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Single-molecule kinetics of an enzyme in the phosphorylation-dephosphorylation cycle

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We consider different reaction mechanisms to study the substrate phosphorylation process catalyzed by the activated ERK2 enzyme. Such reaction schemes are constituted by three Michaelis-Menten (MM) reactions namely the ERK2 activation (phosphorylation), deactivation of phosphorylated ERK2 (dephosphorylation) and substrate phosphorylation catalyzed by the activated ERK2. We theoretically examine and analyze the phosphorylation/dephosphorylation networks to probe dynamic disorder which is a manifestation of multiple competing reaction timescales. We apply the waiting time distribution formalism based on the chemical master equation approach to obtain exact analytical expressions for the turnover time distribution for the substrate phosphorylation event from which we can obtain the mean reaction time and randomness parameter for the quantification of the temporal fluctuations on the different reaction pathways.

Keywords: Single molecule study, phosphorylation-dephosphorylation kinetics, dynamic disorder.

Introduction

Enzymes are the efficient dynamical bio-catalysts performing several vital functions necessary for the sustenance of human life like digestion of food, energy production, cell growth, cell differentiation, and many others[1]. Mitogen-activated protein kinase ERK2 also known as extracellular signal regulatory kinase works at the integration of several biochemical signals assisting various cellular processes like cell proliferation, transcription control and development[2]. Most of the physiological enzymatic actions involve phosphorylation and dephosphorylation cycle (PdPC). In phosphorylation, phosphoryl group attachment with an amino acid residue occurs via phosphoester, phosphoramidate or anhydride linkages. ERK2 activation requires phosphorylation carried out by MAP/ERK kinases (MEK)[3]. For the substrate phosphorylation, the phosphorylated/activated enzyme[4] goes into the nucleus of the stimulated cell and phosphorylates the target. The corresponding dephosphorylation is the detachment of phosphoric ester or anhydride through reversible hydrolysis catalyzed by phosphatases (a kind of hydrolases) which leads to the deactivation of the activated enzyme. This PdPC[5] is very common in post-translational modification occurring in proteins where a covalent enzymatic modification is followed by protein biosynthesis. Experimental and theoretical investigations on the mechanistic action of ERK2 enzyme have shown light on how the initial occupancy of the docking site introduces conformational modifications which is followed by the specific substrate binding happening at the active site[6–8]. Recently, Kolomeisky and co-workers have provided a quantitative network model[9] for theoretically investigating an unexpected enhanced activity caused by the mutations[10] in the D-site of ERK2 enzyme. As per the purposed mechanism firstly, the inactive ERK2 enzyme binds with an activator (MEK). The activator bound enzymatic state irreversibly dissociates leading to the formation of the activated/phosphorylated ERK2. The phosphorylated enzyme can execute the substrate phosphorylation or simply get dephosphorylated following the deactivation route. This biochemical network has the coupled Michaelis-Menten (MM) reactions associated with each of the reaction routes namely, the phosphorylation of the inactive ERK2 (activation), dephosphorylation of the active ERK2 (deactivation) and phosphorylation of the substrate by the active ERK2.

In general, the mechanism of the enzyme action can be understood from the celebrated Michaelis-Menten (MM) reaction[11], $E + S \rightleftharpoons ES \rightarrow E + P$ where an enzyme E rever-

sibly binds with the substrate S to form the enzyme-substrate complex ES which can either irreversibly dissociate to form the product P with the regeneration of the free enzyme E or can revert from the bound state releasing the substrate. These events are characterized by the rate constants k_1, k_2 and k_{-1}, respectively. In the quasi-steady state, we can obtain the velocity expression as

$$v = \frac{k_2[E_0][S]}{k_M + [S]} \tag{1}$$

Here $[E_0]$ is the total enzyme concentration ($[E_0] = [E] + [ES]$), $[S]$ is the substrate concentration and K_M is the Michaelis-Menten constant $\left(K_M = \frac{k_{-1} + k_2}{k_1}\right)$.

With the emergence of the single-molecule fluorescence microscopy[12–14], one can analyze the catalytic activity of a single enzyme in real time. The waiting time distributions associated with the catalytic turnover events in single enzyme studies showed multiexponential decay profiles[15,16] at higher substrate concentration which can be attributed to the enzymatic conformational fluctuations occuring on timescales comparable or longer than the timescale of the catalytic reaction. This can lead to a distribution of reaction rate constants, a phenomenon known as dynamic disorder[17]. In any renewal process, the PDF (Probability Distribution Function)[18] between the consecutive turnover events is a fundamental quantity to be measured. For various kinds of single molecule enzyme reactions like the enzyme inhibition reactions[19], different binding mechanisms in the presence of multiple substrates[20], we can obtain the exact rate expression by applying a theoretical formalism which agrees with that derived in the deterministic limit. Provided that the detailed balance condition holds good[21,22], for the systems with different number of internal states with conformational modulations, the analytical expressions for the reaction velocity shows a substrate concentration dependence which is in accordance with the MM law. The average rate of the substrate phosphorylation by the active (phosphorylated) ERK2, shows the MM behavior[9].

Experimental studies are restricted to rate analyses but theoretical treatments can give dynamical interpretation of the properties which are usually hidden from the ensemble measurements. In order to quantify the temporal fluctuations in the reactions rates, we calculate the randomness parameter[15,18] which is defined as the ratio of dimensionless variance to that of the mean square. Mathematically, it can be represented as

$$R = \frac{\langle t^2 \rangle - \langle t \rangle^2}{\langle t \rangle^2} \tag{2}$$

Here, t is the time between two consecutive successful events. If the value of R is found to be unity then there is a single rate determining step else there are multiple competing reaction timescales. Its value also qualitatively predicts the shape of the PDF[23]. Multiexponentiality in the PDF reflects the existence of several rate governing steps. We ask the following questions: though the average rate of substrate phosphorylation catalyzed by the active ERK2 enzyme shows the hyperbolic dependence with the substrate concentration but what is the nature of the corresponding waiting time distribution? Is dynamic disorder present in such PdPC reaction networks? If so, how the randomness changes under different physical scenarios subjected to change in the magnitude of the rate constants constituting the parameter space and concentrations of the activator, deactivator, substrate? How the introduction of one more activator bound internal state affects the functional form of the reaction velocity and system randomness?

In this study, we have considered three different types of PdPC reaction networks and we have applied the CME (Chemical Master Equation)[24] approach which represents the time evolution of the joint probability distribution. This technique has been employed earlier to differentiate between parallel and off-pathway mechanisms[25] and to calculate the reaction flux[26], the Fano factor and other significant statistical quantities associated with single molecule enzyme reactions. The formulated waiting time distribution focuses on the substrate phosphorylation catalyzed by the active ERK2 enzyme. In different physical scenarios depending on the magnitudes of the rate constants and concentrations of the activator, deactivator, and substrate, the rate-determining step changes. We obtain the analytical expression for the waiting time distribution, the mean turnover time and the randomness parameter for the reaction schemes taken under consideration. This study provides platforms for understanding the dynamical aspects of the phosphorylation-dephosphorylation networks.

