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KINETIC ANALYSIS OF BIPHASIC PROTEIN MODIFICATION REACTIONS

COOPERATIVE EFFECTS

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A mathematical treatment of protein modification reactions is presented, and it is shown that in these cases protein modification is described by a summation of exponential functions of reaction time, the number of exponentials being equal to the number of modified protein species. It is shown that, in cases of protein modification cooperativity, there is a strict dependence of the coefficients of the multiexponential modification equation on the constants of the same equation. The conditions necessary for a reduction of a multiexponential protein modification equation to one of a summation of two exponentials only are examined. The possible formulae for the coefficients of a two-exponential-summation equation, used to describe the modification of protein models with two, three or four modifiable residues (as well as some aspects of models with five and six modifiable residues) per protein molecule are derived. It is seen that the number of such coefficients is severely limited. The most frequently obtained formula for the lower stoichiometric coefficient of a two-exponential-summation equation is $Ak_a/(k_a-k_b)$, where k_a and k_b are the constants of the two exponentials of the equation, and A is a constant. The value most frequently arrived at for A is $(n-1)/n$, where n is the number of modifiable residues per protein molecule, while values such as $1/n$, or a/n (where a is an integer, and also where $a < n$) are also possible. In most of the cooperative protein modification models worked out, k_a is identical with k_n, viz., k_a is identical with the rate constant for the first stoichiometric protein modification.

1. Introduction

Protein modification cooperativity (irreversible inhibitor binding cooperativity) is the modification of one or more protein reactive residues in such a manner that the partially modified protein species possess different reactivity towards the modifying agent [1–5]. It has been shown that, for a protein with two reactive residues per protein molecule, the presence of site-oriented protein modification cooperativity manifests itself as a process which is described by a three-exponential-summation equation, with the concentration of unmodified protein reactive residues as the dependent variables, and reaction time as the independent variable [2]. It has also been shown that, for a two-sited protein, when the modification equation reduces to one which is the summation of two exponentials, a strict dependence of the coefficients on the constants of this equation applies [5]. In this manner the value of the coefficients can be calculated, once the rate constants of the modification process are known. This property of protein modification cooperativity is in contrast to other protein modification processes which manifest themselves as biphasic protein modification reactions, viz., which are described by two-exponential-summation equations. Such processes occur in protein conformational isomerism, and also in the formation of protein-ligand complexes [5]. It seemed of interest

to explore the question of the dependence of the coefficients on the constants of a two-exponential-summation equation, when the modification reaction described involves a protein with more than one modifiable residue per protein molecule. A mathematical treatment of this case is presented here, and the dependence of coefficients on rate constant formulae are derived for various possible stoichiometric models of protein modification cooperativity.

2. Models and rate equations

A protein with n intrinsically identical modifiable residues per protein molecule is considered. The stepwise stoichiometric modification of this protein, to produce the fully modified protein, may be written as follows:

$$E_n \xrightarrow{k_n} E_{n-1} \xrightarrow{k_{n-1}} E_i \xrightarrow{k_i} \ldots E_1 \xrightarrow{k_1} E_0 \qquad (1)$$

whee E_i is the protein species possessing i mol unmodified residues per mol protein, and k_i the rate constant for the modification of this species, to produce the species with $i-1$ moles unmodified residues per mol protein. The assumption is made that the modifying agent concentration is well in excess of protein concentration, so that the modification rate constants of eq. 1 are pseudo-first-order reaction constants.

The rate of change of the concentration of protein modified residues, $[E]_{mod}$, with reaction time as the independent variable, is:

$$[E]'_{mod} = \sum_{i=1}^{n} k_i[E]_i \qquad (2)$$

where $[E]_n = [E]_{n(0)} \exp(-k_n t)$, and also where $[E]_{n(0)}$ is the concentration of E_n at $t = 0$. Clearly, $([E]_{mod}/[E]_n) \leqq n$.

The rate of change of $[E]_i$ is:

$$[E]'_i = k_{i+1}[E]_{i+1} - k_i[E]_i \qquad (3)$$

Taking the Laplace transforms of eqs. 2 and 3, and solving for the Laplace transform of $[E]_{mod}$:

$$[\bar{E}]_{mod} = ([E]_{n(0)}/p) \sum_{m=1}^{n} \prod_{i=m}^{n} k_i/(p + k_i) \qquad (4)$$

where $[\bar{E}]_{mod}$ is the Laplace transform of $[E]_{mod}$, and also where $1/(p + k_i)$ is the Laplace transform of $\exp(-k_i t)$.

