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Polymerization Site in the β Chain of Fibrin: Mapping of the Bβ1-55 Sequence†

Bharat V. Pandya,‡ Jerome L. Gabriel,§ Joseph O'Brien,§ and Andrei Z. Budzynski*,§
Department of Biochemistry, Kirksville College of Osteopathic Medicine, Kirksville, Missouri 63501, and Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140
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ABSTRACT: The formation of a fibrin clot occurs through binding of putative complementary sites, called fibrin polymerization sites, located in the NH_2- and COOH-terminal domains of fibrin monomer molecules. In this study, we have investigated the structure of the NH_2-terminal fibrin polymerization site by using fibrinogen-derived peptides and fragments. Fibrinogen was digested with *Crotalus atrox* protease III, to two major molecular species: a M_r 325 000 derivative (Fg325) and a peptide of M_r 5000. The peptide and its thrombin-cleavage product were purified by ion-exchange and reverse-phase HPLC; the authenticity of the Bβ1-42 and β15-42 peptides, respectively, was confirmed by amino acid sequencing. Since Fg325 had decreased thrombin coagulability, we addressed the question of whether the peptide Bβ1-42 contained a fibrin polymerization site. In other to identify and map the site, the peptides Bβ1-42 and β15-42 were tested for their ability to inhibit fibrin monomer polymerization. In addition the following peptides prepared by chemical synthesis were also tested: β15-18, β15-26, β24-42, β40-54, β50-55, and α17-19-Pro. While Bβ1-42 had no inhibitory activity, the peptide devoid of fibrinopeptide B, β15-42, was a strong inhibitor. The peptides β15-18, β15-26, and β15-42 decreased the rate of fibrin polymerization by 50% at a molar excess of the peptide to fibrin monomer of 500, 430, and 50, respectively. The peptides β24-42, β40-54, and β50-55 were inactive. Computer-aided prediction of probable secondary structures in Bβ1-55, together with polymerization inhibition by β15-55-derived peptides, suggested that the polymerization site in the amino-terminal domain of the β chain might be composed of noncontiguous amino acids. However, the NH_2-terminal disulfide knot of fibrin, which contains amino termini of the α, β, and γ chains, not only was a stronger inhibitor of polymerization than β15-42, but it also had a higher affinity for binding to fibrin monomer. Therefore, it is proposed that the fibrin polymerization site in the NH_2-terminal domain of fibrin may be composed of sequence derived from both the α and β chains.

Human fibrinogen is a large and complex plasma glycoprotein made up of pairs of three nonidentical polypeptide chains: Aα, Bβ, and γ (Doolittle, 1984; Budzynski, 1986). Conversion of fibrinogen to fibrin, which involves cleavage of fibrinopeptide A and fibrinopeptide B from the NH_2 termini of the Aα and Bβ chains of fibrinogen, respectively, is essential for blood clot generation. The formation of an insoluble fibrin clot can be divided into three steps: (a) the removal of fibrinopeptides A and B to form fibrin monomer, (b) the self-polymerization of fibrin monomers to form a soluble clot, and (c) the formation of covalent cross-links by factor XIIIa to form a stable clot. The exact sites of the actions of thrombin and factor XIIIa are known. However, the mechanism of polymerization and amino acid residues of the fibrinogen molecule involved in the polymerization reaction remain to be described. Binding phenomena of fibrinogen, fibrin monomer, and their degradation products have enabled development of a model for the mechanism of fibrin polymerization (Kudryk et al., 1974; Blombäck et al., 1978; Olexa & Budzynski, 1979, 1980). An essential concept of this model is that the thrombin-induced cleavage of the Aα and Bβ chains in the NH_2-terminal domain exposes binding sites, called fibrin polymerization sites, that are complementary to the binding sites preexistent in the COOH-terminal domain of the fibrinogen molecule (Budzynski et al., 1983). A polymerization site localized at the COOH-terminal domain of fibrinogen was postulated to be present within the sequence γ303-411 (Southan et al., 1985; Varadi & Scheraga, 1986). The preservation of the native conformation is essential for expression of polymerization sites in both the NH_2 (Budzynski et al., 1983) and the COOH terminus (Cierniewski et al.,

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‡ Kirksville College.
§ Temple University.

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1986). A series of short peptides corresponding to the NH_2-terminal sequences of the α and β chains of a fibrin monomer molecule had been synthesized (Laudano & Doolittle, 1978). The peptides based on the sequence Gly-Pro-Arg from the α chain were effective inhibitors of fibrin polymerization (Laudano & Doolittle, 1980, 1981) and interfered with polymerization if used at a great excess (Furlan et al., 1982). A conclusion from these studies was that a primary polymerization site, complementary to the site located in the COOH terminus of fragment D, was present at the NH_2 terminus of the α chain of fibrin.

