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REVIEW ARTICLE

Kinetics of protein modification reactions

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Introduction

Protein modification is any transformation involving the formation or the rupture of a covalent, or partially covalent, bond of the protein under study (Cohen, 1970), hence also the more general term 'covalent modification' (Brocklehurst, 1982). Protein modification studies have so far aimed at: (*a*) the determination of the kind and number of reactive groups of the protein molecule under study, (*b*) the development of site-specific protein modifying agents, and (*c*) the elucidation of enzyme protein catalytic function. None of these objectives may be fully realized without a thorough kinetic analysis of the chemical reactions of the protein modification process. In this article a review of the kinetic approaches so far developed in the study of protein modification reactions will be presented. Emphasis will mainly be placed on formalistic descriptions of actual or hypothetical cases of protein modification, but aspects of molecular kinetics (influence of temperature on the rate of chemical modification) will also be considered.

Modification of enzyme protein is frequently accompanied by loss of enzyme catalytic activity (irreversible enzyme inhibition), and in such cases enzyme protein modification and inactivation are best studied simultaneously. Loss of enzyme activity may result if the protein molecule is modified in ways other than a straightforward derivatization of amino acid residues. Loss of protein tertiary and quaternary structure, modification of enzyme-bound cofactors, ligand binding and conformational isomerism of the protein are important from the kinetics point of view, in that these factors may determine the shape of the plot of concentration of modified residues versus reaction time. Enzyme activity loss, consequent to protein modification, may be complete or partial. In the latter case, inactivation may be the expression of a diminished V, or an increased K_m, of the enzyme, or both. It is because of considerations such as these that the kinetics of chemical modification of proteins cannot be considered entirely apart from the kinetics of such processes as heat denaturation of proteins, photoinactivation and radiation-mediated inactivation of enzymes, and high-ionic-strength-mediated protein unfolding.

Chemical modification of proteins, and irreversible enzyme inhibition, have been the subject of several monographs and reviews (Baker, 1967; Singer, 1967; Cohen, 1970; Shaw, 1970; Means & Feeney, 1971; Aldridge & Reiner, 1975; Glazer *et al.*, 1975; Glazer, 1976; Kenyon & Bruice, 1977; Seiler *et al.*, 1978; Brocklehurst, 1979; Sandler, 1980). Radiation inactivation of enzymes has been reviewed by Kempner & Schlegel (1979). An account of the denaturation of proteins by heat, urea, guanidinium salts, acids, bases, etc., is given by Laidler & Bunting (1973).

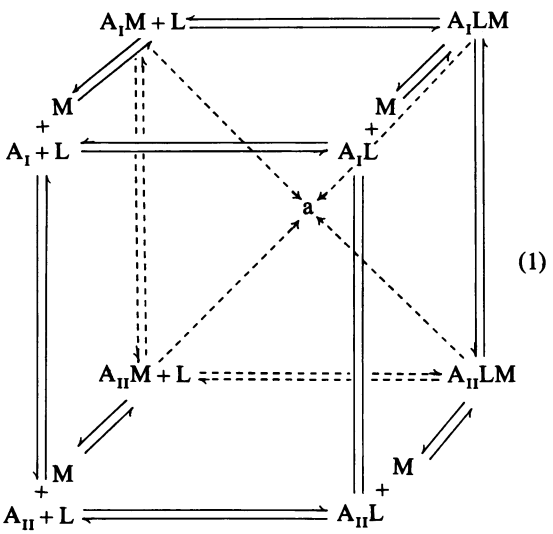
Description of protein modification reactions by the use of rate equations

The primary concern of the enzymologist, who is faced with the analysis of protein modification reactions, is to determine the formalism of the mathematical description of the case in question, and then to formulate the possible models of reaction mechanism which are consistent with the formalism. The mathematical description of the course of protein modification reactions is with the concentration of modified protein reactive groups as the dependent variable and reaction time as the independent variable. Accordingly, it becomes of overriding importance to ascertain whether the dependent variable is or is not linearly dependent on the independent variable used. This is done by performing a number of modification reactions at different initial protein concentrations. If the dependence is a linear one, the fractional concentration of protein reactive groups modified is the same for all initial protein concentrations used, at any chosen reaction time interval from the start of the reaction. A large excess of modifying agent over protein concentration ensures an effectively constant modifying agent concentration, and removes the most common cause of non-linearity of the equations describing protein modification reactions.

