis in 0.1% SDS–10% cross-linked polyacrylamide gels. [c] Values expressed as molar ratio of burst release of *p*-nitrophenol per thrombin. The percent is the change upon converting α-thrombin (before) to ζ-thrombin (after). [d] Expressed in U.S. NIH kiloclotting units per gram of protein. [e] The [^{14}C]*i*-Pr$_2$F-labeled fragments of γ- and ζ-thrombin comigrate. [f] Average values for different ζ-thrombin preparations made from the same α-thrombin preparation. [g] Preparation 10 was made at pH 7.4 (see text); values for preparations 5 and 10 were very close. [h] Chymotrypsin–agarose was washed with soybean trypsin inhibitor prior to use. [i] Preparations of α-thrombin were chosen for high β-thrombin contents.

(Livermore, CA). Amberlite CG-50 resin (200–400 mesh) came from Mallinckrodt Chemical Works (St. Louis, MO), CNBr-activated Sepharose 4B from Pharmacia Fine Chemicals (Piscataway, NJ), and NPGB from ICN Pharmaceuticals, Inc. (Cleveland, OH). The buffer salts HEPES, MES, and Tris were obtained from Research Organics, Inc. (Cleveland, OH); all other materials were from sources indicated in publications cited below.

Thrombin Preparations. Human α-thrombin was prepared from fraction III paste (Fenton et al., 1977a,b). It was converted into γ-thrombin by controlled passage through trypsin-immobilized Sepharose 4B (Bing et al., 1977; Fenton et al., 1977b) or into ζ-thrombin by passage through chymotrypsin-immobilized Sepharose 4B (see below), whereas γ-like thrombin was made from ζ-thrombin by passage through trypsin-immobilized Sepharose 4B (see below). Throughout, an absorption coefficient of 1.75 or 1.83 mL mg^{-1} cm^{-1} at 280 nm was used for all thrombin forms in 1 M acetic acid or 0.10 M NaOH, respectively, and an M_r of 36 500 was assumed for these forms. Thrombin preparations were evaluated (Table I) and stored at −70 °C in 0.75 M NaCl until used (Fenton et al., 1977a,b).

Preparations were evaluated by (i) specific clotting activity with fibrinogen, expressed in U.S. NIH equivalent kiloclotting units per gram of protein, (kCU/g), (ii) percent esterolytically active enzyme by active-site titration with NPGB, and (iii) percent active thrombin form by labeling with [^{14}C]*i*-Pr$_2$P-F and electrophoresing in 0.1% SDS containing 10% cross-linked polyacrylamide gels (Fenton et al., 1977a). Throughout, clotting activities were based on the mean of at least three determinations. Salt gradient ion-exchange chromatography of thrombins on CG-50 resin was performed as described elsewhere (Fenton et al., 1988). Kinetic parameters were determined with Spectrozyme-TH essentially as described elsewhere (Witting et al., 1987) except that substrate concentrations were determined with 9167 M^{-1} cm^{-1} as the extinction coefficient for the isosbestic absorbance at 342 nm for Spectrozyme and *p*-nitroaniline as determined under the present experimental conditions of 0.15 M NaCl, 10 mM HEPES, and 10 mM Tris at pH 7.4 and 24 °C. For each of nine substrate concentrations, at least three determinations were made and averaged in three or four experiments (see Table III).

Chymotrypsin- or Trypsin–Sepharose 4B. The resin, 1.5 g of CNBr-activated Sepharose 4B, was swollen in 20 mL of 1 mM HCl and subsequently washed with 200 mL of the same. It was then washed with 8 mL of coupling buffer (25 mM Na_3BO_3 and 20 mM $CaCl_2$ at pH 10.2) and immediately transferred to 3 mL of coupling buffer containing 3 mg of chymotrypsin. The resin was then slowly stirred overnight at 4 °C, gently filtered, and then washed in succession with the following: (i) 200 mL of 25 mM Na_3BO_3 at pH 10.2, (ii) 200 mL of 0.2 M glycine and 0.1 M Na_3BO_3 at pH 8.0, (iii) 200 mL of 1 M NaCl and 0.1 M sodium acetate at pH 4.0, and (iv) 100 mL of 0.1 M NaCl and 10 mM sodium acetate at pH 4.0. The coupled resin was stored in the final washing solution containing 1% sodium azide at 4 °C. Trypsin–Sepharose 4B was prepared in a similar manner as previously described (Bing et al., 1977; Fenton et al., 1977b).

