Residue Asp-189 Controls both Substrate Binding and the Monovalent Cation Specificity of Thrombin

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Residue Asp-189 plays an important dual role in thrombin: it defines the primary specificity for Arg side chains and participates indirectly in the coordination of Na⁺. The former role is shared by other proteases with trypsin-like specificity, whereas the latter is unique to Na⁺-activated proteases in blood coagulation and the complement system. Replacement of Asp-189 with Ala, Asn, Glu, and Ser drastically reduces the specificity toward substrates carrying Arg or Lys at P1, whereas it has little or no effect toward the hydrolysis of substrates carrying Phe at P1. These findings confirm the important role of Asp-189 in substrate recognition by trypsin-like proteases. The substitutions also affect significantly and unexpectedly the monovalent cation specificity of the enzyme. The Ala and Asn mutations abrogate monovalent cation binding, whereas the Ser and Glu mutations change the monovalent cation preference from Na⁺ to the smaller cation Li⁺ or to the larger cation Rb⁺, respectively. The observation that a single amino acid substitution can alter the monovalent cation specificity of thrombin from Na⁺ (Asp-189) to Li⁺ (Ser-189) or Rb⁺ (Glu-189) is unprecedented in the realm of monovalent cation-activated enzymes.

The molecular origin of protease specificity remains an elu-

site issue in enzymology. Studies on trypsin have revealed the complexity of converting the primary specificity of this enzyme into that of chymotrypsin (1–4) or elastase (5). The conversion is never complete and requires substitutions at residue 189 in the S1 site, as well as swaps of the 186- and 220-loops that contribute to the architecture of the primary specificity pocket but make no direct contact with substrate (6). However, turning chymotrypsin into trypsin using the same strategy proves to be unsuccessful (7). The underlying conclusion from these studies is that primary substrate specificity is a “distributed” property of the protein structure and may require ad hoc changes in each system under study.

The discovery of the molecular basis of the Na⁺-dependent allosteric activation of serine proteases like thrombin (8, 9) has added further complexity to the mechanism of protease specificity. Thrombin binds Na⁺ in close proximity to the primary specificity pocket and thereby enhances its catalytic activity toward synthetic and natural substrates (8, 10, 11). This property is shared by several other enzymes involved in blood coagulation and the immune response, but not by digestive enzymes like trypsin (9), and played a crucial role in the seg-

regation of function in serine proteases during evolution (12, 13). The Na⁺ coordination shell is formed by water molecules and protein backbone atoms (10, 14). One of the ligating water molecules bridges Na⁺ to the side chain of Asp-189 in the primary specificity pocket. When Na⁺ is released from its site, this water molecule is relocated; the side chain of Asp-189 acquires mobility and rearranges in a position no longer opti-

mal for electrostatic coupling with the guanidinium group of the Arg side chain at the P1 position of substrate (15). In Na⁺-activated allosteric proteases like thrombin, substrate recognition is influenced by the presence of Na⁺ that fixes the orientation of residue Asp-189. Studies on the role of Asp-189 in allosteric proteases must, therefore, evaluate the effect of mutations at this position not only upon substrate binding and specificity, as is done in the case of trypsin, but also upon the ability of the enzyme to bind Na⁺ and to transduce this event into enhanced catalytic activity.

MATERIALS AND METHODS

Site-directed mutagenesis of human thrombin was carried out in an HPc4-pNUT expression vector, using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Expression of mutants and wild-type thrombins was carried out in baby hamster kidney cells. The enzyme was activated with the prothrombinase complex for 30 min at 37 °C or with the immobilized snake venom enzyme ecarin. Activated thrombin was purified to homogeneity by fast protein liquid chromato-

tography 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. The active site concentration was determined by titration with hirudin and was found to be >95% in all cases. All assays of amidolytic activity were carried out under experimental conditions of 5 mM Tris, 0.1% PEG, pH 8.0 at 25 °C. The salt concen-

tration, NaCl or ChCl, was 200 mM. The chromogenic substrates H-D-Phe-Pro-Arg-p-nitroanilide (FPR) and H-D-Phe-Pro-Lys-p-nitroanilide (FPK) were purchased from Midwest Bio-Tech (Carmel, IN), and H-D-

