Negatively Co-operative Ligand Binding

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Simple systems are considered in which the binding of a ligand at a single site exhibits a doubly sigmoid curve when saturation is plotted against the logarithm of ligand concentration, i.e. where a fraction of the site exhibits one dissociation constant and the rest exhibits another. The condition for this to occur is that the ligand should also combine at another site on the binding molecule with comparable affinity and that the binding at one site should markedly lower affinity at the other. The protonation of simple compounds such as cysteine and 3-hydroxypyridine is taken as an example of this process and the equations derived are also applied to the binding of substrates to enzymes.

Part 1: Hydrogen-Ion Equilibria

It is highly convenient to specify the ionizing properties of a group by stating its pK . Nevertheless, there are severe limitations in doing so, because a group in a molecule possesses a particular pK value (the group or microscopic pK) only in one particular ionic form of the substance that contains it (Cohn & Edsall, 1943; Benesch & Benesch, 1955; Edsall & Wyman, 1958). Hence the pK of the group changes with pH as the other charges in the molecule are changed. Nevertheless, pK values can prove adequate to specify the ionizing properties of a group, if different pK values are attributed to different fractions of the group. This is shown below for the particular case where only one other ionizing group is close enough in the molecule to the group under consideration appreciably to affect its ionization. Section 1 recapitulates and applies some of the standard treatment of multiple ionizations (Michaelis, 1914; Edsall & Wyman, 1958; Dixon & Webb, 1958) and Sections 2 and 3 draw some conclusions.

Section 1: concentrations of particular ionic species

Scheme 1 represents the ionizations of a substance that contains two ionizing groups, $-AH$, the group under consideration, and $-BH$, the group that influences $-AH$. K_A and K_B are the dissociation constants of these groups. Since their sum, K_1 , called the molecular or macroscopic dissociation constant, must also be a constant, it is clear from their definitions

$$K_A = \frac{[H^+][A^- - BH]}{[HA - BH]}$$

and

$$K_B = \frac{[H^+][HA - B^-]}{[HA - BH]}$$

that

$$K_1 = \frac{[H^+][A^- - BH] + [HA - B^-]}{[HA - BH]}$$

and hence that the sum of the concentrations of $A^- - BH$ and $HA - B^-$ is related to the concentration of $HA - BH$ by the usual dissociation equation.

The ionization of the group $-BH$ changes the dissociation constant of $-AH$ to K'_A , and similarly the ionization of $-AH$ changes that of $-BH$ to K'_B , as shown in Scheme 1. The factor $K_A/K'_A = K_B/K'_B$ is usually above unity if the groups are close or are part of a single conjugated system, sometimes greatly so (it is about 800 in 3-hydroxypyridine; Metzler & Snell, 1955), but mechanisms are conceivable whereby it could fall below unity, i.e. whereby the binding of protons by $A^- - B^-$ could be positively co-operative. It may easily be shown that there is a molecular dissociation constant, K_2 , for the loss of the second proton, and that $1/K_2 = 1/K'_A + 1/K'_B$.

Fig. 1(a) shows the dependence on pH of the concentration of each of the ionic forms of the substance.

$$K_1 = K_A + K_B; 1/K_2 = 1/K'_A + 1/K'_B; K_A/K'_A = K_B/K'_B$$

In it K_A has been arbitrarily set at $4K_B$, so that $K_1 = 5K_B$. Hence pK_1 is 0.7 pH unit below pK_B and 0.1 unit below pK_A . To simplify the situation by letting the dissociation of the first proton be largely complete before the dissociation of the second proton is appreciable, pK_2 has been placed 5 units above pK_1 . These values fix $pK'_A - pK_A$, which is equal to $pK'_B - pK_B$, at 4.2. It is noteworthy that although pK_A and pK_B are the pH values at which the concentrations of A^-BH and of $HA-B^-$ respectively are equal to the concentration of $HA-BH$, pK_1 is the pH at which all three of the species $HA-BH$, A^-BH and $HA-B^-$ are at one-half their maximum values.

