The Determination of Binding Parameters when the Total and Free Substrate Concentrations are Not Approximately Equal

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(Received 29 January 1979)

By using a standard graphical method values for K_m and V may be found that are independent of the conditions and assumptions that the total substrate concentration approximates to its free concentration and that K_m is much larger than the enzyme concentration. The procedure is also applicable to the determination of equilibrium binding parameters of a ligand to a macromolecule.

For the analysis of enzyme kinetics it is convenient, and usually justified, to make the assumption that, if the substrate concentration is very much larger than the enzyme concentration, then the relationship between the velocity of a reaction and the substrate concentration is approximately hyperbolic. The substrate concentration giving half the maximum velocity is usually termed K_m (IUPAC, 1973). In a recent paper 'a more general definition of K_m ' (Griffiths, 1978) was proposed, and a new parameter, $K_{m(G)}$, was introduced. It was shown under the special conditions where $v = V/2$ that the substrate concentration was equal to $K_{m(G)} + ([E_t]/2)$. Eqn. (8) of Griffiths's (1978) paper:

$$v = \frac{V \cdot [S_t]}{K_{m(G)} + [S_t] + [E_t] \cdot [1 - (v/V)]}$$

does not describe a rectangular hyperbola. It is therefore not possible to derive K_m or $K_{m(G)}$ directly from a double-reciprocal plot or its equivalent (Dixon & Webb, 1964; Neame & Richards, 1972; Eisenthal & Cornish-Bowden, 1974) unless $[E_t] \cdot [1 - (v/V)]$ is negligible compared with $K_{m(G)}$. In this case K_m and $K_{m(G)}$ are indistinguishable. From the above equation it may be seen that the assumed hyperbolic relationship between v and $[S_t]$ holds only if $K_{m(G)}$ as well as $[S_t]$ is much greater than $[E_t]$. Methods for the determination of K_m when the enzyme concentration is of a similar magnitude to, or is greater than, $K_{m(G)}$ have been described (Haas *et al.*, 1940; Reiner, 1968; Dixon, 1965, 1972; Kilroe-Smith, 1966; Smith *et al.*, 1977; discussed in Griffiths, 1979). Apart from that of Smith *et al.* (1977), which needs

a curve-fitting routine, these methods require a prior knowledge of V .

In this present paper a graphical procedure is described from which $K_{m(G)}$, K_m and V can be derived and which is independent of a prior knowledge or estimate of V . It is also independent of the condition that the total substrate concentration approximates to its free concentration. The procedure is also applicable to a ligand binding to a macromolecule, in which case it is possible to determine the dissociation constant and a constant that relates the function measured (e.g. fluorescence, absorbance) to unit concentration of ligand saturated by macromolecule. The number of binding sites may also be determined if the molecular weight of the macromolecule or enzyme is known.

Theory and Model

The velocity ($[ES] \cdot k_{+2}$ or v) of an irreversible enzymic reaction for which the following process may be assumed:

may be described by eqn. (1):

$$[ES] \cdot k_{+2} = \frac{(n[E_t] - [ES]) \cdot ([S_t] - [ES]) \cdot k_{+2}}{(k_{+2} + k_{-1})/k_{+1}} \quad (1)$$

where n is the number of catalytic binding sites per oligomer of enzyme. If $(k_{+2} + k_{-1})/k_{+1}$ is redefined as $K_{m(G)}$ (Griffiths, 1978), then eqn. (1) may be rearranged and rewritten as:

$$[ES] \cdot k_{+2} = n[E_t] \cdot k_{+2} - \frac{[ES]}{[S_t]} \cdot k_{+2} \cdot (K_{m(G)} + n[E_t] - [ES]) \quad (2)$$

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Fig. 1. (a) Velocity curves for the titration of an enzyme (E) at several fixed concentrations with a substrate (S), and (b) and (c) plots for the determination of $K_{m(G)}$, k_{+2} and n from the intercept values of plot (a)

