the monomer, $k \simeq 1\ M^{-1}\ s^{-1}$) electron transfer rate constants. This is related to the general problem of comparing intra- and intermolecular catalyses which result in different reaction order processes. It can be estimated that the half-life for disproportionation of 6 mM concentrations of myohemerythrin is about the same as that for octameric hemerythrin. This suggests that the orientation of the two semimetmyohemerythrin molecules in an adduct or transition complex might resemble that for two adjacent monomer units within the octamer where the distances between binuclear iron units are ~28 Å (Harrington & Wilkins, 1981). It is apparent that, quite surprisingly, the behavior of monomeric protein, particularly in its reduction reactions, is more complicated than that of the octameric form.

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Structure of Fragment E Species from Human Cross-Linked Fibrin†

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ABSTRACT: Fragments E_1, E_2, and E_3 are plasmic derivatives of fibrin encompassing the NH_2-terminal region of the molecule. The first two species, but not the third, can bind to fragment DD, forming a (DD)E complex, and therefore probably contain binding sites involved in the polymerization of fibrin. For localization of these sites the structure of the fragments was determined by establishing the NH_2- and COOH-terminal boundaries of the molecules and using the published amino acid sequence of fibrinogen. Fragment E_1 encompasses Gly-α17 to Lys-α78, Gly-β15 to Lys-β122, and Tyr-γ1 to Lys-γ62, this representing the intact NH_2-terminal region of fibrin. Fragment E_2 is an asymmetric molecule which is lacking the sequence Gly-β15 to Lys-β53 in one β-chain remnant. This fragment E_2 also lost Lys-β122 from the COOH terminal of the β chain as compared with fragment E_1. These cleavages did not affect the ability of fragment E_2 to bind to fragment DD. Fragment E_3 was heterogeneous, the main species encompassing Val-α20 to Lys-α78, Lys-β54 to Leu-β120, and Tyr-γ1 to Lys-γ53. Thus, the loss of the binding function involved in the formation of fibrin clot was associated with the removal of small fragments from all three polypeptide chains: α17–19 (Gly-Pro-Arg), β15–53 from the remaining half of the molecule, β121 (Leu), and γ54–58 (Thr-Ser-Glu-Val-Lys).

The study of binding sites on the fibrin molecule involved in polymerization of monomers and formation of a clot indicated that the NH_2-terminal region of the parent molecule participates in this reaction. The cleavage of fibrinopeptide A was requisite to express the binding site since intact fibrinogen or fibrinopeptide A containing fibrinogen derivatives were nonreactive (Heene & Matthias, 1973; Kudryk et al., 1973; Kudryk 1974; Laudano & Doolittle, 1978, 1980; Matthias & Heene, 1973; Matthias et al., 1973; Olexa & Budzynski, 1979b, 1980). Two lines of evidence indicated that the location of this binding site is on the α chain in the amino acid sequence following fibrinopeptide A. First, peptides containing the amino acid sequence which corresponds to the sequence α17–19, bound to fibrin and inhibited polymerization of fibrin monomer (Laudano & Doolittle, 1978, 1980).

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However, some peptides containing this sequence followed by amino acids not corresponding to the fibrinogen sequence are more potent inhibitors of polymerization. Second, abnormal fibrinogen Detroit, having a single amino acid substitution in the same chain, α19 Arg → Ser, had a polymerization defect localized in the NH_2-terminal region of the molecule (Kudryk et al., 1976).

Three molecular species of fragment E, that is, E_1, E_2, and E_3, were isolated from plasmic digestions of human crosslinked fibrin (Olexa & Budzynski, 1979a). Fragments E_1 and E_2, but not fragment E_3, bound specifically with fragment DD forming a (DD)E complex (Olexa & Budzynski, 1979a,b). The mechanism of the binding involves sites participating in the polymerization of fibrin monomers (Olexa & Budzynski, 1980). Fragments E_1, E_2, E_3 as derivatives of the NH_2-terminal region of fibrin do not contain fibrinopeptides and thus have functionally expressed binding sites which appear to be the same as those involved in the propagation of fibrin polymerization.

In this work the primary structure of fragments E_1, E_2, and E_3 was studied by establishing the NH_2- and COOH-terminal boundaries of the molecules and using the published amino acid sequence of fibrinogen (Henschen & Lottspeich, 1977; Watt et al., 1978; Doolittle et al., 1979; Gårdlund 1977a,b; Henschen at al., 1979). Particular attention was focused on differences in the amino acid sequence between fragments E_2 and E_3. The elucidation of structural differences of these two should provide evidence about the localization of a polymerization site in the NH_2-terminal region of human fibrin.

