Cooperative Association of Plasminogen with Fibrinogen†

Marc S. Lewis, Franco Carmassi,‡ and Soo Il Chung*

ABSTRACT: We have examined the association of both Glu- and Lys-plasminogen to fibrinogen by ultracentrifugal sedimentation equilibrium in neutral isotonic buffer in the presence of aprotinin. The fibrinogen and plasminogens, which were homogeneous, did not exhibit any self-association. In each association study, five different molar ratios of plasminogen to fibrinogen were examined. The data were analyzed by mathematical modeling using nonlinear least-squares curve fitting. Analyses of molecular species present showed that up to 4 mol of either Glu- or Lys-plasminogen was associated with each mol of fibrinogen. For the binding of Glu-plasminogen, the values of the successive changes of the standard free energy of association were found to be −5.48, −7.73, −8.88, and −11.41 kcal/mol (K_a = 2.16 × 10^4, 1.32 × 10^6, 1.06 × 10^7, and 1.08 × 10^9 M^{-1}). For the experimental conditions used here, the association of Lys-plasminogen appears to be described by virtually the same fitting parameters. The very marked cooperativity of association found here would appear to imply that there are significant alterations of the structure of fibrinogen as a result of each successive molecule of plasminogen bound.

In the hemostatic and inflammatory processes, fibrin formation is important in protecting surrounding tissue, in repairing tissue, and in providing "temporary connective tissue" (Glynn, 1963; Astrup, 1968; Blombäck et al., 1976). These events are followed by the selective degradation of fibrin (fibrinolysis) catalyzed by the proteolytic enzyme plasmin (Collen, 1980). The mechanism and regulation of fibrinolysis have been the subject of intensive investigation during the last 3 decades (Milstone, 1941; Astrup, 1956; Fearnley, 1973). Recent studies have demonstrated that the rate of fibrinolysis not only is dependent on the concentrations of the interacting enzyme systems (plasminogen and plasminogen activator) and the fast-reacting plasmin inhibitor α_2-antiplasmin but also is regulated by the extent of α_2-antiplasmin cross-linked to fibrin molecules by factor XIIIa (Folk & Finlayson, 1977; Sakata & Aoki, 1980; Tamaki & Aoki, 1981; Carmassi & Chung, 1982). In addition, observations of the rapid inactivation in vivo of generated free plasmin by antiplasmin and of the lesser susceptibility to antiplasmin of plasmin which is bound to the fibrin surface have led to a suggestion that plasmin must be bound to fibrin for rapid fibrinolysis to occur (Rakoczi et al., 1978; Thorsen, 1975).

Two forms of plasminogen have been described: the native form, Glu-plasminogen, which has a glutamic acid as the amino-terminal amino acid, and Lys-plasminogen, which has a lysine as an amino-terminal amino acid as the result of plasmin cleavage of 76 residues from the amino-terminal sequence. Recently, several investigators have demonstrated that plasminogen is bound to fibrin and can be eluted by lysine analogues (Cederholm-Williams, 1977; Suenson & Thorsen, 1981, Juhan-Vague et al., 1981; Lucas et al., 1983). Lucas et al. (1983), using sucrose density gradient ultracentrifugation, reported that Lys-plasminogen, but not native Glu-plasminogen, appeared to bind weakly to fibrinogen. A significant indication of an association between plasminogen and fibrinogen is seen during the preparation of fibrinogen. The preparation of plasminogen-free fibrinogen requires the application of an afffinity purification procedure involving either the passage of the fibrinogen solution through a lysine–agarose matrix or the precipitation of the fibrinogen in the presence of excess amounts of a lysine analogue, ϵ-aminocaproic acid (EACA). A selective affinity between interacting molecules as a means of regulating a specific reaction in a physiological system has been well recognized; i.e., antigen–antibody reactions, factor XIII binding to fibrinogen (Chung et al., 1979; Greenberg & Shuman, 1982), and plasminogen activator binding to fibrin (Hoylaerts et al., 1982).

We have examined the association of both native and modified plasminogen to fibrinogen by sedimentation equilibrium in neutral isotonic buffer. The data, analyzed by mathematical modeling using nonlinear least-squares curve fitting, showed that 4 mol of plasminogen was associated with each mol of fibrinogen, that the association was strong, and that there appeared to be marked cooperativity of association.

Experimental Procedures

Preparation of Lysine–Agarose. Lysine was coupled to cyanogen bromide activated agarose (Sepharose CL4B, Pharmacia) by the method of Chibber et al. (1974).

