# Calcium-Dependent Unmasking of Active Center Cysteine during Activation of Fibrin Stabilizing Factor

C. G. Curtis, K. L. Brown, R. B. Credo, R. A. Domanik, A. Gray, P. Stenberg, and L. Lorand*

ABSTRACT: Limited proteolysis by thrombin of the a subunit of fibrin stabilizing factor (coagulation factor XIII of plasma) is by itself not sufficient to bring about the conversion of this zymogen to a functional enzyme. The active center cysteine residue required for transamidase or esterase activities still remains buried on the thrombin-modified subunit and becomes susceptible to alkylation by iodoacetamide only after exposure of the protein to calcium ions. These ions were shown to exercise a precise control over the degree of unmasking of the critical thiol group under a variety of experimental conditions. The specific effect of a given concentration of calcium ions can be augmented by increasing general ionic strength. Unmasking appears to be maximal in the pH 7–8.5 range with an apparent $pK_a$ of 6. Other group 2a ions could be substituted for calcium with an order of efficacy of $Sr^{2+} > Ba^{2+} > Mg^{2+}$. At $\mu = 0.2$ and pH 7.5, approximately 25 mM calcium ions are needed to cause a 50% unmasking of the thiol. The coupled event of dissociation of the heterologous subunit structure of the thrombin-modified zymogen shows a similar calcium ion dependence. Possible physiological significance of calcium regulation in activating the fibrin stabilizing factor is discussed.

Fibrin stabilizing factor (FSF or factor XIII) is a zymogen precursor in plasma for a transamidating enzyme (fibrinoligase, FSF* or factor $XIII_a$) which is generated at the time of clotting for the purpose of catalyzing the formation of covalent bridges between fibrin units to increase the elasticity of the clot network (see Lorand, 1972). Thus, conversion of the zymogen to the enzyme is an integral part of normal blood coagulation. Genetic lack of the zymogen (Duckert *et al.*, 1960; Lorand *et al.*, 1970) or the accidental occurrence of a circulating inhibitor which specifically affects only activation of the zymogen (without interfering with the transamidase itself) causes very severe hemorrhagic conditions (Lorand *et al.*, 1972a).

It has been known for some time (Lorand and Konishi, 1964) that activation of the plasma zymogen required the presence of both thrombin and calcium ions. Similar to its mode of action in fibrinogen (Lorand, 1951), the effect of thrombin on factor XIII proved to be one of limited proteolysis (as suggested by the work of Konishi and Takagi, 1968; and of Lorand *et al.*, 1968) resulting in the shortening of one of the two subunits (a to a′) of the protein (Bohn, 1970; Schwartz *et al.*, 1971; Takagi and Konishi, 1972) through scission of an N-terminal peptide fragment (Mikuni *et al.*, 1973; Takagi and Doolittle, 1974).

We have recently shown (Curtis *et al.*, 1973) that the thrombin-catalyzed step by itself is not sufficient to generate transamidase activity and that formation of the active center of the enzyme requires a specific interaction of the thrombin-modified zymogen with calcium ions. This second, calcium-dependent step brings about both the unmasking of a buried cysteine residue in the a′ subunit and the coupled dissociation of the heterologous subunit structure of the protein (Lorand *et al.*, 1974). The present paper deals primarily with various aspects of the unmasking of the active center sulfhydryl group in the course of zymogen conversion, as measured by reaction with [$^{14}$C]iodoacetamide.

## Materials and Methods

The zymogen form of factor XIII was prepared from citrated, outdated blood-bank human plasma by the salt gradient elution DEAE-cellulose chromatographic procedure of Lorand and Gotoh (1970), with 1 mM EDTA included in all solutions used. As a final step, 2–6 ml of the purified protein solution was applied to a 2.5 × 90 cm column of Sepharose 6-B, equilibrated with 50 mM Tris-chloride of pH 7.5 containing 1 mM EDTA. On elution with the same at a rate of 20 ml/hr, monitored at 280 nm, the active peak emerging at $V_e/V_0 = 1.4$ was concentrated by precipitation with 40% saturation of ammonium sulfate; it was then dialyzed against 50 mM Tris-chloride (pH 7.5)–1 mM EDTA and stored at 4° as a 1.7% protein solution. A smaller peak ($V_e/V_0 = 1.8$) contained only b subunits.

Bovine thrombin, used for the activation of factor XIII, was obtained by applying the contents of three vials of Parke-Davis Thrombin Topical to a cellulose phosphate column (12 × 2.4 cm) which had been equilibrated with 50 mM potassium phosphate (pH 7.0) (Chou, 1970). On elution with this buffer, a strong yellow band was removed at about 80 ml of effluent and the column was further treated with 250 ml of the buffer. Thrombin was eluted by applying a mixture of 250 mM potassium phosphate and 0.5 M sodium chloride at pH 7.0. The enzyme was dialyzed against 1.0 M potassium chloride (pH 7.0) and was stored at 4°.

The limited proteolytic activation (Lorand and Konishi, 1964; Lorand *et al.*, 1968; Schwartz *et al.*, 1971) of the isolated factor XIII zymogen was typically brought about by allowing 1.8–2.0 mg of this protein to react with 6–7 NIH units of thrombin in 0.3–0.5-ml solutions of 50 mM Tris-acetate buffer of pH 7.5. After an activation period of 20 min (at 25°), aliquots (*ca.* 20 μl) corresponding to approximately 70 μg of the original zymogen were taken for alkylation with iodoacetamide.