In our efforts to elucidate the mechanism of fibrinogen anticoagulation by *Crotalus atrox* venom, we purified and characterized four distinctly different proteases from the venom (Pandya & Budzynski, 1984). *C. atrox* protease III, upon limited proteolysis, cleaved human fibrinogen to generate two molecular species: a M_r 325 000 derivative (Fg325)[1] with impaired thrombin coagulability (Pandya et al., 1985) and a peptide of M_r 5000 derived from the NH_2 terminus of the Bβ chain. On the basis of the amino acid sequencing of purified polypeptide chains of Fg325, the peptide cleaved from the NH_2 terminus of the Bβ chain was thought to be Bβ1-42 (Pandya et al., 1985). The fact, that Fg325 displayed impaired functionality as compared to the parent fibrinogen molecule corroborated earlier postulates (Shainoff & Dardik, 1979; Laudano & Doolittle, 1980) that the NH_2-terminal segment of the β chain of fibrin contained a fibrin polymerization site. The goal of the present investigation was to purify and characterize the peptides Bβ1-42 and β15-42 and to identify and map the fibrin polymerization site in the NH_2 terminus of the β-chain.

Materials and Methods

Reagents. Human fibrinogen was isolated from normal citrated plasma by precipitation with ammonium sulfate (0.86 M) and glycine (2.1 M) followed by gel filtration on Sepharose CL-6B and another ammonium sulfate (0.65 M) precipitation as described previously (Pandya et al., 1985). Protease III was purified from *C. atrox* venom as described (Pandya & Budzynski, 1984) and fibrinogen digestion was done according to a previous protocol (Pandya et al., 1983).

Peptides used in this study were obtained from the following sources: Bβ1-42 and β15-42 were purified from a protease III digest of human fibrinogen as described below. Gly-His-Arg-Pro and Gly-Pro-Arg-Pro were obtained from Serva Fine Biochemicals Inc., Westbury, NY. β24-42 was obtained from Bachem Inc., Torrance, CA. β15-26, β40-54, and β50-55 were synthesized in an automated peptide synthesizer (Model 430A, Applied Biosystems, Foster City, CA) by using the solid-phase method (Barany & Merrifield, 1980). An NH_2-terminal disulfide knot was isolated from a cyanogen bromide digest of fibrinogen by a modification of the method of Blombäck and colleagues (1978).

Preparative Methods. Purification of Bβ1-42 and β15-42 Peptides. Fibrinogen, 20 mg/mL in 0.05 M ammonium bicarbonate and 2.0 mM EDTA, pH 7.8, was digested with 20 μg/mL of protease III at 37 °C for 90 min, until fibrinogen was incoagulable as determined by the thrombin clotting time assay. The digestion was inhibited by addition of PMSF to a final conconcentration of 1 mM and high molecular weight species were precipitated by 1.23 M ammonium sulfate.

FPLC, Anion Exchange. The peptide-containing supernate was dialyzed against distilled water (4 × 1 L), freeze-dried, and dissolved in 0.05 M ammonium bicarbonate, pH 7.8. Crude Bβ1-42 was prepared by anion-exchange FPLC on a Mono Q column (Pharmacia, Piscataway, NJ) equilibrated with the same buffer. The peptide was eluted isocratically with the same buffer. Contaminating fibrinogen and Fg325 were eluted with a linear gradient of 0–0.5 M NaCl in 0.05 M ammonium bicarbonate, pH 7.8.

FPCL, Cation Exchange. Bβ1-42 for functional studies was purified from the anion-exchange peptide fraction by cation-exchange FPLC (Mono S Column, Pharmacia, Piscataway, NJ) on a Waters (Westbury, MA) HPLC system. Peptide, dialyzed against 0.05 M MES buffer, pH 5.5, was injected onto the column and eluted with a linear gradient of 0–0.5 M NaCl in 0.05 M MES buffer, pH 5.5. Flow rate was 1.0 mL/min and detection was at 205 nm; 0.5-mL fractions were collected. Alternatively, the crude peptide mixture was treated with thrombin; the resulting peptide, β15-42, was purified as described above.

HPLC Reverse Phase. For structural analysis, β15-42 obtained from cation-exchange FPLC was fractionated by reverse-phase HPLC on a Synchropak RP-P (Synchrom, Inc., Linden, IN) in the above-described Waters system. The peptide was dialyzed against 0.1% TFA, injected onto the column, and eluted with a linear gradient of 0–40% acetonitrile in 0.1% TFA. The flow rate was 1.0 mL/min and detection was at 205 nm; 0.5-mL fractions were collected. Peak fractions were dried under vacuum (Speed-Vac concentrator, Savant Instruments, Farmingdale, NY), analyzed for purity by the same reverse-phase HPLC, and used for amino acid analysis and sequencing.

Analytical Procedures. Protein concentration was determined either by a microbiuret method (Itzhaki & Gill, 1969) or by spectrophotometric determination at 280 nm using an absorption coefficient of 1.5 for fibrinogen at 1 mg/mL, in aqueous solution at pH 7.0.

Amino acid composition was determined in an automatic analyzer (Model MM-70, Glenco, Houston, TX). The hydrolysis of the peptide was carried out for 18, 36, and 54 h in HCl/propionic acid (50:50 v/v) (Pierce Chemical Co., Rockford, IL) at 110 °C in vacuum (Hirs, 1983).

The amino acid sequence of the entire β15-42 peptide was determined by Edman degradation in an automatic gas-phase sequencer (Hewick et al., 1981) (Model 470 A, Applied Biosystems, Foster City, CA). Phenylthiohydantoin (PTH) derivatives of amino acids released during each cleavage cycle were identified and quantified by HPLC.