Section 2: the pK values of groups

It is clear from Fig. 1(a) that a fraction $a = K_A/(K_A + K_B)$ of the group $-AH$ titrates with a pK of pK_1 . The rest titrates with a pK of pK_2 . Thus the group titrates in two 'steps', whose heights are in the ratio K_A/K_B .

This description is only correct when pK_2 is much higher than pK_1 . Since:

$$K_1/K_2 = (K_A/K'_A)(2 + K_A/K_B + K_B/K_A) \quad (1)$$

as follows from the above definitions of K_1 and K_2 , pK_2 can only be far above pK_1 if either $K_A \gg K'_A$ or K_A and K_B are very different. If K_A and K_B differ greatly, one of the steps will be very small, so the double sigmoid curve will not be seen. Although K_A/K'_A can be large when the groups are adjacent, it is rarely over 100–1000. Hence particular cases need to be examined to find if the description is useful. Nevertheless from eqn. (1) $pK_2 - pK_1$ exceeds $pK'_A - pK_A$ by at least 0.6.

The true total amount of $-A^-$, expressed as a fractional concentration, is derived from the Michaelis (1914) functions as:

$$\frac{a}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}} + \frac{1}{1 + \frac{[H^+]}{K_2} + \frac{[H^+]^2}{K_1 K_2}} \quad (2)$$

where a is, as above, $K_A/(K_A + K_B)$, and if K_2/K_1 is called n and $[H^+]/K_2$ is called x this total simplifies to:

$$\frac{ax + 1}{nx^2 + x + 1} \quad (3)$$

The fractional concentration of $-A^-$ predicted from the description that a fraction a has pK_1 and $1-a$ has pK_2 is:

$$\frac{a}{1 + \frac{[H^+]}{K_1}} + \frac{1-a}{1 + \frac{[H^+]}{K_2}} \quad (4)$$

which put into the terms of expression (3) is:

$$\frac{ax(1-n) + nx + 1}{nx^2 + x + nx + 1} \quad (5)$$

Expressions (3) and (5) differ only in terms containing n . Hence the error involved in using the description of two pK values is negligible for most purposes if $n < 0.01$, i.e. if pK_2 exceeds pK_1 by two units.

For cysteine (Fig. 1b) $pK'_A - pK_A$ is only 1.50 (Benesch & Benesch, 1955) and $pK_2 - pK_1$ is 2.17. This is so small that the bell-shaped curve of the concentration of the partially protonated forms is appreciably distorted from the shape of two separate titrations curves with a plateau between them; indeed when the pK values differ by 2.17 units the

Scheme 2. Mechanism for an enzyme with two binding sites for the same substrate, when only one of them is catalytic

Fig. 1. pH -dependence of the concentrations of various ionic forms

(a) A hypothetical dibasic acid whose $-AH$ group has a dissociation constant (K_A) four times that (K_B) of its $-BH$ group. Ionization of either group diminishes the dissociation constant of the other by a factor of $10^{4.2}$ (this is a larger factor than usually found) to K'_A and K'_B respectively. The various resultant pK values are also marked. Hence 80% of $-AH$ titrates with a pK of pK_1 and 20% with a pK of pK_2 , where K_1 and K_2 are the molecular dissociation constants for the first and second protons to dissociate. (b) Cysteine, from the results and Figs. 2 and 3 of Benesch & Benesch (1955). The pK_1 is 8.36, made up to 8.53 for the thiol group and 8.86 for the amino group; the pK_2 is 10.53, made up of 10.03 for the thiol group and 10.36 for the amino group. Hence 68% of the thiol group titrates with a pK of 8.36 and 32% with a pK of 10.53. (c) 3-Hydroxypyridine, from the results of Metzler & Snell (1955). The pK_1 is 5.10, made up of 5.37 for the phenolic hydroxyl group and 5.44 for the pyridinium group; the pK_2 is 8.60, made up of 8.26 for the hydroxyl group and 8.33 for the pyridinium group. Hence 54% of the phenolic hydroxyl titrates with a pK of 5.10 and 46% with one of 8.60.

width of the curve at one-half its height is 0.25 unit greater than this (Alberty & Massey, 1954; Dixon, 1973). Nevertheless, the description that 68% of the thiol group has a pK of 8.36 and 32% has a pK of 10.53 gives an error that is never above 0.0025 in the fraction of the total that is present as $-S^-$. Hence this form of description is useful down to differences in the molecular pK values of about 2 units. Fig. 1(c) gives a real case where pK_2 and pK_1 differ more.

