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Dissecting substrate recognition by thrombin using the inactive mutant S195A☆

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Abstract

The catalytically inactive mutant S195A was used to study the interaction of thrombin with substrates under equilibrium conditions. By monitoring changes in intrinsic fluorescence, we measured dissociation constants for a variety of synthetic substrates, PAR peptides and the inhibitor PPACK. The S195A mutant retains the Na^+-binding properties of the wild type, and substrate binding to the mutant is enhanced by the presence of Na^+. Temperature dependence studies allowed calculation of the thermodynamic parameters of substrate binding at the active site and showed a negligible ΔC_p. Titration of synthetic substrates carrying substitutions at the P1–P3 positions revealed energetics consistent with the specificity hierarchy identified in hydrolysis by the wild type. Titration with PAR peptides, which interact with both the active site and exosite I of thrombin, also showed consistency with the results obtained with the wild type at steady state. These findings demonstrate that inactive mutants of enzymes make it possible to dissect the equilibrium components linked to substrate binding and complement information on the kinetic properties of the wild type.

Keywords: Molecular recognition; Serine protease; Site-specific cooperativity; Thrombin

1. Introduction

Substrate binding to enzymes such as serine proteases is not easily accessible to experimental measurements under equilibrium conditions. Because substrates are hydrolyzed, inactivated forms of the enzymes are required for these studies. Anhydro-derivatives of thrombin, chymotrypsin and trypsin, in which the catalytic S195 is chemically modified to dehydroalanine, have been synthesized and characterized [1–4]. Anhydrothrombin has been studied in terms of its interactions with both natural substrates and inhibitors. It binds the substrates factor VIII and fibrinogen in the nanomolar range and with higher affinity in the presence of Na^+ [4], and it binds the inhibitor hirudin with a minor (2.6-fold) increase in disso-

☆ It is a privilege to contribute to the special issue honoring the memory of John T. Edsall. Ours is a small tribute to one who was a great scientist, mentor and friend.

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ciation constant compared to the wild type [3]. These studies indicate that anhydrothrombin has properties analogous to the wild type. Mutants of thrombin, trypsin and tissue plasminogen activator in which the catalytic S195 is mutated to Ala have been used as tools in the analysis of how natural inhibitors bind to serine proteases. Binding kinetics of the high-affinity reversible inhibitor hirudin to S195A thrombin are similar to those for the wild type [5]. S195A thrombin can induce conformational changes in antitrypsin upon binding, although the changes are not as pronounced as for the wild type [6]. However, S195A mutants bind serpin-type covalent inhibitors poorly, indicating that covalent binding to S195 is crucial for stable inhibitor–protease complexes [7,8]. Therefore, inactive derivatives seem to be adequate substitutes in interactions with substrates and non-covalent inhibitors, but they do not emulate wild-type interactions with large-molecule irreversible inhibitors.

Thrombin possesses nine Trp residues, and is thus amenable to experiments utilizing the intrinsic fluorescence of the enzyme. Four of these residues, W60d, W96, W148 and W215 (chymotrypsinogen numbering), are located near or within the active site and unprimed specificity pockets [9]. Therefore, intrinsic fluorescence measurements represent an ideal method for studying the interaction of thrombin with both substrates and active site inhibitors. An additional advantage of intrinsic fluorescence measurements is that exogenous fluorescence labels need not be used; such moieties might alter the ligand specificity of the enzyme or block the access of substrates to the active site. Here we demonstrate that intrinsic fluorescence can be used to study the interaction of small-molecule substrates and inhibitors with thrombin under equilibrium conditions. This approach reveals how the process of equilibrium binding without catalysis contributes to molecular recognition by the active enzyme. The form of the enzyme used is the mutant S195A. The structure of this mutant is nearly identical to that of the wild type [10]. The dissociation constants obtained for synthetic chromogenic substrates, PAR peptides and the inhibitor PPACK give key insights into ligand recognition by thrombin and shed light upon previous results obtained by kinetic and structural experiments.

