Supporting Information: High-order Michaelis-Menten equations allow inference of hidden kinetic parameters in enzyme catalysis

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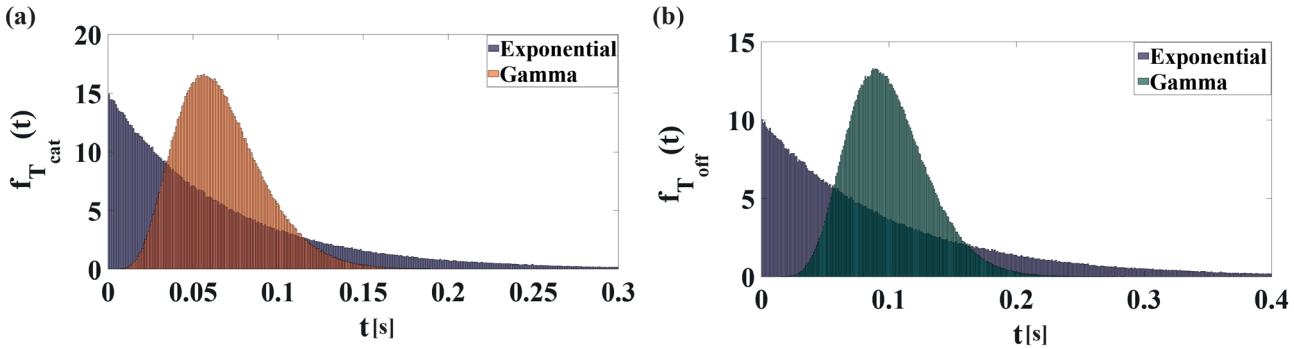
1. Histograms of characteristic kinetic times for the three different models that were discussed in Figs. 2,3, and 4.



Supplementary Fig. 1: Histograms of characteristic kinetic times. Histograms of the (a) catalysis times, T_{cat} and (b) unbinding times, T_{off} that were used in the three different models that were discussed in Figs. 2,3, and 4. These numerical histograms give the probability density functions of catalysis, $f_{T_{cat}}(t)$ and unbinding, $f_{T_{off}}(t)$, respectively. In the first model, the exponential catalysis time (Exponential; blue) in panel (a) was combined with the exponential unbinding time (Exponential; blue) in panel (b). In the second model, the Gamma catalysis time (Gamma; orange) in panel (a) was combined with the exponential unbinding time (Exponential; blue) in panel (b). In the third model, the Gamma catalysis time (Gamma; orange) in panel (a) was combined with the Gamma unbinding time (Gamma; green) in panel (b). Details of the kinetic parameters are provided in the Methods section. Codes employed for generating these PDFs have been supplied as Supplementary Software.

2. Derivation of Eq. (7)

Using Eq. (1) and the conditional waiting times defined in Eqs. (2) and (3) in the main text, we obtain

$$f_{T_{turn}}(t) = \phi_{cat} \int_0^t f_{T_{on}}(t')\, f_{W_{ES}^{cat}}(t-t')\, dt' + (1-\phi_{cat}) \int_0^t g(t')\, f_{T'_{turn}}(t-t')\, dt' , \qquad (1)$$

where $g(t') = \int_0^{t'} f_{T_{on}}(t'') f_{W_{ES}^{off}}(t'-t'')\, dt''$. The first integral on the right-hand-side of Supplementary Equation (1) is the convolution of the binding time distribution, $f_{T_{on}}(t)$ with the conditional catalysis time distribution $f_{W_{ES}^{cat}}(t)$. In the second integral, we have $g(t)$ which itself is the convolution of the binding time distribution with the conditional unbinding time distribution $f_{W_{ES}^{off}}(t)$. Since unbinding is followed by a fresh turnover cycle, the function $g(t)$ should also be convolved with the probability distribution, $f_{T'_{turn}}(t)$, of a new turnover time. Laplace transforming Supplementary Equation (1) we obtain

$$\hat{f}_{T_{turn}}(k) = \phi_{cat}\, \hat{f}_{T_{on}}(k) \hat{f}_{W_{ES}^{cat}}(k) + (1-\phi_{cat})\, \hat{f}_{T_{on}}(k) \hat{f}_{W_{ES}^{off}}(k) \hat{f}_{T'_{turn}}(k), \qquad (2)$$

where we used

$$\hat{g}(k) = \int_0^\infty e^{-kt} \left(\int_0^t f_{T_{on}}(t') f_{W_{ES}^{off}}(t-t')\, dt' \right) dt = \hat{f}_{T_{on}}(k) \hat{f}_{W_{ES}^{off}}(k). \qquad (3)$$

Furthermore, since T'_{turn} is an identical and independent copy of T_{turn} we have $\hat{f}_{T_{turn}}(k) = \hat{f}_{T'_{turn}}(k)$. The Laplace transform of the turnover time distribution can then be written as

$$\hat{f}_{T_{turn}}(k) = \frac{\phi_{cat}\, \hat{f}_{T_{on}}(k)\hat{f}_{W_{ES}^{off}}(k)}{1 - (1 - \phi_{cat})\, \hat{f}_{T_{on}}(k)\hat{f}_{W_{ES}^{off}}(k)}. \tag{4}$$

After some rearrangements, Supplementary Equation (4) can be rewritten as

$$\hat{f}_{T_{turn}}(k) = \frac{\phi_{cat}\hat{f}_{W_{ES}^{cat}}(k)}{\frac{1}{\hat{f}_{T_{on}}(k)} - (1 - \phi_{cat})\hat{f}_{W_{ES}^{off}}(k)}. \tag{5}$$

To proceed, we Laplace transform Eq. (6) in the main text. This gives

$$\hat{f}_{W_{ES}}(k) = \phi_{cat}\, \hat{f}_{W_{ES}^{cat}}(k) + (1 - \phi_{cat})\hat{f}_{W_{ES}^{off}}(k), \tag{6}$$

and using this relation to substitute for $(1 - \phi_{cat})\hat{f}_{W_{ES}^{off}}(k)$ in Supplementary Equation (5) gives

$$\hat{f}_{T_{turn}}(k) = \frac{\phi_{cat}\hat{f}_{W_{ES}^{cat}}(k)}{\frac{1}{\hat{f}_{T_{on}}(k)} + \phi_{cat}\hat{f}_{W_{ES}^{cat}}(k) - \hat{f}_{W_{ES}}(k)}. \tag{7}$$

Eq. (7) in the main text follows by assuming that substrate binding times are exponentially distributed with rate $k_{on}[S]$. Under this assumption, we have

$$\frac{1}{\hat{f}_{T_{on}}(k)} = 1 + \frac{k}{k_{on}[S]}, \tag{8}$$

and substituting back into Supplementary Equation (7) gives Eq. (7).

