Protein-Protein Interactions in Blood Clotting

THE USE OF POLARIZATION OF FLUORESCENCE TO MEASURE THE DISSOCIATION OF PLASMA FACTOR XIIIa

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1. Human plasma Factor XIII (the precursor of fibrin-glutamine-fibrin-lysine endo- γ -glutamyltransferase) was randomly labelled by incubation with fluorescein isothiocyanate. The biochemical properties of the system were unaltered by the label. The polarization of the fluorescein fluorescence attached to the plasma protein was measured and the following conclusions were reached. 2. Factor XIII (α_2b_2) does not dissociate in the protein-concentration range 10–500 μ g/ml either with or without added Ca^{2+} . 3. Factor XIIIa (α'_2b_2) does not dissociate in the absence of Ca^{2+} in the protein-concentration range 10–500 μ g/ml. 4. Additions of Ca^{2+} to Factor XIIIa result in a decreased polarization of fluorescence as the tetramer dissociates. The decrease in polarization was the same amplitude at protein concentrations 10–500 μ g/ml and Ca^{2+} concentrations 2–66 mM and indicates that the overall process is essentially irreversible. The decrease in polarization consisted of fast and slow exponential phases. Both the rate of the fast phase and the proportion of the reaction it represented increased with Ca^{2+} concentration. 5. A comparison of the rate of dissociation measured by fluorescence polarization and the rate of appearance of enzyme activity in the presence of a protein substrate suggests that the Factor XIII is autoactivated by a soluble α -subunit-containing molecule forming a tight complex with the substrate.

In the preceding paper (Lewis *et al.*, 1978) we established that low concentration of Ca^{2+} will bind to human plasma-clotting Factor XIII. However, at such low concentrations there is a lag phase in the continuous fluorimetric assay for Factor XIII (Cooke & Holbrook, 1973, 1974a). This lag phase can be abolished if the Factor XIIIa is preincubated at very high concentrations of Ca^{2+} (30 mM) and then assayed at low Ca^{2+} concentration (Cooke, 1974). Thus, although the enzyme can be active at low Ca^{2+} concentrations, high Ca^{2+} concentrations are required to convert the initially inactive α'_2b_2 enzyme into its final active form.

In the present paper we investigate the state of aggregation of the active enzyme at concentrations within the range normally present in human plasma. We are presented with the problem of measuring the state of assembly of a protein at a physiological concentration that is 100-fold less than can be measured in the ultracentrifuge (Cooke & Holbrook, 1974b).

Weber (1953) pointed out that it is possible to

Abbreviation used: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

learn something of the state of assembly of protein molecules in solution by studying their Brownian motion. When such protein molecules are labelled with fluorescent dyes, the fluorescence of the dyes attached to the surface of proteins is polarized (Weber, 1952a,b). The partial polarization of the fluorescence depends on two factors, the interval between excitation and emission (the lifetime of the excited state) and the speed of rotation of the protein molecule. For a given dye in a given environment (at constant viscosity and temperature) the polarization of fluorescence increases with the size of the protein molecule. Shore & Chakrabarti (1976) have used the technique of polarization of fluorescence to study the state of assembly of mitochondrial malate dehydrogenase as a function of the protein concentration. Johnson & McIntosh (1976) have re-evaluated this technique and point out that the method can, in theory, give information on the association-dissociation of a protein molecule within the lifetime of the excited state of the attached dye. In the present paper we use changes in the polarization of fluorescence of fluorescein to investigate the protein complexes of Factor XIII.

Experimental

The preparation of Factor XIII and the sources of most chemicals were as described by Lewis *et al.* (1978).

Factor XIIIa was prepared by incubation of Factor XIII (8–10 mg/ml) with 15 N.I.H. units of thrombin/ml in 0.1 M-Tris/1 mM-EDTA adjusted to pH 7.5 (with 10 M-HCl) for 40 min at 25°C. After this time, the Factor XIIIa activity has attained a steady, maximum, value. Factor XIIIa was stored at 0°C for up to 8 h without any loss of activity.