Model I:

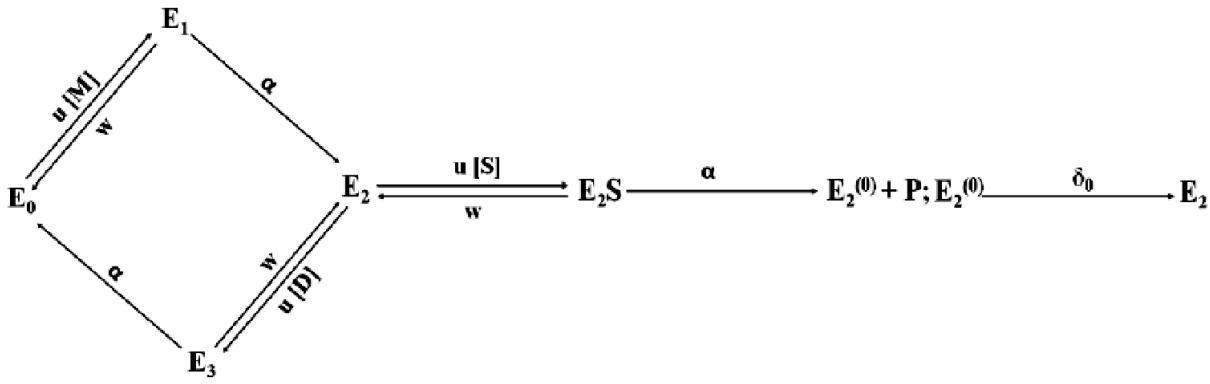

Fig. 1. Schematic representation showing the activation/phosphorylation of the inactive ERK2, deactivation/dephosphorylation of the active ERK2 and substrate phosphorylation carried out by the activated ERK2 enzyme.

In the above schematic, there are three reaction routes namely, the activator (MEK) assisted activation/phosphorylation, the deactivator (phosphatases) involved deactivation/dephosphorylation and the substrate phosphorylation pathway catalyzed by the active ERK2 enzyme. Firstly, the inactive free enzyme (E_0) binds reversibly with an activator (M) to form an activator bound enzymatic state (E_1) which irreversibly dissociates to give the phosphorylated/activated state (E_2). Now, this phosphorylated enzyme (E_2) can access the two probable pathways. Following the deactivation route, E_2 can bind reversibly with the deactivator (D) to form the deactivator bound enzymatic state (E_3) which can irreversibly regenerate the starting unphosphorylated free enzyme state E_0. Following the substrate phosphorylation pathway, E_2 can reversibly bind with the substrate (S) forming the enzyme-substrate complex (E_2S). Further, E_2S irreversibly dissociates to give the product and regenerated enzyme $E_2^{(0)}$. The enzymatic state $E_2^{(0)}$ instantaneously converts to E_2 through a transition characterized by the rate constant δ_0. In the given reaction mechanism, all binding events associated with the three routes namely, the phosphorylation of the inactive ERK2, the dephosphorylation of the active ERK2 and the substrate phosphorylation catalyzed by the active ERK2 are characterized by the rate constant u. All the unbinding processes beginning from the enzymatic bound states (E_1, E_3, E_2S) are designated by the rate constant w, and all the irreversible rate processes are designated by the rate constant α. For this reaction mechanism, the reaction rate statistics in terms of the time evolution of the joint probability of the number of each species involved in the chemical reaction is described by the chemical master equation (CME) approach. The variables constituting the probability distributions are $n_{E_0}, n_{E_1}, n_{E_2}, n_{E_3}, n_{E_2S}, n_{E_2^{(0)}}$ and n_p representing the number of enzyme molecules present in the state E_0, E_1, E_2, E_3, E_2S, $E_2^{(0)}$ and P respectively and n_p is the number of product molecules formed at a time t.

$$
\begin{aligned}
&\partial_t P\left[n_{E_0}, n_{E_1}, n_{E_2}, n_{E_3}, n_{E_2S}, n_{E_2^{(0)}}, n_p; t\right] \\
&= \Big(u[M](n_{E_0}+1)Y_{E_0}Y_{E_1}^{-1} + w(n_{E_1}+1)Y_{E_0}^{-1}Y_{E_1} \\
&+ \alpha(n_{E_1}+1)Y_{E_1}Y_{E_2}^{-1} \\
&+ u[D](n_{E_2}+1)Y_{E_2}Y_{E_3}^{-1} + w(n_{E_3}+1)Y_{E_2}^{-1}Y_{E_3} \\
&+ \alpha(n_{E_3}+1)Y_{E_0}^{-1}Y_{E_3} \\
&+ u[S](n_{E_2}+1)Y_{E_2}Y_{E_2S}^{-1} + w(n_{E_2S}+1)Y_{E_2}^{-1}Y_{E_2S} \\
&+ \alpha(n_{E_2S}+1)Y_{E_2S}Y_{E_2^{(0)}}^{-1}Y_P^{-1} \\
&- \Big(u([M]n_{E_0} + ([D]+[S])n_{E_2}\Big) \\
&+ (w+\alpha)(n_{E_1}+n_{E_3}+n_{E_2S})\Big)\Big) \\
&P\left[n_{E_0}, n_{E_1}, n_{E_2}, n_{E_3}, n_{E_2S}, n_{E_2^{(0)}}, n_p; t\right] \qquad (3)
\end{aligned}
$$

where Y is the step operator when operated[27] on any function $f(x)$ gives the following form $Yf(x) = f(x + 1)$ and $Y^{-1} f(x) = f(x - 1)$. For example when it is applied on the first term of the CME it will give $Y_{E_0} Y_{E_1}^{-1} \; P\left[n_{E_0}, n_{E_1}, n_{E_2}, n_{E_3}, n_{E_2S}, n_{E_2^{(0)}}, n_p; t\right] = P\left[n_{E_0}+1, n_{E_1}-1, n_{E_2}, n_{E_3}, n_{E_2S}, n_{E_2^{(0)}}, n_p; t\right]$.

Owing to the mutual exclusivity of different enzymatic states i.e. at a given instant of time an enzyme can exist only in a particular state we can write the above CME as a set of coupled differential equations:

$$\frac{\partial P_{E_0}(t)}{\partial t} = -u[M]P_{E_0}(t) + wP_{E_1}(t) + \alpha P_{E_3}(t) \tag{4.a}$$

$$\frac{\partial P_{E_1}(t)}{\partial t} = u[M]P_{E_0}(t) - (w+\alpha)P_{E_1}(t) \tag{4.b}$$

$$\frac{\partial P_{E_2}(t)}{\partial t} = \alpha P_{E_1}(t) - u[D]+[S]P_{E_2}(t) + wP_{E_3}(t) + wP_{E_2S}(t) + \delta_0 P_{E_2^{(0)}}(t) \tag{4.c}$$

$$\frac{\partial P_{E_3}(t)}{\partial t} = u[D]P_{E_2}(t) - (w+\alpha)P_{E_3}(t) \tag{4.d}$$

$$\frac{\partial P_{E_2S}(t)}{\partial t} = u[S]P_{E_2}(t) - (w+\alpha)P_{E_2S}(t) \tag{4.e}$$

$$\frac{\partial P_{E_2^{(0)}}(t)}{\partial t} = \alpha P_{E_2S}(t) - \delta_0 P_{E_2^{(0)}}(t) \tag{4.f}$$

