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Comparison of Structures of Various Human Fibrinogens and a Derivative Thereof by a Study of the Kinetics of Release of Fibrinopeptides†

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ABSTRACT: The kinetics of the thrombin-induced release of fibrinopeptides from several variants of human fibrinogen, and from the plasmin digestion fragment E thereof, have been studied by using an HPLC technique to separate the reaction products. The data were analyzed in terms of a Michaelis–Menten mechanism in which the Aα and Bβ chains compete for thrombin. Phosphorylation of Ser-3 of the Aα chain appears to increase the rate of release of the corresponding phosphorylated peptide A from fibrinogen, due to enhanced binding of thrombin (lower value of the Michaelis–Menten constant K_M). However, phosphorylation does not affect the rate of release of the unphosphorylated A or B peptides. Increase in the length of the γ chain (at the C-terminus) does not affect the rate of release of any of the fibrinopeptides. The rate of release of the A peptide from fragment E (which is devoid of the B peptide) is similar to that for the complete fibrinogen molecule. These results are in agreement with an earlier conclusion [Martinelli, R. A., & Scheraga, H. A. (1980) *Biochemistry 19*, 2343] that the Aα and Bβ chains behave independently in their competition for thrombin; i.e., the hydrolyzable Arg–Gly bonds of the Aα and Bβ chains are both accessible to thrombin.

We are carrying out kinetic studies of the thrombin–fibrinogen reaction to determine the size of the active site of thrombin and the nature of its interactions with the fibrinogen substrate (Scheraga, 1977; Marsh et al., 1983). Various substrates are used in these investigations, viz., fibrinogen and several fragments thereof, and the efficiency with which thrombin hydrolyzes these substrates is expressed in terms of the ratio of the Michaelis–Menten parameters, k_{cat}/K_M. The values of k_{cat}/K_M for the hydrolysis of Arg–Gly bonds of the Aα and Bβ chains of fibrinogen itself, leading to the release of fibrinopeptides A and B (FpA and FpB),[1] respectively, serve as a reference against which to judge the suitability of various fibrinogen derivatives as substrates for thrombin. We have previously determined the values of k_{cat}/K_M for bovine fibrinogen (Martinelli & Scheraga, 1980), and we report here the corresponding values for human fibrinogen.

Besides comparing the kinetic data for the bovine and human species, we carried out this investigation primarily for three additional purposes, to determine (i) whether the reported effect of phosphorylation of human FpA on the clotting time (Blombäck et al., 1963) has its origin in the proteolytic action of thrombin on fibrinogen [which might imply an interaction of (phosphorylated) Ser-3 of FpA with thrombin or with other parts of the fibrinogen molecule to induce a more favorable conformation around the hydrolyzable Arg–Gly bond], (ii) whether the variable length at the C-termini of the γ chains of human fibrinogen (Mosesson et al., 1972; Francis et al.,

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[1] Abbreviations: F, fibrinogen; Aα and Bβ, the peptide chains of fibrinogen that release fibrinopeptides A and B, FpA and FpB, respectively, upon hydrolysis by thrombin; AP (or FpAP), a phosphorylated form of FpA; γ, the third type of chain (of variable length) in fibrinogen; D and E, C- and N-terminal fragments of F, produced by plasmin digestion; DSK, disulfide knot (Blombäck et al., 1972); CNBrAα and CNBrBβ, the Aα and Bβ chains, respectively, of the DSK; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; PEG 6000, polyethylene glycol (used to inhibit surface adsorption of thrombin); ε-ACA, ε-aminocaproic acid (a plasmin inhibitor); Trasylol, pancreatic trypsin inhibitor (used to inhibit trypsin, chymotrypsin, and other proteolytic enzymes); SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; ODS, octadecylsilane.

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1980, 1983; Wolfenstein-Todel & Mosesson, 1981) affects the rate of release of FpA and FpB by thrombin, and (iii) whether fragment E,[1] produced by plasmin degradation of human fibrinogen (Marder & Budzynski, 1974; Gaffney, 1977), is as good a substrate as fibrinogen for thrombin.

Experimental Procedures

Materials

Human thrombin, generously provided by J. W. Fenton II, had an α-thrombin concentration of >99.5% and a specific activity of 3000 NIH units/mg. It was diluted 1:100 with 0.02 Tris, 0.15 M NaCl, and 0.625% PEG 6000, pH 7.4, and stored frozen in 50-μL aliquots at −76 °C. Thrombin activity was determined by the clotting assay of Fenton & Fasco (1974) and Fenton et al. (1977) using standard NIH thrombin (lot J) and bovine fibrinogen (Sigma, type 1, fraction I) as a calibration standard.

