Titration and Subunit Localization of Active Center Cysteine in Fibrinoligase (Thrombin-Activated Fibrin Stabilizing Factor).

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SUMMARY: Fibrinoligase activity, measured in a fully synthetic substrate system, was shown to be inhibited by prior incubation of the enzyme with iodoacetamide. Using 1-^{14}C-iodoacetamide, the amount of incorporated radioactivity increased in proportion to the loss of enzyme activity. Labelling of the enzyme was totally dependent on the addition of calcium ions and, under the conditions described, occurred predominantly in the a' protomer. Acid hydrolysis of labelled fibrinoligase gave rise to S-carboxymethylcysteine as the only radioactive amino acid derivative.

Fibrinoligase (or thrombin-activated fibrin stabilizing factor or plasma Factor $XIII_a$) is a calcium-dependent transamidase essential to the last step of blood coagulation (1). Recently developed rate assays for the enzyme using fully synthetic substrate systems (2), together with a chromatographic procedure for obtaining the pure zymogen from human plasma (3), make it now possible to titrate and explore the active center region of the enzyme.

The present report shows that ^{14}C-iodoacetamide inhibits and labels fibrinoligase by reacting with a cysteine residue in the enzyme in a totally calcium dependent manner. Specificity of labelling is underlined by the fact that ^{14}C-iodoacetamide reacts almost exclusively with the a'subunit, one of the two protomers of the a'b, or perhaps $(a'b)_2$, enzyme structure (4-6).

MATERIALS AND METHODS

The Factor XIII zymogen was prepared from citrated human plasma by the DEAE-cellulose chromatographic procedure described by Lorand and Gotoh (3), with 1 mM EDTA included in all solvents and using a Biogel A-0.5 column

as a final step in the purification. The zymogen was stored at 4° as a 1.7% protein solution in 50 mM Tris-chloride buffer of pH 7.5 containing 1 mM EDTA. Activation of Factor XIII to fibrinoligase was carried out by the addition of thrombin (3.75 NIH units per mg Factor XIII) which was purified from bovine thrombin (Parke Davis, Topical) by a cellulose-phosphate chromatographic procedure (7).

β-Phenylpropionyl thiocholine iodide and N-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulphonamide (or dansylcadaverine) were used as substrates (2) for measurement of enzyme activities. 1-^{14}C-Iodoacetamide (58 mCi/mmole) was purchased from the Radiochemical Centre, Amersham.

Labelling of fibrinoligase with ^{14}C-iodoacetamide for Fig. 1 was carried out at various concentrations of this reagent, in a 50 mM Tris-acetate buffer of pH 7.5 and a volume of 50 µl containing 0.035 mg protein. The reaction was initiated by the addition of 100 mM calcium chloride and was allowed to proceed for 20 min at 25°, when 10 µl aliquots were tested for residual enzymatic activity and for incorporation of isotope.

Enzyme activity was assayed in a 100 µl reaction mixture containing 2 mM β-phenylpropionylthiocholine iodide, 1 mM dansylcadaverine, 100 mM calcium chloride and 50 mM Tris-chloride buffer at pH 7.5. The reaction was initiated by the addition of the thiolester and the formation of the water-insoluble fluorescent coupling product, $C_6H_5(CH_2)_2CONH(CH_2)_5NHSO_2C_{10}H_6N(CH_3)_2$, and was followed by continuous extraction into 2 ml heptane and monitored in an Aminco-Bowman Spectrophotofluorometer (excitation at 340 nm, emission at 460 nm).

In order to measure protein-bound radioactivity, 10 µl samples were applied to Whatman 3 mM filter paper (1cm^2) and free radioactivity was removed by successive washes in 10% (30 min) and 5% (3 x 30 min) trichloroacetic acid, then ethanol-acetone (1:1 v/v for 10 min) and acetone (10 min). The isotope remaining on the dried paper was measured by liquid scintillation counting (8).

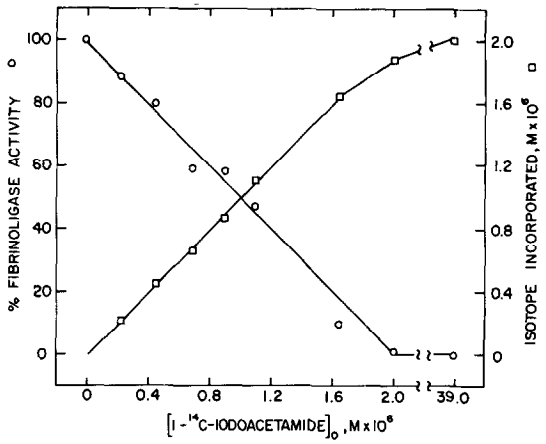

Fig. 1. Simultaneous inhibition and labelling of fibrinoligase with 1-^{14}C-iodoacetamide (for details see text).