Reactions of the thrombin-activated factor XIII with [1-$^{14}$C]iodoacetamide (Amersham-Searle; 58 Ci/mol) were carried out in 0.1-ml incubation mixtures (25°) under the condi-

† From the Department of Biochemistry and Molecular Biology, Northwestern University, Evanston, Illinois 60201. *Received February 11, 1974.* This work was aided by a USPHS Research Career Award (HL-K6-3512) as well as by grants (HL-02212 and HL-16346) from the National Institutes of Health. A Fulbright Travelling Fellowship to C. G. C. is gratefully acknowledged.

tions of pH, ionic strength, calcium ion, iodoacetamide concentration, and time periods specified for each experiment.

Extent of labeling of the protein upon alkylation with [1-$^{14}$C]iodoacetamide was followed by taking 10-$\mu$l aliquots of the incubation mixtures and applying them to 1-cm$^2$ segments of Whatman 3MM filter paper as previously described (Curtis *et al.*, 1973). Free radioactivity was removed by successive washes with 10% (30 min) and 5% aqueous trichloroacetic acid (3 × 10 min), 1:1 (v/v) ethanol–acetone (10 min), and finally acetone (10 min). The dry filter papers were placed in vials with 10 ml of toluene-based scintillation fluid (containing 3.0 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter), and the protein bound radioactivity was measured by counting in a Packard TriCarb liquid scintillation spectrometer.

In order to examine the distribution of the $^{14}$C label in the alkylated protein by means of dodecyl sulfate electrophoresis, suitable aliquots (corresponding to 50–60 $\mu$g of factor XIII) of the incubation mixtures were precipitated with 7.5% cold trichloroacetic acid. The centrifuged precipitates were washed with 10% trichloroacetic acid (3 × 0.5 ml) and acetone (2 × 0.5 ml). The dry sediments were solubilized in 50-$\mu$l mixtures containing 9.0 M urea and 3% sodium dodecyl sulfate in 0.1 M sodium phosphate (pH 7.1) (Schwartz *et al.*, 1973) by heating to 38° for 30 min. Samples of 35 $\mu$l were subjected to disc gel electrophoresis (Weber and Osborn, 1969) and were also assayed for radioactivity. Polyacrylamide (0.6 × 7 cm; 5%) gels were run in a Buchler Polyanalyst apparatus with 0.1 M sodium phosphate–0.1% sodium dodecyl sulfate of pH 6.9, by applying 50 V to 12 gels until the Bromophenol Blue tracking dye moved to within 1 cm of the bottom of the gels (about 2.5 hr). Staining was performed with 0.25% Coomassie Brilliant Blue (dissolved in a mixture of 50% methanol and 10% acetic acid) either for 1 hr at 60° or for 2–3 hr at room temperature; destaining by diffusion was carried out in a mixture of 14% acetic acid and 7% methanol. The distribution of stained protein bands was evaluated by scanning at 553 nm in a Beckman DU spectrophotometer equipped with Gilford optical density converter and linear transport (Model 2410). In order to locate radioactivity after electrophoresis, approximately 2–3-mm slices of the gels were placed in plastic scintillation vials and dissolved in 30% hydrogen peroxide at 50–60° for 5 hr as described by Tischler and Epstein (1968). After cooling, 10 ml of scintillation fluid (toluene–Triton-X-100–1,4-bis[2-(5-phenyloxazolyl)]benzene-2,5-diphenyloxazole, 2.4 1:1.2 1:0.24 g: 0.6 g) was added and isotope content was measured.

To facilitate the measurements regarding the time-dependent generation of transamidase activity (see Figure 5), 1.3 mg of the factor XIII zymogen was mixed with a relatively small amount of thrombin (0.25 NIH units).in a reaction volume of 0.38 ml (50 mM Tris-acetate buffer, pH 7.5). From 0 to 60 min, as indicated in the figure, 20-$\mu$l aliquots were removed and added to an equal volume of hirudin (Sigma Chemical Corp.; 0.07 unit, dissolved in the Tris-acetate buffer) for the purpose of instantaneous termination of thrombin action. Samples of 10–20 $\mu$l from these hirudin-quenched mixtures were then subjected to alkylation with [1-$^{14}$C]iodoacetamide for 20 min at pH 7.5 and 25° in the presence of 0.1 M calcium chloride as described, and were also tested for transamidase activity generated. The latter was assayed (at 25°) in 100-$\mu$l reaction mixtures containing 2.0 mM $\beta$-phenylpropionylthiocholine iodide, 1.0 mM *N*-(5 aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide [*i.e.*, dansylcadaverine], and 100 mM calcium chloride in 50 mM Tris-chloride buffer (pH 7.5). Formation of the water-insoluble fluorescent amide coupling product (Lorand *et al.*, 1972b; Curtis *et al.*, 1973) was followed by direct extraction into 2 ml of heptane layered over the reaction mixture, and was monitored in an Aminco-Bowman spectrophotofluorimeter (excitation at 340 nm; emission at 460 nm). Continuous and rapid passage of the product through the water–heptane interface was ensured by continuous stirring of the aqueous layer at 500 rpm.

