Graphical Determination of Equilibrium Constants

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1. A rapid graphical method is described for determining equilibrium constants of combinations of the type $A + B \rightleftharpoons AB$. The method can be extended to give other quantities for the system.

A recurrent problem in biochemistry, and especially in enzymology, is the determination of the equilibrium constant of a reversible combination of the type $A + B \rightleftharpoons AB$ by keeping the concentration of B constant, varying the concentration of A and measuring the concentration of AB (or some quantity directly related to it, such as spectrophotometric extinction or the rate of some reaction catalysed by AB). In a typical case A might be an activator of an enzyme B; for example, AB might be a flavoprotein oxidase, B its apoenzyme, A its activator FAD, and the available data would be represented by a curve relating the observed rate of the oxidase reaction to the amount of FAD added to a constant amount of the apoenzyme. Frequently neither the absolute concentration of B nor the value of the velocity constant of the reaction catalysed by AB (or the extinction coefficient of AB, as the case may be) is known. In fact the only quantity known in absolute terms is the total amount of A added for each observation.

In cases where the affinity is comparatively low, so that the amount of A added is large in comparison with the amount of enzyme present, one of the graphical methods depending on the Michaelis equation, e.g. the Lineweaver–Burk plot, may be applicable. The Michaelis equation is expressed in terms of the concentration of free A, not the total amount of A added, but if the amount of enzyme is negligible the amount of A bound by it is very small, and the total concentration of A may be taken as giving the concentration of free A.

On the other hand, in experiments with larger amounts of enzyme, or when the affinity is high and much lower concentrations of A must therefore be used, an appreciable fraction of A will be bound by the enzyme. In this case the free concentration will differ widely from the total concentration, and a Lineweaver–Burk plot in terms of total A will not give correct values of the constant. It may be possible to calculate the constant, but the procedure is rather laborious. There is, however, a very rapid graphical method, as follows.

Method

Let us assume that we have a curve relating the velocity (v) of the reaction catalysed by the enzyme to the total added concentration (a_t) of the activator A, with a constant amount of apoenzyme (see Fig. 1). A horizontal line is first ruled to represent V, the maximum velocity obtained with excess of A. From the origin a tangent to the curve is drawn and produced to cut the V-line. Another line is ruled from the origin through the point on the curve at which v is half V, and this is also produced to cut the V-line. The distance between the two intersection points on the V-line then gives the value of the equilibrium constant of the combination of A with B. In Fig. 1, $K = 2 \cdot 0 \times 10^{-7}$M.

Theory

Let a, b and p be the concentrations of A, B and AB respectively, and let the subscript t denote total concentrations (i.e. the sum of the concentrations of the free and bound forms of the substance), the absence of subscript denoting concentrations of free forms.

Then
$$a_t = a + p \tag{1}$$
$$b_t = b + p \tag{2}$$

and if k is the velocity constant of the reaction catalysed by AB
$$v = kp \tag{3}$$
and
$$V = kb_t \tag{4}$$

since the limiting value of p will be reached when the whole of the B is converted into AB.

The equilibrium constant is given by:

$$K = \frac{ab}{p} = \frac{(a_t - p)(b_t - p)}{p} = \left(\frac{a_t}{p} - 1\right)(b_t - p) \tag{5}$$

Now when a_t is very small, p is negligible in comparison with b_t, and
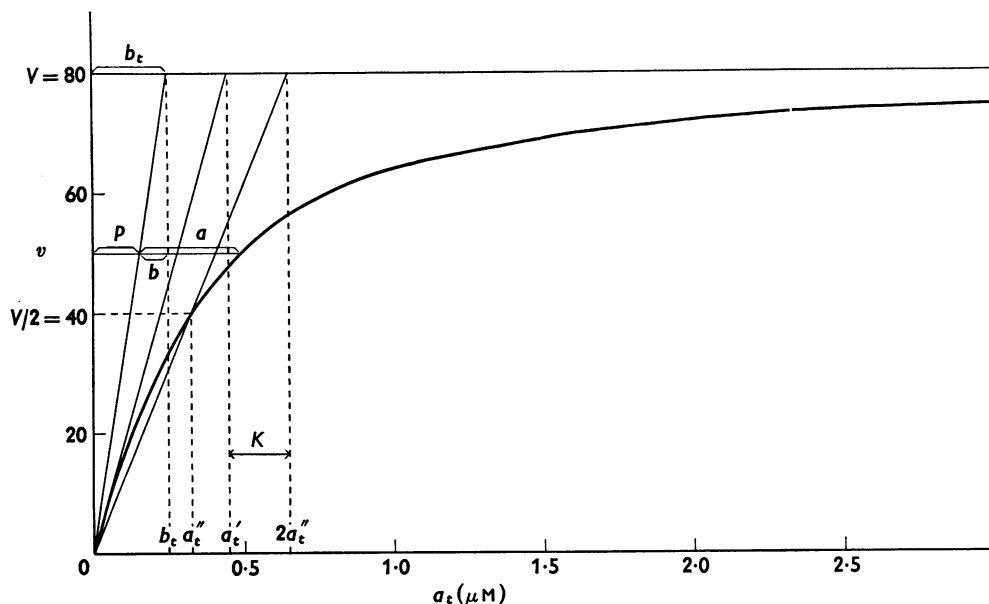
$$b_t - p = b_t = \frac{V}{k}$$

