A novel approach to distinguish between enzyme mechanisms: quasi-steady-state kinetic analysis of the prostaglandin H synthase peroxidase reaction

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A method of analysis for steady-state kinetic data has been developed that allows relationships between key partial reactions in the catalytic cycle of a functioning enzyme to be determined. The novel approach is based on a concept of scalar and vector ‘kinetic connectivities’ between enzyme intermediates in an arbitrary enzyme mechanism. The criterion for the agreement between experimental data and a proposed kinetic model is formulated as the kinetic connectivity of intermediate forms of the enzyme. This concept has advantages over conventional approaches and is better able to describe the complex kinetic behaviour of prostaglandin H synthase (PGHS) when catalysing the oxidation of adrenaline by H2O2. To interpret the experimental data for PGHS, a generalized model for multi-substrate enzyme reactions was developed with provision for irreversible enzyme inactivation. This model showed that two enzyme intermediates must undergo inactivation during the catalytic cycle. These forms are proposed to be PGHS compound I and a compound I–adrenaline complex.

Key words: enzyme inactivation, integral kinetics, kinetic analysis, multi-substrate enzyme reaction, qualitative criteria of kinetic behaviour, unbranched and branched mechanisms.

INTRODUCTION

Enzyme kinetics allow the quantitative description of the change in substrate, intermediate and product concentrations as a function of time provided that the reaction mechanism is known. The solution of the inverse task, i.e. the development of a kinetic model of enzyme action based on a set of experimental data, is a challenging fundamental problem because any particular set of experimental data can invariably be fitted into a large class of kinetic models. In order to determine which particular class of model is appropriate, it would be necessary to determine the kinetic properties of all possible particular models for the most general kinetic schemes and develop criteria of identity/equity between the array of kinetic schemes and the particular kinetic behaviour of the system under investigation.

The need to develop methods of analysis for general kinetic schemes became evident with the discovery of multi-substrate enzyme-catalysed reactions, bi-functional enzymes and enzymes undergoing activation or inactivation during the reaction course. As early as 1959, the steady-state rate equations were derived for a generalized model of a reversible unbranched enzymic reaction [1] with any number of intermediates produced, but with only one substrate consumed and one product formed. For two-substrate reactions, several mechanisms were described including an ordered mechanism with ternary complex formation, a double substitution or Ping-Pong mechanism, a random equilibrium mechanism [2] and the Theorell–Chance mechanism [3].

To elucidate the reaction mechanism from the experimental data a number of classification and graphic approaches have been developed. The hyperbolic function of the rate of the enzymic reaction on the substrate concentration is linear in double-reciprocal plots and can be presented as an additive function of the reciprocal values using the Dalziel parameters [4]. Cleland [5,6] proposed a convenient classification based on the method of King and Altman [7], where the basis for classification was the number of substrates and reaction products and the sequence of their association/dissociation. In the case of a steady-state random-order mechanism (the Ferdinand model), the dependence of the enzymic reaction rate on the substrate concentration deviates from the hyperbolic law; several effects can be observed including substrate activation and/or inhibition [8].

Schemes for a two-substrate enzymic reaction with independent substrate- and product-binding sites have also been developed. In the majority of cases, the dependence of the enzymic reaction rate on the substrate concentration is described by non-hyperbolic equations [9]. Likewise, the allosteric behaviour of enzymes is often characterized by their sigmoidal kinetic profiles [10,11]. The kinetics of enzyme inactivation during the reaction course have been analysed for a one-substrate two-step scheme when inactivation of both free enzyme and the enzyme–substrate complex occurs [12]. A three-step scheme has been used to describe the kinetics of enzyme inactivation by a suicidal substrate [13–15].

Integral kinetics of substrate consumption have been analysed for two-step and three-step, irreversible, one-substrate enzymic reactions involving inactivation of all three intermediate enzyme forms. A procedure for the graphical determination of the inactivation rate constants was developed by Duggleby [16] using the degree of substrate conversion achieved upon the complete inactivation of the enzyme. The non-steady-state integral kinetics of product accumulation have been analysed for a two-step enzymic reaction accompanied by inactivation of the intermediate...
enzyme forms at a constant concentration of substrate [17]. In the general case, product accumulation is described by the sum of two exponentials. A procedure for the determination of the kinetic constants from the experimental data was also described. The form of the dependence of maximal yield of product on the substrate concentration was shown to be of diagnostic significance. The function is hyperbolic if both intermediate enzyme forms are inactivated. However, if the maximal yield of the product does not depend on the substrate concentration, only the enzyme-substrate complex undergoes inactivation. The function is linear if only free enzyme is subject to inactivation [17]. The pre-steady-state kinetics of a two-step Michaelis–Menten scheme have been analysed for an unstable free enzyme and/or enzyme-substrate complex [18].

The theory of complex enzymic reactions began to develop intensely after algorithms based on graph theory for calculating the rates of enzymic reactions had been developed [19–24]. Notwithstanding numerous studies, at present there, to our knowledge, no common algorithms for the determination of the kinetic mechanism of a multi-substrate enzymic reaction. The current procedure essentially comprises the construction of a set of kinetic models followed by a comparison of their properties with the experimentally obtained data.

In this paper we introduce a concept of scalar and vector ‘kinetic connectivity’ of enzyme intermediates and demonstrate that this concept is applicable as a criterion to distinguish kinetic mechanisms. We analyse the integral kinetics of multi-substrate enzymic reactions accompanied by enzyme inactivation in the reaction course for both unbranched and branched mechanisms. To illustrate the power of the approach, the steady-state kinetics of the peroxidase reaction catalysed by prostaglandin H synthase (PGHS) have been studied. PGHS catalyses two consecutive enzymic steps: oxygenation of arachidonic acid with two dioxygen molecules to yield prostaglandin G2 peroxide followed by two-electron reduction of the 15-hydroperoxyl group of dioxygen molecules to yield prostaglandin G2 peroxide [26]. In spite of numerous studies, no common algorithms for the determination of the peroxidase reaction catalysed by PGHS have been studied in 50 mM Tris/HCl (pH 8.0) containing 0.1% Tween 20 and 1 μM haem, with various concentrations of adrenaline and H2O2 at 31 °C. The reaction was initiated by enzyme addition and monitored spectrophotometrically at 480 nm by the accumulation of the oxidized form of adrenaline (adrenochrome; ε = 4000 M−1·cm−1) [29] with a Shimadzu UV-1601 spectrophotometer (Kyoto, Japan). The concentrations of other reagents in the reaction mixture are given in the Figure legends.

