Structural Basis of Na⁺ Activation Mimicry in Murine Thrombin*

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Unlike human thrombin, murine thrombin lacks Na⁺ activation due to the charge reversal substitution D222K in the Na⁺ binding loop. However, the enzyme is functionally stabilized in a Na⁺-bound form and is highly active toward physiologic substrates. The structural basis of this peculiar property is unknown. Here, we present the 2.2 Å resolution x-ray crystal structure of murine thrombin in the absence of inhibitors and salts. The enzyme assumes an active conformation, with Ser-195, Glu-192, and Asp-189 oriented as in the Na⁺-bound fast form of human thrombin. Lys-222 completely occludes the pore of entry to the Na⁺ binding site and positions its side chain inside the pore, with the Nε atom H-bonded to the backbone oxygen atoms of Lys-185, Asp-186b, and Lys-186d. The same architecture is observed in the 1.75 Å resolution structure of a thrombin chimera in which the human enzyme carries all residues defining the Na⁺ pore in the murine enzyme. These findings demonstrate that Na⁺ activation in thrombin is linked to the architecture of the Na⁺ pore. The molecular strategy of Na⁺ activation mimicry unraveled for murine thrombin is relevant to serine proteases and enzymes activated by monovalent cations in general.

Binding of Na⁺ to human thrombin results in enhanced catalytic activity due to faster substrate diffusion into the active site and faster acylation (1–3). The activating effect of Na⁺ is allosteric, or Type II (3, 4), and is linked to significant changes in recognition of physiologic substrates. Na⁺ binding is required for optimal cleavage of the procoagulant substrates fibrinogen (2, 5, 6) and factors V (7), VIII (8), and XI (9) and also for the cleavage of the prothrombotic/signaling factors PAR1, PAR3, and PAR4 (10, 11). On the other hand, Na⁺ binding has no significant effect on activation of the anticoagulant protein C (2, 5, 6). Under physiologic conditions of temperature and [NaCl], thrombin is partitioned almost equally between the Na⁺-free slow form (40%) and the Na⁺-bound fast form (60%) due to the strong temperature dependence of its Na⁺ affinity (1, 12, 13). Any effect that perturbs Na⁺ binding has the potential to drastically affect the procoagulant, prothrombotic, and signaling functions of the enzyme. Integrity of the Na⁺ binding site and allosteric transduction due to Na⁺ activation are therefore necessary conditions to preserve viability of thrombin function in vivo (2, 14).

Much has been learned on the molecular basis of Na⁺ binding to human thrombin (15). Recent measurements of the kinetic mechanism of Na⁺ binding have offered a paradigm shifting scenario by revealing an involvement of the structure of the enzyme as a whole in response to Na⁺ binding (13). Site-directed mutagenesis has identified specific residues linked energetically to Na⁺ binding (15, 16). Na⁺ binding is severely compromised (>30-fold decrease in binding affinity) upon mutation of Asp-189, Glu-217, Asp-222, and Tyr-225. Asp-189 in the primary specificity pocket is within 5 Å of the bound Na⁺ and provides an important link between the Na⁺ site and the P1 residue of substrate (17). Glu-217 makes polar contacts with Lys-224 and Thr-172 that stabilize the 220-loop in the Na⁺ site. The ion pair between Arg-187 and Asp-222 latches the 186-loop onto the 220-loop to stabilize the Na⁺ site and the pore of entry to the Na⁺ site (12, 15, 18); Tyr-225 plays a crucial role in determining the Na⁺-dependent allosteric nature of serine proteases (19) by allowing the correct orientation of the backbone oxygen of residue 224 (20), which contributes directly to the coordination of Na⁺. The side chain of Tyr-225 also secures the integrity of the water channel embedding the primary specificity pocket (20), and the backbone around Tyr-225 is oriented like the selectivity filter of the KcsA K⁺ channel (3, 4, 21). Of these four residues, Glu-217 and Tyr-225 are conserved in thrombin from all species sequenced to date (22). Asp-189 is Ser in the sturgeon, and the D189S mutant of human thrombin has impaired Na⁺ binding and substrate recognition (17). Asp-222 is the least conserved residue among the four. It is Ser in the sturgeon, Lys in the mouse, and Asn in the rat (22). Because of the D222K substitution, murine thrombin lacks Na⁺ activation, which is partially restored by the reverse K222D mutation (23).

