

Active Site Titration, Inhibition

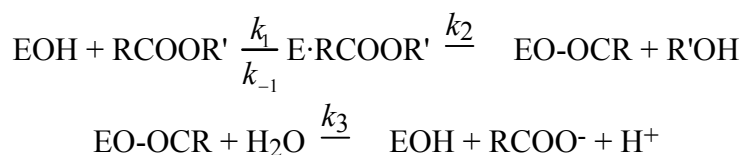
Today I want to start by distinguishing reversible and irreversible inhibition. Then I'm going to cover active site titration, which is a special case of irreversible inhibition and of pre-steady state kinetics, closely linked to the acyl-enzyme mechanism which I talked about last time; so I want to cover it now. If time permits I shall continue with reversible inhibition, which is in the syllabus to be covered next class. I'd like to cover the algebra of it now, then return to its role in Cleland's system for prediction of kinetic effects.

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. Irreversible modifications generally happen slowly, and if so you can see kinetic behavior **change with time**, though in the case of active site titration this change may be very fast. 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 .

My understanding of active site titration dates back to work I did as a post-doc in 1967 and to a paper on enzyme titration by M.L. Bender and 10 coworkers published in the Journal of the American Chemical Society in 1966. But it is only a special case of the general problem of detecting enzyme-substrate intermediates and measuring their rates of formation and breakdown, as described at length by Fersht. One wants to do this to understand the intermediates - which may also be intermediates in protein folding - as well as for the specific purpose of active site titration, described by Fersht on pp. 155-8.

Measuring the rate of formation of enzyme-substrate complexes (non-covalent, usually rapidly reversible) and intermediates (slower, usually irreversible in the sense of not going back to starting materials) are both problems in **pre-steady state** kinetics: one is trying to look at the formation of these enzyme forms which in the ordinary catalytic assay are present at steady-state concentrations. This poses several problems: 1) one is looking at the enzyme as a reactant, $E + S \rightleftharpoons E \cdot S$, and consequently needs to use substrate quantities of enzyme in order to do this; 2) the reactions are usually faster than our usual enzyme assay procedures can handle, since it takes a couple of seconds to mix enzyme and substrate, put the cuvette in the spectrophotometer, and turn it on; 3) the math is somewhat more complicated, it leads to differential equations, and I never took differential equations. So I depend on Fersht and others for the math - though I don't trust his book to be typographically correct; for instance, eqn. 4.20 on p. 141 contains an extra factor of k_f ; compare eqn. 4.23 on the next page. Fersht describes on pp. 135-138 technical ways of handling fast reactions; one can sometimes use other tricks to slow a reaction down to be able to measure it in an ordinary spectrophotometer, and indeed the most straightforward way, though also not technically completely simple, is to carry out the reaction at a very low temperature, as low as -40° in dimethyl sulfoxide - water mixtures, to slow down subsequent reactions. The amount of enzyme used depends on the sensitivity of measurement; using a spectrophotometrically measurable product such as *p*-nitrophenol one needs product concentration and thus enzyme concentration of the order of 10^{-5} M, 0.25 mg/ml in the cuvette if the enzyme is chymotrypsin with a mol. wt. of 25,000. Fluorimetric measurements lower the level needed by a couple of orders of magnitude, and still greater sensitivity can be achieved with radioactive reagents.

Let us start from the acyl-enzyme mechanism,



where R'OH in the original and frequent case is *p*-nitrophenol - more generally call it P₁. Assume for the moment - in the most useful cases it is true - that *k*₃ is essentially = 0, the enzyme accumulates as the acyl-enzyme. You can see that one stoichiometric equivalent of *p*-nitrophenol is released at the *k*₂ step, the acylation. The molar concentration of enzyme active sites is thus measured as equal to the concentration of *p*-nitrophenol released. Of course if *k*₃ is not 0 and free enzyme is released, it goes back to the beginning and releases another equivalent of *p*-nitrophenol. The plot of P₁ released vs. time then approaches a straight line with a positive slope, corresponding to the steady-state rate of the enzyme's action, but with a non-zero intercept on the product axis at the zero time of mixing of enzyme and substrate; see Fersht Fig. 4.10, p. 156. This non-zero intercept is approximately equal to the molar concentration of enzyme [E]₀, and is called the burst (of *p*-nitrophenol release) and symbolized π.

π is equal to [E]₀ only if [S]₀ >> K_{m(app)} and *k*₂ >> *k*₃, though the latter condition favors the former because K_{m(app)} = $\frac{K_s k_3}{k_2 + k_3}$, where K_s = $\frac{k_1}{k_{-1}}$, the dissociation constant of the E·S complex, and if *k*₂ >> *k*₃ K_{m(app)} is << K_s. In the case I worked with, the reaction of trypsin with *p*-nitrophenyl *p*-guanidinobenzoate, I could calculate indirectly that K_{m(app)} was about 10⁻¹¹ M. More generally, how-

ever, $\pi = [\text{E}]_0 \frac{\left[\frac{k_2}{k_2+k_3}\right]^2}{\left[1 + \frac{K_{m(\text{app})}}{[\text{S}]_0}\right]^2}$; a plot of $\frac{1}{\sqrt{\pi}}$ vs. $\frac{1}{[\text{S}]_0}$ will yield as y intercept $\frac{1}{\sqrt{[\text{E}]_0}}$ In practice, if

you are using a titrant where [S]₀ is not large compared to K_{m(app)}, as may be the case if both binding and solubility are poor, you do this plot once, determine the fudge factor by which π at some usable [S]₀ must be multiplied to give the true [E]₀, and ever afterward use that [S]₀ and that fudge factor.

