BIOCHEMICAL LETTERS

Kinetics of protein modification, and/or enzyme inactivation, reactions by an unstable modifying agent

Protein modification, and/or enzyme inactivation, reactions are studied by an analysis of the time dependence of these processes, or alternatively, by an analysis of the dependence of the end point of the protein modification, or enzyme inactivation, reaction on initial modifying agent concentration (Rakitzis, 1984). The latter procedure has been criticized by Topham (1986), who used computer simulations of reaction kinetics with unstable agents to test the validity of the integrated rate equations applicable to the cases under consideration. Topham (1986) has concluded that 'the computer simulation studies show data obtained at the end point of a reaction of an unstable inhibitor with enzyme conform poorly in many situations with relationships derived from integrated rate equations'. It is the purpose of the present letter to comment on the criticism of Topham (1986), and also on the general validity of methods of kinetic analysis, based on studies of the dependence of reaction end point on the initial concentration of modifying agent.

The analysis presented by Topham (1986) is mainly restricted to a study of two basic mechanisms of protein modification, i.e. reaction of protein (enzyme) (E) with modifying agent (inhibitor) (I) to form modified protein (inactive enzyme) (E*), such reaction proceeding: (i) by a simple bimolecular mechanism (eqn. 1) or (ii) through the intermediacy of a protein-modifying agent adsorptive complex (EI) (eqn. 2):

where I* is hydrolysed modifying agent, and also where k_1 , k_{+1} , k_{-1} , k_{+2} and k' are the reaction rate constants. The differential equations describing reactions (1) and (2), as well as the integrated expressions arising therefrom, are given in summary form by Topham (1986). One basic assumption in the development of these equations is that eqn. (3) applies, i.e., that modifying agent disappearance is a first-order process throughout the course of the reaction. Clearly, this is only possible if reaction (3) is a much faster process than either reaction (1) or (2). If this is not the case, i.e. if the rate of modifying agent disappearance is of the same order of magnitude as the rate of protein modification, then modifying agent disappearance is described by:

i.e. it is described by a nonlinear differential equation. In the hypothetical cases of protein modification studied by Topham (1986) protein reaction rates are generally comparable to modifying agent hydrolysis rates. It follows that Topham (1986) has tested the validity of the

Table 1. Stoichiometry of enzyme inactivation to protein modifying agent hydrolysis

The value $d[E^*](0)/d[I^*](0)$ is equal to $[E]_0 k_1/k'$, for reaction scheme (1), and to $[E]_0 k_{+1} k_{+2}/k_{-1} k'$, for reaction scheme (2) (where $[E]_0 = [E]$ at $t = 0$).

Reaction, protein concentration, rate constants, and reference	$d[E^*](0)/d[I^*](0)$
Inactivation of (i) cathepsin D by 1,1-bis(diazoacetyl)-2-phenylethane: $[E]_0 = 4.5 \times 10^{-7}$ M, $(k_1/k') = 7$ mM ⁻¹ , and (ii) pepsin C by diazoacetyl-DL-norleucine methyl ester: $[E]_0 = 3.33 \times 10^{-5}$ M, $(k_1/k') = 2.14$ mM ⁻¹ (Kay & Ryle, 1971; Rakitzis, 1974).	(i) 0.00315 (ii) 0.0713
Inactivation of eel acetylcholinesterase by diphenylphosphochloridate: $[E]_0 = 10^{-8}$ M, $(k_1/k') = 416$ mM ⁻¹ (Maglothlin <i>et al.</i> , 1975).	0.00416
Inactivation of cathepsin D by dithiophosgene: $[E]_0 = 4.5 \times 10^{-7}$ M, $(k_{-1}/k_{+1}) = 96.1$ μM, $k_{+2} = 3.38$ s ⁻¹ , $k' = 8.33$ s ⁻¹ (Rakitzis & Malliopoulou, 1976).	0.0190
Inactivation of RNA polymerase by acetimidate: $[E]_0 = 4.4 \times 10^{-7}$ M, $(k_1/k') = 0.05$ mM ⁻¹ (Makoff & Malcolm, 1980).	0.000022
Computer simulation data: (i) $[E]_0 = 2 \times 10^{-5}$ M, $k_1 = 10$ M ⁻¹ ·s ⁻¹ , $k' = 10^{-4}$ s ⁻¹ (Fig. 1, curve c), (ii) $[E]_0 = 2.5 \times 10^{-5}$ M, $k_{+1} = 10^7$ M ⁻¹ ·s ⁻¹ , $k_{-1} = 10$ s ⁻¹ , $k_{+2} = 5 \times 10^{-4}$ s ⁻¹ , $k' = 4 \times 10^{-4}$ s ⁻¹ (Fig. 3, curve e) (Topham, 1986).	(i) 2 (ii) 31.25

integrated equations describing protein modification by unstable agents by selecting conditions violating the very assumptions on which these equations are based. Hence the inevitable discrepancy between theory and computer simulation data.

