

Measuring Binding

We often want to measure the binding of a ligand, another molecule, usually small, to a protein molecule, whether the protein is an enzyme and the ligand is the first substrate, or the protein is something else whose behavior is affected by binding of the ligand. How do we measure binding? What do we want to measure? In the simplest case we are concerned with the binding of ligand L to protein P, $P + L \rightleftharpoons PL$, more generally $P + nL \rightleftharpoons PL_n$. We want to measure the dissociation constant $K_d = [P][L]/[PL]$ and the number of sites n. With a pure protein of well-defined mol. wt. n expresses the number of sites per protein molecule, with a fairly pure protein and an assumption as to the number of sites n expresses the concentration of the protein and thus its purity; with a crude preparation n expresses the number of sites per mg protein in the preparation, for instance a membrane preparation containing bound receptor proteins.

Besides the concentrations [P], [L] of **free** protein and ligand, we are also concerned with the **total** concentrations of protein and ligand, $[P]_0$ and $[L]_0$. These are typically more easily measured than the concentrations of **free** protein and ligand, particularly the latter when $[P]_0 \cong [L]_0$. $[P]_0 = [P] + [PL]$ and $[L]_0 = [L] + [PL]$, so a measurement of any two thus measures the third.

There are two general ways of measuring binding: measuring [PL] directly by some specific property, and measuring $[L]_0 = [L] + [PL]$ and separately free [L], and thus [PL] by difference. Each of these has advantages and disadvantages. In the former case, the complex can be measured in presence of a large excess of free L - indeed one often can assume $[L] \cong [L]_0$ - and frequently with low concentrations of protein. One "property" of the complex may be protecting the protein against some sort of inactivation - heat or reaction with a chemical modifying reagent; the rate of inactivation will depend on the concentration of **free** protein [P]. If the protein is inactivated at some rate k_0 in absence of the protecting ligand, then the rate k_{obs} in presence of some concentration [L] gives us $[P]/[P]_0 = k_{obs}/k_0$, and $[PL]/[P]_0 = 1 - k_{obs}/k_0$.

More frequently, [PL] is measured by some physical property, such as greatly enhanced fluorescence of L when bound to the protein. This can happen in two ways: 1) binding puts L into a more hydrophobic environment, in which the lifetime of the excited state is longer, hence it is more likely to fluoresce; if the quantum yield of L free in solution is low, a large enhancement may be possible. Very often binding of NADH or NADPH is measured this way. 2) If the ligand binds sufficiently close to a tryptophan or a chemically attached fluorescent group, and absorbs in the range where tryptophan or attached group fluoresces, it can pick up energy from the tryptophan or attached group and fluoresce at **its** normal wavelength of fluorescence. Thus excitation at 290 nm may result in fluorescence of NADH at 425 nm, where normally it would be excited at 340 nm, where tryptophan fluoresces. In some cases, as with the rare earth metal ion Tb^{+++} , whose own absorbance is low, the protein absorbs light much better than the ligand, and if it is close enough to a tryptophan, as with Tb^{+++} binding to trypsin in what is normally a Ca^{++} -binding site, the enhancement of fluorescence can be very large. If the ligand does not itself fluoresce, its binding may still be measured by **quenching** of protein fluorescence if excited tryptophans transfer energy to the ligand rather than fluorescing themselves. Other physical properties which can be used are absorption by specific complexes (for instance the alcohol dehydrogenase - NAD^+ - pyrazole complex) and changes in the circular dichroism spectrum of the enzyme - or induction of a CD spectrum for the ligand when it binds in an asymmetric environment.

These methods have one big drawback: one does not know the molar yield of the complex, i.e. the molar extinction coefficient if one is measuring absorbance. If one knew the concentration of bound ligand one could readily determine the molar yield, but the concentration of bound ligand is what one set out to measure in the first place. If one can make the measurement when free ligand is in large excess one can define a maximal yield, for instance if one is measuring fluorescence the maximum yield is

ΔF_{\max} . If one knew the molar concentration of enzyme - or rather of binding sites - one could define the molar yield, but frequently one is also trying to determine the concentration of binding sites. Sometimes when binding is very tight one can look at ΔF when $[P]_0 > [L]_0$ and ΔF is increasing linearly as L is added. Then one can assume that $[PL] = [L]_0$ and calculate the molar fluorescence yield. If $K_d \gg [P]_0 \approx [L]_0$, the plot of ΔF will go up linearly, then flatten off, with only a little curvature near the "corner". In this case one can extrapolate the flat plateau and the linear increase; the x value of their intersection is the molar concentration of $[P]_0$. See Fig. 6.6 in Fersht, p. 206. But this situation is not always available, and when it is the K_d is difficult to determine.

