

## Kinetics of Interacting Sites

The usual shorthand title for what I here describe is ‘allosteric enzymes’, from the Greek  $\alpha\lambda\lambda\omicron\varsigma$ , other and  $\sigma\tau\epsilon\rho\epsilon\omicron\varsigma$ , rigid, solid, or shape. That is to say, the enzyme – or other protein, hemoglobin is a classic case of such shape changes – exists in two or more conformations, with different activities. But the term originally and basically refers to effects caused by an inhibitor or activator – the general term is effector - binding at a site other than the active site. Thus an allosteric effect can occur in a single-subunit enzyme if the binding of an effector at a site different from the active site changes the  $K_m$  for one of the substrates or the  $V_{max}$ . If such an inhibition could be complete, i.e. binding of the inhibitor completely prevents activity, it would be difficult to distinguish from ordinary competitive inhibition, short of a three-dimensional structure showing that the inhibitor bound at a site other than the catalytic site. More usually the inhibition would be only partial, i.e. the  $K_m$  increases or the  $V_{max}$  decreases to a finite level, and a replot of slopes or intercepts of Lineweaver-Burk plots versus  $[I]$  will be a hyperbola rather than a straight line.

But the term ‘allosteric’ is closely linked with ‘cooperativity’, the observation that in a multisubunit protein the binding of a ligand to one subunit not only changes its conformation, but that of other subunits; the subunits cooperate in some way. Such cooperativity is called homotropic if it refers to the binding of substrate at one active site affecting the binding of the same substrate at other active sites, heterotropic if it refers to binding of an effector at a site other than the active site. Heterotropic allosteric inhibition or activation is quite common in enzymes, especially those of biosynthetic pathways where the cell wants to turn off the enzyme because it has plenty of the pathway product. The original and classic allosteric enzyme is aspartate transcarbamylase, which catalyzes the first committed step of synthesis of pyrimidine nucleotides and is inhibited by the eventual pathway product CTP. Another is chorismate mutase, a key enzyme in the synthesis of tyrosine and phenylalanine. It is inhibited by these amino acids and activated by tryptophan. For a thorough review of what is known about allosteric changes of *E. coli* aspartate transcarbamylase and yeast chorismate mutase, see Helmstaedt et al., *Microbiology and Molecular Biology Reviews* **65**:404-421 (2001).

First consider the case in which the subunits *do not* cooperate, i.e. binding and catalysis at identical sites on identical subunits occur independently. For instance, in a tetrameric enzyme complexes  $E_4S$ ,  $E_4S_2$ ,  $E_4S_3$  and  $E_4S_4$  are formed. The intrinsic dissociation constant  $K_s$  is the same for all sites, but for statistical reasons there are effective dissociation constants for binding of successive substrate molecules. For instance, the first S has four free E subunits to bind to, but only one way for  $E_4S$  to dissociate; consequently the effective  $K_s$  is  $K_s/4$ . For binding of the second S there are three free E to bind to, two ways for S to dissociate, consequently the effective  $K_s$  is  $2 K_s/3$ . For similar reasons the third and fourth are  $3 K_s/2$  and  $4 K_s$ . The velocity equation is then

$$\frac{v}{E_t} = \frac{k_{cat} \left( \frac{4[S]}{K_s} + \frac{6 \cdot 2[S]^2}{K_s^2} + \frac{4 \cdot 3[S]^3}{K_s^3} + \frac{4[S]^4}{K_s^4} \right)}{1 + \frac{4[S]}{K_s} + \frac{6[S]^2}{K_s^2} + \frac{4[S]^3}{K_s^3} + \frac{[S]^4}{K_s^4}}; \text{ the first numerator term is because there are four}$$

different  $E_4S$  complexes (S on each of the four subunits), the second because there are six different  $E_4S_2$  complexes, each able to produce product twice as fast as an  $E_4S$ , the third because there are four different  $E_4S_3$  complexes, each producing P three times as fast, and the fourth

because while there is only one  $E_4S_4$  complex, it produces product four times as fast as an  $E_4S$ .

