The Affinity of Protein C for the Thrombin-Thrombomodulin Complex Is Determined in a Primary Way by Active Site-dependent Interactions*

Received for publication, January 24, 2005, and in revised form, February 8, 2005
Published, JBC Papers in Press, February 10, 2005, DOI 10.1074/jbc.M500881200

Genmin Lu‡, Sotheavy Chhum‡, and Sriram Krishnaswamy‡§¶

From the ‡Joseph Stokes Research Institute, Children's Hospital of Philadelphia and the §Department of Pediatrics, University of Pennsylvania, Philadelphia, Pennsylvania 19104

The interaction of thrombin (IIa) with thrombomodulin (TM) is essential for the efficient activation of protein C (PC). Interactions between PC and extended surfaces, likely contributed by TM within the IIa-TM complex, have been proposed to play a key role in PC activation. Initial velocities of PC activation at different concentrations of PC and TM could be accounted for by a model that did not require consideration of direct binding interactions between PC and TM. Reversible inhibitors directed toward the active site of IIa within the IIa-TM complex behaved as classic competitive inhibitors of both peptidyl substrate cleavage as well as PC activation. The ability of these small molecule inhibitors to block PC binding to the enzyme points to a principal role for active site-dependent substrate recognition in determining the affinity of IIa-TM for its protein substrate. Selective abrogation of active site docking by mutation of the P1 Arg in PC to Gln yielded an uncleavable derivative (PCR15Q). PCR15Q was a poor inhibitor (Ki ≈ 30 μM) of PC activation as well as peptidyl substrate cleavage by IIa-TM. Thus, inhibition by PCR15Q most likely results from its ability to weakly interfere with active site function rather than by blocking extended interactions with the enzyme complex. The data suggest a primary role for active site-dependent substrate recognition in driving the affinity of the IIa-TM complex for its protein substrate. Interactions between PC and extended surfaces contributed by IIa and/or TM within the IIa-TM complex likely contribute in a secondary or minor way to protein substrate affinity.

Thrombin (IIa) plays a central role in the hemostatic process by catalyzing proteolytic reactions in both the procoagulant and anticoagulant pathways of blood coagulation (1–4). The range of procoagulant reactions catalyzed by IIa is largely accomplished with high catalytic efficiency without requiring a cofactor or accessory protein (3–5). In contrast, IIa function in the anticoagulant pathway requires the endothelial cell protein, thrombomodulin (TM) (2, 6). The binding of IIa to TM greatly enhances the ability of IIa to catalyze the activation of protein C (PC) while inhibiting the action of IIa on its procoagulant substrates (6–8). Therefore, TM plays an essential role in modulating IIa function and maintaining the hemostatic balance.

Structure-function studies and a series of x-ray structures have established a principal role for anion binding exosite I of IIa in interactions with its procoagulant substrates (4, 5). Inhibition of the action of IIa on these substrates can be explained by the intimate and occlusive contacts between TM and anion binding exosite I of IIa within the IIa-TM complex (5). This explanation also points to the utilization of different strategies in the recognition of the procoagulant substrates by IIa relative to the recognition of PC by the IIa-TM complex (6).

The mechanisms by which TM greatly enhances PC activation remain incompletely understood. The increased Vmax of PC activation following the interaction of IIa with TM has been attributed to a cofactor-mediated allosteric change in the active site of the proteinase (9, 10). A variety of lines of evidence, including mutagenesis studies, point to a significant contribution from TM-dependent changes in the active site of IIa to the Ca2+ dependence of PC activation and also potentially the enhanced function of the enzyme complex (11). However, a principal role for such allosteric phenomena in stabilizing the transition state is questioned by the finding that TM yields only minor effects on the hydrolysis of peptidyl substrates by IIa (12–14). Furthermore, the x-ray structure of IIa bound to a TM fragment has not yielded obvious evidence for an altered active site in comparison to free IIa (15).

Important contributions from binding interactions between PC and the fourth epidermal growth factor-like domain of TM have been proposed on the basis of the x-ray structure of IIa complexed with a TM fragment (15). This idea is supported by mutagenesis studies and the structural requirements for TM to bind IIa versus its ability to also enhance PC activation (6). The functional significance of this interaction has also been documented by charge reversal mutagenesis studies on putatively complementary interacting surfaces in TM and PC (16). These lines of evidence suggest that interactions between PC and extended surfaces within the enzyme complex represent a principal determinant of substrate affinity and IIa-TM function (15, 16).

This paper is available on line at http://www.jbc.org

* This work was supported by National Institutes of Health Grants HL-47465 and HL-74124 (to S. K.). 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.

To whom correspondence should be addressed: Joseph Stokes Research Institute, Children's Hospital of Philadelphia and the Department of Pediatrics, University of Pennsylvania, Philadelphia, Pennsylvania 19104

‡Joseph Stokes Research Institute, Children's Hospital of Philadelphia and the Department of Pediatrics, University of Pennsylvania, Philadelphia, Pennsylvania 19104

§Department of Pediatrics, University of Pennsylvania, Philadelphia, Pennsylvania 19104

¶From the Joseph Stokes Research Institute, Children's Hospital of Philadelphia and the Department of Pediatrics, University of Pennsylvania, Philadelphia, Pennsylvania 19104

The abbreviations used are: IIa, human α-thrombin; APC, activated protein C; BSA, bovine serum albumin; Gla, 4-carboxyglutamic acid; 125I-SNAP, N-2-dansyl-(p-guanidino)phenylalanineperidode hydrochloride; PAB, 4-aminobenzamidin; PC, human protein C; PCPL, human protein C isolated from plasma; PCR15Q, recombinant human protein C with P1 Arg mutated to Gln; PCTM, recombinant wild type human protein C; Pefablope 5P, 4-aminophenylpyruvate; FFPB-CH2-Cl, l-phenylalanil-l-prolyl-l-arginine chloromethylketone; S2288, H-n-isonucley-l-prolyl-l-arginyl-γ-nitroanilide; S2366, l-pyroglutamyl-l-prolyl-l-arginyl-γ-nitroanilide; sTM, recombinant soluble human thrombomodulin comprising residues 1–497 and lacking chondroitin sulfate; TM, human thrombomodulin; Bis-Tris, 2-[bis(2-hydroxyethyl)aminol]-2-(hydroxymethyl)propane-1,3-diol.
Recognition of Protein C by the Thrombin-Thrombomodulin Complex

However, the low affinity \((K_d \approx 10^{-4} \text{ M})\) measured for the binding of PC to a TM fragment questions the functional role of such an interaction.

