Supporting Information

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SI Text

Supporting Discussion. General, closed-form solutions for the mean and variance. We consider kinetic models of the form:

\[ E_i \xrightarrow{k_i} E_{i+1} \xrightarrow{k_{i+1}} \cdots \xrightarrow{k_{N-1}} E_N \xrightarrow{k_N} E_1, \]

where \( E_i \) corresponds to the \( i \)th kinetic state of the enzyme, \( k_i \) is the forward transition rate from \( E_i \) to \( E_{i+1} \), \( q_i \) is the reverse transition rate from \( E_{i+1} \) back to \( E_i \), and \( N \) is the total number of kinetic states in the cycle. At one or multiple points in the cycle, one of these transitions corresponds to the binding of a substrate molecule, \( S \). We label the binding state \( m \) and the substrate bound state \( m + 1 \). The transition rate for substrate binding is proportional to the inverse of the substrate concentration, which we can combine into the factor \( q_m / k_m[S] \); thus, the forward rate, \( k_m \), is a pseudo-first-order binding constant. Finally, each cycle ends with an irreversible transition that corresponds to a physically detectable signal.

As was shown first by Derrida (1) and later adopted by Chemla et al. (2), the mean cycle completion time, or dwell time, for this kinetic scheme is

\[ \langle \tau \rangle = \sum_{i=1}^{N} \frac{1}{k_i} \Gamma(i + 1, N), \]  

[S2]

where we define the useful combination of rate constants

\[ \Gamma(i, n) \equiv 1 + \sum_{j=0}^{n-j} \prod_{l=0}^{j} \Lambda_l ; \]

[S3]

and \( \Lambda_l \equiv q_{i-1,j} / k_l \). Adopting results from Chemla et al. (2), we find that the variance in the cycle completion times is

\[ \langle \tau^2 \rangle - \langle \tau \rangle^2 = \left( \sum_{i=1}^{N} \frac{1}{k_i} \Gamma(i + 1, N) \right)^2 - 2 \sum_{i=2}^{N} \sum_{j=1}^{i-1} \frac{1}{k_i k_j} \Gamma(j + 1, i - 1) \Gamma(i + 1, N). \]  

[S4]

Eq. S2 and Eq. S4 define the functional dependence of the mean and variance of the cycle completion times on the individual rate constants for all possible kinetic schemes of the form in [S1]. We will use these forms to derive the general substrate dependence of \( n_{\text{min}} \).

Necessary and sufficient conditions for a Michaelis-Menten substrate dependence. We start by considering a cycle in which only a single substrate molecule binds during the transition from state \( m \) to state \( m + 1 \). Notice that the specific form of the mean dwell time, Eq. S2, does not involve the product of a given rate constant with itself. This observation implies that the mean dwell time will have two types of terms, terms proportional to the inverse of the substrate concentration, which we can combine into the factor \( \eta \), and terms independent of the substrate concentration, which we can combine into the factor \( \xi \), yielding

\[ \langle \tau \rangle = \xi + \eta \frac{1}{[S]}. \]  

[S5]

\[ \xi = \sum_{i=1}^{m-1} \frac{1}{k_i} \Gamma(i + 1, m - 1) + \sum_{i=m+1}^{N} \frac{1}{k_i} \Gamma(i + 1, N) \]  

[S6]

and

\[ \eta = \Gamma(m + 1, N) \sum_{j=1}^{m} \prod_{l=1}^{j} \Lambda_l, \]  

[S7]

where in deriving the final property we have used the fact that

\[ \Gamma(i, n) = \Gamma(i - 1, j) + \Gamma(j + 1, n) \prod_{l=i}^{j} \Lambda_l \]  

[S8]

to split the portions of the \( \Gamma(i, n) \) functions that are proportional to or independent of \([S]^{-1}\). Comparison of Eq. S5 with the Michaelis-Menten expression, Eq. 3 in the main text, implies that

\[ \frac{1}{{K}_{\text{cat}}} = \xi \]  

[S9]

and

\[ \frac{K_M}{{K}_{\text{cat}}} = \eta. \]  

