Characterization of the Kinetic Pathway for Liberation of Fibrinopeptides during Assembly of Fibrin*

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The time dependence of the release of fibrinopeptides from fibrinogen was studied as a function of the concentration of fibrinogen, thrombin, and Gly-Pro-Arg-Pro, an inhibitor of fibrin polymerization. The release of fibrinopeptides during fibrin assembly was shown to be a highly ordered process. Rate constants for individual steps in the formation of fibrin were evaluated at pH 7.4, 37 °C, [3/2] = 0.15. The initial event, thrombin-catalyzed proteolysis at Arg-Aa16 to release fibrinopeptide A (Ka/kM = 1.09 × 10^6 M^-1 s^-1) was followed by association of the resulting fibrin I monomers. Association of fibrin I was found to be a reversible process with rate constants of 1 × 10^6 M^-1 s^-1 and 0.064 s^-1 for association and dissociation, respectively. Assuming random polymerization of fibrin I monomers, the equilibrium constant for fibrin formation is 1.0 mg/ml. Association of fibrin I monomers was shown to result in a 6.5-fold increase in the susceptibility of Arg-BB14 to thrombin-mediated proteolysis. The 6.5-fold increase in the observed specificity constant from 6.5 × 10^5 M^-1 s^-1 to 4.2 × 10^6 M^-1 s^-1 upon association of fibrin I monomers and the rate constant for fibrin association indicates that most of the fibrinopeptide B is released after association of fibrin I monomers. The interaction between the pair of polymerization sites in fibrin I dimer was found to be weaker than the interaction of fibrin I with Gly-Pro-Arg-Pro and weaker than the interaction of fibrin I with fibrinogen.

The plasma protein fibrinogen is converted to the insoluble fibrin matrix of blood clots by a multistep process (1-14), wherein the fibrinogen molecule which is comprised of 2 Aa-, 2 Bβ-, and 2 γ-polypeptide chains (15-25) undergoes limited proteolysis and aggregation. Much evidence has accumulated to support the view that fibrin assembly is a highly ordered process which is initiated by thrombin-catalyzed hydrolysis at Arg-Aa16 to form fibrin I monomer and release a 16-aminoacyl residue peptide (FPA) from the N-terminus of each Aa-chain. This event is thought to be followed by end to end aggregation of the resulting fibrin I monomers to form fibrin I oligomers (protofibrils). Association of fibrin I appears to be followed by thrombin-mediated hydrolysis at Arg-Bβ14 to form fibrin II and release a 14-aminoacyl residue peptide (FPB) from the amino terminus of each of the Bβ-chains. The resulting fibrin II protofibrils have a greater propensity than fibrin I protofibrils to undergo lateral interactions which result in fiber formation (9, 10). A reaction sequence for release of fibrinopeptides and fiber formation is shown in Scheme 1, wherein Aaβ2γ and Aβ2γ represent half-molecule units and (aBβγγ)2 represents a pair of interacting half-molecules in dimers and higher oligomers of fibrin I.

The observation that FPA is released more rapidly from fibrinogen than is FPB and the observation that inhibitors of fibrin I aggregation inhibit release of FPB without inhibiting release of FPA suggests that the reactions denoted by the rate constants k3 and k4 are minor pathways for the release of FPB. Observations by others (26, 27) suggest, however, that premature release of FPB via the k4 and k5 reactions occurs to some extent. In this regard, kinetic analysis at low concentrations of fibrinogen of the time dependence of thrombin-catalyzed release of FPA and FPB indicates that k1 > 30 k4 so that at least 97% of the time FPA is released prior to FPB (14). Thus, release of FPB via the k4 step can often be neglected in kinetic studies of the release of fibrinopeptides. The value of k3 and the relative contribution of this step to the time-dependent release of FPB has never been determined, however.

More importantly, Scheme 1 has never been put to a rigorous quantitative test. For example, it has never been determined whether a single set of rate constants could account for the time dependence of the release of FPA and FPB at high thrombin concentrations where the rate of release of FPB should be limited by the rate of association of fibrin I as well as at low thrombin concentrations where the rate of proteolysis should limit the rate of release of FPB. In this work we report for the first time a set of rate constants (k1, k2, k3, k4, k5, and k6) evaluated at the physiological conditions of pH 7.4, 37 °C, and [3/2] = 0.15, which shows that Scheme 1 can quantitatively account for the time-dependent release of fibrinopeptides over a wide range of thrombin and fibrinogen concentrations.

