Interaction of Fibrinogen with Its Platelet Receptor: Kinetics and Effect of pH and Temperature†

Gerard A. Marguerie‡ and Edward F. Plow*

ABSTRACT: The interaction of fibrinogen with its ADP-induced receptor on human platelets involves an initial reversible binding of fibrinogen followed by a stabilization of platelet-bound fibrinogen. In this study, kinetic analyses were performed to provide an independent characterization of the reversible binding of fibrinogen to its platelet receptor. In addition, the effects of temperature and pH on the initial reversible interaction and the subsequent stabilization of platelet-bound fibrinogen were examined. Kinetic analysis of the reaction indicated that the associated events of platelet stimulation, receptor induction, and stabilization of the fibrinogen–receptor complex were not rate-determining steps and that the interaction of fibrinogen with platelets was adequately described by the rate constants of the initial reversible binding of fibrinogen. The rate constant of association, k_{+1}, was 2-fold greater in the presence of 1 mM Ca^{2+} than of 1 mM Mg^{2+}, suggesting a slight differential effect of these cations on the overall reaction. Affinity constants, K_a, of 2.0×10^6 M^{-1} and 1.5×10^6 M^{-1} were derived from the rate constants of association and dissociation in the presence of Ca^{2+} and Mg^{2+}, respectively. These affinity constants were very similar to those obtained from Scatchard analyses at equilibrium and from the rate of platelet aggregation. Minimal fibrinogen binding occurred at pH 6.5, and lowering the pH to 6.5 did not result in dissociation of platelet-bound fibrinogen. The rate constant of association did not differ significantly at 10, 22, or 37 °C, but fewer fibrinogen molecules were bound at 37 °C than at the lower temperatures. Stabilization of platelet-bound fibrinogen occurred to an equal extent at 10 and 22 °C, suggesting that cluster formation and mobility of lipids or proteins in the platelet membrane are not major factors in regulating fibrinogen–platelet interaction.

Fibrinogen is an essential component of the coagulation system and plays a bifunctional role in hemostasis. The formation of fibrin from fibrinogen has been the primary focus of studies of the molecule in the hemostatic process, but numerous investigations have documented that fibrinogen also participates in platelet aggregation induced by ADP[1] [for review, see Mustard & Packham (1970) and Bang et al. (1972)]. Only recently has a molecular basis for the role of fibrinogen in platelet aggregation been provided by the demonstration of a direct interaction of the molecule with the cell (Mustard et al., 1978; Marguerie et al., 1979; Bennett & Vilaire, 1979; Niewiarowski et al., 1980; Figures et al., 1980; Peerschke et al., 1980; Harfenist et al., 1980). This interaction involves the binding of fibrinogen to sites on the platelet induced by ADP, and these sites exhibit many of the characteristics of a discrete and saturable receptor system (Marguerie et al., 1979; Bennett & Vilaire, 1979). Divalent ions and the continuous presence of ADP are required for fibrinogen binding and platelet aggregation, and the dissociation of fibrinogen from the platelet is correlated with platelet disaggregation. The specific binding of fibrinogen to ADP-stimulated platelets was best described by a linear Scatchard plot suggesting a single class of fibrinogen binding sites with an apparent association constant of $K_a = 2 \times 10^6$ M^{-1}. Approximately 40 000 fibrinogen molecules were bound per platelet in the presence of 1 mM calcium (Marguerie et al., 1980).

The interaction between fibrinogen and its platelet receptor has been tentatively integrated into a multistep reaction model which entails the following sequence of events: (1) primary stimulation of the platelet by ADP, (2) induction of the fibrinogen receptor in the presence of ADP and divalent cations, (3) reversible fibrinogen–receptor interaction, and (4) stabilization of fibrinogen–platelet interaction in an apparently irreversible step (Marguerie et al., 1980). In this study, we have attempted to derive the kinetic data for the binding of fibrinogen to ADP-stimulated platelets. Since a multistep mechanism is apparently involved in this interaction, kinetic analysis should provide a clear and independent estimation of the affinity constant of this interaction. Additionally, the influence of temperature and pH on the specific binding of fibrinogen to the platelet has been characterized.

Experimental Procedures

Isotopes were purchased from the following sources: hydroxy[^{14}C]tryptamine creatinine sulfate (50 μCi/μmol) from Radiochemical Centre, Amersham, England; sodium chromate (^{51}Cr 114 μCi/μg) and carrier-free Na^{125}I from Commissariat a l'Energie Atomique, Orsay, France. Other materials used were purchased from the following sources: ADP from Sigma Chemical Co., St. Louis, MO; bovine serum albumin from Calbiochem, La Jolla, CA; chloramine T from Merck & Co., Inc., Rahway, NJ; hirudin from Laboratoire Stago, Stago, France; Aprotinin (Iniprol) from Laboratoire Choay, Choay, France; Chelex 100 from Bio-rad, Richmond, CA; and Sepharose CL-2B from Pharmacia, Uppsala, Sweden.

Preparation of Platelets. Venous blood (60 mL) was collected into acid–citrate–dextrose (5 mL), and the platelets were isolated by differential centrifugation followed by gel filtration on Sepharose CL-2B (Marguerie et al., 1979). The isolation

† From the Unité INSERM U. 143 Institute de Pathologie Cellulaire, Hôpital de Bicêtre, 94270 Le Kremlin-Bicêtre, France (G.A.M.), and the Department of Molecular Immunology, The Research Institute of Scripps Clinic, La Jolla, California 92037 (E.F.P.). *Received June 10, 1980.* This is Publication No. 2149 from The Research Institute of Scripps Clinic. This investigation was supported by NATO Research Grant No. 1852, by INSERM Research Grant 805029, and by HL-16411.

‡ G.A.M. is Chargé de Recherche au Centre National de la Recherche Scientifique.

[1] Abbreviations used: ADP, adenosine 5′-diphosphate; [^{14}C]5HT, 5-hydroxy[2-^{14}C]tryptamine.

