Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 5977–5981, June 1995
Biophysics

An allosteric switch controls the procoagulant and anticoagulant activities of thrombin

(allostery/fibrinogen/protein C/thrombomodulin)

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Communicated by Carl Frieden, Washington University School of Medicine, St. Louis, MO, March 2, 1995 (received for review December 6, 1994)

ABSTRACT **Thrombin is an allosteric enzyme existing in two forms, slow and fast, that differ widely in their specificities toward synthetic and natural amide substrates. The two forms are significantly populated *in vivo*, and the allosteric equilibrium can be affected by the binding of effectors and natural substrates. The fast form is procoagulant because it cleaves fibrinogen with higher specificity; the slow form is anticoagulant because it cleaves protein C with higher specificity. Binding of thrombomodulin inhibits cleavage of fibrinogen by the fast form and promotes cleavage of protein C by the slow form. The allosteric properties of thrombin, which has targeted two distinct conformational states toward its two fundamental and competing roles in hemostasis, are paradigmatic of a molecular strategy that is likely to be exploited by other proteases in the blood coagulation cascade.**

The physiologic response to a vascular lesion entails a number of sequential enzymatic steps catalyzed by distinct serine proteases (1, 2). The cascade of events culminates in the generation of thrombin, which then catalyzes the conversion of fibrinogen into fibrin monomers, leading to clot formation. Efficiency of the coagulation system requires that the response be rapid, localized at the injury site, and then readily terminated. This result is accomplished through a complex network of regulatory interactions. The components of this network share a surprisingly high degree of structural homology (3). Hence, a crucial question arises as to the molecular origin of specificity in the cascade. Is there an underlying molecular strategy shared by different members of the cascade or is specificity accomplished differently by each protease? Understanding the physicochemical and structural basis of protease specificity in the coagulation cascade would provide new tools for engineering proteases of desired specificity and eventually lead to more efficient pharmacological strategies for controlling hemostasis.

Thrombin function conveys most of the complexity of the entire coagulation cascade. This enzyme accomplishes two opposite roles in hemostasis. The procoagulant role entails conversion of fibrinogen into the fibrin clot and enhancement of its own conversion from prothrombin through feed-back activation of proteases upstream in the cascade. The anticoagulant role encompasses activation of protein C (PC) with participation of thrombomodulin (TM), a cofactor on the membrane of endothelial cells (4–6). Activated protein C (APC) rapidly inactivates factors Va and VIIIa, both of which are involved in thrombin generation. Thrombin, therefore, finely tunes its own production in the blood through the balance between positive and negative feed-back loops, and much interest now exists in understanding the molecular mechanism that allows this enzyme to accomplish its dual role in hemostasis (7, 8).

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We recently demonstrated that thrombin is an allosteric enzyme, present in two distinct forms in equilibrium: the slow and fast forms (9, 10). These forms were so defined originally from their ability to interact with and cleave small chromogenic amide substrates; the slow form shows a significantly reduced catalytic efficiency. Because the two forms are significantly populated under physiological conditions (9, 11), assessing their role in the procoagulant and anticoagulant activities of the enzyme is important.

MATERIALS AND METHODS

Human α-thrombin was purified and tested for activity as described (9, 12). Human fibrinogen, PC, APC, and rabbit lung TM of the highest purity and activity were from Hematologic Technologies (Essex Junction, VT). These reagents yielded results consistent with published data (see *Results*). The chromogenic substrate S2266 (H-D-Val-Leu-Arg-*p*-nitroanilide), specific for APC, was from Chromogenix (Molndal, Sweden).

The slow and fast forms were studied under experimental conditions of 5 mM Tris/0.1% PEG (pH 8.0) at 25°C. Ionic strength was kept constant at 200 mM with NaCl, when studying the fast form, or with choline (Ch) chloride, when studying the slow form (9, 10). In studies involving PC, the slow form of thrombin was studied under conditions of 195 mM ChCl/5 mM NaCl (see below).

