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Short Communication

INTERPRETATION OF BIPHASIC PROTEIN MODIFICATION AND MODIFICATION-INDUCED ENZYME INACTIVATION REACTION PLOTS

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Protein modification and/or modification-induced enzyme inactivation reactions are, under certain conditions, linearly dependent on reaction time, and are accordingly described by a summation of exponentials, i.e., by an equation of the form:[1,2]

$$\frac{[A_t]}{[A_0]} = c_1 \exp(-m_1 t) + c_2 \exp(-m_2 t) + \ldots + c_n \exp(-m_n t) \qquad (1)$$

where $[A_t]/[A_0]$ is the fraction of unmodified protein reactive groups, or the fraction of enzyme protein that is catalytically active, at reaction time t, and also where c_1, $c_2, \ldots c_n$ and $m_1, m_2, \ldots m_n$ are constants. Log fractional protein groups concentration, or log fractional enzyme activity, vs reaction time plots are in such cases biphasic, since the final portion of the plot, corresponding to the situation where all but the slowest exponential of the reaction equation have vanished, is rectilinear the rest of the plot appearing as an initial, curvilinear portion. Analysis of protein modification, or of modification-induced enzyme inactivation, data into a summation of exponential functions of reaction time may be carried out graphically.[3] However, before an analysis of a protein modification, or enzyme inactivation, reaction in accordance with eqn. (1) is undertaken, care should be taken to rule out reaction conditions which lead to a nonlinear dependence of the variable considered on reaction time. Such conditions include a concentration of the modifying agent comparable to the protein concentration used, the case where the modifying agent is unstable in solution, dissociation of the protein into subunits during the modification event, and mechanism-based enzyme inactivation (k_{cat} inhibitors, suicide substrates).[1]

Protein covalent modification reactions in accordance with eqn. (1) are frequently interpreted to the effect of the protein preparation under study presenting with two, or more than two, modifiable groups per protein molecule with different reactivity towards the modifying agent used, or to the effect that the protein preparation consists of a mixture of different protein forms (isoenzymes). Since in the case of the existence of two noninteracting classes of reactive groups in the preparation under

study, the equations describing the protein modification, and/or enzyme inactivation, processes are composed of the sum (rather than the difference) of two exponential functions of reaction time, the protein modification, and/or enzyme inactivation, reaction plots may only be concave upwards. Accordingly, the finding of concave downwards protein modification, and/or enzyme inactivation, plots *ipso facto* rules out the existence of only two classes of noninteracting groups. Concave downwards plots are of frequent occurrence in cases of enzyme photoinactivation reactions.[4–6]

In the case of a concave upwards reaction plot an interpretation of the plot to the effect of the existence of two, or more than two, classes of noninteracting groups in the preparation under study, may legitimately be put forward if alternative interpretations of reaction data are properly ruled out. The alternative interpretations, worked out to date, to explain the biphasicity or multiphasicity of protein modification reaction plots (whether concave upwards or downwards), include the following cases:

i) Formation of a protein-modifying agent adsorptive complex, the first-order reaction of which results in protein covalent modification. However, in this case the biphasicity of the reaction plot is not apparent unless fast reaction techniques are used.[7,8]

ii) The existence of two modifiable sites on the enzyme protein, modification of only one (but not both) of which does not result in the loss of catalytic function. This situation has been postulated by Ray and Koshland[2] and also by Tsou,[9] and has been invoked to explain the peculiar findings of the modification of choline acetyltransferase with sulfhydryl reagents.[10] A mathematical treatment of this case is presented in the Appendix.

iii) Protein conformation isomerism, i.e., the existence of the protein under study in two, or more than two, conformationally isomeric forms, with different reactivity towards the modifying agent used.[7]

iv) Protein ligand binding with different reactivity towards the modifying agent used, when the protein-ligand complex is compared to the ligand-free protein.[7] Protein-ligand binding may result in an increased reactivity (sensitising effect) or in a decreased reactivity (protective effect) towards the modifying agent used.

v) Protein modification cooperativity, i.e., the case where the protein presents with more than one modifiable group per protein molecule, and where the partially modified protein species possesses different reactivity towards the modifying agent used.[11,12]

vi) Modification-induced protein unfolding, i.e., the case where the number of modifiable groups per protein molecule is a function of the extent of modification.[13]

Cases (i) through (vi) may be differentiated from the case where the reaction medium consists of a mixture of noninteracting protein forms, by bringing about a change in the concentration of the modifying agent used, and noting whether the coefficients of the exponentials of the reaction equation (c_1 through c_n) acquire new values with the change in the values of the reaction constants (m_1 through m_n). If the coefficients of the reaction equation are not functions of the constants of this same equation, the interpretation of the modification reaction as a result of the modification of a population of noninteracting protein species may provisionally be accepted.

