

# Quantitative full time course analysis of nonlinear enzyme cycling kinetics

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## Supplementary Information

*Theory.*

*Exact solution:* A minimal mechanism describing the catalytic cycle of many enzymes (e.g. hydrolases (EC3) including ATPases<sup>1-3</sup> and nucleotide pyrophosphatase/phosphodiesterase (NPP) family enzymes<sup>4</sup>) is comprised of a single substrate ( $S$ ) that is converted by enzyme ( $E$ ) into two products ( $P_1$  and  $P_2$ ) that dissociate independently (Figure A1). This scheme differs from that assumed by Michaelis and Menten for the glucose hydrolase activity of invertase<sup>5,6</sup> in that it explicitly considers substrate to product transformation<sup>7-9</sup>.

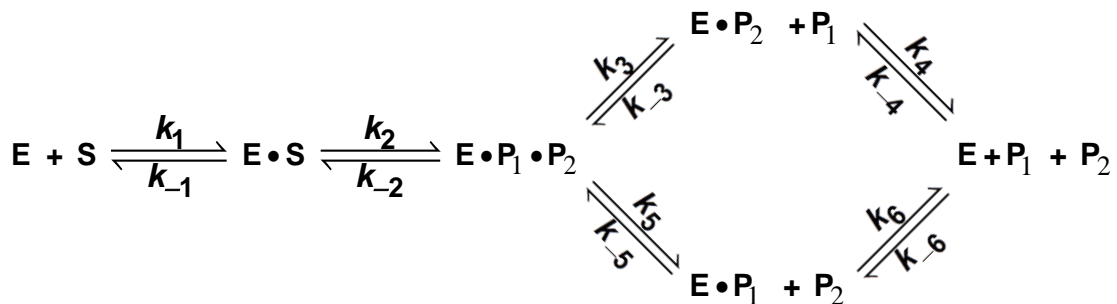


Figure A1. Reaction scheme describing the catalytic cycle of enzymes in which a single substrate ( $S$ ) is converted by enzyme ( $E$ ) into two products ( $P_1$  and  $P_2$ ) that dissociate independently.

For the chemical reaction depicted in Figure A1, there are 4 independent differential equations:

$$\frac{d[E\cdot P_2]}{dt} = k_3[E\cdot P_1\cdot P_2] + k_{-4}[E][P_2] - k_{-3}[E\cdot P_2][P_1] - k_4[E\cdot P_2] \quad A1'$$

$$\frac{d[E\cdot P_1]}{dt} = k_5[E\cdot P_1\cdot P_2] + k_{-6}[E][P_1] - k_{-5}[E\cdot P_1][P_2] - k_6[E\cdot P_1] \quad A2'$$

$$\frac{d[E\cdot P_1\cdot P_2]}{dt} = k_2[E\cdot S] + k_{-3}[E\cdot P_2][P_1] + k_{-5}[E\cdot P_1][P_2] - (k_{-2} + k_3 + k_5)[E\cdot P_1\cdot P_2] \quad A3'$$

$$\frac{d[E\cdot S]}{dt} = k_1[E][S] + k_{-2}[E\cdot P_1\cdot P_2] - (k_{-1} + k_2)[E\cdot S] \quad A4'$$

During steady-state cycling, the steady-state condition requires all the intermediates remain

constants, i.e. in Eqs. A1'-A4',  $\frac{d[E\cdot S]}{dt} = \frac{d[E\cdot P_1\cdot P_2]}{dt} = \frac{d[E\cdot P_1]}{dt} = \frac{d[E\cdot P_2]}{dt} = 0$ . After rearranging

terms, Eqs. A1'-A4' become

$$k_{+3}[E\cdot P_1\cdot P_2] + k_{-4}[E][P_2] = k_{-3}[E\cdot P_2][P_1] + k_{+4}[E\cdot P_2] \quad A1$$

$$k_{+5}[E\cdot P_1\cdot P_2] + k_{-6}[E][P_1] = k_{-5}[E\cdot P_1][P_2] + k_{+6}[E\cdot P_1] \quad A2$$

$$k_{+2}[E\cdot S] + k_{-3}[E\cdot P_2][P_1] + k_{-5}[E\cdot P_1][P_2] = (k_{-2} + k_{+3} + k_{+5})[E\cdot P_1\cdot P_2] \quad A3$$

$$k_{+1}[E][S] + k_{-2}[E\cdot P_1\cdot P_2] = (k_{-1} + k_{+2})[E\cdot S] \quad A4$$

Solving  $[E \cdot P_2]$  in Eq. A1,  $[E \cdot P_1]$  in Eq. A2 and  $[E \cdot S]$  in Eq. A4, and substituting in Eq. A3

yields:

$$k_{+2} \frac{k_{+1}[E][S] + k_{-2}[E \cdot P_1 \cdot P_2]}{k_{-1} + k_{+2}} + k_{-3}[P_1] \frac{k_{+3}[E \cdot P_1 \cdot P_2] + k_{-4}[E][P_2]}{k_{-3}[P_1] + k_{+4}} + k_{-5}[P_2] \frac{k_{+5}[E \cdot P_1 \cdot P_2] + k_{-6}[E][P_1]}{k_{-5}[P_2] + k_{+6}}$$

$$= (k_{-2} + k_{+3} + k_{+5})[E \cdot P_1 \cdot P_2]$$

which can be rearranged and simplified to

$$[E] = \frac{k_{-2} + k_{+3} + k_{+5} - \frac{k_{+2}k_{-2}}{k_{-1} + k_{+2}} - \frac{k_{+3}k_{-3}[P_1]}{k_{-3}[P_1] + k_{+4}} - \frac{k_{+5}k_{-5}[P_2]}{k_{-5}[P_2] + k_{+6}}}{\frac{k_{+1}k_{+2}[S]}{k_{-1} + k_{+2}} + \frac{k_{-3}k_{-4}[P_2][P_1]}{k_{-3}[P_1] + k_{+4}} + \frac{k_{-5}k_{-6}[P_2][P_1]}{k_{-5}[P_2] + k_{+6}}} [E \cdot P_1 \cdot P_2]. \quad A5$$