Ala-Ala-Pro-Phe-p-nitroanilide (AAPF) was obtained from Sigma. The values of Kₘ and k₉cat were obtained from the analysis of progress curves of the release of p-nitroaniline as a function of substrate concentration taking into account product inhibition (16). Because of the drastic loss of substrate specificity, individual values of Kₘ and k₉cat could not be obtained for all mutants under all conditions of interest.

The interaction of thrombin with the inhibitor hirudin was studied from analysis of progress curves as detailed elsewhere (17, 18). Experimental conditions were determined were 5 mM Tris, 200 mM NaCl, 0.1% PEG-8000, pH 8.0 at 25 °C. The release of fibrinopeptide A subsequent to cleavage of fibrinogen, activation of protein C, cleavage of the thrombin receptors PAR1, PAR3, and PAR4, and inhibition by antithrombin were studied as reported (19–22) under experimental conditions of 5 mM Tris, 145 mM NaCl, 0.1% PEG-8000, pH 7.4 at 37 °C.

Equilibrium dissociation constants for monovalent cation (M⁺) bind-

ing were determined by fluorescence titration using a FluoroMax-3

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FIG. 1. Architecture of the Na\(^+\) binding site of thrombin taken from the crystal structure 2THF (14). The bound Na\(^+\) is represented as a cyan ball and is coordinated octahedrally by four water molecules (red balls) and two backbone oxygen atoms from Arg-221a and Lys-224 (shown as CPK models). The side chain of Asp-189 is also shown and couples electrostatically to the guanidinium group of the Arg residue of substrate at P1 (yellow) on one side and one of the water molecules in the Na\(^+\) coordination shell (*) on the other side. When Na\(^+\) is released, the side chain of Asp-189 re-orients as shown in orange (15) and moves away from its optimal coordination of the Arg side chain of substrate. H-bonds refer to the Na\(^+\)-bound form and are shown by broken lines in green. Only residue Asp-189 in the crystal structure 1MHO of the Na\(^+\)-free form is shown for clarity.

SPEX spectrophotometer. Fluorescence titrations took place under experimental conditions of 5 mM Tris, 0.1% PEG-8000, pH 8.0 at 10 °C. The salt concentration was 800 mM. Titrations were carried out by adding aliquots of thrombin in a solution containing [M\(^+\)] = 800 mM Cl\(^-\) salt to a solution containing the enzyme in 800 mM ChCl. In this experiment, the ionic strength (800 mM), enzyme concentration (50 nM), and [Cl\(^-\)] (800 mM) were held constant, while [M\(^+\)] was varied. Excitation was at 295 nm and emission was measured at 333 nm. The value of thrombin intrinsic fluorescence, \(F\), as a function of [M\(^+\)] was fit according to the equation (16)

\[
F = \frac{F_0 + F_1 [M^+] + K_d}{1 + [M^+]/K_d}
\]  
(Eq. 1)

where \(F_0\) and \(F_1\) are the values of \(F\) in the absence and under saturating monovalent cation concentration, and \(K_d\) is the equilibrium dissociation constant for M\(^+\) binding. To ensure the uniqueness of the binding site, binding experiments for Rb\(^+\) (D189E) and Li\(^+\) (D189S) were also carried out at constant 1 – 800 mM in the presence of fixed amounts of NaCl. The apparent \(K_d\) for Rb\(^+\) or Li\(^+\) binding gave a linear dependence upon Na\(^+\) as expected for competitive binding to a single site.

