Kinetics of protein-modification reactions

Stoichiometry of modification-produced enzyme inactivation: modification of rhodanese by 2,4,6-trinitrobenzenesulphonic acid

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A mathematical treatment is presented for the dependence of enzyme activity loss on the numbers and reactivities of the groups essential for catalytic function, when enzyme protein modification is carried out by the use of concentrations of protein reactive groups well in excess of that of modifying agent. Experimentally obtained data on the modification of rhodanese (thiosulphate sulphurtransferase, EC 2.8.1.1) by 2,4,6-trinitrobenzenesulphonic acid are presented, and it is shown that, at pH 9.00, the fractional concentration of rhodanese groups, or of rhodanese group reactivities, essential for enzyme catalytic function is 0.88; this value is found to decrease with decreasing pH of the reaction medium. The possibility that rhodanese inactivation by 2,4,6-trinitrobenzenesulphonic acid is brought about by modification of groups other than amino groups is ruled out by a comparison of the enzyme-inactivation and protein-modification stoichiometries, for putative reaction models for enzyme and modifying agent.

Protein-modifying agents exhibit overlapping specificities (Means & Feeney, 1971; Glazer *et al.*, 1975). The group specificity of a protein-modifying agent is usually determined by amino acid analysis of the products of protein modification. However, conclusions reached in this manner are not always straightforward, because modification products may be hydrolysed, or otherwise removed from the protein, during amino acid analysis (Glazer *et al.*, 1975). A causal connection between protein modification and enzyme inactivation is usually sought by a comparison of the rates of protein modification and of enzyme activity loss (Ray & Koshland, 1961), or by an examination of the dependence of fractional enzyme activity on the extent of protein modification (Tsou, 1962). However, enzyme inactivation may be due to the modification of an unsuspected kind of reactive group, the rate of modification of which happens to be equal to, or a reciprocal of an integral multiple of, the rate of activity loss (Rakitzis, 1984b). A causal connection between protein modification and enzyme inactivation may be established by a comparison of the enzyme-inactivation and protein-modification stoichiometries (Gomi & Fujioka, 1982). In the present paper procedures are described for establishing (a) the dependence of enzyme activity loss on the numbers and reactivi-

ties of the groups essential for enzyme catalytic function, and (b) the involvement of a particular kind of reactive group in the enzyme-inactivation event. Examples are provided from the modification of rhodanese by 2,4,6-trinitrobenzenesulphonic acid.

Theoretical

Protein-modification reactions are described by second-order non-linear differential equations, with concentration of the protein reactive groups being modified as the dependent variable, and reaction time as the independent variable. Linearization of the equations describing protein-modification reactions is usually accomplished by the use of concentrations of modifying agent well in excess of protein concentration (see Rakitzis, 1984a). The same objective may be realized by the use of concentrations of protein reactive groups well in excess of that of the agent (Rakitzis & Malliopoulos, 1978). Two cases are distinguished, as follows.

(a) A protein with n identical reactive groups per protein molecule is considered. The reaction scheme is:

where E_i is the stoichiometric protein species with

i mol. of unmodified groups per mol of protein, M is modifying agent, $E_{\text{mod.}}$ is modified protein and k_d is a constant. Brackets are used below to indicate concentrations. Clearly, eqn. (1) applies only in cases where $n[E]_0 \gg [M]$, since, under these circumstances, the individual reactions of eqn. (1) are parallel first-order reactions, k_d being the sum of the respective reaction rate constants (Frost & Pearson, 1961). The value of k_d is given by the relationship:

$$k_d = k_{\text{mod.}} \cdot n[E]_{n(0)} \quad (2)$$

where $k_{\text{mod.}}$ is the rate constant for modification of protein groups, and also where $[E]_{n(0)}$ is $[E]$ at the start of the reaction. The assumption is made that the process under study is not one of protein-modification co-operativity (see Rakitzis, 1984a). The concentration of modifying agent is given by the relationship:

$$[M] = [M]_0 \cdot e^{-k_d t} \quad (3)$$

where t is reaction time, $[M]$ is the concentration of modifying agent at time t and $[M]_0$ is $[M]$ at $t = 0$. With reaction time as the independent variable:

$$-[E]' = k_{\text{inact.}} \cdot [E] \cdot [M]_0 \cdot e^{-k_d t} \quad (4)$$

where $[E]'$ is the first derivative of $[E]$, and $k_{\text{inact.}}$ is a constant. It is assumed that at all times $[EM] \ll [E]$, where $[EM]$ is the concentration of the reversible enzyme-modifying agent complex, the first-order reaction of which leads to enzyme inactivation. Separating variables in eqn. (4), and integrating between zero and t :

$$\ln([E]_0/[E]) = (k_{\text{inact.}}/k_d) \cdot [M]_0 \cdot (1 - e^{-k_d t}) \quad (5)$$

