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PAR-3 is a low-affinity substrate, high-affinity effector of thrombin☆

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Abstract

A polypeptide corresponding to the extracellular domain of protease-activated receptor 3 (PAR-3) is hydrolyzed by thrombin slowly because of high K_M (>100 μM). However, thrombin is found to bind two PAR-3, one without catalyzing hydrolysis or blocking the active site, while the other is hydrolyzed. In a solvent lacking Na^+, hydrolysis of a nitroanilide substrate is enhanced 1.6-fold by addition of PAR-3 polypeptide, with half-saturation at 2.5 μM. In contrast, the fibrinogen clotting activity of thrombin is inhibited completely by PAR-3, with a K_I of 3 μM. None of the activities of thrombin are affected by addition of 50 μM PAR-4 polypeptide. Thus, PAR-3 in low concentrations binds thrombin in a configuration that blocks the anion-binding exosite but not the catalytic site, while hydrolysis of PAR-3, PAR-4, and other substrates that do not interact with exosite I persists. The allosteric effect of PAR-3 is characteristic of that of Na^+.

Thrombin, the controlling protease in hemostasis and thrombosis, signals platelets by cleavage of their protease-activated receptors (PARs), a small (4-member) family of serpentine G-protein-coupled receptors, each activated by proteolysis near the N terminus of its extracellular exodomain [1]. Human platelets have PAR-1 and PAR-4 on their surface, whereas murine platelets lack PAR-1 but have PAR-3 and PAR-4. Here we report that thrombin occupied at an allosteric site by PAR-3 exodomain, bound in a manner that does not lead to its own proteolysis, can cleave some substrates, including additional PAR-3 exodomain from solution but, not fibrinogen. Recombinant [2] or synthetic [3] PAR-1 exodomain is a sensitive and selective substrate for thrombin ($k_{CAT}/K_M > 10^7\ M^{-1}\ s^{-1}$). This exceptional catalytic efficiency was anticipated from the presence in PAR-1 of an acidic segment, P′ (toward the C-terminus) of the cleavage site, that interacts with exosite 1 on thrombin [4]. PAR-3 likewise has a canonical P′ thrombin-exosite-recognition sequence, but in contrast to all known thrombin substrates, bears a lysyl rather than an arginyl residue at the putative scissile bond [5]. PAR-4 has a thrombin cleavage site but, in contrast to both PAR-1 and PAR-3, has no exosite-recognition sequence [6]. Not surprisingly then, catalytic efficiencies for hydrolysis of synthetic polypeptide segments of PAR-3 and PAR-4 exodomains by thrombin are 1/100 of that for hydrolysis of PAR-1 peptides [3]. Blockade or knockout of PAR-3 partially inhibits thrombin responses of murine platelets [6,7], whereas knockout of PAR-4 abolishes responses at all thrombin concentrations [8]. In cell culture systems, co-transfection of PAR-3 with PAR-4 augments signaling through PAR-4 by enhancing PAR-4 cleavage [9]. The present investigation, undertaken to explore the role of soluble PAR-3 and PAR-4 exodomains as a composite thrombin substrate, has revealed an unanticipated disconnection between PAR-3 recognition and hydrolysis.

Methods

The polypeptides having sequences of the extracellular amino-terminal domains of human PAR-3 and PAR-4, each beginning with the first residue after the putative signal peptide, were synthesized and HPLC-purified by the Mayo Foundation Protein Core Facility:

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PAR-3: M_1ENDTNNLAKPTLPIKTFRGAPPNSFEEFPFSALE GWTGAC$_{41}$-NH_2

PAR-4: S_1TGGGDDSTPSILPAPRGYPGQVCANDS$_{28}$-NH_2

Thrombin coagulant activity was assayed at 30 °C by clotting a solution of 2 mg/mL (6 μM) of bovine fibrinogen dissolved in 0.1 M NaCl, 0.05 M Hepes, pH 7.4. The clotting time is proportional to the reciprocal of thrombin concentration in the range 30–200 nM of human thrombin. Hydrolysis of the tripeptidylnitroanilide substrate SpectrozymeTH was measured ($dA_{405\,nm}/dt$) in solutions as indicated and buffered with 50 mM Hepes, pH 7.4, and containing 1 mg/mL of bovine albumin.

