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Clotting of Bovine Fibrinogen. Kinetic Analysis of the Release of Fibrinopeptides by Thrombin and of the Calcium Uptake upon Clotting at High Fibrinogen Concentrations†

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ABSTRACT: Kinetic data on the release of fibrinopeptides A and B from bovine fibrinogen by human thrombin were obtained at high fibrinogen concentrations, within the 0.8–8.8% range (0.227×10^{-4} to 2.60×10^{-4} M), at 25 °C, pH 7.26, 0.30 ionic strength, and 10^{-4} M free Ca^{2+} concentration. Release of fibrinopeptide A followed strictly first-order kinetics at all concentrations, in spite of the fact that the highest concentration was 26 times larger than the value of K_M found in the literature. This behavior can be explained by inhibition of thrombin by the reaction products, with $K_I = K_M$. The equation describing the course of the reaction under these conditions can be rearranged into a linear relationship between $1/k_{obsd}$ and substrate concentration. The slope of the line is equal to $1/k_{cat}$ and the intercept to K_M/k_{cat}. The data points fell accurately on a straight line, and with the parameters of the latter, k_{cat} and K_M were calculated as $(6.3 \pm 0.11) \times 10^{-10}$ M s^{-1} (unit of thrombin)$^{-1}$ L^{-1} and $(11.0 \pm 3.0) \times 10^{-6}$ M, respectively. These values agree well with those found in the literature. Release of fibrinopeptide B follows complex kinetics. Higgins et al. [Higgins, D. L., Lewis, S. D., & Shafer, J. A. (1983) *J. Biol. Chem. 258*, 9276-9282] suggested that it can be described as the result of two consecutive reactions, the first one being the release of fibrinopeptide A and the second one of fibrinopeptide B from those molecules that have already lost fibrinopeptide A in the previous step. An alternate model was also considered which allowed removal of fibrinopeptide B directly from the native molecule, running parallel to the consecutive reactions. These and several other mathematical models were tested by nonlinear least-squares fitting to our experimental data. Best fit was obtained with the model of two consecutive first-order reactions. Release of fibrinopeptide B from the native molecules, i.e., without previous removal of fibrinopeptide A, if at all possible, was nearly an order of magnitude slower. The rate constants for the release of fibrinopeptide B with the two consecutive reactions model were used as with fibrinopeptide A to obtain k_{cat} equal to $(3.5 \pm 0.2) \times 10^{-10}$ M s^{-1} (unit of thrombin)$^{-1}$ L^{-1} and K_M equal to $(6.0 \pm 8.5) \times 10^{-6}$ M. Data points for fibrinopeptide B were more scattered than those for fibrinopeptide A and hence the uncertainty of K_M. Uptake of calcium associated with the clotting reaction followed the same mechanism as the release of fibrinopeptide B, and the rates of the two processes were also similar.

Determination of the time course of the release of fibrinopeptides A and B was presented in the preceding paper (Mihalyi, 1988) to correlate the conformational change and calcium uptake during clotting of fibrinogen by thrombin with release of fibrinopeptide B. Similar data, however, may contribute also to the resolution of the controversy regarding the kinetics of release of the two fibrinopeptides. Therefore, the experiments were expanded and are reported in the present paper.

Detailed kinetic analysis of this process has been undertaken by several authors. The initial fibrinogen concentration was an order of magnitude below the K_M of the thrombin-catalyzed removal of the fibrinopeptides from fibrinogen in some of these studies (Higgins et al., 1983; Lewis et al., 1985) and at about the value of K_M in others (Martinelli & Scheraga, 1980; Hanna et al., 1984). Moreover, in the latter case, data were restricted to the initial phase of the reaction, before clot formation. Our data were obtained under conditions in which the substrate was partially or nearly completely saturating the enzyme, the initial fibrinogen concentration in some runs being up to 26 times higher than K_M, and the whole course of the reaction was followed. From these data, some conclusions can be drawn about whether thrombin releases fibrinopeptide B from native fibrinogen, as suggested by the studies of Martinelli and Scheraga (1980), or only after prior removal of fibrinopeptide A, or after the polymerization ensuring the release of fibrinopeptide A, as suggested by Blombäck et al. (1978) and Shafer and collaborators (Higgins et al., 1983; Lewis et al., 1985). A puzzling observation was the strict adherence in all our kinetic curves to first-order kinetics, in spite of the high fibrinogen concentration that would have mandated zero-order kinetics. A possible explanation for this is also offered in this paper.

† This is paper 4 in the series "Clotting of Fibrinogen". For paper 3, see Mihalyi (1988).

