Maeda, H., Matsumura, Y., & Kato, H. (1988) *J. Biol. Chem. 263*, 16051–16054.
Matsuura, E., Igarashi, Y., Fujimoto, M., Ichikawa, K., & Koike, T. (1990) *Lancet* (July 21), 177–178.
McNeil, H. P., Simpson, R. J., Chesterman, C. N., & Krilis, S. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4120–4124.
Muta, T., Miyata, T., Misumi, Y., Tokunaga, F., Nakamura, T., Toh, Y., Ikehara, Y., & Iwanaga, S. (1991) *J. Biol. Chem. 266*, 6554–6561.
Polz, E., & Kostner, G. M. (1979) *FEBS Lett. 102*, 183–186.
Schousboe, I. (1988) *Eur. J. Biochem. 176*, 629–636.
Schousboe, I., & Rasmussen, M. S. (1988) *Int. J. Biochem. 20*, 787–792.
Steinkasserer, A., Estaller, C., Weiss, H., Sim, R. B., & Day, A. J. (1991) *Biochem. J. 277*, 387–391.
Sueyoshi, T., Miyata, T., Hashimoto, N., Kato, H., Hayashida, H., Miyata, T., & Iwanaga, S. (1987) *J. Biol. Chem. 262*, 2768–2779.

Reconstitution of Catalytically Competent Human ζ-Thrombin by Combination of ζ-Thrombin Residues A1–36 and B1–148 and an *Escherichia coli* Expressed Polypeptide Corresponding to ζ-Thrombin Residues B149–259

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ABSTRACT: Human ζ-thrombin, a catalytically competent serine proteinase, arises from a single chymotryptic cleavage at Trp-148 in α-thrombin to generate two nonconvalently associated polypeptide segments designated ζ1-thrombin (the 36-residue A-chain disulfide linked to B-chain residues B1–148) and ζ2-thrombin (B149–259). We report here the expression of recombinant ζ2-thrombin in *Escherichia coli* and the reconstitution of catalytically competent ζ-thrombin by combination of ζ1-thrombin with recombinant ζ2-thrombin. A DNA fragment encoding ζ2-thrombin was cloned into a pATH2 expression vector as a *trpE*–ζ2 fusion gene, in which a factor Xa cleavage site was inserted between the *trpE* and the ζ2-thrombin gene. High-level expression of this fusion protein was achieved under the control of the *E. coli trp* promoter. The expressed ζ2-thrombin was liberated from the fusion protein by factor Xa cleavage, reduced with DTT, and purified to homogeneity by reverse-phase HPLC. Oxidation of the reduced ζ2-thrombin in the presence of 80 μM $CuSO_4$ and 6 M urea at pH 8.15 yielded material that was indistinguishable on HPLC from ζ2-thrombin isolated by resolution of human ζ-thrombin. Catalytically active ζ-thrombin was generated by combination of recombinant ζ2-thrombin with ζ1-thrombin that was isolated by resolution of human ζ-thrombin. Recombinant ζ-thrombin displayed catalytic activities, toward a small chromogenic substrate and fibrinogen, that were similar to those of α-thrombin prepared from human blood plasma and ζ-thrombin obtained by treatment of α-thrombin with chymotrypsin. This result indicates that the information for formation of a catalytically competent conformation resides in the primary structure of ζ-thrombin and suggests that studies of variants of ζ-thrombin produced by site-directed mutagenesis of ζ2-thrombin could facilitate identification of the structural and functional determinants of the interactions of thrombin that are important in blood coagulation.

α-Thrombin is a serine protease that plays a central role in hemostasis. It converts (via limited proteolysis) fibrinogen to fibrin monomers that polymerize spontaneously to form the insoluble fibrin matrix of blood clots (Blomback, 1978; Shafer & Higgins, 1988). α-Thrombin also catalyzes conversion of factor XIII to factor XIIIa, a transglutaminase that stabilizes fibrin clots by cross-linking fibrin to itself and other plasma proteins (Lorand & Konishi, 1964; Takagi & Doolittle, 1974; Lewis et al., 1987). Additionally, thrombin regulates the reaction cascade responsible for its generation. α-Thrombin activates factor V, factor VIII, and platelets so as to induce an explosive increase in the rate of generation of thrombin during blood coagulation (Coleman, 1969; Nesheim & Mann, 1979; Hoyer & Trabold, 1981; Mann et al., 1988; Berndt et al., 1986). When thrombin enters the microcirculation (where the concentration of the endothelial cell-surface receptor, thrombomodulin, becomes substantial), thrombin exerts a negative regulatory effect by forming a complex with thrombomodulin which in turn activates protein C (Kisiel, 1979; Esmon et al., 1986). Activated protein C together with its cofactors modulates the thrombin-generating cascade by proteolytically inactivating factor Va and factor VIIIa (Esmon, 1987). Besides its involvement in blood coagulation, thrombin displays a variety of effects in other biological systems. It functions as (i) a growth factor (Bar-shavit et al., 1986), (ii) a promoter of endothelial cell adhesion (Bar-shavit et al., 1991), and (iii) an activator of prostacyclin release from endothelial cells (Pearson et al., 1983).