The solution of eq. 4 is:

$$[(n[E]_{n(0)} - [E]_{mod})/n[E]_{n(0)}] = \sum_{i=1}^{n} C_i \exp(-k_i t) \qquad (5)$$

where

$$C_n = (1/n)\left(1 + \sum_{m=1}^{n-1} \prod_{i=m}^{n-1} \frac{k_i}{k_i - k_n}\right) \qquad (6)$$

$$C_{n-1} = (1/n)\frac{k_n}{k_n - k_{n-1}} \sum_{m=1}^{n-2} \prod_{i=m}^{n-2} \frac{k_i}{k_i - k_{n-1}} \qquad (7)$$

$$C_1 = (1/n) \prod_{i=2}^{n} \frac{k_i}{k_i - k_1} \qquad (8)$$

It will be seen that, if no protein modification cooperativity is present, i.e., if the values of the stoichiometric modification rate constants are such that $k_i = ik_1$, eq. 5 reduces to:

$$[(n[E]_{n(0)} - [E]_{mod})/n[E]_{n(0)}] = \exp(-k_1 t) \qquad (9)$$

This is because when ik_1 is substituted for k_i, eq. 8 reduces to:

$$C_1 = (1/n)\frac{n!}{(n-1)!} = 1 \qquad (10)$$

Since $C_1 + C_2 + \ldots + C_n$ in eq. 5 must be equal to unity, it follows that the coefficients $C_2, C_3, \ldots C_n$ must necessarily be equal to zero. This situation is the one in which the successive protein modifications are noninterdependent. In any other case the number of exponentials used to describe sequential protein modification reactions is larger than one, and the modification reactions involved can be said to be cooperative, although the terms 'cooperative interactions' and 'cooperativity' are generally used to describe properties of systems at equilibrium [2].

For eq. 5 to reduce to a two-exponential-summation equation, one of two conditions has, on a prima facie basis, to be met: (1) The constants $k_n \ldots k_1$ must assume one of two numerical values, both of which values must be different from zero, or (2) all but two of the coefficients $C_n \ldots C_1$ must reduce to zero.

2.1. Case 1

The constants $k_n \ldots k_1$ assume one of two values. Substituting k_g and k_h for the two alternative

values assumed by the constants $k_n \ldots k_1$, and proceeding as before:

$$[E]_{mod} = C_u \sum_{r=1}^{u} t^{r-1} \exp(-k_g t) + C_w \sum_{q=1}^{w} t^{q-1} \exp(-k_h t) \quad (11)$$

where C_u, C_w, u, w, w, r and q are constants, and also where $u + w = n$. Since eq. 11 is not, strictly speaking, an exponential-summation equation, case 1 is ruled out as a diagnostic eventuality.

2.2. Case 2

All but two of the coefficients of eq. 5 reduce to zero. The conditions necessary for a reduction of this sort, for cases with n values from 2 to 4 (as well as some aspects of cases with n values of 5 and 6), are given in summary form in table 1. It will be seen that a value of $Ak_a/(k_a - k_b)$ for the lower stoichiometric coefficient, where k_a and k_b are the constants of the two exponentials of the modification equation, and also where A is a constant, is the value most frequently found among the possible situations listed in table 1. It will be noted that this value is the outcome of diverse experimental eventualities such as (a) a group of the stoichiometric rate constants of the modification process conforming with the requirement of $k_i = ik_1$, (b) some of the modification rate con-

Table 1
Conditions for all but two of the coefficients of eq. 5 to be equal to zero

