Kinetics of protein modification reactions

Plot of fractional enzyme activity versus extent of protein modification in cases where all modifiable groups are essential for enzyme activity

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The plot of fractional enzyme activity versus extent of protein modification, for cases where all enzyme modifiable groups of a certain kind are essential for activity, is found to be nearly independent of the number, per enzyme active site, of modifiable groups involved. Such plots usually, by a fallacious extension of the initial portion of the plot on the extent-of-modification axis, are interpreted to mean the modification of one single group per enzyme active site (or per enzyme molecule). The possible relevance of these findings to cases in the literature is discussed.

Modification of enzyme protein reactive groups may result in the loss of catalytic activity. It has been shown by Tsou (1962) that if i groups, per enzyme active site, are essential for activity, i.e. destruction of any one of these groups results in the loss of catalytic function, then the relation of fractional enzyme activity, a , to the relative concentration of groups modified, p , is:

$$a = (1 - p/n)^i \quad (1)$$

where n is the total number of the same kind of groups per enzyme active site; the assumption is made that all of the n groups react at the same rate with the modifying agent. The dependence of a on p is linearized by plotting the $a^{1/i}$ value versus p (Tsou, 1962). It is the purpose of the present paper to draw attention to the peculiar properties of the a -versus- p plot when $i = n$, i.e. when all modifiable groups of a certain kind are essential for activity. This is a likely eventuality in cases where all of the n groups are part of, or are located very near to, the enzyme active site, and also in cases where the all-or-none concerted model of ligand-induced conformational changes applies (Monod *et al.*, 1965; Koshland *et al.*, 1966). Since, in this model of co-operative interaction, enzyme ligand-binding sites are stabilized into one of two conformational states because of the non-covalent reversible attachment of one single ligand molecule on the protein molecule, it is to be expected that the covalent modification of a suitable site will *a fortiori* produce a generalized conformational change on the entire protein molecule. Examples of modification-induced changes in the ligand-binding

capacity of proteins are (a) a 3-fold increase in the K_m of β -D-glucosidase consequent to modification of tyrosine residues with *N*-acetylimidazole (Kiss *et al.*, 1981), (b) a 5-fold increase in the K_m of aspartase, and a shift in the pH optimum for the activity towards acidic pH, brought about by the modification of thiol groups of this enzyme with *N*-ethylmaleimide (Mizuta & Tokushige, 1975), and (c) a 2-fold increase in the K_m for coenzyme of glutamate dehydrogenase, consequent to modification of arginine residues with cyclohexane-1,2-dione (Blumenthal & Smith, 1975). An example of enzyme inactivation by modification far from the active site is the finding that cyanylation of the active-site thiol groups in two of the four identical subunits of glyceraldehyde-3-phosphate dehydrogenase with 2-nitro-5-thiocyanobenzoic acid results in the total loss of catalytic activity (Byers & Koshland, 1975).

Plots of a versus p , in accordance with eqn. (1), with the restriction of $n = i$, are shown in Fig. 1. It will be seen that, for values of n between 2 and 10, the a -versus- p plots are confined within narrow limits, and, indeed, as n approaches infinity, a approaches the limiting value of e^{-p} (Batschelet, 1979). Experimental situations of enzymes presenting with different numbers of modifiable residues, all of which are essential for activity, would thus be virtually indistinguishable on the a -versus- p plot. Clearly, cases of this sort may be misinterpreted in two entirely different ways, as follows.

(1) Because experimental points would be available over a wide range of values, the plot of fractional enzyme activity versus extent of protein

Fig. 1. Plot of fractional enzyme activity versus extent of protein modification in cases where all modifiable groups of a certain kind are essential for enzyme activity

Plots of fractional enzyme activity versus extent of protein modification are according to eqn. (1), with the restriction of $n = i$, for the cases where $n = 2$ (●), $n = 5$ (○) and $n = 10$ (□). The --- line is the e^{-p} value [where p is groups modified (mol/mol of protein)]. Inset: data for the case where $n = 10$ and re-plotted with values of i equal to 2 (●) and to 3 (○).

modification would be manifestly non-linear. Recourse might be had to a Tsou plot, i.e. to re-plotting the data by using the $a^{1/i}$ value in place of the a value, where i would be any small integer producing the best fit of the data, on the assumption that i groups per enzyme active site are essential for activity (Tsou, 1962). It will be seen from the inset of Fig. 1 that data generated by using an $n = 10$ value in eqn. (1), and also setting $n = i$, will produce a reasonably good fit when re-plotted on the assumption that $i = 3$.

(2) Because experimental points over a wide range of values would not be available, the plot of fractional enzyme activity versus extent of protein modification would be ostensibly linear or quasi-linear. By extending the initial portion of the a -versus- p plot on the p axis, the cases shown in Fig. 1 produce intercepts that are of a value slightly larger than one group modified per molecule of protein. Such plots may easily be interpreted to mean the modification of one group only per enzyme active site, a conclusion that is certainly true if the modification reaction is considered stoichiometrically. The fallacy of drawing conclusions from an extension of the initial portion of the a -versus- p

plot on the p axis for situations other than the one referred to above has amply been dealt with by various authors (see Rakitzis, 1984).