The lack of Na⁺ activation in human thrombin would produce an enzyme with little catalytic activity toward fibrinogen and PAR1, incompatible with biological function (14). Murine thrombin has solved this conundrum by mimicking functionally the Na⁺-bound fast form of the human enzyme, thereby retaining high activity toward fibrinogen, PAR1, and PAR4 and without compromising activation of protein C (23). Such molecular mimicry of Na⁺ activation may have turned beneficial during evolution to counter the effect of numerous mutations that in humans destabilize Na⁺ binding and cause bleeding (24–29). There are few but biologically relevant examples of monovalent cation activation mimicry (3, 4). Actin folds similarly to the K⁺-activated enzyme Hsc70 (30), but the requirement for K⁺ to bridge the Pα and Pβ of ADP is fulfilled by the
**Structure of Murine Thrombin**

**TABLE 1**

| Amino acid differences between human and murine thrombin |
|----------------|----------------|
| Replacements underlined (nine total) indicate non-conservative charge substitutions. The thrombin chimera was constructed by replacing residues 184a, 186, 186b, 186c, and 222 in human thrombin with those of the murine enzyme. |

| 1e | 13 | 14a | 14d | 14h |
|----|----|-----|-----|-----|
| Human | S | E | K | R | E |
| Murine | L | K | T | K | D |
| 90 | 106 | 126 | 127 | 129 |
| Human | I | M | R | E | A |
| Murine | V | L | K | O | T |
| 149e | 150 | 170 | 184a | 186 |
| Human | K | G | D | Y | P |
| Murine | F | I | A | F | V |

**TABLE 2**

| Crystallographic data of murine thrombin(2OCV) and the thrombin chimera(2OD3) |
|----------------|----------------|
| Data collection | |
| Wavelength | 0.9 | 0.9 |
| Space group | C2 | P212121 |
| Unit cell dimensions (Å) | | |
| a = 136.8 | a = 44.9 |
| b = 47.5 | b = 74.1 |
| c = 43.2 | c = 103.6 |
| α = 90° | α = 90° |
| β = 95.5° | β = 90° |
| γ = 90° | γ = 90° |
| Molecules/asymmetric unit | 1 | 1 |
| Resolution range (Å) | 40.0-2.2 | 40.0-1.75 |
| Observations | 85787 | 269728 |
| Unique observations | 13687 | 34880 |
| Completeness | 95.5 (80.3) | 98.2 (96.8) |
| Rmerge (%) | 7.8 (30.1) | 9.5 (36.0) |
| I/σ(I) | 19.3 (4.0) | 18.0 (2.4) |

**Refinement**

| |
|----------------|----------------|
| Resolution (Å) | 40.0-2.2 | 40.0-1.75 |
| Rmerge(Rfree) | 0.186, 0.246 | 0.199, 0.230 |
| Reflections (working/test) | 12219/622 | 3249/1694 |
| Protein atoms | 2374 | 2293 |
| Solvent molecules | 157 | 271 |
| Inhibitor (PPACK) | 0 | 1 |
| r.m.s.d. bond lengths (Å) | 0.007 | 0.012 |
| r.m.s.d. angles (°) | 1.4 | 1.7 |
| r.m.s.d. ΔF values (Å²) (mc/sc)⁴ | 3.32/5.05 | 1.56/2.46 |
| (B) protein (Å²) | 42.9 | 28.0 |
| (B) solvent (Å²) | 46.4 | 39.7 |

**Ramachandran plot**

Most favored (%) | 98.8 | 100.0 |
Generously allowed (%) | 1.2 | 0.0 |
Disallowed (%) | 0.0 | 0.0 |

Non-atom of Lys-18 (31). We therefore surmised that Lys-222 in murine thrombin could play an important structural role in securing functional mimicry of Na⁺ activation.