Release of fibrinopeptides A and B (FpA and FpB) by the slow and fast forms was quantified by reverse-phase HPLC, using a Waters 717plus Autosampler for 1-ml injections into the HPLC system equipped with a Waters C_{18} column (4.6 × 250 mm). Elution was done at a flow rate of 1 ml/min, with a gradient containing 80 mM sodium phosphate buffer, pH 3.1 (solvent A) and acetonitrile (solvent B). Optimal separation was obtained by using 17% of solvent B from 0 to 15 min, followed by a 10-min linear gradient to 40% of solvent B. The effluent was monitored at 205 nm. Extinction coefficients for FpA and FpB were derived by calibration and quantitative amino acid analysis of highly pure standards. The sequential release of FpA and FpB was analyzed according to the mechanism of Shafer and coworkers (13) with the expressions

$$[FpA] = [FpA]_\infty(1 - e^{-at}) \qquad [1]$$

and

$$[FpB] = [FpB]_\infty\left(1 - \frac{ae^{-bt} - be^{-at}}{a - b}\right), \qquad [2]$$

where $[FpA]_\infty$ and $[FpB]_\infty$ are the asymptotic values of the concentrations of FpA and FpB, while a and b are equal to the k_{cat}/K_m ratio for FpA and FpB, respectively, times the enzyme concentration.

Abbreviations: Ch, choline; FpA, fibrinopeptide A; FpB, fibrinopeptide B; PC, protein C; APC, activated protein C; TM, thrombomodulin.

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Binding of TM to thrombin was quantified from the inhibition of hirudin binding. The equilibrium dissociation constant for hirudin binding to thrombin, K_i, was measured as described (9) as a function of TM concentration. The value of K_i was found to increase linearly with the TM concentration, as expected for competitive inhibition. The equilibrium dissociation constant for TM, K_d, was derived from the equation

$$K_i = {}^0K_i\left(1 + \frac{[TM]}{K_d}\right), \quad [3]$$

where ${}^0K_i$ is the value of K_i in the absence of TM.

The interaction of thrombin with PC was studied by using progress curves of substrate hydrolysis. This strategy is simple, accurate, and reliable. It has the advantage of monitoring PC activation continuously and greatly minimizes the amounts of expensive reagents needed for determination of the kinetic parameters. The general reaction scheme for catalytic conversion of PC is

$$T + PC \overset{K_m}{\rightleftarrows} TPC \overset{k_{cat}}{\longrightarrow} T + APC, \quad [4]$$

$$APC + S \overset{{}^CK_m}{\rightleftarrows} APCS \overset{{}^Ck_{cat}}{\longrightarrow} APC + P, \quad [5]$$

and

$$T + S \overset{{}^TK_m}{\rightleftarrows} TS \overset{{}^Tk_{cat}}{\longrightarrow} T + P. \quad [6]$$

The product P is the *p*-nitroaniline released from a chromogenic substrate (S2266) specific for APC and provides the signal to be followed at 405 nm as a function of time. The release of P depends on three reactions: cleavage of S2266 by APC (Eq. **5**), activation of PC by thrombin (Eq. **4**), and cleavage of S2266 by thrombin (Eq. **6**). Eqs. **5** and **6** were studied directly in separate experiments done under identical solution conditions. The kinetic parameters pertaining to Eq. **4**, which is the one of interest, were decoupled from analysis of the progress curve started by adding thrombin to a solution containing PC and S2266, using the information derived independently on Eqs. **5** and **6**. This operation was done by using KINSIM and FITSIM, two modular programs developed to simulate and fit progress curves for any kinetic mechanism (14, 15). Activation of PC was studied under conditions where thrombin was in either the slow or fast forms and also in the presence of 5 mM $CaCl_2$ and 10 mM TM. Control experiments were done to check the effect of Ca^{2+} and TM on thrombin and APC. The activity of thrombin under 0.2 mM NaCl or ChCl was found to be independent of Ca^{2+} concentration, up to 67 mM, in agreement with previous results (16) and with the fact that thrombin, unlike prothrombin, has no binding sites for Ca^{2+} (3, 17). Thrombomodulin at 10 nM was found to affect the slow form but not to affect the fast form. The amidolytic activity of APC was found to strongly depend on Na^+, as reported (18), to the point that no appreciable activity could be measured in 0.2 M ChCl. This effect was not due to enzyme denaturation because addition of small aliquots of Na^+, to a final concentration as low as 5 mM, rapidly induced a drastic enhancement of the hydrolysis rate. For this reason, hydrolysis of PC by the slow form of thrombin was studied under conditions of 195 mM ChCl and 5 mM NaCl. At this Na^+ concentration thrombin is ≈90% in the slow form, which mimics the conditions of 0.2 M NaCl, where thrombin is ≈90% in the fast form (9).

