Aims of this Laboratory:

For the current lab, you will explore several aspects of ligand binding. Ligand binding can address questions as to whether a particular substrate or allosteric effector alters 1) the binding of substrate or cofactor, or 2) whether the native and mutant forms of the protein have the same or different affinities for a ligand or have altered allosteric interactions triggered by, for example, cofactor binding. In these studies, we will explore several different ligand binding methodologies. The following introductory material describes theoretical aspects of ligand binding studies and the ligand binding process, as well as, examples of how to set up various types of ligand binding experiments involving equilibrium dialysis, fluorescence measurements and a typical "protection" type ligand binding experiment. Worked examples of how to quantitatively analyze the data are also included. You will also find a number of problem sets that will test your ability to appropriately analyze and display ligand binding data.

Introduction to ligand binding studies

A property shared by all proteins and many other functional macromolecules is their ability to bind to another molecule. Understanding and quantitating such binding processes is critical to all of biochemistry and molecular biology and is an essential tool in the armory of a well-trained biochemist. Just as with enzyme kinetics, which is used to quantitate the catalytic abilities of enzymes and becomes a tool to be used in a wide variety of structure-function studies of enzymes, ligand binding studies are the primary tool of the remainder of biochemistry and molecular biology. In addition, ligand binding studies provide a valuable complement to enzyme kinetic studies.

The binding process is quite simple:

$$P + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} PL \qquad \text{eq. 1}$$

Where P is the protein and L is the ligand [a small molecule or another protein]. There is a rate of binding, k_{+1}, and a rate of dissociation of the ligand, k_{-1} and of course an equilibrium constant, which usually in ligand binding studies is defined as a dissociation constant, K_D:

$$K_D = \frac{[P][L]}{[PL]} \qquad \text{eq. 2}$$

$$K_D = \frac{k_{-1}}{k_{+1}}$$

eq. 3

Where [P] is the concentration of free protein at equilibrium, [L] is the concentration of free ligand at equilibrium and [PL] is the concentration of protein bound ligand at equilibrium. Another way of thinking about ligand binding is encompassed in the concept of saturation, Y, where Y is defined as:

$$Y = \frac{[PL]}{[P] + [PL]}$$

eq. 4

The saturation, Y, can be redefined by substituting for [PL] from the equilibrium equation for a dissociation constant:

$$[PL] = \frac{[P][L]}{K_D}$$

eq. 5

Hence

$$Y = \frac{\left(\frac{[P][L]}{K_D}\right)}{([P] + [PL])}$$

eq. 6

Divide the top and bottom of this equation by [P]:

$$Y = \frac{\left(\frac{[L]}{K_D}\right)}{\left(1 + \frac{[PL]}{[P]}\right)}$$

eq. 7

Multiplying both top and bottom by K_D we get:

$$Y = \frac{[L]}{\left(K_D + \frac{K_D[PL]}{[P]}\right)}$$

eq. 8

Solving the equilibrium equation for a dissociation constant for [L] and substituting into the above equation, yields:

$$Y = \frac{[L]}{K_D + [L]}$$

eq. 9

Which is a saturation function described by a rectangular hyperbola for a plot of Y versus [L]. The maximum value of Y describes the maximum binding capacity of the protein for the particular ligand. Hence forth to distinguish this maximum binding capacity from the above saturation function this parameter will be referred to as B_{max}. K_D is the dissociation constant for the ligand binding as described above.

There are a variety of experimental approaches for determining the dissociation constant and maximum binding capacity of a ligand binding to a protein. It must be emphasized that, in the treatments developed here, the binding is assumed to be in a free equilibrium on the time scale at which the experiment is performed. As discussed in the module on enzyme kinetics, such equilibrium binding studies are of extreme importance in elucidating kinetic mechanisms of enzymes and establishing the basis of nonlinear kinetics in systems that show non-Michaelis-Menton behavior.