RESULTS

Recent structural work (14, 15) has documented the dual role of Asp-189 in controlling both substrate and Na\(^+\) binding to thrombin (Fig. 1). The side chain of Asp-189 engages the guanidinium group of the P1 Arg of incoming substrate. At the same time, it H-bonds to a water molecule that constitutes one of the six ligands of the bound Na\(^+\). When Na\(^+\) is released from its site, this water molecule is dislodged and the side chain of Asp-189 rearranges and moves away from its optimal interaction with Arg at P1. The movement of Asp-189 linked to Na\(^+\) release contributes to the lower specificity of the Na\(^+\)-free form of thrombin for substrates like FPR carrying Arg at P1 (Fig. 2). The movement also affects recognition of Lys and Phe side chains in the S1 pocket, because Na\(^+\) enhances significantly the \(k_{cat}/K_m\) of less specific substrates like FPK and AAPF. Therefore, we examined the effect of amino acid substitutions on the linkage between Na\(^+\) binding and substrate recognition mediated by residue 189.

Replacement of Asp-189 compromises the specificity of thrombin toward FPR measured as \(k_{cat}/K_m\) up to four orders of magnitude. The Ser substitution has the mildest effect on FPR hydrolysis, whereas Ala at position 189 is the most deleterious. Interestingly, the isosteric replacement D189N also results in severe loss of activity. When measurements of individual values of \(K_m\) and \(k_{cat}\) were possible, the defect was found to reside mostly in substrate binding (typically 1000-fold reduction) and, to a lesser extent, in substrate hydrolysis (10-fold reduction). Equally compromised is the hydrolysis of FPK that is less specific for thrombin than FPR. The D189A and D189N mutations abrogate the ability of thrombin to preferentially cleave Arg versus Lys at the P1 position of substrate (Fig. 2). In the case of the chymotrypsin-specific substrate AAPF, which shows only marginal specificity for thrombin, the mutation of Asp-189 has a more modest effect on substrate hydrolysis. This results in a drastic rebalancing of the Arg versus Phe preference for the P1 position of substrate; it drops up to five orders of magnitude in the case of the D189N mutant in the absence of Na\(^+\) (Fig. 2). The mutation D189S introduces the chymotrypsin residue in the primary specificity pocket of thrombin and shows a gain of specificity toward AAPF compared with wild type in the absence of Na\(^+\). The values of \(k_{cat}/K_m\) however, remain too small to consider the change a significant gain of function. In conclusion, mutation of Asp-189 affects the ability of thrombin to interact with specific substrates like FPR and FPK and reduces drastically the Arg versus Phe preference for the P1 position of substrate. This change, however, is due predominantly to loss of trypsin-like specificity rather than to a gain in chymotrypsin-like specificity. The loss of specificity toward Arg and Lys residues at P1 and the lack of discrimination between charged and hydrophobic P1 residues are consistent with the results of mutations of Asp-189 in trypsin (4).

Natural substrates of thrombin experience reduced cleavage by mutants of Asp-189 to an extent comparable with that seen for the hydrolysis of small chromogenic substrates (Fig. 3). Fibrinogen, protein C, and the PARs are cleaved with a specificity reduced by 3–4 orders of magnitude. The reduction is similar for all substrates with the exception of PAR3 that is less affected than PAR1 and PAR4 by mutation of Asp-189 to Ala. As a result, D189A cuts PAR1 (Arg at P1) and PAR3 (Lys at P1) with comparable specificity, as seen for the hydrolysis of FPR and FPK (Fig. 2). All thrombin natural substrates, except protein C, make ancillary contacts with exosite I, which resides about 20 Å away from the active site (11). The similarity of the perturbations in the recognition of chromogenic and natural substrates is conducive to an effect of the mutation of Asp-189 around the primary specificity pocket and the S2-S3 specificity sites. This conclusion is reinforced by the effect of mutations of Asp-189 on the inhibition by antithrombin (Fig. 3), which contacts primarily the active site of thrombin (23). Interestingly, in the case of this inhibitor, mutation of Asp-189 to residues other than Ser practically abrogates the interaction, suggesting that binding to the S1 pocket is a major, irreplaceable determinant of recognition for the serpin. Recent mutagenesis studies of the P1 residue of antithrombin also vouch for the essential role of the P1-S1 interaction between thrombin and antithrombin (24). Consistent with these findings, the inhibition by hirudin is unaffected by mutation of Asp-189. The crystal structure of the thrombin-hirudin complex (25) has documented the unusual binding mode of hirudin that cleverly avoids making contacts with the S1 specificity pocket of the enzyme to retain integrity of its backbone structure. Mutagenesis studies of hirudin lend support to the contention that positively charged residues of the inhibitor do not contact the enzyme active site (26). Based on the results of the crystal structure and previous mutagenesis studies of hirudin, we expected mutations of Asp-189 to be largely inconsequential on hirudin recognition. This expectation is confirmed by the values of \(K_d\) for hirudin inhibi-
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Mutations of Asp-189 have pronounced effects on the Na⁺-induced enhancement of catalytic activity of thrombin. In the case of FPR, a significant enhancement is seen only for the D189S mutant (Fig. 2). The abrogation of the Na⁺ effect in the D189A mutant is consistent with the fact that most of the Na⁺-induced change on residue 189 involves a reorientation of the carboxylate group of Asp (15). The reduction of the Na⁺ effect seen in the D189E and D189N mutants calls for alternative explanations. The significant decrease in the Na⁺ effect can be analyzed in terms of the expression that links Na⁺ binding to the value of $s = k_{cat}/K_m$, i.e. (16, 27)

