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Letter to the Editor

A revisit of the two-form kinetic model of prothrombinase: A rebuttal

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Dear Editor,

Prothrombinase is a multi-component enzyme that is responsible for the activation of zymogen prothrombin (PT) to thrombin (IIa) [1]. Prothrombinase consists of four major components—enzyme factor Xa (FXa), cofactor factor Va (FVa), negatively charged phospholipid surface, and calcium ions. Cleavage of PT at Arg271 and Arg320 is required for its conversion to IIa [2]. As such, two pathways exist for its activation depending on which of these bonds is cleaved first. Initial cleavage at Arg271 results in the formation of fragment 1.2 and prethrombin-2, while the subsequent cleavage results in the conversion of prethrombin-2 to IIa. In contrast, initial cleavage at Arg320 results in the formation of the intermediate meizothrombin, while the subsequent cleavage results in the release of fragment 1.2, thereby generating IIa. The exact mechanism by which prothrombinase activates PT is still actively under investigation.

In an attempt to resolve the ongoing debate regarding the mechanism of PT activation by prothrombinase, Lee et al. consolidated the empirical data reported by the Nesheim [3] and Krishnaswamy groups [4], and used this information to generate average time course data for PT and its intermediates. The modified mathematical model was then fit to the 'average' data. Based on their analysis, the authors conclude that a one-form model adequately describes the time course data, thereby rendering unnecessary both channeling [5], a process whereby the PT is converted to IIa without the intermediate release, and ratcheting [6], conformational change of PT upon initial cleavage by prothrombinase that makes the secondary cleavage site on PT readily accessible for full activation. Although it may be tempting to average the data sets from two different groups, such an approach is prone to complications, particularly when considering the different reaction conditions. For example, we used low levels of FXa (70 pM) to form prothrombinase, whereas the Krishnaswamy group used FXa at a concentration of 1 nM. The difference in the enzyme levels is important because it affects not only the overall rate of PT consumption, but also the levels of the intermediates.

Furthermore, Lee et al. made an error when estimating the levels of PT, meizothrombin, prethrombin-2 and IIa based on the time course profiles that we reported [3]. We provided time courses for PT, fragment 1.2:A-chain (representing meizothrombin), prethrombin-2 and the B-chain. The observed B-chain level comes from two sources—meizothrombin and IIa. When Lee et al. estimated the total concentration of all species, they simply added all of the parameters that we reported. This led them to erroneously include the level of meizothrombin twice, which resulted in an apparent increase in the total levels of substrate/intermediate/product (Fig. 2 of their manuscript). In contrast, our data revealed a conserved total substrate/intermediate/product level of around 1.4 μM throughout the time course [3].

We realize, however, that there was an error on our part; the non-steady state equations for free concentrations of the substrate and intermediates contained an extra term (Supplementary Materials in Ref. [3]). We added this term because our time course data is based on gel densitometry; therefore, each species reflects the sum of both the free form as well as those in complex with enzyme. In response, we have re-analyzed our data [3] using the modified equations suggested by Lee et al. [7]. As before [3], we determined the concentrations of PT and its intermediate species over time by numerical integration using Berkeley Madonna Software (University of California, Berkeley, CA). Parameter values were optimized by the software using the Simplex procedure. Fig. 1 shows fits of the non-steady state model to our data for both the one-form (panel A) and two-form (panel B) models, in the absence of channeling or ratcheting [5,6]. Although the modifications to our rate equations improve the fit of the prethrombin-2 time course with both models, the trends are very similar to what we reported previously [3]. Contrary to the findings of Lee et al., in the absence of ratcheting and channeling, neither the meizothrombin time course data nor the initial lag phase in the PT consumption fit the one-form model (Fig. 1A), where the total loss of the fit of the model to the data was 0.4880. With the two-form model, in the absence of ratcheting and channeling, the model fit the data better with a total

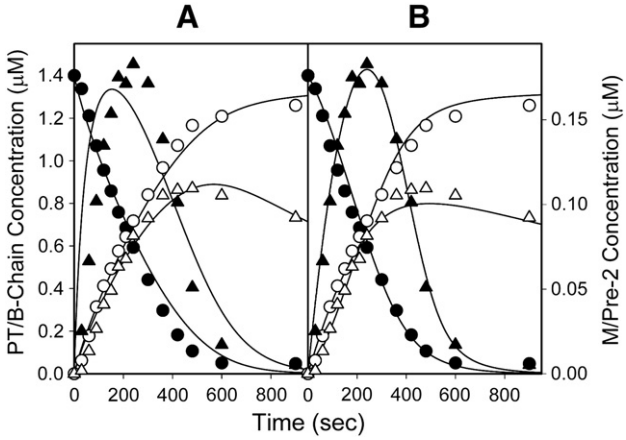

Fig. 1. Fits of the modified one- and two-form models with no steady state assumption to the time course data of PT, meizothrombin (M), prethrombin-2 (Pre-2), and the B-chain in the absence of both channeling [5] and ratcheting [6]. The time course data were previously reported by our group [3], where each point represents the average concentration ($n = 4$) of PT (closed circle) and B-chain (open circle) in accordance with the scale on the left side, as well as M (closed triangle) and Pre-2 (open triangle) in accordance with the scale on the right side.

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Table 1
Best fit rate constants from non-steady state analysis of the full time course data. (average ± SD, $n = 4$).