Ligand binding studies play an important role in many aspects of protein chemistry and enzymology. Many proteins must be assayed based on their specific ligand binding since they have no enzymatic activity. In initial rate kinetic studies, the independently determined dissociation constants can be used in conjunction with kinetically determined dissociation constants as a test of the validity of a proposed kinetic mechanism. In addition, ligand binding studies are essential when examining systems that exhibit nonhyperbolic kinetics.

In any binding study, two parameters describe ligand binding: B_{max}, the maximum number of ligand molecules bound per mole of protein, and K_D, the dissociation constant of the reversible binding process. A variety of techniques have been developed to study ligand binding, although some cannot give a value for B_{max} because of the nature of the assumptions made in analyzing the data. Such methods have great use, however, often in terms of ease and accuracy, and can be employed where knowledge of K_D but not B_{max} is important. In other instances, B_{max} may be the parameter whose value is primarily required, and of course techniques must be chosen that will yield such information.

Approaches for studying ligand binding can be divided into two categories: direct and indirect. In addition to experimental methods for determining ligand binding, a number of ways of presenting ligand binding have been developed, and depending on the information being sought, different types of data presentation are more appropriate. In the context of the graphical representation of ligand binding data, we examine the effects of systems that do not follow hyperbolic saturation.

Methods to study ligand binding
Direct Methods
Equilibrium Dialysis Method
The various direct methods for estimating the amount of ligand bound to a protein have all evolved from equilibrium dialysis and depend on physically separating bound ligand from free. Assuming that there are direct methods for quantitating the amount of ligand bound and free in solution, these techniques all give unequivocal (except for experimental error) estimates of B_{max} and K_D. In equilibrium dialysis, a typical setup of which is shown in Figure 1, the free ligand is allowed to reach an equilibrium across a semipermeable membrane that separates the protein from the bulk phase of the solution.

At equilibrium, the concentration of ligand in the non-protein-containing compartment equals the concentration of *free* ligand (L_{free}) in the protein-containing compartment. If the *total* ligand concentration (L_{total}) in the protein-containing compartment is known (from experimental determination or, if this is not possible, by subtraction of the amount of ligand in the non-protein-containing compartment from the total amount of ligand initially added), the amount bound to the protein (L_{bound}) can be calculated by subtracting the contribution of free ligand: $L_{bound} = L_{total} - L_{free}$. From a single experiment one can find K_D by substitution into

$$K_D = \frac{[P][L]}{[PL]} \qquad \text{eq. 2}$$

where $L_{free} = [L]$ and is experimentally determined; $L_{bound} = [PL]$; and the concentration of [P] is calculated from [PL] and P_{total} which is presumably known. Application of this equation provides a value for K_D but does not give a value for B_{max}. In the simple situation where hyperbolic saturation of a single class of sites occurs, K_D is an accurate estimate. To obtain a value for B_{max} in addition to K_D, the dialysis experiment is repeated with a series of different total ligand or protein concentrations. Results in the simple case are fit to the saturation function and values for B_{max} and K_D obtained. If the binding situation is more complex than one ligand binding to one macromolecule with a single dissociation constant, this type of experiment also reveals such complexity and the data are then plotted using one of the graphical methods described later.

