Identification of a Binding Site for Quaternary Amines in Factor Xa[†]

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ABSTRACT: In the process of characterizing the Na^+-binding properties of factor Xa, a specific inhibition of this enzyme by quaternary amines was identified, consistent with previous observations. The binding occurs with K_i in the low millimolar range, with trimethylphenylammonium (TMPA) showing the highest specificity. Binding of TMPA inhibits substrate hydrolysis in a competitive manner, does not inhibit the binding of *p*-aminobenzamidine to the S1 pocket, and is positively linked to Na^+ binding. Inhibition by TMPA is also seen in thrombin and tissue plasminogen activator (tPA), though to a lesser extent compared to factor Xa. Computer modeling using the crystal structure of factor Xa suggests that TMPA binds to the S2/S3 specificity sites, with its hydrophobic moiety making van der Waals interactions with the side chains of Y99, F174, and W215, and the charged amine coupling electrostatically with the carboxylates of E97. Site-directed mutagenesis of factor Xa, thrombin, and tPA confirms the predictions drawn by docking calculations and reveal a dominant role for residue Y99. Binding of TMPA to factor Xa is drastically (25-fold) reduced by the Y99T replacement. Likewise, the Y99L substitution compromises binding of TMPA to tPA. On the other hand, the affinity of TMPA is enhanced 4-fold in thrombin with the substitution L99Y. The identification of a binding site for quaternary amines in factor Xa has a bearing on the rational design of selective inhibitors of this clotting enzyme.

Serine proteases with Tyr or Phe at position 225 enhance allosterically their catalytic activity upon binding of Na^+ between the 220 and 180 loops (*1*). This class of Na^+-activated enzymes comprises the vitamin K-dependent proteases thrombin, activated protein C, factors Xa, IXa, and VIIa, enzymes of the lectin and classical pathways of the complement system, and enzymes of embryonic development. The Na^+-binding site of thrombin has been identified (*2*) and the effects of Na^+ on the properties of this clotting enzyme have been elucidated (*3*). Specifically, Na^+ promotes the procoagulant functions of thrombin and facilitates cleavage of fibrinogen and the platelet receptor PAR-1. In the case of activated protein C, Na^+ binding promotes the physiological function of the enzyme and facilitates cleavage and inactivation of factor Va (*4*). The Na^+-binding site of coagulation factor Xa has also been identified (*5*) and is located, as expected, in the same position as in thrombin. Factor Xa is the enzyme component of the prothrombinase complex that catalyzes the conversion of prothrombin into thrombin and has recently become an important target in the development of new anticoagulants. The role of Na^+ on the physiological interactions of factor Xa has been described recently (*6*).

Specific interactions of monovalent cations with proteins are studied by replacing the cation of interest with a bulky, "inert" quaternary amine. This keeps constant both the ionic strength and the concentration of the counterion, usually Cl^-, and minimizes complicating effects due to changes in the properties of the solution. The quaternary amines of choice in these studies are Ch[1] or TMA. The necessary condition for using these cations as inert components is that they do not interfere with the properties of either the enzyme or the substrate/ligand interacting with it. The inert nature of Ch and TMA has been verified in the case of thrombin (*7*) and activated protein C (*4*). On the other hand, factor Xa is inhibited by Ch either free or in the prothrombinase complex (*8, 9*). This observation complicates the characterization of Na^+ binding to factor Xa and brings about the need of identifying the structural components responsible for the inhibition.

MATERIAL AND METHODS

Recombinant factor Xa and the mutants F174N and Y99T, recombinant thrombin and the mutant L99Y, recombinant tPA and the mutant Y99L were prepared as described (*10*−*12*). All quaternary amines were obtained from Sigma as chloride salts. The chromogenic substrate RGR specific for factor Xa, FPR specific for thrombin and IPR specific for

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[1] Abbreviations: Ch, choline; IPR, H-D-Ile-Pro-Arg-*p*-nitroanilide; FPR, H-D-Phe-Pro-Arg-*p*-nitroanilide; RGR, H-L-Arg-Gly-Arg-*p*-nitroanilide; TBA, tetrabutylammonium; TEA, tetraethylammonium; TMA, tetramethylammonium; tPA, tissue plasminogen activator.