3. Derivation of Eq. (10)

Expanding the left-hand side of Eq. (9) from the main text in Taylor series, we get

$$\frac{1}{\hat{f}_{T_{turn}}(k)} = \sum_{n=0}^{\infty} \frac{\partial^n}{\partial k^n}\left(\frac{1}{\hat{f}_{T_{turn}}(k)}\right)\Big|_{k=0} \frac{k^n}{n!}. \tag{9}$$

Now, we differentiate each term on the right-hand side of Supplementary Equation (9) with respect to k. This gives Supplementary Equation (10).

$$\frac{1}{\hat{f}_{T_{turn}}(k)} = \frac{1}{\hat{f}_{T_{turn}}(k)}\bigg|_{k=0} - \frac{1}{\left(\hat{f}_{T_{turn}}(k)\right)^2}\left(\frac{\partial}{\partial k}\hat{f}_{T_{turn}}(k)\right)\bigg|_{k=0}\frac{k}{1!}$$
$$+\left\{-\frac{1}{\left(\hat{f}_{T_{turn}}(k)\right)^2}\left(\frac{\partial^2}{\partial k^2}\hat{f}_{T_{turn}}(k)\right)\bigg|_{k=0}\right.$$
$$\left.+\frac{2}{\left(\hat{f}_{T_{turn}}(k)\right)^3}\left(\frac{\partial}{\partial k}\hat{f}_{T_{turn}}(k)\right)^2\bigg|_{k=0}\right\}\frac{k^2}{2!}$$
$$+\left\{-\frac{1}{\left(\hat{f}_{T_{turn}}(k)\right)^2}\left(\frac{\partial^3}{\partial k^3}\hat{f}_{T_{turn}}(k)\right)\bigg|_{k=0}\right. \tag{10}$$
$$+\frac{6}{\left(\hat{f}_{T_{turn}}(k)\right)^3}\left(\frac{\partial}{\partial k}\hat{f}_{T_{turn}}(k)\right)\left(\frac{\partial^2}{\partial k^2}\hat{f}_{T_{turn}}(k)\right)\bigg|_{k=0}$$
$$\left.-\frac{6}{\left(\hat{f}_{T_{turn}}(k)\right)^4}\left(\frac{\partial}{\partial k}\hat{f}_{T_{turn}}(k)\right)^3\bigg|_{k=0}\right\}\frac{k^3}{3!}+\cdots$$

The normalization of the turnover time probability distribution function (PDF) and known relations between the Laplace transform of a random variable and its moments, give the following equations:

$$\hat{f}_{T_{turn}}(k)\big|_{k=0} = 1 \tag{11}$$

and

$$\langle T_{turn}^n\rangle = (-1)^n\left(\frac{\partial^n}{\partial k^n}\hat{f}_{T_{turn}}(k)\right)\bigg|_{k=0}. \tag{12}$$

Using the above set of equations, we rewrite Supplementary Equation (10) as

$$\frac{1}{\hat{f}_{T_{turn}}(k)} = 1 + \langle T_{turn}\rangle k + \left(\langle T_{turn}\rangle^2 - \frac{\langle T_{turn}^2\rangle}{2}\right)k^2 \tag{13}$$
$$+\left(\frac{\langle T_{turn}^3\rangle}{6} - \langle T_{turn}\rangle\langle T_{turn}^2\rangle + \langle T_{turn}\rangle^3\right)k^3 + \cdots$$

Similarly, the first and second terms on the right-hand side of Eq. (9) of the main text can be written as follows

$$\frac{1}{\phi_{cat}k_{on}}\left(\frac{k}{\hat{f}_{W_{ES}^{cat}}(k)}\right) = \frac{1}{\phi_{cat}k_{on}}\sum_{n=0}^{\infty}\frac{\partial^n}{\partial k^n}\left(\frac{k}{\hat{f}_{W_{ES}^{cat}}(k)}\right)\bigg|_{k=0}\frac{k^n}{n!} \tag{14}$$

and

$$\frac{1-\hat{f}_{W_{ES}}(k)}{\phi_{cat}\hat{f}_{W_{ES}^{cat}}(k)}=\frac{1}{\phi_{cat}}\sum_{n=0}^{\infty}\frac{\partial^n}{\partial k^n}\left(\frac{1-\hat{f}_{W_{ES}}(k)}{\hat{f}_{W_{ES}^{cat}}(k)}\right)\Bigg|_{k=0}\frac{k^n}{n!}. \tag{15}$$

Equation (10) follows from equating equal order k terms on both sides of Eq. (9).

4. Derivation of A_n and B_n in Table I

To obtain the expressions in Table I, we differentiate the terms on the right-hand side of Supplementary Equation (14) with respect to k. This gives

$$\begin{aligned}\frac{1}{\phi_{cat}k_{on}}\left(\frac{k}{\hat{f}_{W_{ES}^{cat}}(k)}\right)=\frac{1}{\phi_{cat}k_{on}}\Bigg[&\left\{\frac{1}{\hat{f}_{W_{ES}^{cat}}(k)}\Bigg|_{k=0}-\frac{k}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^2}\left(\frac{\partial}{\partial k}\hat{f}_{W_{ES}^{cat}}(k)\right)\Bigg|_{k=0}\right\}\frac{k}{1!}+\\
&\left\{\frac{2k}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^3}\left(\frac{\partial}{\partial k}\hat{f}_{W_{ES}^{cat}}(k)\right)^2\Bigg|_{k=0}-\frac{2}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^2}\left(\frac{\partial}{\partial k}\hat{f}_{W_{ES}^{cat}}(k)\right)\Bigg|_{k=0}-\right.\\
&\left.\frac{k}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^2}\left(\frac{\partial^2}{\partial k^2}\hat{f}_{W_{ES}^{cat}}(k)\right)\Bigg|_{k=0}\right\}\frac{k^2}{2!}+\left\{-\frac{6k}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^4}\left(\frac{\partial}{\partial k}\hat{f}_{W_{ES}^{cat}}(k)\right)^3\Bigg|_{k=0}+\right.\\
&\frac{6}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^3}\left(\frac{\partial}{\partial k}\hat{f}_{W_{ES}^{cat}}(k)\right)^2\Bigg|_{k=0}+\frac{6k}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^3}\left(\frac{\partial}{\partial k}\hat{f}_{W_{ES}^{cat}}(k)\right)\left(\frac{\partial^2}{\partial k^2}\hat{f}_{W_{ES}^{cat}}(k)\right)\Bigg|_{k=0}-\\
&\left.\frac{3}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^2}\left(\frac{\partial^2}{\partial k^2}\hat{f}_{W_{ES}^{cat}}(k)\right)\Bigg|_{k=0}-\frac{k}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^2}\left(\frac{\partial^3}{\partial k^3}\hat{f}_{W_{ES}^{cat}}(k)\right)\Bigg|_{k=0}\right\}\frac{k^3}{3!}+\cdots\Bigg].\end{aligned} \tag{16}$$