Identification of the amino acid residue modified in the protein by [$^{14}$C]iodoacetamide was carried out by the following procedure. Approximately 0.7 mg of thrombin-activated factor XIII was reacted in an 0.8-ml solution (50 mM Tris-acetate, pH 7.5, 24°) with 27 $\mu$M of [$^{14}$C]iodoacetamide in the presence of 62 mM calcium chloride. After the 20-min period allowed for labeling, the protein was precipitated in 7.5% cold trichloroacetic acid, then washed three times with the same and twice with acetone by centrifugation, and finally dried in a stream of nitrogen. Hydrolysis was performed in 1 ml of 5.7 N hydrochloric acid (108°, 24 hr) and, after lyophilization, the sample was taken up in 0.25 ml of 0.2 M citrate buffer (pH 3.25). A 10-$\mu$l aliquot was removed for measurement of isotope content (2000 cpm) and the remainder was applied to a Custom AA-15 resin column (0.9 × 60 cm) in the Beckman 120C amino acid analyzer at a flow rate of 67 ml/hr at 56°. Aliquots of 0.1 ml were taken from each fraction (1.1 ml) of the eluate for measurement of radioactivity and amino acids were assayed by the fluorescent ninhydrin procedure described by Samejima *et al.* (1971).

Reference *S*-(carboxymethyl)cysteine was purchased from Nutritional Biochemicals Corp. and *S*-[$^{14}$*C*]carboxymethylcysteine was prepared by reacting [1-$^{14}$C]iodoacetamide with a tenfold excess of cysteine hydrochloride in 50 mM Tris-acetate buffer (pH 8.0) for 4 hr at 25°, followed by acid hydrolysis.

Fingerprints of $^{14}$C-labeled peptides from the alkylated fibrinoligase were obtained as follows. Tryptic digests (TPCK, Worthington Biochemicals Corp., 20 units/10 mg of alkylated fibrinoligase) were run in 2 ml of 0.1 M ammonium bicarbonate (pH 8.0) for 24 hr at 37°. This digestion was repeated and, after evaporation to dryness, chymotryptic digestion (Worthington Biochemicals Corp., 0.45 unit/10 mg of alkylated fibrinoligase) was carried out under the same conditions. After evaporation almost to dryness, water (1 ml) was added and the digest lyophilized. It was then dissolved in 0.2 M pyridine–acetate buffer of pH 3.1 and a sample (corresponding to approximately 13 nmol of [$^{14}$C]carbamido derivative) was applied to a Dowex AG 50W-X2 200–325 mesh column (0.9 × 20 cm) equilibrated with the same buffer. The column was washed with 62 ml of 0.2 M pyridine–acetate of pH 3.1 at 20 ml/hr followed by a 300-ml buffer gradient ranging between 0.2 M pyridine–acetate of pH 3.1 and 2 M pyridine–acetate of pH 5.1. The radioactivity (95%) was recovered as a single peak eluted between 172 and 192 ml, and after evaporation to dryness the labeled material was dissolved in pyridine–acetic acid–water (200:8:1800 of pH 6.4). Fingerprints showing the distribution of radioactivity were obtained by subjecting aliquots (1–5 $\mu$l) to electrophoresis on Polygram Cel 300 sheets (20 × 20 cm) in pyridine–acetic acid–water (200:8:1800 of pH 6.4) for 1 hr at 500 V followed by ascending chromatography using 1-butanol–acetic acid–water (100:16:38) as solvent and autoradiography.

The calcium dependent dissociation of the subunit structure of thrombin-activated factor XIII was studied by means of disc gel electrophoresis employing the procedure of Rodbard and Chrambach (1971). This utilizes 7% acrylamide gels and a buffer system of pH 7.8 containing TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid), Bis-tris (bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane and cacodylic acid

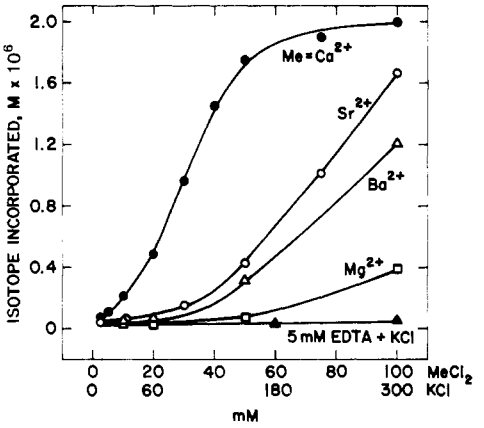



FIGURE 1: Effect of calcium and other group 2a metal ions on the reaction of thrombin-activated fibrin stabilizing factor with [$^{14}$C]iodoacetamide. Alkylation of the protein was carried out in 0.1 ml of 50 mM Tris-acetate buffer of pH 7.5 containing 10 $\mu$l of the metal (Me) chloride salt solution to give the concentrations shown on the abscissa (upper scale); 10 min after the addition of the thrombin-activated zymogen (0.07 mg added in 20 $\mu$l), alkylation was initiated with 20 $\mu$l of a solution of [$^{14}$C]iodoacetamide (to give a final concentration of 35 $\mu$M) and was allowed to proceed at 25° for 20 min. At that time, 10-$\mu$l aliquots were withdrawn and assayed for protein bound radioactivity (ordinate). The control contained only 5 mM EDTA with ionic strength adjusted by appropriate concentrations of potassium chloride (abscissa; lower scale).