What I have just described is called enzyme titration, the measurement of the concentration of active enzyme as the production of one molar equivalent of product P₁. Obviously this is less sensitive than a catalytic assay; what are its advantages? For one thing, the assay is standardized in terms of the extinction coefficient, fluorescence yield, or specific activity of a small molecule, which can be determined unequivocally, without being subject to the effects of pH, temperature, activators, etc. to which the catalytic activity is subject. However, if the product is *p*-nitrophenol, one must remember that its pL_a is 7.15; the pH of the assay solution must be controlled very carefully, since it affects the effective molar extinction coefficient of the product. When I did this I used veronal buffer at pH 8.3, better than a full pH unit above the pK_a; veronal because unlike amine buffers it does not react with nitrophenyl esters non-enzymatically - but it is a 'controlled substance' that you now cannot readily buy.

One is also not subject to uncertainty as to whether 'pure' enzyme was really fully active; inactive enzyme is the most difficult thing to purify away from active enzyme. The purity of the enzyme can be defined as

$$\frac{\text{molarity by active site titration}}{\text{molarity as protein } \left(\frac{\text{mg/ml}}{\text{mol. wt.}}\right)}$$

If one is carrying out a chemical modification reaction, a catalytic assay has an ambiguity: a preparation retaining 20% of the initial activity may be *all* a form with 20% of native activity, due to decrease in V_{max} or increase in K_m; or it may be 20% unmodified enzyme, 80% completely inactive enzyme; or

anything in between. Active site titration removes the ambiguity, as it measures all enzyme with *any* catalytic activity (100% in the first case, 20% in the second), and thus allows distinction between the two cases. The same argument applies to mutant forms of the enzyme created by site-specific mutagenesis, and to variations in rate among isozymes.

There are also enzyme titrations which involve measurement of some stoichiometric complex of enzyme with a small molecule, for instance the complex of liver alcohol dehydrogenase with NAD⁺ and pyrazole. In this case one needs to know the extinction coefficient or fluorescence yield of the complex. If the key ligand (here pyrazole) is tightly enough bound, one can titrate excess enzyme with known amounts of ligand and determine the molar extinction coefficient of the complex by assuming that at low ligand concentrations all the ligand is bound and the change in absorbance represents a stoichiometric amount of E·NAD⁺·pyrazole formed.

Now let us return to the curved part of the plot, where the concentration of product P₁ is still approaching the straight line whose equation is P₁ = π + k_{cat}t. Take first the case where k₃ = 0 and [P₁] approaches a horizontal line. The reaction is just EOH + S ⇌ E-P₂ + P₁, a first-order chemical reaction.

The rate of this reaction (assuming [S]₀ >> [E]₀) is just $\frac{d[E]}{dt} = -bt$, where b is an observed rate constant - the papers on titration use this symbol. Then [E]_t = [E]₀e^{-bt}, or ln[E] = ln[E]₀ - bt. [E] in this case is the amount of enzyme remaining *unmodified*, the distance between the plot of [P₁] and the horizontal line with y intercept = π. Since π = [E]₀, at least in this case, and [E] = π - [P₁], we can write ln(π - [P₁]) = lnπ - bt, and b, the rate constant of acylation, is determined. If k₃ > 0, there is turnover of the enzyme, the plot of [P₁] approaches a straight line whose slope is the rate of turnover k_{cat}, which we earlier found to be $\frac{k_2 k_3}{k_2 + k_3}$. One then plots ln[π + k_{cat}t - [P₁]_t] vs. t to get the observed rate constant b.

Fersht describes this situation more generally as two consecutive irreversible reactions, using k₁ and k₂ where I use k₂ and k₃. In my terms [B] = (EO-COR) = $\frac{[S]_0 k_2}{k_3 - k_2} e^{-k_2 t} - e^{-k_3 t}$; but I can't figure out how this comes out positive when k₂ > k₃. But this is the more general equation describing the concentration as the steady state decays as well as when it forms.

