Interactions of Human Fibrinogens with Factor XIII: Roles of Calcium and the γ' Peptide†

Maia Moaddel,‡,§ David H. Farrell,‖ Margaret A. Daugherty,‡ and Michael G. Fried*,‡

Department of Biochemistry and Molecular Biology, Penn State University College of Medicine, 500 University Drive, Hershey, Pennsylvania 17033, and Department of Oral Molecular Biology, School of Dentistry, Oregon Health Sciences University, 611 Southwest Campus Drive, Portland, Oregon 97201

Received January 19, 2000; Revised Manuscript Received April 4, 2000

ABSTRACT: Plasma factor XIII is the zymogen of the transglutaminase factor XIIIa. This enzyme catalyzes the formation of isopeptide cross-links between fibrin molecules in nascent blood clots that greatly increase the mechanical stability of clots and their resistance to thrombolytic enzymes. We have characterized the solution interactions of factor XIII with two variants of fibrinogen, the soluble precursor of fibrin. Both the predominant fibrinogen γ_A/γ_A and the major variant γ_A/γ' form complexes with a 2 fibrinogen:1 factor XIII ratio. The absence of detectable concentrations of 1:1 complexes in equilibrium mixtures containing free factor XIII and 2:1 complexes suggests that this interaction is cooperative. Factor XIII binds fibrinogen γ_A/γ' ~20-fold more tightly than fibrinogen γ_A/γ_A, and the interaction with fibrinogen γ_A/γ' (but not fibrinogen γ_A/γ_A) is accompanied by a significant release of Ca^{2+}. Taken together, these results suggest that the strikingly anionic γ' C-terminal sequence contains features that are important for factor XIII binding. Consistent with this notion, a synthetic 20-residue polypeptide containing the γ' sequence was found to associate with factor XIII in a 2:1 molar ratio and act as an efficient competitor for fibrinogen γ_A/γ' binding.

Fibrinogen is a plasma protein of M_r ~340 000 that functions as the major structural clotting factor. It is a disulfide-bonded dimer of three polypeptide chains, α (M_r ~65 000), β (M_r ~56 000), and γ (M_r ~47 000), with the stoichiometry $(\alpha\beta\gamma)(\alpha\beta\gamma)$ (*1*). The serine protease thrombin converts soluble fibrinogen to fibrin, which spontaneously polymerizes to form an insoluble clot (*2*). The polymerized strands of fibrin are cross-linked by a transglutaminase, factor XIIIa, that catalyzes the formation of amide linkages between glutamyl and lysyl side chains (*3*). Without these cross-links, the fibrin clot is significantly less resistant to degradation by the fibrinolytic enzymes (*4*). The importance of these cross-links in vivo is graphically illustrated by individuals with factor XIII deficiencies, who can have severe lifelong bleeding tendencies and abnormal wound healing (*5*).

Plasma factor XIII is a protein of M_r ~320 000 composed of two polypeptide *a* subunits of M_r ~83 000 and two polypeptide *b* subunits of M_r ~80 000 (*6*−*8*). Factor XIII circulates as a zymogen until it is activated by the cleavage of a 4000 Da activation peptide from each *a* subunit by thrombin, which is followed by the dissociation of the *b* subunits (*9*). Activation is relatively slow in the absence of fibrin, but is accelerated in the presence of fibrin (*10*), indicating that a significant amount of factor XIII activation occurs after fibrin formation. Fibrin therefore serves as a positive modulator of factor XIII activation.

An alternatively processed form of the mRNA encoding the fibrinogen γ chain gives rise to a variant chain termed γ', γ_B, or $\gamma^{57.5}$ (*11*−*15*) that comprises approximately 7−15% of the total fibrinogen found in plasma (*16*). In the γ' variant, a 20 amino acid sequence replaces the carboxyl-terminal 4 amino acids of the γ chain (*17*). Fibrinogen that contains the γ' chain is a heterodimer with the stoichiometry $(\alpha\beta\gamma)$-$(\alpha\beta\gamma')$ and is referred to as γ_A/γ' fibrinogen, peak II fibrinogen (*16*), γ_A/γ_B fibrinogen (*18*), or $F\gamma^{50,57.5}$ (*15*). Although the function of the γ' chain was unclear for many years following its discovery (*13*, *14*), studies by Siebenlist et al. (*19*) have shown that γ_A/γ' fibrinogen binds noncovalently to the zymogen form of factor XIII, suggesting that γ_A/γ' fibrinogen serves as a carrier protein for factor XIII in plasma. Recent evidence has also shown that cross-linked γ_A/γ' fibrin is more resistant to fibrinolysis than γ_A/γ_A fibrin, implying that the interaction of factor XIII with γ_A/γ' fibrinogen influences clot stability (*20*).

Here, we report some physical properties of the complexes formed between factor XIII and fibrinogens γ_A/γ' and γ_A/γ_A. Both fibrinogens are bound by factor XIII in a 2:1 molar ratio, although fibrinogen γ_A/γ' is bound with significantly greater affinity than fibrinogen γ_A/γ_A. The binding of the two forms of fibrinogen is further distinguished by different sensitivities to EDTA and to changes in $CaCl_2$ concentration. A 20-residue polypeptide containing the sequence of the γ'

† Supported by a Student Award (to M.M.) and a Postdoctoral Fellowship (to M.A.D.) from the American Heart Association, Pennsylvania Affiliate, by Grants-in-Aid from the American Heart Association (to D.H.F. and to M.G.F.), and by NIH Grant R29HL53997 (to D.H.F.).

* Corresponding author. Telephone: (717) 531-5250. Telefax: (717) 531-7072. Email: mfried@psu.edu.

‡ Penn State University College of Medicine.

§ Present address: Diabetes Branch−Experimental Diabetes, Metabolism and Nutrition Section, NIDDK, NIH.

‖ Oregon Health Sciences University.

10.1021/bi000098u CCC: $19.00
Published on Web 05/12/2000

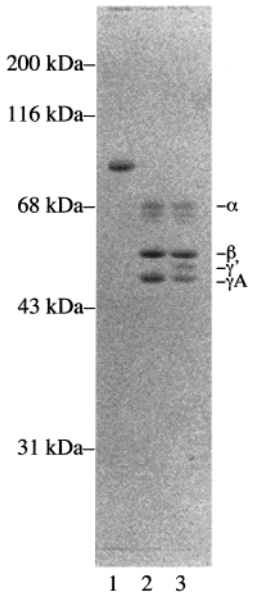

FIGURE 1: SDS−polyacrylamide gel electrophoresis characterization of fibrinogen and factor XIII samples. Samples contained 2 μg of factor XIII (lane 1), fibrinogen γ_A/γ_A (lane 2), or fibrinogen γ_A/γ' (lane 3). Samples were denatured in buffer containing SDS and 2-mercaptoethanol and resolved on a 10% polyacrylamide gel. The factor XIII *a* and *b* subunits comigrate under these conditions. The fibrinogen γ_A/γ_A sample shows the characteristic α, β, and γ_A chain bands, while the fibrinogen γ_A/γ' sample shows the α, β, γ_A, and γ' chain bands.

extension competes with fibrinogen γ_A/γ' for factor XIII binding, indicating that this sequence contains features that are sufficient for functional binding. Taken together, these results support models in which the γ' extension plays a central role in recruiting factor XIII from solution and transporting it to a nascent clot for activation.

