ISOLATION AND IMMUNOLOGICAL CHARACTERIZATION OF A DISULFIDE LOOP REGION IN HUMAN FIBRINOGEN α CHAIN

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1. Introduction

A large portion of the α chain could be isolated from cyanogen bromide (CNBr) cleaved bovine fibrinogen in the peptide F-CB3 which contains a single disulfide bridge [1]. Biochemical [2,3] and immunological [4,5] evidence indicated that large parts of the F-CB3 moiety should be located on the surface of the intact fibrinogen molecule. This agrees with previous observations (reviewed in [6]) on a particular lability of the α chain during degradation of fibrinogen by plasmin. Characterization of structural and immunological properties of F-CB3 may therefore aid in a better understanding of the process of fibrinolysis.

The present study describes the isolation of peptide F-CB3 from human fibrinogen which is similar in structure but not in antigenicity to the previously reported bovine counterpart. A radioimmune assay and other serological methods demonstrated lack of cross-reaction with fragments D and E known to be the stable end products of plasmic degradation.

2. Experimental

Human fibrinogen was obtained from Kabi AB, Stockholm, and purified to 97% clottability by established procedures [7,8]. Cleavage by CNBr and purification of peptide F-CB3 followed essentially the methods described for bovine fibrinogen [1]. Reduction and alkylation was carried out as described [9]. The constituent chains of human fibrinogen were isolated by CM-cellulose chromatography [10]. Plasmic fragments D and E and respective rabbit antisera were a gift of Dr H. Karges, Behringwerke, Marburg. Amino acid composition was determined as described previously [9] on a Durrum D 500 analyzer. The tryptophan content was measured on the amino acid analyzer after hydrolysis with methane-sulfonic acid [11]. Polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate (SDS) [12] was used for molecular weight determination utilizing reduced and alkylated globular proteins for calibration [1].

Three rabbits were immunized with human F-CB3 by a schedule used previously for fibrinogen [4]. Gel precipitation and passive hemagglutination was done according to standard procedures and details have been described elsewhere [4,9]. Labeling of human F-CB3 with ^{125}I, titration of antisera in a radioimmune assay and inhibition tests were the same as described for bovine F-CB3 [5] except that the immune complexes were precipitated with 40% saturated ammonium sulfate [13] instead of with a goat antiserum to rabbit IgG. Results were calculated as suggested by Minden and Farr [13].

3. Results

Recently three large CNBr peptides, F-CBl, F-CB2 and F-CB3, could be isolated and characterized from bovine fibrinogen [1]. Comparison of CNBr cleaved human and bovine fibrinogen by SDS disc electrophoresis showed the same three major bands (fig.1) which, however, had a slightly higher mobility in case of the human preparations. Purification of F-CB3 was achieved by initial chromatography on

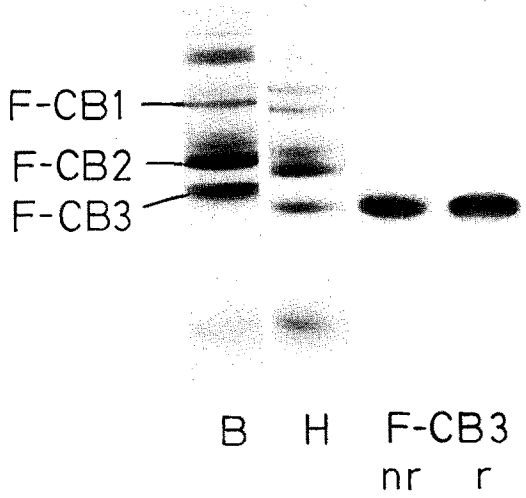

Fig.1. Disc electrophoresis pattern of CNBr digested human (H) and bovine (B) fibrinogen, of the purified human peptide F-CB3 in its native (nr) and reduced (r) form. Positions of individual CNBr peptides from bovine fibrinogen [1] are indicated on the left hand side. Anode at the bottem.

