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 apposite, 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

Abbreviation used: PGHS, prostaglandin H synthase.

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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 are, to our knowledge, no common algorithms for the determination of the

the peroxidase reaction catalysed by PGHS were studied in 50 mM Tris/HCl (pH 8.0) containing 0.1 % Tween 20 and 1 μ M haemin, with various concentrations of adrenaline and H_2O_2 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; $\varepsilon = 4000 \text{ M}^{-1} \cdot \text{cm}^{-1}$) [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:

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 G_2 peroxide followed by two-electron reduction of the 15-hydroperoxyl group of prostaglandin G_2 to form prostaglandin H_2 [25]. In addition to this physiological activity, the enzyme exhibits conventional peroxidic activity towards a wide variety of alternative electron-donor substrates. PGHS is irreversibly inactivated in the course of both catalytic processes [26]. In spite of numerous studies on the structure and function of PGHS, the precise mechanism remains obscure. Our novel approach has allowed us to formulate the minimum requirements for the kinetic mechanism of the peroxidase reaction catalysed by PGHS with accompanying enzyme inactivation.

EXPERIMENTAL

The following reagents were used: 40 % H_2O_2 and L-adrenaline from Merck (Darmstadt, Germany), Tween 20 from Ferak (Berlin, Germany) and Tris, GSH and Fe-protoporphyrin IX (haemin) from Sigma (St. Louis, MO, U.S.A.).

PGHS was isolated from sheep’s vesicular glands that had been stored at -40°C and purified to homogeneity as described previously [27]. Enzyme concentrations were determined by a modified Lowry method [28]. The kinetics of

Here E_i (where $1 \leq i \leq n$, here and elsewhere) are catalytically active enzyme intermediates, E^* is the inactivated enzyme, and α_i and β_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 α_i and β_i terms are products of second-order rate constants multiplied by the substrate or product concentration, respectively, in which case $\alpha_i > 0$ and $\beta_i \geq 0$. 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 $\lambda_i \geq 0$.

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

$$d[E_i]/dt = \alpha_{i-1}[E_{i-1}] + \beta_i[E_{i+1}] - \alpha_i[E_i] - \beta_{i-1}[E_i] - \lambda_i[E_i] \quad (2)$$

$$d[E^*]/dt = \sum_i \lambda_i[E_i] \quad (3)$$

$$v = \alpha_n[E_n] - \beta_n[E_1] \quad (4)$$

$$e_0 = e + [E^*] \quad (5)$$

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 :

$$\alpha_i \gg \lambda_i \quad (6)$$

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. α_i and β_i 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:

$$\alpha_{i-1}[E_{i-1}] + \beta_i[E_{i+1}] - \alpha_i[E_i] - \beta_{i-1}[E_i] = 0 \quad (7)$$

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:

$$d[e]/dt = - \sum_i \lambda_i [E_i] \quad (8)$$

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 α_i and β_i . To analyse a kinetic scheme with the help of graph theory, one of the graph vertices (E_i) needs to be chosen as the base vertex. The set of edges passing through all the vertices of the graph and directed towards the base comprise the 'base tree'. The base tree contains no cycles. The magnitude of the base tree is equal to the product of the magnitudes of the edges making up this base tree. In our case for eqn (1) in the steady state the magnitudes of the base trees (b_{ij}) for the base vertex E_i are defined by eqns (9):

$$\begin{aligned} b_{ij} &= \alpha_1 \alpha_2 \dots \alpha_{i-1} \beta_i \beta_{i+1} \dots \beta_{j-1} 1 \alpha_{j+1} \dots \alpha_n \quad (i < j) \\ b_{ij} &= \alpha_1 \alpha_2 \dots \alpha_{i-1} 1 \alpha_{i+1} \dots \alpha_n \quad (i = j) \\ b_{ij} &= \beta_1 \beta_2 \dots \beta_{j-1} 1 \alpha_{j+1} \dots \alpha_{i-1} \beta_i \dots \beta_n \quad (i > j) \end{aligned} \quad (9)$$

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:

$$\frac{[E_i]}{\sum_i [E_i]} = \frac{\sum_j b_{ij}}{\sum_i \sum_j b_{ij}} \quad (10)$$