Experimental Procedures

Materials and Methods. The materials and methods used were the same as those described in detail in the preceding paper (Mihalyi, 1988). The study of the release of the fibrinopeptides covered approximately the same concentration range as previously, but more intermediate points were obtained. The experimental procedure was slightly modified: Bovine fibrinogen solution of varying concentrations, in 0.3 M NaCl, was adjusted to pH 7.26 and 10^{-4} M free Ca^{2+} concentration. Samples of 0.5 mL from these were reacted with human thrombin in Beckman, Ultra Clar, 8 × 49 mm centrifuge tubes, thermostated to 25 °C. After various incubation periods, the tubes were placed in a boiling water bath. Coagulation occurred in approximately 35 s. After coagulation, the tubes were centrifuged in a Beckman L2-65B ultracentrifuge in Rotor 50 TI at 23 000 rpm for 20 min. Estimation of the fibrinopeptides in the supernatants by high-performance liquid chromatography (HPLC) was performed as described previously.

Results and Discussion

Kinetics of the Release of Fibrinopeptide A. The first-order character of the overall reaction of clotting, i.e., formation of insoluble fibrin from fibrinogen brought about by thrombin, was first demonstrated by Laki (1942). This was confirmed by numerous other studies that need not be enumerated here. Other facets of this reaction, such as heat evolution (Sturtevant et al., 1955) and liberation of hydrogen ions (Mihalyi & Billick, 1963), also proved to be first order. However, the reaction was found to be more complex, and again, Laki (1951) was the first to demonstrate the multistep nature of this process. Thus, the apparent first-order course of the overall reaction is the result of the behavior of the first and rate-limiting step in the chain of events. When suitable methods became available, it was demonstrated that the first of the enzymatic steps of the clotting reaction, release of fibrinopeptide A by thrombin, was strictly first order (Higgins et al., 1983). This indicated that at least under certain conditions of the reaction with respect to thrombin and fibrinogen concentrations, pH, and ionic strength, the rate-limiting step in the overall reaction should be the release of fibrinopeptide A. Recent reports that release of the second fibrinopeptide A from fibrinogen may be 16–40 times faster than of the first one (Landis & Waugh, 1975; Bale et al., 1982) do not affect this conclusion. It modifies only to the extent that in this case release of the first fibrinopeptide A alone would be the rate-limiting factor and the second one would follow immediately after this, leaving the first-order character undisturbed. The polymerization reaction occurs also in several steps. First, a soluble intermediate is formed (Ferry et al., 1954) that later was named the protofibril (Hantgan & Hermans, 1979). Formation of the protofibrils is fast under most conditions and is limited by the release of fibrinopeptide A. However, if insoluble fibrin formation is monitored, there is initially a lag period, reflecting the time interval necessary for a sufficient buildup of the protofibril concentration before they associate into the insoluble thick fibers. The overall reaction may deviate also later from the first-order course when the lateral association into fibers is slow compared to the release of fibrinopeptide A. We limit the overall reaction in this discussion to formation of the protofibrils. Release of fibrinopeptide B, that is slower and of a more complex nature, does not seem to be involved up to this point.

The kinetic studies of release of fibrinopeptide A by thrombin provided the Michaelis–Menten parameters (Bando et al., 1972; Nossel et al., 1976; Martinelli & Scheraga, 1980; Higgins et al., 1983). With both human and bovine fibrinogen, K_M was of the order of 10^{-5} M. Thus, it is evident that in some of the kinetic studies the fibrinogen concentration was equal to or larger than K_M. In spite of this, the kinetics did not deviate from the first-order course. Figure 1 shows first-order plots of a series of experiments encompassing the initial fibrinogen concentration range from 0.227×10^{-4} M (7.71 mg/mL) to 2.60×10^{-4} M (88.63 mg/mL). The data were normalized with respect to both extent of reaction and reaction time, the latter to 1 NIH unit/mL thrombin concentration, assuming an inverse relationship between reaction time and thrombin concentration. As was mentioned in the preceding paper (Mihalyi, 1988), the fibrinopeptide estimates with time reach a maximum and then decline slowly. Therefore, the late points in the reaction were omitted, and the extent of the reaction was obtained by nonlinear least-squares fittings of the points up to 90–95% completion of the reaction. The figure shows that the first-order course is followed up to this level of completion even with the highest fibrinogen concentration, in spite of the fact that this concentration exceeds K_M 26-fold. Under these conditions, if Michaelis–Menten kinetics were obeyed, the reaction should have been of zero order, i.e., linear, for approximately 70% of its course. This is clearly not the case with the experiment depicted in Figure 1 and similar ones not shown, performed in this concentration range.

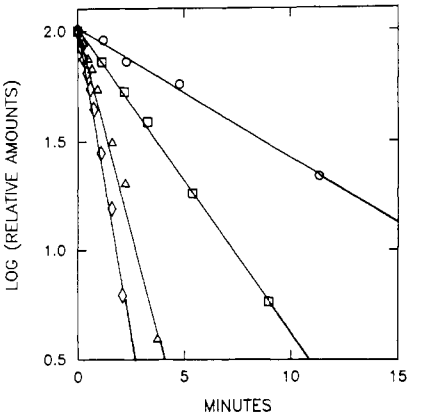

FIGURE 1: First-order plots for the release of fibrinopeptide A by thombin at pH 7.26, 25 °C, and 0.3 ionic strength. Reaction time was normalized to 1 NIH unit/mL. Fibrinogen concentrations were 7.7 mg/mL (◇), 15.3 mg/mL (△), 43.9 mg/mL (□), and 88.6 mg/mL (○).