Section 3: consequences and generalizations

The fact that a group may titrate in two (or more) steps, because of the influence on it of neighbouring groups, compels caution in interpreting curves of pH-dependence. The observation that some parameter shows more than one sigmoid step in such a curve does not necessarily mean that more than one group contributes directly to the parameter in question. Thus, for example, a spectrophotometric titration could show more than one rise of the extinction with pH even though only one form of one group was contributing significantly to the extinction measured. Similarly, the rate of reaction of the group $-AH$ could rise with pH in two steps. In this case the steps need not be in the height ratio of K_A/K_B , since the reactivity of the $-A^-$ group may differ in the A^-BH and A^-B^- species.

Part 2: Enzyme Ligands

This explanation of a multiple sigmoid curve can be applied to ligands other than H^+ ions. Double-sigmoid curves have been found with a number of enzymes, including trypsin, when the initial rate of reaction is plotted against the logarithm of the substrate concentration (Trowbridge *et al.*, 1963; Bechet & Yon, 1964; Nakata & Ishii, 1972). All these authors also attributed the second step to binding of substrate at another site, but they did not make clear that binding at the auxiliary site need not influence the catalytic activity at the active site. The doubly sigmoid curves could simply be due to the fact that the substrate partitioned between the two sites and that binding at either site lowered the affinity of the other. Hence only some of the catalytic sites would be filled in the binary complex, and the rest would be filled up when the ternary complex was formed.

If the binding of substrates in the system represented by Scheme 2 is at equilibrium, the fractional activity of the enzyme can be expressed in terms similar to expression (3) as:

$$\frac{v}{V} = \frac{b \frac{K_{II}}{s} + 1}{\frac{K_I K_{II}}{s^2} + \frac{K_{II}}{s} + 1} \quad (6)$$

where v is the rate of the catalysed reaction when the concentration of free substrate is s , V is the rate when both sites of the enzyme are saturated with substrate, K_I and K_{II} are molecular dissociation constants related to the dissociation constants, K_a etc., by the relationships:

$$1/K_I = 1/K_a + 1/K_b \quad \text{and} \quad K_{II} = K_c + K_d$$

and b represents the ratio k/k' (see Scheme 2) multiplied by the concentration of the ES complex expressed as a fraction of the total concentration of binary complexes, so that:

$$b = \frac{k[ES]}{k'([ES] + [SE])} = \frac{kK_b}{k'(K_a + K_b)} \quad (7)$$

If the binding of a substrate molecule to either of the two sites on the enzyme markedly decreases the affinity at the other site, i.e. $K_{II} \gg K_I$, two limiting cases of eqn. (6) may be considered.

(i) When $s \ll K_{II}$ the equation will simplify to:

$$\frac{v}{V} = \frac{b}{\frac{K_I}{s} + 1}$$

which will describe a rectangular hyperbola which asymptotically approaches a value of b .

(ii) When $s \ll K_{II}$, then since $K_{II} \gg K_I$, $s \gg K_I$. Under these conditions eqn. (6) becomes:

$$\frac{v}{V} = \frac{b \frac{K_{II}}{s} + 1}{\frac{K_{II}}{s} + 1}$$

which may be rearranged to give:

$$\frac{v}{V} = b + \frac{1-b}{1 + \frac{K_{II}}{s}}$$

That is, as in the case where H^+ ions were considered, there appear to be two species of enzyme in solution, a fraction b which exhibits the apparent dissociation constant K_I and a fraction $1-b$ which exhibits the apparent dissociation constant K_{II} .

