A STRUCTURAL ASPECT OF HUMAN FIBRINOGEN SUGGESTED BY ITS PLASMIN DEGRADATION

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1. Introduction

Since the discovery [1] that fibrinogen following treatment with plasmin (EC 3.4.4.14) produces an anticoagulant activity and the report that fibrin deposition plays a vital role in the growth of some malignant tumours [2], considerable interest has been shown in the mechanism of proteolysis of fibrinogen by plasmin and thrombin (EC 3.4.4.13). During plasmin digestion of fibrinogen a large fragment (molecular weight 160,000), released early in the reaction, has been shown to have a maximum anticoagulant effect while the high molecular weight end-products of digestion exhibit a low but significant inhibition of the clotting process [3]. The end or core fragments, named D and E, have been physico-chemically and immunologically characterized [3–6]. The gross structure of intact fibrinogen has been described as three pairs of polypeptide chains designated α (A), β (B) and γ* [7, 8]. The presence of different α (A), β (B) and γ chains in different populations of fibrinogen molecules from a single donor has been suggested recently [9]. Little is known concerning the amino acid sequence of the polypeptide chains and their location in the molecule. This paper describes the subunit molecular weights of human fibrinogen and various fragments released during its hydrolysis with plasmin. The location of the subunits of the anticoagulant core fragment, D, in native fibrinogen is suggested. The spatial positions of the three polypeptide chains in the intact fibrinogen molecule are discussed.

* This nomenclature for the three chains has been recommended by the International Committee on Hemostasis and Thrombosis (Washington, 1967).

2. Materials and methods

Human fibrinogen, KABI, Grade L, Lot no. 45959 A.B. KABI, Stockholm, Sweden) prepared from the plasma of 4000 donors by the ethanol precipitation method of Blombäck and Blombäck [10] was used in these experiments.This fibrinogen was pure by acrylamide gel electrophoresis and was over 90% clottable by the method of Laki [11]. Fibrinogen degradation products (FDP) were prepared at 25° by the addition of 0.14 CTA human plasmin** units per mg of fibrinogen (10 mg/ml in 0.9% NaCl–0.4% sodium citrate, pH 7.3 solution). Aliquots were removed at various time intervals, the digestion in them stopped with 0.2 M e-amino-caproic acid (e-ACA) and the samples frozen in a methanol–CO_2 bath until ready for SDS-acrylamide analysis. Overnight (15 hr) digestion yielded two plasmin resistant core fragments which stained with Amido Black on gel electrophoresis and a mixture of low molecular weight, non-staining peptides [6]. This report deals with molecular weight

** This plasmin was supplied by Dr. A.J. Johnson, New York University Medical Center, School of Medicine, N.Y., USA. The preparation was heterogeneous on acrylamide electrophoresis, was autoactivated in 50% glycerol and contained 10 CTA units per ml. One CTA (Committee for Thrombolytic Agents) unit releases 0.1 μmole of tyrosine equivalents per minute from Lot GC 1-16 α-casein (Report of the International Committee on Hemostatis and Thrombosis, Washington, 1967).