The probability for a single enzymatic turnover takes place in time t and $t + \Delta t$ is $f(t)\Delta t$ and this is the time required for product formation to occur in the same time interval, $f(t)\Delta t = \Delta P_P(t) = \alpha P_{E_2S}(t)\,\Delta t$. In the limit of infinitesimal Δt, the turnover time distribution is

$$f(t) = \frac{\partial P_P(t)}{\partial t} = \alpha P_{E_2S}(t) \tag{5}$$

The instantaneous step representing the regeneration of free enzyme from the state $E_2^{(0)}$ is very fast such that $\frac{\partial P_{E_2^{(0)}}(t)}{\partial t} \approx 0$. At the beginning of the reaction, the enzyme exists in the free-state conformer E_0 only such that $P_{E_0}(0) = 1$, $P_{E_1}(0) = 0$, $P_{E_2}(0) = 0$, $P_{E_3}(0) = 0$, $P_{E_2S}(0) = 0$, $P_{E_2^{(0)}}(0) = 0$ and $P_P(0) = 0$. Also, at any instant of time, the condition $P_{E_0}(t) + P_{E_1}(t) + P_{E_2}(t) + P_{E_3}(t) + P_{E_2S}(t) = 1$ should always be satisfied. Thus, the above set of coupled differential equations can be solved by taking the Laplace transform and applying appropriate initial conditions and normalization constraints we get the following matrix

$$\begin{bmatrix} s+[M]u & -w & 0 & -\alpha & 0 & 0 \\ -[M]u & s+w+\alpha & 0 & 0 & 0 & 0 \\ 0 & -\alpha & s+u([D]+[S]) & -w & -w & -\delta_0 \\ 0 & 0 & -u[D] & s+\alpha+w & 0 & 0 \\ 0 & 0 & -u[S] & 0 & s+w+\alpha & 0 \\ 0 & 0 & 0 & 0 & 0 & s \end{bmatrix} \begin{bmatrix} \hat{P}_{E_0}(s) \\ \hat{P}_{E_1}(s) \\ \hat{P}_{E_2}(s) \\ \hat{P}_{E_3}(s) \\ \hat{P}_{E_2S}(s) \\ \hat{P}_{E_2^{(0)}}(s) \end{bmatrix} = \begin{bmatrix} 1 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \end{bmatrix} \tag{6}$$

where "s" is the Laplace parameter, $\hat{f}(S)$ is the Laplace transform of the function $f(t)$ given as $\hat{f}(S) = \int_0^\infty e^{-st} f(t)\,dt$.

Solving this matrix by taking an inverse gives

$$\begin{bmatrix} \hat{P}_{E_0}(s) \\ \hat{P}_{E_1}(s) \\ \hat{P}_{E_2}(s) \\ \hat{P}_{E_3}(s) \\ \hat{P}_{E_2S}(s) \\ \hat{P}_{E_2^{(0)}}(s) \end{bmatrix} = \begin{bmatrix} \frac{s^3 + A s^2 + B s + C}{s^4 + E s^3 + F s^2 + G s + H} \\ \frac{[M]u s^2 + I s + J}{s^4 + E s^3 + F s^2 + G s + H} \\ \frac{\alpha[M]u s + K}{s^4 + E s^3 + F s^2 + G s + H} \\ \frac{\alpha[D][M]u^2}{s^4 + E s^3 + F s^2 + G s + H} \\ \frac{\alpha[M][S]u^2}{s^4 + E s^3 + F s^2 + G s + H} \\ 0 \end{bmatrix} \tag{7}$$

where

$A = ([D] + [S])u + 2(\alpha + w)$,

$B = \alpha([D] + [S])u + (\alpha + w)(\alpha + ([D] + [S])u + w)$,

$C = \alpha([D] + [S])u(\alpha + w)$,

$E = A + [M]u$,

$F = \alpha^2 + 2\alpha([D] + [S])u + ([M]u + w)(2\alpha + ([D] + [S])u + w)$,

$G = 2J + C + K$,

$H = \alpha^2 [M][S]u^2$,

$I = [M]u(\alpha + [D] + [S])u + w)$,

$J = \alpha [M]([D] + [S])u^2$, and

$K = \alpha[M]u(\alpha + w)$.

From the obtained solution, the waiting time distribution function (s) in the Laplace domain

$$\hat{f}(s) = \alpha \tilde{P}_{E_2S}(S) = \frac{\alpha^2[M][S]u^2}{s^4 + Es^3 + Fs^2 + Gs + H} \quad (8)$$

From the first moment (n = 1) of the waiting time distribution, the mean waiting time can be obtained using the relation

$$\langle t^n \rangle = (-1)^n \left(\frac{d^n \hat{f}(s)}{ds^n} \right)_{s \to 0} \quad (9)$$

$$\langle t \rangle = \frac{(\alpha + w)([D] + [M]) + 2[M][D]u}{\alpha[M][S]u} + \frac{\alpha + 2[M]u + w}{\alpha[M]u} \quad (10)$$

For the reaction scheme represented in Fig. 1, the average waiting time for the substrate phosphorylation shows a linear relationship with the inverse of the substrate concentration i.e. it follows the MM law. To quantify the temporal fluctuations, we calculate the randomness parameter shown in eq. (2). The analytical expression for the given reaction scheme was found to be

$$R = 1 - \frac{2[M][S]\xi_1}{\xi_2} \quad (11)$$

where

$$\xi_1 = \left(\alpha^2 + ([M]u + w)(([D] + [S])u) + w + 2\alpha(([D] + [M] + [S])u) + w \right), \text{ and}$$

$$\xi_2 = ((\alpha + w)([D] + [M] + [S]) + 2[M]([D] + [S])u)^2.$$

When eq. (11) is subjected to the limit $[M] \to 0$, then R attains the value one as the phosphorylation of the enzyme via E_1 formation becomes the slowest step. The substrate would be phosphorylated only after the activation of the enzyme. In eq. (11) if we put the limit $[S] \to 0$, R goes to unity. In this physical scenario, the binding of the substrate with the active ERK2 enzyme becomes the rate determining step.

Model II:

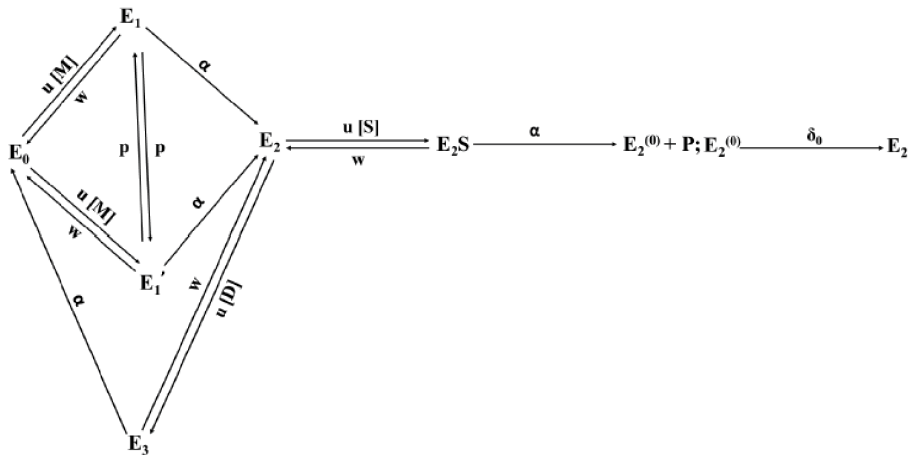

Fig. 2. Schematic representation showing the activation/phosphorylation of the inactive ERK2 assisted by the two different activator-bound enzymatic states (E_1 and E_1' both can directly access the phosphorylated state of the ERK2), deactivation/dephosphorylation of the active ERK2 and substrate phosphorylation carried out by the activated ERK2 enzyme.