This ratio is a function of the constants α_i and β_i 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:

$$v_0 = e_0 \frac{\alpha_1 \alpha_2 \alpha_3 \dots \alpha_n - \beta_1 \beta_2 \beta_3 \dots \beta_n}{\sum_i \sum_j b_{ij}} \quad (11)$$

$$v = e \frac{\alpha_1 \alpha_2 \alpha_3 \dots \alpha_n - \beta_1 \beta_2 \beta_3 \dots \beta_n}{\sum_i \sum_j b_{ij}} \quad (12)$$

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:

$$e = e_0 \exp(-\Lambda t) \quad (13)$$

$$v = v_0 \exp(-\Lambda t) \quad (14)$$

$$\Lambda = \sum_i \Lambda_i \quad (15)$$

$$\Lambda_i = \frac{\lambda_i \sum_j b_{ij}}{\sum_i \sum_j b_{ij}} \quad (16)$$

Λ is the apparent enzyme-inactivation rate constant in the reaction course and Λ_i is the contribution made to the value of Λ 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:

$$S_0 - [S] = [P] - P_0 = P_\infty \{1 - \exp(-\Lambda t)\} \quad (17)$$

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

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

$$P_\infty = v_0 / \Lambda \quad (18)$$

'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_l 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_l 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_l and E_m , (l, m) = 1, and of 0 for unconnected intermediates E_l and E_m , (l, m) = 0.

With vector kinetic connectivity or downstream kinetic connectivity, intermediates E_l and E_m ($l \neq m$) are 'downstream connected' if there is no irreversible step on the way from intermediate E_l towards intermediate E_m downstream in the reaction course.

Figure 1 Scalar kinetic connectivity of intermediate enzyme forms as a criterion of kinetic behaviour of a multi-substrate enzymic reaction

Analysis using the Lineweaver–Burk plot. (A) Substrate S participates in eqn (1) twice, interacting with connected (curve 1) or unconnected (curve 2) intermediate forms. (B) Substrates S_1 and S_2 interact with unconnected intermediate enzyme forms in eqn (1). (C) Substrates S_1 and S_2 interact with connected intermediate enzyme forms in eqn (1).

Otherwise intermediates E_l 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_l and E_m , $[l, m] = 1$, and of 0 for downstream unconnected intermediates E_l 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:

$$\beta_n = 0 \quad (19)$$

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

$$b_{ij} = 0 (i > j) \quad (20)$$

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

$$1/v_0 = (1/e_0) \sum_i \sum_j a_{ij} \quad (21)$$

where

$$a_{ij} = b_{ij}/\alpha_1\alpha_2\alpha_3 \dots \alpha_n \quad (22)$$

The values of terms a_{ij} in this case are defined by eqns (23):

$$\begin{aligned} a_{ij} &= \beta_i\beta_{i+1} \dots \beta_{j-1}/\alpha_i\alpha_{i+1} \dots \alpha_j (i < j) \\ a_{ij} &= 1/\alpha_i (i = j) \\ a_{ij} &= 0 (i > j) \end{aligned} \quad (23)$$

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_l , 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:

$$\begin{aligned} 1/v_0 &= c_0 + c_1/[S] \\ c_0 > 0, c_1 > 0 \end{aligned} \quad (24)$$

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_l 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 :

$$\begin{aligned} 1/v_0 &= c_0 + c_1/[S] + (l, m)c_2/[S]^2 \\ c_0 > 0, c_1 > 0, c_2 > 0 \end{aligned} \quad (25)$$

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_1 with E_l and S_2 with E_m ($l \neq m$), then eqn (21) for $[S_1]$ and $[S_2]$ will have the form:

$$\begin{aligned} 1/v_0 &= c_0 + c_1/[S_1] + c_2/[S_2] + (l, m)c_{12}/[S_1][S_2] \\ c_0 > 0, c_1 > 0, c_2 > 0, c_{12} > 0 \end{aligned} \quad (26)$$