Measurements of protein and fluorescein concentrations

Unlabelled protein concentrations were determined by absorbance measurements with a Pye-Unicam SP.1800 spectrophotometer. The $A_{280}^{1\%}$ used was 13.8 (Schwartz *et al.*, 1973). Fluorescein concentrations were determined by measuring the A_{490} , by using an absorption coefficient of 3.4×10^4 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ (Churchich, 1967). For fluorescein-labelled protein the A_{280} from fluorescein at pH 7.5 is about half of the A_{490} and this correction factor was applied for the determination of labelled protein concentrations by A_{280} . Labelled protein concentrations were also determined by the method of Lowry *et al.* (1951), and both methods gave about the same results.

Labelling of Factor XIII with fluorescein

Just before use a 15 mM stock solution was prepared by dissolving fluorescein isothiocyanate (BDH Chemicals, Poole, Dorset, U.K.) in acetone. Factor XIII (1 ml; 8 mg/ml) was dialysed against 500 ml of 0.1 M-Tris/1 mM-EDTA adjusted to pH 9.0 (with 10 M-HCl) for 12 h at 4°C. A 6-fold molar excess [with respect to the protein-subunits molarity, based on an average molecular weight of 80000 (Cooke & Holbrook, 1974b)] of fluorescein isothiocyanate was added to the protein solution at pH 9.0. After incubation, which varied from 30 to 90 min in the dark at room temperature, the labelling reaction was stopped by filtering the reaction mixture through a Sephadex G-50 (coarse grade) column (1 cm $\times$ 30 cm) equilibrated in 0.1 M-Tris/1 mM-EDTA adjusted to pH 7.5 with 10 M-HCl. Protein preparations labelled with fluorescein are referred to below as Factor XIII^F and Factor XIIIa^F. Labelled Factor XIII (Factor XIII^F) solutions of 2 mg/ml were routinely obtained in the protein peak eluted from the column. This solution was either dialysed for 12 h at 4°C against 200 vol. of 0.1 M-Tris/2 mM-EDTA containing 2% (w/v) active charcoal [Sigma (London) Chemical Co., London S.W.6, U.K.] adjusted to pH 7.5 (with 10 M-HCl) or, when necessary, the protein solution was adjusted to 40% saturation with solid (NH₄)₂SO₄ and the precipitate was

collected by centrifugation at 10000g for 20 min. This precipitate was resuspended into a small volume of pH 7.5 buffer and dialysed against 2×100 vol. of the above charcoal-containing buffer for 12 h at 4°C. Before use the solutions of labelled enzyme were dialysed against 500 vol. of 0.1 M-Tris adjusted to pH 7.5 (with 10 M-HCl) for 10 h at 4°C. The enzyme labelled at pH 9 with fluorescein had unchanged specific enzyme activity in the dansyl-cadaverine-casein assay. At pH 7.5 or 8.0 the enzyme activity was inhibited by the isothiocyanate under the more extreme conditions needed for appreciable labelling.

Labelled Factor XIIIa was obtained from Factor XIII^F exactly in the same way as Factor XIIIa is obtained from Factor XIII.

Analytical ultracentrifuge studies

The sedimentation of a protein sample was measured in a Spinco model E ultracentrifuge at 25°C by using a Ti An-F Rotor at 56100 rev./min in 12 mm 2° Kel-F cells, one with a +1° wedge upper window to separate images, by using schlieren optics. The positions of the peaks were measured from photographs at 8 min intervals by using an optical precision travelling microscope, and the uncorrected sedimentation coefficients were evaluated by using a standard program on a Wang 720B desk-top programmable calculator. The fluorescein-labelled Factor XIII at 10 mg/ml contained a major schlieren peak (9.0S, 80% of the total area), followed by a minor peak (6.1S, 20% of the total area). On addition of 33 mM-Ca²⁺ and incubation for only 1 h the area under the 9.0S peak decreased by 50% and that under the 6.1S peak increased by 50%.

Sodium dodecyl sulphate/polyacrylamide-gel slab electrophoresis

The procedure was that described by Cooke & Holbrook (1974b) except that we now use slab polyacrylamide gels of 8% (w/v) polyacrylamide (dimensions of the gel 13 cm $\times$ 13.5 cm $\times$ 0.2 cm). We did not routinely use a reducing agent in the sample preparation buffer, as this impairs the separation of the α - and β -subunits (Schwartz *et al.*, 1973).

