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A Kinetic Approach to Defining the Role of Chemically Modifiable Residues at the Active Sites of Enzymes

Leonie K. Ashman[A] *and D. Bruce Keech*

Department of Biochemistry, University of Adelaide, Adelaide, S.A. 5001.
[A]Present address: Department of Surgery, University of Adelaide, Adelaide, S.A. 5001.

Abstract

Initial velocity studies can be used to define the function of chemically modifiable residues in the active site of a multisubstrate enzyme. Since the method relies on measuring biological activity, it has the advantage that it can be used with small amounts of relatively impure enzyme, but requires that modified enzyme molecules have some residual catalytic activity. The kinetic analysis of the modified enzyme can be carried out in the presence of some unmodified enzyme molecules.

Consideration of examples of single- and multisubstrate enzymic reactions shows that interpretation of changes in apparent K_m and apparent V values following chemical modification, in terms of effects on substrate binding or catalysis or both, requires a detailed knowledge of the kinetic reaction sequence. In the case of multisubstrate enzymes, it is necessary to ensure that, during the kinetic investigation, the concentrations of the non-varied substrates remain at near-saturating levels. For this reason, and because modification may induce changes in the rate-limiting step, a full kinetic analysis of the modified enzyme is advisable.

Introduction

By taking advantage of the enhanced reactivity which frequently distinguishes catalytically important amino acids, and using group specific reagents, selective covalent modification of residues at the active centre of enzymes can be achieved. While a loss of biological activity may indicate that the affected residue is in or near the active centre, the precise function of the residue is not immediately obvious.

In cases where total inactivation results from chemical modification, no information on the role of the residue in the reaction mechanism can be deduced from a kinetic approach (Knowles 1965). However, where an enzyme can be modified so that its catalytic activity is decreased, but not destroyed, it may be possible to assign a role to the modified residue by a kinetic analysis of the residual activity. This approach, originally suggested by Ray and Koshland (1961), has been used by Knowles (1965) who showed that a modifiable methionine residue at the active centre of α-chymotrypsin is involved in binding the aromatic side chain of the substrate. Previous work in this laboratory (Edwards and Keech 1967) has indicated the usefulness of carrying out initial velocity studies at several stages during the modification process in order to define the function of a reactive cysteinyl residue in propionyl-CoA carboxylase. However, a theoretical basis for the interpretation of the results of such studies and an assessment of the potential of the method has been lacking.

Since modification of a unique residue in all of the enzyme molecules is approached exponentially, it is not always possible to carry out complete modification without significantly affecting other less reactive residues. Furthermore, modified and

unmodified enzyme molecules may be difficult to separate physically. In this paper a method for determining the kinetic properties of the modified enzyme in the presence of unmodified enzyme molecules is presented. The observed changes in apparent kinetic constants arising from chemical modification are interpreted in terms of a substrate binding or catalytic function of the affected residue.

Theory

Kinetic Properties of a System Containing Modified and Unmodified Enzyme Molecules

The following treatment assumes that a decrease in catalytic activity is due to reaction of the modifying reagent with one specific amino acid per active site. The kinetic criteria for ascertaining the number of catalytically significant groups per active centre which are affected during a modification reaction have been defined by Ray and Koshland (1961) and extended by others (Levy *et al.* 1963; Keech and Farrant 1968). It is appreciated that reactions between the reagent and other residues may occur but provided such reactions do not affect the catalytic activity then they will not influence the final conclusions. If, however, modification of a group away from the active centre affects the enzymic activity then it will automatically come into consideration either as a non-specific denaturant or as influencing a particular rate constant. Whether or not an essential residue is actually located in the active site can be determined by using substrates, products or analogues of these to protect the active site against chemical modification.

A system which contains a mixture of native and chemically modified enzyme molecules having different kinetic properties is analogous to one in which there are two different enzymes (isozymes) present, both catalysing the same reaction. Provided Michaelis–Menten kinetics are obeyed by both species, the initial catalytic velocity, v_{obs}, as a function of substrate concentration, S, is given by

$$v_{obs} = EkS/(K+S)+E'k'S/(K'+S), \tag{1}$$

where E, k, K, E', k' and K' are enzyme concentration, the rate constant for product formation and apparent K_m values for the native and modified enzymes respectively.