Kinetic data were analysed by Microcal Origin 6.0 (Microcal Software, Northampton, MA, U.S.A.).

RESULTS AND DISCUSSION

Kinetic analysis of unbranched enzyme reaction

The mechanism for an unbranched multi-substrate enzyme reaction with intermediates undergoing irreversible inactivation has the following form:

\[
\text{E}_1 \xrightarrow{\alpha_1} \text{E}_2 \xrightarrow{\alpha_2} \text{E}_3 \xrightarrow{\alpha_3} \ldots \xrightarrow{\alpha_{n-1}} \text{E}_n \xrightarrow{\alpha_n} (E_1)
\]

Here \( E_i \) (where \( 1 \leq i \leq n \), here and elsewhere) are catalytically active enzyme intermediates, \( E^* \) is the inactivated enzyme, and \( \alpha_i \) and \( \beta_i \) (\( 1 \leq i \leq n \)) are rate constants for the first-order (or pseudo-first-order) inter-conversion of these intermediates. Some of the \( \alpha_i \) and \( \beta_i \) terms are products of second-order rate constants multiplied by the substrate or product concentration, respectively, in which case \( \alpha_i > 0 \) and \( \beta_i > 0 \). It is assumed that the reaction proceeds in a forward direction, running from left to right in eqn (1), from substrates to products. The process of irreversible inactivation of intermediates is characterized by the first-order (or pseudo-first-order) rate constants \( \lambda_i > 0 \).

Eqn (1) is described by the following set of equations:

\[
\begin{align*}
\frac{d[E_i]}{dt} &= \alpha_{i-1}[E_{i-1}] + \beta_i[E_{i+1}] - \alpha_i[E_i] - \beta_i[E_i] - \lambda_i[E_i] \\
\frac{d[E^*]}{dt} &= \sum \lambda_i[E_i] \\
v &= \alpha_i[E_i] - \beta_i[E_i] \\
e_0 &= e + [E^*]
\end{align*}
\]

where \( v \) is the reaction rate, \( e_0 \) is the total enzyme concentration and \( e = \sum [E_i] \) is the sum of concentrations of catalytically active intermediates \( E_i \).

Experimental observations of the nature of enzyme reactions and a rational selection of initial conditions allow certain constraints on the parameters in eqn (1) to be made. These permit the following simplifications. (i) The rate constant of each elementary step of the forward enzyme reaction substantially prevails over the rate constant for irreversible inactivation of each enzyme form \( E_i \):

\[
\alpha_i \gg \lambda_i
\]
This restriction is always valid for enzyme reactions with a turnover number much higher than unity, i.e. the enzyme retains its catalytic properties for a significant number of cycles. (ii) The time needed to achieve a steady state with respect to a catalytically active intermediate \( E_i \) is much shorter than the overall time of the enzyme reaction (eqn 1). (iii) The concentrations of substrates and products are essentially unchanged over the observation period, i.e. \( \alpha \) and \( \beta \) are invariant. This condition can be realized with an appropriate selection of the initial enzyme concentration and/or the period of observation.

Under the above conditions, the steady-state equation for catalytically active intermediates follows:

\[
\alpha_{i-1} [E_{i-1}] + \beta_i [E_{i+1}] - \alpha_i [E_i] - \beta_{i-1} [E_i] = 0
\]

The term ‘steady state’ in this article means the maintenance of the steady-state equations for catalytically active intermediates.

The inactivation of the enzyme during the reaction course is described by the equation:

\[
d[\alpha]/dt = -\sum_i \lambda_i [E_i]
\]

with the initial conditions \( e = e_0 \) at \( t = 0 \), and the values of \([E_i]\), as condition (ii) stipulates (see above), to satisfy eqn (7).

The analysis of eqn (1) in the steady state (i.e. under conditions when eqn 7 is valid) can be greatly simplified by using graph theory [19–21]. The graph presents a set of points (vertices) connected by lines (edges). In kinetic terms, the points represent intermediates and each edge of the graph corresponds to a partial reaction with a numerical value assigned to it. The latter is the first-order (or pseudo-first-order) rate constant for the corresponding partial reaction of the catalytic cycle. In our case, we have two reaction types with the corresponding rate constants \( \alpha \) and \( \beta \). To analyse a kinetic scheme with the help of graph theory, one of the base trees (\( b_i \)) for the base vertex \( E_i \) is defined by eqns (9):

\[
\begin{align*}
\beta_i & = \alpha \beta \ldots \alpha \beta \ldots \alpha (\beta \alpha \ldots \alpha (i < j)) \\
\beta_i & = \alpha \beta \ldots \alpha \beta \ldots \alpha (i = j) \\
\beta_i & = \beta \alpha \beta \ldots \beta \alpha \ldots \beta (i > j)
\end{align*}
\]

Here \( i \) is the number of the base, \( j \) is the ordinal number of the base tree. For convenience, the unity denotes the factor with subscript \( j \), which is absent in the product.

The above statements allow the ratio of the steady-state concentrations of the catalytically active enzyme forms in eqn (1) to be represented in a form convenient for analysis:

\[
\frac{[E_i]}{\sum_i [E_i]} = \frac{\sum_j b_j}{\sum_i \sum_j b_j}
\]

This ratio is a function of the constants \( \alpha \) and \( \beta \) and remains unchanged by enzyme inactivation during the reaction course.