MATERIALS AND METHODS

Proteins. Approximately 100 mg of lyophilized plasminogen-free human plasma fibrinogen (Calbiochem) was dissolved in 100 mL of 39 mM Tris−phosphate (pH 8.6 at 4 °C) containing 5 mM ϵ-aminocaproic acid (ϵ-ACA) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). This was dialyzed against three 1 L changes of the same buffer over a period of 3 days at 4 °C. The γ_A/γ' fibrinogen was resolved from the predominant γ_A/γ_A component by DEAE-cellulose chromatography, and both forms were further purified by chromatography using an affinity resin consisting of glycine-L-proline-L-arginine-L-proline-L-cysteine (GPRPC) covalently linked to agarose (*21*). The resulting purified fibrinogens were dialyzed against 150 mM NaCl, 10 mM Tris HCl (pH 7.5 at 4 °C) and stored at −70 °C. Fibrinogen concentrations were determined spectrophotometrically using the extinction coefficient $\epsilon_{280} = 5.15 \times 10^5$ M^{-1} cm^{-1} (*22*).

Purified plasma factor XIII was the kind gift of Dr. M. Mosesson (Blood Center of Southeastern Wisconsin, Milwaukee, WI), or was obtained from Enzyme Research Laboratories (South Bend, IN). Specific activities of the thrombin-activated factor XIII preparations ranged from 1131 to 1500 units/mL according to the assay of Loewy et al. (*23*). Samples were judged to be free of contaminants detectable by SDS−polyacrylamide gel electrophoresis (Figure 1). The concentration of factor XIII was determined spectrophotometrically using the extinction coefficient $\epsilon_{280} = 4.42 \times 10^5$ M^{-1} cm^{-1} (*6*−*8*).

The polypeptide VRPEHPAETEYDSLYPEDDL, corresponding to the C-terminal 20 residues of the fibrinogen γ' chain, was the kind gift of Dr. Kathleen Berkner (The Cleveland Clinic). Its purity was verified by protein sequencing. Peptide concentrations were determined spectrophotometrically using the extinction coefficient $\epsilon_{280} = 1.79 \times 10^3$ M^{-1} cm^{-1} (Farrell, D. H., unpublished results).

Sedimentation Equilibrium. Samples of fibrinogen and factor XIII were dialyzed against Buffer A [10 mM Tris (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 1 μM leupeptin]. In a few experiments, buffer compositions differ from those specified here. Where different buffers were used, these conditions are identified explicitly in the text. Sedimentation equilibrium experiments were performed using a Beckman XL-A analytical ultracentrifuge fitted with an AN-60Ti rotor, operating at 4.0 ± 0.1 °C. Six-sector centerpieces were used, allowing nine samples of varying protein concentration, $CaCl_2$ concentration, or fibrinogen/factor XIII ratio to be analyzed simultaneously. For each set of solution conditions, data sets were collected at two rotor speeds (typically 6000 and 8000 rpm, although other speeds ranging from 4000 rpm to 16 000 rpm were used on occasion). Absorbance values were measured at 280 nm as functions of radial position. Five scans were averaged for each sample at each rotor speed. The approach to equilibrium was considered to be complete when replicate scans separated by ≥6 h were indistinguishable.

For analysis of the independent sedimentation properties of fibrinogen and factor XIII, experimental data sets relating absorbance at a single wavelength to radial position were fit to models based on eq 1, in which the proteins sedimented as homogeneous, ideal monomers. For a solution containing *n* independent species at equilibrium in a centrifugal field, the absorbance is specified at radial position *r* by

$$A(r) = \sum_n \alpha_{0,n} \exp[\sigma_n(r^2 - r_o^2)] + \zeta \qquad (1)$$

Here $A(r)$ is the absorbance at radial position r, $\alpha_{0,n}$ is the absorbance of species n at reference position r_o, σ_n is the reduced molecular weight of species n, $\sigma_n = M_n(1 - \overline{v_n}\rho)\omega^2/2RT$, M_n is the species molecular weight, $\overline{v_n}$ its partial specific volume, ρ the solvent density, ω the rotor angular velocity, R the gas constant, and T the absolute temperature. The baseline offset term (ζ) is used to compensate for slight position-independent variations in the optical properties of individual samples and/or cell assemblies. Solvent densities were measured using a Mettler density meter. The partial specific volumes of fibrinogen and factor XIII are 0.715 and 0.730 mL/g, respectively (*8*, *24*).

For a system in which two components are in equilibrium, such as n(fibrinogen) + m(factor XIII) ⇄ (fibrinogen)$_n$·(factor XIII)$_m$, the absorbance distribution at sedimentation equilibrium is given by

$$A(r) = \alpha_F \exp[\sigma_F(r^2 - r_o^2)] + \alpha_{XIII} \exp[\sigma_{XIII}(r^2 - r_o^2)] + \alpha_{F\cdot XIII} \exp[\sigma_{F\cdot XIII}(r^2 - r_o^2)] + \zeta \qquad (2)$$

Here, the absorbances of fibrinogen, factor XIII, and the fibrinogen−factor XIII complex at r_o are represented by α_F,

α_{XIII}, and $\alpha_{F\cdot XIII}$, respectively, and the reduced molecular weights of fibrinogen, factor XIII, and the fibrinogen−factor XIII complex are, respectively, $\sigma_F = M_F(1 - \overline{\upsilon_F}\rho)\omega^2/2RT$, $\sigma_{XIII} = M_{XIII}(1 - \overline{\upsilon_{XIII}}\rho)\omega^2/2RT$, and $\sigma_{F\cdot XIII} = (aM_F + bM_{XIII})(1 - \overline{\upsilon_{F\cdot XIII}}\rho)\omega^2/2RT$. The partial specific volumes of fibrinogen−factor XIII complexes were estimated using

$$\overline{\upsilon_{F\cdot XIII}} = (aM_F\overline{\upsilon_F} + bM_{XIII}\overline{\upsilon_{XIII}})/(aM_F + bM_{XIII}) \quad (3)$$

Here, the numbers of fibrinogen and factor XIII molecules present in the complex are represented by *a* and *b*, respectively. Equation 3 contains the assumption that the partial specific volume of a complex is a weight-average of the partial specific volumes of the constituent species and, hence, that there is no significant volume change upon association. Although we do not know whether a volume change occurs, it seems reasonable that any change would be concentrated in the interacting domains and have little effect on the greater part of each protein that is distant from the interacting surfaces.