Sephadex G-100 (fig.2). The rear portion of the peak containing F-CB3 and some F-CB2 was rechromatographed on Sephadex G-200 at pH 8.6 [1] or on Bio-Gel P.60 in 0.2 M formic acid. The final preparation of F-CB3 was then pure as judged

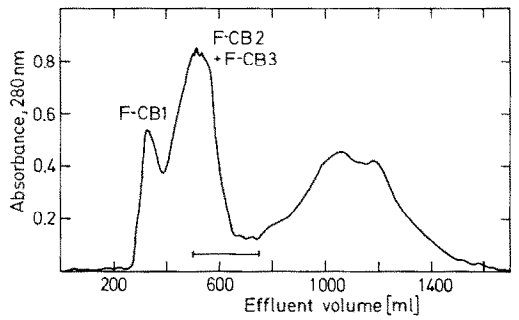

Fig.2. Separation of 450 mg CNBr peptides of human fibrinogen on Sephadex G-100. The column (5 × 76 cm) was eluted with 0.2 M ammonium formate pH 2.5. The pool used for further purification of F-CB3 is indicated on the base line as a horizontal bar.

by electrophoresis (fig.1). The amino acid composition of the peptide, given in table 1, revealed characteristic features found previously for bovine F-CB3 [4] like a high content of glycine, serine and threonine and low tyrosine values. Human F-CB3 also contained two half cystine residues, between one and two homoserine residues and small amounts of methionine.

Electrophoretic properties of F-CB3 were not changed after reduction and alkylation (fig.1). A mol. wt of 29 700 ± 1000 could be determined for reduced F-CB3 by SDS disc electrophoresis. Chromatography of the reduced peptide on Bio-Gel P-10 in 0.2 M ammonium bicarbonate pH 8.6 revealed a single peak, emerging with the void volume which contained more than 99% of the material recovered

Table 1
Amino acid composition of peptide F-CB3 from human fibrinogen [a]

	Native peptide	Reduced peptide
Half cystine	2 (1.6)	2 (2.1)[b]
Aspartic acid	25	23
Threonine	37	34
Serine	56	61
Homoserine	1 (1.5)	1 (1.4)
Glutamic acid	27	28
Proline	20	18
Glycine	49	49
Alanine	8 (7.9)	10
Valine	11	10
Methionine	1 (0.5)	1 (0.8)
Isoleucine	4 (3.8)	4 (3.8)
Leucine	5 (5.4)	6 (5.7)
Tyrosine	3 (3.0)	3 (3.3)
Phenylalanine	3 (2.8)	3 (2.8)
Histidine	5 (4.9)	5 (5.0)
Lysine	12	11
Arginine	15	15
Tryptophan	7 (7.4)	n.d.[c]
Total	291	284

a Given as residues per mole peptide assuming a mol. wt of 29 700. Values are rounded off to the nearest whole number, actual values are given in brackets for less than 10 residues. Values for threonine and serine are corrected by factors 1.12 and 1.25, respectively [9]. Average values from 4 to 6 different preparations.

b As carboxymethyl cysteine.

c Not determined.

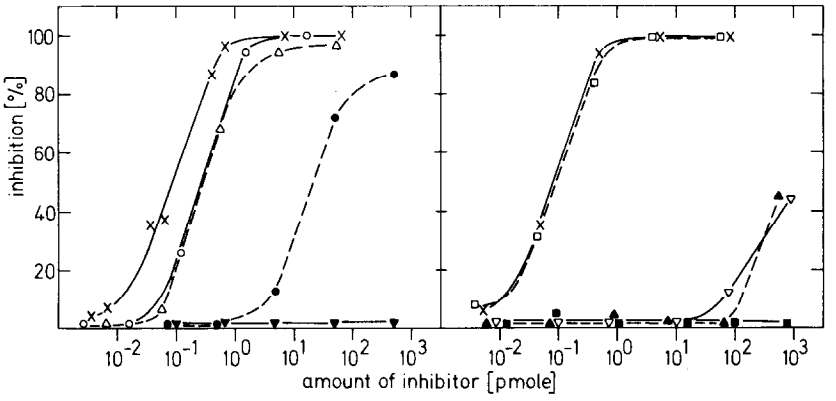

Fig.3. Inhibition of binding of ^{125}I-labeled human F-CB3 to antibodies against F-CB3 by different antigens. Inhibitors used were human F-CB3 (X——X), human fibrinogen (○——○) and its constituent α(△--△), β(●--●) and γ chain (▽——▽), reduced and alkylated human F-CB3 (□--□), human plasmic fragments D (▽——▽) and E (▲--▲) and bovine F-CB3 (■——■).