Cases (i) through (vi) are not mutually exclusive, and may indeed all apply in any one particular instance. Since the number of exponential functions of reaction time, into which any particular experimental situation is to be analysed, is equal to the number of unmodified protein species present in the reaction mixture, protein modification reactions may present as a summation of a large number of exponentials. In

practice, however, protein modification, or modification-induced enzyme inactivation reaction plots are analysed into a summation of two or three exponential functions of reaction time. The conditions under which cases of protein modification cooperativity may present as a summation of only two or three functions of reaction time have been delineated.[11] It is also to be noted that cases (iii) through (vi) may present as the sum or as the difference of two exponential functions of reaction time. Contrariwise, cases (i) and (ii) may only present as a difference of two exponential functions of reaction time, and accordingly these cases necessarily present with a reaction plot that is concave downwards.[7,8]

In distinction to reactions where the fraction of unmodified protein groups is studied as a function of time, reactions of modification-induced enzyme inactivation presenting as a summation of exponential functions of reaction time may not be interpreted on the assumption that the enzyme protein molecule possesses two, or more than two, modifiable groups per protein molecule, each group being essential for enzyme catalytic function. In the case where an enzyme protein possesses groups 1, 2, ... n, the psuedo first-order reaction of which, with the modifying agent used, is carried out with rate constants k_1, k_2, ... k_n, and all of which groups are essential for enzyme catalytic function, the pseudo first-order enzyme inactivation rate constant is equal to $k_1 + k_2 + \ldots + k_n$ (parallel first-order reactions).[3] Accordingly, the finding of a biphasic enzyme inactivation reaction plot is indicative of either the presence of two or more than two isoenzymes (or of two or more than two enzyme subunits, the catalytic action of which is not interdependent) in the preparation under study, or of one or more of the situations (i) through (vi) above.

Cases presenting with biphasic modification kinetics, which have been interpreted on the assumption of the existence of two, or more than two, kinds of groups on the protein under study, have been discussed previously.[7] Cases of modification-induced enzyme inactivation, which have been interpreted on the assumption that the biphasicity of the log fractional enzyme activity vs reaction time plot observed is due to the existence of two, or more than two, groups per protein molecule, with different reactivity towards the modifying agent used (or where the correct interpretation is lacking), include the following:

1) The case of the inactivation of membrane-bound succinate dehydrogenase by three maleimide derivatives.[14] The authors have attributed the biphasicity of the log fractional enzyme activity vs reaction time plots to the existence of "two cases of sulfhydryl groups, with quite different reactivities, which groups were essential for catalytic activity". As mentioned above, however, the existence of more than one group, per enzyme protein molecule, which groups are essential for catalytic function does not necessarily result in the production of biphasic enzyme inactivation reaction plots.

2) The inactivation of the sulfhydryl groups of peptidyl-prolyl *cis–trans* isomerase by *p*(hydroxymercuri)-benzoate.[15,16] The enzyme has a molecular weight of 17 000, and accordingly is probably a monomer. Fischer *et al.* report on the time dependence of the peptidyl prolyl *cis–trans* isomerase sulfhydryl group covalent modification by *p*(hydroxymercuri)-benzoate. The authors observed a very fast sulfhydryl group modification phase, which occurred within the dead-time of mixing, and which these authors attributed to the presence of dithioerythritol in the enzyme solution. The rest of the modification reaction was found by Fischer *et al.* to be described by a summation of two exponentials equation:

$$[SH] = 1.95 \exp(-0.015t) + 0.8 \exp(-0.0028t) \quad (2)$$

where [SH] is the number of unmodified sulfhydryl groups per molecule of enzyme protein, and t is reaction time, in seconds. In the presence of 1.44 μM cyclosporin A, peptidyl-prolyl *cis–trans* isomerase modification is described by the equation:

$$[SH] = 1.89 \exp(-0.015t) \quad (3)$$

Eqn. (2) indicates that peptidyl-prolyl *cis–trans* isomerase possesses 2.75 sulfhydryl groups, per molecule of protein, at zero reaction time. In the presence of cyclosporin A this number reduces to 1.89 (eqn. (3)), hence Fischer *et al.* conclude that, "one sulfhydryl group of peptidyl-prolyl *cis–trans* isomerase is specifically protected against modification by *p*(hydroxymercuri)-benzoate when cyclosporin A is bound to the enzyme. In the absence of cyclosporin A the modification reaction is triphasic. After a fast phase with *p*(hydroxymercuri)-benzoate two peaks are observed in which two and one sulfhydryl group per peptidyl-prolyl *cis–trans* isomerase molecule, respectively, are modified. The slowest phase is not observed in the presence of cyclosprin A. Apparently, binding of cyclosporin A protects the most slowly reactive cystein residue of peptidyl-prolyl *cis–trans* isomerase against modification by *p*(hydroxymercuri)-benzoate". However, in the findings of Fischer *et al.*, the log fractional sulfhydryl groups, and the log fractional enzyme activity, vs reaction time plots are identical, indicating that the biphasicity of the protein modification reaction is necessarily due to one or more of the cases (i) through (v) described above, and also that one sulfhydryl group only, per enzyme protein molecule, is essential for catalytic function.[9,17] Also, the finding of a change in the extent of the reaction on addition of cyclosporin A to the medium, indicated by eqn. (3), may be explained as an effect of this ligand on modification-induced protein unfolding.[13]

