Platelet Stimulation by Thrombin and Other Proteases†

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ABSTRACT: The mechanism of stimulation of platelets by thrombin and other proteases was studied by following kinetics of secretion of Ca^{2+} or ATP. The progress-time curves of secretion were analyzed for rate and total amount released. The reaction of thrombin was perturbed by addition of hydroxylamine or a competitive inhibitor and by variation of pH and it was compared with the reactions of other proteases. Trypsin and papain, with specificities for arginyl residues, induced secretion with a time course that was nearly identical with that induced by thrombin when saturating levels of enzyme were used. At low levels of enzyme, trypsin and papain gave extended lags in the progress-time curves. Higher concentrations of trypsin and papain were required for saturation of the measured parameters. Human plasmin (lysyl specificity) and bovine chymotrypsin (aromatic amino acid specificity) failed to induce platelet secretion. Active site inhibited thrombin was also ineffective. Both yield and kinetics depended on pH, with the pH profile for each enzyme similar to its profile for hydrolysis of synthetic substrates. Studies at low pH also showed that the early part of the reaction undergoes a change in rate-determining step from enzyme dependent at low enzyme to enzyme independent at high enzyme. Hydroxylamine, a nucleophile that would be expected to accelerate hydrolytic reactions, actually decreased both the rate of initial reactions and yield. A competitive inhibitor of thrombin also decreased both rate and yield; a calculated inhibition constant was in agreement with the value for a synthetic substrate, suggesting that the interaction of thrombin with platelets is analogous to reaction with substrates. A modification of our previous model is proposed in order to accommodate the results described here and to reconcile the apparent contradictions that enzyme was found not to turn over in the reaction (Detwiler, T. C., and Feinman, R. D. (1973), *Biochemistry 12,* 282), that catalytic activity is required (Davey, M. G., and Luscher, E. F. (1967), *Nature (London) 216,* 875; this paper), and that the reaction is characterized by an apparent equilibrium binding (Tollefsen, D. M., Feagler J. R., and Majerus, P. W. (1974), *J. Biol. Chem. 249,* 2646). The essential feature is a reversible catalytic step with no dissociation of enzyme from product. This is followed by irreversible, thrombin-independent platelet processes leading to secretion, with yield dependent on the equilibrium concentration of the thrombin product. The model thus has aspects of catalysis, stoichiometry, and an agonist-receptor equilibrium.

Platelets respond in a variety of ways to several different types of stimuli (Marcus, 1969; Mustard and Packham, 1970). One of the important stimuli is thrombin, a proteolytic enzyme that plays a central role in coagulation and hemostasis. The stimulation of platelets by thrombin is both a physiologically significant process and an example of an unusual agonist-receptor reaction, but little is known about the mechanism of action or about the receptor. Although Dip-F[1] or $PhCH_2SO_2F$ inactivated thrombin will not stimulate platelets (Davey and Luscher, 1967; Tollefsen et al., 1974; Phillips, 1974) and evidence has been presented for possible proteolysis of platelet membranes (Nachman, 1965; Baenziger et al., 1971, 1972; Phillips and Agin, 1973), the catalytic nature of thrombin's action on platelets has not been proved. This is especially important because of the recent demonstration that in the reaction of thrombin with platelets there is essentially no turnover of enzyme (Detwiler and Feinman, 1973a); thus thrombin does not appear to have the characteristics of a true catalyst. In addition there is an apparent inconsistency between this lack of turnover and the equilibrium binding recently described by Tollefsen et al. (1974) and by Ganguly (1974).

This paper describes studies of the enzyme involvement in protease stimulation of platelets. We reasoned that any part of the thrombin-induced secretion that depends on the catalytic activity of thrombin should be perturbed by variations in the reaction conditions in the same way that the reaction of thrombin with synthetic substrates would be. The perturbing conditions chosen were variations of pH and inclusion of a competitive inhibitor or hydroxylamine, conditions that have been extensively studied with proteases (Bender and Kezdy, 1965). We also compared the reactions of different proteases with platelets; the enzymes used were thrombin, trypsin, chymotrypsin, plasmin, and papain. The first four enzymes belong to the serine protease family and have a substantial degree of structural homology, but different side chain specificities (Blow, 1971; Keil, 1971; Magnusson, 1971), while papain is a sulfhydryl protease with no structural homology with thrombin but with a similar side chain specificity (Glazer and Smith, 1971). These enzymes thus permit evaluation of the relative contributions of side chain specificity and tertiary structure in the stimulation of platelets by proteases. An active site inhibited thrombin, $PhCH_2SO_2$-thrombin, was used to evaluate the role of nonactive site binding, and soybean trypsin inhibitor was used to relate trypsin-dependent steps to the time course of secretion.

The results permit elaboration of a previously proposed kinetic model of the reaction. The main feature of the new proposal, which reconciles the discrepancy between equilib-

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[1] Abbreviations used are: Dip-F, diisopropyl fluorophosphate; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; NphOGdnBz, *p*-nitrophenyl *p'*-guanidinobenzoate · HCl; NphOPrOBzamPhSO$_3$, *p*-(*p'*-nitrophenoxypropoxy)benzamidine benzenesulfonate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

rium binding and lack of thrombin turnover, is a reversible reaction of thrombin with the receptor.

