Substrate-Assisted Catalysis of the PAR1 Thrombin Receptor
ENHANCEMENT OF MACROMOLECULAR ASSOCIATION AND CLEAVAGE*

Suzanne L. Jacques, Meredith LeMasurier, Paul J. Sheridan, Stacy K. Seeley, and Athan Kuliopulos‡

From the Molecular Cardiology Research Institute, Division of Hematology/Oncology, New England Medical Center and Departments of Medicine and Biochemistry, Tufts University School of Medicine, Boston, Massachusetts 02111

Platelet activation and aggregation are mediated by thrombin cleavage of the exodomain of the PAR1 receptor. The specificity of thrombin for PAR1 is enhanced by binding to a hirudin-like region (Hir) located in the receptor exodomain. Here, we examine the mechanism of thrombin-PAR1 recognition and cleavage by steady-state kinetic measurements using soluble PAR1 N-terminal exodomains. We determined that the primary role of the PAR1 Hir sequence is to reduce the kinetic barriers to formation of the docked thrombin-PAR1 complex rather than to form high affinity ground-state interactions. In addition, the exosite I-bound Hir motif facilitates the productive interaction of the PAR1 \(^{30}\)LDPR/SFL \(^{44}\) sequence with the active site of thrombin. This locking process is the most energetically unfavorable step of the overall reaction. The subsequent irreversible steps of peptide bond cleavage are rapid and allosterically enhanced by the presence of the docked Hir sequence. Furthermore, the C-terminal exodomain product of thrombin cleavage, corresponding to the activated receptor, binds tightly to thrombin. This would suggest that an additional role of the Hir sequence in the thrombin-activated receptor is to sequester thrombin to the platelet surface and modulate cleavage of other platelet receptors such as the PAR4 thrombin receptor, which lacks a functional Hir sequence.

Cellular responses to thrombin are regulated by a novel class of seven transmembrane protease-activated receptors (PARs).\(^1\) PAR1 plays an important role in platelet activation and blood coagulation and has been implicated in the pathological processes leading to heart disease and stroke (1). PAR1 and PAR4 mediate platelet aggregation in humans, whereas PAR3 and PAR4 are responsible for platelet aggregation in mice (2–4). PAR1 and PAR4 have markedly different kinetics of activation by thrombin that contribute to their distinct roles in signaling processes leading to heart disease and stroke (1). PAR1 and PAR4 have markedly different kinetics of activation by thrombin that contribute to their distinct roles in signaling processes leading to heart disease and stroke (1).

Thrombin activates PAR1 by binding and cleaving the N-terminal exodomain at LDPR \(^{41}\) \(\rightarrow\) SFL \(^{42}\) generating a new N-terminus (SFLRRN). The new N-terminus functions as a tethered ligand, which activates PAR1 by binding to the body of the receptor (8, 9). The N-terminal exodomain also contains a sequence, K \(^{51}\)YPF \(^{55}\) (Hir), that resembles the C-tail of the leech anti-coagulant protein, hirudin. The PAR1 Hir sequence interacts with exosite I of thrombin as indicated by x-ray structural analysis (10). Mutagenesis studies indicate that the presence of the Hir sequence in the PAR1 exodomain confers significant enhancements in the efficiency of thrombin cleavage (11–13). In analogy to the dual interactions of hirudin and serpins with thrombin (14, 15), it is possible that the Hir sequence may increase the probability that productive orientations occur at the active site by anchoring the LDPR region and by an induced fit mechanism. Thus, PAR4, which lacks a functional Hir sequence (16), is activated 20- to 70-fold slower by thrombin than PAR1 on human platelets (5).

An unusual feature of thrombin-PAR interactions is that stoichiometric amounts of thrombin, rather than catalytic amounts, are required for platelet activation (5). This raises the possibility that the low turnover of thrombin could be due to tight binding and a slow off-rate from cleaved receptors. However, since fast enzymic reactions are not favored by strong ground-state interactions (17, 18), it is paradoxical that thrombin activation of PAR1 on platelets is extremely rapid (5). Indeed, previous studies (19) established that the N-terminal exodomain of PAR1 (TR78) expressed in soluble form is also cleaved with high efficiency by thrombin.