[S10]

Now, consider the case that multiple substrate molecules bind in a single cycle. Let these binding events occur during the transitions from state \( m \) to state \( m + 1 \) and from state \( m' \) to state \( m' + 1 \). Note that the general form of each of the terms in the mean dwell time \( k_i^{-1} \Gamma(i + 1, N) \) depends on all rate constants between states \( i \) and \( N \). Thus, if there are two binding states between states \( i \) and \( N \), there will be terms that involve the product of the pseudo-first-order binding constants for each of these binding events, and there will be terms proportional to \([S]^{-2}\). Thus, the general substrate dependence for the mean dwell time will not follow the Michaelis-Menten expression, Eq. 3. However, the general form of each of the terms in Eq. S2 reveals that the terms proportional to \([S]^{-2}\) will involve not just the pseudo-first-order rate constants \( k_m \) and \( k_{m'} \) but also the product of all of the \( \Lambda_l \) for states between \( m \) and \( m' \), i.e.,

\[ \prod_{l=m+1}^{m'} \Lambda_l = A_{m+1} A_{m+2} \cdots A_{m'-1} \frac{q_{m} q_{m+1} \cdots q_{m'-3} q_{m'-2}}{k_{m+1} k_{m+2} \cdots k_{m'-2} k_{m'-1}} \]  

[S11]

Because all of the non-Michaelis-Menten terms are proportional to this common factor, if it is zero, then all non-Michaelis-Menten terms will be zero. In this case, the system will display the simple substrate dependence for the mean dwell time, Eq. 3, despite the fact that each cycle requires the binding of multiple substrate molecules.

For finite forward rates, this term can only be zero if one or more of the reverse rates \( q_{m-l} \) through \( q_{m'-3} \) is zero. This observation implies that starting with the first binding event, an irreversible transition anywhere between the first and the second binding event will set the expression in Eq. S11 to zero. Thus, it is both necessary and sufficient for every binding event to be
separated by an irreversible transition for a kinetic scheme of the form in [S1] to display a Michaelis-Menten-like substrate dependence, Eq. 3. These irreversible transitions also include the final irreversible transition \((N \to 1)\) assumed in the models we consider.

**Kinetic blocks.** Irreversible transitions separate the kinetic scheme in [S1] into distinct kinetic blocks—different sets of reversibly connected kinetic states. For example, if there is an irreversible transition between state \(n\) and state \(n+1\), then the kinetic scheme is separated into two kinetic blocks:

\[
E_1 \cdots E_m \xrightarrow{k_{\text{eq}}} E_{m+1} \cdots E_n \xrightarrow{k_{\text{eq}}} E_{m+1} \cdots E_{n-1} \rightarrow E_1,
\]

Kinetic Block 1  
Kinetic Block 2

where states \(m\) and \(m'\) are substrate binding states and the forward transitions out of states \(n\) and \(n'\) are irreversible transitions.

Since the dynamics in one kinetic block are separated from another block by an irreversible transition, the statistics of these blocks are distinct, and the statistical properties of the full cycle will be built from the statistical properties of these isolated blocks. For example, if \((\bar{t}_b)\) is the mean dwell time of the \(b\)th kinetic block and \((\bar{t}_b)^2\) is the variance of this block, then

\[
\langle \tau \rangle = \sum_{b=1}^{N_B} \bar{t}_b \quad \text{and} \quad \langle \tau^2 \rangle - \langle \tau \rangle^2 = \sum_{b=1}^{N_B} (\bar{t}_b^2 - \bar{t}_b^2), \quad \text{[S12]}
\]

where \(N_B\) is the number of kinetic blocks. The validity of Eq. S13 can be checked by using the fact that \(\Lambda_{n+1} = 0\) for all irreversible transitions, which implies that if a state index \(i\) is in a specific kinetic block, i.e., \(n < i \leq n'\), then \(\Gamma(i+1,N) = \Gamma(i+1,n')\). Using this fact, it is possible to show that Eqs. S2 and S4 reduce to Eq. S13.

