

Reversible Inhibition

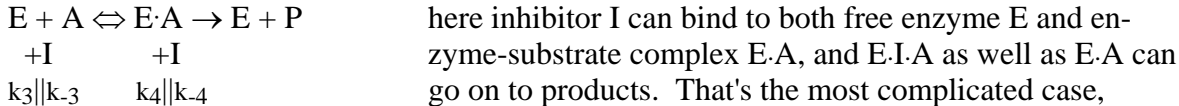
To students, 2004: this is a fuller treatment of simple inhibition than I expect to have time to give, developing the equations of competitive and uncompetitive inhibition.

Inhibition

One can of course consider two sorts of inhibition: **reversible**, in which some ligand binds non-covalently and reversibly to an enzyme to block or modify its activity, and **irreversible**, some sort of covalent modification which is **not** reversible, at least on the time scale of an initial velocity experiment. We really have already talked about irreversible modifications, and all that needs to be said is that they generally happen slowly, and if so you can see kinetic behavior **change with time**, and that their effect is to **remove** active enzyme from the situation, lower E_t and hence V_{max} , so that the effect on a Lineweaver-Burk plot is to increase $1/V_{max}$ **and** increase the slope K_m/V_{max} ; both effects will generally increase with time, i.e. if the modification is slow enough so that you can do several complete Lineweaver-Burk plots in the course of one modification experiment, you will see increasing slopes and intercepts, still the same K_m .

Reversible inhibitors can be divided into **product** inhibitors, normal products of the reaction which bind as they would as substrates for the reverse reaction, **dead-end** inhibitors, which bind to the enzyme generating a form which **cannot** go on to products, and **modifiers**, which bind and cause the normal reaction to follow a different pathway which is slower or even faster than usual. Product inhibition comes up mainly in reactions with two or more substrates and products, where you can run the reaction in presence of one of several products. For the moment let us consider dead-end inhibition and modifiers.

Consider a general scheme for inhibition of a one-substrate enzyme:



here inhibitor I can bind to both free enzyme E and enzyme-substrate complex E·A, and E·I·A as well as E·A can go on to products. That's the most complicated case,

E·I + A \rightleftharpoons E·I·A \rightarrow E·I + P but I want to develop the simple cases first.

If only the k_3 limb must be considered, that is to say that a dead-end complex E·I is formed but goes nowhere else, we have what is called **competitive** inhibition, which E can bind either substrate A or inhibitor I, but not both at the same time, i.e. A and I compete, probably but not certainly for the same site. We can write a dissociation constant $K_i = k_{-3}/k_3 = \frac{[E][I]}{[E \cdot I]}$, the enzyme conservation equation $[E]_t = [E] + [E \cdot A] + [E \cdot I]$, and the rate equation $v = k_2[E \cdot A]$. As usual $k_1[E][A] = (k_{-1} + k_2)[E \cdot A]$, $[E] = [E \cdot A] \frac{k_{-1} + k_2}{k_1[A]}$. Since $[E \cdot I] = \frac{[E][I]}{K_i}$, $[E \cdot I] = [E \cdot A] \left(\frac{k_{-1} + k_2}{k_1[A]} \right) \left(\frac{[I]}{K_i} \right)$, $[E]_t = [E \cdot A] \left(1 + \frac{k_{-1} + k_2}{k_1[A]} \right) \left(1 + \frac{[I]}{K_i} \right)$.

note the term $1 + \frac{[I]}{K_i}$, it will become familiar.

$$\text{Then } [E \cdot A] = \frac{[E]_t}{1 + \frac{k_{-1} + k_2}{k_1[A]} \left(1 + \frac{[I]}{K_i} \right)} = \frac{[E]_t[A]}{[A] + K_m \left(1 + \frac{[I]}{K_i} \right)}, \quad v = k_2[E \cdot A] = \frac{k_2[E]_t[A]}{[A] + K_m \left(1 + \frac{[I]}{K_i} \right)}. \quad \text{This is}$$

identical to the standard Briggs-Haldane form of the Michaelis-Menten equation except that K_m is multiplied by this term $1 + [I]/K_i$; in effect, as $[I]$ increases K_m increases. This affects the **slope** of a Lineweaver-Burk plot, K_m/V_{max} , while V_{max} and thus the intercept does not change. Competitive inhibition is then that in which the **slope** of a Lineweaver-Burk plot is affected **but not the y intercept**; and any such Lineweaver-Burk plot is defined as showing competitive inhibition. The slopes may be

replotted vs [I]; the y intercept of such a plot, when [I] = 0, is K_m/V_m , and the slope is $(K_m/V_m)([I]/K_i)$. The x intercept is $-K_i$, since when [I] = $-K_i$ the term $[I]/K_i = -1$, and the term $1+[I]/K_i = 0$, so the slope is zero.