2. Materials and methods

Site-directed mutagenesis of human α-thrombin was carried out in a HPC4-pNUT expression vector, using the Quikchange site-directed mutagenesis kit from Stratagene. Expression of S195A thrombin was carried out in baby hamster kidney cells, as previously described [11]. S195A and wild-type thrombin were activated with the prothrombinase complex for 60 min at 37 °C. Further activation of S195A was carried out using the immobilized snake-venom enzyme ecarin for 5 h at 37 °C, with rotation. Enzymes used in activation were supplied by American Diagnostica. Activated S195A and wild-type thrombin were purified to homogeneity by FPLC using Resource Q and S columns with a linear gradient from 0.05 to 0.5 M choline chloride (ChCl), 5 mM MES, pH 6, at room temperature.

Fluorescence experiments were carried out using a QM-1 PTI spectrophotometer. Fluorescence titration of S195A and wild-type thrombin with Na^+ took place under experimental conditions of 5 mM Tris, 0.1% poly(ethyleneglycol) (PEG), 800 mM ionic strength, pH 8.0, at 25 °C. Titrations were carried out as follows: a solution containing 100 nM enzyme, 5 mM Tris, 0.1% PEG and 800 mM NaCl was incrementally added to a solution containing 100 nM enzyme, 5 mM Tris, 0.1% PEG and 800 mM ChCl. Ionic strength and enzyme concentration were held constant, while the Na^+ concentration was varied. Excitation was at 284 nm and emission was measured at 333 nm. Appropriate corrections were made for buffer fluorescence and photobleaching. The value of thrombin intrinsic fluorescence as a function of Na^+ concentration was fitted according to the equation:

$$F = \frac{F_0 + F_1 \frac{x}{K_d}}{1 + \frac{x}{K_d}} \quad (1)$$

where F is the intrinsic fluorescence at a particular Na^+ concentration, F_0 is the intrinsic fluorescence

in the absence of Na^+, F_1 is the intrinsic fluorescence under saturating Na^+, x is the Na^+ concentration and K_d is the equilibrium dissociation constant for Na^+ binding.

Ligands were obtained as follows: FPR (H–D-Phe–Pro–Arg–*p*-nitroanilide), FPK (H–D-Phe–Pro–Lys–*p*-nitroanilide), FGR (H–D-Phe–Gly–Arg–*p*-nitroanilide), FGK (H–D-Phe–Gly–Lys–*p*-nitroanilide), VPR (H–D-Val–Pro–Arg–*p*-nitroanilide), VPK (H–D-Val–Pro–Lys–*p*-nitroanilide), VGR (H–D-Val–Gly–Arg–*p*-nitroanilide) and VGK (H–D-Val–Gly–Lys–*p*-nitroanilide) from Midwest Biotech; AAPF (*N*-succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide) from Sigma; protease-activated receptor (PAR) peptides [12] from Biomolecules Midwest; and PPACK (H–D-Phe–Pro–Arg–chloromethylketone) from Calbiochem. Fluorescence titration of S195A with ligands took place under experimental conditions of 5 mM Tris, 0.1% PEG, 800 mM salt, pH 8.0, at 25 °C. Salts used included LiCl, NaCl, KCl and ChCl. Experiments measuring ionic strength dependence of FPR binding varied the NaCl concentration from 100 mM to 800 mM. Experiments measuring temperature dependence of FPR binding were carried out at pH 8.0 over the temperature range from 5 to 45 °C. Titrations were carried out as follows: a solution containing 50 nM enzyme, ligand, 5 mM Tris, 0.1% PEG and 800 mM salt was incrementally added to a solution containing 50 nM enzyme, 5 mM Tris, 0.1% PEG and 800 mM salt. Enzyme concentrations of 10 nM were used when measuring dissociation constants below 10 nM. Thus, ionic strength and enzyme concentration were held constant, while the ligand concentration was varied. Excitation was at 284 nm and emission was measured at 333 nm. Appropriate corrections were made for buffer fluorescence, photobleaching and absorbance or intrinsic fluorescence of ligands. Since dissociation constants were frequently of the same order of magnitude as enzyme concentrations, the value of thrombin intrinsic fluorescence as a function of ligand concentration was fitted according to the equations:

$$F = F_0 + \theta(F_1 - F_0) \tag{2}$$

$$\theta = \frac{x_{tot} - K_d - e_{tot} + \sqrt{(x_{tot} - K_d - e_{tot})^2 + 4K_d x_{tot}}}{x_{tot} + K_d - e_{tot} + \sqrt{(x_{tot} - K_d - e_{tot})^2 + 4K_d x_{tot}}} \tag{3}$$

where F is the intrinsic fluorescence at a particular total ligand concentration, x_{tot}, F_0 is the intrinsic fluorescence in the absence of ligand, F_1 is the intrinsic fluorescence under saturating ligand, K_d is the equilibrium dissociation constant for ligand binding and e_{tot} is the total thrombin concentration.