Preparation of ζ-Thrombin or γ-like Thrombin. A column of ~5 mL of swollen chymotrypsin–Sepharose 4B was prepared in a 5-mL plastic syringe barrel plugged with glass wool, and the resin was washed at 24 °C with 20 mL of 0.68 M NaCl buffered with 10 mM MES at pH 6.2 (or 10 mM HEPES at pH 7.4). Human α-thrombin in 0.75 M NaCl was diluted with 0.10 volume of 0.10 M MES at pH 6.2 (or 0.10 M HEPES at pH 7.4) and applied to the resin at a flow rate of 20–100 mL/h. The absorbancy at 280 nm was monitored with a Pharmacia single-path UV-1 monitor, and eluates with absorbancies of >1 were typically collected. The resulting ζ-thrombin pools were evaluated like other thrombin preparations (Table I).

In a similar manner, γ-like thrombin was made by passage of ζ-thrombin through trypsin-immobilized Sepharose 4B (see Table IV). In one instance, the chymotrypsin resin was treated with 2.4 mg of soybean trypsin inhibitor in 0.68 M NaCl and 10 mM MES at pH 6.2; in another instance, the resin was equilibrated with 0.68 M NaCl and 10 mM HEPES at pH 7.4 (see Table I).

Stability at 37 °C. Preparations of α-, γ-, and ζ-thrombins were chosen to have protein concentrations of ~2 mg/mL and relatively high enzymic activities. In an initial experiment,

Table II: Sequence Identity of the Cathepsin G Generated and Chymotrypsin-Generated Proteolysis Fragments of Human α-Thrombin[a]

	149 Thr	150 Ala	151 Asn	152 Val	153 Gly	154 Lys	155 Gly	156 Gln	157 Pro	158 Ser
cathepsin G[b]	0.44	0.93	0.89	0.96	0.82	0.87	0.92	0.76	0.92	0.61
chymotrypsin[c]	0.49	0.91	0.94	0.88	0.86	0.94	0.80	0.81	0.73	0.54

[a] In addition to the sequences shown, the NH_2-terminal sequences for the A-chain (Thr-Phe-Gly-Ser-Gly-Glu-Ala-Asp-Cys-Gly-) and the B-chain (Ile-Val-Glu-Gly-Ser-Asp-Ala-Glu-Ile-Gly-) were quantitatively recovered. Numbering corresponds to the position of this sequence within the human thrombin B-chain. Values listed below each amino acid correspond to the molar ratio of this residue to that identified in the same cycle for the B-chain. Values for Thr_{149} and Gly_{153} are presented as half-recoveries, since these residues are also released from the A-chain by the same degradative cycle. Both serine and threonine are low-recovery residues. Residue recoveries are uncorrected for operational losses. [b] Preparation was prepared by scaling up cathepsin G digestion conditions, employing chloromethyl ketone inhibitors (see Experimental Procedures). [c] Preparation 4 of ζ-thrombin in Table I.

α- and ζ-thrombins with clotting activities of ~2500 kilounits/g were diluted ~4.5-fold to a protein concentration of 12 μM in a final salt concentration of 0.16 M NaCl, containing 20 mM Tris and 1 mg/mL PEG 6000 at pH 7.4. In addition to α- and ζ-thrombin samples, a 1:1 mixture was prepared, and samples were incubated at 37 °C with aliquots periodically taken for measurement of clotting activities (see above). Precipitates (e.g., precipitated thrombin) were observed after 50 and 75 min for ζ- and α-thrombins, respectively. However, upon dilution with the clotting assay diluent (Fenton et al., 1977a), resuspended precipitates gave visibly clear solutions.

To assess how clotting activity was lost, samples of α-, γ-, and ζ-thrombins were prepared as above and incubated at 37 °C for 3.5 h. Clotting activities of each form were measured before and after incubation, and samples were chromatographed on CG-50 resin developed with a linear NaCl gradient, as described elsewhere (Fenton et al., 1988).

Cathepsin G Digestion. Human polymorphonuclear leukocyte cathepsin G was obtained as a byproduct of neutrophil elastase isolated from outdated white-cell concentrates or from purulent sputum (Martodam et al., 1979; Brower et al., 1987). In a typical experiment, 0.25-mL samples of human α-thrombin at 3.25 mg/mL were dialyzed for 20 min with two changes of 500 mL of 0.15 M NaCl and 20 mM sodium phosphate at pH 7.4 and 4 °C. To 0.2 mL of the dialyzed sample were added 8 μL of cathepsin G at 5.99 mg/mL (or 2.50 μM) and 5 μL of 70 mM MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl (0.5 mg in 30 μL of ethanol), and the mixture was incubated at 37 °C for 60 min. These were then quenched by addition of 10 μL of 20 mM Z-Gly-Leu-Phe-CH_2Cl (0.5 mg in 50 μL of dimethyl sulfoxide) and incubated at 37 °C for another 10 min. Typically, 15-μL aliquots were taken for electrophoresis in SDS-containing 9–18% cross-linked polyacrylamide gels, and clotting activities were performed as previously described (Brower et al., 1987).