0006-2960/81/0420-1074$01.25/0

was carried out in Tyrode–2% albumin buffer (pH 7.4) freed from divalent ions on Chelex 100 columns as previously described (Marguerie et al., 1980). Platelets were counted electronically and adjusted to a concentration of (1×10^8)/mL. The washed platelets exhibited well-defined shape change when stimulated with ADP at 1–10 μM levels, but aggregation only occurred when both fibrinogen and Ca^{2+} and/or Mg^{2+} were present. Platelets were labeled with ^{51}Cr or [^{14}C]5HT by incubation of platelet-rich plasma at 37 °C for 30 min in the presence of either 200 μCi of ^{51}Cr (Cazenave et al., 1975) or 0.8 μCi of [^{14}C]5HT per 10 mL of plasma (Greenberg et al., 1975).

Fibrinogen. Fresh human blood was collected on acid–citrate–dextrose solution containing hirudin (0.04 unit/mL), 6-aminohexanoic acid (0.02 M), and aprotonin (50 units/mL), and fibrinogen was purified by ether precipitation (Kekwick et al., 1955). Hirudin and aprotonin concentrations were maintained throughout the purification. Characterization of the purified fibrinogen and freedom from detectable factor VIII, prothrombin, factor XIII, plasminogen, and fibrin monomers has been previously described (Marguerie et al., 1979). The protein concentration was measured spectrophotometrically by using an extinction coefficient $E_{0.1\%}^{280\ nm}$ of 1.51 (Mihalyi, 1968).

The purified fibrinogen was radioiodinated by using a modified chloramine T method as previously published (Marguerie et al., 1979) and routinely exhibited the following characteristics: (1) a specific activity of 0.3–1.5 μCi/mg, (2) a precipitability of radioactivity in 20% trichloracetic acid of ≥99%, (3) a coagulability of radioactivity by thrombin of 97%, and (4) an immunoprecipitability of radioactivity by a monospecific antifibrinogen of 92%. Autoradiographic display of the radiolabeled fibrinogen following vertical slab gel electrophoresis in 7.5% polyacrylamide in the presence of 1% sodium dodecyl sulfate and 2% 2-mercaptoethanol indicated the labeling of all three constituent chains without alteration of mobility from the position of unlabeled chains. From densitometric scans of the autoradiogram, 99% of the radioactivity was associated with the constituent chains of fibrinogen, and the intensities of radioactivity in the Aα, Bβ, and γ chains were 33%, 54%, and 13%, respectively.

Binding Analysis. Direct association of [^{125}I]fibrinogen with platelets was analyzed by centrifugation through sucrose with slight modification of the published technique (Marguerie et al., 1979). Typically, 50 μL of the [^{125}I]fibrinogen–platelet suspension was layered onto 200 μL of a 15% sucrose solution in the Tyrode–2% albumin buffer in a 500-μL Beckman microfuge tube. Bound [^{125}I]fibrinogen was separated from free [^{125}I]fibrinogen by centrifugation in a Beckman microfuge at 11 750 rpm. For the kinetic analysis, the reaction was initiated by the addition of ADP at time zero, and the time of reaction was the time point at which centrifugation was begun. Each data point was determined in duplicate or triplicate.

In this study, the binding of [^{125}I]fibrinogen to the platelet is expressed as the "specific" binding which is a derivative value obtained by subtracting the "nonspecific" binding from the "total" binding. These terms are defined according to the nomenclature of Cuatrecasas & Hollenberg (1976). Accordingly, the nonspecific binding is the residual binding of [^{125}I]fibrinogen in the presence of at least a 100-fold excess of nonlabeled fibrinogen, and the total binding is that which occurs in the presence of [^{125}I]fibrinogen alone. In experiments in which displacement of the platelet-bound [^{125}I]fibrinogen by unlabeled fibrinogen was assessed, at least a 100-fold excess of unlabeled fibrinogen relative to [^{125}I]fibrinogen added initially to the platelets was routinely utilized. The sole exception to this was the experiment performed at pH 6.5 (Figure 7) in which the limited solubility of fibrinogen at this pH permitted addition of only a 10-fold excess. In all experiments, the molecules of fibrinogen bound per platelet was calculated from the specific activity of the [^{125}I]fibrinogen and by using a molecular weight of 340 000.

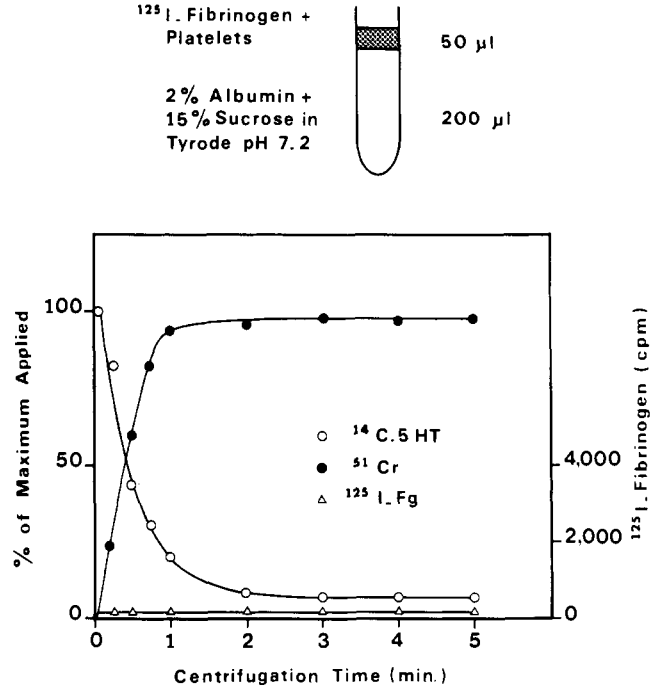

FIGURE 1: Determination of optimal conditions for the fibrinogen–platelet binding assay, achieving phase separation by centrifugation through a 15% sucrose solution in Tyrode–2% albumin buffer (pH 7.2). Washed human platelets [50 μL, (3×10^8)/mL], labeled with either ^{51}Cr or [^{14}C]5HT, were layered onto 200 μL of the sucrose solution, and the recovery of ^{51}Cr (●) in the centrifuge tube tip and [^{14}C]5HT (○) in the supernatant were plotted as a function of centrifugation time. In parallel experiments, [^{125}I]fibrinogen (10^6 cpm) was layered onto the sucrose without platelets and centrifuged under the same conditions, and the recovery of ^{125}I in the tip of the tube (△) was measured.