Materials and Methods

Purification of Fragments E. Human fibrinogen (Grade L, A. B. Kabi, Stockholm, Sweden) was enriched with factor XIII (Loewy et al., 1961) and clotted, as described previously (Marder et al., 1976). One gram of cross-linked fibrin was suspended in 20 mL of prewarmed (37 °C) 0.15 M Tris[1]-HCl buffer, pH 7.8, containing 5 mM calcium chloride and 0.02% sodium azide. An aliquot of either 0.5 or 10.0 mL of human plasmin (kindly supplied by Dr. David Aronson, Bureau of Biologics, Food and Drug Administration, Rockville, MD., containing 10.0 CTA[1] units/mL, 12.7 CTA units/mg of protein) was added to fibrin and digestion allowed to proceed for 24 h at 37 °C with gentle agitation. Digestion was inhibited by the addition of 0.05 or 1.0 mL of trasylol (aprotinin, 10000 KIU[1]/mL; Mobay Chemical Corp., New York). Approximately 500 mg of the cross-linked fibrin digest was applied to a Sepharose CL-6B (Pharmacia, Piscataway, NJ) column (3.5 × 190 cm) developed in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.028 M sodium citrate, 0.1 M sodium chloride, 25 units/mL trasylol, and 0.02% sodium azide. Fractions which contained fragment E species were diluted with an equal volume of 6 M urea and 0.05 M sodium citrate, pH 5.5, and incubated at 37 °C for 1 h, and then 100 mg of the sample was rechromatographed on a Sepharose CL-6B column (2.5 × 170 cm) in the original Tris-citrate buffer, pH 7.4. This procedure dissociated the (DD)E complex and allowed purification of the fragment E species. The subspecies of fragments E were further separated by preparative isoelectric focusing in a 110-mL column (LKB, Bromma, Sweden) on a 4.0–6.5 pH gradient (pharmalytes, Pharmacia, Uppsala, Sweden) stabilized by a sucrose gradient of 5–50%. Approximately 20 mg of protein was applied, and the column was equilibrated at 800 V, 4 °C, for 24 h. Fractions (1 mL) were removed from the column at a flow rate of 20 mL/h and monitored for absorbance at 280 nm and for pH. Ampholytes were removed by dialyzing the pooled fractions against 2 500-fold volumes of 1.0 M sodium chloride and 2 500-fold volumes of 0.15 M sodium chloride followed by 4 500-fold volumes of distilled water, and the fragments were freeze-dried.

Polyacrylamide Gel Electrophoresis. Proteins were analyzed in two electrophoretic systems: 7% polyacrylamide gels containing 0.1% $NaDodSO_4$ according to the method of Weber & Osborn (1969) and Tris–glycine gels (9%) by the method of Davis (1964). Approximately 10 μg of protein was applied per gel, and staining was done according to the procedure of Fairbanks et al. (1971).

Binding of Fragments E and DD. Fragment DD was purified as described previously (Olexa & Budzynski, 1979a). Each isolated fragment E and fragment DD were mixed in a 1:1 molar ratio, diluted to 0.4 mg/mL (final total concentration) in 0.15 M Tris-HCl buffer, pH 7.4, and incubated at room temperature for 1 h. An aliquot was diluted, with an equal volume of 0.05 M Tris-HCl buffer, 0.38 M glycine, and 40% sucrose, pH 8.6, and 50 μL (10 μg) was electrophoresed on Tris–glycine (9%)–polyacrylamide gels.

Cyanogen bromide cleavage of fragments E was carried out in 70% formic acid for 7.5 h followed by freeze-drying. The NH_2-terminal region of E knot was separated from the peptides by gel filtration on a Sephadex G-50 (Pharmacia, Piscataway, NJ) column (2.5 × 50 cm) in 1% acetic acid. The peptides were further separated by low-voltage paper electrophoresis at pH 6.5 as described previously (Takagi & Doolittle, 1974; Doolittle et al., 1977). The E knot was reduced and alkylated (Doolittle et al., 1977), and the carboxymethylated chains were separated by gel filtration on a Sephadex G-50 column (2.5 × 44 cm, in 10% acetic acid); the α chain portin was desalted on a Sephadex G-25 column (2.5 × 30 cm) in 0.1 M ammonium bicarbonate, pH 8.2.

Amino acid compositions were determined by hydrolysis in constant-boiling hydrochloric acid for 24, 48, and 72 h at 110 °C under vacuum. The hydrolysates were dried and then dissolved in 0.1 M citrate–chloride buffer, pH 2.1. Amino acid analyses were performed on a Spinco Model 119 automatic amino acid analyzer (Beckman, Palo Alto, CA). Values were obtained by extrapolation to zero time.

The NH_2-terminal amino acid sequence was determined by the thioacetylation procedure introduced by Mross & Doolittle (1971) and as modified by Takagi & Doolittle (1974). In some cases the NH_2-terminal amino acid was determined by using the dansylation method of Hartley (1970).

Results

Purification of Fragments E. The digestion of cross-linked fibrin by plasmin and the preliminary steps in the purification of the fragments E have been described (Olexa & Budzynski, 1979a). In the present work the use of preparative isoelectric focusing instead of ion-exchange chromatography provided a better separation of the Fragment E subspecies.