**MATERIALS AND METHODS**

Murine thrombin and the thrombin chimera in which murine thrombin carries the substitutions D222K/Y184aF/Pro-Arg-CH₂Cl (PPACK)² (32), with and without Na⁺ present (15), murine thrombin consistently failed to produce diffracting quality crystals in the presence of PPACK and under every buffer condition in the screens containing Na⁺. Crystals of the thrombin chimera were grown in 2 weeks with space group C2 and unit cell parameters 43.2 Å, and 74.1 Å, and 103.6° (Table 2). Crystals of the thrombin chimera were monoclinic, with one molecule in the asymmetric unit, and grew in 2 weeks with space group P2₁,2₁,2₁ and unit cell parameters a = 136.8 Å, b = 47.5 Å, c = 43.2 Å, and β = 95.5° (Table 2). Crystals of the thrombin chimera were orthorhombic, with one molecule per asymmetric unit, and grew in 1 week, with space group P2₁,2₁,2₁ and unit cell parameters a = 44.9 Å, b = 74.1 Å, and c = 103.6 Å (Table 2). Prior to flash-freezing in liquid nitrogen, murine thrombin crystals were cryoprotected with paratone-N oil, whereas crystals of the thrombin chimera were put in a solution similar to the reservoir buffer with 15% glycerol. X-ray data were collected at 100 K on an ADSC Quantum-315 CCD detector at the Biocars Beamline 14-BM-C of Advanced Photon Source, Argonne National Laboratories (Argonne, IL). Data processing including indexing, integration, and scaling was performed using HKL 2000 (33). The results of data collection are shown in Table 2.

Structures were solved by molecular replacement with MOLREP from the CCP4 package (34) and using the coordinates of the PPACK-bound slow form of human thrombin, 1SHH (15), as the starting model. Refinement and electron density map calculations were carried out using CNS (35), and 5% of the reflections were selected randomly and set aside as a test set for cross validation. Model building and analysis of the structures were carried out with TURBO-FRODO (32) and O (36). Rigid body refinement following several cycles of positional, temperature factor, and simulated annealing refinement, including bulk solvent correction, was performed. Thereafter, water molecules were added interactively at the end of each refinement cycle using Fobs - Fcalc peaks over 3σ. Residues

² The abbreviations used are: PPACK, H-o-Phe-Pro-Arg-Ch₂Cl; MES, 4-morpholineethanesulfonic acid; FPR, H-o-Phe-Pro-Arg-p-nitroanilide; r.m.s., root mean square; r.m.s.d., r.m.s. deviation.
of the autolysis loop from 148 to 149 were not included because of their weak density. Residues from 74 to 78 in exosite I were missing in the murine thrombin structure, most likely due to autoproteolytic cleavage at Arg-77a and Arg-73, whose side chain is not visible in the density map. Ramachandran plots were calculated with PROCHECK (37), and the results are listed in Table 2. The coordinates of the structures reported in this study have been deposited in the Protein Data Bank (accession codes 2OCV for murine thrombin, 2OD3 for the thrombin chimera).

Monovalent cation activation profiles were determined as reported elsewhere (19) using the chromogenic substrate H-D-Phe-Pro-Arg-p-nitroanilide (FPR). The values of $k_{cat}/K_m$ for the hydrolysis of FPR were determined in the presence of 200 mM LiCl, NaCl, KCl, or RbCl under experimental conditions of 5 mM Tris, 0.1% polyethylene glycol, 25 °C. Reference values were determined in the presence of 200 mM choline chloride.

RESULTS

There are 32 total replacements between human and murine thrombin (Table 1), five in the A chain and 27 in the catalytic B chain. The net result of the replacements is very significant; murine thrombin has a net charge difference relative to human thrombin of +6. This comes from the introduction of two positively charged residues (I24K, Q131R) and the loss of one positively charged residue (K149eE), three negatively charged residues (E127Q, D170A, D186aN), and three charge reversal substitutions (E13K, K149eE, D222K). The drastic charge difference explains some of the distinct properties of the two enzymes, most notably the different ability to stick to ion exchange columns, the different procedures needed for activation from zymogen, and especially the difficulty for murine thrombin to crystallize under solution conditions containing even minimal amounts of salts. Among the non-conservative substitutions, D186aN and D222K affect residues around the pore of entry of Na$^+$ to its binding site (12).