Human fibrinogen was purified from outdated plasma by the procedure of Doolittle (1980). Purified fibrinogen (2.0 g) was dissolved in 80 mL of buffer [0.039 M Tris–phosphate, 0.02 M ϵ-ACA, and 0.02% sodium azide (a bacteriocidic agent)] and 10 units/mL Trasylol, pH 8.6, and dialyzed twice against 4 L of this buffer at 4 °C. The fibrinogen solution was fractionated further by layering it on a DEAE-Sephacel (Pharmacia Chemicals) ion-exchange column (2.8 × 45 cm) and eluted with a linear pH gradient and quadratic salt gradient (using a nine-chamber apparatus, MRA Corp., Clearwater, FL), as described by Francis et al. (1983).

Cross-linked fibrin was prepared as described previously (Francis et al., 1980). Samples of purified human FpA, FpAP, and FpB were prepared by incubating 1 g of fibrinogen in 50 mL of 0.05 M sodium phosphate, 0.15 M KCl, and 0.1 M ϵ-ACA, pH 8.0, with 50 NIH units/mL thrombin at 25 °C for 6 h. The resulting clot was dissolved by addition of 2% v/v formic acid. Within 2 h at room temperature, the solubilized fibrin monomers aggregated to an insoluble precipitate. The solution was centrifuged at 1650*g* at 4 °C, and the supernatant was treated on Sep-pak C_{18} cartridges (Water Associates) as described by Martinelli & Scheraga (1980). Following lyophilization and re-solution in 10 mL of H_2O, the peptides were fractionated by HPLC by a modification of the procedure of Martinelli & Scheraga (1979, 1980).

Fragment E was prepared from Kabi human fibrinogen which contains enough plasminogen so that, when the Kabi material was dissolved in 40 mL of 5 mM $CaCl_2$ (aq) containing 20000 units streptokinase, the fibrinogen was proteolytically degraded to fragments D and E in 3.5 h at 37 °C. The reaction was terminated by addition of 40 mg of soybean trypsin inhibitor (Marder et al., 1969; Budzynski et al., 1974). Large fragments were separated by gel filtration on a Sephacryl S-200 (Pharmacia) column (2.7 × 160 cm) equilibrated with 0.01 M $NaHCO_3$ and 0.02% sodium azide, pH 8.9. Then fragments D and E were separated by chromatography on a DEAE-Sephacel column (2.5 × 25 cm) (Doolittle et al., 1977) by elution with a linear gradient from 0.01 M $NaHCO_3$/0.02% sodium azide, pH 8.9, to 0.01 M $NaHCO_3$/0.3 M NaCl/0.02% sodium azide, pH 8.0. Fragment E, prepared in this manner, lacks residues 1–42 from the N-terminus of the Bβ chain and ~80% of FpA from the Aα chain (Budzynski et al., 1974; Takagi & Doolittle, 1975).

Methods

HPLC. An SP 8000 microprocessor-controlled high-performance liquid chromatograph (Spectra-Physics) equipped with an SF-770 Spectraflow variable-wavelength UV detector was used for separations of fibrinopeptides, following the procedure of Martinelli & Scheraga (1979, 1980). The stainless steel column (0.46 × 25 cm; Bodman Chemicals) was slurry-packed with Partisil-10 ODS-3 C_{18} reverse-phase packing material (Whatman, Inc.) under 10000 psi pressure (3500 plates/25 cm). The separation of FpA, FpAP, and FpB was accomplished isocratically with 30 mM sodium phosphate, pH 4.5, and acetonitrile (87:13 v/v) (glass-distilled acetonitrile was purchased from Burdick & Jackson Laboratories, Inc.). The fibrinopeptides were detected spectrophotometrically at 205 nm in order to obtain high sensitivity (Martinelli & Scheraga, 1980). Although Kehl et al. (1981) separated the different fibrinopeptides by HPLC, using gradient elution, we developed this isocratic procedure for more rapid analyses during the kinetic studies. This isocratic procedure, however, failed to separate FpA from des-Ala-FpA, which are combined here as the coeluted FpA peak.

Amino Acid Analysis. The fibrinopeptides were hydrolyzed in distilled 6N HCl for 24 h at 110 °C under vacuum, and the amino acids were chromatographed on a Technicon TSN automated amino acid analyzer.

Nitrogen Analysis. The concentrations of standard solutions of the several fibrinopeptides were determined by semiautomated micro-Kjeldahl nitrogen analysis (Lang, 1958; Noel & Hambleton, 1976a,b; Horwitz, 1980).

Phosphorus Analysis. Analyses of standard solutions of fibrinopeptides A and AP for phosphorus were carried out by the microprocedure of Chen et al. (1956). The peptide samples and phosphorus standards were treated with 10% sulfuric acid and ashed as described by Bartlett (1959).

SDS Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis across a polyacrylamide gradient (Margolis & Kenrick, 1967) utilizing the sulfate–borate discontinous buffer system described by Neville (1971) was carried out as described previously (Francis et al., 1979).