Value of n	Coefficients with zero value	Conditions	Value of lower stoichiometric coefficient [a]
2	None	$k_1, k_2 \neq 0$	$(1/2)k_2/(k_2 - k_1)$
3	C_1	$k_1 \gg k_2, k_3$	$(2/3)k_3/(k_3 - k_2)$
3	C_1	$k_2 = 0$	2/3
3	C_2	$k_2 = 2k_1$	$(2/3)k_3/(k_3 - k_1)$
3	C_2	$k_2 \gg k_1, k_3$	$(1/3)k_3/(k_3 - k_1)$
3	C_3	$k_3^2 - (k_1 + 2k_2)k_3 + 3k_1k_2 = 0$	to be computed
4	C_1, C_2	$k_1 \gg k_2, k_3, k_4$ and $k_2 \gg k_3, k_4$	$(3/4)k_4/(k_4 - k_3)$
4	C_1, C_2	$k_3 = 0$	3/4
4	C_1, C_2	$k_1 \gg k_3, k_4$ and $k_2 = 2k_1$	$(3/4)k_4/(k_4 - k_3)$
4	C_1, C_2	$k_1 \gg k_3$ and $k_4 = 2k_3$ and $k_2 = 2k_1$	$(3/4)k_4/(k_4 - k_3)$
4	C_1, C_3	$k_3 \gg k_4$ and $k_2 = 0$	2/4
4	C_1, C_3	$k_1 \gg k_2, k_3, k_4$ and $k_3 = 3k_2$	$(3/4)k_4/(k_4 - k_2)$
4	C_1, C_3	$k_1 \gg k_2, k_3, k_4$ and $k_3 \gg k_2, k_4$	$(2/4)k_4/(k_4 - k_2)$
4	C_1, C_4	$k_4 = 2k_3$ and $k_2 = 0$	2/4
4	C_1, C_4	$k_1 \gg k_2, k_3, k_4$ and $k_4 = 2k_3$ $k_4 \gg k_2$	2/4
4	C_1, C_4	$k_1 \gg k_2, k_2, k_4$ and $k_4^2 - (k_2 + 2k_3)k_4 + 4k_2k_3 = 0$	to be computed
4	C_2, C_3	$k_2 = 2k_1$ and $k_3 = 3k_1$	$(3/4)k_4/(k_4 - k_1)$
4	C_2, C_3	$k_2 \gg k_1, k_3, k_4$ and $k_3 = (3/2)k_1$	$(3/4)k_4/(k_4 - k_1)$
4	C_2, C_4	$k_2 \gg k_1, k_3, k_4$ and $k_4^2 - (k_1 + 3k_3)k_4 + 4k_1k_3 = 0$	to be computed
4	C_2, C_4	$k_2 \gg k_1, k_3, k_4$ and $k_1 \gg k_4$ and $k_4 = 4k_3$	$k_3^2/k_1(k_3 - k_1)$
4	C_3, C_4	$k_3 \gg k_4$ and $k_4^3 + (k_1 - k_2 - k_3)k_4^2 - (k_1k_3 + k_1k_2 + 2k_2k_3)k_4 + 3k_1k_2k_3 = 0$	to be computed
5	C_1, C_2, C_4	$k_1 \gg k_2, k_3, k_4, k_5$ and $k_2 \gg k_3, k_4, k_5$ $k_4 \gg k_3, k_5$	$(3/5)k_5/(k_5 - k_3)$
6	C_1, C_2, C_4 and C_5	$k_1 \gg k_2, k_3, k_4, k_5, k_6$ and $k_2 \gg k_3, k_4, k_5, k_6$ $k_5 \gg k_3, k_4$ and $k_4 \gg k_3, k_6$	$(3/6)k_6/(k_6 - k_3)$
n	$C_1 \ldots C_{n-2}$	$k_1 \gg k_2 \gg k_3 \ldots \gg k_{n-1}$	$((n-1)/n)k_n/(k_n - k_{n-1})$
n	$C_2 \ldots C_{n-1}$	$k_i = ik_1$ and $k_n = nk_1$	$((n-1)/n)k_n/(k_n - k_{n-1})$

[a] The coefficient sequentially closest to C_1, unless it is C_1 itself.

stants being far larger than some other modification rate constants, and (c) a combination of cases a and b. It will also be seen from table 1 that the value most frequently arrived at for the constant A is $(n-1)/n$, while other values such as $1/n$, $(1/2)n$ or a/n (where n is an integer, and also where $a < n$) are possible. The value of $(1/2)n$ for the constant A is of particular interest, since it might reflect the fact that the modification of the protein molecule takes place in two equally spaced steps: one step for half the reactive residues, and the other step for the other half of the reactive residues of the protein. It will be seen from table 1 that this is indeed the case, since the requirement for the lower stoichiometric coefficient being $(2/4)k_a/(k_a - k_b)$ is that $k_1 \gg k_2$, k_3, k_5 and $k_3 \gg k_2$, k_4. It is reasonable to assume that a value of $(1/2)k_a/(k_a - k_b)$ will be obtained in cases with an n value larger than 4, in which the stoichiometric rate constants of the process of modification can be arranged into two sets, as in the example with an n value of 4. It will be seen that this is actually the case for an example with an n value of 6 (table 1). It is remarkable, however, that in other cases, where protein modification is effected in two equally spaced steps (e.g., when $k_1 \gg k_3$, k_4 and $k_2 = 2k_1$, and also in cases where $k_1 \gg k_2$, k_3, k_4 and $k_3 = 3k_2$) the value for the lower stoichiometric coefficient is not $(1/2)nk_a/(k_a - k_b)$ but is $((n-1)/n)k_a/(k_a - k_b)$. The value for the lower stoichiometric coefficient given by second- or higher-order algebraic equations represents improbable situations of relations among modification rate constants. It is apparent that the lower stoichiometric coefficient can assume any real positive value that is larger than unity (when $k_a > k_b$), or any real negative value that is smaller than negative unity (when $k_a < k_b$), depending on the mechanism of cooperativity present.