Purification of Fibrinogen. Human fibrinogen was prepared from fresh frozen plasma by a modification of the methods of Kazal et al. (1963), Mosesson (1962), and Finlayson (1968). The barium citrate adsorbed plasma was brought to 2.1 M with glycine in the presence of 0.1 M EACA at 4 °C. The precipitate, collected by centrifugation at 20000*g* for 20 min, was taken up in a volume (one-fourth that of the plasma) of sodium citrate buffer, pH 6.8, containing 10 units/mL aprotinin and 5 mM benzamidine. The glycine precipitation of fibrinogen was repeated at room temperature, and the precipitate was dissolved in 0.05 M tris(hydroxymethyl)aminomethane (Tris)–acetate buffer, pH 7.5, containing 10 units/mL aprotinin and 5 mM benzamidine. The fibrinogen solution was then dialyzed against three changes of the same buffer at 4 °C, and the dialyzate was clarified by centrifugation at 20000*g* for 20 min at 2 °C. The fibrinogen solution was then adsorbed onto a DE-52 (Whatman) column (2.5 × 20 cm) which had been equilibrated with 0.5 M Tris–acetate buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid (EDTA) and was then eluted with that buffer. When 1.0–1.2 g of fibrinogen was applied, the relatively factor XIII free fibrinogen was eluted in the wash fractions. The column was then eluted with 2 L of a linear salt gradient (0–0.4 M

† From the Biomedical Engineering and Instrumentation Branch, Division of Research Services, and the Laboratory of Oral Biology and Physiology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205. *Received November 4, 1983.*

* Correspondence should be addressed to this author at the Laboratory of Oral Biology and Physiology, National Institute of Dental Research.

‡ Present address: Second Medical Clinic, University of Pisa, Pisa, Italy.

NaCl) in the same buffer. The fractions containing above 96% clotability with thrombin were pooled and concentrated by precipitation with ammonium sulfate at 25% of saturation. The small residual amount of plasminogen in the fibrinogen solution was removed by passage over a lysine–agarose column. The purified fibrinogen showed no lytic activity after 2 days of incubation with urokinase.

Purification of Plasminogen. Native human Glu-plasminogen was prepared from fresh frozen plasma by affinity chromatography on a lysine–agarose column by the method of Deutsch & Mertz (1970) and was further purified by chromatography on a column of DEAE-Sephadex by the method of Robbins & Summaria (1976). This plasminogen appeared homogeneous when examined by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Following conversion to plasmin by urokinase, this preparation showed 70–80% activation by active-site titration using *p*-nitrophenyl *p*-guanidinobenzoate (Chase & Shaw, 1967). The purified Lys-plasminogen was the generous gift of Dr. Genesio Murano, FDA, Bethesda, MD.

Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed on 7.5% polyacrylamide gels as described by Weber & Osborn (1969). Glu-plasminogen, Lys-plasminogen, and fibrinogen were also examined for purity in the absence of SDS on polyacrylamide gels at pH 8.9 (Ornstein, 1964) and at pH 3.5 (Panyim & Chalkley, 1969).

Analytical Ultracentrifugation. Analytical ultracentrifugation was performed by using a standard Beckman Model E analytical ultracentrifuge. All experiments were carried out at 4 °C. Column lengths of approximately 7 mm were used in order to obtain good resolution. Rotor speeds were 9000 rpm for studies on the plasminogens alone, 6000 rpm for studies on fibrinogen alone, and 4400 rpm for studies on the mixtures of fibrinogen and plasminogen. For the studies on the plasminogens and the fibrinogen alone, three concentrations of each protein were run simultaneously: 0.15, 0.10, and 0.05 mg/mL for each protein, respectively. Five different molar ratios of plasminogen to fibrinogen were run simultaneously for the studies on plasminogen–fibrinogen interaction. The total fibrinogen concentration in each cell was constant at 0.10 mg/mL, and the plasminogen:fibrinogen molar ratios were approximately 1.8:1, 1.2:1, 0.9:1, 0.6:1, and 0.3:1. The buffer was 0.01 M Tris, pH 7.4, 0.15 M NaCl, and 100 units/mL aprotinin. Ultracentrifugal equilibrium was considered to have been attained when three successive scans at a wavelength of 280 nm taken at 24-h intervals were invariant. This usually required 7–9 days. Data were obtained by manually digitizing the scans and were analyzed by mathematical modeling using MLAB, an interactive system operating on a DEC-10 computer (Knott, 1979). Since the development of appropriate mathematical models is intimately related to the results obtained, this will be described in detail under Results.

Results

Figure 1 shows the results of the gel electrophoresis examination of fibrinogen and the plasminogens, demonstrating their essential homogeneity. This homogeneity was also demonstrated by the ultracentrifugal analyses of fibrinogen and the plasminogens alone. Additionally, the latter studies demonstrated that these proteins did not undergo any self-association, a desirable condition, since the presence of any polymer of either of the constituent proteins would have represented an additional complication in the analytical procedure.

As the first step in the mathematical modeling of an association reaction, it is necessary to know that molecular species are present. Thus, for a mixture of fibrinogen and plasminogen, denoting fibrinogen as F and plasminogen as P, the only species which could be present are F, P, FP, FP_2, F_2P, etc., depending upon the stoichiometry. The concentration (c) of any molecular species at a radial position r is given by

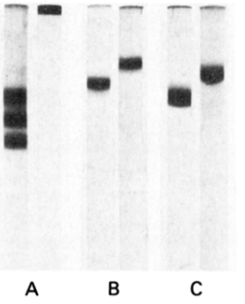

FIGURE 1: SDS–polyacrylamide gel electrophoretic patterns of purified fibrinogen and plasminogens: (A) fibrinogen; (B) Glu-plasminogen; (C) Lys-plasminogen. In each case, the gel on the left has protein which was reduced with dithiothreitol prior to electrophoresis.

$$c_r = c_b \exp[AM(r^2 - r_b^2)] \quad (1)$$

and the total concentration at any radial position r is given by

$$c_r = \sum_{i=1}^{n} c_{b,i} \exp[A_i M_i (r^2 - r_b^2)] + \epsilon \quad (2)$$

Equation 2 contains a term for each molecular species that might be present: M_i = the molecular weight, $A_i = [(1 - \bar{V}_i\rho)\omega^2]/(2RT)$, and $\bar{V}_i$ = the partial specific volume, all for the ith component; ρ = the solution density, ω = the rotor angular velocity, R = the gas constant, T = the absolute temperature, and ϵ is a small error term to adjust for base-line error (Johnson et al., 1981).

