Evidence of the E*-E Equilibrium from Rapid Kinetics of Na+ Binding to Activated Protein C and Factor Xa*

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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 microsecond 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 millisecond 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.

Introduction

Activation of trypsin-like proteases requires proteolytic processing of an inactive zymogen precursor that occurs at the identical position in all known members of the family, i.e., between residues 15 and 16 (chymotrypsin numbering). The nascent N-terminus induces conformational change in the enzyme through formation of an ion-pair with the highly conserved D194 that organizes both the oxyanion hole and substrate binding cleft. Existing structures of the zymogen forms of trypsin, chymotrypsin, and chymase document the lack of ion-pair interaction between D194 and the N-terminus of the catalytic chain. The zymogen-protease conversion is classically associated with the onset of catalytic activity 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 coagulation, 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 activated protease for which Na+ binding has led to the discovery of a key site.11,22

Binding to Activated Thrombin

Na+ binding to thrombin is not a simple one-step process but gives rise to two kinetic phases, one fast (in the microsecond time scale) due to Na+ binding to E to produce E:Na+ and another considerably slower (in the millisecond time scale) that reflects a pre-equilibrium between E and E* according to the kinetic scheme.

The Na+-free form of thrombin, originally defined as the “slow” form, 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+. Of these forms, E interacts with Na+ with a rate constant kA to populate the Na+-bound form E:Na+, originally defined as the “fast” form, that may dissociate into the parent components with a rate constant kA. Na+ binds to a site between the 186- and 220-loops, located >15 Å away from residues of the catalytic triad, and converts the low activity E form to the high activity E:Na+ form. The E* form, on the other hand, is inactive and cannot bind Na+ or substrate to the active site. Recent alternative models of thrombin allostery show considerable confusion about the nature of the slow form, which is defined as E*, zymogen-like, inactive, or a continuum of zymogen-like states, and another considerably slower (in the millisecond time scale) that reflects a pre-equilibrium between E and E* according to the kinetic scheme.

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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 E:Na+ forms, the side chain of W215 is positioned >10 Å 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+ coordination.21 Small but significant differences exist between E and E:Na+. In the E form, the H-bound 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 E: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 hole,29 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+ replacement21 has facilitated the subsequent identification of the analogous Na+ binding sites in factors Xa,47,48 VIIa,49 and activated protein C.50 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 Na+ binding to thrombin run under conditions of separation of time scales,27 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

\[
F = \frac{F_0 + F_1 K_{app}[Na^+]}{1 + K_{app}[Na^+]} \tag{1}
\]

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

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

is the apparent Na+ binding affinity that depends on the intrinsic Na+ binding affinity \(K_A = ([E:Na^+]\div([E])[Na^+])\) and the equilibrium constant for the E*–E interconversion \(r = ([E^*] \div [E]) = (k_- \div k_+).\) 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

\[
\begin{align*}
\frac{d[E^*]/dt}{d[E]/dt} &= \begin{pmatrix} -k_+ & k_+ \\ -k_- & -k_- - k_A[Na^+] \end{pmatrix} \times \\
\frac{d[E]/dt}{d[E:Na^+]/dt} &= \begin{pmatrix} 0 & k_A[Na^+] \\ 0 & -k_A \end{pmatrix} \begin{pmatrix} [E^*] \\ [E] \end{pmatrix} \times \\
\end{align*}
\tag{3}
\]

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

\[
\begin{align*}
\lambda_{1,2} &= -\{k_- + k_+ + k_A[Na^+]\} \pm \{[k_- + k_+ + k_A[Na^+]]^2 - 4k_-k_A[Na^+] - 4k_-k_A[Na^+]^{1/2}\}^{1/2} \\
\end{align*}
\tag{4}
\]

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

\[
k_{fast} = -\lambda_1 = k_+ + k_A[Na^+] \tag{5}
\]

\[
k_{slow} = -\lambda_2 = k_+ + k_+ - \frac{1}{1 + K_A[Na^+]} \tag{6}
\]