The data for plot (a) have been calculated from the quadratic form of eqn. (2) by using concentrations of substrate from 0.1 to 1.0 unit in 0.1-unit steps ($\bullet$) and from 1.25 to 32 units ($\circ$) at concentrations of enzyme from 0.05 to 0.5 unit in 0.05-unit steps with a value for k_{+2} in units of time $^{-1}$, for n of 2 and for $K_{m(G)}$ of 0.5 unit. For plot (b) the reciprocal of the intercepts from the abscissa of plot (a) are multiplied either by the theoretical concentration of the enzyme $[E_t]$ ($\circ$) or by the value V ($\bullet$) taken from the intercepts on the ordinate of plot (a); these values are then plotted against $[E_t]$ ($\circ$) or V ($\bullet$). For plot (c) the intercept values from the abscissa of plot (a) are plotted against the same value divided either by $[E_t]$ ($\circ$) or by V ($\bullet$).

or as:

$$v = V - \frac{v}{[S_t]} \cdot (K_{m(G)} + n[E_t] - [ES]) \quad (3)$$

The family of curves in Fig. 1(a) is derived by plotting v against $v/[S_t]$ (Eadie *et al.*, 1949; Hofstee, 1959). Details of the calculation of v are given in the legend of Fig. 1. The resulting curves show that the relationship is not hyperbolic. However, at both low and high v the curves in the plot are approximately linear. At low v it is possible to extrapolate towards the abscissa. As $[S_t]$ and therefore v tend to zero eqn. (3) approximates to:

$$V = \frac{v}{[S_t]_{[S_t] \rightarrow 0}} \cdot (K_{m(G)} + n[E_t]) \quad (4)$$

and the intercept:

$$\frac{v}{[S_t]_{[S_t] \rightarrow 0}} = \frac{V}{K_{m(G)} + n[E_t]}$$

At high v it is possible to extrapolate towards the ordinate. It can be seen from eqn. (3) that as $[S_t]$ approaches infinity the value of v approaches V . The lines in Fig. 1(a) are drawn by eye. The intercepts on the abscissa are all overestimates (by less than 3%) of the calculated values of $V/(K_{m(G)} + n[E_t])$. The intercepts on the ordinate are also overestimates (by less than 5%) of the model values of V .

The parameters $K_{m(G)}$, k_{+2} and n may be found from the following two plots, shown in Fig. 1(b): the first gives k_{+2} and $K_{m(G)}/n$, the second $K_{m(G)}$. If the concentration of the enzyme, $[E_t]$, is divided by the corresponding intercept value from the abscissa of Fig. 1(a) it yields $(K_{m(G)} + n[E_t])/n \cdot k_{+2}$. This when plotted against $[E_t]$ gives a straight line with a slope of $1/k_{+2}$ and an intercept value on the abscissa equal to $K_{m(G)}/n$. The line drawn through the open circles in Fig. 1(b) is such a plot; the slope and intercepts have the model values. The parameter $K_{m(G)}$ may be found in an analogous manner if the values of V taken from the intercepts on the ordinate of Fig. 1(a) are substituted for the corresponding values of $[E_t]$. The resulting plot of $K_{m(G)} + n[E_t]$ against V is shown by the line, which has the model values for the slope and intercepts, drawn through the filled-in circles in Fig. 1(b).

Alternatively the parameter $n/K_{m(G)}$ may be derived by plotting the intercept values $v/[S_t]$, taken from the abscissa of Fig. 1(a), against the same value divided by $[E_t]$ (Fig. 1c, filled-in circles). The justification of this plot is found by rearrangement of eqn. (4), to give:

$$\frac{v}{[S_t]_{[S_t] \rightarrow 0}} = k_{+2} - \frac{K_{m(G)}}{n[E_t]} \cdot \frac{v}{[S_t]_{[S_t] \rightarrow 0}}$$

This rearrangement, which is in the form for a

Hofstee plot, shows that the relationship between $v/[S_t]_{[S_t] \rightarrow 0}$ and $[E_t]$ is hyperbolic. The data derived by this procedure from Fig. 1(a) are plotted in Fig. 1(c) (open circles). The line drawn through these data has the model intercept of k_{+2} on the ordinate and $k_{+2} \cdot n/K_{m(G)}$ on the abscissa. The parameter $1/K_{m(G)}$ may be found by the same procedure if the appropriate intercept value V taken from the ordinate of Fig. 1(a) is used instead of the value $[E_t]$. The line drawn through the filled-in circles in Fig. 1(c) is such a plot of the data derived from Fig. 1(a). The slope and the intercepts are the model ones.