EXPERIMENTAL PROCEDURES

Materials—Fibrinogen was purified from outdated plasma by the method of Jakobsen and Kierulf (28), except that the fibrinogen

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1 The abbreviations used are: FPA, fibrinopeptide A; FPB, fibrinopeptide B; HPLC, high performance liquid chromatography; PEG, polyethylene glycol 6000.
solvent used to redissolve fibrinogen was the citrate saline solution of Staudenhauer and Wagner (29), and final dialysis was against 0.3 M NaCl. This fibrinogen preparation behaved identically with the fibrinogen prepared and stored in this laboratory (29). All fibrinogen solutions were stored at -70 °C prior to use. An E_{280} of 15.1 and a molecular weight of 340,000 were used to calculate fibrinogen concentrations. Fibrinogen was prepared by allowing 6 ml of a solution of fibrinogen (2.5 mg/ml) in 0.15 M NaCl, 3 mM phosphate buffer, pH 6.8, to react overnight with 0.01 ml of a solution of Reptilase-R, which was obtained by addition of 1 ml of water to a vial containing 34 mg of lyophylized Reptilase-R from Abbott Laboratories. The resulting clot was collected on a glass rod, rinsed with water, and dissolved in 1 ml of 0.02 M acetic acid. An E_{280} of 14.0 and an M_r of 340,000 were used to calculate the concentration of fibrinogen in 0.02 M acetic acid. Pure human α-thrombin with a specific activity of 24 thrombin units/μg (using the method of Lewis and Shafer (31)) was generously supplied by John W. Fenton II, New York State Department of Health. The thrombin which was greater than 92% active by active site titration (32) was stored at 0.3 M NaCl at -70 °C. α-Thrombin accounted for >99% of the thrombin. Values of k_{cat} and k_{cat}/K_M reported in this work are based on the concentration of thrombin determined from its absorbance at 280 nm using an E_{280} of 18.3 in 0.1 M NaOH and an M_r of 36,500 (33). No correction was made for the deviation (<7%) of the thrombin activity from 100% in the active site titrations. Gly-Pro-Arg-Pro-2HAc (M_r = 545) was from Vega Biochemicals.

HPLC—Elution conditions were those described previously (14), except for analysis of reaction mixtures containing <0.1 μM fibrinogen. In these runs, 1.2-ml samples were injected and eluted from a Spherisorb ODS-2, 3-μ (0.46 x 10 cm) column isocratically using 16% CH_3CN in 0.083 M phosphate buffer. The isocratic system produced flatter baselines and the 3-μ column sharpened the FBP peaks considerably at the usual detection limit of 0.02 absorbance unit full scale at 205 nm. After FBP was eluted, a 30-min linear gradient to 40% acetonitrile was used to rinse the column. The column was reequilibrated at 18% CH_3CN before injection of another sample.

Reaction Kinetics—The rates of release of FPA and FBP were measured at pH 7.4, 37 °C, in 137 mM NaCl, 2.5 mM KCl, and 9.47 mM sodium phosphate containing 0.001% or 0.1% PEG. At low thrombin concentrations (0.07-1.4 nM), the kinetics were carried out essentially as already described (9), except that at the lowest fibrinogen concentration a 1.2-ml aliquot of reaction mixture was injected into the HPLC system. Additionally, at 1.4 nM thrombin, individual 1.5-ml microfuge tubes containing the reaction mixture (0.8-1.2 ml) were preequilibrated at 37 °C before an aliquot of thrombin was rapidly added with mixing to each tube. At appropriate times, 0.1 ml of 5 M HClO_4 was added with vigorous mixing to one of the tubes and the volume was adjusted to a known final value (1.3-1.5 ml) with H_2O. The resulting solution was centrifuged and an appropriate volume (1.0-1.2 ml) was injected in the HPLC system. For the runs carried out in the presence of calcium chloride, 18.9 mM sodium phosphate was added with chelating agent to an equal volume of 274 mM NaCl, 0.2% PEG containing 2 mM CaCl2. This procedure avoided precipitation of calcium phosphate. Rates of fibrinopeptide release at 40 nM thrombin were measured using a flow system consisting of a multichannel Rheeva-Angel peristaltic pump which was placed in a 37 °C room. Two channels of the pump contained the fibrinogen and thrombin feed lines which led to a mixing chamber. The mixing chamber was similar to one which has been described previously (34). The peristaltic pump was fitted with tygon tubing to which was connected 1/8-inch outside diameter Teflon tubing equipped with appropriate fittings for connection to the mixing chamber. The solutions of fibrinogen and thrombin were pumped at equal flow rates into the mixing chamber. The concentration of fibrinogen and thrombin were twice the value desired in the reaction. Upon exiting the mixing chamber, the reaction mixture was allowed to flow through a single piece of 0.8-mm inside diameter Teflon tubing of known volume to another mixing chamber. A third channel of the pump contained a solution of HClO_4 which was added with mixing to one of the tubes and a value of the 0.8-mm inside diameter Teflon tubing was used to calculate the flow rates. The volume of the connecting tube was determined from the weight of distilled water it held. By varying the tube length from 5 cm to 4 m, reaction times from 0.17 to 20 s could be obtained at the flow rates used. Prior to each run, the precise flow rate for each line was determined (and adjusted) by measuring the volume of feed liquid utilized over a known time interval. The correct functioning of the flow system was indicated by observation of pseudo-first order kinetics for the release of FPA wherein the quotient of the pseudo-first order rate constant and the thrombin concentration yielded a specificity constant for FPA release of 10.9 M_s^{-1}s^{-1} which was within experimental error (7%) of the rate constant observed for this constant at lower thrombin concentrations in a static system. It is important to note that whereas a flow system might be designed to give lower reaction times by increasing the flow rate and decreasing the diameter of the reaction tube, a practical limit to this approach is reached wherein high shear forces cause fragmentation of fibrinogen. Fibrinogen fragmentation results in the appearance of several peptides during HPLC which are independent of the presence of thrombin.