Estimation of Equilibrium Constants. Data presented below are consistent with models of fibrinogen−factor XIII association in which one factor XIII molecule binds two of fibrinogen. For this model, the absorbance of the fibrinogen−factor XIII complex depends on those of free fibrinogen and factor XIII according to $\alpha_{F\cdot XIII} = K'(\alpha_F)^2(\alpha_{XIII})$, in which K' is the apparent association constant of the binding reaction, with concentrations given in absorbance units. Substitution of this identity into eq 3 gives an expression that contains too many adjustable terms to allow statistically meaningful fitting to a single data set, and to our knowledge, the currently available software for the simultaneous ("global") analysis of multiple data sets is not capable of treating heteroassociation reactions. However, because the reduced molecular weights of fibrinogen and factor XIII are nearly equal ($\sigma_F/\sigma_{XIII} = 1.12$), the equilibrium described by eq 2 can be approximated, with small error, as a reversible trimerization:

$$A(r) = \alpha \exp[\sigma(r^2 - r_o^2)] + \alpha^3 K' \exp[3\sigma(r^2 - r_o^2)] + \zeta \quad (4)$$

in which α represents the absorbance of an effective monomer (of $M_r \sim 336\,000$) at r_o and other terms are defined as described above. We have used the average extinction coefficient $\epsilon_{ave} = (2\epsilon_F + \epsilon_{XIII})/3 = 4.91 \times 10^5\ M^{-1}\ cm^{-1}$ as the extinction coefficient of the effective monomer. This choice, justified by the observation (described below) of a 2:1 fibrinogen/factor XIII complex, leads to a small (~5%) overestimate of free fibrinogen concentrations and a modest (~10%) underestimate of free factor XIII concentrations. Simultaneous least-squares fitting of this expression to multiple data sets was performed with the NONLIN program (*25*), running on a Macintosh computer.[1] Typical analyses contained six data sets, corresponding to two samples differing in total protein concentration (or fibrinogen:factor XIII ratio), equilibrated at three rotor speeds.

RESULTS

Association States of Fibrinogen and Factor XIII. Equilibrium analytical ultracentrifugation was used to characterize the association states of fibrinogens γ_A/γ_A and γ_A/γ' at 4 °C in Buffer A. Shown in Figure 2A are representative sedimentation profiles; the solid curves through the data are global least-squares fits of the expression for a single macromolecular species (eq 1 with $n = 1$) to data obtained at two nominal protein concentrations and at least two rotor speeds. The small, relatively symmetric residuals demonstrate the compatibility of the single-species model with these data sets. The values of M_r returned by these analyses were γ_A/γ_A fibrinogen, 338 900 ± 5800; and γ_A/γ' fibrinogen, 343 800 ± 6300. These values are in good accord with estimates of the molecular weight of γ_A/γ_A fibrinogen calculated from its amino acid and carbohydrate compositions [$M_{r,calc} = 337\,897$ (*26*); 342 620 (*27*−*29*)], and together they demonstrate the absence of significant concentrations of aggregation or degradation products in these fibrinogen preparations.

Parallel experiments were carried out with purified factor XIII. Representative data are shown for this protein, at sedimentation equilibrium in Buffer A containing 10 mM EDTA, or 2 mM $CaCl_2$ (Figure 2B). Both data sets are well fit by the expression for a single macromolecular species (eq 1 with $n = 1$), with values of M_r of 325 400 ± 4400 and 319 200 ± 4800, respectively. A closely similar value (M_r = 325 700) can be calculated from the amino acid and carbohydrate compositions of the factor XIII a_2b_2 tetramer (*30*). The observation that factor XIII dissociates at elevated [$CaCl_2$] (*9*, *31*) raised the question of whether the protein is partially dissociated at 2 mM $CaCl_2$, or even in the presence of EDTA. To test this notion, we fit data sets obtained in the presence of either 10 mM EDTA (0 mM added Ca^{2+}) or 2 mM $CaCl_2$ (0 mM added EDTA) to a two-species version of eq 1 in which the molecular weights were fixed at 325 000 and 155 000 (the latter is the average of predicted molecular weights for a_2 and b_2 species). In both cases, this analysis returned concentrations of the lower molecular weight (dissociated) populations that were, within error zero (result not shown). This indicates that our samples of factor XIII are not significantly dissociated when [$CaCl_2$] ≤2 mM. This concentration range overlaps that of free Ca^{2+} in human serum [1.05 mM ≤ [Ca^{2+}] ≤ 1.3 mM (*32*)]. These observations support the previous conclusion that the predominant quaternary form of factor XIII under physiological ionic conditions is the a_2b_2 tetramer (*6*−*8*).

Factor XIII Binds Two Equivalents of Fibrinogen γ_A/γ'. At sedimentation equilibrium, solutions containing fibrinogen γ_A/γ' and factor XIII contained mixtures of species that gave weight-average molecular weights significantly greater than those of fibrinogen or factor XIII, alone. To better characterize this molecular system, we tested the two-species sedimentation model (eq 1 with $n = 2$) against the data. The globally best fit of this model to the data was one in which $M_{r,1} = 342\,480 \pm 21\,070$ and $M_{r,2} = 1\,084\,000 \pm 89\,700$ (Figure 3A). The small, symmetrical fitting residuals (upper panel) demonstrate the compatibility of this model with the data over the range of protein concentrations present in the sample. (Assuming an average extinction coefficient for fibrinogen and factor XIII of $\epsilon_{280} = 4.8 \times 10^5\ M^{-1}\ cm^{-1}$, the sum of fibrinogen and factor XIII concentrations in this representative sample ranged from ~10.0 nM near the meniscus to ~3.7 μM near the bottom of the sample cell.) In parallel experiments, data sets obtained from solutions

[1] NONLIN for the Macintosh was obtained from the website http://bioc02.uthscsa.edu/.biochem/xla2.html.

