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KINETICS OF PROTEIN MODIFICATION REACTIONS: ANALYSIS OF MODIFICATION-INDUCED PROTEIN UNFOLDING

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A mathematical treatment of a two-sited, modification-induced protein unfolding model is presented, and it is shown that the dependence of the concentration of modified protein groups on reaction time is described by a linear, second-order, differential equation with nonzero right hand side. The analytic solution of this equation consists of a summation of exponential functions of reaction time. By assigning arbitrary values to the modification and isomerization rate constants of these equations, simulated cases of protein modification are presented, and the apparent end-point of the reaction is determined graphically. It is found that the apparent end-point of the reaction is, in most cases studied, different from the true value of two groups modified per protein molecule, and is a function of both the modification, and isomerization rate constants of the model. The first derivative of the protein modification reaction, at the start of the reaction, $[E]'_{mod}(0)$, is determined, for the same simulated cases of protein modification, by two different analytical methods. It is found that the $[E]'_{mod}(0)$ value, obtained from graphical and numerical analysis data, is in most cases in good agreement with the value expected from first principles. Finally, the different irreversible enzyme inhibition forms, contingent upon the different kinds of the enzyme inactivation–protein modification relationships of the protein modification model under consideration, are presented and discussed.

KEY WORDS: Protein modification, protein unfolding, kinetics, modification-induced unfolding.

INTRODUCTION

Protein covalent modification may result in conformational state changes, i.e., disruption of protein tertiary and quaternary structure, as evidenced by protein unfolding and subunit dissociation.[1–19] In the present communication a mathematical treatment of a two-sited, modification-induced protein unfolding model is presented. It seemed of particular interest to examine: (a) the possible effect of modification-induced protein unfolding on the graphically determinable end-point of the protein modification reaction, and (b) the use of the first derivative of the modification process, at initial reaction conditions, as a measure of the effect of modifying agent concentration on the protein under study.[20]

MODEL AND RATE EQUATIONS

A protein, E_{AB}, presenting with two modifiable sites per protein molecule labelled A and B, is considered. It is assumed that at the start of the reaction, only site A is accessible to the modifying agent, M. The partially modified protein species, E_{aB1}, undergoes a conformational change involving protein unfolding or subunit dissociation, and is transformed into the species E_{aB2} with a rate constant of k_1, while

species E_{aB2} is reconverted to species E_{aB1} with a rate constant of k_2. Species E_{aB2} is susceptible to modification with the same rate constant as species E_{AB}, i.e., k_a. These changes may be written as follows:

$$E_{AB} \xrightarrow{k_a} E_{aB1} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{aB2} \xrightarrow{k_a} E_{ab} \tag{1}$$

where E_{ab} is fully modified protein. It is assumed that k_a is a function of modifying agent concentration. With time as the independent variable, the rate equations describing eqn. (1) are:

$$-[E]' = k_a([E]_{AB} + [E]_{aB2}) \tag{2}$$

$$[E]'_{aB1} = k_a[E]_{AB} + k_2[E]_{aB2} - k_1[E]_{aB1} \tag{3}$$

$$[E]'_{aB2} = k_1[E]_{aB1} - (k_a + k_2)[E]_{aB2} \tag{4}$$

where [E] is the concentration of unmodified protein groups. Differentiating eqn. (2), and eliminating $[E]_{AB}$, $[E]_{aB1}$ and $[E]_{aB2}$, by means of eqns (3) and (4), and also by the use of the conservation relationship $[E] = 2[E]_{AB} + [E]_{aB1} + [E]_{aB2}$:

$$[E]'' + (k_1 + k_2 + k_a)[E]' + k_1k_a[E] = 0.5k_a(k_1 - k_2)[E]_0 \exp(-k_at) \tag{5}$$

where $[E]_0$ is [E] at the start of the reaction. The solution of eqn. (5) is:

$$([E]/[E]_0) = C_1 \exp(m_1t) + C_2 \exp(m_2t) + C_3 \exp(-k_at) \tag{6}$$

where t is reaction time, m_1 and m_2 are the roots of the characteristic equation of eqn. (5), and also where:

