Kinetic analysis of protein modification reactions at equilibrium

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A kinetic analysis is presented of reactions of protein modification, and/or of modification-induced enzyme inactivation, which can formally be described by a single exponential function, or by a summation of two exponential functions, of reaction time plus a constant term. The reaction schemes compatible with the kinetic formalism of these cases are given, and a simple kinetic criterion is described whereby the identification of one of these cases, strong negative protein modification co-operativity, may be carried out. The treatment outlined in this paper is applied to a case from the literature, the inactivation of glyceraldehyde-3-phosphate dehydrogenase by butane-2,3-dione [Asriyants, Benkevich & Nagradova (1983) *Biokhimiya* (Engl. Transl.) 48, 164–171].

INTRODUCTION

Studies of the equilibrium relation of the reaction of protein and modifying agent, to produce covalently modified protein, have been carried out in a number of cases involving the modification-induced enzyme inactivation by pyridoxal 5'-phosphate (Piskiewicz & Smith, 1971*a,b*; Chen & Engel, 1975*a,b*; Frerman *et al.*, 1977; Marshall & Cohen, 1977; Ogawa & Fujioka, 1980; Poulou & Kolattukudy, 1980). Chen & Engel (1975*a,b*) determined, by graphical analysis of the enzyme inactivation data obtaining at equilibrium, in addition to the equilibrium constant of the overall enzyme modification reaction, also the dissociation constant of the reversible protein-modifying agent adsorptive complex, the first-order reaction of which results in protein covalent modification, i.e. in modification-induced enzyme inactivation. The purpose of the present paper is to examine the different possible reaction schemes of protein covalent modification in which there is an experimentally determinable modification-free portion of the protein at equilibrium. The cases considered are only those where time-dependency is described by a single exponential function, or by a summation of two exponential functions, of reaction time plus a constant term.

REACTION MODELS AND RATE EQUATIONS

The general kinetic formalism of the reaction schemes under study is presented first, followed by an analysis of the individual cases consistent with the formalism. The reversible conversion of the species A_1 into A_2 and of this into A_3 is considered. The reaction scheme is:

where k_{12} , k_{23} , k_{21} and k_{32} are the reaction rate constants. The reaction of eqn. (1) is described by a third-order linear differential equation with constant coefficients, with the concentration of any one of the species of eqn. (1) as the dependent variable, and reaction time as the independent variable. The characteristic algebraic equation of this differential equation is (Frost & Pearson, 1961):

$$-m^3 + (k_{32} + k_{23} + k_{21} + k_{32})m^2 - (k_{12}k_{23} + k_{21}k_{32} + k_{12}k_{32})m = 0 \quad (2)$$

The roots of eqn. (2) are:

$$m_1 = 0 \quad (3)$$

$$m_2 = \frac{1}{2}(p + q) \quad (4)$$

$$m_3 = \frac{1}{2}(p - q) \quad (5)$$

where $p = k_{12} + k_{23} + k_{21} + k_{32}$ and $q = [p^2 - 4(k_{12}k_{23} + k_{21}k_{32} + k_{12}k_{32})]^{1/2}$. With the concentration of unmodified protein or of catalytically active enzyme $[A_{act.}]$ defined as $[A_1] + [A_2]$, the solution of the differential equation describing eqn. (1) is:

$$\frac{[A_{act.}]}{[A_0]} = C_1 + C_2 \cdot e^{-m_2 t} + C_3 \cdot e^{-m_3 t} \quad (6)$$

where $[A_0]$ is initial protein concentration, and also where C_1 , the modification-free fraction of enzyme protein, is (Frost & Pearson, 1961):

$$C_1 = \frac{k_{32}(k_{12} + k_{21})}{m_2 m_3} = \frac{k_{32}(k_{12} + k_{21})}{k_{32}(k_{12} + k_{21}) + k_{12}k_{23}} \quad (7)$$

The individual reaction schemes of cases of protein modification consistent with the formalism of eqns. (1)–(5) are as follows.

