CYANOGEN BROMIDE CLEAVAGE OF BOVINE FIBRINOGEN. IDENTIFICATION OF A DIMERIC N-TERMINAL PEPTIDE AND TWO OTHER DISULFIDE CONTAINING FRAGMENTS

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1. Introduction

Considerable efforts have been made in the last ten years to characterize the individual polypeptide chains of fibrinogen obtained upon cleavage of disulfide bonds (reviewed in [1]). Other chemical methods like cyanogen bromide (CNBr) treatment were employed far less frequently. Such an approach led to the discovery of a N-terminal disulfide knot in human fibrinogen by Blombäck et al. [2]. The amino acid sequence of the three constituent chains was elucidated recently [3–5]. The disulfide content of this fragment, although very high, does not account for all the cysteine residues in the protein and suggests the existence of other large fragments in CNBr digests of fibrinogen which might have also a multiple chain structure. Several large CNBr peptides were at least indicated by studies on isolated polypeptide chains [6]. The interest in characterizing such fragments originates from our previous findings [6] that a considerable number of the antigenic determinants of fibrinogen require an intact disulfide structure. For their better characterization respective CNBr peptides obtained from non-reduced bovine fibrinogen were isolated in the present study.

2. Experimental

Bovine fibrinogen was purified by established procedures [7, 8] and was finally lyophilized [6]. About 1 g was dissolved in 100 ml 70% formic acid, flushed with nitrogen and after addition of 3.2 g CNBr (sublimated before use) incubated for 4 hr at 30°. The reaction was stopped by addition of 3–4 vol of distilled water followed by lyophilization. The peptide mixture was separated at 4° on a column of Sephadex G-100 equilibrated with ammonium formate pH 2.5 (38 ml formic acid and 2.5 ml 33% aqueous ammonia diluted to 5 ℓ). In the experiments with Bio-Gel P-150 0.2 M ammonium bicarbonate pH 8.5 was used as eluant. Ion-exchange chromatography was carried out at room temp. in the presence of 8 M or 6 M urea which was deionized prior to use [6]. For CM-cellulose a concave gradient (300 × 260 ml) from 0.005 to 0.1 M sodium acetate pH 5.3 was employed. The phosphocellulose columns were equilibrated in 0.001 M sodium acetate pH 3.6 and were eluted with a concave gradient from 0 to 0.6 M NaCl. The peptides were directly lyophilized if chromatographed in a volatile buffer or desalted on Bio-Gel P-2 columns equilibrated in 0.1 M formic acid.

The amino acid composition was determined as described [6]. This method was used to quantitate the peptide content in the fractions obtained upon molecular sieve chromatography. For homoserine analyses the hydrolysates were treated with 1% aqueous piperidine (1 hr, 37°) to open the lactone ring. Half cystine was determined after performic acid oxidation to cysteic acid [9].

Reduction with 0.02 M dithioerythritol in 8 M urea and alkylation with sodium iodoacetate followed a previously described procedure [6]. The reagents were removed on Bio-Gel P-2. Thrombin treatment to demonstrate fibrinopeptides was performed as recommended [2] except that for chromatography Bio-Gel P-10 was used.

Polyacrylamide gel electrophoresis was carried out in the presence of sodium dodecylsulfate (SDS) omitting reducing agents [10]. For molecular weight deter-

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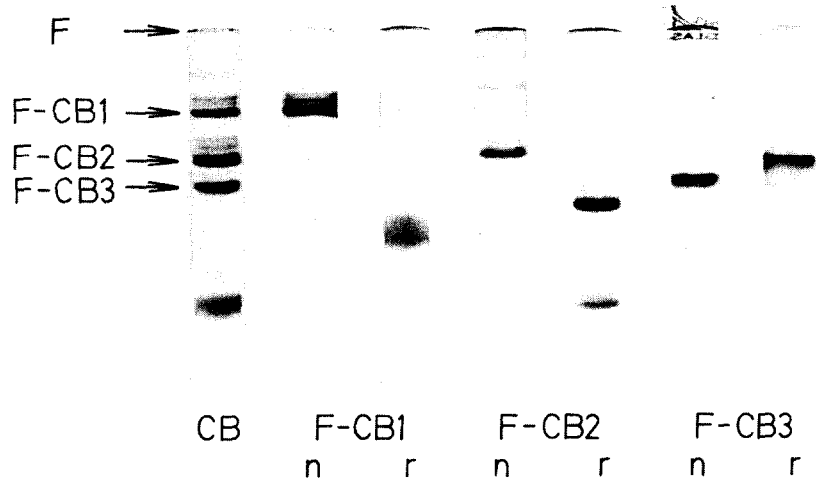