The ionic strength dependence of FPR binding to S195A was analyzed according to the expression:

$$-\ln K_d = A_0 + \Gamma \ln[\text{salt}] \tag{4}$$

This is the Taylor expansion of the function $-\ln K_d$ around the logarithm of [salt] = 1 M, where $-\ln K_d = A_0$. Γ is the phenomenological coefficient quantifying the change in $-\ln K_d$ due to a change in ln [salt].

Site-specific thermodynamic parameters were derived from the dissociation constants as follows [13,14]:

$$\Delta G_1 = RT \ln \frac{K_d^{FPK}}{K_d^{FPR}} \tag{5a}$$

$$\Delta G_2 = RT \ln \frac{K_d^{FGR}}{K_d^{FPR}} \tag{5b}$$

$$\Delta G_3 = RT \ln \frac{K_d^{VPR}}{K_d^{FPR}} \tag{5c}$$

$$\Delta G_{12} = RT \ln \frac{K_d^{FGK} K_d^{FPR}}{K_d^{FPK} K_d^{FGR}} \tag{5d}$$

$$\Delta G_{13} = RT \ln \frac{K_d^{VPK} K_d^{FPR}}{K_d^{FPK} K_d^{VPR}} \tag{5e}$$

$$\Delta G_{23} = RT \ln \frac{K_d^{VGR} K_d^{FPR}}{K_d^{FGR} K_d^{VPR}} \tag{5f}$$

$$\Delta G_{123} = RT \ln \frac{K_d^{VGK} K_d^{FPR} K_d^{FPR}}{K_d^{FPK} K_d^{FGR} K_d^{VPR}} \tag{5g}$$

where R is the gas constant and T the absolute temperature. All changes were calculated relative to FPR to avoid any ambiguity in the definition of the absolute free energy under conditions of equilibrium with the substrate. ΔG_1, ΔG_2 and ΔG_3 are

the changes in binding energy due to single-site substitutions at P1, P2 and P3, respectively. ΔG_{12}, ΔG_{13} and ΔG_{23} are the second-order coupling free energies for substitutions made at the three possible pairs of sites, and ΔG_{123} is the third-order coupling free energy for the triple substitution. These coupling energies reflect interactions between substitutions made at different sites that may reduce ($\Delta G>0$) or enhance ($\Delta G<0$) binding beyond simple additivity.

The equilibrium dissociation constant reflecting product inhibition, K_p, for the hydrolysis of FPR by wild-type thrombin was determined from progress curves of *p*-nitroaniline release at 405 nm in 800 mM salt, 5 mM Tris–HCl, 0.1% PEG, pH 8.0, at 25 °C. The relevant Michaelis–Menten scheme, involving the conversion of substrate S into product P by the enzyme E in the presence of product inhibition is:

$$\mathrm{E}+\mathrm{S}\underset{k_{-1}}{\overset{k_1}{\rightleftarrows}}\mathrm{ES}\xrightarrow{k_2}\mathrm{E}+\mathrm{P}+\mathrm{P}' \tag{6a}$$

$$\mathrm{E}+\mathrm{P}\underset{k_{-3}}{\overset{k_3}{\rightleftarrows}}\mathrm{EP} \tag{6b}$$

where k_1 is the second-order rate constant for substrate binding, k_{-1} is the first-order rate constant for substrate dissociation, k_2 is the first-order rate of turnover and the ratio k_{-3}/k_3 defines the product inhibition constant K_p. P′ denotes the substrate leaving group, *p*-nitroaniline, that serves as a chromophore for the analysis. The velocity of the reaction in the presence of product inhibition, under the steady-state approximation, is:

$$\begin{aligned} v &= \frac{\mathrm{d}P(t)}{\mathrm{d}t} = -\frac{\mathrm{d}S(t)}{\mathrm{d}t} = k_2\mathrm{ES}(t) \\ &= k_{\mathrm{cat}}e_{\mathrm{tot}}\frac{S(t)}{K_{\mathrm{m}}\left[1+\dfrac{P(t)}{K_{\mathrm{p}}}\right]+S(t)} \\ &= k'_{\mathrm{cat}}e_{\mathrm{tot}}\frac{S(t)}{K'_{\mathrm{m}}+S(t)} \end{aligned} \tag{7}$$

where $P(t)$ and $S(t)$ are concentrations of product and substrate at time t, e_{tot} is the total enzyme concentration and

$$K'_{\mathrm{m}} = K_{\mathrm{m}}\frac{1+\dfrac{S(0)}{K_{\mathrm{p}}}}{1-\dfrac{K_{\mathrm{m}}}{K_{\mathrm{p}}}} \tag{8a}$$

$$k'_{\mathrm{cat}} = k_{\mathrm{cat}}\frac{1}{1-\dfrac{K_{\mathrm{m}}}{K_{\mathrm{p}}}} \tag{8b}$$

Eq. (7) cannot be integrated to yield a closed-form solution for $P(t)$ or $S(t)$ as an explicit function of time t. However, the value of $P(t)$ or $S(t)$ can be calculated at any time t from the absorbance readings via the extinction coefficient, and the derivative in Eq. (7) can be numerically calculated from the slope of the progress curve at any point. Hence, at any given time t, a progress curve can return both the value of $P(t)$ or $S(t)$ and the derivative $\mathrm{d}P(t)/\mathrm{d}t$ or $\mathrm{d}S(t)/\mathrm{d}t$. This yields a Michaelis–Menten plot of the velocity v of the reaction vs. the substrate concentration S at any time along the progress curve in a straightforward manner, without the need for numerical integration. Progress curves obtained at different substrate concentrations and transformed for analysis according to Eq. (7)–Eq. (8b) yield the Michaelis–Menten parameters for substrate hydrolysis corrected for product inhibition, and the value of K_p for the equilibrium dissociation constant of product binding to the enzyme.

3. Results and discussion

Na^+ binding to thrombin elicits an increase (10–15%) in the intrinsic fluorescence of the protein, allowing direct measurement of a titration curve [15]. Fluorescence titration of Na^+ binding yields dissociation constants (K_d) of 19 and 15 mM for S195A and the wild type, respectively. Furthermore, the fluorescence signal of S195A is comparable to that of the wild type. Therefore, S195A exerts little or no perturbation on the solution structure or monovalent cation binding properties of thrombin. As a result, we can distinguish between the slow (Na^+-free) and fast (Na^+-bound) forms of S195A under equilibrium conditions.

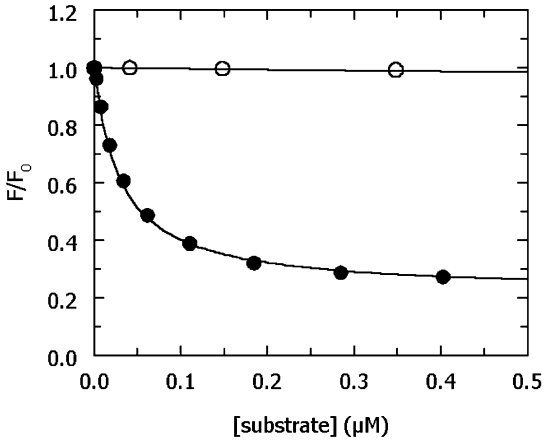

Fig. 1. Representative titration curves of the chromogenic substrates FPR (●) and AAPF (□) with S195A thrombin. Saturating concentrations of AAPF, a chymotrypsin substrate, reduce thrombin intrinsic fluorescence by approximately 9%, whereas thrombin-specific substrates such as FPR cause decreases of at least 50%. Best-fit parameters were determined from Eqs. (2) and (3) in the text: FPR, $F_1/F_0 = 0.219 \pm 0.004$ and $K_d = 26.5 \pm 0.8$ nM; AAPF, $F_1/F_0 = 0.91 \pm 0.01$ and $K_d = 2.5 \pm 0.3$ μM. F_1/F_0 values indicate the fractional intrinsic fluorescence change in thrombin that would be caused by saturating ligand.