Human cross-linked fibrin digested with 5 units of plasmin/g of fibrin will produce primarily the (DD)E complex with Fragments E_1 and E_2 while the addition of 100 units of plasmin/g of fibrin results in the formation of fragments DD and E_3 (Olexa & Budzynski, 1979a). The former digest, gel filtered on a Sepharose CL-6B column, had a predominant peak containing the (DD)E complex. After urea treatment to dissociate the complex and rechromatography on the Sepharose column, a preparation containing approximately 50%

[1] Abbreviations used: CTA, Committee on Thrombolytic Agents; KIU, Kallikrein inhibitor units; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

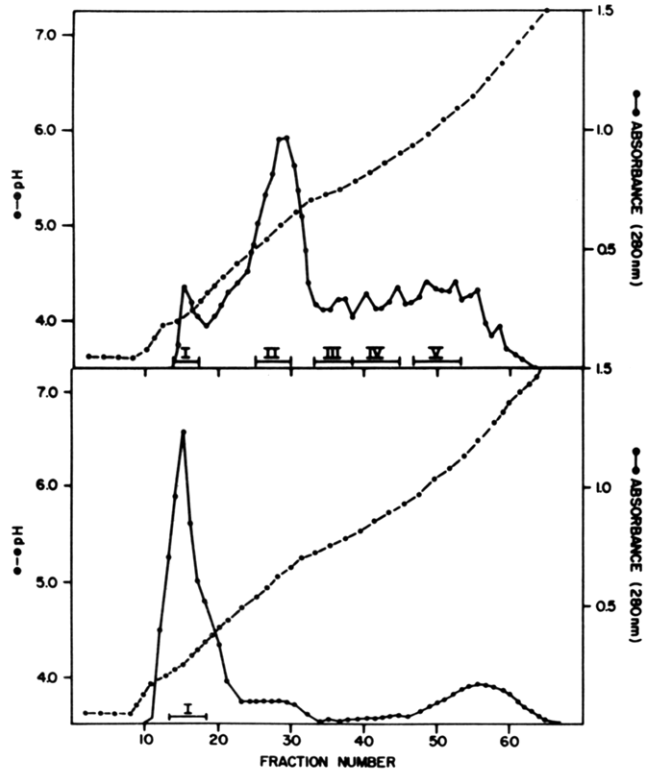

FIGURE 1: Profiles of isoelectric focusing of fragments E from cross-linked fibrin. A mixture of approximately 50% fragment E_1, 50% fragment E_2, and trace amounts of fragment E_3 (upper panel) or fragment E_3 contaminated by α-polymer remnants (lower panel) was separated by preparative isoelectric focusing in a 110-mL column on a 4.0–6.5 pH gradient. Fractions (1 mL) were analyzed for pH and absorbance at 280 nm.

E_1, 50% E_2, and trace amounts of E_3, as determined by $NaDodSO_4$–polyacrylamide (7%) gel electrophoresis, was obtained. Isoelectric focusing of this preparation revealed that there was much heterogeneity in these species (Figure 1, upper panel). In Tris–glycine (9%) gel electrophoresis, it can be seen that the fragment E preparation was separated into five distinct subclasses (Figure 2). In $NaDodSO_4$ (7%) gel electrophoresis, the protein in peak I had the same mobility as fragment E_3, while that in peak VI migrated similarly to fragment E_1. The protein in peaks II–V all migrated as fragment E_2 in $NaDodSO_4$ (7%) gel electrophoresis. Upon reduction, carboxymethylation, and $NaDodSO_4$ (12.5%) gel electrophoresis, the protein in peaks II–V, the subspecies of fragment E_2, each were made up of four different chains of approximate molecular weights 6300 (γ/), 8300 (α/), 9400 (/β/) and 14000 (/β). Therefore, these subspecies were named E_{2_a}, E_{2_b}, and E_{2_c} in order of increasing anodal mobility on Tris–glycine gels. The isolation of these five subclasses of fragment E, i.e., E_1, E_{2_a}, E_{2_b}, E_{2_c}, and E_3, was highly repeatable.

Cross-linked fibrin digested by 100 units of plasmin/g of protein and gel filtered on a Sepharose CL-6B column had a major peak of fragment DD and a peak containing fragment E_3 contaminated by α-polymer remnants and a trace amount of fragment DD. After urea treatment and rechromatography of the fragment E peak, the fragment DD contamination was removed. The profile of the isoelectric focusing of this preparation (Figure 1, lower panel) had one major peak of fragment E_3 (peak I) and a peak containing the α-polymer remnants. The protein in peaks I from the upper and lower profiles (Figure 1) had the same mobility on $NaDodSO_4$ gels and on Tris–glycine gels (Figure 2). After the isoelectric focusing procedure the recovery of fragment E_1 was only ~38% while