Cases described by linear differential equations

Differential equations may be classified according to order. The order of a differential equation is

the order (power) of the highest derivative which occurs (Bronson, 1973). Differential equations may be further classified by whether the coefficients of all derivatives are functions of the independent variable or are constants (Bronson, 1973). Molecular events determining the time dependence of protein modification reactions are: (*a*) modifying agent binding on the protein, to produce a reversible protein–modifying agent complex; (*b*) ligand binding on the protein; (*c*) isomerization of the protein (production of conformational isomers of the protein); (*d*) protein modification co-operativity, i.e., modification of one or more reactive groups in such a manner that the different partially modified, or unmodified, protein species possess different reactivities towards the modifying agent. Setting aside protein modification co-operativity for the moment, the reaction of a protein that is capable of presenting with conformationally isomeric forms with modifying agent and with ligand may be written as follows:



(1)

where A_I and A_{II} are two conformational isomers of the protein, M is modifying agent, L is ligand, and a is modified protein. It will be seen that eight species of unmodified protein are produced, of which four are in the form of a reversible protein–modifying agent complex, the first-order reaction of which leads to the production of modified protein. The time dependence of the transformation reactions of the unmodified protein species of eqn. (1) determines the order of the differential equations describing the course of these reactions, while the dependence of the modification rate constants of this same equation on modifying agent concentration, ligand concentration, or on the state of isomerization of each particular protein species, is entirely a matter of the partition of the protein species into different classes by the time-dependent protein-species-transformation steps present, and the equilibrium within each class in which such protein species participate.

Cases described by linear first-order differential equations with constant coefficients. These may be distinguished into:

(*a*) Cases where a rapid equilibrium exists among all the unmodified protein species of eqn. (1). In this case each protein species may be related to the concentration of total protein reactive groups by appropriate equilibrium relationships, while the four modification rate constants of eqn. (1) will present as one modification rate constant, since all modification reactions of eqn. (1), under the conditions specified above, are parallel reactions. Eqn. (1) reduces to:

$$A \xrightarrow{\quad k \quad} a \qquad (2)$$

where A is protein reactive groups concentration, and k is the sum of the separate modification rate constants multiplied by the appropriate equilibrium relationship governing the interconversion of the individual unmodified protein species. With time as the independent variable, the differential equation describing this situation is:

$$[a]' = k([A]_0 - [a]) \qquad (3)$$

where $[A]_0$ is [A] at $t = 0$. Eqn. (3) may be rearranged to give:

$$[a]' + k[A] = k[A]_0 \qquad (4)$$

a linear first-order differential equation with constant coefficients. Eqn. (4) is solved for [a] by the use of an integrating factor (Bronson, 1973). The integrating factor for eqn. (4) is e^{kt}. Multiplying both sides of eqn. (4) by the integrating factor, and integrating both sides of the equation so produced with respect to t:

$$([A]_0 - [A])/[A]_0 = e^{-kt} \qquad (5)$$

A plot of $\ln\{([A]_0 - [a])/[A]_0\}$ versus reaction time gives a straight line, with a slope of $-k$, passing through the origin of the graph. The description of protein modification, or enzyme inactivation reactions, by the use of eqn. (5) was first performed by Aldridge (1950) in connection with the inactivation of acetylcholinesterase by paraoxon, and has since been used in a large number of protein modification reactions. In fact, a graph of logarithm of concentration of residual protein reactive groups versus reaction time is the starting point of any kinetic analysis of protein modification data.

(*b*) Cases where some of the interconversion reactions of eqn. (1) are nonexistent, so that the unmodified protein species of this equation are

separated into two noninteracting sets. Eqn. (1) reduces to:

$$A_1 \xrightarrow{k_1} a \quad (6)$$

$$A_2 \xrightarrow{k_2} a \quad (7)$$

where A_1 and A_2 are the two sets of unmodified protein species, and k_1 and k_2 are the corresponding modification rate constants. The integrated expression for the sum of eqns. (6) and (7) is:

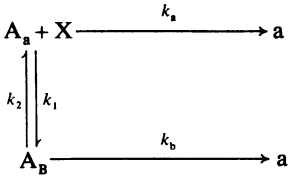
$$([A]_0-[a])/[A]_0 = c_1e^{-k_1t}+c_2e^{-k_2t} \quad (8)$$

where c_1 and c_2 are the fractional concentrations, at $t=0$, of A_1 and A_2, respectively. A plot of $\ln\{([A]_0-[a])/[A]_0\}$ versus reaction time yields a curve the final portion of which is rectilinear (after the exponential with the larger k value has become negligible). It is of interest that the coefficients of eqn. (8) are independent of the values of the constants of this equation, i.e., they are independent of the values for k_1 and k_2. The situation described by eqn. (8) may be extended to cover cases presenting with three or more sets of reactive groups. A procedure for the graphical analysis of curves described by multiexponential equations has been given by Defares & Sneddon (1960). Ray & Koshland (1961) pointed out that an analysis of a protein modification curve into a summation of exponential functions of reaction time provides a measure for the number of amino acid residues of each reactive set, if the number of residues corresponding to $[A]_0$ and the relative molecular mass of the protein are known. In this connection it is of interest that, although a plot of $\ln\{([A]_0-[a])/[A]_0\}$ versus reaction time cannot be constructed without a prior knowledge of the value for $[A]_0$, it is possible to obtain, by graphical analysis, the value for k, as well as for $[A]_0$, by using values of the modification product concentration, [a], at different reaction times (Guggenheim, 1926; Kezdy *et al.*, 1958; Swinbourne, 1960; Glick *et al.*, 1978; Schwartz, 1981) or for certain first order consecutive or parallel reactions, by using absorbance–time measurements at two different wavelengths (Lachmann *et al.*, 1980*a*,*b*).