We have studied PC activation by thrombin complexed with the soluble extracellular domain of TM to investigate the mechanisms underlying substrate recognition by the IIa-TM complex. We have applied related kinetic approaches to distinguish the relative contributions of active site docking versus binding of the protein substrate to extended surfaces within the kinetic complex for PC activation.

EXPERIMENTAL PROCEDURES

Materials—The peptideyl substrates H₃-isoleucyl-l-prolyl-l-arginyl-p-nitroanilide (S2288) and l-prolylarginyl-arginyl-p-nitroanilide (S2366) and inhibitor N²-dansyl-(g-guanido)-l-phenylalanine-piperidide (I2581) were from Chromogenix (West Chester, OH). Stock solutions were prepared in water, and concentrations were determined using \(E_{420} = 8,270 \text{ M}^{-1} \text{ cm}^{-1}\) for the substrates and 4,105 \text{ M}^{-1} \text{ cm}^{-1}\) for I2581 (18, 19). Stock solutions of 4-aminobenzamidine (PAB, Aldrich) were prepared in water, and concentrations were determined using \(E_{280} = 15,000 \text{ M}^{-1} \text{ cm}^{-1}\) (20). Pefabloc SP (4-aminophenylpyruvic acid) was a gift from Merck (Norwalk, CT), and L-arginine (p-toluenesulfonate) was from Calbiochem. Fresh solutions of Pefabloc SP (41 mM) were prepared in Me₂SO and diluted into buffer to yield working solutions. Unless otherwise noted, all kinetic measurements were performed at 25 °C in 20 mM Hepes, 150 mM NaCl, 2 mM Ca²⁺, 0.1% (v/v) polyethylene glycol-8000, 0.1% (w/v) BSA, pH 7.4 (Assay Buffer).

Proteins—The recombinant extracellular domain of human thrombomodulin (residues 1–497, sTM) and lacking chondroitin sulfate (21), was a gift from Bruce Gerlitz, Eli Lilly (Indianapolis, IN). Human prothrombin and PC were isolated using plasmapheresis plasma from the Phasmapheresis Unit of the Hospital of University of Pennsylvania as described (22). Thrombin was produced by proteolysis of prothrombin and purified as described (23). Protein C isolated from plasma (PC₃), was a gift from Dr. George Long, University of Vermont. This fragment was ligated into plasmid pcDNA3.1(−) (Stratagene, La Jolla, CA) and primers 5’-TGATGGGA-3’. Mutagenesis was done using the QuikChange mutagenesis kit (Stratagene). PCR products were isolated from the venom of Puffinid spider (H. venator) using the QuikChange kit (Stratagene) equilibrated in the same buffer. Fractions containing APC were pooled and precipitated with 80% (NH₄)₂SO₄. The precipitate was dissolved in 50 mM NaCl, 2 mM Ca²⁺, 0.1% (w/v) polyethylene glycol-8000, 0.1% (w/v) BSA, pH 7.4 (sTM). The protein C activator was prepared from the venom of Aghistrodon Contortrix as described (25). Activated protein C (APC) was prepared by incubating PC (5 μM, 18 ml) in 20 mM Hepes, 150 mM NaCl, 0.1% (w/v) polyethylene glycol-8000, pH 7.4 with the venom activator (5 μg/ml) for 30 min at 37 °C. The volume of the mixture was reduced to ~2 ml by centrifugal ultrafiltration and applied to a column (1.5 × 110 cm) of Sephacryl S-100 (Amersham Biosciences) equilibrated in the same buffer. Fractions containing APC were pooled and precipitated with 50% (NH₄)₂SO₄. The precipitate was collected by centrifugation (53,000 × g, 20 min), dissolved in Assay Buffer lacking BSA and reapplied to a HPC4 column equilibrated in the same buffer to remove traces of remaining zymogen. The unbound fraction was fractionated with (NH₄)₂SO₄ as before, dissolved in 50% (v/v) glycerol, and stored at –20 °C.

Protein concentrations were determined using the following molecular weights and extinction coefficients (\(E_{420} = 0.5 	ext{ M}^{-1} \text{ cm}^{-1}\); SPC, 0.14; PAB, 0.17; hirudin, 6.2). SDS-PAGE analysis of reaction progress was performed as above. Various concentrations of APC and of Pefabloc SP present in the initial reaction mixture had no detectable effect on the secondary measurements of S2288 hydrolysis. Inhibition by residual PAB in measurements of S2288 hydrolysis was accounted for by interpolation from the dependence of the S2288 cleavage on known concentrations of APC in the presence of appropriate concentrations of PAB.

Progress Curves for the Activation of PC Derivatives—Full progress curves for the activation of PC₄, PC₅, or PC₁₅M were determined at 37 °C in Assay Buffer. Reaction mixtures in Minisorp tubes (Nunc) were initiated by mixing 200 μl of a solution containing the PC variant with 200 μl of a solution containing IIa and sTM to yield final concentrations of 400 nM PC variant, 10 nM IIa, and 100 nM sTM. Aliquots (25 μl) were withdrawn at various times (0–90 min) from reaction mixtures maintained at 37 °C and quenched with 145 μl of Quench Buffer containing 80 mM hirudin. Concentrations of APC formed were determined from discontinuous measurements of S2288 hydrolysis as described above.