Subsequent experiments were carried out in a similar manner except that neither of the chloromethyl ketone proteinase inhibitors was used since they were also found to inhibit α-thrombin in control samples. Clotting assays and electrophoresis were performed as presently described.

Amino Acid Sequence Analysis. Samples of digested thrombins were dialyzed against 0.1% TFA and freeze-dried. These were then redissolved in 0.1% TFA, and 700 pmol was analyzed with an Applied Biosystems Model 470 gas-phase sequenator equipped with a 120A HPLC PTH analyzer. Individual PTH residues were quantitated on a Nelson analytical chromatography data system (Walz et al., 1988). Duplicate sample values were averaged, and the predicted residues for the A- and B-chains of human α-thrombin (Degen et al., 1983) were subtracted.

Results

Initial studies were carried out with human neutrophil cathepsin G isolated as a byproduct of neutrophil elastase purification (Brower et al., 1987). In such experiments, human α-thrombin was incubated with cathepsin G under physiologically relevant conditions of pH 7.4 and 37 °C. Clotting activities were found to decline over 2–4 h to ~60–80% of initial activities, while electrophoresis revealed the formation of two protein fragments similar to those formed upon elastase cleavage of α-thrombin (Brower et al., 1987). Under nonreducing conditions, both α-thrombin and cathepsin G electrophoresed as essentially single components, and the digests migrated predominantly as two major fragments (data not shown). Sequence degradation of cathepsin G digested α-thrombin showed that a single cleavage occurred in the thrombin B-chain (Table II).

Subsequent experiments confirmed similar losses in clotting activity upon incubation of α-thrombin with cathepsin G, and the fragments generated were found to electrophorese with M_r values of ~26 000 and 15 000, where the latter was labeled with [^{14}C]*i*-Pr_2P-F for the active-site serine (Ser_{195} in chymotrypsin) fragment (data not shown).[2] In these experiments, the chloromethyl ketone inhibitors MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl and Z-Gly-Leu-Phe-CH_2Cl for elastase and cathepsin G, respectively (Powers et al., 1977), were omitted, since we found that they were effective thrombin inhibitors (e.g., 51 vs 78% loss of clotting activity at 37 °C for 2 h in the absence vs presence of chloromethyl ketones, respectively), like other heretofore unsuspected inhibitors of the enzyme (Witting et al., 1988).

Because cathepsin G cleavage of the thrombin B-chain implied a chymotrypsin-like specificity, we reasoned that chymotrypsin might carry out the same cleavage under controlled conditions. Passage of α-thrombin through chymotrypsin–Sepharose 4B in 0.68 M NaCl at pH 6.2 and ~24 °C caused essentially no loss of clotting activity. However, upon electrophoresis, essentially complete fragmentation occurred. Subsequent quantitation of 15 preparations of ζ-thrombin established >95% conversion with ~100% retention of NPGB-titratable active sites and >85% clotting activity (Table I). Sequence analysis revealed the same cleavage as for cathepsin G (Table II), and the appropriate-sized fragment of M_r ~15 000 was labeled with [^{14}C]*i*-Pr_2P-F for that containing the active-site serine (Figure 1). In addition to retaining its catalytic sites by NBPG titration and most of its fibrinogen clotting activity, the kinetic parameters (k_m, k_{cat}, and k_{cat}/K_m) for α- vs ζ-thrombin did not significantly differ (±1 SEM) with the synthetic substrate Spectrozyme-TH (Table III). Chromatography of ζ-thrombin on CG-50 resin with a salt gradient further showed that it was retained slightly more than α-thrombin (Figure 2A and 2B), in contrast to γ-thrombin (Figure 2D), thereby demonstrating that the anion-binding

[2] These values sum to ~41 000, which is in accord with the aberrantly high value of 38 500 ± 700 for human α-thrombin electrophoresed under nonreducing conditions (Fenton et al., 1977a).

