Curves of Ligand Binding

THE USE OF HYPERBOLIC FUNCTIONS FOR EXPRESSING TITRATION CURVES

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1. The dependences of the concentrations of the non-ligated, uni-ligated and bi-ligated forms of a molecule that binds two molecules of ligand are expressed as functions of the logarithm of free ligand concentration by means of hyperbolic functions. Expressions are also given for the saturation both of an individual site and of the molecule as a whole. This form of expression allows derivation of the following points. 2. The sharpness of bell-shaped curves of concentration of the uni-ligated form is analysed in terms of the heights of their points of inflexion; these can rise to $1/\sqrt{2}$ of the height of the curve. 3. A single group can exhibit a doubly sigmoid saturation curve if this group and another have comparable affinities for a ligand, and if ligand binding at one of them diminishes the affinity at the other. If the molecular pK values pK_1 and pK_2 for the first and second molecules of ligand are called $pK^* \pm \log m$, so that $K^{*2} = K_1 K_2$ and $m^2 = K_1/K_2$, then the doubly sigmoid curve can be represented by the sum of two independent one-site saturation curves, in general of unequal height, of pK values $pK^* \pm \log \frac{1}{2}[m + \sqrt{(m^2 - 4)}]$. The error in such representation is small either if the mutual interaction between the groups (i.e. m) is large, or if the groups have very similar affinities for the ligand. 4. The sum of two one-site saturation curves, again of pK values of $pK^* \pm \log \frac{1}{2}[m + \sqrt{(m^2 - 4)}]$ but of equal heights, gives a precise value for the total saturation, provided that the binding of one molecule does not promote the binding of a second, i.e. providing that $m \geq 2$. Hence determinations of saturation cannot distinguish interacting and possibly identical sites from independent and different ones.

Although Michaelis (1914) worked out expressions for the dependence of concentrations of particular ionic species on H^+ concentration, and plotted such concentrations against pH , he and most who have followed him gave $[H^+]$ rather than pH in these expressions. This distinction might appear to be trivial, since the term $[H^+]$ can be replaced by 10^{-pH} ; nevertheless, as shown below, simplification of the expressions can be achieved by using as independent variable an expression linearly related to the logarithm of the concentration of the ligand being considered.

The hyperbolic functions (Fig. 1) prove useful for expressing these concentration dependences. Their use was suggested by the appearance of the term $[H^+]/H_{opt.} + H_{opt.}/[H^+]$ [where $H_{opt.} = \sqrt{(K_1 K_2)}$] in the expression for the concentration of the intermediate (monoprotonated) form of a dibasic acid (Dixon, 1973), since this is $2 \cosh x$ if $x = (\ln 10)(pH - pH_{opt.})$, i.e. x is linearly related to pH as required above. Irving & Williams (1949) had also noted that $\tanh x$ represents the one-site titration curve.

The advantage of using the hyperbolic functions is that expressions containing them can be manipulated easily because of the simplicities of the relations between them, such as $d(\sinh x)/dx = \cosh x$. The points

below illustrate the usefulness of this particular method of expression.

Saturation of a Single Site

Irving & Williams (1949) expressed the percentage of a substance that was protonated as $50 [1 - \tanh 1.15(pH_+ - pH)]$; 1.15 is $\frac{1}{2}(\ln 10)$. In slightly different terms, if y is the fractional concentration of the unprotonated form, which is also the degree of release of bound protons, then:

$$y = \frac{1}{2} + \frac{1}{2} \tanh \frac{1}{2}x \quad (1)$$

where x is defined as $\ln K - \ln [H^+]$. This definition makes x linearly related to pH , since $x = (\ln 10)(pH - pK)$. To define the variable $\frac{1}{2}x$ directly would be inconvenient in many of the cases treated below; $\frac{1}{2}x$ is avoided in the alternative form of eqn. (1) obtained by writing $(\sinh x)/(1 + \cosh x)$ for $\tanh \frac{1}{2}x$. Since $\tanh \frac{1}{2}x$ rises from -1 to $+1$ (Fig. 1), the factor of $\frac{1}{2}$ restricts the range of the rise to unity, and the initial term adjusts the height of the horizontal axis to give $y = 0$ when $x = -\infty$.