Fluorescence polarization measurements

Fluorescein fluorescence was excited by using a Kodak Wratten filter no. 98 (maximum transmission at about 430 nm). Emission filters were Kodak Wratten gelatin filters no. 12 (maximum transmission from 540 nm). Excitation and emission polarizers were from Industrial Optics, Visual Products Division, 3M Centre, St. Paul, MN 55101, U.S.A. Filtered excitation light is conducted by a light-guide (Faseroptik, Schott-Mainz, Germany) from the light-source (XBO 250 Xe arc; Osram G.m.b.H., Berlin, Germany) to the excitation polarizer and then to the

1 cm × 1 cm quartz fluorescence cuvette. The contents of the cuvette were stirred constantly, allowing us to add reagents with a micro-syringe without optical or electrical perturbation of the measurement system. The data were stored (see Fig. 1) in one channel of a dual-channel recycling digital store (Transidyne General Corp., Ann Arbor, MI, U.S.A.) as 1024 10-bit words. After the experiment signals from store are reconverted into voltages every 20 ms and displayed on an oscilloscope. Between the store and the oscilloscope it is possible to interpose simple analogue circuits (*a*) to display log(amount remaining) to analyse first-order kinetics and (*b*) to stimulate and subtract an exponential phase (when a reaction is biphasic).

Promising preliminary polarization experiments were made with the instrument of Shore & Chakrabarti (1976). All the results reported in the present paper were obtained with a similar, but simpler, instrument constructed in Bristol and described in Fig. 1. The degree of polarization $P = (I_{||} - I_{\perp}) / (I_{||} + I_{\perp})$ where $I_{||}$ and $I_{\perp}$ are the intensities of fluorescence transmitted when the analysing polarizers are respectively parallel and perpendicular to the plane of the exciting light) was calculated from the ratio $R = I_{||} / I_{\perp}$ when required. In the experiments reported here the maximum relative change in polarization ($\Delta P / P_0$) was 8%, and this corresponds to a relative change in ratio ($\Delta R / R_0$) of 4%. (P_0 and R_0 are the initial values of P and R as defined above.)

With such small changes $d(\Delta R) = \frac{-2}{(1+P_0)^2} d(\Delta P)$.

Since $(1+P_0)^2$ is a constant, $d(\Delta R)/dt$ is directly proportional to $d(\Delta P)/dt$ and the same kinetic analysis may be applied to either parameter. All the experiments described here have been performed at 25°C in 0.1 M-Tris adjusted to pH 7.5 with 10 M-HCl. After addition of various reagents the final volume in the fluorescence cuvette was 3 ml.

Results

Properties of fluorescein-labelled Factor XIII

The determination of the amount of fluorescein bound per subunit molecule of Factor XIII revealed an average of 0.25 mol of fluorescein bound per mol of subunit (mol.wt. 80 000) when the incubation time of Factor XIII and label was 30 min, and 1 mol of fluorescein bound per mol of subunit when the standard incubation time was 75 min. Although these estimates will not be very accurate, they are sufficiently precise to ensure that the protein was not so over-labelled as to give intramolecular energy transfer (Weber & Young, 1964).

Distribution of fluorescein among α - and β -subunits

To interpret our experiments it was important to discover whether the fluorescein label was randomly

Fig. 1. The simple instrument used to measure polarization of fluorescence

The contents of the cuvette were excited with light at about 430 nm selected by a Kodak Wratten filter 98. The exciting light was polarized in the horizontal plane and the balance control used to equalize the response of the two photomultipliers. The exciting polaroid was rotated through 90° and vertically polarized light was then used for the measurement of the ratio of horizontally to vertically polarized fluorescence (H/V). The fluorescence was selected by a gelatine filter, F (Kodak Wratten no. 12). The apparatus was maintained at 25°C.

distributed between *a*- and *b*-subunits or whether one kind of subunit was preferentially labelled. Fluorescein-labelled Factor XIIIa (8 mg/ml) was incubated with 50 mM- Ca^{2+} for 12 h. Under these conditions all the *a'*-subunits are precipitated (Cooke & Holbrook, 1974b); the supernatant contains only *b*-subunits. The supernatant, separated from a yellow-green precipitate by centrifugation at 12000g for 10 min, contained about 50% of the A_{490} of the original Factor XIIIa^F. This showed that 50% of the label was on the *b*-subunits, and consequently the other half was bound to the precipitated *a'*-subunits. When an unstained sodium dodecyl sulphate/polyacrylamide slab electrophoresis gel was viewed in the dark with a hand u.v. lamp, two bands of approximately equal fluorescence were seen at positions that subsequently stained with Coomassie Blue for the *a*- and *b*-subunits. This again suggests that the fluorescein is randomly distributed on both kinds of subunit in the tetramer.

