

Letter to the Editor

Mechanism of Na^+ binding to thrombin resolved by ultra-rapid kineticsStefano Gianni ^{a,*}, Ylva Ivarsson ^a, Alaji Bah ^b, Leslie A. Bush-Pelc ^b, Enrico Di Cera ^{b,*}^a *Istituto di Biologia e Patologia Molecolari del CNR, Dipartimento di Scienze Biochimiche, Università di Roma “La Sapienza”, 00185 Roma, Italy*^b *Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Box 8231, St. Louis, MO 63110, United States*

Received 14 September 2007; received in revised form 24 September 2007; accepted 24 September 2007

Available online 29 September 2007

Abstract

The interaction of Na^+ and K^+ with proteins is at the basis of numerous processes of biological importance. However, measurement of the kinetic components of the interaction has eluded experimentalists for decades because the rate constants are too fast to resolve with conventional stopped-flow methods. Using a continuous-flow apparatus with a dead time of 50 μs we have been able to resolve the kinetic rate constants and entire mechanism of Na^+ binding to thrombin, an interaction that is at the basis of the procoagulant and prothrombotic roles of the enzyme in the blood.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Thrombin; Sodium; Rapid kinetics; Allostery; Monovalent cation activation

Enzymes activated by monovalent cations (M^+ s) [1] require Na^+ or K^+ for optimal activity and utilize M^+ as a cofactor or allosteric effector [2,3]. The allosteric effect of Na^+ on thrombin activity and specificity carries significant physiological relevance [4–6], is at the basis of the procoagulant and prothrombotic roles of the enzyme in the blood and has been studied in great detail both functionally and structurally [7,8]. However, aspects of this physiologically important interaction remain unresolved because Na^+ binding and dissociation occur on a time scale too fast to measure with conventional stopped-flow techniques [5]. A similar limitation applies to the study of other well characterized M^+ -activated enzymes like Trp synthase [9,10], pyruvate kinase [11,12], Hsc70 [13], β -galactosidase [14,15] and inosine monophosphate dehydrogenase [16]. Indeed, no protein system characterized to date has offered direct information on the kinetic rate constants for Na^+ or K^+

binding/dissociation, therefore leaving a gap in our understanding of how M^+ s in general regulate protein function [3].

To circumvent the limitations of standard stopped-flow systems, we used a continuous-flow instrument equipped with an ultra-rapid mixing device and extended our recent characterization of Na^+ interaction with thrombin [8] into the μs time scale. To avoid autoproteolysis at the high concentrations (40 μM) needed for the measurements, we inactivated thrombin with the S195A mutation, prepared as described [6,7,17], to selectively knock out the catalytic Ser-195 with minimal structural perturbation [18,19]. As for wild-type thrombin, the fluorescence increase observed upon Na^+ binding to S195A has an initial rapid phase that cannot be resolved within the dead time (<0.5 ms) of the stopped-flow apparatus, followed by a single exponential slow phase (Fig. 1) with a k_{obs} that decreases as $[\text{Na}^+]$ increases (Fig. 2). The decrease of k_{obs} is conducive to the existence of a conformational change that precedes Na^+ binding [8]. Of crucial importance is to establish whether the fast phase reports just the binding/dissociation of Na^+ with thrombin or also depends on additional conformational changes. In the former case, k_{obs} should change linearly with $[\text{Na}^+]$. In the latter, a hyperbolic dependence of k_{obs} on $[\text{Na}^+]$ should be observed.

The rapid phase was resolved with the continuous-flow apparatus (Fig. 1) and yielded a single exponential time course

* Corresponding authors. Gianni is to be contacted at Istituto di Biologia e Patologia Molecolari del CNR, Dipartimento di Scienze Biochimiche, Università di Roma “La Sapienza”, 00185 Roma, Italy. Tel.: +39 06 4991 0548; fax: +39 06 444 0062. Di Cera, Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Box 8231, St. Louis, MO 63110, United States. Tel.: +1 314 362 4185; fax: +1 314 747 5354.