Methods

Platelet Preparation. Suspensions of washed human platelets were prepared as previously described (Detwiler and Feinman, 1973a).

Measurement of Platelet Secretion. The reactions of enzymes with platelets were measured by recording the enzyme-induced secretion of either Ca^{2+} or ATP. Released Ca^{2+} was measured spectrophotometrically by its reaction with the metallochromic indicator murexide (Detwiler and Feinman, 1973a) and ATP was measured by its luminescent reaction with firefly extract (Detwiler and Feinman, 1973b). For Ca^{2+} secretion, platelet concentrations ranged from 2 to 4×10^8 platelets/ml and for ATP secretion from 1 to 3×10^8.

Analysis of Data. As previously demonstrated (Detwiler and Feinman 1973a), the thrombin-induced secretion of Ca^{2+} has the form of a series first-order reaction (cf. reaction 1 in Discussion) and the essential features of the progress–time curves are a lag followed by an exponential phase (see Figure 1). It was further shown that the rate constant for the second step of the reaction, k_2, was thrombin independent and could be calculated from a log plot of the exponential phase. The lag is quantitated as t_i, the time to the inflection, and is used to calculate a rate constant for the thrombin dependent first reaction, k_1(app), from the relationship $t_i = \ln (k_1(\text{app})/k_2)/(k_1(\text{app}) - k_2)$ derived from the rate expression for appearance of product in eq 1. The rate constant k_1(app) is complex, and may include terms in thrombin concentration and the dissociation constant of the thrombin–platelet complex, K (cf. reaction 2 in Discussion). Thus, k_1(app) is a complex rate constant for the initial steps of the reaction and increases as t_i decreases. While secretion of ATP does not strictly follow the series first-order mechanism (Detwiler and Feinman, 1973b), the approximation is close enough to permit the same analysis. We also measured the amount of Ca^{2+} or ATP secreted, or "yield", which was shown to be a function of both platelet and thrombin concentrations.

This paper deals with the initial steps of thrombin-induced secretion. The parameters measured are t_i, from which k_1(app) is calculated, and yield, expressed as percent of amount secreted at saturating levels of enzyme or under specified conditions.

Reagents. Human thrombin and plasmin were gifts from Drs. J. W. Fenton, II, and T. J. Ryan of the Division of Laboratories and Research, New York State Department of Health, Albany, N.Y. The thrombin preparations were made from Cohn fraction III paste essentially as reported by Fasco and Fenton (1973). These preparations had specific clotting activities of 2800 and 2300 N.I.H. units/mg of protein, were 89 and 90% active by esterase titration with NphOGdnBz (Chase and Shaw, 1970), and electrophoresed almost exclusively as α-thrombin. The plasmin preparation was made by column urokinase-agarose activation (Wiman and Wallen, 1973) of lysyl-agarose purified plasminogen (Deutch and Mertz, 1970) from fraction III paste. The activated plasminogen was electrophoretically pure; however, the plasmin obtained was only about 60% active by esterase titration with NphOGdnBz. Trypsin, chymotrypsin, and papain were the 2× crystallized products, and soybean trypsin inhibitor the lyophilized product, of Worthington Biochemical Corp., Freehold, N.J. Molar enzyme concentrations were determined by active site titrations using *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate for papain as described by Bender et al. (1966) and NphOGdnBz for trypsin. $PhCH_2SO_2$-thrombin was prepared by the method of Lundblad (1971); after reaction, excess reagent and any unreacted thrombin were removed by passing the solution through a benzamidine-agarose column (Schmer, 1972). Enzyme solutions were made in Tris incubation buffer (Detwiler and Feinman, 1973a) with 5 m*M* cysteine and 2 m*M* EDTA added for papain solutions.

The competitive inhibitor *p*-(*p'*-nitrophenoxypropoxy)-benzamidine benzenesulfonate ($NphOPrOBzamPhSO_3$) was synthesized by Dr. T. J. Ryan according to Baker and Erickson (1968). This compound was used in preference to benzamidine because it has been observed to be 20–25 times more effective an inhibitor of the clotting and esterase activities of human thrombin (J. W. Fenton, II, personal communication).

Buffers used were Mes for pH 5.15–7.0, Hepes for pH 6.42–8.25, and Tris-HCl for pH 7.42–9.3; concentrations were 25 m*M* and were made isotonic by addition of NaCl. Hydroxylamine-HCl was dissolved in water and adjusted to pH 7.4 with NaOH for each day's experiments.

