Association of the A Subunits of Recombinant Placental Factor XIII with the Native Carrier B Subunits from Human Plasma†

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ABSTRACT: Interactions of a recombinant human placental protein (rA_2) expressed in yeast and considered to be identical to the catalytic A_2 subunits of factor XIII, the fibrin stabilizing factor zymogen, were examined with the native carrier subunits (B_2) of the factor isolated from human plasma. Nondenaturing electrophoresis and HPLC gel-filtration experiments showed a tight binding of rA_2 to B_2 for forming an ensemble similar to that of plasma factor XIII (A_2B_2). In the presence of excess B_2, however, some higher ordered oligomers (rA_2B_n, where $n > 2$) were also seen in electrophoresis. The same technique revealed a microheterogeneity in the rA_2 preparation; nevertheless, all isoforms could bind to B_2. By employing an ELISA procedure for measuring free B_2 in mixtures with rA_2, an apparent binding constant of 4×10^7 M^{-1} was derived for the association of rA_2 with B_2. Fluorescence depolarization was used to monitor the heterologous association of rA_2 with fluorescein-labeled B_2^F as well as the dissociation of the $rA_2B_2^F$ structure. The former was characterized by an increase, and the latter by a decrease, in the fluorescence anisotropy of the system. Binding of rA_2 to B_2^F (pH 7.5, $\mu = 0.315$, 37 °C) was not influenced by low concentrations of Ca^{2+} ($\leq$30 mM), and $rA_2B_2^F$ proved to be quite stable under these conditions. Much higher concentrations of Ca^{2+}, as well as higher ionic strengths, were required to dissociate this assembly. By contrast, release of B_2^F from the thrombin-modified $rA_2'B_2^F$ occurred rapidly in the presence of low concentrations of Ca^{2+} at low ionic strength. An apparent binding constant of 5.3×10^7 M^{-1} was obtained for the association of rA_2 with B_2^F. A considerably lower value of 3.4×10^6 M^{-1} was measured for the association of thrombin-activated rA_2' with B_2^F, indicating that the thrombin-catalyzed removal of the activation peptide from rA_2 weakens the strength of heterologous association to the carrier subunits by about 2 kcal/mol. The release of B_2^F subunits from the thrombin-modified $rA_2'B_2^F$ ensemble could also be readily elicited with inclusion of monomeric fibrin. Activation by fibrin is attributed to the fact that thrombin-modified $rA_2'^F$ binds to monomeric fibrin ($K_{assoc} = 0.4 \times 10^6$ M^{-1}).

Blood coagulation factor XIII or fibrin stabilizing factor (for a review, see Lorand et al. (1980)) is the plasma zymogen of the enzyme responsible for strengthening the clot network (Roberts et al., 1973; Mockros et al., 1974; Shen et al., 1974; Shen & Lorand, 1983) and for endowing it with a higher resistance to lysis (Lorand & Jacobsen, 1962; Bruner-Lorand et al., 1966; Lorand & Nilsson, 1972). Under physiological conditions, activation of the zymogen is brought about by thrombin and Ca^{2+} (Lorand & Konishi, 1964). Generation of the enzyme (denoted in the literature as factor $XIII_a$ or XIIIa), a transamidase, occurs in two consecutive steps readily resolvable in the test tube into a Ca^{2+}-independent hydrolysis by thrombin followed by a Ca^{2+}-specific, but thrombin-independent, change of the proteolytically cleaved factor XIII′ intermediate:

$$\text{XIII} \xrightarrow{\text{thrombin}} \text{XIII}' \xrightarrow{Ca^{2+}} \text{XIIIa}$$

Factor XIII is made up of two different subunits in an AB protomeric structure thought to associate into an A_2B_2 tetramer (Schwartz et al., 1971, 1973; Bohn, 1972; Carrell et al., 1989). Only the A subunits are modified by thrombin, releasing an N-terminal activation peptide (AP). The primary cleavage site in the A subunit (Mikuni et al., 1973; Nakamura et al., 1974; Takagi & Doolittle, 1974) is at Arg-37; secondary cleavage sites for thrombin were reported (Takahashi et al., 1986) at Lys-513 and Ser-514. Both α- and γ-thrombin can activate factor XIII, but the specificity constant for the release of AP with γ-thrombin is about 5 times lower than with α-thrombin (Lewis et al., 1987).

The hydrolytically cleaved A subunit, denoted as A′, carries the active site cysteine necessary for the expression of the transamidating activity. However, in the heterologous $A_2'B_2$ combination of factor XIII′, the critical cysteine residues are still buried and unmasking requires Ca^{2+} ions (Curtis et al., 1973, 1974; Lorand et al., 1974). The effect of Ca^{2+} is 2-fold; first, it brings about the heterologous dissociation of $A_2'B_2$, and then it promotes a conformational change in the separated A_2' species with induction of the enzymatically active configuration (designated as A_2^*):

$$A_2'B_2 \xrightarrow{Ca^{2+}} A_2' + B_2 \xrightarrow{Ca^{2+}} A_2^*$$

Thus, the physiological conversion of factor XIII to XIIIa may be represented as

$$A_2B_2 \xrightarrow{\text{thrombin}} A_2'B_2 + 2AP \xrightarrow{Ca^{2+}} A_2' + B_2 \xrightarrow{Ca^{2+}} A_2^*$$

Human platelets and placenta contain a protein thought to be identical to the A_2 subunits of plasma factor XIII (Schwartz et al., 1971, 1973; Bohn, 1972). This protein too can be

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activated by thrombin and Ca^{2+} to cross-link fibrin (Buluk et al., 1961), but its physiological function remains unclear. Also, its activation kinetics is quite different from that of plasma factor XIII. However, upon combination with the B_2 subunits purified from plasma, the activation properties become indistinguishable from those of the A_2B_2 ensemble of plasma factor XIII (Lorand et al., 1974).

The amino acid and cDNA sequences for the human placental A_2 protein are known (Grundmann et al., 1986; Ichinose et al., 1986; Takahashi et al., 1986), and recombinant products expressed in yeast were obtained by several investigators (Bishop et al., 1990; Jagadeeswaran & Haas, 1990; Rinas et al, 1990). Such a material was kindly furnished to us by Dr. Paul Bishop of ZymoGenetics (Seattle, WA). We developed various procedures for probing the interaction of the recombinant protein (rA_2) with the native B_2 carrier protein isolated from human plasma (Lorand et al., 1981). The measurements are all the more important because they represent the first set of data for quantitatively evaluating the heterologous interaction of factor XIII subunits. Our results indicate a very tight binding between rA_2 and B_2 in forming the rA_2B_2 structure and also show that cleavage of rA_2 by thrombin greatly weakens the heterologous association of subunits.