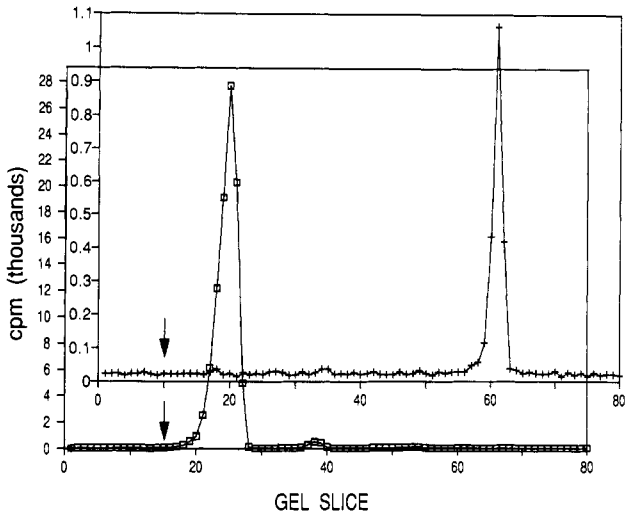

FIGURE 1: Radioactivity profile of human α-thrombin preparation 307 (□) and ζ-thrombin preparation 11 (+) labeled with [^{14}C]*i*-Pr_2P-F and electrophoresed in 0.1% SDS–10% cross-linked polyacrylamide gels under nonreducing conditions (Fenton et al., 1977a). Gel slices were 1 mm. The first peak corresponds to the labeled product of α-thrombin (the intact protein), the second to that of β-thrombin [the B_2 fragment in Fenton et al. (1979)], and the third to that of γ- or ζ-thrombin (the B_4 fragment). The gels were aligned by the migration of bovine serum albumin employed as an internal standard (↓). The M_r of the labeled component of ζ-thrombin is ~15 000.

Table III: Kinetic Parameters for α- versus ζ-Thrombin Determined with Spectrozyme-TH[a]

thrombin form	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
α-thrombin (n = 4)[b]	1.52 ± 0.60	51.9 ± 2.9	39.3 ± 17.6
ζ-thrombin (n = 3)[c]	1.39 ± 0.18	35.8 ± 6.4	26.0 ± 5.5

[a] Determined in 0.15 M NaCl, 10 mM HEPES, and 10 mM Tris at pH 7.4 and 25 °C. See text for experimental details. [b] Determined with α-thrombin preparation 312 (Table I) at 1.18, 1.55, and 1.43 mM concentration. [c] Determined from two determinations with ζ-thrombin in preparations 1 and from one determintion with preparation 4 (Table II) at 1.35 and 1.43 mM concentrations.

exosite was intact for ζ-thrombin (Fenton et al., 1988).

To assess if the ζ-cleavage was independent of the β-cleavage(s), ζ-thrombin was passed through trypsin–Sepharose 4B under conditions for forming β- and γ-thrombins. This caused a small drop in the NPGB-titratable site (e.g., proteolysis to inactive products), while <5% of the clotting activity was recovered (Table IV). This result agrees with other data which show that the β-cleavage(s) cause a marked drop in clotting activity (Sonders & Fenton, 1986; Bezeaud & Guillin, 1988; Hofsteenge et al., 1988). Since the β-cleavage(s) are located in the anion-binding exosite (Fenton, 1986, 1988), the resulting γ-like thrombin from tryptic cleavage(s) was chromatographed on CG-50 resin with a salt gradient and was found to elute like γ-thrombin (panel C vs panel D of Figure 2), showing that the exosite was modified (Fenton et al., 1988). The fact that γ-like thrombin eluted as a bimodal peak, although primarily like γ-thrombin, indicates that more than a single cleavage occurred.