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tPA were synthesized as described (*13*). The values of k_{cat}, K_m, and $s = k_{cat}/K_m$ for substrate hydrolysis were determined from analysis of progress curves of the release of *p*-nitroaniline at 405 nm. Curves run at different substrate concentrations allowed for determination of the kinetic parameters corrected for product inhibition, when present. The K_i for binding of quaternary amines and *p*-aminobenzamidine was determined from the competitive inhibition of substrate hydrolysis under experimental conditions of 5 mM Tris, 0.1% PEG, 5 mM $CaCl_2$, 200 mM NaCl, pH 8.0 at 25 °C.

Na^+ binding to factor Xa was characterized from fluorescence titrations and equilibrium linkage with the binding of TMPA under experimental conditions of 5 mM Tris, 0.1% PEG, 5 mM $CaCl_2$, pH 8.0 at 25 °C, with the ionic strength kept constant at 800 mM with NH_4Cl. The high ionic strength was required by the low affinity of factor Xa for Na^+. Binding measurements carried out at an ionic strength of 200 mM consistently failed to show saturation. Fluorescence titrations were carried out as described (*7*) using a QM-1 PTI spectrofluorometer, using an excitation wavelength of 280 nm and recording emission spectra between 300 and 450 nm. Titrations were performed by acquiring the changes in fluorescence intensity at the peak of the emission spectrum at 340 nm. No significant shift in this peak was observed upon Na^+ binding. The $[Na^+]$ was changed by adding successive amounts of buffer containing 800 mM NaCl to the protein sample kept in buffer at constant ionic strength of 800 mM with NH_4Cl. The fluorescence intensity was corrected for the contribution of the buffer and the dilution of the protein sample and was found to change linearly with the protein concentration in the range studied. Correction for bleaching was minimal, as assessed by control titrations obtained by adding buffer containing 800 mM NH_4Cl to the protein sample. The increase in intrinsic fluorescence corrected for buffer, protein dilution and bleaching, was found to obey the following equation (*7*):

$$F = \frac{F_0 + F_1 \frac{[Na^+]}{K_d}}{1 + \frac{[Na^+]}{K_d}} \quad (1)$$

where F_0 and F_1 are the values of the intrinsic fluorescence F in the absence and under saturating concentrations of Na^+ and K_d is the equilibrium dissociation constant for Na^+ binding. In addition, Na^+ binding was determined from the linkage with TMPA binding using the linkage expression (*7*)

$$\frac{1}{K_i} = \frac{\frac{1}{{}^0K_i} + \frac{1}{{}^1K_i}\frac{[Na^+]}{K_d}}{1 + \frac{[Na^+]}{K_d}} \quad (2)$$

where ${}^0K_i$ and ${}^1K_i$ are the values of K_i in the absence and under saturating concentrations of Na^+. To enable direct comparison with the results from fluorescence titrations, the linkage experiments were carried out under identical solution conditions, with the ionic strength kept constant at 800 mM with NH_4Cl. The $[Na^+]$ was changed by replacing NH_4Cl with NaCl, as discussed elsewhere (*7*).

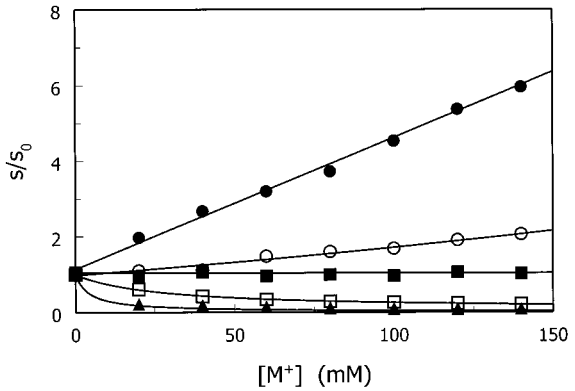

FIGURE 1: Effect of Na^+ (●), K^+ (○), NH_4^+ (■), Ch (□), and TMPA (▲) used as Cl^- salts on the $s = k_{cat}/K_m$ value for the hydrolysis of 222L by factor Xa. Values are expressed relative to that obtained in the absence of monovalent cation (s_0). Experimental conditions are 5 mM Tris, 5 mM $CaCl_2$, 0.1% PEG, pH 8.0, 25 °C. The effect of Li^+ (data not shown) is identical to that of NH_4^+.