The situation described by eqn. (8) may readily be identified by graphical analysis of the protein modification curves at different modifying agent concentrations. The intercept of the final rectilinear portion of the graph of $\ln\{([A]_0-[a])/[A]_0\}$ versus reaction time on the axis representing fractional reactive groups concentration gives the value for the coefficient of the slow exponential, while the intercept on the same axis of the corrected $\ln\{([A]_0-[a])/[A]_0\}$ values gives the value for the coefficient of the fast exponential. If several protein modification experiments are performed, at different modifying agent concentrations, the intercepts of all the slowly reacting components of the reaction are the same, as also happens with the intercepts of all fast-reacting components {this is the case for the reaction of the two essential cysteines of muscle pyruvate kinase with 5'-[*p*-(fluorosulphonyl)benzoyl]guanosine (Tomich *et al.*, 1981)}. The analysis of protein modification data by the method of Ray & Koshland (1961) is often used to identify the, presumably, noninteracting sets of protein reactive groups, without the precaution of performing the modification reaction at two or more different modifying agent concentrations (e.g. by Martinez-Carrion *et al.*, 1967; Chu & Bergdoll, 1969; Schirmer *et al.*, 1970; Grouselle & Pudles, 1977).

Cases described by linear second, or higher, order differential equations with constant coefficients. If, apart from the protein modification reactions, one or more of the interconversion reactions of eqn. (1) is not a process of rapid equilibrium, but instead it is measurably time-dependent, within the framework of the experimental procedure used, protein modification is described by a differential equation of an order higher than one. The same applies to cases of protein modification co-operativity, i.e., cases where protein modification reactions are in the form of a catenary (every species transformation step is also a modification step). In all of these cases, protein modification reaction curves may be seen to consist of summations of exponentials, the coefficients of which are functions of the rate constants of modification (and also, where this is applicable, of protein species interconversion). Accordingly, plots of $\ln\{([A]_0-[a])/[A]_0\}$ versus reaction time of these cases present with final rectilinear portions which, when extended on the axis representing fractional protein reactive groups concentration, do not meet on this axis [a notable case of this kind is the carboxamidomethylation of phosphoglucose isomerase by iodoacetamide (Schnackerz & Noltmann, 1970)].

When one of the species transformation reactions of eqn. (1) is not a process of rapid equilibrium, this equation reduces to (Rakitzis, 1980*a*):



(9)

where A_a and A_b are the two sets of species into which the protein species of eqn. (1) are divided by the one measurably time-dependent step, X is modifying agent or ligand, and k_1, k_2, k_a and k_b are the respective transformation or modification rate constants. In the case where the time-dependent step is an isomerization step, X is equal to unity. With time as the independent variable, the differential equations corresponding to eqn. (9) are:

$$-[A]' = k_a[A_a] + k_b[A_b] \quad (10)$$

$$[A_a]' = k_2[A_b] - (k_1[X] + k_a[A_a]) \quad (11)$$

Differentiating eqn. (10) and eliminating $[A_a]$ and $[A_b]$ by means of the conservation relationship $[A] = [A_a] + [A_b]$, and also by means of eqn. (11):

$$[A]'' + (k_1[X] + k_2 + k_a + k_b)[A]' + (k_a k_2 + k_a k_b + k_b k_1[X])[A] = 0 \quad (12)$$

Eqn. (12) is a second-order linear differential equation with constant coefficients. The solution of an equation of this form is (Bronson, 1973):

$$([A]/[A]_0) = C\, e^{m_1 t} + (1 - C)\, e^{m_2 t} \quad (13)$$

where m_1 and m_2 are the roots of the characteristic equation of the differential equation in question. The value of C is found by applying the initial conditions of the experimental situation described by eqns. (10), (11) and (13). Eqn. (13) may be analysed graphically by means of the relationships:

$$-(m_1 + m_2) = k_1[X] + k_2 + k_a + k_b \quad (14)$$

$$(m_1 + m_2)^2 - (m_1 - m_2)^2 = 4(k_a k_2 + k_a k_b + k_b k_1[X]) \quad (15)$$

An analysis of the data of Sanner & Tron (1975), on the modification of the two fast-reacting sulphhydryl groups of phosphorylase *b* by 5,5'-dithiobis-(2-nitrobenzoic acid), by the procedure outlined above, has been presented (Rakitzis, 1980*a*). Some aspects of the relationship described by eqn. (9) have been studied by Childs & Bardsley (1975). The assumption was made by these authors that the modified protein may further react to produce unmodified protein and X (reversible modification of the protein). The mathematical treatment of this situation involves the solution of a third-order differential equation with constant coefficients. Some of the conclusions of the paper of Childs & Bardsley (1975) have been criticized by Cornish-Bowden (1979). Studies of a two-site site-oriented model of protein modification co-operativity, with stable as well as with unstable modifying agents, have been presented (Ray & Koshland, 1961; Rakitzis, 1977, 1978*b*). A study of the different possible models of stoichiometric protein modification co-operativity, presenting with a modification curve representing the summation of two exponentials, while the number of residues modified per protein molecule is greater than two, has been made (Rakitzis, 1983*a*). Quite often protein modification data, which when plotted according to the method of Ray & Koshland (1961) give different values for the 'fast' and 'slow' reacting sets of residues, for different modifying agent concentrations, have been interpreted to mean the existence of two or more independently reacting sets, i.e., have been interpreted along the lines of eqns. (6)–(8) (see Rakitzis, 1980*a*).