Some of these experiments were described at meetings of FASEB and the Biophysical Society (Radek et al., 1990; Radek & Lorand, 1992).

MATERIALS AND METHODS

Human factor XIII was purified from outdated blood bank plasma (Lorand & Gotoh, 1970; Curtis & Lorand, 1976; Lorand et al., 1981). The recombinant protein rA_2 (Bishop et al., 1990) was stored at 4 °C in a buffer of 75 mM Tris-HCl, 1 mM glycine, 0.5 mM EDTA, and 0.2% sucrose, pH 7.5. The carrier B_2 subunits were isolated from human plasma according to Lorand et al. (1981). Human α-thrombin (a gift of Dr. J. W. Fenton, II of the New York State Department of Health, Albany, NY) was stored in 75 mM Tris-HCl, pH 7.5 (500 NIH units/mL). Thromstop was purchased from American Diagnostica Inc. (Greenwich, CT). Human fibrinogen was obtained as a gift from A. B. Kabi (lot 65036). One gram, dissolved in 50 mL of 75 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 1 mM EDTA, was dialyzed (2 × 2 L, overnight at 4 °C) in the same buffer and stored at −20 °C before use. The GPRP tetrapeptide was purchased from Oz Chemicals Ltd. (Jerusalem, Israel). Protein concentrations (Lowry et al., 1951) are expressed in terms of a Pierce Chemical Co. (Rockford, IL) bovine serum albumin standard.

Activation of rA_2 (200 μg in 180 μL) to rA_2' was carried out by addition of 28 NIH units of human thrombin (56 μL) for 5 min at 37 °C followed by Thromstop to 9 μM (13.5 μL). In parallel experiments (Figure 5), rA_2 was similarly diluted with 75 mM Tris-HCl, pH 7.5, and Thromstop without prior addition of thrombin.

Nondenaturing electrophoresis was performed at 15 °C employing the PhastSystem (Pharmacia, Uppsala, Sweden, Separation Technique File No. 120), using gels with a continuous gradient from 8 to 25% and 2% cross-linking. The gel buffer was 0.112 M Tris at pH 6.4; the upper and lower buffer strips were 2% agarose in 0.88 M L-alanine and 0.25 M Tris, pH 8.8. Samples were prepared in 2.5 mM glycine, 1.3 mM EDTA, and 0.5% sucrose at pH 7.6, with 0.01% bromophenol blue added. Separations were obtained with approximately 6 mA of current (after 325 V·h). Mixtures of rA_2 and B_2 subunits were incubated for 15 min at room temperature prior to application to the gels.

HPLC gel-filtration was carried out by using two 0.75 × 30 cm TSK-3000 SW columns in series, equipped with a 0.75 × 10 cm GSWP guard column (Beckman Industries, Berkeley, CA). Samples (5–10 μL) were injected through a Beckman 210 injector, and elution was performed at 0.8 mL/min with a buffer of 20 mM sodium acetate and 0.15 M sodium chloride at pH 6.5 using a LDC minipump (Rainin Instrument Co., Woburn, MA). The effluent was monitored at 220 nm with a Waters Associates 450 variable-wavelength detector (Milford, MA) connected to a Hewlett-Packard 3390A reporting integrator (Palo Alto, CA) set to an attenuation of 6. The premixed samples were incubated for 35 min at room temperature prior to analysis.

Peaks which eluted from the HPLC column were collected and analyzed by SDS–PAGE. Samples were first dialyzed against three changes of water for 15 h at 4 °C and then brought to dryness with a Speed Vac concentrator (Savant Instruments, Inc., Hicksville, NY). Electrophoresis was performed in the PhastSystem (Pharmacia Separation Technique File No. 110) using gels with a continuous gradient from 10 to 15% and 2% cross-linking. The gel buffer was 0.112 M sodium acetate and 0.112 M Tris, pH 6.4, while the upper and lower buffer strips were 2% agarose containing 0.2 M tricine, 0.2 M Tris (pH 7.5), and 0.55% SDS. Samples were prepared in 10 mM Tris-HCl and 1 mM EDTA, pH 8.0, with 2.5% SDS and were heated to 100 °C for 10 min prior to application to the gel. Electrophoretic separations were carried out at about 10 mA (70 V·h). The gel was silver stained for protein using the photochemical procedure of Oakley et al. (1980) and the Bio-Rad Silver Stain Kit (Bio-Rad, Richmond, CA).

Immunological Procedures. For *immunoblotting*, various amounts of rA_2 (0–1700 ng) were spotted onto a 0.6 × 9.5 cm nitrocellulose sheet (BA83, 0.2-μm pore, Schleicher & Schuell, Keene, NH). Prior to overlaying with B_2 subunits, the sheet was immersed for two periods of 20 min in 4 mL of 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS; 10 mM sodium phosphate buffer plus 140 mM NaCl, pH 7.4). It was then incubated for 1 h with a 3-mL solution of 0.5% BSA in PBS containing 30 μg of B_2. This was followed by washing in 4 mL of 0.5% BSA in PBS (3 × 10 min). Bound B_2 was assayed with a polyclonal rabbit antibody (IgG fraction) raised against B_2. Dilutions of this antibody (in 4 mL of PBS with 0.5% BSA) were applied overnight, and the sheet was then washed with 3 × 4 mL of 0.5% BSA in PBS (10 min each time). An anti-rabbit IgG–alkaline phosphatase-conjugated goat IgG (Promega, Madison, WI) was used as secondary antibody (1:5000 in 4 mL of PBS with 0.5% BSA, incubated for 1 h). The paper was washed 3 × 10 min with BSA–PBS and rinsed with alkaline phosphatase buffer (100 mM Tris-HCl, 100 mM NaCl, and 5 mM $MgCl_2$, pH 9.5). Color development (Blake et al., 1984) was initiated by immersion in 0.37 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.39 mM nitro blue tetrazolium in alkaline phosphatase buffer and was stopped by placing the sheet in water.