At infinite reaction time the value for $\ln([E]_0/[E])$ is:

$$\ln([E]_0/[E]_\infty) = (k_{\text{inact.}}/k_d) \cdot [M]_0 \quad (6)$$

Substituting $\ln([E]_0/[E]_\infty)$ for $(k_{\text{inact.}}/k_d) \cdot [M]_0$ in eqn. (5), and taking logarithms of both sides of the equation so produced:

$$\ln \{ \ln([E]_0/[E]_\infty) \} = \ln \{ \ln([E]_0/[E]_\infty) \} - k_d t \quad (7)$$

Eqn. (7) may be solved graphically. It will be noted that an equation of the same form as eqn. (7) has been developed by Ashani *et al.* (1972) for the case where modifying agent is unstable in solution. The value of the enzyme-inactivation second-order rate constant is given by the relationship:

$$k_{\text{inact.}} = i \cdot k_{\text{mod.}} \quad (8)$$

where i is the number of enzyme protein reactive groups essential for catalytic activity (Rakitzis, 1978). Substituting eqns. (2) and (8) into eqn. (6):

$$\ln([E]_0/[E]_\infty) = (i/n) \cdot [M]_0/[A]_0 \quad (9)$$

(b) Enzyme protein reactive groups are divided into two or more classes of groups with different reactivities towards the modifying agent. Reaction may again be described by eqn. (1), with E_i standing for the i th class of differently reacting groups. The value of k_d is given by the relationship:

$$k_d = [A]_0 \cdot \sum_j a_j \cdot k_{\text{mod.}(j)} \quad (10)$$

where a_j is the number of groups with a modification rate constant of $k_{\text{mod.}(j)}$. Clearly, eqns. (1) and (10) apply only in cases where $a_j \cdot [A]_0 \gg [M]$. The value of the enzyme-inactivation second-order (or pseudo-first-order, under the experimental conditions used) rate constant is given by the relationship:

$$k_{\text{inact.}} = \sum_j b_j \cdot a_j \cdot k_{\text{mod.}(j)} \quad (11)$$

where b_j is the fraction of a_j groups that are essential for enzyme activity. Substituting the values for $k_{\text{inact.}}$, as well as for k_d , into eqn. (6):

$$\ln([E]_0/[E]_\infty) = \frac{\sum_j b_j \cdot a_j \cdot k_{\text{mod.}(j)}}{\sum_j a_j \cdot k_{\text{mod.}(j)}} \cdot [M]_0/[A]_0 \quad (12)$$

It follows that, if all protein reactive groups, of whatever kind, are essential for enzyme activity, i.e. $(i/n) = 1$, for case (a), and $b_j = 1$, for case (b), then eqns. (9) and (12) both reduce to:

$$\ln([E]_0/[E]_\infty) = [M]_0/[A]_0 \quad (13)$$

Accordingly, if all protein reactive groups are essential for activity, a plot of $\ln([E]_0/[E]_\infty)$ versus $[M]_0/[A]_0$ should yield a straight line with a slope of unity. Eqn. (13) may also be arrived at by an extension of the probabilistic treatment given by Tsou (1962). Generalizing eqn. (15) of Tsou (1962):

$$([E]/[E]_0) = \prod_{i,j} (1 - p_j/n_j)^i \quad (14)$$

where p_j is the relative concentration of groups modified for the class of groups containing n_j groups, and also where i is the number of groups essential for activity, per enzyme active site, in each instance. Assuming that $n_j = i$, and also that n_j approaches infinity (this last assumption being equivalent to the assumption that $n_j \cdot [A]_0 \gg [M]_0$), eqn. (14) reduces to:

$$([E]_0/[E]_\infty) = \prod_j e^{p_j} = e^{[M]_0/[A]_0} \quad (15)$$

Experimental

Rhodanese, crystallized from bovine spleen, and 2,4,6-trinitrobenzenesulphonic acid were pur-