Since the values of the molecular weights of each of the possible species are known, the value of the partial specific volume for each of the species can be calculated by using

$$\bar{V}_{F_iP_j} = (iM_F\bar{V}_F + jM_P\bar{V}_P)/(iM_F + jM_P) \quad (3)$$

where i and j have integral values reflecting the possible stoichiometries of fibrinogen and plasminogen in a complex. It should be noted that this equation implicitly assumes that the partial specific volume of a complex is a weight average of the partial specific volumes of the constituent species and thus also assumes that there is no significant volume change upon association. We have no basis for predicting whether or not such as volume change might occur and what magnitude it might have. The accurate measurement of such a change appears to be a very different undertaking. Since the partial specific volumes of fibrinogen and plasminogen are fairly similar (0.705 and 0.715, respectively), minor deviations of the actual value from the calculated value would have no effect upon the stoichiometry of the reaction and probably relatively little effect upon the values obtained for the equilibrium constants for the binding. Thus, the only fitting parameters possible are the values of c_b, concentrations at the cell bottoms of the various species which might be present, and the value of ϵ, the scanner error term. These concentrations are subject to the constraint that they may not be negative. Thus, a zero value for such a parameter implies the absence of the molecular species which it represents. On this basis, all of the data sets showed the presence of fibrinogen, plasminogen, and fibrinogen–plasminogen complexes. The binding stoichiometry is demonstrated in Table I. Fibrinogen, plasminogen, and FP_4 were the predominant species observed. Either zero or very small values were found for the concentrations of FP_1, FP_2, and FP_3, and since they were below the levels of rea-

Table I: Concentrations of Fibrinogen, Plasminogen, and Fibrinogen–Plasminogen Complex Found at the Cell Bottom

cell	initial P:F molar ratio	concn (μmol/L)[a] F	P	FP_4
		Glu-Plasminogen		
1	0.3:1.0	0.691	0.086	0.048
2	0.6:1.0	0.568	0.139	0.063
3	0.9:1.0	0.430	0.157	0.185
4	1.2:1.0	0.369	0.164	0.239
5	1.8:1.0	0.295	0.214	0.283
		Lys-Plasminogen		
1	0.3:1.0	0.667	0.110	0.049
2	0.6:1.0	0.565	0.149	0.086
3	0.9:1.0	0.465	0.164	0.137
4	1.2:1.0	0.364	0.175	0.197
5	1.8:1.0	0.299	0.237	0.302

[a] The concentrations of FP_1, FP_2, and FP_3 were all below 0.001 μmol/L.

sonable precision, they are not presented. These values at least suggest the presence of small finite concentrations of these species. No species involving an association of more than four molecules of plasminogen per molecule of fibrinogen or more than one molecule of fibrinogen per molecule of plasminogen were found. The data fitted to appropriate mathematical models failed to show the presence of any thermodynamic nonideality in the concentration range used.

Having established the stoichiometry, it is next necessary to demonstrate a reversible interaction and to measure the values of the equilibrium constants which describe the reactions. We can postulate the following reactions, with molar equilibrium constants K_i:

$$F + P \rightleftharpoons FP \qquad K_1 = [FP]/([F][P]) \tag{4a}$$

$$FP + P \rightleftharpoons FP_2 \qquad K_2 = [FP_2]/([FP][P]) \tag{4b}$$

$$FP_2 + P \rightleftharpoons FP_3 \qquad K_3 = [FP_3]/([FP_2][P]) \tag{4c}$$

$$FP_3 + P \rightleftharpoons FP_4 \qquad K_4 = [FP_4]/([FP_3][P]) \tag{4d}$$

The molar concentration of each of the species is given by

$$[FP] = K_1[F][P] \tag{5a}$$

$$[FP_2] = K_2[FP][P] = K_1K_2[F][P]^2 \tag{5b}$$

$$[FP_3] = K_3[FP_2][P] = K_1K_2K_3[F][P]^3 \tag{5c}$$

$$[FP_4] = K_4[FP_3][P] = K_1K_2K_3K_4[f][P]^4 \tag{5d}$$

This represents the most general model which can be fit, i.e., where the four equilibrium constants are independent of each other. Such a model can represent four nonidentical binding sites, four identical binding sites with cooperativity, four nonidentical binding sites with cooperativity, or various other combinations such as two sets of two identical binding sites where the two sets of sites have different intrinsic binding constants and where there is cooperativity.