Results

Optimization of the Binding Assay. The direct binding of [^{125}I]fibrinogen to washed human platelets was examined by using centrifugation through sucrose to achieve separation of platelet bound from free ligand. Optimal conditions for the binding assays were sought which would rapidly and quantitatively recover platelets in the absence of significant contamination by unbound fibrinogen to undertake kinetic analyses. Additionally, the phase separation should not result in platelet stimulation, i.e., the release reaction. For evaluation of these questions, the recovery of washed platelet, labeled with either ^{51}Cr or [^{14}C]5HT and in a 50-μL volume, was measured as a function of centrifugation time through 200 μL of a 15% sucrose solution. After centrifugation, the recovery of ^{51}Cr was measured in the tip of the centrifuge tube, and [^{14}C]5HT was measured in the supernatant. As shown in Figure 1, after a 1-min centrifugation, more than 95% of the total ^{51}Cr-labeled platelets was recovered in the tip of the tubes, and [^{14}C]5HT in the supernatant was less than 5% of the total applied. This indicated that centrifugation of platelets through a 15% sucrose solution at a high speed led to quantitative recovery without inducing cell lysis or release of dense granule constituents. In parallel experiments, the level of [^{125}I]fibrinogen in the tip in the presence or absence of nonstimulated (no added ADP)

Table I: Rate Constant of Association, k_{+1}, Estimated from Initial Rates of Fibrinogen Binding

[^{125}I]-fibrinogen (μM)	rate constant (M^{-1} min^{-1})	
	1 mM Ca^{2+}	1 mM Mg^{2+}
0.04	0.60×10^6	0.38×10^6
0.08	0.60×10^6	0.30×10^6
0.16	0.54×10^6	0.30×10^6
mean ± SD	$0.58 \pm 0.03 \times 10^6$	$0.33 \pm 0.05 \times 10^6$

platelets was determined. With or without platelets, less than 0.005% of the total [^{125}I]fibrinogen layered on top of the 15% sucrose solution was recovered in the tip of the tubes at centrifugation times of up to 5 min. In replicate experiments, the average difference (five determinations) in [^{125}I]fibrinogen recovered in the tip with and without platelets indicated the binding of less than 450 molecules/cell in the absence of ADP. Furthermore, when platelets stimulated with 10 μM ADP and [^{125}I]fibrinogen for 5 min were centrifuged for 2 min and the tips were then amputated and counted at either 1 or 30 min after centrifugation, the difference in radioactivity within these tips was less than 0.01%. This indicates minimal dissociation of platelet-bound [^{125}I]fibrinogen after separation of bound from free ligand. Consequently, subsequent binding assays were performed by using a centrifugation time of 1.5 min.

Kinetic Analysis. Kinetic analysis was undertaken to determine the rate of approach to equilibrium of the specific binding of [^{125}I]fibrinogen to ADP-stimulated platelets and to assess the effect of Ca^{2+} and Mg^{2+} on the rate of interaction. In these experiments, washed platelets at a final concentration of (1×10^8)/mL were prepared in divalent cation free Tyrode–2% albumin buffer at pH 7.4 (Marguerie et al., 1980). Ca^{2+} or Mg^{2+} was added at optimal (1 mM) concentrations in the presence of low, nonsaturating concentrations of [^{125}I]fibrinogen (<0.2 μM), and the reaction was initiated by stimulation of the platelets with 11 μM ADP. After the initial mixing, the reaction proceeded at 22 °C under nonstirring conditions. Only the specific binding of the fibrinogen to the platelet was considered and was derived by subtracting the binding observed in the presence of a large excess of unlabeled fibrinogen (2.5×10^{-5} M) [considered as to be nonspecific (Cuatrecasas & Hollenberg, 1976)] from the binding observed in the presence of [^{125}I]fibrinogen alone. Nonspecific binding never exceeded 10% of the total binding. The time courses of the specific binding at three different [^{125}I]fibrinogen concentrations in the presence of either 1 mM Ca^{2+} or 1 mM Mg^{2+} are shown in Figure 2. At the fibrinogen concentrations used, the specific binding reached equilibrium within 20 min with either Ca^{2+} or Mg^{2+} present.

For interpretation of these data in terms of the initial kinetics of the interaction between fibrinogen (F) and its platelet receptor (P) to form a complex (PF), the reaction was described by eq 1 where k_{+1} and k_{-1} are the rate constants of

$$P + F \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} PF \qquad (1)$$

association and dissociation, respectively. The k_{+1} may be estimated from the initial rate of binding (v_i) according to eq

$$v_i = k_{+1}[P_0][F_0] \qquad (2)$$

2 where $[P_0]$ and $[F_0]$ are the concentrations of the receptor and the [^{125}I]fibrinogen initially present in the reaction. Values of k_{+1} obtained from the first 5 min of the binding reaction at the three different [^{125}I]fibrinogen concentrations with platelets from the same donor are listed in Table I. Average values of $(0.58 \pm 0.03) \times 10^6$ M^{-1} min^{-1} and $(0.33 \pm 0.05) \times 10^6$ M^{-1} min^{-1} were obtained over a 4-fold range of fibrinogen concentrations in the presence of 1 mM Ca^{2+} and 1 mM Mg^{2+}, respectively. Thus, the k_{+1} values in the presence of Ca^{2+} and Mg^{2+} were quite similar, differing by less than 2-fold.