One can factor out  $4k_{\text{cat}}[E]_t = V_{\text{max}}$  to get  $\frac{v}{V_{\text{max}}} = \frac{\frac{[S]}{K_s} + \frac{3[S]^2}{K_s^2} + \frac{3[S]^3}{K_s^3} + \frac{[S]^4}{K_s^4}}{1 + \frac{4[S]}{K_s} + \frac{6[S]^2}{K_s^2} + \frac{4[S]^3}{K_s^3} + \frac{[S]^4}{K_s^4}}$ ; fortunately this

reduces to  $\frac{v}{V_{\text{max}}} = \frac{\frac{[S]}{K_s} \left(1 + \frac{[S]}{K_s}\right)^3}{\left(1 + \frac{[S]}{K_s}\right)^4} = \frac{\frac{[S]}{K_s}}{1 + \frac{[S]}{K_s}} = \frac{[S]}{K_s + [S]}$ , the ordinary Michaelis-Menten equation.

This demonstrates that as expected, when  $K_s$  or more generally  $K_m$  is the same for all sites, the equation reduces to the ordinary Michaelis-Menten equation, as we would expect. The only point to remember is the statistical factors for the  $K_s$  or  $K_m$ .

But what we are interested in is the case where the binding of a substrate or effector molecule *changes* the  $K_s$  for the remaining sites. We might say that the first S to bind changes the  $K_s$  for the three remaining sites by a factor a, the second changes  $K_s$  for the two remaining sites by a factor b, and the third changes  $K_s$  for the remaining site by a factor c. The rate

equation is then  $\frac{v}{V_{\text{max}}} = \frac{\frac{[S]}{K_s} + \frac{3[S]^2}{aK_s^2} + \frac{3[S]^3}{a^2bK_s^3} + \frac{[S]^4}{a^3b^2cK_s^4}}{1 + \frac{4[S]}{K_s} + \frac{6[S]^2}{aK_s^2} + \frac{4[S]^3}{a^2bK_s^3} + \frac{[S]^4}{a^3b^2cK_s^4}}$ . In this general model, if a, b and c

are small (much less than 1, each decreases  $K_s$  considerably), it turns out that essentially all the enzyme is present either as free enzyme  $E_4$  or as the complex  $E_4S_4$ . The effect of this on a plot of  $v$  vs.  $[S]$  is that instead of getting a hyperbola, with an increase in saturation from 10% to 75% requiring a 27-fold increase in  $[S]$ , the plot is S-shaped, usually referred to as **sigmoidal**. It increases slowly at low  $[S]$ , where only a little  $E_4S$  is formed, then suddenly increases to approach the hyperbola over a relatively narrow range of substrate concentration – in an example in Segel's text a 2.3-fold range. The point of such sigmoidal  $v$  vs  $[S]$  plots is clear: they allow a much stronger control of rate by substrate concentration over a narrow range, presumably the physiological range of substrate concentration. The first such plot observed was not one of  $v$  vs.  $[S]$ , but of the binding of oxygen to hemoglobin, where it allows the binding of  $O_2$  in the lungs and its release in the tissues to occur essentially completely even though the difference in local  $[O_2]$  is not great, four-fold. Studies of cooperativity of  $O_2$  binding to hemoglobin have been so important to similar studies with enzymes that hemoglobin is sometimes termed a honorary enzyme, even though it does not have a catalytic function.

When a, b and c are low and the protein is thus in either the  $E_4$  or  $E_4S_4$  states almost exclusively, the above equation can be approximated by the **Hill equation**,  $\frac{v}{V_{\text{max}}} = \frac{[S]^n}{K' + [S]^n}$ , with  $n = 4$  in this case.  $K' = a^3b^2cK_s^4$ , but is not  $[S]$  at  $v/V_{\text{max}} = 1/2$ ; this is the  $n^{\text{th}}$  root of  $K'$ . In practice is usually an *empirical* equation, as Hill originally observed it; the less cooperative the enzyme, i.e. the larger the values of a, b and c, the less the observed empirical value of n will approach the true number of interacting sites. The empirical value of n is determined by recasting the

equation in a different form,  $V_{\max}[S]^n = vK' + v[S]^n$ ,  $\frac{[S]^n(V_{\max} - v)}{v} = K'$ ,  $n \log[S] + \log\left(\frac{V_{\max} - v}{v}\right) = \log K'$ ,  $\log\left(\frac{V_{\max} - v}{v}\right) = n \log[S] - \log K'$ . In principle a plot of  $\log\left(\frac{V_{\max} - v}{v}\right)$  vs  $\log[S]$  will be a straight line with slope  $n$ ; in practice it is always somewhat S-shaped, with maximal slope =  $n$  in the middle, and smaller slope at extreme values of  $[S]$ , since at low  $[S]$  complexes with less than  $n$  molecules of  $S$  bound contribute to velocity or average number of molecules of  $S$  bound per enzyme oligomer, and at high values of  $[S]$  the enzyme is nearly saturated and changes in  $v$  are difficult to determine. At that  $[S]$  where  $v = V_{\max}/2$ ,  $v/V_{\max} - v = 1$ , the logarithm = 0,  $n \log[S] = \log K'$ ,  $\log[S] = 1/n \log K'$ ,  $K'$  is the  $n^{\text{th}}$  root of that  $[S]$ .