RESULTS

In this study the concentration of fibrinogen was kept below 0.3 μM to simplify the kinetic analysis. As shown previously (14) under these conditions, the fractional saturation of thrombin is low (i.e. [S] ≤ 10 K_M) so that the Michaelis-Menten equation

\[
\frac{d[S]}{dt} = \frac{k_{cat}}{1 + K_M} [S]
\]

reduces to

\[
\frac{d[S]}{dt} = \frac{k_{cat}[S]}{K_M}
\]

and thrombin-catalyzed steps become first order with respect to the concentrations of thrombin (e) and substrate [S] (i.e. the concentration of AA- or BB-chains). Integration of Equation 2 yields an expression (Equation 3) for

\[
[FPA] = [AaBPy]_o (1 - e^{-kt})
\]

the time-dependent release of FPA, wherein the pseudo-first order rate constant, k_t, is the product of the thrombin concentration and the specificity constant (k_{cat}/K_M) for thrombin-catalyzed hydrolysis at Arg-Aa16 to release FPA and [AaBPy]_o, is the initial concentration of half-molecule units. Previous studies (14) revealed that in the range of thrombin concentrations studied (0.1-1.7 nM), the time dependence of [FPA] could be represented by Equation 4.

\[
[FBP] = [AaBPy]_o + [AaBPy]_o e^{-kt} \frac{k_t}{k_t - k_b} - [AaBPy]_o e^{-kt} \frac{k_t}{k_t - k_b}
\]

Equation 4 is the standard equation for the time dependence of product in a simple first order reaction of the type A → B → C. This equation would be expected to describe the release of FPA in Scheme 1, when polymerization is rapid and complete. Under such conditions, k_b is equivalent to the product of the thrombin concentration and the specificity constant (k_{cat}/K_M) for thrombin-catalyzed hydrolytic release of FBP from aAβPy_2 ensembles, where the subscript "a" denotes self-associated fibrin I as the substrate. The time dependence for the release of FBP at 0.14 μM fibrinogen and 0.28 nM thrombin is shown in Fig. 1A. The solid line was calculated from Equation 4 using the previously determined value (14) of 4.2 × 10^6 M_s^{-1}s^{-1} for k_{cat}/K_M and a value of 1.09 × 10^5 M_s^{-1}s^{-1} determined for k_{cat}/K_A from the fit of FPA release to Equation 3. This value is in reasonable agreement with the previously reported value of 1.16 × 10^5 M_s^{-1}s^{-1} (14).

To verify that k_{cat}/K_M reflects the specificity constant for thrombin-catalyzed release of FBP from fibrin I polymer rather than from some other species, fibrin I polymer was...
isolated and its interaction with thrombin was characterized. To obtain fibrin I, fibrinogen was treated with Reptilase-R, an enzyme which catalyzes the release of FPA, but not FPB, from fibrinogen (5). The resulting clot was dissolved in dilute acetic acid and a sample of this solution was transferred to the reaction mixture which was adjusted to pH 7.4, 37°C, and 0.14 μM fibrin I. After a 20-min incubation to ensure formation of fibrin I polymer, thrombin was added. The resultant release of FPB followed a pseudo-first order rate law wherein the ratio of the pseudo-first order rate constant and the thrombin concentration yielded a value for the specificity constant for the release of FPB from fibrin I polymer which was within 5% of the value of $k_{\text{catBa}}/K_{\text{mBa}}$ determined by fitting the time dependence of the release of FPB to Equation 4 when thrombin was reacted with fibrinogen. Thus, the kinetic properties of isolated fibrin I polymer are consistent with a reaction pathway for the conversion of fibrinogen to fibrin wherein essentially all of the FPB is released from fibrin I polymer.

Although Equation 4 with $k_{\text{catA}}/K_{\text{mA}} = 1.09 \times 10^7$ M$^{-1}$s$^{-1}$ and $k_{\text{catB}}/K_{\text{mB}} = 4.2 \times 10^6$ M$^{-1}$s$^{-1}$ fits the time dependence of release of FPB over a wide range of thrombin concentrations reasonably well, substantial deviations from Equation 4 become apparent at very high thrombin concentrations. These deviations are exemplified in Fig. 1B where the solid line is the fit of the data to Equation 4 with the values of $k_{\text{catA}}/K_{\text{mA}}$ and $k_{\text{catB}}/K_{\text{mB}}$ set at $1.09 \times 10^7$ and $4.2 \times 10^6$ M$^{-1}$s$^{-1}$, respectively. The deviations from Equation 4 are consistent with Scheme 1. According to Scheme 1, a change in the rate-determining step from proteolysis to polymerization should occur as the thrombin concentration is increased. When polymerization becomes rate-controlling, deviations from Equation 4 occur, because the rate of release of FPB will be limited by the rate of association of fibrin I rather than proteolysis, provided of course $k_0 < k_b$.