SDS-PAGE analysis of reaction progress was performed as above except that the reaction mixtures were quenched with 200 μl of Assay Buffer lacking BSA. Aliquots (15 μl) were withdrawn at various times and quenched by mixing with 10 μl of 4x NuPAGE LDS sample buffer (Invitrogen) either without or with 50 mM dithiothreitol. Protein bands were resolved with 10% NOVEX Bis-Tris gels (Invitrogen) either without or with 50 mM dithiothreitol. SDS-PAGE analysis of reaction progress was performed as above except that the reaction mixtures were quenched with 200 μl of Assay Buffer lacking BSA. Aliquots (15 μl) were withdrawn at various times and quenched by mixing with 10 μl of 4x NuPAGE LDS sample buffer (Invitrogen) either without or with 50 mM dithiothreitol. Protein bands were resolved with 10% NOVEX Bis-Tris gels (Invitrogen) and visualized by staining with Coomassie Brilliant Blue R-250.
containing Ila or Ila plus sTM in Assay Buffer to achieve the indicated final concentrations. Initial rates of peptidyl substrate hydrolysis were determined by continuously monitoring \( A_{\text{abs}} \) at 25 °C in a kinetic plate reader. When necessary, initial rates were expressed in concentration terms using \( P_{\text{abs}} = 9887 \text{ M}^{-1} \text{ cm}^{-1} \) (18), and an effective path length of 0.59 cm for a 200-μl reaction volume.

For inhibition studies, the substrate solution contained increasing concentrations of S2366 in the presence of different fixed concentrations of inhibitor. These solutions were supplemented with appropriate volumes of 10× Assay Buffer to compensate for pH and ionic strength changes arising from the use of S2366 and inhibitor stock solutions prepared in H₂O. For studies with inhibitor stock solutions prepared in Me₂SO, control experiments established that the solvent carried over into the assay had a negligible effect on the rate of peptidyl substrate hydrolysis.

Inhibitory effects of PC₁₅₈₁ on S2366 hydrolysis by Ila or Ila plus sTM were measured with a fixed concentration of S2366 and increasing concentrations of PC variant. Control experiments established that any possible contribution of cleavage products of PCR₁₅₈₁ to the rate of S2366 hydrolysis was below detectable limits.

Binding of Active Site-directed Inhibitors to Ila or Ila-sTM—Measurements of the binding of I2₅₈₁ to either Ila or Ila saturated with sTM were performed at 25 °C in a PTI QuantaMaster fluorescence spectrophotometer (Oakville, Canada). Two reaction mixtures in 1 × 1 cm stirred quartz cuvettes were used for each titration. For binding studies with Ila, sample A (2.5 ml) contained 50 nM Ila in Assay Buffer lacking BSA and sample B (2.5 ml) contained 50 nM Ila treated with 10 μM FPR-CH₂Cl to irreversibly occlude the active site of the proteinase. Following incremental additions of I₂₅₈₁ to each sample, fluorescence intensity was determined by integrating ratiometric emission spectra collected between 470 and 650 nm using a 450-nm filter (KV-450, Schott, Duryea, PA) in the emission beam. Following minor corrections for dilution, the fluorescence change arising from the active site-dependent interaction of I₂₅₈₁ with Ila was inferred from the difference in integrated intensity between samples A and B. For binding studies with Ila saturated with sTM, an equivalent approach was used except that the reaction mixtures contained 50 nM Ila plus 100 nM sTM.

Equilibrium dissociation constants for the binding of Pefabloc SP to Ila and Ila saturated with sTM were inferred from competitive binding measurements with I₂₅₈₁. Fluorescence intensity was measured using the same experimental design described above except that initial reaction mixtures contained either 50 or 100 nM I₂₅₈₁. The active site-dependent component of the fluorescence signal was determined as described above following incremental additions of Pefabloc SP.

Data Analysis—The initial rate of APC formation at increasing concentrations of PC at different fixed concentrations of sTM was globally fitted to a two-site saturation model using the program Dynafit (29), generously provided as a gift by Petr Kuzmic (Biokin, Pullman, WA). Reported errors in the fitted parameters correspond to linear approximations of the 95% confidence limits.

All remaining kinetic and binding studies were conducted either in the absence of sTM or in the presence of sTM in excess of the concentration of Ila and well above the equilibrium dissociation constant for Ila binding. Thus, in either configuration, the concentration of enzyme was considered to approximate the total concentration of Ila present.

Steady-state kinetic constants were extracted by fitting initial velocity data to the Henri-Michaelis-Menten equation or to the rate expression for classic competitive inhibition by weighted nonlinear least squares regression analysis, using the Levenberg-Marquardt algorithm (30, 31). Alternate models for reversible inhibition were tested for and eliminated on the basis of established criteria (32). Some data are illustrated in double reciprocal form solely to provide visual confirmation of the conclusions derived from nonlinear regression analyses. Fitted steady-state constants are reported ± 95% confidence limits.

Measurements of fluorescence intensity as a function of increasing concentrations of I₂₅₈₁ were analyzed according to equations previously described (33). Analyses were performed assuming a stoichiometry of 1 mol of I₂₅₈₁ bound per mol of enzyme at saturation to yield fitted values of \( K_d \) and \( I_{\text{max}} \), the signal in the absence of ligand (Offset), and signal at saturating ligand (\( I_{\text{max}} \)). Competitive binding measurements were analyzed as previously described (34, 35), assuming stoichiometries of 1 mol of I₂₅₈₁ or 1 mol of Pefabloc SP bound per mol of enzyme at saturation. This approach yielded fitted values for \( K_d \) and \( K_{\text{Comp}} \), reflecting the equilibrium dissociation constants for I₂₅₈₁ and Pefabloc SP, respectively.