Preliminary polarization of fluorescence measurements

The degree of polarization of fluorescence (*P*) obtained for Factor XIII^F was 0.25. This value remained unchanged over a protein concentration range 10–500 µg/ml and over a range of labelling 0.25–1 molecule of dye per molecule of subunit. Below an average of 0.25 molecule of dye per molecule of subunit of Factor XIII^F the ratio of signal/noise was decreased and light-scattering was a source of error. Above an average of 1.2 molecules of dye per molecule of subunit the *P* value obtained for Factor XIII^F was generally smaller than 0.25; such an observation is characteristic of over-labelling, because under such conditions energy transfer could take place between chromophores on the same protein molecule surface, and this results in a decrease in the degree of polarization of fluorescence (Weber & Young, 1964). After these preliminary measurements we chose to label Factor XIII with an average of 1 molecule of fluorescein per molecule of subunit to obtain the best signal/noise ratio at low protein concentrations and to avoid energy transfer between fluorophores.

In the presence of 33 mM- Ca^{2+} the value of the degree of polarization of fluorescence for Factor XIII^F over a protein concentration range of 10–500 µg/ml remained constant at 0.25; for Factor XIIIa^F in this same protein-concentration range but in the absence of Ca^{2+} and in the presence of 1 mM-EDTA it was also 0.25.

The degree of polarization measured for the b_2^F -subunits, which are in solution in the supernatant after the precipitation of the *a'*^F-subunits, was 0.23 in the protein-concentration range 10–200 µg/ml in the presence or absence of 33 mM- Ca^{2+} .

Dissociation of Factor XIIIa^F in the presence of Ca^{2+}

Fig. 2 is an example of the use of polarization of fluorescence. At the start of the experiment Factor XIII^F (40 µg/ml) has a degree of polarization of fluorescence *P* = 0.25. If this solution is pre-incubated for 2000 s (or for hours in other experiments) in the presence of 33 mM- Ca^{2+} , there is no change in the *P* value. As soon as we introduce thrombin (26.6 N.I.H. units/ml) we initially observe a rapid but small increase in the degree of polarization immediately followed by a larger but slow decrease in the *P* value from 0.253 to 0.234. Such experiments suggest that neither the local environment of the dye in Factor XIII nor the fluorescence lifetime is affected by the presence of Ca^{2+} . The viscosity and the temperature are constant in these experiments and cannot be responsible for the degree of polarization decrease. We thus interpret the decrease in the degree of polarization of fluorescence as being the result of a dissociation of the Factor XIII molecule into subunits *a'*_m^F. As shown in the experiments described below, 33 mM- Ca^{2+} will cause Factor XIIIa^F to dissociate as soon as it is formed by thrombin cleavage. The rate observed is the rate at which thrombin converts Factor XIII^F into Factor XIIIa^F and shows the typical initial zero-order kinetics of the action of an enzyme on its substrate at saturation.

Kinetics of the Ca^{2+} -induced dissociation of Factor XIIIa^F

In two series of experiments the Factor XIIIa^F concentrations were 16 µg/ml and 33 µg/ml, and the

Fig. 2. Decrease in polarization of fluorescein fluorescence when Factor XIII^F is cleaved by thrombin in the presence of a high concentration of Ca^{2+}

Fluorescein-labelled Factor XIII (40 µg/ml, 3 ml) was incubated at 25.0°C. At the first arrow shown, CaCl_2 was added to 33 mM. The small change in polarization is a mixing artifact. At the second arrow the cleavage of Factor XIII to XIIIa was initiated by the addition of thrombin (26.6 N.I.H. units/ml, of a sample which was 95 N.I.H. units/mg of protein). The initial small increase in polarization was reproducible. The results of the experiment were displayed on an oscilloscope.