Various derivatives of thrombin have been obtained from the products of either autolysis or limited proteolysis of α-thrombin, a protein comprised of a 36-residue A-chain disulfide linked to a 259-residue B-chain. Cleavage of α-thrombin at Arg-73, Ala-150, and Trp-148, yields β-, ϵ-, and ζ-thrombin,

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respectively (Mann et al., 1973; Fenton et al., 1977; Braun et al., 1988; Elion et al., 1986; Kawabata et al., 1985; Brezniak et al., 1990). Cleavage of α-thrombin at both Arg-73 and Lys-154 results in the formation of γ-thrombin (Elion et al., 1986; Fenton et al., 1977). β-Thrombin and γ-thrombin are capable of catalyzing the hydrolysis of small peptide substrates, with an efficiency similar to that of α-thrombin, but exhibit markedly diminished fibrinogen-clotting activity (Bezeaud & Guillin, 1987; Hofsteenge et al., 1987; Lewis et al., 1987). ε-Thrombin and ζ-thrombin, however, exhibit activities toward both small substrates and fibrinogen that are similar to those of α-thrombin (Hofsteenge et al., 1987; Brezniak et al., 1990). Although the physiological significance (if any) of the various thrombin derivatives is unknown, they have been extremely useful in identifying domains of thrombin important in its interactions with fibrinogen (Brezniak et al., 1990; Hosfsteenge et al., 1987; Brower et al., 1987).

The observation that proteolytically nicked thrombin derivatives are catalytically active at subnanomolar concentrations indicates that the polypeptide fragments comprising the nicked thrombin derivatives are held together by strong noncovalent interactions. Thus, studies of the resolution and reconstitution of proteolytically nicked α-thrombin derivatives and variants thereof could provide important information regarding the interactions responsible for maintaining the quaternary structure of the derivatives and, by inference, the tertiary structure of α-thrombin. Moreover, much additional information concerning physiologically important interactions of thrombin could be obtained from systematic point mutants of recombinant thrombin. In the present study of human ζ-thrombin and its two components ζ1-thrombin[1] (the 36-residue A-chain disulfide linked to B1–148, encompassing the active site residues H43 and D99) and ζ2-thrombin (B149–259, encompassing the active site residue S205), we demonstrate expression in *Escherichia coli* of recombinant ζ2-thrombin fused with the trpE protein (via a factor Xa cleavage site). After factor Xa catalyzed release from the fusion protein and oxidation, recombinant ζ2-thrombin with correctly formed disulfide bonds was purified to homogeneity and combined with ζ1-thrombin (isolated by resolution of human ζ-thrombin) to generate catalytically active recombinant ζ-thrombin capable of clotting fibrinogen.

Experimental Procedures

Materials. Bovine factor Xa and Chromozym-TH (tosGPR-pna) were from Boehringer Mannheim. Human fibrinogen (essentially plasminogen-free) was purchased from Sigma. *E. coli* RR1 was from BRL. Human α- and ζ-thrombin were kindly provided by Dr. John W. Fenton II (New York State Department of Health). The pATH2 expression vector was the generous gift of Drs. T. J. Koerner and A. Tzagaloff (Columbia University). The human thrombin cDNA was provided by Kathleen Berkner (Zymogenetics). The C4 reverse-phase HPLC column was from Vydac. The Bio-Sil SEC-125 HRLC column was purchased from Bio-Rad. All the other reagents are either HPLC grade or analytical grade.

Isolation of Native ζ2-Thrombin and ζ1-Thrombin. ζ1- and ζ2-thrombin were prepared by resolution of human ζ-thrombin by C4 reverse-phase HPLC with a polyphasic acetonitrile gradient (0–36.5% acetonitrile in 1 min; 36–40% acetonitrile in 35 min; 40–100% acetonitrile in 5 min at a flow rate of 1.5 mL/min) in the presence of 0.1% trifluoroacetic acid. Both ζ1- and ζ2-thrombin were purified to homogeneity as judged by SDS–PAGE.

Construction of trpE–ζ2 Expression Vector. A pair of synthetic oligonucleotides (TCGACCATCGAAGGTCGT and ACGACCTTCGATGG) encoding a factor Xa cleavage site at the 3′ end and containing a *Sal*I site at the 5′ end was prepared. The cDNA sequence encoding ζ2-thrombin was amplified by PCR using the oligonucleotides ACAGCCAACGTTGGTAAGGGG and TTAAGCTTCACGGGATTGGTTCCAGGA as 5′-end and 3′-end primers, respectively. *Hin*dIII digestion of the amplified DNA generated a sticky end in the *Hin*dIII site at the 3′ end of the DNA. The factor Xa fragment and amplied ζ2 DNA were ligated into a pATH2 vector that had been digested by *Sal*I and *Hin*dIII. The DNA sequences of the ζ2-thrombin and factor Xa site were verified by nucleotide sequence analysis.