$$s = \frac{[Na^+] + s_0}{1 + \frac{[Na^+]}{K_d}}$$  (Eq. 2)

where $s_0$ and $s_1$ are the values of $k_{cat}/K_m$ in the absence of and under saturating [Na⁺]. The value of $s_0$ is derived in the presence of the inert cation Ch⁺, which mimics the Na⁺-free form of the enzyme, whereas the value of $s_1$ refers to the extrapolation [Na⁺] → ∞. The important feature of Equation 2 is that the three parameters $s_0$, $s_1$, and the equilibrium dissociation constant for Na⁺ binding, $K_d$, are independent. This implies that the magnitude of cation activation $(s_1/s_0)$ is not necessarily linked to the strength of cation binding $(K_d)$. Hence, if a mutant shows a reduction of Na⁺-induced enhancement of $s$, the effect could be due either to a reduction of the $s_1/s_0$ ratio without effect upon Na⁺ binding and/or to reduced Na⁺ binding. The two possibilities are easily distinguished from direct measurements of Na⁺ binding.

The Na⁺ affinity is significantly compromised upon substitution of Asp-189 with Ala and Asn (data not shown), underscoring the crucial anchoring role of the carboxylate of Asp-189 in the Na⁺ coordination shell (Fig. 1). The Glu and Ser substitutions of Asp-189 also reduce Na⁺ binding to a considerable extent (Fig. 4) and perturb the monovalent cation specificity profile (Fig. 5). The D189E and D189S mutants show a change in monovalent cation specificity from Na⁺ to the larger cation Rb⁺ (D189E) or to the smaller cation Li⁺ (D189S). In both cases, the monovalent cations were found to compete for the same site as Na⁺ (Fig. 6), ruling out the possibility that mutation of Asp-189 had created additional sites for Rb⁺ or Li⁺. It should be pointed out that the binding affinity of D189S and D189E toward their preferred cation is weaker than the Na⁺ affinity of wild type. The changes at residue 189 do not ensure optimal adaptation to a smaller (Li⁺ for D189S) or larger (Rb⁺ for D189E) cation compared with Na⁺. Furthermore, the $k_{cat}/K_m$ toward FPR and other substrates is not optimal in the presence of Li⁺ for D189S or Rb⁺ for D189E (Fig. 7). This result echoes the properties of the quadA’ mutant of thrombin (28); although its monovalent cation specificity shifts from Na⁺ to K⁺, the mutant is not a K⁺-activated enzyme.