Next we consider another reaction route where we consider one more internal state (E_1') which can be formed when the starting enzyme conformer E_0 reversibly binds with the activator. E_1' can irreversibly dissociate to the activated/phosphorylated enzyme (E_2). We also consider the presence of conformational fluctuations between these two activator bound enzymatic conformers E_1 and E_1' characterized by the rate constant p. Just like the previous reaction scheme, the reaction rate statistics can be described by the CME approach. The variables constituting the probability distributions are $n_{E_0}, n_{E_1}, n_{E_1'}, n_{E_2}, n_{E_3}, n_{E_2S}, n_{E_2^{(0)}}$ and n_p representing the number of enzyme molecules present in the state E_0, E_1, E_1', E_2, E_3, E_2S, and $E_2^{(0)}$, respectively and n_p is the number

of product molecules formed at a time *t*.

$$\begin{aligned}
&\partial_t P\left[n_{E_0}, n_{E_1}, n_{E_1'}, n_{E_2}, n_{E_3}, n_{E_2S}, n_{E_2^{(0)}}, n_p; t\right] \\
&= \Big(u[M](n_{E_0}+1)Y_{E_0}Y_{E_1}^{-1} + w(n_{E_1}+1)Y_{E_0}^{-1}Y_{E_1} \\
&+ \alpha(n_{E_1}+1)Y_{E_1}Y_{E_2}^{-1} + u[M](n_{E_0}+1)Y_{E_0}Y_{E_1'}^{-1} \\
&+ w(n_{E_1'}+1)Y_{E_0}^{-1}Y_{E_1'} + \alpha(n_{E_1'}+1)Y_{E_1'}Y_{E_2}^{-1} \\
&+ p(n_{E_1}+1)Y_{E_1}Y_{E_1'}^{-1} + p(n_{E_1'}+1)Y_{E_1}^{-1}Y_{E_1'} \\
&+ u[D](n_{E_2}+1)Y_{E_2}Y_{E_3}^{-1} + w(n_{E_3}+1)Y_{E_2}^{-1}Y_{E_3} \\
&+ \alpha(n_{E_3}+1)Y_{E_0}^{-1}Y_{E_3} + u[S](n_{E_2}+1)Y_{E_2}Y_{E_2S}^{-1} \\
&+ w(n_{E_2S}+1)Y_{E_2}^{-1}Y_{E_2S} + \alpha(n_{E_2S}+1)Y_{E_2S}Y_{E_2^{(0)}}^{-1}Y_P^{-1} \\
&- \Big(2u[M]n_{E_0} + (w+p+\alpha)n_{E_1} + (w+p+\alpha)n_{E_1'} \\
&+ u([D]+[S])n_{E_2S} + (\alpha+w)n_{E_3} + (\alpha+w)n_{E_2S}\Big)\Big) \\
&P\left[n_{E_0}, n_{E_1}, n_{E_1'}, n_{E_2}, n_{E_3}, n_{E_2S}, n_{E_2^{(0)}}, n_p; t\right]
\end{aligned} \tag{12}$$

As shown in Appendix 1, the probability distribution function for the substrate phosphorylation event

$$(s) = \alpha\tilde{P}_{E_2S}(S) = \frac{2\alpha^2[M][S]u^2}{s^4 + Xs^3 + Ys^2 + Zs + 2H} \tag{13}$$

From the first moment of the waiting time distribution, we get the mean reaction time

$$\langle t \rangle = \frac{\alpha + 4[M]u + w}{2\alpha[M]u} + \frac{(\alpha + w)([D] + 2M) + 4[M][D]u}{2\alpha[M][S]u} \tag{14}$$

Thus, the mean time for the substrate phosphorylation follows the MM equation. The analytical expression for the randomness parameter for this reaction scheme was found to be

$$R = 1 - \frac{4[M][S]\xi_3}{\xi_4} \tag{15}$$

where

$$\xi_3 = \Big(\alpha^2 + w + 2\alpha\big(([D]+2[M]+[S])u + w\big) + u(2[M]([D]+[S])u + ([D]+2[M]+[S])w)\Big), \text{ and}$$

$$\xi_4 = ((\alpha+w)([D]+2[M]+[S]) + 4[M]([D]+[S])u)^2.$$

In eq. (15), in the limit $[S] \rightarrow 0$, R attains the value unity as the substrate binding event becomes the rate determining step. Also, in the limit, $[M] \rightarrow 0$, the activation of ERK2 enzyme becomes the slowest step so $R = 1$.

Model III:

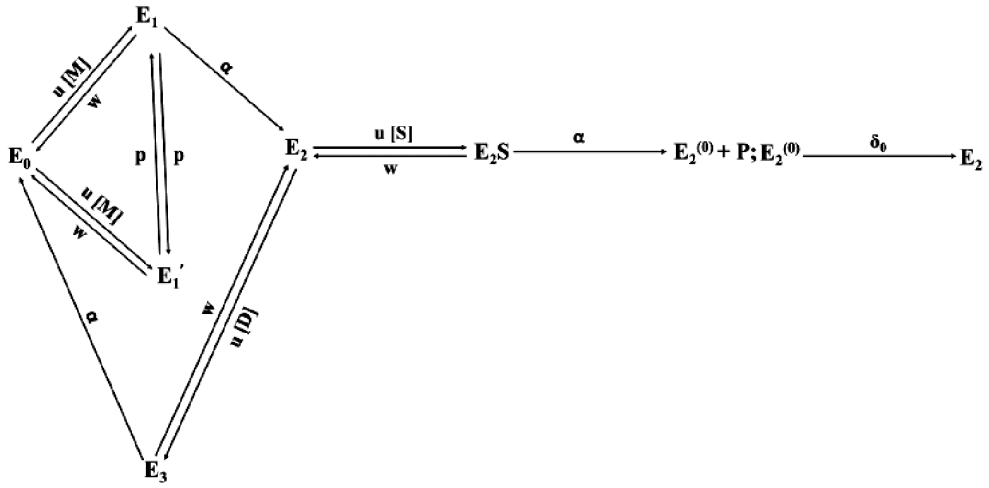

Fig. 3. Schematic representation showing the activation/phosphorylation of the inactive ERK2 assisted by the two different activator-bound enzymatic states (E_1' cannot directly reach the phosphorylated state of the ERK2), deactivation of the active ERK2 and substrate phosphorylation carried out by the activated ERK2 enzyme.

In the above schematic, the activator bound state E_1', cannot directly undergo an irreversible transition leading to the activated state E_2. E_1' can make a conformational flip to E_1, followed by reaching E_2. The rest of Model III is same as that of the reaction Model II as represented in Fig. 2.

Using the CME approach as given in Appendix 2, the probability distribution function for the substrate phosphorylation event

$$\hat{f}(s) = \alpha \hat{P}_{E_2S}(S) = \alpha^2 \left(\frac{[M][S]u^2 s + [M][S]u^2(2p+w)}{s^5 + qs^4 + xs^3 + ys^2 + zs + \lambda_1} \right) \quad (16)$$

From the first moment of the waiting time distribution we get the mean reaction time

$$\langle t \rangle = \frac{\alpha\,(p + [M]u + w) + (2p + w)(3[M]u + w)}{\alpha[M]u(2p + w)}$$

$$+ \frac{(2p + w)(3[D][M]u + ([D] + [M])w) + \alpha([M](2p + w) + [D](p + [M]u + w))}{\alpha[M][S]u(2p + w)} \quad (17)$$

The mean time for the substrate phosphorylation follows the MM equation.