FIGURE 1. A–C, surface rendering of the pore of entry to the Na$^+$ binding site of human thrombin in the structure 1SG8 (15) (A) when compared with the same region in murine thrombin (B) and the thrombin chimera (C). Residues lining the pore are color-coded according to their physical properties (red = positively charged, blue = negatively charged, orange = hydrophobic, white = all others). In the human enzyme, the pore is defined by residue Asp-222 in the 220-loop and the sequence PDEGKR from Pro-186 to Arg-187 in the 186-loop (Table 1) (A). In murine thrombin (B), residue 222 is Lys, and the corresponding sequence in the 186-loop is VNDEKTR (Table 1). The side chain of Lys-222 completely occludes the pore. The side chain of Asn-186a is glycosylated (NAG). Occlusion of the pore is also seen in the thrombin chimera (C), in which the human enzyme carries all residues around the pore as in murine thrombin. There is no glycosylation of Asn-186a in the chimera. D–F, architecture of the pore of entry to the Na$^+$ binding site in the same orientation as shown in the surface rendering (A–C), with relevant residues rendered in Corey-Pauling-Koltun model (carbon in yellow) and the $2Fo-Fc$ electron density maps contoured at the 0.7 $\sigma$ level for the structures presented in this study (E and F). The human enzyme (D) shows the pore wide open, whereas Lys-222 in murine thrombin (E) occludes the pore and positions the N$\delta$ atom within H-bonding distance from Lys-185, Asp-186b, and Lys-186d. The backbone oxygen atom of residue 186b is flipped relative to the position assumed in the fast form of the human enzyme. Also shown is the indole side chain of Trp-20, which is Ser in human thrombin, as a structural signature of the murine enzyme. Lys-222 in the thrombin chimera (F) is positioned as in the murine thrombin structure.
Structure of Murine Thrombin

The structure of murine thrombin was solved at 2.2 Å resolution, free of inhibitors and salts, after many unsuccessful attempts involving the active site inhibitor PPACK and different concentrations of Na\(^+\), K\(^+\), or Li\(^+\) in the crystallization conditions. Experimental conditions under which human thrombin readily crystallizes at high resolution in the presence of PPACK (15, 32) failed to yield crystals of the murine enzyme. The conformation of murine thrombin in its free form has the active site and primary specificity pocket readily accessible to substrate. The r.m.s. deviation at the C\(\alpha\) atoms is only 0.42 Å relative to the fast form of the human enzyme (15). Because the enzyme used was free of inhibitors and in its wild-type form, autoproteolytic digestion at exosite I caused the entire segment 74–78 to be missed in the electron density map. Autoproteolytic attack of Arg-77a followed by a second cleavage at Arg-73 produces \(\beta\)-thrombin from \(\alpha\)-thrombin in the human enzyme (38). The substantial perturbation of exosite I shifts the backbone around His-71 and makes room for a flip of the indole ring of Trp-141, as observed in the inactive monomer of the human thrombin structure solved in the presence of KCl (39, 40). The flipped conformation is also stabilized by a rearrangement of the side chain of Met-32. Whether the flipped conformation of Trp-141 is constitutive in murine thrombin remains to be established. Interest in this residue stems from its involvement in the fluorescence change linked to Na\(^+\) binding to human thrombin (13). The autolysis loop spanning residues 144–150 (32) is disordered, as typically seen in human thrombin even at high resolution (15).