The rate constant ($k_0$) for the thrombin-catalyzed release of FPB from unassociated αBβγ ensembles was determined from measurements of the time dependence of [FPB] in the presence of saturating concentrations of the tetrapeptide Gly-Pro-Arg-Pro. This peptide competes with the Gly-Pro-Arg-Pro Val sequences at the N termini of the α-chains for the polymerization sites in the D-domain in fibrin I (12). As shown in Fig. 2, the release of FPB is increasingly inhibited as the concentration of tetrapeptide is increased until increases in tetrapeptide concentration (above 670 μM) no longer further inhibit FPB release. This limited extent of inhibition of FPB release by high concentrations of tetrapeptide was first observed by Nossal and his co-workers (27). The limiting rate of FPB release is attributed to the unenhanced rate of FPB release from αBβγ half-molecules. Under these conditions, Equation 5 should represent the time dependence of [FPB].

$$[\text{FPB}] = [\alpha\text{Bβγ}]_0 + \frac{[\alpha\text{Bβγ}]_0 k_0 e^{-k_0 t}}{k_1 - k_0} - \frac{[\alpha\text{Bβγ}]_0 k_0 e^{-k_0 t}}{k_1 - k_3}$$ (5)

where $k_0$ is the product of the thrombin concentration and the specificity constant ($k_{\text{catA}}/K_{\text{mA}}$) for the release of FPB from an unassociated monomer. A nonlinear least squares fit of the data in Fig. 2 to Equation 5 yielded a value of 0.65 × 10$^{-3}$ M$^{-1}$s$^{-1}$ for $k_{\text{catA}}/K_{\text{mA}}$. Comparison of the values of the specificity constants $k_{\text{catA}}/K_{\text{mA}}$ and $k_{\text{catB}}/K_{\text{mB}}$ indicates that self-association of αBβγ ensembles results in a 6.5-fold enhancement in the susceptibility of Arg-B614 to attack by thrombin.

To evaluate $k_0$ and $k_{\text{catA}}$ in Scheme 1, the time dependence of the release of FPB was observed over a wide range of thrombin concentrations in the presence and absence of the polymerization inhibitor Gly-Pro-Arg-Pro. The tetrapeptide (T) by binding to αBβγ but not to (αBβγ)$_2$ should shift the association equilibrium as shown in Equation 6.

$$2T + 2\alpha\text{Bβγ} \xrightarrow{K_0} (\alpha\text{Bβγ})_2$$

(6)

If one assumes that the binding of T to αBβγ is rapid so that the equilibrium denoted by $K_0$ is maintained throughout the
reaction, we may write

$$[\text{aB}B\gamma][T] = K_0$$

(7)

$$[\text{aB}B\gamma][T] = [\text{aB}B\gamma] + [\text{aB}B\gamma][T] = [\text{aB}B\gamma]\left(1 + \frac{T}{K_0}\right)$$

(8)

$$[\text{aB}B\gamma] = K_0[\text{aB}B\gamma]\left(1 + \frac{T}{K_0}\right)$$

(9)

For determination of rate constants, experimental data were fit by Runge-Kutta digital integration of the set of differential equations 10–16 which describes the rate of change of the species of Scheme 1 (neglecting the $k_t$ step).

$$\frac{d([\text{aB}B\gamma])}{dt} = -k_p[\text{aB}B\gamma]$$

(10)

$$\frac{d([\text{aB}B\gamma]_{\text{tot}})}{dt} = k_p[\text{aB}B\gamma] - k_p[\text{aB}B\gamma]_{\text{tot}} - 2k_p([\text{aB}B\gamma])$$

(11)

$$\frac{d([\text{aB}B\gamma]_{\text{tot}})}{dt} = k_p([\text{aB}B\gamma]_{\text{tot}})$$

(12)

$$\frac{d([\text{aB}B\gamma]_{\text{tot}})}{dt} = k_p[\text{aB}B\gamma]_{\text{tot}}$$

(13)

$$\frac{d([\text{aB}B\gamma]_{\text{tot}})}{dt} = 2k_p([\text{aB}B\gamma]_{\text{tot}})$$

(14)

$$\frac{d([\text{aB}B\gamma]_{\text{tot}})}{dt} = 2k_p([\text{aB}B\gamma]_{\text{tot}})$$

(15)

$$\frac{d([\text{aB}B\gamma]_{\text{tot}})}{dt} = 2k_p([\text{aB}B\gamma]_{\text{tot}})$$

(16)

where

$k_p = k_p\left(\frac{K_0}{[T] + K_0}\right)$

(17)

To fit the experimental data, the values of $k_1, k_2$, and $k_3$ were fixed at their predetermined values and $k_p$ and $k_p$ were systematically varied until the best fit to the experimental data was obtained.