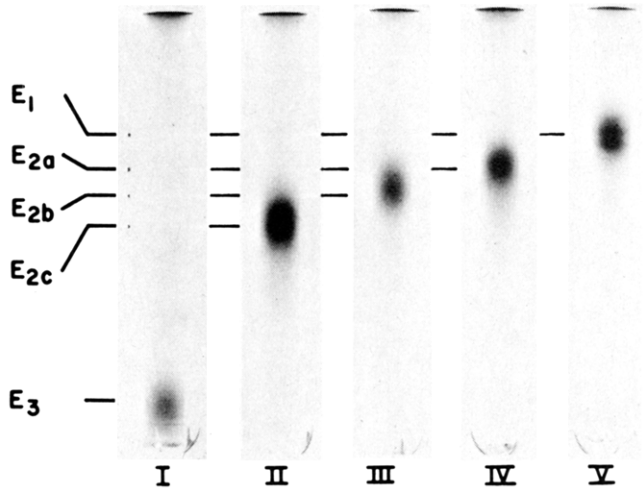

FIGURE 2: Tris–glycine–polyacrylamide (9%) gel electrophoresis of fractions from preparative isoelectric focusing of fragments E (Figure 1).

the recovery of fragment E_2 was ~95% and that of E_3 was >80%.

Isoelectric focusing of a mixture of fragment E species provides evidence that more than three subspecies of fragment E exist. The highest molecular weight species, that is, fragment E_1, has an isoelectric point in the pH range 5.8–6.4. Fragment E_2 was separated into three subclasses, E_{2_a} with an isoelectric point of 5.5–5.8, E_{2_b}, 5.25–5.5, and E_{2_c}, 4.7–5.25. Fragment E_3 had an isoelectric point of 4.1–4.15. It is very likely that microheterogeneity exists in each of the species isolated.

Binding to Fragment DD. The fragment E species isolated by isoelectric focusing were tested for binding to fragment DD. Fragments DD and E were mixed in a 1:1 molar ratio, incubated at room temperature for 1 h and then electrophoresed on Tris–glycine gels to qualitatively detect complex formation (Olexa & Budzynski, 1979b). All of the fragments E from peaks II–V bound to fragment DD forming a (DD)E complex; however, the fragment E in peak I did not complex with fragment DD. These data are in agreement with previous observations that fragments E_1 ane E_2 are capable of forming a (DD)E complex while fragment E_3 is not (Olexa & Budzynski, 1979b).

Analysis of Intact Fragments E. The conversion of fragment E_2 to E_3 appears to remove or destroy the binding site for fragment DD. Therefore, the present studies focused on fragment E_1, as the most undegraded species, fragment E_{2_c}, as the most degraded species which maintains the ability to bind to fragment DD, and fragment E_3, as the terminal species.

Fragments E were hydrolyzed in constant-boiling hydrochloric acid, and the total amino acid composition was determined (Table I). In the conversion from fragment E_1 to E_{2_c} changes occurred in the content of alanine, valine, lysine, and arginine. The cleavage of E_{2_c} to E_3 resulted in differences in aspartic acid (or asparagine), threonine, glutamic acid (or glutamine), proline, glycine, alanine, valine, leucine, isoleucine, lysine, and arginine. The loss of arginine and lysine and the relative increase of aspartic and glutamic acid correponded well with the increase of the negative charge of fragments E during progressive degradation. The changes in valine as fragment E is degraded cannot be easily explained; however, the result was obtained consistently. The molecular weight of the fragments E was calculated on the basis of the total amino acid composition for a dimeric molecule with carbohydrate contributing an additional molecular weight of ~5000.

Table I: Total Amino Acid Composition of Fragments E_1, E_{2c}, and E_3

amino acid	fragment E_1 (res/ fragment)[c]	fragment E_{2c} (res/ fragment)[c]	fragment E_3 (res/ fragment)[c]
Asp[a]	31.7	31.4	27.1
Thr	10.5	10.5	8.5
Ser	17.2	16.9	15.0
Glu[a]	30.3	29.7	24.2
Pro	16.3	14.9	8.7
Gly	15.4	14.3	10.0
Ala	16.3	13.4	8.1
Cys	8.6	9.6	8.7
Val	9.1	11.4	7.5
Met	1.4	1.6	1.8
Ile	6.3	6.0	3.9
Leu	17.7	17.2	13.7
Tyr	9.3	8.9	8.5
Phe	6.5	6.2	5.7
His	4.4	3.8	3.2
Lys	17.7	16.1	11.0
Arg	13.3	11.8	6.8
Trp	+	+	+
total residues	233	223	178
mol wt[b]	56 754	54 614	45 082

[a] Aspartic acid and glutamic acid values include asparagine and glutamine, respectively. [b] Minimum molecular weight calculated for a dimeric fragment E containing carbohydrate accounting for a molecular weight of ~5000. [c] Expressed as residues per fragment.