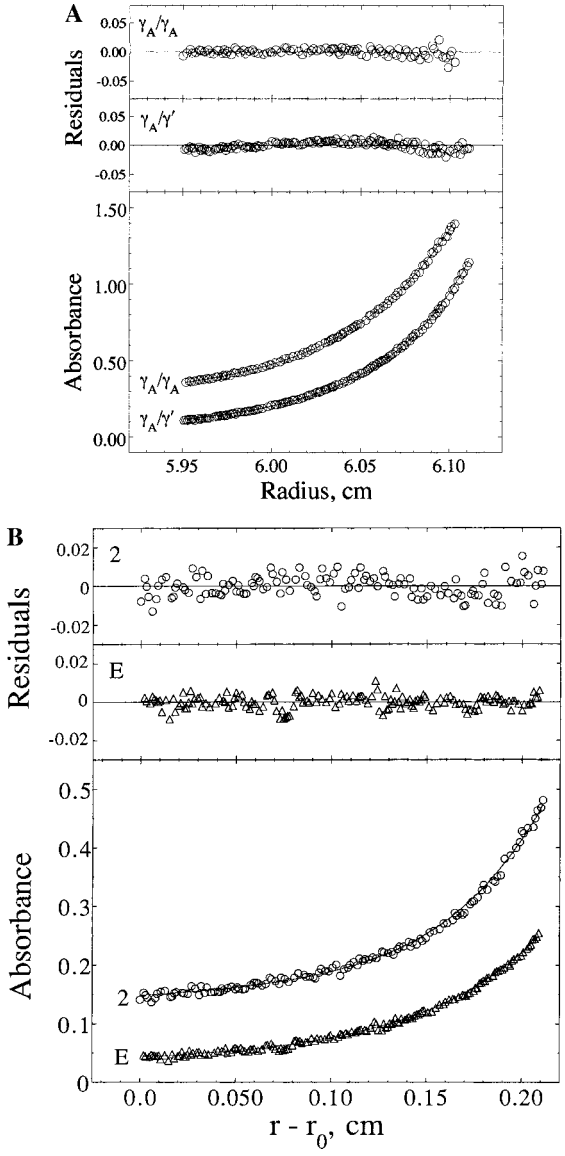

FIGURE 2: Sedimentation equilibrium characterization of fibrinogen and factor XIII samples. (A) Fibrinogens γ_A/γ_A and γ_A/γ' centrifuged to equilibrium at 8000 rpm and 4 °C in Buffer A. The solid lines represent global fits of the single-species model (eq 1) to the ensemble of data sets obtained with each protein as described under Materials and Methods. These fits returned values of $M_r = 338\,900 \pm 5800$ for fibrinogen γ_A/γ_A and $M_r = 343\,800 \pm 6300$ for fibrinogen γ_A/γ'. In both cases, the curve-fitting residuals (upper panels) are small and, to a reasonable approximation, symmetrically distributed, demonstrating that the single-species model is consistent with the mass distributions present in these samples. (B) Factor XIII sediments as an a_2b_2 tetramer in the presence of EDTA and 2 mM $CaCl_2$. Sedimentation equilibrium data were obtained at 8000 rpm and 4 °C. In this case, the data are plotted as a function of the reduced radial position ($r - r_0$), to accommodate samples centrifuged in different sectors of a 6-sector centerpiece. The sample buffers were 10 mM Tris (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 2 mM $CaCl_2$, 1 μM leupeptin (Buffer A modified to contain 2 mM $CaCl_2$; ○) or 10 mM Tris (pH 7.4), 137 mM NaCl, 10 mM EDTA, 2.7 mM KCl, 1 μM leupeptin (Buffer A modified to contain 10 mM EDTA and no $CaCl_2$; △). The solid lines represent global fits of the single-species model (eq 1) to the ensemble of data sets obtained with each protein as described under Materials and Methods. These fits returned values of $M_r = 319\,200 \pm 4800$ for samples in 2 mM $CaCl_2$ and $M_r = 325\,400 \pm 4400$ for samples in 10 mM EDTA. In both cases, the curve-fitting residuals (upper panels) are small and symmetrically distributed, demonstrating that a single-species model is consistent with the mass distributions present in these samples.

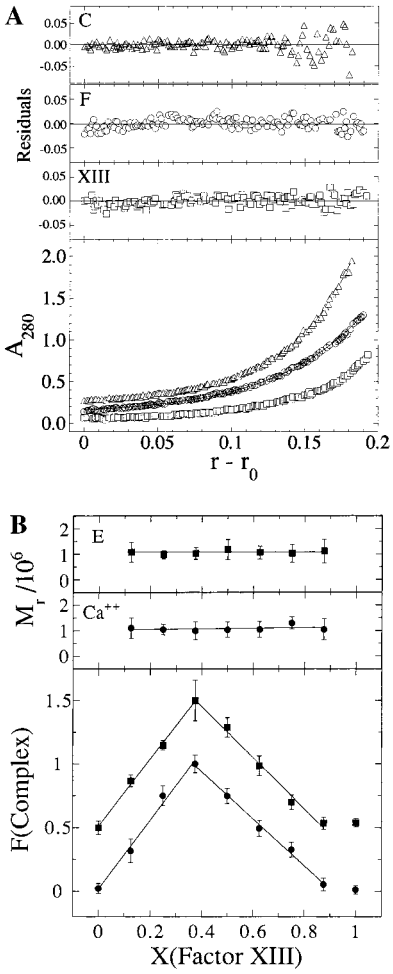

FIGURE 3: Factor XIII binds 2 equiv of fibrinogen γ_A/γ'. (A) Lower panel: Sedimentation equilibrium data for factor XIII alone (□), fibrinogen alone (○), and a mixture of factor XIII and fibrinogen γ_A/γ' (△). The data sets for fibrinogen γ_A/γ' and the factor XIII−fibrinogen mixture are offset vertically for clarity. The data are plotted as a function of the reduced radial position ($r - r_0$), to accommodate samples centrifuged in different sectors of a 6-sector centerpiece. All samples shown here were brought to equilibrium at 8000 rpm in Buffer A at 4 °C. The solid lines represent global fits to six data sets for each protein or protein mixture, as described under Materials and Methods. The factor XIII and fibrinogen samples are fit by the single-species model, which returned $M_{r,factorXIII} = 322\,300 \pm 5800$ and $M_{r,fibrinogen\ \gamma A/\gamma'} = 343\,800 \pm 6300$, respectively. Samples containing factor XIII and fibrinogen γ_A/γ' were fit by the two-species model (eq 1 with $n = 2$), which returned $M_{r,1} = 342\,480 \pm 21\,070$ and $M_{r,2} = 1\,084\,000 \pm 89\,700$. The small, symmetrically distributed residuals demonstrate the good agreement of these models with the experimental mass distributions. (B) Job plots of fibrinogen γ_A/γ' binding factor XIII. Lower panel: Plots of relative concentration of fibrinogen−factor XIII complex (normalized against the largest value in the series) as functions of the nominal mole fractions of factor XIII in the mixtures. The error bars indicate the 95% confidence limits for each value. The data were obtained from sedimentation equilibrium analyses such as the ones shown in panel A. Samples were brought to equilibrium in Buffer A (which contains 1 mM $CaCl_2$; ●) or in Buffer A minus $CaCl_2$, containing 10 mM EDTA (■). The 10 mM EDTA data are offset vertically by a factor of 0.5 to enhance clarity. The solid lines are intended as guides only. Upper panels: relative molecular weights of fibrinogen−factor XIII complexes obtained at the corresponding mole fractions of factor XIII. The error bars indicate the 95% confidence limits for each value. The solid lines are intended as guides only.