Since the first-order course is progressively slower than the zero-order one, one factor that should be considered is the inhibition of the reaction by the reaction products. In the following equations, [E], [S], and [P] stand for the concentrations of free enzyme, free substrate, and free products, respectively, and [ES] and [EP] for the corresponding complexes; t is time and K_M and k_{cat} are the customary parameters of the Michaelis–Menten equation, the apparent equilibrium constant for the dissociation of the enzyme–substrate complex and the rate constant for the formation of products from the latter, while k_{obsd} is the observed rate. It is assumed that the inhibition by the products is the result of a true reversible reaction in which the nonproductive enzyme–product complex is formed:

$$K_I = [E][P]/[EP] \quad (1)$$

The course of the reaction with no inhibition by products is given by the integrated simple Michaelis–Menten equation:

$$[E]k_{cat}t = K_M \ln ([S_0]/[S]) + ([S_0] - [S]) \quad (2)$$

while the course of the reaction with inhibition by the reaction

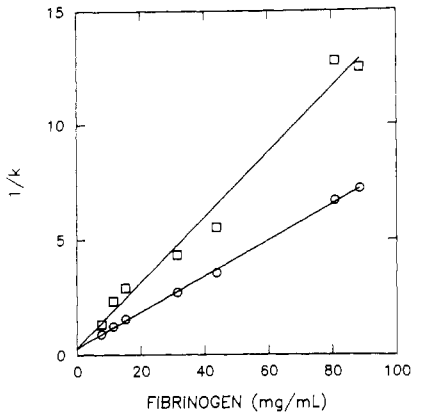

FIGURE 2: Plot of $1/k_{obsd}$ against fibrinogen concentration for the release of fibrinopeptide A (O, lower line) and fibrinopeptide B (□, upper line). Rate constants, obtained by curve fitting to experimental points by using model 2, were normalized to 1 NIH thrombin unit/mL. Other reaction conditions were the same as for Figure 1.

products is given by a modified Michaelis–Menten equation in which the competition of substrate and inhibitor for the enzyme is incorporated.

$$[E]k_{cat}t = K_M\left(1 + \frac{[S_0]}{K_I}\right)\ln\frac{[S_0]}{[S]} - \frac{K_M - K_I}{K_I}([S_0] - [S]) \quad (3)$$

As pointed out by Neurath and Schwert (1950), the last equation is reduced to a first-order one for $K_I = K_M$. In this case:

$$[E]k_{cat}t = (K_M + [S_0])\ln([S_0]/[S]) \quad (4)$$

The reaction course deviates from this for either $K_M < K_I$ or $K_M > K_I$, in the first case being faster and in the second one slower than the first-order course. Further, according to eq 4, the observed first-order rate constant is given by

$$k_{obsd} = k_{cat}/(K_M + [S_0]) \quad (5)$$

Thus, according to the reciprocal of eq 5, a plot of $1/k_{obsd}$ vs $[S_0]$ should give a straight line with $1/k_{cat}$ the slope and K_M/k_{cat} the intercept. The first-order relationship, on the other hand, should hold strictly, regardless of the initial substrate concentration. This means that because the enzyme does not distinguish between the susceptible and the spent substrate, the two will be bound in a ratio equal to the ratio of their concentrations in the solution. This results in a rate proportional to the concentration of the free susceptible substrate, regardless of the degree of saturation of the enzyme; that is to say that the reaction will be first order with respect to the substrate.

The reciprocal rate constants calculated for the release of fibrinopeptide A in the experiments shown in Figure 1 and some others are plotted against fibrinogen concentration in Figure 2. This figure also presents similar data for fibrinopeptide B which will be discussed in the following section. The linear plots obtained confirm the validity of eq 5. From the slope and intercept of the line, k_{cat} and K_M were calculated as $(6.3 \pm 0.1) \times 10^{-10}$ M s^{-1} at 1 NIH unit of thrombin/L and $(11.0 \pm 3.0) \times 10^{-6}$ M, respectively. The accuracy of k_{cat} depends on the accuracy of the activity estimates of the thrombin stock solution. This relies on the stability of the standard thrombin used to calibrate the clotting assay. Substantial error may be introduced by the latter factor. The value of K_M, on the other hand, is determined by the intercept, which is small and consequently has a large relative error. The k_{cat} and K_M values estimated here compare favorably with the ones in the literature (Martinelli & Scheraga, 1980; Higgins et al., 1983; Hanna et al., 1984), although the reaction conditions were slightly different: 25 vs 37 °C, 0.30 vs 0.15 ionic strength, and pH 7.2 vs pH 7.4 or 8.00. All these differences in the reaction conditions tend to increase the rate of thrombin action (Dietler et al., 1985; Shulman & Ferry, 1950; Mihalyi & Billick, 1963), and this may be the reason for the slightly lower k_{cat} and higher K_M observed in our studies. Consequently, our specificity constant (k_{cat}/K_M) is somewhat lower, 5.9×10^{-5} s (unit of thrombin)$^{-1}$ L^{-1}, than the one reported by Martinelli and Scheraga (1980) and Higgins et al. (1983), but equal to that of Hanna et al. (1984). Species differences do not seem to affect to a large extent these parameters. Bovine fibrinogen was used in the experiments of Martinelli and Scheraga and human fibrinogen in the others with homologous thrombin, while bovine fibrinogen and human thrombin were used in our studies.