Docking calculations of TMPA on factor Xa were carried out using the crystal structure 1HCG (*14*). TMPA was built with Biopolymer (MSI, San Diego, CA) and docked on the protein with AutoDock 2.4 (A. Olson, Scripps Research Institute, La Jolla, CA). Ten runs with different starting points of 100 cycles each produced 1000 models/enzyme−ligand complex. During the docking process, the protein backbone was kept rigid and TMPA was free to move. The best models were energy-minimized with Discover 2.9 (MSI) using 200 steepest descent followed by 3000 conjugate gradient steps. All atoms were set free in the presence of water molecules and Na^+ as indicated in the crystal structure.

RESULTS

Serine proteases carrying Tyr or Phe at position 225 are activated by Na^+ and, to a lesser extent, by other monovalent cations (*1*). The activation is measured relative to an "inert" cation, usually a tetralkylammonium salt (*1*) that is too bulky to interact with the protein. The value of the specificity constant $s = k_{cat}/K_m$ for the hydrolysis of a chromogenic substrate by factor Xa as a function of salt concentration is shown in Figure 1. The data show that factor Xa is activated by monovalent cations such as Na^+ and K^+, is not sensitive to NH_4^+ or Li^+, and is inhibited significantly by Ch and TMPA. The lack of inhibition by NH_4^+, as opposed to the effect seen with Ch and TMPA, suggests that the hydrophobic moiety of the quaternary amine plays a role in the interaction with the enzyme. This prompted investigation of the mechanism of inhibition of factor Xa activity by a number of quaternary amines bearing different hydrophobic groups (Figure 2). In all cases, the quaternary amine was found to affect the value of K_m but not k_{cat} (see below), indicating a competitive inhibition of substrate hydrolysis. A size preference for TEA and TMPA is evident, as these compounds bind with higher affinity than the smaller TMA or Ch and the larger TBA.

Because Na^+ activates factor Xa (Figure 1), it obviously does not compete with substrate binding. Analysis of the effect of Na^+ on the binding of TMPA to factor Xa reveals the presence of positive linkage (Figure 3). The affinity for

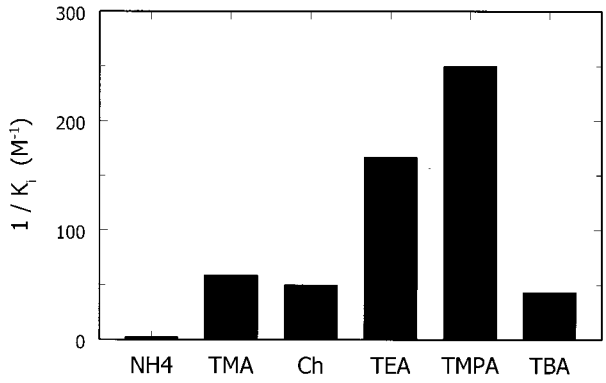

FIGURE 2: Affinity of quaternary amines for factor Xa, expressed as $1/K_I$. Quaternary amines are listed in order of increasing ionic radius, from NH_4^+ to TBA. The size preference for TEA and TMPA is evident from the plot. Experimental conditions are 5 mM Tris, 5 mM $CaCl_2$, 200 mM NaCl, 0.1% PEG, pH 8.0, 25 °C.

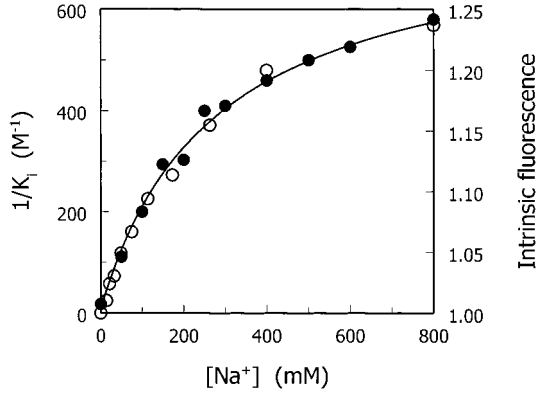

FIGURE 3: Na^+-binding curve of factor Xa obtained from the linkage between Na^+ and TMPA binding (●) or direct fluorescence titration (○), under experimental conditions of 5 mM Tris, 5 mM $CaCl_2$, 0.1% PEG, pH 8.0 at 25 °C. The ionic strength was kept constant at 800 mM using NH_4Cl. The K_d for Na^+ binding is derived from the change in $1/K_i$ as a function of [Na^+] according to the linkage eq 2 in the text. An analogous expression, eq 1, is used to analyze the binding isotherm obtained from fluorescence titration. The continuous line depicts the best fit to the data using eqs 1 and 2 with a value of $K_d = 280 \pm 30$ mM.