Table II: NH_2-Terminal Sequence of Intact Fragments E

fragment E_1	α	^{17}Gly-Pro-Arg-Val-Val
	β	^{15}Gly-His-Arg-Pro-Leu
	γ	^{1}Tyr-Val-Ala-Thr-Arg
fragment E_{2c}	α	^{17}Gly-Pro-Arg-Val-Val
	β_1	^{15}Gly-His-Arg-Pro-Leu
	β_2	^{54}Lys-Val-Glu-Arg-Lys
	γ	^{1}Tyr-Val-Ala-Thr-Arg
fragment E_3	α_1	^{20}Val-Val-Glu-Arg-His
	α_2	^{24}His-Gln-Ser-Ala-Cys
	β	^{54}Lys-Val-Glu-Arg-Lys
	γ	^{1}Tyr-Val-Ala-Thr-Arg

The molecular weight of the carbohydrate moiety was estimated by adding the molecular weights of each sugar residue (Iwanaga et al., 1968; Mills & Liener, 1969). By use of these data the difference in molecular weight between E_1 and E_{2c} was on the order of 2000, however, the change in molecular weight from E_{2c} to E_3 was ~9000. These molecular weights compare favorably to those determined by $NaDodSO_4$ gel electrophoresis of reduced fragments E_1, E_2, and E_3 as 58 900, 55 500, and 49 500, respectively (Olexa & Budzynski, 1979b).

The NH_2-terminal sequence of fragments E was determined for five cycles (Table II). Although three amino acids were expected to be released at each cycle, the assignment of the amino acids to a polypeptide chain was relatively simple since the entire sequence of fibrinogen has been elucidated (Henschen & Lottspeich, 1977; Watt et al., 1978; Gårdlund 1977a,b; Doolittle et al., 1979; Henschen et al., 1979). In fragment E_1 the NH_2-terminal amino acids of the α, β, and γ chains are the same as those of the intact fibrin monomer molecule, the α and β chains beginning with the sequence that immediately follow the fibrinopeptides. Fragment E_{2c} is an asymmetric molecule containing two β-chain remnants, one beginning at Gly-β15 and the other at Lys-β54. This heterogeneity of the β chain will induce a substantial error in the calculation of molecular weight from the total amino acid composition of the fragment (Table I). Fragment E_3 was found to be heterogeneous in the α-chain remnant (Table II). It is possible that another subspecies of fragment E_3 (E_{3_b}, perhaps) is formed which has both α-chain remnants beginning at His-α24.

Cyanogen Bromide Cleavage of Fragments E. Fragments E were treated with cyanogen bromide in order to determine the COOH-terminal boundaries of the α and β chains. This procedure will cleave the α chain at Met-α51 and the β chain at Met-β118. The γ-chain remnant contains no methionine in the fragment E domain, so this chain is not affected by cyanogen bromide. The released peptides were separated from E knot by gel filtration on Sephadex G-50 in 1% acetic acid. The peptides were further separated by low-voltage paper electrophoresis and then hydrolyzed in constant-boiling HCl at 110 °C for 24 h, and the total amino acid composition was determined.

The amino acid compositions of the peptides from the COOH terminal of the α-chain remnant were compared to the known amino acid sequence of the fibrinogen α chain 52–78 (Doolittle et al., 1979). There are no significant differences between the peptides released from the α chain of the three species of fragment E and the residues α52–78 (Table III). Therefore, it appears that the α-chain remnant of all three species of fragment E terminates with Lys-α78.

Peptides isolated from the COOH terminal of the β chain correspond to Tyr-Leu-Leu-Lys, β119–β122 from fragment E_1, Tyr-Leu-Leu, β119–β121 from fragment E_{2c}, and Tyr-Leu, β119–β120 from fragment E_3. Therefore, the COOH-terminal amino acid of the β-chain remnants of fragments E_1, E_{2c}, and E_3 are Lys-β122, Leu-β121, and Leu-β120, respectively. The cleavage of a Leu–Lys or a Leu–Leu bond is unusual for plasmin; however, this phenomenon has been recognized (Takagi & Doolittle, 1975a,b). The cleavage could have been caused by a contamination by a carboxypeptidase B like enzyme (Gårdlund, 1977a,b).

Analysis of E Knot. After CNBr cleavage of fragments E, the knot portion of the molecule was reduced and carboxymethylated. The α-chain remnant was separated from the β and γ chains by gel filtration on a Sephadex G-25 column equilibrated in 0.1 M ammonium bicarbonate, pH 8.2. The α-chain remnant and the mixture of β and γ chains were hydrolyzed, and the total amino acid composition was determined. The NH_2-terminal amino acid of the α chain was determined by using the dansylation technique (Hartley, 1970).