**DISCUSSION**

An important conclusion that emerged from previous studies is that the primary specificity of proteases like trypsin and chymotrypsin cannot be reengineered by single amino acid substitutions at position 189 (1–7). The results reported in the present study on thrombin confirm this view and extend it to the realm of Na⁺-activated proteases. The D189S substitution does not confer thrombin a chymotrypsin-like specificity: although it drastically reduces the Arg versus Phe preference at position P1 of substrate, the D189S mutant remains more specific for Arg side chains at P1.

Because thrombin is a Na⁺-activated enzyme and residue 189 participates directly in the stabilization of the Na⁺ coordination shell, our mutagenesis study also offers insights into the origin of monovalent cation specificity. Substitutions of Asp-189 with Ala, Asn, Glu, and Ser generally results in a drastic loss of Na⁺ binding and the enhancement of catalytic activity. Importantly, the D189A and D189N mutants share striking similarities in their interaction with substrates like FPR, FPK, and AAPF, as well as natural substrates and inhibitors, and have lost the ability to bind monovalent cations. The properties of the D189A mutant are easily rationalized in terms of the lack of the carboxylate group that is required for both substrate and Na⁺ binding (Fig. 1). On the other hand, the properties of the D189N mutant are more difficult to explain. It is not clear why Asn-189 would not allow for significant Na⁺ or substrate binding if oriented as Asp-189. Electrostatic coupling between the guanidinium group of the P1 residue of substrate and the carboxylate of Asp-189 is an important component of substrate recognition in proteases with trypsin-like specificity (4), but the Asp → Asn substitution at position 189 should not weaken this coupling by four orders of magnitude. More importantly, it is difficult to envision how the Asp → Asn substitution of residue 189 could produce the same deleterious effect on substrate recognition as the more drastic Asp → Ala substitution that completely abrogates the polar nature of the side chain. One
possibility is that the side chain of Asn-189 becomes engaged into a polar interaction with a neighbor residue, which would de facto compromise interaction with substrate or Na⁺. This possibility is supported indirectly by the observation that complement factor D uses the side chain of Arg-218 to ion-pair with Asp-189 and to self-inhibit itself (29). Alternatively, Asn-189

FIG. 3. Interactions of wild-type and mutant thrombins of residue 189 with natural substrates and inhibitors. Shown are the values of $s = k_{cat}/K_m$ for the hydrolysis of fibrinogen (FpA), protein C (PC), and the thrombin receptors PAR1, PAR3, and PAR4. Also shown are the values of $h_{\text{inc}}$ for the inhibition of thrombin by antithrombin (AT). Experimental conditions were 5 mM Tris, 145 mM NaCl, 0.1% PEG, pH 7.4 at 37 °C. Data for PC were collected in the presence of 5 mM CaCl₂ and 50 nM thrombomodulin. Data for AT were collected in the presence of 0.5 USP/ml of heparin. The specificity of thrombin toward all substrates is reduced by 3–4 orders of magnitude, with a pattern similar for all mutants and reminiscent of that seen for the small chromogenic substrates FPR and FPK (see Fig. 2). The interactions with PAR3 and AT show a departure from the trend. PAR3 cleavage seems to be compromised to a lesser extent by the mutation of 189, whereas AT inhibition is practically abrogated in mutants of Asp-189 other than D189S.

