THEORETICAL INTERPRETATION OF THE DECREASE OF ACTIVITY OF ENZYME DURING ITS CHEMICAL MODIFICATION

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A mathematical description of the decrease of the activity of enzyme during its chemical modification, based on probability theory as well as on chemical kinetics and considering both stochastically independent as well as stochastically dependent chemical modifications of any number of essential amino acid residues of the enzyme is proposed. Two types of the stochastically dependent chemical modification, *i.e.* with negative cooperative effects and with positive cooperative effects were studied. A direct correlation was found to exist between the relative decrease of enzyme activity on the one hand and the number of essential amino acid residues as well as their role and relative mutual positions on the other. The procedure derived can be used for computer simulation of curves representing the decrease of the activity of enzyme during its chemical modification or for the nonlinear regression analysis of such curves.

Chemical modifications of amino acid residues belong to the experimentally most simple and most frequently used methods of studying the relationship between enzyme structure and function. Aldridge was the first to suggest[1] in 1950 a theoretical approach to the description of the decrease of the activity of the enzyme during its chemical modification, an approach based on a model of irreversible enzyme inhibition. This approach was developed further by Rakitzis[2,3]. The model of irreversible enzyme inhibition, however, is considerably complicated because it requires the solution of a system of differential equations to be carried out. If the binding of several molecules of the chemical modifying reagent to several amino acid residues of the enzyme is studied it is difficult to obtain an analytical expression appropriately describing the real situation.

A kinetic approach based on the assumption that the chemical modification of the enzyme at an excess of the modifying reagent proceeds as a pseudomonomolecular reaction was developed by Ray and Koshland[4] and successfully verified with phosphoglucomutase[5]. The Ray and Koshland's method makes it possible to analytically describe, on the basis of the knowledge of the mechanism of action of the modifying reagent, several types of chemical modifications: *a*) independent chemical modification of more than two essential amino acid residues*; *b*) modification of two amino acid residues whose action in the active site of the enzyme is complementary;

* In this article the term of *essential amino acid residue* will be used as in the original paper by Ray and Koshland[4] for such an amino acid residue the modification of which leads to the loss of activity of the modified enzyme molecule. Moreover, a distinction will be made between modification of *fully* and *partially* essential amino acid residues leading either to full or to partial loss of activity of the enzyme, respectively. The modification of a *non-essential* amino acid residue is regarded as having no influence on the enzyme activity.

c) modification of one amino acid residue of the enzyme accompanied by progressive denaturation of the enzyme; *d*) modification of one or two amino acid residues of the enzyme during which enzyme molecules are formed with relative activity smaller than one and at the same time higher than zero.

An advantage of the kinetic approach of Ray and Koshland is that it enables not only the type of the essential amino acids to be modified but also their number to be determined. However, from the mathematical viewpoint the formulation of these authors can be extended because of its generality, to incorporate also other modification types (*e.g.* modification with cooperative effect, combined modification, *etc.*).

Several authors have tried to determine the number of essential amino acid residues from plots of relative enzyme activity *versus* the number of amino acid residues modified[6-8]. These authors, however, have also pointed to the drawbacks of the method due to an insufficient analytic description of the real processes taking place in the molecule of the enzyme during its chemical modification. The problem of the determination of the number of essential amino acid residues of the enzyme has been elucidated by Tsou[9] also using the stochastic approximation.

In this paper the axioms of probability theory have been used, in combination with kinetic equations for first order reactions, to derive a mathematical description permitting the analytical form of enzyme activity decrease as function of modification of any number of essential amino acid residues and of any chemical modification type to be described.

Mathematical Formulation of Problem

Chemical modification of *i*-th amino acid residue of an enzyme is defined as a consecutive chemical transformation of a given cluster of enzyme molecules according to the following scheme

$$E - X_1X_2 \dots X_i \dots X_n \xrightarrow{k_i} E - X_1X_2 \dots X_iM \dots X_n , \qquad (1)$$

where $X_1, X_2, \dots, X_i, \dots, X_n$ is a cluster of amino acid residues in the enzyme molecule, k_i the rate constant of the transformation and X_iM the product of the modification reaction. It is assumed that the chemical reaction takes place at a large excess of the modifying reagent, *i.e.* that it is pseudomonomolecular and only one molecule of the reagent reacts with one amino acid residue.

