Mutation of W215 Compromises Thrombin Cleavage of Fibrinogen, but Not of PAR-1 or Protein C†

Daniele Arosio, Youhna M. Ayala, and Enrico Di Cera*

Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Box 8231, St. Louis, Missouri 63110

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ABSTRACT: W215 is a highly conserved residue that shapes the S3 and S4 specificity sites of thrombin and participates in an edge-to-face interaction with residue F8 of the fibrinogen Aα chain. Protein C and the platelet receptor PAR-1 carry an acidic residue at P3 and bind to the active site of thrombin without making contact with W215. This suggested that mutation of W215 could dissociate the cleavage of fibrinogen from that of protein C and PAR-1. Replacement of W215 with Phe produces modest effects on thrombin function, whereas the W215Y replacement compromises significantly the catalytic activity toward all chromogenic and natural substrates that are tested. Replacement of W215 with Ala almost obliterates Na^+ binding, reduces the level of fibrinogen cleavage 500-fold, but decreases the levels of protein C activation and PAR-1 cleavage only 3- and 25-fold, respectively. The W215A mutant cleaves PAR-1 with a specificity constant that is more than 13-fold higher than that of fibrinogen and protein C and is the first thrombin derivative to be described that functions as an almost exclusive activator of PAR-1. The environment of W215 influences differentially three physiologically important interactions of thrombin, which should assist in the study of each of these functions separately in vivo.

Thrombin plays two important and opposing functions in the blood (*1*). It acts as a procoagulant when it cleaves fibrinogen and promotes the formation of a fibrin clot. This action is reinforced and amplified by activation of the transglutaminase factor XIII and inhibition of fibrinolysis, the activation of factors V, VIII, and XI upstream in the coagulation cascade, and the cleavage of the platelet receptor PAR-1[1] that leads to platelet activation and aggregation. In addition to its procoagulant function, thrombin also functions as an anticoagulant when it cleaves and activates protein C with the assistance of the endothelial receptor thrombomodulin. Activated protein C initiates a natural anticoagulant pathway by cleaving and inactivating factors Va and VIIIa, two essential cofactors of the intrinsic pathway, thereby downregulating both the amplification and progression of the coagulation cascade. Inactivation of thrombin in the blood is accomplished by the serpin antithrombin III in the presence of heparin.

The multiple roles of thrombin in hemostasis raise the possibility that individual functions, like the cleavage of fibrinogen, PAR-1, or protein C, could be dissociated. This would broaden our understanding of each separate function of thrombin in the biological context explored in transgenic animal models.

It has long been known that thrombin has a preference for aromatic and hydrophobic residues at the P3 and P4 positions of the substrate (*2*). The crystal structure of thrombin inhibited with PPACK (*3*) provides a molecular basis for this preference. The aromatic ring of H-D-Phe at P3 in PPACK participates in an edge-to-face interaction with W215 of thrombin. Residue F8 of the fibrinogen Aα chain participates in a similar interaction with W215 (*4*−*6*). However, both protein C and PAR-1 have an acidic residue at P3. The crystal structure of thrombin covalently inhibited at the active site with a fragment of the thrombin receptor PAR-1 shows that Asp at P3 forms a water-mediated contact with the side chain of R221a in the Na^+ binding loop (*7*) and points away from W215. No structural information about the interaction of thrombin with protein C is currently available, but it is reasonable to assume that the contacts documented in the thrombin−PAR-1 structure at the level of the P3 position may be relevant to protein C as well.

The different contribution made by W215 to fibrinogen and PAR1 recognition, and the similarity of P3 residues between protein C and PAR-1, suggested that mutation of W215 could afford a dissociation of thrombin functions. Residue W215 is absolutely conserved in thrombins from hagfish to humans (*8*) and is also highly conserved in serine proteases, regardless of their specific function or species (*9*). Here we show that mutation of W215 has profound effects on substrate recognition by thrombin and differentially affects the cleavage of fibrinogen, PAR-1, and protein C.

MATERIALS AND METHODS

Site-directed mutagenesis of human α-thrombin was carried out in a HPC4-pNUT expression vector, using overlap extension PCR with two mutant oligonucleotides [upstream, GGG CAT CGT CTC AXX XGG TGA AGG CTG TG; downstream, CAC AGC CTT CAC CXX XTG AGA CGA

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* To whom correspondence should be addressed. Telephone: (314) 362-4185. Fax: (314) 362-7183. E-mail: enrico@caesar.wustl.edu.

[1] Abbreviations: Ch, choline; FPR, H-D-Phe-Pro-Arg-*p*-nitroanilide; LDPR, H-L-Leu-Asp-Pro-Arg-*p*-nitroanilide; PAR-1, protease activated receptor 1; PEG, poly(ethylene glycol); *p*-NA, *p*-nitroaniline; PPACK, H-D-Phe-Pro-Arg-CH_2Cl; Tris, tris(hydroxymethyl)aminomethane.