For computational purposes with this model, it is easier to deal with equilibrium constants based on a mass per unit volume scale, optical density at 280 nm in this case, and eq 5a–d may then be written

$$c_{FP} = k_1c_Fc_P \tag{6a}$$

$$c_{FP_2} = k_1k_2c_Fc_P^2 \tag{6b}$$

$$c_{FP_3} = k_1k_2k_3c_Fc_P^3 \tag{6c}$$

$$c_{FP_4} = k_1k_2k_3k_4c_Fc_P^4 \tag{6d}$$

In eq 6a–d, the values of the k_i's are related to those of the K_i's by the following:

$$K_1 = k_1E_FE_P/E_{FP} = k_1E_FE_P/(E_F + E_P) \tag{7a}$$

$$K_2 = k_2E_{FP}E_P/E_{FP_2} = [k_2(E_F + E_P)E_P]/(E_F + 2E_P) \tag{7b}$$

$$K_3 = k_3E_{FP_2}E_P/E_{FP_3} = [k_3(E_F + 2E_P)E_P]/(E_F + 3E_P) \tag{7c}$$

$$K_4 = k_4E_{FP_3}E_P/E_{FP_4} = [k_4(E_F + 3E_P)E_p]/(E_F + 4E_p) \tag{7d}$$

where E_F is the molar extinction coefficient of fibrinogen (5.15×10^5 M^{-1} at 280 nm) and E_p is the molar extinction coefficient of plasminogen [1.564×10^5 M^{-1} at 280 nm for Glu-plasminogen (Violand & Castellino, 1976) and 1.448×10^5 M^{-1} at 280 nm for Lys-plasminogen (Barlow et al., 1969)]. These equations assume that the molar extinction coefficient of a complex is given by the sum of the molar extinction coefficients of its constituents and that complex formation has no significant effect upon the absorbancies of the chromophores.

Equation 2 may then be written in the following form:

$$\begin{aligned} c_r = {} & c_{b,F}\exp[A_FM_F(r^2 - r_b^2)] + c_{b,P}\exp[A_PM_P(r^2 - r_b^2)] \\ & + k_1c_{b,F}c_{b,P}\exp[A_{FP}M_{FP}(r^2 - r_b^2)] \\ & + k_1k_2c_{b,F}c_{b,P}^2\exp[A_{FP_2}M_{FP_2}(r^2 - r_b^2)] \\ & + k_1k_2k_3c_{b,F}c_{b,P}^3\exp[A_{FP_3}M_{FP_3}(r^2 - r_b^2)] \\ & + k_1k_2k_3k_4c_{b,F}c_{b,P}^4\exp[A_{FP_4}M_{FP_4}(r^2 - r_b^2)] + \epsilon \end{aligned} \tag{8}$$

The data in the form of optical density as a function of cell radius can be fit to this model with the values of $c_{b,F}$, $c_{b,P}$, k_1, k_2, k_3, k_4, and ϵ as fitting parameters. Fitting a model of this type with highly interdependent parameters presents some difficulties, since unless constraints are used, it is possible to obtain convergence in local minima with negative values for the concentrations or the equilibrium constants. This problem may be most readily avoided by fitting the natural logarithms of the equilibrium constants and the concentrations (Johnson et al., 1981) using the following:

$$\begin{aligned} c_r = {} & \exp[\ln c_{b,F} + A_FM_F(r^2 - r_b^2)] \\ & + \exp[\ln c_{b,P} + A_PM_P(r^2 - r_b^2)] \\ & + \exp[\ln k_1 + \ln c_{b,F} + \ln c_{b,P} + A_{FP}M_{FP}(r^2 - r_b^2)] \\ & + \exp[\ln k_1 + \ln k_2 + \ln c_{b,F} + 2\ln c_{b,P} \\ & + A_{FP_2}M_{FP_2}(r^2 - r_b^2)] \\ & + \exp[\ln k_1 + \ln k_2 + \ln k_3 + \ln c_{b,F} + 3\ln c_{b,P} \\ & + A_{FP_3}M_{FP_3}(r^2 - r_b^2)] \\ & + \exp[\ln k_1 + \ln k_2 + \ln k_3 + \ln k_4 + \ln c_{b,F} + 4\ln c_{b,P} \\ & + A_{FP_4}M_{FP_4}(r^2 - r_b^2)] + \epsilon \end{aligned} \tag{9}$$

It is entirely appropriate to use the logarithms of the equilibrium constants and the concentrations as fitting parameters since the values of the standard free energy changes are given by

$$\Delta G_i^\circ = -RT\ln K_i \tag{10}$$

and thus, in effect, we are using these as fitting parameters.

It can be readily seen that eq 2 and 9 are equivalent, and thus if only a single data set is fit, a nonreversible mixture of fibrinogen, plasminogen, and complexes could be fit as if it were in reversible equilibrium. However, this problem can be solved by simultaneously fitting five data sets resulting from initial loadings of five different molar ratios of fibrinogen and plasminogen which have been centrifuged to equilibrium under otherwise identical conditions. With this experimental design, the values of $\ln c_{b,F}$, $\ln c_{b,P}$, and ϵ are unique for each cell, but the values of the $\ln k_i$'s must be common for all five data sets. Obtaining good fits under such conditions represents a stringent test for reversible equilibrium (Roark, 1976).