A special situation is when one of the stoichiometric modification rate constants is equal to zero. In this case the lower stoichiometric coefficient is free of modification rate constants (see examples in table 1 where the lower stoichiometric coefficient assumes values such as 2/3, 2/4 or 3/4). When the stoichiometric rate constant with a value of zero is $k_{0.5n}$, modification of half of the reactive residues of the protein prevents the modification of the other half of the residues. This situation has been observed experimentally, and has been termed 'half of the sites reactivity' [6–10]. It has been common practice to attribute cases where the protein modification equation is of the form: $[E]_{mod} = A + \exp(-kt)$, where A is a constant, to the presence of an intrinsically unreactive group of residues in the protein preparation. In cases where the parameter followed is loss of enzyme activity, rather than concentration of modified protein residues, an alternative explanation for an inactivation resistant portion of enzyme activity is that protein modification may bring about a decrease in the V_{max} value, or an increase in the K_M value of the enzyme under study [1].

3. Discussion

In this communication a mathematical treatment of sequential, stoichiometric protein modification reactions is presented. It is seen that protein modification is described by a summation of exponential functions of reaction time, the constant of each exponential being identical with one of the modification rate constants, while the coefficient of each exponential is a function of modification rate constants only. This property of protein modification cooperativity may be used to distinguish this from other situations of protein modification presenting multiexponential descriptions of reaction sequence, when the concentration of unmodified residues is used as the dependent variable, and reaction time as the independent variable. Such situations are: (a) the presence of ligand-protein complexes, and (b) conformational isomerism of the protein. In these cases protein modification is described by a summation of two or more exponentials if the inactivation rate constants of the ligand-free and of the ligand-complexed protein, or of the different conformational isomers of the protein, are different. [5]. An interesting feature of this dependence of the coefficients on the constants of the equation describing the sequential modification of a protein is the possibility of repeatedly testing for such a dependence by performing a number of different modification experi-

ments at different modifying agent concentrations. If a situation of protein modification cooperativity is involved, the dependence of the coefficients on the rate constant formula should be the same, regardless of the values assumed by the rate constants of the modification reaction in each instance. In this connection it is of interest that, when the cooperative modification equation reduces to a summation of two exponentials, one of the two coefficients of this reduced equation most frequently assumes the formula $Ak_a/(k_a - k_b)$, where k_a and k_b are the two experimentally measurable rate constants of the modification reaction, and A is a constant (table 1).

An application of the theoretical treatment described in this communication may be seen in the modification by iodoacetamide of the four sulfhydryl groups of glyceraldehyde-3-phosphate dehydrogenase [9]. A biphasic protein modification curve was obtained, with the following values: $k_a = 0.693\ \text{min}^{-1}$, $k_b = 0.042\ \text{min}^{-1}$, $C_a = 0.50$ and $C_b = 0.50$ [9]. Substitution of these values into the equation $C_b = Ak_a/(k_a - k_b)$ (where C_b is the lower stoichiometric coefficient) gives an A value of 0.47, in conformity with the model described by the conditions $k_1 \gg k_2$, k_3, k_4 and $k_3 \gg k_2$, k_4 (table 1). This model is one of positive protein modification cooperativity. After separation of the doubly modified enzyme protein, the reactivity of the two remaining residues was determined [9]. After appropriate corrections were made, the second-order stoichiometric modification rate constants, as calculated 'by standard kinetic methods' [9] are: $k_1 = 0.3\ \text{M}^{-1}\ \text{min}^{-1}$, $k_2 = 2500\ \text{M}^{-1}\ \text{min}^{-1}$, $k_3 = 21\,000\ \text{M}^{-1}\ \text{min}^{-1}$, $k_4 = 42\,000\ \text{M}^{-1}\ \text{min}^{-1}$ (where k_1–k_4 are as defined for eq. 1). The conclusion has been drawn from these findings that the modification of glyceraldehyde-3-phosphate dehydrogenase by iodoacetamide is a process of stoichiometric negative protein modification cooperativity [9]. However, when the values for the constants k_1–k_4, referred to above, are substituted into eq. 5, the values for the coefficients C_1–C_4 become: $C_1 = 0.250$, $C_2 = 0.302$, $C_3 = 0.432$ and $C_4 = 0.016$. Since the modification of glyceraldehyde-3-phosphate dehydrogenase by iodoacetamide exhibits a curve which is very different from the one described by these rate constants and coefficients, it is concluded that the values for the stoichiometric modification rate constants, as calculated by the authors [9], are not compatible with stoichiometric protein modification cooperativity.

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