RESULTS AND DISCUSSION

Kinetic Pathway for PC Activation—The possible interactions between Ila (E), sTM (C), and PC (S) that lead to APC (P) formation are presented in Scheme I. Abundant evidence developed from kinetic and physical approaches has established the ability of Ila to bind reversibly to sTM and catalyze PC activation with greatly increased catalytic efficiency (36). Consequently, Steps 1, 2, and 7 (Scheme I) are expected to contribute in a primary way to APC formation in the presence of sTM. The rate of APC formation catalyzed by Ila in the absence of sTM is extremely slow (36). Thus, Steps 3 and 8 are expected to contribute in a minor but detectable way to product formation. In the presence of sTM, binding of the cofactor to the Ila-PC complex (Step 4, Scheme I) must also represent an alternate pathway for ternary complex formation. An important role for a direct interaction between PC and sTM is suggested by mutagenesis studies, molecular modeling, and the x-ray structure of the complex of Ila with a TM fragment (15, 16, 37). Thus, the ECS ternary complex can also potentially be formed through an initial interaction between PC and sTM followed by Ila binding (Steps 5 and 6, Scheme I). Scheme I, justified by these independent lines of evidence, provides the formal basis for the reaction.
testing of the contributions of the various possible pathways to APC formation by IIa and sTM.

Global Analysis of the Kinetics of APC Formation—The ability of steps illustrated in Scheme I to account for APC formation was assessed in initial velocity studies of product formation using increasing concentrations of PC and different fixed concentrations of sTM (Fig. 1). The rate of APC formation increased saturably with increasing concentrations of PC at any concentration of sTM or with increasing concentrations of sTM at any non-zero concentration of PC (Fig. 1).

Numerical analysis according to Scheme I and applying the rapid equilibrium assumption yielded an adequate description of the data (Fig. 1). Fitted constants obtained from this analysis are presented in Table I. In agreement with previously published findings, the enhanced rate of PC activation in the presence of saturating concentrations of sTM was found to result from a ~250-fold enhancement in $K_{cat}$ with a relatively minor increase in substrate affinity (Table I). The inferred equilibrium dissociation constant for the interaction of IIa with sTM ($K_{EC}$, Table I) was also in agreement with previously reported values (21, 38, 39). Surprisingly, inclusion of Steps 5 and 6 did not further improve the quality of the fit and instead yielded highly correlated fitted parameters with large (>100%) errors. Thus, the inclusion of steps wherein the ternary complex assembles through an initial interaction between PC and sTM are not necessary to adequately account for the rate of APC formation in these kinetic measurements.

Equivalent conclusions have been reported from kinetic studies of thrombin-activated fibrinolysis inhibitor activation by IIa+sTM (39). Our findings are also consistent with the weak interaction measured between PC and TM ($K_{i} > 10^{-4} M$) in analytical ultracentrifugation studies (17). However, a potentially important functional role for direct binding interactions between PC and sTM could readily be obscured in our work by a failure of the rapid equilibrium assumption, limitations of data precision or the use of a relatively narrow range of reactant concentrations. Finally, even if Steps 5 and 6 (Scheme I) can be securely eliminated from further consideration, this experimental approach cannot exclude important contributions from direct interactions between the substrate and cofactor within the ECS complex.

Kinetics of Inhibition by Active Site-directed Reversible Inhibitors—Further evidence for a functional role for interactions between PC and extended surfaces in IIa and sTM was sought from studies with active site-directed reversible inhibitors. This strategy relies on qualitative differences in the kinetic mechanism of inhibition of peptidyl substrate versus protein substrate cleavage to infer the contribution of extended interactions to the affinity of the enzyme for the protein substrate (40–42).

Initial velocity studies of S2366 cleavage by IIa saturated with sTM established I2581 as a classic competitive inhibitor of peptidyl substrate cleavage by the enzyme complex (Fig. 2A). This finding yields the foregone conclusion that I2581 and S2366 bind in a mutually exclusive way to the active site of IIa within the IIa-sTM complex. The interaction of I2581 with the active site of the proteinase in the IIa-sTM complex was also directly assessed by fluorescence binding measurements (Fig. 2B). The equilibrium dissociation constant inferred from this approach was in agreement with the kinetically determined $K_i$ (Table II). The close agreement between affinities determined by these two approaches independently verifies the rapid equilibrium assumption used in the interpretation of the inhibition studies.

Equivalent findings were established with Pefabloc SP and PAB (Table II). In each case, the $K_i$ correlated well with the directly measured equilibrium dissociation constant determined from competitive binding measurements (Pefabloc SP) or previously reported for PAB (20). Thus, the peptidyl substrate and active site-directed ligands exhibiting a wide range of affinities bind in a mutually exclusive fashion to the enzyme.

The full range of peptidyl substrate studies was performed with IIa alone and with IIa saturated with sTM (Table II). The presence of sTM had no detectable effect on steady-state kinetic constants for S2366, the kinetic mechanism for inhibition or inhibitor affinity. Thus, obvious changes in the active site of IIa induced by sTM are not detectable by this series of active site-directed probes. Previous studies have documented only minor TM-dependent perturbations, at best, in peptidyl substrate hydrolysis by IIa (12–14). The data suggest that the large changes in $V_{max}$ for PC activation associated with the interaction of sTM with IIa (Table I) are unlikely to derive, in a principal way, from a cofactor-driven conformational change in the active site of the proteinase as previously suggested (9, 10). Initial velocity studies revealed that I2581 also acted as a classic competitive inhibitor of PC activation of IIa saturated with sTM (Fig. 3). The $K_i$ determined in these studies was also in agreement with the measured equilibrium dissociation constant (Table II). The findings imply that the reversible occlusion of the active site of the proteinase within the IIa-sTM complex is sufficient to block the binding of PC. This conclusion was sustained by kinetic studies with the two other reversible inhibitors (Table II). Although inhibition by Pefabloc SP was also characterized by $K_i \sim K_{d}$, the $K_i$ for inhibition by PAB was reproducibly ~2-fold greater than the measured $K_{d}$ (Table II). Rather than suggesting a fundamental problem with the interpretation of the inhibition studies, this phenomenon most likely reflects kinetic complexities associated with the ability of PAB to also bind with good affinity to APC.