In this paper, we examine the mechanism of thrombin-PAR1 recognition and cleavage by conducting steady-state kinetic measurements with soluble PAR1 N-terminal exodomains. We determined that the PAR1 Hir sequence is essential for rapid association with thrombin to form a Hir-docked complex and does not provide tight ground-state binding. The subsequent step, whereby the LDPR/SFL sequence locks into the active site of thrombin, presents the highest energy barrier to the overall reaction. Peptide bond cleavage at Arg\(^{41}\)–Ser\(^{42}\) is allosterically enhanced by the docked Hir sequence. Interestingly, the cleaved exodomain product, TR62, which retains the Hir sequence, binds tightly to thrombin. Thus, the Hir sequence may play an additional role in preserving thrombin association with the cleaved PAR1 receptor, consequently favoring a low turnover of thrombin and tethering the protease near adjacent naive PAR receptors on the platelet surface.

EXPERIMENTAL PROCEDURES

Materials—Pure human z-thrombin was obtained from Haematologic Technologies (Essex Junction, VT) (specific activity = 3290 NIH units/mg). CBS 34.47 (CBS), H-D-cyclohexylglycyl-l-\(\alpha\)-aminobutyryl-l-arginine-p-nitroanilide; CBS, CBS 34.47 chromogenic substrate for thrombin; PBS, phosphate-buffered saline; PEG, polyethylene glycol.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Supported by National Institutes of Health Grant R01HL57905 and by Scholar Awards from the Pew Scholars Program in the Biomedical Sciences and from the American Society of Hematology. To whom correspondence should be addressed: Fax: 617-636-4833; E-mail: akuliopu@opal.tufts.edu.
subcloning the TR78 encoding the expression plasmid pET31MØTR78. This plasmid was generated by the Tufts University School of Medicine Peptide Core Facility. TR78 was produced using *E. coli* and Smith (21). The sequences encoding the exodomains were subdigested with NcoI and ligated to the NcoI restriction fragment from pET22KTRMH-D and pET22KTRMH-Hir1 and ligated to the NcoI restriction sites to generate the N-terminal product (TR16) at 222 nm. TR16 was completely resolved from undigested exodomain. Initial rates were fit to the Michaelis-Menten equation (Eq. 1) by non-linear least-squares regression to generate the kinetic parameters $k_{cat}$ and $K_m$.

$K_{cat} = k_{cat}S/(K_m + S)$  

$K_m = k_{cat}S/(K_m + S)$

**Kinetics of Thrombin Cleavage of Soluble PAR1 Exodomains**—Thrombin cleavage of PAR1 exodomains was monitored by reverse-phase high pressure liquid chromatographic analysis. Identity of peptide fragments was confirmed by matrix-assisted laser desorption ionization mass spectrometry. Lyophilized domains were reconstituted in 20 mM potassium phosphate, pH 7.5, and 150 mM NaCl (PBS), and the concentration was determined by UV absorption ($\varepsilon_{278}$ (TR78) = 15,200 M$^{-1}$ cm$^{-1}$; $\varepsilon_{278}$ (TR78Hir) = 13,800 M$^{-1}$ cm$^{-1}$; $\varepsilon_{278}$ (TR78R41Q) = 8300 M$^{-1}$ cm$^{-1}$; $\varepsilon_{278}$ (TR26) = 6900 M$^{-1}$ cm$^{-1}$). Cleavage assays containing 5–570 nM exodomain in PBS were conducted at 37 °C and were initiated by the addition of human α-thrombin freshly diluted in ice-cold PBS/0.1% PEG 8000 (final thrombin concentration = 60 μM). PEG 8000 was included in the thrombin diluent to eliminate nonspecific binding of thrombin to the walls of the polystyrene tubes (22, 23). The final concentration of PEG 8000 in all of the assays was 0.009%.

Initial rates ($k_{obs}$) were obtained by analyzing base-quenched samples taken at early time points where the progress curves were linear. Quantitation of cleavage products was performed by integrating peak areas and comparing them with calibration curves of fully digested samples (19). Thrombin cleavage of TR78, TR78HirAla, and TR26 was monitored by monitoring the generation of the C-terminal product (TR62, TR62HirAla, and TR20, respectively) at 278 nm. Thrombin cleavage of TR78HirAla and TR59 was monitored by measuring the kinetic parameters $k_{cat}$ and $K_m$ to the Michaelis-Menten equation (Eq. 1) by non-linear least-squares regression to generate the kinetic parameters $k_{cat}$ and $K_m$.