SDS–PAGE was performed according to the procedure of Laemmli (1970). Bβ1-42 and β15-42 peptides from a cation-exchange Mono S column and from a reverse-phase column were analyzed by SDS–PAGE in 10–20% gradient gels.

Fibrin Monomer Polymerization. Fibrin monomer was prepared according to the method of Belitser and colleagues (1968). Polymerization was measured spectrophotometrically at 350 nm as described (Budzynski et al., 1979) for 15 min or until the reaction reached a plateau. Each reaction contained 0.1 mL of 1.5 mg/mL fibrin monomer. Peptides to be tested in the fibrin polymerization reaction were dissolved in 0.05 M Tris-HCl and 0.1 M NaCl, pH 7.55, the buffer, in which the reaction was performed. To measure the rate of polymerization of fibrin monomer alone (100% of the maximum rate plotted on the ordinate), 0.1 mL of fibrin monomer

[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; PTH, phenylthiohydantoin; tNDSK, thrombin-treated NH_2-terminal disulfide knot; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Fg325, M_r 325 000 derivative of fibrinogen; TFA, trifluoroacetic acid; FPLC, fast protein liquid chromatography; HPLC, high-pressure liquid chromatography; I_{50}, molar ratio of a peptide to fibrin monomer that causes a 50% inhibition of the maximum rate of fibrin polymerization.

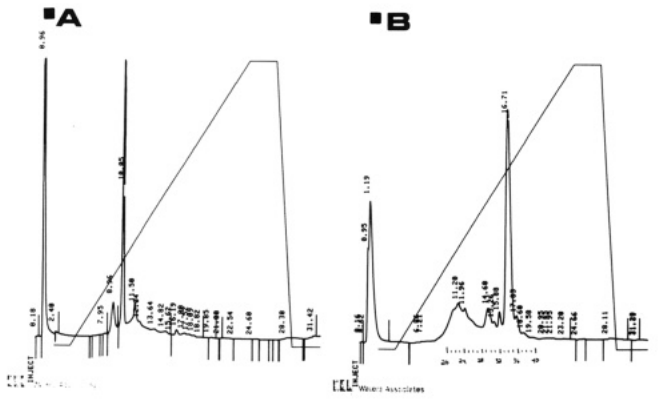

FIGURE 1: Purification of Bβ1–42 and β15–42 by cation-exchange HPLC. The fall-through peak from an anion-exchange Mono Q column was applied on a cation-exchange Mono S column in 0.05 M MES, pH 5.5, and eluted with 0–0.5 M NaCl gradient. (A) One milligram of peptides was applied. The major peak contained Bβ1–42. (B) Part of the peptides from the Mono Q column were first digested with thrombin to cleave fibrinopeptide B, applied on a Mono S column, and eluted with a 0–0.5 M NaCl gradient. The major peak eluting at 16.71 min contained β15–42. Fibrinopeptide B eluted at 1.19 min.

was added to 0.9 mL of buffer. The maximum rate of polymerization was calculated from the slope of the steepest part of the curve and expressed as percent of that of fibrin monomer alone.

Binding of Peptides to Immobilized Fibrin Monomer. Coupling of human fibrinogen to cyanogen bromide activated Sepharose 4B and its conversion to fibrin monomer by treatment with thrombin were performed as described previously (Heene & Matthias, 1973). The efficiency of coupling of fibrinogen was greater than 95% and approximately 10 mg of fibrinogen was coupled to 1 g dry weight of the resin. The conversion of immobilized fibrinogen to fibrin monomer was accomplished by incubating with thrombin and verified by the ability of fibrin monomer to bind fragment D_1 as compared to fibrinogen (Olexa & Budzynski, 1979). Peptides applied on the Sepharose–fibrin monomer column were in 0.05 M sodium phosphate and 0.1 M NaCl, pH 7.4. The peptides bound to the immobilized fibrin monomer were eluted by 0.5 M acetic acid.

RESULTS

Purification of Bβ1-42 and β15-42 Peptides. *C. atrox* protease III is a serine protease of a unique and very limited substrate specificity (Pandya & Budzynski, 1984). We have previously shown that protease III cleaved only the NH_2 terminus of the Bβ chain of fibrinogen, leaving the Aα and γ chains intact (Pandya et al., 1985). The principal cleavage products, therefore, we expected to be Fg325 and a peptide of M_r 5000. To isolate the latter, the protease III digest of fibrinogen was first treated with 1.23 M ammonium sulfate to precipitate Fg325. The dialyzed supernate was applied on an anion-exchange Mono Q column. Several protein peaks representing small amounts of unprecipitated Fg325, undegraded fibrinogen, and unidentified peptides were resolved. SDS–PAGE showed that Bβ1-42 was present in the peak containing polypeptides that did not bind to the Mono Q column (data not shown). The next step in the purification protocol involved cation-exchange chromatography on a Mono S column. The peptide was bound to the column and eluted as a single major peak at 10.05 min in a linear gradient of sodium chloride (Figure 1A).