Always remember that the slope of this plot, referred to as the **Hill coefficient**, is only an approximation, less than or rarely equal to the number of interacting sites. An enzyme with four interacting subunits might show a Hill coefficient of 2.5, but that is enough greater than 2 to say that it is an interacting tetramer, not a pair of interacting dimers.

So far I have covered only cases where binding of substrate (or other ligand such as  $O_2$ ) to its usual site on one subunit affects equivalent sites on other subunits, **homotropic** interactions. Of course there are cases where a non-substrate effector binds to affect multiple substrate sites; this is a **heterotropic** interaction. Of course, the binding of the effector may itself show homotropic effects, binding of effector to one subunit may affect its binding to others.

The foremost theory or model for explaining homotropic interactions is that of Monod, Wyman and Changeux, MWC for short. They make a number of simplifying assumptions:

- 1) The enzyme exists in two states, generally termed T (taut) and R (relaxed). The T state has a lower affinity for the ligand in question.
- 2) It exists only as one state or the other,  $T_4$  or  $R_4$ ; **mixed states**, with some subunits in one state, some in the other, **do not occur**.
- 3) All subunits in a given state have the same dissociation constant,  $K_T$  or  $K_R$  respectively.

The sigmoidal dependence of  $v$  or substrate binding is then calculable in terms of these two dissociation constants and the allosteric constant  $L$ , which is the ratio of the concentrations of T and R when no ligand is present,  $L = [T]/[R]$ . For convenience the substrate concentration is used as  $\alpha = [S]/K_R$ , and  $K_T$  is used in the form  $c = K_R/K_T$ .  $L$  expresses how little R is present in absence of ligand, and  $c$  expresses how much better the binding of ligand is to R than to T; what happens is that ligand binds to available R, converting it to  $RS_n$ , and the equilibrium shifts to provide more R, until eventually all the enzyme is in the R form. Cooperativity will be high when  $c$  is very small and  $L$  fairly large; if  $L$  is too large cooperativity will drop again. The equation is  $\frac{v}{V_{\max}} = \frac{Lc\alpha(1 + c\alpha)^{n-1} + \alpha(1 + \alpha)^{n-1}}{L(1 + c\alpha)^n + (1 + \alpha)^n}$ ;  $L$  and  $c$  are generally evaluated by computer-aided curve-fitting, i.e. adjusting values of  $L$  and  $c$  until plots of  $v/V_{\max}$  calculated from the equation fit the data well. Such plots express the binding of  $O_2$  to hemoglobin and the variation of  $v$  with  $[S]$  for many allosteric enzymes quite well. The effect of heterotropic effectors is typically to shift the equilibrium between R and T by binding to only one of them. An activator A binds to the R form and, assuming that S binds to RA as well as to R, provides more R for it to

bind to. When  $c$  is very small and the above equation reduces to  $v/V_{\max} = \frac{\alpha(1+\alpha)^{n-1}}{L+(1+\alpha)^n}$ , the effect of an activator is to decrease  $L$  by dividing it by a term  $(1+\gamma)^n$ , where  $\gamma = [A]/K_A$  and  $n$  indicates the cooperativity of binding of  $a$ . If  $\gamma$  is large enough it eliminates  $L$  from the equation and you have  $v/V_{\max} = \frac{\alpha(1+\alpha)^{n-1}}{(1+\alpha)^n} = \frac{\alpha}{1+\alpha}$ , simple hyperbolic binding; the activator shifts the binding curve to lower  $[S]$  and in the extreme abolishes sigmoidicity. An inhibitor multiplies  $L$  by a factor  $(1+\beta)^n$  and shifts the binding curve to the right. There are allosteric inhibitors which affect rate by affecting  $V_{\max}$ , by binding to a T form which has a lower  $V_{\max}$  than the R form. In this case there is no cooperativity in substrate binding and the curve of  $v$  vs  $[S]$  is normal hyperbolic, though *inhibitor* binding may be cooperative. These are called V systems, in contrast to these where  $[S]_{0.5}$  is affected, which are called K systems.