For *ELISA*, the wells of a microtiter plate (Schleicher & Schuell) were coated with rA_2 (1 μg in 100 μL of PBS) for 2 h at room temperature on an orbit shaker (Lab Line, Melrose Park, IL). Simultaneously, B_2 (300 ng in 50 μL of PBS) was mixed with 50 μL of 0–500 ng of rA_2 and incubated for 30 min at room temperature on the orbit shaker. Each mixture B_2 with rA_2 was diluted with 900 μL of 0.5% BSA in PBS,

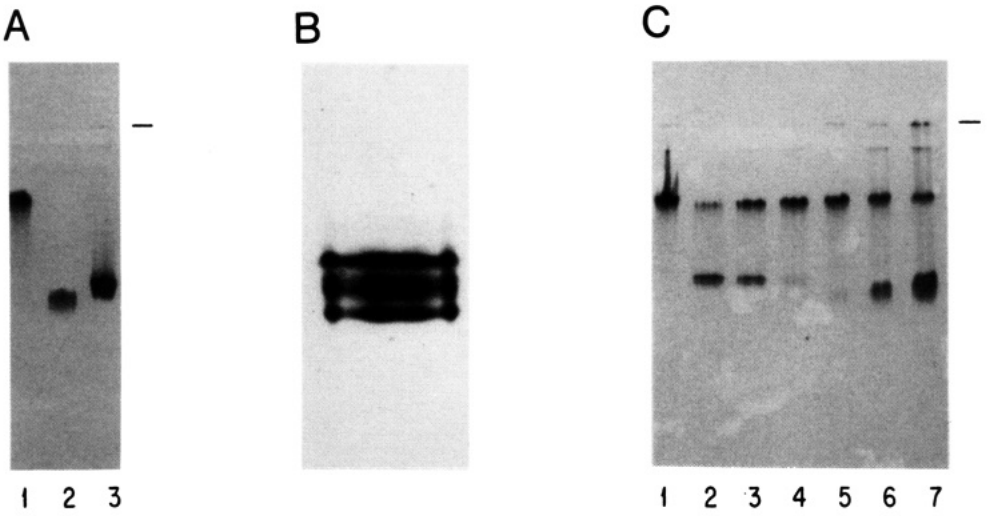

FIGURE 1: Association of the recombinant protein rA_2 with its native B_2 carrier from plasma studied by nondenaturing electrophoresis: (A) lane 1, reference plasma factor XIII (2.4 pmol); lane 2, reference B_2 protein purified from human plasma (1.5 pmol); lane 3, rA_2 (ZymoGenetics, Seattle, WA; 1.5 pmol); (B) enlargement of the band seen with specimens of rA_2 showing evidence of microheterogeneity for the recombinant product; (C) lane 1, reference human plasma factor XIII (2.4 pmol); lanes 2–7, analysis of mixtures of 1.5 pmol of rA_2 with varying amounts of B_2 of 0.4, 0.8, 1.0, 1.5, 3.0, and 6.0 pmol, respectively. Formation of the rA_2B_2 structural equivalent of factor XIII seems to be optimal in lanes 4 and 5. In addition, higher ordered complexes (rA_2B_n, where $n > 2$) form in the presence of excess B_2 (lanes 6 and 7).

and 100-μL aliquots were applied to the rA_2-coated wells. After 1 h, the wells were washed (3 × 5 min) with 200 μL of 0.5% BSA in PBS. Rabbit antibody to B_2 subunit was diluted 1:2 000 000 with 0.5% BSA in PBS, and 200 μL of the diluted antibody was added to each well. The plate was incubated for 1 h and washed (3 × 5 min) with 0.5% BSA in PBS. This was followed by a 1-h incubation (200 μL per well) with a goat anti-rabbit IgG–alkaline phosphatase conjugate diluted 1:5000 with 0.5% BSA in PBS. The plate was washed (2 × 5 min) with 0.5% BSA in PBS and rinsed with alkaline phosphatase buffer (100 mM Tris-HCl/100 mM NaCl/5 mM $MgCl_2$, pH 9.5; 200 μL per well) (Blake et al., 1984). The *p*-nitrophenyl phosphate substrate (100 μL) (Sigma, St. Louis, MO; dissolved to 1 mg/mL in the alkaline phosphatase buffer) was added to each well. After 40 min at room temperature, absorbancy was measured at 410 nm on a Dynatech plate reader (Chantilly, VA). Wells with 0.5% BSA in PBS instead of rA_2B_2 mixture were used as blanks.

Fluorescence Depolarization. The rA_2 and B_2 subunits (200–300 μg) were dialyzed separately against 100 mM Tris-HCl and 1 mM EDTA at pH 9.0 overnight at 4 °C. A 6-fold molar excess of FITC (Sigma Chemical Co., St. Louis, MO) was allowed to react (Freyssinet et al., 1978) with the proteins for 90 min at room temperature in the dark, and the unbound reagent was removed by filtration through a Sephadex G-50 column (1 × 17 cm; medium grade beads; Sigma) equilibrated in 100 mM Tris-HCl and 1 mM EDTA, pH 7.5. The labeled proteins were then concentrated on an Amicon PM30 filter (Amicon Corp., Lexington, MA) and dialyzed overnight against 75 mM Tris-HCl and 1 mM EDTA, pH 7.5, at 4 °C with 2% activated charcoal (Norit A, MGB, Norwood, OH).

Labeling efficiency (ca. 2 or 3 mol of fluorescein per mole of rA_2 or B_2 of 2 × 80 000 each, respectively) was estimated by measuring A_{490} using an absorbancy of 3.4×10^4 L/mol·cm (Churchich, 1967).

Fluorescence polarization measurements were carried out on an SLM 8000C double emission spectrofluorometer (SLM Aminco, Urbana, IL) equipped with Glan-Thompson calcite prism polarizers.

The labeled proteins (3–10 μg/mL in a 2-mL volume) were placed in a 1 × 1 cm quartz cuvette at 37 °C and stirred at ca. 1500 rpm. Additions were made at specified times through an injection port with a glass syringe (Hamilton, Reno, NV). The total volume was not allowed to increase from the initial 2 mL by more than 0.27 mL upon additions. Excitation was fixed at 490 nm, and emissions were measured at 530 nm. Polarizers in the two emission channels were set perpendicular to each other; only the polarizer on the excitation side was rotated.

Polarization (P) was calculated using the following equation: $P = [(R_V/R_H) - 1]/[(R_V/R_H) + 1]$, where R_V is the ratio of intensities in the emission channels when the excitation polarizer is in the vertical position and R_H is the ratio when the excitation polarizer is set in the horizontal position. Anisotropy is defined as $2P/(3 - P)$. Data were subjected to a smoothing routine as recommended by the SLM Operator's Manual (1987, pp 150–152) by using 10 passes of a fixed bandwidth (16 nm), sharp cutoff, and three-point low-pass linear digital filter. An IBM Personal System 2 Model 50Z computer, with software provided by SLM Aminco, was employed for data collection and storage.

