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On the Preparation and Properties of Disulfide-Reduced, Alkylated Fibrinogen and Fibrin

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Recently we have described the preparation and some properties of sulfitolysed fibrinogen and fibrin¹. In order to evaluate the completeness and specificity of the sulfite reaction of these proteins, it was desirable to obtain other disulfide-modified derivatives and to compare their properties. In the present investigation, we have chosen to cleave the disulfide bonds by reduction and to block the resulting sulfhydryl groups with an alkylating reagent. Different reducing agents were tried under different conditions.

Bovine fibrinogen and fibrin, purified according to the method of Blombäck and Blombäck², were used. Two types of reagents were employed for reduction: sodium borohydride or the mercaptans thioglycollic acid and mercapto-ethanol. Sodium borohydride was used mainly as described by Moore *et al.*³ The reaction was performed in an 8 M urea solution at a pH between 9

and 11 at + 40°C. Protein and reagent concentrations were about 0.4 %. Sometimes versene or tris were added to the reaction mixture. Thioglycollic acid and mercapto-ethanol were used mainly as described by Sela *et al.*⁴ and by Anfinsen and Haber⁵ and Crestfield⁶, respectively. The reaction mixture was 8 M in urea, 0.4 M in tris and 5×10^{-3} M in versene at a pH of 8.5 and room temperature. Protein concentration was 0.3–1.0 %. Mercaptan was added in approximately 150-fold molar excess regarding the half-cystine content; the solution was kept in a sealed vessel for 4 to 5 h. Alkylation was performed according to the methods of the same authors³⁻⁶. Only iodoacetic acid was tried. When borohydride was used for reduction, the excess was destroyed by acidification. The reagents and the protein-product were separated by gel-filtration on Sephadex G-50⁷, by dialysis or by precipitation with acetone. The number of unchanged disulfide bonds or freed sulfhydryl groups was determined by electrometric titration. The yields of protein in the procedures were calculated from spectrophotometric values⁸ and from the weights of the lyophilized material.

The reduction procedure with sodium borohydride never acted both quantitatively and selectively on the disulfide bonds. Under the conditions described by Moore *et al.*³ a complete cleavage of the disulfide bonds could be obtained. At the same time, however, some peptide bonds were split as revealed by the formation of new N-terminal amino acids. This side reaction occurred even in the presence of versene. In all these experiments with borohydride the final pH was 10 to 11. In order to obtain a more constant and slightly lower pH some reductions were performed in the presence of tris or with successive additions of hydrochloric acid in a pH-stat. In these cases no complete cleavage of the disulfide bonds was obtained.

The reduction procedure with the mercaptans thioglycollic acid and mercapto-ethanol acted quantitatively on the disulfide bonds as determined by electrometric titration after alkylation and isolation. In spite of the absence of a nitrogen barrier there was no sign of (not reversed) reoxidation. N-Terminal amino acid analysis of the products gave the same results as with the unmodified proteins. The derivatives obtained by mercaptolysis and alkylation of fibrinogen and fibrin were almost quantitatively recovered. For these reasons, the mercaptolysed, alkylated products were

termed S-carboxymethyl-fibrinogen and S-carboxymethyl-fibrin. Using Sephadex gels of various molecular sieve properties, no separation of different fractions of the S-carboxymethyl-proteins could be obtained even in 6 M urea solution. Preliminary ultracentrifuge studies (kindly performed by I. Björk) showed homogeneity of the substances and a decrease in molecular weight as compared to untreated fibrinogen.

Both the S-carboxymethyl-proteins were, when obtained by dialysis or gel filtration, completely soluble at low concentrations in distilled water. They showed, however, a marked tendency to aggregate when the ionic strength of the solution was increased or its pH was lowered. After lyophilization the products were no longer soluble in water, but dissolved easily in solutions of, e.g., urea. Solutions of the S-carboxymethyl-proteins in distilled water or 0.15 M sodium chloride did not exhibit the precipitability at $+58^{\circ}\text{C}$, which is characteristic of untreated fibrinogen.

The S-carboxymethyl-proteins were studied by means of paper and starch gel electrophoresis. In all buffer systems tried with paper electrophoresis only a single, strong band was demonstrable, even when the buffers contained reagents, such as urea, tergitol NP 35, sodium dodecyl sulfate or dimethylformamide, supposed to be capable of splitting hydrogen bonds. In some preparations of S-carboxymethyl-derivative one or two, extremely faint, additional bands could be distinguished. Starch gel electrophoresis in 6–7 M urea pH 9.2 gave the same pattern, with a single, strong band and, in some preparations, two faint additional bands. The main components showed a slightly higher mobility than the corresponding native proteins. The other components had a considerably greater mobility. Thus, the electrophoretic findings were quite different from those obtained with the S-sulfo-derivatives, regarding both the number of components and their mobilities¹.

The reaction of the modified fibrinogen with thrombin was examined. S-Carboxymethyl-fibrinogen was found to be a substrate for thrombin. As with the S-sulfo-derivative no coagulum but a fine precipitate was formed². The precipitation reac-

tion between S-carboxymethyl-fibrinogen and thrombin was unchanged after heating of the derivative to $+60^{\circ}\text{C}$. After incubation of S-carboxymethyl-fibrinogen with thrombin two peptides could be isolated in the non-protein supernatant. These peptides showed the same electrophoretic mobilities at pH 4 and gave the same color shades with ninhydrine as the corresponding peptides released from native, bovine fibrinogen. After incubation of S-carboxymethyl-fibrin with thrombin no peptides were found. N-Terminal amino acid analysis indicated that the peptides were released from the fibrinogen derivative in the same order and at about the same rate as from native fibrinogen³.

The possible re-formation of a saline-soluble, thrombin-coagulable fibrinogen after reduction and reoxidation of the disulfide bonds was studied⁵. Reoxidation in saline and in urea at different dilutions of the reduced fibrinogen was tried. In all instances the protein product was completely insoluble in dilute salt solutions.

The present investigation has shown, that the properties of the S-sulfo-derivative and of the S-carboxymethyl-derivative of fibrinogen and fibrin are very similar except for their behavior in electrophoresis.

This work will be described and discussed in detail in the near future.

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