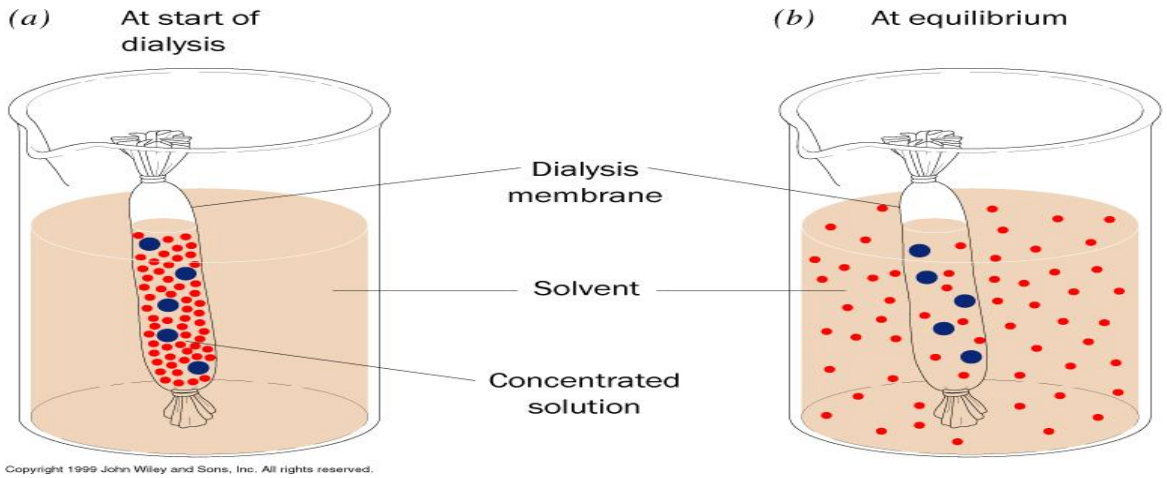


Figure 1. Scheme for equilibrium dialysis. Copyright John Wiley and Sons 1999.

Before moving on to some of the other direct methods for studying ligand binding, we examine in more detail the experimental procedures of equilibrium dialysis. A number of problems may be encountered:

1. True equilibrium of the free ligand may not be achieved. This is usually controlled for in two ways: (a) a duplicate experiment is set up with no protein in either compartment. If equilibrium is reached in the time period used in the experiment, the ligand concentrations in each compartment will be equal; and (b) duplicate determinations in the presence of protein are made, but in one the ligand is initially placed in the compartment containing the protein, while in the other the ligand is initially placed in the solvent compartment. If an effective equilibrium is established during the time course of the experiment, identical results will be obtained.

2. During the time required to reach equilibrium (often 10 to 15 hours), either the ligand or the protein may decay to inactive forms that interfere with the concentration determination. A more serious problem exists if the ligand decays to a form that competes with the true ligand at the protein binding site. The former artifacts can be controlled for by assaying the protein activity and the effective total ligand concentration before and after dialysis.

3. In many cases the free ligand concentration is conveniently assayed by spectrophotometric measurements. If the bound ligand has different spectral properties from the free ligand, the total ligand concentration in the protein-containing compartment will be incorrectly estimated. This can be overcome by denaturing the protein after equilibrium has been achieved and determining the concentration of the total ligand in that compartment when it is free in solution. As will be discussed later, it is often possible to determine an extinction coefficient for the bound ligand via spectral titrations. If it is known, the concentration can be determined directly from the total absorbance of the protein-containing compartment after subtraction of the absorbance of the free ligand (which is known from the compartment lacking protein). These problems are overcome if a ligand is radioactively labelled.

4. In situations where the total ligand concentration is varied (usually the case), one reaches a situation as B_{max} is approached where the amount bound is calculated by subtraction of one large number (for free ligand concentration) from a slightly larger number for $[L_{free}] + [L_{bound}]$. This situation can lead to a large experimental error.

Having considered these potential pitfalls, we now examine some of the other "direct" methods for following ligand binding together with some of their limitations.

Forced Dialysis Method

In a number of variants of equilibrium dialysis, there is physical separation of free from bound ligand via a semipermeable membrane. The ligand and protein are initially in the same compartment, separated from a collection vessel by the semipermeable membrane

At the start of the experiment, pressure is applied to the protein-containing compartment to cause a "forced" dialysis. Solvent, containing free ligand solute but not the protein solute, is forced through the membrane, collected, and the concentration of free ligand determined. In the early versions of such schemes, the forced dialysis was continued until the protein (and its bound ligand) remained associated with the inside of the semipermeable membrane. The assumption was made that the solvent, containing free solute ligand, was in equilibrium with the protein-containing compartment, and hence the concentration of ligand in the forced dialysate was equal to the concentration of free ligand in the protein-containing compartment. To avoid problems that might arise from concentrating the protein, the experiment is terminated at a fixed point so that a constant protein concentration can be used.