Expression and Preparation of the trpE–ζ2 Fusion Protein. The expression vector was used to transform an *E. coli* RR1 strain. A single transformed colony was used to inoculate 2 mL of M9-CA medium with ampicillin and was allowed to grow for 16 h at 29 °C with shaking (400 rpm). The resulting 2-mL suspension of bacteria was mixed with 0.86 mL of glycerol to form a bacterial stock that was stored at −70 °C. This stock was used to inoculate agar plates for colony growth. For large-scale preparation of trpE–ζ2 fusion protein, 1 L of M9-CA medium with ampicillin was inoculated with freshly grown bacteria on an 8-cm petri dish (containing about 300 colonies), and incubated for 16 h at 29 °C with shaking (400 rpm). The cell pellet was collected by centrifugation and suspended in 20 mL of sonication buffer (25 mM sodium phosphate buffer, pH 6.7, 2.5 mM EDTA). The cell suspension was sonicated for 4 min with a Braun-Sonic 2000 sonicator at a power output reading of 130. The sonicated *E. coli* suspension was centrifuged at 10000*g* for 20 min. The pellet was resuspended in 10 mL of sonication buffer and sonicated for an additional 3 min under the same conditions. The sonicated supernatant solutions were pooled, and ammonium sulfate was added to 30% saturation. After being cooled for 10 min in an ice bath, the suspension was centrifuged (at 4 °C) at 10000*g* for 20 min. The resulting pellet containing the fusion protein was dissolved in 8 M urea. The solubilized protein solution was made 10 mM in DTT and adjusted to pH ~8.5 with 2 M Tris. Insoluble material was removed by centrifugation at 10000*g* for 20 min at 4 °C. The supernatant (10 mL) was loaded onto a Sephadex G-50 column (2.5 × 60 cm) and eluted with 20 mM Na_3PO_4, pH 6.7, and the void volume fraction (50 mL) was collected.

Factor Xa Digestion of the trpE–ζ2 Fusion Protein. The fusion protein in the void volume from the Sephadex G-50 chromatography was digested with factor Xa at 14 °C for 16 h in the presence of 0.1 M NaCl, 1 mM $CaCl_2$, and 70 mM Tris-HCl, pH 8.0, using a protein to factor Xa ratio of 100. The resulting digest was stored at −70 °C until use.

Purification of Reduced ζ2-Thrombin. A 5-mL aliquot of the factor Xa digest was reduced with 10 mM DTT in the presence of 5 M urea (pH 8.0) at room temperature for 5 min and immediately loaded onto a C4 reverse-phase HPLC

[1] Abbreviations: DTT, dithiothreitol; HPLC, high-performance liquid chromatography; HRLC, high-resolution liquid chromatography; IAA, 3β-indoleacrylic acid; PCR, polymerase chain reaction; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide; PEG, poly(ethylene glycol); FPA, fibrinopeptide A; FGN, fibrinogen; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate hydrochloride; ζ1-thrombin, the A-chain of α-thrombin (residues 1–36) disulfide linked to residues 1–148 of the B-chain of α-thrombin; ζ2-thrombin, residues 149–259 of the B-chain of α-thrombin.

column (Vydac, 1 × 25 cm) equilibrated with 0.1% TFA. The proteins were fractionated by using the indicated acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. The purification was monitored by 15% SDS–PAGE. The fraction containing r-ζ2-thrombin (as judged by comigration with reduced ζ2-thrombin obtained by resolution of human ζ-thrombin) was immediately lyophilized on a SpeedVac evaporator.

Oxidation and Purification of Recombinant ζ2-Thrombin. Disulfide bonds were formed by oxidation of the reduced r-ζ2-thrombin in the presence of 80 μM $CuSO_4$, 6 M urea, and 70 mM Tris-HCl, pH 8.15, for 45 min at 20 °C at a protein concentration of 0.1 mg/mL. The material that comigrated, on C4 reverse-phase HPLC (Figure 5), with ζ2-thrombin isolated by resolution of ζ-thrombin was collected.

Reconstitution of ζ-Thrombin. Recombinant ζ2-thrombin (500 μg in 150 μL of 0.1% TFA) was combined with ζ1-thrombin (750 μg in 300 μL of 0.1% TFA) in the presence of 0.5 M NaCl, 0.05% PEG 8000, and 50 mM sodium phosphate, pH 6.6, with a final volume of 12 mL. The protein concentrations of ζ1-thrombin and ζ2-thrombin in the reconstitution reaction mixture were 0.063 mg/mL and 0.042 mg/mL, respectively, which gave a molar ratio of ζ2-thrombin to ζ1-thrombin of 1.1. The reconstitution reaction was allowed to proceed at room temperature for 5 min and then on ice for 10 min, after which time reconstitution was complete. The resulting reconstituted ζ2-thrombin was concentrated by Centricon-30 filtration at 4 °C. The concentrated recombinant ζ-thrombin was purified by size-exclusion HRLC on a Bio-Sil SEC-125 column (300 × 7.5 mm).

Assays. Thrombin activity against the small chromogenic substrate tos-GPR-pna (Chromozym-TH) was measured spectrophotometrically at 405 nm. The reactions were carried out at 25 °C in the presence of 142 μM tos-GPR-pna, 40 mM NaCl, 0.1% PEG 8000, and 40 mM sodium phosphate, pH 7.4. One unit of thrombin is defined as the amount of thrombin that will catalyze the hydrolysis of 1 μmol of tos-GPR-pna/min under the above conditions.