containing factor XIII and fibrinogen γ_A/γ_A were also fit well by the two-species model, with globally optimal values of

$M_{r,1} = 338\,200 \pm 17\,350$ and $M_{r,2} = 1\,060\,000 \pm 73\,600$ (Figure 3A). The inclusion of a third species in the sedimentation model did not significantly improve the quality of the fits to either data set. In particular, fitting the data with the expression for the model in which the molecular weight of the third species was that of factor XIII a_2 or b_2 dimers (M_r ~155 000) returned reference concentrations for this species that were within error the same as zero (results not shown).

What is the identity of the large species present in our samples? The molecular weight ($M_{r,2} = 1\,084\,000 \pm 89\,700$) is incompatible with complexes formed by 1:1 association of factor XIII with fibrinogen ($M_{r,predicted}$ ~669 200) or the dimerization of fibrinogen ($M_{r,predicted}$ ~687 600) or factor XIII ($M_{r,predicted}$ ~650 800). On the other hand, this molecular weight is only narrowly distinguishable from the molecular weight predicted for the trimerization of factor XIII ($M_{r,predicted}$ ~976 200), and it is fully compatible with the molecular weights predicted for a trimer of fibrinogen ($M_{r,predicted}$ ~1 031 400) and for 2:1 and 1:2 fibrinogen/factor XIII complexes ($M_{r,predicted}$ ~1 013 000 and ~994 600, respectively). To distinguish among these alternatives, we performed a continuous variation (Job) analysis (*33*). As shown in Figure 3B, maximal complex formation is obtained at a mole fraction of factor XIII of ~0.37, equivalent to an optimal combining ratio of ~1.7 fibrinogens per factor XIII. The molecular weight of the complex is the same, within error, at all mole fractions of factor XIII, consistent with the notion that only one type of complex is formed at all molar ratios. This optimal combining ratio is strongly consistent with a 2:1 fibrinogen/factor XIII complex, and rules out complexes with the inverse (1:2) molar ratio or complexes containing a single protein (trimers of fibrinogen or factor XIII). A parallel experiment carried out in the presence of 10 mM EDTA gave closely similar results (optimal combining ratio of ~1.7 fibrinogens per factor XIII, $M_r = 1\,066\,000 \pm 65\,500$). These results indicate that the fibrinogen/factor XIII stoichiometry of the complex does not depend on the presence of a physiological concentration of free Ca^{2+} ions. An intriguing feature of these results is the absence of detectable quantities of 1:1 fibrinogen/factor XIII complexes in mixtures that contain free proteins and the 2:1 complex. This association pattern strongly suggests that the binding of fibrinogen by factor XIII is cooperative.

The γ′ Sequence Influences Factor XIII Binding Affinity in a [Ca^{2+}]*-Dependent Manner*. As a first step in evaluating the role of the γ' sequence in factor XIII−fibrinogen interactions, we compared the affinity of factor XIII for fibrinogen γ_A/γ_A (which lacks the γ' sequence) to that for fibrinogen γ_A/γ'. Under our standard 1 mM $CaCl_2$ conditions (Buffer A), factor XIII binds 2 equiv of fibrinogen γ_A/γ' with an apparent association constant $K_a = 3.6 \times 10^9\ M^{-2}$ [95% confidence range $(1.6-7.3) \times 10^9\ M^{-2}$; Figure 4]. This value is increased somewhat in samples equilibrated in Buffer A lacking $CaCl_2$, and significantly reduced when the $CaCl_2$ concentration is increased to 2 mM. The importance of divalent cations to the association reaction is revealed by the ~20-fold enhancement in affinity (relative to samples in Buffer A) that is obtained when samples are brought to equilibrium in the absence of added $CaCl_2$ and the presence of 10 mM EDTA.

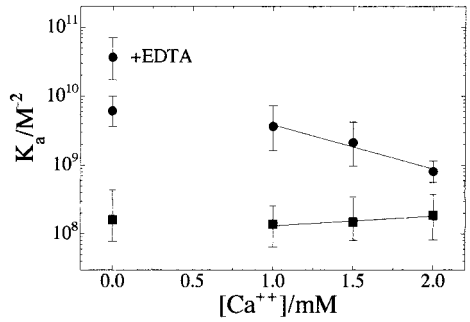

FIGURE 4: Binding of factor XIII to fibrinogens γ_A/γ' and γ_A/γ_A. Dependence of equilibrium association constants on [Ca^{2+}]. Samples contained factor XIII and fibrinogen γ_A/γ' (●) or factor XIII and fibrinogen γ_A/γ_A (■). They were brought to sedimentation equilibrium at 4 °C in 10 mM Tris (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 1 μM leupeptin buffer containing the indicated concentration of $CaCl_2$. The data indicated by +EDTA were obtained in the same buffer modified to contain 10 mM EDTA. The values shown represent equilibrium constants (and 95% confidence limits) returned by global fits of eq 4 to multiple data sets, as described under Materials and Methods.

Sharply contrasting behavior is seen with fibrinogen γ_A/γ_A. In Buffer A, its affinity for factor XIII is significantly less than that of fibrinogen γ_A/γ' [$K_{a,\gamma A\gamma A} = 1.3 \times 10^8\ M^{-2}$; range $(0.65-2.6) \times 10^8\ M^{-2}$]. This affinity is, within experimental error, independent of the concentration of added $CaCl_2$ (Figure 4). In addition, we were unable to detect fibrinogen γ_A/γ_A−factor XIII complexes in the absence of added $CaCl_2$ and the presence of 10 mM EDTA, although control samples brought to equilibrium at the same time without added $CaCl_2$ (and lacking EDTA) gave detectable binding, as did samples equilibrated in parallel at 1.0, 1.5, and 2.0 mM $CaCl_2$. Although the lack of detectable binding in the presence of EDTA limits our ability to interpret this observation, its contrast to the enhanced binding of fibrinogen γ_A/γ' indicates that the chelation of divalent cations has significantly different consequences for fibrinogens γ_A/γ_A and γ_A/γ'. Since fibrinogens γ_A/γ_A and γ_A/γ' differ in the presence of the γ' sequence, these results support the conclusion that the γ' sequence plays a significant role in factor XIII binding and that [Ca^{2+}] modulates factor XIII−γ' interactions.