Kinetic Experiments. The rate of reaction of thrombin with fibrinogen *before the gel point* was monitored by following the formation of FpA, FpAP, and FpB at various times. Fibrinogen stock solutions were dialyzed against 0.05 M sodium phosphate and 0.15 M KCl, pH 8.0, and their concentrations were determined by nitrogen analysis, spectrophotometrically at 280 nm, using an extinction coefficient $E^{1\%}_{1cm}$ = 15.0 (Doolittle, 1975), and by the stoichiometric concentrations of FpA plus FpAP and of FpB produced by the end of the reaction with thrombin.

For each individual run, the fibrogen stock solutions were diluted to concentrations of 1–14 μM (2–28 μM peptides) with phosphate–KCl buffer and brought to 0.1 M in ϵ-ACA; 8 mL of each diluted solution was incubated with 0.01 NIH unit/mL thrombin at 25 °C, and aliquots of 1.0 mL were removed at 0, 5, 10, 15, 20, 30, and 60 min. The reaction was terminated (and the fibrinogen and thrombin precipitated) by heating in a boiling water bath for 2 min. Infinite-time points were obtained by incubating 1 mL of the diluted fibrinogen solution with 40.0 NIH units/mL thrombin at 25 °C for 6 h and then heating in the boiling water bath for 2 min. The samples were cooled immediately in an ice bath, brought to pH 2.5 with phosphoric acid, and centrifuged at 2800 rpm at 4 °C. The supernatant (100 μL) was chromatographed by HPLC to determine the concentrations of FpA, FpAP, and FpB. The procedure for HPLC fractionation was the same as that described for the preparation of the standard solutions of these peptides. At the fibrinogen and thrombin concentrations used in the kinetic studies, the solutions clotted in ~2 h. Thus, there was no visible formation of a clot up to 1-h incubation; however, a clot was visible in the infinite-time reaction. When

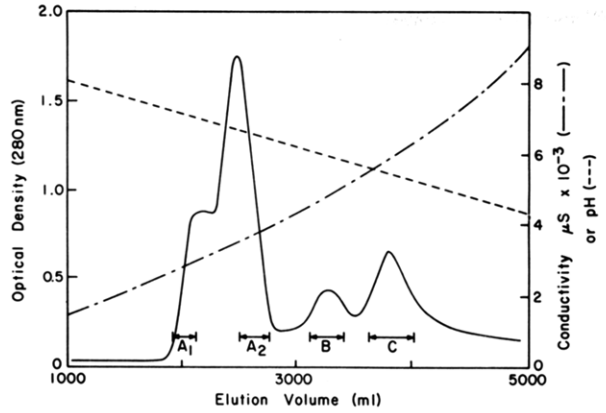

FIGURE 1: DEAE-Sephacel fractionation of purified fibrinogen: column size 2.8 × 45 cm and elution rate 60 mL/h at room temperature. The pH and salt gradient are described under Materials. Peak A was pooled separately as A_1 and A_2 as indicated.

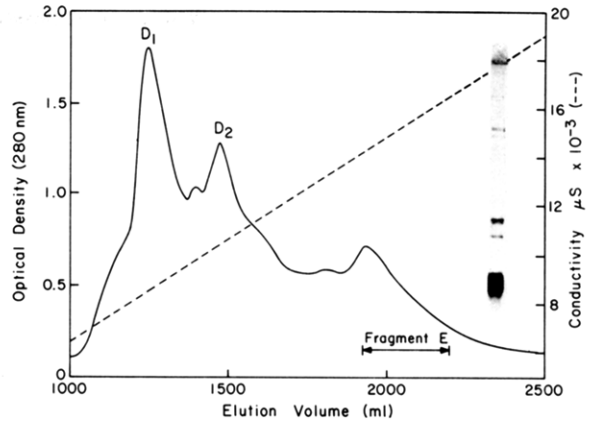

FIGURE 2: DEAE-Sephacel fractionation of the D + E pool from Sephacel S-200 chromatography: column size 2.5 × 25 cm and elution rate 60 mL/h at room temperature. The buffer and salt gradient are described under Materials. Fraction E was pooled as indicated. The inset is the result of SDS gel electrophoresis of the pooled E fraction, showing one dominant (fragment E) and two faint (unidentified) bands.

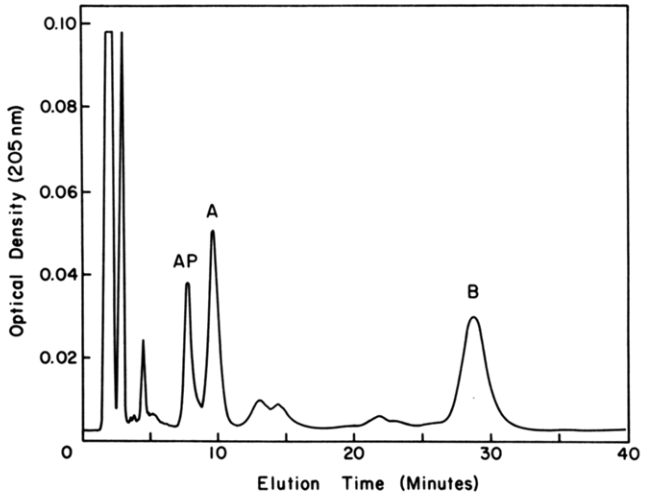

FIGURE 3: Typical HPLC elution profile, from a kinetic run: C_{18} reverse-phase column (0.46 × 25 cm) and flow rate 2 mL/min at room temperature. Isocratic elution with 30 mM sodium phosphate, pH 4.5/acetonitrile (87:13) resulted in the separation of the peaks FpAP, FpA, and FpB. Peptides were detected spectrophotometrically at 205 nm.