Fibrinogen-clotting activity was determined using a BRL FibroSystem apparatus at 37 °C. The fibrinogen (Sigma) solution contained 6 μM human fibrinogen and 35 mM sodium phosphate, pH 7.4. The enzyme solution prepared in siliconized tubes contained 40 mM sodium phosphate, pH 7.4, and the indicated thrombin derivative. To start the clotting, 100 μL of thrombin solution was mixed with 200 μL of fibrinogen solution. Clotting time was automatically recorded by the apparatus. That amount of thrombin which will form a clot in 15 ± 0.5 s is normalized to the NIH unit of thrombin as described previously (Lewis & Shafer, 1984).

Active Site Titration. The active site contents of the thrombin derivatives were determined by titration with 4-methylumbelliferyl *p*-guanidinobenzoate hydrochloride (MUGB) as described by Jameson et al. (1973), with some modifications. The titration was carried out in 10 μM MUGB in 100 mM NaCl/0.1% poly(ethylene glycol) 8000/40 mM sodium phosphate, pH 7.4. A small volume of MUGB (in DSMO) was added to the reaction buffer, and the spontaneous hydrolysis was monitored for a few minutes. Thrombin was then added (at approximately 0.1 μM), and the pre-steady-state burst of fluorescence (365-nM excitation, 445-nM emission) was measured at 25 °C. Active site concentrations were determined from a standard curve that was constructed to relate fluorescence to the concentration of methylumbelliferone.

Fibrinopeptide A Release. Thrombin-catalyzed release of fibrinopeptide A from fibrinogen was measured by reverse-phase HPLC as described previously (Lewis & Shafer, 1984).

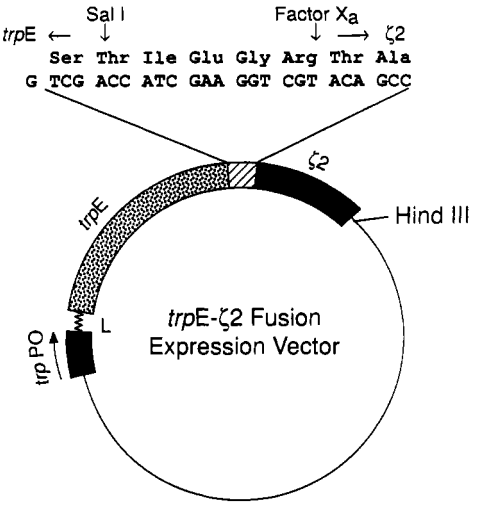

FIGURE 1: Structure of *trpE–ζ2* fusion expression vector. *E. coli* tryptophan promoter–operator sequences are shown as a black box with the direction of transcription indicated by an arrow. The leader sequence between the tryptophan promoter and the *trpE*-coding sequence (stippled box) is indicated by a jagged line. The factor Xa (Ile-Glu-Gly-Arg-) site is shown on the top of the vector. The ζ2-thrombin gene (black box) with its factor Xa sequence was cloned into *Sal*I and *Hind*III sites of pATH2 plasmid.

Amino Acid Sequence Analysis. Amino acid sequence analysis was performed on an Applied Biosystems 477A protein sequencer.

Protein Determination. Protein concentrations were determined from their absorbance at 280 nm using values for $\epsilon_{280}^{1\%}$ of 18.7 for ζ1-thrombin and 16.0 for ζ2-thrombin in 0.1% TFA (S. D. Lewis and J. A. Shafer, unpublished data) and 18.3 for α-thrombin and ζ-thrombin in 0.1 N NaOH (Fenton et al., 1977).

RESULTS

Construction of the trpE–ζ2 Expression Vector. Initial attempts to express ζ2-thrombin in *E. coli* as a simple polypeptide were unsuccessful, suggesting the possibility that the small eukaryotic ζ2-thrombin polypeptide was unstable in the bacterial host as noted for other eukaryotic proteins (Tanguchi et al., 1980; Lennick, 1987; Gan et al., 1989). We were therefore prompted to utilize a strategy commonly used to circumvent this problem wherein the gene of interest is fused to a nucleotide sequence coding for a bacterial protein. This approach often produces a fusion protein containing a stabilized eukaryotic protein. Thus, ζ2-thrombin was expressed as a trpE fusion protein (with an intervening factor Xa cleavage site) in a pATH2 expression vector (Figure 1) that has been used successfully to express fusion proteins at high levels (Gilmore et al., 1988).

Expression of trpE–ζ2. In the course of expression of *trpE–ζ2*, we found that induction with indoleacrylic acid (IAA), an antagonist of tryptophan biosynthesis and an inducer of the *trp* promoter, did not significantly increase the expression level of the fusion protein when M9-CA medium was used. Thus, expression was carried out in M9-CA medium without IAA induction. To maximize production of the soluble fusion protein, *E. coli* were grown at 29 °C (Shein, 1988). Figure 2 illustrates the time-dependent expression of the trpE–ζ2 fusion protein as analyzed by 12% SDS–PAGE. After 10 h of growth in M9-CA medium, increased amounts of a polypeptide with the size expected (~50 kDa) for the trpE–ζ2 fusion protein were observed. Cell lysate containing pATH2 without the ζ2-thrombin gene exhibited less 50-kDa protein