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TGC CC] and two flanking oligonucleotide primers [*Bgl*II (upstream), GAA GAT CTA CAT CCA CCC CAG G; *Eco*RI (downstream), TGA CCA TGA TTA CGA ATT C]. Expression was carried out in baby hamster kidney cells as described previously (*10*). The enzyme was activated with the prothrombinase complex for about 30 min at 37 °C and then purified to homogeneity by monoS FPLC using a linear gradient of 0.05 to 0.5 M NaCl in 5 mM MES (pH 6) at room temperature. Mutants were further checked for incomplete refolding or autolytic digestion by N-terminal amino acid sequencing. Electrospray mass spectrometry yielded molecular weights consistent with the mutations introduced and indicated identical glycosylation in wild-type and mutant constructs. The active site concentration was determined by titration with hirudin and was found to be >90% in all cases.

All assays were carried out under experimental conditions of 5 mM Tris, 0.1% PEG, and 145 mM NaCl at pH 7.4 and 37 °C, unless otherwise specified. The chromogenic substrates FPR and LDPR were synthesized by solid phase HPLC, purified by HPLC, and analyzed by mass spectrometry. Progress curves of the release of *p*-NA following the hydrolysis of the chromogenic substrate were measured at 405 nm as a function of substrate concentration and analyzed to extract the values of k_{cat}/K_m and k_{cat}, after proper correction for product inhibition. The activation of protein C in the presence of 100 nM rabbit thrombomodulin and 5 mM $CaCl_2$, the release of fibrinopeptide A from fibrinogen and fibrinopeptide B from fibrin, and the inhibition of thrombin by antithrombin III in the presence of 0.5 USP unit/mL of heparin were carried out as described previously (*11*).

The interaction of thrombin with the platelet receptor PAR-1 was studied using the extracellular portion of the receptor spanning residues 33−62 (TR^{33-62}). The fragment has the sequence A^{33}TNATLDPRSFLLRNPNDKYEPFWEDEEKN^{62} and is cut by thrombin between R41 and S42 (*12*). TR^{33-62} and its product of cleavage (TR^{33-41}) are significantly different in size and could be separated by reverse phase HPLC. Product formation and substrate depletion were monitored with a C_{18} Waters Novapak column (4.6 mm × 250 mm, 4 μm). Optimal separation was achieved with a sodium phosphate buffer/acetonitrile linear gradient over the course of 30 min, at a flow rate of 1 mL/min. Extinction coefficients for TR^{33-62} and its products of cleavage were derived by calibration and quantitative amino acid analysis of highly pure standards. Products were also analyzed by electrospray mass spectroscopy and N-terminal amino acid sequencing. The concentration of TR^{33-62} was 2 μM, whereas enzyme concentrations ranged from 0.1 to 10 nM depending on the activity. Reactions were stopped at different times by addition of perchloric acid to a final concentration of 0.2 M. No degradation of TR^{33-62} occurred in the absence of thrombin under all the conditions that were tested. The concentrations of the product TR^{33-41} and the substrate TR^{33-62} measured as a function of time were analyzed according to the kinetic equations

$$[TR^{33-62}] = [TR^{33-62}]_0 \exp(-se_T t) \quad (1)$$

$$[TR^{33-41}] = [TR^{33-41}]_\infty [1 - \exp(-se_T t)] \quad (2)$$

where s ($=k_{cat}/K_m$) is the specificity constant for the cleavage of TR^{33-62} by thrombin and e_T is the thrombin concentration. These equations are valid when the substrate concentration is less than K_m. Attempts to measure the value of K_m indicated a value of >10 μM. The excellent fit of the progress curves of both substrate consumption and product release (see Results) confirms the validity of eqs 1 and 2.

Fluorescence spectra were measured using a QM-1 PTI spectrofluorometer, using an excitation wavelength of 282 nm and recording emission between 296 and 420 nm. Because W215 is responsible for the increase in intrinsic fluorescence upon Na^+ binding (see Results), fluorescence titrations of Na^+ binding in the W215 mutants could not be performed. The binding of Na^+ in these mutants was assessed from the linkage with hirudin binding (*13*), under experimental conditions of 5 mM Tris and 0.1% PEG at pH 8.0 and 25 °C, with the ionic strength kept constant at 800 mM with ChCl. The high ionic strength was required by the low Na^+ affinity of some mutants. The linkage expression used for the analysis of hirudin binding as a function of Na^+ concentration is (*13*)

$$\frac{1}{K_i} = \frac{\frac{1}{{}^0K_i} + \frac{1}{{}^1K_i}\frac{[Na^+]}{K_d}}{1 + \frac{[Na^+]}{K_d}} \quad (3)$$

where ${}^0K_i$ and ${}^1K_i$ are the values of K_i for hirudin in the absence and under saturating concentrations of Na^+, respectively. [Na^+] was changed by replacing NaCl with ChCl, as discussed elsewhere (*13*).

RESULTS

Na^+ binding to thrombin elicits a significant increase in the intrinsic fluorescence of the protein that can be used to measure a titration curve directly (*13*). The Trp residue responsible for this effect has not been identified. W215 is located about 10 Å away from the bound Na^+ in the crystal structure and is the Trp residue closest to the monovalent cation. Mutation of W215 to Phe practically abolishes the change in intrinsic fluorescence due to Na^+ binding (Figure 1), whereas it does not compromise the monovalent cation sensitivity of the enzyme (Figure 2). This proves that W215 is the chromophore responsible for the fluorescence change upon Na^+ binding.