fragment E was used as the substrate, the kinetic experiments were carried out in a similar manner. The range of concentrations of fragment E was 4–48 μM (1.3–14 μM FpA, since only 16% of FpA remained in the fragment E used in these experiments).

The concentrations of FpA, FpAP, and FpB were determined by converting both peak heights and peak areas from the HPLC chromatograms to concentrations by using calibration curves obtained by plotting the concentrations of a standard solution of each fibrinopeptide vs. either peak height in centimeters or peak area in centimeters squared. Both types of calibration curves were linear for all three fibrinopeptides examined. The reported concentrations of fibrinopeptides in these kinetic studies are the averages of four calculated concentrations from peak height and peak area from two HPLC runs.

Control experiments were performed to assure that there were no losses in fibrinopeptides during the heating or acidification steps.

The initial rates of release of the different fibrinopeptides were calculated from the slopes of the linear least-squares lines through the points obtained during the first 20 min of reaction, during which 30% or less of the substrate was hydrolyzed; however, the reaction was also followed for a longer time (up to 1 h), although only the data during the first 20 min were used to calculate initial rates.

Results

When purified fibrinogen was subjected to DEAE-Sephacel fractionation, the chromatogram exhibited three main peaks, A, B, and C (Figure 1). However, these peaks, in particular peak A, show a leading edge which indicates additional heterogeneity. The fibrinogen eluting in peaks A, B, and C, and cross-linked fibrin prepared from these fibrinogen peaks, was analyzed by SDS–polyacrylamide gel electrophoresis after reduction of the disulfide bonds. Peaks B and C contained elongated γ_{55} and $\gamma_{57.5}$ chains, respectively, in addition to the γ_{50} species, as described by Francis et al. (1983); the fibrinogen in peaks B and C also exhibited some degradation at the C-termini of the Aα chains. Peak A was pooled separately as two fractions, the early fraction A_1 and the late fraction A_2, as indicated in Figure 1. Samples of fibrinogen A_1, A_2, and C were treated with thrombin as described under Methods to obtain the infinite-time points. A_1 showed a trace amount (<0.05 μmol of FpAP/1.0 μmol of F) of FpAP produced, and the concentrations of both FpA and FpB produced corresponded to the stoichiometric values within an error of ~5% (viz., 2 mol of FpA and 2 mol of FpB per mol of fibrinogen), whereas A_2 and C showed the production of both FpAP, FpA, and FpB, where the sum of the concentrations of the first two species, as well as that of the third, corresponded to the stoichiometric values within the same limits of error. The ratio of FpAP to total fibrinopeptide A was 40 ± 2% and 30 ± 2% from fibrinogen A_2 and C, respectively. According to Blombäck et al. (1966), the phosphorus group is on Ser-3 of the Aα chain. Fibrinogens A_1, A_2, and C were >90% clottable.

The purified fragment E obtained from DEAE-Sephacel chromatography (Figure 2) was shown to consist primarily of one band on SDS gel electrophoresis (see inset of Figure 2). Treatment of this fraction with thrombin (as described for the infinite-time point) produced 0.35 mol of FpA/mol of fragment E and a trace of FpAP; FpB was not detected. This is in agreement with the report of Budzynski et al. (1974) that all of the FpB is hydrolyzed and that FpAP is hydrolyzed preferentially to FpA, during the early stages of plasmin digestion of fibrinogen.

Figure 3 shows a typical HPLC elution profile from a kinetic run, where the order of elution is FpAP, FpA, and FpB. Table

Table I: Amino Acid Composition of Fibrinopeptides[a]

amino acid	FpAP	FpA	FpB
Ala	2.01 (2)[b]	1.81 (2)[b]	1.12 (1)[b]
Arg	0.96 (1)	1.03 (1)	1.12 (1)
Asp	2.04 (2)	2.08 (2)	2.96 (3)
Glu	2.02 (2)	2.09 (2)	3.05 (3)
Gly	5.08 (5)	4.64 (5)	2.18 (2)
Leu	1.00 (1)	1.00 (1)	
Phe	1.00 (1)	1.05 (1)	2.00 (2)
Ser	0.82 (1)	1.02 (1)	1.34 (1)
Val	1.02 (1)	1.00 (1)	0.97 (1)

[a] Hydrolysis was performed in 6 N HCl at 110 °C for 24 h under vacuum. The reported values (given as moles of amino acid per mole of peptide) have not been corrected for hydrolysis losses. [b] The theoretical values for FpA and FpB were reported by Blombäck et al. (1966).