Reaction	Constants	One-form model		Two-form model	
		None	Ratcheting and channeling	None	Ratcheting and channeling
$E(1)+P \Leftrightarrow (E(1)\cdot P)_{(1)}$	k_1	146.28 ± 3.63	198.87 ± 10.74	217.32 ± 17.85	289.18 ± 41.53
	j_1	$(3.07 \pm 0.79)\times 10^{-6}$	$(1.41 \pm 0.42)\times 10^{-2}$	$(1.44 \pm 0.32)\times 10^{-2}$	0.52 ± 0.08
$P \rightarrow M$	k_2	71.59 ± 15.33	26.51 ± 7.87	570.71 ± 49.36	190.79 ± 44.76
$M \rightarrow M\ 1$	k_9	–	$(7.76 \pm 0.31)\times 10^{-2}$	–	$(3.61 \pm 1.66)\times 10^{-2}$
$E(2)+M1 \Leftrightarrow E(2)\cdot M1$	k_3	–	314.56 ± 9.75	–	706.71 ± 97.64
	j_3	–	$(1.50 \pm 0.66)\times 10^{-4}$	–	$(1.94 \pm 0.62)\times 10^{-6}$
$E(2)+M \Leftrightarrow E(2)\cdot M$	k_{3a}	785.14 ± 299.00	0.79 ± 0.08	855.21 ± 203.98	1.16 ± 0.56
	j_{3a}	$(2.40 \pm 1.36)\times 10^{-5}$	$(1.27 \pm 0.58)\times 10^{-2}$	1.63 ± 0.75	0.52 ± 0.02
$M\ 1 \rightarrow T$	k_4	–	250.88 ± 22.26	–	417.35 ± 32.76
$M \rightarrow T$	k_{4a}	895.47 ± 127.54	0.87 ± 0.14	860.32 ± 24.17	429.33 ± 57.94
$E(2)+P \Leftrightarrow (E(2)\cdot P)_{(2)}$	k_5	16.69 ± 2.74	21.27 ± 1.62	15.48 ± 3.66	26.64 ± 2.26
	j_5	$(1.27 \pm 0.42)\times 10^{-4}$	$(8.87 \pm 1.78)\times 10^{-2}$	$(1.18 \pm 0.38)\times 10^{-2}$	$(9.99 \pm 3.79)\times 10^{-2}$
$P \rightarrow P\ 2$	k_6	39.87 ± 6.50	85.39 ± 11.87	13.83 ± 1.39	9.21 ± 1.83
$P\ 2 \rightarrow P\ 21$	k_{10}	–	$(1.18 \pm 0.21)\times 10^{-4}$	–	$(1.57 \pm 0.60)\times 10^{-3}$
$E(1)+P21 \Leftrightarrow E(1)\cdot P21$	k_7	–	0.22 ± 0.03	–	21.19 ± 1.75
	j_7	–	0.65 ± 0.10	–	0.60 ± 0.12
$E(1)+P2 \Leftrightarrow E(1)\cdot P2$	k_{7a}	11.66 ± 1.83	9.99 ± 1.81	9.50 ± 0.84	1.10 ± 0.31
	j_{7a}	$(8.48 \pm 1.95)\times 10^{-3}$	$(1.48 \pm 0.41)\times 10^{-4}$	$(9.85 \pm 0.83)\times 10^{-2}$	0.66 ± 0.08
$P\ 21 \rightarrow T$	k_8	–	114.19 ± 0.23	–	132.98 ± 20.82
$P\ 2 \rightarrow P\ T$	k_{8a}	125.16 ± 15.18	113.75 ± 1.10	117.17 ± 2.42	122.33 ± 11.62
$E\ 1 \rightarrow E\ 2$	k_{11}	–	–	48.88 ± 14.00	224.93 ± 27.74
$E\ 2 \rightarrow E\ 1$	k_{12}	–	–	51.11 ± 20.83	231.29 ± 29.68
$(E(1)\cdot P)_{(1)} \rightarrow T$	k_{C1}	–	37.23 ± 9.91	–	208.74 ± 33.23
$(E(2)\cdot P)_{(2)} \rightarrow T$	k_{C2}	–	$(1.95 \pm 0.48)\times 10^{-4}$	–	0.19 ± 0.06
	DT	0.002	0.002	0.003	0.003

The units for all association rate constant (k_1, k_3, k_{3a}, k_5, k_7, k_{7a}) values are $\mu M^{-1}\ s^{-1}$. The units for all dissociation rate constant (j_x) values, turnover rate constants (k_2, k_4, k_{4a}, k_6, k_8, k_{8a}, k_{C1}, k_{C2}), and k_{11} and k_{12} are s^{-1}. The units for DT (integration time interval) values are in seconds.

loss of 0.3405 (Fig. 1B). However, with the addition of channeling alone, but not ratcheting alone, the one-form model was able to fit the data. Both one-form and two-form models fit the data equally well with the addition of ratcheting and channeling, where the total losses were 0.2749 and 0.2641, respectively (data not shown). The best fit parameter values for either the one-form or the two-form model, with or without ratcheting and channeling, are provided in Table 1. Although the modification of the rate equations resulted in minimal differences in most of the rate constants, the largest differences were observed for rate constants that determine IIa generation (*i.e.*, k_4, k_{4a}, k_{8a}, k_{C1}, and k_{C2}).

Despite the improvements in fitting our time course data, especially the prethrombin-2 time course, provided by the modified models, our current findings are similar to those we reported previously. Therefore, our interpretation of the data remains unchanged. In conclusion, we disagree with the conclusions of Lee et al. Furthermore, channeling and/or ratcheting should be included when modeling the mechanism of prothrombinase activation of PT.

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