Fig. 3. Analysis of a reaction in which fluorescein-labelled Factor XIIIa (33 µg/ml) was induced to dissociate by the presence of CaCl₂ (16.5 mM)

(a) Original trace of polarization of fluorescence against time. (b) Original trace, a log plot of the slow phase (1 decade = 2 divisions), and the end point of the reaction. (c) Fast phase and its log plot (1 decade = 1 division) after the corresponding and simulated slow phase has been subtracted from the original reaction.

Fig. 4. Variations of the apparent rate constants (k) of the fast phase and the slow phase in the decrease of polarization of fluorescence of Factor XIIIa^F with Ca²⁺

Results are given for two protein concentrations (Δ, ▲, Factor XIIIa^F 16 µg/ml; ○, ●, Factor XIIIa^F 33 µg/ml) as a function of Ca²⁺ concentration. The filled symbols are the slow phase (right-hand scale); the open symbols are the fast phase (left-hand scale). Experiments were similar to Fig. 3.

variations in the rate of decreased polarization of fluorescence due to the dissociation with added Ca²⁺ were measured. Fig. 3(a) shows a typical result with 33 µg of Factor XIIIa^F/ml in the presence of 16.5 mM-Ca²⁺. The dissociation is biphasic. Fig. 3(b) shows that the second, slower, phase can be represented as an exponential decrease. Fig. 3(c) shows

that when the slow second exponential phase is subtracted from the original there remains an initial rapid exponential decrease. We were routinely able to analyse the biphasic decrease in polarization as rapid and slow exponential phases over a wide range of protein and Ca²⁺ concentrations. At low Ca²⁺ and high protein concentrations, where long incubation times were needed, the system was not stable enough to evaluate whether a third very slow linear decrease in polarization was present. We were able to measure the respective apparent rate constants of the fast and slow phases and their respective relative amplitudes at various protein concentrations and over a range of Ca²⁺ concentrations of 4–50 mM. Control experiments revealed that Mg²⁺ does not induce the dissociation of Factor XIIIa^F and neither is it able to induce the appearance of the catalytic activity of Factor XIIIa (Cooke *et al.*, 1974; Lewis *et al.*, 1978). Thus the dissociation does not seem to be wholly a simple ionic-strength effect. Fig. 4 represents the variations of the apparent rate constants of the fast and slow phases at two protein concentrations in the range 4–50 mM-Ca²⁺. Table 1 shows that the rate constant of the fast phase decreases slightly at a given Ca²⁺ concentration if we increase the protein concentration; the contrary is observed for the rate constant of the slow phase.

In all these measurements the important observed fact was that the overall degree of polarization of fluorescence change is constant at about $\Delta P = -0.02$ over a protein concentration range 10–500 µg/ml and a Ca²⁺ concentration range 4–50 mM. The relative amplitudes of the fast and slow phases of the decrease in the degree of polarization vary with the Ca²⁺ concentrations; Fig. 5 shows that at 50 mM-Ca²⁺ the reaction is about 85–90% fast phase and at 4 mM-Ca²⁺ the reaction is about 85–90% slow phase.

Table 1. Variations of the apparent first-order rate constants for depolarization of Factor XIIIa^F fluorescence

The rate constants for the fast and slow phases at 25 mM-Ca²⁺ in a protein concentration range 8–66 µg/ml were measured from experiments similar to that shown in Fig. 3.

Rate constants	[Protein] (µg/ml)	...	8	16	25	33	50	66
k_{fast} (min ⁻¹)			6.0	4.6	3.9	3.45	2.75	2.10
k_{slow} (min ⁻¹)			0.48	0.66	0.73	0.78	0.90	1.00

Fig. 5. Relative amplitudes of the fast (○) and slow (△) phases in the Ca²⁺-induced depolarization of Factor XIIIa^F fluorescence

The protein concentration was 33 µg/ml. At all Ca²⁺ concentrations the overall polarization change was constant at about $\Delta P = -0.02$. Experiments were those from Fig. 4.

Thrombin–Factor XIII complex

Careful examination of Fig. 2 shows that on adding thrombin to Factor XIII there is a small, but reproducible, increase in polarization before the dissociation reaction. This would be in agreement with the formation of a high-molecular-weight enzyme–substrate complex. When the same experiment was performed in the absence of Ca²⁺ and in 1 mM-EDTA there was also a small increase in polarization (Fig. 6). The magnitude of the increase with 40 µg of Factor XIII/ml was proportional to the amount of thrombin: 13.3 N.I.H. units of thrombin/ml gave $\Delta P = +0.0015$; 26.6 N.I.H. units/ml gave $\Delta P = +0.003$ and 40 N.I.H. units/ml gave $\Delta P = +0.0045$. Over 10 min, when the Factor XIII^F had been converted into Factor XIIIa^F, the initially increased polarization decreased to its starting value. Less than 10% of the protein in the thrombin preparation was active enzyme.

