Steady-State Kinetic Study of the Bovine Thrombin–Fibrinogen Interaction†

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ABSTRACT: Thrombin converts fibrinogen to fibrin monomer by hydrolyzing an Arg–Gly bond in each of the two Aα and two Bβ chains of fibrinogen. This action releases one molecule of fibrinopeptide A and one of fibrinopeptide B from each Aα and Bβ chain, respectively. By use of a high-performance liquid chromatography assay, with bovine thrombin and bovine fibrinogen, the rates of the simultaneous release of these two peptides (prior to the gel point) have been measured for a range of fibrinogen concentrations. From these data, values of the Michaelis–Menten parameters, k_{cat} and K_M, for the interaction of bovine thrombin with the Aα and Bβ chains of bovine fibrinogen were determined. For the Aα chain, $k_{cat} = 7.3 \times 10^{-10}$ M [(NIH unit/L)s]$^{-1}$ and $K_M = 9.2 \times 10^{-6}$ M and, for the Bβ chain, $k_{cat} = 1.15 \times 10^{-10}$ M [(NIH unit/L)s]$^{-1}$ and $K_M = 11.3 \times 10^{-6}$ M. The mechanistic implications of these results were also investigated, and a model is proposed which is consistent with the following experimental observations on the release of the fibrinopeptides from fibrinogen: (1) fibrinopeptide B is released from the *start* of the reaction at some low but nonzero rate, (2) after this initial low rate of release of fibrinopeptide B, the rate is accelerated at the lower concentrations of fibrinogen examined, and (3) the biphasic release of fibrinopeptide B was not observed at the higher concentrations of fibrinogen examined during the time period when the reaction was monitored.

The mechanism of action of thrombin on fibrinogen is being investigated in this laboratory by using an active-site mapping approach (Scheraga, 1977). Peptides of varying size which are analogous to portions of the fibrinogen molecule around the thrombin-susceptible Arg–Gly bonds have been prepared, either by chemical cleavages of fibrinogen (Hageman & Scheraga, 1974, 1977) or by synthetic methods (Andreatta et al., 1971; Liem et al., 1971; Liem & Scheraga, 1973, 1974; van Nispen et al., 1977). These peptides were used as substrates in kinetics experiments in which their Arg–Gly bonds were hydrolyzed by thrombin. The values of the specificity constants, k_{cat}/K_M, obtained for these hydrolyses were to be compared with the value for the natural substrate, fibrinogen, in order to determine, among other things, the minimum-size peptide which possesses all the residues essential for proper interaction with thrombin.

In this approach, it is necessary to have reliable values of k_{cat} and K_M for the interaction of thrombin with fibrinogen. A number of studies have been carried out to determine the rate of release of fibrinopeptides A and B[1] (Blombäck & Vestermark, 1958; Blombäck, 1958; Bando et al., 1972; Blombäck & Blombäck, 1972; Bilezikian et al., 1975; Nossel et al., 1976), from which values of the Michaelis–Menten parameters could be obtained. These studies, however, were carried out under different conditions (temperature, pH, and ionic strength) from those used in the active-site mapping experiments performed in this laboratory. More importantly, no systematic steady-state kinetic study of the thrombin–fibrinogen interaction over a range of fibrinogen concentrations (prior to the gel point) has been reported.

In order to determine the values of the Michaelis–Menten parameters under the desired conditions (25 °C; pH 8; 0.15 M ionic strength), steady-state kinetics experiments were performed, utilizing a rapid and sensitive LC assay for bovine fibrinopeptides which has been developed recently (Martinelli & Scheraga, 1979). With this technique, it was possible to make accurate measurements of the initial rates of the simultaneous release of both fibrinopeptides A and B from fibrinogen as a function of fibrinogen concentration, from which the Michaelis–Menten parameters for the Aα and Bβ chains of bovine fibrinogen could be determined.

In addition to providing reliable values of the kinetic constants for the thrombin–fibrinogen interaction, the results of these experiments enabled some conclusions to be drawn about the mechanism of the release of the fibrinopeptides.

Experimental Section

Materials. Thrombin was prepared from bovine prothrombin (Sigma Chemical Co.; Lot 65-C-8400) as described earlier (Hageman & Scheraga, 1974). Bovine fibrinogen, 95% clottable, was obtained from Miles Laboratory (Lot 24). Since this material was actually only 90% clottable, it was purified further as described previously (Hageman & Scheraga, 1977) to obtain a product that was >96% clottable. Samples of purified bovine fibrinopeptides A and B were prepared from fibrinogen (Blombäck & Vestermark, 1958), and the purity of these peptides was determined by LC and by amino acid analysis. Imidazole, 99%, was obtained from Aldrich Chemical Co. and was recrystallized twice from benzene prior to use in the buffer for clotting assays. Polyamide microlayer sheets were purchased from Analtech, and dansyl chloride and dansyl amino acid standards were obtained from Schwarz/Mann. For use as LC solvents, glass-distilled acetonitrile was purchased from Burdrich and Jackson Laboratories, and water was doubly distilled and deionized. LC solvents were degassed and filtered through Millipore filters (5 μM; Type LS) prior to use.

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[1] Abbreviations used: LC, high-performance liquid chromatography; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH (TCA in figures), trichloroacetic acid; fibrinopeptides A and B, the N-terminal portions of the Aα and Bβ chains, respectively, of fibrinogen that are released upon interaction with thrombin; v_A and v_B, initial velocities of release of fibrinopeptides A and B, respectively; CNBr Aα and CNBr Bβ, the N-terminal CNBr fragments of the Aα and Bβ chains, respectively, of fibrinogen; Ac, the acetyl blocking group for primary amines; p-Glu, pyroglutamic acid.

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All other chemicals were reagent grade or better and used without further purification.

LC Apparatus. A modular liquid chromatograph, consisting of a Model No. 6000A pump, a Model U6K universal injector, and a Model No. 450 variable-wavelength detector, all from Waters Associates, was used to assay samples in the kinetic runs. A reversed-phase μBondapak C_{18} column, 4 mm × 30 cm, also from Waters Associates, was used for separations. LC samples were pretreated on Sep-Pak C_{18} cartridges from Waters Associates (see below). Injections were made with a Pressure Lok syringe from Precision Sampling Corp.