Similarly, differentiating the terms on the right-hand side of Supplementary Equation (15) gives

$$
\begin{aligned}
\frac{1-\hat{f}_{W_{ES}}(k)}{\phi_{cat}\hat{f}_{W_{ES}^{cat}}(k)} = \frac{1}{\phi_{cat}}\Bigg[& \Bigg\{ -\frac{1}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)}\left(\frac{\partial}{\partial k}\hat{f}_{W_{ES}}(k)\right)\Bigg|_{k=0} - \frac{\left(1-\hat{f}_{W_{ES}}(k)\right)}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^2}\left(\frac{\partial}{\partial k}\hat{f}_{W_{ES}^{cat}}(k)\right)\Bigg|_{k=0} \Bigg\}\frac{k}{1!} \\
& + \Bigg\{ \frac{2}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^2}\left(\frac{\partial}{\partial k}\hat{f}_{W_{ES}^{cat}}(k)\right)\left(\frac{\partial}{\partial k}\hat{f}_{W_{ES}}(k)\right)\Bigg|_{k=0} \\
& - \frac{1}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)}\left(\frac{\partial^2}{\partial k^2}\hat{f}_{W_{ES}}(k)\right)\Bigg|_{k=0} + \frac{2\left(1-\hat{f}_{W_{ES}}(k)\right)}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^3}\left(\frac{\partial}{\partial k}\hat{f}_{W_{ES}^{cat}}(k)\right)^2\Bigg|_{k=0} \\
& - \frac{\left(1-\hat{f}_{W_{ES}}(k)\right)}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^2}\left(\frac{\partial^2}{\partial k^2}\hat{f}_{W_{ES}^{cat}}(k)\right)\Bigg|_{k=0} \Bigg\}\frac{k^2}{2!} \\
& + \Bigg\{ \frac{3}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^2}\left(\frac{\partial}{\partial k}\hat{f}_{W_{ES}^{cat}}(k)\right)\left(\frac{\partial^2}{\partial k^2}\hat{f}_{W_{ES}}(k)\right)\Bigg|_{k=0} \\
& - \frac{6}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^3}\left(\frac{\partial}{\partial k}\hat{f}_{W_{ES}}(k)\right)\left(\frac{\partial}{\partial k}\hat{f}_{W_{ES}^{cat}}(k)\right)^2\Bigg|_{k=0} \\
& + \frac{3}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^2}\left(\frac{\partial}{\partial k}\hat{f}_{W_{ES}}(k)\right)\left(\frac{\partial^2}{\partial k^2}\hat{f}_{W_{ES}^{cat}}(k)\right)\Bigg|_{k=0} \\
& - \frac{1}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)}\left(\frac{\partial^3}{\partial k^3}\hat{f}_{W_{ES}}(k)\right)\Bigg|_{k=0} - \frac{6\left(1-\hat{f}_{W_{ES}}(k)\right)}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^4}\left(\frac{\partial}{\partial k}\hat{f}_{W_{ES}^{cat}}(k)\right)^3\Bigg|_{k=0} \\
& + \frac{6\left(1-\hat{f}_{W_{ES}}(k)\right)}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^3}\left(\frac{\partial}{\partial k}\hat{f}_{W_{ES}^{cat}}(k)\right)\left(\frac{\partial^2}{\partial k^2}\hat{f}_{W_{ES}^{cat}}(k)\right)\Bigg|_{k=0} \\
& - \frac{\left(1-\hat{f}_{W_{ES}}(k)\right)}{\left(\hat{f}_{W_{ES}^{cat}}(k)\right)^2}\left(\frac{\partial^3}{\partial k^3}\hat{f}_{W_{ES}^{cat}}(k)\right)\Bigg|_{k=0} \Bigg\}\frac{k^3}{3!} + \cdots \Bigg]
\end{aligned}
\tag{17}
$$

Then, given the normalization of $\hat{f}_{W_{ES}^{cat}}(t)$ and $\hat{f}_{W_{ES}}(t)$ and known relations between the Laplace transform of a random variable and its moments, we get the following equations

$$
\hat{f}_{W_{ES}^{cat}}(k)\Big|_{k=0} = 1, \tag{18}
$$

$$\langle (W_{ES}^{cat})^n \rangle = (-1)^n \left(\frac{\partial^n}{\partial k^n} \hat{f}_{W_{ES}^{cat}}(k) \right) \Big|_{k=0}, \tag{19}$$

$$\hat{f}_{W_{ES}}(k)\big|_{k=0} = 1, \tag{20}$$

$$\langle W_{ES}^n \rangle = (-1)^n \left(\frac{\partial^n}{\partial k^n} \hat{f}_{W_{ES}}(k) \right) \Big|_{k=0}. \tag{21}$$

Substituting supplementary equations (18) - (21) into supplementary equations (16) and (17) we obtain

$$\frac{1}{\phi_{cat} k_{on}} \left(\frac{k}{\hat{f}_{W_{ES}^{cat}}(k)} \right) = \frac{1}{\phi_{cat} k_{on}} \left[k + \langle W_{ES}^{cat} \rangle k^2 + \left\{ \langle W_{ES}^{cat} \rangle^2 - \frac{\langle (W_{ES}^{cat})^2 \rangle}{2} \right\} k^3 + \cdots \right], \tag{22}$$

and

$$\begin{aligned} \frac{1 - \hat{f}_{W_{ES}}(k)}{\phi_{cat} \hat{f}_{W_{ES}^{cat}}(k)} = \frac{1}{\phi_{cat}} \Bigg[& \langle W_{ES} \rangle k + \left\{ \frac{2\langle W_{ES}^{cat} \rangle \langle W_{ES} \rangle - \langle W_{ES}^2 \rangle}{2} \right\} k^2 \\ & + \left\{ \frac{\langle W_{ES}^3 \rangle}{6} - \frac{\langle W_{ES}^{cat} \rangle \langle W_{ES}^2 \rangle}{2} + \langle W_{ES} \rangle \langle W_{ES}^{cat} \rangle^2 - \frac{\langle W_{ES} \rangle \langle (W_{ES}^{cat})^2 \rangle}{2} \right\} k^3 + \cdots \Bigg]. \end{aligned} \tag{23}$$

Collecting coefficients corresponding to the same power of k from Supplementary Equations (22) and (23) we obtain the analytical expressions for A_n and B_n in Table I. Therefore, the presented mathematical procedure enables Supplementary Equations (13), (22), and (23) that eventually establish Eq. (10) as the general form of Eqs. (8), (11) and (12) of the main text.

5. Proof that $f_{W_{ES}^{cat}}(t)$, $f_{W_{ES}^{off}}(t)$ and $f_{W_{ES}}(t)$ are identical for Markovian enzymes

For the classical Michaelis-Menten reaction, where all the kinetic processes are exponential, the PDFs of substrate unbinding and catalysis times read

$$f_{T_{off}}(t) = k_{off}\, e^{-k_{off} t}, \tag{24}$$

and

$$f_{T_{cat}}(t) = k_{cat}\, e^{-k_{cat} t}.$$

Substituting Supplementary Equation (24) into Eqs. (4) and (5) of the main text gives the following conditional waiting times distributions

$$f_{W_{ES}^{cat}}(t) = \frac{k_{cat}\, e^{-k_{cat}t} e^{-k_{off}t}}{\int_0^\infty k_{cat}\, e^{-k_{cat}t} e^{-k_{off}t} dt} = \left(k_{cat} + k_{off}\right) e^{-(k_{cat}+k_{off})t}, \tag{25}$$

and

$$f_{W_{ES}^{off}}(t) = \frac{k_{off}\, e^{-k_{off}t}\, e^{-k_{cat}t}}{\int_0^\infty k_{off}\, e^{-k_{off}t}\, e^{-k_{cat}t}\, dt} = \left(k_{cat} + k_{off}\right) e^{-(k_{cat}+k_{off})t}. \tag{26}$$