chased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Rhodanese activity was determined by the method of Sörbo (1953), with thiosulphate and cyanide as substrates. Thiocyanate production was found to be linearly dependent on rhodanese concentration, at least up to an absorbance reading of 1200cm^{-1} at 460nm , under the experimental conditions used (Sörbo, 1953). Studies of the inactivation of rhodanese by 2,4,6-trinitrobenzenesulphonic acid, an arylating agent specific for primary amines (Fields, 1971; Means & Feeney, 1971; Glazer *et al.*, 1975), were carried out by preparing an enzyme/modifying agent mixture, the final composition of which, for the pH 7.50 and pH 8.25 samples, was: 0.1M -sodium phosphate buffer, of the required pH, 0.01M -sodium thiosulphate, $30\mu\text{M}$ -rhodanese and 2,4,6-trinitrobenzenesulphonic acid as indicated in Figs. 1 and 2. The pH 9.00 sample consisted of 0.05M -sodium borate buffer, in addition to the solution components referred to above. After incubation of the enzyme/modifying agent mixture in a water bath at 25°C , for time periods sufficient to bring about complete reaction of 2,4,6-trinitrobenzenesulphonic acid with rhodanese (these time periods varying from 90 to 180 min, for the cases shown in Figs. 1 and 2), the preparation was used for determination of rhodanese activity. The extent of reaction of 2,4,6-trinitrobenzenesulphonic acid was monitored by taking absorbance readings at 345nm (Goldfarb, 1966).

Plots of rhodanese-inactivation data according to eqn. (7), at pH 9.00, and at different 2,4,6-trinitrobenzenesulphonic acid concentrations, are shown in Fig. 1. It is seen from Fig. 1 that plots of $\ln\{\ln([E]/[E]_\infty)\}$ versus reaction time, at four different 2,4,6-trinitrobenzenesulphonic acid concentrations, have the same slope, i.e. exhibit the same k_d value, for the cases shown. It is further seen that all four plots are rectilinear, and extend up to the zero time point, indicating that disappearance of the modifying agent is a one-exponential process, in accordance with eqn. (3). This establishes the validity of the model for modification-produced enzyme inactivation described in the present paper.

The dependence of rhodanese activity loss on the numbers and reactivities of the groups essential for enzyme catalytic function is shown by plotting the $\ln([E]_0/[E]_\infty)$ value versus the $[M]_0/[A]_0$ value, in accordance with eqn. (12). It is seen from Fig. 2 that, at pH 9.00, a slope of 0.88 is obtained, indicating the fraction of rhodanese group reactivities essential for enzyme catalytic function. The slopes obtained at pH values of 8.25 and 7.50 are 0.81 and 0.60 respectively, indicating that as the pH of the reaction medium is decreased the

Fig. 1. Inactivation of rhodanese by 2,4,6-trinitrobenzenesulphonic acid: determination of the first-order rate constant for disappearance of modifying agent

Plots of $\ln\{\ln([E]/[E]_\infty)\}$ versus reaction time, where $[E]$ is rhodanese activity at time t and $[E]_\infty$ is rhodanese activity at infinite reaction time, in accordance with eqn. (7). Reaction of enzyme and modifying agent was at a 2,4,6-trinitrobenzenesulphonic acid initial concentration of $30\mu\text{M}$ ($\circ$), $24\mu\text{M}$ ($\bullet$), $18\mu\text{M}$ ($\square$) and $12\mu\text{M}$ ($\blacksquare$). For details of the experimental procedure used see the text.

fraction of rhodanese group reactivities essential for enzyme activity becomes smaller. Apparently, protonation of rhodanese nucleophilic groups brings about a decrease in the high degree of cooperativity obtaining at alkaline pH values. A decrease, or complete loss, of enzyme catalytic function consequent upon enzyme protein modification at regions far from the active site has been postulated in several cases of modification-produced enzyme activity loss (see Rakitzis, 1984b).

The question arises whether 2,4,6-trinitrobenzenesulphonic acid brings about the co-operative inactivation of rhodanese by modification of free amino groups, or by some other kind(s) of group trinitrophenylation that does not bring about an increase in absorbance at 345nm . A likely candidate is the single persulphide group of the rhodanese molecule, involvement of which in the catalytic mechanism of this enzyme is well established (Westley, 1973; Schlesinger & Westley, 1974). Utilization of 2,4,6-trinitrobenzenesulphonic acid for the modification of groups other than free amino groups would produce a decrease in the apparent absorption coefficient of trinitrophenylated rhodanese, and indeed the value obtained is $11500\text{M}^{-1}\cdot\text{cm}^{-1}$, a value considerably lower than the generally accepted value of