The total amino acid compositions of the α-chain remnants from E_1, E_{2c}, and E_3 are very similar; the only significant differences are in the content of Gly, Pro, and Arg in the conversion of E_{2c} to E_3 (Table IV). The NH_2-terminal amino acids of the α-chain remnants are Gly for fragments E_1 and E_{2c} but Val and His for fragment E_3. These data are in agreement with the NH_2-terminal sequence of the entire fragment E molecule (Table II). Therefore, the α-chain remnants of fragments E_1 and E_{2c} extend from Gly-α17 to Lys-α78. In fragment E_3 there is heterogeneity of the α-chain remnant: one appears to contain the sequence Val-α20 to Lys-α78 and the other His-α24 to Lys-α78. At present it is not known if fragment E_3 contains internal heterogeneity with one copy of each variant of the α-chain remnant. It is likely that both possibilities exist, that is, a fragment E_3 with a homogeneous population of α chains of Val-α17 to Lys-α78

Table III: Amino Acid Composition of α-Chain COOH-Terminal Remnant

amino acid	fragment E_1 (res/peptide)[b]	fragment E_{2_c} (res/peptide)[c,b]	fragment E_3 (res/peptide)[b]	Aα 52–78[a] (res/peptides)[b]
Asp[c]	6.7	7.2	7.0	6
Thr	1.0	1.0	1.0	1
Ser	1.6	1.4	1.5	1
Glu[c]	3.9	3.6	3.6	4
Pro				0
Gly	1.5	1.1	1.3	1
Ala				0
Cys				0
Val	0.6	0.8	0.8	1
Met				0
Ile	1.4	1.7	1.6	2
Leu	2.1	2.6	2.5	3
Tyr	1.2	0.8	0.9	1
Phe	2.0	1.8	2.0	2
His				0
Lys	4.1	4.1	4.1	4
Arg	0.9	0.9	0.8	1
Trp				0
total	27.0	27.0	27.1	28
amino terminal	Lys	Lys	Lys	

[a] Based on the amino acid sequence of the Aα chain of fibrinogen as represented by Doolittle et al. (1979). [b] Expressed as residues per peptide. [c] Aspartic acid and glutamic acid values include asparagine and glutamine, respectively.

Table IV: Total Amino Acid Composition of α-Chain Remnant

amino acid	fragment E_1 (res/peptide)[e]	fragment E_{2_c} (res/peptide)[c,e]	fragment E_3 (res/peptide)[e]
Asp[a]	5.5 (5)	5.2 (5)	5.1 (5)
Thr	0.6 (0)	0.5 (0)	0.6 (0)
Ser	3.7 (4)	3.5 (4)	3.5 (4)
Glu[a]	3.9 (3)	3.9 (3)	3.8 (3)
Pro	2.7 (3)	2.9 (3)	2.3 (2)
Gly	2.5 (2)	2.3 (2)	1.7 (1)
Ala	1.3 (1)	1.3 (1)	1.4 (1)
Cys[b]	2.7 (4)	3.1 (4)	3.2 (4)
Val[c]	1.2 (2)	1.4 (2)	0.9 (2)
Met[d]	0.6 (1)	0.5 (1)	0.6 (1)
Ile	0.3 (0)	0.3 (0)	0.3 (0)
Leu	0.7 (0)	0.7 (0)	0.7 (0)
Tyr	1.0 (1)	1.2 (1)	1.1 (1)
Phe	1.4 (1)	1.3 (1)	1.1 (1)
His	1.0 (1)	0.9 (1)	0.9 (1)
Lys	2.4 (2)	2.3 (2)	2.2 (2)
Arg	2.6 (3)	2.6 (3)	1.8 (2)
Trp	(+1) (1)	(+1) (1)	(+1) (1)
total	35.0 35	34.9 33	32.2 32

[a] Aspartic acid and glutamic acid values include asparagine and glutamine, respectively. [b] Cys measured as (carboxymethyl)-cysteine. [c] Val–Val bond. [d] Met measured as homoserine. [e] Expressed as residues per peptide. Expected values are given in parentheses.

is cleaved asymmetrically to form an intermediate fragment E_3, followed by cleavage to a terminal fragment E_3 with both α-chain remnants of His-α24 to Lys-α78. Since the difference in molecular weight of the two chains is minor and there is not charge gain or loss after the cleavage of the peptide Val-Val-Glu-Arg, the separation of the variants will be complicated and this question will be difficult to resolve.

The mixture of β and γ chains from the reduced and carboxymethylated E knot region of the three species of fragment E was hydrolyzed and the amino acid composition determined. The total amino acid composition of fragment E_1 knot β and γ remnants correlated very closely with the amino acid sequence β15–118 and γ1–62. The fragment E_3 knot β and γ remnants had an amino acid composition very similar to the compositions of β54–118 and γ1–53, respectively. The amino acid composition of the β and γ remnants from E_{2_c} knot was compared to the sequence β15–118, β54–118, γ1–62, and γ1–53, assuming equal amounts of each chain in the mixture. The values correlated relatively well; however, some disagreements are present. Since fragment E_{2_c} is a transient intermediate species between E_1 and E_3, it is very likely that minor heterogeneity may be present in the preparation. The NH_2-terminal amino acids of the β and γ chain of fragment E_1 were Tyr and Gly; for E_{2_c}, Tyr, Gly, and Lys were present; for E_3, Tyr and Lys were measured (Table II). These data indicate that the β-chain remnant of fragment E_1 is Gly-β15 to Lys-β122 and fragment E_3 β chain extends from Lys-β54 to Leu-β120. In fragment E_{2_c} heterogenity of the β chain exists. This dimeric molecule probably contains one β-chain remnant extending from Gly-β15 to Leu-β121 and one encompassing the sequence Lys-β54 to Leu-β121. The cleavage of the sequence β15–β53 from the NH_2 terminal of one of the β-chain remnants of fragment E_1 can account for the decrease in molecular weight as fragment E_{2_c} is formed. Similarly, the cleavage of the other β-chain remnant in the conversion of E_{2_c} to E_3 accounts for the change in the molecular weight of this species. Futher, reduced and carboxymethylated fragments E_{2_a}, E_{2_b}, and E_{2_c} analyzed on $NaDodSO_4$–polyacrylamide (12.5%) gels show that all species of fragment E_2 are made up of four polypeptide chains. Therefore, it is very likely that the fragment E_2 species are heterogeneous molecules containing two varieties of β-chain remnants.