The γ′ Peptide Competes with Fibrinogen γ_A/γ' for Binding Factor XIII. The difference in the affinities of factor XIII for γ_A/γ' and γ_A/γ_A fibrinogens is consistent with binding models in which the γ' sequence plays a crucial role. It was therefore of interest to determine whether a 20-residue polypeptide containing the γ' sequence VRPEHPAETEYDSLYPEDDL can present at least the minimum set of features necessary for factor XIII binding. As a consequence of its low molecular weight (2375), the binding of this polypeptide to factor XIII could not be directly detected by the sedimentation equilibrium method (result not shown). However, appropriate factor XIII binding should make the polypeptide an effective competitor of fibrinogen interactions. Such a binding competition can be represented as shown in eq 5:

$$2F + XIII{\cdot}P_n \rightleftarrows F_2{\cdot}XIII + nP \qquad (5)$$

Here F, XIII, and P represent fibrinogen, factor XIII, and peptide, respectively, and n is the peptide's binding stoichi-

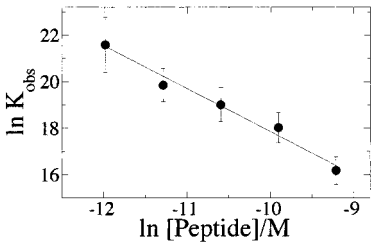

FIGURE 5: The γ' peptide competes with fibrinogen γ_A/γ' for factor XIII interaction. Plot of ln K_{obs} against ln [peptide]. Measurements were made at 4 °C in Buffer A, supplemented with the indicated concentrations of the 20-residue γ' polypeptide. The error bars represent 95% confidence intervals for the values of K_{obs}. As described in eq 6, the slope is a measure of the peptide stoichiometry of the reaction. Its value is -1.86 ± 0.56, suggesting that ~2 equiv of γ' peptide are released when 2 equiv of fibrinogen γ_A/γ' bind factor XIII.

ometry. The [peptide] dependence of the apparent macromolecular association constant for this reaction ($K_{obs} = [F_2 \cdot XIII]/[F]^2[XIII \cdot P_n]$) is given by (*34*)

$$\frac{\partial \ln K_{obs}}{\partial \ln [P]} = -n \qquad (6)$$

Shown in Figure 5 is a graph of the dependence of ln K_{obs} on ln [peptide] for the binding of fibrinogen γ_A/γ' by factor XIII. The slope (-1.84 ± 0.56) indicates that ~2 molecules of peptide are released when fibrinogen binds factor XIII. This value is equal, within error, to the fibrinogen fibrinogen γ_A/γ' stoichiometry of the reaction and is thus consistent with the competition model.

DISCUSSION

The existence of a complex between factor XIII and fibrinogen was postulated nearly 3 decades ago (*23*). We have examined the binding of fibrinogens γ_A/γ' and γ_A/γ_A by factor XIII over a wide range of protein concentrations and molar ratios and over a narrow, but physiologically relevant, range of Ca^{2+} concentrations. Both the molecular weights of the observable complexes and the optimal combining ratios of fibrinogen to factor XIII are consistent with the formation of complexes with a 2:1 fibrinogen:factor XIII molar ratio. The release of ~2 equiv of γ' polypeptide in the fibrinogen/γ' peptide competition assay is also consistent with the binding of two ligands by factor XIII. Assemblies of significantly larger or smaller molecular weight than that predicted for the 2:1 fibrinogen/factor XIII complex were not detected. The absence of 1:1 fibrinogen/factor XIII complexes is a significant feature, because 1:1 complexes should be present at detectable levels in equilibrium mixtures containing free proteins and the 2:1 complex, unless the binding reaction is highly cooperative. Such cooperativity implies communication between the fibrinogen γ_A/γ' binding sites of factor XIII. Since activated factor XIII efficiently cross-links fibrin γ-chain C-terminal segments (*3*, *35*), it seems likely that these portions of the fibrin D-domain are in close proximity in the fibrinogen$_2$·factor XIII complex. One assembly model that combines these features is shown schematically in Figure 6. Sedimentation velocity experiments to test hydrodynamic predictions of this model are currently underway.

The elevated affinity with which factor XIII binds fibrinogen γ_A/γ' and the ability of the γ' polypeptide to compete as a ligand of factor XIII are both strong evidence for the participation of the γ' sequence in the binding interaction. On the other hand, the ability of fibrinogen γ_A/γ_A to form 2:1 complexes with factor XIII indicates that other fibrinogen sequences are bound by factor XIII. If these sequences were bound independently of the γ' sites, one might expect the stoichiometry of the fibrinogen γ_A/γ' complex to be greater than that of the fibrinogen γ_A/γ_A complex. Because it is not, we favor models in which both the γ' region and D-domain sequences present in γ_A/γ_A fibrinogen contribute to the structure that is bound by factor XIII.

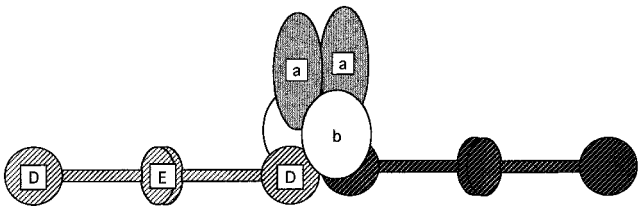

FIGURE 6: Schematic model of a 2:1 fibrinogen−factor XIII complex. Shapes are arbitrary and are not drawn to scale. The trinodular structure of fibrinogen (hatched figures) is consistent with the original electron microscopic results of Hall and Slayter (*43*) and with the recent crystal structure of the fibrinogen D-dimer solved by Spraggon et al. (*44*). Fibrinogen D- and E-domains are labeled. Our sedimentation equilibrium data do not specify which subunit(s) of factor XIII associate(s) with fibrinogen. The *b* subunit is modeled as the interacting subunit on the basis of results showing that plasma factor XIII (the a_2b_2 tetramer) binds fibrinogen γ_A/γ' while placental factor XIII, which contains only *a* chains, does not (*19*).