The kinetic data, therefore, support our hypothesis of inhibition of thrombin action by the reaction products, with $K_I = K_M$. Fibrinopeptides in equal molar concentration to fibrinogen did not affect the kinetics of fibrinopeptide release (unpublished experiment), suggesting that these are not the inhibitory products. Thus, the remaining large molecular weight moiety, fibrin monomer or fibrin polymer, should be the species involved. Inhibition of fibrinogen clotting by reaction products was suggested a long time ago by Waugh and Livingstone (1951) and elaborated more recently by Landis and Waugh (1975), but these papers did not receive the attention they deserve.

The conclusion from the kinetic data is that fibrin should bind thrombin. This was indeed demonstrated conclusively by Seegers et al. (1945). These experiments were repeated in a more sophisticated way and resulted in dissociation constants of 1.7×10^{-6} and 1.3×10^{-6} M (Liu et al., 1979, 1980). The constants are for dissociation from the fibrin gel. Kaminski and McDonagh (1983) performed experiments with fibrin monomer bound to Sepharose that yielded a K_D close to the above one of 2.0×10^{-6} M and one thrombin binding site per monomer. Further experiments suggested that the integrity of the active site of thrombin is not necessary but probably a secondary site is involved in binding to fibrin (Wilner et al., 1981). It may be mentioned here that an extended binding site was suggested also by kinetic studies with peptide segments of various sizes from the Aα chain of fibrinogen (Meinwald et al., 1980). The effect of inhibitors and activators also indicated that there are at least two spatially distinct binding subsites in thrombin (Conery & Berliner, 1983). The prevalence of these subsites, if these interact predominantly with the fibrin monomer segment and not the fibrinopeptide side, explains the unchanged binding constant for substrate and product. In view of a possibly more complicated system than anticipated and the quite different techniques used, one kinetic and the other of equilibrium, the 5-fold difference between K_I and K_M does not seem to discredit our assumption that these two quantities should be equal.

Kinetics of the Release of Fibrinopeptide B. Release of fibrinopeptide B is initially much slower than that of fibrinopeptide A, but after a lag period, it is accelerated appreciably to approximately one-fourth of the level of the latter. This behavior cannot be anticipated by restricting the observations to the initial portion of the reaction and seeking to establish the kinetic parameters on the basis of the initial velocities. The analysis of the reaction must be extended to its whole course. As a result of the two different perspectives, there are two competing models. One maintains that at least in the early

part of the reaction both peptides are liberated from native fibrinogen in two concurrent and independent reactions, but the rate of release of fibrinopeptide B is about 6 times slower than that of A (Martinelli & Scheraga, 1980; Hanna et al., 1984). The other one contends that release of fibrinopeptide B is preconditioned by that of fibrinopeptide A and/or polymerization. Consequently, the reaction can be described as the result of two consecutive steps (Blombäck et al., 1978; Higgins et al., 1983; Lewis et al., 1985). The decisive argument rests on whether fibrinopeptide B is released or not from native fibrinogen, and the question of what causes the acceleration of this release in the later stages of the reaction is not addressed in this controversy.

We have analyzed our data in terms of consecutive and other reaction mechanisms for release of fibrinopeptide B. With all the mathematical models tested, it was assumed that release of fibrinopeptide A follows strict, independent, first-order kinetics:

$$\text{FPA} = 100(1 - e^{-k_1 t}) \quad (6)$$

This is amply demonstrated in Figure 1. No attempt was made to modify this model. For the release of fibrinopeptide B, on the other hand, various mechanisms were assumed. With models 1 and 4, the release of the two fibrinopeptides is described by independent reactions. With all the others, appearance of fibrinopeptide B is linked to release of fibrinopeptide A. All the reactions depicted in these models are first order, or combinations of first-order reactions. The reaction curves were all normalized to 100. FPA and FPB stand for relative proportions of the two fibrinopeptides; k_1 is the rate constant for release of fibrinopeptide A and k_2 for that of fibrinopeptide B. Since release of fibrinopeptide A with all these models follows the same kinetics, described by eq 6, this is not repeated below in the description of each one of these. Thus, the models in reality pertain only to the release of fibrinopeptide B.