RESULTS

Nondenaturing Electrophoresis and HPLC Gel-Filtration Show Complex Formation between rA_2 and B_2. Nondenaturing electrophoresis was employed earlier for studying the heterologous dissociation of the FXIII ensemble during activation of the zymogen, and the same method was used for demonstrating the phenomenon of association between the recombinant rA_2 and the native B_2 subunits. Under the conditions described in the Materials and Methods section, the reference specimen of FXIII (i.e., A_2B_2) moved much slower (Figure 1A, lane 1) than either B_2 (lane 2) or rA_2 (lane 3); B_2 was slightly ahead of rA_2. Higher magnification of the stained band in lane 3 revealed a certain degree of microheterogeneity within rA_2 itself (Figure 1B). Whatever consequences this circumstance might entail for the potential enzyme activity which might be generated from the rA_2 material (i.e., are all subspecies of rA_2 equally active?), as demonstrated by the results presented in Figure 1C, all components in the preparation were able to associate with native B_2 subunits. When rA_2 and B_2 were mixed prior to

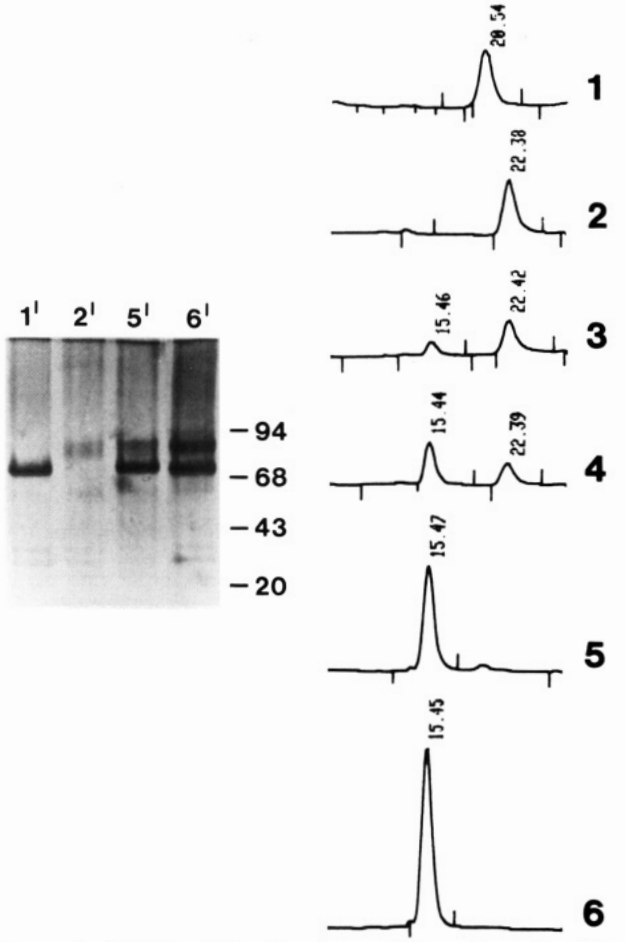

FIGURE 2: HPLC gel filtration also provides evidence for tight complex formation between rA_2 and native B_2. Elution profiles 1 and 2 pertain to 31.2 pmol of the B_2 and rA_2 proteins, whereas those shown in lanes 3–5 represent mixtures of 31.2 pmol of rA_2 with increasing amounts of B_2, i.e., 6.2, 15.6, and 31.2 pmol, respectively. A reference profile for human plasma factor XIII is shown in graph 6. The SDS–PAGE patterns in the insert (indicated with molecular weight markers $\times 10^{-3}$) reflect the protein composition of some of the peaks collected from the HPLC column, i.e., lane 1′, 20.54-min peak from graph 1, lane 2′, the 22.38-min peak from graph 2, lane 5′, the 15.47-min peak from graph 5, and lane 6′, the 15.45-min peak from graph 6. The protein composition of the complex of rA_2 and B_2, isolated as in graph 5, seems to be qualitatively similar to that of human plasma factor XIII.

electrophoresis in various mole ratios (i.e., 1.5 pmol of rA_2 to 0.4, 0.8, 1.0, 1.5, 3.0, and 6.0 pmol of B_2, shown in lanes 2–7), a slow-moving species indistinguishable from the reference FXIII (lane 1) appeared. In lanes 2 and 3 an excess of rA_2 was evident, whereas in lanes 6 and 7 free B_2 remained, suggesting that conditions for the one-to-one complexing of rA_2 and B_2 to yield rA_2B_2 were best met with the mixtures shown in lanes 4 and 5. However, with excess carrier B_2, the electrophoretic gels (lanes 6 and 7) also showed the presence of higher orders of oligomers (rA_2B_n, where $n > 2$).

Complex formation between rA_2 and B_2, just as between platelet factor XIII and the B subunits (Chung et al., 1974), could also be readily demonstrated by gel filtration (Figure 2). In the HPLC system employed (see Materials and Methods), when 31.2 pmol of rA_2 was mixed prior to injection with increasing amounts of B_2 (6.2, 15.6, and 31.2 pmol, shown in graphs 3–5), there was a progressive generation of a faster emerging peak (ca. 15.5 min) which, in fact, eluted at the position of reference FXIII (graph 6). Moreover, as the mole ratio of B_2 to rA_2 increased, the free rA_2 (which according to graph 2 would appear at ca. 22.3 min) diminished in graphs

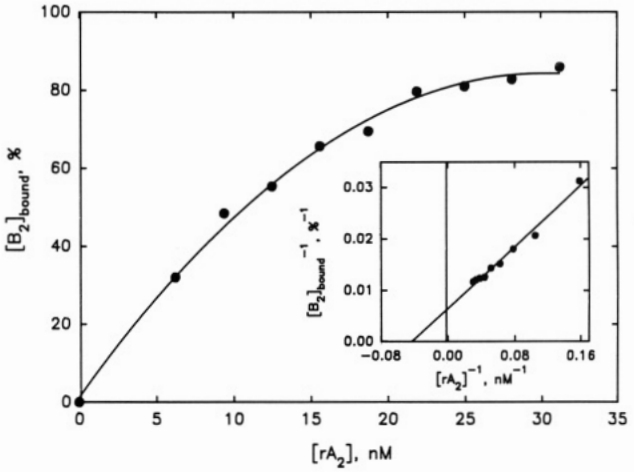

FIGURE 3: ELISA procedure for measuring the association of rA_2 with B_2. Various amounts of rA_2 (0–500 ng) were incubated with a fixed amount of B_2 (300 ng) for 30 min at room temperature. The concentrations of rA_2 in mixtures with B_2 are given on the abscissa of the graph. The residual amount of free B_2 remaining in mixtures with rA_2 was determined by the immunoassay described in the text, and from this the percentage of B_2 complexed to rA_2, presented on the ordinate as $[B_2]_{bound}$, was calculated. Measurements were normalized for development of color without rA_2. Wells with 0.5% BSA in PBS instead of the rA_2B_2 mixture were used as blanks. The insert is a double reciprocal presentation of the data.

