The activation of enzymes by metal ions

Attempts have been made to interpret quantitatively metal ion activation data in terms of dissociation constants for metal-enzyme interactions[1,2,3,4,5,6,7], obtained by use of equations of the type:

$$K = \frac{[\text{Enzyme}]\,[\text{Metal}]}{[\text{Metal-enzyme}]} = \frac{V_{max}-V}{V} \times [M] \qquad (1)$$

where V is the initial velocity, or some function related to it, at a metal ion concentration $[M]$ and V_{max} is the maximum attainable velocity with increasing metal ion concentration but all other factors constant. For such an equation to be valid certain criteria must be satisfied. The kinetic order of the reaction must remain unchanged for all metal ion concentrations and $[M]$ must refer to the concentrations of *free* metal ions — including hydrated forms, but excluding forms such as MOH^+. Further, it must be demonstrated that equation (1) is compatible with the kinetic behaviour of the system.

Investigations are being carried out in this laboratory on the cobaltoustion-activated glycylglycine dipeptidase from rat muscle and human uterus. In this system the number of metal ions chelated by the enzyme protein is negligible compared with the number chelated by the substrate since the effective molar ratio of substrate to protein is at least 100:1. In the pH region where $[H^+]$ is very much less than the carboxyl dissociation constant of glycylglycine, the concentrations of free metal ions and of various chelate complexes may be calculated from the equations:

$$\frac{[M_0^{2+}]}{[M^{2+}]} = 1 + [L^-]\,K_1 + [L^-]^2\,K_1 \cdot K_2 \qquad (2)$$

$$[L^-] = \frac{[L_0] - \bar{n}\,[M_0^{2+}]}{1 + [H^+]/K_a} \qquad (3)$$

where $\bar{n}$ represents the average number of ligands bound per total metal ion, L_0 and L^- the total and anionic species of glycylglycine, and M_0^{2+} and M^{2+} the total and free metal ion. K_a is the amino group dissociation constant and K_1 and K_2 are the first and second association constants of the metal-glycylglycine system. These equations may be solved by successive approximations.

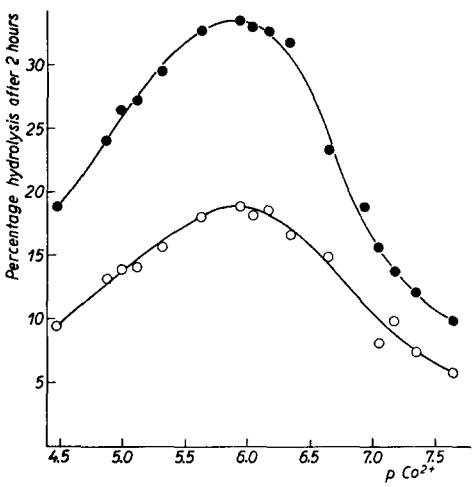

Fig. 1. pCo^{2+} activity curves of glycylglycine dipeptidase at pH 8.0, initial glycylglycine concentration 0.05 M and 25° C. The enzyme preparation was a rat muscle extract, ● Final dilution 1:20; ○ Final dilution 1:40.

Calculation of the free metal ion concentration by these methods enables pMetal-activity curves, analogous to the familiar pH-activity curves, to be obtained for metal-activated enzymes. Two such curves for glycylglycine dipeptidase are illustrated in Fig. 1. They have the typical bell-shape so often observed for pH-activity curves. The low pCo^{2+} portions reflect the experimental observation that concentrations of cobaltous ions greater than a critical level are inhibitory[8]. From the high pCo^{2+} portions, K of equation (1) may be calculated to be $1.1 \cdot 10^{-7} M$, assuming that the results can be interpreted in this manner. SMITH[1] gives a value of $2.8 \cdot 10^{-5} M$ at pH 8.0 and 40° C. The divergence between these values illustrates the magnitude of the error introduced by ignoring the chelation of metal ions by the substrate. SMITH's value may be corrected using the apparent thermodynamic data at 40° C for glycylglycine[9], $pK_a = 7.86$, $\log K_1 = 3.08$, $\log K_2 = 2.37$. The corrected value thus obtained is $K = 9.7 \cdot 10^{-8}$, in reasonable agreement with the value obtained in this laboratory.