**Michaelis-Menten parameters for multiblock cycles.** In principle, the number of kinetic blocks in a given cycle is equal to the number of irreversible transitions. However, for our purposes, we will group kinetic blocks such that each composite block contains one and only one substrate binding event. In this fashion, \(N_B\) will also be the number of substrate binding events in each cycle. Then because each composite block is a version of the nearest-neighbor kinetic cycle considered in [S1], i.e., a \(n\)-state system with one binding state located at state \(m\), the expressions for the mean dwell time derived above are valid for each kinetic block. In other words, \(\xi \to \xi_b\) and \(\eta \to \eta_b\). The indexes in these terms, \(\eta_b\) and \(\xi_b\), are now particular to the individual kinetic block: the index 1 corresponds to the first state in the block, \(m\) corresponds to the position of the binding state within that block, and \(n\) or \(N\) in Eqs. S6 and S7, correspond to the last state within that block. The numerical value of these indexes need not match the position of the state in the overall cycle.

Because the mean dwell time is the sum of the mean for each kinetic block, it will have the same Michaelis-Menten form as in Eqs. 3 and S5. However, the Michaelis constants will be the sum of the individual parameters of each kinetic block

\[
\frac{1}{K_{\text{cat}}} = \sum_{b=1}^{N_B} \frac{1}{k_{\text{cat},b}} = \sum_{b=1}^{N_B} \frac{1}{k_{\text{cat},b}} \quad \text{[S14]}
\]

and

\[
\frac{K_M}{k_{\text{cat}}} = \sum_{b=1}^{N_B} n_b = \sum_{b=1}^{N_B} \frac{K_{M,b}}{k_{\text{cat},b}} \quad \text{[S15]}
\]

where \(k_{\text{cat},b}\) and \(K_{M,b}\) are the Michaelis constants for the \(b\)th kinetic block. Because the binding of multiple substrate molecules per cycle likely involves the action of multiple subunits in the enzyme, these parameters can just as easily be thought of as the Michaelis constants of the \(b\)th subunit.

**Substrate dependence of the variance.** Since the variance of the individual kinetic blocks add to produce the total variance for the system, we need only consider the variance for a single kinetic block. In this case,

\[
\langle \bar{t}_b^2 \rangle - \langle \bar{t}_b \rangle^2 = \left( \sum_{i=1}^{n} \frac{1}{k_i} \Gamma(i+1,n) \right)^2 - 2 \sum_{i=2}^{n} \sum_{j=1}^{i-1} \frac{1}{k_i} \frac{1}{k_j} \Gamma(j+1,i-1) \Gamma(i+1,n). \quad \text{[S16]}
\]

The first term is simply the mean squared, so its coefficients have already been determined above. We now focus on the second term. Notice again that this term does not involve the production of a given rate with itself. This observation implies that there will only be two types of terms: terms proportional to \([S]^{-1}\) and terms independent of \([S]^{-1}\). Thus,

\[
2 \sum_{i=2}^{n} \sum_{j=1}^{i-1} \frac{1}{k_i} \frac{1}{k_j} \Gamma(j+1,i-1) \Gamma(i+1,n) = \chi_b + \sigma_b \left( \frac{1}{[S]} \right). \quad \text{[S17]}
\]

where we have combined all terms proportional to the inverse of the substrate concentration or independent of this concentration into \(\sigma_b\) and \(\chi_b\), respectively. The subscript \(b\) represents the fact that there will be a similar term for each kinetic block in the cycle.

By expanding Eq. S17 and collecting terms proportional or independent of \([S]^{-1}\), it is possible to show that

\[
\chi_b = \sum_{i=2}^{m-1} \sum_{j=1}^{i-1} \frac{1}{k_i} \frac{1}{k_j} \Gamma(i+1,m-1) \Gamma(j+1,i-1)
+ \sum_{i=m-1}^{n} \sum_{j=1}^{i-1} \frac{1}{k_i} \frac{1}{k_j} \Gamma(i+1,n) \Gamma(j+1,m-1)
+ \sum_{i=m}^{n} \sum_{j=m+1}^{n} \frac{1}{k_i} \frac{1}{k_j} \Gamma(i+1,n) \Gamma(j+1,i-1) \quad \text{[S18]}
\]