Since standard size aliquots are removed for assay during the course of a chemical modification experiment, then the total amount of enzyme used per assay ($E+E'$) is constant and equation (1) may be written as

$$v_{obs} = xVS/(K+S)+(1-x)V'S/(K'+S), \tag{2}$$

where x is the proportion of the enzyme in the native form, V is the maximum velocity when all of the enzyme is in the native form, and V' is the maximum velocity when all of the enzyme is in the modified form.

Curves of the form of equations (1) and (2) are not true hyperbolae and do not give linear double reciprocal plots (Cleland 1970) except in the limiting cases where (i) no modification has occurred, i.e. where $x = 1$, (ii) complete modification has occurred, i.e. $x = 0$, (iii) no change occurs in the Michaelis constant during modification, i.e. $K = K'$, or (iv) $V' = 0$, i.e. where the modified enzyme is completely

inactive. In other cases, double reciprocal plots approximate linearity only over limited ranges of substrate concentrations. Failure to recognize this behaviour could lead to erroneous conclusions about the effect of modification on the kinetic constants of the enzyme. A theoretical example is depicted in Fig. 1, where curves are generated

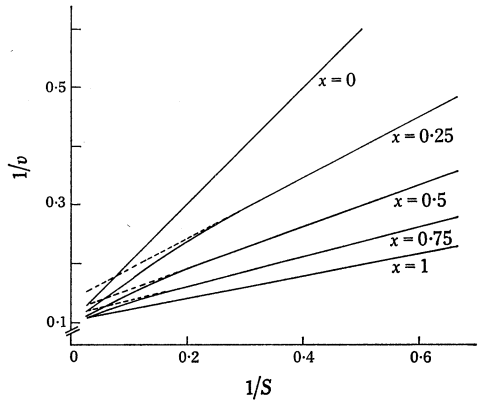


Fig. 1. Theoretical $1/v$ versus $1/S$ plots for a mixture of two enzyme species catalysing the same reaction but having different K_m values. The curves were computed according to equation (2) for different values of x and for S in the range 1·5–40, taking $V = V' = 10$, $K = 2$ and $K' = 10$. The solid lines are the computed curves, while the dotted lines indicate the deviation from linearity for $0 < x < 1$.

for different proportions of the enzyme in the unmodified and modified forms and taking $V = V' = 10$, $K = 2$ and $K' = 10$. This represents the situation where the V is unchanged by chemical modification but the K_m value increases fivefold. It should

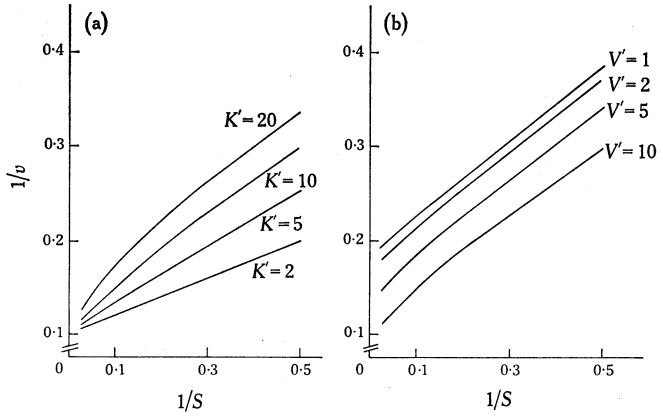


Fig. 2. Theoretical $1/v$ versus $1/S$ plots, computed according to equation (2), illustrating the effect of (*a*) the value of K' and (*b*) the value of V' on the extent of deviation from linearity. For (*a*), $V = V' = 10, K = 2, x = 0{\cdot}5$. For (*b*), $V = 10, K = 2, K' = 10$, $x = 0{\cdot}5$.

be noted that the extent of deviation from linearity depends on the relative values of K_m and V for the native relative to the modified enzyme. In general, it will be more pronounced where K' is much greater than K and less when V' is small relative to V. These points are illustrated in Figs 2*a* and 2*b*.