The term of *modifiable amino acid residue* will be used for such amino acid residues which will be modified with probability $p \to 1$ at time $t \to \infty$. The chemical modification of any modifiable amino acid residue in a statistic cluster of enzyme molecules is a *random event* because only such collisions of modifiable amino acid residues with the modifying reagent lead to chemical reaction which occur with a sufficient energy and at the accurate orientation of the components. The random modification of X_i can depend, however, also on another random event, *i.e.* on the modification of amino acid residues localized in space close to each other. The rate

constant of the modification k_i is therefore determined *à priori* by the primary, secondary and tertiary structure of the enzyme and by the sum of all microscopic effects determined by the experimental conditions given. Because of the influence of the amino acid residues localized in space close to X_i it is possible to distinguish between two basic types of chemical modifications, *a*) *stochastically independent modification*, *i.e.* a modification of any modifiable amino acid residue of the same enzyme molecule which has no effect on the modification of X_i, and *b*) *stochastically dependent modification*, *i.e.* a modification of some modifiable amino acid residues of the same enzyme molecule affecting the modification of X_i.

RESULTS

The stochastically independent modification of one amino acid residue can be described by the following equation

$$x_i^t = x_E^0 \,.\, [1 - \exp(-k_i t)], \tag{2}$$

where x_E^0 is the molar enzyme concentration at modification time $t = 0$, x_i^t is the molar concentration of the modified amino acid residue X_i at modification time t and k_i is the rate constant of the modification. If N is the total number of the enzyme molecules and N_i^t is the number of the enzyme molecules in which X_i has been modified, then ratio x_i^t/x_E^0 represents the probability (pX_i) of occurrence of N_i^t molecules of the enzyme at time t in which X_i has been modified. The probability of the opposite event, *i.e.* when $N - N_i^t$ molecules of the enzyme remain unmodified in the reaction mixture at time t, will be described by $p\bar{X}_i$. Clearly $pX_i + p\bar{X}_i = 1$. If X_i is a fully essential amino acid residue, the probability of occurence of $N - N_i^t$ of enzyme molecules with unmodified X_i equals the probability of occurence of the same number of active enzyme molecules, *i.e.* $p\bar{X}_i = A_R$, where A_R is the relative activity of the enzyme. Hence,

$$A_R = \exp(-k_i t). \tag{3}$$

If X_i is a partially essential amino acid, A_R depends also on the formation of enzyme molecules with altered enzyme activity. The contribution of such molecules to the measurable enzyme activity is F_i $(0 < F_i < 1)$ and they are formed with probability $pX_i \,.\, F_i$. The relative enzyme activity can then be expressed by the following equation

$$A_R = p\bar{X}_i + F_i \,.\, pX_i = F_i + \exp(-k_i t) \,.\, (1 - F_i). \tag{4}$$

If two partially essential amino acid residues of the enzyme X_i, X_j are modified, the probability of the occurrence of active enzyme molecules is given by the sum of probabilities of the following events: *1.* The occurrence of enzyme molecules in which no

essential amino acid residue has been modified (probability $p\bar{X}_i \,.\, p\bar{X}_j$). *2.* The occurrence of enzyme molecules with X_i modified and at the same time with X_j unmodified. The enzyme molecules with altered activity $0 < F_i < 1$ are formed with probability $pX_i \,.\, p\bar{X}_j \,.\, F_i$. *3.* The occurrence of enzyme molecules with X_j and X_i at the same time unmodified. The enzyme molecules with altered enzyme activity $0 < F_j < 1$ are produced with probability $p\bar{X}_i \,.\, pX_j \,.\, F_j$. *4.* The occurrence of enzyme molecules with both X_i and X_j modified. The enzyme molecules with altered enzyme activity $0 < F_{ij} < 1$ are formed with probability $pX_i \,.\, pX_j \,.\, F_{ij}$. The occurence of active molecules of the enzyme at time t of the modification process is then given by

$$A_R = p\bar{X}_i \,.\, p\bar{X}_j + pX_i \,.\, p\bar{X}_j \,.\, F_i + p\bar{X}_i \,.\, pX_j \,.\, F_j + pX_i \,.\, pX_j \,.\, F_{ij} \,. \qquad (5)$$

After the substitution similar to that used for Eq. *3* we obtain

$$A_R = \exp\left[-(k_i + k_j) \,.\, t\right] \,.\, (1 + F_{ij} - F_j - F_i) + \\ + \exp\left(-k_i t\right) \,.\, (F_j - F_{ij}) + \exp\left(-k_j t\right) \,.\, (F_i - F_{ij}) + F_{ij} \,. \qquad (6)$$