The observed rate constant b is related to substrate concentration in much the same way as v in simple Michaelis-Menten kinetics. It is defined as

$$b = k_3 + \frac{k_2}{1 + K_s/[S]_0} = k_3 + \frac{k_2[S]_0}{K_s + [S]_0} = k_3 \frac{K_s + [S]_0}{K_s + [S]_0} + \frac{k_2[S]_0}{K_s + [S]_0} = \frac{[S]_0(k_2 + k_3) + K_s k_3}{K_s + [S]_0}$$

where the denominator in the second term of the first expression represents how much of [E]₀ is present as the non-covalent E·S complex as acylation starts. This can be simplified if one stipulates that

[S]₀ >> K_{m(app)}, or [S]₀ >> $\frac{K_s k_3}{k_2 + k_3}$, or [S]₀(k₂ + k₃) >> K_sk₃, so that one can eliminate the last term in the

equation above, leaving $b \approx \frac{(k_2 + k_3)[S]_0}{K_s + [S]_0}$. This can be inverted like a L-B plot, $\frac{1}{b} = \frac{1}{k_2 + k_3} +$

$\frac{K_s}{k_2 + k_3} \frac{1}{[S]_0}$; a plot of $\frac{1}{b}$ vs. $\frac{1}{[S]_0}$ will have intercept $\frac{1}{k_2 + k_3}$ and slope $\frac{K_s}{k_2 + k_3}$. Given any two of

these quantities one can determine the third. If you can determine from steady state kinetics the K_{m(app)}

= $K_s \frac{k_3}{k_2 + k_3}$, which = k₃ times the slope of this plot, you can determine k₃ in this way. If k₃ is too small to be determined in this way, as with trypsin and p-nitrophenyl p'-guanidinobenzoate where K_{m(app)} is

about 10^{-11} M, you can isolate the acyl enzyme and measure its rate of hydrolysis, which is k_3 , as the rate of recovery of activity - it took two days for full recovery in this case. K_s is determined by dividing slope by intercept, exactly as with a Lineweaver-Burk plot.

Note how in this expression the intercept is the *sum* of the two rate constants. This is similar to the situation when determining the rate constants k_f , k_r of a *reversible* reaction; the observed rate constant, analogous to b above, is the sum of the two constants, even when you are observing the first stage of approach to equilibrium. Fersht calls this observed rate constant $1/\tau$, the reciprocal relaxation constant, a term which comes from temperature jump studies where a sudden change in temperature changes the equilibrium of the reaction and the approach to the new equilibrium is observed. In a simple $A \rightleftharpoons B$ reaction the individual rate constants k_f , k_r cannot be determined unless what he calls an amplitude factor $\frac{k_f}{k_f+k_r}$ is also known, analogous to $\frac{k_3}{k_2+k_3}$ above. But in a case where there is also substrate, such as binding of substrate to enzyme, if this is slow enough to be observed at least by stopped-flow methods, the on rate depends also on the substrate concentration, $1/\tau = k_{off} + k_{on}[S]$; a plot of the observed rate constant vs. substrate concentration will have slope k_{on} , intercept k_{off} , see Fig. 4.7, p. 144 of Fersht.

It may be difficult to determine the rate constant b , even with stopped-flow instrumentation. But in presence of a competitive inhibitor I , whose K_i , dissociation constant for $EI \rightleftharpoons E+I$, has been determined by competition with the ordinary catalytic reaction, one can slow down the reaction. The expression for $1/b$ is $\frac{1}{b} = \frac{1}{k_2+k_3} + \frac{K_s(1+[I]/K_i)}{(k_2+k_3)[S]_0}$. One can keep $[S]$ constant and vary $[I]$, the plot of $\frac{1}{b}$ vs $[I]$ has intercept $\frac{1}{k_2+k_3} + \frac{K_s}{(k_2+k_3)[S]_0}$ and slope $\frac{K_s}{(k_2+k_3)K_i[S]_0}$. Multiplying the latter by K_i gives $\frac{K_s}{(k_2+k_3)[S]_0}$, which can be subtracted from the intercept; if you still have an appreciable quantity you can calculate k_2+k_3 , which is approximately k_2 . Knowing k_2 and k_3 (from turnover) you can calculate K_s and thence $K_{m(app)}$; you then know if $[S]_0 \gg K_{m(app)}$ and the extrapolation of $1/\sqrt{\pi}$ vs. $1/[S]_0$ is unnecessary. The value of $K_{m(app)} = 10^{-11}$ M for *p*-nitrophenyl *p'*-guanidinobenzoate as titrant for trypsin was calculated in this way.

Another use of an inhibitor is described by Fersht on pp. 220-221. The inhibitor proflavin increases its fluorescence when it binds to an enzyme active site, decreases it when it is displaced. The E·proflavin complex is in equilibrium with free E, and thus with E·S; this equilibrium is reached immediately on addition of S. But as $E \cdot S \rightleftharpoons E-S$, the acyl-enzyme or equivalent intermediate, enzyme is pulled out of the other complexes, including E·proflavin. Thus a further decrease in proflavin fluorescence is seen, whose observed rate constant is the same observed constant b described above. An advantage is that the same inhibitor can be used with many substrates, to characterize the K_s and k_2 in each case, without having to make new nitrophenyl esters. Displacement of a fluorescent ligand can be used similarly to measure the dissociation constant of other ligands from the enzyme, even metal ions which displace the fluorescent ion terbium; I will give you a sheet on that at a later class.