TMPA increases more than 10-fold upon Na^+ binding to Xa. Linkage principles enable the derivation of the K_d for Na^+ binding to factor Xa from the data in Figure 3 using eq 2. The estimated value is 280 ± 30 mM and shows that factor Xa binds Na^+ with a weaker affinity compared to its cognate allosteric proteases thrombin (*3*) and activated protein C (*4*). The binding of Na^+ to factor Xa also elicits a significant increase in the intrinsic fluorescence of the enzyme that affords measurement of an accurate titration curve, as in the case of thrombin (*7*). The resulting binding isotherm overlaps well with the linkage data in Figure 3 and is consistent with the same value of K_d for Na^+ binding when the data are analyzed according to eq 1.

Molecular-docking experiments were carried out to identify the structural determinants for the binding of TMPA to factor Xa. These experiments suggest binding of TMPA to the region around the S2 and S3 sites (Figure 4). The S2 pocket of factor Xa is very shallow because of the presence of Y99 that causes a strong preference for Gly at P2. The S3 pocket contains the aromatic residues F174, W215, and Y99, and an anionic hole made by the backbone carbonyl O atoms of E97, T98, and I175, with partial contribution coming from the carboxylates of E97 (*15*). This gives the S3 site of factor Xa similar charge properties to the S1 site and explains the high specificity of factor Xa for bis-amidine inhibitors and substrates containing the RGR sequence (*16*). TMPA fits snugly into the S3 site, making van der Waals contacts with Y99, F174, and W215, whereas the charged amine is in a position to couple favorably with the carboxylates of E97.

TMPA also inhibits thrombin, though to a much lesser extent compared to factor Xa. Unlike factor Xa, the inhibition is specific to TMPA because other quaternary amines such as Ch and TMA are known not to interact with thrombin (*5*, *7*). The reduced affinity for TMPA binding in thrombin may be due to the differences in the S2/S3 sites (*15*). Notably, thrombin has a Glu positioned like E97 of Xa in the S2/S3 sites, but carries a less bulky Leu at position 99. The L99Y mutant of thrombin shows increased inhibition by TMPA compared to wild-type (Figure 5), with an affinity close to that seen for factor Xa. The W215A mutant of thrombin is inhibited by TMPA with an affinity comparable to wild-type ($K_i = 66 \pm 6$ mM, data not shown), ruling out a dominant factor for this residue in the energetics of TMPA binding. On the other hand, the substitution Y99T in factor Xa almost abrogates TMPA binding (Figure 6). Binding of the quaternary amine is also compromised in the F174N mutant, but not as in the case of the Y99T mutant. Similar results are obtained with tPA, that carries Y99 in the S2/S3 sites (Figure 7). Binding of TMPA is greatly compromised with the Y99L substitution. Unlike factor Xa, tPA does not carry E97 in the S2/S3 sites, suggesting that the electrostatic coupling between the charged amine and the carboxylates of E97 may not contribute significantly to the binding affinity. In all cases, binding of TMPA inhibits substrate hydrolysis in a competitive manner, as indicated by the linear increase in 1/*s* as a function of [TMPA] coupled to a constant value of k_{cat}. Furthermore, binding of *p*-aminobenzamidine, that is known to interact with the S1 site of serine proteases such as factor Xa (*17*), does not affect the affinity of TMPA (Figure 6). This rules out the possibility that quaternary amines bind to the primary specificity pocket. On the basis of the results of our mutagenesis studies and the molecular-docking experiments, we conclude that the S2/S3 sites of factor Xa provide the locale for the binding of quaternary amines to this enzyme. The van der Waals interactions of the hydrophobic moiety of the quaternary amine with the aromatic ring of Y99 are the dominant driving force in determining the binding affinity. In fact, a high affinity binding site for TMPA can be engineered in thrombin with the introduction of Tyr at position 99.