Results and discussion

Analyzed directly by capillary electrophoresis for peptide hydrolysis by thrombin, PAR3$_{1–41}$ and PAR-4$_{1–28}$ behave as first order (i.e., non-saturating) substrates in concentrations as high as 0.1 mM, so K_M for PAR-3 and PAR-4 lie near or above that value. The observed second order rate constants for hydrolysis, $2 \times 10^4\ M^{-1}\ s^{-1}$ for PAR-3$_{1–41}$ and $8 \times 10^4\ M^{-1}\ s^{-1}$ for PAR-4$_{1–28}$, are representative of non-specific hydrolysis of peptides containing a thrombin cleavage sequence [10]. These rate constants are smaller than those observed with PAR-3 and PAR-4 exodomain peptides having truncated N-terminal sequences [3], so the presence of the full leaving peptide may have a modest impact on proteolysis kinetics.

As an alternative approach to estimating very high K_M, the PAR oligopeptides were used as competitive inhibitors of hydrolysis of a tripeptidylnitroanilide substrate. Unexpectedly, PAR-3$_{1–41}$ *increased* the rate of nitroanilide hydrolysis 1.6-fold with an EC50 of 2.5 μM (Fig. 1, triangles). This effect is on k_{CAT}, as the rate increase was observed with 50 μM nitroanilide substrate, or 20 times K_M. This effect on k_{CAT} was observed only in KCl solutions. In NaCl solution (Fig. 1, squares) the k_{CAT} for *p*-nitroanilide hydrolysis is 1.6 times that in KCl and is insensitive to PAR3$_{1–14}$ (n.b., the effect of Na^+ is strictly on the k_{CAT} for nitroanilide hydrolysis, data not shown). The stimulation by PAR-3$_{1–41}$ of hydrolysis of the nitroanilide is mirrored by *inhibition* of thrombin coagulant activity by PAR-3$_{1–41}$ in NaCl solution, with an IC50 of 3 μM (Fig. 1, circles). In contrast, neither clotting of fibrinogen nor hydrolysis of nitroanilide was affected by PAR-4$_{1–28}$ in concentrations as high as 50 μM. When tripeptidylnitroanilide concentration was lowered to 5 μM (twice the K_M) and thrombin was saturated with 50 μM (10 times the IC_{50}) PAR-3$_{1–41}$, the addition of 50 μM PAR-4$_{1–28}$ had no effect on substrate hydrolysis, so binding of PAR-3$_{1–41}$ to thrombin in solution does not impart specificity for PAR-4$_{1–28}$. The anticoagulant effect of PAR-3$_{1–41}$ is clearly the result of binding to thrombin, as clotting of fibrinogen with reptilase (*Bothrops atrox* coagulant) was unaffected by PAR-3$_{1–41}$ (not shown).

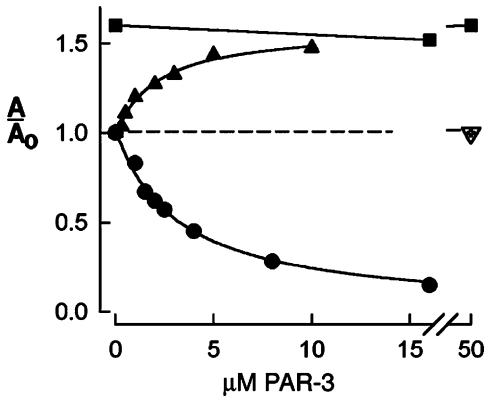

Fig. 1. Effect of PAR-3 on thrombin activities. Hydrolysis of 50 μM tripeptidylnitroanilide by 1 nM thrombin was measured in 0.1 M KCl (triangles) and in 0.1 M NaCl (squares). Relative activities (A/A_0) are shown as the quotient of the activity, without peptide, in KCl solution for the nitroanilide data. Clotting activity (circles) was measured in 0.1 M NaCl and is shown as the quotient of that without peptide. The open triangle/dashed line denotes addition of 50 μM PAR-4 to the tripeptidylnitroanilide hydrolysis assay. Curves through solid triangles and circles are non-linear least squares fits to rectangular hyperbolae.