The Michaelis–Menten parameters in Eqs. **4–6** were determined by analysis of progress curves of substrate hydrolysis using KINSIM and FITSIM. The values of ${}^TK_m$ and ${}^Tk_{cat}$ (see Eq. **6**) for the hydrolysis of S2266 by thrombin in the various solution conditions were as follows: 210 ± 10 μM and 26 ± 1 s^{-1} (0.2 M NaCl), 490 ± 20 μM and 29 ± 1 s^{-1} (195 mM ChCl, 5 mM NaCl), 270 ± 20 μM and 29 ± 1 s^{-1} (195 mM ChCl/5 mM NaCl/10 nM TM). No effect of Ca^{2+} at 5 mM was found under all conditions examined, and likewise 10 nM TM had no effect on the hydrolysis of S2266 by the fast form. The values of ${}^CK_m$ and ${}^Ck_{cat}$ (see Eq. **5**) for the hydrolysis of S2266 by APC in the various solution conditions were as follows: 300 ± 10 μM and 11.3 ± 0.2 s^{-1} (0.2 M NaCl), 1.0 ± 0.1 mM and 2.0 ± 0.1 s^{-1} (195 mM ChCl/5 mM NaCl), 1.0 ± 0.1 mM and 6.0 ± 0.3 s^{-1} (195 mM ChCl/5 mM NaCl/5 mM $CaCl_2$). Thrombomodulin at 10 nM had no effect on activity of APC, regardless of solution conditions, and 5 mM Ca^{2+} had no effect under all conditions studied in 0.2 M NaCl. Progress curves of S2266 hydrolysis were measured to derive the specificity (k_{cat}/K_m ratio for Eq. **4**) of the slow and fast forms toward PC. The S2266 concentration was 50 μM in all cases, and PC was used at 300 nM, well below estimated K_m values (19). The thrombin concentration was 2 nM. Under these conditions release of *p*-nitroaniline due to cleavage by thrombin and APC was extremely sensitive to the k_{cat}/K_m ratio for PC activation, thereby allowing accurate measurements of this parameter; this was due to the constraints imposed by the parallel Eqs. **5** and **6** that were studied independently. The robustness of the results was checked extensively by KINSIM with various combinations of kinetic rates for substrate binding, dissociation, acylation, and deacylation for thrombin and APC. All combinations consistent with the ${}^Tk_{cat}/{}^TK_m$ and ${}^Ck_{cat}/{}^CK_m$ ratios determined independently led to the same value of k_{cat}/K_m for PC activation by thrombin. Activation of PC was practically complete under all conditions studied. Product inhibition was carefully checked and ruled out by independent measurements of ${}^Tk_{cat}$, ${}^TK_m$, ${}^Ck_{cat}$, and ${}^CK_m$ using initial velocity determinations. These measurements yielded specificity ratios in agreement with those determined by progress curves.

RESULTS

The functional properties of the slow and fast forms of thrombin are summarized in Tables 1 and 2. Thrombomodulin binds to the fast form with an affinity about four times higher (see Fig. 1). The coupling free energy term, ΔG_c, quantifies the linkage between the binding of a ligand to thrombin and the allosteric, slow→fast transition (10). For TM, ΔG_c is different from that of the hirudin tail, hir-$^{(55-65)}$, thereby implying that the region of thrombin responsible for binding TM may contain residues sensitive to the allosteric transition of the