Determination of Kinetic Constants for the Modified Enzyme

For any given amino acid residue in the protein, assuming that the concentration of the modifying reagent remains constant (i.e. it is present in excess), the proportion of that residue remaining unmodified (x) at any given time (t) is given by

$$x = e^{-kt}, \tag{3}$$

where k is the pseudo first-order rate constant for the modification reaction. Thus, from equations (2) and (3), at any time t after the initiation of a modification reaction, the observed catalytic activity, v_t, for an aliquot of the enzyme removed for assay at a fixed substrate concentration, S, is given by

$$v_t = e^{-kt} v_0 + (1 - e^{-kt}) v_\infty, \tag{4}$$

where v_0 is the catalytic activity at time zero, i.e. $VS/(K+S)$, and v_∞ is the catalytic activity at infinite time, i.e. $V'S/(K'+S)$. Rearranging equation (4) leads to

$$v_t = (v_0 - v_\infty)e^{-kt} + v_\infty. \tag{5}$$

Thus the value of k (and of v_∞) can be obtained by fitting a curve of this form to a set of (t, v_t) data points,* and then using equation (3) the value of x at any time t can be determined.

K and V of equation (2) can be readily determined from a set of (S, v_{obs}) data for the t_0 enzyme sample (i.e. when $x = 1$). Then for any specific values of S and t, the contribution of the unmodified enzyme to the observed reaction velocity (v_{obs}), i.e. $xVS/(K+S)$, can be evaluated. The velocity, v', which is due to the modified enzyme can be calculated from

$$v' = v_{obs} - xVS/(K+S) = (1 - x)V'S/(K'+S); \tag{6}$$

substituting v'' for $v'/(1 - x)$, gives

$$v'' = V'S/(K'+S), \tag{7}$$

which is a hyperbolic function of the form of the Michaelis–Menten equation enabling calculation of V' and K'.

* Many general non-linear fitting programs are available for a large computer. A method which can be used with a programmable desk computer is as follows:

Determine a reasonable range for v_∞, say $a < v_\infty < b$. Then with v_0 known, the data can be transformed

$$(v_t - a)/(v_0 - a) = e^{-kt},$$

i.e.

$$\ln[(v_t - a)/(v_0 - a)] = -kt,$$

and k can be determined by the usual linear least squares procedure, with a residual sum of squares. The process is then repeated with a series of increasing values for v_∞. The best estimate of v_∞ is that which gives rise to the minimum residual sum of squares. Using this value the final estimate of k is obtained. Several methods for fitting an equation of this form are described by Draper and Smith (1966).

Thus K, V, K' and V' can be determined from two v_{obs} versus S curves; one for the unmodified enzyme and another at a time during the modification reaction provided k (and hence x) is determined separately.

Interpretation of Changes in Apparent Kinetic Constants

Single-substrate systems—Case 1

A single-substrate single-product enzyme which obeys classical Michaelis–Menten kinetics can be described by the following reaction sequence:

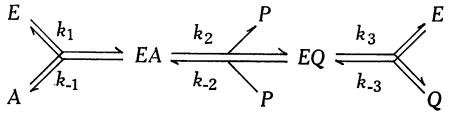
$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_2} E + P$$

where E, A, EA and P are the concentrations of free enzyme, free substrate, enzyme–substrate complex and product respectively, and k_1, k_{-1} and k_2 are the rate constants for the reactions indicated. In this case, $K_m = (k_{-1}+k_2)/k_1$ and $V = k_2[E_t]$, where $[E_t]$ is the total enzyme concentration.

If on modification a substrate-binding residue or a residue close to the binding site is modified in such a way as to influence the value of k_1 or k_{-1} or both, then changes in the value of K_m but not V will result. If a catalytic residue is modified resulting in changes in k_2, then changes in both K_m and V will occur. If $k_1 \simeq k_{-1} \simeq k_2$, then significant changes in K_m will be observed; however, if the value of k_2 is very much smaller than k_1 (and k_{-1}), (i.e. where product formation is the rate-limiting step) then $K_m \simeq k_1/k_{-1}$ and changes in the value of K_m will be less pronounced. If the modification is non-specific corresponding to general denaturation of the enzyme with a decrease in the effective enzyme concentration $[E_t]$, the result obtained will be a decrease in V with no change in the value of K_m.