Proteolysis of PAR-3 does not proceed via a typical thrombin–substrate complex in which both the scissile (P1) residue and the canonical exosite-recognition sequence contribute to substrate recognition (Fig. 2, reaction I). With PAR-3$_{1–41}$ in concentrations that saturate thrombin allosterically (Fig. 1), proteolysis of PAR3$_{1–41}$ remains first order in substrate. For this behavior to occur, PAR-3$_{1–41}$ bound in the site that inhibits fibrinogen hydrolysis and enhances nitroanilide hydrolysis cannot itself be hydrolyzed but rather, in the manner of a subunit of the enzyme, binds in the high-affinity site in an orientation that neither occupies nor hinders the catalytic center (Fig. 2, reaction II). This effect of PAR-3$_{1–41}$ on nitroanilide hydrolysis has a counterpart observed for a shorter hirudin-like peptide derived from PAR-1 [11]. Exosite I is promiscuous for hirudin-like acidic sequences, so PAR-3 might conceivably be bound in reverse orientation. The anomalous relationship of PAR-3 and thrombin lends credence to the proposals by Coughlin and co-workers [9] that the mechanism of the cofactor activity of PAR-3 could depend on a dimeric substrate, where PAR-3 provides affinity and PAR-4, lacking a thrombin recognition sequence, provides the cleavage site (Fig. 2, reaction III). The mechanism fits with the finding that, at least in solution, PAR-3$_{1–41}$ does not directly enhance affinity of thrombin for PAR-4$_{1–28}$.

The reduced rate of thrombin-catalyzed nitroanilide hydrolysis when [Na^+] is low, and its reversal by addition of PAR-3$_{1–41}$ is a rational consequence of PAR-3 occupation of the anion-binding exosite in thrombin. This Na^+-specific behavior has been characterized as a switch between "slow" and "fast" conformations of the catalytic site and specificity cleft [12]. The fast form of the enzyme is stabilized by Na^+ or by occupancy of the

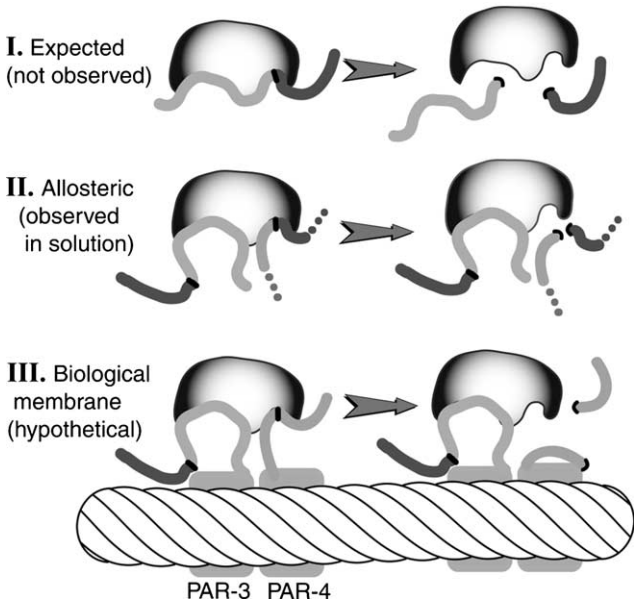

Fig. 2. Models for interaction of PAR-3 with thrombin. (I) Conventional enzyme–substrate complex yielding products. This reaction is representative of that with PAR-1 or fibrinogen, which interacts both with the catalytic center and exosite. (II) The exosite is occupied by PAR-3 in a configuration that does not obstruct the catalytic center, which can then accept any substrate, including a second PAR-3, which can bind without interacting with the exosite. Fibrinogen, however, cannot. (III) A hypothetical dimer in the platelet membrane in which PAR-4 provides the cleavage site and PAR-3 provides specificity (affinity). This hypothetical reaction, not able to occur in solution, is consistent with data from model systems, transgenic mice and the data in Fig. 1.

anion binding exosite. In any case, the dynamics of interaction of thrombin with PAR-3 on the platelet membrane cannot be inferred from the solution analysis. Quantitative analysis of PAR-3 cleavage on the platelet will be needed to settle the issue of whether PAR-3 is hydrolyzed in the course of thrombin-catalyzed murine platelet activation. Interestingly, rat PAR3 [13] has a glutamyl residue at P1, which is very unlikely to be hydrolyzed at all by thrombin. The issue becomes even more intriguing with the findings by Ofosu et al. [14] that an endogenous platelet protease contributes to, and may account for, a major share of, PAR-1 hydrolysis, and by Ramakrishnan et al. [15] that cleavage of glycoprotein V by thrombin imparts a non-proteolytic thrombin receptor function to platelets.

To summarize the principal finding, PAR-$3_{1–41}$ binds to thrombin exosite I with high affinity, in a configuration that does not block the catalytic center. Thrombin bearing the bound PAR-$3_{1–41}$ can hydrolyze additional PAR-$3_{1–41}$, PAR-$4_{1–28}$ and tripeptidylnitroanilides, but not fibrinogen.

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