Table 1. Properties of slow and fast forms of thrombin: K_d values

	K_d			
	Slow	Fast	r	ΔG_c, kcal/mol
Fibrinogen	22 ± 3 μM	1.3 ± 0.3 μM	17 ± 4	−1.7 ± 0.1
Hirudin	6.9 ± 0.7 pM	0.39 ± 0.06 pM	18 ± 3	−1.7 ± 0.1
Hirudin-(55–65)	6.0 ± 0.8 μM	2.6 ± 0.3 μM	2.3 ± 0.4	−0.5 ± 0.1
TM	14 ± 2 nM	3.2 ± 0.1 nM	4.4 ± 0.6	−0.88 ± 0.08

Experimental conditions are as follows: 5 mM Tris/0.1% PEG, pH 8.0 at 25°C, and 0.2 M NaCl for the fast form or 0.2 M ChCl for the slow form. The third column reflects the ratio of affinity or specificity between fast and slow forms; the last column gives the coupling free energy $\Delta G_c = -RT \ln r$ (10).

Table 2. Properties of slow and fast forms of thrombin: k_{cat}/K_m values

	k_{cat}/K_m			
	Slow	Fast	r	ΔG_c, kcal/mol
FpA	$1.88 \pm 0.04\ \mu M^{-1}\cdot s^{-1}$	$13.4 \pm 0.2\ \mu M^{-1}\cdot s^{-1}$	7.1 ± 0.2	-1.16 ± 0.02
FpB	$2.00 \pm 0.06\ \mu M^{-1}\cdot s^{-1}$	$7.9 \pm 0.2\ \mu M^{-1}\cdot s^{-1}$	4.0 ± 0.2	-0.82 ± 0.03
PC	$24 \pm 1\ mM^{-1}\cdot s^{-1}$	$3.5 \pm 0.1\ mM^{-1}\cdot s^{-1}$	0.15 ± 0.01	1.13 ± 0.04
PC (+ TM)	$120 \pm 10\ mM^{-1}\cdot s^{-1}$	$28 \pm 1\ mM^{-1}\cdot s^{-1}$	0.23 ± 0.02	0.87 ± 0.05
PC (+ Ca^{2+})	$0.18 \pm 0.01\ mM^{-1}\cdot s^{-1}$	$0.15 \pm 0.04\ mM^{-1}\cdot s^{-1}$	0.8 ± 0.2	0.1 ± 0.1
PC (+ Ca^{2+} and TM)	$140 \pm 10\ mM^{-1}\cdot s^{-1}$	$97 \pm 9\ mM^{-1}\cdot s^{-1}$	0.69 ± 0.08	0.22 ± 0.07

Experimental conditions are as for Table 1; in the experiments with PC the slow form was studied under conditions of 195 mM ChCl/5 mM NaCl. Concentrations of Ca^{2+} and TM are 5 mM and 10 nM, respectively. The third and last columns are as for Table 1.

enzyme but that are not responsible for binding of the hirudin tail. This conclusion agrees with recent structural data (20). The difference in ΔG_c between fibrinogen, hirudin, and TM is from the contribution of the region around the catalytic pocket of the enzyme that is sensitive to the allosteric transition and interacts with the N-terminal domains of hirudin and the fibrinogen Aα chain but does not interact with TM.

Previous kinetic studies indicated that the fast form cleaves a number of synthetic amide substrates with higher efficiency (9). Consistent with these findings, FpA and FpB are cleaved by the fast form with higher specificity (Fig. 2), indicating that this form is responsible for the procoagulant activity. Both forms obey Shafer's sequential mechanism for release of fibrinopeptides, with FpB being released after FpA. The value of ΔG_c in Table 2 is related to the specificity ratio and reflects linkage between the stabilization of the transition state and the slow→fast transition. The differences between FpA and FpB contain important clues as to the structural components sensitive to the allosteric switch in the transition state.