$K_{cat} = k_{cat}S/(K_m + S)$  

$K_m = k_{cat}S/(K_m + S)$

**Kinetics of Thrombin Cleavage of Soluble PAR1 Exodomains**—Cleavage of chromogenic substrates was determined by continuously monitoring the increase in absorbance at 405 nm at 37 °C in a 96-well format using a SPECTRAMax 340 microplate spectrophotometer. Cleavage assays were comprised of 5–400 μM substrate (CBS, H$_2$N-LDPR-pNA, or N-acetyl-LDPR-pNA) in 20 mM Tris-HCl, pH 8.3, and 150 mM NaCl (TBS) and were initiated by the addition of thrombin freshly diluted in ice-cold TBS/0.1% PEG 8000 (final thrombin concentration = 208 μM). Initial rates were determined by taking the initial slopes of the progress curves using SOFTmax PRO version 2.1. Quantification of the p-nitroaniline product was determined with the extinction coefficient ($\epsilon_{405}$ = 3800 M$^{-1}$ cm$^{-1}$), and data were fit to the Michaelis-Menten equation. Inhibition of thrombin cleavage of CBS was performed as above, with the exception that PAR1 exodomains were added at four to five different concentrations ranging from 5 to 300 nM, and assays were performed at 24 °C. Inhibition data were tested against 12 inhibition models (linear, hyperbolic, and parabolic models of competitive, noncompetitive, uncompetitive, and full-noncompetitive inhibition) by non-linear least-squares regression analysis. The model of best fit was determined by visual inspection of the slope and intercept replots, by the “goodness of fit” criterion, which is a normalized Akaike information criterion test, using EZFit 5.0 (Porelli Scientific, Inc., Amherst, NH) and by F-test using Graft 3.0 (Erithacus Software Ltd., Staines, UK). Three patterns...
emerged from the inhibition of CBS cleavage by PAR1 exodomains: linear competitive (C) inhibition represented by Eqs. 2 and 3, linear noncompetitive (NC) inhibition, represented by Eqs. 4 and 5, and linear uncompetitive (UC) inhibition, represented by Eqs. 6 and 7:

\[ k_{\text{obs}} = k_{\text{cat}} S / (K_s + S) \] (Eq. 2)

\[ k_{\text{obs}} = k_{\text{cat}} S / (K_s + S) \] (Eq. 3)

where \( I \) is inhibitor concentration, \( K_s \) is the slope inhibition constant, and \( K_c \) is the intercept inhibition constant.

Effect of Solution Viscosity on PAR1 Exodomain Cleavage by Thrombin—Assays were performed as above in the presence of 0–30% sucrose by relative viscosity. Substrate stickiness was determined by plotting substrate prior to conversion to product, will decrease relative to PBS alone for the PAR1 exodomains or TBS alone for the chromogenic substrates in quadruplicate using an Ostwald viscometer. Initial velocities were fitted to the Michaelis-Menten equation, and the kinetic parameters were analyzed as a function of relative viscosity. Because solution viscosity affects the rates of diffusion-controlled steps, such as substrate association and dissociation, one can define the parameters shown in the following one-substrate reaction:

\[ k_{\text{obs}} = k_{\text{cat}} S / (K_s + S) \] (Eq. 4)

\[ k_{\text{obs}} = k_{\text{cat}} S / (K_s + S) \] (Eq. 5)

\[ E + S \rightleftharpoons ES \rightarrow E + P \] (Eq. 6)

\[ E + S \rightleftharpoons ES \rightarrow E + P \] (Eq. 7)

where \( k_s \) is the rate constant of substrate association, \( k_d \) is the rate of substrate dissociation, \( k_s \) is the rate of conversion to product, E is enzyme, S is substrate, \( K_{es} \) is the enzyme-substrate complex, and P is product. Ideally, rates of steps not controlled by diffusion such as substrate association and dissociation, one can define the parameters shown in the following one-substrate reaction:
where the slope, \( \frac{k_{\text{cat}}(K_m)^n}{K_m} = 1 + k_2/k_1 \), is equal to 1 for a sticky substrate and 0 for a nonsticky substrate, and \( (k_{\text{cat}}/K_m) \) is determined in the absence of viscogen. The “stickiness” ratio \( (S_p = k_2/k_1) \) approaches zero for a nonsticky substrate, whereas it is large for sticky substrates. Association rate constants and \( S_p \) were determined by linear regression analysis of the viscosity data of fits to Eq. 10.

\[
\frac{1}{\eta_{\text{rel}}} = \frac{k_{\text{cat}}/K_m}{(k_{\text{cat}}/K_m)} + \frac{(k_2/k_1)/k_3}{h_{\text{rel}}}, \tag{10}
\]