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 Λ (the apparent enzyme-inactivation rate constant) and P_∞ (the final yield of the product) to be determined. In the case of an irreversible reaction ($\beta_n = 0$) all the terms containing β_n transform to zero in the expressions for Λ (eqn 15) and P_∞ (eqn 18). The expression for P_∞ (eqn 18) acquires the form:

$$1/P_\infty = (1/e_0) \sum_i \frac{1}{\pi_i} \quad (27)$$

$$1/\pi_i = \lambda_i \sum_j a_{ij} \quad (28)$$

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 ($1 \leq i \leq n$):

$$e_0/P_\infty \geq 1/\pi_i \geq \lambda_i/\alpha_i \quad (29)$$

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_∞/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 Λ and $1/P_\infty$ (eqns 15 and 27), then the dependence of Λ 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_r , undergoes inactivation, i.e. $\lambda_r \neq 0$, $\lambda_i = 0$ 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 Λ and $1/P_\infty$ have the form:

$$\Lambda = \frac{c_0 + c_1[S]}{d_0 + d_1[S]} \quad (30)$$

$$d_0 > 0, d_1 > 0$$

$$1/P_\infty = f_0 + f_1/[S] \quad (31)$$

where d_0 is the sum of terms that do not contain $[S]$ and d_1 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_1 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 Λ 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 $c_0 > 0$, $c_1 > 0$ in eqn (30) and $f_0 > 0$, $f_1 > 0$ 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 $c_0 = 0$, $c_1 > 0$ in eqn (30) and $f_0 > 0$, $f_1 = 0$ in eqn (31). (iii) The substrate S interaction point undergoes inactivation, i.e. $r = l$. In this case $c_0 > 0$, $c_1 = 0$ in eqn (30) and $f_0 = 0$, $f_1 > 0$ in eqn (31).

In the case of eqn (1), if a number of enzyme intermediates are subject to inactivation, then the expressions for Λ and $1/P_\infty$ can be written in the form of eqns (30) and (31), respectively, and the type of the dependence of Λ 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_r). Since the contribution of each inactivated form to the final

Figure 2 Vector kinetic connectivity between intermediate enzyme forms as a criterion for the enzyme-inactivation mechanism in the catalytic cycle

Typical functions of the apparent enzyme-inactivation rate constant during reaction (Λ) and maximal yield of the product of the enzymic reaction (P_∞) versus substrate concentration. (A) All intermediate enzyme forms inactivated in eqn (1) are downstream unconnected with the substrate S interaction point. (B) Only the substrate S interaction point is inactivated. (C) All other cases.

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 $c_0 = 0$, $c_1 > 0$ in eqn (30) and $f_0 > 0$, $f_1 = 0$ in eqn (31), the dependence of Λ on $[S]$ is linear in double-reciprocal plots and P_∞ is independent of $[S]$ (Figure 2A). This kinetic pattern represents the case with the condition $[r, l] = 0$ fulfilled, with all of the enzyme forms undergoing inactivation E_r .

Pattern 2: if $c_0 > 0$, $c_1 = 0$ in eqn (30) and $f_0 = 0$, $f_1 > 0$ in eqn (31), the dependence of Λ on $[S]$ is linear in a $1/\Lambda$ versus $[S]$ plot and P_∞ 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 Λ on $[S]$ cannot be represented as a straight line in any plot and the dependence of P_∞ 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_r ($[r, l] = 1$) undergoes inactivation (in addition to E_r , inactivation may involve any number of other intermediate forms) and (ii) the substrate S donation point E_l 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_r) 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 , Λ 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

* and ** indicate the links of the unordered part (see Figure 3), for which vector connectivity is assumed. It is clear that the vector connectivity $[p, q]$ can be determined through * or ** paths, necessitating the designation $[p, q]^*$ and $[p, q]^{**}$.