Methods. (1) Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out on samples of bovine fibrinogen and bovine thrombin (Weber & Osborn, 1969); the gels were 7.5% acrylamide and 0.25% bis(acrylamide). β-Mercaptoethanol was omitted from the thrombin samples. The gels were run at 8 mA/gel and stained with Coomassie Brilliant Blue.

(2) N-Terminal Analysis. N-Terminal group analysis was carried out on purified fibrinogen using dansyl chloride (Woods & Wang, 1967). Dansyl amino acids were identified by two-dimensional polyamide microlayer chromatography (Woods & Wang, 1967), and standard dansyl amino acids were cochromatographed with the samples.

(3) Nitrogen Analysis. The concentrations of fibrinopeptides A and B in the standard solutions were determined by micro-Kjeldahl nitrogen analysis (Lang, 1968; Noel & Hambleton, 1976a,b).

Kinetics Experiments. Kinetics experiments were carried out at 25 °C in 0.15 M KCl and 0.05 M sodium phosphate, pH 8.00. The concentrations of bovine fibrinogen stock solutions were determined spectrophotometrically (Ehrenpreis & Scheraga, 1957). The reaction mixtures were then prepared by quantitative dilutions of the fibrinogen stock solutions. The fibrinogen concentrations used in the kinetics experiments were in the range of 0.805×10^{-5} to 6.24×10^{-5} M. The thrombin concentration, determined by clotting assay (Liem et al., 1971) and spectrophotometrically (Liem & Scheraga, 1973), was 0.01 NIH unit/mL ($\sim 10^{-10}$ M).

After addition of thrombin, digestion proceeded for various time intervals, and each reaction solution was then quenched by addition of formic acid (to 2% v/v). In the runs at each of these time intervals, a separate sample of fibrinogen was incubated with thrombin, the total volume of each reaction solution being 10 mL. Zero-time points were obtained by adding formic acid before addition of thrombin. Infinite-time points were obtained by using larger thrombin concentrations (40 NIH units/mL) and allowing the reaction to proceed for ~6 h (very much longer than the time intervals at which the rates of hydrolysis were measured).

In the kinetics experiments (at a thrombin concentration of 0.01 NIH unit/mL), the rates were measured over a time period of 0 to ~1–1.5 h. Since the clotting time (at this thrombin concentration) is ~2 h, no clots were present in the kinetics runs, although intermediate polymers are expected to have formed. On the other hand, in the experiments at a thrombin concentration of 40 NIH units/mL (to obtain the infinite-time points), clots were present.

Immediately after the reaction was stopped by addition of formic acid (in the kinetics experiments), all protein material present was precipitated by addition of an equal volume of ice-cold 15% Cl_3AcOH solution. These samples were then centrifuged at 20000 rpm for 20 min at 0 °C.

The supernatant after centrifugation, which contained the fibrinopeptides, was treated on Sep-Pak C_{18} cartridges, prior to analysis by LC. The supernatant was passed through these cartridges to bind the fibrinopeptides to the resin in the cartridges. The cartridge was then washed with ~5 mL of 0.083 M ammonium phosphate buffer, pH 3.10, to remove most of the salts, buffer components, and Cl_3AcOH. The fibrinopeptides were then eluted with ~9 mL of a solvent consisting of acetonitrile–0.083 M ammonium phosphate buffer, pH 3.10 (35:65). This solution of fibrinopeptides was then diluted to 10 mL with water, and 25 μL was analyzed by LC.

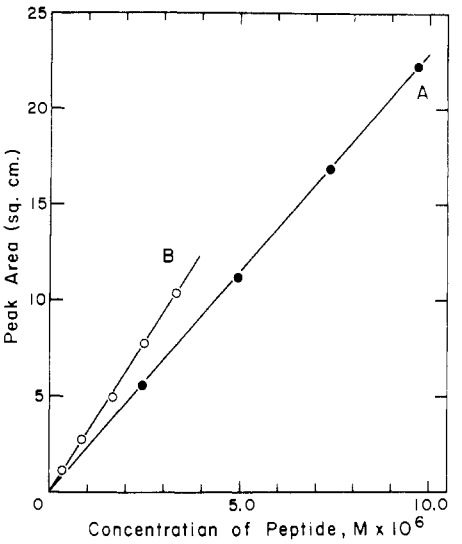

FIGURE 1: Peak area (at 205 nm) vs. concentration of fibrinopeptide A (●) and fibrinopeptide B (○). These are the calibration curves for the LC determination of unknown amounts of the fibrinopeptides. The concentrations of the fibrinopeptide standards were determined by micro-Kjeldahl nitrogen analysis.

The LC technique used to assay these samples for fibrinopeptides A and B was essentially the same as that reported earlier (Martinelli & Scheraga, 1979). The only change was that the chromatograms were analyzed by absorbance at 205 nm rather than at 210 nm in order to increase the sensitivity. A calibration curve was prepared by using purified samples of fibrinopeptides A and B (Figure 1). The concentrations of the fibrinopeptides in these standards were determined by micro-Kjeldahl nitrogen analysis.

The areas under the peaks in the chromatograms of the kinetics samples corresponding to fibrinopeptides A and B were determined and converted to concentration of fibrinopeptide by using the calibration curves. The initial rates of release of fibrinopeptides A and B at each concentration of fibrinogen were then determined from the slope of the least-squares line through a plot of (moles of peptide released per liter) vs. time (see Figures 4 and 5 below). Only those points for which the concentration of substrate hydrolyzed was less than 25% of the initial substrate concentration were used in the determination of initial velocities.

Appropriate control experiments were performed to ensure that there were no losses of fibrinopeptides during the precipitation with Cl_3AcOH and to ensure that recovery from the Sep-Pak cartridges was quantitative. In addition, experiments were carried out to demonstrate the linearity of the rate of release of fibrinopeptide A with thrombin concentration. The fibrinogen concentration used in this experiment was 4.03×10^{-5} M, and the thrombin concentrations were in the range of 0.0011–0.05 NIH unit/mL. Digestion with thrombin proceeded for 20 min when the reaction was quenched and worked up in the manner described above.