Model 1 assumes independent first-order reactions for the liberation of the two fibrinopeptides. Release of fibrinopeptide B is given by

$$\text{FPB} = 100(1 - e^{-k_2 t}) \quad (7)$$

Model 2 is based on the integrated form of the rate equation for two first-order consecutive reactions (Harcourt & Esson, 1866). It is assumed here that k_1 is the rate constant for the release of fibrinopeptide A and k_2 is that for the release of fibrinopeptide B from those molecules that have already lost fibrinopeptide A. The course of the reaction is described by

$$\text{FPB} = 100\left(1 - \frac{k_2}{k_2 - k_1}e^{-k_1 t} + \frac{k_1}{k_2 - k_1}e^{-k_2 t}\right) \quad (8)$$

Model 3 allows that, besides the consecutive order for release described above, fibrinopeptide B can be released also directly from the native molecule with a rate denoted by k_2'. The equation for this combined mechanism was derived by Higgins et al. (1983):

$$\text{FPB} = 100\left(1 + \frac{k_2 - k_2'}{k_1 - k_2 + k_2'}e^{-(k_1 + k_2')t} - \frac{k_1}{k_1 - k_2 + k_2'}e^{-k_2 t}\right) \quad (9)$$

Model 4, as well as the following ones, explores the possibility of a mechanism different from the A → B → C type utilized in models 2 and 3. It assumes that liberation of fibrinopeptide B requires cleavage of n number of bonds. This type of reaction is common with production of multichain fragments from proteins and excision of a peptide segment from a loop of a polypeptide chain (Mihalyi, 1978). If these bonds are cleaved, all with the same rate, the course of the reaction is given by

$$\text{FPB} = 100(1 - e^{-k_2 t})^n \quad (10)$$

Release of fibrinopeptide B is quite independent in this model from that of A.

Model 5 is similar to model 4; it also assumes independent cleavage of two bonds, but one of these has a rate equal to that of the release of fibrinopeptide A. Thus, liberation of fibrinopeptide B is linked to that of A, not in a consecutive but in a simultaneous fashion. The equation for a reaction of this type is

$$\text{FPB} = 100(1 - e^{-k_1 t})(1 - e^{-k_2 t}) \quad (11)$$

Model 6 is an elaboration of model 5, that allows direct release of fibrinopeptide B from the native molecule with cleavage of a single bond and a rate constant of k_2', running parallel with the release through cleavage of two bonds. The equation for this is

$$\text{FPB} = 100(1 - e^{-k_1 t})(1 - e^{-k_2 t}) + e^{-k_1 t}(1 - e^{-k_2' t}) \quad (12)$$

Three data sets were analyzed by nonlinear least-squares curve fittings to the above models, using MLAB, an interactive computer program developed at NIH (Knott, 1979). These were the low fibrinogen concentration run shown in Figure 8 of the preceding paper (Mihalyi, 1988) and the lowest and highest concentration runs described in this paper. There were only minor differences between the results with all of these. Model 2 was used as a reference, since this is the most likely one. Figure 3A,B shows the curve fittings to the lowest and highest fibrinogen concentration runs with this model. The time scale with both was normalized to 1 NIH unit/mL thrombin concentration, assuming an inverse relationship between reaction time and enzyme concentration. It is apparent that, although there is more than 10-fold difference in fibrinogen concentration between the two runs, release of both fibrinopeptides A and B follows accurately their particular reaction course at both concentrations: first-order reaction for A and two consecutive firt-order reactions for B. Subsequently, each model was matched to each set of data. In Figure 4A, model 2 is compared to model 1, and in Figure 4B to models 3 and 5. The poor fit of fibrinopeptide B release to a simple first-order reaction course is exemplified in the first one and the reasonably good fits to the two other models in the second one. These figures were obtained with the data of the low fibrinogen concentration run described in the preceding paper (Mihalyi, 1988). The rate constants and their errors obtained with the other two sets of data are given in Table I. The computed errors may be compared within the respective set, but not between the two sets, because of the different number of data points in each one of these.

The poor fit of the data to model 1 came as no surprise, since it was obvious from the shape of the curve with a lag period that a first-order course could not describe this. The model with two consecutive first-order reactions (model 2), on the other hand, fitted well the experimental points. Although the fit could not be improved, this model appeared to tolerate to a small extent liberation of fibrinopeptide B without prior release of fibrinopeptide A (model 3), with two of the data sets. However, the rate constant for the direct release from native fibrinogen was an order of magnitude smaller than that for the species with fibrinopeptide A already released, and the standard error was nearly as large as the rate constant itself. With the experiment with low fibrinogen concentration given in the table, the rate for this reaction was zero. All this