$$C_1 = \frac{m_1 + k_1 + k_2 + 0.5k_a}{m_1 - m_2} + \frac{0.5k_a(k_1 - k_2)}{(k_a + m_1)(m_1 - m_2)} \tag{7}$$

$$C_2 = \frac{m_2 + k_1 + k_2 + 0.5k_a}{m_2 - m_1} + \frac{0.5k_a(k_1 - k_2)}{(k_a + m_2)(m_2 - m_1)} \tag{8}$$

$$C_3 = \frac{0.5k_a(k_1 - k_2)}{(k_a + m_1)(k_a + m_2)} = \frac{0.5(k_2 - k_1)}{k_2} \tag{9}$$

It is assumed that at $t = 0$, $[E]_{aB1}$, $[E]_{aB2}$ and $[E]_{ab}$ are equal to zero.

SIMULATED CASES AND DISCUSSION

In this communication a mathematical analysis of a two-sited modification-induced protein unfolding model is presented. It is shown that a description of this situation is accomplished by means of a second-order, linear differential equation with constant coefficients and nonzero right hand side, with the concentration of protein unreacted groups as the dependent variable, and reaction time as the independent variable (eqn. (5)). Solution of this equation is effected by a summation of three exponential functions of the reaction time equation (eqn. (6)). The constants and coefficients of eqn. (6), for different hypothetical cases of modification-induced protein unfolding, are given in Table I. The question arises of how actual situations of protein modification, in conformity with the data of Table I, will appear to the experimentalist. It is assumed that the experimentally measurable quantity is $[E]_{mod}$, the concentration of modified protein groups. The fractional concentration of unmodified protein groups,

TABLE I
Dependence of the constants and coefficients of eqn. (6) on the isomerization and modification rate constants of the modification-induced protein unfolding model. Isomerization and modification rate constants, as well as the constants of eqn. (6) are in min^{-1}

Case No.	k_1	k_2	k_a	$-m_1$	$-m_2$	C_1	C_2	C_3
1	1	1	1	2.6180	0.38197	0.0492	0.9508	0
2	1	1	2	3.4142	0.58579	0.1464	0.8534	0
3	1	1	5	6.1926	0.80472	0.3143	0.6860	0
4	1	1	10	11.0990	0.90098	0.4019	0.5981	0
5	1	1	15	16.0664	0.93363	0.4339	0.5661	0
6	1	1	25	26.0399	0.96006	0.4601	0.5399	0
7	5	1	1	6.1926	0.80742	0.0145	2.9856	−2
8	10	1	1	11.0990	0.90098	0.0044	5.4957	−4.5
9	1	5	1	6.8541	0.14590	0.0017	0.5981	0.4
10	1	10	1	11.9161	0.08392	0.0004	0.5497	0.45
11	1	10	5	15.6812	0.3189	0.0049	0.5451	0.45
12	1	10	10	20.5125	0.4875	0.0115	0.5385	0.45