I shows the results of amino acid analyses and the theoretical values for the material from each peak. Phosphorus analysis indicated that FpAP has 0.92 mol of P/mol of FpAP and that FpA has 0.05 mol of P/mol of FpA.

Kinetic Equations. In a study of the thrombin–fibrinogen reaction with bovine materials, Martinelli & Scheraga (1980) showed that FpA and FpB are released simultaneously from the *start* of the reaction, with both Aα and Bβ competing for the active site of thrombin. However, the rate of release of FpA is greater than that of FpB. We have found here that the same behavior is observed with human materials. Hurlet-Jensen et al. (1982) using a radioimmunoassay to detect very small amounts of FpB in the early stage of the thrombin–fibrinogen reaction reported similar results with human materials. Before presenting the experimental data, we derive the kinetic equations for competitive and sequential mechanisms [by analogy with the derivations of Martinelli & Scheraga (1980)] because we encounter here the release of three, instead of two, fibrinopeptides, viz., FpA, FpAP, and FpB.

We may represent the competitive mechanism by the following equations:

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

$$AP\alpha + T \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} AP\alpha T \xrightarrow{k_{AP}} AP + \alpha + T \quad (2)$$

$$B\beta + T \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} B\beta T \xrightarrow{k_B} B + \beta + T \quad (3)$$

where T is thrombin, AαT, APαT, and BβT are the enzyme–substrate complexes for Aα, APα, and Bβ, and A, AP, and B are the reaction products FpA, FpAP, and FpB. α and β are the corresponding fibrin monomer chains formed after release of the fibrinopeptides. We assume that these enzyme–substrate complexes are present at steady-state concentrations in the early stages of the reaction and that they then proceed to form the corresponding products irreversibly. The Michaelis–Menten constants K_M and k_{cat} for these reactions are defined in terms of the parameters of eq 1–3 by Martinelli & Scheraga (1980).

Following Martinelli & Scheraga (1980), we obtain

$$v_A = \frac{k_A[A\alpha][T]_0}{K_{M_A} + [A\alpha] + (K_{M_A}/K_{M_{AP}})[AP\alpha] + (K_{M_A}/K_{M_B})[B\beta]} \quad (4)$$

$$v_{AP} = \frac{k_{AP}[AP\alpha][T]_0}{K_{M_{AP}} + [AP\alpha] + (K_{M_{AP}}/K_{M_A})[A\alpha] + (K_{M_{AP}}/K_{M_B})[B\beta]} \quad (5)$$

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

where the v's are the *initial* velocities and $[T]_0$ is the *total* enzyme concentration.

It can be shown that

$$(K_{M_A})_{app} + (K_{M_{AP}})_{app} = (K_{M_B})_{app}$$

where $(K_{M_A})_{app} = G_A K_{M_A}$, and similarly for $(K_{M_{AP}})_{app}$ and $(K_{M_B})_{app}$, where

$$G_A = \frac{(1-x)K_{M_{AP}}K_{M_B}}{(1-x)K_{M_{AP}}K_{M_B} + K_{M_A}K_{M_{AP}} + xK_{M_A}K_{M_B}}$$

where $x = [AP\alpha]$, $1 - x = [A\alpha]$, and $[B\beta]$ is normalized to 1; i.e., this expression for G_A involves the various true K_M's and the concentrations of the various substrates. Similar expressions hold for G_{AP} and G_B. Such a simple expression for the equality of the apparent K_M's does not necessarily hold for the true K_M's. Likewise, $(k_{cat_A})_{app} = G_A(k_{cat_A})$. If one were to plot $1/v_A$ vs. $1/[A\alpha]$, one would determine $(K_{M_A})_{app}$, and likewise for $(K_{M_{AP}})_{app}$ and $(K_{M_B})_{app}$. However, a plot of $1/v_A$ vs. $G_A/[A\alpha]$ [or, equivalently, treatment of the data by the method of Cornish-Bowden & Koshland (1970) and Wharton et al. (1974)] gives the true K_{M_A} and k_{cat_A}. In addition

$$\frac{k_{cat_A}}{K_{M_A}} = \frac{(k_{cat_A})_{app}}{(K_{M_A})_{app}}$$

We may represent the sequential mechanism by the following equations:

$$ABf + T \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ABfT \xrightarrow{k_A} A + Bf + T \quad (7)$$

$$APBf + T \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} APBfT \xrightarrow{k_{AP}} AP + Bf + T \quad (8)$$

$$Bf + T \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} BfT \xrightarrow{k_B} B + f + T \quad (9)$$

where ABf and APBf are half of the fibrinogen molecule in which the A peptide is unphosphorylated or phosphorylated, respectively, and Bf is half of the fibrinogen molecule from which FpA or FpAP has been released. ABfT, APBfT, and BfT are the corresponding enzyme–substrate complexes. In this model we assume that FpA and FpAP are released competitively, whereas FpB is released sequentially to FpA and FpAP.