and

\[
\sigma_b = \sum_{i=2}^{m-1} \sum_{j=1}^{i-1} \frac{1}{k_i} \frac{1}{k_j} \Gamma(m+1,n) \Gamma(j+1,i-1) \prod_{l=i+1}^{m} A_l
+ \sum_{i=m-1}^{n} \sum_{j=m+1}^{n} \frac{1}{k_i} \frac{1}{k_j} \Gamma(i+1,n) \Gamma(m+1,i-1) \prod_{l=m+1}^{m} A_l \quad \text{[S19]}
\]

When combined with [S3], Eqs. S5, S6, S13, S16, and S17, these complicated sums of products of rate constants completely determine the way in which the individual rate constants of a specific kinetic model combine to form the variance of the cycle completion time.

**General form for the substrate dependence of \(\eta_{\text{bar}}\).** With the substrate dependence of the mean and the variance of the individual
kinetic blocks, Eqs. S5–S7 and S17, S18, and an understanding of how these statistical properties of the individual blocks compose the statistical properties of the full cycle, Eq. S16, we can now calculate the general substrate dependence of \( n_{\text{min}} \). Using the definition of \( n_{\text{min}} \) and the expressions derived above

\[
\begin{align*}
n_{\text{min}} &= \frac{\left( \sum_{b=1}^{N_b} \xi_b \right)^2}{\sum_{b=1}^{N_b} \xi_b^2} = \frac{\left( \sum_{b=1}^{N_b} (\xi_b + \eta_b[S])^{-1} \right)^2}{\sum_{b=1}^{N_b} (\xi_b + \eta_b[S])^{-1} - 2 \left( \sum_{b=1}^{N_b} \xi_b + \sigma_b[S]^{-1} \right)}.
\end{align*}
\]

With some additional algebra, it is possible to show that this expression reduces to

\[
\begin{align*}
n_{\text{min}} &= \frac{N_L N_S \left( 1 + \frac{[S]}{K_S} \right)^2}{N_L \left( \frac{[S]}{K_S} \right)^2 + 2 \alpha \frac{[S]}{K_S} + N_S},
\end{align*}
\]

which is Eq. 4 in the main text. In this process we have used Eqs. S14 and S15 to define the Michaelis constant for the full cycle,

\[
K_S = \sum_{b=1}^{N_b} \frac{\eta_b}{\xi_b},
\]

and we have defined the following “macroscopic” constants

\[
N_L = \frac{\left( \sum_{b=1}^{N_b} \eta_b \right)^2}{\sum_{b=1}^{N_b} \eta_b^2},
\]

\[
N_S = \frac{\left( \sum_{b=1}^{N_b} \xi_b \eta_b \right)^2}{\sum_{b=1}^{N_b} \xi_b \eta_b \sum_{b=1}^{N_b} \xi_b \eta_b},
\]

and

\[
a = \frac{\sum_{b=1}^{N_b} (\xi_b \eta_b - \sigma_b)}{\sum_{b=1}^{N_b} \eta_b \sum_{b=1}^{N_b} \eta_b N_L N_S}.
\]

The final expressions, Eqs. S23–S25, completely determine the macroscopic parameters of the general substrate dependence of \( n_{\text{min}} \) from the individual kinetic rates that compose the cycle.

**Properties of \( N_L \).**starting with Eq. S23, which relates \( N_L \) to the specific kinetic rates in the underlying kinetic cycle, we now derive the properties of \( N_L \) listed in the main text.