**RESULTS**

The PAR1 Hirudin-like Sequence Increases the Rates of Macromolecular Association and Cleavage by Thrombin—To determine the mechanism by which the Hir sequence enhances thrombin-PAR1 interactions, we measured the effects of the Hir sequence on steady-state kinetic parameters and substrate association/dissociation rates using an array of soluble PAR1 exodomain mutants (Fig. 1). The full-length PAR1 exodomain, TR78 (Ala26-Thr102), is an effective substrate for thrombin with a \( k_{\text{cat}} \) of 58 s\(^{-1} \) and a \( K_m \) of 26 \( \mu \)M (Table I). Deletion of the 51-KEYPF55 Hir sequence (TR78Har) or substitution with 51-AAAAK55 (TR78HarAla) causes an 8- to 30-fold decrease in catalytic efficiency \( k_{\text{cat}}/K_m \). The \( K_m \) for both Hir mutants increased by 3-fold, whereas \( k_{\text{cat}} \) decreased by 2.6-fold for TR78Har and by 10-fold for TR78HarAla.

The contribution of the Hir sequence to the binding events preceding PAR1 exodomain cleavage was obtained from the effects of viscosity on the reaction kinetics. Most strikingly, the viscosity experiments revealed that the association rate constant \( k_1 \) for TR78Har is 73-fold slower than the \( k_1 \) for wild-type TR78 (Table II). We also determined the effect of the Hir sequence on the stickiness of the PAR1 exodomain to thrombin. A sticky substrate is a substrate with a slow rate of dissociation \( k_{\text{cat}}/K_m \) compared with the forward rate of conversion to product \( k_1 \) (Eq. 8). As shown in Fig. 2, the catalytic efficiency for thrombin cleavage of TR78 was not significantly affected by changes in relative viscosity as illustrated by the slope of \( (k_{\text{cat}}/K_m)/K_m \) versus \( \eta_{\text{rel}} \). These data were plotted as a linear regression analysis of the data. Each data point is a least-squares fit of the data. Each data point is from \( k_{\text{cat}}/K_m \) values derived from five different substrate concentrations.

**Thrombin-Binds Tightly to the Cleaved PAR1 Exodomain Product**—The events following PAR1 cleavage by thrombin are germane to thrombin-platelet interactions, because the Hir sequence remains behind as part of the cleaved PAR1 receptor. Thus, following proteolysis and dissociation of the N-terminal PAR1 cleavage fragment TR16, thrombin may remain bound to the cleaved PAR1 and kept in proximity to adjacent naive PAR1 and PAR4 receptors on the platelet surface. To determine the affinity of thrombin for the two TR78 cleavage products, TR16 (Ala26-Arg102) and TR62 (Ser42-Thr102), inhibition of cleavage of a small thrombin-optimized chromogenic substrate (CBS) was measured (Table III). The N-terminal cleavage product, inhibition kinetics, because the large concentrations of sucrose may lead to nonspecific effects such as changes in dielectric constant and volume exclusion. However, since sucrose had no effect on the cleavage rates of wild-type TR78, \( H_2N-\text{LDPR-pNA} \) and N-acetyl-LDPR-pNA peptides are surprisingly good substrates for thrombin with catalytic efficiencies similar to TR78 and 3- to 5-fold higher than those exhibited by the P3-P3 peptide DPR/SFL (Table I). The \( k_{\text{cat}} \) values (120 s\(^{-1} \) for thrombin cleavage of the LDPR substrates are 2-fold larger than for TR78 and \( K_m \) values (66–113 \( \mu \)M) are similar to TR78Har and TR78HarAla. Although these minimal LDPR substrates lack the Hir sequence, they demonstrate rapid rates of association and dissociation with thrombin and are nonsticky substrates. Association rate constants for the LDPR substrates obtained from viscosity measurements are 5-fold faster relative to TR78 and 330- to 460-fold faster relative to TR78Har (Table II). The 16-fold size difference between LDPR-pNA and TR78 predicts a 2.4-fold difference in rates of association and dissociation. Therefore, appending a large exodomain to the LDPR sequence dramatically decreases the rate of productive binding of the P3-P3 residues to the active site (the locking step) unless offset by prior docking via the Hir sequence in wild-type PAR1.
uct, TR16, was unable to inhibit thrombin at concentrations as high as 290 μM despite containing the LDPR sequence. In contrast, the C-terminal cleavage product, TR62, corresponding to the activated PAR1 receptor, is an excellent inhibitor of thrombin with a $K_i$ of 9.4 μM. Because TR62 lacks P$_1$-$P_2$ active site binding residues, the competitive pattern of inhibition (Eq. 7) indicates that the Hir sequence of TR62 binds to exosite I of thrombin and allosterically inhibits cleavage of the CBS substrate. Indeed, TR78HirAla is only able to inhibit the active site of thrombin with a $K_i$ of 35 μM in a competitive pattern (Eq. 3), and TR78ΔHir does not inhibit cleavage of CBS at 105 μM (Table III). The lower affinity of TR78HirAla for thrombin may be a result of repulsive forces caused by the shifted WEDEE residues. Therefore, the presence of the exosite I-binding Hir motif is required for the formation of an ESI complex and inhibition of CBS cleavage.