FIG. 4. Monovalent cation binding curves of wild-type (●, ▲, ■) and mutant thrombins D189S (○, □) and D189E (○, △) determined from changes in intrinsic fluorescence as a function of cation concentration [M⁺], under experimental conditions of 5 mM Tris, 0.1% PEG, pH 8.0 at 10 °C, I = 800 mX. Data are expressed as fractional changes of the value of fluorescence to enable direct comparison. Continuous lines were drawn by using the expression $\theta = (F - F_0)(F_1 - F_0)/F_0 = [M^+] \left[ K_{d} + [M^+] \right]$, which is a rearrangement of Equation 1 in the text. The best-fit parameter values are (left) $K_{d,\text{Li}} = 250 \pm 20$ mm (■) and $K_{d,\text{Na}} = 14 \pm 1$ mm (●) for wild-type, $K_{d,\text{Li}} = 56 \pm 4$ mm (□) and $K_{d,\text{Na}} = 300 \pm 30$ mm (○) for D189S; (right) $K_{d,\text{Rb}} = 130 \pm 10$ mm (▲) and $K_{d,\text{Na}} = 14 \pm 1$ mm (○) for wild-type, $K_{d,\text{Rb}} = 71 \pm 6$ mm (△) and $K_{d,\text{Na}} = 210 \pm 20$ mm (○) for D189E. Note how the cation specificity of D189S and D189E shifts from Na⁺ (wild type) to Li⁺ or Rb⁺, respectively.
may simply orient itself away from optimal interactions with substrate and Na\(^+\) because of its different polarity compared with Asp-189. Indeed, the crystal structures of S198D chymotrypsin and D189S trypsin mutants show an orientation of residue 189 that is largely incompatible with optimal substrate binding (30, 31). Changes in the polarity of residue 189 have been invoked as the source of these drastic structural rearrangements.

The findings reported in this study support the recent structural observations that the side chain of Asp-189 reorients upon Na\(^+\) binding to thrombin and that this movement contributes to the Na\(^+\)-induced allosteric enhancement of catalytic activity by bringing the carboxylate of Asp-189 in optimal electrostatic coupling with the side chain of Arg at P1 of substrate (15). Asp-189 is strategically positioned for linking Na\(^+\) and substrate binding and any alterations in its side chain may easily compromise its dual recognition function. Movement of Asp-189 at the bottom of the primary specificity pocket must have an effect on the shape of the pocket itself, because a Na\(^+\) effect is also seen in the wild type with substrates like AAPF that carry Phe at P1 and cannot couple electrostatically with Asp-189. This conclusion is consistent with recent structural studies (15) that document long range effects propagating from the Na\(^+\) site to the S1 specificity site up to the catalytic S195 when Na\(^+\) binds to thrombin.

An unanticipated feature of the D189E and D189S mutants is that their impaired Na\(^+\) binding is replaced by acquired specificity toward Rb\(^+\) or Li\(^+\), respectively. The affinity is weaker than that of wild type toward Na\(^+\) or that acquired by the quadA mutant toward K\(^+\) (28). The observation that a single amino acid substitution can alter the monovalent cation specificity of an enzyme from Na\(^+\) (Asp-189) to Li\(^+\) (Ser-189) or Rb\(^+\) (Glu-189) is unprecedented. We have recently demonstrated that thrombin can be converted into a K\(^+\)-specific enzyme (28), but this involved a quadruple amino acid insertion into the 186-loop. The inverse correlation observed between the size of the side chain of residue 189 and the ionic radius of the preferred cation suggests a key role for the hydration shell of the bound cation in determining specificity. In the wild type, Asp-189 allows for an intervening water molecule to bridge the carboxylate side chain and Na\(^+\) (Fig. 1). Larger cations like K\(^+\) and Rb\(^+\) could not fit in the Na\(^+\) cage without distorting the coordination shell. Indeed, the Rb\(^+\) bound structure of thrombin (10) has one water molecule replaced by the backbone oxygen of Tyr-184a. Likewise, Li\(^+\) may be too small to ensure optimal H-bonding of the coordination shell with protein atoms. The D189E replacement most likely displaces the intervening water molecule, thus weakening the linkage with the bound Na\(^+\). The larger cations K\(^+\) and Rb\(^+\) can compensate for the increased room in the Na\(^+\) cage, thereby explaining the change in monovalent cation preference of this mutant. Most likely, the side chain of Glu-189 directly links to the bound Rb\(^+\) or Li\(^+\), a hypothesis that will be tested by x-ray crystallographic studies. Finally, in the case of D189S, the shorter side chain may weaken its linkage with the intervening water molecule coordinating the Na\(^+\). In this case, that water molecule would not be displaced and additional solvent molecules could fill the room in the Na\(^+\) cage. This would favor the binding of a highly hydrated cation like Li\(^+\), thereby explaining the acquired monovalent cation preference of this mutant.