In particular, we use the Cauchy-Schwarz inequality to prove that \( N_L \) is bounded from above by the number of substrate molecules that are bound each cycle and from below by 1. The Cauchy-Schwarz inequality states that for all real numbers \( x_i \) and \( y_i \)

\[
\left( \sum_{i=1}^{N} x_i y_i \right)^2 \leq \sum_{i=1}^{N} x_i^2 \sum_{i=1}^{N} y_i^2.
\]

If we let \( x_i = \eta_b \) and \( y_i = 1 \) for all i, then this expression becomes

\[
\left( \sum_{b=1}^{N_b} \eta_b \right)^2 \leq \sum_{b=1}^{N_b} \eta_b^2 \sum_{b=1}^{N_b} 1 = N_B \sum_{b=1}^{N_b} \eta_b^2
\]

which proves the bound

\[
\frac{\left( \sum_{b=1}^{N_b} \eta_b \right)^2}{\sum_{b=1}^{N_b} \eta_b^2} = N_L \leq N_B.
\]

This expression is Eq. 10 in the main text. A corollary of the Cauchy-Schwarz inequality is that the equality only holds if all \( x_i \) are proportional to all \( y_i \) with the same proportionality constant for all \( i \), i.e., \( x_i = \alpha y_i \) for all \( i \). Because the \( y_i = 1 \) in our argument above, \( N_L = N_B \) if and only if the \( \eta_b \) for each kinetic block are identical. Interestingly, Eq. S7 indicates that, in general, the \( \eta_b \) are complicated functions of the number of kinetic states and their rates, yet Eq. S28 does not depend on these details, only the numerical value of \( \eta_b \). Thus, one might imagine a case in which the binding events are quite different in each kinetic block, but the \( \eta_b \) happen to be identical, in which case, \( N_L = N_B \). It is worth further emphasizing that \( N_L = N_B \) if all \( \eta_b \) are the same, even if the kinetic events involved in binding are reversible. Thus, reversibility in binding or other connected kinetic transitions need not lower \( N_L \) from \( N_B \). (This requirement is in contrast to what we will prove below for \( N_S \).)

Eq. S28 also indicates that \( N_L \) is bounded from below from 1. If the kinetic scheme involves the binding of only a single substrate molecule, then Eq. S28 shows trivially that \( N_L = 1 \). However, even if multiple substrate molecules are bound, Eq. S28 requires that \( 1 \leq N_L \), with \( N_L \) only approaching 1 in the limit that one of the \( \eta_b \) is much larger than all other \( \eta_b \). Thus, a measured value of \( N_L \) less than 1 indicates that one of our assumptions must not apply to the kinetic mechanism of the specific enzyme. The most likely assumption would be that the kinetic mechanism has more complicated catalytic pathways than included in Eq. S1.

Finally, it is worth noting that the individual \( \eta_b \) correspond to the ratio of \( K_M \) to \( k_{\text{cat}} \) for each kinetic block. This ratio is the inverse of what is commonly known as the catalytic efficiency of the enzyme for a given substrate molecule (3). Given the complexity of the different kinetic constants observed above, it is perhaps remarkable that \( N_L \) would depend in a simple fashion on such an important kinetic parameter.

**Properties of \( N_S \).** In order to derive the properties of \( N_S \) listed in the main text, it is first necessary to simplify the denominator of Eq. S24: \( \xi_b^2 - 2\xi_b \). Expanding terms in \( \xi_b \), reindexing to match terms in \( \chi_b \), and using the identity in Eq. S8, it is possible to show that this denominator can be written as

\[
\begin{align*}
\xi_b^2 - 2\xi_b &= \sum_{i=1}^{m} \left( \frac{1}{k_i} \Gamma(i + 1, m - 1) \right)^2 + \sum_{i=1}^{n} \left( \frac{1}{k_i} \Gamma(i + 1, n) \right)^2 + 2 \sum_{i=2}^{m} \frac{1}{k_i} \Gamma(i + 1, m - 1) \sum_{j=1}^{i-1} \frac{1}{k_j} \prod_{l=j+1}^{i} A_l \quad [S29]
\end{align*}
\]

Note that the first two terms in this expression are simply the sum of the squares of the terms that compose \( \xi_b \) (see Eq. S6).
To prove the bound on \(N_S\), we first define a related quantity
\[
N' = \left( \sum_{i=1}^{N_{\text{other}}} \xi_i \right)^2, \tag{S30}
\]
where each \(\xi_i\) can be identified with a term in each of the \(\xi_i^k\) from each of the different kinetic blocks. Explicitly, \(x_i = k_i^{-1}\Gamma(i + 1, m - 1)\) for \(i < m\) and \(x_i = k_i^{-1}\Gamma(i + 1, n)\) for \(i > m\) for each kinetic block. The sum in Eq. S30 contains \(N_{\text{other}}\) terms since there is an \(x_i\) for each of the nonsubstrate binding states (see the bounds on the sum in Eq. S6).