A separate experiment was also performed in order to determine the early events in the action of thrombin on fibri-

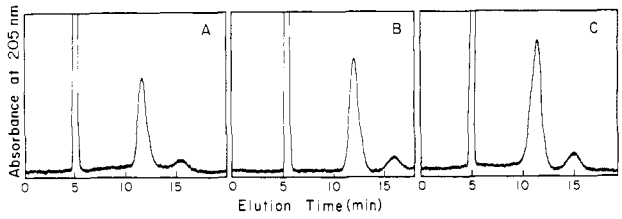

FIGURE 2: Sample LC chromatograms from the kinetic experiments at 25 °C in 0.15 M KCl and 0.05 M sodium phosphate, pH 8.00. The fibrinogen concentration was 6.24×10^{-5} M and the thrombin concentration was 0.01 NIH unit/mL. (A) 30, (B) 40, and (C) 50 min after addition of thrombin. Fibrinopeptides A and B eluted at ~12 and ~16 min, respectively, and Cl_3AcOH at ~5 min.

nogen. In this experiment, a low thrombin concentration was used (0.0011 NIH unit/mL) to digest samples of fibrinogen (4.03×10^{-5} M).

Results

Purity of Fibrinogen and Thrombin. $NaDodSO_4$ gel electrophoresis of samples of reduced fibrinogen gave three bands, corresponding to the Aα, Bβ, and γ chains, while unreduced thrombin gave only one; thrombin was not reduced because α-, β-, and γ-thrombins can be distinguished even when the disulfide bonds are intact (Mann et al., 1971). N-Terminal analysis of purified bovine fibrinogen gave only (the proper) Glu and Tyr as N-terminal amino acids (Lorand & Middlebrook, 1952; Bettelheim & Bailey, 1952; Blombäck & Yamashina, 1958) [the Bβ chain having the undetectable p-Glu as the N-terminal residue (Doolittle & Blombäck, 1964)]. These results indicated that the fibrinogen and thrombin were pure (see also LC Assay).

LC Assay. The LC assay was useful for detecting alterations in thrombin activity since α-thrombin will release fibrinopeptides A and B from fibrinogen whereas its degradation products (β- and γ-thrombin) do so less efficiently (Lundblad et al., 1975). Thus, as thrombin degrades, fibrinopeptide becomes less easily detectable by LC assay. This is a more sensitive assay than a clotting assay since, under some conditions, the clotting time was unaltered even though the LC assay indicated a reduction in thrombin activity, attributed to degradation from α- to β-thrombin. All kinetic runs were made under conditions such that no degradation of α-thrombin was detectable.

Because of this difference in sensitivity between the LC and clotting assays, it may be worthwhile to consider the adoption of the LC method as a routine assay for thrombin activity. An additional advantage is that the LC method measures the rate of release of fibrinopeptides A and B (i.e., the *direct* result of thrombin action) whereas the clotting assay measures the *net* effect of proteolysis of fibrinogen plus polymerization and cross-linking of fibrin monomers and intermediate polymers.

Kinetic Runs. By use of the LC assay, the rates of release of fibrinopeptides A and B could be monitored (see Figure 2). Results of the control experiments demonstrated that no losses of fibrinopeptides occurred in the Cl_3AcOH precipitation step or in the treatment on the Sep-Pak C_{18} cartridges. The linear dependence of the initial rate of release of fibrinopeptide A on thrombin concentrations from 0.0011 to 0.05 NIH unit/mL was also demonstrated (see Figure 3).

The results of the kinetics experiments are summarized in Figures 4 and 5. The lines drawn through the plots of (moles of peptide released per liter) vs. time were determined by least-squares analysis.

There is a small difference (~10–15%) between the infinite-time concentrations of the fibrinopeptides and the concentrations expected on the basis of the spectrophotometrically determined concentrations of fibrinogen (the former should

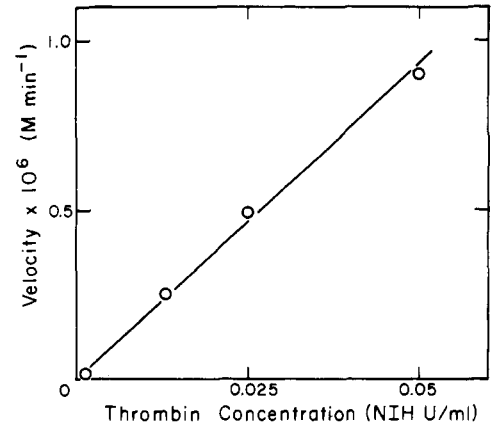

FIGURE 3: Dependence of the initial rate of release of fibrinopeptide A on thrombin concentration (fibrinogen concentration = 4.03×10^{-5} M) at 25 °C in 0.15 M KCl and 0.05 M sodium phosphate, pH 8.00.

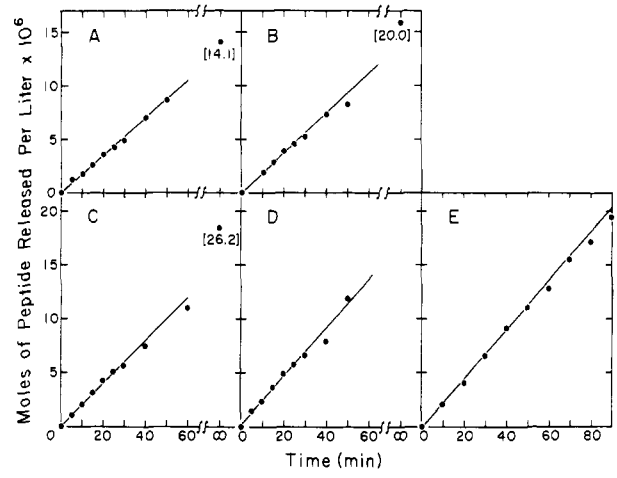

FIGURE 4: Data for the release of fibrinopeptide A at 25 °C in 0.15 M KCl and 0.05 M sodium phosphate, pH 8.00. Thrombin concentration was 0.01 NIH unit/mL. Fibrinogen concentrations were (A) 0.805×10^{-5}, (B) 1.04×10^{-5}, (C) 1.56×10^{-5}, (D) 3.22×10^{-5}, and (E) 6.24×10^{-5} M. The lines drawn through these points were obtained by least-squares analysis. The data in brackets are the infinite-time points.