Solving  $[E \cdot P_2]$  in Eq. A1,  $[E \cdot P_1]$  in Eq. A2, and  $[E \cdot S]$  in Eq. A4 and substituting them with

$[E]$  (Eq. A5) into the mass balance equation for the total enzyme  $[E]_{tot}$  yields:

$$[E]_{tot} = [E] + [E \cdot S] + [E \cdot P_1 \cdot P_2] + [E \cdot P_2] + [E \cdot P_1]$$

$$= \left( 1 + \frac{k_{+1}[S]}{k_{-1} + k_{+2}} + \frac{k_{-4}[P_2]}{k_{-3}[P_1] + k_{+4}} + \frac{k_{-6}[P_1]}{k_{-5}[P_2] + k_{+6}} \right) [E] + \left( 1 + \frac{k_{-2}}{k_{-1} + k_{+2}} + \frac{k_{+3}}{k_{-3}[P_1] + k_{+4}} + \frac{k_{+5}}{k_{-5}[P_2] + k_{+6}} \right) [E \cdot P_1 \cdot P_2]$$

$$= \left( \left( 1 + \frac{k_{+1}[S]}{k_{-1} + k_{+2}} + \frac{k_{-4}[P_2]}{k_{-3}[P_1] + k_{+4}} + \frac{k_{-6}[P_1]}{k_{-5}[P_2] + k_{+6}} \right) \frac{k_{-2} + k_{+3} + k_{+5} - \frac{k_{+2}k_{-2}}{k_{-1} + k_{+2}} - \frac{k_{+3}k_{-3}[P_1]}{k_{-3}[P_1] + k_{+4}} - \frac{k_{+5}k_{-5}[P_2]}{k_{-5}[P_2] + k_{+6}}}{\frac{k_{+1}k_{+2}[S]}{k_{-1} + k_{+2}} + \frac{k_{-3}k_{-4}[P_2][P_1]}{k_{-3}[P_1] + k_{+4}} + \frac{k_{-5}k_{-6}[P_1][P_2]}{k_{-5}[P_2] + k_{+6}}} \right) [E \cdot P_1 \cdot P_2]$$

$$+ \left( 1 + \frac{k_{-2}}{k_{-1} + k_{+2}} + \frac{k_{+3}}{k_{-3}[P_1] + k_{+4}} + \frac{k_{+5}}{k_{-5}[P_2] + k_{+6}} \right) [E \cdot P_1 \cdot P_2] \quad A6$$

and allows for  $[E \cdot P_1 \cdot P_2]$  in Eq. A6 to be solved according to:

$$\begin{aligned}
[E \cdot P_1 \cdot P_2] = & \frac{[E]_{tot}}{\left(1 + \frac{k_{+1}[S]}{k_{-1} + k_{+2}} + \frac{k_{-4}[P_2]}{k_{-3}[P_1] + k_{+4}} + \frac{k_{-6}[P_1]}{k_{-5}[P_2] + k_{+6}}\right) \left(k_{-2} + k_{+3} + k_{+5} - \frac{k_{+2}k_{-2}}{k_{-1} + k_{+2}} - \frac{k_{+3}k_{-3}[P_1]}{k_{-3}[P_1] + k_{+4}} - \frac{k_{+5}k_{-5}[P_2]}{k_{-5}[P_2] + k_{+6}}\right)} \\
& + \frac{k_{+1}k_{+2}[S]}{k_{-1} + k_{+2}} + \frac{k_{-3}k_{-4}[P_1][P_2]}{k_{-3}[P_1] + k_{+4}} + \frac{k_{-5}k_{-6}[P_1][P_2]}{k_{-5}[P_2] + k_{+6}} \\
& + 1 + \frac{k_{-2}}{k_{-1} + k_{+2}} + \frac{k_{+3}}{k_{-3}[P_1] + k_{+4}} + \frac{k_{+5}}{k_{-5}[P_2] + k_{+6}}
\end{aligned} \tag{A7}$$

During steady-state cycling the two product release rates are equal and given by:

$$\begin{aligned}
\frac{d[P_2]}{dt} &= \frac{d[P_1]}{dt} = k_{+6}[E \cdot P_1] - k_{-6}[E][P_1] + k_{+3}[E \cdot P_1 \cdot P_2] - k_{-3}[E \cdot P_2][P_1] \\
&= (k_{+3} + k_{+5})[E \cdot P_1 \cdot P_2] - k_{-5}[E \cdot P_1][P_2] - k_{-3}[E \cdot P_2][P_1] \\
&= k_{+2}[E \cdot S] - k_{-2}[E \cdot P_1 \cdot P_2] \\
&= \frac{k_{+1}k_{+2}}{k_{-1} + k_{+2}}[E][S] - \frac{k_{-1}k_{-2}}{k_{-1} + k_{+2}}[E \cdot P_1 \cdot P_2] \\
&= \frac{\left(\frac{k_{+1}k_{+2}}{k_{-1} + k_{+2}}[S] \left(k_{-2} + k_{+3} + k_{+5} - \frac{k_{+2}k_{-2}}{k_{-1} + k_{+2}} - \frac{k_{+3}k_{-3}[P_1]}{k_{-3}[P_1] + k_{+4}} - \frac{k_{+5}k_{-5}[P_2]}{k_{-5}[P_2] + k_{+6}}\right) - \frac{k_{-1}k_{-2}}{k_{-1} + k_{+2}} \left(\frac{k_{+1}k_{+2}[S]}{k_{-1} + k_{+2}} + \frac{k_{-3}k_{-4}[P_1][P_2]}{k_{-3}[P_1] + k_{+4}} + \frac{k_{-5}k_{-6}[P_1][P_2]}{k_{-5}[P_2] + k_{+6}}\right)\right)[E]_{tot}}{\left(1 + \frac{k_{+1}[S]}{k_{-1} + k_{+2}} + \frac{k_{-4}[P_2]}{k_{-3}[P_1] + k_{+4}} + \frac{k_{-6}[P_1]}{k_{-5}[P_2] + k_{+6}}\right) \left(k_{-2} + k_{+3} + k_{+5} - \frac{k_{+2}k_{-2}}{k_{-1} + k_{+2}} - \frac{k_{+3}k_{-3}[P_1]}{k_{-3}[P_1] + k_{+4}} - \frac{k_{+5}k_{-5}[P_2]}{k_{-5}[P_2] + k_{+6}}\right)} \\
& + \left(1 + \frac{k_{-2}}{k_{-1} + k_{+2}} + \frac{k_{+3}}{k_{-3}[P_1] + k_{+4}} + \frac{k_{+5}}{k_{-5}[P_2] + k_{+6}}\right) \left(\frac{k_{+1}k_{+2}[S]}{k_{-1} + k_{+2}} + \frac{k_{-3}k_{-4}[P_1][P_2]}{k_{-3}[P_1] + k_{+4}} + \frac{k_{-5}k_{-6}[P_1][P_2]}{k_{-5}[P_2] + k_{+6}}\right)
\end{aligned} \tag{A8}$$

Eqs. A2, A4, A5 and A7 were substituted in derivation of Eq. A8. Accordingly, when  $t \rightarrow 0$ ,  $[P_1], [P_2] \rightarrow 0$  and  $[S] \rightarrow [S]_0$ , the initial substrate concentration, and the initial steady-state velocity simplifies to:

$$\begin{aligned}
\frac{d[P_1]}{dt} \Big|_{t \rightarrow 0} &\rightarrow \frac{\left( \frac{k_{+1}k_{+2}}{k_{-1} + k_{+2}} [S]_0 \left( k_{-2} + k_{+3} + k_{+5} - \frac{k_{+2}k_{-2}}{k_{-1} + k_{+2}} \right) - \frac{k_{-1}k_{-2}}{k_{-1} + k_{+2}} \left( \frac{k_{+1}k_{+2}[S]_0}{k_{-1} + k_{+2}} \right) \right) [E]_{tot}}{\left( 1 + \frac{k_{+1}[S]_0}{k_{-1} + k_{+2}} \right) \left( k_{-2} + k_{+3} + k_{+5} - \frac{k_{+2}k_{-2}}{k_{-1} + k_{+2}} \right) + \left( 1 + \frac{k_{-2}}{k_{-1} + k_{+2}} + \frac{k_{+3}}{k_{+4}} + \frac{k_{+5}}{k_{+6}} \right) \left( \frac{k_{+1}k_{+2}[S]_0}{k_{-1} + k_{+2}} \right)} \\
&= \frac{k_{+1}k_{+2}[S]_0 (k_{+3} + k_{+5}) [E]_{tot}}{k_{-1}k_{-2} + (k_{+3} + k_{+5})(k_{-1} + k_{+2}) + k_{+1}[S]_0 \left( k_{-2} + k_{+3} + k_{+5} + k_{+2} + \frac{k_{+2}k_{+3}}{k_{+4}} + \frac{k_{+2}k_{+5}}{k_{+6}} \right)} \\
&= \frac{k_{cat} [E]_{tot} [S]_0}{K_M + [S]_0}
\end{aligned} \tag{A9}$$

which is the familiar Briggs-Haldane equation for the initial substrate concentration-dependent enzyme cycling in the absence of product inhibition and substrate depletion, with the enzymatic reaction parameters

$$\begin{aligned}
k_{cat} &= \frac{k_{+2} (k_{+3} + k_{+5}) k_{+4} k_{+6}}{k_{+4} k_{+6} (k_{+2} + k_{-2} + k_{+3} + k_{+5}) + k_{+2} (k_{+3} k_{+6} + k_{+4} k_{+5})} \\
K_M &= \frac{k_{+4} k_{+6} (k_{-1} k_{-2} + (k_{+3} + k_{+5}) (k_{-1} + k_{+2}))}{k_{+1} (k_{+4} k_{+6} (k_{+2} + k_{-2} + k_{+3} + k_{+5}) + k_{+2} (k_{+3} k_{+6} + k_{+4} k_{+5}))}
\end{aligned} \tag{A10}$$

The steady-state  $k_{cat}$  and  $K_M$  expressions in Eq. A10 are identical to those obtained in the absence of product inhibition <sup>4</sup>.

