Published in final edited form as:

J Phys Chem B. 2010 December 16; 114(49): 16125–16130. doi:10.1021/jp105502c.

Evidence of the E*-E equilibrium from rapid kinetics of Na⁺ binding to activated protein C and factor Xa*

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Abstract

Na⁺ binding to thrombin enhances the procoagulant and prothrombotic functions of the enzyme and obeys a mechanism that produces two kinetic phases: one fast (in the μ s time scale) due to Na⁺ binding to the low activity form E to produce the high activity form E:Na⁺, and another considerably slower (in the ms time scale) that reflects a pre-equilibrium between E and the inactive form E*. In this study we demonstrate that this mechanism also exists in other Na⁺-activated clotting proteases like factor Xa and activated protein C. These findings, along with recent structural data, suggest that the E*-E equilibrium is a general feature of the trypsin fold.

Keywords

Thrombin; activated protein C; factor Xa; Na⁺ binding; rapid kinetics; E* form

Introduction

Activation of trypsin-like proteases requires proteolytic processing of an inactive zymogen precursor¹ that occurs at the identical position in all known members of the family, i.e., between residues 15 and 16 (chymotrypsinogen numbering). The nascent N-terminus induces conformational change in the enzyme through formation of an ion-pair with the highly conserved Asp¹⁹⁴ that organizes both the oxyanion hole and substrate binding cleft^{2,3}. Existing structures of the zymogen forms of trypsin³, chymotrypsin⁴ and chymase⁵ document the lack of ion-pair interaction between Asp¹⁹⁴ and the N-terminus of the catalytic chain. The zymogen→protease conversion is classically associated with the onset of catalytic activity^{6,7} and provides a useful paradigm for understanding key features of protease function and regulation.

Recent structural and functional work on thrombin, a trypsin-like protease involved in blood coagulation⁸, has added complexity to this widely accepted scenario. Thrombin is a Na⁺-activated protease for which Na⁺ binding significantly enhances activity toward synthetic and physiological substrates. The interaction of Na⁺ with thrombin is physiologically relevant and promotes cleavage of the substrates fibrinogen and PAR1⁹⁻¹¹, coagulation factors V¹², VIII¹³ and XI¹⁴, but has no effect on activation of the anticoagulant protein C^{10,11}, which makes Na⁺ an exquisite procoagulant/prothrombotic cofactor of thrombin. Indeed, several naturally occurring mutations like prothrombin Frankfurt (E146A)¹⁵, Salakta (E146A)¹⁶, Greenville (R187Q)¹⁷, Scranton (K224T)¹⁸, Copenhagen (A190V)¹⁹ and Saint Denis (D221E)²⁰ that affect residues linked to Na⁺ binding²¹ are associated with

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bleeding. Furthermore, anticoagulant thrombin mutants have been engineered rationally by perturbing the Na^+ site^{11,22-26}. Investigation of the kinetic mechanism of Na^+ binding has led to the discovery of a key allosteric equilibrium that involves the free enzyme²⁷. Na^+ binding to thrombin is not a simple one-step process but gives rise to two kinetic phases, one fast (in the μs time scale) due to Na^+ binding to E to produce $\text{E}:\text{Na}^+$ ^{27,28} and another considerably slower (in the ms time scale) that reflects a pre-equilibrium between E and E^* according to the kinetic scheme^{8,27,29}. The Na^+ -free form of thrombin, originally defined as the “slow” form³⁰, is not a single species or equivalently an ensemble of species in rapid equilibrium but a mixture of E^* and E that interconvert with kinetic rate constants k_r and k_{-r} . Of these forms, E interacts with Na^+ with a rate constant k_A to populate the Na^+ -bound form $\text{E}:\text{Na}^+$, originally defined as the “fast” form³⁰, that may dissociate into the parent components with a rate constant k_{-A} . Na^+ binds to a site between the 186- and 220- loops, located $>15 \text{ \AA}$ away from residues of the catalytic triad^{21,31}, and converts the low activity E form to the high activity $\text{E}:\text{Na}^+$ form. The E^* form, on the other hand, is inactive and cannot bind Na^+ or substrate to the active site^{27,32}. Recent alternative models of thrombin allostery³³⁻³⁶ show considerable confusion about the nature of the slow form, which is defined as E^* , zymogen-like, inactive or as a continuum of zymogen-like states³³⁻³⁶, deny the existence of the low activity E form and basically predict a one-step kinetic mechanism for Na^+ binding, with the enzyme transitioning directly from an inactive Na^+ -free form to the active Na^+ -bound form. These alternative models of thrombin allostery fail to account mathematically for the kinetic mechanism of Na^+ binding and activation, as we have shown repeatedly^{8,29,37}, and are directly refuted by the kinetic mechanism in scheme 1. Existence of the active E form is strongly supported by kinetic^{29,30} and structural^{21,38-40} data. Under physiological conditions, E^* only makes a minuscule ($<1\%$) contribution to the $\text{E}^*-\text{E}-\text{E}:\text{Na}^+$ equilibria that are dominated by the active forms E (40%) and $\text{E}:\text{Na}^+$ (60%)²⁷. E^* becomes appreciably populated only at temperatures $<25^\circ\text{C}$ and in the absence of Na^+ , or as a result of specific mutations^{26,29,41}.