DISCUSSION

Previous studies have documented the inhibitory effect of Ch on factor Xa and the prothrombinase complex (*8*, *9*). In the present study, we have identified the binding site responsible for this effect. The site has high affinity for molecules such as TMPA, with a K_I in the low millimolar range, but also binds smaller molecules such as Ch and TMA. TMPA is also found to inhibit thrombin, for which Ch and TMA are inert (*7*), and tPA. Computer modeling using the crystal structure of factor Xa suggests a critical interaction of the quaternary amine with the phenyl ring of Y99 and the indole ring of W215. Site-directed mutagenesis studies

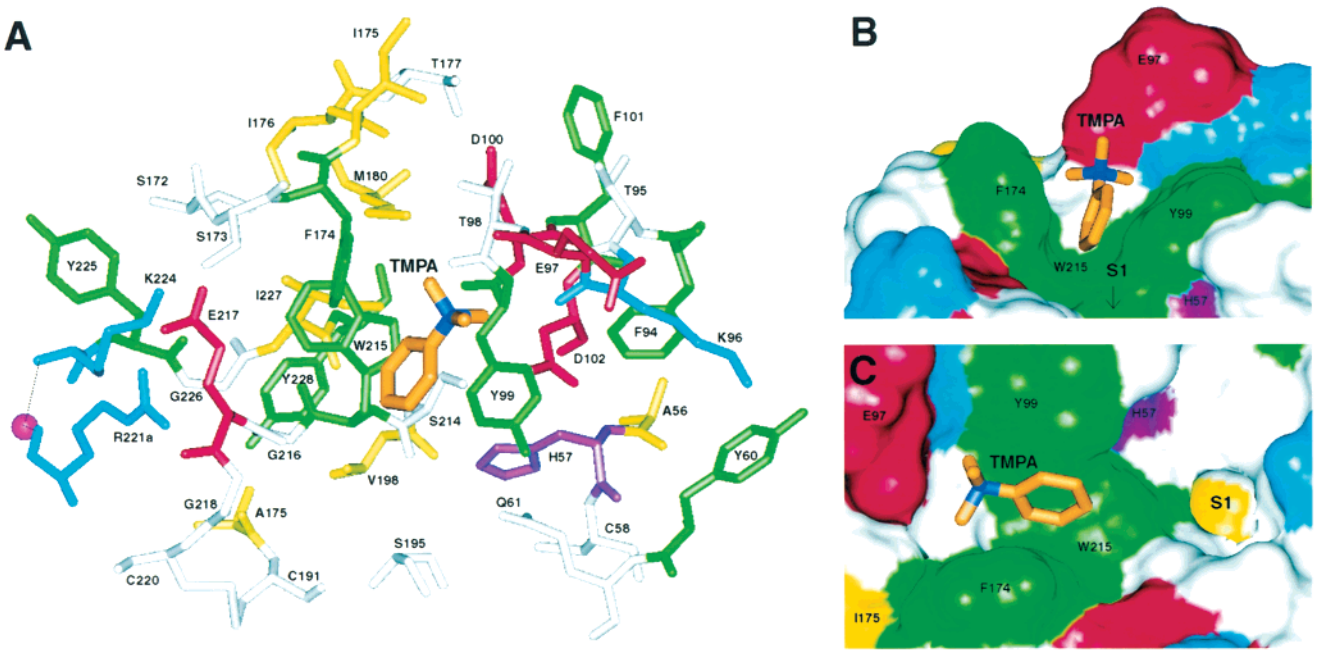

FIGURE 4: Computer model of the complex of factor Xa with TMPA displayed as a stick model, with orange carbons and blue nitrogens. The bound Na^+ is shown as a purple ball (panel A). Panel A shows residues of factor Xa located <10 Å from the bound TMPA, color coded as follows: acidic (D, E), red; basic (K, R), blue; aromatic (F, W, Y), green; hydrophobic (A, I, L, M, V), yellow; neutral (C, G, N, P, Q, S, T), white; H, purple. The accessible surface of the binding site of TMPA is shown in panels B and C with the same color scheme. Panel B shows a side view of the active site, with the S1 site (marked) in the front and the S3 site in the back. Panel C shows a view of the active site from the top, with the S1 cavity on the right and the S2/S3 sites on the left. TMPA binds to the S2/S3 sites of factor Xa making van der Waals interactions with the aromatic rings of Y99, F174, and W215 through their hydrophobic moieties. The side chain of E97 is within range for electrostatic coupling with TMPA. The model is consistent with both kinetic and mutagenesis data. Binding of quaternary amines to the S2/S3 sites inhibits substrate hydrolysis in a competitive manner by occluding access to these recognition sites.