The most important features of the murine thrombin structure involve the pore of entry to the Na\(^+\) binding site (Fig. 1). The ion pair between Asp-222 and Arg-187 is a hallmark of the Na\(^+\)-bound fast form in the human enzyme (15, 18) and contributes to the architecture of the pore. In murine thrombin, the presence of Lys-222 pushes the guanidinium group of Arg-187 away. The side chain of Lys-222 occupies the interior of the pore and completely occludes its access from the solvent (Fig. 1). The N\(\gamma\) atom of Lys-222 penetrates the pore and sits ~5 Å away from where Na\(^+\) binds in the fast form (15), within H-bonding distance of the backbone oxygen atoms of Lys-185 (2.85 Å), Asp-186b (3.34 Å), and Lys-186d (2.71 Å). The backbone oxygen atom of residue 186b, recruited by the N\(\gamma\) atom of Lys-222, is flipped relative to the position assumed in the fast form of the human enzyme. Other changes of interest around the pore region include glycosylation of Asn-186a, which is never observed in the human enzyme. Trp-20, which is Ser in the human enzyme, is identified unequivocally from the density map and offers a unique fingerprint of the murine structure (Fig. 1). The aromatic residue sits only 4.7 Å away from Lys-186d in the pore region and 14 Å away from where Na\(^+\) binds in the human enzyme. That is about the same distance as Trp-215 in the aryl binding site, which is a major fluorophore reporting Na\(^+\) binding to human thrombin (41). Trp-20 in murine thrombin could therefore interfere with the fluorescence change linked to Na\(^+\) binding that is difficult to detect even after Na\(^+\) activation is partially restored with the K222D mutation (23).

Oclusion of the Na\(^+\) pore by the side chain of Lys-222 is linked to a conformation similar to the Na\(^+\) -bound fast form of the human enzyme. That is revealed by inspection of three markers of the allosteric slow \(\rightarrow\) fast transition of thrombin (2, 15). Glu-192 at the entrance of the active site, Ser-195 in the active site, and Asp-189 in the primary specificity pocket are oriented as in the fast form (Fig. 2). Ser-195 is within H-bonding distance (3.05 Å) of the catalytic His-57. This H-bond is present in the fast form of the human enzyme (3.09 Å) but is broken (3.70 Å) in the Na\(^+\)-free slow form (15). The side chain of Asp-189 in the primary specificity pocket is oriented optimally for coordination of Arg of substrate, seen in the fast form. The conformations of Asp-189 and Ser-195 are maintained by H-bonding interactions mediated by water molecules, as in the fast form of the human enzyme. However, only seven water molecules (red balls) are present in this region of the murine thrombin structure, as opposed to Na\(^+\) (green ball) and 11 water molecules (cyan balls) present in the fast form of the human enzyme (15). The presence of Lys-222 in murine thrombin pushes Arg-187 away and closer (2.55 Å to Asp-221. The N\(\gamma\) atom of Lys-222 and the O\(\alpha\) atom of Arg-221a H-bond to water w153, which in turn stabilizes water w51, in a position equivalent (<1 Å away) to the bound Na\(^+\) in the fast form (green ball) and in contact with the backbone oxygen atoms of Arg-221a (2.77 Å) and Lys-224 (2.61 Å). The H-bonding network around water w51 mimics that seen around the bound Na\(^+\) in the fast form of the human enzyme (15) and establishes a connection to the O\(\epsilon\)2 atom of Asp-189 via water w97. The O\(\epsilon\)1 atom of Asp-189 is held in place by an H-bond with water w55 (2.74 Å); Ser-195 is fixed in its orientation by a water-mediated contact with the O\(\epsilon\)1 atom of Glu-192, with water w63 positioned 3.19 Å away from the O\(\gamma\) atom of Ser-195 and 2.82 Å away from the O\(\epsilon\)1 atom of Glu-192. The only two water molecules, w141 and w142, between Asp-189 and Ser-195 are too far away from either residue. Thus, murine thrombin lacks the connectivity between the primary specificity pocket and the catalytic triad seen in the fast form of the human enzyme.
around water w51 mimics that found around the bound Na\textsuperscript{+} (2.77 Å) and Lys-224 (2.61 Å). The H-bonding network is likewise coordinated by the backbone oxygen atoms of Arg-189 and Glu-192 that would provide connectivity from the bound Na\textsuperscript{+} to the catalytic Ser-195 (15). The only two water molecules, w141 and w142, in the active site between Asp-189 and Ser-195 are fixed in their orientation by a water-mediated contact with the Oe1 atom of Glu-192, with water w63 positioned 3.19 Å away from the Oy atom of Ser-195 and 2.82 Å away from the Oe1 atom of Glu-192. Unlike the fast form of human thrombin, there is no obvious water-mediated linkage between Asp-189 and Glu-192 that would provide connectivity from the bound Na\textsuperscript{+} to the catalytic Ser-195 (15). The two only water molecules, w141 and w142, in the active site between Asp-189 and Ser-195 are too far away from either residue. Therefore, the conformations of the critical residues Asp-189 and Ser-195 in murine thrombin have been optimized independently of each other, with Lys-222 sealing the Na\textsuperscript{+} pore and contributing indirectly to stabilization of the Na\textsuperscript{+} binding site via waters w153 and w51.