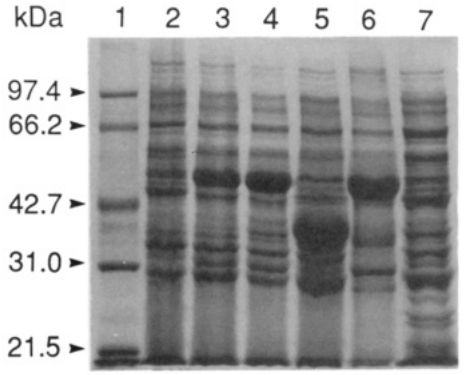

FIGURE 2: SDS–PAGE analysis of expression of the recombinant ζ2 fusion protein. *E. coli* RRI containing the *trpE–ζ2* expression vector was grown in M9-CA medium with 100 μg/mL ampicillin at 29 °C. Aliquots (50 μL) of the culture were taken at different times and centrifuged in an Eppendorf centrifuge. The pellet was suspended in SDS–PAGE sample buffer and heated at 100 °C for 3 min. Lysates equivalent to the same amount of protein were analyzed by 12% SDS–PAGE. Lane 1, Bio-Rad low molecular weight standard; lanes 2–4, trpE–ζ2 culture at 7, 10, and 16 h, respectively; lane 5, 16-h culture of trpE control culture with no ζ2-thrombin gene; lane 6, ammonium sulfate pellet at 16 h; lane 7, ammonium sulfate supernatant at 16 h.

(Figure 2). The expressed fusion protein was accumulated to a high level accounting for about 20% of total cell protein after 16 h. In the large-scale expression of the fusion protein, trpE–ζ2 was soluble after sonication, suggesting that no inclusion bodies were formed under the expression conditions. The fusion protein was very insoluble in ammonium sulfate, however. At 30% saturation, little or no trpE–ζ2 fusion protein appeared to be in the supernatant fraction as evidenced by the fact that the intensity of the 50-kDa band for this fraction was similar to that observed for cell lysates from cells without a ζ2-thrombin gene (Figure 2).

Purification and Cleavage of the trpE–ζ2 Fusion Protein. Ammonium sulfate fractionation of sonicated *E. coli* paste was used to remove much of the contaminating proteins (Figure 2, lanes 6 and 7). To eliminate potential factor Xa inhibitors and low molecular weight proteins, the ammonium sulfate pellet was dissolved in 10 mL of 8 M urea and loaded onto a Sephadex G-50 column after being reduced by DTT (see Experimental Procedures). The trpE–ζ2 fusion protein from the Sephadex G-50 void volume fraction (50 mL) was treated with factor Xa to release reduced ζ2-thrombin. The HPLC profile of the factor Xa cleaved fusion protein is shown in Figure 3. A 1-L overnight culture of *E. coli* yielded 11 mg of purified reduced recombinant ζ2-thrombin. Reduced ζ2-thrombin was purified to homogeneity as judged by 15% SDS–PAGE (Figure 4). The identity of reduced recombinant ζ2-thrombin was established by its size on SDS–PAGE (Figure 4) and amino acid sequence analysis. Sequence analysis revealed that the first 25 N-terminal residues of purified, reduced recombinant ζ2-thrombin were identical to that of reduced ζ2-thrombin obtained by resolution of human ζ-thrombin. Additionally, the elution behaviors on HPLC of the recombinant and naturally occurring polypeptides were identical.

Oxidation of Reduced r-ζ2-Thrombin. Separate studies of the behavior on HPLC of native ζ2-thrombin and reduced native ζ2-thrombin indicated a difference in elution position (data not shown) which prompted us to monitor oxidation of recombinant reduced ζ2-thrombin by HPLC. In the presence of 80 μM $CuSO_4$ and 70 mM Tris-HCl, pH 8.15, urea markedly affected oxidation of reduced ζ2-thrombin. At 2 M urea, very little reduced recombinant ζ2-thrombin was converted to the desired oxidized form (Figure 5A), whereas

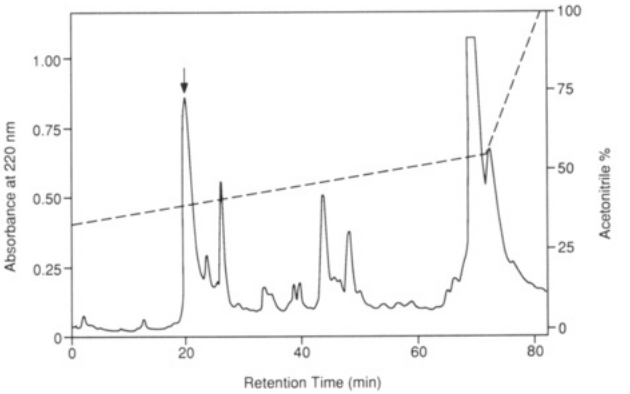

FIGURE 3: Purification of recombinant ζ2-thrombin. A C4 reverse-phase HPLC column was equilibrated with 0.1% trifluoroacetic acid. Factor Xa digested fusion protein was fractionated on the column by acetonitrile gradients in the presence of 0.1% trifluoroacetic acid as indicated by the dashed line. The recombinant reduced ζ2-thrombin peak is shown by the arrow.