Also, in this case the splitting probabilities for catalysis and unbinding are $\phi_{cat} = \frac{k_{cat}}{k_{cat}+k_{off}}$ and $1 - \phi_{cat} = \frac{k_{off}}{k_{cat}+k_{off}}$, respectively. Now, substituting Supplementary Equations (25) and (26) into Eq. (6) in the main text, we obtain the PDF of the waiting time distribution function in the ES state. This is given by

$$f_{W_{ES}}(t) = \left(k_{cat} + k_{off}\right) e^{-(k_{cat}+k_{off})t}. \tag{27}$$

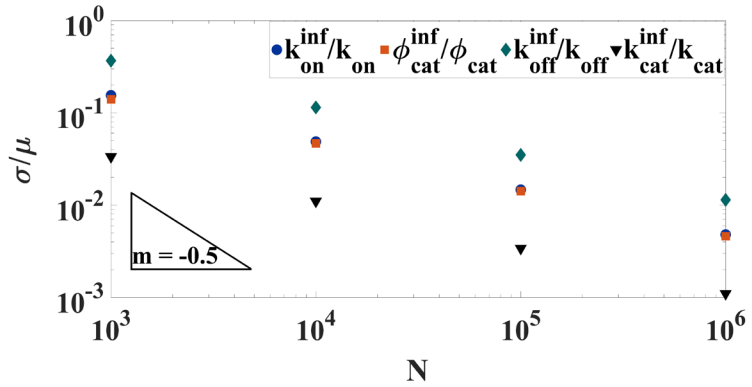
Supplementary Equations (25), (26) and (27) show that within the classical framework, the conditional and unconditional PDFs, $f_{W_{ES}^{cat}}(t)$, $f_{W_{ES}^{off}}(t)$ and $f_{W_{ES}}(t)$, are identical. Hence, we have $\langle (W_{ES}^{cat})^n \rangle = \langle W_{ES}^n \rangle = \langle \left(W_{ES}^{off}\right)^n \rangle$.

6. Proof that B_2, A_3 and B_3 in table I are zero for Markovian enzymes

Following Supplementary Equations (25) and (27), we can calculate the mean conditional catalytic time and the mean time spent by the enzyme in the bound state, ES. Since their functional forms are the same, we get $\langle W_{ES} \rangle = \langle W_{ES}^{cat} \rangle = 1/\left(k_{cat} + k_{off}\right)$. Utilizing basic properties of the exponential distribution we also have $\langle W_{ES}^2 \rangle = \langle (W_{ES}^{cat})^2 \rangle = 2/\left(k_{cat} + k_{off}\right)^2$ and $\langle W_{ES}^3 \rangle = \langle (W_{ES}^{cat})^3 \rangle = 6/\left(k_{cat} + k_{off}\right)^3$. With these expressions at hand, it is easy to see that for Markovian enzymes $B_2 = 2\langle W_{ES}^{cat} \rangle \langle W_{ES} \rangle - \langle W_{ES}^2 \rangle = 0$. Similarly, $A_3 = \langle W_{ES}^{cat} \rangle^2 - \frac{\langle \left(W_{ES}^{cat}\right)^2 \rangle}{2} = 0$ and $B_3 = \frac{\langle W_{ES}^3 \rangle}{6} - \frac{\langle W_{ES}^{cat} \rangle \langle W_{ES}^2 \rangle}{2} + \langle W_{ES} \rangle \langle W_{ES}^{cat} \rangle^2 - \frac{\langle W_{ES} \rangle \langle \left(W_{ES}^{cat}\right)^2 \rangle}{2} = 0$.

7. Normalized standard deviations of the estimates in Fig. 5 obey $\sim 1/\sqrt{N}$ scaling law

To quantify the improving accuracy trend of our inference procedure, we plot in Supplementary Fig. 2 the normalized standard deviation $\left(\frac{\sigma}{\mu}\right)$ of the data presented in the box plots of Fig. 5 as a function of the number of simulated turnover cycles per substrate concentration, (N). We find that the normalized standard deviations of our estimates obey the typical $\sim 1/\sqrt{N}$ scaling law.



Supplementary Fig. 2: Relative standard deviations as a function of the number of turnover cycles. Log-log plot presenting the normalized standard deviations of the inferred (inf) to the actual kinetic parameters: k_{on}^{inf}/k_{on} (blue circles), $\phi_{cat}^{inf}/\phi_{cat}$ (orange squares), k_{off}^{inf}/k_{off} (green diamonds), and k_{cat}^{inf}/k_{cat} (black triangles) vs. the number of simulated turnover cycles per substrate concentration, N. Here, the binding rate, the splitting probability of catalysis, the rates of unbinding, and catalysis are given by k_{on}, ϕ_{cat}, k_{off}, and k_{cat}, respectively. The substrate concentration values lie in the range: $0.2K_M - 4K_M$, where K_M is the Michaelis-Menten constant. Refer to Methods for further details. The standard deviations (σ) are normalized by the corresponding mean values (μ). All normalized standard deviations obey the typical scaling law, $\sigma/\mu \sim 1/\sqrt{N}$. They have the same slope, $m = -0.5$. Source data are provided as a Source Data file. Codes employed for generating these data sets have been supplied as Supplementary Software.

8. Mapping the kinetic scheme in Fig. 6a onto the renewal kinetic scheme in Fig. 1a

To describe the kinetic scheme in Fig. 6a using the renewal approach to enzyme catalysis, we first need to find the corresponding times for binding, T_{on}, unbinding, T_{off}, and catalysis, T_{cat} (Fig. 1a in the main text). By construction, the binding time distribution in Fig. 6a is exponential with rate as $k_{on}[S]$. We thus have

$$f_{T_{on}}(t) = k_{on}[S]e^{-k_{on}[S]t}. \tag{28}$$

The substrate binding event leads to the first bound state ES_1 which is further irreversibly transferred to the second enzyme-substrate complex, ES_2. Noting that the substrate unbinds from both these enzyme-substrate complexes at the same rate, k_{off}, we have

$$f_{T_{off}}(t) = k_{off}e^{-k_{off}t}, \tag{29}$$

for the unbinding time distribution. Finally, we note that the product can only be formed from ES_2. Thus, the PDF of catalysis times, $f_{T_{cat}}(t)$, is a convolution of two exponentially distributed processes with rates k_{cat}^1 and k_{cat}^2, respectively. We thus have

$$f_{T_{cat}}(t) = \int_0^t \left(k_{cat}^1 e^{-k_{cat}^1 t'}\right) k_{cat}^2 e^{-k_{cat}^2 (t-t')}\, dt' = \frac{\left(e^{-k_{cat}^2 t} - e^{-k_{cat}^1 t}\right) k_{cat}^1 k_{cat}^2}{k_{cat}^1 - k_{cat}^2}. \tag{30}$$

In this way we have coarse grained the two bound enzymatic states, ES_1 and ES_2, into a single bound state ES, thus transforming from the sequential description of Fig. 6a to the renewal description of Fig. 1a.