E-mail addresses: Stefano.gianni@uniroma1.it (S. Gianni), enrico@wustl.edu (E. Di Cera).

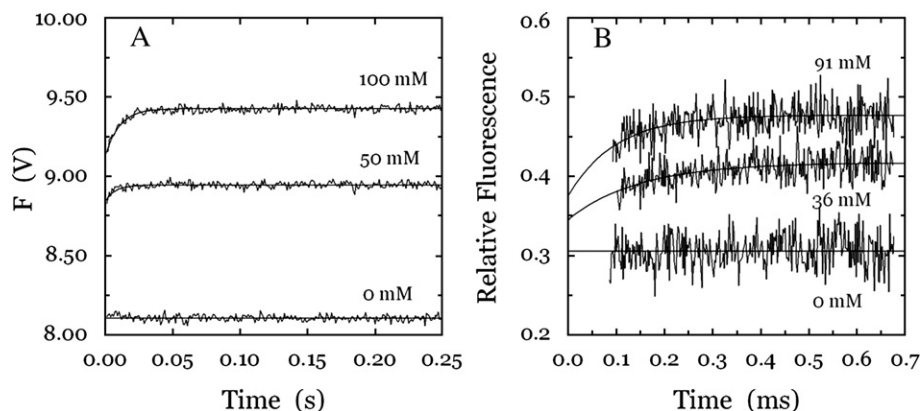


Fig. 1. A, B (A) Kinetic traces of Na^+ binding to human thrombin in the 0–250 ms time scale. Shown are the traces obtained at 50 and 100 mM Na^+ with the stopped-flow method using an Applied Photophysics SX20 spectrometer, with an excitation of 280 nm and a cutoff filter at 305 nm [8]. Traces are averages of three determinations. 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 $[\text{Na}^+]$ (Fig. 2A). (B) Kinetic traces of Na^+ binding to human thrombin in the 0–700 μs time scale. Shown are the traces obtained at 36 and 91 mM Na^+ with the continuous-flow method as single determinations with an exposure time of 3 s. We built an ultra-rapid mixer [23] of similar design and methodology to that described by Shastry and Roder [24,25]. The flow cell was purchased from Hellma (Germany). The mixed sample was illuminated with an A1010B Mercury-Xenon lamp (PTI, UK) at 280 nm, using a Model 101 Monochromator (PTI, UK). Fluorescence was recorded with a Micromax CCD camera (Princeton Instrument, USA), with a typical exposure time of 1–3 s and employing different emission glass filters. An original, pneumatically driven, loading syringes unit was designed. Data were recorded at 3 bars, leading to a linear velocity in the flow cell of about 16 m/s. Experimental conditions were identical to those used in the stopped-flow experiments. The dead time of the instrument was determined by fluorescence using the quenching of *N*-acetyl-tryptophanamide (NATA) by *N*-bromo-succinamide (NBS) [26]. The pseudo-first-order quenching reaction of NATA by NBS was measured at NBS concentrations from 3.5 to 33 mM. The observed fluorescence traces obtained at different NBS concentrations yielded single exponential time courses extrapolating to a common point near $f_{\text{rel}}=1$, the expected initial fluorescence of NATA in the absence of quencher. The time delay from this point to the first data point that falls on the fitted exponential provided an estimate of the dead time, 40–50 μs , of the instrument. Binding of Na^+ in the 0–700 μs time scale obeys a single-exponential phase with a k_{obs} increasing linearly with $[\text{Na}^+]$ (Fig. 2B). This resolves the fast phase detected with the stopped-flow method and shown in (A). Experimental conditions for the two methods are: 5 mM Tris, 0.1% PEG8000, pH 8.0 at 25 $^{\circ}\text{C}$. The thrombin concentration was 50 nM for the stopped-flow measurements and 40 μM for the continuous-flow measurements. The $[\text{Na}^+]$, as indicated, was changed by keeping the ionic strength constant at 400 mM with choline chloride. Continuous lines were drawn using the expression $a+b\exp(-k_{\text{obs}}t)$ with best-fit parameter values: (A) $a=8.944\pm0.001$ V, $b=-0.10\pm0.01$ V, $k_{\text{obs}}=111\pm9$ s^{-1} ($[\text{Na}^+]=50$ mM); $a=9.427\pm0.001$ V, $b=-0.29\pm0.01$ V, $k_{\text{obs}}=96\pm6$ s^{-1} ($[\text{Na}^+]=100$ mM). (B) $a=0.417\pm0.002$, $b=-0.072\pm0.02$, $k_{\text{obs}}=7\pm2$ ms^{-1} ($[\text{Na}^+]=36$ mM); $a=0.477\pm0.002$, $b=-0.10\pm0.03$, $k_{\text{obs}}=10\pm2$ ms^{-1} ($[\text{Na}^+]=91$ mM). All data were collected at least in duplicate.