The thrombin digest of Bβ1-42 was also fractionated on the Mono S column. β15-42 eluted from this column later than Bβ1-42 in the gradient, at 16.71 min (Figure 1B). Fibrinopeptide B did not bind to the column and was eluted at 1.19 min. In order to confirm its purity, β15-42 peptide from a cation-exchanged Mono S column was applied to a reverse-phase column and eluted by an acetonitrile gradient. There were two peaks of β15-42 at 18.31 and 19.41 min (Figure 2); two peaks were also observed by other investigators (Koehn et al., 1983; Birken et al., 1984; Skogen & Wilner, 1986). The isolation of β15-42 in two different peaks on reverse-phase HPLC was not due to any heterogeneity at either NH_2 or COOH termini. Amino acid sequencing by Edman degradation confirmed that the sequence of both peptides corresponded exactly to that of β15-42. SDS–PAGE was performed to assess the purity of HPLC-purified peptides. Both Bβ1-42 and β15-42, from either cation-exchange or reverse-phase HPLC, were found to be homogeneous (Figure 3). The molecular weights of Bβ1-42 and β15-42, calculated from their

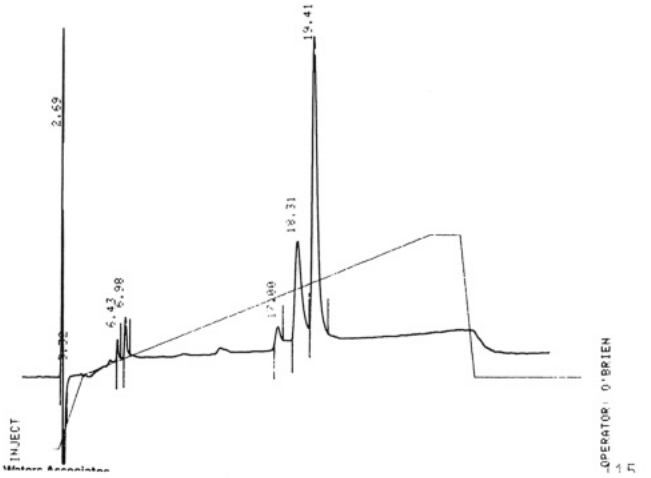

FIGURE 2: Purification of β15–42 by reverse-phase HPLC. The Bβ15–42 peptide isolated by a cation-exchange column, Mono S, was applied onto a reverse-phase Synchropak RP-P column in 0.1% trifluoroacetic acid (TFA) and eluted with a linear gradient of 0–40% acetonitrile in 0.1% TFA. Two peaks of β15–42 peptides at 18.31 and 19.41 min were resolved. Upon amino acid sequencing, both peptides were proven to have the sequence β15–42.

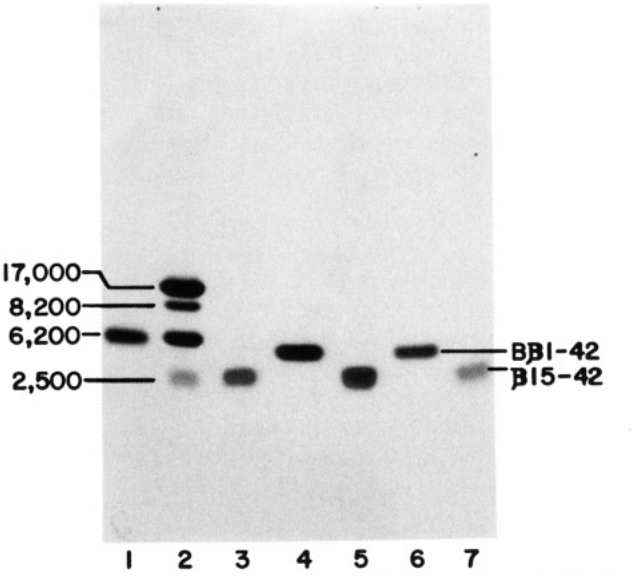

FIGURE 3: SDS–PAGE of HPLC-purified Bβ1–42 and β15–42 peptides. SDS–PAGE was performed in slab gels according to the method of Laemmli (1970) in a gel containing a 10–20% linear gradient of acrylamide. The stacking gel contained 4.5% acrylamide. The following samples are shown: lane 1, Trasylol; lane 2, standards of M_r 17000, 8200, 6200, and 2500; lane 4, Bβ1–42 from reverse-phase HPLC; lanes 3 and 5, β15–42 from reverse-phase HPLC; lane 6, Bβ1–42 from a cation-exchange Mono S column; lane 7, β15–42 from a cation-exchange Mono S column.

Table I: Effect of Peptides on Fibrin Polymerization

molar ratio[a]	maximum rate of fibrin polymerization (%)								
	α17–19-Pro	β15–18	β15–26	β15–42	Bβ1-42	β24–42	β40–54	β50–55	tNDSK[b]
1	100	100	100	100	100	99	101	98	100
10	89	95	92	70	93	100	89	102	50
100	60	68	65	39	100	89	100	95	
1000	10	34	32			100	99	100	
I_{50}[c]	190	500	430	50					10

[a] Molar ratio is expressed as the ratio of peptide to fibrin monomer. [b] tNDSK is a fragment of fibrin-containing remnants from amino-terminal sequences of all three polypeptide chains. The polypeptide chain composition of tNDSK is $(\alpha 17\text{–}51, \beta 15\text{–}118, \gamma 1\text{–}78)_2$. [c] I_{50} defines the molar ratio of a peptide to fibrin monomer that causes a 50% inhibition of the maximum rate of fibrin polymerization.

mobilities in SDS–PAGE, were 5000 and 2500, respectively; these molecular weights were in agreement with the expected molecular weights of 4568 and 3000 for Bβ1-42 and β15-42, respectively, calculated from their amino acid sequences.