3) The inactivation of bovine liver glutathione *S*-transferase by modification of arginine residues with phenylglyoxal.[18] From the finding that two arginine residues are modified per molecule of enzyme protein, and the fact that the enzyme has been shown to be composed of two identical or nearly identical subunits, the authors conclude that modification of one arginine residue per subunit had occurred. However, the enzyme modification-induced inactivation curve is biphasic, with an intercept of 0.30 on the fractional activity axis, suggesting that the occurrence of two intrinsically different, catalytically active enzyme subunits may be ruled out as an explanation for the biphasicity of the enzyme inactivation curve (had this been the case the intercept would be equal to 0.50). As in cases 1 and 2 above, explanation of the biphasicity of the enzyme inactivation reaction is by one or more of cases (i) through (vi).

4) The inactivation of choline acetyltransferase with sulfhydryl reagents.[10] The authors have attributed some peculiarities of enzyme inactivation kinetics to the existence of two modifiable sulfhydryl residues, modification of either of which (but not both) does not result in the loss of catalytic function (case (ii) above). However, as will be noted from the Appendix, this case always presents with log fractional activity vs reaction time plots that are concave downwards. Since in this experimental situation log fractional activity vs time plots are concave upwards, explanation of the findings has to be by some mechanism other than case (ii) above.

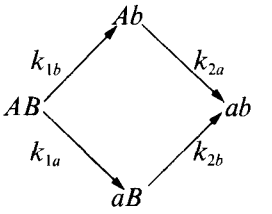
It may be concluded that the interpretation of the biphasicity of enzyme inactivation reaction plots is incumbent upon, and may be complementary to, the interpretation of the protein modification reaction of the preparation under study. In distinction to cases of protein modification, enzyme inactivation reactions present with questions involving the number of protein reactive groups essential for catalytic function, and

also, in cases where enzyme activity is retained, the effect of protein modification on the enzyme kinetic parameters (K_M, V_{max} etc). The number of groups, per protein molecule, which are essential for enzyme catalytic function may be determined by a comparison of the time-dependence of the enzyme inactivation and of the protein modification reaction rate constants.[9,17] Concerning the interpretation of multiphasic protein modification reactions, it should be noted that the occurrence of interconvertible protein species during the modification event, points to the existence of protein regulatory mechanisms of probable physiological significance.

APPENDIX

Modification-induced inactivation of an enzyme possessing two reactive groups per molecule of protein, either one (but not both) of which groups may be modified without loss of enzyme catalytic function.

The reaction scheme is:[12]



(4)

where A and B are the two modifiable enzyme groups. Upper case letters are, in eqn. (4), used to indicate unmodified groups, and lower case letters are used to indicate covalently modified groups, while k_{1a}, k_{1b}, k_{2a} and k_{2b} are the relevant rate constants. In the absence of protein modification cooperativity, $k_{1a} = k_{2b}$, and $k_{1b} = k_{2a}$. Since either of sites A and B is able to support enzyme catalytic function, the species AB, Ab, aB are catalytically active, while the species *ab* is inactive. Assuming that the concentration of the modifying agent used is in large excess over enzyme protein concentration, the concentration of the species ab at reaction time t is given by the relationship:[12]

$$[ab] = [E_0]\{1 - e^{-k1at} - e^{-k1bt} + e^{-(k_{1a}+k_{1b})t}\} \quad (5)$$

where $[E_0]$ is initial enzyme protein concentration. Since the concentration of catalytically active enzyme protein is equal to $[E_0]$–$[ab]$, the fraction of active enzyme protein is given by the relationship:

$$\frac{[E_{act}]}{[E_0]} = e^{-k1at} + e^{-k1bt} - e^{-(k_{1a}+k_{1b})t} \quad (6)$$

In the case where sites A and B are intrinsically identical $k_{1a} = k_{1b} = k$, Eqn. (6) reduces to:

$$\frac{[E_{act}]}{[E_0]} = 2e^{-kt} - e^{-2kt} \quad (7)$$

It will be noted from eqns (4) through (7) that the title situation will present with a three exponentials equation, while in the case where the two enzyme protein groups are identically reactive, description will be by a two exponentials equation. However.

as will be noted from eqns (6) and 7) description of the enzyme inactivation reaction will be by a difference of exponential functions of reaction time, i.e., the log fractional activity vs reaction time plot will be concave downwards.

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