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Comments on the kinetic analysis of enzyme reactions involving an unstable irreversible modifier

Important enzyme systems regulating metabolic pathways are themselves controlled by modifiers, some of which irreversibly denature in the same time range as that of the enzymic cycle. Recently Topham (1990), in a comprehensive treatment, has carried out a kinetic analysis of the model in Scheme 1 of an enzyme-catalysed reaction which proceeds in the presence of an unstable, irreversible modifier, X. This author made assumptions which permitted the derivation of the explicit expression of the time course of the product formation. However, the first of the assumptions made is inappropriate, thus rendering the validity of his analysis uncertain.

The equation describing the disappearance of the modifier X is:

$$\frac{d[X]}{dt} = -(k' + k_{\text{mod.}}[E] + k'_{\text{mod.}}[ES])[X] \quad (1)$$

According to eqn. (1), the modifier concentration approximately varies as:

$$[X] \approx [X]_0 e^{-k't} \quad (2)$$

where $[X]_0$ is the initial concentration of X. The condition that:

$$k' \gg k_{\text{mod.}}[E] + k'_{\text{mod.}}[ES] \quad (3)$$

must be verified for any moment of the reaction. Since during the whole course of the reaction $[E]_0 \geq [E] \geq [ES]$, we have:

$$(k_{\text{mod.}} + k'_{\text{mod.}})[E]_0 \geq k_{\text{mod.}}[E] + k'_{\text{mod.}}[ES] \quad (4)$$

Therefore, a sufficient condition for the fulfilment of the inequality (3) is that:

$$k' \gg (k_{\text{mod.}} + k'_{\text{mod.}})[E]_0 \quad (5)$$

Scheme 1.

S, substrate; P, product; R, decomposition product of X; E, enzyme.

where $[E]_0$ is the total concentration of the enzyme. Condition (5), and therefore condition (3), are easy to reach experimentally by lowering the initial concentration of the enzyme. Topham assumes, to obtain eqn. (2) (which is necessary for the derivation of all of his kinetic equations), that the concentration of the modifier, $[X]$, at any time is much higher than $[E]_0$, i.e.:

$$[X] \gg [E]_0 \quad (6)$$

but he does not show how this condition leads to the equations. We have proved that the necessary and sufficient condition for the validity of eqn. (2), and therefore of that of all of the kinetic equations of Topham (1990), is that of eqn. (3). The condition (6) is not a sufficient condition for the derivation of the equations of Topham.

In Fig. 1(a), it is shown that even though condition (6) is not fulfilled (but assuming that Topham's other assumptions are

Fig. 1. Plots showing that the assumption $[X] \geq [E]_0$ during the reaction is invalid

(a) $[P]$ and $[X]$ curves have been obtained by numerical integration and from the plot of Topham's eqn. (11) together with eqn. (2). In each case, the curves overlap. (b) The thin and heavy lines for $[P]$ represent the product accumulation curves obtained by numerical integration and from Topham's eqn. (11) resp. The curves for $[X]$ obtained from numerical integration and from eqn. (2) overlap. The broken lines represent the value of $[E]_0$. The conditions were: $k_{+1} = 10^9 \text{ M}^{-1} \text{ s}^{-1}$, $k_{-1} = 10^6 \text{ s}^{-1}$, $k_{\text{mod.}} = 500 \text{ M}^{-1} \text{ s}^{-1}$, $k'_{\text{mod.}} = 510 \text{ M}^{-1} \text{ s}^{-1}$, $k_{+2} = 10^7 \text{ M}^{-1} \text{ s}^{-1}$, $k_{-2} = 10^5 \text{ s}^{-1}$, $[S]_0 = 2 \times 10^{-3} \text{ M}$, $[P]_0 = 0$ and (a): $k' = k_{+2} = 10 \text{ s}^{-1}$, $k_{+2}^* = 5 \text{ s}^{-1}$, $[E]_0 = 10^{-7} \text{ M}$, $[X]_0 = 10^{-6} \text{ M}$. (b): $k' = 10^{-5} \text{ s}^{-1}$, $k_{+2} = 100 \text{ s}^{-1}$, $k_{+2}^* = 50 \text{ s}^{-1}$, $[E]_0 = 10^{-8} \text{ M}$, $[X]_0 = 10^{-6} \text{ M}$.

sound), eqn. (11) of Topham (1990) is valid, since, although $[X]$ is not $\gg [E]_0$ during the whole course of the reaction, the condition (5) is fulfilled. Fig. 1(b) shows that if the condition (6) (and the remaining assumptions of Topham), but not condition (5), is satisfied, then eqn. (11) of Topham (1990) fails. The numerical integration to obtain the simulated curves in Fig. 1 was carried out by the use of the predictor-corrector algorithm of Adams-Moulton, starting with the fourth order Runge-Kutta method (Gerald, 1978). Before plotting eqn. (11) of Topham (1990), we corrected the mistake: $e^{ik't}$ instead of $e^{-ik't}$. For the acquisition of the points necessary to plot this equation, we elaborated a computer program using the BASIC language. This program is available to interested readers on request. It allows the choice of the number of terms in the infinite series in the kinetic equations. We progressively increased this number from 1 and, in all cases, the difference between the successive results vanished after less than 20 steps. For the plots of eqn. (11) of Topham (1990) in Fig. 1, we employed 20 terms. The algorithm for the numerical integration and the program elaborated by us were implemented and compiled in TurboBASIC 1.0 on an INVES PC-640A computer (IBM AT-compatible) with an Intel 80287 arithmetic coprocessor.