Results

Enzyme Comparative Study. Of the enzymes tested, plasmin (2×10^{-7} *M*), chymotrypsin (8×10^{-7} *M*), and $PhCH_2SO_2$-thrombin (3.4×10^{-7} *M*) did not induce secretion from platelets. In addition, $PhCH_2SO_2$-thrombin or plasmin had no effect on release induced by subsequent addition of less than saturating levels of active thrombin or trypsin. Both trypsin and papain did induce secretion of Ca^{2+} and ATP; typical progress–time curves of secretion of Ca^{2+} induced by thrombin, trypsin and papain are shown in Figure 1. The curves obtained with trypsin or papain are nearly identical with that of thrombin at saturating levels of enzyme, suggesting basically similar mechanisms. In contrast, there are striking differences at lower levels of enzymes (Figure 1b). With thrombin, the progress curve can still be closely approximated by a theoretical series first-order curve, but with trypsin and papain the very long lag precludes this simple mechanism, indicating an additional step(s), probably not first order. In addition to this qualitative difference at less than saturating levels of enzyme, there are substantial differences in concentration dependence (Figure 2). Three hundred times more papain than thrombin is required for saturation of yield and kinetic parameters; with trypsin about 50 times more is required to saturate kinetic parameters while yield is saturated at less than twice the level for thrombin. In addition it is clear with trypsin and especially clear with papain that the enzyme vs. yield plot is sigmoidal. This behavior, suggestive of cooperative interactions, was postulated with thrombin but was difficult to establish conclusively because such low amounts are effective.

Soybean trypsin inhibitor affected the trypsin-induced secretion the same way that hirudin, a potent thrombin inhibitor, has been shown to affect thrombin-induced secretion (Detwiler and Feinman, 1973a). Addition of inhibitor before enzyme completely blocked the reaction while addition of inhibitor 5 sec after enzyme had no effect, demonstrating that only the initial part of the curve represents enzyme dependent steps.

Effect of pH. The pH dependence of t_i and yield of protease-induced secretion (Figure 3) are in general similar to

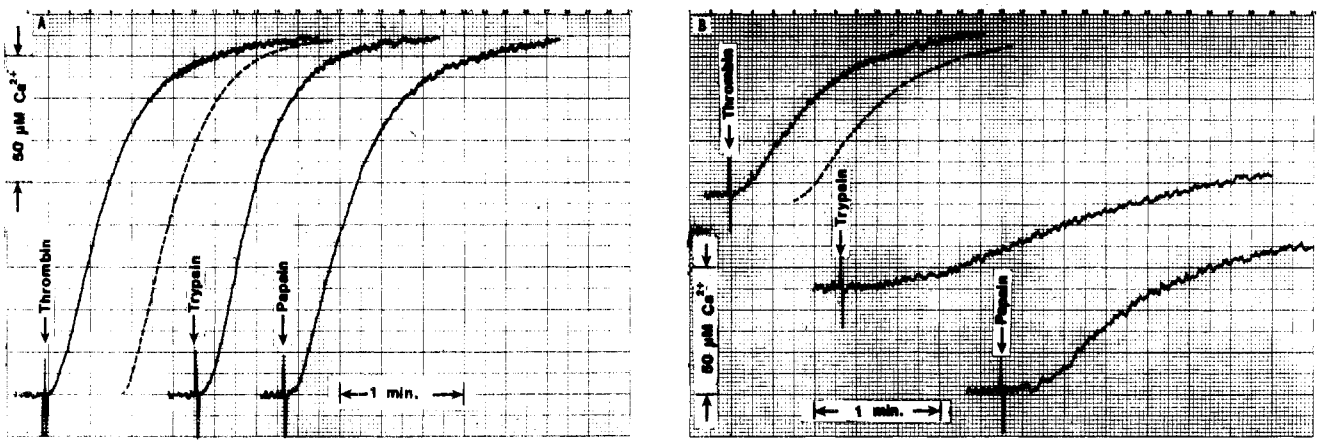


FIGURE 1: Progress-time curves of protease-induced secretion of Ca^{2+}. (A) Reactions were initiated with saturating levels of enzyme. The concentrations used were: thrombin, 8.3×10^{-8} *M*; trypsin, 8.5×10^{-7} *M*; papain, 6.5×10^{-6} *M*. The broken line is an analog computer simulation of a series first-order reaction with rate constants $k_1 = 0.26$ and $k_2 = 0.049$. (B) Reactions were initiated with less than saturating levels of enzymes. The concentrations used were: thrombin, 2.6×10^{-9} *M*; trypsin, 7.0×10^{-9} *M*; and papain, 1.0×10^{-6} *M*. The broken line is a theoretical curve for a series first-order reactions calculated as described by Detwiler and Feinman (1973a), with rate constants $k_1 = 0.08$ sec^{-1} and $k_2 = 0.043$ sec^{-1}.