(i) The protein forms with the modifying agent a reversible complex, the first-order reaction of which results in protein covalent modification:

where A is unmodified protein, M is modifying agent, AM is the reversible protein-modifying agent adsorptive complex and a is covalently modified protein, and where k_{+1} , k_{+2} , k_{-1} and k_{-2} are the binding and modification rate constants. If $[M] \gg [A]$, eqn. (8) is consistent with the formalism of eqns. (1)–(7). Substituting the constants $k_1[M]$, k_{+2} , k_{-1} and k_{-2} for the constants k_{12} , k_{23} , k_{21} and k_{32} (in the same order) and rearranging:

$$\frac{C_1}{1 - C_1} = \frac{1}{K_{eq}[M]} + \frac{1}{K_i K_{eq}} \quad (9)$$

A plot of $C_1/(1 - C_1)$ versus $1/[M]$ will give a straight-line relationship, with a slope of $1/K_{eq}$ and an intercept on the $1/[M]$ axis of $-1/K_i$ (Chen & Engel, 1975*a*).

It should be noted that the reaction described by eqn.

(1) is an addition reaction. Substitution reactions, i.e. reactions of the form:

where P is the second product of the reaction, are amenable to the treatment outlined in this paper, provided that $[P] \gg [a]$ at all times, i.e. provided that P, in sufficiently high concentration, is present in the initial reaction mixture.

In the case where $m_2 \gg m_3$ (or vice versa) eqn. (6) reduces to a single exponential equation plus a constant term. The reaction scheme of eqn. (1) reduces to:

and the time-dependence of active enzyme $[A_{\text{act.}}]$ is given by:

$$\frac{[A_{\text{act.}}]}{[A_0]} = \frac{k_2}{k_1 + k_2} + \frac{k_1}{k_1 + k_2} \cdot e^{-(k_1 + k_2)t} \quad (12)$$

where the dependence of k_1 on $[M]$ is given by:

$$k_1 = k_{1(0)}[M]/(K_1 + [M]) \quad (13)$$

where $k_{1(0)}$ is equal to the value of the protein modification rate constant when all of the protein is reversibly complexed with modifying agent.

(ii) The protein exists in two conformationally isomeric forms, of which one only is susceptible to modification:

where A_I and A_{II} are the two protein conformational isomers and a is a modified protein, and where k_I , k_{II} , k_{III} and k_{IV} are the isomeric transformation and modification rate constants. The reaction scheme of eqn. (14) indicates that M is in a state of rapid equilibrium with A_{II} . If the dissociation constant of the adsorptive complex A_{II} -M is K'_1 , then the equation equivalent to eqn. (9) is:

$$\frac{C_1}{1 - C_1} = \frac{k_{IV}(k_I + k_{II})}{k_I k_{III(0)}} + \frac{k_{IV}(k_I + k_{II}) K'_1}{k_I k_{III(0)}[M]} \quad (15)$$

where $k_{III(0)}$ is the value for k_{III} when all of A_{II} is reversibly complexed with modifying agent. It will be seen from eqn. (15) that a plot of $C_1/(1 - C_1)$ versus $1/[M]$ will give a straight-line relationship, with an intercept on the $1/[M]$ axis equal to $-1/K'_1$ and a slope equal to the equilibrium constant of the reaction the reactants of which are A_I and M and the product is modified protein. It should be noted that, if the protein species A_I is susceptible to modification, then description of this situation will be by a differential equation of an order higher than three, and accordingly this situation is not considered here. A kinetic analysis of protein modification in cases where both conformational isomers are susceptible to modification, but where the reactions leading from covalently modified to catalytically active protein are ignored, has been presented elsewhere (Rakitzis, 1986a). As in case (i), if $m_2 \gg m_3$ (or vice versa) description of the reaction will be by a single exponential function of reaction time plus a constant term. The dependence of susceptibility to modification

on conformational isomerism has been established for the modification of the two fast-reacting thiol groups of phosphorylase *b* by 5,5'-dithiobis-(2-nitrobenzoic acid), and has been implicated as the most probable cause of the biphasicity of several cases of protein modification (Sanner & Tron, 1975; Rakitzis, 1980a). In connection with eqn. (15) it should be mentioned that protein modification isomerism may also imitate formation of a protein-modifying agent adsorptive complex when the parameter studied is the effect of modifying agent concentration on the protein modification first-order reaction rate constant (Rakitzis, 1986a).