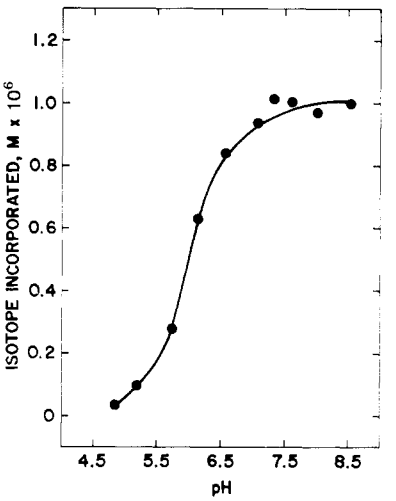



FIGURE 2: pH dependence of the reaction of [$^{14}$C]iodoacetamide with fibrinoligase. Alkylation of the enzyme was carried out in 0.1 ml of 50 mM Tris-maleate buffer (pH ranging between 4.8 and 8.5) containing 50 mM calcium chloride (added in 10 $\mu$l). Thrombin-activated fibrin stabilizing factor (0.033 mg in 10 $\mu$l) was then added and, following incubation at 25° for 10 min, alkylation was initiated by the addition of [$^{14}$C]iodoacetamide (16 $\mu$M, added in 10 $\mu$l). Aliquots (10 $\mu$l) were withdrawn after 20 min and were assayed for protein bound radioactivity.

at an ionic strength of 0.01. For these experiments the zymogen was activated by thrombin for 20 min at 25° and thrombin action was quenched by addition of fivefold excess of hirudin. Aliquots corresponding to 1.3 mg/ml of the thrombin-modified protein were then incubated for 30 min at 38° at $\mu$ = 0.2 in solutions containing calcium chloride of 0–50 mM, just prior to electrophoresis.

## Results

Figure 1 shows the reactivity (at 25° and pH 7.5) of a fibrin stabilizing factor (factor XIII) preparation with iodoacetamide after it has been fully activated by thrombin. Inspection of the activated zymogen using sodium dodecyl sulfate disc gel electrophoresis revealed complete conversion of a to a′ subunits (Schwartz *et al.*, 1973). The extent of isotope incorporation into the protein from [$^{14}$C]iodoacetamide (given for 20 min time points in the figure) depends on the concentration of calcium chloride in the medium; in the absence of calcium ions no reaction occurred and even a compensatory increase in ionic strength by potassium or sodium chloride was entirely without effect (see control in Figure 1). However, calcium could be partially replaced by other group 2a cations with an apparent order of efficacy of $Ca^{2+} > Sr^{2+} > Ba^{2+} > Mg^{2+}$. It may be mentioned that under the conditions described, reaction of the protein with iodoacetamide in the presence of calcium ions (particularly for concentrations of 50 mM or higher) was virtually complete within 10 min.

Furthermore, it could be demonstrated that cysteine was the only amino acid residue modified by treating the protein with iodoacetamide. All the radioactivity present in the acid hydrolysate was recovered in a single ninhydrin positive peak which eluted between 35 and 42 ml from the amino acid analytical column at the same position where in parallel runs authentic *S*-(carboxymethyl)cysteine,both the unlabeled material purchased and the radioactive product synthesized by us, were shown to emerge. The specificity of the reaction of [$^{14}$C]iodoacetamide with the protein was further substantiated by fingerprints of tryptic and chymotryptic digests of the alkylated enzyme which, by autoradiography, showed essentially a single radioactive spot (Curtis *et al.*, 1973).

The pH dependence of alkylation with [$^{14}$C]iodoacetamide was examined in the pH 4.8–8.5 range at 50 mM calcium chloride concentration, with measurements made at reaction times from 10 to 60 min. Below pH 5, even with the longest time, no incorporation of the isotope could be seen. Controls in which the calcium chloride was replaced by 5 mM EDTA showed no reactivity whatsoever toward iodoacetamide in the entire pH range of 4.8–8.5. Figure 2 presents the data for 20-min time points and shows a plateau of maximal reactivity between pH 7 and 8.5. It may be mentioned that changing the composition of buffer at pH 7.5 from 50 mM Tris-acetate to Tris-maleate did not affect the level of alkylation of the protein. From the curve in Figure 2 an apparent $pK_a$ of 6.0 was calculated for the uptake of the isotope.

The fact that the extent of alkylation is apparently maximal around pH 7.5 made it possible to use iodoacetamide as a specific titrant to evaluate the functional normality of fibrinoligase. The relevant data for seven different enzyme preparations obtained in our laboratory during the past year are summarized in Table I. It is seen that the percentage of active protein molecules varied from 41 to 75%.

The results given in Figure 1 did not separate the specific effects of group 2a cations from their general contributions to ionic strength. Further experiments with calcium (Figure 3a and b) proved that unmasking of the sulfhydryl group was a combined function of both specific ionic effects and of the general ionic strength. It is seen that raising the latter from 0.05 to 0.35, with 2.5 and 10 mM calcium ions included, increased the availability of iodoacetamide titratable sites. A double reciprocal plot for the reaction of the protein with iodoacetamide in 20 min at varying calcium ion concentrations but at a constant ionic strength of 0.2, pH 7.5 (Figure 4), showed that 25 mM calcium chloride was needed to achieve a degree of half-maximal alkylation.