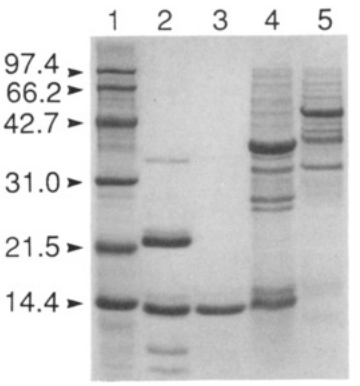

FIGURE 4: SDS–PAGE of reduced recombinant ζ2-thrombin: lane 1, Bio-Rad low molecular weight standard; lane 2, native reduced ζ-thrombin; lane 3, purified reduced recombinant ζ2-thrombin; lane 4, factor Xa cleavage mixture; lane 5, uncleaved trpE–ζ2 fusion protein. The samples were analyzed on a 15% reducing gel.

the desired oxidized form of ζ2-thrombin became a major product when oxidation was carried out in the presence of 6 M urea (Figure 5C). It is interesting to note that the increased yields of the desired form of recombinant ζ2-thrombin with increasing urea concentration appeared to occur at the expense of material eluting at about 50 min. This late-eluting material may well be aggregated ζ2-thrombin. The desired oxidized form of ζ2-thrombin was purified by HPLC. Figure 6 shows that the HPLC retention time of native ζ2-thrombin is identical to that of the ζ2-thrombin isolated by resolution of ζ-thrombin.

Reconstitution of Catalytically Competent ζ-Thrombin by Combination of ζ1-Thrombin and ζ2-Thrombin. Recombinant ζ2-thrombin was used to titrate native ζ1-thrombin. The titration was monitored by an assay for thrombin activity using the chromogenic substrate tos-GPR-pna. The specific activity of the reconstituted recombinant ζ-thrombin depicted in Figure 7 was markedly enhanced upon further purification by size-exclusion HRLC.

Biological Activity of Recombinant ζ2-Thrombin. Examination of the data in Table I shows that reconstituted recombinant ζ-thrombin that was further purified by size-exclusion HRLC (see Experimental Procedures) is similar to native ζ-thrombin and α-thrombin with respect to (i) active site content, (ii) catalytic efficiency in the hydrolysis of the small substrate tos-GPR-pna, and (iii) activity toward its physiological substrate fibrinogen as determined by clotting time and the rate of FPA release.

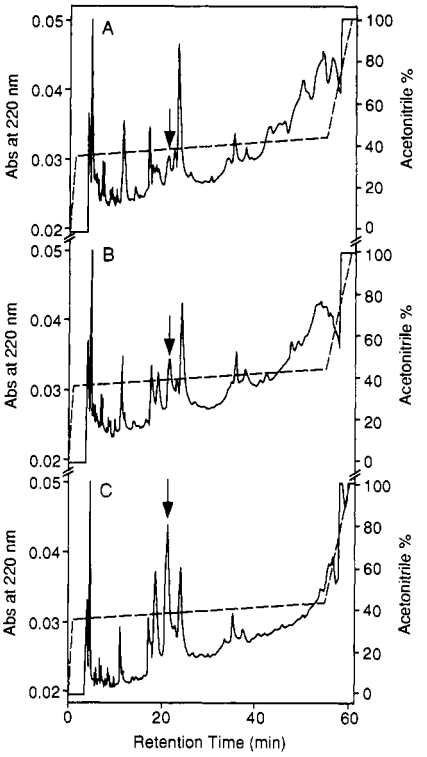

FIGURE 5: Oxidation of recombinant ζ2-thrombin. Reduced recombinant ζ2-thrombin was oxidized at a protein concentration of 0.1 mg/mL in the presence of 80 μM $CuSO_4$, 70 mM Tris-HCl, pH 8.15, and urea at concentrations of 2 M (A), 4 M (B), and 6 M (C), respectively. After 45 min of oxidation at 20 °C, the reaction mixture was immediately loaded onto a C4 reverse-phase HPLC column. The acetonitrile gradients in the presence of 0.1% trifluoroacetic acid are given by dashed lines. The peaks corresponding to the elution position of ζ2-thrombin prepared by resolution of ζ-thrombin without reduction of disulfide bonds are indicated by arrows.

Table I: Activities of Thrombin Derivatives

form of thrombin	enzymatic activity: chromo-zym-TH hydrolysis (units/mg)	enzymatic activity: FGN clotting (NIH units/mg)	enzymatic activity: FPA release (k_{cat}/K_m) ($10^6 \times M^{-1} s^{-1}$)	active site content: mol/mol[a]	active site content: % α[b]
α-thrombin[c]	22.2	2764	12.8	0.96 ± 0.01	100
ζ-thrombin[c]	24.3	2513	12.8	1.16 ± 0.02	120
r-ζ-thrombin[c]	21.3	2342	9.7	0.99 ± 0.09	103

[a] Moles of active site per mole of protein. Average and standard deviations for three assays on a single solution of protein for each thrombin form. [b] These values are relative to the active site content of α-thrombin. In light of possible errors in the determination of protein concentration, not reflected in the listed standard deviations, the differences between the active site contents of the thrombin forms are not significant. [c] All three thrombin forms were purified by size-exclusion HRLC.

Discussion

One of the attractive features of the expression procedure we used to produce recombinant ζ2-thrombin is that the process from cell growth to reconstituted ζ2-thrombin can be completed in only three days. Hence, the methodology provides a simple and quick approach for producing thrombin variants for structural and functional studies. Expression of prothrombin in SV40-transformed African green monkey kidney cells and in baby hamster kidney cells by the recently reported methods of Kim et al. (1990) and Le Bonniec et al. (1990) should provide a method for obtaining variants of intact α-thrombin for complementary studies of the structure–function relationship for thrombin.