(iii) The protein forms a reversible complex with a ligand in the medium, with only the ligand-complexed protein species being susceptible to modification. This case is formally identical with case (ii), with k_I being a function of ligand concentration. The effect of ligands on the kinetics of protein modification is exemplified in numerous cases of protection of the protein from modification [e.g. in the case of the glucose-mediated protection from inactivation of hexokinase by *N*-bromoacetylglucosamine (Connolly & Trayer, 1979)], and also in the increase in the rate of modification (or of modification-induced inactivation) of phosphoenolpyruvate carboxykinase by phenylglyoxal, mediated by Mn^{2+} (Malebran & Cardemil, 1987), of the tryptophan residues of *Abrus* agglutinin by *N*-bromosuccinimide, mediated by lactose (Patanjali *et al.*, 1987), and of the increase in the rate of glycosylation of, and of keto-aldehyde formation from, bovine serum albumin, mediated by phosphate (Wolff & Dean, 1987).

(iv) Negative protein modification co-operativity, i.e. the case where the protein presents with more than one reactive group per protein molecule, and where the partially modified protein species are less reactive towards the modifying agent used. Protein modification co-operativity may be distinguished as stoichiometric co-operativity or site-oriented co-operativity (Rakitzis, 1977, 1983a,b,c). Examples of stoichiometric protein modification co-operativity are: (1) the carbamoylation of the four terminal valine residues of human haemoglobin (positive co-operativity) and (2) the photo-oxidation of the methionine residues of phosphoglucumutase (negative co-operativity) (Rakitzis, 1983a, 1984a). Examples of site-oriented co-operativity are: (1) the carboxymethylation of the histidine hydantoin derivative (Lennette & Plapp, 1979; Rakitzis, 1983c) and (2) the carboxymethylation of either His-119 or His-12, never both, of ribonuclease A (Heinrikson *et al.*, 1965). A special case of negative modification co-operativity has been termed 'half-of-the sites reactivity' (Stallcup & Koshland, 1973a,b; Schwendimann *et al.*, 1976). The cases to be examined are as follows.

(a) Stoichiometric negative protein modification co-operativity (Rakitzis, 1983a,b,c). The reaction scheme is:

where upper-case letters are used to indicate unmodified protein groups and lower-case letters are used to indicate covalently modified groups. The protein AA presents with two intrinsically identical reactive groups per protein molecule. The first stoichiometric modification generates the species Aa, and the second stoichiometric modification generates the species aa. The rate constants k_{+1a}

and k_{+2a} are functions of modifying agent concentration, in such a way that:

$$k_{+1a} = k_{+1a(0)}[M]/(K_{i1} + [M]), k_{+2a(0)}[M]/(K_{i2} + [M]) \quad (17)$$

where $k_{+1a(0)}$ and $k_{+2a(0)}$ are the rate constants obtaining when the species AA and Aa are fully complexed with modifying agent, and where K_{i1} and K_{i2} are the dissociation constants of these same protein species-modifying agent adsorptive complexes, in the order mentioned. The equation equivalent to eqns. (9) and (15) is:

$$\frac{C_1}{1 - C_1} = \frac{2 + k_{+1a}/k_{-1a}}{(k_{+1a}k_{+2a}/k_{-1a}k_{-2a}) - 1} \quad (18)$$

where C_1 is the modification-free portion and/or the catalytically active portion of the protein under study (if every protein modifiable site also is an enzyme active site). It will be noted from eqn. (18) that plots of $C_1/(1 - C_1)$ versus $1/[M]$ will not necessarily be rectilinear. It will also be noted from the reaction scheme of eqn. (16) that stoichiometric protein modification co-operativity will present as a summation of two exponentials plus a constant term equation. As in cases (i)–(iii), when $m_2 \gg m_3$ (or vice versa) the protein modification equation will, in this case as well, reduce to a single exponential plus a constant term equation.

(b) Site-oriented strong negative protein modification co-operativity (Rakitzis, 1977). The reaction scheme is:

where upper-case and lower-case letters are used as in (a). Group A belongs to the enzyme active site, and group B belongs to a modulator site. Accordingly, the species AB and Ab are catalytically active, whereas the species aB is inactive. In this case the fraction of unmodified protein groups is not equal to the fraction of enzyme molecules that are catalytically active. The term 'strong negative protein modification co-operativity' indicates that the partially modified protein species are unreactive towards the modifying agent used. The dependence of the concentration of enzyme that is catalytically active $[E_{act.}]$ on reaction time is:

$$\frac{[E_{act.}]}{[E_0]} = \frac{k_{+b}}{k_{+a} + k_{+b}} + \frac{k_{+a}}{k_{+a} + k_{+b}} \cdot e^{-(k_{+a} + k_{+b})t} \quad (20)$$