Taken out of context, the binding affinities that we have measured may appear to be too small to be consistent with the proposal (*19*) that γ_A/γ' fibrinogen serves as a carrier protein for factor XIII in plasma. For example, at 1 mM $CaCl_2$, $K_{a,\gamma A/\gamma'} \sim 3.6 \times 10^9$ M^{-2} and $K_{a,\gamma A/\gamma A} \sim 1.3 \times 10^8$ M^{-2} (Figure 4). Since the plasma concentrations of factor XIII, fibrinogen γ'/γ_A, and fibrinogen γ_A/γ_A are ~11 μg/mL (~34 nM), 200−400 μg/mL (0.6−1.2 μM), and 2.0−4.0 mg/mL (6−12 μM), respectively (*16*, *36*, *37*), these equilibrium constants predict that only 0.1−0.5% of the total factor XIII should be bound by fibrinogen γ_A/γ' and only 0.4−1.8% should be bound by fibrinogen γ_A/γ_A. However, our measurements were made in dilute solutions, where protein activity coefficients are close to unity. These solutions do not replicate the crowded conditions that prevail in plasma. Minton and colleagues have shown that at a serum albumin concentration of ~80 mg/mL, roughly equal to the *total* concentration of protein in plasma (*38*, *39*), the activity coefficient of dilute fibrinogen is ~10 (*40*). When this factor is taken into account, our equilibrium constants predict that 11−34% of the total factor XIII should be bound by fibrinogen γ_A/γ' and 32−65% should be bound by fibrinogen γ_A/γ_A. These values are clearly consistent with models in which fibrinogen acts as a carrier for factor XIII in plasma[2] (*19*, *23*).

One prediction of this analysis is that, at equilibrium, a greater fraction of the total plasma factor XIII is likely to

[2] These values were calculated with the assumption that the activity coefficient of fibrinogen in plasma is ~10 as described by Rivas et al. (*40*). Smaller fractional occupancies should occur in less concentrated solutions.

be associated with γ_A/γ_A fibrinogen than with the γ_A/γ' protein. This is a consequence of the fact that the ratio of equilibrium constants $K_{a,\gamma A/\gamma'}/K_{a,\gamma A/\gamma A}$ is smaller than the ratio of fibrinogen concentrations [fibrinogen γ_A/γ_A]/[fibrinogen γ_A/γ']. This outcome was unexpected because factor XIII has been isolated from plasma as a complex with fibrinogen γ_A/γ' (*19*), but not, to our knowledge, as a complex with fibrinogen γ_A/γ_A. On the other hand, the elevated affinity of factor XIII for γ_A/γ' fibrinogen leads to the prediction that the mole fraction of γ_A/γ' fibrinogen bound by factor XIII (~0.019) will be greater than that of γ_A/γ_A fibrinogen (~0.004). This may result in more facile detection of factor XIII complexes with γ_A/γ' fibrinogen than with the γ_A/γ_A protein. We emphasize that our results do not address any differences in the *kinetic* stabilities of γ_A/γ_A and γ_A/γ' complexes that may be responsible for the different isolation efficiencies of these complexes. Ultimately, the stronger association of factor XIII with γ_A/γ' fibrinogen may be responsible for the more extensive cross-linking of γ_A/γ' fibrin by factor XIIIa (*20*; Moaddel et al., manuscript in preparation), and may contribute to the increased resistance of γ_A/γ' fibrin clots to fibrinolysis.

The linked release of $CaCl_2$ with fibrinogen γ_A/γ' binding (but not fibrinogen γ_A/γ_A binding) and the significant increase of binding affinity for fibrinogen γ_A/γ' in the presence of excess EDTA (Figure 4) suggest that the γ' motif contains at least one binding site for Ca^{2+}. The presence of 8 carboxylate groups (7 aspartyl and glutamyl side chains plus the C-terminus) and 2 potential sulfotyrosine residues (*41*) in the 20-residue γ' sequence is consistent with this idea. We speculate that calcium binding by the γ' sequence may play a role in factor XIII activation that goes beyond the modulation of binding affinities shown in Figure 4. The dissociation of factor XIII *a* and *b* subunits and the exposure of the active site cysteines in the *a* subunits are both calcium-dependent events (*9*, *31*). Since calcium is released during factor XIII binding (Figure 4), the γ' sequence may act as a reservoir of calcium ions that are made available for factor XIII activation. Efficient factor XIII activation may account for enhanced cross-linking of γ_A/γ' fibrin (*20*; Moaddel et al., manuscript in preparation), and may contribute to the increased fibrinolytic resistance of γ_A/γ' fibrin.

Caveats. The experiments described here were performed under dilute solution conditions, using purified fibrinogen and factor XIII proteins. Accordingly, our results do not reflect the consequences of molecular crowding, nor the presence of other macromolecular interactions that may compete with factor XIII binding to fibrinogens γ_A/γ_A and γ_A/γ' in vivo. In particular, we have not yet characterized the binding of factor XIII with fibrinogen γ'/γ'. Although this variant is likely to comprise only 0.49−2.25% of all fibrinogen molecules, the resultant plasma concentration may exceed that of factor XIII. If the association constant is high, a significant fraction of factor XIII may be associated with this variant in plasma. Finally, the use of highly purified proteins, that have been dialyzed to equilibrium, eliminates the effects of low molecular weight species (including polypeptides, polysaccharides, and inorganic ions) that are present in plasma but not represented in Buffer A. An understanding of these factors will be necessary before our results should be considered as fully representative of the analogous interactions that take place in blood plasma.

Several of the results discussed above raise important questions for future research. First, why is factor XIII binding to fibrinogen γ_A/γ_A limited to a 2:1 stoichiometry? Fibrinogen γ_A/γ_A molecules have two identical D-domains which, all things being equal, should be capable of binding factor XIII. Since factor XIII binds two fibrinogen molecules, the result might be expected to be a linear polymerization, yielding a heterogeneous population of complexes with stoichiometries greater than 2:1. The fact that we obtained a single, well-defined optimal combining ratio and a single molecular weight for the factor XIII−fibrinogen γ_A/γ_A complex suggests the existence of a cooperative effect that inhibits the binding of 2 equiv of factor XIII to a fibrinogen γ_A/γ_A molecule. Such long-range cooperativity has at least one precedent: the binding of plasminogen to its sites in the D- and E-domains of fibrinogen has also been found to be cooperative (*24*). A second question is the following: Which residues in the γ' sequence are important for factor XIII binding? It is evident that this 20 amino acid C-terminal sequence presents at least the minimum set of surface features necessary for stable binding and competition with fibrinogen γ_A/γ' under our solution conditions. Since clots formed with γ_A/γ' fibrinogen are efficiently cross-linked by factor XIII to form structures with enhanced resistance to fibrinolysis (*20*), inhibitors based on γ' peptide structures may be useful for the artificial control of clot stability. A related question is: What γ' residues are important for the $CaCl_2$ modulation of factor XIII binding? Although crystal structures of fibrin(ogen) fragments containing the γ' sequence have been published (*42*), the carboxyl terminus of the γ' chain was not visible, so any potential interactions of the γ' chain with Ca^{2+} were not evident. Finally, what is the relationship between fibrinogen binding and factor XIII activation? Many noncovalent interactions take place in plasma, and an important challenge for the future will be to identify their roles in the essential pathways of thrombosis and thrombolysis.