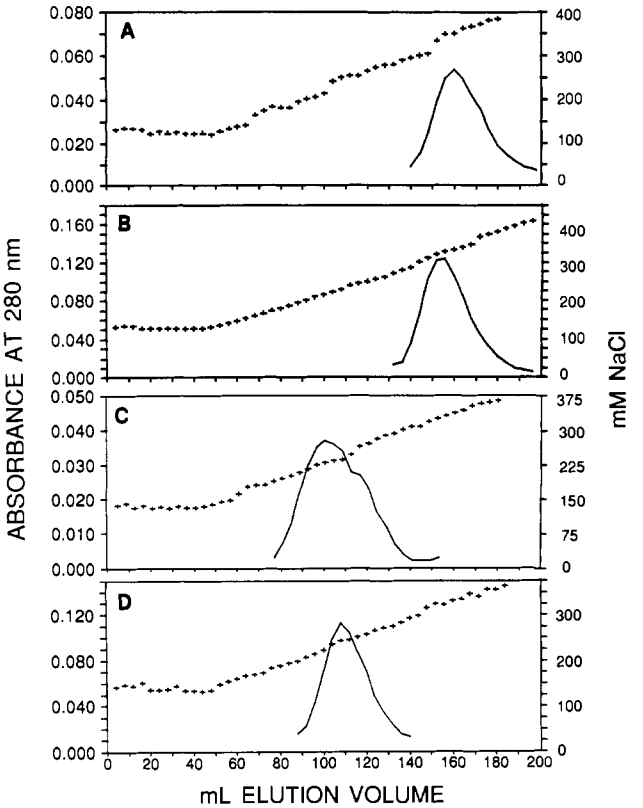

FIGURE 2: Elution profiles of thrombin forms from Amberlite CG-50 resin [200–400-mesh cross-linked poly(methacrylic acid)]. Salt gradient chromatography was carried out with a linear NaCl gradient in 50 mM Tris at pH 7.4 and ~24 °C (Fenton et al., 1988). (A) ζ-Thrombin preparation 5 with midpeak at 350 mM NaCl; (B) α-thrombin 311 at 330 mM; (C) γ-like thrombin (Table IV) at 260 and 310 mM; (D) γ-thrombin 72 at 240 mM. Absorbances not shown did not significantly differ from those of the base line.

Laboratory handling of ζ-thrombin indicated that it was less stable than α-thrombin (e.g., lost clotting activity). In stability experiments at 37 °C, ζ-thrombin indeed lost clotting activity more rapidly than α-thrombin (Figure 3) and did so independently of the presence of α-thrombin (i.e., the 1:1 mixture of α- and ζ-thrombin). When incubated at 37 °C for 3.5 h and chromatographed on CG-50 resin with a salt gradient, α-thrombin eluted as 5% denatured (void volume), 20% autolyzed (intermediately retained), and 75% native form, where the recovery of the native form correlated with the clotting activity retained (Table V). In contrast, ζ-thrombin eluted as 92% denatured and 8% native form, and γ-thrombin eluted as 94% denatured and 6% native form. Since γ-thrombin readily denatured, any autoproteolytic conversion of ζ- to γ-like thrombin could not have been detected in this experiment. However, in another experiment where ζ-thrombin was incubated for 30 min at 37 °C, 72 and 28% were found in the

Table IV: Conversion of Human α- to ζ-Thrombin and Subsequently to γ-like Thrombin by Passage through Chymotrypsin- and Trypsin–Agarose Resins[a]

material	thrombin form[b]			NPGB titration[c]	fibrinogen clotting	
	α	β	γ/ζ		kCU/g	% retained
α-thrombin (original prepd)[d]	97.4	2.6	0.00	1.12	2424	100
ζ-thrombin (through chymotrypsin–agarose)[d]	2.8	0.3	96.9	1.20	2347	96.8
γ-like thrombin (through trypsin–agarose)	0.0	4.6	95.4	0.78	109	4.5

[a] See text or experimental procedures. [b] The [^{14}C]*i*-Pr_2P-F-labeled, catalytic serine containing fragments of either γ- or ζ-thrombin are of similar size and are not well resolved upon SDS electrophoresis in 10% cross-linked polyacrylamine gels. [c] Molar ratio of burst release of *p*-nitrophenol per thrombin. [d] Preparations α-311 and ζ-5 in Table I, respectively.

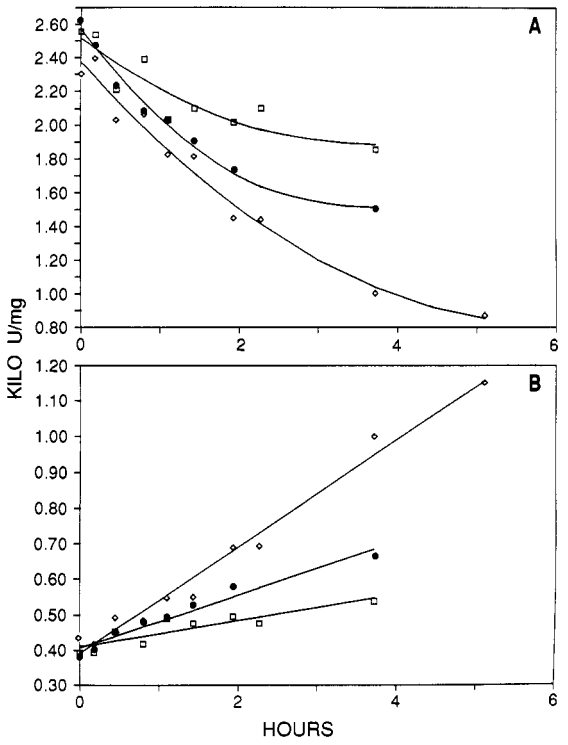

FIGURE 3: Stability of α-thrombin 317 (□) vs ζ-thrombin 12 (◊) vs a 1:1 mixture of the α- and ζ-thrombins (●) in 20 mM Tris–0.15 M NaCl containing 1 mg/mL PEG 6000 at pH 7.4 and 37 °C. Specific activities were measured by clotting activities. Panel A shows the direct plot, whereas panel B gives that for the reciprocal activity. The slopes in panel B multiplied by the zero-time specific clotting activities gave rates of 0.096, 0.198, and 0.351 h^{-1} for α-, α- + ζ-, and ζ-thrombins, respectively.

native and denatured forms, respectively, with no indication of γ-like thrombin, suggesting that ζ-thrombin may be directly denaturing into inactive forms. Such denaturation of ζ-thrombin at 37 °C would further account for the loss of clotting activities in our initial studies with cathepsin G.