where $[E_0]$ is the enzyme concentration at $t = 0$. The values for k_{+a} and k_{+b} may be obtained graphically from a plot of $\log([E_{act.}]/[E_0])$ versus reaction time (Frost & Pearson, 1961). Since both k_{+a} and k_{+b} are functions of modifying agent concentration, it follows that this case may be differentiated from cases (i) and (ii), when these cases present as a single exponential function of reaction time plus a constant term; in these cases the constant k_2 is not a function of modifying agent concentration [eqns. (11) and (12)]. It will also be noted from eqn. (19) that the reactions leading from covalently modified protein to unmodified protein are ignored. If these reactions are taken into account, the reaction scheme becomes similar to the reaction schemes described by eqns. (1), (14) and (16):

where k_{+a} , k_{+b} , k_{-a} and k_{-b} are the reaction rate constants. The equation equivalent to eqns. (9), (15) and (18) is:

$$\frac{C_a}{1 - C_a} = \frac{k_{+b}k_{-a}}{k_{-b}k_{+a}} + \frac{k_{-a}}{k_{+a}} \quad (22)$$

where C_a is the protein fraction that is catalytically active, and where the constants k_{+a} and k_{+b} are defined by the relationships:

$$k_{+a} = k_{+a(0)}[M]/(K_{iAB} + [M]), \quad k_{+b} = k_{+b(0)}[M]/(K_{iAB} + [M]) \quad (23)$$

where $k_{+a(0)}$ and $k_{+b(0)}$ are the rate constants obtaining when the species aB and Ab are produced from the species AB, when this species is fully complexed with modifying agent, and also where K_{iAB} is the dissociation constant of the species AB-modifying agent adsorptive complex (the binding of the species AB with two molecules of M to produce a reversible adsorptive complex is assumed to be a non-co-operative process). It will be noted from eqn. (22) that, as with stoichiometric negative protein modification co-operativity, plots of $C_1/(1 - C_1)$ versus $1/[M]$ should not necessarily be expected to be rectilinear. It is likewise easy to see that the same conclusion applies to the case of site-oriented modification co-operativity described by eqn. (19).

(v) The preparation consists of a mixture of two proteins (isoenzymes), of which one only is susceptible to modification. Description of the dependence on reaction time of the concentration of unmodified protein (or catalytically active enzyme species $[A_{act.}]$) is given by:

$$\frac{[A_{act.}]}{[A_0]} = \frac{c_2}{c_1 + c_2} + \frac{c_1}{c_1 + c_2} \cdot e^{-k_1 t} \quad (24)$$

where $[A_0]$ is the total concentration of protein at $t = 0$ and where c_1 and c_2 are the concentrations of the individual proteins of the preparation under study, k_1 is the modification rate constant of the protein presenting with concentration c_1 and the modification rate constant of the protein presenting with concentration c_2 is equal to zero. It will be noted that case (iv)(b) is kinetically indistinguishable from case (v) if k_{+a} and k_{+b} are identically dependent on modifying agent concentration.

RELEVANCE TO EXPERIMENTAL DATA AND DISCUSSION

In this paper a kinetic analysis is presented of protein modification reactions in which the dependence of unmodified protein concentration on reaction time is given by a single exponential function, or by a summation of two exponential functions, of reaction time plus a constant term [eqns. (1)–(7)]. It is shown that several protein modification situations are compatible with the formalism of the reaction scheme of eqn. (1). It is also shown that a study of the time-dependence of protein modification may be complementary to, and may thus be of help towards the interpretation of, studies of protein modification (or of modification-induced enzyme inactivation) reactions at equilibrium.

A case from the literature, to which the treatment outlined in this communication may be applied, is the inactivation of glyceraldehyde-3-phosphate dehydrogenase by butane-2,3-dione (Asriyants *et al.*, 1983). This reagent modifies arginine residues. The products of the

reaction have not been identified (Glazer *et al.*, 1975). It may accordingly be presumed that the reaction of butane-2,3-dione with glyceraldehyde-3-phosphate dehydrogenase is an addition-type reaction. Asriyants *et al.* (1983) have found, by amino acid analysis of the modified protein, that two arginine residues are modified per subunit of enzyme protein. From the finding that the order of the inactivation reaction is unity, the authors conclude that only one of the two modifiable arginine residues, per subunit, is essential for enzyme activity. This conclusion, however, is patently wrong. An order of unity of an enzyme inactivation reaction indicates that this is the order for the reaction of the inactivation of the single protein species whose modification is the rate-limiting step in the overall reaction (Rakitzis, 1985, 1986b). It may accordingly be seen that the modification-induced inactivation data of Asriyants *et al.* (1983), as well as any conclusions to be derived therefrom, apply to the inactivation of this single protein species, modification of which is rate-limiting.