The C-Terminal Region of the PAR1 Exodomain Modulates Thrombin Binding to the LDPR and Hir Regions—Results from previous studies (12, 25) indicate that small PAR1 exodomain fragments appear to be better substrates for thrombin than the full-length TR78 exodomain. Furthermore, removal of the Hir sequence produces surprisingly more severe effects on the cleavage efficiency of short PAR1 substrates lacking residues to the C-terminal side of the Hir sequence (TR28–60 versus TR28–45) as compared with the larger TR78 (Table I). These results suggest that the C-terminal half of the PAR1 exodomain modulates interactions with thrombin. Hence, we investigated the role of the C-terminal region of the exodomain (Fig. 1) on the kinetics of thrombin cleavage. We compared the catalytic efficiency of full-length PAR1 exodomain, TR78, to those of C-terminally truncated domains TR59 (Ala$^{26}$-Leu$^{84}$) and TR26 (Ala$^{36}$-Glu$^{60}$), a minimal PAR1 exodomain analogous to TR28–60 (12). As shown in Fig. 3, TR59 and TR26 are cleaved ~2-fold faster than TR78 with 2- to 5-fold lower $K_m$ values (Table I). The $k_{cat}/K_m$ of TR26 cleavage is only 1.5-fold faster than the reported cleavage of TR28–60 (12), attesting to the reproducibility of these cleavage studies using soluble exodomains. Altogether, deletion of the C-terminal portion of the PAR1 exodomain results in a 4- to 9-fold increase in $k_{cat}/K_m$. Because no further increase in cleavage rate was seen with TR26 as compared with TR59, we can conclude that the increase in catalytic efficiency is due to the removal of the C-terminal residues Pro$^{85}$-Thr$^{102}$.

To determine whether the C-terminal region was directly inhibiting thrombin, the peptide LBS-1, which contains residues Pro$^{85}$-Leu$^{96}$ (Fig. 1), was tested for inhibition of CBS or TR78 cleavage. This region comprises a portion of the intramolecular ligand binding site as shown by mutagenesis (8, 9) and NMR studies. The LBS-1 peptide did not inhibit thrombin activity at 100–300 μM concentrations. Because the C-terminal region of the PAR1 exodomain does not directly inhibit thrombin cleavage, it is likely that this region confers additional structural constraints when bound to thrombin, which are lacking in the smaller exodomains TR26 and TR59.

To further delineate the linkage between the C-terminal residues and the Hir sequence, the ability of the truncated PAR1 exodomains to inhibit cleavage of the chromogenic substrate, CBS, was examined. TR78, TR59, and TR26 are all potent noncompetitive inhibitors of thrombin activity with similar inhibition constants ($K_i = 2.3–7.6$ μM) due to formation of an EI complex with Hir and LDPR regions bound to thrombin (Table III). The noncompetitive patterns of inhibition (Eq. 5) reflect an additional ESI complex ($K_{ia} = 7.6–21$ μM) with simultaneous binding of CBS at the active site and the Hir-containing PAR1 exodomains at exosite I of thrombin. However, a point mutation (R41Q) at the P$_1$ site of TR26 causes a 25-fold loss in binding ($K_{ia}$) versus only 1.3- to 1.5-fold loss due to mutation of the P$_1$ site in the full-length exodomain (TR78R41Q, TR78R41S). Therefore, the R41Q mutation at the crucial P$_1$ position disrupts thrombin binding to the C-terminally deleted TR26 PAR1 fragment but does not appreciably affect binding in the context of full-length TR78. This confirms that residues located to the C-terminal side of the Hir region in the PAR1 exodomain provide hitherto unappreciated binding/structural determinants for thrombin complexation and cleavage of PAR1.