Since fibrinoligase is formed from an oligomeric zymogen

TABLE I: Functional Purity of Fibrin Stabilizing Factor Preparations Obtained by the Method of Lorand and Gotoh (1970) from Human Plasma.

| Preparation | Protein[a] (M × $10^6$) | Sulfhydryl Groups Titrated[b] (M × $10^6$) | (Reactive Protein/ Total Protein) × 100 |
|---|---|---|---|
| 1 | 106 | 54 | 51 |
| 2 | 81 | 45 | 56 |
| 3 | 101 | 63 | 62 |
| 4 | 73 | 35 | 48 |
| 5 | 92 | 69 | 75 |
| 6 | 66 | 27 | 41 |
| 7 | 79 | 43 | 54 |

[a] Protein concentrations were computed from absorbancy at 280 nm [$A_{1\ cm}(1\%) = 13.8$; Schwartz *et al.* 1973] by assuming one active site per 160,000 g of protein [Lorand *et al.*, 1968; Schwartz *et al.*, 1973]. [b] Reactivity of sulfhydryl groups was measured by 20-min alkylation of the thrombin-activated zymogen with [$^{14}$C]iodoacetamide in the presence of 100 mM calcium chloride at pH 7.5 (25°) with the filter paper assay described in Methods.

comprising two different types of subunits (a and b; Schwartz *et al.*, 1971), it was important to identify which of these two carried the catalytically essential cysteine side-chain functionality. In specific terms, with the thrombin-activated zymogen in which the a subunit has already undergone limited hydrolysis, the problem was to define whether the a′ or the b or both subunits of the protein would become alkylated by iodoacetamide. Results obtained from preparations which were first fully activated by thrombin with all a subunits converted to a′ and then were reacted with an excess of [$^{14}$C]iodoacetamide under different ionic strength (0.07–0.33) and calcium concentration (0–53 mM) showed that, no matter what the degree of alkylation, uptake of the isotope always occurred preponderantly into the a′ subunit. This was, of course, fully in accord with our earlier conclusion obtained under a single set of conditions (Curtis *et al.*, 1973), that the active center cysteine residue of fibrinoligase is derived exclusively from the a′ subunit.

In addition to these situations in which the fibrin stabilizing factor zymogen was maximally activated by thrombin so as to yield a complete conversion of a subunits to a′, experiments were also performed in which the extent of a to a′ conversion was controlled by using relatively small amounts of thrombin for the activation of the zymogen and by terminating the action of thrombin at exact time points with addition of hirudin. Formation of the a′ species was evaluated by means of sodium dodecyl sulfate disc gel electrophoresis, then the partially activated zymogen was exposed to calcium ions (100 mM) and was examined both for transamidase activity generated and for isotope incorporation with [$^{14}$C]iodoacetamide. Enzyme activity was measured in a fully synthetic substrate system, involving reaction between β-phenylpropionylthiocholine and dansylcadaverine (Lorand *et al.*, 1972b; Curtis *et al.*, 1973). For purposes of easier comparison, results in Figure 5 are expressed on fractional scales with the values obtained after 60 min of thrombin treatment taken as unity. These correspond to the maximally obtainable enzyme activity and to the greatest degree of unmasking of thiol groups on the native protein, respectively. It is seen that there is a precise correlation between the extent of enzyme activity generated and the number of groups which became accessible to titration with iodoacetamide.

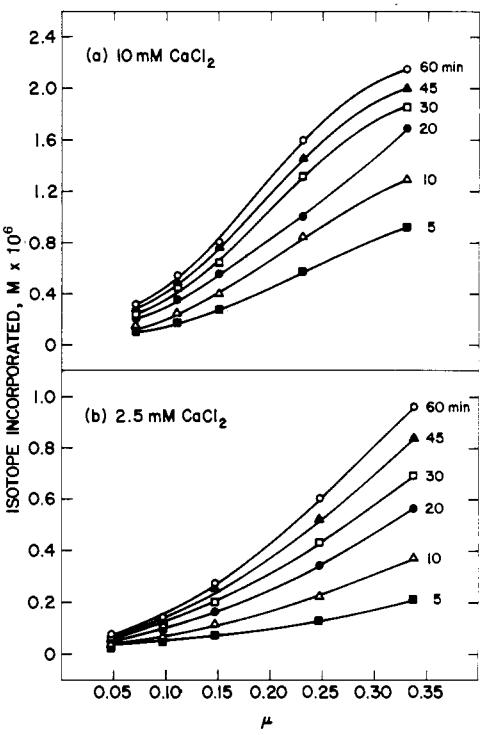



FIGURE 3: Influence of ionic strength on the unmasking of iodoacetamide titratable sites in thrombin-activated fibrin stabilizing factor at fixed concentrations of calcium ions. Alkylation of the protein was carried out in 0.1 ml of 50 mM Tris-acetate buffer of pH 7.5 containing either (a) 10 mM or (b) 2.5 mM calcium chloride. The ionic strength of the medium was adjusted with sodium chloride to give the values shown on the abscissa. The concentration of [$^{14}$C]iodoacetamide was 8.5 μM and alkylation at 25° was initiated by the addition of 0.07 mg of the thrombin-activated zymogen. Aliquots (10 μl) were removed at times ranging between 5 and 60 min as shown and assayed for protein bound radioactivity (ordinate).

Taking advantage of the different degrees of labeling obtained by alkylating the fractionally activated zymogen samples, the question of relative distribution of the isotope among the protein subunits could be examined in relation to partial

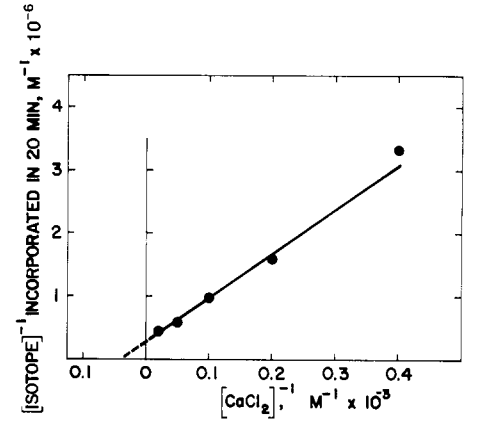



FIGURE 4: A double reciprocal plot showing the extent of alkylation of thrombin-activated fibrin stabilizing factor after 20 min of reaction with [$^{14}$C]iodoacetamide at varying concentrations of calcium chloride but at fixed ionic strength. Alkylation of the protein was carried out as in Figure 4 but with calcium chloride concentrations ranging between 2.5 and 53 mM and with the ionic strength maintained at 0.2 by addition of sodium chloride.