It will be noted (Table 1 and Fig. 1) that plots of $C_1/(1 - C_1)$, as well as of $1/k_1$, versus $1/[M]$ are rectilinear; moreover, in all cases the lines pass through the origin of the graph. This finding is consistent with, in each case, a high value for the dissociation constant of the protein-modifying agent adsorptive complex, whether the correct equations to use are eqns. (9) and (12) [case (i)] or eqn. (15) [case (ii)]. It will also be noted from Table 1 that the calculated value for the constant k_2 is reasonably uniform at all butane-2,3-dione concentrations used. Accordingly, strong negative modification co-operativity, in which both rate constants [e.g. k_{+8} and k_{+9} , in case (iv)(b) or k_1 and k_2 of Table 1] should be subject to changes in modifying agent concentration, may be ruled out. Of the remaining possibilities, it is not clear whether the data of the inactivation of

Fig. 1. Plots of the data of Asriyants *et al.* (1983) on the inactivation of glyceraldehyde-3-phosphate dehydrogenase by butane-2,3-dione

Data for the rabbit enzyme (●) and for the rat enzyme (○) are plotted in accordance with eqn. (9). Also shown are plots of the reciprocal of k_1 , calculated from the first-order enzyme inactivation rate constant, and the time-independent term of the inactivation equation (Table 1) for the rabbit enzyme (■) and for the rat enzyme (□).

Table 1. Kinetic analysis of the data of Asriyants *et al.* (1983) on the inactivation of glyceraldehyde-3-phosphate dehydrogenase by butane-2,3-dione

The enzyme inactivation data of Asriyants *et al.* (1983) have been analysed by these authors as a single exponential function of reaction time plus a constant term. The first-order inactivation rate constant k_{inact} and the constant term $[E_{\text{eq}}]$ (fractional enzyme activity at equilibrium) are from Figs. 1 and 2 of Asriyants *et al.* (1983). The values for k_1 and k_2 have been calculated by graphical analysis of the enzyme inactivation data, in accordance with eqn. (12) of the present paper. $[M]$ is butane-2,3-dione concentration.

		Reaction rate constants (min ⁻¹)		
[M] (mM)	[E _{eq.}]	k _{inact.}	k ₁	k ₂
Rabbit muscle enzyme				
3	0.54	0.115	0.053	0.062
7.5	0.30	0.204	0.143	0.061
15	0.18	0.363	0.298	0.065
30	0.10	0.741	0.667	0.074
Rat muscle enzyme				
9	0.45	0.133	0.073	0.060
18	0.20	0.230	0.184	0.046
30	0.15	0.446	0.379	0.067
60	0.11	0.700	0.623	0.077
120	0.06	1.570	1.480	0.094

glyceraldehyde-3-phosphate dehydrogenase by butane-2,3-dione should be interpreted on the basis of an equilibrium relation between enzyme protein and modifying agent [case (i)] or of an additional equilibrium involving the isomerization of two protein conformations [case (ii)]. In either one of these two situations, however, the equilibrium constant of the overall modification-induced enzyme inactivation reaction, for both the rabbit and the rat enzymes, is 29.7 M^{-1} .

In conclusion, the kinetic analysis presented in this paper makes use of the fraction of unmodified protein, or of the inactivation-free portion of enzyme, obtaining at equilibrium, to distinguish among the different reaction models that have been developed in order to explain the biphasicity of many protein modification and enzyme inactivation reactions (Ray & Koshland, 1961; Rakitzis, 1977, 1980a,b, 1983a,b, 1984b). In addition, it is made clear that the determination of the thermodynamic equilibrium constant of the overall protein modification reaction may not be carried out simply by a plot based on the dependence of the fraction of unmodified protein obtaining at equilibrium on the concentration of the modifying agent used. The determination of the equilibrium constant of the reaction must rest, instead, on a thorough kinetic analysis of the protein covalent modification process.

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