Fig. 1. SDS-polyacrylamide gel electrophoresis of CNBr peptides of bovine fibrinogen (CB) and of purified components (F-CB1, F-CB2 and F-CB3), either in their native (n) or reduced and alkylated (r) state. F denotes the positions of untreated fibrinogen. 7.5% gels were employed.

minations immunoglobulin G, serum albumin, ovalbumin, chymotrypsinogen and myoglobin served as calibrating substances either native or after reduction and alkylation in relation to the state of the peptide investigated. To account for possible anomalous behaviour [11] 7.5% and 5% gels were employed in parallel experiments. Both, however, gave essentially the same results. It should also be considered that molecular weight estimates on non-reduced proteins by this method might involve an error up to 20% [12].

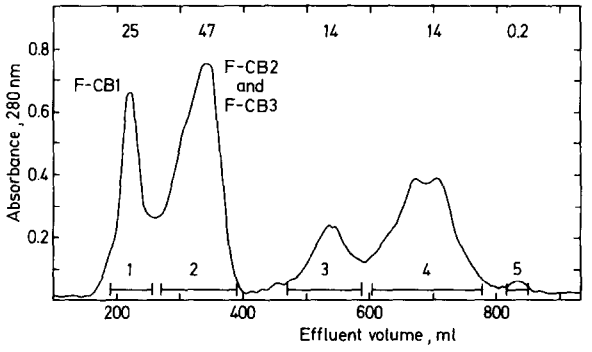

Fig. 2. Separation of 150 mg CNBr peptides of bovine fibrinogen on Sephadex G-100 in ammonium formate pH 2.5. On the baseline of the diagram the pools subsequently used are indicated. At the top the yield of peptide in the single pools is given in percent. The overall recovery was 95%. Column size 3.2 × 85 cm, flow rate 15 ml/hr.

3. Results

Cleavage with CNBr in 70% formic acid converted more than 95% of methionine in bovine fibrinogen (19 residues per 1000) into homoserine. Polyacrylamide gel electrophoresis of the digest (fig. 1) revealed four prominent bands with higher mobility than untreated fibrinogen. Three bands contained rather homogeneous peptides and were designated as F-CB1, F-CB2 and F-CB3 in the order of increasing mobility*. Partial separation was achieved on Sephadex G-100 (fig. 2). The first peak contained essentially F-CB1, the other two peptides were found in the second peak.

The peptide F-CB1 was further purified by rechromatography under identical conditions. In electrophoresis a main component accompanied by two weaker bands of slightly slower mobility was observed (fig. 1). A molecular weight of 84,000 was estimated for the prominent band and a high cystine and homoserine content could be demonstrated for the entire material

* The nomenclature employed is analogous to that used for CNBr peptides of collagen [13]. F denotes the origin from entire fibrinogen and may be replaced by α, β or γ if fragments from individual chains are characterized. The numbers assigned to the peptides followed their order upon chromatography and do not necessarily correspond to the order in the molecule.

Table 1
Chemical properties and molecular weight of the large CNBr peptides of bovine fibrinogen.

Peptide	Amino acid composition (moles/1000 mole)		Molecular weight (1×10^{-3}) ± S.D.		Proposed minimal chain number
	Half cystine	Homoserine	Native	Reduced and alkylated	
F-CB1	32	12	84.3 ± 2.8	18.5 ± 0.9	6
F-CB2	28	10	49.4 ± 3.4	27.5 ± 1.6 11.9 ± 0.5	3
F-CB3	6	4	37.9 ± 2.2	35.1 ± 1.5	1

(table 1). A broad heterogeneous peak was obtained by chromatography on CM-cellulose in the presence of 8 M urea. Although the two contaminants could not be removed by this procedure, they appeared more concentrated in the rear portion of the peak. Amino acid analyses performed on different subfractions of the peak did not reveal any significant differences. It is therefore suggested that the peptides in the additional bands contain F-CB1 of longer size owing to some uncleaved methionine residues, a not unusual finding for CNBr peptides of proteins (cf. [13, 14]).