Expression (1) applies equally to ligands other than H^+ ions. Since in that case it is more usual to consider $\log [L]$ than the $-\log [L]$ used in the case of H^+ , the

Fig. 1. Plots of the hyperbolic functions

The definitions of the functions are: $\sinh x = \frac{1}{2}(e^x - e^{-x})$; $\cosh x = \frac{1}{2}(e^x + e^{-x})$; $\tanh x = (\sinh x)/(\cosh x)$. The power series for the first two functions are:

$$\begin{aligned}\sinh x &= x + x^3/3! + x^5/5! + \dots \\ \cosh x &= 1 + x^2/2! + x^4/4! + \dots\end{aligned}$$

same expression applies if y is the fraction ligated and x is $\ln[L] - \ln K$, where K is still the dissociation constant.

If a solute binds n molecules of ligand with so great a degree of positive co-operativity that partially ligated molecules do not exist in appreciable concentrations, then the fractional concentration of the fully ligated form is given by:

$$y = \frac{1}{2} + \frac{1}{2} \tanh \frac{1}{2} n \cdot x \quad (2)$$

This was also pointed out by Irving & Williams (1949).

2. Ligand Binding at Two Sites

Michaelis functions

Michaelis (1914) presented expressions for the concentrations of the species in the equilibrium

as fractions of their sum $[A]_t$, in terms of $[L]$, the concentration of free ligand, and of the two dissociation constants K_1 and K_2 . The expression for $[AL]$ gave, if the binding sites differed, the sum of the concentrations of the two species that had bound one

ligand molecule, one with it on one site and one with it on the other. Auerbach & Smolczyk (1924) and Sillén (1956) greatly simplified the Michaelis expressions by using the geometric mean of the two dissociation constants (here called K^*) and the parameter $m = \sqrt{(K_1/K_2)}$; Waley (1953), Alberty & Massey (1954) and Dixon (1973) found the same parameters useful for expressing the concentration of the uni-ligated species AL . If the variable x is defined as $\ln[L] - \ln K^*$, then the fractional concentrations of the species are given by:

$$\frac{[A]}{[A]_t} = \frac{1}{2} - \frac{1}{2} \frac{m + 2 \sinh x}{m + 2 \cosh x} \quad (3)$$

$$\frac{[AL]}{[A]_t} = \frac{m}{m + 2 \cosh x} \quad (4)$$

$$\frac{[AL_2]}{[A]_t} = \frac{1}{2} - \frac{1}{2} \frac{m - 2 \sinh x}{m + 2 \cosh x} \quad (5)$$

Greatly different pK values

It is well known that the Michaelis functions follow simple one-site titration curves if K_1 and K_2 differ greatly, i.e. if m is large. This can be seen if $2 \cosh d$ is substituted for m in any of the above expressions, e.g. expression (5). It then becomes:

$$\frac{1}{2} + \frac{1}{2} \frac{\sinh x - \cosh d}{\cosh x + \cosh d}$$

If m is large, $\cosh d$ in the numerator can be replaced by $\sinh d$ (see Fig. 1), giving:

$$\frac{1}{2} + \frac{1}{2} \frac{\sinh x - \sinh d}{\cosh x + \cosh d} = \frac{1}{2} + \frac{1}{2} \tanh \frac{1}{2}(x - d) \quad (6)$$

[since $\sinh A - \sinh B = 2 \cosh \frac{1}{2}(A+B) \cdot \sinh \frac{1}{2}(A-B)$ and $\cosh A + \cosh B = 2 \cosh \frac{1}{2}(A+B) \cdot \cosh \frac{1}{2}(A-B)$]. Since m is large it differs little from e^d , so that comparison of expressions (1) and (6) shows that the concentration of bi-ligated species follows a one-site saturation curve of pK equal to $pK^* + \log m$.

Bell-shaped curves

Expression (4) gives the concentration of an intermediate form, and thus the dependence on x of the rate of any reaction that is prevented by binding of ligand at one site and dissociation at another. If expressed as a fraction of its own maximum value it becomes:

$$\frac{m+2}{m+2 \cosh x} \quad (7)$$

and this is essentially the expression $(m+2)/(m+H/H_{opt.}+H_{opt.}/H)$ given by Dixon (1973), who pointed out that provided $m \geq 2$ it also gives the rate of a reaction whose pH-dependence is due to a change with pH of rate-determining step.

Fig. 2. Curves of $z = (m+2)/(m+2 \cosh x)$

These curves show dependence of the concentration of the intermediate (uni-ligated) form on the logarithm of ligand concentration. When $m = 0$ the peak is as sharp as it can be; when $m = 2$ it is as sharp as it can be without co-operative ligand binding. These curves may represent rates of reaction if the rate follows the concentration of a particular form, but sharper dependences of rate are possible if loss or gain of a ligand at one of several sites deactivates the reactant (i.e. if there are more than two binding sites). The lower horizontal scale represents pH units when the ligand is H^+ .