No	Substrate S donation point	Position of inactivated form E_i	Type of dependency of v_0 , Λ and $1/P_\infty$ as a function of $[S]$
1	l	m	$c_0 = 0, c_1 \neq 0, c_2 = 0,$ $f_0 \neq 0$ if $[m, l] = 1, f_1 \neq 0, f_2 = 0,$ $d_0 \neq 0, d_1 \neq 0, d_2 = 0$
2	l	t	$c_0 = 0, c_1 \neq 0, c_2 = 0,$ $f_0 \neq 0$ if $[q, l]([t, q] + [p, q]^*) \neq 0, f_1 \neq 0, f_2 = 0,$ $d_0 \neq 0, d_1 \neq 0, d_2 = 0$
3	l	p	$c_0 = 0, c_1 \neq 0, c_2 = 0,$ $f_0 \neq 0$ if $([p, q]^* + [p, q]^{**})[q, l] \neq 0,$ $f_1 \neq 0, f_2 = 0, d_0 \neq 0, d_1 \neq 0, d_2 = 0$
4	l	q	$c_0 = 0, c_1 \neq 0, c_2 = 0,$ $f_0 \neq 0$ if $[q, l] \neq 0, f_1 \neq 0, f_2 = 0,$ $d_0 \neq 0, d_1 \neq 0, d_2 = 0$
5	q	l	$c_0 = 0, c_1 \neq 0, c_2 = 0,$ $f_0 \neq 0$ if $[l, p][p, q]^*[p, q]^{**} \neq 0,$ $f_1 \neq 0, f_2 = 0, d_0 \neq 0, d_1 \neq 0, d_2 = 0$
6	q	t	$c_0 = 0, c_1 \neq 0, c_2 = 0,$ $f_0 \neq 0$ if $[t, q] + [p, q]^* \neq 0,$ $f_1 \neq 0, f_2 = 0, d_0 \neq 0, d_1 \neq 0, d_2 = 0$
7	q	p	$c_0 = 0, c_1 \neq 0, c_2 = 0,$ $f_0 \neq 0$ if $[p, q]^* + [p, q]^{**} \neq 0,$ $f_1 \neq 0, f_2 = 0, d_0 \neq 0, d_1 \neq 0, d_2 = 0$
8	q	q	$c_0 = 0, c_1 \neq 0, c_2 = 0,$ $f_0 \neq 0, f_1 = 0, f_2 = 0,$ $d_0 \neq 0, d_1 \neq 0, d_2 = 0$
9	p and n	m	$c_0 = 0, c_1 \neq 0, c_2 \neq 0,$ $f_0 \neq 0$ if $[p, n][m, p] \neq 0,$ $f_1 \neq 0, f_2 \neq 0, d_0 \neq 0, d_1 \neq 0, d_2 \neq 0$
10	p and n	p	$c_0 = 0, c_1 \neq 0, c_2 \neq 0,$ $f_0 \neq 0$ if $[p, n] \neq 0,$ $f_1 \neq 0, f_2 = 0, d_0 \neq 0, d_1 \neq 0, d_2 \neq 0$
11	p and n	q	$c_0 = 0, c_1 \neq 0, c_2 \neq 0,$ $f_0 = 0, f_1 \neq 0, f_2 \neq 0,$ $d_0 \neq 0, d_1 \neq 0, d_2 \neq 0$
12	p and n	t	$c_0 = 0, c_1 \neq 0, c_2 \neq 0, f_0 = 0,$ $f_1 \neq 0$ if $[t, q] + [p, n] \neq 0,$ $f_2 \neq 0, d_0 \neq 0, d_1 \neq 0, d_2 \neq 0$
13	p and n	o	$c_0 = 0, c_1 \neq 0, c_2 \neq 0,$ $f_0 = 0, f_1 \neq 0,$ $f_2 \neq 0$ if $[o, q] \neq 0, d_0 \neq 0, d_1 \neq 0, d_2 \neq 0$
14	p and o	n	$c_0 = 0, c_1 \neq 0, c_2 \neq 0,$ $f_0 \neq 0$ if $[n, o] \neq 0,$ $f_1 \neq 0, f_2 = 0, d_0 \neq 0, d_1 \neq 0, d_2 \neq 0$
15	p and n	n	$c_0 = 0, c_1 \neq 0, c_2 \neq 0,$ $f_0 \neq 0, f_1 \neq 0$ if $[n, q] \neq 0,$ $f_2 = 0, d_0 \neq 0, d_1 \neq 0, d_2 \neq 0$
16	n and t	m	$c_0 = 0, c_1 \neq 0$ if $[p, n] + [p, t] \neq 0,$ $c_2 \neq 0, f_0 \neq 0$ if $[m, p][p, n][p, t] \neq 0,$ $f_1 \neq 0$ if $[p, n] + [p, t] \neq 0,$ $f_2 \neq 0, d_0 \neq 0$ if $[p, n] + [p, t] \neq 0,$ $d_1 \neq 0, d_2 \neq 0$
17	n and t	p	$c_0 = 0, c_1 \neq 0$ if $[p, n] + [p, t] \neq 0$ $c_2 \neq 0, f_0 \neq 0$ if $[p, t][p, n] \neq 0,$ $f_1 \neq 0$ if $[p, t] + [p, n] \neq 0, f_2 \neq 0$ $d_0 \neq 0$ if $[p, t] + [p, n] \neq 0,$ $d_1 \neq 0, d_2 \neq 0$
18	n and t	q	$c_0 = 0, c_1 \neq 0$ if $[p, t] + [p, n] \neq 0$ $c_2 \neq 0, f_0 = 0,$ $f_1 \neq 0$ if $[p, t] + [p, n] \neq 0,$ $f_2 \neq 0, d_0 \neq 0$ if $[p, t] + [p, n] \neq 0,$ $d_1 \neq 0, d_2 \neq 0$
19	o and t	n	$c_0 = 0, c_1 \neq 0$ if $[p, t] + [p, o] \neq 0$ $c_2 \neq 0, f_0 \neq 0$ if $[p, t][n, o] \neq 0,$ $f_1 \neq 0$ if $[p, t] + [n, o] \neq 0,$ $f_2 \neq 0, d_0 \neq 0$ if $[p, t] + [p, n] \neq 0,$ $d_1 \neq 0, d_2 \neq 0$