3 and 4 and essentially disappeared with the approximately equimolar mixing of rA_2 and B_2 in graph 5. However, a very small peak corresponding to the position of free B_2 (ca. 20.5 min, see graph 1) could still be seen in graph 5. SDS–PAGE analysis (insert to Figure 2) confirmed that the new ensemble created by the near-equimolar mixing of rA_2 and B_2, isolated by gel-filtration chromatography, really contained both types of subunits.

Immunological Tests for Measuring the Binding of rA_2 to B_2. Using the dot-blot assay described in the Materials and Methods section, the binding of B_2 to rA_2 (≥50 ng) could be detected reliably with a 1:2 000 000 dilution of the antibody to B_2.

Results with an ELISA procedure for measuring the association of rA_2 with B_2 are presented in Figure 3. Various amounts of rA_2 (0–500 ng) were incubated with a fixed amounts of B_2 (300 ng) for 30 min at room temperature. Residual amounts of free B_2 remaining in the mixtures were determined by the immunoassay described in the Materials and Methods section, and the percentage of B_2 complexed to rA_2 was calculated. From the double reciprocal insert in Figure 3, an apparent binding constant of about 4×10^7 M^{-1} was derived for the association of rA_2 with B_2.

Fluorescence Depolarization Studies. Earlier experiments using fluorescein isothiocyanate (FITC) labeled plasma factor XIII (i.e., $A_2^FB_2^F$) showed the applicability of depolarization techniques for studying the thrombin plus Ca^{2+}-dependent dissociation of the labeled zymogen ensemble (Freyssinet et al., 1978), $A_2'^FB_2^F$. We have explored this methodology to examine the association of the recombinant rA_2 protein and its thrombin-modified form, rA_2', with the FITC-labeled native plasma carrier (Radek et al., 1990; Radek & Lorand, 1992), B_2^F.

(*i*) *Association of rA_2 with Fluorescein-Labeled B_2 (i.e., B_2^F) and the Thrombin Plus Ca^{2+}-Mediated Release of B_2^F Subunits from the Ensemble.* When rA_2 was mixed with fluorescein-labeled B_2, a marked increase in fluorescence anisotropy was observed; its magnitude, up to a point of saturation, depended on the mole ratio of rA_2 added. The

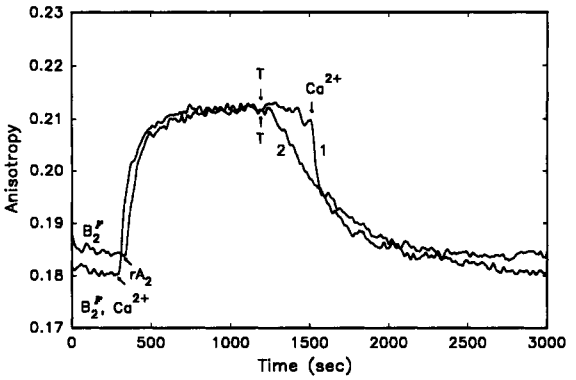

FIGURE 4: Fluorescence anisotropy change accompanying the association of B_2^F with rA_2 and also showing the thrombin plus Ca^{2+}-dependent release of B_2^F from $rA_2B_2^F$. To solutions (2 mL) of fluorescein-labeled B_2^F (29 nM in 75 mM Tris-HCl, pH 7.5, 0.15 M NaCl) containing either 1 mM EDTA (graph 1) or 30 mM $CaCl_2$ (graph 2) was added 31 nM rA_2 (8.6 μL) (at 325 s in graph 1 and at 300 s in graph 2; marked by arrows). There was a rapid and similar increase in the anisotropies of the systems upon mixing rA_2 with B_2^F. At the arrows marked T (1200 s), human thrombin (16 NIH units in 32 μL) was injected, and in the experiment in graph 1, $CaCl_2$ (15 μL) was added at 1500 s (arrow marked Ca^{2+}) to a concentration of 30 mM. In both experiments, anisotropy values returned close to that of the original with B_2^F alone, indicating the thrombin plus Ca^{2+}-dependent release of B_2^F from its association with rA_2. Pretreatment of the ensemble by thrombin before the addition of Ca^{2+} (as in graph 1) caused a more rapid drop in anisotropy.

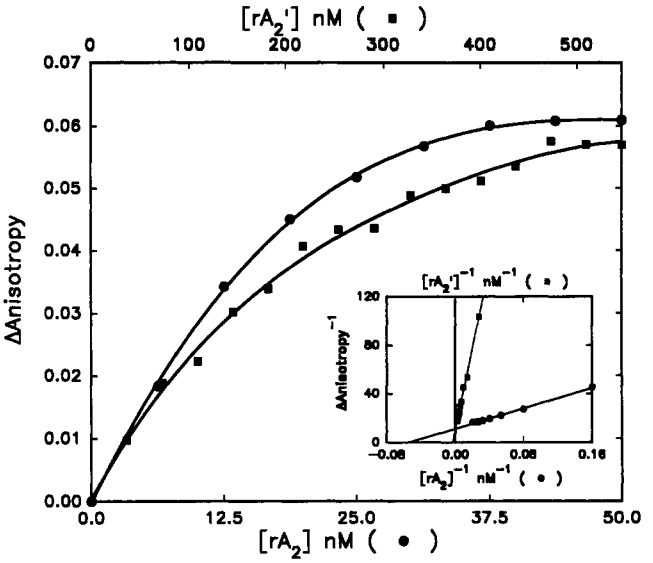

FIGURE 5: Modification of rA_2 by thrombin, weakening its association to B_2^F. The ordinate shows the measured increase (Δanisotropy) from the initial anisotropy value (0.1865) of the starting B_2^F solution (19 nM in 2 mL of 75 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.5 mM EDTA). Aliquots of rA_2 (3 μL; ●, bottom abscissa) or thrombin-activated rA_2' (18 μL; ■, top abscissa) were injected and changes in anisotropy were recorded each time after 5 min. The insert is a double reciprocal presentation of the data. Activation of rA_2 to rA_2' by thrombin is described in Materials and Methods.

experiment shown in Figure 4 presents the data obtained with the addition of about equimolar amounts of rA_2 to B_2^F. With regard to the increase in anisotropy, it did not seem to make much difference whether or not 30 mM Ca^{2+} was present at this stage of the experiment (compare graphs 1 and 2), and the increase can clearly be interpreted as being due to the formation of the larger $rA_2B_2^F$ structure.

