ISOLATION AND CHEMICAL CHARACTERIZATION OF REDUCED AND AMINOETHYLATED POLYPEPTIDE CHAINS OF BOVINE FIBRINOGEN

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1. Introduction

Purification of the individual polypeptide chains of the fibrinogen molecule is a task still encountered with some difficulties. Sulfitolysis and separation of the S-sulfo derivatives on CM-cellulose has been initially applied for this purpose [1,2]. Incomplete cleavage and/or reoxidation caused the occurence of polymerized α-chains which is considered as a serious handicap of this procedure [3,4]. Reduction of fibrinogen by thiol reagents followed by alkylation with iodoacetic acid furnished a complete cleavage to the constituent chains [4–6]. Although chromatographic separation of the alkylated chains could be demonstrated on CM-cellulose the presence of probably degraded α-chains caused problems in achieving a good and quantitative separation of α- and β-chains [7]. This seems to be of particular importance for bovine fibrinogen [4]. Yet, the alkylation of methionine residues is a disadvantage of this method especially when it is intended to perform further structural studies. This difficulty may be circumvented by the use of less than stoichiometric amounts of the alkylating agent [6].

Alkylation of cysteine residues in proteins can also be accomplished by ethylene imine [8,9]. Application of this technique to bovine fibrinogen proved to have several advantages in the present study. The polypeptide chains could be essentially purified by two chromatographic steps. Characterization of the chains by their amino acid composition indicated more reliable values than hitherto reported for cysteine and methionine in preparations of bovine origin.

2. Materials and methods

Bovine fibrinogen was purchased from the Behringwerke, Marburg, and was purified and lyophilized as described previously [4]. A 0.5% solution of fibrinogen in 0.08 M Tris–HCl pH 8.6, 8 M urea was flushed with nitrogen prior to reduction with 20 mM dithioerythritol for 4 hr at 37°C. The urea solution was each time deionized before use [4]. After completion of the reduction the cysteine residues were blocked by addition of ethylene imine monomer (Roth, Karlsruhe) in several portions to a final concentration of 200 mM. The solution was stirred for an additional 4 hr at room temperature and the pH maintained at 8 by addition of HCl. Finally, the solution was dialyzed in the cold room against the starting buffer used in DEAE-cellulose chromatography.

About 150 to 200 mg of reduced and aminoethylated fibrinogen was chromatographed at room temperature on a DEAE-cellulose column (2.5 × 6.5 cm) equilibrated with 0.005 M Tris–HCl pH 8.9, 8 M urea. After application of the sample elution was achieved with a concave gradient from 0 to 0.08 M NaCl (550/350 ml) at a flow rate of 60 ml/hr. Appropriate fractions were pooled according to their purity as judged by electrophoresis of individual fractions. The pools were lyophilized after dialysis against dilute aqueous ammonia pH 9.2. If required final desalting was achieved on appropriate Bio-Gel P-2 columns equilibrated in 0.2 M formic acid. A second chromatography was carried out on CM-cellulose in 6 M urea, 0.01 M sodium acetate pH 5.3 applying a

gradient from 0.01 to 0.08 sodium acetate [4]. The recoveries from the DEAE-cellulose and CM-cellulose columns were in the order of 95%.

Polyacrylamide gel electrophoresis (7.5% gels) in the presence of sodium dodecyl sulfate was carried out under non-reducing conditions and followed a procedure described previously [10]. Estimates of molecular weights were obtained after calibration with reduced and alkylated marker proteins as indicated elsewhere [4].

The amino acid composition was determined after hydrolysis with 6 M HCl in the presence of 0.06% mercaptoethanol under an atmosphere of nitrogen for 24 hr at 110°C. Analyses were run on analyser models Unichrom and Multichrom from Beckman, Munich. For quantitation of aminoethyl cysteine a sample purchased by Cyclo Chemical, Los Angeles, was used as reference. No corrections were made for loss during hydrolysis but this loss seems to be minimal for serine and threonine in aminoethylated proteins [8,9].

3. Results

Complete cleavage of fibrinogen to α, β and γ-chains could be achieved by reduction and aminoethylation as judged by electrophoresis (fig. 1). The absence of any uncleaved fibrinogen which does hardly penetrate the gel was indicated by these findings. Amino acid analyses showed a complete conversion of cystine to aminoethyl cysteine thus supporting the electrophoretic data. Molecular weight estimates either for the mixture of chains or the purified components revealed values of 63 500 ± 1500, 56 800 ± 600 and of 48 200 ± 1000 for α, β and γ-chains, respectively.