For disc-gel electrophoretic studies, 43 μM of ^{14}C-iodoacetamide was allowed to react with 0.86 mg of fibrinoligase in the presence of 50 mM calcium chloride, 0.05 M Tris-acetate buffer of pH 7.5 in a volume of 0.5 ml with continuous stirring at 25° for 15 min. The modified protein was precipitated by the addition of 7.5% trichloroacetic acid and washed successively with the same and also with acetone to remove free radioactivity. The dry protein precipitate was finally taken up in 0.8 ml of a mixture of 9 M urea and 3% sodium dodecylsulfate in 100 mM sodium phosphate buffer of pH 7.1, and was subjected to polyacrylamide disc-gel electrophoresis as described by Weber and Osborn (9). The gels were stained with Coomassie Brilliant Blue (0.25%) and after destaining in 14% acetic acid - 7% methanol the protein bands were cut out, dissolved in 30% hydrogen peroxide at 50° and assayed for ^{14}C activity by liquid scintillation counting.

RESULTS

Inactivation of fibrinoligase by ^{14}C-iodoacetamide (Fig. 1) is accompanied by a proportional uptake of the isotope, indicating that alkylation of the enzyme had taken place. It should be emphasized that, under the conditions employed, no labelling of the inactive zymogen (i.e., Factor XIII

itself) could be observed; hence, thrombin-activation appears to be a prerequisite for the unmasking of iodoacetamide-reactive sites. By appropriate selection of reagent concentration, enzyme activity can be totally abolished and, at this point, the extent of isotope uptake also approaches a maximal value. In the experiment given in Fig. 1, the end point was reached at 2 μM iodoacetamide which may be taken as a measure of the titratable active sites of the enzyme. Based on an extinction coefficient of 13.8 (10) and assuming one active site per 160,000 g of enzyme protein (10 ; 11), the maximum possible concentration of the enzyme was calculated as 4.3 μM. On this basis,

Table I. Calcium dependence of the alkylation of fibrinoligase with ^{14}C-iodoacetamide.

Solute concentrations during reactions with iodoacetamide	^{14}C-Carbamidomethyl group incorporated, M x 10^6
5 mM EDTA	0.03
5 mM EDTA+ 300 mM KCl	0.06
100 mM $CaCl_2$	2.00

Table I. Fibrinoligase (0.07 mg) was incubated for 10 min at 25° in an 80 μl solution containing 50 mM Tris-acetate buffer at pH 7.5 and the solutes listed in the Table. Then 20 μl of C^{14}-iodoacetamide was added to a concentration of 39 μM and was allowed to react for 20 min at which time 10 μl aliquots were spotted onto filter paper and assayed for isotope incorporation.

approximately 47% of the active sites in this particular preparation (derived from a zymogen stored for one month) appeared to be functional.

Alkylation with iodoacetamide was found to be totally calcium dependent and, as shown in Table I, this seems to be due to a specific ion effect since calcium chloride could not be replaced by potassium chloride of equal ionic strength.

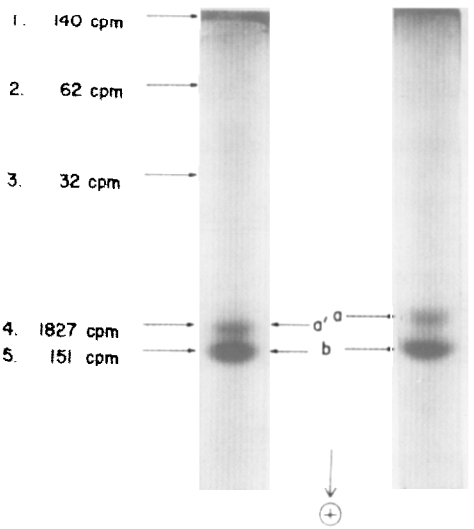

Fig. 2. Distribution of isotope following treatment of fibrinoligase with ^{14}C-iodoacetamide in 50 mM calcium chloride and separation of constituent chains by sodium dodecylsulphate disc-gel electrophoresis. After destaining, 3 mm long segments were cut out of the gel at the points marked from 1 to 5. These points correspond to the centers of segments and to actual migration distances (from the point of application at the top) of 1.5, 15, 33, 58 and 62 mm respectively. The distribution of isotope is given as cpm per segment. The gel to the right of the figure is included for reference to show the protein subunits of the inactive zymogen, unreactive towards iodoacetamide.

The specific nature of the reaction of fibrinoligase with iodoacetamide was also evident by examination of the chains separated on disc-gel electrophoresis (Fig. 2) which showed that with the fully activated enzyme, labelling occurred predominantly in the a' protomer. It will be recalled that it is this subunit which arises from the a structure following thrombin-activation of the zymogen (4). These results support the view that the a' protomer is the catalytic subunit in the a'b dimeric enzyme.

Acid hydrolysis (5.7 N hydrochloric acid, 24 hr at 108°) of the isotopically labelled enzyme gave rise to a single radioactive peak on the Beckman Amino Acid Analyzer, appearing at the elution volume of an authentic S-carboxymethylcysteine marker. Thus, fibrinoligase may be classified as a thiol enzyme with cysteine as its functional group, located in its a' subunit.

Tryptic and chrymotryptic digestion of the alkylated enzyme permitted

recovery of most of the radioactivity in a peptide which could be isolated by chromatography on AMINEX AG 50W-X2, 200-325 Mesh and fingerprinting. Amino acid sequencing of this active center peptide is under investigation.

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