In a similar way, as shown for the competitive model, we can derive the kinetic equations for the *initial* rate of release of FpA and FpAP:

$$v_A = \frac{k_A[T]_0[ABf]}{K_{M_A} + [ABf] + (K_{M_A}/K_{M_{AP}})[APBf]} \quad (10)$$

$$v_{AP} = \frac{k_{AP}[T]_0[APBf]}{K_{M_{AP}} + [APBf] + (K_{M_{AP}}/K_{M_A})[ABf]} \quad (11)$$

Following Martinelli & Scheraga (1980), we obtain the following equation for the *initial* rate of release of FpB:

$$d[FpB]/dt = v_B = (v_A + v_{AP})ct \quad (12)$$

where t is time and

$$c = \frac{k_{cat_B}}{1 + (K_3/[T]_0)(1 + [ABf]/K_{M_A} + [APBf]/K_{M_{AP}})}$$

and K_3 is the equilibrium constant of the first step in eq 9. By integrating eq 12, we obtain

$$[FpB] = \left(\frac{1}{2}\right)(v_A + v_{AP})ct^2 \quad (13)$$

Table II: Kinetic Constants for the Hydrolysis of Arg–Gly Bonds of Human Fibrinogen Derivatives by Thrombin at pH 8.0 and 25 °C

	$K_M \times 10^6$ (M)	$k_{cat} \times 10^{11}$ [M [(NIH units/L) s]$^{-1}$]	$k_{cat}/K_M \times 10^7$ [[(NIH units/L)s]$^{-1}$]
fibrinogen Aα (bovine)[a,b]	9.2	73	795
fibrinogen Bβ (bovine)[a,b]	11.3	11.5	100
fibrinogen A_1[b]			
Aα	9.5 ± 0.5[d]	45.0 ± 5.0[d]	475 ± 50[d]
Bβ	16.0 ± 0.5	16.5 ± 0.5	105 ± 10
fibrinogen A_2[b,c]			
Aα	9.5 ± 0.5	50.0 ± 5.0	525 ± 50
APα	4.8 ± 0.3	32.0 ± 3.0	670 ± 30
Bβ	15.5 ± 0.5	16.0 ± 0.5	105 ± 10
fibrinogen C[b,c]			
Aα	10.0 ± 0.5	55.0 ± 5.0	550 ± 50
APα	4.5 ± 0.3	31.0 ± 3.0	690 ± 30
Bβ	15.0 ± 0.5	16.5 ± 0.5	110 ± 10
fragment E (Aα)	9.0 ± 0.5	37.0 ± 3.0	410 ± 50
CNBr Aα[e]	47	48	100
CNBr Bβ[f]	189	6.2	3.3

[a] Determined by Martinelli & Scheraga (1980). [b] These data pertain to a single chain. [c] Account was taken of the actual AP content in analyzing the data for A_2 and C. [d] The errors given in the table are from the nonlinear least-squares analysis. (The errors in the absolute values are probably much larger.) [e] Determined by Hageman & Scheraga (1974). [f] Determined by Hageman & Scheraga (1977).

that is, the rate of release of FpB is linear in time, and the amount of FpB produced is a quadratic function of time.

The competitive and sequential models indicate that v_B should be independent of t or linear in t (eq 6 and 12, respectively). Since our data, like those of Martinelli & Scheraga (1980), indicate that v_B is independent of t for the first 20–30 min of reaction, before significant polymerization of fibrin monomer occurs (less than 30% FpA and less than 6% FpB produced), we conclude that the mechanism is a competitive one and use the competitive model to compute K_M's and k_{cat}'s.

Equations 4–6 were used to analyze the data for fractions A_2 and C. For fraction A_1, where [FpAP] = 0, eq 4 and 6 [which reduce to those of Martinelli & Scheraga (1980)] were used. In the case of fragment E, where [FpAP] = 0 and [FpB] = 0, a simple Michaelis–Menten rate equation was used, namely

$$v_A = \frac{k_A[A\alpha][T]_0}{K_{M_A} + [A\alpha]} \quad (14)$$

As has been suggested by Lee & Wilson (1971), [Aα], [APα], and [Bβ] are taken as the average values of the corresponding concentrations over the time period of the kinetic run. Equations 15–17 express these values in terms of the initial concentrations $[A\alpha]_0$, $[AP\alpha]_0$, and $[B\beta]_0$:

$$[A\alpha] = [[A\alpha]_0 + ([A\alpha]_0 - [A])]/2 \quad (15)$$

$$[AP\alpha] = [[AP\alpha]_0 + ([AP\alpha]_0 - [AP])]/2 \quad (16)$$

$$[B\beta] = [[B\beta]_0 + ([B\beta]_0 - [B])]/2 \quad (17)$$

Kinetic Results. The initial rates of release of FpA, FpAP, and FpB (over the first 20 min of reaction time) were measured at 10 different concentrations of each fibrinogen derivative in the range of $0.1K_M$ to $2K_M$ for each peptide.