COOH Terminal of the γ Chain. Since the γ-chain component of fragment E is not cleaved by cyanogen bromide, because there are no methionines in this sequence, an alternative method was used to determine the COOH-terminal amino acid sequence of this chain. The mixture of the β- and γ-chain remnants from the E knot preparations was treated with trypsin, then the peptides were separated by paper electrophoresis, and the total amino acid composition was determined. Since the boundaries of the β-chain remnant and the NH_2-terminal of the γ-chain remnant have been elucidated, the tryptic peptides could be mapped into the known sequence. For fragments E_1 and E_{2_c} peptides corresponding to Tyr-γ1 to Lys-γ62 have been mapped. However, in fragment E_3 the γ-chain sequence extends only from Tyr-γ1 to Lys-γ53. Therefore, the γ-chain remnants of fragments E_1 and E_{2_c} are from Tyr-γ1 to Lys-γ62, but in fragment E_3 the

Table V: NH_2- and COOH-Terminal Amino Acids in Fragments E

polypeptide chain	digested material	fragment	terminal amino acid		ref
			NH_2–	–COOH	
Aα	fibrinogen	late E	Ala-1		Kowalska-Loth et al. (1973)
	fibrinogen	early E	Ala-1		Takagi & Doolittle (1975a)
	fibrinogen	late E	Gly-17	Lys-78	Takagi & Doolittle (1975a,b)
	fibrinogen	very late E	Val-20		Takagi & Doolittle (1975a)
	fibrinogen	late E	Ala-1	Lys-81	Gårdlund (1977)
	cross-linked fibrin	E_1	Gly-17	Lys-78	this work
	cross-linked fibrin	E_{2c}	Gly-17	Lys-78	this work
	cross-linked fibrin	E_3	Val-20	Lys-78	this work
Bβ	fibrinogen	late E	Lys-54		Kowalska-Loth et al. (1973)
	fibrinogen	early E	Lys-54		Takagi & Doolittle (1975a)
	fibrinogen	late E	Lys-54	Lys-122	Takagi & Doolittle (1975a,b)
	fibrinogen	very late E	Lys-54	Leu-121	Takagi & Doolittle (1975a,b)
	fibrinogen	late E	Lys-54	Leu-121	Gårdlund (1977)
	cross-linked fibrin	E_1	Gly-15	Lys-122	this work
	cross-linked fibrin	E_{2c}	Gly-15/Lys-54	Leu-121	this work
	cross-linked fibrin	E_3	Lys-54	Leu-120	this work
γ	fibrinogen	late E	Tyr-1	Lys-58	Kowalska-Loth et al. (1973)
	fibrinogen	early E	Tyr-1		Takagi & Doolittle (1975a)
	fibrinogen	late E	Tyr-1	Lys-53	Takagi & Doolittle (1975a)
	fibrinogen	very late E	Tyr-1		Takagi & Doolittle (1975a)
	fibrinogen	late E	Tyr-1	Lys-58	Gårdlund (1977)
	cross-linked fibrin	E_1	Tyr-1	Lys-62	this work
	cross-linked fibrin	E_{2c}	Tyr-1	Lys-62	this work
	cross-linked fibrin	E_3	Tyr-1	Lys-53	this work

γ chain remnant is shorter: Tyr-γ1 to Lys-γ53. A schematic representation of the degradation pathway, $E_1 \rightarrow E_{2c} \rightarrow E_3$, is shown in Figure 3.

Discussion

Studies on the amino acid sequence of fragment E indicated multiple cleavages in products obtained from human fibrinogen digested with plasmin under a variety of conditions (Table V). In addition, minor heterogeneities were observed in fibrinogen fragments E (Gårdlund, 1977; Kowalska-Loth et al., 1973; Takagi & Doolittle, 1975a,b) and in fibrin fragments studied in this work. The major difference in the degradative pathway of fibrinogen and cross-linked fibrin by plasmin is the protection of fragment E moiety against proteolysis. Upon lysis of clots composed of cross-linked fibrin, the association of fragments E and DD in the (DD)E complex is apparently responsible for the protective effect (Olexa & Budzynski, 1979a,b).