Single-substrate systems—Case 2

A reaction involving two products and two transition-state complexes can be described by the following sequence:



Expressions for K and V in terms of rate constants for the individual steps can be derived using the method of King and Altman (1956). Using initial velocity conditions where $P = Q \simeq 0$, the following expressions are obtained:

$$K_m = (k_2k_3+k_{-1}k_3)/(k_1k_2+k_1k_3)$$

and

$$V = k_2k_3[E_t]/(k_2+k_3).$$

If the rate-limiting step is much slower than the others, some simplification is possible (Zerner and Bender 1964). Assuming that k_2 is rate limiting so that $k_2+k_3 \simeq k_3$ and $k_1, k_{-1} \gg k_2$, then

$$K_m \simeq k_{-1}/k_1,$$

and

$$V \simeq k_2[E_t].$$

This is analogous to case 1. However, where k_3 is rate limiting, so that $k_2+k_3 \simeq k_2$,

$$K_m \simeq (k_2k_3+k_{-1}k_3)/k_1k_2$$

and

$$V \simeq k_3[E_t].$$

Thus, alteration of k_1 or k_{-1} or both will again affect K_m without changing V. A decrease in k_2 will also affect K_m but not V (unless k_2 becomes rate limiting), and a decrease in k_3 will decrease both K_m and V in such a way that K_m/V will remain constant. Thus, determination of the step affected requires a detailed knowledge of the reaction sequence and recognition of the fact that modification may cause a different step in the process to become rate limiting. In addition, in view of the fact that binding and catalysis are not necessarily mutually exclusive functions, a situation may arise where the rate constants for both binding and catalysis are changed on modification. For some mechanisms, an effect of modification on both catalysis and binding may be difficult to distinguish from a specific effect on a catalytic step.

Multisubstrate systems

For a multisubstrate system, the situation is more complex since the expressions for the apparent K_m value for a particular substrate and apparent V contain terms relating to the fractional saturation of the enzyme with the other substrates. For example, for a two-substrate two-product system with a non-sequential (ping pong) sequence

$$\text{app } K_a = K_a/(K_b/B+1)$$

and

$$\text{app } V = V/(K_b/B+1).$$

If substrate B is at saturating concentrations ($B \gg K_b$), then the denominator in each case approaches unity and app $K_a \simeq K_a$, and app $V \simeq V$. If modification alters K_b so that the fixed level of substrate B is no longer saturating, then the denominator for both expressions becomes greater than unity, and both app K_a and app V will be decreased although the ratio of app K_a/app V will be unaltered.

For a two-substrate system with a sequential mechanism,

$$\text{app } K_a = K_a[(K_{ia}K_b/K_aB)+1]/(K_b/B+1)$$

and

$$\text{app } V = V/(K_b/B+1).$$

Once again, app $K_a \simeq K_a$ and app $V \simeq V$ if $B \gg K_b$, and if modification increases the value of K_b such that B is no longer saturating, app V will decrease and app K_a may increase, decrease or remain constant depending on the values of K_a and K_{ia}. In this case,

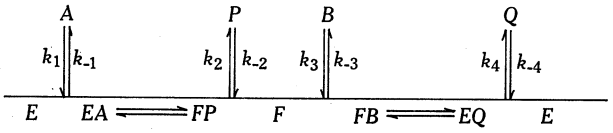
$$\text{app } K_a/\text{ app } V = K_a[(K_{ia}K_b/K_aB)+1]/V$$

will not be constant since it contains the term K_b/B.

In the case of a multisubstrate enzyme, K_m and V are more complex functions of rate constants than in the case of a single-substrate enzyme system. For any given mechanism, these kinetic constants can be expressed in terms of the rate constants for the individual steps. From a consideration of which kinetic constants are changed or

unchanged by modification, and which rate constants they have in common, it should be possible to determine which particular rate constants have been affected. However, as for the single-substrate system, it is necessary to assess the relative contributions of the various rate constants to the values of K_m and V, and this depends on which step is rate limiting. Thus, a knowledge of the relative magnitudes of the different rate constants, as well as the reaction mechanism, is necessary for the valid interpretation of experimental data.