The occurrence of aminoterminal sequences in F-CB1 was studied by thrombin digestion [2]. Subsequent chromatography on Bio-Gel P-10 revealed two peaks. A large peptide appeared with the void volume exhibiting properties not very different from that of the starting material except for a slight decrease in size. The second peak exactly resembled in amino acid composition an equimolar mixture of fibrinopeptide A and B (see [15]) and was recovered in a yield of 2.2 moles per mole F-CB1. More extensive degradation of F-CB1 after reduction and alkylation was revealed by electrophoresis (fig. 1) and demonstrated a single broad band of molecular weight 18,000. However, three fragments were observed on phosphocellulose (fig. 3) which differed considerably from each other in amino acid composition. The electrophoretic behaviour of the component in the first peak, though not identified in the entire mixture, suggested a molecular weight below 10,000. Since amino acid analysis indicated a minimal size of 52 residues, it might resemble the N-terminal α-chain fragment of human fibrinogen [5]. The peptides in the last two peaks had molecular weights of about 18,000.

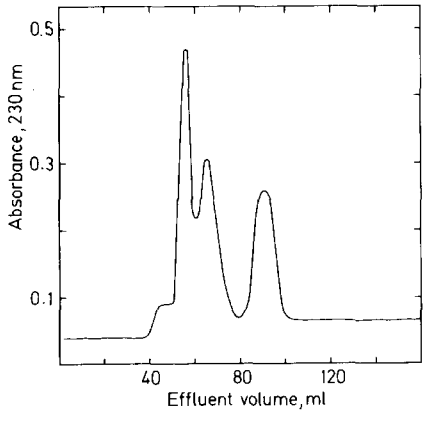

Fig. 3. Chromatography of reduced and alkylated F-CB1 on phosphocellulose equilibrated in 0.001 M sodium acetate pH 3.6, 6 M urea. Elution was performed with a concave gradient (100/100 ml) from 0–0.6 M NaCl.

Separation of F-CB2 from F-CB3 was achieved on Bio-Gel P-150 (fig. 4), and revealed quite distinct differences in homoserine and cystine content (table 1). Calibration of the column with proteins of known size clearly indicated that F-CB2 (molecular weight 49,000, table 1) emerged near the void volume and must be considered as aggregated. Part of this aggregated material obviously became stabilized by disulfide bonds (perhaps generated by disulfide exchange) since a SDS-resistant, slower moving band still remained on electrophoresis (fig. 1). The peptide F-CB3 appeared essentially pure in electrophoresis but three incompletely resolved peaks were observed on CM-cellulose having an identical amino acid composition.

Reduction and alkylation of F-CB2 (molecular weight 49,000) yielded two electrophoretic bands (fig. 1) with a size of 27,000 and 12,000 daltons, respectively. A third, smaller component was suggested by phosphocellulose chromatography (not shown) and amino acid analysis. Reduced and alkylated F-CB3

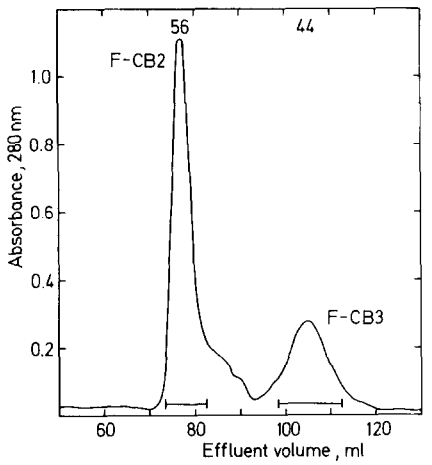

Fig. 4. Separation of F-CB2 and F-CB3 (20 mg of pool 2, fig. 2) on Bio-Gel P-150 equilibrated with 0.2 M ammonium bicarbonate pH 8.5. At the top the relative peptide content of the two pools is given in %. Column size 1.5 × 128 cm, flow rate 6 ml/hr.

revealed still a single electrophoretic band but a reduction in molecular weight of about 7% (table 1). This difference must be considered as within the limits of analytical error. Thrombin digestion of a mixture of F-CB2 and F-CB3 provided no evidence for the occurrence of fibrinopeptides (yield less than 0.05 moles).