The change in monovalent cation specificity documented for D189E and D189S is not translated into a preferential activation of thrombin in the presence of Rb\(^+\) (D189E) or Li\(^+\) (D189S). A similar result was recently obtained for mutants of thrombin in the 186-loop (28). Cation binding and enhancement of catalytic activity are processes not necessarily linked, because the former is a property of the free enzyme, and the

**FIG. 5.** Monovalent cation specificity profile for wild-type thrombin (●) and the mutants D189E (○) and D189S (□). Shown are the values of the binding free energy $\Delta G = RT \ln K_d$, determined for Li\(^+\), Na\(^+\), K\(^+\), and Rb\(^+\) by fluorescence titrations under experimental conditions of 5 mM Tris, 0.1% PEG, pH 8.0 at 10 °C, I = 800 mM. Note how the specificity of D189E and D189S are shifted toward cations of larger and smaller ionic radius, respectively, compared with wild type. Continuous curves are spline interpolations of the data.

**FIG. 6.** Measurements of Li\(^+\) (●) and Rb\(^+\) (○) binding to the thrombin mutants D189S and D189E, as a function of [Na\(^+\)], under experimental conditions of 5 mM Tris, 0.1% PEG, pH 8.0 at 10 °C, I = 800 mM. The linear dependence of the apparent $K_d$ for Li\(^+\) or Rb\(^+\) binding, as a function of [Na\(^+\)] demonstrates the uniqueness of the cation binding site. Curves were drawn according to the equation for competitive inhibition $K_d = K_{d,M} \cdot (1 + [\text{Na}^+] / K_{d,\text{Na}})$, where $K_{d,M}$ is the equilibrium dissociation constant for Li\(^+\) or Rb\(^+\) binding, and $K_{d,\text{Na}}$ is the analogous quantity for Na\(^+\). Best-fit values of these parameters are (D189S) $K_{d,M} = 54 \pm 4$ mM, $K_{d,\text{Na}} = 240 \pm 25$ mM; (D189E) $K_{d,Rb} = 67 \pm 4$ mM, $K_{d,\text{Na}} = 170 \pm 16$ mM. The values of these parameters agree with those determined from direct fluorescence titrations (see Fig. 4) under identical solution conditions.

**FIG. 7.** Monovalent cation activation profile for wild-type and mutant thrombins of Asp-189. Shown are the values of $s = K_{d,M}/K_d$ for the hydrolysis of FPR in the presence of different monovalent cations, expressed in units of the value calculated in ChCl. Experimental conditions were 5 mM Tris, 0.1% PEG, pH 8.0 at 25 °C. The salt concentration was 200 mM LiCl (gray), NaCl (white), KCl (black), or RbCl (hatched).
latter depends upon the enzyme-substrate complex. Residues responsible for cation binding and/or transduction of this event into enhanced catalytic activity can be identified by site-directed mutagenesis. Residue Asp-189 is involved in both processes, because its mutation to Ala compromises Na\textsuperscript{+} binding and the Na\textsuperscript{+}-induced enhancement of $k_{cat}/K_{m}$. However, the side chain of Asp-189 is more critical for allosteric transduction, because its mutation to Ala compromises Na\textsuperscript{+} binding, but have practically lost the K\textsuperscript{+} activation effect. The results of recent mutagenesis studies (28), together with the role uncovered here for Asp-189, demonstrate that the monovalent cation specificity of thrombin can be redesigned, which is a unique accomplishment in the realm of monovalent cation-activated enzymes. Future studies should build on this important result to identify residues that do not participate directly in cation binding, but play a key role in the process that links cation binding to substrate recognition. At that point, the task of turning thrombin into a K\textsuperscript{+}- or Li\textsuperscript{+}-activated enzyme should become possible.