From the Cauchy-Schwarz inequality, it follows, as above, that
\[
N' \leq N_{\text{other}}. \tag{S31}
\]
Since the only difference between \(N_S\) and \(N'\) is that the denominator of \(N_S\) contains additional positive terms (the final two terms in Eq. S29), \(N_S \leq N'\); thus, we can immediately conclude that
\[
N_S \leq N_{\text{other}}. \tag{S32}
\]

The bound stated in [9] in the main text.

Furthermore, \(N_S = N'\) only in the limit that the additional terms in the denominator, the final two terms in Eq. S29, are zero. Inspection of these two terms reveals that they are proporional to products of \(A_l\), where all \(A_l\) are present except for the \(A_{m+1}\), which contain the rate for unbinding substrate, \(q_m\). Thus, if all of the nonbinding transitions are irreversible, these terms will be zero, and \(N_S = N'\). Furthermore, the corollary to the Cauchy-Schwarz inequality, discussed above, implies that \(N' = N_{\text{other}}\) if and only if all of the forward transition rates are identical. Combining these results, we conclude that \(N_S = N_{\text{other}}\) if and only if all nonbinding transitions are irreversible and ii) all forward transition rates are identical.

The specific form for \(N_S\), Eq. S24, also reveals that for the linear, nearest-neighbor models considered here \(N_S\) is always bounded from below by 1. Again, this result implies that measurement of a value for \(N_S\) less than 1 is statistical proof that the kinetic mechanism in question must have features not considered here, e.g., parallel catalytic pathways or off-pathway states.

**Properties of \(\alpha\).** The general form of \(\alpha\) in Eq. S25 provides several limits on its possible values. We start by showing that \(\alpha\) is bounded from above by \(N_SN_L\). In particular, we show that
\[
\sum_{b=1}^{N_S} (\xi_b\eta_b - \alpha_b) \leq \frac{1}{\eta_b} \sum_{b=1}^{N_S} \xi_b. \tag{S33}
\]

To prove this inequality, we recognize that since \(\sigma_b\) is nonnegative (it is a function of rate constants which must all be nonnegative),
\[
\sum_{b=1}^{N_S} (\xi_b\eta_b - \alpha_b) \leq \sum_{b=1}^{N_S} \eta_b \xi_b \leq \sum_{b=1}^{N_S} \eta_b \sum_{b=1}^{N_S} \xi_b. \tag{S34}
\]
Reindexing the bottom products yields
\[
\sum_{b=1}^{N_S} \eta_b \sum_{b=1}^{N_S} \xi_b = \sum_{b=1}^{N_S} \eta_b \sum_{j=1}^{N_S} \xi_j = \sum_{b=1}^{N_S} \eta_b \sum_{b=1}^{N_S} \xi_b + \sum_{i=2}^{N_S} \sum_{j=1}^{N_S} \eta_j \xi_j. \tag{S35}
\]
The first term in the final expression is simply the numerator and the additional terms must be positive; thus,

\[
\sum_{b=1}^{N_S} (\xi_b\eta_b - \alpha_b) \leq \frac{1}{\eta_b} \sum_{b=1}^{N_S} \xi_b, \tag{S36}
\]
which implies that
\[
\alpha \leq N_SN_L. \tag{S37}
\]