Discussion

Procoagulant α-thrombin not only converts fibrinogen into clottable fibrin by cleaving fibrinopeptides A and B but also becomes actively incorporated into fibrin clots (Liu et al., 1979; Wilner et al., 1981) and is found in thrombi (Francis et al., 1983). Blood clots or thrombi have an architecture consisting of coarse fibrin rods in the interior covered with a fine mesh fibrin canopy (Galanakis et al., 1987). As such, thrombi provide protective environments shielding their constituents and further contain blood cells, such as erythrocytes, neutrophils, and activated platelets.

Table V: Thermal Stabilities of Thrombin Forms[a]

products	thrombin preparation		
	α-317[b]	ζ-14[b]	γ-77
denatured protein	5.11	91.9	94.4
γ-thrombin or γ-like thrombin	20.1	0	5.63
α- or ζ-thrombin	74.8	8.10	0

[a] Relative peak areas (%) obtained from the degradation products of thrombin forms after heating at 37 °C for 3.5 h. Samples were chromatographed on CG-50 resin developed with a linear salt gradient, and protein fractions were monitored by their absorbances at 280 nm (see text for details). Denatured proteins eluted in the void volume, whereas γ-thrombin or γ-like thrombin eluted with <300 mM NaCl and α- or ζ-thrombin with >300 mM NaCl in 50 mM Tris at pH 7.4 and 24 °C. [b] After heating, 77.8 and 6.1% of the initial clotting activities were retained for the α- vs ζ-thrombin preparations, respectively. These retained clotting activities agree closely with the values of 74.8 and 8.10% for the relative amounts of α- or ζ-thrombin eluted from CG-50 resin.

During thrombus maturation, fibrin becomes cross-linked via thrombin-activated factor XIII, clot contraction occurs, and the aging process proceeds. In thrombin-induced pulmonary edema, neutrophil activation is a hallmark of the onset of edema and vascular injury (Malik, 1986). Activated neutrophils release not only oxidants but also proteinases, which partially degrade fibrin (Francis & Marder, 1986). Of these, we have previously shown that neutrophil elastase will convert human α- into ϵ-thrombin by cleaving the Ala_{150}–Asn_{151} bond in the thrombin B-chain (Brower et al., 1987). In the present study, we have further shown that neutrophil cathepsin G will convert α- to ζ-thrombin by cleaving the Trp_{148}–Thr_{149} bond in the B-chain (Table II). These cleavages occur two residues apart and within an insertion peptide when the B-chain amino acid sequence is aligned to that of chymotrypsin (Figure 4).

Whereas pancreatic elastase (Kawabata et al., 1985) carries out the same cleavage as neutrophil elastase (Brower et al., 1987) and does so in either human or bovine thrombins [Figure 1 in Fenton and Bing (1986)], we presently found that chymotrypsin mimics neutrophil cathepsin G in carrying out the identical corresponding cleavage (Table II). Although not verified by sequence analysis, we also have prepared the bovine thrombin homologue, which behaves the same as human ζ-thrombin upon labeling with [^{14}C]*i*-Pr_2P-F and electrophoreses in 0.1% SDS containing polyacrylamide gels (Table I, footnote *a*). The fact that the bovine thrombin B-chain sequence is the same as that of human thrombin at the ζ-cleavage site (Figure 4) strongly suggests that homologous forms are produced in either species. Interestingly, both of the ϵ- and ζ-cleavage sites are located in an insertion peptide, as shown when the thrombin B-chain is compared to chymotrypsin (Figure 4). This insertion further occurs where α-chymotrypsin is auto-