When PC is used as substrate, the slow form cleaves with higher specificity. PC is the first substrate found to be more specific for the slow thrombin form (see Fig. 3, where the effect is illustrated in the form of progress curves of S2266 hydrolysis). Release of *p*-nitroaniline is more pronounced when thrombin is in the fast form, but this is due to a higher specificity of thrombin and APC toward S2266 with Na^+ (see *Materials and Methods*). The contribution of PC activation (Eq. **4**) must be decoupled from the progress curves using the information derived independently on substrate hydrolysis by thrombin and APC (Eqs. **5** and **6**). When this decoupling is done, specificity of thrombin for PC is greater in the slow form. This conclusion is illustrated directly by simulated progress curves. The formation of *p*-nitroaniline can be simulated by swapping the k_{cat}/K_m values obtained for PC activation in Eq.

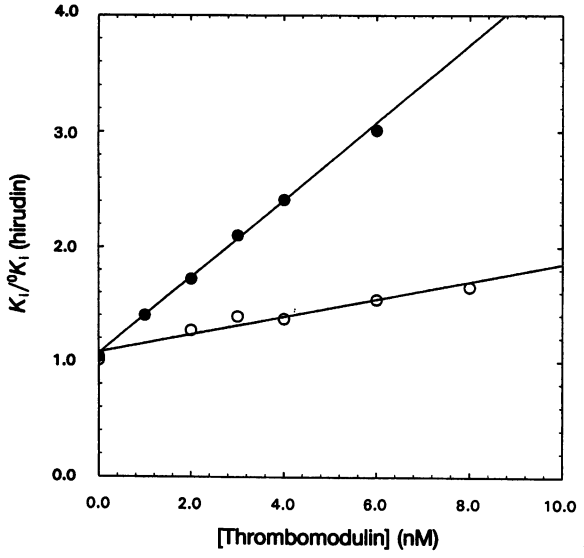

FIG. 1. Effect of TM on the equilibrium dissociation constant (K_i) of hirudin. Experimental conditions are as follows: 5 mM Tris/0.1% PEG, pH 8.0 at 25°C, and 0.2 M NaCl (●) or 0.2 M ChCl (○). The continuous lines were drawn according to Eq. **3**; parameter values for K_d are listed in Table 1.

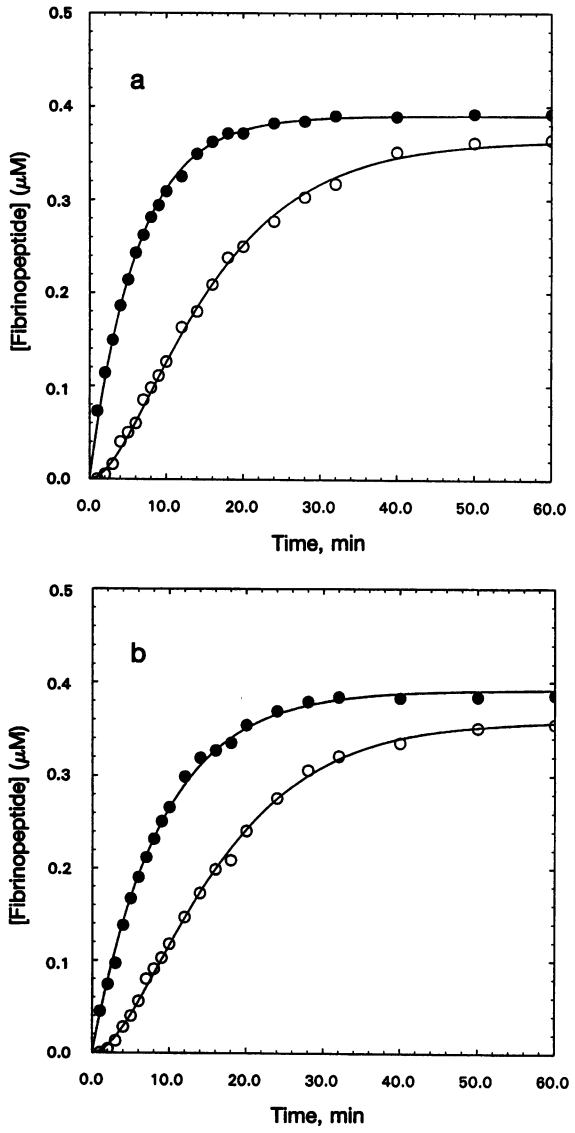

FIG. 2. (*a* and *b*) Progress curves for release of FpA (●) and FpB (○) by the fast (*a*) and slow (*b*) thrombin forms. Experimental conditions are as follows: 0.2 μM fibrinogen/5 mM Tris/0.1% PEG, pH 8.0 at 25°C, and 0.2 nM thrombin/0.2 M NaCl (*a*) or 1 nM thrombin/0.2 M ChCl (*b*). Continuous lines were drawn according to Eqs. **1** and **2**; parameter values for k_{cat}/K_m are listed in Table 2.