Fluorescence titration of the synthetic thrombin substrates FPR, FPK, FGR, FGK, VPR, VPK, VGR and VGK with S195A brings about a substantial decrease (50–75%) in the intrinsic fluorescence of the protein, allowing direct measurement of titration curves (Fig. 1). Substrate binding does not appreciably alter the peak emission or excitation wavelengths of the enzyme. The magnitude of the fluorescence change is similar for the fast and slow forms. Use of saturating concentrations of Na^+ (800 mM) for experiments involving the fast form ensured that any changes in occupancy of the Na^+ site were too small to measurably affect titration curves. Titration with the chymotrypsin substrate AAPF, towards which thrombin has poor specificity, brings about a negligible fluorescence change compared to FPR, the most specific thrombin substrate examined (Fig. 1). The sequences of the eight thrombin substrates titrated against S195A allow determination of the relative contributions of the P1–P3 substrate subsites to binding at equilibrium (Table 1). The dissociation constants of the substrates increase with the number of subsite perturbations, approximately paralleling the decrease in enzyme specificity observed going from FPR to VGK [16]. Calculation of the free energy values due to perturbation of the P1–P3 residues of the substrate, however, reveals that the positive subsite cooperativity observed for the transition state by kinetic measurements does not exist for equilibrium binding (Table 2). In fact, the third-order coupling energies for the fast and slow forms of S195A are strongly negative, whereas the corresponding parameters are strongly positive for the wild-type transition state. Substrate binding at equilibrium thus appears to be characterized in part by negative cooperativity.

The affinity of FPR for thrombin was measured as a function of ionic strength in the range from 100 to 800 mM NaCl (Fig. 2). The dissociation constant increases minimally with ionic strength; the value of Γ in Eq. (4) is -0.25, implying a small contribution from electrostatic forces to substrate binding at the active site. This contribution

Table 1
Values of K_d for S195A thrombin and k_{cat}/K_m for wild type

	FPR	FPK	FGR	VPR	FGK	VGR	VPK	VGK
Site(s) perturbed	None	P1	P2	P3	P1, P2	P2, P3	P1, P3	P1, P2, P3
K_d, fast form (μM)	0.026	0.35	6.4	0.26	65	30.5	3.4	15.3
k_{cat}/K_m, fast form ($\mu M^{-1}\ s^{-1}$)	90	7.9	2.0	100	0.021	0.34	2.1	0.0047
K_d, slow form (μM)	0.28	6.3	18.2	2.6	62	62	5.1	42
k_{cat}/K_m, slow form ($\mu M^{-1}\ s^{-1}$)	3.0	0.35	0.86	6.7	0.0026	0.17	0.11	0.00079

Conditions for dissociation constant determination were 800 mM NaCl (fast form) or 800 mM ChCl (slow form), 5 mM Tris, pH 8.0 at 25 °C, 0.1% PEG. k_{cat}/K_m values are those reported by Vindigni et al. [16]; conditions were 200 mM NaCl (fast form) or 200 mM ChCl (slow form), 5 mM Tris, pH 8.0 at 25 °C, 0.1% PEG. Errors are ±10% or less.

Table 2
Free energy of perturbation of subsites P1–P3 of FPR

	Free energy (kcal mol^{-1})						
	ΔG_1	ΔG_2	ΔG_3	ΔG_{12}	ΔG_{13}	ΔG_{23}	ΔG_{123}
K_d, fast form	1.5	3.3	1.4	−0.2	0.0	−0.5	−2.4
k_{cat}/K_m, fast form	1.4	2.3	−0.1	1.3	0.8	1.1	2.2
K_d, slow form	1.9	2.5	1.3	−1.2	−1.5	−0.6	−3.2
k_{cat}/K_m, slow form	1.3	0.7	−0.5	2.2	1.2	1.4	3.3

Free energy values were calculated from the dissociation and specificity constants of the substrates listed in Table 1 using Eq. (5a)–Eq. (5g) in the text. Errors are ±0.1 kcal mol^{-1} or less. k_{cat}/K_m values are from Vindigni et al. [16].

likely comes from the salt bridge interaction formed between the side chain of the S1 residue D189 and the side chain of the P1 residue (Arg) of the substrate. A Γ value of −0.25 is also observed for the interaction of Na^+ with thrombin [17], a binding event that also involves a single charge.