The MWC model however makes some rather restrictive assumptions, in particular the Lincolnian assumption that no oligomer half R and half T can exist. One consequence is that this model cannot include **negative cooperativity**, the case where binding of a ligand to one subunit *decreases* binding at other subunits (increases  $K_s$ ), since negative cooperativity states that mixed forms such as TRS and  $T_2R_2S_2$  not only exist but are favored. In contrast, what is known as the Koshland-Nemethy-Filmer model – Nemethy did the mathematics, Filmer the experiments and Koshland the publicity – allows ‘mixed’ oligomers and variation of  $K_s$  from one complex to the next. It is an extension of Adair’s 1925 paper describing the binding of  $O_2$  to hemoglobin in terms of four *different*  $K_s$ , and hence is sometimes called the Adair-Koshland model. The MWC model is a special case of the KNC model, and it in turn is a special case of a still more general model described by Eigen, which however is too general to be useful.

The KNF model assumes that while free enzyme (subunit) is in a conformation A, binding of ligand S to it converts it to conformation B. Here the dissociation of S from B is described by the association constant  $K_s = \frac{[BS]}{[B][S]}$ , and a transformation constant  $K_t$  describes the equilibrium between A and B,  $K_t = [B]/[A]$ . The overall reaction  $A + S \rightarrow BS$  is described by the product of these constants,  $K_s K_t = [BS]/[S][A]$ , whether B or AS is an intermediate in formation of BS. (The Eigen model allows both B and AS to be present in appreciable concentrations, the KNF model doesn’t.) Next equilibrium constants are described for the interaction between subunits; the interaction between two vacant subunits, called  $K_{AA}$ , is set = 1, and other interactions are stronger ( $>1$ ) or weaker ( $<1$ ) than this.  $K_{AB}$  represents the interaction between a filled and an unfilled site, and  $K_{BB}$  that between two filled sites. They may be thought of as the equilibrium constants for the hypothetical reactions  $AA + B \rightleftharpoons AB + A$  and  $AB + B \rightleftharpoons BB + A$  respectively. Each step of a binding series (to successive subunits) includes a  $K_t$  term for the transformation of A to B, a  $K_s$  term for binding of a substrate molecule, and interaction constants for the new interactions formed (in the numerator) and the old ones lost (in the denominator). If a given complex, such as ABS, occurs more than once (either subunit of AA can become B), the appropriate statistical factor is introduced. Both ABS and BSA are represented by  $K_t K_s K_{AB} [S][A_2]$ , and  $B_2S_2$  by  $K_t^2 K_s^2 K_{BB} [S]^2 [A_2]$ . The fraction of the total enzyme in each state is the appropriate term over the sum of all terms, and the velocity is equal to  $k_{\text{cat}}[BSA] + k_{\text{cat}}[ABS] + 2 k_{\text{cat}}[B_2S_2]$ , so that 
$$\frac{v}{[E]_t} = \frac{k_{\text{cat}} 2 K_t K_s K_{AB} [S] + 2 k_{\text{cat}} K_t^2 K_s^2 K_{BB} [S]^2}{1 + 2 K_t K_s K_{AB} [S] + K_t^2 K_s^2 K_{BB} [S]^2}$$
 which can be divided

by  $2k_{\text{cat}}[E]_t = V_{\text{max}}$  to give the expression for  $v/V_{\text{max}}$ . Each term in such an expression includes: a factor for the number of ways of arranging the species in question, the product of the transformation and binding constants to reach the species from free enzyme – this will have  $K_t K_s$  to the power of the number of substrate molecules bound in the complex in question – and factors for the AB and BB interactions involved, to the power of the number of such interactions involved (only one in the dimer, which has kept these equations simple), and the rate constant of the reaction, weighted according to the number of substrate molecules in the complex. The velocity equation includes all these terms in the numerator, and all but the rate constant term in the denominator (see the equation for the dimer, above). For oligomers of more than two subunits there are different ways of arranging the subunits, in terms of how many interactions there are; for instance, a tetramer could be arranged in a linear fashion, with two central subunits interacting with two others, the end units with only one other; in square fashion, with each subunit interacting with two neighbors, horizontal or vertical but not diagonal; and the tetrahedral arrangement where each subunit interacts with all three others. These will differ in the type and number of interaction coefficients involved; for instance, in the square model the equilibrium for the first binding of S is  $K_s K_t K_{AB}^2$ , because the BS subunit formed interacts with two AS subunits, while in the tetrahedral arrangement it is  $K_s K_t K_{AB}^3$  because it interacts with all three remaining A subunits. There are two different ways for forming the  $A_2 B_2 S_2$  square complex, depending on whether the BS subunits are next to each other or diagonally across from each other. In the first case there are two AB interactions as before, one BB interaction; since the number of AB interactions does not change  $K_{AB}$  does not appear in the individual equilibrium constant of formation,  $K_{\text{eq}} = K_s K_t K_{BB}$ . In the second case there are four AB interactions, vs two in the  $A_3 B S$  complex, so  $K_{\text{eq}} = K_s K_t K_{AB}^2$ . When interactions disappear they are in the denominator; going from the diagonal or ‘trans’  $A_2 B_2 S_2$  complex to the  $AB_3 S_3$  complex has  $K_{\text{eq}} = K_s K_t K_{BB}^2 / K_{AB}^2$ , since two AB interactions are replaced by BB interactions.