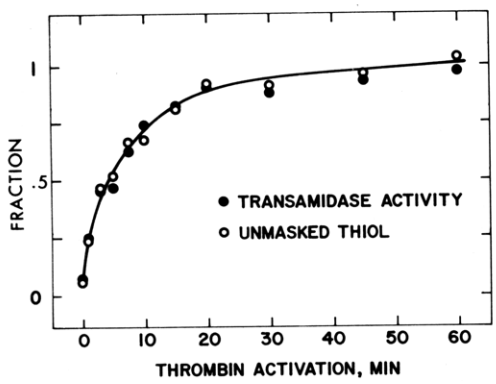
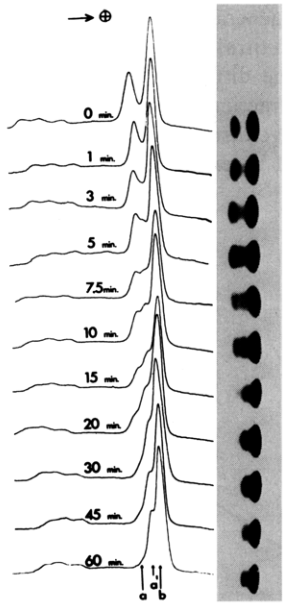



FIGURE 5: Simultaneous unmasking of thiol groups and generation of enzyme activity during zymogen conversion. Following partial activation of fibrin stabilizing factor with thrombin, samples were taken for reaction with [$^{14}$C]iodoacetamide (○) and for measurement of transamidase activity (●) as described in Methods. Points on the abscissa correspond to those in Figure 6, representing times of quenching of zymogen activation by addition of hirudin. The ordinate shows fractional unmasking of thiols and development of enzyme activity, with the 60-min value on the curve taken to signify unity, *i.e.*, total unmasking of thiols and maximal obtainable enzyme activity.

enzyme activation. Figure 6 shows the isotope distribution in relation to the protein bands seen by the sodium dodecyl sulfate disc gel electrophoresis. The zymogen itself (*i.e.*, 0 min of thrombin activation) is refractory to alkylation and uptake of the label into the a′ subunit is seen to increase as zymogen activation progresses. It should be pointed out that the calcium-dependent unmasking of iodoacetamide reactive groups on the thrombin-modified zymogen was easily reversed by the addition of excess EDTA.

The calcium-dependent unmasking of the active center thiol in the a′ subunit of the thrombin-activated zymogen is accompanied by a clear-cut dissociation of this subunit from b even at




| a | a' | b |
|---|---|---|
| cpm | | |
| 49 | 6 | 4 |
| 301 | 237 | 70 |
| 355 | 468 | 98 |
| 442 | 751 | 156 |
| 395 | 631 | 157 |
| 574 | 843 | 189 |
| 422 | 914 | 203 |
| 536 | 1,332 | 294 |
| 486 | 1,303 | 367 |
| 499 | 1,483 | 374 |
| 449 | 1,658 | 351 |

FIGURE 6: Sodium dodecyl sulfate electrophoretograms showing the gradual conversion of a subunits to a′ in fibrin stabilizing factor with low concentration of thrombin, with activation terminated at exact time points by the addition of hirudin. Coomassie Blue stained protein bands (right) are shown with corresponding scans at 553 nm after periods of activations with thrombin for 0 to 60 min. After as complete alkylation of the partially activated zymogen as possible with [$^{14}$C]iodoacetamide, isotope distribution in the excised protein bands is indicated by the numbers (in cpm) to the right.

protein concentrations as high as 1.3 mg/ml. With disc gel electrophoresis, carried out under nondenaturing conditions, it can be demonstrated that both the zymogen and its thrombin-modified form migrate as a single molecular entity. Addition of calcium ions to the thrombin-activated zymogen prior to electrophoresis, however, brings about a dissociation of the quaternary structure of the protein. The electrophoretic patterns reflecting these changes as a function of calcium ion concentration are shown in Figure 7 for a zymogen preparation which has previously been fully activated by thrombin. Protein samples were incubated at 38° for 30 min at $\mu = 0.2$ and various concentrations of calcium chloride (0–50 mM), just prior to electrophoresis at pH 7.8 by the procedure of Rodbard and Chrambach (1971). With increasing concentrations of calcium ions, an increased release of b subunits is seen, represented by the most anodically migrating bands (Lorand *et al.*, 1974). Under the conditions of this experiment, the calcium chloride concentration necessary for producing 50% dissociation of b is estimated to be between 20 and 30 mM.