The only models of plasminogen binding which have been considered so far are the model which has four independent binding sites for plasminogen and those models which have an extent of cooperativity that makes it impossible to distinguish between them and the model with four independent sites with different binding constants. One problem that we have with these models is that the concentrations of the species FP_1, FP_2, and FP_3 appear to be so low that it is difficult to fit for the equilibrium constants for their formation with meaningful precision. However, these models require that those values be obtained. If we are willing to treat the association as if the four plasminogen molecules are bound simultaneously to the fibrinogen molecule, a situation which may be considered to be essentially equivalent to very marked cooperativity, then the reaction and the equilibrium constant are

$$F + 4P \rightleftharpoons FP_4 \qquad K = [FP_4]/([F][P]^4) \quad (11)$$

and the molar concentration of the FP_4 complex is given by

$$[FP_4] = K[F][P]^4 \quad (12)$$

It can be seen, by comparing eq 5d and 12, that $K = K_1K_2K_3K_4$. We then have equations analogous to eq 6 and 7, giving

$$c_{FP_4} = kc_Fc_P^4 \quad (13)$$

$$K = kE_FE_P^4/E_{FP_4} = E_FE_P^4/(E_F + 4E_P) \quad (14)$$

and the fitting equation becomes

$$c_r = \exp[\ln c_{b,F} + A_FM_F(r^2 - r_b^2)] + \exp[\ln c_{b,P} + A_PM_P(r^2 - r_b^2)] + \exp[\ln k + \ln c_{b,F} + 4 \ln c_{b,P} + A_{FP_4}M_{FP_4}(r^2 - r_b^2)] + \epsilon \quad (15)$$

By using this model, we have reduced the number of fitting parameters by three and eliminate the dependency on very low concentration values. For this gain, we give up possible information concerning intermediate steps in the binding of four molecules of plasminogen.

Other possible models of plasminogen binding need to be considered. One such model is that of four identical sites on the fibrinogen molecule with an intrinsic equilibrium constant K. For such a model, the individual equilibrium constants are given by $K_i = [K(n - i + 1)]/i$, where n is the maximum number of identical sites (Van Holde, 1971). This gives $K_1 = 4K$, $K_2 = 3K/2$, $K_3 = 2K/3$, and $K_4 = K/4$. Conversion to mass per unit volume equilibrium constants using these equations and eq 7a–d then gives the following relationships which may be substituted in eq 9: $\ln k_1 = \ln K - 10.32$, $\ln k_2 = \ln K - 11.34$, $\ln k_3 = \ln K - 12.18$, and $\ln k_4 = \ln K - 13.19$. With these substitutions, the fit is then done for the values of the $\ln c$'s for each cell and the common value of $\ln K$. Such a model explicitly excludes the possibility of any cooperativity.

Another possible model involves two sets of plasminogen binding sites per fibrinogen molecule, corresponding to two different domains in the molecule, each having two identical binding sites with each set having its own intrinsic association constant, K_I and K_{II}. This gives $K_1 = 2K_I$, $K_2 = K_I/2$, $K_3 = 2K_{II}$, and $K_4 = K_{II}/2$. Conversion gives $\ln k_1 = \ln K_I - 11.01$, $\ln k_2 = \ln K_I - 12.46$, $\ln k_3 = \ln K_{II} - 11.09$, and $\ln k_4 = \ln K_{II} - 12.50$. These are also substituted in eq 9, and the fit is done as described above except that the values of $\ln K_I$ and $\ln K_{II}$ are common fitting parameters. Such a model also does not permit any cooperativity.

We may also consider a model where there are two sets of sites, as above, with one set having an intrinsic association constant K_I and not exhibiting any cooperativity and the other set of sites exhibiting a significant level of cooperativity; and thus, the two sets should be treated as if they were totally independent. Then, $\ln k_1$ and $\ln k_2$ would be defined as described above, and in the actual fitting procedure, they were obtained by fitting for all four values of $\ln k_i$, but applying the constraint that $\ln k_2 = \ln k_1 - 1.45$. This constraint comes from taking the difference between the relationships $\ln k_2 = \ln K_I - 12.46$ and $\ln k_1 = \ln K_I - 11.01$.

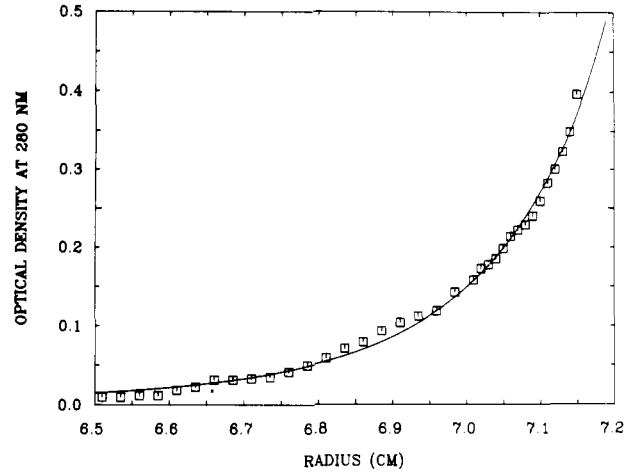

FIGURE 2: Optical density at 280 nm as a function of radial position at equilibrium at 4400 rpm for a mixture of Glu-plasminogen and fibrinogen in a 0.9:1 molar ratio. The fitting line was generated for the model with four independent sites by using the equilibrium constants given in Table II.