All three forms of thrombin have been identified crystallographically. Distinguishing features of E^* are the collapse of the 215-217 segment into the active site that brings the highly conserved W215 in contact with the catalytic H57, a flip of the peptide bond between residues E192 and G193 that disrupts the oxyanion hole, and disorder in the 186- and 220-loops that perturbs the Na^+ binding environment^{26,29,42,43}. The collapse of W215 into the active site precludes access of substrate to the primary specificity pocket and explains, along with perturbation of the Na^+ binding site, why E^* is an inactive form unable to interact with substrate or Na^+ . In the active E and $\text{E}:\text{Na}^+$ forms the side chain of W215 is positioned $>10 \text{ \AA}$ away from the catalytic H57 on the opposite side of the active site cleft, the active site is widely accessible to substrate and the 186- and 220- loops are ordered and correctly positioned for Na^+ coordination²¹. Small but significant differences exist between E and $\text{E}:\text{Na}^+$. In the E form, the H-bond between the catalytic S195 and H57 is weakened or broken, the side chain of D189 in the primary specificity pocket is slightly shifted away from the optimal position for coordination of the Arg residue of an incoming substrate and polar interactions between the 186- and 220- loops in the Na^+ binding site are weakened. An additional important difference between E and $\text{E}:\text{Na}^+$ has been identified recently and involves the E192-G193 peptide bond that is flipped in the E form, leading to disruption of the oxyanion hole²⁹, as also documented in a structure of thrombin free of Na^+ ⁴⁴.

Na^+ binding is a property of other vitamin K-dependent clotting proteases like factors VIIa, IXa, Xa and activated protein C that share high sequence homology with thrombin and carry the necessary Tyr residue at position 225^{45,46}. Structural identification of the Na^+ binding site of thrombin using Rb^+ replacement³¹ has facilitated the subsequent identification of the analogous Na^+ binding sites in factors Xa^{47,48}, VIIa⁴⁹ and activated protein C⁵⁰. Several groups have shown that Na^+ has a significant influence on the activity of factor Xa⁵¹⁻⁵⁷ and

activated protein C⁵⁸⁻⁶², but a modest effect on the properties of factor VIIa^{45,63}. In the case of factor IXa, structural evidence of Na⁺ binding remains controversial⁶⁴, but the effect of Na⁺ on the kinetic properties of the enzyme is well established^{45,65}. The physiological role of Na⁺ in these proteases remains largely unexplored⁶⁶. There is currently no structural information as to whether these enzymes partition among E*, E and E:Na⁺ forms like thrombin and no kinetic information on the mechanism of Na⁺ binding that would clarify the generality of the results reported for thrombin. In this study we present evidence that activated protein C and factor Xa bind Na⁺ through a mechanism that is identical to that of thrombin, thereby supporting the generality of the E*-E equilibrium.