Classic competitive inhibition implies that binding of these reversible inhibitors to the active site of IIa within the IIa-sTM complex effectively eliminates PC binding. Because of limitations inherent in such kinetic approaches, it is also possible that the active site ligands are not purely competitive inhibitors but simply decrease PC affinity to some undetectably low value. This possibility is minimized by the agreement between the $K_i$ and independently measured $K_{d}$ (Table II). However, either interpretation implies that interactions between PC and the active site of the enzyme play a principal role in determining the affinity of the IIa-sTM complex for PC. This conclusion is in line with mechanisms of substrate recognition established for the less selective serine proteinases of the chymotrypsinogen family (43). However, the findings are surprising considering the established and predominant role for exosite interactions in the action of IIa on a range of its procoagulant protein

### Table I

| Step  | Kinetic constant | Value ± S.D. |
|-------|-----------------|--------------|
| 1     | $K_{EC}$ (nM)   | 23.5 ± 1.8   |
| 2     | $K_{EC,S}$ (μM) | 11.8 ± 0.6   |
| 3     | $K_{EC}$ (μM)   | 44.8 ± 4.7   |
| 4     | $K_{EC,S}$ (nM) | 6.2 ± 0.9    |
| 5     | $K_{EC}$ (nM)   | NF           |
| 6     | $K_{EC,S}$ (nM) | NF           |
| 7     | $(V_{max})_{EC}$ (min⁻¹) | 19.6 ± 0.4 |
| 8     | $(V_{max})_{EC}$ (min⁻¹) | 0.08 ± 0.02 |

*a* Step numbers correspond to those illustrated in Scheme I.

*b* Symbolic kinetic constants with units indicated in parentheses.

*c* Parameters and linear approximations of their 95% confidence limits obtained by global analysis of the data in Fig. 1 using Scheme I.

**c** Calculated using $K_{EC} = (K_{EC,S}K_{EC,S}S/K_{EC})$. Uncertainty in the calculated value was determined by propagating errors in the fitted terms.

*NF* = not fitted. These constants were very poorly determined, and the corresponding steps were not included in the global analysis.
Recognition of Protein C by the Thrombin-Thrombomodulin Complex

Fig. 2. Active site-dependent interaction of I2581 with IIa-sTM. A, inhibition of peptideyl substrate cleavage. Initial velocities for S2366 hydrolysis were measured using 2 nM IIa plus 100 nM TM and I2581 fixed at 0 (○), 100 nM (●), 200 nM (▲), or 400 nM (▲). The lines are drawn according to the rate expression for classic reversible competitive inhibition using the fitted constants listed in Table II. B, binding of I2581 to IIa-sTM. Reaction mixtures containing 50 nM IIa and 100 nM sTM with and without 10 μM FPR-CH₂Cl were titrated with increasing concentrations of I2581. Fluorescence was monitored using λₜₐₓ = 330 nm and integrating emission intensity between 470 and 650 nm. The fluorescence change arising from active site-dependent binding of I2581 (F - F₀) was calculated as described under “Experimental Procedures.” The line is drawn following fitting as described under “Data Analysis” assuming a stoichiometry of 1 and using the fitted constants: Kᵣ = 16.4 ± 1.2 nM, Offset = -0.065 ± 0.049, and ΔF_max = 59.0 ± 0.6.

Table II

Kinetics of inhibition by active site-directed reversible inhibitors

| Enzyme species | Substrate | Inhibitor | Inhibition type | Kᵢ = S.D. | V/E = S.D. | Kₛ = S.D. | Kᵢ/Kₛ = S.D. |
|----------------|-----------|-----------|----------------|------------|------------|------------|---------------|
| IIa            | S2366     | None      | NA             | 201 ± 7    | 190 ± 1    | NA         | NA            |
| IIa            | S2366     | I2581     | Competitive    | 192 ± 8    | 187 ± 2    | 26 ± 1     | 25 ± 1        |
| IIa            | S2366     | Pefabloc SP| Competitive   | 211 ± 8    | 197 ± 2    | 580 ± 19   | 651 ± 20      |
| IIa-sTM        | S2366     | None      | NA             | 134 ± 5    | 197 ± 1    | NA         | NA            |
| IIa-sTM        | S2366     | I2581     | Competitive    | 126 ± 6    | 194 ± 2    | 17 ± 1     | 16 ± 1        |
| IIa-sTM        | S2366     | Pefabloc SP| Competitive   | 234 ± 15   | 202 ± 3    | 563 ± 32   | 410 ± 13      |
| IIa-sTM        | PC        | None      | NA             | 12.4 ± 0.8 | 0.28 ± 0.01| NA         | NA            |
| IIa-sTM        | PC        | I2581     | Competitive    | 12.6 ± 0.4 | 0.29 ± 0.01| 21 ± 1     |               |
| IIa-sTM        | PC        | Pefabloc SP| Competitive   | 10.7 ± 0.5 | 0.25 ± 0.01| 470 ± 17   |               |
| IIa-sTM        | PC        | PAB       | Competitive    | 11.8 ± 0.8 | 0.27 ± 0.07| 137,000 ± 7,000|               |