Fig. 2 shows curves of expression (7) (called z below) for values of m of 0, 2 and 10^3 . The curve is given for $m = 2$ because this is its value if the group (microscopic) constants are identical and independent. A value below this reflects co-operative ligand binding. It may be noted that expressions that contain only $\cosh x$ and constants, i.e. do not contain $\sinh x$ (unless it is raised to an even power), are symmetrical about the vertical axis (Fig. 1).

When $m \rightarrow 0$, $z = 1/\cosh x$ and the peak is the sharpest it can be, but of course the fractional concentration of the uni-ligated form (expression 4) then approaches zero, since the second ligand molecule binds infinitely more strongly than the first. The peak in this case appears unusually sharp, because its points of inflexion are so high. Differentiating z twice with respect to x shows that the curves have inflexions when $4 \cosh x = m + \sqrt{(m^2 + 32)}$, i.e. when $z = (2m+4)/[3m + \sqrt{(m^2 + 32)}]$. When $m = 0$, $z = 1/\sqrt{2}$ at these inflexions, so they are at 0.711 of the height. When $m = 2$ and the peak is as sharp as it can be without positively co-operative ligand binding, $z = 2/(1 + \cosh x) = 1/\cosh^2 \frac{1}{2}x$. This curve is also the slope of the one-site saturation curve, i.e. of expression (1), multiplied by 4 (cf. Irving *et al.*, 1949). The inflexions are at $\cosh x = 2$ and two-thirds of the height. As m rises further the inflexions fall towards $z = \frac{1}{2}$. For comparison, the normal distribution curve has inflexions at e^{-2} , i.e. 0.607, of its height.

Doubly sigmoid binding curves

Dixon & Tipton (1973) pointed out that the titration curve of a single group is doubly sigmoid if another group in the molecule titrates over the same range and if the binding of ligand to one group diminishes the affinity at the other. This is shown below from expressions (4) and (5); formulation in these terms makes it simple to demonstrate, and brings out the conditions under which the titration of a single group can be expressed as the sum of two one-site titrations.

In this situation the unligated form binds the first ligand molecule partly at the group under consideration (A) and partly at the auxiliary group (B), according to their group (microscopic) dissociation constants. Thus only a fraction a of the uni-ligated form has ligand bound at group A, but all of the bi-ligated form has. Hence the total fractional concentration of the bound form of group A (Fig. 3) is expression (5) plus a times expression (4), and is:

$$\frac{1}{2} + \frac{am - \frac{1}{2}m + \sinh x}{m + 2 \cosh x} \quad (8)$$

Substitution again of $2 \cosh d$ for m (a substitution that gives real values of d only when $m \geq 2$) converts expression (8) into:

$$\frac{1}{2} + \frac{\sinh x + (2a - 1) \cosh d}{\cosh x + \cosh d} \quad (9)$$

The fraction of group A ligated if a fraction a possessed a fixed pK of $pK^* - \log d$ and a fraction $(1-a)$ had a fixed (independent) pK of $pK^* + \log d$ is:

$$\frac{1}{2} + \frac{1}{2}a \tanh \frac{1}{2}(x+d) + \frac{1}{2}(1-a) \tanh \frac{1}{2}(x-d) \quad (10)$$

and this can be rearranged to give:

$$\frac{1}{2} + \frac{\sinh x + (2a - 1) \sinh d}{\cosh x + \cosh d} \quad (11)$$

This rearrangement makes use of the relation:

$$\begin{aligned} p \tanh(A+B) + q \tanh(A-B) \\ = \frac{(p+q) \sinh 2A + (p-q) \sinh 2B}{\cosh 2A + \cosh 2B} \end{aligned}$$

which follows from the definition of $\tanh$.

Expression (11) differs from expression (9) only by the change of $(2a - 1) \cosh d$ into $(2a - 1) \sinh d$ in the numerator. These terms will differ little either if d (and hence m) is large (Fig. 1) or if a is close to 0.5. In such circumstances there will therefore be little error in representing the saturation of a single site, whose affinity is influenced by binding at another site (expression 9), as the sum of two independent one-site saturations (expression 11).