If the number of measurably time-dependent interconversion steps in eqn. (1) is greater than one, the order of the differential equation describing protein modification is greater than two. In general, the order of the differential equation describing the protein modification event is equal to $n+1$, where n is the number of measurably time-dependent interconversion steps of protein species. It is of interest that algebraic equations, and consequently also differential equations, of an order higher than four cannot be solved analytically (Ayoub, 1982). A notable exception is the case of consecutive reactions, the roots of the characteristic equation for which are identical with the reaction rate constants (Lachman *et al.*, 1980*a*,*b*; Rakitzis, 1983*a*). As well as protein modification co-operativity, protein unfolding and refolding may also be described by means of consecutive reactions. An extensive analysis of the kinetics of protein unfolding and refolding has been given by Ikai & Tanford (1973), Ikai *et al.* (1973), and Tanford *et al.* (1973).

Cases described by linear differential equations with variable coefficients. Variability of coefficients in the differential equations describing protein modification arises in cases where either the modifying agent, or the protein itself, are unstable in solution.

(*a*) The modifying agent is unstable. The assumption is made that the modifying agent disappears in a first-order reaction, so that $[M] = [M]_0 e^{-k_h t}$, where $[M]_0$ is initial modifying agent concentration and k_h is a constant. Eqn. (3) is transformed into:

$$-[A]' = k_{app.}\, e^{-k_h t}[A] \quad (16)$$

where $k_{app.} = k[M]$, i.e., $k_{app.}$ is linearly dependent on [M]. Since cases where $k_{app.}$ is not linearly dependent on [M] are treated in another section of this article, a description of more complicated cases of protein modification by an unstable modifying agent is given in that section. Solving eqn. (16) by means of the integrating factor $\exp(k_{app.} \exp[-k_h t])$:

$$\ln([A]/[A]_0) = ([M]_0 k/k_h)(e^{-k_h t} - 1) \quad (17)$$

As reaction time is increased, a limiting value for $[A]/[A]_0$ is approached:

$$\ln([A]_\infty/[A]_0) = -[M]_0 k/k_h \quad (18)$$

Rearranging eqn. (17) (after a suitable transformation):

$$\ln[\ln([A]/[A]_0 - \ln([A]_\infty/[A]_0)] = \ln\ln([A]_0/[A]_\infty]) - k_h t \quad (19)$$

Eqn. (19) may be used to obtain graphically the value for the rate constant k_h of modifying agent

disappearance. The treatment outlined in eqns. (16)–(19) has been developed by Purdie & Heggie (1969), and by Ashani *et al.* (1972). This treatment has been applied to cases of enzyme inactivation by unstable modifying agents by Ashani *et al.* (1972), Maglothin & Wilson (1974), Rakitzis (1974), Maglothin *et al.* (1975), Barnett & Rosenberry (1978), Johnson & Poisner (1980), and by Makoff & Malcolm (1980, 1981).

(*b*) The protein is unstable. This case is treated by Haldane (1965) and by Laidler & Bunting (1973) for the case where the enzyme is inactivated by its own substrate, and by Rakitzis *et al.* (1978) for the case where the anion transport system in erythrocyte membranes is inactivated by a modifying agent. The reaction scheme is:

$$A + S \rightleftharpoons AS \longrightarrow A + P \qquad (20)$$

$$A + S \rightleftharpoons AS' \longrightarrow a \qquad (21)$$

where S is substrate, P is the product of the enzyme catalytic action, AS is the enzyme–substrate complex at the catalytic site, and AS' is the enzyme–substrate complex outside the catalytic site. If the enzyme or the membrane transport system in question obey first-order kinetics with regard to substrate in product formation, the mathematical description of product formation is very much along the lines of eqns. (16)–(19). A long ago recognized situation of this sort is the inactivation of catalase by hydrogen peroxide (Laidler & Bunting, 1973).

Cases described by non-linear differential equations

Because of the extreme complexity in the classification schemes of non-linear differential equations (Davis, 1962), the brief account of non-linear differential equations describing protein modification reactions given here is based on a factual rather than a formalistic approach.

Cases where the modifying agent concentration is not well in excess of protein concentration. Protein modification is described by the classical equations of second-order reaction kinetics (Frost & Pearson, 1961; Latham & Burgess, 1977). These equations have been applied in the study of the oxidation of a sulphhydryl group of phosphoribosylpyrophosphate synthetase (Roberts *et al.*, 1975), the reactivity of the thiol group in human and bovine albumin with 2,2'-dithiopyridine (Pedersen & Jacobsen, 1980), and the reactivity of sarcoplasmic reticulum adenosinetriphosphatase with a derivative of iodoacetamide (Coan & Keating, 1982).

Cases where the protein dissociates into monomers (or oligomers) during the course of the modification. Vas & Boross (1972) have considered the case where a protein dissociates into two monomers; furthermore, only the monomers are susceptible to modification. Vas & Boross (1972) have used the integrated form of the differential equation describing this situation to compare calculated and experimentally obtained data for the heat inactivation of glyceraldehyde-3-phosphate dehydrogenase.