Experimental Methods

Human thrombin was expressed and purified to homogeneity as reported elsewhere²¹. Glutathione domainless activated protein C was expressed using an HPC4-modified pRc/RSV vector (a gift from Dr. A. Rezaie) using a baby hamster kidney cell line. Purification and activation were carried out as described⁶⁷. Human factor Xa was obtained from Hematologic Technologies Inc (Essex Junction, VT).

Stopped-flow fluorescence measurements over a time range of 250 ms were carried out with an Applied Photophysics SX20 spectrometer, with excitation at 280 nm and a cutoff filter at 305 nm²⁷. Samples of the protein at a final concentration of 100 nM in a buffer of 50 mM Tris, 0.1% PEG8000, pH 8.0 at 15 °C were mixed with an equal volume (60 µl) of buffer containing variable amounts of NaCl, with a final concentration of NaCl in the 0-200 mM range. Due to the inhibitory effect of choline chloride (ChCl) on factor Xa because of specific binding of the cation to the active site of the enzyme^{54,68,69}, this salt was not used to maintain the ionic strength constant as done previously for studies on thrombin²⁷ and the Na⁺ concentration was changed by increasing the concentration of NaCl in the buffer. Stopped-flow measurements of Na⁺ binding to thrombin run under conditions where the ionic strength was not kept constant with ChCl yield results comparable to those run under conditions of constant ionic strength (see Results).

The total fluorescence change observed upon Na⁺ binding obeys the expression²⁷

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

where F_0 and F_1 are the values of F at $[Na^+] = 0$ and $[Na^+] = \infty$, and

$$K_{app} = \frac{[E:Na^+]}{([E^*] + [E])[Na^+]} = \frac{K_A}{1+r} \quad (2)$$

is the apparent Na⁺ binding affinity that depends on the intrinsic Na⁺ binding affinity

$K_A = \frac{[E:Na^+]}{[E][Na^+]}$ and the equilibrium constant for the E*-E interconversion $r = \frac{[E^*]}{[E]} = \frac{k_{-r}}{k_r}$. The value of r is derived from the dependence of the rate constant of the slow phase of fluorescence increase upon Na⁺ binding due to the E*-E interconversion. Scheme 1 leads to the set of differential equations

$$\begin{pmatrix} d[E^*]/dt \\ d[E]/dt \\ d[E:Na^+]/dt \end{pmatrix} = \begin{pmatrix} -k_{-r} & k_r & 0 \\ k_{-r} & -k_r - k_A [Na^+] & k_{-A} \\ 0 & k_A [Na^+] & -k_{-A} \end{pmatrix} \begin{pmatrix} [E^*] \\ [E] \\ [E:Na^+] \end{pmatrix} \quad (3)$$

The two non-zero eigenvalues associated with the 3×3 matrix of kinetic rate constants in eq 3 are

$$\lambda_{1,2} = -\frac{k_{-r} + k_r + k_{-A} + k_A [Na^+] \pm \sqrt{(k_{-r} + k_r + k_{-A} + k_A [Na^+])^2 - 4k_r(k_{-A} + k_A [Na^+]) - 4k_{-r}k_{-A}}}{2} \quad (4)$$

and define the rates associated with the time evolution of $[E^*]$, $[E]$ and $[E:Na^+]$. Under conditions of separation of time scales, where Na^+ binding and dissociation are much faster than the rates for the E^* - E interconversion, the two eigenvalues in eq 4 generate the rate constants

$$k_{fast} = -\lambda_1 = k_{-A} + k_A [Na^+] \quad (5)$$

$$k_{slow} = -\lambda_2 = k_r + k_{-r} \frac{1}{1 + K_A [Na^+]} \quad (6)$$

The value of k_{fast} is too fast to resolve by stopped-flow and requires the use of ultra-rapid kinetic methods²⁸. The value of k_{slow} corresponds to the k_{obs} of the slow exponential phase and is expected to decrease hyperbolically with increasing $[Na^+]$ from $k_r + k_{-r}$ ($[Na^+] = 0$) to k_r ($[Na^+] = \infty$), thereby yielding k_r , k_{-r} and K_A .

