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# The Amino Acid Sequence around the Reactive Cysteine Residue in Human Plasma Factor XIII

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1. Activated human plasma factor XIII was 65% inhibited by iodo[$^{14}$C]acetate and incorporated 0.6mol of label into the $\alpha$ subunit, which eventually was allowed to form a precipitate. 2. All the label was recovered as *S*-carboxymethylcysteine in a tetrapeptide of sequence Gly-Gln-Cys-Trp.

Factor XIII is the zymogen of plasma transglutaminase which, during the terminal stage of blood clotting, cross-links the fibrin gel by eliminating $NH_3$ between glutamine and lysine side chains (Pisano *et al.*, 1968). The enzyme is mechanistically similar to the guinea-pig liver transglutaminase: both enzymes have steady-state kinetics consistent with a Ping Pong acyl-enzyme mechanism (Folk, 1969; Chung & Folk, 1972), both are absolutely dependent on $Ca^{2+}$ for activity, and the activity of both the liver (Folk & Cole, 1966) and the plasma enzymes is associated with a reactive thiol group (Loewy *et al.*, 1961*b*). The appearance of a reactive thiol group in the plasma enzyme and the simultaneous appearance of enzyme activity in the presence of $Ca^{2+}$ (Cooke & Holbrook, 1973) is consistent with an acyl-enzyme intermediate, although it does not preclude a more complex role for $Ca^{2+}$. The liver enzyme differs from the plasma enzyme in that it does not require thrombin and it is much less specific as regards the glutamine acceptor substrate (Matačić & Loewy, 1966). The human plasma enzyme has an $\alpha_2\beta_2$ structure in which all subunits have molecular weight around 80000 (Schwartz *et al.*, 1973).

After cleavage with thrombin and activation with $Ca^{2+}$ the tetramer of the human enzyme dissociates to give a soluble protein containing only $\beta$ chains and a second protein containing only $\alpha$ chains. This second protein is initially soluble, but at 8mg/ml it slowly forms a precipitate which is still active (Cooke & Holbrook, 1972; R. D. Cooke & J. J. Holbrook, unpublished work). Only the $\alpha$ protein, either soluble or insoluble, is active. The present paper reports the characterization of the amino acid sequence around the reactive thiol group of the plasma enzyme. The precipitation of the $\alpha$ subunit enabled specific purification of the labelled $\alpha$ protein and thus no interference in the calculation of label incorporated into the $\alpha$ protein by the soluble $\beta$ protein or by any denatured Factor XIII, which is sometimes present (Curtis *et al.*, 1973).

The Cohn-I fraction (Cohn *et al.*, 1946) from outdated pooled human plasma cryosupernatant was stored at −15°C until Factor XIII was made by the method of Loewy *et al.* (1961*a*) as modified by Lorand *et al.* (1968). The enzyme activity was assayed with dansyl-cadaverine and casein similarly to the method of Lorand *et al.* (1971). The purified zymogen showed only the characteristic $\alpha$ and $\beta$ subunits on polyacrylamide-gel electrophoresis (Schwartz *et al.*, 1973). The protein was determined from $E^{1\%}_{280} = 13.8$ given by these workers.

Factor XIII (160mg in 20ml of 0.1M-Tris–HCl, pH7.5) was activated for 10min by the addition of 0.5M-$CaCl_2$ (2ml) and 1000 N.I.H. units of thrombin (Leo Pharmaceutical Products, Ballerup, Denmark). A solution of iodo[$^{14}$C]acetic acid (1.6ml; 50$\mu$Ci; 32$\mu$Ci/$\mu$mol; The Radiochemical Centre, Amersham, Bucks., U.K.) was added. Compared with a control without iodoacetate, which remained fully active, the sample lost 65% of its initial activity over 60min incubation at 20°C. Activity was then completely inhibited with excess of iodoacetate (55$\mu$mol) and the reaction was terminated 15min later with dithiothreitol (50mg). During the incubation, as expected, the $\alpha$ subunit was slowly precipitated in both the control and the sample, and after a total of 2h the precipitate was sedimented in a bench centrifuge and washed four times by resuspension in 5ml of 1% (w/v) trichloroacetic acid. The extinction of the precipitate dissolved in 8M-urea was measured and the protein content calculated by assuming that $E^{1\%}_{280} = 13.8$ was unchanged by the urea. The radioactivity of a sample was measured in a Nuclear–Chicago Isocap 300 liquid-scintillation counter by using programme B2 in a scintillator containing 9.5ml of a mixture of 75mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 4g of 2,5-diphenyloxazole and 120g of naphthalene in 1000ml of dioxan, mixed with 0.5ml of water. It was calculated that 0.56mol of iodo[$^{14}$C]acetate had been incorporated into each mol of $\alpha$ subunit (80000 relative subunit weight) at 65% inhibition. The chymotryptic peptides obtained below were soluble without urea, and again assuming $E^{1\%}_{280}$ was unchanged by the digestion from its value in the intact protein the combined extinction and radioactivity measurements

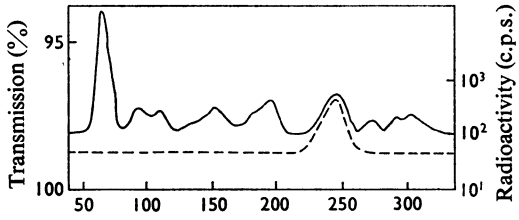


Fig. 1. *Gel filtration of the chymotryptic peptides of* $^{14}$*C-labelled α chain of human plasma Factor XIIIa*

Peptides (18mg) were filtered through a column (2cm × 80cm) of Sephadex G-25 in 30mM-$NH_4HCO_3$. The eluate was continuously monitored for percentage transmission at 253.7nm (——) and for radioactivity (– – – –) with the logarithmic channel of a Nuclear Enterprises (Sighthill, Edinburgh, U.K.) counter with an NE806 flow head. Some 90% of the radioactivity applied to the column, and all that was eluted was in the eluate at 220ml–260ml, which was pooled and evaporated to dryness.