The affinity of FPR for thrombin was measured as a function of temperature in the range from 5 to 45 °C in order to characterize the thermodynamic parameters for substrate binding at the active site (Fig. 3). FPR was chosen for these experiments since it has the highest affinity for S195A thrombin, and thus allows experiments over a larger temperature range. The van't Hoff plot is linear, allowing determination of ΔH (−21 kcal mol^{-1}) and ΔS (−36 cal mol^{-1} K^{-1}) for substrate binding. The enthalpic contribution to substrate binding agrees closely with the binding enthalpy (−25 kcal mol^{-1}) calculated by comparing the kinetically determined activation energy for FPR binding and release by the fast form [18]. The linearity of the temperature dependence indicates that there is no ΔC_p for substrate binding. This is in contrast to the recent finding that active-site inhibitors bind trypsin and the slow form of thrombin with strong negative ΔC_p values, although smaller negative ΔC_p values are observed for the fast form of thrombin [19].

The affinity of FPR for thrombin S195A was measured in LiCl, NaCl, KCl and ChCl, and compared to the values of K_p for FPR binding to

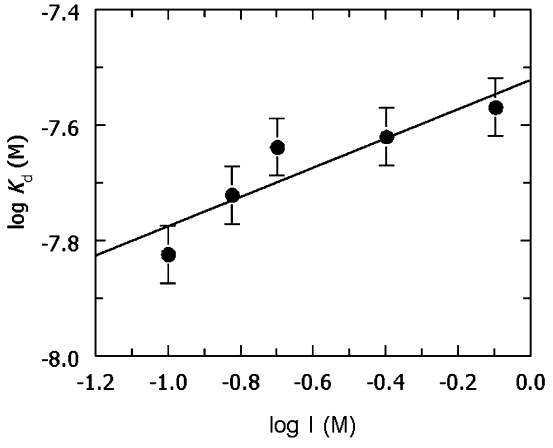

Fig. 2. Salt dependence of the binding of FPR to S195A thrombin. The continuous line was drawn according to Eq. (4) in the text, with best-fit parameter values: $A_0 = 7.52 \pm 0.04$, $\Gamma = -0.25 \pm 0.07$.

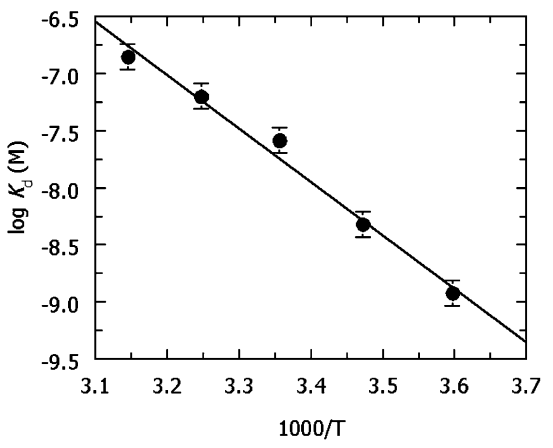

Fig. 3. van't Hoff plot for FPR binding to S195A thrombin. Linear regression of the data yields thermodynamic parameter values $\Delta H = -21 \pm 1$ kcal mol^{-1} and $\Delta S = -36 \pm 5$ cal mol^{-1} K^{-1}.