Table 1 Continued

No	Substrate <i>S</i> donation point	Position of inactivated form E_r	Type of dependency of v_0 , Λ and $1/P_\infty$ as a function of $[S]$
20	<i>n</i> and <i>t</i>	<i>o</i>	$c_0 = 0, c_1 \neq 0$ if $[p, t] + [p, n] \neq 0$ $c_2 \neq 0, f_0 = 0$, $f_1 \neq 0$ if $[p, t] + [p, n][o, q] \neq 0$, $f_2 \neq 0, d_0 \neq 0$ if $[p, t] + [p, n] \neq 0$, $d_1 \neq 0, d_2 \neq 0$
21	<i>n</i> and <i>t</i>	<i>n</i>	$c_0 = 0, c_1 \neq 0$ if $[p, t] + [p, n] \neq 0$, $c_2 \neq 0, f_0 \neq 0$ if $[p, t] \neq 0$, $f_1 \neq 0, f_2 = 0$, $d_0 \neq 0$ if $[p, t] + [p, n] \neq 0$, $d_1 \neq 0, d_2 \neq 0$

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

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

v_0 , Λ and $1/P_\infty$ on $[S]$ are described by the following equations:

$$v_0 = \frac{c_0 + c_1[S] + c_2[S]^2}{d_0 + d_1[S] + d_2[S]^2} \quad (32)$$

$$\Lambda = \frac{f_0 + f_1[S] + f_2[S]^2}{d_0 + d_1[S] + d_2[S]^2} \quad (33)$$

$$1/P_\infty = \frac{f_0 + f_1[S] + f_2[S]^2}{c_0 + c_1[S] + c_2[S]^2} \quad (34)$$

with all of the coefficients greater than or equal to zero (the exact restrictions on these coefficients are listed in Table 1).

Above we considered the simplest case, when only one intermediate undergoes inactivation. When two or more intermediates are subject to inactivation, then the expressions for Λ 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_q) is inactivated during the reaction, then case 11 (Table 1) applies, which together with eqns (32)–(34) gives:

$$v_0 = \frac{c_1[S] + c_2[S]^2}{d_0 + d_1[S] + d_2[S]^2} \quad (35)$$

$$(\Lambda)^q = \frac{f_1^q[S] + f_2^q[S]^2}{d_0 + d_1[S] + d_2[S]^2} \quad (36)$$

$$(1/P_\infty)^q = \frac{f_1^q[S] + f_2^q[S]^2}{c_1[S] + c_2[S]^2} \quad (37)$$

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:

$$v_0 = \frac{c_1[S] + c_2[S]^2}{d_0 + d_1[S] + d_2[S]^2} \quad (38)$$

$$(\Lambda)^n = \frac{f_0^n + f_1^n[n, q][S]}{d_0 + d_1[S] + d_2[S]^2} \quad (39)$$