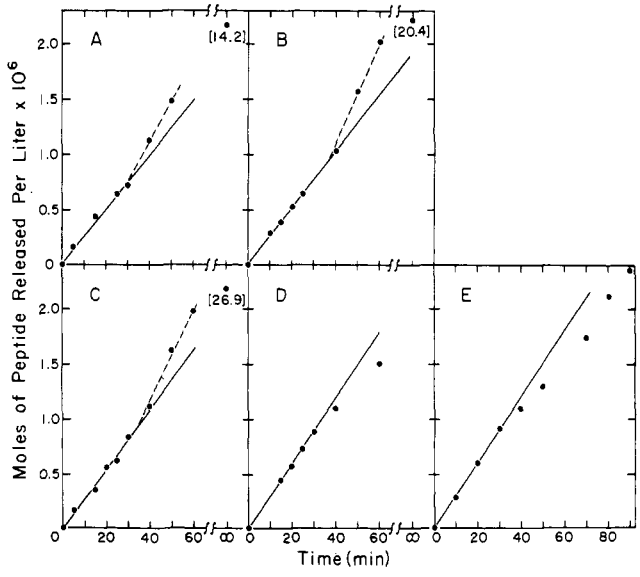

FIGURE 5: Data for the release of fibrinopeptide B. Thrombin and fibrinogen concentrations were the same as those in Figure 4.

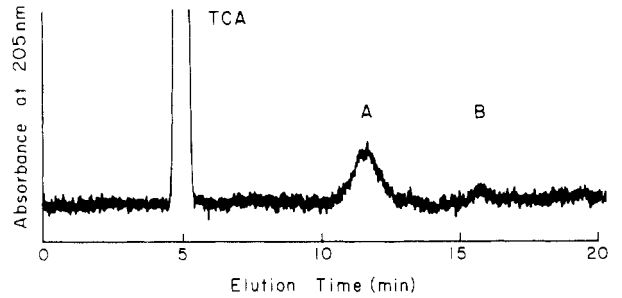

FIGURE 6: LC chromatogram showing release of fibrinopeptides in an early stage of the thrombin–fibrinogen reaction at 25 °C in 0.15 M KCl and 0.05 M sodium phosphate, pH 8.00. Fibrinogen concentration was 4.03×10^{-5} M and thrombin concentration was 0.0011 NIH unit/mL; the chromatogram pertains to 5 min of reaction after the addition of thrombin. A and B refer to the peaks for fibrinopeptides A and B, respectively; the initial large peak pertains to Cl_3AcOH.

be twice the latter). This difference is apparent in Figures 4A,C and 5A,C and can be attributed to two factors. First, it might be difficult for thrombin to diffuse through the gel matrix (in the infinite-time runs) to reach all unhydrolyzed fibrinogen molecules. Second, it was very difficult to dissolve these clots; in fact, it was for this reason that infinite-time values were not determined for the two largest fibrinogen concentrations. For these reasons, the concentrations of fibrinogen used in the calculations were taken as the larger ones based on the spectrophotometric assay.

The results of the kinetic experiments (Figures 4 and 5) demonstrate that, while the release of fibrinopeptide A appears to be linear in time, the release of fibrinopeptide B under the conditions studied exhibits complex behavior. In agreement with earlier studies (Bettelheim, 1956; Blombäck, 1958), fibrinopeptide B is released initially at a slower rate than that of fibrinopeptide A. These experiments also demonstrate that fibrinopeptide B is released from the start of the reaction (Figure 5). To confirm this observation, we carried out a separate experiment, in which a very low thrombin concentration (0.0011 NIH unit/mL) was used in the digestion of solutions of fibrinogen (4.03×10^{-5} M). The results are shown in Figure 6, which indicates that *both* fibrinopeptides A and B are present at 5 min after addition of thrombin to fibrinogen, even at this low thrombin concentration. Finally, these experiments demonstrate that the release of fibrinopeptide B is biphasic, at least at the lower fibrinogen cencentrations examined. This biphasic behavior has also been reported previously (Blombäck et al., 1978). For these lower fibrinogen concentrations, the release of fibrinopeptide B proceeds at a low rate up until 30–40 min, after which the rate appears to increase (Figure 5A–C). This biphasic behavior is absent in the release of fibrinopeptide B at the higher fibrinogen concentrations on the time scale examined here (Figure 5D,E). It should be recalled that all of the data of Figures 4 and 5 were obtained before the clotting time, which was ~2 h in these experiments.

Discussion

The determination of the Michaelis–Menten parameters from the initial velocities of release of fibrinopeptides A and B is not straightforward since the rate of release of each peptide depends on the rate of release of the other peptide (according to the proposed mechanism for the thrombin–fibrinogen interaction). Even if the Lineweaver–Burk plots of reciprocal velocities vs. the reciprocal of the substrate concentrations were linear, the values obtained for the slopes and intercepts from these plots would not correspond simply to the Michaelis–Menten parameters. This can be seen readily from an examination of the proposed mechanism.