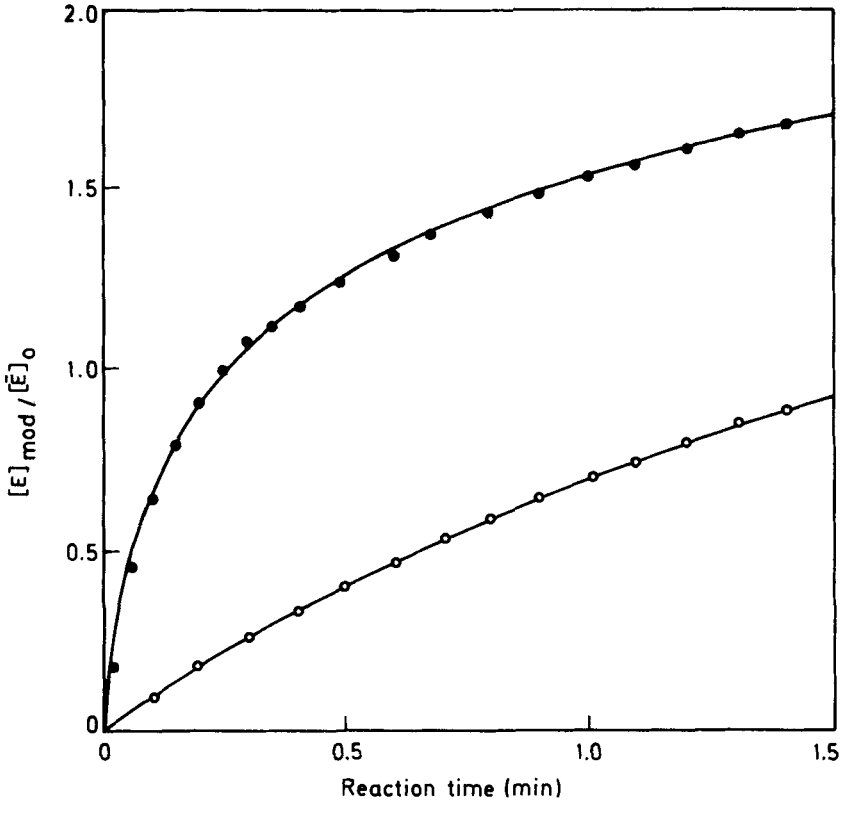

FIGURE 1 Dependence of the fractional concentration of modified protein groups on reaction time. Isomerization rate constants are, for both cases shown, $k_1 = k_2 = 1\,min^{-1}$. The modification rate constant, k_a, is $1\,min^{-1}$ (0), and $10\,min^{-1}$ (●).

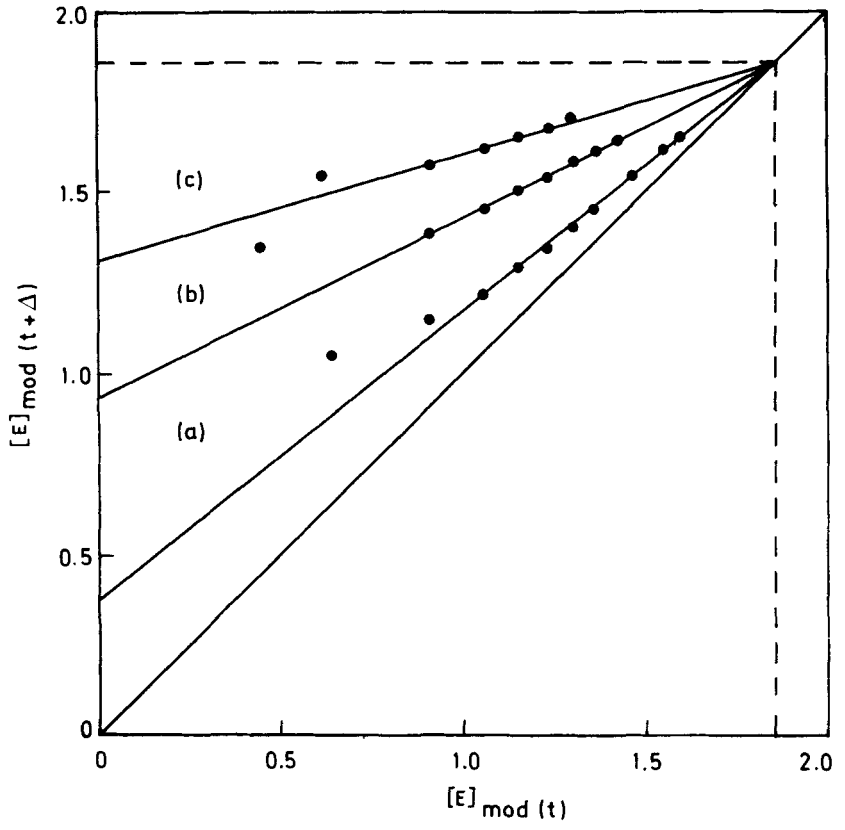

FIGURE 2 Determination, by the Kézdy-Swinbourne procedure, of the end-point of the protein isomerization reaction of case 4 of Tables I, II and III. The constant increment, Δ, between corresponding $[E]_{mod(t)}$ values is, in min: 0.2 (a), 0.6 (b), and 1.0 (c). For details of the graphical analysis procedure used, see text.

i.e., the $[E]/[E]_0$ value of eqn. (6) is $(n[\bar{E}]_0 - [E]_{mod})/n[\bar{E}]_0$, where $[\bar{E}]_0$ is total protein concentration, and n is the number of modifiable groups per protein molecule. Since n is assumed to be unknown to the experimentalist, fitting of the protein modification data to eqn. (6) must perforce be accomplished by the use of a value for n, determinable by some interpolation procedure.