The stoichiometric proportion between the three CNBr peptides was calculated from the amino acid content in the peaks obtained after molecular sieve chromatography (figs. 2 and 4). Assuming the molecular weights given in table 1, the peptides F-CB1, F-CB2 and F-CB3 occur approx. in the molar proportion 1:2:2.

4. Discussion

The three large, disulfide-containing CNBr peptides characterized account for about 75% of the total mass of the fibrinogen molecule. They appeared rather homogeneous by the criteria of electrophoresis and molecular sieve chromatography. Ion-exchange chromatography revealed heterogeneity not reflected by significant differences in amino acid composition. A similar microheterogeneity has already been reported for CNBr peptides of collagen [13, 16] and is probably caused by limited deamidation occurring under the acidic conditions of the CNBr reaction. More concise data on this topic as well as on the properties of subfragments will be given elsewhere [17].

Only the largest peptide F-CB1 contained the N-terminally located fibrinopeptides. It should therefore correspond to the disulfide knot derived from human fibrinogen [2]. Accordingly, three different chains are demonstrated after reduction which, however, just account for half of the molecular weight of the nonreduced peptide. Although final evidence is still lacking the assumption of two identical pairs of three chains is indicated by the stoichiometric yield of fibrinopeptides and supported by the model of a symmetric chain structure of fibrinogen [1, 2]. One should therefore suspect that F-CB1 not only contains disulfide bridges between the different chains but also that region joining the identical halves of the fibrinogen molecule. Similar proposals have been made recently by Blombäck [4] for the corresponding peptide of human fibrinogen. The lower molecular weight of 60,000 found for that peptide (cited in [4]) probably reflects the higher methionine content in human fibrinogen.

A structure involving also three different chains is suggested for the as yet unknown peptide F-CB2. Since the sum of the molecular weights of the constituent chains approaches the size of the nonreduced peptide, a monomeric assembly is indicated (table 1). This is in agreement with the two times higher molar yield if compared with F-CB1. The involvement of fragments from the β- as well as the γ-chains can be supposed from the size patterns found for CNBr peptides of the reduced chains of bovine fibrinogen [6]. In digests of human fibrinogen by plasmin a presumably three-chain fragment D is found [12, 18, 19] which might be related to F-CB2. A similar relationship between the six chain disulfide knot and plasmic fragment E has already been substantiated more definitely [12, 20, 21]. The third CNBr peptide F-CB3 is probably composed of a single chain although it contains one disulfide bridge. Correlation with the CNBr peptide patterns of the individual fibrinogen chains [6] precludes any origin other than from the α-chain. By chemical evidence both peptides must be derived from the central portion of the fibrinogen molecule.

The homoserine content of the peptides F-CB1 and F-CB3 is somewhat higher than expected from the pro-

posed chain structure. At present the occurrence of small additional fragments joined to these CNBr peptides via disulfide bridges cannot be excluded. Such moieties which obviously did not comprise the three chain model of fibrinogen may arise by cleavage of methionine located in intrachainary loops as observed for example in CNBr cleaved serum albumin [13]. A single chain peptide F-CB4 containing one disulfide bond in a sequence of about 50 amino acids was recently identified in a subfraction of pool 4 (see fig. 2; unpublished). Screening of all the other peptide fractions revealed no or only a negligible content of cystine. It is, therefore, likely that these four CNBr peptides account for all the disulfide bridges of bovine fibrinogen. They should serve as an appropriate starting material for elucidating the entire pattern of intramolecular cross-links, and its relation to distinct antigenic specificities.

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