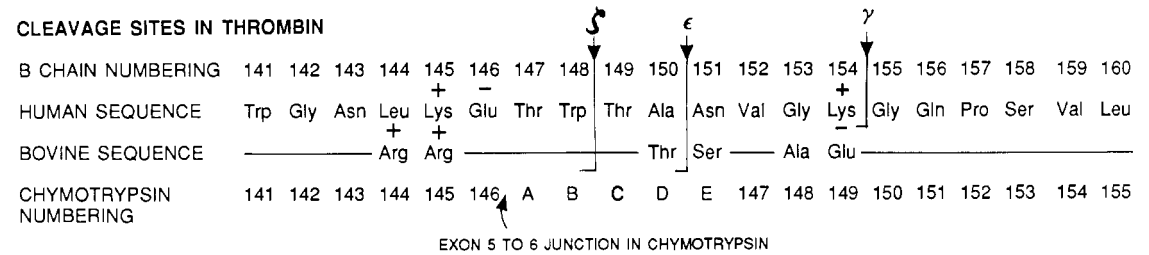

FIGURE 4: γ-, ϵ-, and ζ-cleavage sites in the thrombin B-chain. The γ-site is for autoproteolytic or tryptic cleavage of human α-thrombin (Fenton et al., 1977b). The ϵ-site is for cleavage by neutrophil elastase (Brower et al., 1987) or pancreatic elastase (Kawabata et al., 1985), whereas the ζ-site is for cleavage by neutrophil cathepsin G or chymotrypsin (Table II). Bovine thrombin lacks the γ-site (Fenton et al., 1977b), is cleaved at the ϵ-site by pancreatic elastase (Fenton & Bing, 1981), and presumptively has the ζ-site (D. V. Brezniak and J. W. Fenton II, unpublished data). The 146–147 bond in α-chymotrypsin is autoproteolytically cleaved (Briktoft & Blow, 1972) and corresponds to the exon 5–6 junction in the rat chymotrypsin B gene (Bell et al., 1984). This junction, furthermore, corresponds to the insertion peptide containing the ϵ- and ζ-sites in the thrombin B-chain.

proteolytically cleaved and where an exon junction occurs in the chymotrypsinogen B gene (Fenton & Bing, 1986). However, this insertion does not correspond to the appropriate exon junction in the bovine prothrombin gene; this suggests that the thrombin zymogen is more highly evolved than chymotrypsinogen (Irwin et al., 1988).

Employing chymotrypsin–Sepharose 4B, we have been able to produce 50–100 mg scale preparations retaining ~100% of the enzyme active sites by NPGB titrations and possessing >85% of the clotting activity of the parental α-thrombin preparation. Kinetic parameters determined with the tripeptide chromogenic substrate Spectrozyme-TH revealed no significant differences between α- and ζ-thrombins, implying no major structural differences in the derivative thrombin surrounding the catalytic site (Table III). That the anion-binding exosite necessary for fibrin(ogen) recognition (Fenton et al., 1988) also was retained intact was shown by the salt gradient elution of ζ-thrombin from CG-50 resin (Figure 3). The observation that it eluted with a slightly higher salt concentration than α-thrombin suggests that cleavage in ζ-thrombin might allow the exosite to associate more strongly with this anionic resin than for the native enzyme.

Three residues beyond the insertion peptide is the autoproteolytic or tryptic γ-cleavage site at Lys_{154} in the human thrombin B-chain (Figure 4). This residue is replaced by Glu_{154} in bovine thrombin with the consequence that a counterpart of human γ-thrombin cannot be found (Fenton et al., 1977b). In fact, passing bovine thrombin through trypsin immobilized on Sepharose 4B results in 100% conversion to β-thrombin; this form, like human γ-thrombin, essentially lacks clotting activity (Sonder & Fenton, 1986). The more recent isolation of human β-thrombin from partial digests (Bezeaud & Guillin, 1988; Hofsteenge et al., 1988) has confirmed that this form is a prerequisite to forming γ-thrombin and that loss of clotting activity occurs concurrent to forming β-thrombin (Fenton et al., 1977a,b). The present finding that proteolytic cleavage six residues in front of the γ-cleavage site (Figure 4) does not result in substantial loss of clotting activity (Table I) implies that the protein domain containing the three cleavage sites is not critical for preserving clotting (e.g., the catalytic site and adjacent regions, as well as the anion-binding exosite). Similar conclusions may also be drawn from studies on ε-thrombin (Brower et al., 1987; Hofsteenge et al., 1988).

When human ζ-thrombin was passed through trypsin–Sepharose 4B (under conditions for preparing γ-thrombin), we found, as anticipated, that clotting activity was essentially destroyed (Table IV) and that the γ-like thrombin eluted from the CG-50 resin was more similar to γ- than to α- or ζ-thrombin (Figure 3). The fact that it eluted as a bimodal peak indicates more than a single tryptic cleavage (e.g., various β-cleavages, or the γ-cleavage may or may not have occurred). Since autoproteolysis should occur at the sites cleaved by trypsin (Fenton et al., 1977a,b; Boissel et al., 1984; Chang, 1986), we reasoned that ζ-thrombin might be less stable than α-thrombin, owing to conversion to γ-like thrombins. Indeed, ζ-thrombin was less stable than α-thrombin upon incubation at pH 7.4 and 37 °C (Figure 3). This instability was not attributable to degradation by α-thrombin of ζ-thrombin since the 1:1 mixture declined in activity to half that of either form. However, when samples of 3.5-h incubations were applied to CG-50 resin, they revealed the predominance of denatured materials (Table V). Similarly, after 30-min incubation of ζ-thrombin at 37 °C, no evidence was obtained for its autoproteolytic conversion to γ-like thrombin, suggesting that it directly converts to denatured forms. In this regard, the 50% denaturation temperature for heating human α-thrombin for 5 min is ~50 °C but varies with a number of conditions (Landis et al., 1981), and that of γ-thrombin is ~10 °C lower (Fenton et al., 1979).