In light of what is known about the thrombin and Ca^{2+}-promoted heterologous dissociation of subunits of plasma FXIII (Lorand et al., 1974; Chung et al., 1974; Cooke & Holbrook, 1974), it can be assumed that the further addition of thrombin to the $rA_2B_2^F$ solution which already contained Ca^{2+} would directly lead to a drop in anisotropy. Indeed, as seen in Figure 4 (graph 2), this is what was found. By contrast, addition of thrombin to the $rA_2B_2^F$ solution, initially lacking in Ca^{2+} (graph 1), did not result in a significant change in anisotropy until the latter injection of Ca^{2+} into the system. It should be noted that the specimen which was pretreated with thrombin (i.e., $rA_2'B_2^F$, graph 1) displayed a more rapid decrease in anisotropy.

(ii) Comparison of the Associations of rA_2 and the Thrombin-Modified rA_2' Species with B_2^F. When rA_2 or its thrombin-modified rA_2' form was added in increments to a fixed amount of B_2^F, stepwise increases in fluorescence anisotropy were recorded. Measurements of changes in anisotropy values with each addition yielded the data presented in Figure 5, from which apparent binding constants of $K = 5.3 \times 10^7$ M^{-1} and $K' = 3.4 \times 10^6$ M^{-1} could be calculated for the $rA_2 + B_2^F \rightleftharpoons rA_2B_2^F$ and $rA_2' + B_2^F \rightleftharpoons rA_2'B_2^F$ equilibria, respectively.

(iii) Differences in Ca^{2+} and Ionic Strength Requirements for the Release of B_2^F Subunits from the $rA_2B_2^F$ and Thrombin-Modified $rA_2'B_2^F$ Structures. Figure 6A,B shows the Ca^{2+}-induced decrease in fluorescence anisotropies when various concentrations of the cation were added to solutions of $rA_2B_2^F$ and those of the thrombin-primed $rA_2'B_2^F$ zymogen. Progressive augmentations in the rates of change were evident as the Ca^{2+} concentration was increased from 50 to 200 mM in Figure 6A and from 10 to 30 mM in Figure 6B. Apparently, 30 mM Ca^{2+} at $\mu = 0.315$ caused a near total dissociation of $rA_2'B_2^F$ because 200 mM Ca^{2+} (dotted line in Figure 6B; $\mu = 0.825$), although increasing the rate of release of B_2^F, caused a similar overall change in the anisotropy value of the system. Moreover, it is clear that the effect of Ca^{2+} was specific in either case, because Mg^{2+} could not act as a substitute. It should also be noted that the release of B_2^F from $rA_2B_2^F$, in contrast to that from $rA_2'B_2^F$, required not only higher concentrations of Ca^{2+} but also a much higher ionic strength.

(iv) Monomeric Fibrin-Promoted Release of B_2^F Subunits from the Thrombin-Modified $rA_2'B_2^F$ Ensemble. Changes in fluorescence anisotropy were also examined when fibrin formation, stopped at the monomeric stage by the GPRP tetrapeptide (Laudano & Doolittle, 1980), was allowed to take place in the solution of $rA_2'B_2^F$. It is seen in Figure 7 that the presence of fibrin produced a marked drop in the anisotropy of the system. Inclusion of Ca^{2+} did not appreciably alter the observed pattern of release of B_2^F subunits (data not shown).

Experiments were carried out under the conditions described in Figure 7 to compare the effect of monomeric fibrin on the release of B_2^F from the $rA_2'B_2^F$ structure with that of the similarly large plasma protein, fibronectin. The measured rate of release of B_2^F from the anisotropy drop was more than 7-fold greater with fibrin (2 μM) than with an equimolar concentration of fibronectin in the mixture (data not shown).

The specific effect of fibrin in promoting the release of B_2^F from $rA_2'B_2^F$ could be rationalized by suggesting that fibrin, itself, might preferentially bind the other dissociating partner, rA_2'. This was tested by preparing fluorescein-labeled rA_2^F, converting it to $rA_2'^F$ by treatment with thrombin, and measuring the affinity of the latter species to monomeric human fibrin, as in Figure 8. The experimental data yielded an apparent association constant of about 0.4×10^6 M^{-1} for the binding of $rA_2'^F$ to fibrin.