The process that results in a decrease in affinity at one site when another site is filled need not necessarily involve a conformational change in the protein. In cases where charge interaction constitutes the binding force in the enzyme-substrate complex electrostatic interaction could diminish affinity at the second site as it does in the rigid molecule of fumarate where the difference in pK values is 1.36 units.

A scheme such as that shown can therefore give rise to a doubly sigmoid plot as observed for trypsin. There is no need to assume that the ternary complex

Fig. 2. Eadie plot calculated for eqn. (6)

Values for the constants used were: $K_I = 0.5 \text{ mM}$, $K_{II} = 10 \text{ mM}$, $b = 0.2$ and $k' = V/e = 10 \text{ s}^{-1}$. Values for the intercepts of the two linear portions of the graph are marked. The slope of the steeper tangent will be $-K_{II}$ and that of the less steep $-K_I$.

(SES) forms products any faster than the binary complex (ES) does, as has been postulated by Bechet & Yon (1964) and Nakata & Ishii (1972), since the two steps in the curve could be explained if the catalytic site were only partly filled in the binary complex. Indeed where $b < 1$ kinetics can provide no evidence on whether the rate constants for the breakdown of ES and SES differ. All they can give is K_I , K_{II} and the relative sizes of the two steps, i.e. b and $1-b$. Since $b = kK_b/k'(K_a + K_b)$, it is not possible to discover whether k and k' differ, unless K_a and K_b are known from other evidence. If, however, $b > 1$, k must exceed k' and the curve of v against $\log s$ will show a maximum (cf. Reiner, 1959, pp. 66–69).

Although eqn. (6) is exact, there will be an error in summarizing it by saying that a fraction of the catalytic site has a dissociation constant K_I and the rest K_{II} . This error will depend on K_{II}/K_I and, as discussed in connexion with expressions (2)–(5), it will be negligible if this ratio is over 100. Under these conditions both ES and SE attain one-half their maximum concentrations when $s = K_I$, and SES when $s = K_{II}$. It should be noted that neither of these apparent K_m values can be equated with the dis-

sociation constant of a single enzyme species; each represents a composite value for the two sites.

Fig. 2 shows an Eadie (1942) plot for the variation of initial rate with substrate concentration as calculated for Scheme 2 by using values of k' , K_I and K_{II} comparable with those given by Nakata & Ishii (1972) for the hydrolysis of benzoyl-L-arginine *p*-nitroanilide by trypsin. Such a curve would also be expressed as a downwardly curving double-reciprocal plot, a double-sigmoid plot of v against $\log s$, a plot of v against s with an R_s value (Kirtley & Koshland, 1967) of greater than 81 or as a Hill plot with a value for the Hill coefficient of less than 1. Since these different plots are merely different ways of expressing the same result they do not represent separate criteria for the existence of negative co-operativity, as implied by Soberano & Schoellmann (1972).

At low values of s the double-reciprocal plot will approach a straight line which obeys the equation

$$\frac{1}{v} = \frac{K_I}{V \cdot b} \left(\frac{1}{s} \right) + \frac{1}{V \cdot b}$$

This line will intersect the vertical axis at $1/v = 1/V \cdot b$ and will intersect the horizontal axis when $-1/s = 1/K_I$. The tangent to the double-reciprocal plot at $1/s = 0$ will obey the equation:

$$\frac{1}{v} = \frac{K_{II}(1-b)}{V} \left(\frac{1}{s} \right) + \frac{1}{V}$$

Such a tangent will intersect the vertical axis when $1/v = 1/V$ and its intercept on the horizontal axis will be $-1/s = \frac{1}{K_{II}(1-b)}$. Hence the estimate of the

Michaelis constant obtained by extrapolating the initial part of the reciprocal plot will give $K_{II}(1-b)$ rather than K_{II} itself. Similar conclusions obtain in the case of two enzymes with different K_m values acting on the same substrate (cf. Reiner, 1959, pp. 98–102).

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