Graphical analysis procedures for the determination of the end-point of a reaction, of which product formation is measurable directly, have been developed by several authors.[21-24] While all of these procedures are designed for the determination of the end-point, as well as of the rate constant, of a reaction described by a single exponential function of reaction time, in principle they are also applicable to cases described by a summation of exponential functions of reaction time, i.e., they are applicable to the portion of the reaction dominated by the slowest exponential. In the present investigation the Kézdy–Swinbourne procedure was used since, for the case where the reaction under study is described by a summation of exponential functions of reaction time, this procedure yields the true value for the reaction end-point, while the more widely used Guggenheim method yields the reaction end-point multiplied by

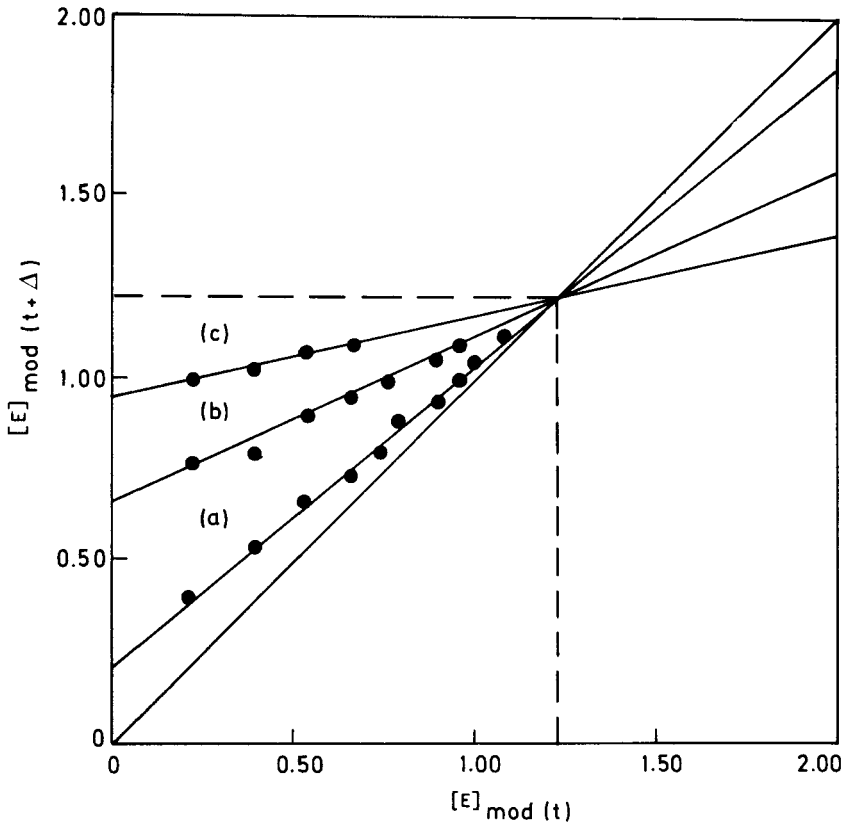

FIGURE 3 Determination, by the Kézdy-Swinbourne procedure, of the end-point of the protein modification reaction of case 10 of Tables I, II and III. The constant increment, Δ, between corresponding $[E]_{mod(t)}$ values is, in min: 0.25 (a), 1.0 (b), and 2.0 (c). For details of the graphical analysis procedure used, see text.

the coefficient of the slowest exponential.[25] The Kézdy-Swinbourne method is based on obtaining one set of reaction product values at times t_1, t_2, t_3, etc., and another set of product values at times $t_1 + \Delta$, $t_2 + \Delta$, $t_3 + \Delta$, etc., where Δ is a constant increment. Repeating the same procedure for different values of Δ, and plotting the pairs of values obtained against each other, a number of plots are obtained, all of which intersect at the value of product equal to the reaction end-point. The dependence of product formation on reaction time, as well as Kézdy-Swinbourne plots, for cases given in Table I, is shown in Figures 1, 2 and 3.