To show that \(\alpha\) is bounded from below by zero, we must prove that \(\xi_b\eta_b - \alpha_b\) is nonnegative. Using Eqs. S6 and S7, we expand \(\xi_b\eta_b\)
\[
\xi_b\eta_b = \biggl( \sum_{i=1}^{m-1} \frac{1}{k_i} \Gamma(i + 1, m - 1) \biggr) + \sum_{i=m+1}^{m} \frac{1}{k_i} \Gamma(i + 1, n) \biggl( \Gamma(m + 1, n) \sum_{i=1}^{m} \frac{1}{k_j} \sum_{j=1}^{m} A_j \biggr)
\]
\[
= \sum_{i=1}^{m-1} \sum_{j=1}^{m} \frac{1}{k_i k_j} \Gamma(i + 1, m - 1) \Gamma(m + 1, n) \sum_{j=1}^{m} A_j
\]
\[
+ \sum_{i=m+1}^{m} \sum_{j=1}^{m} \frac{1}{k_i k_j} \Gamma(i + 1, n) \Gamma(m + 1, n) \sum_{j=1}^{m} A_j, \tag{S38}
\]
where we have again changed the indexing to match the indexing of terms in \(\sigma_b\), Eq. S19. Combining terms yields
\[
\xi_b\eta_b - \sigma_b = \sum_{i=2}^{m-1} \prod_{l=2}^{m} A_l \Gamma(m + 1, n) \sum_{j=1}^{m} \frac{1}{k_l} \Gamma(j + 1, m - 1)
\]
\[
+ \sum_{i=m+1}^{m} \prod_{l=1}^{m} A_l \biggl( \sum_{j=1}^{m} \frac{1}{k_l} \Gamma(j + 1, m - 1) \biggr)
\]
\[
+ \sum_{i=m+1}^{m} \sum_{j=1}^{m} \frac{1}{k_i k_j} \Gamma(i + 1, n) \Gamma(m + 1, n) \sum_{j=1}^{m} A_j, \tag{S39}
\]
where we have removed the first term of the sum in \(\xi_b\eta_b\), \(i = 1\) so that this sum is over the same indices as in \(\sigma_b\), and we have used the property in Eq. S8. This expression, while cumbersome, is positive; thus, \(\alpha\) is bounded by
\[
0 \leq \alpha \leq N_SN_L. \tag{S40}
\]

Inspection of the combined form for \(\xi_b\eta_b - \sigma_b\) in Eq. S39 reveals that all of the terms are proportional to two common factors, \(A_m\) and \(A_{m+1}\). If these two terms are zero, then \(\xi_b\eta_b - \sigma_b = 0\), and if these terms are zero for all of the kinetic blocks that compose the full cycle, then \(\alpha = 0\). Setting \(A_m\) and \(A_{m+1}\) equal to zero corresponds to setting two reverse rates to zero, \(q_{m-1}\) and \(q_m\). The rate at which substrate molecules unbind from the enzyme while \(q_{m-1}\) is the rate at which the substrate binding state transitions into nonsubstrate binding states. Thus, setting these rates equal to zero, implies that the local structure of the kinetic mechanism as substrate loads is
\[
E_{m-1} \rightarrow E_m \rightarrow E_{m+1}. \tag{S41}
\]
Remarkably, since all of the terms in Eq. S39 must be nonnegative, and the only common factors between all of these terms are \(A_m\) and \(A_{m+1}\), the only way that this expression can be zero is if the local structure of binding transition is that seen in [S41]. Thus, \(\alpha = 0\) if and only if the binding state is not in equilibrium with
any other kinetic states. The implication of this result is that such kinetic schemes have fundamentally different fluctuations and, more importantly, careful measurements of the substrate dependence of \( n_{\text{min}} \) can reveal these unique fluctuations.


Fig. S1. Removing \( n_{\text{min}} \) measurements does not bias fit values. Fit values of \( N_S \) (red circles), \( N_L \) (black squares), and \( \alpha \) (blue triangles) determined from fits of the data in Fig. 3B excluding each of the eight measured \( n_{\text{min}} \) values. The light regions correspond to the fit values of these parameters with their uncertainties when all eight values are included, Table 1. All fits were performed with a fixed \( K_M \) of 23 \( \mu \)M. The fact that the fit values show no systematic bias as different points are removed indicates that the fit values are not biased by single measurements rather measurements over the full range of [ATP] constrain these fit values. Error bars represent the standard deviation determined from the nonlinear, least-squares fit to Eq. 4.