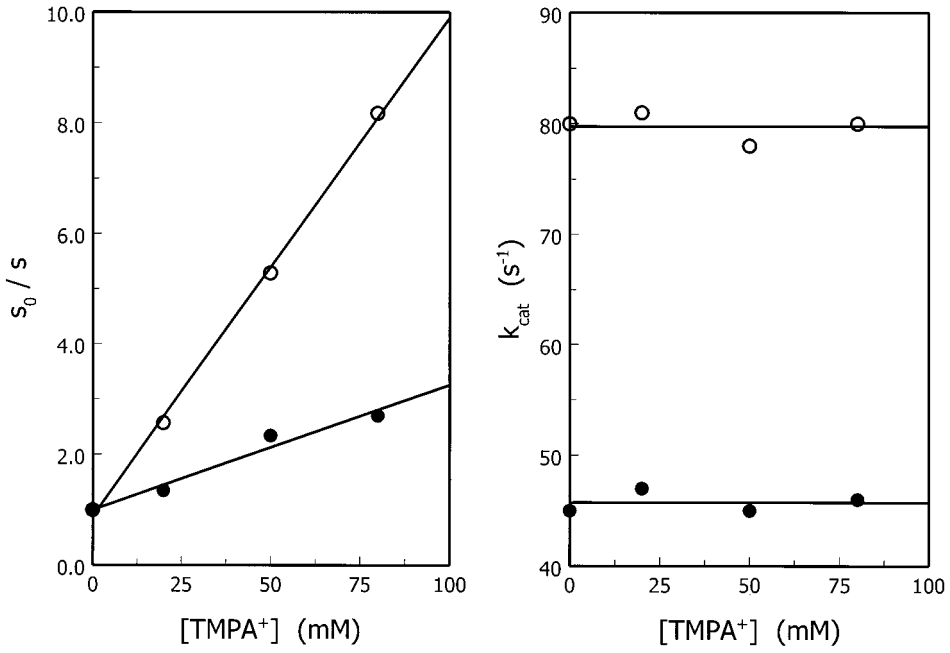

FIGURE 5: Effect of TMPA on the hydrolysis of the chromogenic substrate FPR by thrombin under experimental conditions of 5 mM Tris, 200 mM NaCl, 0.1% PEG, pH 8.0 at 25 °C. Shown are the values of the inverse specificity constant $1/s = K_m/k_{cat}$ relative to $1/s_0$ (left), where s_0 is the value of s in the absence of TMPA and the values of k_{cat} (right). TMPA behaves as a competitive inhibitor because its concentration increases $1/s$ linearly without affecting k_{cat}. Inhibition of the L99Y mutant (○) by TMPA is enhanced significantly compared to wild-type (●), with the K_I for TMPA binding going from 44 ± 10 mM (wild-type) to 10 ± 1 mM (L99Y mutant). The values of s_0 are 89 ± 5 μM^{-1} s^{-1} (wild-type) and 1.8 ± 0.2 μM^{-1} s^{-1} (L99Y mutant).

strongly support this region as the locale for interaction of TMPA with factor Xa and possibly other serine proteases. The affinity of the binding site for quaternary amines is affected decisively by substitution of Y99, that represents the dominant factor for binding. The L99Y mutant of thrombin shows increased affinity for TMPA relative to wild-type, whereas the substitution Y99T in factor Xa and Y99L in tPA practically abrogates TMPA binding.