The kinetics for the inactivation of a homotetrameric protein, as well as of some models involving isomerizable and dissociable–associable oligomeric proteins, were developed by Keleti (1971), and by Fischer *et al.* (1973). These authors used simplifying assumptions which permitted the linearization of the non-linear differential equations describing these complex models. In stoichiometric protein modification co-operativity, the modification rate constants may be determined by the use of the concentration of the monomodified, dimodified or *n*-fold-modified protein species. The method is also applicable when the protein modification reaction is a second-order reaction, as well as when it is a first-order reaction (Rakitzis, 1983*b*).

Cases where the enzyme is inactivated during the course of enzyme action (suicide substrates). If the enzyme–substrate complex at the catalytic site is inactivated at an appreciable rate, the situation can be represented as:

$$A + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} AS \begin{array}{l} \nearrow A + P \\ \searrow a \end{array} \qquad (22)$$

In this situation of interest is the 'partition ratio', i.e., the P/a ratio, which is constant under all circumstances. Since S, as well as A, are diminished during the course of the reaction, the differential equation describing this reaction is nonlinear. Mathematical treatments of this situation have been presented by Waley (1980), and by Tatsunami *et al.* (1981). 'Suicide substrates' have been the topic of a number of communications, as well as of a symposium (Rando, 1974*a*,*b*; Seiler *et al.*, 1978).

Cases where enzyme and modifying agent react in the course of enzyme catalytic action. In this situation the parameter used as a dependent variable is the concentration of the product produced by the catalytic action of the enzyme. If $[AS] \ll [A]$, i.e., if product formation is a first-order process with regard to substrate concentration, this case leads to a description of enzyme protein modification by linear differential equations. However, if the condition $[AS] \ll [A]$ cannot be considered to be valid, the differential equations involved are nonlinear. Treatments of this case have been presented by Hollaway *et al.* (1980), and by Tian & Tsou (1982). These authors, albeit by the use of simplifying assumptions, accomplish the determination of the microscopic rate constants for the protein modification event by an analysis of the data of enzyme activity product–time measurements during the course of protein modification.

Rapid equilibrium reactions affecting the rate of protein modification

As will be seen from eqn. (1), any change in the values of the measurably time-independent (rapid equilibrium) protein species transformation steps will reflect on the overall rate of protein modification. In particular, concerning the situation described by eqn. (9), it will be seen that a change in the value of any of the rate constants involved will result in a change in the m_1 and m_2 values, as well as the C value, of eqn. (13). Macroscopic protein modification rate constants may depend on several parameters.

Modifying agent concentration

The formation of a reversible protein–modifying agent complex, the subsequent first-order reaction of which results in protein modification, was first postulated by Kitz & Wilson (1962) in a study of the inactivation of acetylcholinesterase by methanesulphonic acid esters. Kitz & Wilson (1962) observed a 'rate saturation effect', when enzyme inactivation was studied at different modifying agent concentrations, from which they concluded that the reaction under study was effected by the mediation of a protein–modifying agent adsorptive complex (akin to the enzyme–substrate Michaelis complex):

$$A + M \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} AM \xrightarrow{k_{+2}} a \qquad (23)$$

Kitz & Wilson (1962) were able to determine the values for K_i (defined as k_{-1}/k_{+1}), and k_{+2}, for several methanesulphonic acid esters, by the use of the relationship:

$$k_{app.} = (k_{+2}[M]/[K_i + [M]) \qquad (24)$$

where $k_{app.}$ is the macroscopic modification rate constant of the reaction. A graphical determination of k_{+2} and K_i may be accomplished by the use of the linearized transformation of eqn. (24) (in analogy with the Lineweaver–Burk transformation of the Michaelis–Menten equation):

$$\frac{1}{k_{app.}} = \frac{1}{k_{+2}} + \frac{K_i}{k_{+2}}\left(\frac{1}{M}\right) \qquad (25)$$

The values for k_{+2} and K_i have been determined, for a large number of proteins and modifying agents, by the use of eqn. (25) (see, among others, Kitz & Wilson, 1962; Baker *et al.*, 1962; Schaeffer *et al.*, 1967; Shaw, 1970; Bing *et al.*, 1972; Brake & Weber, 1974; Redkar & Kenkare, 1975; Pavlic & Wilson, 1978; Rakitzis *et al.*, 1978; Connoly & Trayer, 1979).