The analytical expression for the randomness parameter for this reaction scheme was found to be

$$R = 1 - \frac{2[M][S]\xi_8}{\xi_9} \quad (18)$$

where

$$\xi_5 = (2p+w)^2(2[M]u + w)(([D] + [S])u + w \;,$$

$$\xi_6 = \alpha^2(2p^2 + p([D]+2[M]+[S])u - [M]([D]+[S])u^2$$

$$+3pw + [M]uw + w^2)\,,$$

$$\xi_7 = \alpha(2p + w(u(3p([D]+2[M]+[S]) + [M]([D]+[S])u)$$

$$+3p + 2([D]+2[M]+[S])u)w + 2w^2)\,,$$

$\xi_8 = \xi_5 + \xi_6 + \xi_7$, and

$$\xi_9 = ((2p + w(3[M]([D] + [S])u + ([D]+[M]+[S])w)$$

$$+\alpha([S](p+w) + ([D](p + [M]u + w) + [M](2p + [S]u + w)))^2$$

In eq. (18) if we put the limit $[S] \to 0$, then R attains the value unity as the substrate binding event becomes the rate determining step. Also, in the limit, $[M] \to 0$, the activation of ERK2 enzyme becomes the slowest step so $R = 1$.

Results and discussion

Variation of randomness parameter 'R' as a function of substrate concentration '[S]' for Model I

We plot the randomness parameter R as a function of $[S]$ for a given set of kinetic parameters to analyze the effects of u, $[M]$ and $[D]$ as shown in Fig. 4. For all the cases, at low $[S]$, the substrate binding event is the rate-determining step and there is no dynamic disorder and $R = 1$. At higher $[S]$, the enzyme is present in the substrate-bound E_2S state and there is a competition between the product formation event and

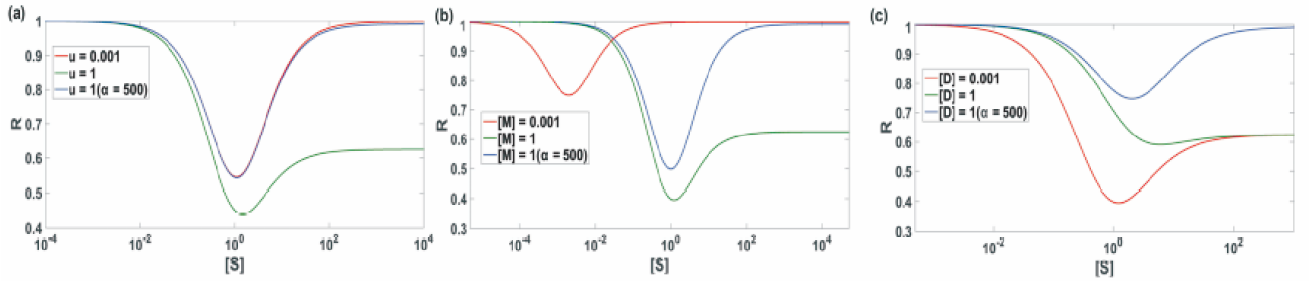

Fig. 4. The plots showing the variation of the randomness parameter (R) as a function of the substrate concentration $[S]$ for the reaction scheme represented in Fig. 1 to analyze the effect of (a) the binding rate constant (u) with the common set of reaction parameters: $w = 1$, $\alpha = 5$, $[M] = 1$ and $[D] = 0.1$, (b) activator concentration ($[M]$) in the common parameter space: $u = 1$, $w = 1$, $\alpha = 5$, and $[D] = 0.001$ and (c) deactivator concentration ($[D]$) for the given set of reaction rate constants: $u = 1$, $w = 1$, $\alpha = 5$ and $[M] = 1$. The red and green solid lines represent the obtained behaviour at two values 0.001 and 1, respectively for $u/[M]/[D]$ for the plots labelled as (a), (b) and (c), respectively. The blue solid line in each plot represents the response for the case when $\alpha = 500$ and the corresponding values of $u/[M]/[D]$ are taken to be unity for the plots labelled as (a), (b) and (c), respectively.

the dissociation of E_2S back to E_2. For comparable α and w, there is competition between these two events contributing to the randomness of the system and R deviates from unity. The competition reduces when one of the rate constant involved in the competition is considerably higher than the other assuring the presence of a single rate-determining step. For example at high values of α the product formation event occurs readily and the value of randomness parameter approaches to unity irrespective of changes in any other system parameters.

At higher [S], when the enzyme is present in the substrate-bound E_2S state, at low values of u, the conversion of E_2 into E_2S is the slowest step and R = 1. With an increase in the value of u, the substrate-binding event is no longer the slowest step. There is a competition between the events starting from E_2S state which can lead to deviation in the value of R from unity.

For a given set of kinetic parameters at low [S], R = 1 irrespective of [M]. At high [S], when [M] is low, the formation of E_1 from E_0 is the rate governing step and R approaches unity. With an increase in [M], a sufficient amount of the activated enzyme E_2 would be formed which will be readily converted to E_2S at moderate to high [S] and R deviates from unity due to competing reaction time scales.

For a given parameter space, at low [S], there is no dynamic disorder irrespective of [D]. For high values of [M], the free enzyme E_0 will go to the E_2 state which can participate either in the substrate phosphorylation or the deactivation route when [S] and [D] are of comparable order. This leads to deviation in the value R from unity. At higher [S], and low to moderate values of [D], the substrate phosphorylation would be favored over the deactivation route and R is independent of [D]. The enzyme will be now in the E_2S state from which product formation and dissociation can happen on comparable timescales and the randomness parameter attains a constant non-unity value irrespective of the value of [D]. Considering a physical scenario where the magnitudes of [D] and [S] are considerably high, we find that irrespective of [D], at low [S], R = 1. At higher [S] and [D], sufficient amount of E_2S and as well as E_3 would be formed which increases the probabilities of the transitions from these bound enzymatic states and thus, $R \neq 1$. Further, at high [S] and at extremely high values of [D], the dephosphorylation route will be favored over the product formation step and thus, the value of R approaches to unity.

Based on Table 1 in Appendix 1, the dependence of the randomness parameter on u, [M] and [D] for Model II is same as in Model I. Also based on the expression of the randomness parameter as given in eq. (15), the randomness parameter is independent of the conformational fluctuation rate p in Model II. When the rate of conformational fluctuations between E_1 and E_1' and the rate of formation of the phosphorylated enzyme from E_1 and E_1' are same, there is no competition among reaction timescales, and the randomness parameter is independent of the conformational fluctuations at a given concentration of [M] and [D]. For Model III, the presence of conformational fluctuations between two activator bound enzymatic states affects the randomness in the system. When the enzyme is in the inactive E_1' state, it has to reach to the E_1 state for the reaction to proceed towards product formation. When the rate constant characterizing these conformational transitions (p) is smaller or comparable in magnitude to the other rate constants then it can lead to dynamic disorder. This is evident from the significant deviation in the value of R from unity (eq.(18)) as shown in Fig. 5.