This is the equation describing the analytical form of the decrease of A_R during stochastically independent chemical modification of two partially essential amino acid residues. By simple extension of this relation to incorporate the stochastically independent modification of *m* partially essential amino acid residues of the enzyme we arrive at

$$A_R = \prod_i^m p\bar{X}_i + \sum_i^m F_i pX_i \prod_{\substack{j \\ j \neq i}}^{m-1} p\bar{X}_j + \sum_{\substack{i,j \\ i \neq j}}^m F_{ij} \,.\, pX_i \,.\, pX_j \prod_{\substack{k \\ k \neq i \neq j}}^{m-2} p\bar{X}_k + F_{12\ldots m} \,.\, \prod_i^m pX_i \,. \qquad (7)$$

Eq. 7 can be reduced for stochastically independent modification of *m* fully essential amino acid residues to the following form

$$A_R = \prod_i^m p\bar{X}_i = \exp\left[(-\sum_i^m k_i) \,.\, t\right] \,. \qquad (8)$$

There are two types of the stochastically dependent modification: modification with a negative cooperative effect and modification with a positive cooperative effect.

Modification with negative cooperative effect. The simplest event of this modification occurs when two modifiable amino acid residues X_i and X_j are localized close to each other in the enzyme molecule. If the molecule of the modifying reagent is large enough and the modifiable amino acid residues close enough to each other, X_i and X_j compete for the modifying reagent. Consequently, the modification of X_i excludes the

modification of X_j in the same enzyme molecule and *vice versa* since

$$\mathrm{E - X_1X_2 \ldots X_i \ldots X_j \ldots X_n} \begin{array}{l} \xrightarrow{k_i} \mathrm{E - X_1X_2 \ldots X_iM \ldots X_j \ldots X_n} \\ \xrightarrow{k_j} \mathrm{E - X_1X_2 \ldots X_i \ldots X_jM \ldots X_n} . \end{array} \tag{9}$$

The probability of modification of X_i or X_j can be found then from kinetic equations of parallel reactions of the first order[10]. It can be regarded as a *conditional probability*. The probability of occurence of enzyme molecules with modified X_i and with X_j simultaneously unmodified can be deduced as

$$p_x X_i = \frac{k_i}{k_i + k_j} . \{1 - \exp[-(k_i + k_j) . t]\} . \tag{10}$$

The probability of the opposite event – the occurrence of enzyme molecules with X_i unmodified is

$$p_x \bar{X}_i = \frac{k_i}{k_i + k_j} + \frac{k_i}{k_i + k_j} . \exp[-(k_i + k_j) . t] . \tag{11}$$

Generalizing our considerations about the modification of n amino acid residues with the negative cooperative effect out of which m are essential, we obtain an equation describing the decrease of the relative enzyme activity A_R during the modification

$$A_R = 1 - \sum_i^m p_c X_i + \sum_i^m F_i . p_c X_i \tag{12}$$

or

$$A_R = 1 + \frac{\sum_i^m k_i . (F_i - 1) . [1 - \exp(-kt)]}{k} , \tag{13}$$

where $k = \sum_j^n k_j$ and $0 \leqq F_i \leqq 1$.

Modification with positive cooperative effect. The simplest variant is the modification of X_j conditioned by the modification of X_i

$$\mathrm{E - X_1X_2 \ldots X_i \ldots X_j \ldots X_n} \xrightarrow{k_i} \mathrm{E - X_1X_2 \ldots X_iM \ldots X_j \ldots X_n} \xrightarrow{k_j} \mathrm{E - X_1X_2 \ldots X_iM \ldots X_jM \ldots X_n} . \tag{14}$$

The modification of X_j can also be conditioned by a suitable conformational change of the enzyme molecule which is described by the same kinetic expression except that k_i is replaced by the rate constant of the conformational change. The conditional probability of occurrence of enzyme molecules with unmodified X_j can be obtained from kinetic equations representing two consecutive first order reactions[10]

$$p_{x_1}\bar{X}_j = \frac{k_j \,.\, \exp(-k_i t) - k_i \,.\, \exp(-k_j t)}{k_j - k_i} \,. \qquad (15)$$

Eq. *15* simultaneously expresses the time profile of A_R on condition that X_j is a fully essential amino acid and its modification is conditioned by the modification of X_i which is non-essential (or by a reversible conformational change of the enzyme molecule).