Under the conditions employed in obtaining the pCo^{2+} activity curves the reaction follows apparent zero-order kinetics with respect to substrate, and no enhancement of the reaction rate can be demonstrated by pre-incubating the enzyme with the metal ion activator[1,8]. It is important to see whether this is compatible with the tacitly assumed kinetic scheme[1],

$$E + A \underset{\text{(active enzyme)}}{\rightleftharpoons} E\text{-}A \quad \text{(equilibrium rapidly attained)}$$

$$E\text{-}A + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-}A\text{-}S \xrightarrow{k_3} E\text{-}A + \text{products}$$

In this scheme it can be shown that the velocity is given by $V = \frac{k_3 \cdot e \cdot x}{K_m(1 + K/a) + x}$ (4)

where e, x and a are the concentrations of enzyme (total), substrate and free metal ion,

$$K_m = \frac{k_2 + k_3}{k_1} \text{ and } K = \frac{[E]\,[A]}{[EA]}$$

From equation (4) it follows that zero-order kinetics can only be observed if $x \gg K_m(1 + K/a)$. Under these circumstances $V = k_3 \cdot e$ and the reaction rate is independent of activator concentration. Thus the simultaneous occurrence of zero-order kinetics and rate dependence on metal ion concentration is inconsistent with the above reaction sequence.

One of a number of reaction schemes which fits the kinetic data is:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \qquad ES + A \underset{}{\overset{K_{D'}}{\rightleftharpoons}} ESA \qquad ESA + A \underset{}{\overset{K_{D''}}{\rightleftharpoons}} ESA_2 \qquad SAE \xrightarrow{k_3} E + A + \text{products}$$

On these assumptions the velocity of the reaction is given by

$$V = \frac{k_3 \cdot e \cdot x}{(k_2 \cdot K_{D'}/a + k_3)/k_1 + (1 + K_{D'}/a + a/K_{D''}) \cdot x} \quad (5)$$

where $K_{D'} = \frac{[A]\,[ES]}{[ESA]}$ and $K_{D''} = \frac{[A]\,[ESA]}{[ESA_2]}$

This reaction scheme implies that the activating metal ion combines with the enzyme substrate complex and the resultant intermediate is decomposed to give the products of the reaction. It is noteworthy that either first- or zero-order kinetics can be followed at varying activator concentrations. The experimental data fit this scheme remarkably well.

Another possibility[10] is that the metal-substrate 1:1 complex is the true substrate for the enzyme. Kinetic equations can be developed for this case. This scheme is difficult to distinguish experimentally from that of equation (5), although the evidence suggests that the activator combines with the enzyme-substrate complex. Further elucidation of the problem must await the availability of purified enzyme preparations and a precise determination, at varying activator concentrations, of the apparent Michaelis parameters k_3' and K_m' obtainable from the LINEWEAVER-BURK plot.

A different type of kinetic evidence is available concerning the role of the metal ion in systems such as leucine aminopeptidase. In this a measurable time reaction between the metal ion activator and the enzyme is observed. It is then clear that the activation phenomenon must involve the formation of a metal-protein complex. If it is assumed that the metal-enzyme equilibrium is not disturbed by the addition of the substrate, then the dissociation constant of the metal-protein complex can be obtained from data under conditions of either first- or zero-order kinetics. The kinetic data for leucine aminopeptidase have been reduced on this basis[2,3].

Thus, considerable care is required in the interpretation of metal-activation data. The first essential is the calculation of the free metal ion concentration. Where initial velocities cannot be obtained further difficulties arise since thermodynamic data for mixed complexes, *e.g.* cobalt-glycine-glycylglycine are unlikely to be available. Kinetic data alone cannot unambiguously establish a postulated reaction sequence but can serve the useful purpose of eliminating some of the possible activation mechanisms.

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