Results

Na^+ binding to thrombin gives rise to a biphasic increase in intrinsic fluorescence (Figure 1) as reported previously²⁷. A fast phase of increase evolves within the dead time (0.5 ms) of the spectrometer. The slow phase obeys a single exponential with k_{obs} decreasing hyperbolically with $[Na^+]$ as predicted from eq 6 (Figure 2). Analysis of k_{obs} for the slow phase yields values of $k_r = 89 \pm 2 \text{ s}^{-1}$, $k_{-r} = 85 \pm 9 \text{ s}^{-1}$ and $K_A = 160 \pm 30 \text{ M}^{-1}$ (Table 1), in agreement with the results reported recently under conditions of constant ionic strength²⁷. The value of $r = 0.95 \pm 0.09$ confirms that E^* and E are equally populated in the absence of Na^+ at 15 °C.

In the case of factor Xa, the observed response to Na^+ binding is biphasic as seen for thrombin, but with some important differences (Figure 1). The total increase in fluorescence is significantly more pronounced (27% vs 15%) (Figure 3) and the dependence of k_{obs} for the slow phase on $[Na^+]$ (Figure 2) reveals a significantly weaker Na^+ affinity ($K_A = 16 \pm 4 \text{ M}^{-1}$) and slightly slower rates of E^* - E interconversion ($k_r = 45 \pm 4 \text{ s}^{-1}$, $k_{-r} = 70 \pm 10 \text{ s}^{-1}$). Both the large total fluorescence change and weak Na^+ affinity were reported in previous studies^{51,52,70}. The value of $r = 1.5 \pm 0.2$ for FXa is slightly higher than that of thrombin and underscores a larger contribution of E^* to the E^* - E equilibrium at 15 °C.

The behavior of activated protein C deserves attention. Na^+ binding to activated protein C has been difficult to monitor by intrinsic fluorescence due to the weak amplitude of the

response^{70,71} (Figure 3). This puzzling observation has always been at odds with the sharp increase in amidolytic activity of activated protein C in the presence of Na⁺^{45,58-62,71,72}. Analysis of Na⁺ binding to activated protein C by rapid kinetics reveals a simple and important explanation for the modest total fluorescence change. Na⁺ binding elicits a biphasic response for activated protein C as seen for thrombin and factor Xa, but the fast phase has a negative amplitude that opposes the positive contribution coming from the slow phase (Figure 1). The peculiar negative amplitude for the fast phase is likely due to a drastically different environment of the major fluorophores of activated protein C compared to thrombin and factor Xa, but conclusive answers can only come from systematic mutagenesis of Trp residues of activated protein C as done for thrombin²⁷. The net result given by the sum of the two phases is a total fluorescence change of small amplitude (Figure 3), with all the relevant information on Na⁺ binding being confined to a transient within the first 50 ms of the reaction. The traces in Figure 1 leave little doubt about the interaction of Na⁺ with activated protein C and support the existence of the allosteric E*-E equilibrium. In fact, analysis of k_{obs} for the slow phase shows an inverse hyperbolic dependence on [Na⁺] (Figure 2) with values of $k_r=25\pm1\text{ s}^{-1}$, $k_{-r}=24\pm3\text{ s}^{-1}$ and $K_A=120\pm10\text{ M}^{-1}$. The Na⁺ binding affinity of activated protein C is comparable to that of thrombin and so is the value of $r=0.95\pm0.08$. However, the rates for the E*-E interconversion are significantly slower. These findings reveal important new information on Na⁺ binding to factor Xa and activated protein C that have eluded experimentalists for over three decades.