The two hypothetical one-site curves that are added to represent the actual curve have dissociation constants of K^*e^d and K^*e^{-d} . The corresponding pK values prove to be $pK^* \pm \log \frac{1}{2}[m + \sqrt{(m^2 - 4)}]$ when m rather than d is used for expressing them. These are

Fig. 3. Saturation of a single site with ligand

The general expression for the filling of a single site when it is influenced by one other site is given in expression (9). The curve drawn is for $a = 0.8$ and $m = 10^{2.5}$; since m is large this resolves into the sum of two one-site saturations of expression (10) (cf. Dixon & Tipton, 1973).

not quite the same as pK_1 and pK_2 (i.e. $pK^* \pm \log m$) used for this purpose by Dixon & Tipton (1973), but they approach pK_1 and pK_2 when m is large. For cysteine, whose pK_1 and pK_2 values are 8.36 and 10.53 (Benesch & Benesch, 1955), $m = 12.2$, so that the difference between $\log m$ and $\log \frac{1}{2}[m + \sqrt{(m^2 - 4)}]$ is less than 0.01. So there is still very little error in representing the dissociation of the thiol group of cysteine by stating that 68% of the group exhibits a pK of pK_1 (8.36) and the remaining 32% one of pK_2 (10.53).

Degree of saturation of two sites

Auerbach & Smolczyk (1924) analysed the curves of proton release from a dibasic acid and Sillén (1956) showed plots of the curves like those in Fig. 4. The equation of these curves, however, has not been explicitly presented. The degree of saturation, y , is expression (5) plus half of expression (4) [i.e. expression (8) when $a = \frac{1}{2}$], and so is given by:

$$y = \frac{1}{2} + \frac{\sinh x}{m + 2 \cosh x} \quad (12)$$

When $m = 0$ this becomes $y = \frac{1}{2} + \frac{1}{2} \tanh x$, which is the particular case of eqn. (2) when $n = 2$. When $m > 2$ the transformation into expression (9) becomes possible, and gives:

$$y = \frac{1}{2} + \frac{1}{2} \frac{\sinh x}{\cosh x + \cosh d} \\ = \frac{1}{2} + \frac{1}{4} \tanh \frac{1}{2}(x + d) + \frac{1}{4} \tanh \frac{1}{2}(x - d)$$

[i.e. the relevant forms of expressions (9) and (11) are identical].

This identity has the important consequence that the saturation curve is precisely the sum of two one-site saturations of independent dissociation constants. Hence determination of the degree of saturation can never distinguish binding to two (possibly identical) sites that interact so that $m > 2$ from binding to two non-identical and independent sites.

The pK values of the two one-site curves that summate to give the actual curves are, as in the previous section, $pK^* \pm \log \frac{1}{2}[m + \sqrt{(m^2 - 4)}]$. Clearly these approach pK_1 and pK_2 (i.e. $pK^* \pm \log m$) when m is large.

Slopes and inflexions of the curve of saturation of two sites

The slope of the saturation equation (12) at $x = 0$ is $1/(m + 2)$, and so it falls from $\frac{1}{2}$ to $\frac{1}{4}$ as m rises from 0 to 2 (Fig. 4). Although the saturation can be expressed as the sum of two one-site saturations when $m > 2$, double differentiation of y shows that their pK values are not far enough apart for y to show further points of inflexion until m exceeds 4 [Auerbach & Smolczyk (1924) demonstrated this, but in a more complex way], i.e. when pK_1 and pK_2 differ by 1.2 units, and the component one-site curves have pK values $\{pK^* \pm \log \frac{1}{2}[m + \sqrt{(m^2 - 4)}]\}$ 1.14 units apart. These points of inflexion are at $\cosh x = m/2 - 4/m$. As m rises from 4 towards infinity and the slope at $x = 0$ falls from $\frac{1}{4}$ towards zero, the slope at the other points of inflexion is $m^2/8(m^2 - 4)$ and so it falls, as they move from $x = 0$ towards $x = \pm \ln m$, from $\frac{1}{4}$ towards $\frac{1}{8}$ (Fig. 4).