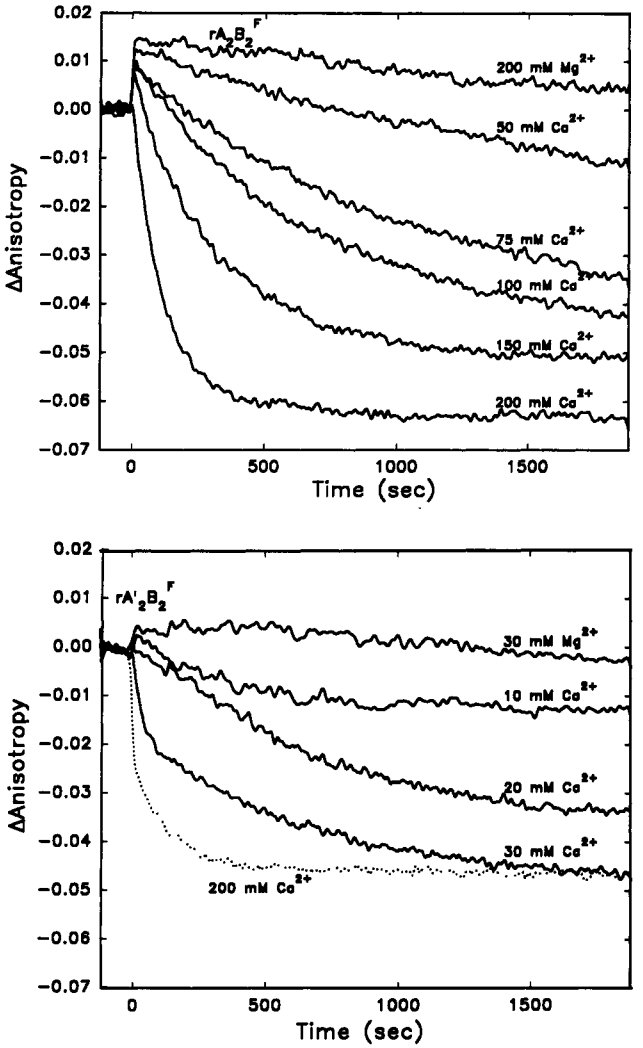

FIGURE 6: Differences in Ca^{2+} and ionic strength requirements for the release of B_2^F from the $rA_2B_2^F$ and $rA_2'B_2^F$ assemblies. (A, upper) Starting mixtures (2 mL) contained 20 nM B_2^F and 22 nM rA_2 in 75 mM Tris-HCl, pH 7.5, and 0.15 M NaCl. Ten minutes later, corresponding to zero time on the abscissa, $CaCl_2$ was injected (100 μL) to yield the concentrations (50–200 mM) indicated on the graphs. Total ionic strength was maintained at 0.825 by appropriate inclusions of $MgCl_2$. The experiment with 200 mM $MgCl_2$, without any Ca^{2+}, underlines the specificity of the Ca^{2+} effect. The ordinate shows changes from the anisotropy of the starting mixture (0.2387). (B, lower) Starting mixtures were formed as above, but human thrombin (3.5 NIH units in 7 μL) was injected 5 min prior to the addition of $CaCl_2$ or $MgCl_2$ to the concentrations shown on the graphs (10–30 mM). In all experiments, except in that shown with the dotted line (200 mM Ca^{2+}, μ = 0.825), total ionic strength was set to 0.315 by appropriate inclusions of $MgCl_2$. The experiment with 30 mM $MgCl_2$ contained no Ca^{2+}, again indicating the specificity of Ca^{2+}. The ordinate shows changes from the anisotropy value (0.2355) of the thrombin-treated mixture prior to injection of any Ca^{2+} or Mg^{2+}.

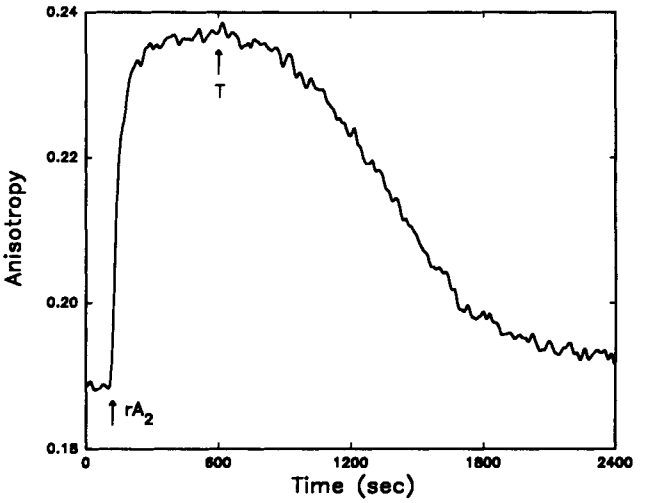

FIGURE 7: Monomeric fibrin promoting the dissociation of the thrombin-modified $rA_2'B_2^F$ ensemble. To a mixture of B_2^F (22.5 nM) and human fibrinogen (2 μM) in 2-mL solutions with 75 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5 mM EDTA, and 5 mM GPRP tetrapeptide was added rA_2 (25 nM) at 120 s (arrow marked rA_2) to form $rA_2B_2^F$ with the attendant increase in fluorescence anisotropy. Injection of human thrombin (1.5 NIH units in 3 μL at 600 s; arrow marked T) caused a gradual return of the anisotropy of the system to near the starting value.

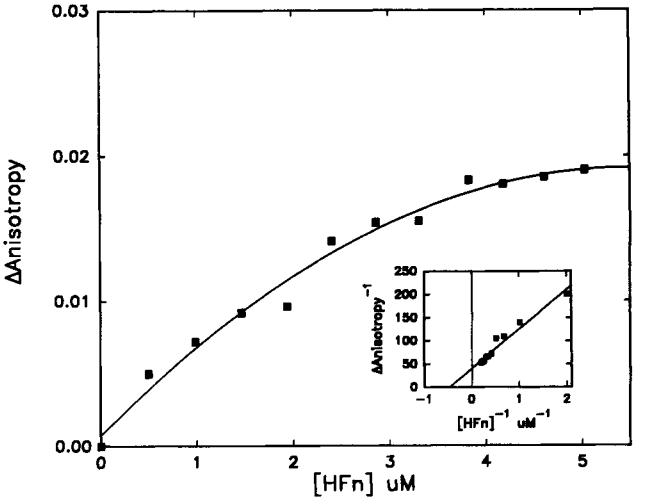

FIGURE 8: Monomeric fibrin binding the thrombin-activated $rA_2'^F$ species. Activation of rA_2^F (30 nM) in a 2-mL solution of 75 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5 mM EDTA, and 5 mM GPRP tetrapeptide was accomplished by treatment with human thrombin (1.5 units in 3 μL) for 5 min (37 °C). Anisotropy increases (ordinate) from the starting value (0.2240) were recorded at 2.5-min intervals upon the repeated injections of human fibrinogen (1 nmol in 18.3 μL). Cumulative concentrations of fibrinogen, converted to fibrin by thrombin in the cuvette, are presented as human fibrin (HFn) on the abscissa. The insert shows a double reciprocal plot of the data.

DISCUSSION

Several experimental approaches were explored to test the ability of the recombinant placental factor XIII preparation expressed in yeast (Bishop et al., 1990; referred to as rA_2) to complex with the native carrier B_2 subunits isolated from human plasma (Lorand et al., 1981). Nondenaturing electrophoresis (Figure 1) and HPLC gel filtration (Figure 2) were used to demonstrate a strong tendency for binding between the two proteins. The electrophoretic experiments were particularly interesting in that they revealed a significant degree of microheterogeneity in the recombinant protein; nevertheless, all isoforms of rA_2 (Figure 1B) could clearly bind to B_2. In mixtures of the two proteins, as the molar ratio of B_2 to rA_2 approached unity, the mobility of the first oligomeric product (rA_2B_2) was indistinguishable from that of reference plasma factor XIII (A_2B_2). However, with an excess of B_2 in the mixtures—even though the rA_2B_2 complex remained the main product of assembly—higher ordered oligomers (rA_2B_n, where $n > 2$) were also clearly visible on the electrophoretic gels (Figure 1C, lanes 6 and 7).