Effect of Bβ1-42 and β15-42 on Fibrin Monomer Polymerization. Bβ1-42 and β15-42 were tested for their ability to inhibit fibrin monomer polymerization. β15-42 inhibited fibrin polymerization in a concentration-dependent manner. The maximum rate of polymerization was determined at various molar ratios of the peptide to fibrin monomer. The plot of percent of maximum rate versus molar ratio of the peptide to fibrin monomer showed that a 50-fold molar excess of the peptide was required to achieve 50% inhibition of the reaction rate (Figure 4). Bβ1-42 did not inhibit polymerization even at a 100-fold molar excess of the peptide to fibrin monomer (Table I). Thus, the cleavage of fibrinopeptide B created a functional inhibitor in the NH_2 terminus of the β chain.

Effect of Synthetic Peptides on Fibrin Polymerization. In order to map this polymerization site, several synthetic peptides were also tested in the polymerization assay (Table I). Short synthetic peptides modeled on the NH_2 termini of the α and β chains of human fibrin have been previously tested for their ability to inhibit fibrin polymerization (Laudano & Doolittle, 1978, 1980). Peptides derived from the NH_2 termini, namely, α17–19-Pro (Gly-Pro-Arg-Pro) and β15–18 (Gly-His-Arg-Pro) were tested as controls along with various peptides derived from the β15-55 sequence. β15–18, β15–26, and α17–19-Pro inhibited the polymerization reaction. However, β24–42, β40–54, and β50-55 were inactive even at a 1000-fold molar excess of the peptide to fibrin monomer. To compare the effect of various peptides, the molar excess of the peptide required for 50% inhibition of the polymerization reaction rate was calculated (I_{50}). The I_{50} values for β15–18, β15–26, and α17–19-Pro were 500, 430, and 190, respectively (Table I).

The most potent inhibitor among all the peptides tested was β15–42, showing the lowest I_{50} value (Table I). The thrombin-treated NH_2-terminal disulfide knot (tNDSK) contains sequences from all three polypeptide chains of fibrin. Specifically, its polypeptide composition is $(\alpha 17\text{–}51, \beta 15\text{–}118, \gamma 1\text{–}78)_2$. The I_{50} value of 10 (Table I) for tNDSK was 5-fold lower than that for β15–42. These results implied that tNDSK should bind to its complementary binding site even tighter than β15–42 would. Therefore, we tested the binding of Bβ1–42, β15–42, and tNDSK to a column of immobilized fibrin monomer. Approximately, 25% and 58% of the applied Bβ1–42 and β15–42 peptides, respectively, bound to the column. On the other hand, approximately 87% of tNDSK applied on the column was bound. These results are consistent with the observation that ligands that bind more tightly to fibrin monomer are stronger inhibitors of fibrin monomer polymerization.

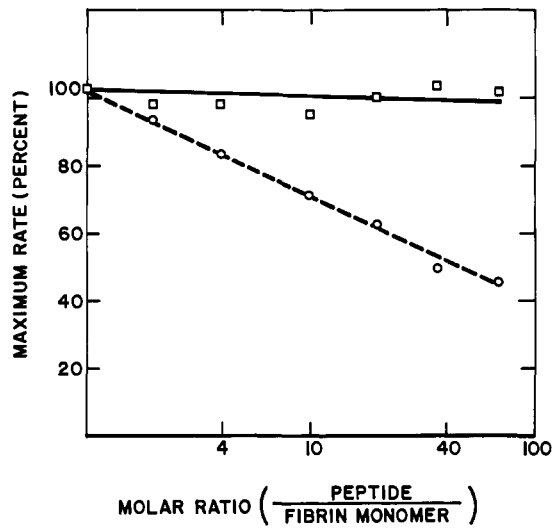

FIGURE 4: Effect of Bβ1–42 and β15–42 on fibrin polymerization. Fibrin polymerization was monitored at 350 nm as an increase in turbidity, for up to 15 min. The rate of the reaction was calculated from the steepest part of the curve and expressed as the percent of that of fibrin monomer alone (maximum rate, percent). The peptides, Bβ1–42 or β15–42, were added to the polymerization reaction at various amounts to achieve the molar ratio of the peptide to fibrin monomer as shown on the abscissa. The rates of the polymerization reaction, as percent of control, in the presence of Bβ1–42 (□) or β15–42 (○) were plotted against the molar ratio of the peptide to fibrin monomer.

Discussion

Fibrinogen fragment Fg325 has impaired thrombin-induced coagulability, undegraded Aα and γ chains, and degraded Bβ chains lacking in the sequence Bβ1–42 (Pandya et al., 1985). Thus, it was logical to postulate that β15–42 may contain amino acid residues involved in fibrin polymerization, particularly since other investigators have concluded that this plasmin-susceptible segment comprises a critical portion of the fibrinopeptide B dependent polymerization site (Shainoff & Dardik, 1979) and that abnormally coagulable fibrinogen New York I contains a deletion of Bβ9–72 (Liu et al., 1985). One way to test this hypothesis was to assess the effect of β15–42 and its derivatives on the process of fibrin polymerization.