Fig. 1. Graphical method for determining the dissociation constant K, as applied to a flavoprotein oxidase. The ordinate shows the velocity (v) of the oxidase reaction in arbitrary units, and the abscissa the total concentration (a_t) of activator FAD. For other quantities see the text.

and substituting from this and eqn. (3) in eqn. (5) we get:

$$\frac{a_t}{v} = \frac{K}{V} + \frac{1}{k} \qquad (6)$$

which is the equation of the tangent. If the value of a_t at which the tangent cuts the V-line (i.e. v becomes V in eqn. 6) is denoted by a_t'

$$a_t' = K + \frac{V}{k} \qquad (7)$$

Considering now the point on the curve at which $v = V/2$, we note from eqns. (3) and (4) that here:

$$p = \frac{b_t}{2} \quad \text{and} \quad b_t - p = \frac{b_t}{2}$$

and if we denote the value of a_t at this point by a_t'', we have from eqns. (5) and (7)

$$K = \left(\frac{2a_t''}{b_t} - 1\right)\frac{b_t}{2} = a_t'' - \frac{b_t}{2}$$

$$= a_t'' - \frac{V}{2k} = a_t' - \frac{V}{k} \qquad (8)$$

Therefore

$$\frac{V}{k} = 2(a_t' - a_t'')$$

and substitution of this in eqn. (7) gives:

$$K = a_t' - 2(a_t' - a_t'') = 2a_t'' - a_t' \qquad (9)$$

Extensions

The method may be extended to the determination of other quantities from the same diagram as follows.

If a distance equal to K is set off to the left from a_t', the absolute concentration of the enzyme is obtained, for from eqns. (4) and (7)

$$b_t = a_t' - K \qquad (10)$$

and, now that both b_t and V are known, eqn. (4) gives the value of the proportionality constant k, i.e. the velocity constant of the enzyme reaction (or, in a spectrophotometric determination, the extinction coefficient of AB).

If a line is ruled from the origin to the point corresponding to b_t on the V-line, the values of a, b and p for any point on the curve can be read off, as shown in Fig. 1. This follows for p from eqns. (3) and (4), for a from eqn. (1), and for b from eqn. (2). Thus all the quantities are determined.

Comments

The validity of the method depends on two assumptions. The first is that A does not combine with other substances in the solution. The second is that one molecule of A combines with one of B.

If the reaction is $2A+B = A_2B$, involving two equilibrium constants (one for each step in the formation of the complex), the situation is much more complicated, and the method has not so far been extended to such systems. If, however, the two combining sites in B are of identical nature and so far apart that they do not influence one another, each will combine with A quite independently of the other, so that only one equilibrium constant is involved and the method is applicable. In this case b_t' is the total concentration of binding sites, rather than of B molecules.

The accuracy of determination of K by the method appears to be sufficient for general purposes, although with precise experimental observations it may be possible to obtain rather greater accuracy by calculation. The ease and speed of the graphical method, however, usually outweigh some loss of accuracy. There is no special difficulty in drawing the tangent, since curves of this nature are comparatively linear near the origin. The main source of inaccuracy is probably a tendency to underestimate the value of V, and it is important to have some experimental points at high values of a_t. A quick check on the correctness of the V-line is given by the rule (derivable from eqn. 5) that v falls short of V by one-nth at a value of a_t one-nth less than $nK+b_t$. For instance in Fig. 1, $10K+b_t$ is $2 \cdot 25 \times 10^{-6}$M; this less one-tenth is $2 \cdot 03 \times 10^{-6}$M, and at this value of a_t the curve reads 72, which is V less one-tenth.

The accuracy of b_t depends on its magnitude in relation to K. Where it is of the same order as K or larger the accuracy is usually adequate, but where it is much smaller than K the accuracy is poor, since any error in drawing the tangent then affects b_t much more than K. However, the value of b_t can be chosen at will and it should be possible to repeat the observations with a sufficiently high value to give good results.