$$(1/P_\infty)^n = \frac{f_0^n + f_1^n[n, q][S]}{c_1[S] + c_2[S]^2} \quad (40)$$

where all of the coefficients are greater than zero and coefficients c_1 , c_2 , d_0 , d_1 and 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_n and E_q) are inactivated simultaneously during the reaction. Λ and $1/P_\infty$ are given by eqns (41) and (42):

$$\Lambda = (\Lambda)^q + (\Lambda)^n \quad (41)$$

$$1/P_\infty = (1/P_\infty)^q + (1/P_\infty)^n \quad (42)$$

Figure 4 Progress curves of the PGHS-catalysed peroxidase reactions

(A) Kinetics of product accumulation (adrenochrome) under conditions of low substrate consumption in the presence of different concentrations of PGHS. ● indicate experimental data and solid lines are simulations of the experimental data with eqns (17) and (18). Solubilized preparation of PGHS ($\mu\text{g/ml}$ protein): curve 1, 0; curve 2, 11.3; curve 3, 18.8; curve 4, 37.7; curve 5, 56.5; curve 6, 75.4; curve 7, 113. (B) Dependence of the values of v_0 (●; in $\mu\text{M/min}$), P_∞ (○; in μM) and Δ (▲; in min^{-1}) on the concentration of PGHS enzyme. Conditions: 1.4 mM adrenaline, 1.05 mM H_2O_2 .

Figure 5 PGHS-catalysed peroxidase reaction

Dependencies of the rate of the peroxidase reaction, v_0 , on the concentrations of substrates, H_2O_2 (A) and adrenaline (B), in double-reciprocal plots. (A) Adrenaline concentrations: 0.086 mM (▲), 0.186 mM (○), 0.466 mM (●) and 1.4 mM (Δ). (B) H_2O_2 concentrations: 0.064 mM (▲), 0.127 mM (○), 0.254 mM (●) and 0.356 mM (Δ). In both panels, solid lines show simulated dependencies using eqn (45). Conditions: solubilized preparation of PGHS with 14 $\mu\text{g/ml}$ protein.

Using eqns (36), (37) and (39)–(42) we obtain:

$$\Delta = \frac{f_0^n + (f_1^q + f_1^n[n, q])[S] + f_2^q[S]^2}{d_0 + d_1[S] + d_2[S]^2} \quad (43)$$

$$1/P_\infty = \frac{f_0^n + (f_1^q + f_1^n[n, q])[S] + f_2^q[S]^2}{c_1[S] + c_2[S]^2} \quad (44)$$

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 Δ 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_∞) did not exceed 15% of the initial concentration of each substrate. The values of v_0 , P_∞ and Δ 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_∞ are proportional to e_0 , whereas the values of Δ are virtually independent of e_0 . This result is entirely consistent with eqns (11), (27) and (28), and (15) and (16).

Figure 6 PGHS inactivation in the course of the peroxidase reaction

The dependence of the inactivation apparent rate constant in the catalytic cycle (Δ) on the concentration of H_2O_2 in a double-reciprocal plot. Adrenaline concentrations: 0.086 mM (●), 0.186 mM (▲), 0.466 mM (○) and 1.4 mM (Δ). 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 H_2O_2 shown by a double-reciprocal plot. Adrenaline concentration: 0.086 mM (Δ), 0.186 mM (○), 0.466 mM (▲) and 1.4 mM (●). Solid lines: simulated dependencies using eqn (46). Conditions: as for Figure 5.

The dependence of the experimentally evaluated parameters ν_0 , P_∞ and Δ on the concentrations of substrates, i.e. H_2O_2 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, ν_0 , on the concentrations of H_2O_2 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

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 (Δ) on the concentration of adrenaline. H_2O_2 concentration: 0.064 mM (Δ), 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. H_2O_2 concentration: 0.064 mM (□), 0.127 mM (○), 0.254 mM (●), 0.356 mM (Δ) and 0.508 mM (▲). Solid line: simulated dependency using eqn (46). Conditions: as for Figure 5.