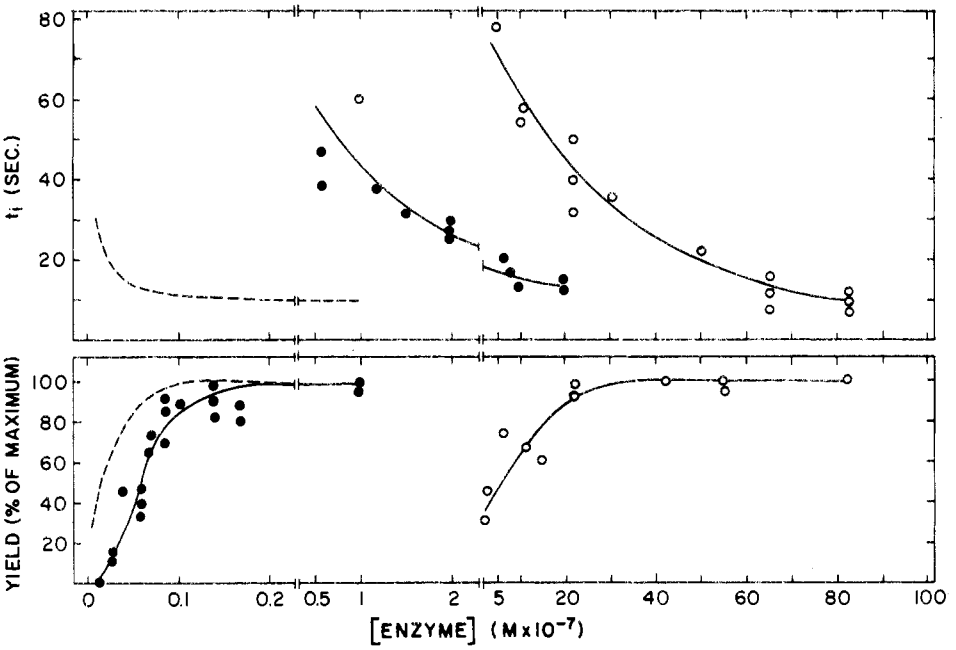


FIGURE 2: Effect of enzyme concentration on t_i and yield for protease-induced secretion of Ca^{2+}. (- - -) Thrombin (from Detwiler and Feinman, 1973a); (●) trypsin; (○) papain.

the pH dependence of catalysis of other substrates by these enzymes, with optima at 7 or greater for thrombin and trypsin and at about 6 for papain. However, saturating values of k_2 and t_i are independent of pH over the range used (i.e., at any pH the reaction can be made identical with that at pH 7.4 by increasing enzyme concentration). The most significant aspects of Figure 3 are (i) pH effects are overcome by increasing concentrations of thrombin or trypsin, suggesting a change in rate-determining step from pH dependent at low enzyme to pH independent at high enzyme, and (ii) yield as well as t_i are dependent on pH, suggesting an interrelationship between yield and kinetic parameters.

Effect of Hydroxylamine. Hydroxylamine and other nucleophiles enhance the rate of proteolytic reactions by competing with water (Bender and Kezdy, 1965). If a hydrolytic step were rate determining in any of the reactions seen here, it should be accelerated by hydroxylamine. Likewise, if the apparently irreversible binding of thrombin noted before (Detwiler and Feinman, 1973a) were due to an acyl-enzyme intermediate, hydroxylamine should increase thrombin turnover causing an increase in yield at low thrombin. For these experiments ATP secretion was measured because hydroxylamine interfered with the murexide reaction used to monitor Ca^{2+} secretion. With a 25% saturating level of thrombin, hydroxylamine was varied from 0 to 100 m*M* with constant ionic strength. The results were surprising; yield was decreased to 20% of control instead of increased and t_i was increased twofold. An effect of hydroxylamine on the receptor cannot be excluded and it has been reported that hydroxylamine inhibits platelet aggregation. However, the effect was not on the secretory process, since secretion induced by the divalent cation ionophore A23187, which induces secretion by a different mechanism (Feinman and Detwiler, 1974; Friedman and Detwiler, 1975), was not inhibited.

