Vol. 84, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 29, 1978 Pages 495-500

SEQUENCE OF AMINO ACIDS COMPRISING THE SINGLE INTRA-CHAIN DISULFIDE LOOP IN THE α-CHAIN OF HUMAN FIBRINOGEN

R. F. Doolittle, B. A. Cottrell, D. Strong and K. W. K. Watt

Department of Chemistry, University of California, San Diego

La Jolla, California 92093

Received August 2,1978

SUMMARY. Amino acid sequence studies have revealed that the largest cyanogen bromide fragment from the α-chain of human fibrinogen contains two cysteine residues which are situated thirty residues apart and near the carboxy-terminal end of that fragment. In contrast to a recent report, no other cysteines exist in this region of the α-chain. The sequence has been compared to disulfide loops in the β- and γ-chains and some very marginal homology suggested.

The α-chains of vertebrate fibrinogens are characteristically the largest of the three non-identical polypeptide chains which comprise these dimeric molecules. In the case of the human fibrinogen molecule, preliminary amino acid sequence studies have indicated that the α-chain consists of 625 ± 20 residues (1). It has been thought that only eight of these are cysteines, six of which exist in the amino-terminal quarter of the chain (1,2). The other two cysteines are located in the largest fragment resulting from cyanogen bromide fragmentation, and several lines of evidence have indicated that they are joined in a single intrachain disulfide loop. For example, brief exposure of native fibrinogen to plasmin results in the release of a large section of the α-chain which includes this same fragment (3,4). Similarly, cyanogen bromide fragmentation of intact fibrinogen (as opposed to isolated α-chains) gives rise to the same fragment also (5).

Recently, however, Fretto et al. (6) have reported that this region of the human fibrinogen α-chain contains two disulfide loops. This finding, if substantiated, would have a significant bearing on the nature of this part of

0006-291X/78/0842-0495$01.00/0

the α-chain, which has a variety of interesting structural and physiological characteristics. Accordingly, we have carefully examined the amino acid sequence of this portion of the cyanogen bromide fragment in question. As in our previous studies, we used α-chains which were labeled on their cysteines by alkylation with [^{14}C] iodoacetic acid. We now report that we have been able to account for all the radioactivity in the fragment in two cysteine residues located 30 residues apart and near the carboxy-terminus of the fragment. Moreover, we have been able to overlap this section of the fragment with the remainder of the carboxy-terminal region, all of which we have sequenced (7).

EXPERIMENTAL

Most of the methodology for this study is reported in detail in our original report on the eleven CNBr[1] fragments of the α-chain (2). The largest of the CNBr fragments (CN-I) served as the starting material for the present investigation. It was further fragmented by treatments with a variety of proteolytic enzymes. All radioactive peptides (i.e., those containing carboxymethyl cysteine) were purified by gel filtration and/or paper electrophoresis. Peptides were analyzed and sequenced by several methods, including thioacetylation stepwise degradation after attachment to glass beads (8) and the Dns-PhNCS procedure (9).

In particular, our strategy began with the digestion of CN-I with plasmin, an operation which we have previously shown splits the fragment into a large non-radioactive piece (PI) and a smaller fragment (PII) containing all the carboxymethylcysteine (2) (Fig. 1). This latter fragment, which consists of 70-90 residues, was subjected to a series of independent digestions with trypsin, staphylococcal protease and chymotrypsin. In each case the radioactivity was carefully monitored and subsequently accounted for in purified peptides containing one of two cysteine residues. As a case in point, the chymotryptic

[1]Abbreviations used: CNBr = cyanogen bromide. Dns-PhNCS = 5-dimethylamino-naphthalene-1-sulfonyl chloride-phenylisothiocyanate.