Table I: Numerical Values from Kinetic Modelings of the Release of Fibrinopeptide B. Rate Constants and Their Errors

	low fibrinogen concentration[a]			high fibrinogen concentration[b]		
	rate constant × 10^2	rms weighted deviation error	weighted sum of squares	rate constant × 10^2	rms weighted deviation error	weighted sum of squares
model 1	$k_2 = 3.42 \pm 0.28$	5.66	576.2	$k_2 = 0.88 \pm 0.07$	4.96	295.7
model 2	$k_2 = 7.70 \pm 0.32$	1.81	59.2	$k_2 = 1.69 \pm 0.11$	2.72	88.6
model 3	$k_2 = 7.70 \pm 0.72$ $k_2' = 0 \pm 0.31$	1.86	59.2	$k_2 = 1.54 \pm 0.20$ $k_2' = 0.12 \pm 0.15$	2.75	83.6
model 4	$k_2 = 7.74 \pm 0.46$ $n = 2.39 \pm 0.18$	1.67	47.7	$k_2 = 1.49 \pm 0.16$ $n = 1.86 \pm 0.26$	2.80	86.2
model 5	$k_2 = 4.72 \pm 0.20$	2.28	94.3	$k_2 = 10.9 \pm 0.07$	2.92	102.3
model 6	$k_2 = 4.72 \pm 0.30$ $k_2' = 0 \pm 0.50$	2.35	94.2	$k_2 = 1.09 \pm 0.09$ $k_2' = 0 \pm 0.20$	3.05	102.3

[a] Fibrinogen concentration, 7.7 mg/mL (0.226 × 10^{-4} M); [thrombin], 0.103 NIH unit/mL; $k_1 = 0.113$/min. [b] Fibrinogen concentration, 88.6 mg/mL (2.61 × 10^{-4} M); [thrombin], 0.216 NIH unit/mL; $k_1 = 0.0278$/min.

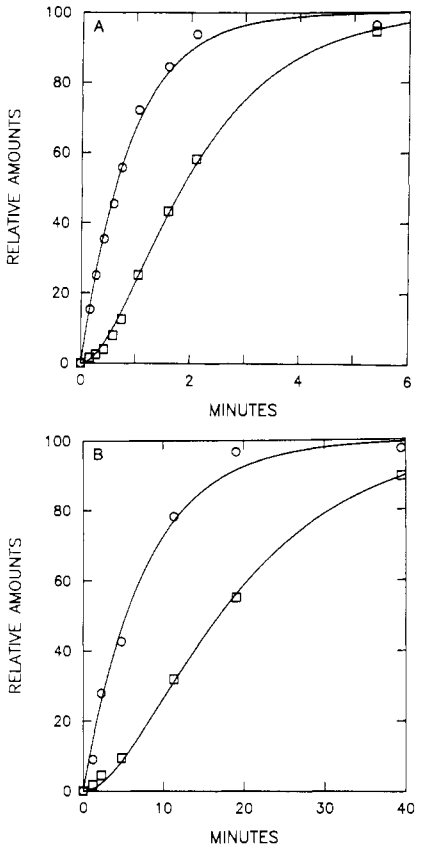

FIGURE 3: Release of fibrinopeptides A (○) and B (□) by thrombin. Reaction curves: first-order course for fibrinopeptide A, two consecutive first-order reactions for fibrinopeptide B (model 2). Fibrinogen concentrations were 7.7 mg/mL (0.227 × 10^{-4} M) for panel A and 88.6 mg/mL (2.60 × 10^{-4} M) for panel B. Thrombin concentrations were normalized to 1 NIH unit of thrombin/mL. Other reaction conditions were the same as for Figure 1.

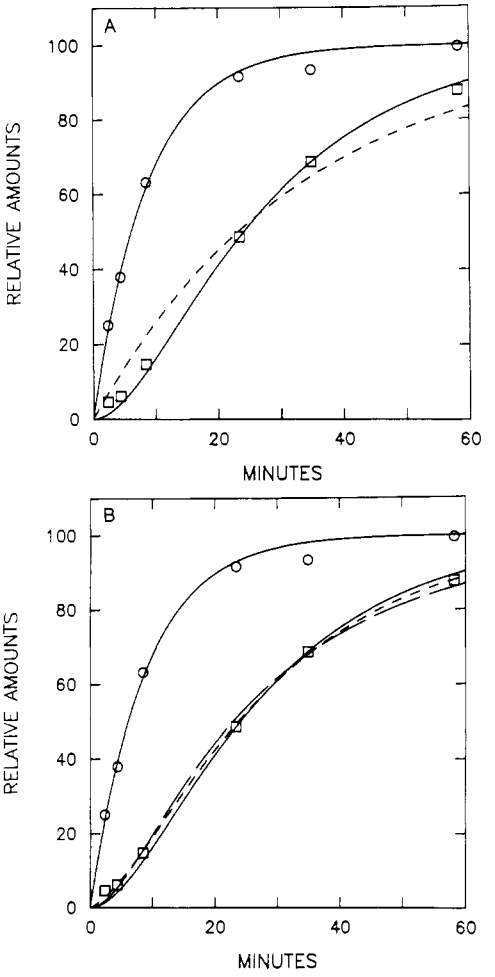

FIGURE 4: Test of the various models for release of fibrinopeptide B by curve fitting. Fibrinopeptide A (○); fibrinopeptide B (□); solid line in both panels, model 2; dashed line in panel A, model 1; dashed line in panel B, model 3; long dashed line in panel B, model 5. Fibrinogen concentration 12.3 mg/mL (0.364 × 10^{-4} M); thrombin concentration 0.2 NIH unit/mL; other reaction conditions the same as in Figure 1.

suggests that the cleavage may occur but has very little significance in the context of the whole reaction.

