AMINO ACID SEQUENCE OF THE CARBOXY-TERMINAL CYANOGEN BROMIDE FRAGMENT FROM BOVINE AND HUMAN FIBRINOGEN γ-CHAINS

J.J. SHARP*, K.G. CASSMAN and R.F. DOOLITTLE†
Department of Chemistry, University of California, San Diego, La Jolla, California 92037, USA

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1. Introduction

Recently we reported the amino acid sequences of the carboxyterminal eicosapeptides of the γ-chains from human and bovine fibrinogen [1]. These sequences were determined as part of a study on fibrin crosslinking sites, and advantage was taken of the factor XIII-catalyzed introduction of a radioactive substitute donor in the isolation of the peptides [2,3]. We now report the isolation of the corresponding cyanogen bromide fragment and its complete amino acid sequence. Essentially the same strategy was employed as in our previous studies [1–4] except that cyanogen bromide fragmentation was substituted for the tryptic digestion and a Sephadex G-75 gel filtering system employed instead of the Sephadex G-25 column used for the smaller tryptic peptides.

2. Materials and methods

Bovine fibrinogen (Armour fraction I) was further purified by ammonium sulfate fractionation [5]. [^{14}C]glycine ethyl ester (New England Nuclear) was incorporated into γ-chains as described previously [2] by clotting fibrinogen with thrombin in the presence of cysteine and calcium ions, taking advantage of the small amounts of contaminating factor XIII in the purified fibrinogen. Labelled fibrin (or unlabelled fibrinogen in some experiments), was sulfitolyzed and the α-, β-, and γ-chains separated on carboxymethyl cellulose at pH 5.2 in 8 M urea [6]. After dialysis and freeze-drying, the γ-chains were subjected to fragmentation by cyanogen bromide (Eastman) in 70% formic acid, for 5 hr at room temp. [7]. At the end of that time the preparations were diluted with distilled water and freeze-dried before being dissolved in 1 M propionic acid and passed over a G-75 column equilibrated with that solvent.

The radioactive peaks (or, in the case of fibrinogen, the cold equivalents) were pooled, concentrated and purified further by paper electrophoresis. Peptides were analyzed on a Spinco automatic amino acid analyzer, Model 117, employing a single column analysis system, after hydrolysis for varying times with 5.7 N HCl at 110°. Further fragmentation of the cyanogen bromide segment was achieved by digestion with TPCK-trypsin (Worthington) and thermolysin (a gift from Dr. H. Matsubara). Asparagine was distinguished from aspartic acid by analysis of the appropriate thermolysin and tryptic peptides after total enzymatic hydrolysis, as well as by considering the electrophoretic mobilities of these peptides. Pronase was obtained from Cal Biochem and leucine amino peptidase from Worthington.

Stepwise degradations were carried out essentially as described by Hartly [8]. DNA-proline was determined after a 4 hr hydrolysis at 110° and DNS-isoleucine after 24, 48, and 72 hr hydrolysis [9].

* Postdoctoral Fellow of American Heart Association.
† Career Development Awardee of U.S.P.H.S.

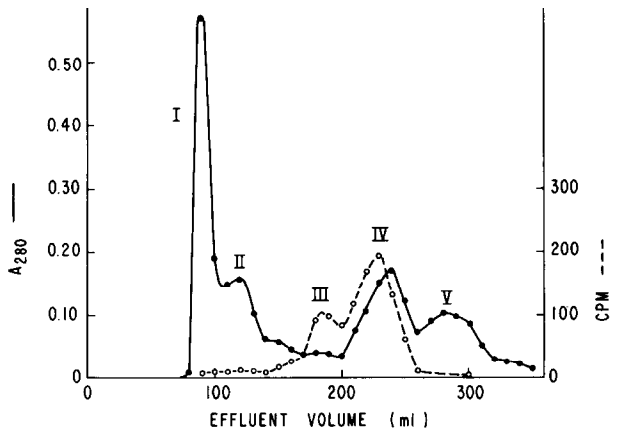

Fig. 1. Elution profile of cyanogen bromide digest of labelled bovine fibrin γ-chains gel filtered through Sephadex G-75 (2.5 × 60 cm) in 1 M propionic acid. Fraction size = 5 ml; 0.2 ml aliquots removed for counting.