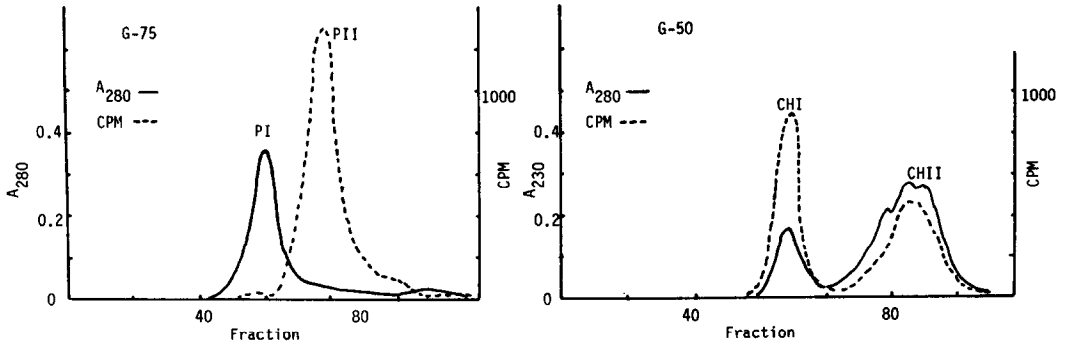

Fig. 1. Gel filtration on Sephadex G-75 (2.5 x 110 cm.) of plasmin-treated cyanogen bromide fragment CN-I (left). The first peak (PI), which contains all the tryptophan and the bulk of the absorbance at λ = 280 nM, has no radioactivity and no evidence of cysteine being present. The second peak (PII) contains all of the radioactivity. The material from pool II was subsequently treated with chymotrypsin and subjected to gel filtration on Sephadex G-50 (2.5 x 90 cm.) (right). Two radioactive peaks were observed. All the radioactivity in peak I was found associated with the carboxy-terminal fragment shown in Fig. 2.

digest was gel filtered on G-50 (Fig. 1, right) and two radioactive peaks obtained, each of which contained half of the total radioactivity. The first peak was a virtually pure 29-residue fragment corresponding to C1 in Fig. 2. Only one cysteine residue was identified in the peptide.

The overlapping nature of the peptides obtained also allowed us to determine the entire amino acid sequence of this region (Fig. 2). The possibility of a repetitive sequence yielding two identical disulfide loops was ruled out on the basis of peptide yields as well as by establishing overlaps with adjacent peptides. As a result of these overlaps, we have tentatively assigned the 38-residue segment shown in Fig. 2 to residues 462-499 of the entire α-chain.

It must be remarked that a number of anomalous cleavages occur in this portion of the molecule which tended to complicate the initial interpretation. Thus, chymotrypsin splits after two different threonine residues (Fig. 2), the second occurring at a relatively slow rate and not going to completion. Similarly, the tryptic peptide containing the cysteine adjacent to a serine (Fig. 2)

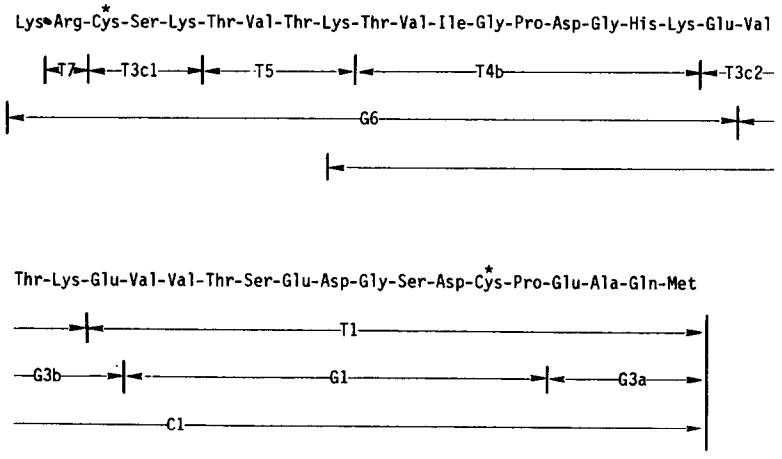

Fig. 2. Summary of data used to determine the sequence of the α-chain region including the single disulfide loop which exists in this chain. T = trypsin; G = staphylococcal protease; C = chymotrypsin. Asterisks (*) indicate cysteine residues labeled with [^{14}C] iodoacetic acid.

appeared in several guises, partly because of the incomplete cleavage of the arginine residue on its amino-terminal side, and partly because of an undetermined extra negative charge associated with the peptide under some circumstances. There is a possibility that some of the serines and threonines in this part of the chain are partially phosphorylated (Jue & Doolittle, unpublished).