9. Derivation of Eq. (19)

To derive Eq. (19), we explicitly write the survival probability functions $\left(\bar{F}_X(t) = 1 - \int_0^t f_X(t')\,dt'\right)$ of the unbinding time

$$\bar{F}_{T_{off}}(t) = e^{-k_{off}t}, \tag{31}$$

and catalysis time

$$\bar{F}_{T_{cat}}(t) = \frac{e^{-k_{cat}^2 t}k_{cat}^1 - e^{-k_{cat}^1 t}k_{cat}^2}{k_{cat}^1 - k_{cat}^2}. \tag{32}$$

Then, we substitute Supplementary Equations (31) and (32) into Eqs. (4), (5), and (6) of the main text to obtain the conditional and unconditional PDFs of the waiting time in the ES state

$$f_{W_{ES}^{cat}}(t) = \frac{e^{-\left(k_{cat}^1+k_{cat}^2+k_{off}\right)t}\left(e^{k_{cat}^1 t}-e^{k_{cat}^2 t}\right)\left(k_{cat}^1+k_{off}\right)\left(k_{cat}^2+k_{off}\right)}{k_{cat}^1-k_{cat}^2}, \tag{33}$$

$$f_{W_{ES}^{off}}(t) = \frac{e^{-\left(k_{cat}^1+k_{cat}^2+k_{off}\right)t}\left(e^{k_{cat}^1 t}k_{cat}^1-e^{k_{cat}^2 t}k_{cat}^2\right)\left(k_{cat}^1+k_{off}\right)\left(k_{cat}^2+k_{off}\right)}{\left(k_{cat}^1-k_{cat}^2\right)\left(k_{cat}^1+k_{cat}^2+k_{off}\right)}, \tag{34}$$

$$f_{W_{ES}}(t) = \frac{e^{-\left(k_{cat}^1+k_{cat}^2+k_{off}\right)t}\left(-e^{k_{cat}^2 t}k_{cat}^2\left(k_{cat}^1+k_{off}\right)+e^{k_{cat}^1 t}k_{cat}^1\left(k_{cat}^2+k_{off}\right)\right)}{k_{cat}^1-k_{cat}^2}. \tag{35}$$

The first moments of these distributions can now be computed. These are given by

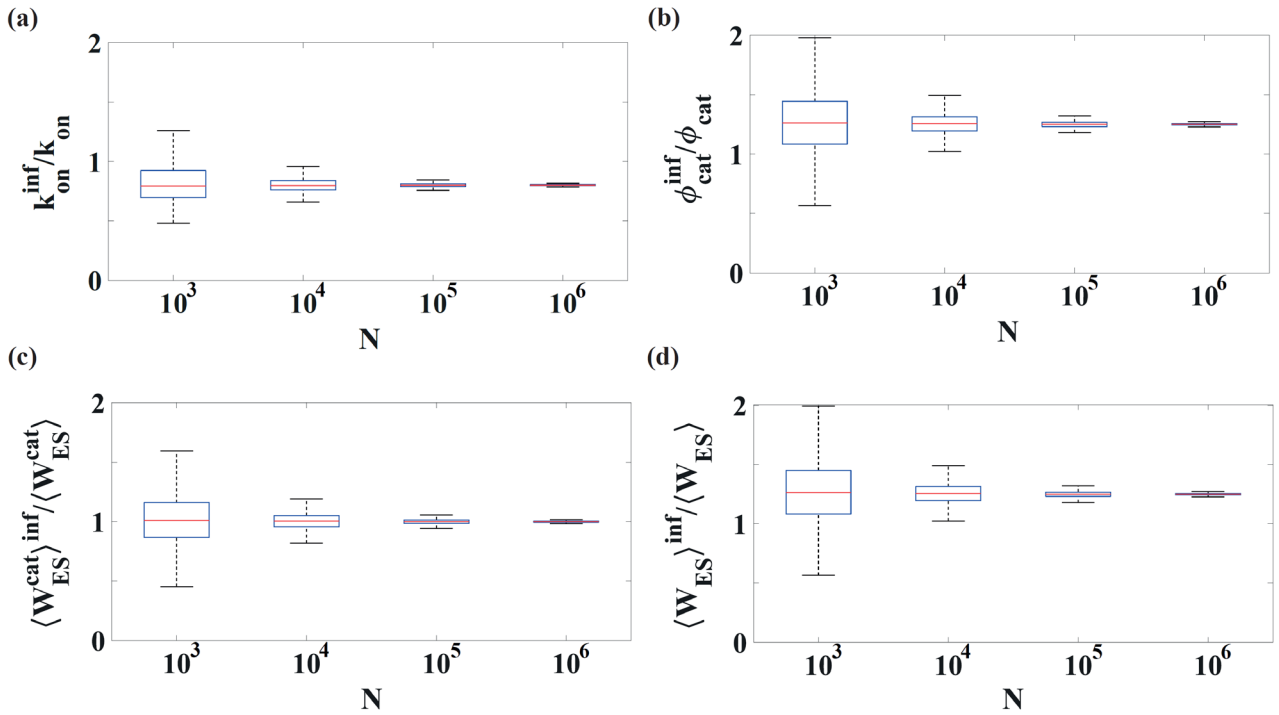
$$\langle W_{ES}^{cat}\rangle = \frac{1}{k_{cat}^1 + k_{off}} + \frac{1}{k_{cat}^2 + k_{off}}, \tag{36}$$

$$\langle W_{ES}^{off}\rangle = \frac{1}{k_{cat}^1 + k_{off}} + \frac{1}{k_{cat}^2 + k_{off}} - \frac{1}{k_{cat}^1 + k_{cat}^2 + k_{off}}, \tag{37}$$

$$\langle W_{ES}\rangle = \frac{k_{cat}^1 + k_{cat}^2 + k_{off}}{\left(k_{cat}^1 + k_{off}\right)\left(k_{cat}^2 + k_{off}\right)}. \tag{38}$$

Equation (19) in the main text follows by taking the ratio of $\langle W_{ES}\rangle$ and $\langle W_{ES}^{cat}\rangle$.

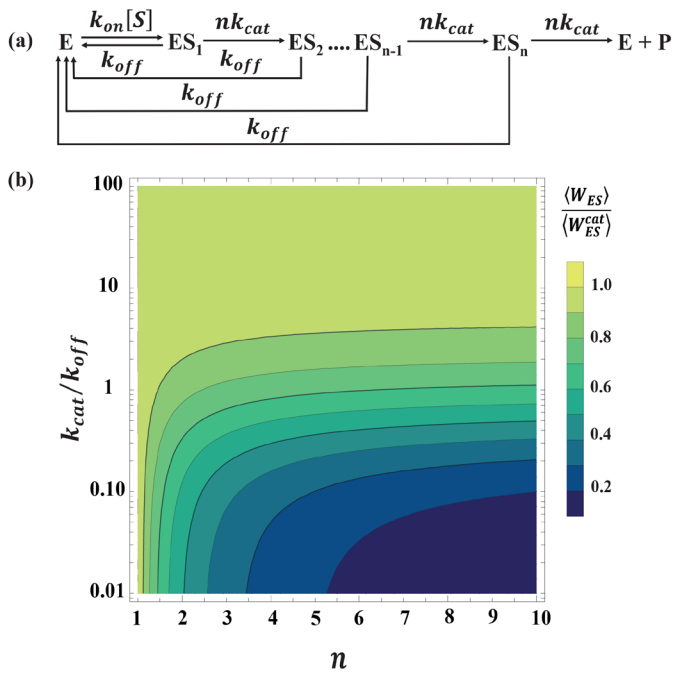
10. Sensitivity of inference procedure for the sequential scheme in Fig. 6a