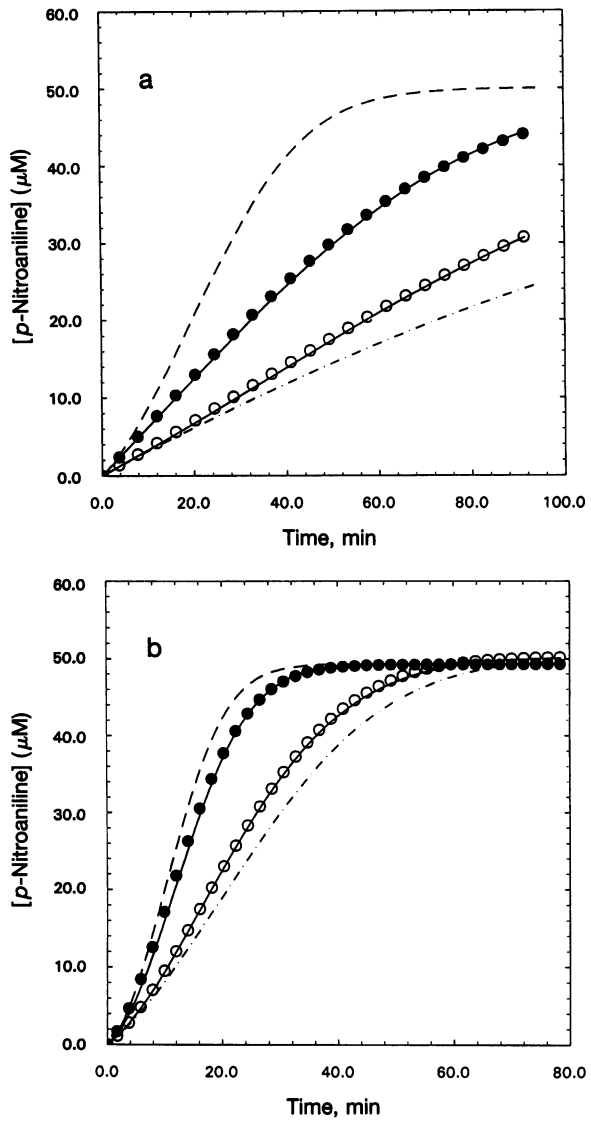

FIG. 3. (*a* and *b*) Progress curves of S2266 hydrolysis due to PC activation by thrombin in the absence (*a*) or presence (*b*) of 5 mM $CaCl_2$/10 nM TM. Experimental conditions are as follows: 2 nM thrombin/300 nM PC/5 mM Tris/0.1% PEG, pH 8.0 at 25°C, and 0.2 M NaCl (●) or 195 mM ChCl/5 mM NaCl (○). The continuous lines were drawn by using FITSIM, according to the kinetic mechanism depicted by Eqs. **4–6** in text. Values of k_{cat}/K_m for PC activation by thrombin in the various experimental conditions are listed in Table 2. Discontinuous lines (– – –) simulate the progress curves that would be observed if the fast form cleaved PC with the same specificity as found experimentally for the slow form (see Table 2). Discontinuous-dotted (–·–) lines simulate the progress curves that would be observed if the slow form cleaved PC with the same specificity as found experimentally for the fast form (see Table 2). The simulated curves show directly that the slow form cleaves PC with higher specificity.