Because there is a large body of data indicating that the three non-identical chains of vertebrate fibrinogen have evolved from a common ancestor (10), it was of interest to compare the amino acid sequence in this disulfide loop region with the disulfide loops which exist in the corresponding regions of the β- and γ-chains (Fig. 3). The cysteines in the disulfide loops in the β- and γ-chains are both 13 residues apart, in contrast with the 30-residue spacing in the α-chain. Whereas the β- and γ-chains are strikingly homolgous in this region (11,12), no convincing homology has yet been observed in this part of the α-chain. Nonetheless, as in the extreme carboxy-terminal portions of the chain (13), there is just the slightest hint of resemblance, including a Cys-Ser-Lys sequence in common with the β-chain and the existence of a histidine residue at the same spot in all three chains (Fig. 3). In general,

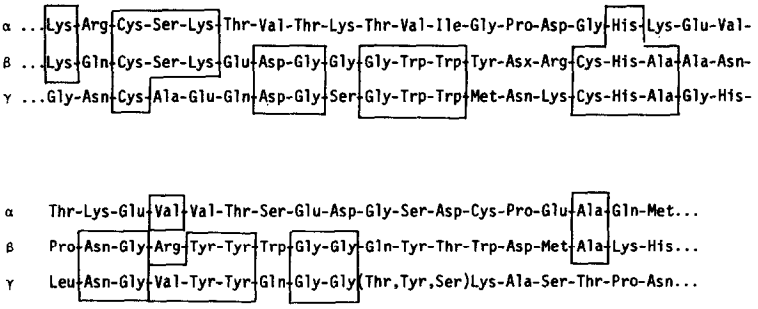

Fig. 3. Comparison of α-, β- and γ-chains in the regions of intra-chain disulfide loops. Note the strong homology between β- and γ-chains (11,12) and the marginal (at best) homology involving the α-chain. The γ-chain section shown corresponds to residues γ-324-361, and the β-chain to residues β-392-429. According to our preliminary numbering system, the α-chain section corresponds to residues 462-499.

however, the α-chain sequence in the disulfide loop exemplifies the uniqueness of this chain in its carboxy-terminal portion.

In summary, the α-chain of human fibrinogen contains eight cysteine residues. As reported previously, six of these are involved in interchain connections in the amino-terminal quarter of the molecule. The other two cysteines are approximately located at positions 464 and 494 and form the single disulfide loop which exists in α-chains. The disulfide loop sequence has just a hint of homology with the sequences observed in the smaller disulfide loops found in the β- and γ-chains.

ACKNOWLEDGMENTS

We thank Marcia Riley and Dennis Trovato for excellent technical assistance in this study. Our work is supported by N.I.H. Grants Nos. HL-18,576 and GM-17,702.

REFERENCES

1. Iwanaga, S., Wallén, P., Gröndahl, M. J., Henschen, A. and Blombäck, B. Eur. J. Biochem., 8:189-199 (1969).
2. Doolittle, R. F., Cassman, K. G., Cottrell, B. A., Friezner, S. J., Hucko, J. T. and Takagi, T., Biochemistry, 16:1703-1709 (1977).

3. Harfenist, E. J. and Canfield, R. E., Biochemistry, 14:4110-4117 (1975).
4. Takagi, T. and Doolittle, R. F., Biochemistry, 14:5149-5156 (1975).
5. Gollwitzer, R. and Timpl, R., FEBS Letters, 58:269-272 (1975).
6. Fretto, L. J., Ferguson, E. W., Steinman, H. M. and McKee, P. A., J. Biol. Chem., 253:2184-2195 (1978).
7. Cottrell, B. A. and Doolittle, R. F., Thromb. Res., In press.
8. Mross, G. A. and Doolittle, R. F., Mol. Biol., Biochem. and Biophys., 25:1-20 (1977).
9. Gray, W., Meth. Enzymol., 25:333-344 (1972).
10. Doolittle, R. F., Federation Proceedings, 35:2145-2149 (1976).
11. Henschen, A. and Lottspeich, F., Thromb. Res., 11:869-880 (1977).
12. Watt, K. W. K., Takagi, T. and Doolittle, R. F., Proc. Natl. Acad. Sci., U.S.A., 75:1731-1735 (1978)
13. Cottrell, B. A. and Doolittle, R. F., Biochem. Biophys. Res. Comm., 71: 754-761 (1976).