*a The enzyme species correspond to thrombin alone or thrombin saturated with sTM. Measurements of S2366 hydrolysis were performed with 2 nM IIa ± 100 nM sTM. Protein C activation was measured with 10 nM IIa plus 100 nM sTM. Equilibrium binding studies were performed with 50 nM IIa ± 100 nM sTM.
*b Initial velocities were measured as illustrated in Figs. 2 and 3 using increasing concentrations of either S2366 or PC in the absence of inhibitors or in the presence of different fixed concentrations of the indicated inhibitor.
*Steady-state kinetic constants are presented ± 95% confidence limits.
*Equilibrium constants were determined by fluorescence measurements as noted under “Experimental Procedures.” The value for PAB binding was taken from Evans, S. A., et al. (20).
*NA, not applicable.
*Competitive denotes classical reversible competitive inhibition.
*ND, not determined.

substrates (5, 44). Moreover, exosite-dependent substrate recognition is also established to play a principal role in determining binding affinity and specificity in the action of other coagulation enzyme complexes on their protein substrates (45).

The data are consistent with the interpretation that it is active site docking rather than interactions between PC and extended surfaces within the IIa-sTM complex that is a principal determinant of substrate affinity. The findings reveal difficulties in documenting a significant role for direct interactions between PC and sTM in the assembly of the ternary ECS complex (Scheme I). Similarly, difficulties are also evident in documenting a major contribution for interactions between PC and extended surfaces within the IIa-sTM in determining substrate affinity as has previously been proposed (15, 16, 37).

Contribution of Active Site-dependent Interactions to the Affinity of IIa-sTM for Protein C—Further studies were pursued using a recombinant derivative of PC (PC₁₁₁₅₄₉) in which the P₁ Arg was mutated to Gln to abrogate the ability of this PC derivative to effectively engage the active site of IIa-sTM.

SDS-PAGE (Fig. 4, inset) indicated that purified recombinant wild type PC (PCWT) and PC₁₁₁₅₄₉ were similar to the protein isolated from plasma (PC₁₁₁₅₄₉) with the exception of a greater abundance of single chain material (46). Accordingly, N-terminal sequencing yielded 8.9 and 9.4 kDa, respectively. SDS-PAGE and mass spectrometry confirmed equivalent molecular weights for the plasma-derived material and recombinant species (not shown). The heavy chain species yielded the expected sequence for the processed two-chain zymogen (47). Mass spectrometry confirmed equivalent molecular weights for the plasma-derived material and recombinant species (not shown). In agreement with published data for PC₁₁₁₅₄₉ (47), chemical analysis of 4-carboxyglutamic acid content yielded 8.9 ± 0.3 and 9.4 ± 0.2 mol of Glu/mol of protein for PCWT and PC₁₁₁₅₄₉, respectively. PC₁₁₁₅₄₉ and PC₁₁₁₅₄₉ possess appropriate post-translational modifications known to be necessary for function.

Progress curves of PC activation by IIa saturated with sTM indicated that PCWT was activated with a similar rate and to
the same extent as PC_{PL} (Fig. 4). In contrast, cleavage of S2288 was not detectable following incubation of PC_{R15Q} with IIa-sTM (Fig. 4). SDS-PAGE analysis of PC_{PL} and PC_{WT} following prolonged treatment with IIa-sTM eliminated the single chain species and yielded heavy chain forms that migrated with slightly lower molecular weights than the α and β heavy chain species observed for the zymogens (Fig. 4, inset). This indicates quantitative activation of PC_{PL} and PC_{WT}. In contrast, PC_{R15Q} was not cleaved by IIa-sTM (Fig. 4, inset), confirming the intent of the substitution of the P1 Arg to yield an uncleavable zymogen species.

An uncleavable zymogen species that is solely defective in its ability to engage the active site of IIa-sTM is expected to retain the ability to engage the enzyme complex through extended interactions. Thus, provided such interactions play a significant role in PC activation by IIa-sTM, PC_{R15Q} is expected to act as an inhibitor of protein substrate cleavage without interfering with peptidyl substrate cleavage by the enzyme complex (45).

Initial velocity studies revealed that PC_{R15Q} was a poor inhibitor of PC_{PL} activation by IIa-sTM (Fig. 5). Assuming competitive inhibition, the $K_i$ for inhibition by PC_{R15Q} was estimated at ≥30 µM (Fig. 5). Thus, abrogation of active site interactions has a deleterious effect on protein substrate affinity. The effects of PC_{R15Q} on PC activation were compared with the effects on peptidyl substrate cleavage by either free IIa or IIa plus sTM using substrate concentrations at equivalent multiples of $K_m$. Under these comparable conditions, inhibition of PC activation by PC_{R15Q} was matched by the inhibition of active site function assessed by the kinetics of cleavage of S2366 (Fig. 5). Taken together with similar findings made with IIa alone, it seems improbable that the inhibitory effects of PC_{R15Q} on PC activation arise from interference with interactions between the protein substrate and extended surfaces within the IIa-sTM complex. Instead, inhibition by this recombinant derivative likely arises from its ability to weakly interfere with active site interactions within the enzyme complex.

Zymogen derivatives, rendered uncleavable by substitution of the P1 Arg, have proved uniquely useful as exosite-directed inhibitors (27, 41, 48). Their use in dissociating the inhibition of protein substrate cleavage from the inhibition of peptidyl substrate cleavage has aided in resolving the principal contribution of exosite binding rather than active site docking to protein substrate affinity in other coagulation enzymes (45). In surprising contrast, equivalent kinetic approaches indicate a primary role for active site-dependent recognition of PC by the IIa-sTM complex. Thus, despite the lines of evidence suggesting a role for interactions between PC and extended surfaces in TM (15, 16, 37), our findings indicate that such potential interactions are likely to contribute only in a secondary way to the affinity of the IIa-sTM complex for PC.