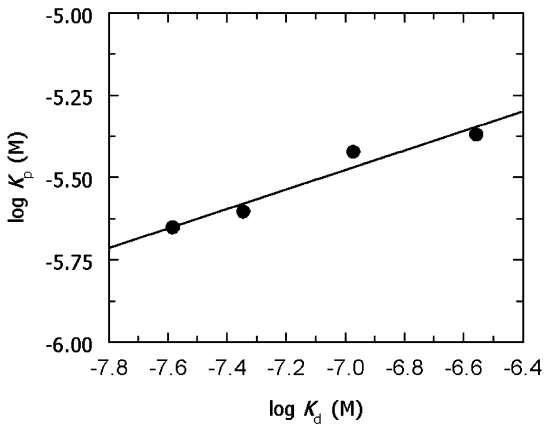

Fig. 4. Comparison of substrate dissociation constants (K_d) and product dissociation constants (K_p) for FPR hydrolysis measured in various chloride salts. From left to right, data points refer to NaCl, KCl, LiCl and ChCl. The correlation in the plot is $r=0.970$.

wild-type thrombin (Fig. 4). The trend of binding constants measured follows that observed for catalytic efficiency in different chloride salts [15]. Furthermore, there is an excellent correlation between the values, indicating that the energetics of substrate binding to S195A are very consistent with the reversible binding of the product H–D-Phe–Pro–Arg to wild-type thrombin. The effect of monovalent cation identity on thrombin specificity is mediated in significant part by substrate binding, and the remainder of the enzyme monovalent-cation specificity is achieved in the transition state. The comparison also offers a quantitative assessment of the contribution of the chromophore leaving group (*p*-nitroanilide) to substrate binding as $\Delta G=RT\ln\left(K_p^{FPR}/K_d^{FPR}\right)$. The values are 2.6 kcal mol^{-1} in NaCl, 2.4 kcal mol^{-1} in KCl, 2.1 kcal mol^{-1} in LiCl and 1.7 kcal mol^{-1} in ChCl.

Fluorescence titration with the inhibitor PPACK also produces a significant fluorescence decrease. PPACK and FPR differ in that FPR contains a *p*-nitroanilide leaving group, whereas PPACK contains a much smaller chloromethylketone group after the P1 residue. Measurement of PPACK and FPR binding allows comparison of binding constants obtained exclusively from S195A. PPACK binds with a dissociation constant of 4.2 ± 0.9 μM in NaCl. The reduced binding affinity is in concordance with the K_p values observed in Fig. 4. The $\Delta G=RT\ln\left(K_d^{PPACK}/K_d^{FPR}\right)$ is 3.0 kcal mol^{-1}, similar to the value calculated using product inhibition constants. PPACK binds the Na^+-bound form of S195A with much higher affinity than it binds the Na^+-free form; the dissociation constant in ChCl was found to be >100 μM, and thus beyond the range of the present technique.

Fluorescence titration of PAR peptides was conducted to characterize the interaction of S195A with those substrates. PAR peptides bind S195A with decreasing affinity in the order PAR1 > PAR3 > PAR4 (Fig. 4). Affinity of PAR peptide binding follows the same order as the K_m values determined for PAR peptide hydrolysis [12]. However, each PAR peptide has a different effect on the intrinsic fluorescence of S195A. Addition of PAR1 brings about a modest increase in intrinsic fluorescence, while PAR3 elicits a smaller increase in signal. PAR4, on the other hand, causes a modest decrease in fluorescence (Fig. 5). The nature of the binding epitopes for the PAR peptides may explain the variety of fluorescence changes observed. Exosite I of thrombin plays an increasingly important role in PAR catalysis in the order PAR4 < PAR3 < PAR1. The active site region, on the other hand, plays an increasingly important role in PAR catalysis in the order PAR1 < PAR3 < PAR4 [12]. PAR4, which interacts primarily with the active site region, elicits a fluorescence decrease, as do the tripeptide chromogenic substrates, which interact exclusively with the active site region. PAR1 and PAR3 have sufficiently strong interactions with exosite I to bring about increases in intrinsic fluorescence. Hirudin, which interacts strongly with exosite I of thrombin, elicits an increase in intrinsic fluorescence in both wild type [20] and S195A [5] of approximately 35%. Residue W141, which lies buried beneath the surface residues of exosite I, is likely connected to this fluorescence increase associated with exosite I binding. Fluorescence titration with the different PAR peptides thus demonstrates the opposing fluorescence changes brought about by interaction with the active site region and exosite I. Accordingly, both the magnitude and sign of a fluorescence change induced by a ligand can give