Under conditions where there is no initial product (i.e.  $[P_1] = [P_2] = 0$  at  $t = 0$ ), and free substrate and product concentrations are much greater than total enzyme (i.e.

$[S], [P_1], [P_2] \gg [E]_{tot}$ ), the two free products are approximately equal ( $[P_2] \sim [P_1] = [P]$ ) and

the free substrate  $[S] \sim [S]_0 - [P_1] = [S]_0 - [P]$ . Eq. A8 can therefore be expressed as:

$$\begin{aligned}
& \left( (k_{+1}k_{+2}k_{+5}k_{+6}[S] - k_{-1}k_{-2}k_{-5}k_{-6}[P_1][P_2]) (k_{-3}[P_1] + k_{+4}) \right. \\
& \left. + (k_{+1}k_{+2}k_{+3}k_{+4}[S] - k_{-1}k_{-2}k_{-3}k_{-4}[P_1][P_2]) (k_{-5}[P_2] + k_{+6}) \right) [E]_{tot} \\
\frac{d[P]}{dt} = & \frac{\left( (k_{+1}(k_{-2} + k_{+2})[S] + k_{-1}k_{-2}) (k_{-3}[P_1] + k_{+4}) (k_{-5}[P_2] + k_{+6}) \right. \\
& + (k_{+3}k_{-4}[P_2]) (k_{-5}[P_2] + k_{+6}) + k_{+5}k_{-6}[P_1] (k_{-3}[P_1] + k_{+4}) \left. \right) (k_{-1} + k_{+2}) \\
& + (k_{-1}k_{-2}k_{-4}[P_2] + k_{+1}k_{+3} (k_{+2} + k_{+4})[S] + (k_{-1} + k_{+2})k_{+3}k_{+4} + (k_{-1} + k_{+2} + k_{-2})k_{-3}k_{-4}[P_1][P_2]) (k_{-5}[P_2] + k_{+6}) \\
& + (k_{-1}k_{-2}k_{-6}[P_1] + k_{+1}k_{+5} (k_{+2} + k_{+6})[S] + (k_{-1} + k_{+2})k_{+5}k_{+6} + (k_{-1} + k_{+2} + k_{-2})k_{-5}k_{-6}[P_1][P_2]) (k_{-3}[P_1] + k_{+4}) \\
& \left. + (k_{+3}k_{+4}k_{-6}[P_1] + k_{-4}k_{+5}k_{+6}[P_2] + k_{-3}k_{-4}k_{+5}[P_1][P_2] + k_{+3}k_{-5}k_{-6}[P_1][P_2]) \right) (k_{-1} + k_{+2}) \\
& \left( (k_{+1}k_{+2}k_{+5}k_{+6} ([S]_0 - [P]) - k_{-1}k_{-2}k_{-5}k_{-6}[P]^2) (k_{-3}[P] + k_{+4}) \right. \\
& \left. + (k_{+1}k_{+2}k_{+3}k_{+4} ([S]_0 - [P]) - k_{-1}k_{-2}k_{-3}k_{-4}[P]^2) (k_{-5}[P] + k_{+6}) \right) [E]_{tot} \\
= & \frac{\left( (k_{+1}(k_{-2} + k_{+2}) ([S]_0 - [P]) + k_{-1}k_{-2}) (k_{-3}[P] + k_{+4}) (k_{-5}[P] + k_{+6}) \right. \\
& + (k_{+3}k_{-4}[P]) (k_{-5}[P] + k_{+6}) + k_{+5}k_{-6}[P] (k_{-3}[P] + k_{+4}) \left. \right) (k_{-1} + k_{+2}) \\
& + (k_{-1}k_{-2}k_{-4}[P] + k_{+1}k_{+3} (k_{+2} + k_{+4}) ([S]_0 - [P]) + (k_{-1} + k_{+2})k_{+3}k_{+4} + (k_{-1} + k_{+2} + k_{-2})k_{-3}k_{-4}[P]^2) (k_{-5}[P] + k_{+6}) \\
& + (k_{-1}k_{-2}k_{-6}[P] + k_{+1}k_{+5} (k_{+2} + k_{+6}) ([S]_0 - [P]) + (k_{-1} + k_{+2})k_{+5}k_{+6} + (k_{-1} + k_{+2} + k_{-2})k_{-5}k_{-6}[P]^2) (k_{-3}[P] + k_{+4}) \\
& \left. + (k_{+3}k_{+4}k_{-6}[P] + k_{-4}k_{+5}k_{+6}[P] + k_{-3}k_{-4}k_{+5}[P]^2 + k_{+3}k_{-5}k_{-6}[P]^2) \right) (k_{-1} + k_{+2}) \\
= & \frac{\alpha_0 + \alpha_1[P] - \alpha_2[P]^2 - \alpha_3[P]^3}{\beta_0 + \beta_1[P] + \beta_2[P]^2 + \beta_3[P]^3} [E]_{tot} \tag{A11}
\end{aligned}$$

where some of the  $\alpha_i$  and  $\beta_i$  ( $i = 0, 1, 2, 3$ ) constants depend on the initial substrate concentration.