A comparison of the rate of reactions (1) and (2) with the rate of reaction (3) is best carried out if the first derivatives of these processes, at the start of the reaction, are compared. The ratio of the first derivative of either reaction (1), or reaction (2), at the start of the reaction, to the first derivative of reaction (3), again at the start of the reaction, $d[E^*](0)/d[I^*](0)$, has the dimensions of a pure number, and corresponds to the stoichiometry of the number of protein groups modified, or of enzyme functional units rendered inactive, per molecule of modifying agent reacted with water. Clearly, this number must have a value much smaller than unity for reaction (3) to be valid. The value of $d[E^*](0)/d[I^*](0)$, for several published cases of enzyme irreversible inhibition by unstable modifying agents, along with the corresponding values for some of the computer simulation cases presented by Topham (1986), are given in Table 1. The computer simulation data of Topham (1986) provide an excellent illustration of the importance of the restrictions assumed in the formulation of reactions (1)–(3). Clearly, with unstable modifying agents presenting with half-lives as long as 116 min ($k' = 10^{-4} \text{ s}^{-1}$), as is the case with the putative modifying agents of the computer simulation data of Topham (1986), the integrated equations arising out of eqns. (1) and (2) are inapplicable. Such modifying agents are best assumed to be stable in solution.

The caution advocated by Topham (1986) in the interpretation of the dependence of the end point of the protein modification, or enzyme inactivation, reactions on initial modifying agent concentration is well taken, however, if it is seen within the framework of the many reaction possibilities known to exist in protein-modifying

agent reactions, in addition to those described by eqns. (1) and (2). Such possibilities include: (i) the presence of two or more noninteracting sets of protein reactive groups, (ii) protein modification co-operativity, (iii) protein conformational isomerism, (iv) ligand binding to the protein, (v) enzyme inactivation co-operativity (Rakitzis, 1980, 1984, 1986). When stable modifying agents are used, cases (ii)–(v) are described by second-order differential equations with constant coefficients, with solutions consisting of summations of exponential functions of reaction time. With unstable modifying agents, however, the corresponding equations are second-order equations with variable coefficients. Such equations are solved by means of power series expansions (Rakitzis & Tsamatos, 1986). Clearly, interpretation of experimental data by means of a particular reaction model, e.g., that of eqn. (1) or eqn. (2), is only feasible if alternative reaction possibilities may properly be ruled out.

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Alternative methods for the determination of rate constants describing enzyme inactivation by an unstable inhibitor

Most studies of irreversible enzyme inhibition are interpreted in terms of two basic mechanisms (eqns. 1 and 2):

The integrated rate equations describing the time-dependent loss of enzyme activity for the case of an unstable inhibitor which decays in a parallel reaction to form product R (eqn. 3) are well established for both mechanisms (Purdie & Heggie, 1970; Rakitzis, 1974; Topham, 1986).

In order to obtain these analytical solutions it is assumed that inhibitor decay is first-order, and so the

concentration of inhibitor at any moment is set equal to $[I]_0 e^{-k't}$, where $[I]_0$ is the initial concentration of inhibitor. First-order kinetics with respect to inhibitor concentration will clearly hold when the relative flux (Q) through reaction (1) or (2) compared to that via reaction (3) $\ll 1$. This condition may, however, be satisfied experimentally when the rates through the two pathways are similar by ensuring that the inhibitor is in large excess over the enzyme during the period of observation. This can be demonstrated by computer simulation, and Fig. 1 shows the concentration of remaining inhibitor ($[I]$), expressed as a fraction of the concentration predicted by a simple first-order decay process, over the time-course of enzyme inactivation for various ratios of initial inhibitor concentration to total enzyme concentration ($[I]_0/[E]_0$) in the range 0.5–50.0 when $Q = 1$ for reactions conforming to eqn. (1). Deviations from first-order kinetics are evidently trivial when $[I]_0/[E]_0 \gg 1$, and the claim by Rakitzis (1987) that first-order inhibitor decay is possible only when $Q \ll 1$ can be refuted. The approach of conducting experiments under pseudo-first-order conditions ($[I]_0/[E]_0 \gg 1$), and analysis of the time-course of inactivation based upon the use of integrated rate equations as described by Topham (1985, 1986) and Gomi & Fujioka (1983) is therefore valid when rates of inactivation are