Mechanisms for Evaluating Parameters. Two models to account for the rates of release of the fibrinopeptides from fibrinogen have been given a preliminary examination (Hageman & Scheraga, 1977). The first model is a competitive one which considers the Aα and Bβ chains of fibrinogen to be competitive inhibitors of each other and assumes that each chain is fully accessible to thrombin. This mechanism is represented as

$$A\alpha + T \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A\alpha T \xrightarrow{k_A} A + \alpha + T \quad (1)$$

$$B\beta + T \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} B\beta T \xrightarrow{k_B} B + \beta + T \quad (2)$$

where T is thrombin, AαT and BβT are the enzyme–substrate complexes, and A, α, B, and β are the products of the hydrolysis of the Aα and Bβ chains. This mechanism assumes that the enzyme–substrate complexes are present at steady-state concentrations and that the breakdown of these complexes to the products is irreversible with first-order rate constants k_A and k_B, respectively. K_{M_A} and K_{M_B} are the Michaelis–Menten constants for each reaction and are defined as

$$K_{M_A} = (k_{-1} + k_A)/k_1 \quad (3)$$

$$K_{M_B} = (k_{-2} + k_B)/k_2 \quad (4)$$

This model leads to the following rate equations for the initial velocities:

$$v_A = \frac{d[A]}{dt} = k_A[A\alpha T] = \frac{k_A[T]_0[A\alpha]}{K_{M_A} + [A\alpha] + (K_{M_A}/K_{M_B})[B\beta]} \quad (5)$$

$$v_B = \frac{d[B]}{dt} = k_B[B\beta T] = \frac{k_B[T]_0[B\beta]}{K_{M_B} + [B\beta] + (K_{M_B}/K_{M_A})[A\alpha]} \quad (6)$$

where $[T]_0$ is the initial thrombin concentration. Following the suggestion of Lee & Wilson (1971), $[A\alpha]$ and $[B\beta]$ are taken as the average values of the concentrations of the Aα and Bβ chains of fibrinogen over the time period of the kinetics experiment:

$$[A\alpha] = [[A\alpha]_0 + ([A\alpha]_0 - [A])]/2 \quad (7)$$

$$[B\beta] = [[B\beta]_0 + ([B\beta]_0 - [B])]/2 \quad (8)$$

Taking the reciprocals of eq 5 and 6 and setting $k_A[T]_0 = v_{max_A}$ and $k_B[T]_0 = v_{max_B}$, we obtain

$$\frac{1}{v_A} = \frac{1}{v_{max_A}} + \frac{K_{M_A}}{v_{max_A}}\left(1 + \frac{[B\beta]}{K_{M_B}}\right)\frac{1}{[A\alpha]} \quad (9)$$

$$\frac{1}{v_B} = \frac{1}{v_{max_B}} + \frac{K_{M_B}}{v_{max_B}}\left(1 + \frac{[A\alpha]}{K_{M_A}}\right)\frac{1}{[B\beta]} \quad (10)$$

Inspection of eq 9 and 10 reveals the problem involved in obtaining values of K_{M_A}, K_{M_B}, v_{max_A}, and v_{max_B} from double-reciprocal plots of these data. Instead of plotting $1/v_A$ vs. $1/[A\alpha]$ and $1/v_B$ vs. $1/[B\beta]$, the correct variables to plot are the reciprocal velocities vs. $(1 + [B\beta]/K_{M_B})(1/[A\alpha])$ and $(1 + [A\alpha]/K_{M_A})(1/[B\beta])$, respectively, but these two quantities contain two of the parameters that are to be determined, K_{M_A} and K_{M_B}.

The second model examined to account for the rates of release of the fibrinopeptides from fibrinogen is a sequential one. The basic assumption of this model is that the Bβ chain of fibrinogen is inaccessible to thrombin until after the Aα

chain in that part of the fibrinogen molecule has been hydrolyzed by thrombin. The equations representing the sequential model are

$$ABf + T \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ABfT \xrightarrow{k_A} T + A + Bf \quad (11)$$

$$Bf + T \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} BfT \xrightarrow{k_B} T + B + f \quad (12)$$

where ABf is half of the fibrinogen molecule (which is considered to be an ABf dimer), Bf is the product of the removal of fibrinopeptide A from ABf, and f is the product of the removal of both fibrinopeptides A and B (fibrin monomer); ABfT and BfT are the enzyme–substrate complexes.

The steady-state approximation can be applied to ABfT but not to BfT, since the concentration of Bf changes with time (increasing continually from zero throughout the *early* period of the reaction). In order to obtain the rate equations for this model, we assume that the second reaction (the release of fibrinopeptide B) is a negligible perturbation of the first reaction. This approximation should hold for the early stages of the reaction, where $v_A > v_B$, which is observed experimentally. Then, since the steady-state approximation holds for the first reaction

$$v_A \approx \frac{v_{max_A}[ABf]}{K_{M_A} + [ABf]} \quad (13)$$

where v_{max_A} and K_{M_A} have the same definition as in the competitive model, and [ABf] is the average substrate concentration over the time period of the kinetics experiment.

The rates of change of [Bf] and [BfT] may then be written as

$$\frac{d[Bf]}{dt} = v_A - k_2[Bf][T] + k_{-2}[BfT] \quad (14)$$

$$\frac{d[BfT]}{dt} = -(k_{-2} + k_B)[BfT] + k_2[Bf][T] \quad (15)$$

Adding these two equations, we obtain

$$\frac{d([Bf] + [BfT])}{dt} = v_A - k_B[BfT] \quad (16)$$

where

$$v_B = k_B[BfT] \quad (17)$$

Since [BfT] is very small in the early part of the reaction, the conservation equation

$$[T]_0 = [T] + [ABfT] + [BfT] \quad (18)$$

may be approximated by

$$[T]_0 \approx [T] + [ABfT] \quad (19)$$

Substituting the steady-state concentration of [ABfT] for the first reaction, this equation becomes

$$[T]_0 \approx [T](1 + [ABf]/K_{M_A}) \quad (20)$$

We may also assume that a rapidly attained equilibrium exists between Bf, T, and BfT; hence

$$K_2 = ([Bf][T])/[BfT] \quad (21)$$

With the aid of eq 20 and 21, we may then write

$$[Bf] + [BfT] = [BfT]\left[1 + \frac{K_2}{[T]_0}\left(1 + \frac{[ABf]}{K_{M_A}}\right)\right] \quad (22)$$

Differentiating eq 22 with respect to t (remembering that the expression in brackets is constant) and substituting in eq 16, we obtain

$$\frac{d[BfT]}{dt} = \frac{v_A - k_B[BfT]}{1 + \frac{K_2}{[T]_0}\left(1 + \frac{[ABf]}{K_{M_A}}\right)} \quad (23)$$

