IDENTIFICATION OF A LARGE FRAGMENT AFTER LIMITED CYANOGEN BROMIDE CLEAVAGE OF FIBRINOGEN

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1. Introduction

The disulfide regions of the fibrinogen molecule are becoming increasingly characterized by the aid of fragments obtained upon cyanogen bromide (CNBr) or plasmin cleavage [1–10]. An interesting relation between two distinct CNBr peptides (called F-CB1 and F-CB2 in case of bovine fibrinogen, [ref. 4]) and plasmic fragments E and D, e.g. final products of the proteolytic treatment, could be substantiated by chemical and immunological evidence [2, 3, 6, 7, 9]. Initial stages of plasmin digestion give rise to large fragments such as fragment X being composed of two fragments D and one fragment E [5–8, 10]. In the present study a similar large fragment was obtained after limited CNBr cleavage in dilute hydrochloric acid.

2. Experimental

Lyophilized bovine fibrinogen was prepared by standard procedure [see 11]. One gram was dissolved in 100 ml dilute hydrochloric acid of pH 1.5. The solution was flushed with nitrogen and after addition of 3.2 g CNBr incubated for 4 hr at 30°C. After dilution with 3–4 vol distilled water the reagents were then removed by lyophilization. Complete CNBr cleavage in 70% formic acid as well as separation of the peptides on Sephadex G-100 followed an earlier described procedure [4]. Reduction with dithioerythritol and alkylation with iodoacetic acid was carried out in 8 M urea [11]. Thrombin treatment for 1 hr [1] was followed by separation of fibrinopeptides from the core protein on Bio-Gel P-10 [4].

Molecular weight estimates were obtained by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) [12]. The markers used for calibration including native fibrinogen have been described earlier [4]. Amino acid analysis was performed as reported previously [4, 11].

Rabbits were immunized with 0.5 mg of respective CNBr peptide dissolved in 0.5 ml 0.2 M ammonium bicarbonate pH 8.5 which was mixed with an equal volume of Freund's complete adjuvant and injected into several intracutaneous sites of the dorsal skin. A booster injection of 0.5 mg antigen without adjuvant was given intraperitoneally 4 weeks later and blood was collected 3 weeks thereafter. For serologic studies gel diffusion in agarose and passive hemagglutination [cf. 11] were employed. In the hemagglutination-inhibition assay the antibody solutions were diluted to 8 agglutinating units [see 13]. The minimal amount of inhibitor required to completely block this constant amount of antibody was determined.

3. Results

Cyanogen bromide cleavage of bovine fibrinogen in dilute hydrochloric acid converted only 77% of methionine to homoserine as opposed to a value of more than 95% found after cleavage in 70% formic acid [4]. This difference is also reflected by a different pattern observed in SDS-polyacrylamide gel electrophoresis (fig. 1). A strong band was unique in the incompletely cleaved material which hardly penetrates 7.5% gels (peptide F-CB large)*. Peptides F-CB1 and F-CB2 could not be demonstrated but are found in the complete digest. In both digests a band

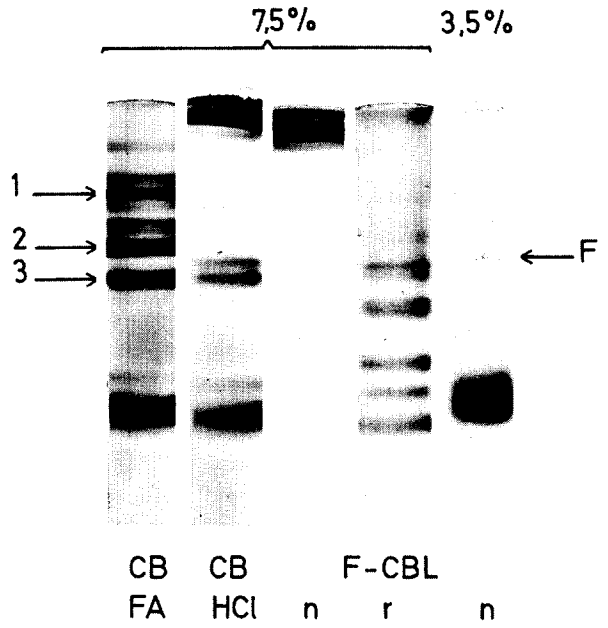

Fig. 1. SDS–polyacrylamide gel electrophoresis of fibrinogen fragments obtained by CNBr cleavage (CB) either in formic acid (FA) or in hydrochloric acid (HCl), and of isolated F-CB large (F-CBL) (fig. 1, pool 1) in its native (n) or reduced (r) state. 7.5% gels were employed except for the pattern at the right (3.5% gel). On the left hand side the position of distinct CNBr peptides (7.5% gel) is indicated by respective numerals; on the right hand side the position of native fibrinogen is given (3.5% gel).

comprising peptide F-CB3 could be detected although it is replaced by a double band (tentatively called F-CB3*) in the incompletely cleaved fibrinogen.