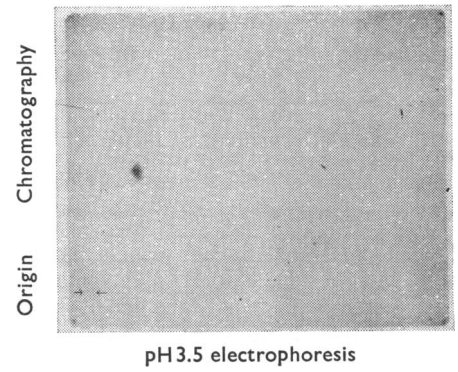


Fig. 2. *Purification of the peptide from a two-dimensional 'map'*

A radioautogram on X-ray film of the material from the Sephadex peak applied at the corner of a sheet of Whatman no. 3 paper and developed at right-angles by chromatography in butanol–acetic acid–water (72:21:107, by vol.) overnight and then by electrophoresis at pH3.5 at 2kV for 2h. Paper containing the single radioactive spot was eluted with aq. 1% (v/v) $NH_3$ and the remainder stained with ninhydrin. There were no spots near the labelled one.

gave 0.63mol of iodo[$^{14}$C]acetate per mol of α subunit which had been 65% inhibited. The precipitate was suspended in 2ml of 30mM-$NH_4HCO_3$ and was digested with one-twentieth of its weight of chymotrypsin (Serva Entwicklungslabor, Heidelberg, W. Germany) for 1h at 37°C and then 16h at 3°C. The peptides, which were all soluble after 1h, were fractionated on Sephadex G-25 (Fig. 1). Some 90% of the radioactivity applied to the column, and all of that eluted, was in a single peak which was further purified by a two-dimensional paper separation (Fig. 2).

A sample of the single radioactive peptide eluted from the 'map' was hydrolysed at 105°C in an $N_2$-flushed evacuated tube for 20h and analysed on a Technicon TSM single-column amino acid analyser. The composition was carboxymethylcysteine (0.9), glutamic acid (1.0) and glycine (1.1). No other residue was present at above 0.05 residue, except tyrosine, which because of uncertainty in the baseline could have been present at up to 0.15 residue. Radioactivity in the hydrolysate migrated at pH6.5 with carboxymethylcysteine. The sequence of about 50nmol of peptide was determined by the dansyl–Edman method described by Gray (1967*a*,*b*). Dansyl derivatives were separated on polyamide layers (10cm × 10cm; from BDH, Poole, Dorset, U.K.) at right-angles with solvents I and II of Woods & Wang (1967). After examination, the layer was then further developed in the solvent described by Crowshaw *et al.* (1967). The end groups detected before the degradation and after each step were respectively glycine (with some tyrosine), glutamic acid (with no trace of glycine), carboxymethylcysteine and a trace of tryptophan (no hydrolysis). The mobility of the peptide at pH6.5 was −0.38 relative to aspartic acid (= −1.0), and thus according to Offord (1966) the glutamic acid arose from glutamine. The presence of a tyrosine end group might have been due to contamination of the tetrapeptide with a pentapeptide similar to that described by Folk & Cole (1966) in guinea-pig liver, which had the sequence Tyr-Gly-Gln-Cys-Trp, although to judge from the amino acid analysis this contaminant could not have been more than 15%. The presence of tryptophan was deduced from the positive reaction given by the peptide when stained with dimethylaminobenzaldehyde on paper (Dalgleish, 1952), the shoulder in the u.v. spectrum at 290nm and the absence from the acid hydrolysate of an amino acid at which chymotrypsin normally cleaves.

These results establish the sequence of the peptide in the α chain of human plasma Factor XIII which contains a reactive thiol group after cleavage with thrombin and activation by $Ca^{2+}$ as Gly-Gln-Cys-Trp. This is identical with the sequence of peptide-I isolated by Folk & Cole (1966) from guinea-pig liver transglutaminase. The stoicheiometry of our labelling (about 0.6mol of label incorporated per mol of α subunit at 65% inhibition) is uncertain to the extent that

it is based on a single experiment with labelled iodoacetate and that it was necessary to assume that the $E^{1\%}_{280}$ of the labelled protein was the same as that of the native protein and that it was not appreciably changed either in 8M-urea or by digestion with chymotrypsin. It also depends on the validity of extrapolating the results obtained at 65% inhibition linearly to predict about 0.9mol incorporated per mol of subunit for complete inhibition. This may not be unreasonable, since the specific radioactivity of the isolated peptide (after completion of the inhibition with unlabelled iodoacetate) was about one-half of that of the labelled iodoacetate used to obtain 65% inhibition. Even given these uncertainties, the result is appreciably greater incorporation than the value of 0.5mol per mol of $\alpha$ subunit which would be expected if the protein exhibited half-of-the-sites reactivity as briefly suggested by Chung & Folk (1973). Curtis *et al.* (1973) attributed their low incorporations to the presence of denatured enzyme in their zymogen. The technique used by us was devised to prevent the presence of either inactive enzyme or $\beta_2$ protein interfering with the incorporation results, since the incorporation was measured on the $\alpha$ protein, which is specifically precipitated after cleavage with thrombin and activation by $Ca^{2+}$.

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