To validate the results observed with the murine enzyme, we engineered a thrombin chimera with the human enzyme carrying all residues around the Na\textsuperscript{+} pore in murine thrombin. The chimera lacks Na\textsuperscript{+} activation, as expected (Fig. 3), and its crystal structure was solved at 1.75 Å resolution in the presence of PPACK and in the absence of Na\textsuperscript{+}. The pore region in the chimera looks practically identical to that of the murine enzyme (Fig. 1), with the side chain of Lys-222 occupying the interior of the pore. This finding proves that the architecture of the pore is determined directly by the residues defining it and is not influenced by features that are unique to the murine thrombin structure, such as glycosylation of Asn-186a in the 186-loop or the missing portion of exosite I. It should be pointed out, however, that even six replacements around the Na\textsuperscript{+} pore in the chimera fail to produce full functional mimicry of Na\textsuperscript{+} activation (Fig. 3). Obviously, other residues replaced in murine thrombin relative to the human enzyme must contribute to the effect. Charged replacements (Table 1) are obvious candidates for future mutagenesis studies. Particularly intriguing is the presence of Trp-20 near the Na\textsuperscript{+} pore, which replaces Ser in human thrombin (Fig. 1). Preliminary data on the chimera carrying the additional substitution S20W show a significantly improved \(k_\text{cat}/K_m\) for FPR hydrolysis in choline chloride to a value (23 \(\mu M^{-1} s^{-1}\)) that is <3-fold lower than that of wild-type murine thrombin and >5-fold higher than that of wild-type human thrombin.

**DISCUSSION**

Murine thrombin has been the subject of detailed genetic analysis in the context of models of human disease (43–46). However, important differences exist in the functional interactions and regulation of thrombin between mice and humans. In addition to the basic differences uncovered in the role of PARs in platelet activation between mice and humans (44, 47), thrombin in the mouse lacks Na\textsuperscript{+} activation (23), which is a hallmark feature of the human enzyme (2). The lack of Na\textsuperscript{+} activation in human thrombin would be inconsequential on the ability to cleave the anticoagulant protein C (2, 5, 6) but would drastically compromise activity toward fibrinogen and the PARs (2, 5, 6, 11) and make the enzyme unable to carry out its procoagulant, prothrombotic, and signaling roles in the blood (14, 48). Several naturally occurring mutations of the prothrombin gene, such as prothrombin Frankfurt (E146A) (24), Salakta (E146A) (25), Greenville (R187Q) (26), Scranton (K224T) (27), Copenhagen (A190V) (28), and Saint Denis (D221E) (29), affect residues linked to Na\textsuperscript{+} binding (15) and are often associated with bleeding. Furthermore, thrombin can be engineered for anticoagulant activity in vitro and in vivo by selectively perturbing the Na\textsuperscript{+} site and adjacent residues with site-directed mutations (6, 49–52). Murine thrombin compensates for the lack of Na\textsuperscript{+} activation by mimicking functionally the Na\textsuperscript{+}-bound form of the human enzyme (23). That ensures high efficiency in the catalytic activity toward fibrinogen, PAR1, and PAR4 and does not compromise protein C activation.