The reaction end-point, i.e., the graphically obtained fractional concentration of protein modified groups, at infinite reaction time $[E]_{mod(\infty)}/[\bar{E}]_0$, for the cases presented in Table I, is given in Table II. It will be noted that the true value for $[E]_{mod(\infty)}/[\bar{E}]_0$, i.e., a value of two groups modified per molecule of protein, is obtained in only one of the cases presented in Tables I and II. It will also be noted that $[E]_{mod(\infty)}/[\bar{E}]_0$ is a function of both the modification rate constant, k_a, and the isomerization rate constants, k_1 and k_2, of eqn. (1). For the group of cases where $k_1 = k_2$ (cases 1–6 of Tables I and II), an increase in the value of k_a may bring about an increase (cases 2–4), or a decrease (cases 1, 5 and 6) of the $[E]_{mod(\infty)}/[\bar{E}]_0$ value. For the group of cases where

TABLE II

Graphical analysis of protein reactive groups modification data generated through eqn. (6), for the cases given in Table I. $[E]_{mod(t)}$, and $[E]_{mod(\infty)}$, are in moles of groups modified per mole of protein, and t is in min. For details of the graphical analysis procedure used, see text

Case No.	$[E]_{mod(\infty)}/[\bar{E}]_0$	$([E]_{mod(\infty)} - [E]_{mod(t)})/[E]_{mod(\infty)}$
1	1.50	$\exp(-0.611t)$
2	1.33	$\exp(-1.49t)$
3	1.38	$\exp(-2.90t)$
4	1.85	$0.27\exp(-13.8t) + 0.63\exp(-1.28t)$
5	1.08	$\exp(-13.3t)$
6	1.08	$\exp(-22.2t)$
7	2.00	$1.20\exp(-0.68t) - 0.20\exp(-2.18t)$
8	2.26	$\exp(-0.484t)$
9	1.40	$\exp(-0.621t)$
10	1.22	$\exp(-0.768t)$
11	1.53	$0.25\exp(-12.9t) + 0.75\exp(-1.19t)$
12	1.80	$0.42\exp(-12.5t) + 0.58\exp(-0.809t)$

$k_2 > k_1$, an increase in the value of k_a brings about an increase in the $[E]_{mod(\infty)}/[\bar{E}]_0$ value (cases 10–12 of Tables I and II). It may accordingly be concluded that the model described by eqn. (1) may, in principle, be used towards an explanation of the mechanism of modification-induced protein unfolding observed in actual experimental situations. In this connection, it should be noted that the model described by eqn. (1) is the simplest possible: the number of modifiable groups per protein molecule is generally larger than two, while protein unfolding may also involve protein modification cooperativity, i.e., modification of the protein in such a manner that the partially modified protein species possess different reactivity towards the modifying agent used.[26–30] It should be apparent, however, that the analytic solution of equations describing models more complicated than that of eqn. (1) would present with formidable difficulties, and would only be practicable if enough simplifying assumptions were made. Modification-induced protein unfolding, as evidenced by an increase in the number per protein molecule of groups modified in consequence to an increase in the concentration of modifying agent used, has been observed in the case of the carbamylation of bovine lens α-crystallin,[31] the nitration of tyrosine residues of cytochrome $P\text{-}450_{scc}$,[32] the citraconylation of bovine lens α-crystallin,[33] and the trinitrophenylation of primary amino groups of bovine liver rhodanese.[15,20]