with H_2O_2 and adrenaline is equal to zero: $(\text{H}_2\text{O}_2, \text{Ad}) = 0$. The curves in Figure 5 were simulated by the following empirical equation, obtained using linear regression:

$$1/\nu_0 = (14 \pm 1) + (6.86 \pm 0.6)/[\text{H}_2\text{O}_2] + (7.28 \pm 0.8)/[\text{Ad}] \quad (45)$$

where $[\text{Ad}]$ is the concentration of adrenaline. The dependencies of P_∞ and Δ on the concentration of substrates are shown in Figures 6–9. The dependence of Δ on the concentration of

Scheme 1 Proposed kinetic mechanism of the PGHS peroxidase reaction with adrenaline as the reducing substrate

[Fe(III)] is the resting enzyme, [Fe(IV)=O]⁺ is compound I, [Fe(III)]...H₂O₂ is the resting enzyme-H₂O₂ complex and [Fe(IV)=O]⁺...Ad is the compound I-adrenaline complex. k_1 and k_3 are the second-order rate constants for the reaction with H₂O₂ and adrenaline, respectively. α_2 , α_4 , β_1 and β_3 are the first-order rate constants; λ_3 and λ_4 are the first-order (or pseudo-first-order) enzyme inactivation rate constants.

H₂O₂ obeys eqn (30) at $c_0 = 0$, $c_1 \neq 0$ and gives a linear double-reciprocal plot (see Figure 6); P_∞ obeys eqn (31) at $f_0 \neq 0$, $c_1 \neq 0$ and is independent of the concentration of H₂O₂ (see Figure 7). The dependence of Λ 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_∞ 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_∞ on the concentration of adrenaline and H₂O₂ in Figures 7 and 9 are described by the following empirical equation, obtained using linear regression:

$$1/P_\infty = (11 \pm 1) + (7.4 \pm 1.5)/[\text{Ad}] \quad (46)$$

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

$$\Lambda = \frac{(11 \pm 1) + (7.4 \pm 1.5)/[\text{Ad}]}{(14 \pm 1) + (6.86 \pm 0.6)/[\text{H}_2\text{O}_2] + (7.28 \pm 0.8)/[\text{Ad}]} \quad (47)$$

It can be seen from Figures 6 and 8 that eqn (47) describes the dependency of Λ on H₂O₂ and adrenaline concentrations.

The experimental kinetic data can be simulated by an unbranched scheme, and thus there is no need to propose a more complex mechanism for the peroxidase reaction catalysed by PGHS.

This analysis now enables the requirements for a kinetic mechanism to be formulated for the peroxidase reaction catalysed by PGHS. Requirement 1: H₂O₂ and adrenaline interaction points are unconnected (H_2O_2 , Ad)=0. Thus, the reaction mechanism cannot have a section connecting these intermediates and consisting only of reversible steps of enzyme transformations. Requirement 2: any inactivated enzyme intermediate form E_r and the H₂O₂ interaction point are downstream unconnected [E_r , H₂O₂]=0. This means that at least one irreversible step must be present in the reaction course between each intermediate E_r undergoing inactivation and the H₂O₂ interaction point. Requirement 3: if only one enzyme form is inactivated, it cannot be at the adrenaline interaction point and the criterion [E_r , Ad]=1 should be satisfied, i.e. there are no irreversible steps

downstream from this inactivated form towards the adrenaline interaction point. Requirement 4: if several enzyme forms undergo inactivation in the reaction course, all of them cannot fulfil the condition [E_r , Ad]=0 simultaneously.

Thus, the experimental data and our novel analysis method show clearly that the PGHS-catalysed oxidation of adrenaline by H₂O₂ occurs by a Ping-Pong mechanism. A previous study on the oxidation of arachidonic acid resulted in a similar conclusion [33]. A Ping-Pong mechanism has also been proposed for catalase, where H₂O₂ acts both as the first and second substrate [34].

It is important to note that the experimentally derived eqns (45)–(47) and the foregoing requirements determine the class of kinetic mechanisms capable of fitting the experimental data. 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:

where α_1 and α_3 are pseudo-first-order rate constants equal to $k_1[\text{H}_2\text{O}_2]$ and $k_3[\text{Ad}]$ respectively (k_1 and k_3 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 complexes shown in eqn (48): E_1 , [Fe(III)]; E_2 , [Fe(III)]...H₂O₂; E_3 , [Fe(IV)=O]⁺ and E_4 , [Fe(IV)=O]⁺...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, $\beta_4 = 0$ (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_r in eqn (48) that satisfy the stipulation that [E_r , Ad]=1. Condition 5, $\lambda_3 \neq 0$ and $\lambda_2 + \lambda_4 \neq 0$. Since requirement 3 is unrealizable, then E_3 must undergo inactivation with at least one other intermediate.