4 by the slow and fast forms (see Table 2), while keeping all other rates in Eqs. **5** and **6** unchanged. This result yields two curves: one (discontinuous line in Fig. 3) depicting the progress curve that would be measured if the fast form cleaved PC with the specificity found experimentally for the slow form, and the other (discontinuous-dotted line) depicting the progress curve that would be measured if the slow form cleaved PC with the specificity determined experimentally for the fast form. The actual progress curve with thrombin in the fast form lies below the simulated one, whereas that of the slow form lies above the simulated curve. Hence, the slow form cleaves PC with higher specificity.

Preferential cleavage of PC by the slow form is a crucial observation for understanding the structural basis of thrombin specificity. The results obtained in the presence of 10 nM TM likely underestimate the preferential cleavage by the slow form because TM binds with higher affinity to the fast form. Measurements done as a function of TM concentration confirm the preferential cleavage of PC by the slow form under saturating concentrations of TM, measurements that are difficult to achieve in practice. The k_{cat}/K_m values obtained for the fast form are consistent with the results of previous studies (19, 21), where the specificity was measured by quenching Eqs. **4–6** at different time intervals and assaying APC activity. The experimental strategy based on the analysis of progress curves, introduced in the present study, provides results that are accurate and reliable and has the desirable practical advantage of requiring smaller amounts of material.

The specificity of thrombin for PC is drastically reduced in the presence of Ca^{2+} in both the slow and fast forms. This result is consistent with previous findings obtained in the presence of 0.1 M NaCl (22, 23). The effect is due to a conformational change of PC induced by Ca^{2+} binding to a high-affinity site in the region homologous to the Ca^{2+} binding loop of chymotrypsin (23, 24). However, Ca^{2+} increases the specificity when TM is present. As a result, the effect of TM in switching thrombin specificity toward PC changes from a factor of ≈5–8 in the absence of Ca^{2+} to a factor of 600–800 in the presence of Ca^{2+}. These results agree with previous findings obtained in the presence of NaCl and in the absence of a phospholipid surface (19, 21).

DISCUSSION

A crucial issue in the study of blood coagulation is to understand the rules for specificity and regulatory interactions that give rise to the efficiency of the hemostatic process. The coagulation cascade was originally conceived as a mechanism to amplify the response to acute vessel injury downstream to the generation of thrombin and formation of the fibrin clot (25, 26). However, efficient coagulation requires that the response be localized to the site of injury, be rapidly achieved, and be terminated. This task is accomplished by carefully articulated mechanisms of regulation (1, 2) and especially by the anticoagulant feed-back mechanisms centered around the action of TM and PC (6). The results reported here broaden our understanding of molecular recognition and regulation in blood coagulation. The observation that antagonistic roles in hemostasis can be targeted toward distinct allosteric forms of a protease is unprecedented. The slow and fast forms of thrombin have been targeted toward the anticoagulant and procoagulant activities of the enzyme, as illustrated in Fig. 4. Fibrinogen is cleaved by the fast form with higher specificity, whereas PC is cleaved by the slow form with higher specificity. By linkage principles, it follows that fibrinogen binding promotes the slow→fast transition, whereas the opposite is seen upon binding of PC. The role of TM in the regulation of thrombin function is crucial and can be understood in terms of the effect on the slow↔fast equilibrium. In the absence of TM, the procoagulant role of thrombin prevails because the fast form is more populated and cleaves fibrinogen with higher efficiency. In the presence of TM, the fast form is preferentially inhibited because TM binds to this form with higher affinity. This mechanism provides the most effective way of switching thrombin into an anticoagulant enzyme. Once the fast form is inhibited and can no longer cleave fibrinogen, the anticoagulant role of the slow form prevails, leading to efficient activation of PC.