Competitive Inhibitors. On the assumption that the early

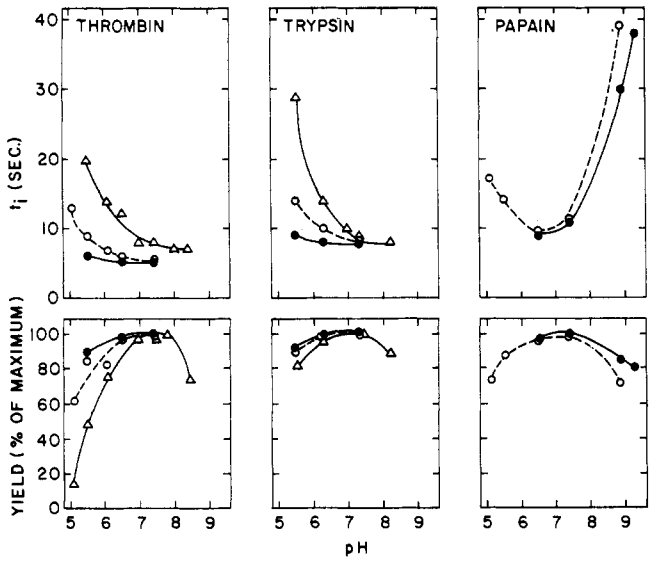


FIGURE 3: Effect of pH on protease-induced secretion of Ca^{2+}; 100 μl of platelets in 154 mM NaCl, 5 mM glucose, and 0.6 mM disodium citrate were added to isotonic buffer containing 5 mM glucose and 0.3 mM murexide 45 sec prior to enzyme addition. Thrombin: (Δ-Δ) 5×10^{-8} M; (O- -O) 3×10^{-7} M; (●——●) 1.2×10^{-6} M. Trypsin: (Δ-Δ) 4×10^{-7} M; (O- -O) 1×10^{-6} M; (●——●) 2×10^{-6} M. Papain: (O- -O) 1×10^{-5} M; (●——●) 2×10^{-5} M. Yield was calculated as percent of maximum secreted by saturating enzyme at pH 7.4.

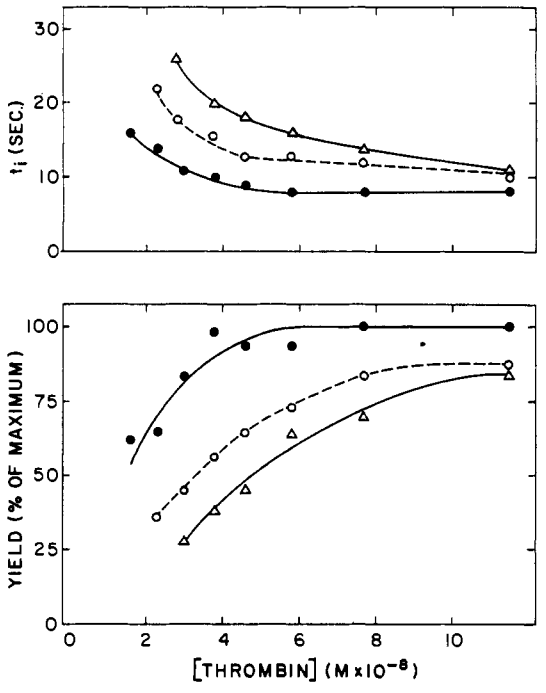


FIGURE 4: Effect of a competitive inhibitor, NphOPrOBzamPhSO$_3$, on thrombin-induced secretion of ATP. Yield was calculated as percent of maximum secretion with saturating thrombin. (●——●) No inhibitor; (O- -O) 0.03 mM NphOPrOBzamPhSO$_3$; (Δ——Δ) 0.05 mM NphOPrOBzamPhSO$_3$.

steps of thrombin stimulation of platelets are analogous to enzyme-substrate interactions, we tested the effects of the competitive inhibitor NphOPrOBzamPhSO$_3$. The inhibition is shown in Figure 4, and as in the case of the pH experiments, yield as well as rate was inhibited.

By analogy with the Michaelis-Menten treatment, these data are also plotted in a double reciprocal form (Figure 5)

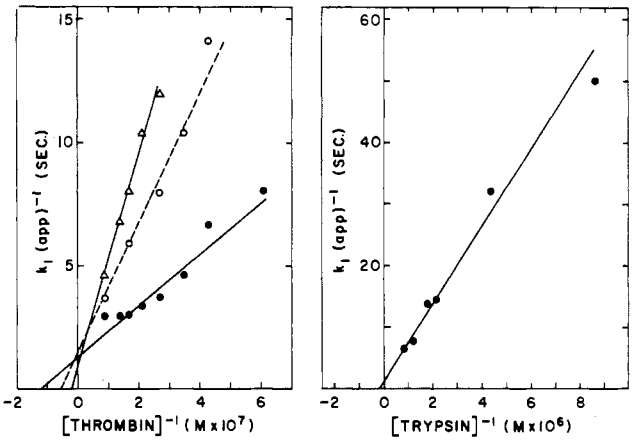


FIGURE 5: Double reciprocal plots of enzyme concentration vs. rate of the initial reaction. Apparent first-order rate constants for the initial steps of protease-induced secretion of ATP were calculated from observed values for t_i and k_2 as described under Methods. Lines are best fit determined by weighted least squares. The data in the left plot are from Figure 4 using the same symbols. The curve for no inhibitor was drawn with omission of the two points at highest thrombin, since these represent saturating values of t_i.

Table I: Kinetic Parameters for the Reaction of Thrombin and Trypsin with Platelets.[a]

	K_m (μM)	k_{cat} (sec^{-1})
Thrombin (human)[b]	0.08	0.8
Thrombin (human)[c]	0.21	1.5
Thrombin (bovine)[d]	0.04	0.5
Trypsin (bovine)[b]	7.60	1.1

[a] Kinetic parameters were obtained from the intercepts of lines of double reciprocal plots as in Figure 5 with lines drawn by weighted least-square analysis. [b] Data from Figure 5. [c] Data from a separate experiment using the same thrombin but a different platelet preparation. [d] Data from Figure 4A of Detwiler and Feinman (1973b).