For example, consider the case of a ping pong mechanism corresponding to the following scheme:



where

$$V = k_2k_4[E_t]/(k_2+k_4),$$

$$K_a = k_4(k_{-1}+k_2)/k_1(k_2+k_4),$$

$$K_b = k_2(k_{-3}+k_4)/k_3(k_2+k_4).$$

As shown for the single-substrate system, if one step in the reaction is much slower than the other, some simplication of these expressions occurs.

(a) Where the formation and release of P is rate limiting, i.e. $k_2 \ll k_4$, then

$$V \simeq k_2[E_t],$$

$$K_a \simeq (k_2+k_{-1})/k_1,$$

and

$$K_b \simeq k_2k_{-3}/k_3k_4+k_2/k_3.$$

(b) Where the formation and release of Q is rate limiting, i.e. $k_2 \gg k_4$, then

$$V \simeq k_4[E_t],$$

$$K_a \simeq k_4k_{-1}/k_1k_2+k_4/k_1,$$

and

$$K_b \simeq (k_{-3}+k_4)/k_3.$$

Thus, in case (b), an increase in K_b with no appreciable change in V or K_a indicates a change in k_3 or k_{-3} or both. A similar experimental result can be explained in case (a) by a decrease in k_4 provided k_4 remains $\gg k_2$.

Discussion

Kinetic studies using chemically modified enzymes which retain some catalytic activity can give information on the function of specific residues in the dynamics of catalysis. The method has the particular advantage that it can be used with small amounts of relatively impure enzyme. While final conclusions on the function of modifiable residues in the catalytic reaction must take account of the effect of the introduction of sometimes bulky groups into the enzyme (Cohen 1970), the usefulness

of the method has been clearly demonstrated by investigations on hydrolytic enzymes such as α-chymotrypsin (Lawson and Schramm 1962; Knowles 1965). The study described here indicates that the method can be extended and used with multisubstrate enzymes, provided the reaction mechanism is known, and may be used even when it is not possible to obtain a homogeneous preparation of modified enzyme. Although we have concentrated on the modification using group-specific amino acid reagents in the foregoing discussion, the general ideas are also applicable to determining the role of residues modified using affinity labels.

Two important conclusions emerge from this study.

(i) A mixture of native and chemically modified partially active enzyme molecules, as occurs during the progress of a limited modification reaction, cannot be considered in the same way as a system containing a reversible inhibitor. In particular, v_{obs} versus substrate concentration profiles are not true hyperbolae and failure to recognize this fact can lead to erroneous conclusions about the effect of modification on the kinetic constants of the enzyme. Where the enzyme modification is complicated by the occurrence of slower side reactions so that the reaction cannot be taken to completion, the following procedure can be used to determine the kinetic constants K' and V' for the modified enzyme. Incubate the enzyme with the modifying reagent, removing samples, quenching the reaction and assaying under standard conditions at t_0 and several other times during the modification reaction. Using equation (5), determine the rate constant, k, for the modification reaction. Then the proportion of native enzyme remaining at any time, t, can be calculated using equation (3). Carry out an initial velocity study on the t_0 sample and the sample taken at time t. (In practice, the samples used will be chosen compromising between the desire to minimize the amount of native enzyme present and yet avoid complications due to secondary, non-specific modification reactions. Also, theoretical examples depicted in Figs 1 and 2 illustrate the need to use a wide range of substrate concentration in these experiments.) Knowing the value of x for that particular sample, the reaction velocity due to the modified enzyme at various substrate concentrations, and hence K' and V', can be determined using equations (6) and (7).

(ii) Changes in K_m and V values resulting from chemical modification must be interpreted with caution. The foregoing analysis makes it clear that, even for a single-substrate enzyme, a knowledge of the mechanism, enabling expression of the kinetic constants in terms of rate constants for the individual steps in the reaction and the rate-limiting step, is essential for the interpretation of results. For a multisubstrate enzyme there is the additional complication that the level of a fixed substrate may be non-saturating for the modified enzyme. This situation should be readily apparent if the effect of modification on the app K_m value for each substrate is examined. However, it is desirable that a complete initial velocity study, varying both substrates, be undertaken before and after modification since this would indicate what changes had occurred in the values for all kinetic constants.

Acknowledgments

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