Integration leads to

$$[BfT] = \frac{v_A}{k_B}(1 - e^{-ct}) \quad (24)$$

where

$$c = \frac{k_B}{1 + \frac{K_2}{[T]_0}\left(1 + \frac{[ABf]}{K_{M_A}}\right)} \quad (25)$$

Approximating the exponential by $1 - ct$ at small t, eq 24 becomes

$$[BfT] = \frac{v_A ct}{k_B} \quad (26)$$

Substitution of eq 26 into eq 17 leads to

$$v_B = v_A ct \quad (27)$$

The two models predict different behaviors of the rate of release of fibrinopeptide B in the *early stages* of the reaction. According to eq 6 (the competitive model), v_B should be independent of time (since [Aα] and [Bβ] are essentially constant). In contrast, in the sequential model (eq 27), v_B increases linearly with time. This is understandable in terms of the nature of the sequential model; i.e., since [Bf] increases with time (according to eq 11), the rate of formation of B, which depends on [Bf], will also increase with time. Since the experimental results (Figure 5) indicate that v_B is independent of time in the *early stages* of the reaction, the competitive model is selected over the sequential one.

Michaelis–Menten Parameters. The true values of the Michaelis–Menten parameters (for the competitive model) were then determined by a nonlinear least-squares curve-fitting procedure (Cornish-Bowden & Koshland, 1970; Wharton et al., 1974). In this analysis, the initial values of these parameters were those obtained from the plots of $1/v_A$ vs. $1/[A\alpha]$ and $1/v_B$ vs. $1/[B\beta]$. Several initial values of the parameters were also used in this program, and, in each case, the final values of the parameters converged close to the values reported below for the Michaelis–Menten parameters.

The values for the Michaelis–Menten parameters are as follows: $K_M = 9.2 \times 10^{-6}$ M and $k_{cat} = 7.3 \times 10^{-10}$ M [(NIH unit/L)s]$^{-1}$ for the Aα chain; $K_M = 11.3 \times 10^{-6}$ M and $k_{cat} = 1.15 \times 10^{-10}$ M [(NIH unit/L)s]$^{-1}$ for the Bβ chain. The sum of the squares of the deviations of the initial velocities (v_A and v_B) calculated by using these values of the kinetics parameters and the experimental initial velocities was 1.09×10^{-17} (M min^{-1})^2; the squares of the experimental initial velocities were in the range of 3.0×10^{-14} to 5.2×10^{-14} (M min^{-1})^2 for v_A^2 and 5.9×10^{-16} to 9.5×10^{-16} (M min^{-1})^2 for v_B^2. Figure 7 shows the fit of the calculated values of the parameters to the data.

CNBr Aα. The results of the previous active-site mapping experiments can now be reinterpreted in light of the Michaelis–Menten parameters determined in this work (see Table I). On the basis of previous estimates of k_{cat} and K_M for the Aα chain of fibrinogen, the N-terminal CNBr fragment of the Aα chain was thought to be just as good a substrate as the intact Aα chain (Hageman & Scheraga, 1974). This con-

Table I: Comparison of the Kinetic Constants for the Hydrolysis of Arg–Gly Bonds by Thrombin at pH 8.0 and 25 °C

substrate		$K_M \times 10^6$ (M)	$k_{cat} \times 10^{11}$ [M [(NIH units/L)s]$^{-1}$]	$(k_{cat}/K_M) \times 10^7$ [[(NIH units/L)s]$^{-1}$]	ref
fibrinogen (bovine):	Aα chain	9.2[a]	73	793	this work
	Bβ chain	11.3[a]	11.5	102	this work
CNBR Aα		47	48	100	Hageman & Scheraga (1974)
CNBr Bβ		189	6.21	3.3	Hageman & Scheraga (1977)
peptide A[b]		630	0.30	0.05	van Nispen et al. (1977)
peptide B[b]		1560	0.32	0.02	van Nispen et al. (1977)
peptide C[b]		680	10.8	1.6	van Nispen et al. (1977)

[a] These data pertain to a single chain. [b] Peptides A, B, and C are as follows:

peptide A: Ac-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-Arg-$NHCH_3$

peptide B: Ac-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-Arg-$NHCH_3$

peptide C: Ac-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-Arg-$NHCH_3$

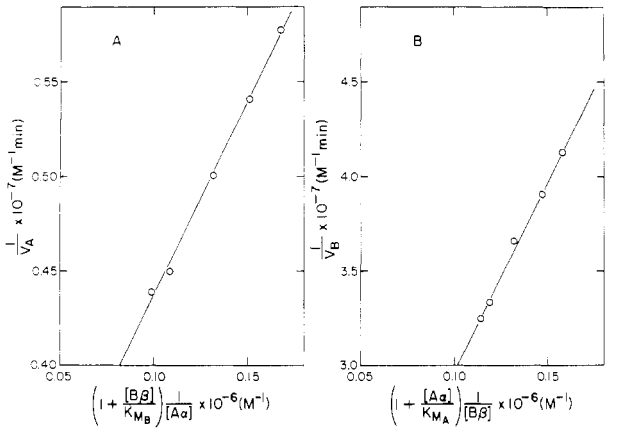

FIGURE 7: Correct double-reciprocal plots for the data of Figures 4 and 5, showing the fit of the calculated values of the Michaelis–Menten parameters to the experimentally observed initial velocities. (A) For the Aα chain; (B) for the Bβ chain.

clusion is now less certain on the basis of the results reported here. Although both k_{cat} and K_M for the CNBr Aα peptide differ by less than 1 order of magnitude from the values of the Aα chain, a comparison of the values for the specificity constant, k_{cat}/K_M, for the two substrates shows a significant difference. There is an ambiguity in these comparisons in that the results reported here pertain to *bovine* fibrinogen, while the earlier study of CNBr Aα used *human* material. Although the N-terminal CNBr fragments of the bovine and human Aα chains show considerable sequence homology (Martinelli et al., 1979), it is conceivable that the differences between the kinetic constants for the Aα chain and CNBr Aα may be a reflection of the variability of the sequence between the two species and not of a lack of essential features in the CNBr Aα peptide. We believe, however, that these differences in sequences are not significant and that CNBr Aα does contain all of the amino acids required for interaction with thrombin. We thus attribute the difference in k_{cat}/K_M between CNBr Aα and fibrinogen Aα to slight perturbation of the three-dimensional structure in CNBr Aα, caused by CNBr cleavage and by carboxymethylation of its Cys residues.