**DISCUSSION**

Thrombin has many substrates and inhibitors, including fibrinogen, prothrombin, protein C, antithrombin III, α$_2$-macroglobulin, and factors V, VII, XI, and XIII, that circulate at concentrations ranging from 30 nM to 10 μM (26). By comparison, PAR1 is a rare substrate for thrombin with only 600–1800 receptors present on the surface of each platelet (27, 28). During the time period prior to clot formation, the prothrombinase complex on the surface of platelets generates 2–15 nM thrombin in whole blood (29). Thus, to successfully compete for these low levels of thrombin generated *in situ*, PAR1 must increase the probability that collision between these macromolecules leads to productive binding and cleavage on the surface of platelets.

---

*S. Seeley, J. Baleja, and A. Kuliopulos, unpublished results.*
Fig. 3. Effect of deletion of the C terminus of PAR1 exodomain on thrombin cleavage. Initial velocity ($k_{\text{obs}}$) of thrombin cleavage was obtained for various concentrations of TR78, TR59, and TR26 as described under "Experimental Procedures." The curves represent the best fits to Eq. 1 by nonlinear least-squares regression analysis.

Fig. 4. Energetics of thrombin interactions and cleavage of PAR1. A two-step dock and lock mechanism is shown for thrombin cleavage of PAR1 on platelets for wild-type (WT-middle) and ΔHir (bottom) receptors. The individual steps of the dock and lock mechanism are illustrated as an energy diagram (top) and are aligned with the mechanisms below. The energy diagram was constructed using Gibb's free energies of activation ($\Delta G^\ddagger_T$) calculated from the equation $\Delta G^\ddagger_T = RT \ln (kT h)/\ln (k)$ (17), using the kinetic parameters $k_{\text{cat}}/K_m$ and $k_{\text{cat}}$ and the individual rates shown in Schemes 1 and 2 for TR78 and TR78ΔHir, respectively. The rate constants of Schemes 1 and 2 were determined as follows: Association rates constants ($k_a$) for TR78 and TR78ΔHir were directly determined in viscosity studies and correspond to $k_1$ and $k_2$, respectively. The maximal cleavage rate ($k_{\text{cat}}$) of TR78 by thrombin is determined by both $k_2$ and $k_3$, whereas it is equal to $k_3$ for TR78ΔHir. As an initial estimate, we assumed that $k_2$ and $k_3$ contribute equivalently to $k_{\text{cat}}$ for TR78 ($k_2 = k_3 = 116 \text{ s}^{-1}$) and that the stickiness ratio ($S_r = 0.12$) is equal to $k_2/k_1$ ($k_1 = 967 \text{ s}^{-1}$). The remaining value, $k_3$, for TR78 and TR78ΔHir was determined by fitting the initial rate data (i.e. Fig. 3) to Michaelis-Menten equation where: $K_m = (k_3/k_2 + k_3/k_1)$ for TR78ΔHir and $K_m = (k_3/k_2 + k_3/k_1)$ for TR78 (21), allowing the other parameters to float from their initial values. The thick lines represent the energetics for WT PAR1. The dotted lines represent the energetics for ΔHir PAR1. The concentration scale on the left depicts the energies of the initial substrate concentrations. $\Delta G^\ddagger_T$ values for H2N-LDPR-pNA (L) were determined using kinetic constants from Tables I and II. $\Delta G^\ddagger_T$ values for fibrinogen (F) were determined from $k_1$ and $K_1$, at a plasma concentration of 10 μM (26, 34). Stopped-flow fluorescence studies resolved the process of association of hirulog to exosite I and the active site of thrombin into four separate steps (33). $\Delta G^\ddagger_T$ for hirulog (H) was determined using $k_1$ and $k_1$, from the four-step mechanism, and $k_3$ was approximated by the slowest of the last three steps (30 s $^{-1}$).
Thrombin-PAR1 Dock and Lock Mechanism

Platelets. Adding to the problem of low abundance, several lines of experimental evidence indicate that the PAR1 exodomain is conformationally mobile. For instance, thrombin-activated PAR1 is able to donate its tethered ligand to adjacent PAR1 receptors and activate them by an intermolecular liganding mechanism (30). Protease-susceptibility studies (19, 31) and NMR structural data also indicate that the PAR1 exodomain is expected to form productive complexes more slowly with thrombin as a result of high entropic barriers due to enhanced rotational and translational degrees of freedom. Our data demonstrate that PAR1 uses the Hir motif in a “dock and lock” mechanism (Fig. 4) to overcome the entropic and kinetic barriers leading to formation of productive thrombin-PAR1 complexes.