The third order polynomial function in the numerator of Eq. A11,

$\alpha_0 + \alpha_1[P] - \alpha_2[P]^2 - \alpha_3[P]^3$ , has three roots, which are a positive root,  $r$ , and either two

negative roots or a complex conjugate pair of roots<sup>10</sup>. Therefore, Eq. A11 can be rearranged as

follows

$$\left( -\frac{\beta_3}{\alpha_3} - \frac{1}{\alpha_3^2} \left( \frac{q}{[P] - r} + \frac{B_1[P] + B_0}{[P]^2 + A_1[P] + A_0} \right) \right) d[P] = [E]_{tot} dt \tag{A12}$$

where the new coefficients  $q, A_0, A_1, B_0,$  and  $B_1$  are combinations of  $\alpha_i$  and  $\beta_i$  ( $i = 0, 1, 2, 3$ ) that may or may not depend on the initial substrate concentration. Integrating both sides of Eq. A12 and incorporating the initial condition  $[P] = 0$  yields:

$$\begin{aligned}
& -\frac{\beta_3}{\alpha_3}[P] + \frac{q}{\alpha_3^2} \ln \frac{r}{r-[P]} + \frac{B_1}{2\alpha_3^2} \ln \frac{A_0}{[P]^2 + A_1[P] + A_0} \\
& + \frac{1}{\alpha_3^2} \left( \frac{A_1 B_1}{2} - B_0 \right) \frac{2}{\sqrt{4A_0 - A_1^2}} \left( \arctan \left( \frac{2[P] + A_1}{\sqrt{4A_0 - A_1^2}} \right) - \arctan \left( \frac{A_1}{\sqrt{4A_0 - A_1^2}} \right) \right) \\
& = [E]_{tot} t, \quad \text{for } A_1^2 < 4A_0
\end{aligned} \tag{A13}$$

or

$$\begin{aligned}
& -\frac{\beta_3}{\alpha_3}[P] + \frac{q}{\alpha_3^2} \ln \frac{r}{r-[P]} + \frac{B_1}{2\alpha_3^2} \ln \frac{A_0}{[P]^2 + A_1[P] + A_0} \\
& + \frac{1}{\alpha_3^2} \left( \frac{A_1 B_1}{2} - B_0 \right) \frac{1}{\sqrt{A_1^2 - 4A_0}} \ln \frac{\frac{2[P]}{A_1 - \sqrt{A_1^2 - 4A_0}} + 1}{\frac{2[P]}{A_1 + \sqrt{A_1^2 - 4A_0}} + 1} \\
& = [E]_{tot} t, \quad \text{for } A_1^2 > 4A_0
\end{aligned} \tag{A13'}$$

Although Eqs. A13 and A13' represent exact analytical solutions of differential equation Eq. A11, the concentration of free enzymatic product  $[P]$  is a complicated implicit function of time ( $t$ ) composed of a linear, two logarithmic and an inverse tangent or additional logarithmic function, and thus it is difficult to extract physically meaningful parameters (e.g.  $k_{cat}$  and  $K_M$ ). An approximation approach to obtain a solution of the concentration of free product  $[P]$  that allows for physically meaningful parameters to be extracted is as follows.

*First order approximation analysis:* Given that the normalized free product  $x = \frac{[P]}{[S]_0} < 1$  and

letting  $k_{+4}' = \frac{k_{+4}}{[S]_0}$ ,  $k_{+6}' = \frac{k_{+6}}{[S]_0}$  (ratio between product release rate constant(s) and initial

substrate concentration) and normalized product release rate  $\frac{dx}{dt} = \frac{1}{[S]_0} \frac{d[P]}{dt}$ , the differential

equation Eq. A11 of  $[P]$  becomes the following differential equation of a *small* variable  $x$ :

$$\begin{aligned} \frac{dx}{dt} = & \frac{\left( (k_{+1}k_{+2}k_{+5}k_{+6}'(1-x) - k_{-1}k_{-2}k_{-5}k_{-6}x^2)(k_{-3}x + k_{+4}') \right. \\ & \left. + (k_{+1}k_{+2}k_{+3}k_{+4}'(1-x) - k_{-1}k_{-2}k_{-3}k_{-4}x^2)(k_{-5}x + k_{+6}') \right) [E]_{tot}}{\left( k_{+1}(k_{-2} + k_{+2})[S]_0(1-x) + k_{-1}k_{-2} \right) (k_{-3}x + k_{+4}') (k_{-5}x + k_{+6}') \\ & + (k_{+3}k_{-4}x(k_{-5}x + k_{+6}') + k_{+5}k_{-6}x(k_{-3}x + k_{+4}')) (k_{-1} + k_{+2}) \\ & + (k_{-1}k_{-2}k_{-4}x + k_{+1}k_{+3}(k_{+2} + [S]_0k_{+4}'))(1-x) + (k_{-1} + k_{+2})k_{+3}k_{+4}' + (k_{-1} + k_{+2} + k_{-2})k_{-3}k_{-4}x^2[S]_0 \Big) (k_{-5}x + k_{+6}') \\ & + (k_{-1}k_{-2}k_{-6}x + k_{+1}k_{+5}(k_{+2} + [S]_0k_{+6}'))(1-x) + (k_{-1} + k_{+2})k_{+5}k_{+6}' + (k_{-1} + k_{+2} + k_{-2})k_{-5}k_{-6}x^2[S]_0 \Big) (k_{-3}x + k_{+4}') \\ & + (k_{+3}k_{+4}'k_{-6}x + k_{-4}k_{+5}k_{+6}'x + k_{-3}k_{-4}k_{+5}x^2 + k_{+3}k_{-5}k_{-6}x^2) (k_{-1} + k_{+2})} \\ = & \frac{Q(x)}{P(x)} [E]_{tot} = f(x) [E]_{tot} . \end{aligned} \tag{A14}$$