The initial steady-state rate \( v_0 \) and the actual steady-state rate after a period of reaction \( v \) are defined by the equations:

\[
v_0 = e_0 \alpha_1 \alpha_2 \ldots \alpha_n - \beta_1 \beta_2 \ldots \beta_n \sum_i \sum_j b_{ij}
\]

(11)

\[
v = e \alpha_1 \alpha_2 \ldots \alpha_n - \beta_1 \beta_2 \ldots \beta_n \sum_i \sum_j b_{ij}
\]

(12)

Eqns (11) and (12) were published previously by us [30,31]. They are much simpler than those published by others [1,32] and have potential as a basis for qualitative analysis.

Taking into account enzyme inactivation occurring during the reaction course (see eqn 8), \( e \) and \( v \) change according to the following equations:

\[
e = e_0 \exp(-\lambda t)
\]

(13)

\[
v = v_0 \exp(-\lambda t)
\]

(14)

\[
\Lambda = \sum_i \Lambda_i
\]

(15)

\[
\Lambda_i = \lambda_i \sum_j b_{ij}
\]

(16)

\( \Lambda \) is the apparent enzyme-inactivation rate constant in the reaction course and \( \Lambda \) is the contribution made to the value of \( \Lambda \) by inactivation of a particular intermediate form \( E_i \).

The progress curves of substrate \( S \) depletion (or product \( P \) accumulation) in the course of the eqn (1) are described by the following equation:

\[
S_0 - [S] = [P] - P_0 = P_\infty [1 - \exp(-\Lambda t)]
\]

(17)

where \( S_0 \) and \( P_0 \) are the initial concentrations of substrate \( S \) and product \( P \) respectively.

The final yield of product, \( P_\infty \) (the concentration of the reaction product after the enzyme inactivation is complete), is given by:

\[
P_\infty = v_0 / \Lambda
\]

(18)

‘Kinetic connectivity’ of enzyme intermediates as a distinguishing criterion

A qualitative analysis of the equations developed above becomes possible with the use of a new concept, kinetic connectivity of enzyme intermediates.

In scalar kinetic connectivity, intermediates \( E_i \) and \( E_m \) (where \( l \neq m \)) are considered ‘connected’ if they are linked by a section in the reaction mechanism, which consists only of reversible steps of enzyme transformations. Otherwise \( E_i \) and \( E_m \) are considered to be ‘unconnected’. The scalar kinetic connectivity \( (l, m) \) is a quantitative descriptor of the kinetic connectivity of all intermediates, having a value of 1 for connected intermediates \( E_i \) and \( E_m \), \((l, m) = 1\), and of 0 for unconnected intermediates \( E_i \) and \( E_m \), \((l, m) = 0\).

With vector kinetic connectivity or downstream kinetic connectivity, intermediates \( E_i \) and \( E_m \) (\( l \neq m \)) are ‘downstream connected’ if there is no irreversible step on the way from intermediate \( E_i \) towards intermediate \( E_m \) downstream in the reaction course.
Otherwise intermediates \( E_i \) and \( E_m \) are considered ‘downstream unconnected’. The extent of vector kinetic connectivity \([l, m]\) is a quantitative descriptor of the vector kinetic connectivity of all intermediates having a value of 1 for downstream connected intermediates \( E_i \) and \( E_m \), \([l, m]=1\), and of 0 for downstream unconnected intermediates \( E_i \) and \( E_m \), \([l, m]=0\).

The analysis of the steady-state rate equation

For eqn (1) to be irreversible, the rate constant for the back reaction of at least one partial reaction must be zero. For convenience, let us assign the subscript \( n \) to this step:

\[
\beta_n = 0
\]

In this case all the terms defined by eqn (9), \( b_{ij} \) (where \( i > j \)), become zero:

\[
b_{ij} = 0 (i > j)
\]

The expression for \( v_0 \) can then be written in the form:

\[
1/v_0 = (1/e_0) \sum_j a_j
\]

where

\[
a_j = b_{ij}/\alpha_i\alpha_j\alpha_{i+1} \ldots \alpha_n
\]

The values of terms \( a_j \) in this case are defined by eqns (23):

\[
a_{ij} = \beta_i\beta_{i+1} \ldots \beta_{j-1}/\alpha_i\alpha_{i+1} \ldots \alpha_j (i < j)
\]

\[
a_{ij} = 1/\alpha_i (i = j)
\]

\[
a_{ij} = 0 (i > j)
\]

The expression for \( 1/v_0 \) has the following properties.

(i) If the substrate \( S \) in eqn (1) takes part in the catalytic cycle only once, i.e. it interacts only with one intermediate enzyme form \( E_i \), then \( a_i = k_i[S] \) where \( k_i \) is the first-order rate constant and [S] is the concentration of substrate \( S \). It should be noted that [S] appears in eqn (21) only as a part of \( a_i \). It can be seen from eqns (21) and (23) that the sum on the right-hand side of eqn (21) contains the [S] term only at the power of 0 or \(-1\); thus, eqn (21) can be written as:

\[
1/v_0 = c_0 + c_1/[S]
\]

\[
c_0 > 0, c_1 > 0
\]  \( \text{ (24) } \)

where \( c_0 \) is the sum of terms that do not contain \( 1/[S] \) and \( c_1 \) is the sum of coefficients at \( 1/[S] \) in eqn (21). Eqn (24) gives linear double-reciprocal plots.