It can be shown that unless polymerization is at least partially rate-determining, the time dependence of release of [FPB] will not yield a unique value for $k_p$ or $k_p$. Thus, high concentrations of thrombin were used to increase the rate of the $k_p$ and $k_p$ steps so that polymerization would become partially rate-determining. Initial experiments done in the presence of high concentrations of thrombin, but in the absence of polymerization inhibitor, yielded data which allowed us to estimate $k_p$ with reasonable precision, but these data did not permit accurate evaluation of $k_p$. The difficulty in fixing $k_p$ arose because at the initial concentration of fibrinogen ($\geq 0.07 \mu M$) required to obtain accurate determinations of FPB, the extent of dissociation of $[\text{aB}B\gamma]_{\text{tot}}$ was too small to permit accurate evaluation of $k_p$. To evaluate $k_p$ accurately, the extent of association of $[\text{aB}B\gamma]_{\text{tot}}$ was decreased by using Gly-Pro-Arg-Pro (7') to decrease selectively the value of $k_p$ (see Equation 17). When the thrombin concentration is sufficiently low so that $k_p$ is substantially larger than $k_p$, the $\text{aB}B\gamma$ half-molecule has sufficient time to fully equilibrate with $[\text{aB}B\gamma]_{\text{tot}}$. Under these conditions, one can apply the analytical methods used by Beatty and Ballou (36) for numerical solutions of systems of enzymatically catalyzed reactions with rapid equilibrium steps. Thus, the relationship

$$[\text{aB}B\gamma][T] + 2([\text{aB}B\gamma]) = [\text{aB}B\gamma][T] + 2K[\text{aB}B\gamma]$$

(18)

(where $K = k_p/k_p = ([\text{aB}B\gamma]_2)/([\text{aB}B\gamma][T]$) can be used to eliminate the $k_p$ and $k_p$ terms in Equations 11 and 12.

Differentiating Equation 18, one obtains

$$d([\text{aB}B\gamma]_{\text{tot}} + 2d([\text{aB}B\gamma]_{\text{tot}}) = d([\text{aB}B\gamma]_{\text{tot}} + 4K[\text{aB}B\gamma]_{\text{tot}})$$

(19)

substituting Equations 11 and 12 in Equation 19 and solving for $d([\text{aB}B\gamma]_{\text{tot}})/dt$ one obtains

$$d([\text{aB}B\gamma]_{\text{tot}})/dt = k_4[\text{aB}B\gamma]_{\text{tot}} - k_4[\text{aB}B\gamma]_{\text{tot}} - 2k_4([\text{aB}B\gamma]_{\text{tot}})$$

(20)

Differentiation of the equilibrium expression for $K$ yields

$$d([\text{aB}B\gamma]_{\text{tot}})/dt = 2K[\text{aB}B\gamma]_{\text{tot}} d([\text{aB}B\gamma]_{\text{tot}})/dt$$

(21)

It follows that a set of differential equations consisting of Equations 10, 20, 21, and 13–16 should fit experimental data when $k_p \gg k_p$.

Time dependencies for the release of FPB at 0.071 nM thrombin ($k_1 = 7.7 \times 10^{-5}$ s$^{-1}$, $k_2 = 3.0 \times 10^{-5}$ s$^{-1}$) and 1.4 nM thrombin ($k_1 = 1.5 \times 10^{-2}$ s$^{-1}$, $k_2 = 5.9 \times 10^{-3}$ s$^{-1}$) are illustrated in Fig. 3. In both cases, the best fit of the data was obtained with $k_p/k_p$ equal to $6.25 \times 10^3$ M$^{-1}$ s$^{-1}$. When the thrombin concentration was increased to 40 nM, however, a good fit to the experimental data could no longer be obtained by integration of Equations 10, 20, 21, and 13–16. This observation was taken to indicate that at 40 nM thrombin (where $k_p = 0.168$ s$^{-1}$), $k_p$ was no longer much larger than $k_2$ and that the steady state value of $[\text{aB}B\gamma]_{\text{tot}}/[\text{aB}B\gamma]_{\text{tot}}$ fell below its equilibrium value so that Equations 20 and 21 could no longer be used in place of Equations 11 and 12.

To evaluate $k_p$ and $k_p$, differential equations 10–16 were numerically integrated keeping $k_1$, $k_2$, $k_3$, and $k_p/k_p$ at their previously determined values while systematically varying $k_p$ so that the best fit of the experimental data at 40 nM thrombin was obtained. As shown in Fig. 4, the best fit of the data yielded a value of $4 \times 10^4$ M$^{-1}$ s$^{-1}$ for $k_p$ and a value of 0.064 s$^{-1}$ for $k_p$. Since the value of $k_p$ should be independent of the presence of tetrapeptide, it was possible to use the values of $k_1$, $k_2$, $k_3$, and $k_p$ to determine $k_p$ as a function of the tetrapeptide concentration. Additionally, these kinetic constants could be used to determine $k_p$ from the time dependence of the release of FPB in the absence of tetrapeptide. The dashed line in Fig. 1B and the data in Fig. 5 show the best fit of the data obtained from numerical integrations of Equations 10–16 wherein $k_2$ was systematically varied while keeping $k_1$.