A typical set of data at 2.15 μM fibrinogen A_2 is shown in Figure 4; it should be realized that the observed rate depends not only on k_{cat} and K_M but also on the concentration of substrate which differs for all three peptides. This is shown more clearly in Figure 5A, where the data of Figure 4 are replotted to take account of the different initial concentrations of FpA and FpAP; i.e., FpAP is intrinsically released faster than FpA. The same conclusion follows from Figure 5B where, in addition, it is seen that the percent of FpA released varies with time under nonsaturating conditions (lower curve) but is independent of time under saturating conditions, i.e., for concentrations higher than K_M (upper curve). The linked equations (4–6) were solved for the corresponding values of k_{cat} and K_M for each type of fibrinopeptide by a nonlinear least-squares curve-fitting procedure (Cornish-Bowden & Koshland, 1970; Wharton et al., 1974), as described by Martinelli & Scheraga (1980). Table II summarizes the values of K_M and k_{cat} for FpA, FpAP, and FpB from different

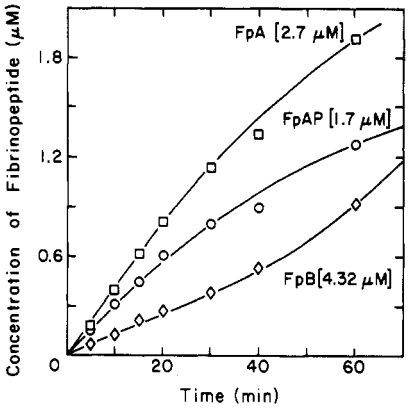

FIGURE 4: Typical experimental data showing rate of release of (□) FpA, (○) FpAP, and (◇) FpB from fibrinogen A_2 at 25 °C. Values in brackets are the concentrations at infinite time. The initial concentration of fibrinogen A_2 was 2.15 μM; thrombin concentration 0.01 unit/mL; 0.05 M sodium phosphate, 0.15 M KCl, and 0.1 M ε-ACA (pH 8.0). Clotting time ~2 h.

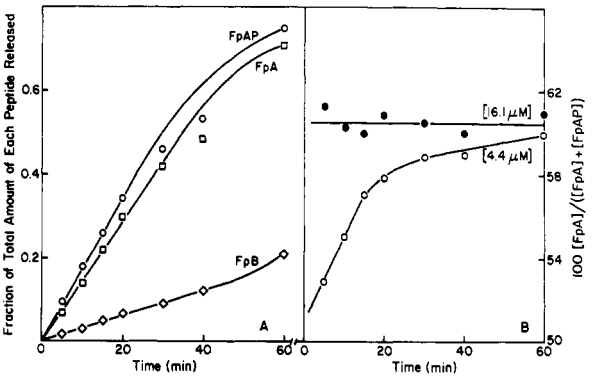

FIGURE 5: (A) Replot of data of Figure 4 as moles released at time t per moles released at infinite time: (□) FpA; (○) FpAP; (◇) FpB, from fibrinogen A_2 at 25 °C. (B) Plot of percent fibrinopeptide A, i.e., 100[FpA]/([FpA] + [FpAP]), vs. time for two different concentrations of fibrinogen A_2: 2.15 μM (○); 8.0 μM (●). Values in brackets are the concentrations of FpA-plus-FpAP at infinite time.

fibrinogen variants and from fragment E.

Discussion

The data of Table II indicate that the kinetic constants for release of FpA and FpB from human A_1, A_2, and C are similar. Therefore, extension of the C-terminus of the γ chain in no way interferes with the action of thrombin on the N-termini of the Aα and Bβ chains. This is in agreement with the finding of Finlayson et al. (1980) that the heavier γ chain of fibrinogen Paris I has no effect on the release of FpA and FpB. In addition, these kinetic constants are similar to those for bovine fibrinogen, within experimental error.

The value of K_M for the release of FpAP from fibrinogen A_2 appears to be lower than that for FpA; i.e., the phosphorylated chain appears to bind thrombin better than the unphosphorylated one. This may possibly be the origin of the observation of Blombäck et al. (1963) that phosphorylated fibrinogen clots faster than the unphosphorylated species. If so, then we must ultimately account for an interaction between Ser-3 (which is quite far removed, in sequence, from Arg-Gly at residues 16 and 17, where hydrolysis takes place) and thrombin (and/or another part of fibrinogen to enhance its binding to thrombin).

Kehl et al. (1982) had concluded that FpA and FpAP are released at the same rate. However, their experiments were carried out at saturating concentrations, greatly exceeding K_M. Therefore, they effectively were observing k_{cat}. Our results agree with theirs that, within experimental error, k_{cat} is essentially the same for FpA and FpAP (upper curve of Figure 5B). However, according to our results, it is K_M that differs for these two peptides, even allowing for a large experimental error.