The NH_2 terminal of fragment E_1 is identical with that of fibrinogen depleted of fibrinopeptides A and B (Tables I–IV, Figure 3). This derivative represents the largest isolated and characterized fragment E. The major structural change upon the conversion of fragment E_1 into E_2 is the loss of 39 amino acid residues from only one β-chain remnant. Thus, the essential structure of fragment E_2 is asymmetric. This asymmetry of the β chain is not associated with a loss of binding of fragment E_2 to fragment DD (Olexa & Budzynski, 1979b). It is not clear if a single intact β-chain segment would be sufficient to enable binding to fragment DD and to promote the polymerization of fibrin. The importance of this polypeptide chain segment for the formation of a clot was recognized while studying thrombin clottability of fragments X from a stage 1 and a stage 2 digests. While the former is well clottable, the clottability of the latter is significantly impaired (Niewiarowski et al., 1977; Tranqui-Pouit et al., 1975), and this difference may be related to the presence of Gly-β15 to Lys-β53 in fragment X (stage 1) and the absence in fragment X (stage 2).

The degradation of fragment E_2 to E_3 affected four places in the molecule removing the following sections: (1) Gly-α17 to Arg-α19 and also partially Gly-α17 to Arg-23, (2) Gly-β15 to Lys-β53 from half of the asymmetric molecule, (3) Leu-β121, and (4) Thr-γ54 to Lys-γ62. Experimental evidence indicated that the NH_2 terminal of fibrin α chain was directly involved in the polymerization reaction (Kudryk et al., 1976),

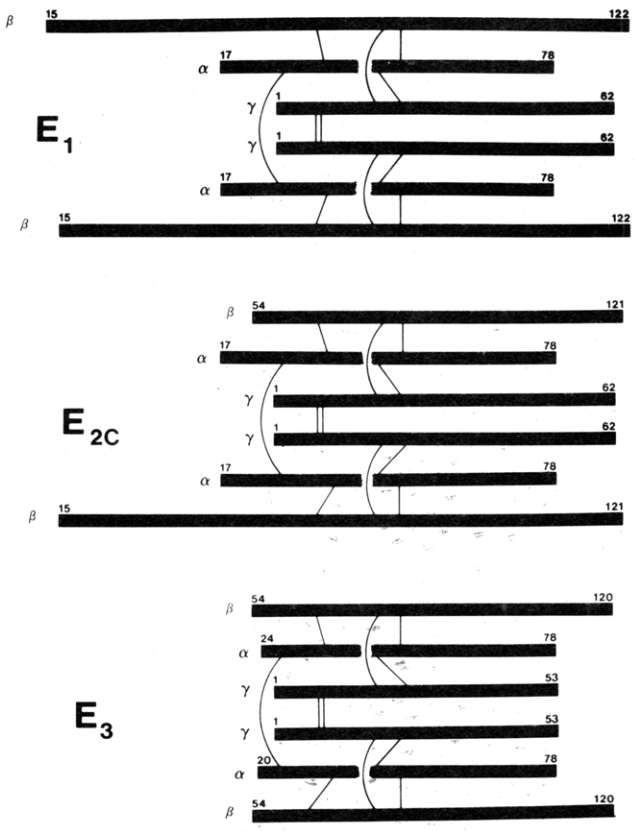

FIGURE 3: Schematic representation of the structure of three species of fragment E. The polypeptide chains are represented by thick dark lines and disulfide linkages, by the thin dark lines joining the chains. The NH_2- and COOH-terminal amino acids are indicated by the numbers above the respective chains and refer to the amino acid sequence of fibrinogen as published (Henschen & Lottspeich, 1977; Watt et al., 1978; Doolittle et al., 1979; Henschen et al., 1979).

and the loss of both α17–19 and the binding function in fragment E_3 is entirely compatible with this idea. The tripeptide Gly-Pro-Arg inhibits fibrin monomer polymerization and binds to fibrin, albeit with a relatively low affinity (Laudano & Doolittle, 1978, 1980), suggesting that other polypeptide segments may cooperate positively in the expression of binding functions. This notion is supported by observations that fragment E from short digests of fibrinogen, which has the Aα-chain remnant terminating with fibrinopeptide A (Table V; Olexa & Budzynski, 1979b), failed after thrombin treatment to bind with fragment DD and to form of (DD)E complex. A possible cooperative segment may be β15–53; however, β121 and γ54–62 should be also taken under consideration.

Fragment E is a structurally dimeric molecule and possesses most likely two sets of polymerization sites. A complementary binding region to each of these sites was indicated to be in the fragment D domain (Kudryk et al., 1973, 1976; Olexa & Budzynski, 1979b, 1980; Matthias et al., 1973). Consequently, one fragment E domain should bind two fragment D domains originating from two fibrin monomer molecules. As such, fragment E should cooperate in the propagation of fibrin polymers, and it has been shown that fragments E_1 and E_2 slightly accelerate polymerization and become incorporated into fibrin clots (Budzynski et al., 1979).

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