Replacement of W215 with Tyr and Ala compromises the monovalent cation sensitivity of thrombin, suggesting a significant drop in Na^+ binding affinity (Figure 2). The lack of a fluorescence signal in the W215 derivatives made it necessary to measure the Na^+ binding affinity from the linkage with hirudin binding (*13*). The results of these measurements are shown in Figure 3 and confirm the expectation drawn from the data in Figure 2 that the W215Y and W215A mutants show a significantly reduced Na^+ affinity compared to those of the wild type and the W215F mutant. The origin of this difference is intriguing, given that Phe and Tyr only differ by a hydroxyl group and residue 215 is not directly involved in Na^+ coordination (*14*).

Practically all mutants of thrombin defective for Na^+ binding reported to date show a gain in anticoagulant activity because of the larger loss of fibrinogen clotting relative to protein C activation (*11, 15*−*17*). The loss of Na^+ binding

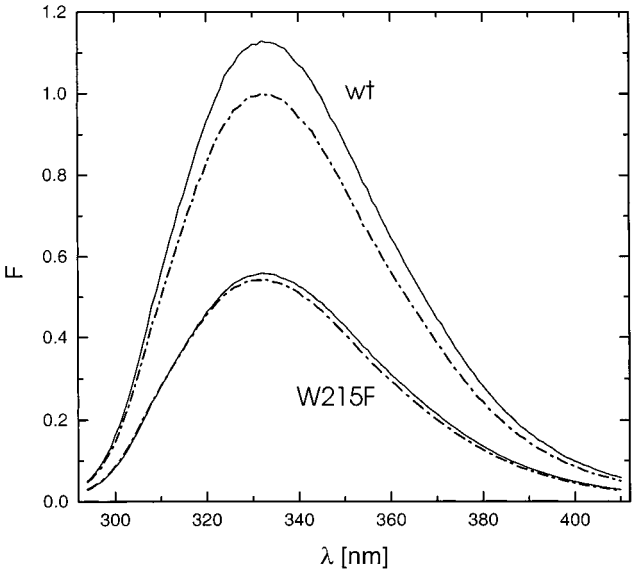

FIGURE 1: Fluorescence spectra of thrombin in NaCl (—) and ChCl (— - —). The binding of Na^+ in the wild type enhances the intrinsic fluorescence by nearly 15% at the peak. The effect is abrogated in the W215F mutant, which nonetheless binds Na^+ (see Figure 2 and Table 1). W215 is the chromophore responsible for the change in the intrinsic fluorescence observed upon Na^+ binding to thrombin. Experimental conditions are 5 mM Tris, 0.1% PEG, pH 8.0, 25 °C, and 800 mM NaCl or ChCl, as indicated.

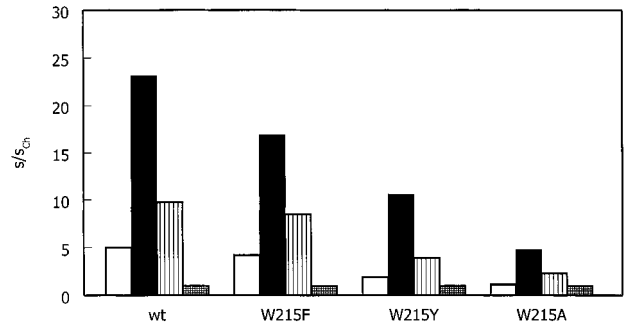

FIGURE 2: Monovalent cation specificity of wild-type and mutant thrombins. The values of *s* for the hydrolysis of FPR are reported in the presence of LiCl (white bars), NaCl (black bars), KCl (hatched bars), and ChCl (cross-hatched bars). Values are expressed relative to the value of *s* in ChCl, s_{Ch}. The monovalent cation sensitivity of thrombin is progressively compromised as W215 is replaced by Phe, Tyr, and Ala. The profiles suggest that mutants W215Y and W215A have lower Na^+ binding affinities than the wild type and the mutant W215F, consistent with the data depicted in Figure 3. Experimental conditions are 5 mM Tris, 0.1% PEG, pH 8.0, 25 °C, and 200 mM LiCl, NaCl, KCl, or ChCl, as indicated.

in the W215Y and W215A mutants suggests that these molecules may be stabilized in the slow form, with a reduced procoagulant activity and unaffected anticoagulant activity (*1*). A mutation of thrombin causes a pure allosteric effect only if it shifts the slow ↔ fast equilibrium without compromising other properties of the enzyme. In this case, the properties of the mutant can be predicted from those of the slow and fast forms of the wild type and the known Na^+ binding affinity. Mutation of a residue that affects Na^+ binding, but that is also involved in protein stability or direct ligand recognition, may generate effects in addition to the perturbation of the slow ↔ fast equilibrium. This is the case of residue W215, which is involved in direct binding of small chromogenic substrates (*3*) and fibrinogen (*4*−*6*). Whereas the W215F substitution does not affect significantly the value