Chromatography of aminoethylated fibrinogen on DEAE-cellulose revealed three main peaks (fig. 2). The cuts indicated in fig. 2 contained chains of a high electrophoretic purity (about 85 to 95%). The prevailing portion of the α-chain peak and the material chromatographing between α and γ-chains contained two chain components of about equal concentration. The reason for the heterogeneous appearance of the γ-chain peak in its trailing portion is not yet clear but may reflect microheterogeneity of γ-chains reported previously [6,11–13].

Final purification of the chains to homogeneous electrophoretic bands (fig. 1) was achieved on CM-

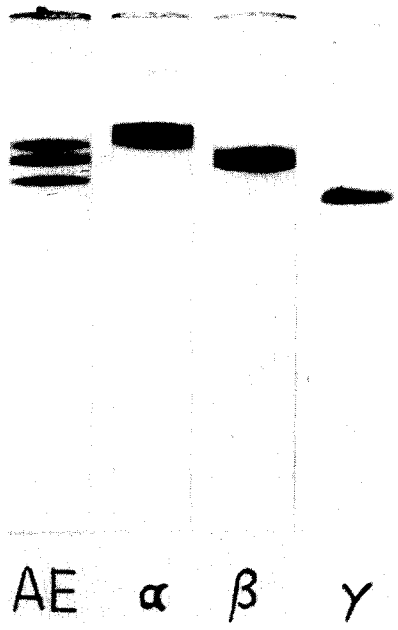

Fig. 1. Polyacrylamide gel electrophoresis pattern of reduced and aminoethylated fibrinogen (AE) and of purified individual chain constituents (α,β,γ). Runs were carried out in the presence of sodium dodecylsulfate. Anode is at the bottom.

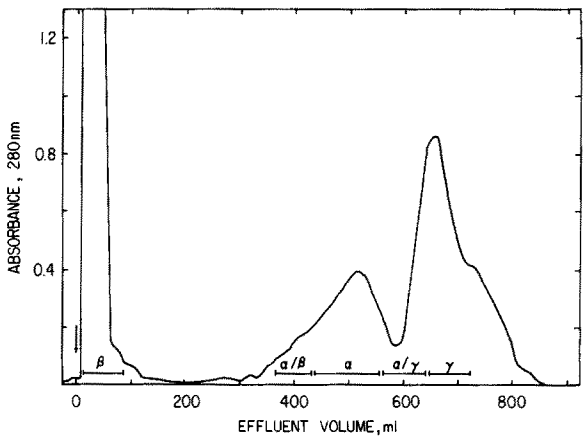

Fig. 2. Separation of reduced and aminoethylated polypeptide chains of bovine fibrinogen on DEAE-cellulose. The column was equilibrated with 0.005 M Tris–HCl pH 8.9, 8 M urea. The start of the gradient (0 to 0.08 M NaCl) is indicated by the arrow. At the baseline the pools subjected to further purifications are designated by the main chain constituents contained in them.

Table 1
Comparison by amino acid composition of aminoethylated (AE) and carboxymethylated (CM) polypeptide chains of bovine fibrinogen*

	AE α-chain	CM α-chain	AE β-chain	CM β-chain	AE γ-chain	CM γ-chain
aspartic acid	66	66	63	63	60	60
threonine	42	43	34	35	30	29
serine	54	54	31	33	28	25
glutamic acid	64	62	61	60	51	51
proline	35	35	28	30	12	11
glycine	63	64	47	49	38	39
alanine	24	24	22	20	22	22
valine	23	26	29	28	18	18
methionine	7	6	12	9	7	5
isoleucine	20	20	21	20	24	25
leucine	33	33	31	31	27	28
tyrosine	6	6	23	23	23	23
phenylalanine	22	21	12	11	18	17
histidine	12	13	8	8	9	9
lysine	34	38	39	40	30	32
arginine	36	32	31	27	17	16
half-cystine**	8	8	11	14	8	12
Total	549	551	503	501	422	422

* Given as residues per peptide chain rounded off to the nearest whole number. Data for carboxymethylated chains are taken from [4].
** Determined as aminoethyl-cysteine or carboxymethyl-cysteine, respectively.

cellulose (not shown). Under the conditions applied each chain was retarded on the column and their emergence from the column was in the order γ, α and β-chains, respectively. The inversion in elution position of aminoethylated α and β-chains if compared with the respective carboxymethylated chains [4] probably reflects the introduction of basic groups by aminoethylation instead of the additional negative charge provided by the iodoacetic acid reagent.