By establishing a value for the end-point of the reaction, the fractional concentration of protein unreacted groups may be calculated, and the description of the reaction as an exponential function or a summation of exponential functions of reaction time, i.e., a description in accordance with eqn. (10), may be accomplished:

$$(([E]_{mod(\infty)} - [E]_{mod(t)})/[E]_{mod(\infty)}) = \Sigma_i c_i \exp(-k_i t) \qquad (10)$$

where c_i are the coefficients, and k_i the constants of the protein modification equation. Concentration of protein modified groups *vs* reaction time data may be fitted to eqn. (10) by graphical analysis.[34] Analyses of two of the cases presented in Table I are shown in Figures 4 and 5, while results of the analyses of all of the cases presented in Table I are given in summary form in Table II. It will be noted from Table II that the phenomenological description of each particular case is quite unrelated to the mathematical description of the case, as deduced from the structure of the model of eqn. (1). Clearly, trying to deduce the kinetic structure of the protein modification

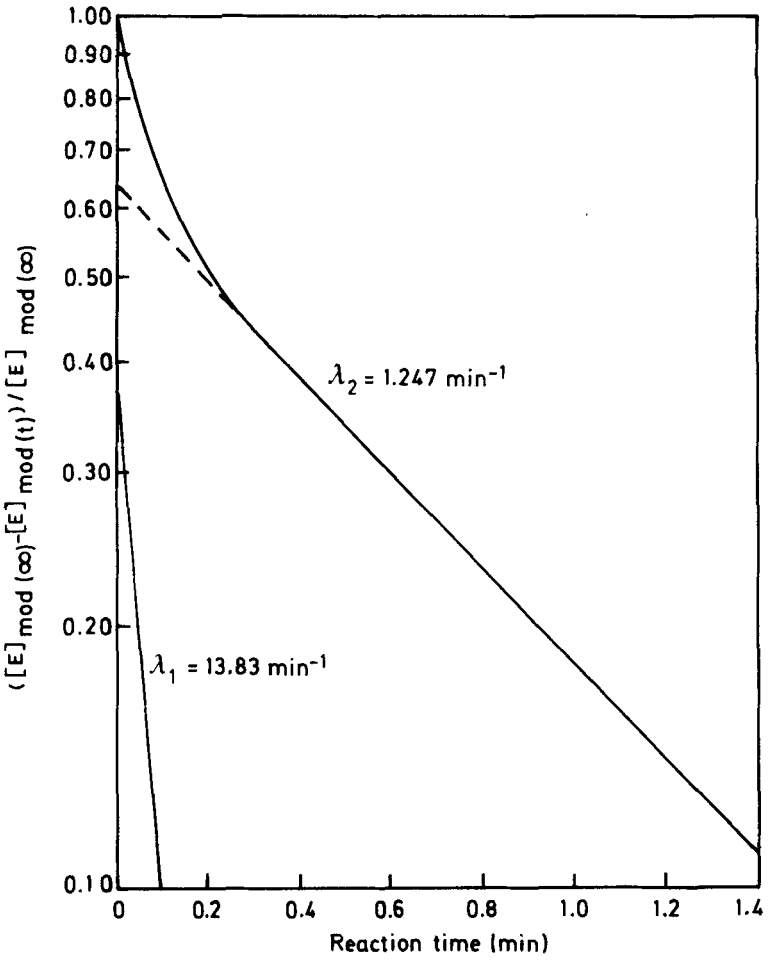

FIGURE 4 Graphical analysis of the protein modification data of case 4 of Tables I and II as a summation of exponential functions of reaction time. The negative values of the constants of eqn. (10) are given as λ_1 and λ_2; the coefficients of the same equation may be read directly from the graph. For details of the analytical procedure used, see text.

system from the phenomenological description of the reaction would, in cases of protein modification which are in accordance with eqn. (1), or indeed with more complicated reaction models than that of eqn. (1), be grossly misleading, unless the effect of modifying agent concentration on the apparent end-point of the reaction was taken into account.