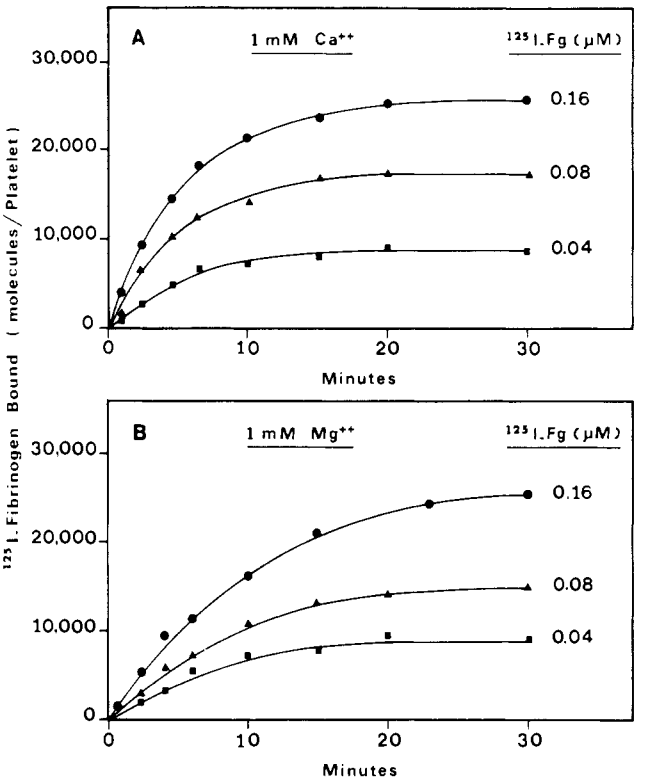

FIGURE 2: Time course of the specific binding of [^{125}I]fibrinogen to ADP-stimulated platelets in the presence of 1 mM Ca^{2+} (A) or 1 mM Mg^{2+} (B). Platelets at 1×10^8/mL in Tyrode–2% albumin solution, free of divalent ions, were stimulated with 11 μM ADP without stirring at 22 °C in the presence of different [^{125}I]fibrinogen concentrations. Divalent ions were added prior to the addition of [^{125}I]fibrinogen and ADP. Specific binding was obtained by substracting the binding observed in the presence of a large excess of unlabeled fibrinogen (2.5×10^{-5} M) from the binding observed in the presence of [^{125}I]fibrinogen alone.

According to eq 1, the rate of formation of a fibrinogen–receptor complex is given by the differential equation (Kahn, 1975):

$$\frac{d[PF]}{dt} = k_{+1}[F][P] - k_{-1}[PF]_e \qquad (3)$$

Integration of this equation results in the expression

$$[PF]_e = [PF]\{1 - \exp[-(k_{+1}[F] + k_{-1})t]\} \qquad (4)$$

where $[PF]_e$ is the concentration of the fibrinogen–receptor complex at equilibrium and $\{1 - \exp[-(k_{+1}[F] + k_{-1})t]\}$ describes the time course with which equilibrium is achieved (Kahn, 1975). Under pseudo-first-order conditions, eq 4 may be solved for $t_{1/2}$ from the half-maximal value of [PF] to obtain the rate constant of approach to equilibrium, k_{obsd}.

$$k_{obsd} = \frac{\ln 2}{t_{1/2}} = k_{+1}[F] + k_{-1} \qquad (5)$$

The k_{obsd} of the fibrinogen–platelet interaction was determined from the slope of the Guggenheim plots (Cornish-Bowden, 1976) shown in Figure 3. The linearity of the plots shown at each fibrinogen concentration indicates that the initial binding of fibrinogen to its platelet receptor followed pseudo-first-order kinetics for the first 5 min of the reaction in the presence of either Ca^{2+} or Mg^{2+}. Values for k_{obsd} were determined with platelets from five individual donors at the three

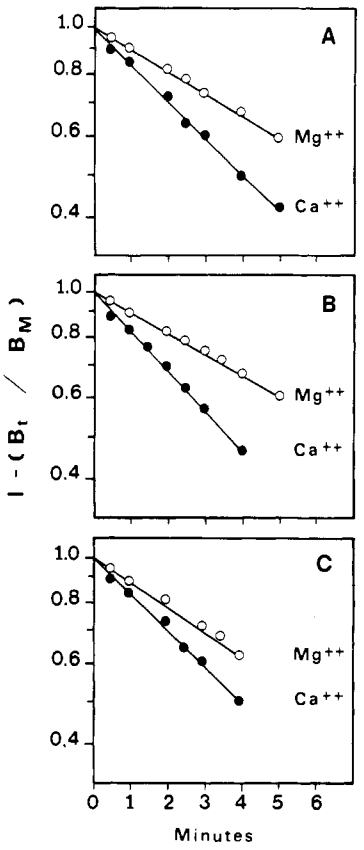

FIGURE 3: Determination of the first-order rate constant for the interaction of [^{125}I]fibrinogen with platelet by means of a Guggenheim plot in which B_t is the specific binding at time t and B_M is the specific binding at equilibrium. ADP-stimulated platelets (1×10^8/mL) were incubated at 22 °C in the presence of 1 mM Ca^{2+} or 1 mM Mg^{2+} and with [^{125}I]fibrinogen at final concentrations of 0.16 μM (A), 0.08 μM (B), and 0.04 μM (C).

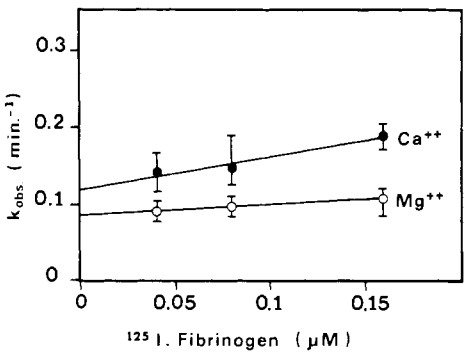

FIGURE 4: Rate of [^{125}I]fibrinogen binding to ADP-stimulated platelets. The pseudo-first-order rate constant for approach to equilibrium, k_{obsd}, is plotted vs. [^{125}I]fibrinogen concentration according to eq 5. Regression lines with correlation coefficients of 0.98 and 0.99 were derived in the presence of 1 mM Ca^{2+} and 1 mM Mg^{2+}, respectively.