The results presented here elucidate the structural basis of Na\textsuperscript{+} activation mimicry in murine thrombin. Previous attempts to mimic functionally the activating effect of Na\textsuperscript{+} in thrombin by replacing Gly-184 with Lys have produced a poorly active enzyme (53). Similarly, mimicry of K\textsuperscript{+} activation in rabbit muscle pyruvate kinase (54) and the ATPase domain of Hsc70 (55) have utilized Lys substitutions near the monovalent cation binding site, but in no case did the mutation result in high catalytic activity. The strategy used by murine thrombin is obviously very effective because the enzyme shows high catalytic activity (15) and are often associated with bleeding. Furthermore, thrombin can be engineered for anticoagulant activity in vitro and in vivo by selectively perturbing the Na\textsuperscript{+} site and adjacent residues with site-directed mutations (6, 49–52). Murine thrombin compensates for the lack of Na\textsuperscript{+} activation by mimicking functionally the Na\textsuperscript{+}-bound form of the human enzyme (23). That ensures high efficiency in the catalytic activity toward fibrinogen, PAR1, and PAR4 and does not compromise protein C activation.
the 186-loop and is ∼5 Å away from where Na⁺ binds in the human enzyme. It is unclear whether the Nζ atom in this position could provide effective electrostatic mimicry of the bound Na⁺. It is reasonable to assume, however, that the side chain of Lys-222 could penetrate the Na⁺ pore farther to position the Nζ atom closer to the site occupied by Na⁺. Nonetheless, the presence of Lys-222 seals the entrance to the Na⁺ site, abrogates sensitivity to monovalent cations (23), and generates an environment within the primary specificity pocket that is optimized for substrate binding. That is accomplished through a network of water molecules that mimics the Na⁺-bound architecture in the proximity of Asp-189. A water-mediated contact, similar to that documented in the fast form of the human enzyme, fixes the orientation of the catalytic Ser-195 with the assistance of the side chain of Glu-192. As a result, both Ser-195 and Asp-189 assume conformations like those in the fast form of human thrombin, thereby accounting for the high catalytic activity of the murine enzyme.

Our structural analysis provides a reasonable scenario for the molecular origin of the Na⁺ activation mimicry in murine thrombin. However, a key question remains as to why murine thrombin lacks Na⁺ activation. The origin of the difference probably resides in an evolutionary advantage to counter the effects of mutations that produce anticoagulant effects by destabilizing Na⁺ binding. It is known that the C-terminal domain of serine proteases dictated functional diversity during evolution (56) and is the locale preferentially targeted by naturally occurring mutations. All residues linked energetically to Na⁺ binding to thrombin reside in the C-terminal domain of the enzyme (15), and so do most of the known naturally occurring mutations of the prothrombin gene in humans (24–29). It would be important to establish whether mutations that cause anticoagulant effects in the human enzyme behave differently in murine thrombin. The Y225P mutation selectively abolishes Na⁺ binding to human thrombin and compromises fibrinogen and PAR1 recognition indirectly by switching the enzyme to the anticoagulant slow form (6, 19). Such mutation in murine thrombin should be inconsequential on fibrinogen and PAR1 recognition. The W215A/E217A mutant of human thrombin features remarkable anticoagulant and antithrombotic properties in vitro and in vivo (51, 52, 57, 58) but is significantly less anticoagulant in its murine version (23).

It has been suggested that the activity of serine proteases devoid of Na⁺ activation can be enhanced by introducing Na⁺ binding (19), based on the observation that the activity of Na⁺-activated proteases decreases when Na⁺ binding is abrogated by mutagenesis (19, 59–65). Na⁺ activation was recently engineered into trypsin (66), but it required extensive amino acid substitutions, and the activity of the mutant remained below that of wild type. The strategy used by murine thrombin to functionally mimic Na⁺ activation provides an alternative route to endow poorly active proteases, such as tissue-type plasminogen activator, with enhanced catalytic activity. The structural template that ensures functional mimicry apparently resides in a small number of residues defining the Na⁺ pore. Future mutagenesis studies in other proteases should consider the intriguing possibility that introduction of Na⁺ activation mimicry as seen in murine thrombin may effectively boost catalytic activity in serine proteases of biotechnological and biomedical relevance.

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