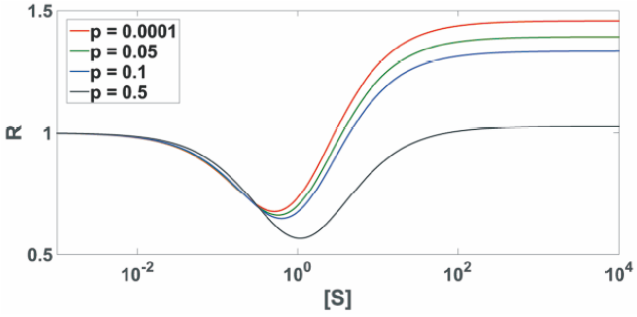

Fig. 5. Plot showing the variation of randomness parameter (R) as a function of substrate concentration ([S]) for the reaction scheme represented in Fig. 3 to analyze the effect of the rate constant p at four different values 0.0001, 0.05, 0.1 and 0.5 represented by the red, green, blue and black solid lines, respectively. The common set of reaction parameters u = 1, w = 1, α = 5, [M] = 10 and [D] = 0.1.

With an increase in the magnitude of p, the E_1' state is readily converted to E_1 state and the dynamic disorder decreases, R is equal to one. As seen from Table 1 in Appendix 2, all other dependencies of R on u, [M] and [D] follows the same trend as observed in Model I.

Conclusions

In this study, we have applied the waiting time distribution formalism to understand the dynamical picture associated with the substrate phosphorylation process catalyzed by the activated ERK2 enzyme. For such reaction systems, we find that the reaction velocity shows a hyperbolic dependence on the substrate concentration as observed in a simple MM type reaction. But there are multiple rate-determining steps in different physical scenarios depending on the given parameter space as well as the concentrations of the activator, substrate, and deactivator. When there is sufficient availability of the activated ERK2 (E_2), and the substrate concentration is considerably higher than the [D], the phosphorylation of the substrate will be favored over the deactivation/dephosphorylation of the active enzyme (E_2). At high [S], the enzyme present in the substrate-bound state can either form the product or E_2S can simply revert back to E_2. Thus, there are multiple rate-determining steps contributing to the randomness of the system. For the cases when the [D] and [S] both are significantly high, the probabilities of transitions from the deactivator bound and substrate-bound enzymatic states increases leading to the deviation in the value of R from unity. For the reaction Model II, the presence of conformational fluctuations between E_1 and E_1' neither affects the reaction rate nor the randomness parameter. If the activated enzymatic state is not directly accessible to one of the activator bound state as shown in reaction Model III, then the enzyme will be trapped in the E_1' state and the formation of the activated ERK2 (E_2) form is hindered. If the conformational fluctuations are faster, then it will readily go back to E_1 state from which the formation of E_2 takes place and the disorder in the system decreases.

The applied theoretical formalism provides justifications to explain the causes contributing to the system randomness and also emphasizes on how the rate determining step changes in different physical situations. This study provides a platform for dynamical interpretations associated with the phosphorylation/dephosphorylation network reactions.

Acknowledgements

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Appendix 1

We write the CME for the reaction Model II as shown in eq. (12) of the manuscript. To solve that we use the mutual exclusivity of different enzymatic states i.e. at a given instant of time an enzyme can exist only in a particular state. This reduces the CME to the following set of coupled differential equations:

$$\frac{\partial P_{E_0}(t)}{\partial t} = -2u[M]P_{E_0}(t) + wP_{E_1}(t) + wP_{E_1'}(t) + \alpha P_{E_3}(t) \quad \text{(A1.1a)}$$

$$\frac{\partial P_{E_1}(t)}{\partial t} = u[M]P_{E_0}(t) - aP_{E_1}(t) + pP_{E_1'}(t) \quad \text{(A1.1b)}$$

$$\frac{\partial P_{E_1'}(t)}{\partial t} = u[M]P_{E_0}(t) + pP_{E_1}(t) - aP_{E_1'}(t) \quad \text{(A1.1c)}$$

$$\frac{\partial P_{E_2}(t)}{\partial t} = \alpha P_{E_1}(t) + \alpha P_{E_1'}(t) - u([D] + [S])P_{E_2}(t) + wP_{E_3}(t) + wP_{E_2S}(t) + \delta_0 P_{E_2^{(0)}}(t) \quad \text{(A1.1d)}$$

$$\frac{\partial P_{E_3}(t)}{\partial t} = u[D]P_{E_2}(t) - bP_{E_3}(t) \quad \text{(A1.1e)}$$

$$\frac{\partial P_{E_2S}(t)}{\partial t} = u[S]P_{E_2}(t) - bP_{E_2S}(t) \quad \text{(A1.1f)}$$

$$\frac{\partial P_{E_2^{(0)}}(t)}{\partial t} = \alpha P_{E_2S}(t) - \delta_0 P_{E_2^{(0)}}(t) \quad \text{(A1.1g)}$$

where $a = w + \alpha + p$ and $b = \alpha + w$.

The turnover time distribution for the reaction Model II is

$$f(t) = \frac{\partial P_P(t)}{\partial t} = \alpha P_{E_2S}(t)\,. \quad \text{(A1. 2)}$$

The instantaneous step representing the regeneration of free enzyme from the state $E_2^{(0)}$ is very fast such that $\frac{\partial P_{E_2^{(0)}}(t)}{\partial t} \approx 0$. At the beginning of the reaction, the enzyme exists in the free-state conformer E_0 only such that $P_{E_0}(0) = 1$, $P_{E_1}(0) = 0$, $P_{E_1'}(0) = 0$, $P_{E_2}(0) = 0$, $P_{E_3}(0) = 0$, $P_{E_2S}(0) = 0$, $P_{E_2^{(0)}}(0) = 0$ and $P_p(0) = 0$. Also, at any instant of time, the condition $P_{E_0}(t) + P_{E_1}(t) + P_{E_1'}(t) + P_{E_2}(t) + P_{E_3}(t) + P_{E_2S}(t) = 1$ should always be satisfied. Thus, the above set of coupled differential equations can be solved by taking the Laplace transform and applying appropriate initial conditions and normalization constraints to obtain the following matrix

$$\begin{bmatrix} s+2u[M] & -w & -w & 0 & -\alpha & 0 & 0 \\ -u[M] & s+a & -p & 0 & 0 & 0 & 0 \\ -u[M] & -p & s+a & 0 & 0 & 0 & 0 \\ 0 & -\alpha & -\alpha & s+u([S]+[D]) & -w & -w & -\delta_0 \\ 0 & 0 & 0 & -u[D] & s+b & 0 & 0 \\ 0 & 0 & 0 & -u[S] & 0 & s+b & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & s \end{bmatrix} \begin{bmatrix} \hat{P}_{E_0}(s) \\ \hat{P}_{E_1}(s) \\ \hat{P}_{E_1'}(s) \\ \hat{P}_{E_2}(s) \\ \hat{P}_{E_3}(s) \\ \hat{P}_{E_2S}(s) \\ \hat{P}_{E_2^{(0)}}(s) \end{bmatrix} = \begin{bmatrix} 1 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \end{bmatrix} \quad \text{(A1. 3)}$$