where  $P(x)$ ,  $Q(x)$  and  $f(x)$  are functions of  $x$  defined as

$$f(x) = \frac{Q(x)}{P(x)} \tag{A15}$$



$$\begin{aligned}
P(x) &= (k_{+1}(k_{-2} + k_{+2})[S]_0(1-x) + k_{-1}k_{-2})(k_{-3}x + k_{+4}')(k_{-5}x + k_{+6}') \\
&+ (k_{+3}k_{-4}x(k_{-5}x + k_{+6}') + k_{+5}k_{-6}x(k_{-3}x + k_{+4}'))(k_{-1} + k_{+2}) \\
&+ (k_{-1}k_{-2}k_{-4}x + k_{+1}k_{+3}(k_{+2} + [S]_0k_{+4}'))(1-x) + (k_{-1} + k_{+2})k_{+3}k_{+4}' + (k_{-1} + k_{+2} + k_{-2})k_{-3}k_{-4}x^2[S]_0)(k_{-5}x + k_{+6}') \\
&+ (k_{-1}k_{-2}k_{-6}x + k_{+1}k_{+5}(k_{+2} + [S]_0k_{+6}'))(1-x) + (k_{-1} + k_{+2})k_{+5}k_{+6}' + (k_{-1} + k_{+2} + k_{-2})k_{-5}k_{-6}x^2[S]_0)(k_{-3}x + k_{+4}') \\
&+ (k_{+3}k_{+4}'k_{-6}x + k_{-4}k_{+5}k_{+6}'x + k_{-3}k_{-4}k_{+5}x^2 + k_{+3}k_{-5}k_{-6}x^2)(k_{-1} + k_{+2})
\end{aligned} \tag{A16}$$

and

$$\begin{aligned}
Q(x) &= (k_{+1}k_{+2}k_{+5}k_{+6}'(1-x) - k_{-1}k_{-2}k_{-5}k_{-6}x^2)(k_{-3}x + k_{+4}') \\
&+ (k_{+1}k_{+2}k_{+3}k_{+4}'(1-x) - k_{-1}k_{-2}k_{-3}k_{-4}x^2)(k_{-5}x + k_{+6}') \\
&= k_{+1}k_{+2}k_{+4}'k_{+6}'(k_{+3} + k_{+5}) + k_{+1}k_{+2}((k_{-3}k_{+5}k_{+6}' + k_{+3}k_{+4}'k_{-5}) - k_{+4}'k_{+6}'(k_{+3} + k_{+5}))x \\
&- (k_{+1}k_{+2}(k_{-3}k_{+5}k_{+6}' + k_{+3}k_{+4}'k_{-5}) + k_{-1}k_{-2}(k_{-3}k_{-4}k_{+6}' + k_{+4}'k_{-5}k_{-6}))x^2 - k_{-1}k_{-2}k_{-3}k_{-5}(k_{-4} + k_{-6})x^3
\end{aligned} \tag{A17}$$

with partial derivatives to  $x$  as  $f'(x)$ ,

$$\begin{aligned}
P'(x) &= (-k_{+1}(k_{-2} + k_{+2})[S]_0)(k_{-3}x + k_{+4}')(k_{-5}x + k_{+6}') \\
&+ (k_{+1}(k_{-2} + k_{+2})[S]_0(1-x) + k_{-1}k_{-2})k_{-3}(k_{-5}x + k_{+6}') \\
&+ (k_{+1}(k_{-2} + k_{+2})[S]_0(1-x) + k_{-1}k_{-2})(k_{-3}x + k_{+4}')k_{-5} \\
&+ (k_{+3}k_{-4}(k_{-5}x + k_{+6}') + k_{+3}k_{-4}xk_{-5} + k_{+5}k_{-6}(k_{-3}x + k_{+4}') + k_{-3}k_{+5}k_{-6}x)(k_{-1} + k_{+2}) \\
&+ (k_{-1}k_{-2}k_{-4} - k_{+1}k_{+3}(k_{+2} + [S]_0k_{+4}')) + 2(k_{-1} + k_{+2} + k_{-2})k_{-3}k_{-4}x[S]_0)(k_{-5}x + k_{+6}') \\
&+ (k_{-1}k_{-2}k_{-4}x + k_{+1}k_{+3}(k_{+2} + [S]_0k_{+4}'))(1-x) + (k_{-1} + k_{+2})k_{+3}k_{+4}' + (k_{-1} + k_{+2} + k_{-2})k_{-3}k_{-4}x^2[S]_0)k_{-5} \\
&+ (k_{-1}k_{-2}k_{-6} - k_{+1}k_{+5}(k_{+2} + [S]_0k_{+6}')) + 2(k_{-1} + k_{+2} + k_{-2})k_{-5}k_{-6}x[S]_0)(k_{-3}x + k_{+4}') \\
&+ (k_{-1}k_{-2}k_{-6}x + k_{+1}k_{+5}(k_{+2} + [S]_0k_{+6}'))(1-x) + (k_{-1} + k_{+2})k_{+5}k_{+6}' + (k_{-1} + k_{+2} + k_{-2})k_{-5}k_{-6}x^2[S]_0)k_{-3} \\
&+ (k_{+3}k_{+4}'k_{-6} + k_{-4}k_{+5}k_{+6}' + 2k_{-3}k_{-4}k_{+5}x + 2k_{+3}k_{-5}k_{-6}x)(k_{-1} + k_{+2})
\end{aligned} \tag{A18}$$

and

$$Q'(x) = k_{+1}k_{+2} \left( (k_{-3}k_{+5}k_{+6} + k_{+3}k_{+4} 'k_{-5}) - k_{+4} 'k_{+6} '(k_{+3} + k_{+5}) \right) - 2(k_{+1}k_{+2} (k_{-3}k_{+5}k_{+6} + k_{+3}k_{+4} 'k_{-5}) + k_{-1}k_{-2} (k_{-3}k_{-4}k_{+6} + k_{+4} 'k_{-5}k_{-6}))x - 3k_{-1}k_{-2}k_{-3}k_{-5} (k_{-4} + k_{-6})x^2 \quad \text{A19}$$