(ii) If substrate \( S \) interacts with the enzyme twice in the catalytic cycle (eqn 1), i.e. with two intermediate forms of the enzyme, \( E_i \) and \( E_m (l \neq m) \), then, using the same reasoning as in the previous paragraph, eqn (21) can be rewritten as a function of \( S \):

\[
1/v_0 = c_0 + c_1/[S] + (l, m)c_2/[S]^2
\]

\[
c_0 > 0, c_1 > 0, c_2 > 0
\]  \( \text{ (25) } \)

This means that if the substrate interacts with two unconnected enzyme intermediates \([l, m]=0\)], then eqn (21) as a function of the concentration of \( S \) will again be linear (eqn 24) in double-reciprocal plots. However, if the substrate interacts with two connected enzyme intermediate forms \([l, m]=1\), then eqn (21) transforms into eqn (25), which gives quadratic parabolic double-reciprocal plots (Figure 1A, curve 1).

(iii) If two substrates \( S_1 \) and \( S_2 \) take part in eqn (1), each interacting with only one enzyme intermediate, \( S_i \) with \( E_i \) and \( S_2 \) with \( E_m (l \neq m) \), then eqn (21) for \([S_1] \) and \([S_2] \) will have the form:

\[
1/v_0 = c_0 + c_1/[S_1] + c_2/[S_2] + (l, m)c_{12}/[S_1][S_2]
\]

\[
c_0 > 0, c_1 > 0, c_2 > 0, c_{12} > 0
\]  \( \text{ (26) } \)

Thus, if substrates \( S_1 \) and \( S_2 \) interact with unconnected enzyme intermediates \([l, m]=0\)], the rate equation gives a set of parallel straight lines for double-reciprocal plots. This is characteristic of a Ping-Pong-type mechanism (Figure 1B). If substrates \( S_1 \) and \( S_2 \) interact with connected enzyme intermediates \([l, m]=1\), the rate equation gives double-reciprocal plots comprising a set of straight lines intersecting at a single point in the second or third quadrant (Figure 1C).

**Enzyme inactivation during the catalytic cycle**

Let us consider again that eqn (1) is irreversible (i.e. eqn 19 is valid). The integral kinetics of eqn (1) described by eqns (9)–(18)
allow the parameters $\Lambda$ (the apparent enzyme-inactivation rate constant) and $P_\infty$ (the final yield of the product) to be determined. In the case of an irreversible reaction ($\beta_i = 0$) all the terms containing $\beta_i$ transform to zero in the expressions for $\Lambda$ (eqn 15) and $P_\infty$ (eqn 18). The expression for $P_\infty$ (eqn 18) acquires the form:

$$1/P_\infty = (1/e_0) \sum \frac{1}{\pi_i} \tag{27}$$

$$1/\pi_i = \lambda_i \sum a_{ij} \tag{28}$$

where $1/\pi_i$ is the contribution that inactivation of the intermediate form $E_i$ makes to the value of $1/P_\infty$, with the $a_{ij}$ values determined by eqns (23).

As follows from eqns (27) and (28) for any value of $i (1 \leq i \leq n)$:

$$e_0/P_\infty \geq 1/\pi_i \geq \lambda_i/\alpha_i \tag{29}$$

If $P_\infty/e_0 \gg 1$, then $\lambda_i \ll \alpha_i$, which means that if the enzyme turnover number before complete inactivation ($P_\infty/e_0$) significantly exceeds 1, then the rate constants for the forward partial reactions prevail over the corresponding inactivation rate constants (see eqn 6).

Since the inactivation of each enzyme intermediate, $E_i$, makes additive contributions to the expressions for $\Lambda$ and $1/P_\infty$ (eqns 15 and 27), then the dependence of $\Lambda$ and $1/P_\infty$ on the substrate concentrations can be analysed (i.e. when several or even all of the enzyme intermediates undergo inactivation).

Let us assume that only one enzyme intermediate in eqn (1), $E_i$, undergoes inactivation, i.e. $\lambda_i \neq 0, \lambda_i = 0$ for all $i \neq r$ and substrate $S$ takes part in eqn (1) only by interacting with the enzyme intermediate $E_r$. In this case, the equations for $\Lambda$ and $1/P_\infty$ have the form:

$$\Lambda = \frac{c_0 + c_i[S]}{d_0 + d_i[S]}$$

$$d_0 > 0, d_i > 0 \tag{30}$$

$$1/P_\infty = f_0 + f_i/[S] \tag{31}$$

where $d_i$ is the sum of terms that do not contain $[S]$ and $d_i$ is the sum of coefficients at $[S]$ containing terms in the denominator of eqn (30), whereas $f_0$ is the sum of terms that do not contain $1/[S]$ and $f_i$ is the sum of coefficients at $1/[S]$ in eqn (31). The following three cases need to be considered in order to classify the observed dependence of $\Lambda$ and $1/P_\infty$ on substrate concentration. (i) The intermediate form of the enzyme undergoing inactivation and the substrate $S$ interaction point are downstream connected, i.e. $[r, l] = 1$. In this case $c_0 > 0, c_i > 0$ in eqn (30) and $f_0 > 0, f_i > 0$ in eqn (31). (ii) The intermediate form of the enzyme undergoing inactivation and the substrate $S$ interaction point are downstream unconnected, i.e. $[r, l] = 0$. In this case $c_0 = 0, c_i > 0$ in eqn (30) and $f_0 > 0, f_i = 0$ in eqn (31). (iii) The substrate $S$ interaction point undergoes inactivation, i.e. $r = l$. In this case $c_0 > 0, c_i = 0$ in eqn (30) and $f_0 = 0, f_i > 0$ in eqn (31).

In the case of eqn (1), if a number of enzyme intermediates are subject to inactivation, then the expressions for $\Lambda$ and $1/P_\infty$ can be written in the form of eqns (30) and (31), respectively, and the type of the dependence of $\Lambda$ and $1/P_\infty$ on the substrate concentration is determined by the kinetic connectivity ratio of each inactivated form with the substrate interaction point ($E_i$). Since the contribution of each inactivated form to the final expression for eqns (15) and (27) is additive, the mechanism of eqn (1) may generate one of the three experimentally observable patterns that are listed below.