The amino acid composition of purified aminoethylated chains is compared in table 1 with values reported previously for carboxymethylated chains [4]. A good agreement between these data within the limits of analytical error is obvious except for larger deviations for some of the cysteine and methionine values.

4. Discussion

The present study demonstrated that reduction of fibrinogen followed by aminoethylation yields a product which can be used for further purification of the individual polypeptide chains. Identification of the chains by molecular weight determination as well as by amino acid analysis is in agreement with previously published data (reviewed in [14]).

Despite the clear assignment of the chains by amino acid composition some deviations in the cysteine and methionine content to previously published results on bovine fibrinogen [4] are worthy of further discussion. Thus, the cysteine yield is apparently lower in the aminoethylated chains and accounted for a total number of 27 residues per half molecule of fibrinogen. This value is in excellent agreement with previous data on the cysteine content of human fibrinogen and fibrinogen chains [6,15] which were obtained after carboxymethylation under conditions preventing alkylation of methionine. Further support for the reliability of the estimate of cysteine is derived from chemical data on cyanogen bromide peptides of bovine fibrinogen [16,17] which indicated the same number of cysteine residues demonstrated for the aminoethylated chains.

The finding of a higher cysteine content in carboxy-

methylated bovine fibrinogen [4] probably reflects the occurrence of alkylated or oxidized methionine derivatives which may chromatograph on the analyzer in the position of carboxymethyl cysteine [18]. This interpretation is in agreement with the higher methionine values found in the aminoethylated chains if compared with the respective carboxymethylated products. The aminoethylation procedure may circumvent problems of incomplete CNBr cleavage due to the partial alkylation of methionine (see ref. [4]). It has the further advantage that aminoethylated cysteine can be cleaved by trypsin at its C-terminal site [8]. Thus, the procedure described in the present study might also be of some aid for a better characterization of the disulfide regions in the fibrinogen molecule which are likely to be invariant for many if not all of the vertebrate fibrinogens.

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References

[1] Clegg, J. B. and Bailey, K. (1962) Biochim. Biophys. Acta, 63, 525.
[2] Henschen, A. (1964) Ark. Kemi, 22, 375.
[3] McKee, P. A., Mattock, P. and Hill, R. L. (1970) Proc. Natl. Acad. Sci. U.S. 66, 738.
[4] Gollwitzer, R., Timpl, R., Becker, U. and Furthmayr, H. (1972) Eur. J. Biochem., 28, 497.
[5] Murano, G., Wiman, B., Blombäck, M. and Blombäck, B. (1971) FEBS Letters, 14, 37.
[6] Henschen, A. and Edman, P. (1972) Biochim. Biophys. Acta, 263, 351.
[7] Murano, G., Wiman, B. and Blombäck, B. (1972) Thromb. Res., 1, 161.
[8] Raftery, M. A. and Cole, R. D. (1966) J. Biol. Chem., 241, 3457.
[9] Fothergill, L. A. and Fothergill, J. E. (1970) Biochem. J., 116, 555.
[10] Furthmayr, H. and Timpl, R. (1971) Anal. Biochem., 41, 510.
[11] Gerbeck, C. M., Yoshikawa, T. and Montgomery, R. (1969) Arch. Biochem. Biophys., 134, 67.
[12] Brummel, M. C. and Montgomery, R. (1970) Anal. Biochem., 33, 28.
[13] Cartwright, T. and Keckwick, R. G. O. (1971) Biochim. Biophys. Acta, 236, 550.
[14] Doolittle, R. F. (1973) Adv. Protein Chem., 27, 1.
[15] Henschen, A. (1964) Ark. Kemi, 22, 355.
[16] Timpl, R. and Gollwitzer, R. (1973) FEBS Letters, 29, 92.
[17] Timpl, R., Hahn, E. and Gollwitzer, R., Eur. J. Biochem. submitted.
[18] Bundlach, H. G., Stein, W. H. and Moore, S. (1959) J. Biol. Chem., 234, 1754.