REFERENCES
1. Hedstrom, L., Szilagyi, L., and Rutter, W. J. (1992) Science 255, 1249–1253
2. Hedstrom, L., Farr-Jones, S., Kettner, C. A., and Rutter, W. J. (1994) Biochemistry 33, 8764–8769
3. Perona, J. J., Hedstrom, L., Rutter, W. J., and Fletterick, R. J. (1995) Biochemistry 34, 1499–1499
4. Graf, L., Jancso, A., Szilagyi, L., Hegyi, G., Pinter, K., Naray-Szabo, G., Hepp, J., Medzhbradsky, K., and Rutter, W. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4961–4965
5. Hung, S. H., and Hedstrom, L. (1998) Protein Eng. 11, 669–673
6. Hedstrom, L. (2002) Chem. Rev. 102, 4501–4524
7. Venekens, I., Szilagyi, L., Graf, L., and Rutter, W. J. (1996) FEBS Lett. 383, 143–147
8. Wells, C. M., and Di Cera, E. (1992) Biochemistry 31, 11721–11730
9. Dang, Q. D., and Di Cera, E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10653–10656
10. Di Cera, E., Guinto, A., Dang, Q. D., Ayala, Y. M., Wuyi, M., and Tulinsky, A. (1995) J. Biol. Chem. 270, 22088–22092
11. Di Cera, E. (2003) Chest 124, 115–178
12. Krem, M. M., and Di Cera, E. (2001) EMBO J. 20, 3036–3045
13. Krem, M. M., and Di Cera, E. (2002) Trends Biochem. Sci. 27, 67–74
14. Guinto, E. R., Caccia, S., Rose, T., Futterer, K., Waksman, G., and Di Cera, E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1852–1857
15. Pineda, A. O., Savvides, S. N., Waksman, G., and Di Cera, E. (2002) J. Biol. Chem. 277, 40177–40180
16. Krem, M. M., and Di Cera, E. (2003) Biophys. Chem. 100, 315–323
17. Stone, S. R., and Hofsteenge, J. (1986) Biochemistry 25, 4622–4628
18. Ayala, Y., and Di Cera, E. (1994) J. Mol. Biol. 235, 735–746
19. Ayala, Y. M., Cantwell, A. M., Rose, T., Bush, L. A., Arosio, D., and Di Cera, E. (2001) Proteins 45, 107–116
20. Dang, O. D., Guinto, A., and Di Cera, E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5977–5981
21. Guinto, A., and Di Cera, E. (1996) Biochemistry 35, 4417–4426
22. Dang, Q. D., Guinto, A., and Di Cera, E. (1997) Nat. Biotechnol. 15, 146–149
23. Olson, S. T., and Chuang, Y. J. (2002) Trends Cardiovasc. Med. 12, 331–338
24. Chuang, Y. J., Swanson, R., Raja, S. M., Bock, S. C., and Olson, S. T. (2001) Biochemistry 40, 6670–6679
25. Rydel, T. J., Tulinsky, A., Bode, W., and Huber, R. (1991) J. Mol. Biol. 221, 583–691
26. Braun, P. J., Dennis, S., Hofsteenge, J., and Stone, S. R. (1988) Biochemistry 27, 6517–6522
27. Di Cera, E., Hofprater, K. P., and Dang, Q. D. (1996) Biophys. J. 70, 174–181
28. Prasad, S., Wright, K. J., Banerjee Roy, D., Bush, L. A., Cantwell, A. M., and Di Cera, E. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 13785–13790
29. Jing, H., Macon, K. J., Moore, D., Delucas, L. J., Volanakis, J. E., and Narayana, S. V. (1999) EMBO J. 18, 804–814
30. Szabo, E., Bocskei, Z., Naray-Szabo, G., and Graf, L. (1999) Eur. J. Biochem. 263, 20–26
31. Szabo, E., Venekei, I., Bocskei, Z., Naray-Szabo, G., and Graf, L. (2003) J. Mol. Biol. 331, 1121–1130
32. Sundararaju, B., Chen, H., Shilcutt, S., and Phillips, R. S. (2000) Biochemistry 39, 8546–8555
33. Phillips, R. S., and Doshi, K. J. (1998) Eur. J. Biochem. 255, 508–515
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