It has been shown that in cases of protein modification where the kinetic structure of the system has not been fully identified, and when studying the effect of changes in modifying agent, ligand or hydrogen ion concentration on reaction velocity, the preferred value to use is the first derivative, at the start of the reaction, of the concentration of modified protein groups, $[E]'_{mod}(0)$. This is so because the $[E]'_{mod}(0)$ value of a protein modification reaction is free of isomerization rate constants, and is only dependent on the rate constants pertaining to the modification of the protein

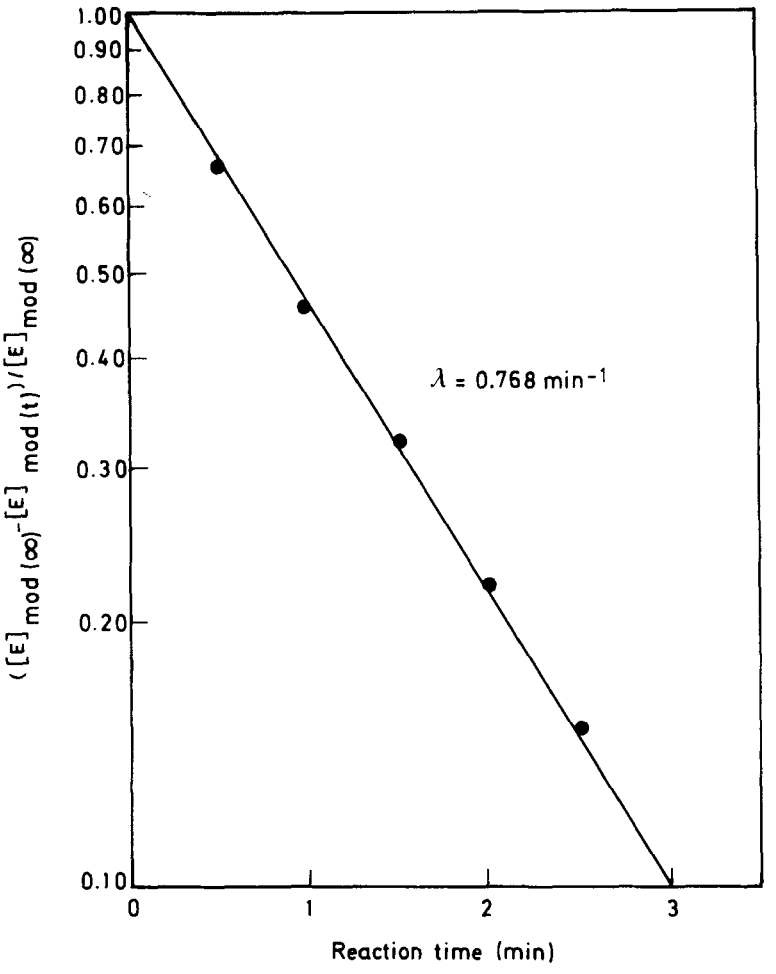

FIGURE 5 Graphical analysis of the protein modification data of case 10 of Tables I and II as a summation of exponential functions of reaction time. The negative value of the constant of the monoexponential modification process is given as λ. For details of the analytical procedure used, see text.

species present at the start of the reaction.[20] In the present investigation, two different procedures for the determination of $[E]'_{mod}(0)$ were used:

a) The operational definition of $[E]'_{mod}(0)$, for a process described by a summation of exponential functions of reaction time:[20]

$$[E]'_{mod}(0) = \Sigma_i c_i k_i \quad (11)$$

where $n = [E]_{mod(\infty)}/[\bar{E}]_0$, and also where c_i and k_i are as defined in eqn. (10).

b) Fitting of the protein modification data to the equation:

$$[E]_{mod(t)} = a_0 + a_1 t + a_2 t^2 \ldots + a_j t^j + \ldots \quad (12)$$

which can be shown to define any single-valued continuous function.[35] Since a_0 for a reaction in accordance with eqn. (1) is equal to zero, the coefficients $a_1, a_2, \ldots a_j$ can

TABLE III

Determination of the first derivative value, at the start of the reaction, of the concentration of modified protein groups, $[E]'_{mod}(0)$, for the cases of modification-induced protein unfolding presented in Tables I and II. $[E]'_{mod}(0)$ is determined: (a) as given in eqn. (11), and by the use of the data of Table II, and (b) by a numerical analysis procedure, from the values of $[E]_{mod(t)}$ generated by the use of eqn. (6). For details of analytical procedures used, see text