**Pattern 1:** if $c_0 = 0, c_i > 0$ in eqn (30) and $f_0 > 0, f_i = 0$ in eqn (31), the dependence of $\Lambda$ on $[S]$ is linear in double-reciprocal plots and $P_\infty$ is independent of $[S]$ (Figure 2A). This kinetic pattern represents the case with the condition $[r, l] = 1$ fulfilled, with all of the enzyme forms undergoing inactivation $E_i$.

**Pattern 2:** if $c_0 > 0, c_i = 0$ in eqn (30) and $f_0 = 0, f_i > 0$ in eqn (31), the dependence of $\Lambda$ on $[S]$ is linear in a $1/\Lambda$ versus $[S]$ plot and $P_\infty$ is directly proportional to $[S]$ (Figure 2B). This kinetic pattern characterizes the case where only one intermediate enzyme form undergoing inactivation interacts with the substrate $S$.

**Pattern 3:** none of the coefficients in eqns (30) and (31) equals zero; the dependence of $\Lambda$ on $[S]$ cannot be represented as a straight line in any plot and the dependence of $P_\infty$ on $[S]$ is linear in double-reciprocal plots (Figure 2C). This kinetic pattern covers all cases other than those noted in patterns 1 and 2, namely (i) at least one intermediate form $E_i ([r, l] = 1)$ undergoes inactivation (in addition to $E_i$, inactivation may involve any number of other intermediate forms) and (ii) the substrate $S$ donation point $E_i$ together with at least one other enzyme intermediate undergo inactivation.

**Kinetic analysis of branched enzyme reactions**

The concept of kinetic connectivity can also be applied to more complex systems such as a branched enzymic reaction. A scheme for a branched reaction [8,9] can always be separated into two parts, i.e. ordered and unordered (see Figure 3). Let us consider that (i) substrate $S$ takes part in the reaction by interacting once with one intermediate in the ordered part, or twice with two intermediates in the unordered part; and (ii) only one intermediate ($E_i$) undergoes inactivation in the course of the reaction. Using graph theory, it can be shown that in this case the dependences of
Table 1 Summary of the types of dependence of $v_0$, $\Lambda$, and $1/P_\infty$ on $[S]$ (eqns 32–34) for various positions of interaction points of substrate $S$, inactivated intermediate $E_i$ and values of kinetic connectivities

<table>
<thead>
<tr>
<th>No</th>
<th>Substrate S donation point</th>
<th>Position of inactivated form $E_i$</th>
<th>Type of dependency of $v_0$, $\Lambda$, and $1/P_\infty$ as a function of $[S]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>l</td>
<td>m</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
</tr>
<tr>
<td>2</td>
<td>l</td>
<td>t</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
</tr>
<tr>
<td>3</td>
<td>l</td>
<td>p</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
</tr>
<tr>
<td>4</td>
<td>l</td>
<td>q</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
</tr>
<tr>
<td>5</td>
<td>q</td>
<td>i</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
</tr>
<tr>
<td>6</td>
<td>q</td>
<td>t</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
</tr>
<tr>
<td>7</td>
<td>q</td>
<td>p</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
</tr>
<tr>
<td>8</td>
<td>q</td>
<td>m</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
</tr>
<tr>
<td>9</td>
<td>p and n</td>
<td>m</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
</tr>
<tr>
<td>10</td>
<td>p and n</td>
<td>p</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
</tr>
<tr>
<td>11</td>
<td>p and n</td>
<td>q</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
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<tr>
<td>12</td>
<td>p and n</td>
<td>t</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
</tr>
<tr>
<td>13</td>
<td>p and n</td>
<td>o</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
</tr>
<tr>
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<td>p and o</td>
<td>n</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
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<tr>
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<td>p and n</td>
<td>n</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
</tr>
<tr>
<td>16</td>
<td>n and t</td>
<td>m</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
</tr>
<tr>
<td>17</td>
<td>n and t</td>
<td>p</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
</tr>
<tr>
<td>18</td>
<td>n and t</td>
<td>q</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
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<tr>
<td>19</td>
<td>o and t</td>
<td>n</td>
<td>$c_0 = 0$, $c_1 \neq 0$, $c_2 = 0$, $t_0 \neq 0$, $t_1 = 0$, $t_2 = 0$, $d_0 = 0$, $d_1 = 0$, $d_2 = 0$</td>
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Table 1 Continued  

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<th>No</th>
<th>Substrate S donation point</th>
<th>Position of inactivated form ( E_i )</th>
<th>Type of dependency of ( v_0, \Lambda ) and ( 1/P_\infty ) as a function of [S]</th>
</tr>
</thead>
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<td>20</td>
<td>( n ) and ( f )</td>
<td>( o )</td>
<td>( c_0 = 0, c_1 \neq 0 ) if ( [p, f] + [p, n] \neq 0 )</td>
</tr>
</tbody>
</table>

\( c_2 \neq 0, t_0 = 0, \)
\( t_1 \neq 0 \) if \( [p, f] + [p, n][o, q] \neq 0, \)
\( t_2 \neq 0, d_0 \neq 0 \) if \( [p, f] + [p, n] \neq 0, \)
\( d_1 \neq 0, d_2 \neq 0 \)
\( c_0 = 0, c_1 \neq 0 \) if \( [p, f] + [p, n] \neq 0, \)
\( c_2 \neq 0, t_0 = 0, \)
\( t_1 \neq 0, t_2 = 0, \)
\( d_0 \neq 0 \) if \( [p, f] + [p, n] \neq 0, \)
\( d_1 \neq 0, d_2 \neq 0 \)

| 21 | \( n \) and \( f \)      | \( n \)                          | \( c_0 = 0, c_1 \neq 0 \) if \( [p, f] + [p, n] \neq 0, \)

\( c_2 \neq 0, t_0 = 0, \)
\( t_1 \neq 0, t_2 = 0, \)
\( d_0 \neq 0 \) if \( [p, f] + [p, n] \neq 0, \)
\( d_1 \neq 0, d_2 \neq 0 \)

\( n_i \) si \( n \) activated during the reaction, then the expression for \( \Lambda \) and \( 1/P_\infty \) can be written in the form of eqns (33) and (34), respectively. Since the contributions of each inactivated form to the final expression for eqns (33) and (34) are additive and the denominators on the right-hand side do not depend on the inactivation parameters, the appropriate forms of eqns (33) and (34) can be easily defined by reference to Table 1 for the relative amounts of inactivated intermediates. This is illustrated by the three examples given below.