from the column (data not shown). Amino acid composition of the peptide contained in this peak resembled closely that of native F-CB3 (table 1) also with respect to the cysteine, homoserine and methionine content.

Rabbit antisera to human F-CB3 did strongly bind ^{125}I-labeled human F-CB3 in a manner as was observed for the corresponding bovine system [5]. Inhibition assays which are shown in fig.3 were used to study the relationship to other antigenic fragments. No differences were observed when comparing native and reduced F-CB3. Native fibrinogen and its constituent α chain were also strong inhibitors except that 3-fold higher amounts were required to obtain the same effect as with F-CB3. Even though this difference is apparently close to the experimental error in this assay, it may still reflect a slight change in antigenic structure. The β chain was a 100-fold less potent inhibitor compared to the α chain whereas γ chain did not have any activity. Lack of inhibitory activity was also indicated for plasmin fragments D and E from human fibrinogen and for the bovine peptide F-CB3. Inhibitory effects observed for the β chain and with fragments D and E at very high concentrations are probably due to contamination by traces of α chains and larger plasmic fragments, respectively, which cannot be detected by usual biochemical methods.

The relation between F-CB3 and fragments D and E was also studied by gel precipitation and passive hemagglutination (data not shown). Both tests revealed a strong reaction between rabbit antibodies to fragments D and E and the antigen used for immunization and no or negligible reactions with F-CB3. Antibodies to F-CB3 showed high agglutination titers (range 1:256 to 1:1024) for F-CB3 but no cross reaction was observed with fragments D and E. Only faint reactions were found with F-CB3 in gel diffusion tests.

4. Discussion

Amino acid composition and immunological evidence for an exclusive relation to the α chain indicate that the CNBr peptide from human fibrinogen which has been characterized in the present study is homologous to the previously described peptide F-CB3 from bovine fibrinogen [1,4]. The sum of methionine and homoserine accounts for two residues in human F-CB3. Presumably, the chain contains an uncleaved methionine which may be partly converted to a component giving rise to homoserine upon acid hydrolysis [14]. Support for this interpretation was obtained by reduction experiments which failed to demonstrate a large change in size and the existence of a disulfide-bonded small peptide entity. Thus, human and bovine F-CB3 have basically the same single chain structure which is folded into a loop by one disulfide bridge. However, the

human peptide is smaller by about 6000 daltons.

Lack of cross-reactivity between human and bovine F-CB3 was corroborated by identical findings with antibodies to the bovine peptide [5]. Both antigens are identical with respect to a complete cross-reaction observed for homologous fibrinogen and reduced F-CB3. The weak activity of human F-CB3 in gel precipitation contrasts with a strong reaction in other serological tests and may be due to the existence of just one or a few antigenic determinants on the peptide. The antigenic structure of bovine F-CB3 is apparently more complex [5].

Several immunological methods failed to show any relation between F-CB3 and plasmin fragments D and E. Observations on a localization of fragment D close to the amino-terminal fragment E [15] suggests that the F-CB3 moiety should originate from the C-terminal portion of α chain. In fact, a recent study of Hessel [16] indicated the release of an α chain fragment with a mol. wt of 50 000 in early phases of plasmic digestion. Upon further cleavage with CNBr a fragment was obtained which resembled peptide F-CB3 in size and amino acid composition. Thus, the sensitive radioimmune inhibition assay described for F-CB3 should be useful for characterizing early products of fibrinolysis in human serum under normal and clinical conditions. Since tryptic cleavage of bovine F-CB3 did not abolish serologic activity [5] one may expect applications even in cases where extensive plasmic degradation has occurred.

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