How can we distinguish between the individual types of chemical modification? As a first approximation it is most suitable to plot semilogarithmically the dependence of A_R *versus* modification time as in Fig. 1. It should be noted, though, that according to Eqs *4* and *11* the analytical expressions of A_R for a stochastically independent modification of a partially essential amino acid residue and for a stochastically dependent modification of a fully essential amino acid residue with negative cooperative effect of the non-essential amino acid residue are identical, namely

$$A_R = A_R^\infty + (1 - A_R^\infty) \,.\, \exp(-kt) \,, \qquad (16)$$

where A_R^∞ is the relative enzyme activity at modification time $t \to \infty$. If these two modification types are to be distinguished from each other the determination of the enzyme activity and also the extent of modification of the amino acid residues must be determined, *e.g.* by methods described in refs[4,11]. In this manner the course of $p\bar{X}_i$ can be compared with the course of A_R. It should be noted that Eq. *16* can be linearized by semilogarithmically plotting $\log[(A_R - A_R^\infty)/(1 - A_R^\infty)]$ *versus* t.

At present facilities are available for computer simulation of the experimentally investigated dependence of the decrease of A_R during the enzyme modification as well as of the extent of modification of the individual amino acid residues. Thus the correctness of the modification model proposed can be visually verified and the modification constants for the individual amino acid residues can be estimated preliminarily. However, an unbiassed judgement of the correctness of the above model especially of the occurrence of the various alternatives should result from nonlinear regression analysis testing the individual models quantitatively. The model proposed should be supported by the knowledge of the chemical properties of the given system, of the reaction conditions and, if necessary, it should be verified by physical methods (mainly by spectral methods).

Comparison of the theory with the experiment. The modification of one histidine residue (rate constant k_H) and of one tyrosine residue (rate constant k_T) of purine specific RNAase U_2 by ethoxyformylanhydride[12] can serve as a suitable example of the stochastically dependent modification. The experimental values of enzyme activity as well as the extent of modification of the individual amino acid residues were read off from the experimental curves (ref.[12]). The incomplete modification of both amino acids indicates that we are dealing with a modification with negative cooperative effect (Fig. 2). Since the time profile of A_R traces the time profile of the probability of occurence of unmodified amino acid residues of histidine ($p\bar{X}_H$) it can be postulated that one amino acid residue modifiable at the given experimental conditions is essential, and moreover, fully essential for the enzyme activity. However, A_R is also influenced by the modification of the other amino acid – most probably by the modification of the nonessential tyrosine residue. The shape of the curve characterizing

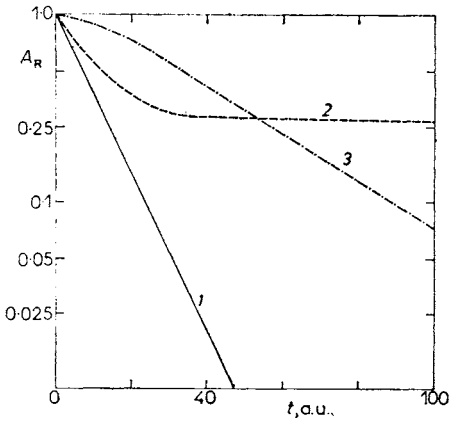

FIG. 1

Semilogarithmic plot of the decrease of relative enzyme activity A_R *versus* time of modification for *1* stochastically independent modification of one fully essential amino acid residue X_i ($k_i = 0{\cdot}1$); *2* stochastically dependent modification of fully essential amino acid residue X_i with negative cooperative effect of modification of non essential amino acid residue X_j ($k_i = 0{\cdot}08$ and $k_j = 0{\cdot}3$); *3* stochastically dependent modification of fully essential amino acid residue X_j with positive cooperative effect of modification of non-essential amino acid residue X_i ($k_i = 0{\cdot}08$, $k_j = 0{\cdot}1$)