Satisfying condition 5 requires that either all three forms of the enzyme, [Fe(III)]...H₂O₂, [Fe(IV)=O]⁺ and

$[\text{Fe(IV)=O}]^{*+} \cdots \text{Ad}$, or the combination of two forms, either $[\text{Fe(III)}] \cdots \text{H}_2\text{O}_2$ and $[\text{Fe(IV)=O}]^{*+}$ or $[\text{Fe(IV)=O}]^{*+}$ and $[\text{Fe(IV)=O}]^{*+} \cdots \text{Ad}$, are inactivated during the catalytic cycle. We consider that if $[\text{Fe(IV)=O}]^{*+}$ is unstable ($\lambda_3 \neq 0$), then its adduct with adrenaline $\{[\text{Fe(IV)=O}]^{*+} \cdots \text{Ad}\}$ must also be unstable ($\lambda_4 \neq 0$). On the other hand, there is no reason to suppose that the adduct of the stable enzyme form, $[\text{Fe(III)}]$, with H_2O_2 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 $[\text{Fe(IV)=O}]^{*+}$ (the adrenaline 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 H_2O_2 would be described by eqn (30) with $c_1 = 0$. 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 H_2O_2 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 H_2O_2 concentration. This has not been observed. Compound I for PGHS can exist in a ferryl haem $[\text{Fe(IV)=O}]$ form with either a haem π -cation radical or protein tyrosyl radical. Thus both compound I and its adrenaline-bound form may contain a tyrosyl radical. We therefore favour the proposal of Lu et al. [39] who suggested that all PGHS intermediates that contain a tyrosyl radical undergo inactivation.

The proposed mechanism (Scheme 1) allows us to evaluate the inactivation rate constants λ_3 and λ_4 . By using eqns (15) and (16) the expressions for Λ in this case are defined by eqn (49):

$$\Lambda = \frac{\lambda_3(k_1[\text{H}_2\text{O}_2]\alpha_2\alpha_4 + k_1[\text{H}_2\text{O}_2]\alpha_2\beta_3) + \lambda_4k_1[\text{H}_2\text{O}_2]\alpha_2k_3[\text{Ad}]}{\alpha_2k_3[\text{Ad}]\alpha_4 + \beta_1k_3[\text{Ad}]\alpha_4 + k_1[\text{H}_2\text{O}_2]k_3[\text{Ad}]\alpha_4 + k_1[\text{H}_2\text{O}_2]\alpha_2\alpha_4 + k_1[\text{H}_2\text{O}_2]\alpha_2\beta_3 + k_1[\text{H}_2\text{O}_2]\alpha_2k_3[\text{Ad}]} \quad (49)$$

If the concentration of adrenaline decreases to zero, the value of Λ (eqn 49) becomes equal to λ_3 . From eqn (47) (see also Figure 8), $\lambda_3 = 1.0 \pm 0.2 \text{ min}^{-1}$. If the concentrations of adrenaline and H_2O_2 increase infinitely, then Λ (eqn 49) becomes equal to $\lambda_4\alpha_2/(\alpha_2 + \alpha_4)$. From eqn (47) (see also Figure 6), $\lambda_4\alpha_2/(\alpha_2 + \alpha_4) = 0.8 \pm 0.1 \text{ min}^{-1}$. Strictly speaking, we must say that $\lambda_3 = 1.0 \pm 0.2 \text{ min}^{-1}$ and $\lambda_4 \geq 0.8 \pm 0.1 \text{ min}^{-1}$. If $\alpha_2 \gg \alpha_4$ then $\lambda_4 = 0.8 \pm 0.1 \text{ min}^{-1}$. In this case the values of λ_3 and λ_4 are essentially the same. This is not unreasonable since the inactivation of $[\text{Fe(IV)=O}]^{*+}$, characterized by λ_3 , and the inactivation of $[\text{Fe(IV)=O}]^{*+} \cdots \text{Ad}$, characterized by λ_4 , 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

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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