[^{125}I]fibrinogen concentrations, and the mean values of k_{obsd} are plotted as a function of [^{125}I]fibrinogen concentration in Figure 4. Straight line plots were obtained in the presence of either cation, indicating that the model of F + P ⇌ PF is applicable. It is noted from the standard of deviations (see Figure 4) that the values of k_{obsd} were donor independent. At each fibrinogen concentration, the k_{obsd} were slightly higher in the presence of Ca^{2+} as compared to Mg^{2+}. The rate constant of association (k_{+1}), the slope of the line, and the rate constant of dissociation (k_{-1}), the intercept, were $k_{+1} = 0.30 \times 10^6$ M^{-1} min^{-1} and $k_{-1} = 0.126$ min^{-1} in the presence of 1 mM Ca^{2+} and $k_{+1} = 0.13 \times 10^6$ M^{-1} min^{-1} and $k_{-1} = 0.086$ min^{-1} in the presence of 1 mM Mg^{2+}. The derived affinity

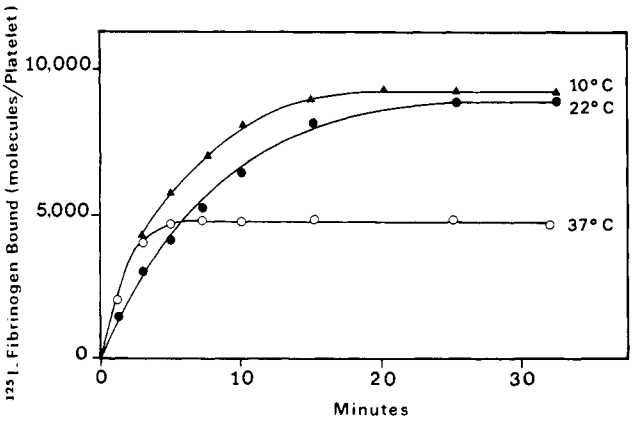

FIGURE 5: Effect of temperature on the specific binding of [^{125}I]-fibrinogen to ADP-stimulated platelets. Washed platelets [(1×10^8)/mL] were suspended in Tyrode–2% albumin solution containing 2 mM Mg^{2+} and 0.5 mM Ca^{2+}. Specific binding was obtained as described in Figure 2. Mixtures were incubated at the different temperatures for 10 min prior to the addition of ADP (11 μM) and [^{125}I]fibrinogen (0.16 μM), and equilibration at each temperature was verified by direct measurements of the mixtures.

Table II: Effect of Temperature on the Rate Constant of Association, k_{+1}, Calculated from the Initial Rate of Fibrinogen Binding[a]

donor	temp (°C)	maximum binding (molecules/platelet)	k_{+1} (M^{-1} min^{-1})
A	37	5 000 ± 200	0.25 ± 0.05 × 10^6
B	37	4 500 ± 100	0.28 ± 0.05 × 10^6
A	22	9 000 ± 200	0.12 ± 0.07 × 10^6
B	22	11 600 ± 1000	0.28 ± 0.05 × 10^6
C	10	9 100 ± 500	0.27 ± 0.05 × 10^6
D	10	15 000 ± 300	0.28 ± 0.08 × 10^6

[a] Washed platelets were suspended in a Tyrode–2% albumin buffer containing 2 mM Mg^{2+} and 0.5 mM Ca^{2+}. Stimulation by 11 μM ADP was carried out in the presence of 0.16 μM [^{125}I]-fibrinogen. Donors A and B were drawn on two different occasions.

constants $K_a = k_{+1}/k_{-1}$ were 2.4×10^6 M^{-1} and 1.5×10^6 M^{-1} in the presence of 1 mM Ca^{2+} and 1 mM Mg^{2+}, respectively.

Effect of Temperature. Washed platelets were suspended in Tyrode–2% albumin buffer containing 2 mM Mg^{2+} and 0.5 mM Ca^{2+}, and the time course of the specific fibrinogen binding was compared at 10, 22, and 37 °C (Figure 5). (The nonspecific component of binding was found to be temperature independent.) The time courses of specific binding at 10 and 22 °C were similar with respect to the initial rate of binding and the maximum binding attained. At 37 °C, the initial binding of fibrinogen was similar to that observed at 22 or 10 °C, but the maximum binding reached at equilibrium was lower. The apparent similarities in the initial rates of binding of [^{125}I]fibrinogen at the different temperatures were confirmed by estimation of the rate constant of association, k_{+1} (Table II). Values of k_{+1} at 10, 22, and 37 °C were markedly similar for different donors. Maximal binding differed for platelets derived from different donors as previously reported (Marguerie et al., 1980), but, in comparing the same donors at 22 and 37 °C, approximately twice as many fibrinogen molecules were bound at 22 °C.

We have previously reported (Marguerie et al., 1980) that with time platelet-bound fibrinogen becomes nondissociable from the platelet by a large excess of unlabeled fibrinogen. The effect of temperature on the progressive stabilization of platelet-bound fibrinogen was assessed by examining the capacity of unlabeled fibrinogen to displace platelet-bound [^{125}I]fibrinogen at 10 and 22 °C. Washed platelets (1 ×

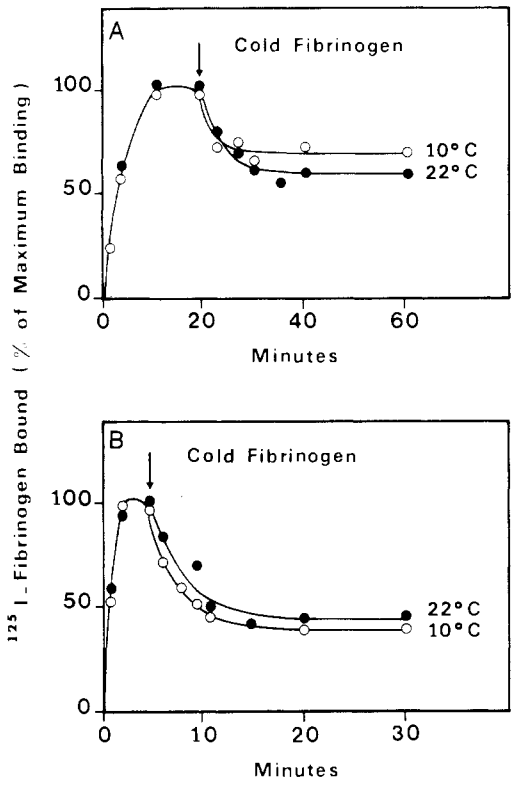

FIGURE 6: Displacement of platelet-bound [^{125}I]fibrinogen by the addition of a 100-fold excess of unlabeled fibrinogen at 10 and 22 °C. Platelets were stimulated in the conditions described in Figure 5. After 20-min (A) or 5-min (B) incubation at either 10 or 22 °C, a 100-fold excess of unlabeled fibrinogen was added to the solution, and dissociation of the platelet-bound radioactivity was measured.