Obviously I don’t expect you to swallow all this and be able to use it. I merely want to show that this model 1) is more general, can express a greater variety of behavior, 2) this behavior includes cases where a later stage is less stable than an earlier one,  $K_{BB} < K_{AB}^2$ , leading to negative cooperativity. If  $K_{BB} > K_{AB}^2$  positive cooperativity is observed. Negative cooperativity doesn’t look like much in a plot of  $v$  vs  $[S]$ , just a hyperbola which doesn’t flatten out but continues to creep upwards, but a  $v$  vs  $\log [S]$  plot will show steps. In Lioneweaiver-Burk plots positive cooperativity gives a concave-upwards line, negative cooperativity a concave-downwards line. It is perfectly possible to have *both* positive and negative cooperativity in an enzyme of three or more subunits. The classic case is glyceraldehyde-3-phosphate dehydrogenase, where the first two dissociation constants for  $\text{NAD}^+$  are  $< 10^{-8}$  M and the binding is cooperative, the third  $\approx 10^{-6}$  M, the fourth  $\approx 10^{-4}$  M, negative cooperativity. Such an enzyme preserves a moderate rate of action over a very wide range of substrate concentration, wider than with a non-cooperative enzyme. Eadie plots ( $v/[S]$  vs  $v$ ) or Sctached plots ( $v/[S]$  vs  $v$ , where  $v$  is the average number of ligand molecules bound, as found in an equilibrium dialysis experiment will be concave-downward in positive cooperativity, concave-upwards in negative cooperativity. It should be added, however, that the plots characteristic of negative cooperativity can be caused by a number of other things: enzyme heterogeneity, different enzyme species having different  $K_{mS}$ ; substrate heterogeneity; random mechanisms, if the preferred pathway switches as  $[S]$  increases – though this has never been observed; substrate-product complexes EBQ where Q dissociates more rapidly than from EQ; and some cases where product and substrate bind to different forms whose equilibration is not rate-limiting.

It is clear that the KNF model, by using more constants, can fit a wider variety of data, even if its original constraints are preserved (S binds only to form B, so that neither AS nor free B are observed; all AB interactions are the same, as are all BB interactions). By generalizing these restrictions (allowing S to bind also to A, and free B to exist), one should be able to fit any data to the resulting complex equation. A variety of types of heterotropic interaction can occur: those where inhibitor and substrate truly exclude each other, those where both can bind to the same subunit, and those where both bind in a required order. Koshland's studies have provided methods for fitting actual data to theoretical equations, and thereby in principle determining what model is followed. But we must remember that as models become more complex, the data must be more and more precise for one to be able to distinguish among the models.

The MWC model is sufficient to explain homotropic interaction in aspartate transcarbamylase. But the heterotropic effectors CTP and ATP immediately complicate the situation. Though they bind at more or less the same site, CTP contracts the site and the quaternary structure of the protein (which comprises six catalytic subunits, in two groups of three, and six regulatory subunits, in three groups of two between the catalytic triads), while ATP expands both, and the effects on the catalytic site are transmitted in different ways. Some mutants are further shifted toward the R or the T state and show no homotropic effects, but still are affected by ATP and/or CTP; some lose sensitivity to one of these effectors, but not the other.

Yeast chorismate mutase is believed to have another subunit conformation, called super-R, and to exist in T-super R and R-super R states in addition to  $T_2$  and  $R_2$  states. The super-R state is induced by binding of the substrate chorismate, and yet other different conformations seem to result from the binding of the activator tryptophan and the inhibitor tyrosine. As complex as the MWC and KNF models are, they seem still to be simplifications of real conditions.