## Discussion

We reported about a year ago (Curtis *et al.*, 1973) that fibrinoligase was a sulfhydryl enzyme, that its active center cysteine was located in the a′ subunit which previously underwent limited proteolysis by thrombin, and, finally, that unmasking of the catalytically significant residue was brought about by a distinct reaction of the thrombin-activated zymogen with calcium ions. All three conclusions were confirmed thereafter by two other groups of researchers (Holbrook *et al.*, 1973); Chung *et al.*, 1974). The present paper deals with the specificity of the mode of action of calcium ions in greater details. These ions seem to exercise a very precise control over the degree of unmasking of the critical thiol group, as measured by accessibility to alkylation with [$^{14}$C]iodoacetamide, which is directly linked to the generation of transamidating enzyme activity. Among the group 2a ions, calcium is by far the most effective, followed in a decreasing order of efficiency by strontium, barium, and magnesium. The specific effect of a given concentration of calcium ions can be augmented, but not replaced, by increasing the general ionic strength of the medium. In this connection the data given in Figure 3 may be of special interest. At the physiological 2.5 mM calcium concentration and the ionic strength of 0.15 existing in human plasma, only a slow and very limited unmasking of the reactive sulfhydryl groups can occur, but the system is well poised to respond to changes in both. This behavior may be of utmost physiological significance in the sense that the chance of generating dangerous transamidase activity in the circulation would be avoided even if an accidental activation of the zymogen by thrombin or some other trypsin-like enzyme did accidentally occur. Control of the calcium-dependent, second step in the activation of the enzyme appears to be physiologically far more feasible than that of the proteolytic step. In Figures 5 and 6 hirudin had to be applied at precise moments to restrict the extent of proteolytic conversion of the zymogen, and it is most unlikely that this sort of time-related shut-off process could be simulated in nature. It is more probable that the reversible, calcium ion dependent step is the actual site of physiological regulation,

$$\text{zymogen} \xrightarrow{\text{thrombin}} \text{zymogen}' \overset{Ca^{2+}}{\rightleftharpoons} \text{enzyme}$$

and only the disruption of platelets or other cells can create favorable conditions for the sudden activation of this enzyme by causing an increase in calcium concentration and in ionic strength locally wherever clotting is to take place. As soon as

the enzyme drifts out of this immediate ion enriched environment into the general plasma milieu, its active center cysteine is reburied and the protein becomes enzymatically inactive again.

The calcium-specific alkylation of the active center made it possible to determine the operational normality and functional purity of various preparations of fibrinoligase (Curtis *et al.*, 1973) which, of course, is a prerequisite for meaningful kinetic work with this enzyme (Curtis *et al.*, 1974). Functional purity of enzyme preparations obtained in our laboratory over the past year varied from 41 to 75% which appears to be well in the range for a number of highly purified enzymes using a variety of active center directed titration methods (Bender *et al.*, 1966). The most active preparation titrated to 0.75 equiv of sulfhydryl groups/160,000 g of zymogen (Table I) which amounts to 1.5 equiv in relation to the $(ab)_2$ structure of 320,000 molecular weight (Schwartz *et al.*, 1973); Chung *et al.*, 1974). Clearly, our findings do not support the claim advanced by Chung *et al.* (1974) that the enzyme functions according to a "half-of-the-sites" type of reactivity.

Disc gel electrophoresis, carried out under nondenaturing conditions, proved to be a simple method for demonstrating calcium ion induced changes in the quaternary structure of the thrombin-modified zymogen which accompany the unmasking of the active center cysteine residue in its a′ subunit and the simultaneous generation of enzyme activity. The most anodically migrating band, increasing in intensity with increasing concentrations of calcium ions (Figure 7), was shown to correspond to catalytically inactive b subunits. As b is released, the calcium-activated a′ subunits (a*), as also noted by Holbrook *et al.* (1973), form aggregates which barely penetrate the gels. The transamidase specific fluorescent activity stain, using dimethylcasein and dansylcadaverine substrates, can be elicited only at the location of the $(a^*)_n$ aggregates on top of the gels and at the position of the center band which diminishes with increasing concentration of calcium ions and corresponds to the zymogen species. Addition of EDTA to the most fully dissociated system is known to reestablish an electrophoretic pattern indistinguishable from the original zymogen itself (Lorand *et al.*, 1974), demonstrating a heterologous reassociation of subunits. Collectively, in terms of the protomeric unit of the thrombin-activated zymogen, these results indicate the following equilibria, with the concentration of calcium ions regulating the degree of dissociation of b subunits.

$$a'b \underset{\text{EDTA}}{\overset{Ca^{2+}}{\rightleftharpoons}} a^* + b \qquad (a^*)_n$$

Recently, Chung *et al.* (1974) concluded that dissociation should be represented as $a'_2b_2 \rightleftharpoons a'_2 + b_2$.

Direct inspection of the b subunit region in the gels, visible perhaps also in the prints (Figure 7), reveals definite microheterogeneity which will have to be taken into account in more penetrating future studies as to the nature of b to b and a′ to b type of associations. We have found that disc gel electrophoresis under isoelectric focusing conditions shows a number of multiple forms not only for the b but also for the a′ type of subunits (Domanik, 1974). Apparently, all forms can participate in heterologous a′ to b type of association–dissociation reactions. From the data in Table I, however, it would seem that only about 41–75% of the a′ population can be typically converted to the enzymatically competent form.

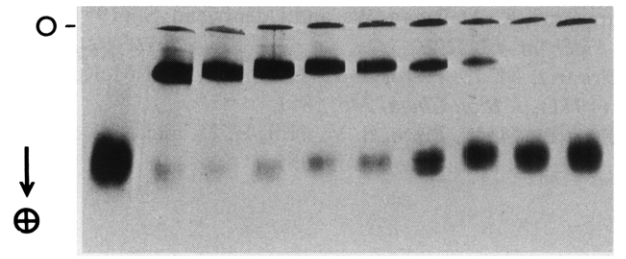



FIGURE 7: Calcium-dependent dissociation of thrombin-activated fibrin stabilizing factor as seen by disc gel electrophoresis (pH 7.8; 7% acrylamide, 1 mA/gel for 4 hr at 4°). Samples of the thrombin-activated zymogen were incubated at 38° for 30 min at $\mu = 0.2$ at various concentrations of calcium chloride (0, 5, 10, 15, 20, 25, 30, 40, and 50 mM, starting from the second gel on the left to right) just prior to electrophoresis with approximately 40 μg of protein applied to each gel. For comparison, the electrophoretic pattern of a pure b subunit preparation is included on the left.