Synthetic Peptides. A series of synthetic peptides (A, B, and C in Table I), analogous to the sequence around the thrombin-susceptible Arg–Gly bond in the Aα chain, have also been examined as substrates for thrombin (van Nispen et al., 1977). The results of these experiments have supported the hypothesis of Blombäck (1967) that a Phe residue, located eight residues away in the N-terminal direction from the Arg–Gly bond, is essential for proper interaction with thrombin. Evidence in support of this hypothesis comes from the observation that k_{cat} for peptide C, the only one of these peptides to have this Phe residue, is much larger than k_{cat} for the other two peptides and of the same order of magnitude as k_{cat} for CNBr Aα. It appears that the Phe residue is necessary for the thrombin–fibrinogen interaction, but it is not yet clear that peptide C has all the residues necessary for this interaction. The same ambiguity (as discussed above) arises in comparing kinetic constants for these synthetic peptides based on the sequence of *human* fibrinogen with those for the bovine Aα chain. On the other hand, conformational differences between peptide C and the corresponding portion of fibrinogen may be the origin of the difference in K_M.

CNBr Bβ. The *N*-terminal CNBr fragment of the Bβ chain, CNBr Bβ, was examined as a model for the Bβ chain (Hageman & Scheraga, 1977). The Arg–Gly bond in CNBr Bβ is clearly hydrolyzed by thrombin less efficiently than the same bond in the Bβ chain in fibrinogen. This same conclusion was reached previously (Hageman & Scheraga, 1977), but now the reason for this observation can be discerned more clearly. Although k_{cat} values for the hydrolysis of CNBr Bβ and the Bβ chain are nearly the same, K_M for CNBr Bβ is larger than K_M for the Bβ chain by a factor of ~20. It appears that thrombin will hydrolyze the specific Arg–Gly bonds in these two substrates at about the same rate once it binds to these two substrates; the binding of the Bβ chain of CNBr to thrombin, however, is not as efficient as the binding of the Bβ chain in fibrinogen. This could be due to a binding site that is not present in CNBr Bβ (possibly in the Aα chain or γ chain in fibrinogen) and/or to nonequivalence of the conformation of CNBr Bβ and the corresponding portion of the Bβ chain in intact fibrinogen. The CNBr Bβ fragment was derived from bovine fibrinogen so that the comparison of kinetic constants with the Bβ chain is less ambiguous than for the Aα chain and its analogues.

Roles of k_{cat} and K_M. Finally, a comparison of the kinetic parameters for the Aα and Bβ chains of fibrinogen indicates that the slow rate of release of fibrinopeptide B compared to fibrinopeptide A is due to the value for k_{cat}, since the values of K_M for these chains are approximately the same. In this connection, it should be noted that the sequence around the Arg–Gly bond in the Bβ chain does not contain a Phe residue analogous to that found eight residues away in the N-terminal direction in the Aα chain.

Role of Conformation. As indicated above, the conformations of the portions of the Aα and Bβ chains that bind to thrombin may play a role in determining k_{cat}/K_M, i.e., in determining the narrow specificity of thrombin compared to, say, trypsin. In support of this idea, it has been found that short peptides having the sequence around the hydrolyzable Arg–Gly bond of the Aα chain do possess a conformation that is recognizable by NMR measurements, viz., a type II β turn

involving a hydrogen bond to the Gly CO group (Von Dreele et al., 1978). Such conformational features may not only position the Arg–Gly bond properly in the active site but also orient the Phe residue properly for its interaction with a complementary portion of the thrombin molecule. Indeed, some short peptides, with Phe placed artificially close to Val–Arg in the sequence, do show enhanced values of k_{cat}/K_M (Claeson et al., 1977), and their ^1H and ^{13}C NMR spectra suggest that the aromatic ring of Phe could be situated near Val-Arg-Gly in the three-dimensional structure of the Aα chain to enhance its binding to thrombin (Rae & Scheraga, 1979).

Mechanistic Implications. On the basis of the results of the steady-state kinetic experiments presented here, it is possible to form a picture of the molecular events in the thrombin-catalyzed release of the fibrinopeptides from fibrinogen. In the early stages of the reaction, the Aα and Bβ chains of fibrinogen compete with each other for the active site of thrombin. Although the affinity of each chain for thrombin is approximately the same during the early stages of the reaction (see the values of K_M in Table I), fibrinopeptide A is released at a faster rate than fibrinopeptide B because the rate constant for the hydrolysis of the specific Arg–Gly bond in the Aα chain is greater than that for the Bβ chain. The competitive nature of this interaction in the early stages of the thrombin–fibrinogen reaction has been supported by the results presented here. In particular, the absence of a time dependence for the rate of release of fibrinopeptide B established a preference for a competitive model over a sequential model as being more consistent with the observed *initial* rates of release of the fibrinopeptides. There is, however, another type of sequential mechanism—one in which thrombin binds to fibrinogen, cleaves the bond to release fibrinopeptide A, and then cleaves the bond to release fibrinopeptide B *without* having dissociated from fibrinogen. It can be shown that, in this mechanism, the rate of release of fibrinopeptide A would be approximately the same as that for fibrinopeptide B; i.e., the release of fibrinopeptide B would be rate limiting. Since, however, we observe that $v_A > v_B$, this alternative sequential model is unacceptable.

The competitive model advanced here is, however, at variance with a previous report that prior release of fibrinopeptide A is necessary for the release of fibrinopeptide B by thrombin (Blombäck et al., 1978). This preference for the sequential model was based on measurements of velocities but on a longer time scale than that used here, viz., one in which clotting occurred. As will be shown below, this may be related to the discrepancy between the conclusions reached as to the mechanism of thrombin action in the *early* stages of the reaction with fibrinogen.