**Example 1**

Assuming that \( S \) interacts with intermediates \( E_p \) and \( E_n \) (Figure 3) and that only one intermediate \( (E_n) \) is inactivated during the reaction, then case 11 (Table 1) applies, which together with eqns (32)–(34) gives:

\[ v_0 = \frac{c_1[S] + c_2[S]^2}{d_0 + d_1[S] + d_2[S]^2} \]

\[ \Lambda = \frac{f_0 + f_1[S] + f_2[S]^2}{d_0 + d_1[S] + d_2[S]^2} \]

\[ 1/P_\infty = \frac{d_0 + d_1[S] + d_2[S]^2}{c_0 + c_1[S] + c_2[S]^2} \]

where all of the coefficients are greater than zero.

**Example 2**

Assuming that \( S \) interacts with intermediates \( E_p \) and \( E_n \) and that only one intermediate \( (E_n) \) is inactivated during the reaction, then case 15 (Table 1) applies, which together with eqns (32)–(34) gives:

\[ v_0 = \frac{c_1[S] + c_2[S]^2}{d_0 + d_1[S] + d_2[S]^2} \]

\[ \Lambda = \frac{f_0 + f_1[S] + f_2[S]^2}{d_0 + d_1[S] + d_2[S]^2} \]

\[ 1/P_\infty = \frac{d_0 + d_1[S] + d_2[S]^2}{c_0 + c_1[S] + c_2[S]^2} \]

where all of the coefficients are greater than zero and coefficients \( c_1, c_2, d_0, d_1, d_2 \) are the same as in eqns (35)–(37).

**Example 3**

Assuming that \( S \) interacts with intermediates \( E_p \) and \( E_n \) and that two intermediates \( (E_p \) and \( E_n) \) are inactivated simultaneously during the reaction, \( \Lambda \) and \( 1/P_\infty \) are given by eqns (41) and (42):

\[ \Lambda = (\Lambda)^\nu + (\Lambda)^\nu \]

\[ 1/P_\infty = (1/P_\infty)^\nu + (1/P_\infty)^\nu \]

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**Figure 3** The mechanism of a branched enzymic reaction with one unordered part

Arrows designate the directions of the enzymic reaction. Asterisks (*) and (**) designate two separate links of an unordered part of the branched enzymic reaction. Intermediates \( E_t \) and \( E_n \) (in the ordered part), intermediates \( E_l \) and \( E_m \) (in the unordered part), bifurcation point \( E_p \) and the point of convergence \( E_q \) are indicated. The ordered part of the enzymic reaction contains at least one irreversible step.

\[ v_0, \Lambda \text{ and } 1/P_\infty \text{ on } [S] \text{ are described by the following equations:} \]

\[ v_0 = \frac{c_0 + c_1[S] + c_2[S]^2}{d_0 + d_1[S] + d_2[S]^2} \]  

\[ \Lambda = \frac{f_0 + f_1[S] + f_2[S]^2}{d_0 + d_1[S] + d_2[S]^2} \]  

\[ 1/P_\infty = \frac{f_0 + f_1[S] + f_2[S]^2}{c_0 + c_1[S] + c_2[S]^2} \]
Using eqns (36), (37) and (39)–(42) we obtain:

\[
\Lambda = \frac{f_0^e + (f_0^e + f_0^s[n, q][S] + f_0^s[S]^2}{d_0 + d_1[S] + d_2[S]^2}
\]

(43)

\[
1/P_\infty = \frac{f_0^e + (f_0^e + f_0^s[n, q][S] + f_0^s[S]^2}{c_1[S] + c_2[S]^2}
\]

(44)

The equation for \(v_0\) is the same as eqn (35) or (38).

Expressions for three or more intermediates undergoing inactivation can be developed in a similar manner with \(\Lambda\) and \(1/P_\infty\) easily deduced by summation of the relevant coefficients given in Table 1.

Thus, the analysis of experimental data in terms of kinetic connectivity provides rigorous constraints on any kinetic model that is developed. These kinetic connectivity relationships define the class of model(s) able to satisfy the experimental data. To describe the experimental data within the established class of generalized models, the simplest model can be selected initially and then this can be developed. This process is shown below for adrenaline oxidation by \(H_2O_2\), catalysed by PGHS isolated from ovine vesicular glands.

**Steady-state kinetics of the PGHS-catalysed peroxidase reaction**

The steady-state kinetics of the peroxidase reaction catalysed by PGHS were studied with \(H_2O_2\) and adrenaline substrates, designated \(S_1\) and \(S_2\), respectively. The integral kinetics of accumulation of the peroxidase reaction product (adrenochrome) for different enzyme concentrations \((e_0)\) are shown in Figure 4(A).

The curves are fitted by eqns (17) and (18) for all concentrations of \(H_2O_2\) and adrenaline under conditions in which the final yield of the reaction product \((P_\infty)\) did not exceed 15% of the initial concentration of each substrate. The values of \(v_0\), \(P_\infty\) and \(\Lambda\) as functions of \(e_0\) obtained from the data presented in Figure 4(A) are shown in Figure 4(B). It can be seen that the values of \(v_0\) and \(P_\infty\) are proportional to \(e_0\), whereas the values of \(\Lambda\) are virtually independent of \(e_0\). This result is entirely consistent with eqns (11), (27) and (28), and (15) and (16).
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Figure 6  PGHS inactivation in the course of the peroxidase reaction
The dependence of the inactivation apparent rate constant in the catalytic cycle (Λ) on the concentration of H2O2 in a double-reciprocal plot. Adrenaline concentrations: 0.086 mM (●), 0.186 mM (▲), 0.466 mM (○) and 1.4 mM (△). Solid lines: simulated dependencies using eqn (47). Conditions: as for Figure 5.