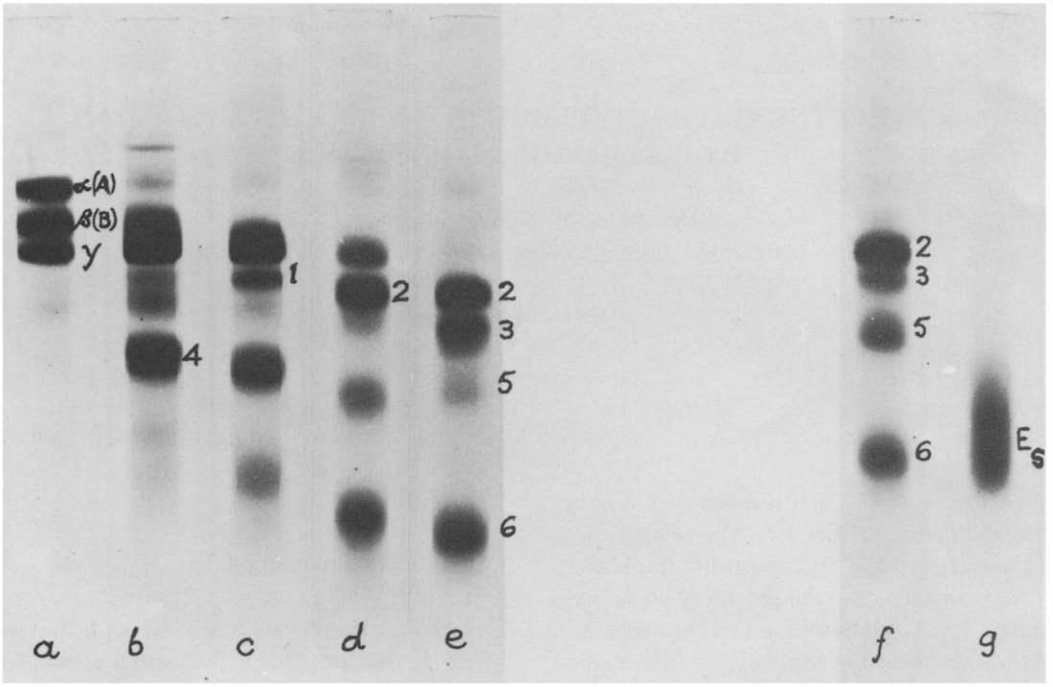

Fig. 1. SDS-acrylamide gel patterns of the subunits of human fibrinogen, its plasmin degradation products at various time intervals and its plasmin resistant 'core' fragments, D and E: (a) fibrinogen; fibrinogen digested with plasmin for (b) 2 min (c) 10 min (d) 30 min (e) 15 hr; (f) purified 'core' fragment D; (g) purified 'core' fragment E. The migration distances of the subunits in (f) and (g) should not be compared to those in (a–e) since they represent a separate electrophoretic run. Numbers (1, 2 etc.) are used to identify components other than the polypeptide chains of the intact fibrinogen molecule (α (A) β (B) and γ) and the E subunits (E_S). The nomenclature used is identical to that used in fig. 2.

measurements and thus the gel electrophoretic heterogeneity of D [12] is not considered since the various D fragments have been shown to have about the same molecular weight [13]. The core fragments, D and E, were isolated from the complete digest (15 hr) of fibrinogen by DEAE chromatography [4] and fragment D was further purified from traces of core fragment E by Sephadex G-200 chromatography [13].

The sodium dodecyl sulphate (SDS) gel electrophoretic technique used was a modification of the method of Shapiro, Vinuela and Maizel [14] as described by Kerr and Martin [15] using 7% polyacrylamide gels. Samples (10–50 μg per gel) were dissolved in 0.01 M phosphate buffer (pH 7.5), 1.0% SDS, 0.1 M β-mercaptoethanol and heated at 95° for 10 min. Before loading on SDS-acrylamide gels the samples were diluted with distilled water to 0.1% SDS. A linear relationship was established between the logarithm of molecular weight and the migration distance of known protein markers. The marker proteins used were pyruvate kinase (molecular weight 57,000), lactic dehydrogenase (molecular weight 33,500), chymotrypsinogen (molecular weight 25,700) and lysozyme (molecular weight 14,300).

3. Results and discussion

Fig. 1 shows the SDS-acrylamide gel patterns of SDS, β-mercaptoethanol treated fibrinogen and some of its plasmin degradation products released at various times during the digestion process.

Molecular weights of 67,000, 58,000 and 47,000 were estimated for the α (A), β (B) and γ polypeptide chains respectively in the intact fibrinogen molecule

and differ from those reported elsewhere [16]. The 2 min digest (fig. 1b) shows the absence of intact α (A) chains with the production of a new fragment [4] (molecular weight 31,600). The minor components between 4 and the γ-chains (fig. 1b) may be intermediate degradation products of α (A) chains. A recent report [17] describing the subunit nature of thrombin clottable protein in human fibrinogen–plasmin digests suggests that there are two highly labile α (A) chains in fibrinogen though little information is given concerning the degree of plasminolysis or the nature of the plasmin induced fragments of the α (A) chain complex. The longest surviving intact chain is γ (fig. 1d) which seems to be degraded to a new fragment [3] of molecular weight 32,700. By inference the β (B) chain is the second polypeptide chain in the intact fibrinogen molecule to be attacked by plasmin and densitometric examination of the acrylamide gels shown in fig. 1b to d supports this inference. In fig. 1c the loss of the β (B) polypeptide chains coincides with the appearance of component 1 (molecular weight 41,300) which degrades to component 2 (molecular weight 37,000) (fig. 1d and e); at the same time component 4 reduces to a final molecular weight of 12,000 (component 6).