	$[E]'_{mod}(0)$ (min^{-1})		
Case No.	Expected	Found by (a)	Found by (b)
1	1	0.916	1.02
2	2	1.99	1.95
3	5	4.00	4.85
4	10	10.9	9.11
5	15	14.4	14.7
6	25	24.0	23.7
7	1	0.760	0.970
8	1	1.094	0.964
9	1	0.869	0.994
10	1	0.937	0.987
11	5	4.12	4.90
12	10	10.3	9.81

be estimated by setting up j simultaneous equations in j unknowns. The $[E]'_{mod}(0)$ value, i.e. a_1, was estimated for the cases of protein modification presented in Table I by using the first three values for $[E]_{mod(t)}$ generated by the use of eqn. (6), when deriving the data for Figures 1–3 and Table II.

The $[E]'_{mod}(0)$ values for all of the cases presented in Table I are given in Table III. It will be noted from Table III that the $[E]'_{mod}(0)$ values, calculated by either one of the procedures used (with the exception of the values calculated by procedure (a) for cases 3, 7 and 11), agree with the value expected from first principles, within a margin of error of 15%. It will also be noted that, of the two analytical procedures used for the determination of $[E]'_{mod}(0)$, the numerical analysis procedure is by far the more accurate.

It is of interest to examine the possible appearance to the experimentalist of the modification-induced protein unfolding model presented in this communication, from the point of view of enzyme activity loss, in the case where the protein undergoing modification also happens to be catalytically active. Three cases may be distinguished: i) Site A, of the model of eqn. (1), is the only enzyme active site. In this case, enzyme residual activity, a, will be a single exponential function of reaction time, with a rate constant of $-k_a$. The number, q, of enzyme protein groups essential for catalytic function may be construed by a $a^{1/q}$ *vs* $[E]_{mod(t)}$ plot (Tsou plot), and is equal to the ratio of the enzyme inactivation first-order rate constant to the enzyme protein modification first-order rate constant.[36] The apparent number of enzyme protein groups essential for catalytic function may, for the cases presented in Table I, be found by comparing the $-k_a$ value of each case with the graphically determined first-order protein modification reaction rate constant, for the cases where the graphically determined modification event consists of a single exponential function of reaction time (Table II). It will be noted from the values given in Tables I and II that in several cases the apparent number of groups essential for enzyme catalytic function will be larger than unity, and, in some cases, will be equal to two groups per enzyme protein

molecule. Clearly such graphically derived values for the number of groups essential for catalytic function will, in many cases, be incorrect.

ii) Both sites A and B are enzyme active-sites. In this case, since site B will be catalytically active only when in the unfolded state, the mathematical description of enzyme activity loss and that of protein groups modification will be identical. Graphical determination of the number of groups per enzyme protein molecule essential for catalytic function will give a value of unity, i.e., will give the correct value.

iii) The enzyme active-site is not structurally associated with either site A or site B. In this case the extent of enzyme inactivation will depend on the nature of interaction between the enzyme protein modifiable site(s) and the enzyme active-site. In the extreme case where modification of any one of the protein reactive groups results in enzyme activity loss, the first-order rate constant of the enzyme inactivation reaction will be j times the first-order rate constant for protein groups modification, i.e., the graphically determined number of groups per enzyme protein molecule essential for catalytic function will be equal to j.[15,20,36,37] This will result, in cases where $j > 1$, in obtaining a grossly incorrect value for the number of groups per enzyme protein molecule essential for catalytic function.

CONCLUSION

The treatment of the modification-induced protein-unfolding model, presented in this communication, indicates that the determination of the end-point of a protein modification reaction is a *sine qua non* requirement for the correct understanding of the kinetics of protein modification, and/or the concomitant enzyme activity loss. The examples of graphical and numerical analysis of specific cases of the model, elaborated in the course of the treatment, establish that for most of the cases presented and, presumably, for most of the cases to be met in practice the first derivative, at initial reaction conditions, of the concentration of modified protein groups is the preferred value to use when evaluating the effect of hydrogen ion or of modifying agent concentration on the extent of protein modification and/or enzyme inactivation.[20]

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