A general approach which deals with these problems has been described by Lodola, Spragg and Holbrook, as explained in detail on the sheets handed out. This method uses **total** ligand $[L]_0$ in the plot, rather than free ligand $[L]$ which may be ill-defined when $[P]_0 \approx [L]_0$. The dependent parameter is called \bar{v} in the paper. $\bar{v} = \Delta F / \Delta F_{\max}$, or one can use any other physical property which is saturable, including $1 - k_{\text{obs}}/k_0$ as described above. ΔF_{\max} can in principle be determined from a Lineweaver-Burk-type plot when $[L] \approx [L]_0 \gg [P]_0$. The equation is $K_d(1/1-\bar{v}) = [L]_0/\bar{v} - [P]_0$; the derivation (with E rather than P) is in the handout. One plots $[L]_0/\bar{v}$ on the y axis, vs. $1/1-\bar{v}$, on the x axis. There are no actual points with $1/1-\bar{v}$ less than 1, since \bar{v} is a fraction, between 0 and 1, and $1-\bar{v}$ cannot be greater than 1, but one can nevertheless extrapolate the plot to the y axis; the y intercept is $[P]_0$, because when $[L]_0/\bar{v} = [P]_0$ the right side of the equation, $[L]_0/\bar{v} - [P]_0$, equals zero, the left side must also equal zero, and one is at zero on the $1/1-\bar{v}$ scale. The slope of the plot is K_d . $[P]_0$ is best determined when it is high, K_d when $[P]_0$ is low. This treatment assumes that all sites measured have the same K_d , which will make the line straight; if they aren't it won't be, but they don't worry about that.

One other problem of fluorescence measurements is that the molar yield, for instance of fluorescence, may not be the same for all molecules of L bound - some may not show any increase of fluorescence. For instance, mitochondrial aldehyde dehydrogenase E2 isozyme, which Dr. Pietruszko has worked on, is a tetramer, but only the first NADH to bind, $K_d = 0.5 \mu\text{M}$, shows fluorescence enhancement, though three others, with increasing K_d s, can be shown by other means to bind. Thus you may be missing significant information if you rely on fluorescence enhancement. On the other hand, a more general method may confuse you with binding which is **not** significant.

Even if the ligand whose binding you want to measure does **not** have a useful characteristic such as fluorescence, you may be able to measure its binding by competition with a fluorescent ligand. The other side of the handout sheet describes this, with measurement of Ca^{++} binding to trypsin in competition with Tb^{+++} as the example. But this is a general treatment usable for any displacement of a signaling ligand by another.

The other general method is to measure $[L]$ when $[L]_0$ is known and calculate $[PL]$, or $n[PL_n]$, by difference. In some cases one can measure free $[L]$ directly, as with a specific ion electrode; if the free $[L]$ is $10 \mu\text{M}$, but one has put in a total of $20 \mu\text{M}$, the rest must be bound. More generally one can use **equilibrium dialysis** or related methods. In equilibrium dialysis one has two chambers, separated by a semipermeable membrane; the protein is in only one of them. The ligand can start out in one chamber or both, but at equilibrium **free** L is at the same concentration in both. One thus measures **total** ligand in both chambers, most often by radioactivity, and the difference between $[L]_0$ in the chamber **with** protein and that in the chamber **without** protein is $[PL]$. In order for this difference to be measured with some accuracy the protein concentration must be large enough so that the difference between the chambers is large, ideally as large as the $[L]$ in the chamber without protein. To achieve this the **molar** concentration of protein, more rigorously concentration of binding sites for L, must be of the same order of magnitude as $[L]_0$ and both must be of the same order of magnitude as the dissociation constant K_d .

This can call for a lot of protein. Another drawback of equilibrium dialysis is that one typically incubates overnight to reach equilibrium - though equilibrium may in fact be reached a lot sooner - and either the protein or the ligand (NADH, for instance) may not be stable for that long a period. In that case one can use some sort of **forced** dialysis. For instance, one could mix protein and ligand in solution, then force some of the solution through an ultrafiltration membrane which does not pass the protein. The total ligand concentration in solution passed through will be $[L]$, that in the solution above the filter will be $[L] + [PL]$. In some cases, especially with membrane preparations, one pushes all the solution through the filter, and measures radioactive ligand retained on the filter, assuming it is $[PL]$; strictly, one should assume some of the free ligand is also retained, the volume of wet protein on the filter not being negligible. If the binding is tight enough one may be able to wash the filter, eluting unbound L but not tightly bound L. Or one may wash the filter with excess non-radioactive ligand, assuming that radioactive ligand which is unbound or weakly bound will rapidly exchange with free ligand, while tightly bound ligand won't. This is true only when the K_d is very low and the exchange is slow.

Another method is that of Hummel and Dryer; it is probably not much used nowadays, but Fersht describes it, p. 203 and Fig. 6.4 on p. 204. One equilibrates a small gel filtration column with a known concentration of L, then applies protein, also in presence of that conc. of L. One collects small fractions of eluate and measures $[L]_0$ in them, as by radioactivity. Where the protein elutes there will be excess L, representing the $[PL]$ present, in equilibrium with the free $[L]$ with which the column was equilibrated. Later there will be a hole, where the $[L]_0$ dips below the level of free L with which the column was equilibrated; this also represents the amount of L bound to the protein and eluting earlier. Or one can do basically the same thing, but with protein sedimenting through a sucrose gradient, with a constant level of free L, in an ultracentrifuge; in the fractions from the gradient there should be excess L where the protein is.