The question arises whether the case where $n = i$ applies is of frequent experimental occurrence. An obvious requirement for this eventuality should be the finding that, of a large number of groups of a certain kind, only one is to be found to be modified per protein molecule (or per enzyme active site). This requirement is met with in a surprisingly large number of cases in the literature. Riordan (1979) lists ten cases, all involving modification of arginine residues, where one or two residues only are modified, although the total number of arginine residues per protein molecule ranges from eight to 26. Riordan (1979) attributes the high selectivity of modification observed to an increased reactivity of one or two residues per protein molecule, such increased reactivity being due to steric factors in and around the active site. It is remarkable that in none of the cases cited by Riordan (1979) has the rate constant for the modification reaction been determined, although the rate constant for the inactivation reaction is given for most of the cases studied. It will be noted that, if as cited by Riordan (1979) one or two arginine residues are modified per enzyme molecule, then the rate of inactivation should, at the most, be twice the rate of protein modification. In the cases cited by Riordan (1979), for which data on the time-dependency of protein modification are supplied, as in the modification of aspartate transcarbamoylase by phenylglyoxal (Kantrowitz & Lipscomb, 1976), it readily becomes apparent that protein modification is a much slower process than enzyme inactivation, since after 300 min from the start of the reaction the plot of number of arginine residues modified versus time is essentially rectilinear, i.e. the plot has not yet started to slope off, whereas enzyme activity loss proceeds with a half-life of 90 min. The plots of fractional enzyme activity versus extent of protein modification in all of the cases cited by Riordan (1979) resemble Fig. 1 in that these plots are linear down to an a value of between 0.4 and 0.2, and that the intercept on the p axis is slightly larger than 1 mol of groups modified/mol of protein; at a values below 0.2 sloping off occurs, so that the overall shape of the plot is concave upwards.

It has been shown (Rakitzis, 1980a) that a -versus- p plots that are concave upwards may be produced if enzyme activity loss is described by a summation of two or more exponential functions of reaction time. However, if enzyme activity loss is described by a single exponential function of reaction time, then the a -versus- p plot may only be of the following kinds: (a) rectilinear, if the rate constant for enzyme inactivation is equal to the

rate constant for protein modification; (b) concave downwards, if enzyme protein modification is described by a summation of two or more exponential functions of reaction time the coefficients of which are positive; (c) concave upwards, if $i > 1$, or if enzyme protein modification is described by a summation of exponential functions of reaction time, at least one of the coefficients of which is negative [protein modification co-operativity, differential ligand and protein conformation effects on modification (Rakitzis, 1977, 1980b)].

Since all cases cited by Riordan (1979), as well as numerous other cases of the same kind (e.g. by Belfort *et al.*, 1980; Bond *et al.*, 1980; Olson & Massey, 1980; Patanjali *et al.*, 1984), provide *a*-versus-*p* plots that essentially are concave upwards (if the portion below an *a* value of between 0.4 and 0.2 is taken into account), and also, since in all of these cases enzyme inactivation is described by a single exponential function of reaction time, it is concluded that these cases should be interpreted, at the present state of knowledge, to be the outcome either of a stoichiometric modification of all accessible modifiable groups, or of protein modification co-operativity and/or differential ligand and protein conformation effects on modification. In any event, these cases should not be interpreted in the simplest way possible, i.e. to the effect that out of all residues of a certain kind only one per enzyme active site is primarily reactive. The final points on the *a*-versus-*p* plot, i.e. the points diverging from the initial rectilinear portion of the plot, should not be interpreted to mean 'the incorporation of more slowly reactive residues, with only a very minor effect on enzyme inactivation' (Bond *et al.*, 1980). Were this the case, then the *a*-versus-*p* plot should be concave downwards (Rakitzis, 1980a). It must be stressed that the plot of fractional enzyme activity versus extent of protein modification is of little use if the final rectilinear portion of the plot is not clearly observable, and also that a detailed study of the kinetics of protein modification is just as important as the study of enzyme inactivation. Clearly, it is by a study of both processes that the causal connection between protein modification and enzyme inactivation may properly be evaluated.

Naturally, a comparison of enzyme inactivation and of protein modification rates is meaningful, in the context of the argument presented here, only if modification of protein reactive groups is described by a single exponential function of reaction time. In cases where protein modification is described by summations of exponentials equations such a comparison should be undertaken only when the various microscopic steps responsible for the summations of exponentials modification event have been fully described and the specific

protein species modification rate constants have been identified (Rakitzis, 1980b).

In this connection it should be noted that protein modification and enzyme inactivation belong to different classes of phenomena, and that what appears as a causal link between these two classes may be sheer coincidence: enzyme inactivation may be caused by the modification of an unsuspected kind of reactive group, while the rate of modification of the groups assumed to be responsible for enzyme activity loss may be equal to (or one-half, one-third etc.) the rate of enzyme inactivation, thereby giving rise to the conclusion that one (or two, three etc.) modifiable group of the kind under examination is essential for enzyme activity. One is reminded of Geulincx's two clocks, both keeping perfect time, one of which will strike whenever the other points to the hour (Russell, 1948).

An example of the correct application of first principles in the elucidation of the causal connection between protein modification and enzyme inactivation is the study of the modification of *S*-adenosylhomocysteinase by iodoacetamide (Gomi & Fujioka, 1982). It was found by these authors that the number of cysteine residues modified, as determined by amino acid analysis, is equal to the number of residues modified, as determined by iodo[1-¹⁴C]acetamide incorporation, thereby establishing that modification of cysteine residues is the necessary and sufficient condition for enzyme inactivation. It was also found that both enzyme inactivation and modification of cysteine residues can be described by single exponential functions of reaction time, the constant for enzyme inactivation being twice the constant for modification of cysteine residues. The plot of fractional enzyme activity versus extent of protein modification is identical with a plot calculated on the assumption, at this stage of the investigation turned into a firm conclusion, that two out of the three modifiable cysteine residues of *S*-adenosylhomocysteinase are essential for enzyme activity.

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