The equally good, or sometimes even better, performance of model 4 is the result of the particular relationship between k_1 and k_2 in these reactions. If $k_1 = 2k_2$, it can be easily shown that eq 8 reduces to eq 10 with $n = 2$. In our experiments, k_1/k_2 varied between 1.6 and 2.4, thus explaining the good fit of the data to either model 2 or model 4. Thus, in this case, curve fitting is not able to distinguish between these two models.

Model 5 does not fit the data as well as model 2 or its variants, but still to an acceptable level. Model 6, that incorporates in model 5 cleavage of fibrinopeptide B without prior removal of fibrinopeptide A, appears to be even less likely than it was with the model of consecutive reactions.

In conclusion, curve fittings appear to favor the two consecutive first-order reactions model, in which first fibrinopeptide A is cleaved off followed by release of fibrinopeptide B from the desA-fibrinogen molecules, with a slight possibility of direct removal of fibrinopeptide B from the intact molecule. However, just from the fittings, one cannot exclude a mechanism involving cleavage of two bonds for release of fibrinopeptide B. There is ample chemical evidence that thrombin, within the time frame of the clotting reaction, cleaves only a single susceptible bond in each of the Aα and Bβ chains of fibrinogen (Doolittle, 1973). Therefore, whatever model we

assume, this has to deal with the cleavages at the established susceptible points of the two chains. The two bonds cleaved could be the ones on the Bβ chains, meaning that fibrinopeptide B can be released only in pairs, or, less likely, that it is liberated only if fibrinopeptide A is also released, either before or after the cleavage of the Bβ chain susceptible bond. All these cleavages are independent and simultaneous, but cleavage of the respective susceptible bond is not sufficient for release of fibrinopeptide B. With either of the two abovementioned possibilities, after cleavage of the Bβ chain susceptible bond, fibrinopeptide B is held back until either of the other peptide is also removed, if this was not removed already. This retention should be only by secondary forces, since the peptide bond holding fibrinopeptide B was already severed. Very tight binding of a peptide segment to a protein is not an impossibility, but this one should be resistant to denaturation by heat or trichloroacetic acid, since these were used to separate the peptide fractions from the protein moiety. If the model with two bonds cleaved is not rejected offhand as another example of the fallacies of kinetic modelings, experiments should be performed for detecting a hysteresis between the number of susceptible bonds cleaved and the amounts of fibrinopeptide B liberated.

The results discussed above are in complete agreement with those of Shafer and collaborators (Higgins et al., 1980; Lewis et al., 1983), which were conducted at less than 0.35 μM initial fibrinogen concentration, nearly 1000 times lower than in our highest concentration experiment. The substrate concentration being much below K_M in the first and much above K_M in the second case, the reaction should have been of first order and of zero order, respectively. Instead, the same kinetics of consecutive first-order reactions applied to both cases. This suggests that release of fibrinopeptide B is determined by the same factors that govern release of fibrinopeptide A; namely, the reaction is inhibited by the reaction products, and K_I equals K_M. Further support for this contention came from the plot of $1/k_{obsd}$ vs initial fibrinogen concentration. This plot is shown in Figure 2 with its counterpart for fibrinopeptide A. The linear plot and the nonzero intercept at the ordinate indicate that the relationships discussed in the preceding section may apply also to the release of fibrinopeptide B. Therefore, k_{cat} and K_M were calculated from the slope and intercept of the line. The value found for K_M of $(6.0 \pm 8.5) \times 10^{-6}$ M is only about half of that found for fibrinopeptide A. However, the error of K_M is much larger than for the latter, a reflection of the more pronounced scatter of the data points with this peptide. The value of k_{cat} for fibrinopeptide B is $(3.5 \pm 0.2) \times 10^{-10}$ M s^{-1} (unit of thrombin)$^{-1}$ L^{-1}, also about half of that for fibrinopeptide A, the ratio k_{cat_A}/k_{cat_B} being 1.79. On the other hand, the specificity constant, k_{cat}/K_M, is 5.7×10^{-5} s unit^{-1} L^{-1}, the same as for fibrinopeptide A. These vlaues may be compared to those of Martinelli and Scheraga (1980) for the B peptide of 11.3×10^{-6} M for K_M and 1.15×10^{-10} M s^{-1} unit^{-1} L^{-1} for k_{cat}. The reaction conditions in the two determinations were different; also, human thrombin was used in one and bovine in the other, but a correction factor can be obtained by comparing the k_{cat} data from the two investigations for liberation of fibrinopeptide A, for which there was no difference in the assumed models. The correction factor is small, 1.15, but increases our value of k_{cat} to 4.1×10^{-10} M s^{-1} unit^{-1} L^{-1}, nearly 4 times the value of Martinelli and Scheraga. The different results are obviously a reflection of the different reaction mechanisms assumed: one was based on initial rates and the other one on the whole course of the reaction. If the course of the reaction is reconstructed with eq 8 and the parameters obtained for fibrinopeptide B, the initial rate is about one-sixth of that of the initial rate of the release of fibrinopeptide A, thus explaining the discrepancy. The fact that the release of fibrinopeptide B initially follows a quasi-linear course from zero time evidently is not a sufficient argument for assuming the release of this peptide from native fibrinogen in a parallel and independent reaction from that of fibrinopeptide A, even in the early phase of the reaction.