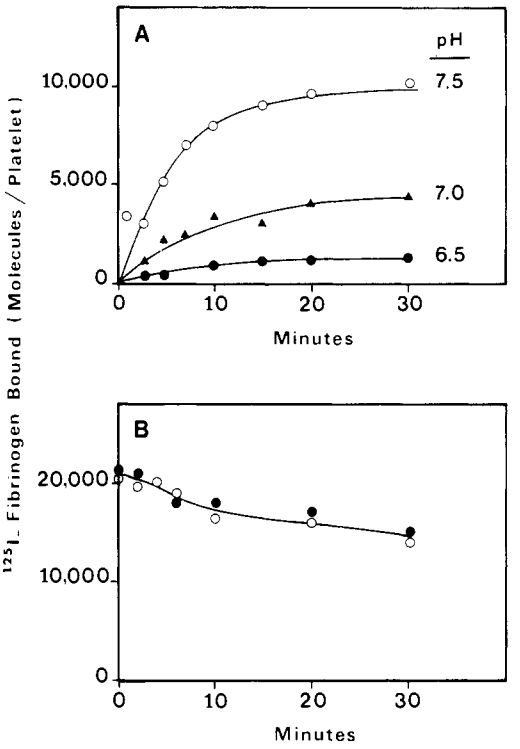

FIGURE 7: Effect of the pH on the specific binding of [^{125}I]fibrinogen to platelet (A) and on the dissociation of the platelet-bound [^{125}I]-fibrinogen (B). The experimental conditions were those of Figure 5. In the displacement experiment, [^{125}I]fibrinogen was bound to ADP-stimulated platelets at pH 7.5 for 20 min, the pH was lowered to 6.5, and then either a 10-fold excess of unlabeled fibrinogen (●) or an equivalent volume of buffer at pH 6.5 (○) was added. The time zero in B corresponds to the time at which the pH was lowered from 7.5 to 6.5.

10^8/mL) were stimulated with 11 μM ADP in the presence of 0.2 μM [^{125}I]fibrinogen, and unlabeled fibrinogen was added at a final concentration of 25 μM after 5 or 20 min. The displacement of [^{125}I]fibrinogen by cold fibrinogen with time at the two temperatures is shown in Figure 6. When unlabeled fibrinogen was added 5 min after the stimulation by ADP, 55% of the bound radioactivity was displaced within 15 min at either 10 or 22 °C. When cold fibrinogen was added 20 min after stimulation, only 30–40% of the platelet-bound radioactivity was displaced within 15 min. Thus, temperature did not significantly alter the progressive stabilization of platelet-bound fibrinogen.

Effect of pH. The effect of pH on the time course of the specific binding of [^{125}I]fibrinogen to ADP-stimulated platelets is illustrated in Figure 7A. In these experiments, the preparation of the platelet was carried out at pH 7.0. The platelet suspension [(1×10^8)/mL] was then adjusted with HCl diluted in Tyrode–2% albumin containing 2 mM Mg^{2+} and 0.5 mM Ca^{2+} to the desired pH and incubated for 10 min prior to the addition of [^{125}I]fibrinogen and ADP. The volumes of all samples were equalized, and the pH of the suspensions was measured several times during the course of the experiment to monitor possible changes in pH. At pH 7.5 a typical binding curve was obtained, whereas very little binding and intermediate binding was observed at pH 6.5 and 7.0, respectively. At 30 min, the binding at pH 6.5 and 7.0 was 13% and 44% of that observed at pH 7.5. Additionally, the effect of pH on the reversibility of the fibrinogen–platelet reaction was examined. Washed platelets were stimulated at pH 7.5 with 8 μM ADP in the presence of 0.2 μM [^{125}I]fibrinogen; after 20 min of incubation, the pH was adjusted to 6.5. Displacement of the platelet-bound [^{125}I]fibrinogen either by dilution with Tyrode–2% albumin buffer at pH 6.5 or by a 10-fold excess ($2\ \mu$M) of unlabeled fibrinogen (a large excess of unlabeled fibrinogen could not be added due to the precipitation of the fibrinogen at pH 6.5) was then measured. As illustrated in Figure 7B, after 30 min, 70% of the bound [^{125}I]fibrinogen was still associated with the platelets, indicating that change in pH did not directly dissociate or did not facilitate the dissociation of the platelet-bound [^{125}I]fibrinogen by a 10-fold excess of unlabeled fibrinogen.

Discussion

We have previously reported (Marguerie et al., 1980) that the interaction of fibrinogen with its platelet receptor may be described by a four-step mechanism:

Reaction 1

$$P_1 + ADP \rightleftharpoons P_2$$

Reaction 2

$$P_2 + ADP \overset{Ca,Mg}{\rightleftharpoons} P_3$$

Reaction 3

$$P_3 + F \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} P_3F$$

Reaction 4

$$P_3F \xrightarrow{k_2} P_4F$$

where F is the fibrinogen and P_1 to P_4 represent different stages of platelet activation. Reaction 1 entails the initial reversible stimulation of platelet by ADP, which results in shape change. Reaction 2 is the direct induction of the fibrinogen receptor in a step which requires divalent ions and ADP. Reactions

Table III: Summary of Affinity Constants, K_a, for the Interaction of Fibrinogen with ADP-Stimulated Platelets

divalent ion	method of estimation	affinity constant [M^{-1}]
1 mM Ca^{2+}	initial rate of binding	2.4×10^6
	Scatchard	2.0×10^6
1 mM Mg^{2+}	initial rate of binding	1.5×10^6
	Scatchard[a]	1.8×10^6
2 mM Mg^{2+}	rate of aggregation[b]	3.3×10^6

[a] Marguerie et al., 1980. [b] Marguerie et al., 1979.