Since  $x = \frac{[P]}{[S]_0} < 1$ , and is therefore a small variable, the normalized product release rate  $\frac{dx}{dt}$  in

Eq. A14 can be expanded at  $x = 0$  using the Taylor series expansion.

$$\frac{dx}{dt} = f(x)[E]_{tot} = \left( f(0) + f'(0)x + O(x^2) \right) [E]_{tot} \quad \text{A20}$$

where  $O(x^2)$  is a very small quantity proportional to the second order of the variable  $x$  that can be ignored in the first order approximation. In Eq. A20, the fact that  $x$  is a normalized free product concentration that always increases (i.e. the rate,  $\frac{dx}{dt} > 0$ ), but the increase gradually slows down due to product inhibition or substrate depletion (i.e. the second derivative of  $x$  to  $t$  is negative) leads to

$$f(0) = \frac{1}{[E]_{tot}} \left. \frac{dx}{dt} \right|_{x=0} > 0$$

$$f'(0) = \left. \frac{\partial f(x)}{\partial x} \right|_{x=0} = \frac{1}{[E]_{tot}} \left. \frac{d^2x}{dt^2} \right|_{x=0} < 0 \quad \text{A21}$$

At the first order approximation, the solution to the linear differential equation Eq. A20 with the initial condition  $x = 0$  is

$$x = \frac{v_0}{[S]_0 \eta} (1 - e^{-\eta t}) \quad \text{A22}$$

or

$$[P] = \frac{v_0}{\eta} (1 - e^{-\eta t}). \quad \text{A23}$$

where  $v_0$  is the initial steady-state velocity defined as

$$\begin{aligned} v_0 &= f(0)[S]_0[E]_{tot} = \frac{Q(0)}{P(0)}[S]_0[E]_{tot} \\ &= \frac{k_{+1}k_{+2}(k_{+3} + k_{+5})k_{+4}k_{+6}[S]_0[E]_{tot}}{k_{+1}((k_{-2} + k_{+2} + k_{+3} + k_{+5})k_{+4}k_{+6} + k_{+2}(k_{+3}k_{+6} + k_{+4}k_{+5}))[S]_0 + (k_{-1}k_{-2} + (k_{-1} + k_{+2})(k_{+3} + k_{+5}))k_{+4}k_{+6}} \\ &= \frac{k_{cat}[S]_0[E]_{tot}}{K_M + [S]_0} \end{aligned} \quad \text{A24}$$

with  $k_{cat}$  and  $K_M$  expressed as in Eq. 10.

We introduce a new term,  $\eta$ , to describe the decay of the steady-state cycling rate due to product inhibition or substrate depletion as:

$$\eta = -f'(0)[E]_{tot} = -\left(\frac{Q'(0)P(0) - Q(0)P'(0)}{P(0)^2}\right)[E]_{tot}$$

which can be expressed in terms of reaction rate constants as follows:

$$\begin{aligned} &k_{+1}k_{+2}[E]_{tot} \left( k_{+1}(k_{+4}k_{+6}(k_{-2} + k_{+2})(k_{+3}k_{-3}k_{+6} + k_{+4}k_{+5}k_{-5}) \right. \\ &+ k_{+2}k_{+3}k_{+5}(k_{+4} - k_{+6})(k_{-3}k_{+6} - k_{+4}k_{-5}))[S]_0^2 \\ &+ k_{+4}k_{+6}(k_{-1}k_{-2}(k_{+3}k_{-3}k_{+6} + k_{+3}k_{-4}k_{+6} + k_{+3}k_{+4}k_{-6} + k_{+4}k_{+5}k_{-5} + k_{-4}k_{+5}k_{+6} + k_{+4}k_{+5}k_{-6}) \\ &+ (k_{-1} + k_{+2})(k_{+3} + k_{+5})^2(k_{-4}k_{+6} + k_{+4}k_{-6}))[S]_0 \\ &+ (k_{+3} + k_{+5})(k_{+4}k_{+6})^2(k_{-1}k_{-2} + (k_{-1} + k_{+2})(k_{+3} + k_{+5})) \left. \right) \\ \eta &= \frac{\quad}{(k_{+1}((k_{-2} + k_{+2} + k_{+3} + k_{+5})k_{+4}k_{+6} + k_{+2}(k_{+3}k_{+6} + k_{+4}k_{+5}))[S]_0 + (k_{-1}k_{-2} + (k_{-1} + k_{+2})(k_{+3} + k_{+5}))k_{+4}k_{+6})^2} \quad \text{A25} \end{aligned}$$

When  $\eta \rightarrow 0$ , from Eq. A23,  $[P] \rightarrow v_0 t$ , i.e. the time course of product formation becomes linear with time. Eq. A23 predicts that  $[P] \rightarrow \frac{v_0}{\eta}$  as  $t \rightarrow \infty$  and the enzymatic reaction reaches equilibrium. In other words, accumulation of product gradually decreases the steady-state cycling rate to 0 (i.e., the substrate, enzyme and product(s) are in equilibrium, with no net conversion of substrate(s) to product(s)).