3. Results and discussion

Amino acid analysis of bovine fibrinogen and fibrin chains revealed that there were eight methionines per 47,000 mol. wt., indicating that theoretically nine fragments should be produced by cyanogen bromide cleavage. The carboxy-terminal fragment was easily located (Peak IV in fig. 1) since it contained the radioactive substitute crosslinking donor, glycine ethyl ester. We have previously established that the bulk of the incorporated radioactivity is located at a site fourteen residues from the carboxy-terminus [1]. The singly crosslinked dimer comprised of two neighbouring terminal segments was also located (peak III in fig. 1) and characterized.

The amino acid composition of the purified fractions revealed that the terminal cyanogen bromide fragment contains 27 residues. Tests for tryptophan and carbohydrate were negative. Limited digestion with thermolysin split the fragment into two pieces, one identical with the eicosapeptide we had previously characterized [1] from tryptic digests, and a heptapeptide corresponding to the amino-terminal region. Further digestion with this enzyme gave rise to a variety of other peptides whose amino acid compositions were all consistent with this arrangement (fig. 2). Trypsin split the peptide at four places, including removal of the amino-terminal lysine residue and an unexpected cleavage at a leucyl–asparaginyl bond (fig. 2). The sequence of the previously unstudied portion of the peptide was unequivocally established by a series of extended stepwise degradations using the DNS-PITC method. A total of 13 steps were performed, thereby passing through the unknown region and into the previously characterized portion. Amino acid analyses of the corresponding human carboxy-terminal cyanogen bromide fragment (and of the peptides obtained from the thermolysin digest of this peptide) indicate it also contains 27 residues. In addition to the previously established threonine/alanine and histidine/glutamine substitutions [1], the analyses indicate a phenylalanine/leucine substitution in the previously uncharacterized amino-terminal portion of the human cyanogen bromide fragment.

The carboxy-terminal regions of γ-chains from vertebrate fibrinogens are functionally important for fibrin stabilization [1,3,4]. Thus, the terminal segments of neighboring molecules in a fibrin gel are oriented in an anti-parallel fashion and become reciprocally bound by a pair of covalent crosslinks between the lysines at 6c and the glutamines at 14c (fig. 2) (the c indicates numbering from carboxy-terminus). We have put forth the suggestion that these same regions are also involved in the polymerization process before the introduction of the covalent links [10]. It is some interest to compare this segment

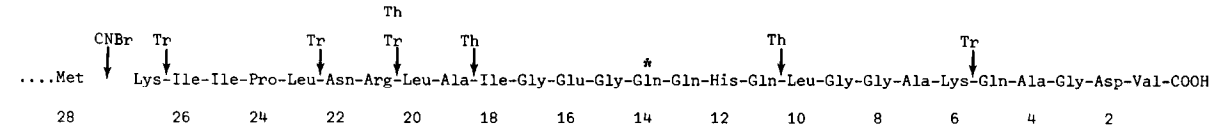

Fig. 2. Amino acid sequence of carboxy-terminal cyanogen bromide fragment from γ-chains of bovine fibrinogen and fibrin. Sites of trypsin and thermolysin cleavage are designated Tr and Th, respectively. In the present study, DNS-PITC degradation steps were carried through 13 successful cycles, crossing the bridge into the previously characterized region comprising the carboxy-terminal eicosapeptide. Numbering is from the carboxy-terminus. *indicates site of incorporation of substitute donor.

Table 1
Amino acid composition of carboxy-terminal cyanogen bromide fragment from γ-chains of bovine fibrinogen (presented as molar ratios).

	Hydrolysis time		
	24 hr	48 hr	72 hr
Aspartic acid	2.1	2.1	2.1
Glutamine acid	5.5	5.4	5.4
Proline	1.0	1.1	1.1
Glycine	5.0	5.0	4.9
Alanine	3.0	3.0	2.9
Valine	1.1	1.0	1.0
Isoleucine	2.4	2.7	2.8
Leucine	3.1	3.0	3.0
Histidine	1.0	0.9	1.0
Lysine	1.9	1.9	1.9
Arginine	0.9	0.9	0.9
Total residues	27.0	27.0	27.0

with the cyanogen bromide carboxy-terminal fragment of calf skin collagen, the structure of which has recently been reported [11], since the peptide is thought to be involved in the crosslinking of collagen. Although there is some vague similarity between the two peptides, there is no real basis for proposing homology. Crosslinking in collagen, of course, although involving lysine sidechains, is fundamentally different from that which occurs in fibrin stabilization [12]. It is a remarkable coincidence, nonetheless, that the carboxy-terminal segments of these two fiber generating proteins should both be the sites of covalent reinforcement.

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