The dock and lock mechanism describes the two-step binding of PAR1 to thrombin. The mechanism is based in part on previous x-ray crystallographic (10) and kinetic studies (11, 12), which showed that thrombin interacts with the PAR1 exodomain substrate at two distant and distinct sites. Thus, in addition to binding to the active site, TR78 is a noncompetitive inhibitor of CBS cleavage as a result of binding to exosite I of thrombin. Hirudin and a related peptide, hirulog, were shown by pre-steady-state kinetics to bind thrombin in an ordered mechanism, where the C-terminal region binds first to exosite I of thrombin followed by binding of the N-terminal region to the active site of thrombin (32, 33).

Based on these observations, the interactions of the PAR1 exodomain with thrombin can be described most simply by a two-step binding model (Scheme 1). In contrast, the interaction of the Hir-deleted PAR1 exodomain (TR78A Hir) with thrombin is better described by a model comprised of a single binding event (Scheme 2), because this exodomain does not bind exosite I of thrombin. As shown in Scheme 1, TR78 rapidly associates with thrombin to form the Hir-docked complex. The docked exodomain is not sticky and dissociates 8-fold faster from thrombin with fibrinogen (F) and hirulog (H) are shown using data acquired under similar conditions (32, 34). A Gibbs free energy scale for 1 nM to 1 M free substrate concentration is shown at the left of Fig. 4. This energy scale illustrates the advantage that fibrinogen has over PAR1 as a competing substrate for thrombin. Thus, the energy barrier to association and formation of a docked complex is at least 5.6 kcal/mol higher for PAR1 (~1 nM) relative to fibrinogen (10 μM) assuming equal partitioning of thrombin between the platelet surface and the fluid phase (29). Quite remarkably, the ΔG‡ for the barrier to association of thrombin to TR78 and fibrinogen are nearly identical, suggesting similar docking mechanisms to their respective Hir-like sequences. Hirulog, on the other hand, associates 21-fold faster than TR78 and has a lower barrier to formation of the docked complex. Once docked to thrombin, WT TR78 lowers the barrier to formation of the LDPR-locked complex by starting from an elevated ground-state and by lowering the transition-state energy of locking. Deletion of the Hir sequence eliminates the first docking step and forces the ΔHir mutant to climb a much higher energy barrier to formation of the LDPR-bound complex. The locking step presents the highest energy barrier for both WT and ΔHir exodomains during the entire cleavage process. Subsequent steps of irreversible cleavage are energetically favorable and help drive the overall cleavage process forward.

Intriguingly, the cleaved exodomain product, TR62, which retains the Hir sequence, binds relatively tightly to thrombin (Kii = 9.4 μM) as a first indication that thrombin may remain transiently associated with cleaved PAR1 receptor on the surface of platelets. These persistent interactions might be employed to keep thrombin tethered to the platelet surface and sequestered from antithrombin molecules present in the fluid phase (α2-macroglobulin, heparin II cofactor, antithrombin III, and heparin). Too high of an affinity for the PAR1 Hir sequence might be detrimental and could seriously impair proteolysis of other thrombin substrates and further reduce thrombin turnover (35). Therefore, the macromolecular interactions of thrombin with PAR1 must strike a balance between the opposing demands of specificity and speed.

Acknowledgments—We thank Odessa Yabut for her assistance in plasmid construction and purification of the TR59 exodomain and the members of the Kuliopulos laboratory for critically reading the manuscript.