Figure 7  PGHS inactivation in the course of the peroxidase reaction
The dependence of the final yield of the reaction product (P∞) on the concentration of H2O2 shown by a double-reciprocal plot. Adrenaline concentration: 0.086 mM (△), 0.186 mM (○), 0.466 mM (▲) and 1.4 mM (●). Solid lines: simulated dependencies using eqn (46). Conditions: as for Figure 5.

Figure 8  PGHS inactivation in the course of the peroxidase reaction
The dependence of the apparent inactivation rate constant in the course of the reaction (Λ) on the concentration of adrenaline. H2O2 concentration: 0.064 mM (△), 0.089 mM (○), 0.127 mM (□), 0.254 mM (●) and 0.508 mM (▲). Solid lines: simulated dependencies using eqn (47). Conditions: as for Figure 5.

Figure 9  PGHS inactivation in the course of the peroxidase reaction
The dependence of the final yield of the reaction product (P∞) on the concentration of adrenaline shown by a double-reciprocal plot. H2O2 concentration: 0.064 mM (□), 0.127 mM (○), 0.254 mM (●), 0.356 mM (△) and 0.508 mM (▲). Solid line: simulated dependency using eqn (46). Conditions: as for Figure 5.

The dependence of the experimentally evaluated parameters ν₀, P∞ and Λ on the concentrations of substrates, i.e. H2O2 and adrenaline, was also determined. Those parts of the experimental integral curves satisfying the condition of low substrate consumption were described by eqns (17) and (18). The dependence of the initial rate of the peroxidase reaction, ν₀, on the concentrations of H2O2 and adrenaline (Figure 5) are a set of parallel straight lines in double-reciprocal plots described by eqn (26) under the condition of (l, m) = 0. This means that the scalar kinetic connectivity of the enzyme intermediates reacting with H2O2 and adrenaline is equal to zero: (H2O2, Ad) = 0. The curves in Figure 5 were simulated by the following empirical equation, obtained using linear regression:

\[
\frac{1}{ν₀} = (14 ± 1) + (6.86 ± 0.6)/[H₂O₂] + (7.28 ± 0.8)/[Ad]
\]

(45)

where [Ad] is the concentration of adrenaline. The dependencies of P∞ and Λ on the concentration of substrates are shown in Figures 6–9. The dependence of Λ on the concentration of
The terms \(E_r\) must be present in the reaction course between each intermediate form of PGHS peroxidase reaction with adrenaline as the reducing substrate. It can be seen from Figures 6 and 8 that eqn (47) describes the linear regression:

\[
\frac{1}{P\infty} = (11 \pm 1) + (7.4 \pm 1.5)/[\text{Ad}]
\]

The terms \(v_0\), \(P\infty\) and \(\Lambda\) are not independent and should obey eqn (18). The empirical equation for the dependence of \(\Lambda\) on the concentration of both peroxidase substrates should obey eqns (18), (45) and (46) and have the form:

\[
\Lambda = \frac{(11 \pm 1) + (7.4 \pm 1.5)/[\text{Ad}]}{(14 \pm 1) + (6.86 \pm 0.6)/[\text{H}_2\text{O}_2] + (7.28 \pm 0.8)/[\text{Ad}]}\]

H\(_2\)O\(_2\) obeys eqn (30) at \(c_0 = 0\), \(c_1 \neq 0\) and gives a linear double-reciprocal plot (see Figure 6); \(P\infty\) obeys eqn (31) at \(f_0 \neq 0\), \(c_1 \neq 0\) and is independent of the concentration of H\(_2\)O\(_2\) (see Figure 7).

The dependence of \(\Lambda\) on the concentration of adrenaline is governed by eqn (30) at \(c_0 \neq 0\), \(c_1 \neq 0\) (see Figure 8); the dependence of \(P\infty\) on the concentration of adrenaline obeys eqn (31) at \(f_0 \neq 0\), \(c_1 \neq 0\) and results in a linear double-reciprocal plot (see Figure 9).

The simulated curves fitting the dependence of \(P\infty\) on the concentration of adrenaline and H\(_2\)O\(_2\) in Figures 7 and 9 are described by the following empirical equation, obtained using linear regression:

\[
1/P\infty = (11 \pm 1) + (7.4 \pm 1.5)/[\text{Ad}]
\]

where \(\alpha_1\) and \(\alpha_2\) are pseudo-first-order rate constants equal to \(k_1[\text{H}_2\text{O}_2]\) and \(k_1[\text{Ad}]\) respectively (\(k_1\) and \(k_2\) are the second-order rate constants). The simplest kinetic scheme, which can serve as a starting point for the creation of a kinetic mechanism of the peroxidase reaction, has the following form:

\[
E_1 \overset{k_1}{\underset{\beta_1}{\rightleftharpoons}} E_2 \overset{k_2}{\underset{\beta_2}{\rightleftharpoons}} E_3 \overset{k_3}{\underset{\beta_3}{\rightleftharpoons}} E_4 \overset{k_4}{\underset{\beta_4}{\rightleftharpoons}} (E_1)
\]

where \(\alpha_1\) and \(\alpha_2\) are pseudo-first-order rate constants equal to \(k_1[\text{H}_2\text{O}_2]\) and \(k_1[\text{Ad}]\) respectively (\(k_1\) and \(k_2\) are the second-order rate constants).