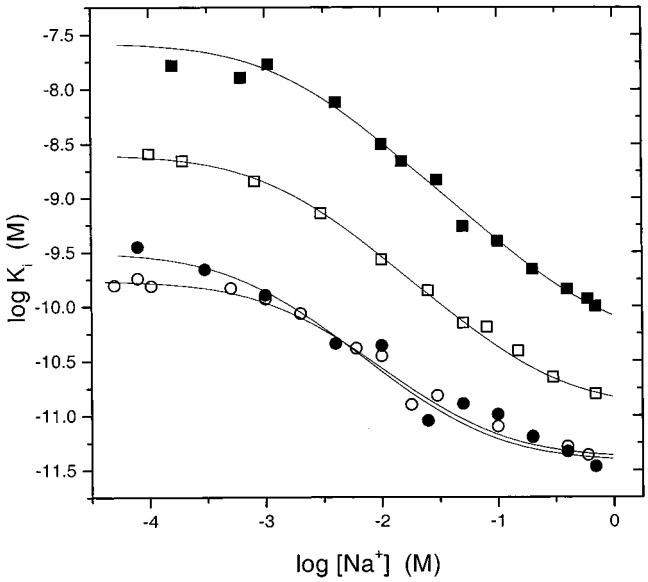

FIGURE 3: Linkage between hirudin and Na^+ binding to the wild type (○) and to thrombin mutants W215F (●), W215Y (□), and W215A (■). Continuous lines were drawn using eq 3 in the text with best-fit parameter values: (○) $^0K_i = 0.17 \pm 0.02$ nM, $^1K_i = 4.0 \pm 0.4$ pM, and $K_d = 66 \pm 3$ mM; (●) $^0K_i = 0.32 \pm 0.3$ nM, $^1K_i = 3.8 \pm 0.4$ pM, and $K_d = 62 \pm 3$ mM; (□) $^0K_i = 2.5 \pm 0.2$ nM, $^1K_i = 12 \pm 2$ pM, and $K_d = 260 \pm 50$ mM; and (■) $^0K_i = 27 \pm 3$ nM, $^1K_i = 63 \pm 9$ pM, and $K_d = 600 \pm 200$ mM. Experimental conditions are 5 mM Tris, 0.1% PEG, pH 8.0, and 25 °C. The ionic strength is 800 mM, kept constant with ChCl.

of *s* for the hydrolysis of the chromogenic substrates FPR and LDPR, the W215Y and W215A mutants exhibit specificity constants perturbed by 20−100-fold (Table 1). The aromatic nature of residue 215 is considered essential for high-affinity substrate binding, and indeed, ablation of this property in the W215A mutant produces a significant increase in K_m, with a smaller effect on k_{cat} in FPR. The presence of the hydroxyl group on the aromatic ring of Tyr also reduces the level of substrate binding relative to that of the Phe derivative through an effect mainly on the K_m, suggesting that the polar group of the Tyr is not well accommodated in the highly hydrophobic environment around W215 in the wild type (*3*, *9*). Finally, the loss of specificity due to substrate binding is more pronounced for FPR, which mimics the interaction of fibrinogen, than for LDPR, which mimics the interaction of PAR-1 and possibly protein C with thrombin. The crystal structure of thrombin inhibited with PPACK, which closely resembles FPR, shows an edge-to-face interaction between H-D-Phe at P3 and W215 (*3*). On the other hand, the structure of thrombin inhibited at the active site with a fragment of the thrombin receptor PAR-1, carrying the sequence LDPR into the active site, shows Asp at P3 pointing away from W215 and making a water-mediated contact with R221a in the Na^+ binding loop (*7*).

The foregoing results presage improved anticoagulant properties of the mutants of W215, similar to those observed for residues around the Na^+ binding loop (*11*, *17*). Indeed, mutation of W215 affects profoundly the ability of thrombin to release fibrinopeptide A from fibrinogen (Table 1). This property is compromised in all mutants and reaches a loss of 500-fold in the W215A construct. The loss is almost entirely due to the replacement of W215 and the disruption of the favorable interaction with fibrinogen. The Na^+ affinity

Table 1: Specificity of Wild-Type and Residue W215 Mutant Thrombins[a]

	wild type	W215F	W215Y	W215A
Na^+ binding K_d (mM)[b]	66 ± 3	62 ± 3	260 ± 50	600 ± 200
FPR[c] k_{cat}/K_m (μM^{-1} s^{-1})	88 ± 4	44 ± 1	5.4 ± 0.4	0.91 ± 0.01
k_{cat} (s^{-1})	56 ± 3	77 ± 8	120 ± 10	43 ± 2
LDPR[c,d] k_{cat}/K_m (μM^{-1} s^{-1})	4.7 ± 0.9	1.2 ± 0.1	0.040 ± 0.002	0.025 ± 0.001
fibrinogen k_{cat}/K_m (μM^{-1} s^{-1})[e]	17 ± 1	2.3 ± 0.1	0.19 ± 0.01	0.034 ± 0.002
fibrin k_{cat}/K_m (μM^{-1} s^{-1})[e]	8.1 ± 0.5	1.4 ± 0.1	0.08 ± 0.01	0.053 ± 0.003
protein C k_{cat}/K_m (μM^{-1} s^{-1})[f]	0.22 ± 0.01	0.13 ± 0.01	0.032 ± 0.008	0.075 ± 0.006
PAR-1 k_{cat}/K_m (μM^{-1} s^{-1})	26 ± 1	8.0 ± 0.3	1.2 ± 0.1	1.0 ± 0.1
antithrombin III k_{on} (μM^{-1} s^{-1})[g]	13 ± 1	12 ± 1	1.2 ± 0.01	0.56 ± 0.04