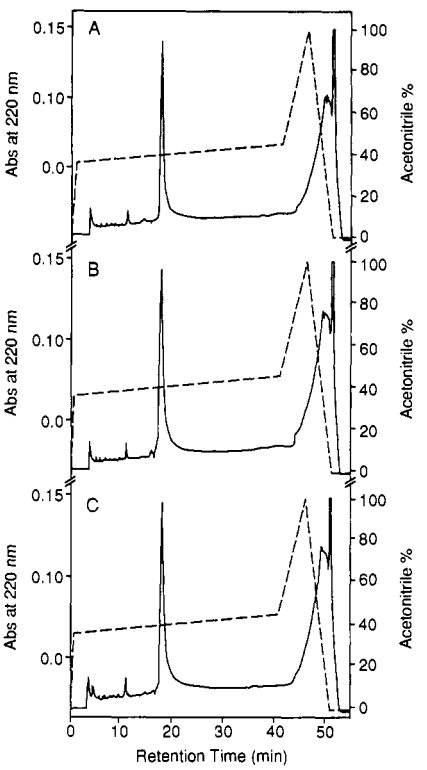

FIGURE 6: Comparison of purified recombinant and native ζ2-thrombin on reverse-phase HPLC. ζ2-Thrombin was analyzed on a C4 reverse-phase HPLC column. The polyphasic gradients are indicated by dashed lines. Panel A, 10 μg of native ζ2-thrombin obtained by resolution of ζ-thrombin; panel B, 10 μg of oxidized recombinant ζ2-thrombin; panel C, a mixture containing 5 μg of native ζ2-thrombin and 5 μg of oxidized recombinant ζ2-thrombin.

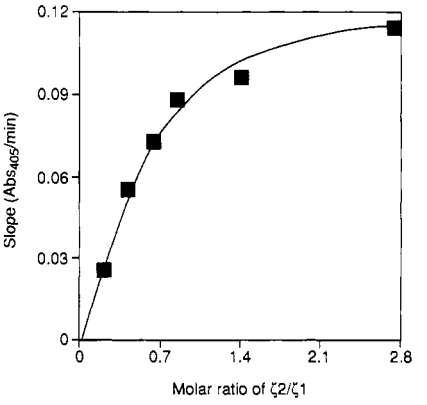

FIGURE 7: Reconstitution of ζ-thrombin from ζ1- and recombinant ζ2-thrombin. Reconstitution of ζ-thrombin was carried out at a fixed concentration of ζ1-thrombin (0.13 mg/mL) in 0.5 M NaCl/0.05% PEG 8000/50 mM sodium phosphate, pH 6.6. The indicated amounts of recombinant ζ2-thrombin were added and incubated for 2 min at room temperature and for 10 min on ice, after which time reconstitution appeared to be complete. The reconstituted reaction mixture was diluted, and an aliquot containing 0.16 μg of ζ1-thrombin was assayed with chromogenic substrate.

The expression of ζ2-thrombin was under the control of the *E. coli trp* promoter which is turned on at low tryptophan levels. We found, however, that addition of the antagonist of tryptophan biosynthesis IAA was not necessary for expression of *trpE–ζ2*. Presumably, tryptophan depletion occurs after limited cell growth ($OD_{600} > \sim 2$) in the presence of good aeration (which may contribute to tryptophan depletion via oxidative reactions) at which time the trpE–ζ2 fusion protein is expressed (Figure 2).

Many eukaryotic cysteine-containing proteins overproduced in *E. coli* are obtained in an inactive form. Renaturation of these proteins is often performed at an alkaline pH under conditions to facilitate disulfide bond formation. We have reoxidized ζ2-thrombin at different pH values in the presence of various oxidants, such as glutathione disulfide and cystamine. None of these conditions, however, gave a good yield of oxidized ζ2-thrombin with correctly paired disulfides. Independent studies (data not shown) had indicated that ζ2-thrombin aggregates in alkaline pH even at a protein concentration of less than 0.1 mg/mL, suggesting that aggregation may well have been responsible for our poor yields. To suppress aggregation, oxidation of ζ2-thrombin was carried out in urea solution. ζ2-Thrombin with correctly paired disulfides was obtained in good yield when reduced recombinant ζ2-thrombin was incubated in the presence of 6 M urea and 80 μM $CuSO_4$ at pH 8.15 for 45 min. Apparently, a substantial fraction of reduced thrombin exists in a conformation in 6 M urea wherein disulfide bond formation between Cys-173 and Cys-187 and between Cys-201 and Cys-231 can occur.

Previous studies of Chang et al. (1980) reported that catalytic activity could be recovered after brief treatment of γ-thrombin with 4 M urea, suggesting that it might be possible to reconstitute a nicked thrombin derivative by the combination of purified component polypeptides. The present study is the first report demonstrating that combination of the isolated polypeptide chains of a proteolytically nicked form of thrombin can reconstitute catalytically competent enzyme. This observation indicates that the information for formation of a catalytically competent conformation resides in the primary structure of ζ-thrombin. The observation that the reconstituted ζ-thrombin exhibited catalytic activities similar to those of native ζ-thrombin suggests that reconstituted ζ-thrombin and variants thereof should be useful in characterizing physiologically important interactions of ζ-thrombin.