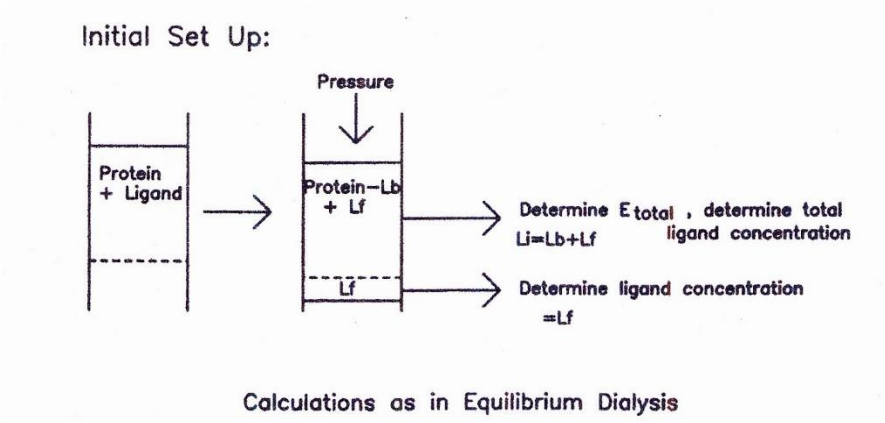
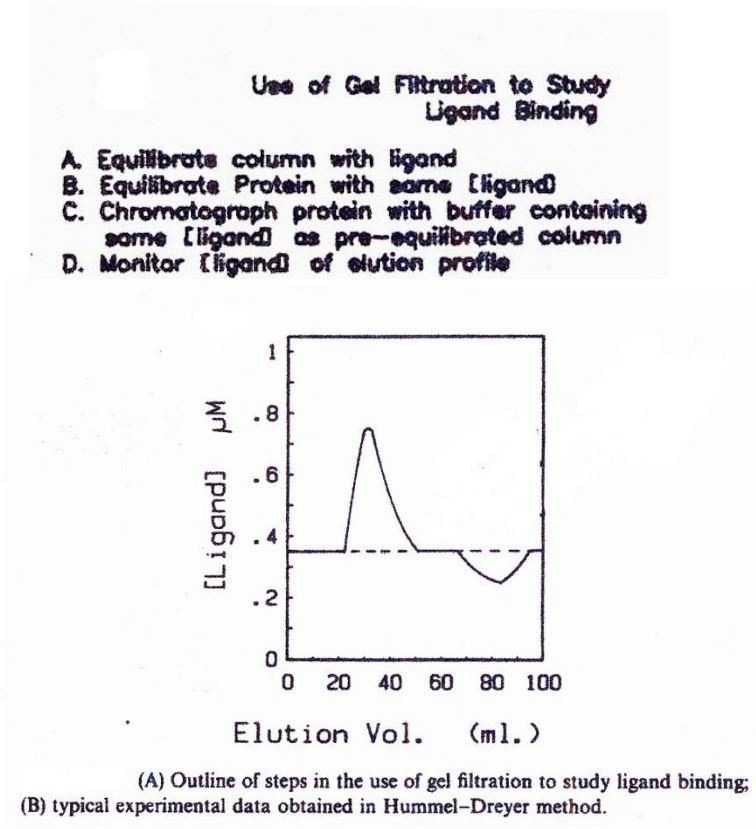


Figure 2. Scheme for forced-dialysis method of determining equilibrium binding.

To achieve multiple determinations, buffer containing ligand is added to the protein containing compartment at the termination of one run and the process repeated, allowing either increasing or decreasing total ligand concentrations to be used.

Protein Transport Methods

Several methods have been developed where protein is transported through a solution containing ligand, and the amount of ligand associated with the region containing protein is compared to regions lacking protein to give the amount of ligand bound to the protein. Two methods, gel filtration and sedimentation, will be discussed.



(A) Outline of steps in the use of gel filtration to study ligand binding; (B) typical experimental data obtained in Hummel–Dreyer method.

Figure 3. Hummel-Dreyer method to study ligand binding.