Separation of the large components of the incomplete digest could be achieved on Sephadex G-100 (fig. 2). F-CB large which appeared in the void volume could be obtained in rather pure form upon rechromatography (see fig. 1). The second peak contained essentially F-CB3* which was purified by the same procedure. Amino acid analysis (table 1) revealed striking differences between F-CB large and fibrinogen especially in cystine content and a close resemblance between F-CB3* and F-CB3.

* A nomenclature using numerals has been suggested for disulfide-containing CNBr peptides of fibrinogen [4]. The designation F-CB large is to be considered tentative and may be replaced by numerals upon complete knowledge of the genuine constituents.

Table 1
Amino acid composition of bovine fibrinogen and respective CNBr peptides[a].

	Fribrinogen	F-CB large	F-CB3	F-CB3*
	moles/1000 mole			
Half cystine	17	24	6.3	5.3
Aspartic acid	124	136	107	107
Threonine	75	65	107	108
Serine	76	74	131	143
Homoserine	–	8.2	4.0	5.3
Glutamic acid	120	131	79	89
Proline	52	49	81	76
Glycine	99	79	155	146
Alanine	44	40	46	44
Valine	52	50	43	35
Methionine	17	6.1[b]	–	4.2
Isoleucine	45	54	17	16
Leucine	65	73	29	32
Tyrosine	36	37	5.7	7.7
Phenylalanine	32	31	47	46
Histidine	17	13	33	33
Lysine	73	71	49	47
Arginine	56	59	60	56

[a] The values are rounded off to the nearest integer if more than 10 residues are found. A dash denotes less than one residue. Tryptophan was not determined.

[b] Mostly as methionine sulfoxide or methionine sulfone.

The molecular weight of F-CB large was estimated as 203 000 ± 11 000. Upon reduction and alkylation 5 main bands appeared on electrophoresis (fig. 1) which range in mol. wt from 7000 to 35 000. Their sum accounts for a mol. wt of 98 000. Thrombin cleavage of F-CB large revealed 2.3 moles of fibrinopeptides A and B, respectively, in 1 mole of peptide as judged from quantitative amino acid analysis.

A second CNBr cleavage of F-CB large performed in 70% formic acid was without success. This result could not be improved if the first cleavage in hydrochloric acid was carried out entirely under nitrogen, possibly because a large part of the residual methionine in F-CB large appears to be oxidized (table 1). Evidence for the involvement of F-CB1 and F-CB2 in F-CB large was therefore provided by immunological aids. In gel diffusion employing antisera to F-CB1 or F-CB2 complete identity was found between the corresponding peptides, and F-CB large respectively (fig. 3). Antisera to F-CB3 gave only a faint reaction with F-CB large but demonstrated that F-CB3 and

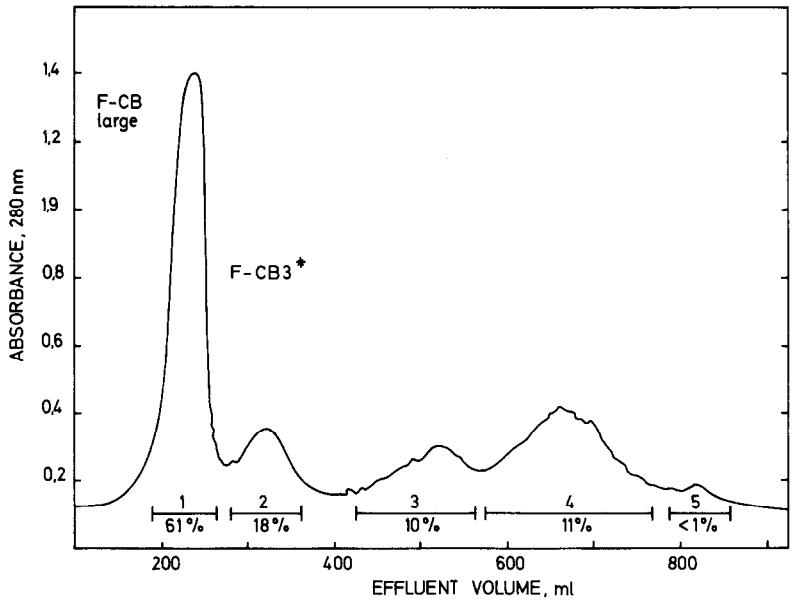

Fig. 2. Chromatographic separation of CNBr peptides (hydrochloric acid) on Sephadex G-100 equilibrated with ammonium formiate pH 2.5. On the basis the pools used are indicated, together with the relative peptide content in % of total recovery as determined by quantitative amino acid analysis after acid hydrolysis.