Discrimination between these models requires the application of appropriate criteria for goodness of fit. Several criteria can be used for discriminating between possible models. The most important of these is that the fitting parameters must have physically meaningful values. If more than one model meets this criterion, then the criteria of minimum root mean square (rms) error and minimum systematic deviation of the data points from the fitting line can be used to assist in determining the most appropriate model.

The model involving four identical sites fails on the basis of the first criterion. This model requires the presence of much higher concentrations of FP_1, FP_2, and FP_3 to be present than were observed; it requires vanishingly small concentrations of both fibrinogen and plasminogen, again in contradiction to what was observed; it gave an intrinsic association constant of 3.5×10^{13}, an improbably high value, for the binding of Glu-plasminogen. The binding of Lys-plasminogen did not differ in any significant way. The model with two sets of sites had the same problem with the concentrations of the components; the intrinsic association constants were 5.8×10^{11} and 2.5×10^{15}, and Lys-plasminogen again behaved in an almost identical manner.

None of these criteria permit discrimination between the model with four independent binding sites with implied cooperativity, the model where four plasminogens are bound without consideration for intermediate species, and the model where the first two sites had a common intrinsic binding constant and no cooperativity and the other two binding sites were independent with implied cooperativity. Figures 2–4 are appropriate for any of these models. Figure 2 shows the fit of data from a 0.9:1 molar ratio mixture of Glu-plasminogen and fibrinogen. Figure 3 shows the deviations of the data from the fitting line for the fit shown in Figure 2. Figure 4 shows the distributions of fibrinogen, plasminogen, and the FP_4 complex and their sum in terms of their optical densities at 280 nm for the model with four independent binding sites.

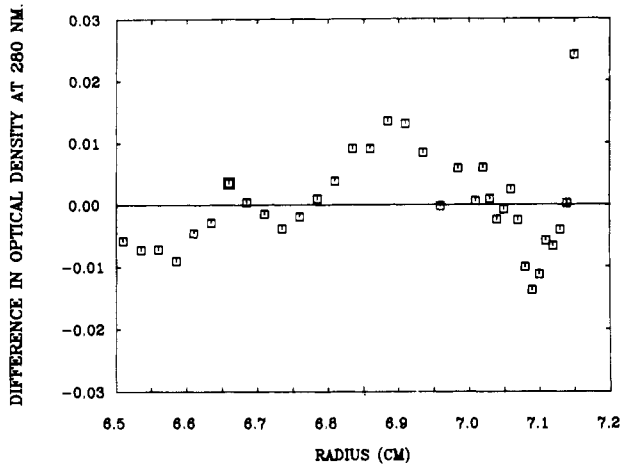

FIGURE 3: Deviation of the data points from the fitting line for the fit shown in Figure 2.

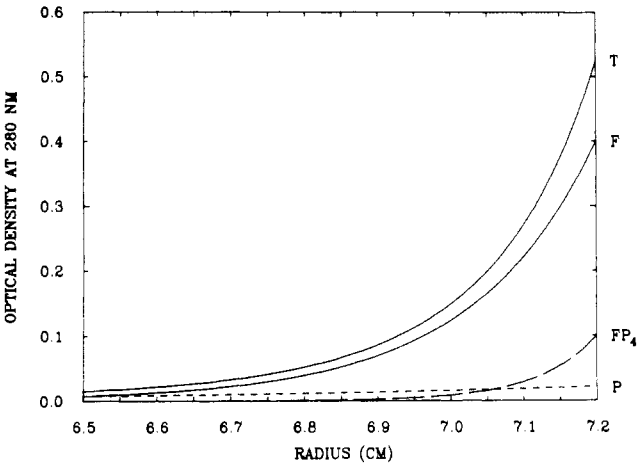

FIGURE 4: Distribution of the various components present at equilibrium in terms of their optical densities at 280 nm. T denotes the total sum of all the optical densities; F denotes that due to fibrinogen; P denotes that due to Glu-plasminogen; FP_4 denotes that due to the FP_4 complex. The quantities of the other complexes present are too low to be effectively shown in this figure.

Because the ratio of the molar extinction coefficients of plasminogen and fibrinogen is significantly less than the ratio of their molecular weights, the quantities of plasminogen and FP_4 appear disproportionally small in comparison to the quantity of fibrinogen. The quantities of the FP_1, FP_2, and FP_3 complexes present are too low to be effectively presented in this figure but are presumed present in sufficient quantity to have some effect upon the fit. The values of the equilibrium constants and the changes of standard free energy for the three different models for the binding of Glu- and Lys-plasminogen to fibrinogen are given in Table II.

Table II

type	site	K_i (L/M)	$\Delta G°_i$ (kcal/M)
Binding of Glu-Plasminogen[a]			
four sites	1	2.16×10^4	-5.45
cooperative	2	1.32×10^6	-7.73
	3	1.08×10^7	-8.88
	4	1.06×10^9	-11.41
two sites	1	2.38×10^5	-6.79
noncooperative	2	5.92×10^4	-6.03
two sites	3	1.04×10^7	-8.87
cooperative	4	1.68×10^9	-11.56
four sites simultaneously		3.27×10^{26}	-33.6
Binding of Lys-Plasminogen[b]			
four sites	1	2.35×10^4	-5.52
cooperative	2	1.24×10^6	-7.75
	3	1.11×10^7	-8.90
	4	9.46×10^8	-11.34
two sites	1	3.39×10^5	-6.99
noncooperative	2	8.42×10^4	-6.22
two sites	3	1.11×10^7	-8.90
cooperative	4	9.46×10^8	-11.34
four sites simultaneously		3.06×10^{26}	-33.6

[a] rms error = 0.025 ODU at 280 nm for any type. [b] rms error = 0.016 ODU at 280 nm for any type.