When $K_i \gg [M]$, $k_{app.}$ is linearly dependent on [M], a situation compatible with the reaction between A and M by a simple bimolecular mechanism, i.e., without the mediation of an adsorptive complex. Brocklehurst (1979) has advanced the view that, in all cases presenting with first-order kinetics with regard to modifying agent concentration, the reaction may proceed through the intermediacy of a protein–modifying agent adsorptive complex, albeit this complex may be characterized by a rather large dissociation constant. Brocklehurst (1979) has pointed out that since k_{+1} has a probable lower limit of $10^7 M^{-1} \cdot s^{-1}$, i.e., a value approaching that of a diffusion-controlled reaction (Hammes & Schimmel, 1970), the value for the second-order rate constant, for most protein modification reactions so far described, is far smaller than k_{+1}, a prerequisite for which is that $k_{+2} \ll k_{-1}$, which is the necessary and sufficient condition for the existence of equilibrium (or one of 'quasi-equilibrium', to avoid equivocation) between [AM], [A] and [M] (eqn. 23). As will be seen from eqn. (12), if $k_{-1} \gg k_{+2}$ (and unless $k_{+2} \gg k_{+1}[M]$) then $m_1 \gg m_2$. This inequality ensures that one of the two exponentials of the equation describing the protein modification reaction disappears early in the reaction, i.e., a steady state sets in, and also that the coefficient of the slower exponential is close to unity. Consequently, the protein modification reaction will, under these circumstances, appear to obey first-order kinetics (Malcolm & Radda, 1970; Cornish-Bowden, 1979; Brocklehurst, 1979, 1982).

The reaction between protein and modifying agent may involve more than one modifying agent molecule per protein molecule. The reversible binding of two or more micromolecules per protein molecule may be described by the Adair (1925) equation, and also by the co-operativity models developed by elaborations on the Adair (1925) equation by Pauling (1935), Monod *et al.* (1965), and by Koshland *et al.* (1966). It appears that, with one notable exception (Levilliers, 1977), protein modification data have not been treated by any of the procedures described by the authors mentioned above, as has been the case with ligand binding on haemoglobin, or a large number of enzyme proteins (Koshland, 1970; Cornish-Bowden, 1976). Levy *et al.* (1963) have used a simplified form of the Hill (1913) equation to study multiple modifying agent binding on protein:

$$k_{app.} = k_{+2}[M]^h / K_i \qquad (26)$$

where h is the Hill coefficient. A plot of $\log k_{app.}$ versus $\log[M]$ (or alternatively, of $\log t_{\frac{1}{2}}$, where $t_{\frac{1}{2}}$ is the half-life for modification of protein reactive groups or enzyme inactivation, versus $\log[M]$), yields a straight line with a slope of h. The treatment of Levy *et al.* (1963) has been applied by these authors to the inactivation of myosin adenosine triphosphatase by 2,4-dinitrophenol ($h = 3$). Eqn.

(26) has also been applied to cases of enzyme inactivation by Cardemil & Eyzaguirre (1979), Petz *et al.* (1979), Belfort *et al.* (1980), Borders *et al.* (1982) and Sönderling & Mäkinen (1983).

Rapid equilibrium reactions are of particular interest in cases of protein modification by unstable modifying agents, since in such cases every expression involving modifying agent concentration is converted into a time-dependent factor in the differential equation describing protein modification. The case where k' in eqn. (16) is not linearly dependent on [M] has been treated by Purdie & Heggie (1969), and by Rakitzis (1974). It was found that, when $[I]_0 \gg K_i$ (where $[I]_0$ is initial modifying agent concentration), a straight line is obtained when $\log([A]_0/[A]_\infty)$ is plotted versus $\log[I]_0$; the intercept of the plot on the $\log[I]_0$ axis is $\log K_i$. A brief account of the kinetics of protein modification reactions by unstable modifying agents has been given by Rakitzis (1981).

Ligand concentration

The effect of ligand on the rate of protein modification depends on whether the modification rate of the protein–ligand–modifying agent complex is faster or slower than the modification rate of the protein–modifying agent complex. In the former case the effect of ligand binding on the protein is sensitizing, in the latter case it is a protective effect. Equations describing protective or sensitizing ligand effects have been developed for the former case by Kitz & Wilson (1962), and for the latter case by Pavlic & Zorko (1978). A method describing the determination of the dissociation constant for ligand binding to enzyme protein, from initial rates of enzyme inactivation and from the total concentration of ligand added, has been developed by Horiike & McCormick (1980).

Hydrogen ion concentration

Protonation of protein nucleophilic groups is a special case of ligand binding. However, as pointed out by Cornish-Bowden (1976): 'there are several differences between protons and other modifiers that make it worthwhile to examine protons separately. Firstly, virtually all enzymes are affected by protons, so that the proton is far more important than any other modifier. It is far smaller than any other chemical species and has no steric effect'. In the simplest case of covalent modification of one group (or one set of identical groups), of which only the unprotonated, or dissociated, form is reactive, the apparent modification rate constant, $k_{app.}$, obeys a simple adsorption relationship:

$$k_{app.} = k/(1 + [H^+]/K_a) \qquad (27)$$

where k is the modification rate constant for the unprotonated form of the group, and K_a is its dissociation constant (Connoly & Trayer, 1979). Cases of protein modification, or enzyme inactivation, compatible with eqn. (27) have been presented by Connoly & Trayer (1979), Belfort *et al.* (1980) and by Sönderling & Mäkinen (1983). If two non-identical acidic groups react with the modifying agent, the concentration of the reactive, unprotonated or dissociated, form is given by the Michaelis pH functions (Cornish-Bowden, 1976), and plots of $k_{app.}$ verus pH are bell-shaped (Lennette & Plapp, 1979). A study of the pH dependence of covalent modification by two-protonic state electrophiles has been presented by Brocklehurst (1982).