In Fig. 1(c) the parameter k_{+2} is determined as $[E_t]$ tends to infinity, and, as $v/[S_t]$ tends to zero, its accuracy is independent of the molar concentration of E_t but is dependent on any error in the concentration of S_t . The parameter $K_{m(G)}/n$ is directly proportional to the error in $[E_t]$. $K_{m(G)}$ and n cannot be determined more accurately than the error involved in extrapolation of the plots in Fig. 1(a) to infinite $[S_t]$. However, the values of $K_{m(G)}$ and n may be found more accurately and without extrapolation to infinite $[S_t]$ by replotting the data in Fig. 1(a) in the form of v against $v/[E_t]$ at various values of $[S_t]$. If the data in Fig. 1(a) represented by the filled-in circles are replotted in this way the resulting plot is identical with that of the filled-in circles in Fig. 1(a) if the abscissa scale is expanded n -fold. The equation describing this plot may be derived by replacing $n[E_t]$ by $[S_t]$ and $[S_t]$ by $n[E_t]$ in eqn. (3). A plot of $(K_{m(G)} + [S_t])/k_{+2}$ against $[S_t]$ (equivalent to the open circles in Fig. 1b) will have a slope of $1/k_{+2}$ and an intercept on the abscissa of $K_{m(G)}$. The corresponding plot to the open circles in Fig. 1(c) will give an intercept on the ordinate of $k_{+2} \cdot n$ and on the abscissa of $k_{+2} \cdot n/K_{m(G)}$. In this plot the parameter $k_{+2} \cdot n$ is determined as $[S_t]$ tends to infinity and as $[E_t]$ tends to zero; its accuracy is therefore dependent on any error in the molar concentration of E_t . The error in $K_{m(G)}$ is directly proportional to any error in the concentration of S_t .

In summary, from these various plots it is possible to determine values for k_{+2} and $K_{m(G)}$ that are as accurate as the molar concentration of the substrate, and a value for n that is as accurate as the molar concentration of the enzyme.

Discussion

In terms of enzyme kinetics the measurement of v at low substrate concentrations may in practice make this means of deriving $K_{m(G)}$, K_m and n unusable. Also, the use of enzyme concentrations similar to $K_{m(G)}$ may not be popular. However, it could prove useful in two instances: firstly for the determination of $K_{m(G)}/n$ in conditions, such as strong product inhibition, where low substrate concentrations have to be used; secondly in cases of substrate depletion

(discussed in Griffiths, 1979). For the estimation of n it has been assumed that the velocity resulting from the saturation of a unit concentration of substrate with enzyme is the same as that resulting from the saturation of a unit concentration of active site with substrate. It has also been assumed that all the enzyme binding sites are catalytic.

Eqns. (1) and (2) of this paper may be rewritten in general terms to describe the equilibrium binding of a ligand to a ligator. The procedures described are then applicable to the determination of equilibrium binding parameters. This applies especially where the affinity is relatively high and where it is necessary to use similar concentrations of ligand and macromolecule. If the concentration of the ligand–ligator complex is only known indirectly (e.g. from fluorescence or absorbance), then a constant may be found that relates the function measured to the concentration of the complex.

The equations are derived for the binding of a substrate to an enzyme with one or more non-interactive sites with the same $K_{m(G)}$. The analysis described is not suitable if these conditions are not met.

I thank Le Centre National de la Recherche Scientifique for financial support, Professor D. Gautheron for inviting me to work in her department and for reading the manu-

script, Dr. A. P. Dawson and Dr. C. Godinot for commenting on the equations and Dr. J. R. Griffiths for a preprint of his review.

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