The identification of the S2/S3 sites as the domain responsible for the specific inhibition of factor Xa by

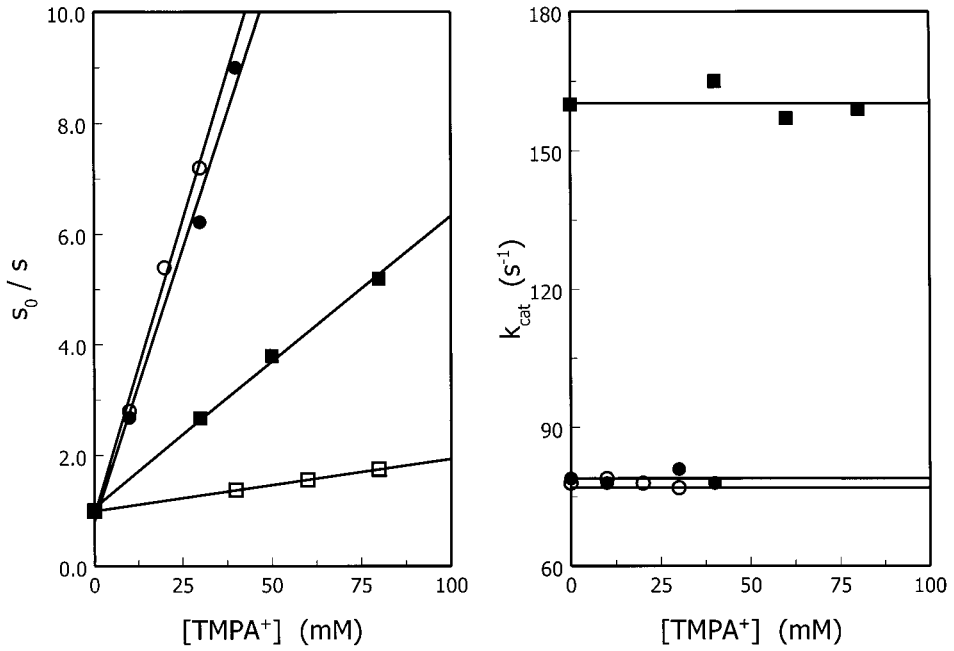

FIGURE 6: Effect of TMPA on the hydrolysis of the chromogenic substrate RGR by factor Xa under experimental conditions of 5 mM Tris, 5 mM $CaCl_2$, 200 mM NaCl, 0.1% PEG, pH 8.0 at 25 °C. Shown are the values of the inverse specificity constant $1/s = K_m/k_{cat}$ relative to $1/s_0$ (left), where s_0 is the value of s in the absence of TMPA, and the values of k_{cat} (right). The values of k_{cat} for the Y99T mutant could not be determined due to the very high value of $K_m > 10$ mM. TMPA behaves as a competitive inhibitor because its concentration increases $1/s$ linearly without affecting k_{cat}. The Y99T substitution (□) changes the K_I for TMPA to 107 ± 8 mM relative to 4 ± 1 mM in the wild-type (●). The F174N substitution (■) decreases TMPA binding to a lesser extent and brings the K_I to 20 ± 2 mM. Also shown are the results obtained in the presence of 100 μM *p*-aminobenzamidine (○), a competitive inhibitor of factor Xa that binds to the S1 site with a $K_i = 85 \pm 9$ μM. The value of K_i for TMPA binding is 5 ± 1 mM, practically identical to that obtained in the absence of *p*-aminobenzamidine. These data support a dominant role for residue 99 in the recognition of TMPA and validate the prediction derived from docking calculations that the S2/S3 sites of factor Xa provide the locale for binding of quaternary amines. The values of s_0 are 1.3 ± 0.1 μM^{-1} s^{-1} (wild-type), 0.038 ± 0.002 μM^{-1} s^{-1} (Y99T mutant), 0.17 ± 0.01 μM^{-1} s^{-1} (F174N mutant), and 0.59 ± 0.02 μM^{-1} s^{-1} (wild-type in the presence of 100 μM *p*-aminobenzamidine).