The significance of the NH_2 terminus of the β chain of fibrin in the polymerization process has been based on several observations. The cleavage of fibrinopeptide B at 14 °C by the copperhead snake venom procoagulant enzyme (Shainoff & Dardik, 1979, 1983) resulted in a clot that, as demonstrated by electron microscopy, contained fibers similar to those in thrombin-derived clots (Weisel, 1986; Mosesson et al., 1987). The synthetic peptide β15–18 bound to immobilized fibrinogen (Laudano & Doolittle, 1978, 1980, 1981) and interfered with fibrin monomer polymerization (Furlan et al., 1982). However, alkylation of the NH_2-terminal βGly15 in synthetic peptides abolished their affinity for fibrin (Hsieh et al., 1981). Photooxidation of βHis16 in tNDSK produced a derivative with impaired binding to immobilized fibrinogen (Shimizu et al., 1983); however, its ability to bind to immobilized fragment DD was retained (Shimizu et al., 1986). Finally, a monoclonal

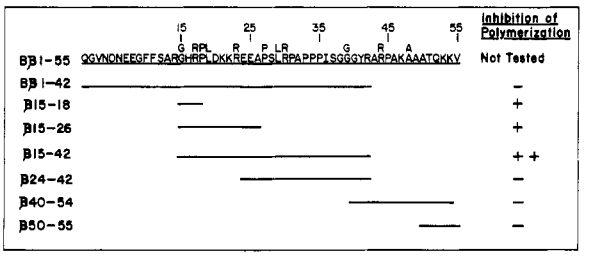

FIGURE 5: Map of the fibrin polymerization site in the NH_2 terminus of the Bβ chain. Straight lines representing different peptides are drawn to scale with respect to Bβ1-55. The sequence Bβ1-55 of human fibrinogen is shown by single-letter codes. The positions of sequence homology with identical amino acids between human and lamprey fibrin β chains are shown by the same letter codes in the appropriate amino acid positions. The effect of each peptide on fibrin monomer polymerization is qualitatively stated by either a positive "+" or a negative "−" symbol.

antibody developed against β15–21 showed that this sequence is exposed in fibrin but not accessible in fibrinogen (Hui et al., 1983). Plasmin-generated derivatives of cross-linked fibrin, fragments E_1, E_2, and E_3, provide some insight into the probable structure of the fibrin polymerization site in the NH_2 terminus of the β chain of fibrin. Fragments E_1 and E_2 bind to fragment DD, whereas fragment E_3, lacking the β15–53 sequence, does not (Olexa & Budzynski, 1979; Olexa et al., 1981). Therefore, we postulated that certain amino acid residues within the β43–53 sequence may be important contributors of the polymerization site, in addition to those within the β15–42 sequence. Figure 5 shows that β15–42 (I_{50} = 50) was a stronger inhibitor than β15–18 (I_{50} = 500) or β15–26 (I_{50} = 430), indicating that some residues within the β27–42 sequence may be involved in the fibrin polymerization site. Yet, β24–42 did not inhibit fibrin polymerization. Therefore, it is possible that the β27–42 sequence enhances the inhibitory potential of β15–26 only when the former is an integral part of the β15–42 sequence. In other words, the folding of β15–42 may bring certain amino acid residues within the β27–42 sequence in close proximity to those within β15–26 that are essential residues of this fibrin polymerization site (Figure 6C). In a similar way the amino acid residues within the β43–53 sequence may play a role in fibrin polymerization, but only when this sequence is contiguous with β15–42. Indeed, synthetic peptides β40–54 and β50–55 failed to inhibit fibrin polymerization. Thus, a peptide of a sequence longer than β15–42, for example, β15–55, ought to be tested to evaluate the role of the β43–53 sequence in the fibrin polymerization site.

Evaluation of amino acid sequence homology between the lamprey β chain (Bohonus et al., 1986) and the human β chain reveals that there are five, four, and two invariant amino acid residues in β15–26, β24–42, and β40–55, respectively (Figure 5). The invariant residues within β24–42 and β40–55 sequences could very well serve an important role in the fibrin polymerization site, but these residues require a proper folding that may be occurring in β15–55 but not in β24–42, β40–54, and β50–55. Since β15–18 (I_{50} = 500) and β15–26 (I_{50} = 430) are less potent inhibitors of polymerization than β15–42 (I_{50} = 50) and since β24–42, β40–54, and β50–55 do not inhibit polymerization, it can be concluded that the secondary and tertiary structures of the β-chain NH_2-terminal polymerization site play a crucial role in the expression of the binding function.