**Implications for IIa-sTM Function**—We have pursued solution-phase studies of PC activation using sTM to probe the putative role of extended interactions between the substrate and the cofactor in the function of the anticoagulant pathway. This approach is prefaced by proposals for such a role on the basis of structural and mutagenesis studies conducted with equivalent forms or even further truncated derivatives of TM (15, 16, 37). The available evidence also suggests that the soluble system likely recapitulates principal features of the activation reaction observed with the intact cofactor species incorporated into phosphatidylcholine membranes (38). Nevertheless, it remains possible that additional interactions, not obvious in studies with sTM, play important roles when the
enzyme complex is assembled with the full-length cofactor incorporated into membranes or with derivatives containing chondroitin sulfate. Furthermore, PC activation on the endothelium is expected to be modulated by membrane binding by the substrate and by its interaction with EPCR, both of which serve to present PC to the enzyme complex (6, 49). The potential contributions of such additional interactions to the recognition of PC by IIa-TM have not been assessed in the present work.

TM-dependent allosteric changes in the active site of IIa represent an obvious candidate explanation for the ability of TM to greatly enhance the $V_{\text{max}}$ for PC or thrombin-activated fibrinolysis inhibitor activation while simultaneously reducing its catalytic efficiency toward a range of procoagulant substrates (6, 36). Indeed, several lines of evidence have been developed in support of this idea (10, 13, 50). Stronger evidence links such allosteric phenomena to the Ca$^{2+}$-dependence of PC activation (11, 51), which may or may not have bearing on the function of TM within the enzyme complex at physiological Ca$^{2+}$. In contrast, our studies with active site-directed ligands and peptidyl substrates provide no obvious indication of changes in the active site of IIa that arise from its interaction with sTM. Although the findings are consistent with previous work (12–14), conclusions from x-ray structures (15), and observations in other coagulation enzyme complexes (45), it remains possible that TM-dependent transition state stabilization that leads to large changes in $V_{\text{max}}$ for PC activation is not mirrored by equivalent changes in peptidyl substrate cleavage.

Multiple lines of evidence, obtained in the present study, indicate that interactions between PC and extended surfaces within the IIa-TM complex do not contribute in a detectable way to substrate affinity. Instead, the data suggest that the affinity of PC for the enzyme complex is determined in a primary way by interactions with the active site of the proteinase. The findings are unexpected considering the strategies utilized by IIa to act on a range of its procoagulant substrates and the burgeoning evidence implicating key exosite interactions in the function of other coagulation enzyme complexes (5, 45). Despite the conclusions from a variety of mutagenesis studies (16, 37, 52), it appears unlikely that TM function in PC activation is principally realized through binding the substrate and facilitating its presentation to the active site of the catalyst.

The two obvious possible explanations do not provide a compelling accounting for the enhanced anticoagulant function of IIa-TM. However, the numerous lines of existing evidence can be accommodated by a model in which TM functions by modulating ground state interactions with the protein substrate without necessarily altering the active site of IIa or contributing directly and in a principal way to substrate affinity. As initially proposed by Esmon (11), and in line with the more recent proposals from Di Cera (53) and Rezaie (51), we speculate that, in the absence of TM, PC interacts with IIa in a mode associated with a low $V_{\text{max}}$ TM binding and occlusion of surfaces in anion binding exosite I could eliminate the non-productive binding mode with PC and provide a concomitant enhancement of productive binding associated with a greater $V_{\text{max}}$. Such TM-dependent modulation of substrate binding to IIa could translate into a large increase in $V_{\text{max}}$ without necessarily affecting $K_{\text{cat}}$. Because both binding modes are associated with product formation and are proposed to be modulated by TM, $k_{\text{cat}}/K_{\text{M}}$ is not necessarily expected to be constant (54–56). We also speculate that these changes arise from steric effects associated with regions of TM not directly associated with IIa binding and by the net result of selective occlusion of side chains on IIa that contribute in a positive and negative way to the various modes in which substrates can engage the enzyme.

Summary—The ability of TM to selectively enhance IIa function in the anticoagulant pathway has alternately been ascribed to a TM-dependent allosteric change in the active site of IIa or the ability of TM surfaces within the IIa-TM complex to bind PC and present the substrate to the active site of the catalyst. In the latter case, extended interactions between PC and surfaces removed from the enzymic active site are expected to contribute in a major way to substrate affinity. A series of kinetic approaches now indicate interactions between PC and the enzymic active site contributes in a primary way to the affinity of PC for the IIa-TM complex. Exosite binding is shown to contribute in a secondary or minor way to substrate affinity. This strategy sets the IIa-TM complex apart from other coagulation enzyme complexes wherein exosite binding is documented to play a principal role in biological function.

Acknowledgments—We thank Dr. Jan Pohl, Emory University, for providing N-terminal sequence analyses, Dr. Bruce Gerlitz, Eli Lilly, for the generous gift of recombinant thrombomodulin, Dr. George Long, University of Vermont, for the cDNA encoding human protein C, and Dr. Petr Kuzmic, Biokin Ltd., for the program DynaFit. We also thank our colleagues Dr. William Church, Rodney Camire, and George Vlasko for critical reading of the manuscript.