The final core fragments, D and E, of fibrinogen digestion with plasmin are of necessity represented in their subunit form in fig. 1e. Other low molecular weight peptides are not seen because they are not stained with Amido-Black. Fig. 1f and g depict the SDS-acrylamide patterns of the subunits of isolated core fragments D and E respectively and their addition should yield a subunit pattern as shown in fig. 1e for the complete digest and this is obviously not so. The subunits of fragment E stain very poorly and required 100 μg to supply the pattern shown in fig. 1g and thus would not be shown in the pattern of the subunits of the complete digest in fig. 1e. Fragment E after reduction at room temperature comprised a single component of molecular weight 44,500 while following heating (95° × 10 min) and reduction it was converted to a diffuse component (E_s) of molecular weight about 14,500. The pattern in fig. 1g could be interpreted as three poorly resolved components with a molecular weight range of 12,000–18,000, rather than a two component system as has been suggested [18]. The relatively inaccessible interchain disulphide bridges of fragment E could explain

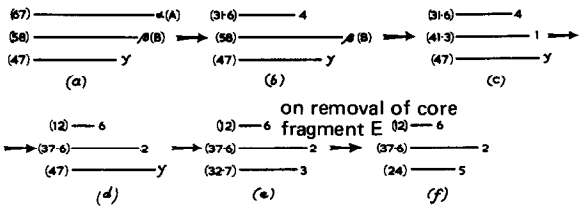

Fig. 2. Proposed sequence of the plasmin degradation of polypeptide chains in the intact fibrinogen molecule. (a) fibrinogen; fibrinogen digested with plasmin for (b) 2 min (c) 10 min (d) 30 min (e) 15 hr; (f) fragment D isolated from 15 hr digest of fibrinogen. Molecular weights (× 10^{-3}) are in parenthesis to the left of each subunit which is denoted by a straight line the length of which is approximately proportional to the molecular weight. To the right of each subunit is an identifying letter or number similar to those used in fig. 1.

the contention [19] that the disulphide bridges in fibrinogen are mainly intrachain. In comparing fig. 1e and f it would seem that the separation of fragment E from fragment D results in a reduction in the molecular weight of one of its subunits from 32,700 (component 3) to 24,000 (component 5). Indeed it has been found that following the removal of core fragment E, the further hydrolysis of subunit 3 to subunit 5 is accelerated by the addition of plasmin or by freezing and thawing. The complete disappearance of fragment 3 occurred after further digestion. Since molecular weights of 68,000 [20] to 83,000 [4] have been reported for unreduced fragment D it seems that this fragment in the whole digest is made up of three disulphide bonded polypeptide chains (components 2, 3, 6 in fig. 1e; molecular weights 37,600, 32,700, and 12,000 respectively) having a total molecular weight of 82,300. After isolation this fragment also contains three disulphide bonded chains (components 2, 5, 6 in fig. 1f; molecular weights 37,600, 24,000 and 12,000 respectively) and has a molecular weight of 73,600. This supports the suggestion [20] that core fragments D and E may be linked by non-covalent bonds since fragment E seemss to inhibit the degradation of one of the chains of fragment D.

These results suggest a sequence of digestion of the polypeptide chains in intact fibrinogen as shown in fig. 2. The molecular weight estimates for the α (A), β (B) and γ chains (as shown in parenthesis in

fig. 2a) suggest a molecular weight for intact fibrinogen of 344,000 assuming a dimeric structure for the molecule [7]. Component 4 (fig. 2b) appears to be the precursor of the low molecular weight fraction (12,000; 6, fig. 2d) and both, together with the 12,000 molecular weight subunit of isolated fragment D (fig. 2f) appear to arise from α (A) chains. Similarly it can be argued that the 37,600 and 24,000 molecular weight fragments of D (fig. 2f) are sections of the β (B) and γ chains respectively. These stable subfragments of D may prove useful for amino acid sequence studies of fibrinogen and for elucidating the inherent heterogeneity of the molecule [9].

The fact that fibrinogen following limited plasminolysis can be clotted with thrombin [21] suggests that γ chains are vital for the end-to-end polymerisation step in the coagulation process. The high degree of interspecies homology of the *N*-terminal region of the γ chain as against that of the α (A) and β (B) chains [22] supports this contention. The susceptibility of the chains to plasmin attack suggests that the lysyl and arginyl residues of the γ chains are initially inaccessible whereas those of the α (A) chains and to a lesser extent those of the β (B) chains, are easily hydrolysed which suggests perhaps that the α (A) chains are located on the outside of the molecule and the β (B) and γ chains on the inside; however, such conclusions require more rigid evidence such as might be supplied by X-ray diffraction analyses in conjunction with sequence analyses.

Concluding on a practical note, fibrin clot lysis methodology [23–25] which is being used to assay clinically used thrombolytic agents such as streptokinase and urokinase should be viewed with caution in view of the above described rapid modification of the α (A) chain of fibrinogen in a fibrinolytic environment.

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