Supplementary Fig. 3: Accuracy of the inferred kinetic parameters as a function of the sample size for the sequential reaction mechanism. Boxplots for the inferred (a) binding rate $\left(k_{on}^{inf}\right)$, (b) splitting probability of catalysis $\left(\phi_{cat}^{inf}\right)$, (c) mean conditional catalysis time ($\langle W_{ES}^{cat}\rangle^{inf}$), and (d) mean waiting time in the bound-enzymatic state ($\langle W_{ES}\rangle^{inf}$) normalized by the corresponding true values ($k_{on}, \phi_{cat}, \langle W_{ES}^{cat}\rangle, \langle W_{ES}\rangle$). Results are plotted as a function of the number of turnover events per substrate concentration, N. For each N, 10^3 independent repetitions were performed. The bottom and top of each box are the 25th and 75th percentiles of the sample, respectively, red lines mark the median value, and whiskers extend to 1.5 times the interquartile range. Source data are provided as a Source Data file. Codes employed for generating these data sets have been supplied as Supplementary Software.

Boxplots in Supplementary Fig. 3, present the distribution of the inferred binding rate, the conditional probability of catalysis, the mean conditional catalysis time, and the mean waiting time in the ES state, against the number of turnover events per substrate concentration, N. Data were obtained by simulating the sequential model in Fig. 6a with $\langle T_{on}\rangle = (k_{on}[S])^{-1} = (5 * [S])^{-1}\ [s], \langle T_{off}\rangle = \left(k_{off}\right)^{-1} = 1/10\ [s]$, and $\langle T_{cat}\rangle = (k_{cat}^1)^{-1} + (k_{cat}^2)^{-1} = (10)^{-1} + (20)^{-1}\ [s]$. Consequently, we have $K_M = \frac{A_1}{B_1} = \frac{1}{k_{on}\langle W_{ES}\rangle} = 3\ [\mu M]$. To ensure consistency in analysis, we take the same substrate concentration values as those used in Figs. 3 and 4.

The quantities Ω_1 and Ω_2 were estimated based on their relation to the turnover time moments (first column of Table 1). They were then plotted as a function of the reciprocal of the substrate concentration, which were taken in the range $0.2K_M - 4K_M$, and fitted to obtain estimates of A_1, B_1, A_2, and B_2. Eqs. (13) to (16), and the closure relation $\frac{\langle W_{ES}\rangle}{\langle W_{ES}^{cat}\rangle} = 1$, were used to obtain estimates of $k_{on}, \phi_{cat}, \langle W_{ES}^{cat}\rangle$, and $\langle W_{ES}\rangle$. To assess the fidelity of this procedure, it was repeated 10^3 times. The presented results show that 10^3 turnover events per substrate concentration were already enough to produce reasonable estimates, and that 10^4 turnover events per substrate concentration gave good estimates. More data leads to an even better



Supplementary Fig. 4: The n-step sequential mechanism. (a) A schematic representation of a model involving n identical catalytic steps in sequence. The free enzyme, E undergoes binding with a substrate to form the first substrate-bound conformer ES_1 that sequentially transitions to the second bound-enzymatic state, ES_2. From this stage of the network, the bound-enzyme conformational interconversions sequentially progress till the nth bound state, ES_n. Exclusively, this conformer can form the product, P through an irreversible catalytic event. From all these bound-structural states, unbinding events can occur that lead back to the free enzyme. The described kinetic processes are characterized by the rates of binding $(k_{on}[S])$ where $[S]$ is the substrate concentration, unbinding events (k_{off}), and the catalytic transitions (nk_{cat}). (b) 2D color map of the error factor $\frac{\langle W_{ES}\rangle}{\langle W_{ES}^{cat}\rangle}$ calculated in the space of the normalized catalytic rate $\frac{k_{cat}}{k_{off}}$ and the number of catalytic steps n. Here $\langle W_{ES}\rangle$ and $\langle W_{ES}^{cat}\rangle$ are the mean waiting times of the bound-enzymatic state and the conditional catalysis times, respectively.

accuracy, such that 10^6 turnover events provide estimates with very little statistical error. Note that here, $\frac{\langle W_{ES}\rangle}{\langle W_{ES}^{cat}\rangle} = 0.8$, which is obtained by substituting the abovementioned rate constants into Eq. (19). Thus, on top of the statistical estimation error, we also expect a systematic estimation error, which is seen clearly in panels (a), (b) and (d) of Supplementary Fig. 3.

11. Error factor for the sequential kinetic scheme with n intermediate states

To further evaluate the range of applicability of the Markovian assumption, $\frac{\langle W_{ES}\rangle}{\langle W_{ES}^{cat}\rangle} \approx 1$, we consider here a sequential kinetic scheme with n identical intermediate steps (see Supplementary Fig. 4a), where each enzyme-substrate complex ES_i can be transformed into the next complex, ES_{i+1}, (or in the case of $i = n$ to the product *P)* with rate nk_{cat}. Alternatively, at each step unbinding can occur with rate k_{off}. Note that rescaling the rate k_{cat} by a factor n is done (without loss of generality) to keep the mean catalytic time equal to $1/k_{cat}$, as in the case of the classical Michaelis-Menten model. This allows for a fair comparison.

To describe the kinetic scheme in Supplementary Fig. 4a using the renewal approach, we first need to find the corresponding times for binding, T_{on} , unbinding, T_{off}, and catalysis, T_{cat}. By construction, the binding time distribution in Supplementary Fig. 4a is exponential with rate $k_{on}[S]$. We thus have $f_{T_{on}}(t) =$

$k_{on}[S]e^{-k_{on}[S]t}$, which is identical to Supplementary Equation (28). As all unbinding process follow an exponential distribution with rate k_{off} we have $f_{T_{off}}(t) = k_{off}e^{-k_{off}t}$, which is identical to Supplementary Equation (29). Finally, note that from ES_1 a chain of irreversible transitions begins to reach the ultimate bound conformer, ES_n, which can be transformed into the product. Therefore, the functional form of $f_{T_{cat}}(t)$ is equivalent to the convolution of n sequential steps where each transition occurs with the same rate, nk_{cat}. The PDF of catalysis time is thus described by a n-step Erlang distribution function (convolution of n independent and identically distributed exponential steps) which reads

$$f_{T_{cat}}(t) = \frac{(nk_{cat})^n t^{n-1} e^{-nk_{cat}t}}{\Gamma[n]}. \tag{39}$$

From the above, it follows that the survival probability functions for $f_{T_{cat}}(t)$ and $f_{T_{off}}(t)$ can be written as

$$\bar{F}_{T_{cat}}(t) = \frac{\Gamma[n, nk_{cat}t]}{\Gamma[n]} \tag{40}$$

and

$$\bar{F}_{T_{off}}(t) = e^{-k_{off}t}. \tag{41}$$

In Supplementary Equation (40), $\Gamma[n, nk_{cat}t] = \int_{nk_{cat}t}^{\infty} x^{n-1}e^{-x}\,dx$ represents the upper incomplete Gamma function. The algebraic sum of $\Gamma[n, nk_{cat}t]$ and the lower incomplete Gamma function, $\gamma[n, nk_{cat}t] = \int_0^{nk_{cat}t} x^{n-1}e^{-x}\,dx$ gives the complete Gamma function, $\Gamma[n] = \int_0^{\infty} x^{n-1}e^{-x}\,dx$.