Discussion

Demonstration of the existence of the E*-E equilibrium has significant implications for factor Xa. This enzyme catalyzes the conversion of prothrombin into thrombin with the assistance of factor Va, Ca²⁺ and phospholipids, altogether defining the prothrombinase complex⁷³. Prothrombin is composed of a Gla domain, two kringle domains and the serine protease catalytic domain. The prothrombinase complex converts prothrombin to the mature enzyme along two pathways by cleaving sequentially at R271 and R320 (prothrombin numbering). Initial cleavage at R320 (R15 in the chymotrypsin numbering) between the A and B chains is the preferred pathway under physiological conditions and generates the active intermediate meizothrombin by triggering formation of the I16-D194 ion-pair, structuring of the active site and oxyanion hole⁷⁴. The alternative initial cleavage at R271 sheds the Gla domain and the two kringles and generates the inactive precursor prethrombin-2 with the R15-I16 peptide bond intact. The precise mechanism of thrombin generation by the prothrombinase complex remains highly controversial, with opposing models postulating existence of two interconverting functional forms of prothrombinase⁷⁵ or of the substrate prothrombin^{76,77}, each favoring one of the two possible cleavages. Pedersen has recently cast doubts on the mathematical rigor of these models⁷⁸ and added renewed concerns about their conceptual validity. The results presented here demonstrate that factor Xa is capable of alternative conformations due to Na⁺ binding and the E*-E equilibrium. The existing model of prothrombinase assuming different conformations of the enzyme directing cleavage at the two possible sites⁷⁵ should be refined to include the effects of Na⁺ and of the E*-E equilibrium on the kinetics of prothrombin cleavage. Given the modest Na⁺ affinity of factor Xa (Figure 3), it is unlikely that the enzyme exists in vivo in a form that is saturated with the cation. It would be of much interest to establish if the E and E:Na⁺ forms of factor Xa play different roles in cleavage at R320 or R271. The role of E* for factor Xa also needs attention, though this conformation may be transiently shifted to E upon interaction with other components of the prothrombinase complex and Ca²⁺. The conformational plasticity of factor Xa, partitioning among E*, E and E:Na⁺ forms, calls into question the main tenet of the alternative model of prothrombinase action where only the substrate prothrombin is assumed to exist in different conformations^{76,77}. On the other hand, conformational plasticity of the substrate along the prothrombin activation pathway is

supported by evidence that meizothrombin partitions among E^* , E and $E:Na^+$ forms⁷⁹, thereby casting doubts on the hypothesis that only prothrombinase assumes different conformational states⁷⁵. A realistic model of prothrombinase function should necessarily allow multiple conformational states for both enzyme and substrate.

The E^* - E equilibrium has equally important implications for activated protein C. The effect of Na^+ on activated protein C has been documented for a long time⁵⁸⁻⁶². Like thrombin, activated protein C is endowed with different physiological functions⁸⁰. The enzyme is a potent anticoagulant when it cleaves and inactivates factor Va with the assistance of protein S, but also acts as a potent cytoprotective agent upon interaction with and cleavage of PAR1 with the assistance of the endothelial cell protein C receptor⁸¹⁻⁸⁷. Earlier studies have shown that Na^+ has no effect on the action of activated protein C on factor Va^{60,62}. Analysis of the effect of Na^+ on cleavage of PAR1 reveals a slow rate in the presence of Na^+ ⁸⁸ but practically no activity in the absence of cation (data not shown). It is therefore possible that the role of Na^+ in activated protein C is to promote the cytoprotective activity of the enzyme through the $E:Na^+$ form. There is currently enormous interest in dissociating the anticoagulant and cytoprotective functions of activated protein C to produce variants that ameliorate inflammatory reactions without interfering with hemostasis^{82,87}. For this strategy to succeed, the Na^+ binding affinity of activated protein C should remain unperturbed. As for factor Xa, the conformational plasticity of activated protein C becomes relevant when considering the documented multiple interactions of this enzyme^{80,84,89}. Existence of multiple conformations may provide an important mechanism for selecting specific functions, as demonstrated by thrombin^{8,10}. It is possible that different conformations of activated protein C may be involved in different physiological roles and even trigger distinct receptor signaling pathways.