Plots of fractional enzyme activity versus extent of protein modification (Tsou plots)

When enzyme protein modification and inactivation studies are performed on the same preparation, it is possible to correlate the two sets of findings in a plot of fractional enzyme activity versus extent of protein modification. Such plots have been used to obtain, by a process of extrapolation, the maximum extent of protein modification, i.e., the total number of reactive groups per protein molecule. It is common practice to construct and interpret such plots of fractional enzyme activity versus extent of protein modification on an intuitive basis, despite the firm theoretical treatment of this topic by Tsou (1962), and the elaborations on this by Horiike & McCormick (1979), Stevens & Colman (1980), and Rakitzis (1978*a*, 1980*b*).

Tsou (1962) has employed a statistical method of studying the relationship between fractional enzyme activity and extent of protein modification. Tsou (1962) has considered the following cases: (*a*) Only one type of equally reactive groups, of which one or more are essential for enzyme activity, are modified. (*b*) Reactive groups are divided into essential type groups, and non-essential type groups, the latter type groups reacting at a markedly different rate than the former. (*c*) The modification of the protein in such a manner that some residual enzyme activity is left after complete modification of a certain essential type of groups. (*d*) Retention of full enzyme activity when any one of several essential type groups remains unaltered. (*e*) Two types of groups are modified, and both types contain essential groups. Since the derivations of Tsou (1962) contain neither a time factor nor a modifying agent concentration factor, the conclusions drawn by an application of this method are free of the restrictions applying to conclusions arrived at by studies of the time dependence of protein modification reactions. On the other hand, time-dependence studies offer the advantage of a greater insight into the possible mechanisms of protein modification. It is best to

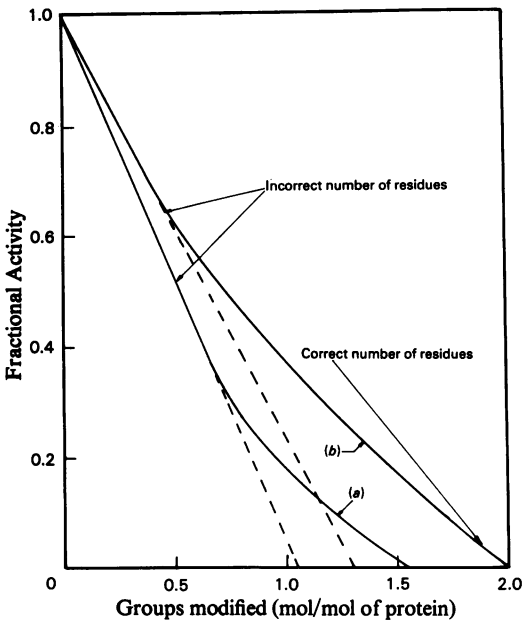


Fig. 1. *Plots of fractional enzyme activity versus extent of protein modification may easily be misinterpreted*
(*a*) Site-oriented protein modification co-operativity in a two-site enzyme protein. The modification rate constants are: $k_{1b} = k_{2b} = 0$, $k_{1a} = 0.5\,min^{-1}$, $k_{2a} = 0.05\,min^{-1}$. For a definition of the notation used, see Rakitzis (1977). (*b*) The enzyme preparation is a mixture of two isoenzymes present in equal molarities. The first isoenzyme possesses one reactive residue per molecule of protein and is modified with a rate constant of $0.5\,min^{-1}$. The second isoenzyme possesses three reactive residues per molecule of protein and is modified with a rate constant of $0.1\,min^{-1}$. [Both cases shown in the Figure have been presented in Rakitzis (1980*b*).]

combine statistical and time-dependence considerations when studying the modification of a catalytically active protein. Rakitzis (1978*a*) has pointed out that, in the case of enzyme protein modification reactions which are described by summations of exponential functions of reaction time, the conclusions of Tsou (1962) can also be arrived at by a juxtaposition of the equation describing protein modification and the equation describing enzyme inactivation. It is accordingly seen that it is wrong to extrapolate the initial portion of the plot of fractional enzyme activity versus number of groups modified per molecule of protein, and interpret the intercept of this on the axis representing the number of groups modified to mean the number of groups of the 'fast' reacting set; an exception to this is strong irreversible binding co-operativity (Rakitzis, 1980*b*). This fundamental mistake has also been pointed out by Horiike & McCormick (1979, 1980), and by Stevens & Colman (1980). A large number of authors have used the plot of fractional enzyme activity versus extent of protein modification to distinguish between 'fast' and 'slow' sets of residues, without a proper analysis of this plot (e.g., Di Pietro *et al.*, 1979; Petz *et al.*, 1979; Bond *et al.*, 1980; de Kok *et al.*, 1980; Huber *et al.*, 1982; Mäkinen *et al.*, 1982; Inano & Tamaoki, 1983, as well as the papers cited by Rakitzis, 1980*b*). Hypothetical cases of enzyme protein modification which may lead to an erroneous interpretation of Tsou plots are shown in Fig. 1. As will be seen from Fig. 1, it may also not be quite safe to use the extrapolation of the final rectilinear portion of the plot on the axis showing number of groups modified, if this last rectilinear portion is not extensive enough: in case (*b*) of Fig. 1 an erroneous value of 1.55 mol of groups modified/mol of protein is obtained, albeit the true value is 2 mol of groups/mol of protein.