Since there was no evidence for dissociation by back-diffusion, i.e, undue streaking of bands during nondenaturing electrophoresis or asymmetrical skewing of peaks during gel filtration, it can be assumed that complexes of rA_2 with B_2 are characterized by a tight heterologous binding of these subunits. A rapid procedure was developed to obtain a more quantitative value for the affinity between rA_2 and B_2. The

residual amount of free B_2, remaining in mixtures of rA_2 with B_2, was measured by immunological means. The free B_2 was allowed to bind to rA_2-coated microtiter wells where its amount was estimated by ELISA methodology using an antibody directed against the B_2 protein (Figure 3). For the complex formation between rA_2 and B_2, an approximate binding constant of 4×10^7 M^{-1} could be calculated.

The technique of fluorescence depolarization offered a most versatile method for studying interactions between rA_2 and B_2. Best results were obtained when the B subunits were labeled with fluorescein (B_2^F). Heterologous association was monitored by the increase in fluorescence anisotropy at a fixed concentration of B_2^F mixed with various concentrations of rA_2. Upon the addition of sufficient rA_2, as seen in Figure 5, the increase in anisotropy reached a limiting value. Interestingly, essentially the same limiting anisotropy difference was registered by adding the thrombin-modified rA_2' to B_2^F. However, the measured affinities of rA_2 and rA_2' for B_2 were markedly different (5.3×10^7 M^{-1} for rA_2 and 3.4×10^6 M^{-1} for rA_2') showing that, although rA_2' binds rather well to B_2^F, the stability of the thrombin-modified $rA_2'B_2^F$ ensemble is appreciably weaker than that of the virgin $rA_2B_2^F$. Thus, it may be concluded that major interaction domains in rA_2 necessary for complexation to B_2^F are still retained in rA_2' after cleavage by thrombin. However, the thrombin-catalyzed removal of the N-terminal activation peptide from rA_2 causes a significant reduction (ca. 2 kcal/mol) in the strength of binding to the carrier subunits.

It may be noted here that treatment with neuraminidase or with *N*-glycosidase F, which caused the removal of sialic acid and carbohydrate moieties from B_2^F, did not affect the ability of the plasma carrier to complex with rA_2 (data not shown). This suggests that the heterologous binding domains of B_2, complementary to those in rA_2, reside in the protein and not in the carbohydrate attachments of B_2.

The observations, presented in Figure 5, that rA_2' binds B_2^F less strongly than rA_2 provide a rationale for the action of thrombin in the physiological mode of activating plasma factor XIII. This is also reflected in the increased sensitivity of the $rA_2'B_2^F$ ensemble, as compared to the intact $rA_2B_2^F$, to release B_2^F. Just as the plasma factor XIII (A_2B_2) zymogen can be induced to dissociate by high enough concentrations of Ca^{2+} in the absence of any treatment with thrombin (Credo et al., 1978; Lorand et al., 1981), the $rA_2B_2^F$ ensemble can also be labilized at high ionic strength ($\mu = 0.825$) to release B_2^F when the concentration of Ca^{2+} in the medium is raised to about $[Ca^{2+}]_{1/2} = 75$ mM (Figure 6A). By contrast (Figure 6B), the thrombin-modified $rA_2'B_2^F$ structure could be dissociated by about a 5-fold lower concentration of the cation ($[Ca^{2+}]_{1/2} = 15$ mM, at much lower total ionic strength: $\mu = 0.315$).

Figure 4 shows a full cycle for associating nearly equimolar amounts of rA_2 with B_2^F and subsequently causing the release of B_2^F from the ensemble by adding thrombin then Ca^{2+} or vice versa. Clearly, the $rA_2B_2^F$ structure was quite stable in 30 mM Ca^{2+}, whereas the thrombin-modified $rA_2'B_2^F$ released its B_2^F component quite readily under these conditions.

Fibrin(ogen) fulfills important forward regulatory functions in the physiological pathway of conversion of plasma factor XIII (Lewis et al., 1985; Greenberg et al., 1987; Naski et al., 1991). In particular, it is known to greatly reduce the Ca^{2+} requirement for the activation of the zymogen (Credo et al., 1981). Fluorescence depolarization seemed to be well suited to examine the influence of monomeric fibrin formation on the release of B_2^F from the thrombin-modified $rA_2'B_2^F$ ensemble. The aggregation inhibitory GPRP tetrapeptide (Laudano & Doolittle, 1980) was added to prevent clot formation in the cuvette. Figure 7 illustrates the dramatic effect of fibrin monomer for promoting the release of B_2^F. A likely explanation for this finding is that fibrin (**F**) competes for rA_2' and thereby shifts the overall balance for producing free B_2^F:

$$rA_2'B_2^F \rightleftharpoons rA_2' + B_2^F$$

$$rA_2' + F \rightleftharpoons rA_2'F$$

With the use of fluorescein-labeled and thrombin-modified rA_2 (i.e., $rA_2'^F$), an apparent association constant of about 0.4×10^6 M^{-1} was measured for its interaction with human fibrin monomer (Figure 8). In the experiments of Hornyak and Shafer (1992), the inactive carbamylmethyl derivative of the same recombinant protein (called, by these authors, platelet factor XIII) gave an identical value for binding to fibrin.

The demonstrated ability of rA_2 to bind to the native plasma carrier B_2 in a combination analogous to that of the plasma factor XIII construct is encouraging for the possible use of the recombinant protein in clinical situations where previously only plasma concentrates or purified fractions were available for therapy. For example, neonates are known to have a significantly lower level of factor XIII (Henriksson et al., 1974), and hemorrhagic complications are well documented in patients with the hereditary absence of factor XIII; recombinant rA_2 preparations might also help to neutralize or remove autoimmune inhibitors against the factor XIII system (Lorand et al., 1980). However, prior to injection of the recombinant product into the circulation, thoughtful attention should be paid to the consequences—if any—of forming certain amounts of the higher ordered rA_2B_n (where $n > 2$) oligomers (shown in Figure 1C, lanes 6 and 7) which are absent from the purified plasma factor XIII preparations.

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