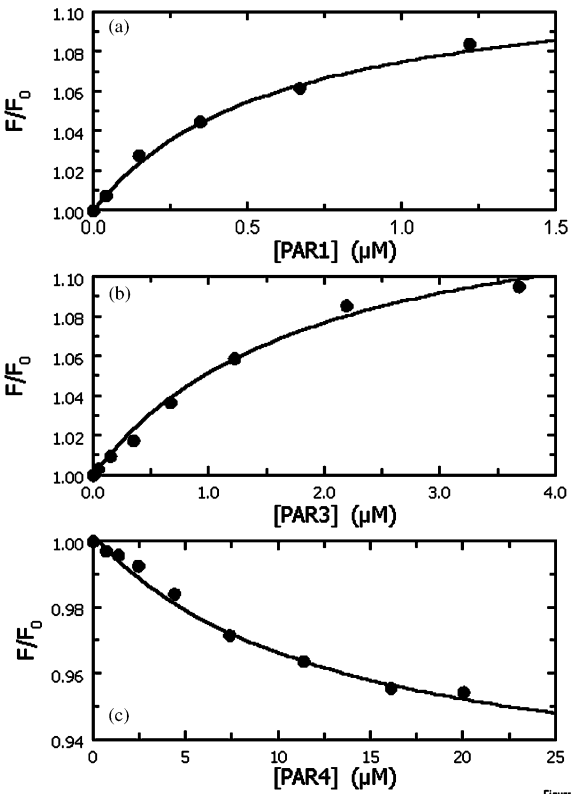

Fig. 5. Representative titration curves of (a) PAR1, (b) PAR3 and (c) PAR4 peptides with S195A thrombin. Curves shown were measured in 800 mM NaCl; curves obtained in 800 mM ChCl were similar. PAR1 and PAR3 cause fluorescence increases, and PAR4 causes a fluorescence decrease. Best-fit parameter values were determined from Eqs. (2) and (3) in the text: PAR1 (NaCl), $F_1/F_0=1.12\pm0.01$ and $K_d=0.5\pm0.1$ μM; PAR1 (ChCl), $F_1/F_0=1.19\pm0.02$ and $K_d=0.6\pm0.1$ μM; PAR3 (NaCl), $F_1/F_0=1.15\pm0.01$ and $K_d=1.8\pm0.4$ μM; PAR3 (ChCl), $F_1/F_0=1.19\pm0.04$ and $K_d=2.5\pm1.0$ μM; PAR4 (NaCl), $F_1/F_0=0.92\pm0.01$ and $K_d=13\pm3$ μM; PAR4 (ChCl), $F_1/F_0=0.84\pm0.04$ and $K_d=30\pm10$ μM.

basic information as to which domains of thrombin are most important for recognizing that ligand.

The inactive mutant S195A, which cannot access the transition state, makes it possible to dissect the separate contributions of the 'ground' and transition states of thrombin to molecular recognition. Equilibrium binding does not fully account for the substrate specificity of the enzyme, as positively cooperative substrate recognition is achieved exclusively in the transition state. In the case of monovalent cation binding, the ground state of the enzyme contributes part, but not all, of the cation specificity. However, for the recognition of PAR peptides, which provide information on active site–exosite I interactions, the ground state of the enzyme has properties practically identical to the catalytic form. Thus, active site–exosite I interactions are determined almost exclusively by the equilibrium ground state of the enzyme.

Intrinsic fluorescence titration with S195A represents a novel method for quantitatively measuring ligand and substrate interactions with thrombin. The range of measurable dissociation constants varies from below 1 nM to 100 μM and perhaps higher, depending on the fluorescence and absorbance properties of the ligands studied. Furthermore, such experiments can be carried out with nanomolar concentrations of enzyme. In addition to ascertaining equilibrium binding data, the method enables the determination of which part of the enzyme plays the most important role in binding a particular substrate. Since S195A has been shown to have similar small- and large-molecule recognition properties as wild-type thrombin, double mutants involving S195A and other residues of interest can be constructed to study the equilibrium binding properties of those mutants.

Acknowledgments

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