and are analyzed as follows. The rate of appearance of product, TR^0 in eq 3 in Discussion, is $d[TR^0]/dt = k_{cat}[T][R]/K$ (neglecting k_{-cat}). If we make the assumption that $[T_0] > [TR]$, at least initially, this expression is approximately equal to $k_{cat}[T_0]/([T_0] + K)[R_0] = k_1(app)[R_0]$. The intercepts of the reciprocal plots in Figure 5 would represent $(k_{cat})^{-1}$ and $(-K_m)^{-1}$. The values for these parameters and for similar parameters for trypsin are shown in Table I. Competitive inhibition can be treated in the usual way and the inhibition constant can be calculated according to the expression K_m (inhib.) = $K_m([I] + K_i)/K_i$. An inhibition constant of 1×10^{-5} M can be calculated for NphOPrOBzamPhSO$_3$, in reasonable agreement with the value of 3×10^{-5} M calculated from inhibition of hydrolysis of N-benzoyl-L-arginine p-nitroanilide (T. J. Ryan and J. W. Fenton II, personal communication).

These experiments were done with measurement of secretion of ATP instead of Ca^{2+} because the greater sensitivity permitted a wider range of activities to be measured accurately. We have shown that higher thrombin concentrations are required to saturate the kinetics of ATP release (Detwiler and Feinman, 1973b) and we have now established that the difference is due to the phosphate used to prevent decay of luminescence in the ATP assay; reciprocal plots for ATP secretion gave K_m values four times higher with

phosphate buffer than with Tris but essentially no difference in k_{cat}. Thus, actual values of K_m will depend on experimental conditions.

Discussion

As previously discussed (Detwiler and Feinman, 1973a), platelet secretion induced by saturating levels of thrombin can be described by the series first-order sequence in reaction 1, where P is a platelet and P* is some intermediate. As

$$\mathrm{P} \xrightarrow{k_1} \mathrm{P}^* \xrightarrow{k_2} \text{secretion (ATP or } Ca^{2+}) \qquad (1)$$

shown in Figure 1, high levels of trypsin and papain induce secretion that can be described by the same simple mechanism and with essentially the same rate constants, so it is reasonable to assume that at high levels of these enzymes the reaction of each with platelets reduces to the same mechanism. To include the observed thrombin dependence at less than saturating thrombin, this model was elaborated as shown in reaction 2, where T is thrombin, K is a dissociation constant,[2] and k_1(app) is a function of K, k_1, and T. However, this model cannot describe all of the results obtained in these experiments and by other workers. The major inconsistencies are itemized below. (i) Whereas reactions of thrombin approximate series first-order reactions at either high *or* low levels of enzyme, reactions with low levels of trypsin and papain cannot be fit by such a simple mechanism because of the very long lags (Figure 1b), suggesting an additional, non-first-order step. (ii) With change in pH there is a change in rate-determining step for k_1(app), also indicating the need for an additional step in reaction 2. It is unlikely that the additional step could simply be the initial complex formation (K of reaction 2) because enzyme–substrate complex formation is usually much faster than the reactions observed here and because K for these proteases is independent of pH in this range (Hess, 1971). It is also unlikely that the observed pH effect is due to a change in a platelet functional group, since secretion is pH independent at high enzyme and since the change is not the same with each enzyme, but follows their pH dependence for catalysis, suggesting a pH dependent catalytic step in stimulation of platelets. (iii) Whereas yield and k_1(app) have the same enzyme dependence with thrombin, as reaction 2 demands, the enzyme dependence of these two parameters is markedly different with trypsin (Figure 2), requiring some mechanism that allows separation of kinetic parameters from yield determination. (iv) In contrast to item iii, when the kinetic parameters are modified in the expected way by changing pH or adding a competitive inhibitor, yield also changes, suggesting a dependence of yield on kinetics in these cases. This is especially clear with the competitive inhibitor; reaction 2 predicts that it would decrease k_1(app) by decreasing free thrombin, but that there would be no effect on yield since eventually all thrombin would be bound because of the irreversible step. (v) Hydroxylamine, a nucleophile that would be expected to accelerate hydrolytic reactions, actually inhibited both kinetic parameters and yield, while reaction 2 predicts either no effect or an increased yield due to faster turnover of bound thrombin. (vi) The apparent equilibrium binding of thrombin (Tollefsen et al., 1974) is inconsistent with the apparent stoichiometric reaction of thrombin (Detwiler and Feinman, 1973a,b) which is explained in reaction 2 by the irreversible k_1 step.

$$n\mathrm{T} + \mathrm{P} \overset{K}{\rightleftharpoons} \mathrm{T}_n\mathrm{P} \xrightarrow{k_1} \mathrm{T}_n\mathrm{P}^* \xrightarrow{k_2} \text{secretion} \qquad (2)$$

(k_1(app) spans the first two steps)