REFERENCES

1. Mann, K. G., Jenny, R. J., and Krishnaswamy, S. (1988) Annu. Rev. Biochem. 57, 915–956
2. Esmon, C. T. (1993) Annu. Rev. Cell Biol. 9, 1–26
3. Davis, E. W., Fujikawa, K., and Kisiel, W. (1991) Biochemistry 30, 10363–10370
4. Di Cera, E. (2003) Chest 124, 118–178
5. Stubbs, M. T., and Bode, W. (1993) Thromb. Res. 69, 1–58
6. Esmon, C. T. (2003) Chest 124, 386–395
7. Esmon, C. T., Eson, N. L., and Harris, K. W. (1982) J. Biol. Chem. 257, 7944–7947
8. Jakobovits, H. V., Kline, M. D., and Owen, W. G. (1986) J. Biol. Chem. 261, 30767–30772
9. Esmon, C. T., Eson, N. L., Le Bennecie, B. F., and Johnson, A. E. (1993) Methods Enzymol. 222, 359–385
10. Musci, G., Berliner, L. J., and Esmon, C. T. (1988) Biochemistry 27, 789–793
11. Esmon, C. T. (1993) Thromb. Haemost. 70, 29–35
12. Hofsteenge, J., Taguchi, H., and Stone, S. R. (1986) J. Biol. Chem. 261, 2345–2351
13. Ye, J., Liu, L. W., Eson, C. T., and Johnson, A. E. (1992) J. Biol. Chem. 267, 11023–11028
14. Vindigni, A., White, C. E., Komives, E. A., and Di Cera (1997) Biochemistry 36, 6674–6681
15. Pictet-Prior, P., Iwanaga, Y., Huber, R., Paglia, R., Rumennik, G., Seto, M., Morser, J., Light, D. R., and Bode, W. (2000) Nature 404, 518–525
16. Yang, L., and Rezaie, A. R. (2003) J. Biol. Chem. 278, 10484–10490
17. Olsen, P. H., Eson, N. L., Eson, C. T., and Lau, T. M. (1992) Biochemistry 31, 746–754
18. Lottenberg, R., and Jackson, C. M. (1983) Biochim. Biophys. Acta 742, 558–564
19. Nesheim, M. E., Prendergast, F. G., and Mann, K. G. (1979) Biochemistry 18, 996–1003
20. Evans, S. A., Olson, S. T., and Shore, J. D. (1982) J. Biol. Chem. 257, 3014–3017
21. Parkinson, J. P., Grinnell, B. W., Moore, R. E., Hoskins, J., Vlahos, C. J., and Parkinson, J. F. (1996) Biochemistry 35, 9273–9282
22. Baugh, R. J., and Krishnaswamy, S. (1996) J. Biol. Chem. 271, 10363–10370
23. Strouse, M., and Johnson, M. L. (1992) Arch. Biochem. Biophys. 297, 87–105
24. Wilkens, M., and Krishnaswamy, S. (2002) J. Biol. Chem. 277, 54927–54936
25. Camire, R. M., Larson, P. J., Stafford, D. W., and High, K. A. (2000) Biochemistry 39, 14322–14329
26. Stearns, D. J., Kurosawa, S., and Esmon, C. T. (1989) J. Biol. Chem. 264, 16126–16134
27. Orcutt, S. J., and Krishnaswamy, S. (2004) J. Biol. Chem. 279, 54927–54936
28. Kuzmic, P. (1996) J. Biol. Chem. 271, 3159–3165
29. Bode, W. (1996) Trends Biochem. Sci. 21, 455–458
30. Straume, M., and Johnson, M. L. (1992) Methods Enzymol. 210, 87–105
31. Wilkens, M., and Krishnaswamy, S. (2002) J. Biol. Chem. 277, 9366–9374
32. Olson, S. T., Bock, P. E., and Sheffer, R. (1991) Arch. Biochem. Biophys. 286, 533–545
33. Betz, A., and Krishnaswamy, S. (1998) J. Biol. Chem. 273, 10709–10718
34. Rezaie, A. R. (2003) J. Biol. Chem. 278, 4743–4746
35. Krohe, K. E., Berndt, B. M., Shen, L., Morser, J., Dahlback, B., and Villoutreix B. O. (1999) Proteins 35, 218–234
36. Stein, D. J., Kurosawa, S., and Esmon, C. T. (1989) J. Biol. Chem. 264, 3352–3356
Recognition of Protein C by the Thrombin-Thrombomodulin Complex

16603–16608
40. Krishnaswamy, S., and Betz, A. (1997) *Biochemistry* **36**, 12080–12086
41. Baugh, R. J., Dickinson, C. D., Raf, W., and Krishnaswamy, S. (2000) *J. Biol. Chem.* **275**, 28826–28833
42. Bokovik, D. S., and Krishnaswamy, S. (2000) *J. Biol. Chem.* **275**, 38561–38570
43. Perona, J. J., and Craik, C. S. (1995) *Protein Sci.* **4**, 337–360
44. Stubbs, M. T., and Bode, W. (1995) *Trends Biochem. Sci.* **20**, 23–28
45. Krishnaswamy, S. (2005) *J. Thromb. Haemost.* **3**, 54–67
46. Grinnell, B. W., Walls, J. D., and Gerlitz, B. (1991) *J. Biol. Chem.* **266**, 9778–9785
47. Kisel, W. (1979) *J. Clin. Invest.* **64**, 761–769
48. Orcutt, S. J., Pietropaolo, C., and Krishnaswamy, S. (2002) *J. Biol. Chem.* **277**, 46191–46196
49. Xu, J., Esmon, N. L., and Esmon, C. T. (1999) *J. Biol. Chem.* **274**, 6704–6710
50. Ye, J., Esmon, N. L., Esmon, C. T., and Johnson, A. E. (1991) *J. Biol. Chem.* **266**, 23016–23021
51. Rezaie, A. R., and Yang, L. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 12051–12056
52. Wang, W., Nagashima, M., Schneider, M., Morser, J., and Nesheim, M. E. (2000) *J. Biol. Chem.* **275**, 22942–22947
53. Xu, H., Bush, L. A., Pineda, A. O., Caccia, S., and Di Cera, E. (2005) *J. Biol. Chem.* **280**, 7956–7961
54. Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol.* **43**, 219–410
55. Jencks, W. P. (1987) *Catalysis in Chemistry and Enzymology*, 1st Ed., Dover Publications Inc., New York
56. Fersht, A. (1977) *Enzyme Structure and Mechanism*, 1st Ed., pp. 84–102 W.H. Freeman and Co., San Francisco