Fig. 3. Determination of the apparent equilibrium constant for self-association of $[\text{aB}B\gamma]_2$ from the time dependence of the release of FPB from fibrinogen (0.30 μM) at low concentrations of thrombin in the presence of 100 μM Gly-Pro-Arg-Pro. Panel A, 0.071 nM thrombin (e); Panel B, 1.4 nM thrombin (e). The reaction conditions were pH 7.4, 37°C, $\gamma/2 = 0.15$, and 0.1% PEG. The lines were obtained by Runge-Kutta digital integration of Equations 10, 20, 21, and 13–16 with $k_1 = 1.09 \times 10^5$ s$^{-1}$, $k_2 = 4.2 \times 10^9$ s$^{-1}$, $k_3 = 6.5 \times 10^5$ s$^{-1}$, and $k_4/k_p = 1.25 \times 10^4$ M$^{-1}$ s$^{-1}$ (---), 6.25 \times 10^4 M$^{-1}$ s$^{-1}$ (----).
FIG. 4. Determination of the apparent rate constants ($k'_p$ and $k_p$) for formation and dissociation of the (aB2γ3) ensemble from the time dependence of the release of FPB from fibrinogen (0.30 μM) in the presence of 100 μM Gly-Pro-Arg-Pro at a high concentration of thrombin. The reaction conditions were 37 °C, Γ/2 = 0.15, 0.1% PEG, and 40 nM thrombin (e). The lines were calculated by Runge-Kutta digital integration of Equations 10-16 with $k_1 = 1.09 \times 10^4$ e s$^{-1}$, $k_2 = 4.2 \times 10^6$ e s$^{-1}$, $k_3 = 8.5 \times 10^6$ e s$^{-1}$, and with $k'_p = 2 \times 10^7$ M$^{-1}$s$^{-1}$, $k_p = 0.032$ s$^{-1}$ (- -); $k'_p = 4 \times 10^6$ M$^{-1}$s$^{-1}$, $k_p = 0.064$ s$^{-1}$ (---); $k'_p = 8 \times 10^6$ M$^{-1}$s$^{-1}$, $k_p = 0.128$ s$^{-1}$ (. . .). Note that the ratio $k'_p/k_p$ was fixed at 6.25 $\times$ 10$^{-6}$ (the value determined for $k'_p/k_p$ in Fig. 3) for the values of $k'_p$ and $k_p$ used to fit the data.

FIG. 5. Determination of the rate constants ($k_n$) for association of aB2γ3 from the time dependence of the thrombin-mediated release of FPB from fibrinogen (0.07 μM) at pH 7.4, 37 °C, Γ/2 = 0.15, 0.1% PEG, 40 nM thrombin (e). Panel A, the lines depicted were obtained by digital integration of Equations 10-16 where $k_1 = 1.09 \times 10^4$ e s$^{-1}$, $k_2 = 4.2 \times 10^6$ e s$^{-1}$, $k_3 = 8.5 \times 10^6$ e s$^{-1}$, $k_p = 0.064$ s$^{-1}$, and $k_p = 1.2 \times 10^6$ M$^{-1}$s$^{-1}$ (---), $k_p = 1.0 \times 10^6$ M$^{-1}$s$^{-1}$ (----); $k_p = 0.76 \times 10^6$ M$^{-1}$s$^{-1}$ (----). The lines depicted in Panel B show the dependence of the fit on the value of $k_n$ where $k_1 = 1.09 \times 10^4$ e s$^{-1}$, $k_2 = 4.2 \times 10^6$ e s$^{-1}$, $k_3 = 8.5 \times 10^6$ e s$^{-1}$, $k_p = 0.064$ s$^{-1}$, $k_p = 4.2 \times 10^6$ e s$^{-1}$ (----), $k_p = 6.5 \times 10^6$ e s$^{-1}$, $k_p = 0.064$ s$^{-1}$, $k_p = 1 \times 10^6$ M$^{-1}$s$^{-1}$ (panel A), $k'_p = 4 \times 10^6$ M$^{-1}$s$^{-1}$ (panels B and C).

Fig. 6. Dependence of the release of FPB on the fibrinogen concentration and the presence of Ca$^{2+}$ and PEG at pH 7.4, 37 °C, Γ/2 = 0.15, 1.4 nM thrombin (e). Panel A, 0.1% PEG, 0.30 μM fibrinogen; (O); 0.07 μM fibrinogen (□). Panel B, 0.1% PEG, 100 μM Gly-Pro-Arg-Pro, 0.30 μM fibrinogen (O), 0.07 μM fibrinogen (□); Panel C, 100 μM Gly-Pro-Arg-Pro, 0.30 μM fibrinogen, 0.1% PEG (O), 0.001% PEG (△), and 0.1% PEG and 1.0 mM calcium chloride (○). The lines were obtained by Runge-Kutta digital integration of the differential equations for Scheme 1 with $k_1 = 1.09 \times 10^4$ e s$^{-1}$, $k_2 = 4.2 \times 10^6$ e s$^{-1}$, $k_3 = 6.5 \times 10^6$ e s$^{-1}$, $k_p = 0.064$ s$^{-1}$, $k_p = 1 \times 10^6$ M$^{-1}$s$^{-1}$ (panel A), $k'_p = 4 \times 10^6$ M$^{-1}$s$^{-1}$ (panels B and C).

$k_0$, $k_n$, and $k_p$ fixed at their previously determined values. The best fit of the experimental data yielded a value of $1.0 \times 10^6$ M$^{-1}$s$^{-1}$ for $k_0$. This value is reasonably close to the value of $1.2 \times 10^6$ M$^{-1}$s$^{-1}$ (at pH 7.4, Γ/2 = 0.14, 23 °C) reported for the rate constant for biomolecular association of fibrin I monomers by Hantgan et al. (10) in their stopped flow light-scattering studies of fibrin assembly. The kinetic parameters determined for Scheme 1 predict that a small variation in the time dependence of the extent of FPB release should be observed at very low fibrinogen concentrations. The correspondence between the observed and predicted time dependencies is shown in Fig. 6, A and B.