3 and 4 are the reversible and irreversible interactions of fibrinogen with its receptor. In this study, a kinetic analysis has been performed as a potential approach for dissecting these reaction steps and specifically characterizing the reversible interaction of fibrinogen with the platelet. For the purpose of the kinetic analysis used in this study, it was assumed that the overall four-step reaction could be simplified and reduced to the bimolecular interaction described by reaction 3. This assumption appears valid since the rate constant of approach to the equilibrium (k_{obsd}) was linear for the first 5 min in a Guggenheim plot. This implies that reactions 1 and 2 are not rate determining relative to reaction 3. This does not prove that the rate constant k_2 of the irreversible reaction 4 is negligible when compared to k_{-1} of reaction 3 but makes it unlikely that k_2 is significantly greater than k_{-1}. From the rate constants of association and dissociation, k_{+1} and k_{-1}, of reaction 3, affinity constants K_a of 2.0×10^6 M^{-1} in the presence of 1 mM Ca^{2+} and 1.5×10^6 M^{-1} in the presence of 1 mM Mg^{2+} were derived. These values are in excellent agreement with the affinity constants estimated from binding experiments at equilibrium by Scatchard analyses (Table III). It is also noteworthy that the affinity constant derived from the rate of platelet aggregation as a function of fibrinogen concentration was 3.3×10^6 M^{-1} (Marguerie et al., 1979). Thus, by three independent approaches, the derived affinity constants for the reaction were very similar, differing by only 2-fold.

Hawiger et al. (1980) have recently reported affinity constants of 6.7×10^6 M^{-1} for the binding of fibrinogen to ADP-stimulated platelets and 5.5×10^6 M^{-1} for thrombin-stimulated platelets. These values were derived from binding experiments in which separation of free from bound ligand was achieved by centrifugation through a mixture of dibutyl phthalate and Apiezon oil C. Thus, affinity constants derived by phase separation through an aqueous solution (sucrose) in this study and through a nonaqueous solution are in general agreement. Bennett & Vilaire (1979) reported an affinity constant of 1.2×10^7 M^{-1} by centrifugation through silicon oil. These differences in values may reflect differences in platelet and ligand preparations as well as in the separation technique.

When two different methods to calculate the rate constants were used, slight differences were noted in the values obtained in the presence of Ca^{2+} or Mg^{2+}. Although the absolute values derived for k_{+1} by the two approaches differed, in each case Ca^{2+} increased the rate constant by approximately 2-fold relative to Mg^{2+}. This suggests that Ca^{2+} may have a slight catalytic effect or Mg^{2+} a slight inhibitory effect on the reaction. The difference in kinetic constants observed with the two divalent ions may reflect a subtle differential effect directly on reaction 3 with either the platelet or the ligand being altered. Alternatively, a differential effect of Ca^{2+} or Mg^{2+} on the preceding event, receptor induction (reaction 2), could also give rise to the observed differences.

Similar values for the rate constant of association, k_{+1}, were obtained at 10, 22, and 37 °C, indicating that temperature does not significantly influence the kinetics of initial binding. The maximum number of fibrinogen molecules bound per platelet was similar at 10 and 22 °C but was consistently lower at 37 °C. Induction of fewer receptors per platelet, stimulation of fewer platelets, or a decrease in the stability of induced receptors could all result in the lower binding observed at 37 °C. Since platelets incubated at 37 °C undergo a progressive loss in reactivity to ADP (Praga & Pogliani, 1973), a more generalized and nonspecific effect may be involved. The time course of specific binding at 22 ant 10 °C was very similar, suggesting that the fluidity of the platelet membrane is not a major factor in the fibrinogen–platelet receptor interaction. Since the dissociation of platelet-bound fibrinogen was also identical at either 10 or 22 °C, the clustering or lateral mobility of lipids and proteins in the plane of the platelet membrane, events which are temperature dependent (Singer, 1974; Vignais, 1976), can be reasonably excluded as essential features of the irreversible binding of fibrinogen to the platelet.

The binding of fibrinogen to platelets was pH dependent, and minimal binding occurred at pH 6.5. This effect may be entirely due to the failure of ADP to appropriately stimulate the platelet at this pH since the second phosphate group of ADP ionizes between pH 6.2 and 6.7. Alternatively, the direct interaction of fibrinogen with its platelet receptor may involve ionizable residues such as histidine and be therefore pH dependent. Since lowering the pH to 6.5 did not dissociate platelet-bound fibrinogen, the same ionizable groups would not be involved in the stabilization of the fibrinogen–platelet complex. When platelet-bound fibrinogen is extracted with sodium dodecyl sulfate and analyzed on polyacrylamide gels under reducing conditions, its constituent chains are of the same apparent size as those of native fibrinogen (Marguerie et al., 1979). This suggests that covalent interactions are not involved in the stabilization of the fibrinogen–platelet complex. Thus, membrane fluidity and ionic or covalent bonding, secondary to the initial reversible binding of fibrinogen, are probably not responsible for the irreversible phase of the interaction. As an alternative, secondary hydrophobic interaction may be considered as a basis for the formation of a stable fibrinogen–receptor complex.

Acknowledgments

We gratefully acknowledge Nadine Thomas for excellent technical assistance and Catherine Jacobson and Roberta Novak for the preparation of the manuscript.

References

Bang, N. U., Heidenreich, R. O., & Trygstad, C. W. (1972) *Ann. N.Y. Acad. Sci. 201*, 280–299.

Bennett, J. S., & Vilaire, G. (1979) *J. Clin. Invest. 64*, 1393–1401.

Cazenave, J. P., Packham, M. A., Guccione, M. A., & Mustard, J. F. (1975) *J. Lab. Clin. Med. 86*, 551–563.

Cornish-Bowden, A. (1976) in *Principle of Enzyme Kinetics*, p 13, Butterworths, London.

Cuatrecasas, P., & Hollenberg, M. D. (1976) *Adv. Protein Chem. 30*, 251–450.

Figures, W. R., Colman, R. F., Morinelli, T. A., Niewiarowski, S., Budzynski, A. Z., & Colman, R. W. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 872.