Proposed Model. We thus propose an elaboration of our previous model that can account for all of these results. This model, which considers a thrombin–receptor reaction followed by thrombin-independent platelet processes, is shown in reaction 3, where R is a membrane receptor and k_{cat} is the rate constant for the catalytic step. This model separates the enzyme-dependent and enzyme-independent steps and includes at least one additional step before P*. We propose that the catalytic steps are reversible while the secretion itself is an irreversible platelet response, with yield determined by the equilibrium concentration of TR^0. Reaction 3 is meant to separate conceptually the receptor reaction, which can be described in terms of enzyme chemistry, from the subsequent physiologic processes. The broken line is not meant to imply a separate kinetic step but to show the dependence of the second process on the first.

$$\begin{array}{l} \mathrm{T} + \mathrm{R} \overset{K}{\rightleftharpoons} \mathrm{TR} \overset{k_{cat}}{\underset{k_{-cat}}{\rightleftharpoons}} \mathrm{TR}^0 \\ \qquad\qquad\qquad\qquad \mathrm{P} \xrightarrow{k_1} \mathrm{P}^* \xrightarrow{k_2} \text{secretion} \end{array} \qquad (3)$$

This model has aspects of both an agonist–receptor equilibrium reaction and an enzyme reaction and reconciles the seemingly contradictory observations that the mode of action of thrombin involves the active site (Davey and Luscher, 1967), does not show any turnover of enzyme (Detwiler and Feinman, 1973a,b), and can be studied under equilibrium conditions (Tollefsen et al., 1974).

To analyze this model in terms of the observed results we consider expressions for k_1(app) and yield. At high levels of enzyme, the rate-determining step for k_1(app) is the k_1 step, a pH-independent and enzyme-independent platelet process, while at low levels of enzyme k_1(app) would be largely influenced by the rate of formation of TR^0 so that $k_1(\mathrm{app})[\mathrm{R}] \cong d[\mathrm{TR}^0]/dt = k_{cat}[\mathrm{TR}] = k_{cat}[\mathrm{T}][\mathrm{R}]/K$.[3] Yield is determined by the equilibrium concentration of TR^0, shown in eq 4. According to the model in reaction 3,

$$[\mathrm{TR}^0] = [\mathrm{T}][\mathrm{R}]k_{cat}/Kk_{-cat} = [\mathrm{T}][\mathrm{R}]/K_{eq} \qquad (4)$$

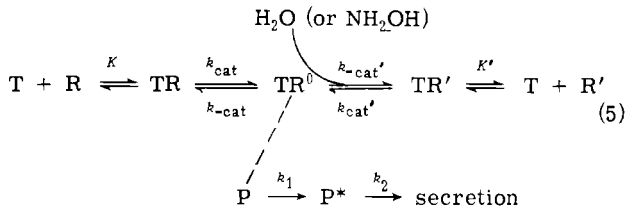
then, the fact that thrombin and trypsin show the same concentration dependence for yield even though K_m is an order of magnitude different could be due to a different k_{-cat} for the two enzymes. Likewise, by modifying k_{cat}, pH would affect yield (even if K is pH independent). A competitive in-

[2] We refer to three K's. K is a dissociation constant, K_m is the experimentally determined Michaelis constant and K_{eq} refers to the equilibrium concentration of TR^0 ($K_{eq} = Kk_{-cat}/k_{cat}$). These parameters are related to those used in serine protease chemistry in the following way. If TR^0 in reaction 3 were, for example, an acyl-enzyme, $K = K_s$ and $k_{cat} = k_2$ in the usual formulation (Bender and Kezdy, 1965). Alternatively, if hydrolysis takes place, k_{cat} might contain both k_2 and k_3. For comparison with the trypsin inhibitor model of Laskowski, our constant $K = K_L = k_{-1}/k_1$, $k_{cat} = k_2$ and $k_{-cat} = k_{-2}$. Our K_{eq} refers only to the single species TR^0 since this is the active form, but K_{eq} is analogous to Laskowski's $(K_{ass})^{-1}$ (Luthy et al., 1973).

[3] The expression $K = [\mathrm{T}][\mathrm{R}]/[\mathrm{TR}]$ contains no higher order terms in thrombin concentration since the appropriate physical model is a surface reaction, so the equilibrium would follow a model such as the Langmuir adsorption isotherm (see, e.g., Moore, 1972). The mathematics would be the same as the Michaelis–Menton model but this must be explicitly considered since a solution reaction would require higher order terms in [T].

hibitor would decrease free thrombin, simultaneously decreasing k_1(app) and yield.

The most novel aspect of this proposal is the reversible catalysis by thrombin. This is similar to the interaction of trypsin with soybean trypsin inhibitor (Laskowski and Sealock, 1971; Luthy et al., 1973; Mattis and Laskowski, 1973) to form a stable, covalent enzyme–inhibitor complex in equilibrium with native inhibitor (which would be analogous to R in reaction 3) and with a modified inhibitor. This type of interaction has also been proposed for the inhibition of thrombin by antithrombin–heparin cofactor (Rosenberg and Damus, 1973) and may be general. It is interesting to consider the possibility that the trypsin inhibitor model described by Laskowski applies totally to the thrombin–platelet reaction. This is shown in reaction 5, which now includes



R′, a modified (hydrolyzed) receptor that also reacts with thrombin. This model maintains the same features described for reaction 3, with the yield determined by the equilibrium concentration of TR^0, but would also explain the results with hydroxylamine. If TR^0 were susceptible to nucleophilic attack by water it would also be attacked by hydroxylamine; the effects would be to *decrease* the rate of formation of TR^0, since the rate of breakdown of TR^0 would be increased, and to decrease the yield by removing TR^0 from the equilibrium.