The major driving force for the slow→fast transition is provided by the binding of Na^+ to a single site (9, 10). The equilibrium between the slow and fast forms of the free enzyme is set by the amount of Na^+ in the blood (9, 11). The

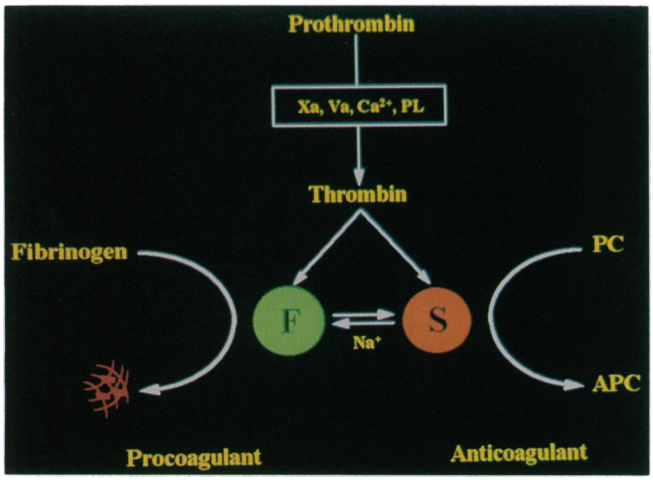

FIG. 4. Schematic of the dual role of thrombin in the blood coagulation cascade partitioned between slow and fast forms. Once generated from prothrombin by prothrombinase complex, formed by factors Xa, Va, Ca^{2+}, and phospholipids (PL), thrombin undergoes the equilibrium between slow and fast forms. The fast form is responsible for cleavage of fibrinogen and procoagulant activity. The slow form cleaves PC and promotes anticoagulant activity. Binding of Na^+ controls partitioning of free enzyme between its two allosteric forms.

role of Na^+ is that of a "passive" allosteric effector that is taken up by thrombin when fibrinogen binds and is released upon binding of PC. The Na^+ concentration in the blood is very close to the K_d for this cation (9), which guarantees optimal conditions for allosteric modulation. The Na^+-binding site of thrombin has recently been identified crystallographically from analysis of 21 different structures (A. Tulinsky, personal communication). The site is shaped by a loop connecting the last two β-strands of the B chain, with the carbonyl oxygens of Arg-221a and Lys-224 providing two of the six ligating groups in the octahedral coordination shell. A similar architecture has been reported for other Na^+-binding proteins (27). The sequence of the Na^+-binding loop is absolutely conserved in thrombins from nine different species (28), which proves the importance of this structural domain in thrombin function and is similar to that observed in other proteases involved in blood coagulation. Hence, the Na^+-induced allosteric regulation discovered for thrombin represents a molecular strategy for specificity that may be shared by many other members of the cascade. Coagulation factors should be studied in the future with this important possibility in mind.

Identification of the Na^+-binding site of thrombin brings about the question of when this site is generated during the activation from prothrombin. The intermediate thrombin forms on the activation pathway from prothrombin have been carefully characterized by Doyle and Mann (29). Meizothrombin is the first active product of prothrombin activation under *in vivo* conditions. This intermediate is produced by a cleavage at the Arg-323 bond, which causes separation of the A and B chains of thrombin. A puzzling property of meizothrombin is that it binds TM and cleaves PC with high efficiency but does not bind and cleave fibrinogen (29). We speculate that this property may be the result of an incomplete formation of the Na^+-binding site. Meizothrombin would have its catalytic machinery stabilized in the slow form. Studies of the effect of Na^+ on meizothrombin should be done to test this hypothesis.

The discovery of thrombin as an allosteric enzyme, which has targeted its two forms toward procoagulant and anticoagulant activities, is a crucial step toward the development of more effective pharmacological strategies. The design of "allosteric effectors" of thrombin is a promising approach to reach a sustained, long-term control of thrombin activity in the blood, as demanded by several thrombotic and hemorrhagic disorders (30, 31). Slow-form stabilizers may act as anticoagulants, whereas fast-form stabilizers may act as procoagulants. Our findings, along with the recent structural identification of the Na^+-binding site and the ongoing work on site-directed mutants of this site and the structure of the slow form, have set the stage for a realistic pursuit of this exciting goal.

We are grateful to Dr. Alexander Tulinsky for sharing unpublished results on the Na^+-binding site of thrombin and for providing the coordinates of several thrombin structures. This work was supported, in part, by National Institutes of Health Grant HL49413, National Science Foundation Grant MCB94-06103, and by grants from the American Heart Association and the Monsanto-Searle Company. E.D.C. is an Established Investigator of the American Heart Association and Genentech.

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