Despite the higher rate of release of FpAP from A_2, the rates of release of FpA and FpB from this species are the same as those from A_1. This supports the conclusion (Martinelli & Scheraga, 1980) that the chains behave independently in their competition for thrombin. The independence of the Aα and Bβ chains, in their competition for thrombin, is also supported by the report of Andes et al. (1982) that FpB is released (at a slightly lower than normal rate) *before* the complete release of FpA in fibrinogen New Orleans (an abnormal fibrinogen).

Henschen et al. (1983) divide abnormal fibrinogens into several categories according to the number of moles of FpA and FpB released per mole of F. Two categories of interest here are those in which 2 mol of FpB and 0 mol of FpA are released per mol of F (e.g., fibrinogen Metz) and those in which 2 mol of FpB and 1 mol of FpA are released per mol of F (e.g., fibrinogens Amsterdam, Frankfurt II and III, London III, and Zurich I). The existence of such abnormal fibrinogens is consistent with our conclusion that FpB is exposed for reaction with thrombin and can be released without the need for prior release of FpA.

The values of K_M and k_{cat} for the release of FpA from fragment E are the same as those for the complete fibrinogen molecule. This not only supports the conclusion that the chains behave independently (and that the rate of release of FpA is independent of the presence or absence of FpB) but also suggests that the conformation around the hydrolyzable Arg–Gly bond of the Aα chain is the same in fragment E and in the intact fibrinogen molecule.

All of these observations support the conclusion (Martinelli & Scheraga, 1980) that the *early* rates of release of FpA and FpB are independent of each other and that both the Aα and Bβ chains compete for the active site of thrombin. This implied surface accessibility of the Arg–Gly bonds of the Aα and Bβ chains is supported by their accessibility to antibodies (Nagy et al., 1982; J. A. Nagy, Y. C. Meinwald, and H. A. Scheraga, unpublished results) and by other evidence (J. A. Nagy, Y. C. Meinwald, and H. A. Scheraga, unpublished results). However, from our observed dependence of the rate of release of FpB *later* than 30 min in the reaction [see also Martinelli & Scheraga (1980)], it appears (Hurlet-Jensen et al., 1982) that polymerization of fibrin monomer may induce a conformational change which increases the rate of release of FpB and makes the reaction *appear* to be sequential (this latter process is of no concern in our study of the *initial* rates of release of these peptides, i.e., during the first 20 min).

In contrast to the conclusion reached here, and previously (Martinelli & Scheraga, 1980), that the hydrolysis of the Aα and Bβ chains of fibrinogen proceeds independently (competitive mechanism), the results of a recent kinetic investigation of the thrombin–fibrinogen reaction have been interpreted as implying a sequential mechanism (Higgins et al., 1983); i.e., the prior release of FpA was thought to be required for the release of FpB. This discrepancy may be attributed to the different conditions under which the kinetic data were obtained. Higgins et al. (1983) obtained their data *later* in the reaction (e.g., the first points for the release of FpB in their Figure 1 correspond to greater than 25% release of total FpA, whereas ours correspond to about 8%). Thus, as indicated above, their data are undoubtedly influenced by the subsequent polymerization of fibrin monomer and the concomitant enhanced release of FpB. The issue of a competitive vs. a sequential mechanism for the release of the fibrinopeptides hinges upon whether a nonzero initial rate of release of FpB from intact fibrinogen can be detected. A nonzero rate was observed in our studies, but in that of Higgins et al. (1983) the value determined under their conditions was zero within experimental error. Under the conditions employed in our experiments, it was possible to detect an experimentally significant nonzero rate of release of FpB from intact fibrinogen. Thus, the competitive mechanism is a more accurate description of the *early* stages of the thrombin–fibrinogen reaction.

It should also be noted that, whereas polymerization in the later stages increases the rate of release of FpB, Hurlet-Jensen et al. (1982) have shown that, in the *early* stages (where presumably very little polymerization of fibrin monomer occurs), the addition of a polymerization inhibitor does not affect the *initial* rates of release of FpA and FpB. Therefore, our initial rates in the first 20 min of the reaction [in contrast to those of Higgins et al. (1983)] were not influenced by any subsequent polymerization.

In summary, we have presented kinetic evidence that the extension of the C-termini of the γ chains has no effect on the action of thrombin at the N-termini of the Aα and Bβ chains of fibrinogen, whereas the phosphate group on Ser-3 of the Aα chain leads to better binding (lower value of K_M) of thrombin to this chain. In addition, we have demonstrated that the Aα and Bβ chains behave independently in their competition for the active site of thrombin. Moreover, we have shown that the plasmin-digestion product, fragment E, possesses all of the reactive sites, in the proper conformation, for the binding of its Aα chain to thrombin.

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