We have also considered the possibility that there is no catalytic step; that is, that formation of TR is sufficient for platelet stimulation with thrombin functioning as a hormone instead of as an enzyme. To be consistent with the data here, this would require that formation of the TR complex (i) be slow, (ii) have the same pH dependence as observed for catalysis by these enzymes, (iii) involve the enzyme active site (a competitive inhibitor blocks), and (iv) not be dependent on other features of the protein (proteins with no structural homology are active). It is significant that enzyme specificity closely parallels specificity for catalysis at arginyl residues but has no correlation with structural homology. Thus, while catalysis, or more precisely, a reaction of the active site of the proteases with the receptor, cannot be proved, there is considerable circumstantial evidence for inclusion of such a step in the model.

Complete description of the reaction of thrombin with platelets will require isolation and characterization of the receptor and this model is pertinent to the many efforts to identify the receptor by searching for a hydrolyzed product. Although this model allows for hydrolysis (reaction 5), its essential features do not demand it (reaction 3).

Protease Specificity. The proteases that induced secretion, thrombin, trypsin, and papain have specificity for peptides of basic amino acids. Thrombin has a high degree of specificity for arginyl side chains (Weinstein and Doolittle, 1972) and, of the three, the greatest reactivity with platelets. Trypsin has little preference for arginyl or lysyl side chains with synthetic substrates (Weinstein and Doolittle, 1972) but a somewhat greater preference for arginyl side chains in protein substrates (Wang and Carpenter, 1967; Bergmann, 1942) and intermediate reactivity with platelets. Papain, which shows the least reactivity of the three enzymes with platelets, has less side chain specificity than either thrombin or trypsin (Glazer and Smith, 1971) but there are other subsites that play a role in selective hydrolysis (Schechter and Berger, 1967). Chymotrypsin, which does not catalyze hydrolysis of peptides with basic amino acid side chains, is not reactive with platelets, nor is plasmin, which shows a preference for lysyl side chains (Weinstein and Doolittle, 1972). The obvious conclusion from these results is that the susceptible peptide of the platelet thrombin receptor contains an arginyl residue. However, it should be recognized that specificity for protein or membrane substrates can be determined by considerations other than immediate side chains.

Our observation of no platelet stimulation by plasmin contradicts the report of Niewiarowski *et al.* (1973) that either trypsin or streptokinase-activated plasminogen induced aggregation and release of serotonin and nucleotides and caused a decreased response to thrombin. Since they do not report the active molar concentration of their enzymes, comparison of experimental conditions is difficult.

Role of the Active Site. It is known that active site inhibited thrombin does not stimulate platelets (Davey and Luscher, 1967), suggesting involvement of the active site. The data presented here are further evidence for the enzymatic nature of the reaction, since the pH–rate profile is similar to that for other substrates and NphOPrOBzamPh-SO_3 inhibition is competitive with nearly the same K_i as for a synthetic substrate. However, it has also been reported (Tollefsen et al., 1974; Phillips, 1974) that while active site inhibited thrombin does not stimulate platelets, it physically binds exactly as does active thrombin and competes with active thrombin for binding but not for stimulation. They also observed that inhibited thrombin slightly enhanced the effect of very low levels of thrombin on induction of secretion or aggregation. We have confirmed that inhibited thrombin, even in a 1000-fold excess, does not compete with low levels of active thrombin for inducing secretion but we have consistently been unable to observe any enhancement. We believe that the slight enhancements reported may be related to the well-known instability of thrombin in dilute solutions (Magnusson, 1970), where thrombin is lost through surface interactions and nonspecific binding; addition of large amounts of inhibited thrombin would simply spare active thrombin. (This might apply more to the work of Phillips (1974), who used platelets in plasma, known to contain antithrombins). The paradox of competition for binding but no competition for activity can most readily be explained by different types of platelet binding sites, with the major part of the bound thrombin at sites other than productive receptors. Various lines of evidence have led to the suggestions of either different types of sites or site interactions (Detwiler and Feinman, 1973a; Tollefsen et al., 1974; Ganguly, 1974; Phillips, 1974), and until such possibilities are more fully understood measurements of binding, either directly or by kinetic studies, must be considered tentative. This is important to our proposed model, which was developed in part to reconcile the apparent contradiction of stoichiometry but equilibrium binding; if the binding studies do not reflect binding to the active receptor there is less evidence to indicate our proposed mechanism. Thus, we make no pretense that the model proposed here is a complete or final description of the reaction but we believe that it defines the problem in a conceptually new way and can serve as a valuable working hypothesis.

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