After further rearrangement and taking an inverse gives

$$\begin{bmatrix} \hat{P}_{E_0}(s) \\ \hat{P}_{E_1}(s) \\ \hat{P}_{E_1'}(s) \\ \hat{P}_{E_2}(s) \\ \hat{P}_{E_3}(s) \\ \hat{P}_{E_2S}(s) \\ \hat{P}_{E_2^{(0)}}(s) \end{bmatrix} = \begin{bmatrix} \frac{s^3 + A\,s^2 + B\,s + C}{s^4 + X\,s^3 + Y\,s^2 + Z\,s + 2H} \\ \frac{[M]u\,s^2 + I\,s + J}{s^4 + X\,s^3 + Y\,s^2 + Z\,s + 2H} \\ \frac{[M]u\,s^2 + I\,s + J}{s^4 + X\,s^3 + Y\,s^2 + Z\,s + 2H} \\ \frac{2\alpha[M]u\,s + 2K}{s^4 + X\,s^3 + Y\,s^2 + Z\,s + 2H} \\ \frac{2\alpha[D][M]u^2}{s^4 + X\,s^3 + Y\,s^2 + Z\,s + 2H} \\ \frac{2\alpha[M][S]u^2}{s^4 + X\,s^3 + Y\,s^2 + Z\,s + 2H} \\ 0 \end{bmatrix} \quad \text{(A1.4)}$$

where

$X = E + [M]u$,

$Y = \alpha^2 + (2[M]u + w)([D] + [S])u + w)$

$+ 2\alpha\,(([D] + 2[M] + [S])u + w)$ and

$Z = \alpha u((\alpha + w)([D] + 2[M] + [S] + 4[M][D] + [S])u$,

Using $\hat{P}_{E_2S}(S)$ from eq. (A1.4) we obtain the expressions for the waiting time distribution (eq. (13)), the mean turnover time (eq. (14)) and the randomness parameter (eq. (15)) as shown in the manuscript.

Table 1

(a) Effect of the binding rate constant u at high [S] for Model II

Kinetic parameters	u	R
$w = 1$, $\alpha = 5$, [M] = 1, [D] = 0.1, [S] = 5×10^4	0.001	~1
	0.5	0.6250
	1	0.4800
	5	0.3786
$w = 1$, $\alpha = 500$, [M] = 1, [D] = 0.1, [S] = 5×10^4	1 (fixed)	~1

(b) Effect of the activator concentration [M] at high [S] for Model II

Kinetic parameters	u	R
$u = 1$, $w = 1$, $\alpha = 5$, [M] = 1, [D] = 0.001, [S] = 5×10^4	0.001	~1
	0.1	0.8906
	1	0.4800
	10	0.4140
$u = 1$, $w = 1$, $\alpha = 500$, [M] = 1, [D] = 0.001, [S] = 5×10^4	1 (fixed)	~1

(c) Effect of the deactivator concentration [D] for Model II

Kinetic parameters	[D]	R
$u = 1$, $w = 1$, $\alpha = 5$, [M] = 1, [S] = 5×10^4	0.001	0.4800
	1	0.4800
	1×10^3	0.4902
	5×10^6	~1
$u = 1$, $w = 1$, $\alpha = 500$, [M] = 1, [S] = 5×10^4	1 (fixed)	~1

Appendix 2

Just like the previous reaction schemes (Model I and Model II), the reaction rate statistics for the reaction Model III can be described by the CME approach. The variables constituting the probability distributions are $n_{E_0}, n_{E_1}, n_{E_1'}, n_{E_2}, n_{E_3}, n_{E_2S}, n_{E_2^{(0)}}$ and n_p representing the number of enzyme molecules present in the state E_0, E_1, E_1', E_2, E_3, E_2S, and $E_2^{(0)}$, respectively. n_p is the number of product molecules formed at a time t.

$$\hat{\partial}_t P\left[n_{E_0}, n_{E_1}, n_{E_1'}, n_{E_2}, n_{E_3}, n_{E_2S}, n_{E_2^{(0)}}, n_p, t\right]$$

$$= \Big(u[M](n_{E_0}+1)Y_{E_0}\,Y_{E_1}^{-1} + w(n_{E_1}+1)Y_{E_0}^{-1}Y_{E_1}$$

$$+ \alpha(n_{E_1}+1)Y_{E_1}\,Y_{E_2}^{-1}$$

$$+ u[M](n_{E_0}+1)Y_{E_0}\,Y_{E_1'}^{-1} + w(n_{E_1'}+1)Y_{E_0}^{-1}Y_{E_1'}$$

$$
\begin{aligned}
&+ p(n_{E_1}+1)Y_{E_1}Y_{E_1'}^{-1} \\
&+ p(n_{E_1'}+1)Y_{E_1'}^{-1}Y_{E_1'} + u[D](n_{E_2}+1)Y_{E_2}Y_{E_3}^{-1} \\
&+ w(n_{E_3}+1)Y_{E_2}^{-1}Y_{E_3} \\
&+ \alpha(n_{E_3}+1)Y_{E_0}^{-1}Y_{E_3} + u[S](n_{E_2}+1)Y_{E_2}Y_{E_2S}^{-1} \\
&+ w(n_{E_2S}+1)Y_{E_2}^{-1}Y_{E_2S} \\
&+ \alpha(n_{E_2S}+1)Y_{E_2S}Y_{E_2^{(0)}}^{-1}Y_P^{-1} \\
&- \Big(2u[M]n_{E_0} + (w+p+\alpha)n_{E_1} + (w+p)n_{E_1'} \\
&+ u([D]+[S])n_{E_2S} \\
&+ (\alpha+w)n_{E_3} + (\alpha+w)n_{E_2S}\Big)\Big) \\
&P\left[n_{E_0}, n_{E_1}, n_{E_1'}, n_{E_2}, n_{E_3}, n_{E_2S}, n_{E_2^{(0)}}, n_P; t\right] \qquad \text{(A2.1)}
\end{aligned}
$$

Owing to the mutual exclusivity of different enzymatic states the above CME as a set of coupled differential equations:

$$\frac{\partial P_{E_0}(t)}{\partial t} = -2u[M]P_{E_0}(t) + wP_{E_1}(t) + wP_{E_1'}(t) + \alpha P_{E_3}(t) \qquad \text{(A2.2a)}$$

$$\frac{\partial P_{E_1}(t)}{\partial t} = u[M]P_{E_0}(t) - aP_{E_1}(t) + pP_{E_1'}(t) \qquad \text{(A2.2b)}$$

$$\frac{\partial P_{E_1'}(t)}{\partial t} = u[M]P_{E_0}(t) + pP_{E_1}(t) - (w+p)P_{E_1'}(t) \qquad \text{(A2.2c)}$$

$$\frac{\partial P_{E_2}(t)}{\partial t} = \alpha P_{E_1}(t) - u([D]+[S])P_{E_2}(t) + wP_{E_3}(t) + wP_{E_2S}(t) + \delta_0 P_{E_2^{(0)}}(t) \qquad \text{(A2.2d)}$$

$$\frac{\partial P_{E_3}(t)}{\partial t} = u[D]P_{E_2}(t) - bP_{E_3}(t) \qquad \text{(A2.2e)}$$

$$\frac{\partial P_{E_2S}(t)}{\partial t} = u[S]P_{E_2}(t) - bP_{E_2S}(t) \qquad \text{(A2.2f)}$$

$$\frac{\partial P_{E_2^{(0)}}(t)}{\partial t} = \alpha P_{E_2S}(t) - \delta_0 P_{E_2^{(0)}}(t) \qquad \text{(A2.2g)}$$

The turnover time distribution for the given reaction Model III is

$$f(t) = \frac{\partial P_P(t)}{\partial t} = \alpha P_{E_2S}(t)\,. \qquad \text{(A2.3)}$$

The above set of coupled differential equations can be solved by taking the Laplace transform and applying appropriate initial conditions and normalization constraints as explained for the reaction Model I and II.