An alternative way of handling inhibition is the **Dixon plot**, in which one determines v at only two values of [A] but at a number of values of [I], and plots $1/v$ vs. [I], getting two lines for the two values of [A] used. Or one can take for one of them the value of V_{max} determined in absence of I; since V_m will not be affected by [I], this infinite concentration of A will give a horizontal line. What we are doing is plotting the inverted equation $\frac{1}{v} = \frac{[A]+K_m(1+[I]/K_i)}{V_m[A]}$, in which the independent variable is [I].

When [I] = $-K_i$ the term $1+[I]/K_i$ is again equal to zero, so the equation reduces to $1/v = [A]/V_m[A] = 1/V_m$, i.e. all plots **whatever** the substrate concentration [A] will **cross** (intersect) at this point where [I] = $-K_i$, which will have a y value of $1/V_m$.

Now consider the case in which only the k_4 limb is operative, [I] combines with E.A - **only** E.A - to give a complex E.I.A which does **not** go ahead to E.I + product. Here $K_i = \frac{[E.A][I]}{[EIA]}$, $[EIA] = \frac{[E.A][I]}{K_i}$,

so we can write the conservation equation $[E]_t = [E]+[EA]+[EIA] = [EA] \left[\frac{k_{-1}+k_2}{k_1[A]} + 1 + \frac{[I]}{K_i} \right]$, $[EA] =$

$\frac{[E]_t}{\left[\frac{k_{-1}+k_2}{k_1[A]} + 1 + \frac{[I]}{K_i} \right]}$, $v = k_2[EA] = \frac{k_2[E]_t[A]}{\left[\frac{k_{-1}+k_2}{k_1} \right] + \left[1 + \frac{[I]}{K_i} \right][A]}$. The **substrate** term in the denominator is now

multiplied by the term $1+[I]/K_i$. This is not such a useful way to think about the equation; it is better to divide top and bottom by this term $(1+[I]/K_i)$, so that

$v = \frac{V_m}{1+[I]/K_i} \frac{[A]}{K_m + [A]}$, **both** V_m and K_m are **decreased** by the same factor $1+[I]/K_i$. If you wonder about

K_m being decreased by an inhibitor, remember how the apparent K_m in the acyl-enzyme mechanism was decreased by a high k_2/K_3 ratio; here also the E.A intermediate is pulled ahead into another intermediate, albeit not a productive one. The fact that K_m and V_{max} are decreased by the **same** factor $1+[I]/K_i$ means that the slope K_m/V_{max} of Lineweaver-Burk plots is **not affected**, only the intercept $1/V_m$; as [I] increases you get a series of **parallel** lines going up the y axis. A replot of intercepts vs [I] has the x intercept at [I] = $-K_i$, as before. This is called **uncompetitive** inhibition.

The third case is the one in which **both** the k_3 and k_4 limbs are operative. Consider first the special case where $k_{-3}/k_3 = k_{-4}/k_4 = K_i$, the affinity of I is **exactly the same** for E and E.A. The result is essentially the addition together of the two previous effects, **both slope and intercept** increase by the term $1+[I]/K_i$. In this special case the lines intersect **on** the x axis of a Lineweaver-Burk plot, and replots of **either** slope or intercept vs [I] give the same K_i . More generally, k_{-3}/k_3 does not equal k_{-4}/k_4 , and k_{-5}/k_5 does not equal k_{-1}/k_1 , though $k_{-3}k_{-5}/k_3k_5$ does equal $k_{-1}k_{-4}/k_1k_4$. We speak of k_{-3}/k_3 as $K_{i(\text{slope})}$ and k_{-4}/k_4 as $K_{i(\text{intercept})}$, determining them from replots of slopes and intercepts respectively. This case is called **mixed** noncompetitive, or sometimes just mixed inhibition, though I stand with Cleland in calling **any** inhibition where **both** slope and intercept are affected **noncompetitive**, and the case where the two K_i s are the same **pure** noncompetitive inhibition.