Data thus obtained is usually plotted by a **Scatchard plot** of $v/[L]$ vs v , where v is properly the ratio of concentration of bound L to molar concentration of protein $[P]_0$, and $[L]$ is free L, normally assumed $= [L]_0$, but this may not be true when a significant fraction of $[L]_0$ is actually bound to the protein. The Scatchard plot is actually an Eadie plot with the axes reversed, and v replacing v ; the x intercept is n , the number of binding sites per mole (or mg) protein, and the slope is $-1/K_d$.

The virtue and vice of the Scatchard plot is that it also shows looser binding of L to P, as points which leave the line before it hits the x axis and go on out to the right looking for a larger value of n . However, if there is significant looser binding even in the range where the tighter binding is being measured, as for instance when n for the looser binding is a much higher number, and thus a significant number of L will be bound loosely even at $[L]$ far below this higher K_d , then amount measured as bound will be larger than predicted by the tight binding, and n for the tight binding will be significantly overestimated. Dr. Kahn has produced computer print-outs demonstrating this. One way of looking at it is that if there is only a single variety of binding, which is saturable, then a plot of n vs **log** $[L]$ will be sigmoidal, S-shaped, leveling off when saturation is nearly achieved. To feel safe in concluding that one is looking at one variety of binding, not affected by a weaker binding, one should plot results in this way; only if the plot actually shows sigmoidal character, leveling off above the mid-point, can one believe that the data really define n well in a Scatchard plot. This situation is described by Klotz, *Science* **217**:1247 [1982].

Also of interest is the measurement of protein-protein interactions, particularly relatively weak interactions which are broken up during purification and generally whenever one does anything to separate the proteins (if one protein binds tightly to another, it should elute from gel filtration in an earlier fraction than where it would elute as a free protein, but if it dissociates it will drop back, the complex will have a long tail of dissociated protein. This is best handled by **frontal analysis**, putting a

slug of mixed proteins on the column and determining how much the front is delayed compared to what you would expect for a non-dissociating complex; the complex would be separable from the individual proteins only if the dissociation were slow. See Winzor, D.J., in *Physical Principles and Techniques of Protein Chemistry*, S.J. Leach ed., Academic Press, 1969, pp. 451-495.) Similar results can be obtained using partition between two phases, as described early in the course; if the two proteins appear in particular fractions of a countercurrent distribution experiment, but shift toward an intermediate position when preincubated together, it is evidence that they complex, even weakly. I doubt that a K_d for the complex can be determined using partition, but it is otherwise an attractive method of demonstrating weak protein-protein interactions.

When the proteins differ significantly in molecular weight, so that one is excluded from a gel filtration material such as Sephadex, one isn't, one can do the experiment in a small tube rather than a column, containing enough gel to leave only a small amount of solution above it. The smaller protein 'sees' a larger volume in the tube than the larger protein, since it can enter the pores of the gel. If the total volume available to small molecules is V_t , and that available to large molecules is V_0 , then the concentration of free L is L/V_t , that of bound L = PL is PL/V_0 ; the total conc. of L outside the gel particles is $L/V_t + PL/V_0$, and since V_0 is smaller than V_t this concentration is greater than the concentration of L outside in absence of P, L/V_t . One can determine V_t and V_0 essentially by the concentration of large and small molecules when known amounts are added to the system separately. All this is described in a paper by Metzger and Stone in *J. Biol. Chem.* for 1969, I don't have the exact reference.

This procedure is not available when the proteins are similar in size, but in this case the complex is significantly bigger than either alone, and other means can be used. As mentioned above, the complex would elute from a gel filtration column earlier, or sediment faster in the ultracentrifuge, than the free proteins; but once it gets ahead of them it will in principle begin to dissociate, and analysis may be difficult.

A method which does not suffer from the problem of separation, allowing measurement of protein-protein interaction at equilibrium without separation, is fluorescence polarization; however, it is likely to depend on chemical modification of one of the proteins with a fluorescent reporter group. It depends on the fact that a large complex tumbles more slowly than smaller molecules. If a fluorescent molecule is excited with polarized light, and does not tumble before it re-radiates, the emitted light will also be polarized; if it tumbles between excitation and emission, the emitted light will not be polarized. Thus one can measure the polarization of the emitted light, which is easy, and see if it increases in presence of another protein to which the protein of interest, probably labeled with a reporter group of carefully chosen fluorescence lifetime, is suspected to bind. There is quite a bit of math involved, which I have never done, but it isn't bad; I suspect the Leach book tells how to do this.