Factor XIIIa^F–protein substrate complex monitored by polarization of fluorescence

At the end of a dissociation reaction of Factor XIIIa^F (0.1 mg/ml in the presence of 16 mM-Ca²⁺) we introduced casein substrate (0.75 mg/ml). After the addition of substrate, P increased from 0.23 to 0.26 over 2–3 min. This indicated the formation of a complex. No change in P value was observed when Factor XIIIa^F (0.1 mg/ml) and substrate (0.75 mg/ml) were incubated in the absence of Ca²⁺. Even with this control experiment, it is difficult to draw precise conclusions, because of the uncertainty about the changed degree of aggregation of casein in the presence of high concentrations of Ca²⁺.

Being a fluorescence technique, the method of polarization is very sensitive and can be used at physiological protein concentrations to obtain kinetic information about the changes in the state of assembly of Factor XIIIa^F in the presence of Ca²⁺. In previous experiments on Factor XIII (e.g. Cooke, 1974) it was assumed that the uncleaved factor was a tetramer, not only at 5 mg/ml, where its properties were examined in the ultracentrifuge, but also at 10–20 µg/ml, the concentration at which it occurs in the human plasma. The assumption that the tetramer does not dissociate is confirmed by the unchanged polarization of fluorescence measured for Factor XIII^F with or without added Ca²⁺ over the protein-concentration range 10–500 µg/ml. The polarization of fluorescence of fluorescein-labelled Factor XIIIa in EDTA is also constant over a wide protein-concentration range. Thus this molecule, which at high concentrations is also a tetramer, does not appreciably dissociate on dilution.

Discussion

The labelling of a protein by a fluorescent dye did not modify its catalytic activity or its general sedimentation behaviour. All our control experiments

Fig. 6. Effect of thrombin on the polarization of fluorescein fluorescence of Factor XIII^F

When thrombin (40 N.I.H. units/ml) was added to labelled Factor XIII^F (40 µg/ml), there was a rapid increase in polarization followed by a slow decrease towards the starting value. The time course of the decrease was similar to the time needed to convert Factor XIII into Factor XIIIa. The buffer was 0.1 M-Tris/1 mM-EDTA adjusted to pH 7.5 with 10 M-HCl.

and measurements have led us to conclude that the changes in polarization observed with Ca^{2+} reflect changes in protein state of assembly rather than modified properties of the dye (Johnson & McIntosh, 1976). Fluorescein could produce polarization changes by resonance energy transfer, but in this present case the polarization changes are in the wrong direction for this to be a possible explanation. Fluorescein is not the ideal fluorescent probe for Factor XIII; its fluorescence is very sensitive to pH and the lifetime of its excited state is only about 4.8 ms. With improved instrumentation it would be desirable to use other dyes.

Addition of a high concentration of Ca^{2+} to Factor XIIIa^F initiates a biphasic decrease in polarization of fluorescein fluorescence. At high protein concentrations one of the products of the dissociation is b_2 -subunits (Cooke & Holbrook, 1974b); the state of aggregation of the a' -subunits which remain is uncertain. At low protein concentrations, where there is no formation of a visible precipitate, the solution of mixed subunits has the same polarization as one of b_2^F ($P = 0.23$) and this could be taken as weak evidence that the a'^F -subunits were also dimeric. This conclusion would be more convincing had our apparatus enabled the same experiment to be performed with a range of dyes of different lifetimes.