There remain cases in which k_6 is **not** zero, where the complex EIA goes ahead to products, perhaps even at a rate **faster** than k_2 , in which case you have activation rather than inhibition. All of these are

called **hyperbolic** inhibition (or activation), because as $[I]$ increases v tends only toward some new limiting values $k_6[E]_t$ rather than toward zero, so that a replot of $1/V_{\max}$ vs $[I]$ is a hyperbola, approaching a limiting value of $1/k_6[E]_t$ rather than a straight line going up indefinitely. In the simplest case $k_6 = k_2$ but $k_5/k_5 = K_s'$ is greater than $k_1/k_1 = K_s$, so that K_m approaches some new value. A replot of the slopes K_m/V_m will be a hyperbola approaching the maximum value K_m'/V_m . This is called **partial** or **hyperbolic** competitive inhibition. Similarly, k_3/k_3 may equal k_4/k_4 , but k_6 be less than k_2 ; in this case the **intercepts** will give a hyperbola when replotted vs. $[I]$. This would be 'simple' intersecting hyperbolic noncompetitive inhibition.. In the most general case $k_3/k_3 = K_i$, $k_4/k_4 = K_i' = \alpha K_i$, $k_6 = \beta k_2$, and **both** replots are hyperbolas. The K_i s can be found by transforming the slopes and intercepts by subtracting the slope or intercept of the uninhibited case, $\Delta\text{slope} = \text{slope at some } [I] - \text{slope at } [I] = 0$, $\Delta\text{intercept} = \text{intercept at some } [I] - \text{intercept at } [I] = 0$, then replotting $1/\Delta\text{slope}$ or $1/\Delta\text{intercept}$ vs $1/[I]$. Both replots have an x intercept of $-\beta/\alpha K_i$, and the intercept replot has a y intercept of $\beta V_m/(1-\beta)$, so α , β and K_i can all be calculated.

pH effects

Generally there will be some pH limits to catalysis, i.e. the ionization of some side chain will generate an inactive form of the enzyme; this may be either a catalytically important group, participating directly in the reaction, or one whose ionization results in conformational change, such as ile¹⁶ in chymotrypsin, deprotonation of which allows asp¹⁸² to fall into the catalytic site.

We can look at ionization states of the enzyme as $EH_1H_2 \rightarrow EH_2 \rightarrow E$, and similarly for the EA complex $EH_1H_2A \rightarrow EH_2A \rightarrow EA$; in both cases only the middle form EH_2 , EH_2A is enzymatically active - only EH_2 binds A, only EH_2A goes to products. (This is of course very simplistic; there are for instance cases where EH_2 binds the substrate, but then ionizes so that EA gives the product.)

The plot of enzyme activity vs pH, corresponding to the fraction of the enzyme in the active form, will be bell-shaped, with activity proportional to $\frac{1}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}}$. The plot will have a maximum at some

$[H^+]_{\text{opt}}$ halfway between the two pK_{as} , $[H^+] =$ the square root of K_1K_2 . The activity will be half maximal at two values $[H^+]_1$, $[H^+]_2$ - which are **not** the pK_{as} , because unless they are widely separated the enzyme is never completely in the active form EH_2 , but the pK_{as} would be where it is **half** in that form. However, it can be shown that $[H^+]_1 + [H^+]_2 = K_1 + 4[H^+]_{\text{opt}}$, so that K_1 and thence K_2 can be determined.

Up to here I have not specified whether the dissociation constants are of E or of EA. If "activity" is V_m , or $k_{\text{cat}} = V_m/[E]_t$, they are the dissociation constants of EA, the form undergoing the rate-limiting step (if there is a well-defined rate-limiting step; there are arguments about just what this means). The K_m s are affected by both sets of dissociation constants, for free E and EA, but k_{cat}/K_m , or V_{\max}/K_m , reflects **only** the ionization constants for **free** enzyme; this is quite general for other properties.

These treatments assume 1) the two ionizations take place sequentially, whereas actually they are independent; 2) the **substrate** does not ionize in this range, or if it does its ionization does not affect its binding or conversion to product. The inaccuracy of assumption 1 is corrected for by adding a factor K_2/K_1 to the denominators of the above equations (with $1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}$ in the denominator); obviously it will be significant only if the two constants are close to each other (K_2 is by definition smaller than K_1). Problems with assumption 2 are corrected for by **multiplying** the denominator by a factor

$\left\{ 1 + \frac{K_{1s}}{[H^+]} + \frac{[H^+]}{K_{2s}} \right\}$, if the substrate ionizes twice and only the middle form is active; leave out one of these terms if only one ionization is involved.