The unnecessary assumption (6) probably led Topham (1990) to propose a kinetic data analysis, which is based on the first instants of the reaction (at which this assumption is fulfilled because the modifier has not yet decayed appreciably), although his equations are valid in the entire time range. Since for short reaction times, the exponential terms cannot be neglected, that approach suffers from both mathematical and experimental difficulties. The assumption (6), which also has been used in earlier papers (Topham, 1987), has been questioned by Rakitzis (1987).

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Making sense of the kinetics of reactions of unstable modifiers with enzymes

The determination of kinetic parameters describing irreversible enzyme modification by unstable reagents may be achieved by analysis of reaction time-courses under conditions of modifier (X) excess over enzyme ($[X] \gg [E]_0$). Appropriate protocols have been devised for the discontinuous monitoring of residual enzyme activity during modification reactions conducted in the absence of substrate (Gomi & Fujioka, 1983; Topham, 1984, 1985, 1988; Topham & Dalziel, 1986) and for the continuous monitoring of enzyme-catalysed reactions in the presence of an unstable modifier (Topham, 1990). In order to derive all the integrated-rate equations described in these studies, it is necessary to assume that modifier decay is first-order. This enables the concentration

of modifier at any moment to be set equal to $[X]_0 e^{-k't}$ where k' is the (pseudo) first-order rate constant for modifier decomposition.

Varón *et al.* (1992) have challenged the validity of the time-course approach as it applies to the analysis of product (P) formation in the presence of an unstable inhibitor according to a direct-encounter mechanism (Schemes 1 of Topham, 1990 and Varón *et al.*, 1992). Their criticism centres on (a) the impropriety of the condition $[X] \gg [E]_0$ as a criterion by which inhibitor decay is adjudged to be first-order and (b) its replacement by the inequality

$$k' \gg (k_{\text{mod.}} + k'_{\text{mod.}})[E]_0$$

where $k_{\text{mod.}}$ and $k'_{\text{mod.}}$ are second-order rate constants for the reaction of the modifier with the free enzyme (E) and the enzyme-substrate (ES) complex, respectively. Varón *et al.* (1992) cite two extreme cases that purport to reveal inconsistencies between the results of their own numerical simulations and the integrated-rate equation for this mechanism [eqn. (11) of Topham (1990)], reproduced below after correction of the typographical error:

$[P] =$

$$v^*t + \frac{(v-v^*)}{k'} \exp\left(\frac{-A[X]_0}{k'}\right) \cdot \left(k't + \sum_{i=1}^{t-\infty} \left(\frac{A[X]_0}{k'}\right)^i \frac{[1 - (e^{-k't})^i]}{i \cdot i!}\right) \quad (1)$$

where v^* and v are the usual Michaelis-Menten steady-state rate expressions for the modified and unmodified forms of the enzyme, respectively [see eqn. (10) of Topham (1990)] and A is an apparent second-order rate constant for modification:

$$A = \frac{k_{\text{mod.}}K_m + k'_{\text{mod.}}[S]}{K_m + [S]} \quad (2)$$

It is helpful to consider reactions of enzymes with unstable modifiers in terms of the relative flux (Q) occurring through reactions of the modifier with the various enzyme-containing species compared to that proceeding via the non-enzymic pathway for modifier decay. The flux ratio Q will decrease as the modification reaction proceeds and is at a maximum ($Q_{\text{max.}}$) in the limit $t \rightarrow 0$. For the mechanism under discussion:

$$Q = (k_{\text{mod.}}[E] + k'_{\text{mod.}}[ES])/k' \quad (3)$$

and

$$Q_{\text{max.}} = A[E]_0/k' \quad (4)$$

The example illustrated in Fig. 1(a) of Varón *et al.* (1992) concerns a highly unstable inhibitor ($t_{1/2} = 0.069$ s). The flux ratio $Q_{\text{max.}}$ for this reaction is $5.07 \times 10^{-5} \ll 1$. Since first-order kinetics with respect to inhibitor concentration clearly hold under these conditions, it is not surprising that eqn. (1) is also obeyed. However, by ensuring that $k' \gg (k_{\text{mod.}} + k'_{\text{mod.}})[E]_0$ while simultaneously permitting the deliberate violation of the condition $[X] \gg [E]_0$ over most of the observation period (~ 0.6 s), Varón *et al.* (1992) have been obliged to consider a system of little or no practical relevance. The inhibitor is so unstable that 99.995 % of it will decompose non-enzymically within one second! Furthermore its efficacy in the reaction mixture of Fig. 1(a) is minimal. It can be calculated [using eqn. 7 of Topham (1990)] that a mere 0.051 % of the total enzyme will have been modified (i.e. exists as E^* or ES^*) at the end of the observation period (taken as 0.6s). When more realistic experimental conditions apply, rates of enzyme inactivation and non-enzymic inhibitor decay can often be expected to be similar and $Q_{\text{max.}}$ approximates to unity [see Topham (1987) for experimental examples]. Ironically Varón *et al.* (1992) make this same point in their opening