However, various substances enhance the thermal stability of thrombin (Landis et al., 1981). Because thrombin in a clot may be in contact with fibrin and other proteins, these might stabilize it from denaturation. The observation that greater chromogenic substrate activities (all thrombin forms) can be obtained from thrombi than can be accounted for by clotting activities (e.g., α- and ζ-thrombins) suggests that α-thrombin may be converted to nonclotting/low clotting activity forms in vivo (Francis et al., 1983). Such forms, along with α-thrombin, might stimulate cellular responses, such as inducing tissue plasminogen activator release (Levin et al., 1986), increasing albumin transport across endothelial monolayers (Garcia et al., 1986), and causing smooth muscle contracture (Walz et al., 1985), as well as being potent mitogens (Glenn et al., 1980; Perdue et al., 1981). This suggests that various thrombin forms may participate in the initiation of recovery or wound healing processes (Fenton, 1988).

Acknowledgments

In addition to those cited in the text for gifts, we thank Kimberly E. Garry in New York for assisting with neutrophil cathepsin G experiments and June Snow in Detroit for operating the gas-phase sequencer, which is part of the Protein Core Facility supported by Wayne State University School of Medicine. We also thank Christine T. Bradley and Mary Jane Boulay in Albany for their secretarial assistance.

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Complete Resonance Assignment for the Polypeptide Backbone of Interleukin 1β Using Three-Dimensional Heteronuclear NMR Spectroscopy†

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Received October 3, 1989; Revised Manuscript Received December 6, 1989

ABSTRACT: The complete sequence-specific assignment of the ^{15}N and ^{1}H backbone resonances of the NMR spectrum of recombinant human interleukin 1β (153 residues, M_r = 17 400) has been obtained by using primarily ^{15}N-^{1}H heteronuclear three-dimensional (3D) NMR techniques in combination with ^{15}N-^{1}H heteronuclear and ^{1}H homonuclear two-dimensional NMR. The fingerprint region of the spectrum was analyzed by using a combination of 3D heteronuclear ^{1}H Hartmann-Hahn ^{15}N-^{1}H multiple quantum coherence (3D HOHAHA-HMQC) and 3D heteronuclear ^{1}H nuclear Overhauser ^{15}N-^{1}H multiple quantum coherence (3D NOESY-HMQC) spectroscopies. We show that the problems of amide NH and C$^{\alpha}$H chemical shift degeneracy that are prevalent for proteins of this size are readily overcome by using the 3D heteronuclear NMR technique. A doubling of some peaks in the spectrum was found to be due to N-terminal heterogeneity of the ^{15}N-labeled protein, corresponding to a mixture of wild-type and des-Ala-1-interleukin 1β. The complete list of ^{15}N and ^{1}H assignments is given for all the amide NH and C$^{\alpha}$H resonances of all non-proline residues, as well as the ^{1}H assignments for some of the amino acid side chains. This first example of the sequence-specific assignment of a protein using heteronuclear 3D NMR provides a basis for further conformational and dynamic studies of interleukin 1β.

Interleukin 1 (IL-1)[1] is a cytokine protein with a variety of pleiotropic effects, interacting with virtually every organ and tissue system in the body, which is secreted from many nucleated cell types, particularly activated monocytes and macrophages. Specific activities identified for IL-1 include thymocyte proliferation via the induction of interleukin 2 release, stimulation of B-lymphocyte proliferation, prostaglandin and collagenase release, induction of acute-phase protein synthesis by hepatocytes, and fibroblast growth factor activity.

† This work was supported by the Intramural AIDS Targeted Antiviral Program of the Office of the Director of the National Institutes of Health (to G.M.C. and A.M.G.).
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[1] Abbreviations: IL-1, interleukin 1; IL-1α, interleukin 1α; IL-1β, interleukin 1β; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; HMQC, heteronuclear multiple quantum coherence spectroscopy; COSY, correlated spectroscopy; P.COSY, primitive COSY; $d_{XY}(i,i+1)$, NOE between proton X on residue i and proton Y on residue i + 1.

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