REFERENCES

1. McKee, P. A., Rogers, L. A., Marler, E., and Hill, R. L. (1966) *Arch. Biochem. Biophys. 116*, 271−279.
2. Blombäck, B. (1996) *Thromb. Res. 83*, 1−75.
3. Chen, R., and Doolittle, R. F. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 420−427.
4. Lorand, L. (1972) *Ann. N.Y. Acad. Sci. 202*, 6−30.
5. Izumi, T., Hashiguchi, T., Castaman, G., Tosetto, A., Rodeghiero, F., Girolami, A., and Ichinose, A. (1996) *Blood 87*, 2769−2774.
6. Chung, S. I. (1972) *Ann. N.Y. Acad. Sci. 202*, 240−255.
7. Schwartz, M. L., Pizzo, S. V., Hill, R. L., and McKee, P. A. (1971) *J. Biol. Chem. 246*, 5851−5854.
8. Schwartz, M. L., Pizzo, S. V., Hill, R. L., and McKee, P. A. (1973) *J. Biol. Chem. 248*, 1395−1407.
9. Chung, S. I., Lewis, M. S., and Folk, J. E. (1974) *J. Biol. Chem. 249*, 940−950.
10. Lewis, S. D., Janus, T. J., Lorand, L., and Shafer, J. A. (1985) *Biochemistry 24*, 6772−6777.
11. Chung, D., and Davie, E. W. (1984) *Biochemistry 23*, 4232−4236.
12. Fornace, A. J., Cummings, D. E., Comeau, C. M., Kant, J. A., and Crabtree, G. R. (1984) *J. Biol. Chem. 255*, 5599−5604.
13. Wolfenstein-Todel, C., and Mosesson, M. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5069−5073.
14. Francis, C. W., Marder, V. J., and Martin, S. E. (1980) *J. Biol. Chem. 255*, 5599−5604.

15. Peerschke, E. I. B., Francis, C. W., and Marder, V. J. (1986) *Blood 67*, 385−390.
16. Mosesson, M. W., Finlayson, J. S., and Umfleet, R. A. (1972) *J. Biol. Chem. 247*, 5223−5227.
17. Wolfenstein-Todel, C., and Mosesson, M. W. (1981) *Biochemistry 20*, 6146−6149.
18. Lawrence, S. O., Wright, T. W., Francis, C. W., Fay, P. J., and Haidaris, P. J. (1993) *Blood 82*, 2406−2413.
19. Siebenlist, K. R., Meh, D. A., and Mosesson, M. W. (1996) *Biochemistry 35*, 10448−10453.
20. Falls, L. A., and Farrell, D. H. (1997) *J. Biol. Chem. 272*, 14251−14256.
21. Farrell, D. H., and Thiagarajan, P. (1994) *J. Biol. Chem. 269*, 226−231.
22. Mosesson, M. W., and Finlayson, J. S. (1963) *J. Lab. Clin. Med. 62*, 663−674.
23. Loewy, A. G., Dahlberg, A., Dunathan, K., Kriel, R., and Wolfinger, H. L. (1961) *J. Biol. Chem. 236*, 2634−2643.
24. Lewis, M. S., Carmassi, F., and Chung, S. I. (1984) *Biochemistry 23*, 3874−3879.
25. Johnson, M. L., Correia, J. J., Yphantis, D. A., and Halvorson, H. R. (1981) *Biophys. J. 36*, 575−588.
26. Henschen, A., Lottspeich, F., Kehl, M., and Southan, C. (1983) *Ann. N.Y. Acad. Sci. 408*, 28−43.
27. Chung, D. W., Chan, W.-Y., and Davie, E. W. (1983a) *Biochemistry 22*, 3250−3256.
28. Chung, D. W., Que, B. G., Rixon, M. W., Mace, J. M., and Davie, E. W. (1983b) *Biochemistry 22*, 3244−3250.
29. Rixon, M. W., Chan, W.-Y., Davie, E. W., and Chung, D. W. (1983) *Biochemistry 22*, 3237−3244.
30. Ichinose, A., Bottenus, R. E., and Davie, E. W. (1990) *J. Biol. Chem. 265*, 13411−13414.
31. Radek, J. T., Jeong, J. M., Wilson, J., and Lorand, L. (1993) *Biochemistry 32*, 3527−3534.
32. Murray, R. K., Granner, D. K., Mayes, P. A., and Rodwell, V. W. (1999) *Harper's Biochemistry*, 25th ed., Appleton & Lange, Stamford, CT.
33. Huang, C. Y. (1982) *Methods Enzymol. 87*, 509−525.
34. Wyman, J., and Gill, S. J. (1990) *Binding and Linkage*, University Science Books, Mill Valley, CA.
35. Ware, S., Donahue, J. P., Hawiger, J., and Anderson, W. F. (1999) *Protein Sci. 8*, 2663−2671.
36. Yorifuji, H., Anderson, K., Lynch, G. W., Van de Water, L., and McDonagh, J. (1988) *Blood 72*, 1645−1650.
37. Chung, D. W., and Ichinose, A. (1995) in *The Metabolic and Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Valle, D., and Sly, W. S., Eds.) 7th ed., pp 3223−3240, McGraw-Hill, New York.
38. Dittmer, D. S., Ed. (1961) *Blood And Other Body Fluids. Biological Handbooks*, FASEB, Washington, DC.
39. Bing, D. H., Ed. (1979) *The Chemistry And Physiology Of The Human Plasma Proteins*, Pergamon Press, New York.
40. Rivas, G., Fernandez, J. A., and Minton, A. P. (1999) *Biochemistry 38*, 9379−9388
41. Farrell, D. H., Mulvihill, E. R., Huang, S., Chung, D. W., and Davie, E. W. (1991) *Biochemistry 30*, 9414−9420.
42. Cote, H. C., Pratt, K. P., Davie, E. W., and Chung, D. W. (1997) *J. Biol. Chem. 272*, 23792−23798.
43. Hall, C. E., and Slayter, H. S. (1959) *J. Biophys. Biochem. Cytol. 5*, 11−16.
44. Spraggon, G., Everse, S. J., and Doolittle, R. F. (1997) *Nature 389*, 455−462.

BI000098U