F-CB3* are serologically identical. Since fibrinogen also showed a line of identity with F-CB3, the reduced reaction with F-CB large could not be due to uncovering of hidden antigenic determinants on F-CB3.

The low activity of F-CB large for antibodies to F-CB3 was reassessed by semiquantitative hemagglutination-inhibition. Thus, on a molar basis it could be estimated that F-CB large contains less than 5% F-CB3.

4. Discussion

Since CNBr cleavage of model peptides does not depend very critically on pH, incomplete cleavage in dilute acids suggested unfolding of the proteins as the crucial step in this kind of degradation [14–16]. This explanation might be applied to the present findings. Thus it may be concluded that F-CB large is mainly composed of F-CB1 which corresponds to the

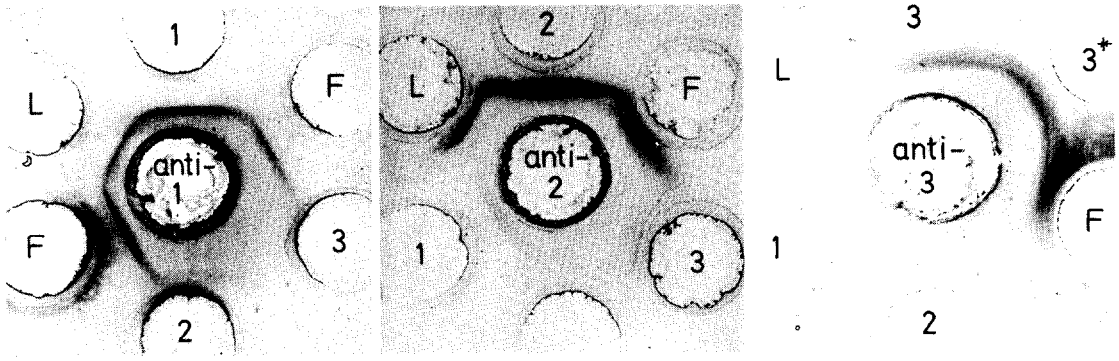

Fig. 3. Comparison by gel diffusion of F-CB large (L) and F-CB3* (3*) with fibrinogen (F), F-CB1 (1), F-CB2 (2) and F-CB3 (3) employing antisera to F-CB1 (anti-1), F-CB2 (anti-2) and F-CB3 (anti-3).

N-terminal disulfide knot of Blombäck et al. [1], and F-CB2, peptides which have been identified recently in the complete digest [4]. This interpretation is supported by the liberation of stoichiometric amounts of fibrinopeptides A and B from F-CB large as well as by the serologic results.

Reduction and alkylation of F-CB large revealed 5 chain fragments which just account for half of the original molecular weight. This suggests a symmetric structure of the fragment in the manner F-CB2/F-CB1/F-CB2, a composition which should have a molecular weight of 182 000 [4]. It cannot be decided as yet if further moieties are involved and which kind of chain (α, β or γ) is responsible for the link between F-CB1 and F-CB2. Recent unpublished results indicate that the yield of the fourth disulfide-containing CNBr peptide F-CB4 being composed of about 50 amino acid residues is also strikingly reduced after incomplete CNBr cleavage. The presence of microheterogeneity of F-CB large which might be caused by a random process of incomplete methionine cleavage remains still to be elucidated.

A single chain peptide F-CB3 of molecular weight 35 000 which is derived from the α-chain and contains one disulfide bridge [4] could be released even by the incomplete cleavage procedure: recovery from the Sephadex G-100 column (fig. 2) indicated a molar yield in the order of 90%. F-CB3* occurs as a double band probably caused by incomplete cleavage at a single point which has already been observed in CNBr cleavage of partially oxidized α-chains [11]. The easy liberation suggests a preferential localization of F-CB3 on the surface of the molecule quite in accordance with the observation of a high reactivity of α-chains with anti-ribrinogen antibodies [11]. A similar high activity has recently been demonstrated for F-CB3 itself (unpublished).

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