The value of \(k_{fast}\) is too fast to resolve by stopped-flow and requires the use of ultrarapid kinetic methods.28 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 previously.\(^{27} \) 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 strength.\(^{72} \) 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 \( E^* \rightarrow E \) 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 \( E^* \rightarrow E \) 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 \( Na^+ \) binding to activated protein C and support the existence of the allosteric \( E^* \rightarrow E \) equilibrium. In fact, analysis of \( k_{obs} \) for the slow phase shows an inverse hyperbolic dependence on \([Na^+]\) (Figure 2) with values of \( k_r = 25 \pm 1 \text{ s}^{-1}, k_r^- = 24 \pm 3 \text{ s}^{-1} \), and \( K_A = 120 \pm 10 \text{ M}^{-1} \). The \( Na^+ \) binding affinity of activated protein C is comparable to that of thrombin, and so is the value of \( r = 0.95 \pm 0.08 \). However, the rates for the \( E^* \rightarrow E \) 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 \( E^* \rightarrow E \) equilibrium has significant implications for factor Xa. This enzyme catalyzes the conversion of prothrombin into thrombin with the assistance of factor Va, Ca\(^{2+} \), and phospholipids, altogether defining the prothrombinase complex.\(^{71} \) 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 IIa-D194 ion-pair and structuring of the active site and oxyanion hole.\(^{74} \) The alternative initial cleavage at R271 sheds the Gla domain and the two kringles and generates the inactive precursor prothrombin-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 prothrombinase58 or of the substrate prothrombin,\(^{56,77} \) each favoring one of the two possible cleavages. Pedersen has recently cast doubts on the mathematical rigor of these models\(^{78} \) 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 \( E^* \rightarrow E \) equilibrium. The existing model of prothrombinase assuming different conformations of the enzyme-directing cleavage at the two possible sites should be refined to include the effects of \( Na^+ \) and of the \( E^* \rightarrow E \) 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. If 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 Ca\(^{2+} \). 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.\(^{56,77} \) 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\(^+ \) forms,\(^{79} \) thereby casting doubts on the hypothesis that only prothrombinase assumes different conformational states.\(^{75} \) A realistic model of prothrombinase function should necessarily allow multiple conformational states for both enzyme and substrate.

The \( E^* \rightarrow E \) equilibrium has equally important implications for activated protein C. The effect of \( Na^+ \) on activated protein

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**TABLE 1: Parameters for \( Na^+ \) Binding to Thrombin, Factor Xa, and Activated Protein C**

<table>
<thead>
<tr>
<th>enzyme</th>
<th>( F_0 (V) )</th>
<th>( F_1 (V) )</th>
<th>( \Delta F/F_0 ) (%)</th>
<th>( K_{app} (M^{-1}) )</th>
<th>( K_A (M^{-1}) )</th>
<th>( k_r (s^{-1}) )</th>
<th>( k_r^- (s^{-1}) )</th>
<th>( r )</th>
</tr>
</thead>
<tbody>
<tr>
<td>thrombin</td>
<td>8.20 ± 0.05</td>
<td>9.39 ± 0.03</td>
<td>15</td>
<td>120 ± 20</td>
<td>160 ± 30</td>
<td>89 ± 2</td>
<td>85 ± 9</td>
<td>0.95 ± 0.09</td>
</tr>
<tr>
<td>factor Xa</td>
<td>8.23 ± 0.05</td>
<td>10.5 ± 0.1</td>
<td>27</td>
<td>10 ± 2</td>
<td>16 ± 4</td>
<td>45 ± 4</td>
<td>70 ± 10</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>APC(^*)</td>
<td>8.15 ± 0.03</td>
<td>8.66 ± 0.02</td>
<td>6.1</td>
<td>59 ± 3</td>
<td>120 ± 10</td>
<td>25 ± 1</td>
<td>24 ± 3</td>
<td>0.95 ± 0.08</td>
</tr>
</tbody>
</table>

\(^*\) From the data in Figure 3 using eq 1 in the text.\(^*\) From the data in Figure 2 using eq 2 in the text.\(^*\) Activated protein 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 [Na⁺].

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 [Na⁺].

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 (gray 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 allostery.33-36 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 $r = k_r/k_a$ is comparable for all enzymes, supporting the conclusion that the distribution of E⁺ and E forms is conserved among these different trypsin-like proteases.

C has been documented for a long time.58-62 Like thrombin, activated protein C is endowed with different physiological functions.60 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 receptor.81-87 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⁺ 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.²⁻⁸ Seventh 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.⁸⁻¹⁰ 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 deacetylase 8.⁸⁰⁻⁸⁴⁻⁸⁹ Existence of multiple conformations may provide an important mechanism for selecting specific functions, as demonstrated by thrombin.⁸⁻¹⁰ It is possible that different physiological roles and even trigger distinct receptor signaling pathways.

In the case of complement factors, kallikreins, tryptase, and inhibitors,⁹¹⁻⁹³ Binding to Activated Protein C and Factor Xa* reveals 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 complex.¹⁰⁹ Indeed, the crystal structure of factor B reveals a conformation with the oxygen hole disrupted by a flip of the 192–193 peptide bond.¹⁰⁰ Similar arguments apply to clotting factor VIIa that is mostly inactive until complexed with tissue factor.¹¹⁰ In the case of complement factor B,¹⁰⁰ hepatocyte growth factor activator,⁹₆ or clotting factor VIIa,⁹⁹ 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 a mechanism.²⁶⁻¹⁰¹

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References and Notes