All the curve fittings described so far in this section were performed with simultaneous use of the equations and data pertaining to release of both fibrinopeptides. However, the data for fibrinopeptide A can be used by themselves, as described in the previous section. For a given run, whether the data were taken alone or in combination with those of fibrinopeptide B and irrespective of the model used for the release of the latter, the kinetic parameter for the release of peptide A was always very nearly the same. Thus, release of fibrinopeptide A is well-defined mathematically and is independent of whatever mechanism is assumed for release of fibrinopeptide B. The same is not true for fibrinopeptide B. When this is taken alone, fitting to eq 8 is by no means well-defined. If the data are extremely good, the resolution into the two steps approximates that of the combined approach, with the rate constants close to those with the latter. However, with a slight scatter of the data, especially in the initial period, there is difficulty in the fitting, with the result being two reactions with practically identical rate constants, i.e., a single first-order reaction. If the constraint $k_1 = 1.79k_2$ is introduced, the impass is naturally resolved, since this is tantamount to using the fibrinopeptide A release reaction, and the correct rate constants are obtained.

Kinetics of Calcium Uptake. Since release of fibrinopeptide B and the calcium uptake associated with clotting run an identical course, as demonstrated by Figures 8 and 10 of the preceding paper (Mihalyi, 1988), it was expected that the calcium binding data can be described also by the equation of two consecutive first-order reactions. However, as with the fibrinopeptide B data taken by themselves, the curve fitting is not straightforward, yielding in most cases two reactions with nearly identical rates, the equivalent of a single reaction. If the constraint $k_1 = 1.79k_2$ derived from the combined fitting of both fibrinopeptide A and B curves is applied, the resolution is good, and the errors of the rate constants are only slightly larger than with the peptide curves. The rate constants for the slower reaction, k_2 in our notation, derived from the calcium uptake curves in Figures 8 and 9 of the preceding paper (Mihalyi, 1988), are nearly identical with those obtained for fibrinopeptide B release. This is a result expected from the inspection of these two figures and gives support for the assumption of identical courses for the two reactions. The reciprocal rate constants obtained for the corrected calcium uptake curves, derived from the recordings shown in Figure 7 of the previous paper (Mihalyi, 1988), were plotted in Figure 5 against the initial fibrinogen concentration. From the slopes and intercepts of the straight lines, corresponding to the two components of the reaction, k_{cat} and K_M were calculated as described in the previous section. The values for k_{cat} of 6.3×10^{-10} and 3.9×10^{-10} M s^{-1} unit^{-1} L^{-1}, respectively, are close to those obtained from the peptide data, but K_M is 4.4×10^{-5} M for both, 4 times larger than that for fibrinopeptide A and 7 times larger than that for fibrinopeptide B. This is a reflection of the much larger intercepts. The scatter of the data is smaller in this experiment than in the ones with the peptides, but apparently a systematic error is introduced that causes the reciprocal rates to be progressively larger than expected as the

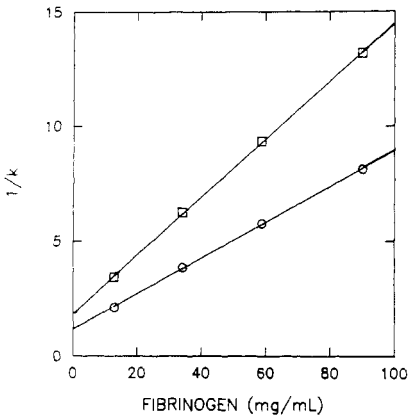

FIGURE 5: Plots of $1/k$ against fibrinogen concentration, derived from the calcium uptake curves shown in Figure 7 of the preceding paper (Mihalyi, 1988). The corrected calcium uptake curves were resolved into two components by fitting to the two consecutive first-order reactions model (model 2). Rate constants were normalized to 1 NIH unit/mL. Other reaction conditions were the same as in Figure 1.

fibrinogen concentration decreases. This may have been caused by an experimental fault, such as a no longer negligible mixing time, or electrode response time, as the rate of the reaction was increasing to half-life times of the order of 1.5 min at the lowest fibrinogen concentration. Progressively increasing errors may have been introduced by the rather involved correction procedure of the calcium uptake data, with successively decreasing heights of the curves. Although less satisfactory than expected, the results are sufficient to substantiate the claim that the courses of Ca^{2+} uptake and release of fibrinopeptide B are close if not identical. Consequently, no attempt was made to correct the uncertainty with respect to K_M.

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