Arranging them in the form of matrix we get

$$\begin{bmatrix} s+2u[M] & -w & -w & 0 & -\alpha & 0 & 0 \\ -u[M] & s+a & -p & 0 & 0 & 0 & 0 \\ -u[M] & -p & s+p+w & 0 & 0 & 0 & 0 \\ 0 & -\alpha & 0 & s+u([S]+[D]) & -w & -w & -\delta_0 \\ 0 & 0 & 0 & -u[D] & s+b & 0 & 0 \\ 0 & 0 & 0 & -u[S] & 0 & s+b & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & s \end{bmatrix} \begin{bmatrix} \hat{P}_{E_0}(s) \\ \hat{P}_{E_1}(s) \\ \hat{P}_{E_1'}(s) \\ \hat{P}_{E_2}(s) \\ \hat{P}_{E_3}(s) \\ \hat{P}_{E_2S}(s) \\ \hat{P}_{E_2^{(0)}}(s) \end{bmatrix} = \begin{bmatrix} 1 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \end{bmatrix} \qquad \text{(A2.4)}$$

After further rearrangement and taking an inverse gives

$$\begin{bmatrix} \hat{P}_{E_0}(s) \\ \hat{P}_{E_1}(s) \\ \hat{P}_{E_1'}(s) \\ \hat{P}_{E_2}(s) \\ \hat{P}_{E_3}(s) \\ \hat{P}_{E_2S}(s) \\ \hat{P}_{E_2^{(0)}}(s) \end{bmatrix} = \begin{bmatrix} \frac{s^4 + h\,s^3 + j\,s^2 + l\,s + n}{s^5 + q\,s^4 + x\,s^3 + y\,s^2 + z\,s + \lambda_1} \\ \frac{[M]u\,s^3 + \lambda_2\,s^2 + \lambda_3\,s + \lambda_4}{s^5 + q\,s^4 + x\,s^3 + y\,s^2 + z\,s + \lambda_1} \\ \frac{[M]u\,s^3 + \lambda_5\,s^2 + \lambda_6\,s + \lambda_7}{s^5 + q\,s^4 + x\,s^3 + y\,s^2 + z\,s + \lambda_1} \\ \frac{\alpha[M]u\,s^2 + \lambda_8\,s + \lambda_9}{s^5 + q\,s^4 + x\,s^3 + y\,s^2 + z\,s + \lambda_1} \\ \frac{\alpha[D][M]u^2\,s + \alpha[D][M]u^2(2p+w)}{s^5 + q\,s^4 + x\,s^3 + y\,s^2 + z\,s + \lambda_1} \\ \frac{\alpha[M][S]u^2 s + \alpha[M][S]u^2(2p+w)}{s^5 + q\,s^4 + x\,s^3 + y\,s^2 + z\,s + \lambda_1} \\ 0 \end{bmatrix} \qquad \text{(A2.5)}$$

where

$$h = 2(\alpha + w) + 2p + w + ([D] + [S])u,$$

$$j = \alpha([D]+[S])u + \alpha(p+w) + w(2p+w) + (\alpha+([D]+[S])u + w)(\alpha + 2(p+)w)\ ,$$

$$l = \alpha([D]+[S])u\,(\alpha + 2(p+)w) + (\alpha+([D]+[S])u + w)(\alpha(p+w) + w(2p)+ w)\ ,$$

$$n = (\alpha+([D]+[S])u(\alpha(p+w) + w(2p)+ w)\ ,$$

$$q = 2p + ([D]+2[M]+[S])u+3w + \alpha\left(2+\alpha[M][S]u^2(2p+\mathrm{w})\right),$$

$$x = \alpha^2 + (2[M]u+ w)(([D]+[S])u)+w\ + (2p+w)$$

$$(([D]+2[M]+[S])u+2w)$$

$$+\alpha(3p+2([D]+2[M]+[S])u+4w),$$

$$y=(2p+w)(2[M]u+w)(([D]+[S])u)+w$$

$$+\alpha^2(p+([D]+2[M]+[S])u+w)$$

$$+\alpha(6[M]pu+([D]+[S])u(3p+4[M]u)$$

$$+3pw+3[D]+5[M]+3[S]uw+2w^2),$$

$$z=\alpha u(2p+w)(3[M]([D]+[S])u+([D]+[M]+[S])w)$$

$$+\alpha([S](p+w)+[D](p+[M])u+w)$$

$$+[M](2p+2[S]u+w))),$$

$$\lambda_1=\alpha^2[M][S]u^2(2p+w),$$

$$\lambda_2=[M]u(\alpha+2p+([D]+[S])u+2w),$$

$$\lambda_3=[M]u(\alpha([D]+[S])u+(2p+w)(b+([D]+[S]u)),$$

$$\lambda_4=\alpha[M]([D]+[S])u^2(2p+w),$$

$$\lambda_5=\lambda_2+\alpha[Mu],$$

$$\lambda_6=\lambda_3+[M]u(\alpha(b+([D]+[S])u),$$

$$\lambda_7=\lambda_4+\alpha^2[M]([D]+[S])u^2,$$

$$\lambda_8=\alpha[M]u(\alpha+2(p+w),\text{ and}$$

$$\lambda_9=\alpha[M]ub(2p+w).$$

Using $\tilde{P}_{E_2S}(S)$ from eq. (A2.5) we obtain the expression for the waiting time distribution (eq. (16)), the mean turnover time (eq. (17)) and the randomness parameter (eq. (18)) as shown in the manuscript.

Table 1

(a) Effect of the binding rate constant u at high [S] for Model III

Kinetic parameters	u	R
w = 1, α = 5, p = 1, [M] = 1, [D] = 0.1, [S] = 5×10^4	0.001	~1
	0.5	0.7174
	1	0.6790
	5	0.7837
w = 1, α = 500, p = 1, [M] = 1, [D] = 0.1, [S] = 5×10^4	1 (fixed)	~1

Table-1 (contd.)

(b) Effect of the activator concentration [M] at high [S] for Model II

Kinetic parameters	u	R
u = 1, w = 1, p = 1, α = 5, [M] = 1, [D] = 0.001, [S] = 5×10^4	0.001	~1
	0.1	0.8940
	1	0.6790
	10	0.8384
u = 1, w = 1, α = 500, p = 1, [M] = 1, [D] = 0.001, [S] = 5×10^4	1 (fixed)	~1

(c) Effect of the deactivator concentration [D] for Model II

Kinetic parameters	[D]	R
u = 1, w = 1, α = 5, p = 1, [M] = 1, [S] = 5×10^4	0.001	0.6790
	1	0.6790
	1×10^3	0.6852
	5×10^6	~1
u = 1, w = 1, α = 500, p = 1, [M] = 1, [S] = 5×10^4	1 (fixed)	~1

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