The simplest way of using gel filtration chromatography to study ligand binding is exemplified by the *Hummel-Dreyer method,* the general principles of which are given in Fig. 3. A gel filtration column is equilibrated with buffer containing a ligand at a defined concentration. Equilibration of the column is determined by the eluent containing the same ligand concentration as the starting buffer. The protein sample, which is pre-equilibrated with the same ligand concentration as the equilibrating buffer, is introduced to the column and the column developed with the equilibrating buffer. Under circumstances where the protein binds the ligand, the sample solvent is depleted with respect to free ligand. As elution proceeds, the protein is separated from ligand-depleted solvent and elutes as a peak with its bound ligand such that the total ligand concentration in the protein peak is higher than the concentration of the equilibration buffer. As a result, a corresponding trough follows this peak. When the ligand concentration is monitored as the column develops, the profile obtained looks like that shown Fig. 3. If the ligand does not bind to the protein, the measured concentration of *ligand* during elution does not deviate from the concentration of the equilibration buffer, the dashed line in Fig.3B.

To achieve the best results, it is important to choose a stationary phase matrix that gives maximum separation between the ligand and the protein. This is usually a matrix that totally excludes the protein. It is also essential that the ligand elution profile has a plateau of constant ligand concentration between the peak and the trough. This indicates that for the flow rate used to elute the column, the bound and free ligand are in effective equilibrium. If this plateau is not observed, the binding equilibria are too slow relative to the flow rate of the column and the experiment must be repeated at a lower flow rate.

The simplest way to calculate the amount of bound ligand is to collect fractions and determine the ligand concentration per fraction. Although in theory the calculation of the amount of bound ligand can be made using either the peak or the trough, in practice it is easier to use the trough, as interference of the ligand concentration determination by the protein is minimized. Assuming that the ligand concentration is determined by absorbance measurements, the concentration of bound ligand is given by

$$\mu\text{Mol}_{\text{bound}} = \frac{(\Sigma(\Delta A_i)(\mu l_i)}{\varepsilon_{\mu M}\ell} \qquad \text{eq. 10}$$

where ΔA_i is the difference in the absorbance of the fraction, i, and the baseline absorbance determined by the equilibrating ligand concentration; $\mu 1_i$ is the volume of fraction I; $\varepsilon_{\mu M}$ is the micromolar extinction coefficient of the ligand; and ℓ is the path length of the spectrophotometer cell. The summation is carried out for each fraction in the trough region of the chromatograph, giving the amount of ligand bound *by the amount of protein* in the initial sample.

Although the dissociation constant for the ligand binding process can be calculated from a single experiment, it is necessary to obtain additional data for more detailed analysis of the binding isotherm. Therefore, the experiment must be

repeated at either different protein concentrations of the loaded sample or, preferably, at different concentrations of the equilibrating ligand. Essentially the same approach is used when bound and free ligand are separated by centrifugation. This method is outlined below.

Outline of Sedimentation Method

1. Sedimentation cell contains ligand.
2. Protein is sedimented through ligand solute.
3. Ligand bound to sedimenting protein is estimated by one of two approaches:
    i. By change in molecular weight of protein at a series of different ligand concentrations. This approach is quite successful even with relatively small ligands (mol.wts *500-600).
    ii. Using absorbance measurements to give an estimate of bound ligand per sedimenting protein molecule.

Protein Precipitation Approaches

In a number of instances, especially where large molecule ligands are involved, it is convenient to study binding by precipitating the protein-ligand complex away from the free ligand and then determining the amount of protein and ligand in the complex. The free ligand concentration is then determined in the supernatant. Although frequently used (out of necessity), it is subject to the criticism that the binding of ligand in the presence of the appropriate precipitant may not accurately reflect that found under normal circumstances. In addition, it is necessary to control for non-bound ligand that is included in the precipitated material. When a radioactive ligand is employed, this is often achieved by repeating the precipitation in the presence of a large excess of unlabeled ligand. In this case, precipitated radioactive material is used as a control blank to be subtracted from the specifically bound ligand.