[a] Experimental conditions are 5 mM Tris, 0.1% PEG, 145 mM NaCl, pH 7.4, and 37 °C, unless otherwise noted. [b] Derived from the linkage with hirudin binding under experimental conditions of 5 mM Tris, 0.1% PEG, pH 8.0, 25 °C, and $I = 800$ mM. [c] Experimental conditions are 5 mM Tris, 0.1% PEG, 200 mM NaCl, pH 8.0, and 25 °C. [d] Values of k_{cat} could not be estimated due to the very high K_m of this substrate. [e] Fibrinopeptide A released from fibrinogen and fibrinopeptide B released from fibrin I monomers. [f] In the presence of 100 nM rabbit thrombomodulin and 5 mM $CaCl_2$. [g] In the presence of 0.5 USP unit/mL heparin.

of the W215A mutant under physiological conditions is too low to measure, and no significant change is observed between the specificity constant of the slow form (obtained in the presence of 145 mM ChCl) and that obtained with 145 mM NaCl. On the other hand, measurements carried out at 25 °C and $I = 800$ mM, as for hirudin binding, enable estimation of the properties of the Na^+-bound fast form of the W215A mutant. Na^+ binding does not restore activity because the release of fibrinopeptide A by the fast form of W215A occurs with a specificity constant that is 300-fold lower than that of the wild type, in close agreement with the drop of 500-fold measured under physiological conditions (Table 1). A comparison with the cleavage of FPR reveals a similar loss of specificity (10-fold) of W215Y relative to that of W215F, again suggesting that the presence of the hydroxyl group on the aromatic ring of Tyr is detrimental to substrate binding. However, the loss of specificity in the W215A mutant relative to that of the wild type is more pronounced in the case of fibrinogen than in the case of FPR. The replacement of Trp with Phe already reduces the specificity constant of fibrinogen 7-fold instead of 2-fold, and addition of the hydroxyl group on the Phe ring produces a further drop of specificity of 12-fold instead of 8-fold, giving rise to a loss of specificity in W215Y relative to the wild type of 90-fold for fibrinogen as opposed to only 16-fold for FPR. When the aromatic side chain of Trp is removed altogether in the Ala substitution, the drop in specificity toward fibrinogen reaches 500-fold.

An interesting feature of the role of W215 in the interaction of thrombin with fibrinogen is that the W215A mutant releases fibrinopeptide A from fibrinogen and fibrinopetide B from fibrin with similar kinetics and rate constants. The Shafer mechanism of release of fibrinopeptides (*18*) states that fibrinopeptide A is released first from fibrinogen, leading to formation of fibrin I monomers. These monomers aggregate to form fibrin I protofibrils, from which fibrinopeptide B is released to give rise to fibrin II protofibrils that form the scaffold of the fibrin clot. Under conditions where the thrombin concentration is rate-limiting, a lag phase occurs after the release of fibrinopeptide A before appreciable amounts of fibrinopeptide B are detected (Figure 4). The mechanism is obeyed by the wild type and the thrombin mutants W215F and W215Y, but does not hold for the W215A mutant. In this case, release of fibrinopeptide B occurs without delay and with a rate constant that is slightly higher than that pertaining to fibrinopeptide A (Table 1). It is conceivable that steric constraints in the S3 and S4 sites of thrombin oppose the release of fibrinopeptide B directly from fibrinogen and that these constraints are removed with the W215A substitution. Most likely, then, both fibrinopeptide A and B can be released from fibrinogen under physiological conditions, but the rate of fibrinopeptide B release is too small to measure and becomes appreciable only after fibrin I protofibrils are formed.

The drastic perturbation of substrate binding seen in the case of fibrinogen is not matched by protein C in the presence of thrombomodulin. This substrate experiences only a modest loss of specificity, as the value of *s* drops 3-fold in the W215A mutant. The loss of specificity toward protein C is even less pronounced than that seen in the case of LDPR, a chromogenic substrate carrying Asp at P3 like protein C. We conclude that the environment of W215 of thrombin is not significantly involved in the binding of protein C. The differential effect on the binding of fibrinogen and protein C makes the W215A mutant the best anticoagulant thrombin reported to date (*17*). The gain in anticoagulant potency is larger than that of the E217K mutant (*16*) and the deletion mutant of the autolysis loop (*20*). E217 makes a tenuous contact with G12 of the fibrinogen Aα chain, and ion-pairs with K224 to stabilize the Na^+ binding environment (*14*). The loss of Na^+ binding in this mutant (*17*), together with the direct effect on fibrinogen binding, brings about compromised cleavage of the procoagulant substrate relative to protein C. A similar effect is seen upon deletion of the autolysis loop of thrombin (*20*), though in this case the structural basis of the effect is less clear because the loop makes no contact with fibrinopeptide A bound to the active site of thrombin (*4*−*6*).