Substituting Supplementary Equations (40) and (41) into Eqs. (4), (5), and (6) in the main text, we obtain the conditional and unconditional PDFs of the waiting time in the ES state

$$f_{W_{ES}^{cat}}(t) = \frac{\left(nk_{off}\right)^n t^{n-1} \left(\frac{1}{n} + \frac{k_{cat}}{k_{off}}\right)^n e^{-nk_{off}\left(\frac{1}{n}+\frac{k_{cat}}{k_{off}}\right)t}}{\Gamma[n]} \tag{42}$$

$$f_{W_{ES}^{off}}(t) = \left(\frac{\Gamma[n, nk_{cat}t]}{\Gamma[n]}\right)\left(\frac{\left(\frac{1}{n} + \frac{k_{cat}}{k_{off}}\right)^n}{\left(\frac{1}{n} + \frac{k_{cat}}{k_{off}}\right)^n - \left(\frac{k_{cat}}{k_{off}}\right)^n}\right) k_{off}e^{-k_{off}t} \tag{43}$$

$$f_{W_{ES}}(t) = \frac{\left((nk_{cat})^n t^{n-1} + k_{off}\Gamma[n, nk_{cat}t]e^{nk_{cat}t}\right)e^{-nk_{off}\left(\frac{1}{n}+\frac{k_{cat}}{k_{off}}\right)t}}{\Gamma[n]} \tag{44}$$

The corresponding means of these distributions are given by

$$\langle W_{ES}^{cat}\rangle = \frac{1}{k_{off}\left(\frac{1}{n}+\frac{k_{cat}}{k_{off}}\right)} \tag{45}$$

$$\langle W_{ES}^{off}\rangle = \frac{1}{k_{off}}\left(1+\frac{1}{\left(\frac{1}{n}+\frac{k_{cat}}{k_{off}}\right)\left(1-\left(\frac{k_{off}}{k_{cat}}\right)^{n}\left(\frac{1}{n}+\frac{k_{cat}}{k_{off}}\right)^{n}\right)}\right), \tag{46}$$

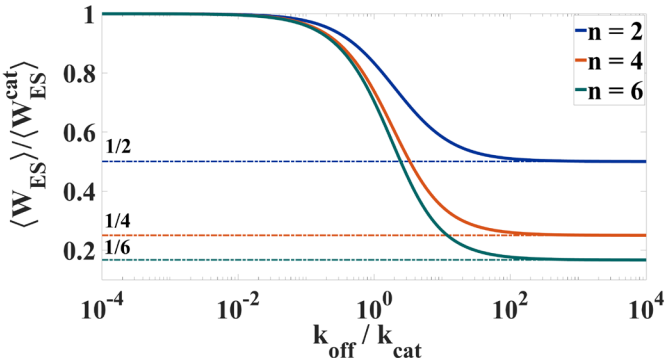
and

$$\langle W_{ES}\rangle = \frac{1}{k_{off}}\left(1-\left(\frac{\frac{k_{cat}}{k_{off}}}{\frac{1}{n}+\frac{k_{cat}}{k_{off}}}\right)^{n}\right). \tag{47}$$

Finally, we obtain the following expression for the ratio $\frac{\langle W_{ES}\rangle}{\langle W_{ES}^{cat}\rangle}$

$$\frac{\langle W_{ES}\rangle}{\langle W_{ES}^{cat}\rangle} = \left(\frac{1}{n}+\frac{k_{cat}}{k_{off}}\right)\left(1-\left(\frac{\frac{k_{cat}}{k_{off}}}{\frac{1}{n}+\frac{k_{cat}}{k_{off}}}\right)^{n}\right). \tag{48}$$

As expected, in the limit of a single catalytic transition ($n = 1$), this equation gives $\frac{\langle W_{ES}\rangle}{\langle W_{ES}^{cat}\rangle} = 1$. However, for $n > 1$, we find that the error factor in Supplementary Equation (48) is smaller than one but larger than $1/n$ (see Supplementary Fig. 5). The latter value is obtained in the limit of $k_{cat} \ll k_{off}$ where we have $\langle W_{ES}\rangle \approx 1/k_{off}$ and $\langle W_{ES}^{cat}\rangle \approx n/k_{off}$; and consequently $\langle W_{ES}\rangle/\langle W_{ES}^{cat}\rangle \approx 1/n$.



Supplementary Fig. 5: The error factor, $\frac{\langle W_{ES}\rangle}{\langle W_{ES}^{cat}\rangle}$, for different values of the number of catalytic steps, n. The blue, orange and green solid lines show the behavior of $\frac{\langle W_{ES}\rangle}{\langle W_{ES}^{cat}\rangle}$ from Supplementary Equation (48) as a function of $\frac{k_{off}}{k_{cat}}$ for three values of n: 2,4, and 6, respectively. Here n is the number of catalytic steps present in the network (see Supplementary Fig. 4a). We see that the ratio $\frac{\langle W_{ES}\rangle}{\langle W_{ES}^{cat}\rangle}$ decreases with $\frac{k_{off}}{k_{cat}}$, and eventually saturates at $1/n$. The blue, orange, and green dot-dashed lines show the limiting values of $\frac{\langle W_{ES}\rangle}{\langle W_{ES}^{cat}\rangle}$ for the corresponding values of $1/n$. $\langle W_{ES}\rangle$ and $\langle W_{ES}^{cat}\rangle$ are the mean waiting times of the bound-enzymatic state and the conditional catalysis times, respectively. The unbinding and catalytic transitions are respectively characterized by k_{off} and k_{cat}.

12. Mapping the kinetic scheme in Fig. 7a onto the renewal kinetic scheme in Fig. 1

To describe the kinetic scheme in Fig. 7a using the renewal approach, we again need to find the PDFs of the binding, T_{on} , unbinding, T_{off}, and catalysis, T_{cat} times (see Fig.1a in the main text). Here, there are two competitive routes for substrate binding, which occur with probabilities p and $1-p$ to enzyme-substrate complexes, ES_1 and ES_2, respectively. Therefore, the binding time PDF can be written as $f_{T_{on}}(t) = pk_{on}[S]e^{-k_{on}[S]t} + (1-p)k_{on}[S]e^{-k_{on}[S]t} = k_{on}[S]e^{-k_{on}[S]t}$, which is the same as in the standard Michaelis-Menten model. Noting that the substrate unbinds from both enzyme-substrate complexes with the same rate, k_{off}, we have $f_{T_{off}}(t) = k_{off}e^{-k_{off}t}$ for the unbinding time PDF. Once the ES_1 or ES_2 complexes are formed, catalysis occurs at the corresponding catalytic rates, namely, k_{cat}^1 or k_{cat}^2. The catalytic transitions occurring via the two pathways are weighted by the splitting probabilities p and $1-p$, respectively. As a result, the PDF of the catalysis time is given by a double-exponential function.

$$f_{T_{cat}}(t) = p\,k_{cat}^1 e^{-k_{cat}^1 t} + (1-p)k_{cat}^2 e^{-k_{cat}^2 t}. \tag{49}$$

In this way we have coarse grained the two bound enzymatic states, ES_1 and ES_2, into a single bound state ES, from which product formation occurs with probabilities p at rate k_{cat}^1 and with probability $1-p$ at rate k_{cat}^2.