with a k_{obs} that increases linearly with $[\text{Na}^+]$ (Fig. 2). The two-step mechanism of Na^+ binding to thrombin solved by combination of the stopped-slow and continuous-flow measurements is found in Scheme 1.

This is the mechanism recently proposed by Bah et al. [8], but validated for the binding/dissociation components of Na^+ by direct resolution of the rapid phase. Thrombin exists in equilibrium between two forms, E^* and E , that interconvert with

kinetic rate constants k_1 and k_{-1} . Of these forms, only E can interact with Na^+ with a rate constant k_A to populate $\text{E}:\text{Na}^+$, that may dissociate into the parent components with a rate constant k_{-A} . The fast phase is due to the $\text{E}-\text{E}:\text{Na}^+$ interconversion involving Na^+ binding/dissociation, and the slow phase is due to the $\text{E}-\text{E}^*$ interconversion that precedes Na^+ binding.

The exact analytical solution of Scheme 1 calls for two eigenvalues that give the k_{obs} for the two exponential transitions

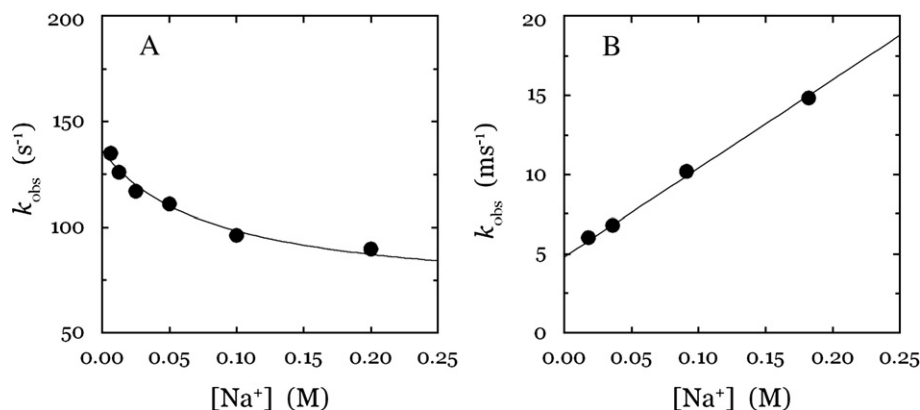


Fig. 2. A, B Values of k_{obs} vs $[\text{Na}^+]$ for the slow and fast phases of fluorescence change due to Na^+ binding to thrombin shown in Fig. 1. Shown are the results pertaining to the stopped-flow (A) and continuous-flow (B) measurements. Note the different time scale between the two panels. Experimental conditions are given in the legend to Fig. 1. Continuous lines were drawn according to eqs 1 and 2 in the text, with best-fit parameter values: $k_1=67\pm7$ s^{-1} , $k_{-1}=69\pm6$ s^{-1} , $k_A=56,000\pm200$ $\text{M}^{-1}\text{s}^{-1}$, $k_{-A}=4,800\pm200$ s^{-1} .

Download English Version:

<https://daneshyari.com/en/article/5372063>

Download Persian Version:

<https://daneshyari.com/article/5372063>

[Daneshyari.com](https://daneshyari.com)