The results obtained in the case of the thrombin receptor PAR-1 are of particular importance. Little is known about the epitopes and energetic signatures of PAR-1 binding to thrombin (*12*, *21*−*23*). Although exosite I is involved in recognition of PAR-1 (*21*), the precise extent of the epitope in this region and whether it overlaps with those of fibrinogen and thrombomodulin have not been established. The crystal structure of thrombin inhibited at the active site with a fragment of PAR-1 reveals a mode of interaction of the P3 residue that differs from that seen in PPACK or fibrinogen. Therefore, the possibility exists that fibrinogen and PAR-1 binding can be dissociated by modifying residues in the active site. The availability of a quantitative assay on the purified, extracellular fragment of PAR-1 enables the direct

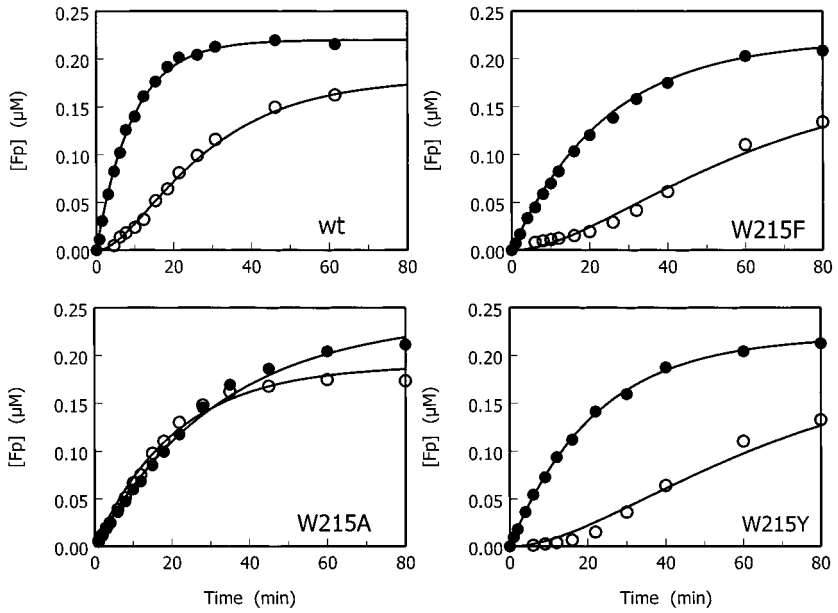

FIGURE 4: Progress curves of the release of fibrinopeptides A (●) and B (○) by wild-type and mutant thrombins. Continuous lines were drawn using eqs 3a and 3b of Vindigni and Di Cera (*19*), with κ_1 and κ_2 expressed as the value of s for the release of fibrinopeptide (s_1 for fibrinopeptide A and s_2 for fibrinopeptide B), times the concentration of thrombin e_T. The best-fit parameter values are as follows: wild type, $[FpA]_\infty = 0.22 \pm 0.01$ μM, $s_1 = 17 \pm 1$ μM^{-1} s^{-1}, $f[FpB]_\infty = 0.18 \pm 0.01$ μM, $s_2 = 8.1 \pm 0.5$ μM^{-1} s^{-1}, and $e_T = 0.1$ nM; W215F, $[FpA]_\infty = 0.22 \pm 0.01$ μM, $s_1 = 2.3 \pm 0.1$ μM^{-1} s^{-1}, $f[FpB]_\infty = 0.18 \pm 0.01$ μM, $s_2 = 1.4 \pm 0.1$ μM^{-1} s^{-1}, and $e_T = 0.3$ nM; W215Y, $[FpA]_\infty = 0.22 \pm 0.01$ μM, $s_1 = 0.19 \pm 0.01$ μM^{-1} s^{-1}, $f[FpB]_\infty = 0.20 \pm 0.01$ μM, $s_2 = 0.08 \pm 0.01$ μM^{-1} s^{-1}, and $e_T = 4$ nM; and W215A, $[FpA]_\infty = 0.24 \pm 0.01$ μM, $s_1 = 0.034 \pm 0.002$ μM^{-1} s^{-1}, $f[FpB]_\infty = 0.19 \pm 0.01$ μM, $s_2 = 0.053 \pm 0.003$ μM^{-1} s^{-1}, and $e_T =$ 15 nM. In the case of W215A, the parameters for the release of fibrinopeptide B refer to an equation of the same form as eq 3a of Vindigni and Di Cera (*19*) because no lag phase was observed. Experimental conditions are 5 mM Tris, 0.1% PEG, 145 mM NaCl, pH 7.4, and 37 °C.

study of the structural components involved in PAR-1 binding. The results are shown in Figure 5. The excellent fit observed using eqs 1 and 2 proves that the kinetics of PAR-1 cleavage is first-order under the conditions tested and that the K_m is greater than 2 μM. This differs from previous data where the cleavage of PAR-1 was studied using a smaller fragment encompassing residues 38−60 of the thrombin receptor, which was found to be cut by thrombin with a higher value of s compared to that of TR^{33-62} (Table 1) and a K_m of 1.3 μM (*22*). Our data agree well with a recent study of the cleavage of the entire extracellular domain of PAR-1, where values of s (7.5 μM^{-1} s^{-1}) and K_m (16 μM) have been reported (*23*), lending further support to the validity of eqs 1 and 2.