REFERENCES
1. Vu, T.-K. H., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) Cell 64, 1057–1068
2. Xu, W.-F., Andersen, H., Whitmore, T. E., Presnell, S. R., Yee, D. P., Ching, A., Gilbert, T., Davie, E. W., and Foster, D. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6642–6646
3. Kahn, M. L., Zheng, Y.-W., Huang, W., Biggins, V., Zheng, D., Molf, S., Fares, E. V., Tam, C., and Coughlin, S. R. (1998) Nature 394, 690–694
4. Kahn, M. L., Nakaniishi-Matsui, M., Shapiro, M. J., Ishihara, H., and Coughlin, S. R. (1999) J. Clin. Invest. 103, 879–887
5. Covic, L., Gresser, A. L., and Kuliopulos, A. (2000) Biochemistry 39,
6. Steinhoff, M., Vergnolle, N., Young, S. H., Tognetto, M., Amadesi, S., Ennes, H. S., Trevisani, M., Hollenberg, M. D., Wallace, J. L., Caughey, G. H., Mitchell, S. E., Williams, L. M., Gepptetti, P., Mayer, E. A., and Bunnett, N. W. (2000) Nat. Med. 6, 151–158
7. Miyata, S., Koshikawa, N., Yasumitsu, H., and Miyazaki, K. (2000) J. Biol. Chem. 275, 4592–4596
8. Bahou, W. F., Kutok, J. L., Wong, A., Potter, C. L., and Coller, B. S. (1994) Blood 84, 4195–4202
9. Nanavicius, T., Ishii, M., Wang, L., Chen, M., Chen, J., Turk, C. W., Cohen, F. E., and Coughlin, S. R. (1995) J. Biol. Chem. 270, 21619–21625
10. Matthews, I. I., Padmanabhan, K. P., Ganesh, V., Tulinsky, A., Ishii, M., Chen, J., Turk, C. E., Coughlin, S. R., and Fenton, J. W. (1994) Biochemistry 33, 3266–3279
11. Liu, L. W., Yu, T., Esmon, C. T., and Coughlin, S. R. (1991) J. Biol. Chem. 266, 10977–10980
12. Vu, T. R. H., Wheaton, V. I., Hung, D. T., Charo, I., and Coughlin, S. R. (1991) Nature 353, 674–677
13. Ishii, K., Gerszten, R., Zheng, Y. W., Welsh, J. B., Turk, C. W., and Coughlin, S. R. (1995) J. Biol. Chem. 270, 16435–16440
14. Stone, S. R., and Hermans, J. M. (1995) Biochemistry 34, 5164–5172
15. Kuliopulos, A., Mildvan, A. S., Record, M. T., and DiCera, E. (1995) J. Biol. Chem. 273, 197–198
16. Nakashima-Matsui, M., Zheng, Y. W., Sulciner, D. J., Weiss, E. J., Ludeman, M. J., and Coughlin, S. R. (2000) Nature 404, 609–613
17. Fersht, A. (1985) Enzyme Structure and Mechanism, pp. 121–346, W. H. Freeman and Co., New York
18. Kuliopulos, A., Walsh, C. T. (1994) J. Am. Chem. Soc. 116, 4599–4607
19. Kuliopulos, A., Talalay, P., and Mildvan, A. S. (1990) Biochemistry 29, 10271–10280
20. Wasiadowski, W., Basco, M. D., Martin, B. M., Detweiler, T. C., and Fenton, J. W. (1976) Thromb. Res. 8, 881–886
21. Kuliopulos, A., Talalay, P., and Mildvan, A. S. (1990) Biochemistry 29, 10271–10280
22. Kuliopulos, A., and Walsh, C. T. (1994) J. Am. Chem. Soc. 116, 4599–4607
23. Kuliopulos, A., Talalay, P., and Mildvan, A. S. (1990) Biochemistry 29, 10271–10280
24. Fersht, A. (1985) Enzyme Structure and Mechanism, pp. 121–346, W. H. Freeman and Co., New York
25. Kuliopulos, A., and Walsh, C. T. (1994) J. Am. Chem. Soc. 116, 4599–4607
26. Kuliopulos, A., Walsh, C. T. (1994) J. Am. Chem. Soc. 116, 4599–4607
27. Kuliopulos, A., Talalay, P., and Mildvan, A. S. (1990) Biochemistry 29, 10271–10280
28. Kuliopulos, A., and Walsh, C. T. (1994) J. Am. Chem. Soc. 116, 4599–4607
29. Kuliopulos, A., Walsh, C. T. (1994) J. Am. Chem. Soc. 116, 4599–4607
30. Kuliopulos, A., Walsh, C. T. (1994) J. Am. Chem. Soc. 116, 4599–4607
31. Kuliopulos, A., Walsh, C. T. (1994) J. Am. Chem. Soc. 116, 4599–4607
32. Kuliopulos, A., Walsh, C. T. (1994) J. Am. Chem. Soc. 116, 4599–4607
33. Kuliopulos, A., Walsh, C. T. (1994) J. Am. Chem. Soc. 116, 4599–4607
34. Kuliopulos, A., Walsh, C. T. (1994) J. Am. Chem. Soc. 116, 4599–4607
35. Kuliopulos, A., Walsh, C. T. (1994) J. Am. Chem. Soc. 116, 4599–4607
36. Kuliopulos, A., Walsh, C. T. (1994) J. Am. Chem. Soc. 116, 4599–4607
37. Kuliopulos, A., Walsh, C. T. (1994) J. Am. Chem. Soc. 116, 4599–4607
38. Kuliopulos, A., Walsh, C. T. (1994) J. Am. Chem. Soc. 116, 4599–4607
39. Kuliopulos, A., Walsh, C. T. (1994) J. Am. Chem. Soc. 116, 4599–4607
40. Kuliopulos, A., Walsh, C. T. (1994) J. Am. Chem. Soc. 116, 4599–4607