The value of  $\eta$  serves as a valuable experimental diagnostic for assessing time course non-linearity, specifically identifying the origin of non-linearity (product inhibition vs. substrate depletion). For non-linearity to be negligible and product formation to be linear across the observed time range  $\tau$ ,  $\eta\tau \ll 1$ ; that is,  $\eta \ll \frac{1}{\tau}$ . When  $[S]_0 \rightarrow 0$ , Eq. A25 simplifies to:

$$\eta \rightarrow \frac{k_{+1}k_{+2}(k_{+3} + k_{+5})[E]_{tot}}{k_{-1}k_{-2} + (k_{-1} + k_{+2})(k_{+3} + k_{+5})} = \frac{k_{cat}}{K_M} [E]_{tot}. \quad A26$$

Under this condition  $\eta$  is independent of product binding and non-linearity arises from substrate depletion, which is proportional to  $\frac{k_{cat}}{K_M}$ . When  $[S]_0 \rightarrow \infty$ , Eq. A25 simplifies to:

$$\eta \rightarrow \frac{k_{+2}(k_{+4}k_{+6}(k_{-2} + k_{+2})(k_{+3}k_{-3}k_{+6} + k_{+4}k_{+5}k_{-5}) + k_{+2}k_{+3}k_{+5}(k_{+4} - k_{+6})(k_{-3}k_{+6} - k_{+4}k_{-5})) [E]_{tot}}{\left( (k_{-2} + k_{+2} + k_{+3} + k_{+5})k_{+4}k_{+6} + k_{+2}(k_{+3}k_{+6} + k_{+4}k_{+5}) \right)^2} \quad A27$$

Under this condition the value of  $\eta$  and any observed non-linearity in enzyme activity time courses are dominated by product inhibition. Note that  $\eta \propto [E]_{tot}$ , which quantitatively predicts

how reducing the enzyme concentration reduces both substrate depletion and product inhibition effects.

The rate equation for product formation (Eq. A20) can be rewritten using the definition of  $v_0$  and  $\eta$  as follows:

$$v = \frac{d[P]}{dt} = v_0 - \eta[P] \quad \text{A28}$$

If the initial velocity  $v_0$  and rate constant of the decrease in product formation  $\eta$  are obtained from the analysis of a non-linear time course of product formation by Eq. A23, Eq. A28 provides a method to calculate the velocity  $v$  at any given product concentration  $[P]$  for all the initial substrate concentration  $[S]_0$ . Subsequently, the plots of  $v$  versus  $[S]_0$  for several different product (the inhibitor in this case) concentrations can be used to analyze the inhibitor (product) binding constant(s) using the standard inhibition analysis technique for enzyme kinetics<sup>7</sup> even though product formation time courses are non-linear, which we demonstrate in the main text.

*Second order approximation analysis:* The time-dependent product formation Eq. A23 was derived from applying the first order approximation to the rate equation (Eqs. A14 and A20). For the second order approximation, the rate equation Eq. A20 becomes

$$\frac{dx}{dt} = f(x)[E]_{tot} = \left( f(0) + f'(0)x + \frac{1}{2} f''(0)x^2 + O(x^3) \right) [E]_{tot} \quad \text{A29}$$

where  $f''(x)$  is the second partial derivative to  $x$ .  $O(x^3)$  is a small quantity proportional to the third order of  $x$  and is ignored at the second order approximation. Integration of Eq. A29 yields

$$\left\{ \begin{array}{l} \frac{f''(0)x + f'(0) - \frac{\Delta}{[E]_{tot}}}{f''(0)x + f'(0) + \frac{\Delta}{[E]_{tot}}} = Ce^{\Delta t}, \quad \text{if } (f'(0)^2 - 2f''(0)f(0))[E]_{tot}^2 = \Delta^2 > 0 \\ 2f''(0)x + f'(0) = \frac{\Delta}{[E]_{tot}} \tan\left(\frac{\Delta(t+C)}{2}\right) \quad \text{if } (f'(0)^2 - 2f''(0)f(0))[E]_{tot}^2 = -\Delta^2 < 0 \end{array} \right. \quad . \text{A30}$$

$C$  in Eq. A30 is an arbitrary constant to be determined by the initial conditions.  $\Delta$  is defined in the equation. Since a tangent function is not a physically meaningful solution for the time course of product formation, the condition  $f'(0)^2 - 2f''(0)f(0) < 0$  would never occur and the solution with a tangent function is disregarded. Eq. A30 with the initial condition  $x = 0$  can be written as

$$\begin{aligned} [P] &= \frac{2f(0)[S]_0(1-e^{-\Delta t})}{\frac{\Delta}{[E]_{tot}} - f'(0) + \left(\frac{\Delta}{[E]_{tot}} + f'(0)\right)e^{-\Delta t}} = \frac{2\frac{v_0}{[E]_{tot}}(1-e^{-\Delta t})}{\frac{\Delta}{[E]_{tot}} + \frac{\eta}{[E]_{tot}} + \left(\frac{\Delta}{[E]_{tot}} - \frac{\eta}{[E]_{tot}}\right)e^{-\Delta t}} \quad \text{A31} \\ &= \frac{2v_0(1-e^{-\Delta t})}{\Delta + \eta + (\Delta - \eta)e^{-\Delta t}} \end{aligned}$$

where  $v_0$ ,  $\eta$  and  $\Delta$  are defined in Eqs. A24, A25 and A30, respectively.

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