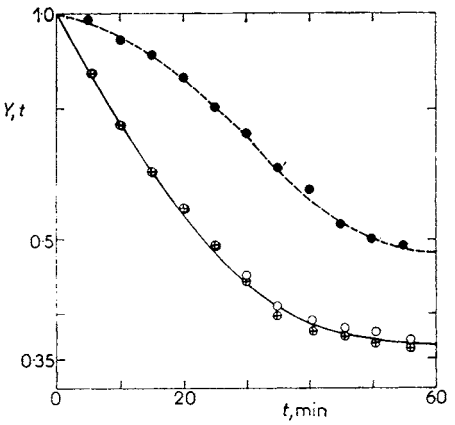

FIG. 2

Comparison of the theory of stochastically dependent modification with negative cooperative effect with experimental data. Ethoxyformylation of RNAase U_2 from *Ustillago sphaerogena*. The values of A_R^{exp}, $p\bar{X}_H$, $p\bar{X}_T$ were calculated from the experimental curves (ref.[12]). ●–●–● $Y(t) = p\bar{X}_T$; ○–○–○ $Y(t) = p\bar{X}_H$; ⊕ ⊙ ⊕ $Y(t) = A_R^{exp}$; ——— $Y(t) = A_R^{theor}$ is the theoretical curve of A_R calculated according to Eq. *17* for $k_T = 0{\cdot}017$ min^{-1}; $k_H = 0{\cdot}038$ min^{-1}

the probability of occurence of unmodified tyrosine residues ($p\bar{X}_T$) indicates that these residues are modified simultaneously with the negative cooperative effect. Hence, A_R can be obtained from Eq. *11* as follows

$$A_R = p_x\bar{X}_H = \frac{k_T}{k_T + k_H} + \frac{k_H}{k_T + k_H} \cdot \exp\left[-(k_H + k_T) \cdot t\right]. \qquad (17)$$

We can see in Fig. 2 that the theoretical curve of A_R^{theor}, calculated from this equation by using $k_T = 0{\cdot}017\ min^{-1}$ and $k_H = 0{\cdot}038\ min^{-1}$, estimated from the linear parts of the semilogarithmic plot, is in good agreement with the experimental values of A_R^{exp}.

An experimental example of stochastically independent modification of two amino acid residues has been given and interpreted by Ray and Koshland[5].

DISCUSSION

The proposed mathematical approach based on the axioms of the probability theory in combination with the first order kinetic equations makes it possible to simply derive an analytical expression describing the decrease of A_R for the various types of chemical modifications. By comparing the calculated curve with the experimental curve by computer simulation or better, by the nonlinear regression analysis, we obtain information on amino acid residues essential for enzyme activity, their type, number, role, and also on their relative mutual orientation.

The basic types of the modification reactions have been described and general equations for A_R characterizing the stochastically independent modification and the stochastically dependent modification with the negative cooperative effect of any number of essential amino acid residues have been derived. The equations describing the stochastically independent modification implicitely include most of the equations of the frequently used Ray and Koshland theory[4]. However, the approach proposed significantly extends this theory in several directions. Firstly, it makes possible a very simple derivation of equations describing the decrease of the enzyme activity for any number of modifiable amino acid residues. Moreover, the modification with a negative cooperative effect which can occur, as documented by the modification of the histidine residue of RNAase U_2 by ethoxyformyl anhydride, is also discussed. The modification of an non-essential amino acid residue has in this case a certain "protective" effect on the modification of an essential amino acid residue which manifests itself by incomplete modification of the latter. Such case can be distinguished, however, from the complete modification of a partially essential amino acid, a modification characterized by the same profile of A_R (Eq. *16*), only by simultaneous measurement of the degree of modification of the individual amino acid residues.

Another advantage of the approach proposed is the possibility of a simple derivation of A_R for any type of chemical modification which can appear as a combination

of several modifications classified above. For example, some essential amino acid residues of the same enzyme molecule can be modified with a negative cooperative effect while other residues with a positive cooperative effect and the remaining residues are modified stochastically independently. Such description, of course, becomes more complicated and its adequacy is much smaller than if simple modification types are considered. One should therefore choose the proper modifying reagent as well as the suitable experimental conditions of the modification reaction in order to minimize the number of modified amino acid residues. In order to further increase the unambiguity of the interpretation it is necessary to choose such a modifying reagent which yields a product measurable spectrophotometrically or by some other physical method. By this approach it is possible to compare the curve describing A_R with curves describing the extent of modification not only qualitatively but also quantitatively by comparing the kinetic constants. However, conclusions about the number, role and relative mutual positions of the essential amino acid residues should be made very prudently since the real course of the modification may be much more complicated than the assumed course (there may occur time dependent changes of the enzyme after the addition of the modifying reagent, or the modification reaction may be accompanied by the formation of poorly defined reaction products, *etc.*). Any conclusions in this respect should be supported therefore by chemical modifications of the enzyme in the presence of the substrate or of a competitive inhibitor. Supporting evidence should be obtained, above all, from the measurement of the spectral characteritics of the modified enzyme and of the native enzyme and also of the enzyme–inhibitor complex.

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