Discussion

In this study, we have measured the affinity of native plasminogen and partly degraded plasminogen for fibrinogen under equilibrium conditions. The affinity for lysine analogues in plasminogen and its fragments (kringles 1–4 and miniplasminogen) has been investigated extensively by using insoluble interacting components such as fibrin or lysine-linked solid matrices (Cederholm-Williams & Swain, 1979; Suenson & Thorsen, 1981; Thorsen et al., 1981; Lucas et al., 1983; Vâradi & Patthy, 1983). However, very little has been reported on the binding properties of plasminogen to fibrinogen, which are the physiological constituents of plasma. A possible association between plasminogen and fibrinogen was evident in the early investigations of fibrinogen, when it was noted that plasminogen constantly accompanied fibrinogen during purification. Isolated pure plasminogen and fibrinogen exhibit significantly different behavior with respect to salt precipitation and gel filtration chromatography; thus, the failure of these procedures to separate these proteins from each other during the process of purification very strongly indicates a marked degree of interaction.

However, because of the difficulty of measuring heterogeneous protein associations under equilibrium conditions, the direct evidence for the association of these proteins has not been demonstrated previously. In this study, we have developed a refined and rigorous procedure for the analysis of such a heterogeneous protein association under equilibrium conditions. This method of analysis of heterogeneous associating systems is a direct extension of the methods developed for the analysis of simple and complex homogeneous associations (Davies et al., 1980). Tindall & Aune (1982) have recently described a similar method. Previous studies of single-site or multi-site binding have been based upon obtaining molecular weight distributions for mixtures of components and then analyzing these distributions for the possible complexes which might produce such distributions (Howlett & Nichol, 1973; Clarke & Howlett, 1979; Howlett et al., 1982). The method we have used here has the advantage that it is based upon a direct analysis of the data, i.e., concentration as a function of radial position in the centrifuge cell, and thus minimizes error by avoiding the propagation of error which results from obtaining molecular weight distributions from the original data and then analyzing these distributions for the molecular species which might be present.

Contrary to the earlier study of Lucas et al. (1983), which reported that, on the basis of sucrose density gradient centrifugation, native plasminogen showed no binding and that only 2 mol of Lys-plasminogen was bound per mol of fibrinogen, we have demonstrated that 4 mol of either form of plasminogen is bound per mol of fibrinogen. These results are consistent with the results of Vâradi & Patthy (1983), who showed that there was an affinity for plasminogen in two different domains of the fibrinogen molecule (fragments E and D). Our study also indicates a progressive increase in the affinity of plasminogen for fibrinogen as the binding sites are

occupied, thus strongly suggesting cooperative binding behavior. Since the equilibrium constant for the first site is relatively small, any factor which decreases the probability of the first binding site being occupied will have the effect of significantly inhibiting the overall binding of plasminogen to fibrinogen. Our study suggests that a conformational change is essential for the occupation of further sites. Thus, any factor which inhibits conformational change is also inhibitory for further binding.

The observation of Rakoczi et al. (1978) that fibrinogen and its fragments interfere with the reaction between plasmin and α_2-antiplasmin is also supporting evidence for the binding of plasminogen by fibrinogen since α_2-antiplasmin competes for plasmin lysine binding sites (Moroi & Aoki, 1976; Wiman & Collen, 1977; Wiman, 1980).

Our results showed that there were no significant differences between the association constants obtained for the binding of either Glu- or Lys-plasminogen to fibrinogen. This result differs from the observations of other investigators who have found that Lys-plasminogen appears to be more stongly bound than Glu-plasminogen. We have considered the possibility that the Glu-plasminogen had a significant conversion to the Lys form during the periods of time involved for centrifugal equilibrium even in the presence of aprotinin. This was ruled out by examination of the solutions by SDS gel electrophoresis following centrifugation to equilibrium with the demonstration of the absence of any detectable amount of conversion of Glu- to Lys-plasminogen. We feel that additional studies of this type on fibrinogen fragments will be required in order to elucidate the mechanism of binding of the different forms of plasminogen to the different sites of fibrinogen in a more definitive manner.

It is difficult to comment in detail on the significance of the observed values of the changes of the standard free energy, since these reflect free-energy changes due to the changes in conformation as well as the free-energy changes due to the binding of plasminogen. The total free-energy change of −33.5 kcal/mol of fibrinogen for the binding of all four molecules of plasminogen suggests that some type of noncovalent binding is involved.

Acknowledgments

We thank Drs. Jules Gladner, Elemer Mihalyi, and Allen Minton for critical evaluations and helpful suggestions.

Registry No. Plasminogen, 9001-91-6.