The data in Fig. 6C show that at 100 μM tetrapeptide and 0.3 μM fibrinogen there is no significant effect of 1 mM Ca$^{2+}$ or 0.1% PEG on the rate of release of FPB. These observations suggest that 1 mM Ca$^{2+}$ or 0.1% PEG has little effect on the equilibrium constant for formation of (aB2γ3). This finding parallels the observation of Shainoff and Dardik (33) that
determined from the plots in Fig. 7 and Fig. 4, as well as the value of $k_p$ (i.e. $k_p$) determined in the absence of Gly-Pro-Arg-Pro ($1 \times 10^8$ M$^{-1}$s$^{-1}$) are plotted according to Equation 22 in Fig. 8. The observed linear dependence of $k_p$ on $[T]$ yields a value of 25.3 $\mu$m for $K_D$. This value is consistent with the value of 25 $\mu$m reported for the binding of Gly-Pro-Arg-Pro to human fibrinogen at 22 °C, pH 7.2, $\Gamma/2 = 0.15$, reported by Laudano and Doolittle (13).

**DISCUSSION**

Scheme 1 depicts the simplest reaction pathway for the conversion of fibrinogen to fibrin II fibers which quantitatively accounts for our observations of thrombin-catalyzed release of fibrinopeptides from normal human fibrinogen at pH 7.4, 37 °C, $\Gamma/2 = 0.15$. It is important to note that previous studies (14) have demonstrated that release of FPB via the $k_3$ step accounts for less than 3% of the FPB and thus release of FPB prior to FPA was neglected in our analysis. Interpretation of the dependence of the release of FPB on the concentration of thrombin, fibrinogen, and Gly-Pro-Arg-Pro in terms of the reaction pathway described in Scheme 1 yielded: (a) a rate constant for fibrin I association which is close to that obtained (10) by direct measurement of self-association of fibrin I to protofibrils using light scattering; (b) a specificity constant for thrombin-catalyzed release of FPB from fibrin I polymer which corresponds to that obtained from independent measurements of thrombin-catalyzed release of FPB from separately prepared fibrin I polymer. These independent verifications of rate constants for putative intermediates indicate that conversion of fibrinogen to fibrin is well represented by the reaction pathway depicted in Scheme 1. Additionally, interpretation of the thrombin-catalyzed release of FPB in terms of this reaction pathway yielded an equilibrium constant for the binding of Gly-Pro-Arg-Pro to fibrin I monomer which is similar to that previously reported (13) for the binding of this tetrapeptide to fibrinogen. This observation is consistent with previous proposals (e.g. Ref. 13) of the existence of an exposed polymerization site in the D-domain of fibrinogen and fibrin I monomer.

Although the time dependencies of the release of FPA and FPB fit a model wherein the reactivity of each half-molecule ensemble is independent of the state of the adjacent half-molecule ensemble on the same molecule, the existence of intramolecular effects has not been ruled out. In this regard, studies of the polymerization of fibrin I subsequent to hydrolysis at Arg-Aa16 suggest that hydrolysis at the second Arg-Aa16 residue in a fibrinogen molecule may be more than an order of magnitude more rapid than the first (37–39). The existence of such an effect would indicate that the value of $k_{3aA}/K_A$ reported in this work is the specificity constant for the first rate-limiting cleavage at Arg-Aa16. Should other intramolecular interactions come to light, it should be possible to utilize the values of rate constants reported here to assign rate constants in amended versions of the reaction pathway.

Since fibrin I can associate with fibrinogen (e.g. Refs. 35, 40, and 41) as well as undergo self-association, it is appropriate to consider whether the kinetics of FPB release might be complicated by formation of complexes between fibrinogen and fibrin I. Substitution of the specificity constants for the sequential release of FPA ($1.09 \times 10^6$ M$^{-1}$s$^{-1}$) and FPB ($4.2 \times 10^8$ M$^{-1}$s$^{-1}$) in Equations 3 and 4 indicate that at a time when 50% of the FPA has been released, the release of FPB is less than 7%. Consequently, when FPB release is greater than 7%, the concentration of $A_0B_3=B_3A$ units is greater than that of the intact $A_0B_3=B_3A$ units. Since the affinity of an $A_0B_3=B_3A$ unit for another $A_0B_3=B_3A$ is $10^7$-fold greater than that for an $A_0B_3=B_3A$ monomer.
unit (35), the concentration of fibrinogen:fibrin I complexes is probably too low to have a detectable effect on the kinetics of FPB release after a few per cent of the FPB have been released.² These considerations together with the low value observed for \( k_3 \) relative to \( k_6 \) and \( k_7 \) and the low value of \( k_8 \) relative to \( k_9 \) suggest that essentially all of the FPB is released from fibrin I polymer. It is important to note, however, that the relative magnitudes of rate and equilibrium constants in Scheme 1 may be functions of the reaction conditions and the structure of fibrinogen. Thus, the degree of sequentiality of the process may be altered by changes in pH, temperature, ionic strength, or by variations in fibrinogen structure such as those associated with many dysfibrinogenemias.