The overall process induced when Ca^{2+} is added to Factor XIIIa is essentially irreversible at the protein concentrations that we have studied. This conclusion stems from the observation that the overall amplitude of the change in the polarization of fluorescence is the same at all protein and Ca^{2+} concentrations. Thus some step(s) in the mechanism, subsequent to the cleavage of Factor XIII by thrombin, must be irreversible in the presence of Ca^{2+} . From previous experiments at high protein and Ca^{2+} concentrations

it is known (Cooke & Holbrook, 1974b) that the formation of the a' -subunit precipitate is largely irreversible. However, this step alone cannot be the only reversible step, as the irreversibility persists at much lower protein concentrations, where there is no visible aggregation. For this reason we shall include an irreversible but uncharacterized rearrangement of soluble a' -subunit protein in the mechanism ($a'_2 \rightarrow a'_m$). We have not yet determined whether the 'irreversible' step can be reversed by removal of Ca^{2+} with EDTA. As would be expected, mixing an impure thrombin preparation with Factor XIII resulted in the increase in polarization expected for a thrombin-Factor XIII enzyme-substrate complex. The observation that the increased polarization was abolished as Factor XIII was converted into Factor XIIIa suggests that the activated factor does not bind to thrombin. This leads to a speculation that much of the thrombin-recognition site is on the 36-residue (Takagi & Doolittle, 1974) activation peptide. Lack of a complex with Factor XIIIa would be supported by the observation that prolonged incubation of Factor XIIIa with thrombin does not result in further digestion of Factor XIIIa.

An advantage of the polarization-of-fluorescence technique is that the kinetics of dissociation can be directly compared with kinetics of the lag phase observed in the appearance of enzyme activity at low concentrations of Ca^{2+} (Cooke & Holbrook, 1973; Cooke, 1974). We observe that at 0.2 mM- Ca^{2+} in the continuous assay medium (10 μg of Factor XIIIa/ml) the rate of the amine incorporation into casein has reached a maximum value after a lag phase of about 5 min. Yet at 0.2 mM- Ca^{2+} , without substrates, there is no detectable dissociation of Factor XIIIa^F in 10 min (Fig. 4). We can explain this contradiction in rates by postulating that the initial reversible equilibrium that is established when 0.2 mM- Ca^{2+}

Scheme 1. Protein complexes that occur during the activation of Factor XIII by thrombin, calcium and substrate (S). The transformation of a'_2 into a'_m and a'_n is essentially irreversible at the protein and calcium concentrations studied. a'_m and a'_n are defined in the text.

is added to Factor XIIIa is heavily in favour of tetramer and that it is pulled to the right when the large protein substrate combines with α_2 -subunits (and this prevents α_2 -subunits recombining with β_2). Scheme 1 rationalizes our present information about the mechanism.

The technique of polarization of fluorescence has provided useful kinetic and equilibrium information about the protein-protein interactions involved in the generation of enzyme activity from human plasma Factor XIII. We suggest that, if planned carefully, polarization experiments will give useful information about other protein-assembly systems at the protein concentrations at which biological activity is expressed.

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References

- Churchich, J. E. (1967) *Biochim. Biophys. Acta* **147**, 511-517
- Cooke, R. D. (1974) *Biochem. J.* **141**, 683-691
- Cooke, R. D. & Holbrook, J. J. (1973) *Biochem. Soc. Trans.* **1**, 1198-1200
- Cooke, R. D. & Holbrook, J. J. (1974a) *Biochem. J.* **141**, 71-78
- Cooke, R. D. & Holbrook, J. J. (1974b) *Biochem. J.* **141**, 79-84
- Cooke, R. D., Pestell, T. C. & Holbrook, J. J. (1974) *Biochem. J.* **141**, 675-682
- Johnson, P. & McIntosh, R. P. (1976) *Biochim. Biophys. Acta* **453**, 521-532
- Lewis, B. A., Freyssinet, J.-M. & Holbrook, J. J. (1978) *Biochem. J.* **169**, 397-402
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Schwartz, M. L., Pizzo, S. V., Hill, R. L. & McKee, P. A. (1973) *J. Biol. Chem.* **248**, 1395-1407
- Shore, J. D. & Chakrabarti, S. K. (1976) *Biochemistry* **15**, 875-879
- Takagi, T. & Doolittle, R. F. (1974) *Biochemistry* **13**, 750-756
- Weber, G. (1952a) *Biochem. J.* **51**, 145-155
- Weber, G. (1952b) *Biochem. J.* **51**, 155-167
- Weber, G. (1953) *Adv. Protein Chem.* **8**, 415-459
- Weber, G. & Young, L. B. (1964) *J. Biol. Chem.* **239**, 1415-1423