The secondary structural features of the Bβ1-55 peptide (Figure 6A) were assessed by using commercially available software from DNASTAR (Madison, WI). The methods of both Chou and Fasman (1978) and Robson and colleagues (Garnier et al., 1978), were used to predict the probable locations of α-helix, β-structure, and random coil. The hydrophobicity of the sequence was computed by the method of Kyte

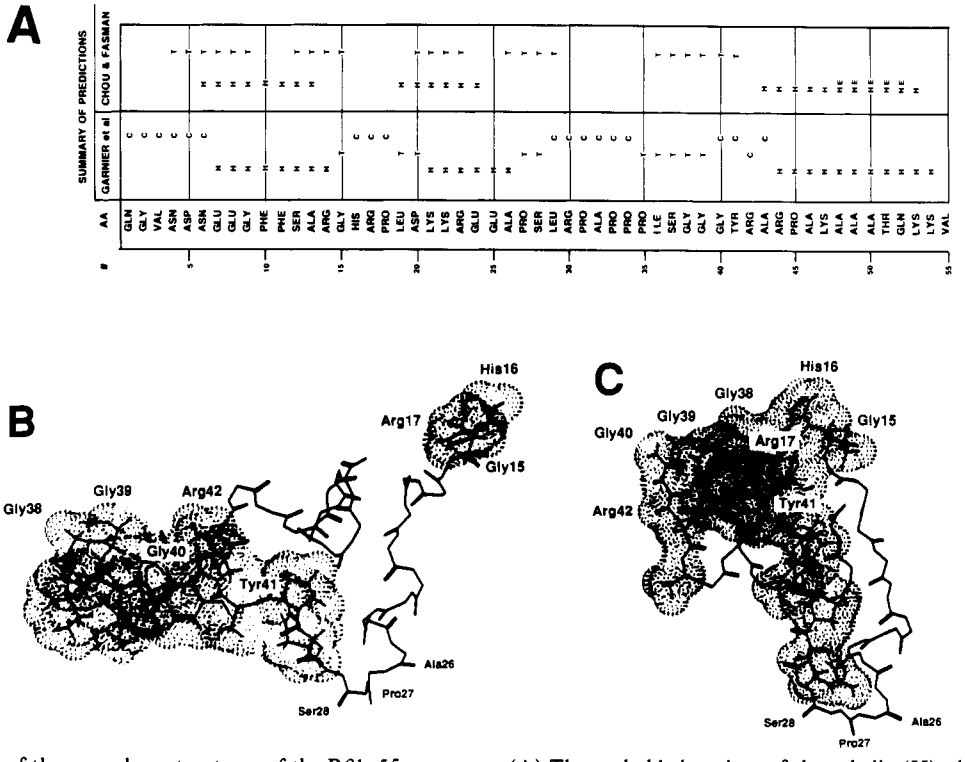

FIGURE 6: Prediction of the secondary structures of the Bβ1–55 sequence. (A) The probable locations of the α-helix (H), the β-structure (E), the random coil (C), and the turn of loop structures between helical and/or β-structures (T) were predicted by algorithms of Chou and Fasman (1978) and Garnier and colleagues (1978). (B) A model showing the predicted tertiary structure of the Bβ1–55 sequence. The fibrinopeptide B sequence Bβ1–14 has not been displayed in this model for clarity and better comparison with β15–55. (C) A model showing the predicted tertiary structure of the β15–55 sequence, that is, after cleavage of fibrinopeptide B. Notably the Gly-His-Arg sequence converged with the Gly-Gly-Gly-Tyr sequence, forming a composite site comprised of noncontiguous amino acid residues.

and Doolittle (1982) and the hydrophobic moment by the method of Eisenberg et al. (1986). The hydrophobicity and probable location of antigenic sites of the sequence were predicted by the method of Hopp and Woods (1981). For the sake of simplicity, we have chosen to show a summary of secondary structural predictions by Chou and Fasman's and Garnier's methods (Figure 6A). A computer printout containing details of all structural predictions will be furnished to any reader upon request. Computer modeling of the tertiary structure of β15–55 was performed by using the Biograf program (Biodesign, Inc., Pasadena, CA) on a Silicon Graphics Personal IRIS computer. The calculations involved energy minimizations, or molecular mechanics, coupled with molecular dynamics simulations in order to generate minimal energy structures (Mayo et al., 1989, 1991). Figure 6B shows a model of β15–55 in fibrinogen, that is, with attached fibrinopeptide B, which is not displayed for clarity. Figure 6C shows the same sequence in fibrin. Both models have been projected in relation to arbitrarily selected segment β26–28. Sphere-marked areas show β15–17 (upper right in Figure 6B) and a loop β30–43 (left in Figure 6B). The following possibilities can be suggested on the basis of structural considerations.

1. There may be three α-helices in the Bβ1–55 sequence, which span Bβ6–13, β19–26, and β43–55. The thrombin cleavage site at Bβ14–15 and the plasmin or *C. atrox* protease cleavage site at β42–43 lie in a random coil or turn structure just next to an α-helix. On the other hand β21–22, the peptide bond that is not easily cleaved by plasmin, lies in the interior of an α-helix.

2. β20–25 is the most hydrophilic region of the peptide and therefore may express an antigenic site.

3. Proper folding of β15–55 may be mediated by two α-helices spanning β19–26 and β43–55 sequences.

4. β24–42, which contains invariant amino acid residues β27Pro, β29Leu, β30Arg, and β39Gly, fails to inhibit polymerization, since the loop β30–43 contains random coil and turn structures that presumably cannot properly fold in the absence of β15-17 and β43-55 sequences.