Fig. 2. Inactivation of rhodanese by 2,4,6-trinitrobenzenesulphonic acid: determination of the fractional concentration of rhodanese groups, or rhodanese group reactivities, essential for catalytic activity

Plots of $\ln([E]_0/[E]_\infty)$ versus $[M]_0/[A]_0$, where $[E]_0$ is rhodanese activity at zero time, $[E]$ is rhodanese activity at infinite reaction time, $[A]_0$ is rhodanese concentration at zero time and $[M]_0$ is 2,4,6-trinitrobenzenesulphonic acid concentration at zero time. Reaction of enzyme and modifying agent was at pH 7.50 (○), pH 8.25 (●) and pH 9.00 (□). Inset: the putative absorption coefficient obtained on the assumption that a portion of the modifying agent, stoichiometrically equal to the portion of inactivated enzyme, is unavailable for reaction with amino groups (○), and the assumption of complete utilization of modifying agent by modification of amino groups (●). For details of the experimental procedure used see the text.

$14000\text{M}^{-1}\cdot\text{cm}^{-1}$ (Means & Feeney, 1971). However, the absorption coefficient for the trinitrophenylated α -amino groups of several amino acids and peptides has been found to vary with chain length (Goldfarb, 1966), and accordingly the lower value for the absorption coefficient of trinitrophenylated rhodanese may well be due to the modification of free amino groups alone. Nevertheless, the possibility of modification of rhodanese groups other than amino groups should not be dismissed lightly, in view of the finding that 2,4,6-trinitrobenzenesulphonic acid reacts with thiol groups, albeit with the production of derivatives that are unstable at physiological pH values (Kotaki *et al.*, 1964; Freedman & Radda, 1968).

To help answer the question of the group involved in the trinitrophenylation-produced loss of rhodanese activity, use was made of the complete utilization of modifying agent, at infinite

reaction time, when the concentration of protein reactive groups is in great excess over that of the modifying agent. Accordingly, a stoichiometric relation may be established between disappearance of modifying agent and enzyme activity loss. The molar absorption coefficient for trinitrophenylated rhodanese, established in this manner, is obviously dependent on the particular enzyme-inactivation model selected, since the stoichiometry of modification-produced enzyme inactivation can be formulated in a number of ways. For the case under study, three main reaction models may be visualized: (a) amino groups only are essential for enzyme activity; (b) groups other than amino groups only are essential for enzyme activity; (c) both kinds of groups are essential for enzyme activity. (Additional reaction models could be formulated: e.g., one in which rhodanese inactivation results from the modification of two groups other than amino groups per enzyme active site.) For reaction model (a) a uniform absorption coefficient, under different conditions of concentration of the modifying agent, pH, temperature etc. is expected if the ΔA_{345} value is divided by the molarity of modifying agent utilized. For model (b) a uniform absorption coefficient is expected if the stoichiometric portion corresponding to enzyme activity loss has been subtracted from the molarity of modifying agent utilized. Model (c) is indistinguishable from model (a) if amino groups, and groups other than amino groups, react with the modifying agent at rates the ratio of which remains constant under all conditions tested. It is seen from the inset of Fig. 2 that a constant absorption coefficient is obtained, at different concentrations of modifying agent, and/or reaction pH values, if the concentration of modifying agent utilized is used without any correction for enzyme activity loss. This establishes model (a) as the correct interpretation of the experimentally obtained data, i.e. rhodanese inactivation due to modification of amino groups only. However, the possibility of model (c), involvement of both amino groups and groups other than amino groups, with the qualification referred to above, may not be dismissed at the present state of knowledge.

A causal connection between protein modification and enzyme inactivation, based on a comparison of the stoichiometry of utilization of modifying agent and modification of protein reactive groups, has been established for the carboxyamidation of *S*-adenosylhomocysteinase by iodoacetamide (Gomi & Fujioka, 1982) and for the oxidation of tryptophan residues of pyruvate dehydrogenase by bromosuccinimide (Korochnik *et al.*, 1984). However, the determination of the extent of modification of protein reactive groups, when performed spectrophotometrically, is based on the

assumption of a universally valid, group-related, absorption coefficient for the reaction products in question, a thesis that may not always be correct. This difficulty is obviated by a comparison of the putative absorption coefficients, obtained by the determination of stoichiometries of protein modification and of enzyme inactivation for the possible reaction models tested. In addition, this procedure permits the establishment of a possible causal link between spectral-change-free group modification and enzyme activity loss, when the kind of groups, modification of which is experimentally determined, is not involved in the enzyme inactivation event.

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