Mutation of W215 affects cleavage of PAR-1 to an extent intermediate between those of fibrinogen and protein C. As for fibrinogen, replacement of Trp with Phe and Tyr progressively reduces specificity, but the Ala mutant behaves like the Tyr mutant. The aromatic ring of Phe preserves the interaction with PAR-1, but introduction of a hydroxyl group in the ring, or removal of the ring altogether, produces a similar loss of cleavage of PAR-1. This lends further support to the notion that the hydroxyl group of Tyr is not accommodated well in the hydrophobic environment of W215 of thrombin and the presence of Tyr at position 215 may compromise the advantageous properties of an aromatic residue at this position. As a result of the replacement of Trp with Ala at position 215, a differential effect on substrate recognition is seen. The W215A has severely compromised clotting activity (500-fold), with moderate loss of PAR-1 cleavage (26-fold) and insignificant loss of protein C cleavage (3-fold). Hence, mutation of W215 with Ala affords a significantly different perturbation of three important functions of thrombin mediated by the cleavage of fibrinogen leading to clot formation, cleavage of PAR-1 leading to platelet aggregation, and cleavage of protein C leading to inactivation of Va and reduced thrombin generation. Whereas the wild type cleaves fibrinogen and PAR-1 with comparable specificity constants, the W215A mutant preferentially cleaves PAR-1 (Table 1). Hence, mutation of W215 to Ala affords a change in specificity of thrombin from fibrinogen to PAR-1.

The peculiar profile seen for PAR-1 cleavage in the W215 mutants is replicated in the interaction of antithrombin III in the presence of heparin (Table 1). In this case, Phe is equivalent to the wild-type Trp, whereas Tyr and Ala afford a decreased interaction of antithrombin III. The loss is only 25-fold and compares well with PAR-1 cleavage.

DISCUSSION

W215 in thrombin interacts differently with different physiologic substrates. Mutation of W215 to Ala compromises significantly fibrinogen binding, and to a lesser extent PAR-1 cleavage, and has almost no effect on protein C cleavage in the presence of thrombomodulin. The molecular origin of this effect resides in the direct involvement of W215 in fibrinogen, but not protein C, recognition. A smaller secondary effect is generated by the loss of Na^+ binding in the W215A mutant and stabilization of the anticoagulant slow form of the enzyme.

We have documented peculiar effects of the substitution of W215 with Phe and Tyr. The presence of a polar group on the aromatic ring of Tyr greatly compromises Na^+ binding, as well as fibrinogen cleavage and chromogenic