5. Cleavage of fibrinopeptide B brings about a dramatic change in conformation since the loop does rotate as β15–17 and the loop β30–43 converge (Figure 6C). A new order of amino acid residues has been formed, Gly40-Gly39-Gly38, Tyr41, Arg17-His16-Gly15-NH_2, that may illustrate a part of a composite polymerization site in the β-chain.

6. In the fibrinogen and fibrin models, the distance between His16 and Tyr41 is 27.4 and 12.4 Å, respectively. It should be noted that Tyr, Arg, His, and the NH_2 group were identified by various authors to be essential for clot formation.

It has been widely accepted that the NH_2 terminus of the fibrin α chain does contain a fibrin polymerization site, because cleavage of fibrinogen by reptilase, which causes release of only fibrinopeptide A, results in clot formation (Blombäck et al., 1957). A peptide modeled on the fibrin α-chain NH_2 terminus, α17–19-Pro, was shown to have an I_{50} = 190 (Table I). Thus, inhibitory activity of this peptide is weaker than that of β15–42 (I_{50} = 50) but stronger than that of β15–26 (I_{50} = 430). Fibrin α-chain-derived peptides longer than α17–20 have not been tested in fibrin polymerization as yet. However, it cannot be excluded that the NH_2-terminal fibrin polymerization site is made up of sequences derived from both the α and β chains of fibrin. To test this hypothesis, tNDSK, which contains NH_2-terminal sequences of all the three chains of fibrin, was tested. tNDSK (I_{50} = 10) was a much stronger inhibitor of polymerization than β15–42 (Table I). Moreover, tNDSK also had a higher affinity for fibrin monomer than β15–42 had, as shown by binding studies using immobilized fibrin monomer. The results of polymerization inhibition and binding studies support the conclusion that the fibrin polymerization site in the NH_2 teminus of fibrin may be composed of noncontiguous amino acid residues derived from both the α and β chains. It cannot be ruled out that NH_2-terminal residues from the γ chain also may be integrated into this polymerization site.

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Characterization of cDNA for Human Tripeptidyl Peptidase II: The N-Terminal Part of the Enzyme Is Similar to Subtilisin†,‡

Birgitta Tomkinson*,§ and Ann-Kristin Jonsson‖

Department of Medical and Physiological Chemistry, Uppsala University, Biomedical Center, Box 575, S-751 23 Uppsala, Sweden, and Department of Cell Research, Swedish University for Agricultural Sciences, Biomedical Center, Box 596, S-751 24 Uppsala, Sweden

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ABSTRACT: Tripeptidyl peptidase II is a high molecular weight serine exopeptidase, which has been purified from rat liver and human erythrocytes. Four clones, representing 4453 bp, or 90% of the mRNA of the human enzyme, have been isolated from two different cDNA libraries. One clone, designated A2, was obtained after screening a human B-lymphocyte cDNA library with a degenerated oligonucleotide mixture. The B-lymphocyte cDNA library and a cDNA library, obtained from human fibroblasts, were rescreened with a 147 bp fragment from the 5′ part of the A2 clone, whereby three different overlapping cDNA clones could be isolated. The deduced amino acid sequence, 1196 amino acid residues, corresponding to the longest open reading frame of the assembled nucleotide sequence, was compared to sequences of current databases. This revealed a 56% similarity between the bacterial enzyme subtilisin and the N-terminal part of tripeptidyl peptidase II. The enzyme was found to be represented by two different mRNAs of 4.2 and 5.0 kilobases, respectively, which probably result from the utilization of two different polyadenylation sites. Furthermore, cDNA corresponding to both the N-terminal and C-terminal part of tripeptidyl peptidase II hybridized with genomic DNA from mouse, horse, calf, and hen, even under fairly high stringency conditions, indicating that tripeptidyl peptidase II is highly conserved.

In 1983, the discovery of a mammalian peptidase, which, at neutral pH, removes tripeptides from the N-terminus of longer peptides, was reported (Bålöw et al., 1983). This peptidase, currently named tripeptidyl peptidase II (TPP II),[1] has since been characterized (Bålöw et al., 1986; Bålöw & Eriksson, 1987; Macpherson et al., 1987; Tomkinson et al., 1987; Tomkinson & Zetterqvist, 1990) and shown to possess a number of unusual properties. TPP II is a high molecular weight serine exopeptidase, consisting of subunits with M_r 135000. The amino acid sequence surrounding the active-site serine residue is similar to the peptidases of the subtilisin class (Tomkinson et al., 1987), while the other mammalian serine peptidases studied so far are of the trypsin class (Neurath, 1984). Furthermore, it has been noted that there is an immunological cross-reactivity between TPP II and the cell binding domain of the extracellular matrix protein fibronectin (Tomkinson & Zetterqvist, 1990).

In order to investigate the nature, as well as the extent, of the similarity of TPP II to subtilisin and fibronectin, respectively, and to reveal possible similarities to other proteins, it was considered necessary to determine the amino acid sequence of the peptidase. The knowledge of part of the amino acid

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* To whom correspondence should be addressed.
§ Uppsala University.
‖ Swedish University for Agricultural Sciences.

[1] Abbreviations: EBV, Epstein-Barr virus; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl and 15 mM sodium citrate; TPP II, tripeptidyl peptidase II.

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