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REFERENCES

Bar-Shavit, R., Kahn, A. J., Mann, K. G., Mann, K. G., & Wilner, G. D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 976–980.

Bar-Shavit, R., Sabbah, V., Lampugnani, M. G., Marchisio, P. C., Fenton, J. W., II, Vlodavsky, I., & Dejana, E. (1991) *J. Cell Biol. 112*, 335–344.

Berndt, M. C., Gregory, C., Dowden, G., & Castaldi, A. (1986) *Ann. N.Y. Acad. Sci. 485*, 374–386.

Bezeaud, A., & Guillin, M.-C. (1988) *J. Biol. Chem. 263*, 3576–3581.

Blomback, B., Hessel, B., Hogg, D., & Therikildsen, L. (1978) *Nature (London) 275*, 501–505.

Braun, P. J., Chang, J. Y., Hofsteenge, J., & Stone, S. R. (1988) *Thromb. Res. 50*, 273–283.

Brezniak, D. V., Brower, M. S., Witting, J. I., Walz, D. A., & Fenton, J. W., II (1990) *Biochemistry 29*, 3536–3542.

Brower, M. S., Walz, D. A., Garry, K. E., & Fenton, J. W., II (1987) *Blood 69*, 813–819.

Chang, T.-L., Bauer, R. S., & Berliner, L. J. (1980) *J. Biol. Chem. 255*, 5904–5906.

Coleman, R. J. (1969) *Biochemistry 8*, 1438–1445.

Eilon, J., Bezeaud, A., Boissel, J. P., Guillin, M. C., Jandrotperrus, M., Lebonniec, B., & Rabiet, M. J. (1986) *Ann. N. Y. Acad. Sci. 485*, 16–26.

Esmon, C. T. (1987) *Science 235*, 1348–1352.

Esmon, C. T., Esmon, N. L., Kurosawa, S., & Johnson, A. E. (1986) *Ann. N.Y. Acad. Sci. 485*, 215–220.

Fenton, J. W., II, Fasco, M. J., Stackrow, A. B., Aronson, D. L., Young, A. M., & Finlayson, J. S. (1977) *J. Biol. Chem. 252*, 3587–3598.

Fenton, J. W., II, Landis, B. H., Walz, D. A., & Finlayson, J. S. (1977b) in *Chemistry and Biology of Thrombin* (Lundblad, R. L., Fenton, J. W., II, & Mann, K. G., Eds.) pp 43–70, Ann Arbor Science, Ann Arbor, MI.

Gan, Z.-R., Condra, J. H., Gould, R. J., Zivin, R. A., Bennett, C. D., Jacobs, J. W., Friedman, P. A., & Polokoff, M. A. (1989) *Gene 79*, 159–166.

Gilmore, R. D., Jr., Engelking, H. M., Manning, D. S., & Leong, J. C. (1988) *Bio/Technology 6*, 295–300.

Hofsteenge, J., Braun, P. J., & Stone, S. R. (1988) *Biochemistry 27*, 2144–2151.

Hoyer, L. W., & Trabold, N. C. (1981) *J. Lab. Clin. Med. 97*, 50–64.

Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, W. S. A., & Elmore, D. T. (1973) *Biochem. J. 131*, 107–117.

Kawabata, S.-I., Morita, T., Iwanaga, S., & Igarashi, H. (1985) *J. Biochem. 97*, 325–331.

Kim, J.-M., Kim, Y. J., Choi, E.-H., Kim, J.-M., Kim, J. (1990) *Korean Biochem. J. 23*, 427–432.

Kisiel, W. (1979) *J. Clin. Invest. 64*, 761–769.

Le Bonniec, B. F., & Esmon, C. T. (1990) *Circulation 82*, 367.

Lennick, M., Haynes, J. R., & Shen, S.-H. (1987) *Gene 61*, 103–112.

Lewis, S. D., & Shafer, J. A. (1984) *Thromb. Res. 35*, 111–120.

Lewis, S. D., Lorand, L., Fenton, J. W., II, & Shafer, J. A. (1987) *Biochemistry 26*, 7597–7602.

Lorand, L., & Konishi, K. (1964) *Arch. Biochem. Biophys. 105*, 58–67.

Mann, K. G., Yip, R., Heldebrant, C. M., & Fass, D. N. (1973) *J. Biol. Chem. 248*, 1868–1875.

Mann, K. G., Jenny, R. J., & Krishnaswamy, S. (1988) *Annu. Rev. Biochem. 57*, 915–956.

Nesheim, M. E., & Mann, K. G. (1979) *J. Biol. Chem. 254*, 1326–1334.

Pearson, J. D., Carleton, J. S., & Hutchings, A. (1983) *Thromb. Res. 29*, 115–124.

Schein, C. H., & Noteborn, M. H. M. (1988) *Bio/Technology 6*, 291–294.

Shafer, J. A., & Higgins, D. L. (1988) *CRC Crit. Rev. Clin. Lab. Sci. 26*, 1–41.

Takagi, T., & Doolittle, R. F. (1974) *Biochemistry 13*, 750–756.

Taniguchi, T., Guarente, L., Roberts, T. M., Kimelman, D., Douhan J., III, & Ptashne, M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5230–5233.