In order to account for this apparent discrepancy, we must first consider a feature of the hydrolysis that has not yet been discussed, viz., the acceleration of the rate of release of fibrinopeptide B (see Figure 5A–C) when the concentration of fibrinogen molecules that have lost one or both molecules of fibrinopeptide A (but still retain fibrinopeptide B) increases. This increase in rate may be due to a conformational change occurring either upon the release of fibrinopeptide A or upon polymerization of fibrin monomers to form (soluble) intermediate polymers. On the basis of evidence provided by Blombäck et al. (1978), it seems that the latter explanation is the more likely one. In any case, the conformational change (no matter what its origin is) enhances the susceptibility of the Arg–Gly bond in the Bβ chain to be cleaved by thrombin. It is not clear whether this enhancement in the rate of release of fibrinopeptide B is reflected in a change in K_M, k_{cat}, or both. The data obtained in this work are too scanty for speculation. Several points, however, are clear from this work: (1) the enhancement in the rate of release of fibrinopeptide B comes *before* the appearance of a clot and (2) this rate enhancement was observed at the lower fibrinogen concentrations but not at the higher concentrations. This latter observation is understandable only in the light of the competitive nature of the action of thrombin on fibrinogen. Early in the reaction (i.e., only when small amounts of the fibrinopeptides have been released—about the first 30 min under the conditions used here) the Aα and Bβ chains compete for hydrolysis by thrombin. Then, when a substantial amount of fibrinopeptide A has been released and the monomers start to polymerize with the concomitant conformational change, this in effect causes the appearance of a "new substrate": the unhydrolyzed Bβ chains in the polymerized monomers. This would account for the acceleration in the rate of release of fibrinopeptide B. This new substrate, however, must still compete with the Aα and Bβ chains in the remaining intact fibrinogen, so that the rates of release of these two peptides will now depend on the relative concentrations of fibrinogen and polymerized fibrin monomer. For the three lowest fibrinogen concentrations examined here, the relative concentrations of these species are such that the increase in rate of release of fibrinopeptide B is observed. For the two highest concentrations of fibrinogen, however, this acceleration in the rate of release of fibrinopeptide B was not observed over the time course of the experiment. Evidently, the amount of unhydrolyzed fibrinogen is high enough for it to compete better than the polymerized fibrin for hydrolysis by thrombin during this time period.

According to the above explanation, the necessary condition for the *increase* in the rate of release of fibrinopeptide B by thrombin (later in time compared to its slower rate observable at the *beginning* of thrombin action) is the prior release of fibrinopeptide A [and possibly the formation of (soluble) intermediate polymers]. This conclusion is also supported by the results of stopped-flow light-scattering experiments on fibrin polymerization, which correlate the release of fibrinopeptide B with the formation of protofibrils (Hantgan & Hermans, 1979). A sensitive technique such as LC can detect *both* the initial slow and later faster releases of fibrinopeptide B, but a less sensitive one [such as the Edman method (Blombäck & Blombäck, 1972)] would not detect fibrinopeptide B until later in the reaction. Thus, the less sensitive method would see the competitive mechanism as a sequential one.

The rate equations (eq 5 and 6) derived for the competitive model apply *only to the early stages of the reaction* and have been shown to be inconsistent with the observed acceleration of the rate of release of fibrinopeptide B *later* in the reaction (Hageman & Scheraga, 1977). A mechanism which would account for *both* the initial slow release of fibrinopeptide B and its later larger rate at relatively low fibrinogen concentrations, together with the faster rate of release of fibrinopeptide A, can be summarized as

$$A\alpha B\beta\gamma + T \underset{}{\overset{K_{M_A}}{\rightleftharpoons}} A\alpha B\beta\gamma T \xrightarrow{k_A} A + \alpha B\beta\gamma + T \quad (28)$$

$$A\alpha B\beta\gamma + T \overset{K'_{M_B}}{\rightleftharpoons} (A\alpha B\beta\gamma T)' \xrightarrow{k'_B} B + A\alpha\beta\gamma + T \quad (29)$$

$$A\alpha\beta\gamma + T \overset{K_{M_A}}{\rightleftharpoons} A\alpha\beta\gamma T \xrightarrow{k_A} A + \alpha\beta\gamma + T \quad (30)$$

$$\alpha B\beta\gamma + T \overset{K_{M_B}}{\rightleftharpoons} \alpha B\beta\gamma T \xrightarrow{k_B} B + \alpha\beta\gamma + T \quad (31)$$

$A\alpha B\beta\gamma$ represents half of the dimeric fibrinogen molecule.

$\alpha B\beta\gamma$ and $\alpha\beta\gamma$ may represent either fibrin monomers or intermediate polymers. K_{M_A} and k_A are the kinetic parameters for the release of fibrinopeptide A from the Aα chain, assumed to be the same in all species containing the Aα chain. K'_{M_B} and k'_B are the kinetic parameters for the *slow* release of fibrinopeptide B from Bβ chains in unhydrolyzed fibrinogen, while K_{M_B} and k_B are the parameters for the *fast* release of fibrinopeptide B from the Bβ chain in the intermediate polymers.

The mechanism in eq 28–31 is consistent with the data reported here, and further work is in progress to test its validity. In particular, if we could isolate thrombin-digested fibrinogen molecules which have a molecule of fibrinopeptide B removed but still retain both molecules of fibrinopeptide A, we would obtain unambiguous support for this mechanism.

In this connection, it is of interest that some of the polymerization sites of fibrin monomer are close to the N termini of the α and β chains that are exposed after cleavage of their Arg–Gly bonds by thrombin. This is indicated by the following facts. The fibrin monomer from Fibrinogen Detroit, which has -Gly-Pro-Ser- in place of -Gly-Pro-Arg- at the N terminus of the α chain, aggregates more slowly than normal fibrin monomer (Blombäck et al., 1978). Shainoff & Dardik (1979) showed that the preferential removal of fibrinopeptide B from normal fibrinogen by the procoagulant enzyme from copperhead snake venom leads to a polymerizable product; however, the removal of a somewhat larger fragment than fibrinopeptide B by plasmin (early in the reaction before any cleavage occurs in the Aα chain) leads to a product that does not polymerize when thrombin is added.

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