A consideration of the relevant literature [35–38] allows the following forms of PGHS to be assigned to the intermediate enzyme–substrate complexes shown in eqn (48): \(E_1\), \([\text{Fe(III)}]\); \(E_2\), \([\text{Fe(II)}]\) \(\cdots\) \(H_2\text{O}_2\); \(E_3\), \(\text{[Fe(IV) = O]^{+}}\) and \(E_4\), \(\text{[Fe(IV) = O]^{+} \cdots Ad}\) (see Scheme 1).

To meet all of the requirements described above, eqn (48) must satisfy the following conditions. Condition 1, \(\beta_2 = 0\) (requirement 1). Condition 2, \(\beta_1 = 0\) (requirements 1 and 2). Condition 3, \(\lambda_1 = 0\) (requirement 2). Condition 4, the number of inactivated intermediates must exceed 1. This results from the contradiction of requirement 3 and condition 1 for eqn (48).

In other words, there are no intermediates \(E_i\) in eqn (48) that satisfy the stipulation that \([E_i, \text{Ad}] = 1\). Condition 5, \(\lambda_2 \neq 0\) and \(\lambda_2 + \lambda_4 \neq 0\). Since requirement 3 is unrealizable, then \(E_1\) must undergo inactivation with at least one other intermediate.

Satisfying condition 5 requires that either all three forms of the enzyme, \([\text{Fe(III)}] \cdots H_2\text{O}_2\), \(\text{[Fe(IV) = O]^{+}}\) and
predicted the characteristic features of the peroxidase reaction catalysed by PGHS with adrenaline as the reducing substrate. Its future application to the cyclo-oxygenase reaction catalysed by PGHS may provide new insights into the kinetic relationship between the two active sites in PGHS, i.e. the haem iron and the arachidonic acid-binding site that contains the catalytically active tyrosine residue.

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[Fe(IV) = O]+···Ad, or the combination of two forms, either [Fe(III)]·H2O2 or [Fe(IV) = O]+ and [Fe(IV) = O]+···Ad, are inactivated during the catalytic cycle. We consider that if [Fe(IV) = O]+ is unstable (λ3 ≠ 0), then its adduct with adrenaline {[Fe(IV) = O]+···Ad} must also be unstable (λ3 ≠ 0). On the other hand, there is no reason to suppose that the adduct of the stable enzyme form, [Fe(III)], with H2O2 undergoes inactivation. We consider that the simplest and most probable kinetic mechanism satisfying all the above requirements and fitting our experimental data is that shown in Scheme 1.

The nature of the intermediates that undergo inactivation in the peroxidase cycle of PGHS has been discussed previously [36,39,40]. Smith et al. [36,40] concluded that [Fe(IV) = O]+ (the adenine interaction point) is the only enzyme form subject to inactivation in the peroxidase cycle. However, if this were true, the apparent rate constant for enzyme inactivation at a fixed concentration of H2O2 would be described by eqn (30) with c1 = 0. This contradicts requirement 3 for our mechanism that is based on experimental data and is represented by the empirical eqn (47). The inactivation of haem-containing peroxidases in the absence of a donor substrate has been studied by Rodriguez-Lopez et al [41]: the interaction of compound I with H2O2 or m-chloroperoxybenzoic acid was found to cause irreversible inactivation. However, if this were also true for the PGHS peroxidase reaction, our experimental data would have exhibited a square dependence for parameter Λ on H2O2 concentration. This has not been observed. Compound I for PGHS can exist in a ferryl haem [Fe(IV) = O] form with either a haem π-cation radical or protein tyrosyl radical. Thus both compound I and its adduct with adrenaline {[Fe(IV) = O]+···Ad} must also be unstable (λ3 ≠ 0). On the other hand, there is no reason to suppose that the adduct of the stable enzyme form, [Fe(III)], with H2O2 undergoes inactivation. We consider that the simplest and most probable kinetic mechanism satisfying all the above requirements and fitting our experimental data is that shown in Scheme 1.

The proposed mechanism (Scheme 1) allows us to evaluate the inactivation rate constants λ3 and λ4. By using eqns (15) and (16) the expressions for Λ in this case are defined by eqn (49):

\[ Λ = \frac{λ_3[k_1[H_2O_2]α_2α_4 + k_1[H_2O_2]α_2β_1] + λ_4k_1[H_2O_2]α_4k_4[Ad]}{α_2k_4[Ad]α_4 + β_1k_4[Ad]α_4 + k_1[H_2O_2]k_4α_4 + k_1[H_2O_2]α_4α_4 + k_1[H_2O_2]α_4β_1 + k_1[H_2O_2]α_4k_4[Ad]} \]  

If the concentration of adrenaline decreases to zero, the value of Λ (eqn 49) becomes equal to λ3. From eqn (47) (see also Figure 8), λ3 = 1.0 ± 0.2 min⁻¹. If the concentrations of adrenaline and H2O2 increase infinitely, then Λ (eqn 49) becomes equal to λ3(α2 / (α2 + α4)). From eqn (47) (see also Figure 6), λ3α2 / (α2 + α4) = 0.8 ± 0.1 min⁻¹. Strictly speaking, we must say that λ3 = 1.0 ± 0.2 min⁻¹ and λ3 > 0.8 ± 0.1 min⁻¹. If α2 > α4 then λ3 = 0.8 ± 0.1 min⁻¹. In this case the values of λ3 and λ4 are essentially the same. This is not unreasonable since the inactivation of [Fe(IV) = O]+, characterized by λ3, and the inactivation of [Fe(IV) = O]+···Ad, characterized by λ4, is most probably the result of the same chemical reaction involving free-radical-induced oxidation of the haem.

Thus our new concept of scalar and vector kinetic connectivity of enzyme intermediate forms has been shown to be capable of providing the criteria necessary to test the consistency of a proposed kinetic mechanism with the experimentally observed kinetics of a multi-substrate reaction that is accompanied by irreversible enzyme inactivation. The application of this novel approach and formulation of these criteria predicts the key properties of any kinetic model. This new method has successfully
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