The data presented in this study support the generality of the E^* - E equilibrium in clotting proteases and structural studies of activated protein C and factor Xa are underway to trap these alternative conformations as done for thrombin. The E^* - E equilibrium is relevant to other enzymes and has recently been invoked to explain the mechanism of K^+ activation of histone deacetylase⁸⁹. We have proposed that the E^* - E equilibrium is a basic feature of the trypsin fold and offers a simple mechanism of allosteric regulation of protease activity after the irreversible zymogen→protease conversion has taken place⁹¹. The collapse of the 215-217 segment into the active site and/or disruption of the oxyanion hole, have been observed in the inactive form of α I-tryptase⁹², the high-temperature-requirement-like protease⁹³, complement factor D⁹⁴, granzyme K⁹⁵, hepatocyte growth factor activator⁹⁶, prostate kallikrein⁹⁷, prostasin^{98,99}, complement factor B¹⁰⁰, the arterivirus nsp4¹⁰¹, the epidermolytic toxin A^{102,103} and clotting factor VIIa⁴⁹. Stabilization of E^* obliterates activity of the enzyme, thereby keeping it in a zymogen-like form until binding of a suitable cofactor elicits conversion to the active form E . The new paradigm established by the E^* - E equilibrium has obvious physiological relevance. In the case of complement factors, kallikreins, tryptase and some coagulation factors activity must be kept to a minimum until binding of a trigger factor ensues. Stabilization of E^* may afford a resting state of the protease waiting for action, as seen for other systems¹⁰⁴⁻¹⁰⁸. Stabilization of E^* is particularly important in maintaining the resting state of complement factor D that does not have a zymogen form or known natural inhibitors⁹⁴. In this case, binding of substrate induces the transition to the active E form. Factor B is mostly inactive until binding of complement factor C3 unleashes catalytic activity at the site where amplification of C3 activation is most needed prior to formation of the membrane attack complex¹⁰⁹. Indeed, the crystal structure of factor B reveals a conformation with the oxyanion hole disrupted by a flip of the 192-193 peptide bond¹⁰⁰. Similar arguments apply to clotting factor VIIa that is mostly inactive until complexed with tissue factor¹¹⁰. In the case of complement factor B¹⁰⁰, hepatocyte growth factor activator⁹⁶ or clotting factor VIIa⁴⁹, stabilization of E^* is

achieved after the zymogen→protease conversion has taken place and transition to the active E form relies on the binding of substrate and/or cofactors. Finally, stabilization of E* offers a rational strategy to engineering “allosteric switches” that become activated “on demand” upon binding of cofactors. A number of anticoagulant thrombin mutants have been shown to actually exploit such mechanism^{26,41}.

Acknowledgments

This work was supported in part by the National Institutes of Health Research Grants HL49413, HL58141 HL73813 and HL95315 (to E.D.C.)

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Figure 1. Kinetic traces of Na^+ binding to thrombin (A), factor Xa (B) and activated protein C (C)

In all cases binding of Na^+ obeys a two-step mechanism, with a fast phase completed within the dead time (<0.5 ms) of the spectrometer, followed by a single-exponential slow phase.

The k_{obs} for the slow phase decreases with increasing $[\text{Na}^+]$ (see also Fig. 2). Experimental conditions are: 100 nM enzyme, 50 mM Tris, 0.1% PEG8000, pH 8.0, at 15 °C. Continuous

lines were drawn using the expression $b - a \exp(-k_{\text{obs}} t)$ with best-fit parameters:

(A) $a=0.000 \pm 0.000$ V, $b=8.239 \pm 0.001$ V (0 mM Na^+); $a=0.000 \pm 0.000$ V, $b=8.470 \pm 0.001$ V (3.25 mM Na^+); $a=0.175 \pm 0.002$ V, $k_{\text{obs}}=120 \pm 4$ s $^{-1}$, $b=8.942 \pm 0.001$ V (12.5 mM Na^+); $a=0.392 \pm 0.001$ V, $k_{\text{obs}}=96 \pm 1$ s $^{-1}$, $b=9.340 \pm 0.002$ V (200 mM Na^+). (B) $a=0.000 \pm 0.000$ V, $b=8.112 \pm 0.001$ V (0 mM Na^+); $a=0.000 \pm 0.000$ V, $b=8.601 \pm 0.001$ V (20 mM Na^+); $a=0.261 \pm 0.002$ V, $k_{\text{obs}}=64 \pm 4$ s $^{-1}$, $b=9.391 \pm 0.001$ V (120 mM Na^+); $a=0.488 \pm 0.001$ V, $k_{\text{obs}}=60 \pm 1$ s $^{-1}$, $b=9.915 \pm 0.002$ V (400 mM Na^+). (C) $a=0.000 \pm 0.000$ V, $b=8.485 \pm 0.001$ V (0 mM Na^+); $a=0.162 \pm 0.001$ V, $k_{\text{obs}}=31 \pm 1$ s $^{-1}$, $b=8.501 \pm 0.001$ V (curve in the middle not labeled, 25 mM Na^+); $a=0.458 \pm 0.001$ V, $k_{\text{obs}}=27 \pm 1$ s $^{-1}$, $b=8.608 \pm 0.001$ V (300 mM Na^+).

The concentration of Na^+ was changed in all cases by adding NaCl without keeping the ionic strength constant. The amplitude of the slow phase for factor Xa and activated protein C becomes less pronounced in the presence of Ca^{2+} (data not shown), reflecting the positive linkage between Na^+ and Ca^{2+} binding in these proteases^{51,52,54-58}. Binding of Ca^{2+} likely shifts the E*-E equilibrium in favor of E. In the case of thrombin, Ca^{2+} has no effect on Na^+ binding.

Figure 2. Na⁺ dependence of the k_{obs} of the slow phase of Na⁺ binding to thrombin (open circles), factor Xa (black circles) and activated protein C (grey circles)

The values were obtained from analysis of the kinetic traces (see also Figure 1) and analyzed according to eq 6 in the text with best-fit parameter values listed in Table 1. Note how k_{obs} features an inverse hyperbolic dependence on $[Na^+]$, thereby proving the existence of the E*-E equilibrium preceding Na⁺ binding. This demonstrates directly that the Na⁺-free slow form of thrombin is not a single species or a single ensemble of species, contrary to the basic assumption of alternative models of thrombin allostery³³⁻³⁶. The Na⁺ affinity is highest for thrombin and lowest for factor Xa. The rate constants pertaining to the E*-E interconversion are fastest for thrombin and slowest for activated protein C. However, the value of the equilibrium constant $r=k_{-r}/k_r$ is comparable for all enzymes, supporting the conclusion that the distribution of E* and E forms is conserved among different trypsin-like proteases.

Figure 3. Na⁺ binding to thrombin (open circles), factor Xa (black circles) and activated protein C (grey circles)

Na⁺ binding curves were obtained from the total change in intrinsic fluorescence determined by stopped-flow kinetics. Experimental conditions are: 100 nM enzyme, 50mM Tris, 0.1% PEG8000, pH 8.0, at 15 °C. Continuous lines were drawn according to eq 1 in the text with best-fit parameter values listed in Table 1. Note how the Na⁺ affinity is highest for thrombin and lowest for factor Xa, which however shows the largest change in intrinsic fluorescence upon Na⁺ binding.

SCHEME 1.

Table 1

Parameters for Na^+ binding to thrombin, factor Xa and activated protein C

Enzyme	F_0 (V) ^a	F_1 (V) ^a	$\Delta F/F_0$ (%) ^a	K_{app} (M ⁻¹) ^a	K_A (M ⁻¹) ^b	k_t (s ⁻¹) ^b	k_{-t} (s ⁻¹) ^b	r^2 ^b
Thrombin	8.20±0.05	9.39±0.03	15	120±20	160±30	89±2	85±9	0.95±0.09
Factor Xa	8.23±0.05	10.5±0.1	27	10±2	16±4	45±4	70±10	1.5±0.2
APC ^c	8.15±0.03	8.66±0.02	6.1	59±3	120±10	25±1	24±3	0.95±0.08

^aFrom the data in Figure 3 using eq 1 in the text.

^bFrom the data in Figure 2 using eq 2 in the text.

^cActivated protein C.