Thermodynamics of protein modification reactions

The temperature dependence of reaction rate constants yields the values for the enthalpy, as well as for the entropy change, of the formation of the activated complex of the reaction under study (Frost & Pearson, 1961; Gutfreund, 1972; Laidler & Bunting, 1973). It has been pointed out by Lennette & Plapp (1979) that, in contrast with studies of enzyme catalysis, the thermodynamic parameters for the rate constants of the reaction of modifying agents with proteins can always be compared with the parameters of the reaction of the same modifying agent with small molecules containing the same functional groups as those with which they react in the intact protein. 'Active-site directed reagents resemble substrates in their behaviour towards enzymes: they bind to the active site and their rates of reaction with the enzyme are facilitated, presumably by one or more catalytic factors. Hence, active-site directed reagents can be used to compare an enzymatically facilitated reaction with the same, uncatalysed, chemical reaction.' Consequently, enzyme protein modification may be used to probe into the nature of enzyme catalysis.

Whitaker & Lee (1972) studied the reaction of 2-chloroacetamide with ficin, the rate of alkylation of which is 4–7 times faster in the presence of the inhibitor benzoyl-D-arginine ethyl ester than in its absence. This rate enhancement was found to be due to a more favourable $\Delta S\ddagger$, and may accordingly be considered to be due to a conformational change in the enzyme–inhibitor complex. Pavlic (1973) studied the temperature dependence of the tetraethylammonium-mediated sensitization of the methanesulphonylation of acetylcholinesterase; sensitization is due to an increase in $\Delta S\ddagger$ and may thus be attributed to structural alterations in the

active site. By studying the effect of monovalent ions of the Hofmeister series on the methanesulphonylation of acetylcholinesterase, Pavlic (1980) found a good correlation between the acceleration of methanesulphonylation and the extra entropy of hydration of the enzyme. Halasz & Polgar (1976) used methyl iodide and iodoacetamide to probe the microenvironment of the active sites of thiosubtilisin and papain. A comparison of the thermodynamic activation parameters leads to the conclusion that 'the –SH group of thiosubtilisin is located in an environment less polar than water. The concentration of methyl iodide in this nonpolar layer is higher than in the bulk solution, which results in an enhanced reaction rate'.

The free energy change difference between a co-operative protein modification reaction, and the hypothetical reaction of the same reactants to form the same products in the absence of co-operative interactions, is a function of the microscopic rate constants of these two reactions (Rakitzis, 1983*c*). The free energy change difference between the reaction of a modifying agent and a protein, and the reaction between the same modifying agent with a small molecule or another protein, containing the same functional group, is one example of the more general case of 'linear free energy change differences in a series of related phenomena' (Frost & Pearson, 1961). A special case of linear free energy change differences is the Brönsted relationship, i.e., the correlation of the intrinsic reactivity of a functional group with its pK_a, i.e., with its nucleophilicity. Brönsted plots have been presented by Freedman & Radda (1968), and by Fields (1971), for the reaction of amino acids and peptides with 2,4,6-trinitrobenzenesulphonic acid, and by Amitai *et al.* (1976) for the inhibition of cholinesterase by 1,3,2-dioxaphosphorinane 2-oxide derivatives. Inasmuch as studies of this kind allow a distinction between nucleophilic catalysis and general base catalysis, when the attacking nucleophile is part of a catalytic reaction sequence, it is to be expected that studies of free energy change relationships, in cases of covalent modification, will be of help in the elucidation of the mechanism of enzyme catalytic action, as well as in the development of high-affinity active-site-directed irreversible enzyme inhibitors.

Conclusion

The kinetics of protein modification, developed over the past 30 years, have mostly dealt with the time dependency of modification reactions rather than, as has been the case with enzyme catalytic activity reactions, with mechanisms involving rapid equilibria. As is seen from the material of this review, the problems of the kinetics of protein modification have developed along the lines of, and share the same peculiarities with, the solution of differential equations, both linear and non-linear. Clearly, the major problems in the kinetic analysis of protein modification reactions are identification problems, i.e., problems of fitting a set of experimental data to a number of alternative systems of differential equations, representing alternative hypotheses.

The major area of research where further developments in the kinetic analysis of protein modification reactions are to be expected is in the formalistic mathematical treatment concerning problems of subunit association–dissociation with concomitant protein denaturation, i.e., problems formulated with non-linear differential equations. Also, the thermodynamics approach to protein modification will offer further insight into the relationship between chemical structure of modifying agents, as well as of protein reactive groups, and the formalistic kinetics describing the protein modification event. Inevitably, the structure–reactivity problems which always have provided, and will continue to provide, the stimulus for the performance of the highly exacting work involved in kinetic studies, are leading, despite the formidable difficulties involved, to a widespread use of quantum mechanical considerations (Höltje, 1974; Richards, 1977).

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