EXPERIMENT 1

An Equilibrium Dialysis Study of NAD^+ Binding to Glutamate Dehydrogenase

Glutamate Dehydrogenase has six identical subunits, each with a single active site and has the ability to utilize NAD^+ as its cofactor in the oxidative deamination of glutamate. A simple question that could be asked is whether or not there are six binding sites on the enzyme for NAD^+ and whether or not the binding sites show any cooperativity. Since NAD^+ does not demonstrate any fluorescence, it is convenient to follow binding, especially where one wants to quantitate the number of binding sites, by equilibrium dialysis. The following experiment illustrates the design set up of such an experiment.

2/16 Blue: Enzyme
2/16 Purple: Enzyme + 40 mM glutarate
2/18 Green: Enzyme + 0.5 mM ADP

Reagents:

0.04M Sodium Phosphate Buffer, pH 8.0, containing 20μM EDTA
Glutamate Dehydrogenase at 2mg/mL in 0.04 M sodium phosphate buffer, pH 8, 20 μM EDTA
1mM NAD^+ in H_2O: the concentration of this solution should be accurately determined by absorbance measurements at 260nm using a millimolar extinction coefficient for NAD^+ of 18 cm^{-1}
400mM glutarate in H_2O
50 mM ADP in H_2O

Materials/Equipment:

1cm width dialysis tubing with a 10-12 Kda cut-off: soaked and stored in deionized water
Screw cap tubes capable of holding 12-15mL of total volume
Rocker or rotator device capable of holding the screw cap tubes
Cold Room or Chromatography cabinet

Overview of the Experiment:

You will use small sections of dialysis tubing capable of holding 1-2mL total volume to contain 1mL of enzyme solution. You will dialyze against 10mL of a buffer solution containing the appropriate combination of ligands and concentration range of ligands. Dialysis will continue until the next lab period. You will retrieve the tubing to obtain its contents and also analyze the residual dialysis buffer. The contents of the tubing represent the protein-ligand complex, PL, and the free ligand, L, while the residual dialysis buffer represents free ligand, L. Each tube will contain different concentrations of the ligand whose binding you are studying and fixed concentrations of all other reagents such that you are studying the binding of only one component.

To design the concentration ranges of ligand to use in a binding experiment, you must have some idea of the ligand affinity, or conduct a preliminary experiment to establish an approximate dissociation constant. For example, binding studies suggest that the dissociation constant for NAD^+ binding to free bovine mitochondrial enzyme is on the order of 470 μM (Dalziel and Egan. The binding of oxidized coenzymes by GDH and the effect of glutarate and purine nucleotides. Biochem. J. (1972) 126:975-984) but that substrate or a suitable substrate analog increases the affinity by approximately 3 fold. This means that to achieve an effective range of saturations, from 10% to maybe 90% you would need to use a range of ligand concentrations from 50 μM to 5000 μM in the absence of a substrate analog, and 15 μM to 1500 μM in the presence of a substrate analog. The range of free and total concentrations of NAD^+ that will need to be measured during the experiment will range from less than 15 μM up to over 1mM. Using the millimolar extinction coefficient for NAD^+ at 260nm of 18 cm^{-1}, one can calculate that such measurements should be possible by spectrophotometric measurement of the NAD^+ concentrations inside and outside of the dialysis tube. Although, at the higher NAD+ concentrations, it will clearly be necessary to dilute the sample significantly: 100μM NAD^+ will have an absorption of 1.8: above the usually accepted "accurate" range of 0.05 to 1.0 for a spectrophotometer. *Note*: The linearity

of detection on the spectrophotometer in use can easily be established using a Beer's Law titration of a stock solution of NAD^+.

For the experiment here, we will use six concentrations of NAD^+ in the range 15 - 500μM and set up each measurement in duplicate. You will set up one tube with no NAD+. You should reserve 15 ml of buffer for zeroing the spectrophotometer at the end of the equilibrium dialysis experiment: thus there will be 7 conditions, each setup in duplicate.