Greenberg, J., Packham, M. A., Cazenave, J. P., Reimers, M. J., & Mustard, J. F. (1975) *Lab. Invest. 32*, 476–484.

Harfenist, E. J., Packham, M. A., & Mustard, J. F. (1980) *Blood 56*, 189–198.

Hawiger, J., Parksinson, S., & Timmons, S. (1980) *Nature (London) 283*, 195–197.
Kahn, C. R. (1975) *Methods Membr. Biol. 3*, 81.
Kekwick, R. A., McKay, M. E., Nance, M. H., & Record, B. R. (1955) *Biochem. J. 60*, 671–683.
Marguerie, G. A., Plow, E. F., & Edgington, T. S. (1979) *J. Biol. Chem. 254*, 5357–5363.
Marguerie, G. A., Edgington, T. S., & Plow, E. F. (1980) *J. Biol. Chem. 255*, 154–161.
Mihalyi, E. (1968) *Biochemistry 7*, 208–223.
Mustard, J. F., & Packham, M. A. (1970) *Pharmacol. Rev. 22*, 97–187.
Mustard, J. F., Packham, M. A., Kinlough-Rathbone, R. L., Perry, D. W., & Regoeczi, E. (1978) *Blood 52*, 453–466.
Niewiarowski, S., Morinelli, T., & Budzynski, A. Z. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 543.
Peerschke, E. I., Zucker, M. B., Grant, R. A., Egan, J. J., & Johnson, M. M. (1980) *Blood 55*, 841–847.
Praga, C. A., & Pogliani, E. M. (1973) *Thromb. Diath. Haemorrh. 29*, 183–189.
Singer, S. J. (1974) *Annu. Rev. Biochem. 43*, 805–833.
Vignais, P. M. (1976) in *Mitochondria, Bioenergetics, Biogenesis, and Membrane Structure* (Packer, L., Ed.) pp 367–379, Academic Press, New York.

Spectral Evidence for a Rapidly Formed Structural Intermediate in the Refolding Kinetics of Hen Egg-White Lysozyme†

Shingo Kato,‡ Motoyoshi Okamura, Nobuo Shimamoto, and Hiroyasu Utiyama*

ABSTRACT: For investigation of the conformation of the unfolded species and its role in the refolding kinetics, refolding kinetic measurements were made on hen egg-white lysozyme by using the stopped-flow method at 25 °C in the four sets of initial and final folding condition: (1) 4 M guanidinium chloride (GdmCl) and 0.5 M GdmCl; (2) 40% acetic acid (HOAc) and 5% HOAc; (3) 4 M GdmCl and 0.5 M GdmCl–5% HOAc; (4) 40% HOAc and 0.5 M GdmCl–5% HOAc. The kinetic results as measured by absorbance at three wavelengths, 301, 292, and 250 nm, agreed with each other and indicated strict biphasic behavior without exception. The kinetic parameters were determined only by the final refolding conditions. The spectral properties of the unfolded species at the end of stopped-flow mixing were investigated by comparing the total kinetic amplitude with the difference between the static absorbance of the native molecule in the final refolding conditions and that of the unfolded molecule in the initial unfolding conditions. The solvent effect was considered in the comparison. It was concluded that the unfolded species assumed a new transient conformation in the mixing process and that the transformation was completed within the mixing time.

In kinetic studies of the refolding of small proteins stopped-flow technique is often used, where the denatured protein solution is quickly brought to the refolding condition by mixing with some diluent and the refolding process taking place thereafter is followed by spectral measurements. The most extensive investigation was made by the Baldwin group on bovine pancreatic ribonuclease A (RNase A)[1] [review by Baldwin (1975, 1978)]. It was shown that, in the direct folding reaction (folding of the fast-folding form), the small protein refolded from the denatured and extended conformation into the native and compact one essentially in a single kinetic process. This result indicates that the refolding process involves highly cooperative interactions among many parts of the molecule. The refolding rate, ranging from 10 to 100 s^{-1}, is therefore probably much slower than the rates of local conformational changes. The regular secondary structure is also formed more rapidly. The rate of α-helix formation in synthetic homopolymers ranges from 10^7 to 10^8 sec^{-1} (Schwarz, 1965; Hammes & Roberts, 1969).

In this paper we focus our attention on the conformation of the unfolded protein, by means of stopped-flow refolding studies. The above discussion suggests that, when unfolded molecules start folding within the mixing process of the stopped-flow apparatus, the molecules may acquire, at the end of mixing, a new local secondary structure different from the one in the initial unfolding conditions. The unfolded molecule with the new secondary structure would exist only transiently as a refolding intermediate, and the new local secondary structure would be characterized by the refolding condition and might be the principal factor determining the refolding rate. If this is correct, the refolding kinetics should be determined solely by the final refolding condition, independent of the initial unfolding condition. The first purpose of this study is to show by spectral data that the unfolded conformation in refolding condition is different from that in the initial unfolding condition.

The second purpose concerns the refolding kinetics of hen egg-white lysozyme (HEWL). The three-species model $U_1 \rightleftharpoons U_2 \rightleftharpoons N$ (N = native, U_1, U_2 = unfolded) has been well established for the refolding process of RNase A (Garel & Baldwin, 1973, 1975a,b; Hagerman & Baldwin, 1976; Nall et al., 1978). It is not established, however, that the model is valid for other globular proteins. Even for HEWL, which has been widely used in physical studies of proteins, some experimental results now available (Tanford et al., 1973) are in apparent contradiction to the model: (1) the unfolding

† From the Life Science Group, Faculty of Integrated Arts and Sciences, Hiroshima University, Hiroshima 730, Japan. *Received June 6, 1980.* This work was supported in part by Research Grants B247134 and C458122 to H.U. from the Ministry of Education, Science and Culture of Japan.

‡ Permanent address: Institute for Chemical Research, Kyoto University, Uji 611, Japan.

[1] Abbreviations used: RNase A, bovine pancreatic ribonuclease A with disulfide bonds intact; HEWL, hen egg-white lysozyme with the intact disulfide bonds; GdmCl, guanidinium chloride; HOAc, acetic acid.

0006-2960/81/0420-1080$01.25/0