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# Characterization of Protein Kinases Forming Acid-Labile Histone Phosphates in Walker-256 Carcinosarcoma Cell Nuclei†

Donald L. Smith, Chi-Ching Chen, Berndt B. Bruegger, Steven L. Holtz, Richard M. Halpern, and Roberts A. Smith*,‡

ABSTRACT: Two histone kinases, each of which catalyzes the transfer of the γ-phosphoryl group from ATP to a specific histone fraction forming acid-labile histone phosphates, have been partially purified from nuclei of Walker-256 carcinosarcoma cells. One of these enzymes preferentially phosphorylates histone IV (f2a1) at an optimum pH of 9.5 while the other preferentially phosphorylates histone I (f1) at an optimum pH of 6.5 [Smith, D. L., Bruegger, B. B., Halpern, R. M., and Smith, R. A. (1973), *Nature (London) 246,* 103]. Some of the properties of these two enzymes have been studied and compared. Both enzymes have an absolute requirement for $Mg^{2+}$, which could not be replaced by $Mn^{2+}$ or $Ca^{2+}$, and both enzymes showed similar saturation levels for ATP and their respective histone substrates. Neither enzyme was stimulated by the presence of added cyclic AMP or cyclic GMP. The pH 9.5 kinase was strongly inhibited by relatively low concentrations of GTP or CTP and was also moderately inhibited by dATP and dGTP, although incubation of the enzyme with [γ-$^{32}$P]GTP showed GTP to be a poor phosphoryl donor. The pH 6.5 kinase was specific for ATP and unaffected by other nucleoside triphosphates. Histone IV from which the 18 carboxy-terminal amino acids had been removed by treatment with CNBr could also be phosphorylated by the pH 9.5 kinase.

In a previous study we presented evidence for the existence of at least two new histone kinases in cell nuclei of several normal rat tissues and Walker-256 carcinosarcoma (Smith *et al.*, 1973). All tissues studied had one or both of these histone kinases. These enzymes were unique in that the reactions they catalyzed led to the formation of acid-labile phosphoprotein linkages rather than the acid-stable phosphomonoesters that were formed by previously studied histone kinases (for example, Walsh *et al.*, 1968; Langan, 1968). Acid-labile bonds would have been destroyed in enzyme studies using the conventional trichloroacetic acid assay procedure for histone kinase or in whole animal experiments using acids to extract the histones. We reported that one of the new enzymes had a pH optimum of 9.5 and phosphorylated histone IV (f2a1)[1] most extensively, whereas the other enzyme had a pH optimum of 6.5 and phosphorylated histone I (f1) most extensively. Further investigation of the phosphorylated products has shown that the pH 9.5 kinase phosphorylates both of the histidine groups in histone IV to form 3-phosphohistidine and that the pH 6.5 kinase phosphorylates lysine groups in histone I (Smith *et al.*, 1973; B. B. Bruegger *et al.*, manuscript in preparation).

Both of these histone kinases were present at relatively high levels in the nuclei of Walker-256 carcinosarcomas, so the purified enzymes from this rapidly dividing tissue were deemed suitable for characterization. The comparison of these enzymes is presented in this study.

## Materials and Methods

*[γ-$^{32}$P]ATP and [γ-$^{32}$P]GTP.* [γ-$^{32}$P]ATP was prepared (Glynn and Chappell, 1964) using carrier-free [$^{32}$P]orthophosphoric acid obtained from ICN Pharmaceuticals. The specific radioactivity of the [γ-$^{32}$P]ATP ranged from $3.1 \times 10^9$ to $9.5 \times 10^9$ cpm/μmol. [γ-$^{32}$P]GTP, with a specific radioactivity of $3.1 \times 10^{10}$ cpm/μmol, was obtained from New England Nuclear. Concentration of these compounds was determined by absorbance at 259 nm, and purity was ascertained by descending paper chromatography in ethanol–1 M ammonium acetate (7:3).

*Histones.* Whole histone and histone I (f1) were obtained from Sigma Chemical Co., and the mixture of histones IV (f2a1) and IIb1 (f2a2) from calf thymus was obtained from Sigma Chemical Co. or isolated from whole histone (Oliver *et*

† From the Departments of Chemistry and Medicine and the Molecular Biology Institute, University of California, Los Angeles, California 90024. *Received February 7, 1974.* This work was supported in part by U. S. Public Health Service Grant CA-13196, Biochemistry Research Training Grant GM-00463, and a grant from the Julius and Dorothy Fried Foundation. Chemistry Department Publication No. 3272.

‡ Department of Chemistry.

[1] Abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; pH 6.5 kinase, histone kinase which forms acid-labile phosphates at pH 6.5; pH 9.5 kinase, histone kinase which forms acid-labile phosphates at pH 9.5. Histone nomenclature used is that of Rasmussen *et al.* (1962). An alternative nomenclature (Johns and Butler, 1962) is shown in parentheses when used.