References

Astrup, T. (1956) *Blood 11*, 781–806.
Astrup, T. (1968) in *Chemical Biology of Inflammation* (Houck, J. C., & Forcher, B. K., Eds.) pp 241–246, Pergamon Press, New York.
Barlow, G. H., Summaria, L., & Robbins, K. C. (1969) *J. Biol. Chem. 244*, 1138–1141.
Blombäck, B., Hogg, D. H., Gardlund, B., Hessel, B., & Kudryk, B. (1976) *Thromb. Res. 8 (Suppl. II)*, 329–346.
Carmassi, F., & Chung, S. I. (1983) *Prog. Fibrinolysis 4*, 281–285.
Cederholm-Williams, S. A. (1977) *Thromb. Res. 11*, 421–423.
Cederholm-Williams, S. A., & Swain, A. (1979) *Thromb. Res. 16*, 705–713.
Chase, T., Jr., & Shaw, E. (1967) *Biochem. Biophys. Res. Commun. 29*, 508–512.
Chibber, A. K., Deutsch, D. G., & Mertz, E. T. (1974) *Methods Enzymol. 34*, 424–432.
Chung, S. I., Folk, J. E., & Finlayson, J. S. (1979) *Thromb. Haemostasis 42*, 226.
Clarke, R. G., & Hewlett, G. J. (1979) *Arch. Biochem. Biophys. 195*, 235–242.
Collen, D. (1980) *Thromb. Haemostasis 43*, 77–89.
Davies, P. J. A., Wallach, D., Willingham, M., Pastan, I., & Lewis, M. S. (1980) *Biochemistry 19*, 1366–1372.
Deutsch, D. G., & Mertz, E. T. (1970) *Science (Washington, D.C.) 170*, 1095–1096.
Fearnley, G. R. (1973) *Adv. Drug Res. 7*, 107–163.
Finlayson, J. S. (1968) in *Fibrinogen* (Laki, K., Ed.) pp 39–59, Marcel Dekker, New York.
Folk, J. E., & Finlayson, J. S. (1977) *Adv. Protein Chem. 31*, 1–133.
Glynn, L. E. (1963) *Bull. Rheum. Dis. 14*, 323–326.
Greenberg, C. S., & Shuman, M. A. (1982) *J. Biol. Chem. 257*, 6096–6101.
Howlett, G. J., & Nichol, L. (1973) *J. Biol. Chem. 248*, 619–621.
Howlett, G. J., Dickson, P. W., Birch, H., & Schreiber, G. (1982) *Arch. Biochem. Biophys. 215*, 309–318.
Hoylaerts, M., Riken, D. C., Lijnen, H. R., & Collen, D. (1982) *J. Biol. Chem.* 257, 2912–2919.
Johnson, M. L., Correia, J. J., Yphantis, D. A., & Halvorson, H. (1981) *Biophys. J. 36*, 575–588.
Juhan-Vague, I., Calas, M. F., Roux, F., Juhan, C., Durand-Dessemon, F., de Laforte, C., & Serradimigni, A. (1981) *Thromb. Haemostasis 45*, 154–157.
Kazal, L. A., Amsel, S., Miller, O. P., & Tocantins, L. M. (1963) *Proc. Soc. Exp. Biol. Med. 113*, 989–992.
Knott, G. D. (1979) *Comput. Programs Biomed. 10*, 271–280.
Lucas, M. A., Fretto, L. J., & McKee, P. A. (1983) *J. Biol. Chem. 258*, 4249–4256.
Milstone, H. (1941) *J. Immunol. 42*, 109–116.
Moroi, M., & Aoki, N. (1976) *J. Biol. Chem. 251*, 5956–5965.
Mosesson, M. W. (1962) *Biochim. Biophys. Acta 57*, 204–213.
Ornstein, L. L. (1964) *Ann. N.Y. Acad. Sci. 121*, 321–349.
Panyim, S., & Chalkley, R. (1969) *Arch. Biochem. Biophys. 130*, 337–346.
Rakoczi, I., Wiman, B., & Collen, D. (1978) *Biochim. Biophys. Acta 540*, 295–300.
Roark, D. E. (1976) *Biophys. Chem. 5*, 185–196.
Robbins, K. C., & Summaria, L. (1976) *Methods Enzymol. 45*, 257–273.
Sakata, Y., & Aoki, N. (1980) *J. Clin. Invest. 65*, 6614–6621.
Suenson, E., & Thorsen, S. (1981) *Biochem. J. 197*, 619–628.
Tamaki, T., & Aoki, N. (1981) *Biochim. Biophys. Acta 661*, 280–286.
Thorsen, S. (1975) *Biochim. Biophys. Acta* 393, 55–65.
Thorsen, S., Clemmensen, S. I., Sottrup-Jensen, L., & Magnusson, S. (1981) *Biochim. Biophys. Acta 668*, 377–387.
Tindall, S. H., & Aune, K. C. (1982) *Anal. Biochem. 120*, 71–84.
Van Holde, K. E. (1971) in *Physical Biochemistry*, pp 51–78, Prentice-Hall, Englewood Cliffs, NJ.
Váradi, A., & Patthy, L. (1983) *Biochemistry 22*, 2440–2446.
Violand, B. N., & Castellino, F. J. (1976) *J. Biol. Chem. 251*, 3906–3912.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Wiman, B. (1980) *Biochem. J. 191*, 229–234.
Wiman, B., & Collen, D. (1977) *Eur. J. Biochem. 78*, 19–26.