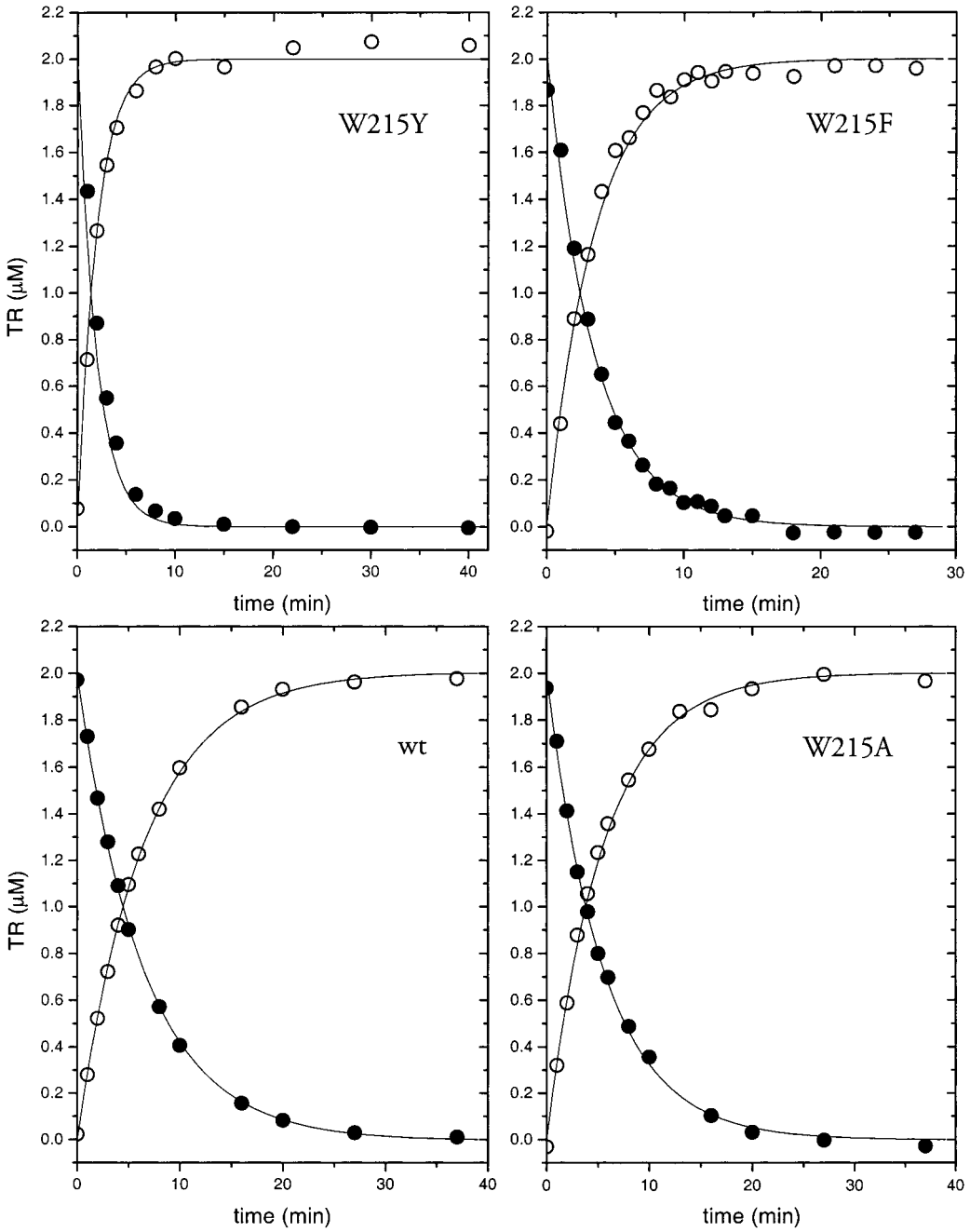

FIGURE 5: Progress curves of the hydrolysis of TR^{33-62} (●) and the release of the product TR^{33-41} (○) by wild-type and mutant thrombins. Continuous lines were drawn using eqs 1 and 2 in the text with best-fit parameter values: wild type, $[TR^{33-62}]_0 = [TR^{33-41}]_\infty = 2.0 \pm 0.1$ μM, $s = 26 \pm 1$ μM^{-1} s^{-1}, and $e_T = 0.1$ nM; W215F, $[TR^{33-62}]_0 = [TR^{33-41}]_\infty = 2.0 \pm 0.1$ μM, $s = 8.0 \pm 0.3$ μM^{-1} s^{-1}, and $e_T = 0.6$ nM; W215Y, $[TR^{33-62}]_0 = [TR^{33-41}]_\infty = 2.0 \pm 0.1$ μM, $s = 1.2 \pm 0.1$ μM^{-1} s^{-1}, and $e_T = 6.9$ nM; and W215A, $[TR^{33-62}]_0 = [TR^{33-41}]_\infty = 2.0 \pm 0.2$ μM, $s = 1.0 \pm 0.1$ μM^{-1} s^{-1}, and $e_T = 3$ nM. Experimental conditions are 5 mM Tris, 0.1% PEG, 145 mM NaCl, pH 7.4, and 37 °C.

substrate hydrolysis. It is not clear how such a seemingly tenuous perturbation of the aromatic ring would produce large functional consequences without invoking changes in the stability of the enzyme. It is possible that the presence of a polar group in the hydrophobic environment around W215 is not well tolerated and may actually position the side chain of Tyr in a different conformation relative to Phe. W215 is almost completely buried, and a Tyr at position 215 may gain solvent exposure and cause steric hindrance for incoming substrates. The effect on Na^+ binding remains unexplained. Residues 215−217 play a very important role in thrombin and other serine proteases (*1*, *3*, *9*). The role of W215 has been documented here by site-directed mutagenesis. Residue 216 is a Gly in practically all proteases because a side chain at this position would hinder access to the active site and the primary specificity pocket. Residue 217 is Glu in thrombin and ion-pairs to K224 on the neighbor antiparallel β-strand defining the wall of the Na^+ channel and the primary specificity pocket (*1*, *14*). Breakage of the ion pair with the Ala substitution of either E217 or K224 results in almost identical effects due to the loss of Na^+ binding and reduced fibrinogen clotting (*11*, *15*, *17*). A reasonable explanation for the effects seen in the W215Y and W215A mutants is that perturbation of residue 215 propagates to the neighbor residues G216 and E217, producing changes in the access to the S1 site and reduced Na^+ binding.

The moderate effect on PAR-1 cleavage observed in the W215 mutants is noteworthy. PAR-1 binds to exosite I via a sequence that closely resembles the acidic C-tail of hirudin (*12*). This interaction also mimics a similar interaction of the fibrinogen Aα chain downstream from the site of cleavage by thrombin (*4*). Perturbation of exosite I by enzymatic digestion with trypsin to produce γ-thrombin abrogates both fibrinogen and PAR-1 cleavage (*12*). However, significant differences exist in the way fibrinogen and PAR-1 contact the active site of thrombin, with W215 making a direct interaction with fibrinogen but not PAR-1. This observation is corroborated by our results and shows that the W215 environment is predominantly involved in the clotting function of thrombin. Consequently, the W215A mutation produces a thrombin that cleaves PAR-1 with a specificity constant that is 20-fold higher than that of fibrinogen or protein C.

These results broaden our understanding of thrombin specificity and the structural determinants that are involved in substrate recognition. Further studies are necessary to identify more precisely the epitopes for protein C and PAR-1 binding. The penultimate β-strand of the B chain hosts highly conserved residues such as W215 and G216 whose mutation affects both the specificity and catalytic activity of the enzyme. This region represents an important target for future mutagenesis studies.

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