It is interesting to consider further the dimerization of fibrin I as represented in Equation 23

\[
2\alpha B\gamma \rightleftharpoons k_p (\alpha B\gamma)_2
\]

Self-association of fibrin I involves formation of two sets of interactions involving the two N termini of the \( \alpha \)-chain in the E-domain and two polymerization sites in the D-domain. The standard unitary free energy change³ for the process is given by Equation 24

\[
\Delta G_f = -RT \ln K = -7.98T = -12,700 \text{cal/mol}
\]

Thus, the unitive free energy change for one D-domain interacting with one E-domain should be -6350 cal/mol (i.e. 0.5 \( \times -12,700 \text{cal/mol} \)). Substitution of this value of \( \Delta G_f \) in Equation 25 yields a value of

\[
\Delta G_f = -RT \ln K = -7.98T
\]

5.3 \( \times 10^2 \) for \( K \), the equilibrium constant for the association of an E-domain with a D-domain. This value is 75 times smaller than the equilibrium constant (4.0 \( \times 10^4 \text{ M}^{-1} \)) for the association of Gly-Pro-Arg-Pro with the D-domain in fibrinogen (23) or fibrinogen (13) and 19 times smaller than the equilibrium constant (1.0 \( \times 10^5 \text{ M}^{-1} \)) for association of Gly-Pro-Arg-Val with the D-domain of fibrinogen (13). An even greater discrepancy (~19 \( \times 10^5 \)-fold) appears to exist between the strength of the interactions between the D- and E-domains in fibrin I dimer and in fibrinogen:fibrinogen complexes, since the equilibrium constant for binding of fibrinogen to fibrin I is \( -10^6 \text{ M}^{-1} \) (35, 40). Further work is necessary to determine the structural basis for the higher affinity of fibrinogen for fibrinogen I and the reason why the D-E interactions in fibrin I dimer are weaker than those found when fibrin I complexes with Gly-Pro-Arg-Pro and fibrinogen.

It is important to note that the value of 1.56 \( \times 10^7 \text{ M}^{-1} \) determined for the apparent equilibrium constant \( (k_p/k_{-p}) \) indicates that aggregation of fibrin I monomers to protofibrils should be reversible and dependent on the concentration of fibrinogen. The size distribution of linear fibrin protofibrils should be given by the equation of Flory (43) for polymerization of a bifunctional monomer.

\[
w(i) = \frac{1}{(1-p)^p} \]

where \( w(i) \) is the weight fraction of monomeric units in a protofibril containing \( i \) monomers and \( p \) is the fraction of filled polymerization sites. Thus

\[
p = 1 - \frac{[\alpha B\gamma y]}{[\alpha B\gamma \beta y]} = \frac{k_p}{2[\beta \gamma ]^2} - k_{-p}
\]

If the equilibrium constant for addition of each fibrin I monomer to a protofibril is equal to \( k_p/k_{-p}, \) \( p \) can be determined by using the equilibrium expression

\[
[\alpha B\gamma \beta y] = [\alpha B\gamma y] = k_p
\]

to calculate \( [\alpha B\gamma y] \) in Equation 27. At the highest fibrinogen concentration used in this work (0.3 \( \mu \text{M} \)), \( [\alpha B\gamma \beta y] = 0.6 \mu \text{M} \), \( [\alpha B\gamma y] = 0.124 \mu \text{M} \), and \( p = 0.79 \). Calculation of the distribution of \( w(i) \) as a function of the degree of polymerization \( i \) with Equation 26 and \( p \) set at 0.79 indicates that only 30% of the fibrin monomers should be present in oligomers with more than 10 monomer units.

At a 10-fold higher concentration of fibrinogen (3.0 \( \mu \text{M} \)), Equations 26–28 indicate that over 80% of the fibrin I monomers should be present in protofibrils more than 10 monomeric units in length (at 37°C, pH 7.4, \( 1/2 = 0.15 \)). Since long protofibrils would be expected to have a greater propensity to participate in the side to side interactions involved in fiber growth, fiber formation should serve to drive the polymerization of fibrin I monomer toward completion. In this regard, it is interesting to note that Shainoff and Dardik (35) in their studies of the effect of fibrinogen on the elution position of fibrin I during gel filtration have estimated an equilibrium constant of \( -10^8 \text{ M}^{-1} \) for incorporation of a fibrin I monomer into a fibrin I fiber. Side to side interactions which are present in fibrin I fibers, but absent in fibrin I protofibrils, may account for this equilibrium constant for self-association of fibrin I being larger than the equilibrium constant of 1.56 \( \times 10^7 \text{ M}^{-1} \) reported in this work for fibrin I dimerization.

It is also important to realize that interactions between adjacent D-domains which exist in the trimer and higher oligomers, but not in the dimer (10), might cause the value of the equilibrium constant for dimerization of fibrin I monomer to be less than that for addition of a fibrin I monomer to a growing protofibril. If such were the case, the measured value of \( k_p/k_{-p} \) would be an average value, which could cause us to underestimate somewhat the size of protofibrils. Further work is necessary, however, to determine whether interactions between adjacent D-domains contribute significantly to the stability of protofibrils.

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