Note on the meaning of m

If the two ligand-binding sites considered above are identical and independent $m = 2$. When $m < 2$ the binding must be positively co-operative. When $m > 2$ it is usual to describe the binding as negatively co-operative, but it should be noted that this is not necessarily true at the group (microscopic) level, since $\sqrt{m} = K_1/K_2 = (2 + K_A/K_B + K_B/K_A)K_A/K'_A$, where K_A and K_B are the dissociation constants for the two groups when the other group is ligated, and K'_A is the constant for group A when group B is unligated (see, e.g., Dixon & Tipton, 1973). Hence K_1/K_2 can exceed 4, i.e. m can exceed 2, even if K_A does not exceed K'_A , i.e. if binding ligand at one site does not diminish affinity at the other. This is only so if K_A and K_B differ, so it does not apply to identical sites. Except for K_A , K'_A and K_B mentioned in this section, all the dissociation constants mentioned above are molecular (macroscopic, titration) constants, since the Michaelis functions contain only such constants. They apply to the first and second ligand molecules to bind, in whatever way each of these partitions between different sites.

3. Saturation of Four Sites

An expression corresponding to eqn. (12) in the two-site case can be obtained for the saturation of four sites if two major simplifying assumptions are made. The first is that the four binding sites are identical and the second is that the binding of each molecule of ligand increases the group dissociation constant at

Fig. 4. Saturation of a two-site binding molecule

The curves are of $y = \frac{1}{2} + (\sinh x)/(m+2 \cosh x)$; this represents saturation if $x = \ln [L] - \frac{1}{2} \ln K_1 K_2$ and $m = \sqrt{(K_1/K_2)}$ (cf. Auerbach & Smolczyk, 1924; Sillén, 1956).

m	Co-operativity	Notes
0	Infinite	$y = \frac{1}{2} + \frac{1}{2} \tanh x$
<2	Positive	
2	Zero	$y = \frac{1}{2} + \frac{1}{2} \tanh \frac{1}{2} x$
>2	Negative	y is the sum of two one-site saturation curves
>4		y shows three points of inflexion: at $x = 0$ and at $\cosh x = m/2 - 4/m$

any other site by the same factor, called b^2 . Such assumptions could be true in the case of tetrahedral symmetry. Then if the group dissociation constant of the first ligand molecule bound is called K , the molecular constants for the four bound are $K/4$, $\frac{3}{2}b^2K$, $\frac{3}{2}b^4K$ and $4b^6K$. If x is defined as $\ln [L] - \ln K - 3 \ln b$ (so that $x = 0$ when $[L] = b^3 K = K^*$), the saturation y is given by:

$$y = \frac{1}{2} + \frac{\sinh 2x + 2b^3 \sinh x}{\cosh 2x + 4b^3 \cosh x + 3b^4}$$

$$= \frac{1}{2} + \frac{(\sinh x)(\cosh x + b^3)}{2 \cosh^2 x + 4b^3 \cosh x + 3b^4 - 1} \quad (13)$$

When $b = 0$, $y = \frac{1}{2} + \frac{1}{2} \tanh 2x$ (a particular case of expression 2); when $b = 1$, the sites are independent and $y = \frac{1}{2} + \frac{1}{2} \tanh \frac{1}{2} x$; when $b > 1$, $y = \frac{1}{2} + \frac{1}{2} \tanh \frac{1}{2} (x - g) + \frac{1}{2} \tanh \frac{1}{2} (x + g) + \frac{1}{2} \tanh \frac{1}{2} (x - h) + \frac{1}{2} \tanh \frac{1}{2} (x + h)$ where $\cosh g$ and $\cosh h$ are given by

$$b^3 \pm \sqrt{(b^6 - \frac{3}{2}b^4 + \frac{1}{2})},$$

i.e. $b^3 \pm (b^2 - 1)\sqrt{(b^2 + \frac{1}{2})}$, so that the curve is the sum of four one-site saturations. When b is large enough for $\cosh g$ and $\cosh h$ to be taken as $b^3 \pm \sqrt{(b^6 - \frac{3}{2}b^4 + \frac{1}{2})} = b^3 \pm (b^3 - \frac{3}{2}b)$ and for them to approach $\frac{1}{2}e^g$ and $\frac{1}{2}e^h$, then g and h approach $\ln(4b^3)$ and $\ln(\frac{4}{3}b)$, i.e. $\ln(K^*/K_1)$ and $\ln(K^*/K_2)$ respectively, so that the pK values of the one-site curves approach pK_1 , pK_2 , pK_3 and pK_4 , as expected. The usefulness of expression (13) is limited both by its complexity and by the re-

strictive assumptions made to render it as simple as possible. It is therefore presented partly to show the limits of the approach.

Note added in proof (received 26 November 1973)

Most of the above expressions are simplified if written in terms of $\alpha = \frac{1}{2}m$ rather than of m .

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