13. Derivation of Eq. (20)

To derive Eq. (20), we explicitly write the survival probability functions $\left(\bar{F}_X(t) = 1 - \int_0^t f_X(t')\,dt'\right)$ of the catalysis and unbinding times as

$$\bar{F}_{T_{cat}}(t) = pe^{-k_{cat}^1 t} + (1-p)e^{-k_{cat}^2 t} \tag{50}$$

$$\bar{F}_{T_{off}}(t) = e^{-k_{off}t} \tag{51}$$

Substituting Supplementary Equations (50) and (51) into Eqs. (4), (5) and (6) of the main text, we obtain the following equations

$$f_{W_{ES}^{cat}}(t) = \frac{e^{-\left(k_{cat}^1+k_{cat}^2+k_{off}\right)t}\left(k_{cat}^1+k_{off}\right)\left(k_{cat}^2+k_{off}\right)\left(pk_{cat}^1 e^{k_{cat}^2 t}+(1-p)k_{cat}^2 e^{k_{cat}^1 t}\right)}{pk_{cat}^1\left(k_{cat}^2+k_{off}\right)+(1-p)k_{cat}^2\left(k_{cat}^1+k_{off}\right)}, \tag{52}$$

$$f_{W_{ES}^{off}}(t) = \frac{e^{-\left(k_{cat}^1+k_{cat}^2+k_{off}\right)t}\left(k_{cat}^1+k_{off}\right)\left(k_{cat}^2+k_{off}\right)\left(pe^{k_{cat}^2 t}+(1-p)e^{k_{cat}^1 t}\right)}{p\left(k_{cat}^2+k_{off}\right)+(1-p)\left(k_{cat}^1+k_{off}\right)}, \tag{53}$$

$$f_{W_{ES}}(t) = e^{-\left(k_{cat}^1+k_{cat}^2+k_{off}\right)t}\left(pe^{k_{cat}^2 t}\left(k_{cat}^1+k_{off}\right)+(1-p)e^{k_{cat}^1 t}\left(k_{cat}^2+k_{off}\right)\right). \tag{54}$$

The corresponding means of these distributions are given by

$$\langle W_{ES}^{cat} \rangle = \frac{\left(k_{cat}^1 + k_{off}\right)\left(k_{cat}^2 + k_{off}\right)\left(\frac{pk_{cat}^1}{\left(k_{cat}^1 + k_{off}\right)^2} + \frac{(1-p)k_{cat}^2}{\left(k_{cat}^2 + k_{off}\right)^2}\right)}{pk_{cat}^1\left(k_{cat}^2 + k_{off}\right) + (1-p)k_{cat}^2\left(k_{cat}^1 + k_{off}\right)}, \tag{55}$$

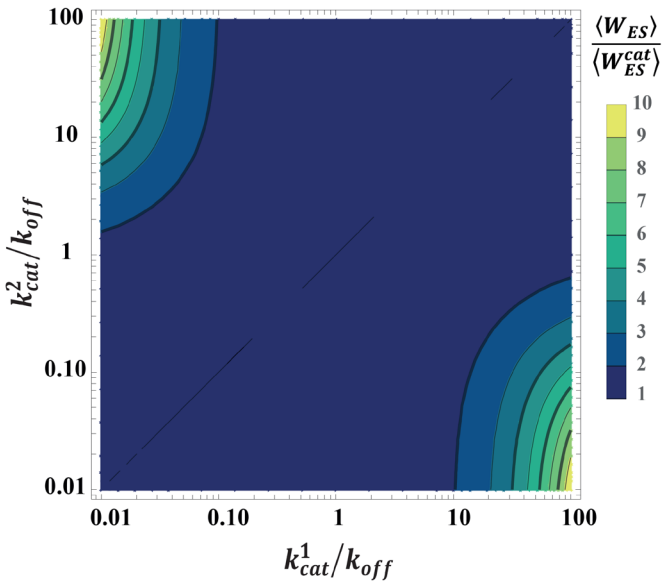
$$\langle W_{ES}^{off} \rangle = \frac{\left(k_{cat}^1 + k_{off}\right)\left(k_{cat}^2 + k_{off}\right)\left(\frac{p}{\left(k_{cat}^1 + k_{off}\right)^2} + \frac{(1-p)}{\left(k_{cat}^2 + k_{off}\right)^2}\right)}{p\left(k_{cat}^2 + k_{off}\right) + (1-p)\left(k_{cat}^1 + k_{off}\right)}, \tag{56}$$

$$\langle W_{ES} \rangle = \frac{p}{\left(k_{cat}^1 + k_{off}\right)} + \frac{(1-p)}{\left(k_{cat}^2 + k_{off}\right)}. \tag{57}$$

Using the above expressions, we arrive at Eq. (20) for the ratio of $\langle W_{ES} \rangle$ and $\langle W_{ES}^{cat} \rangle$ that is presented in the main text.

14. Error factor for the parallel kinetic scheme with probability $p = 0.9$

As noted in the main text, when the splitting probability is different from 0.5, one catalytic pathway is preferred over the other and deviations of the error factor $\frac{\langle W_{ES} \rangle}{\langle W_{ES}^{cat} \rangle}$ from unity become less significant. We illustrate this in Supplementary Fig. 6 for a splitting probability $p = 0.9$ which provides a strong bias towards the first catalytic pathway that leads to the ES_1 complex. In this case for $\frac{k_{cat}^1}{k_{off}}$ and $\frac{k_{cat}^2}{k_{off}}$ that differ from each other by less than four orders of magnitude, the error factor is < 10.



Supplementary Fig. 6: Error factor for the parallel kinetic scheme with $p = 0.9$. 2D color map of the error factor $\frac{\langle W_{ES}\rangle}{\langle W_{ES}^{cat}\rangle}$ calculated in the space of the normalized catalytic rates $\frac{k^1_{cat}}{k_{off}}$ and $\frac{k^2_{cat}}{k_{off}}$. $\langle W_{ES}\rangle$ and $\langle W_{ES}^{cat}\rangle$ are the mean waiting times of the bound-enzymatic state and the conditional catalysis times, respectively. The kinetic processes are characterized by the rates of unbinding (k_{off}), the first catalytic transition (k^1_{cat}), and the second catalytic process (k^2_{cat}). The calculations were performed for a splitting probability of binding, $p = 0.9$.