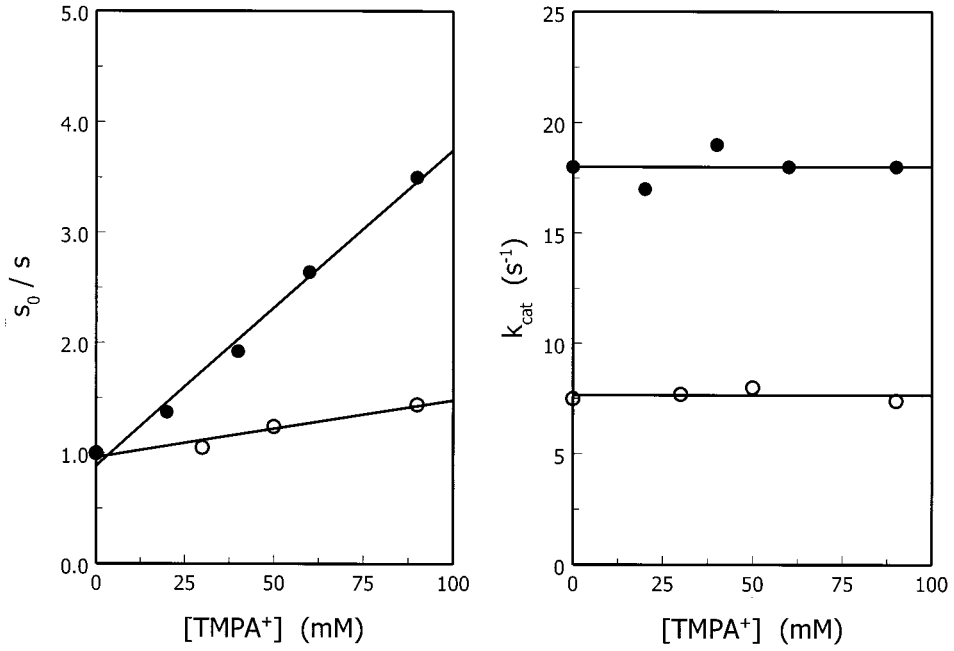

FIGURE 7: Effect of TMPA on the hydrolysis of the chromogenic substrate IPR by tPA under experimental conditions of 5 mM Tris, 200 mM NaCl, 0.1% PEG, pH 8.0 at 25 °C. Shown are the values of the inverse specificity constant $1/s = K_m/k_{cat}$ relative to $1/s_0$ (left), where s_0 is the value of s in the absence of TMPA, and the values of k_{cat} (right). TMPA behaves as a competitive inhibitor because its concentration increases $1/s$ linearly without affecting k_{cat}. Inhibition of the Y99L mutant (○) by TMPA is decreased significantly compared to wild-type (●), with the K_I for TMPA binding going from 30 ± 4 mM (wild-type) to 170 ± 20 mM (Y99L mutant). The values of s_0 are 0.086 ± 0.004 μM^{-1} s^{-1} (wild-type) and 0.21 ± 0.01 μM^{-1} s^{-1} (L99Y mutant).

quaternary amines may have significant implications for drug design. The particular architecture of the S2/S3 sites of factor Xa resembles that of the primary specificity site S1 and has led to the design of bivalent inhibitors that could couple

electrostatically to both sites (*16*). Examples of these inhibitors are the bis-benzamidine derivatives capable of binding to the S1 site and to the quaternary amine hole shaped by Y99, F174, and W215. The identification of the binding site for quaternary amines reported in this study raises the issue of selectivity of molecules designed to interact with this moiety in factor Xa. Usually, inhibitors of factor Xa are tested for selectivity against thrombin and trypsin (*16*). However, these enzymes carry L99 in the S2 site and are expected to interact weakly with quaternary amine substituents binding to the S2/S3 sites. Other proteases with Y99 should be included in selectivity screens in view of the results reported in this study. Among the notable proteases carrying Y99 in the S2/S3 sites are the blood clotting enzymes factors IX and XII and plasma kallikrein, and the complement enzymes C1r, factors B, C2, and I. Inhibitors of factor Xa should be tested for activity against these clotting and complement enzymes because specific interactions may elicit fatal side effects in vivo as demonstrated by a thrombin inhibitor cross reacting with complement factor I (*18*).

Factor Xa was originally identified as a Na^+-activated enzyme by Orthner and Kosow (*19*). The results reported here confirm that Na^+ binding enhances the catalytic activity of factor Xa, but show that the Na^+ affinity is only 280 mM at 25 °C, in the presence of 5 mM $CaCl_2$. This raises the question of the physiologic role of Na^+ in factor Xa. Because the Na^+ affinity decreases significantly by increasing temperature in the physiologic range (*20*), it is expected that the Na^+-bound form of factor Xa be minimally populated in vivo where the Na^+ concentration is 145 mM. Therefore, Na^+ may have only a small effect on the activity of the prothrombinase complex. Recent mutagenesis studies of factor Xa where Na^+ binding was abrogated with the Y225P replacement nicely confirm this expectation (*6*).

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