Tube	**Outside Solution**						**Dialysis tubing solution**			
#	*Buffer*	*NAD+*	*[NAD+]*	*Effector*	*[effector]*	*H2O*	*Enzyme*	*NAD+*	*effector*	*H2O*
	mL	*mL*	*μM*	*mL*	*μM*		*mL*	*mL*	*mL*	*mL*
		Stock or diluted stock				*To vol. of 12 mL*				*To vol. of 1 mL*
1	6	0.24	20				0.5			
2	6	0.6	50				0.5			
3	6	1.2	100				0.5			
4	6	2.4	200				0.5			
5	6	3.6	300				0.5			
6	6	6	500				0.5			
7	6	0	0				0.5	0		

Place the outside solution into the screw capped tube

To a suitable length of the soaked dialysis tubing, knot one end. With a transfer pipet, introduce the 1mL solution containing the enzyme. Knot the tubing as close to the top of the contained solution as possible, removing air from the tubing. Place the knotted bag into the appropriate screw capped tube.

Place all of the tubes with their dialysis bags in the Cold Room (~4°C) on the rocker/rotator and allow to equilibrate until the next lab period.

At the completion of the dialysis period, you will need to accurately measure the protein concentration and NAD^+ concentration of the dialysis tubing contents and the NAD^+ concentration of the outside solution. This is best done by measuring the absorption spectrum from 240nm to 350nm using an appropriate dilution of each solution. The spectrum of the outside solution is then subtracted from that of the dialysis tubing solution to give a spectrum of the bound nucleotide plus protein. The no NAD+ control spectrum is then subtracted from the bound nucleotide plus protein spectrum to yield the NAD+ bound spectrum.

Data Analyses: Calculate $[L_{free}]$ for each dialysis reaction. Calculate [PL], which is equivalent to $[L_{bound}]$ for each dialysis reaction. Remember to correct for protein contribution to absorption spectrum. Create a Klotz plot from these data. Determine the K_D and $[PL_{max}]$.

Indirect Methods

Spectroscopic Methods

These depend on either a change in the ligand's or the protein's spectral properties upon complex formation, and are probably the most widely employed techniques for studying ligand binding. They have the advantages of being rapid, reproducible, and quite accurate. *However,* there are some potential sources of deception in what is an otherwise simple approach, and also (in general), these methods do not give a value for $\mathbf{B_{max}}$ only for $\mathbf{K_D}$.

Consider two cases, both involving fluorescence measurements, although the same arguments that we will use could be made for any other spectral parameter. In the first, we assume that the fluorescence of a ligand is enhanced upon binding to the protein, as depicted in Figure 4.

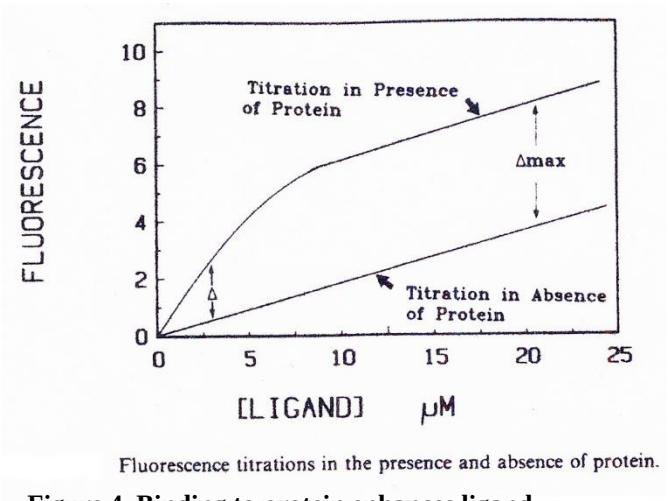


Figure 4. Binding to protein enhances ligand fluorescence.

A. Ligand Binding Studies

Consider the case of the addition of NADH aliquots to bovine glutamate dehydrogenase shown in Figure 5. Defining the following terms, we can derive an equation for the amount of bound NADH at any given point in the fluorescence titration. Let F_m be the experimentally determined fluorescence in the presence of protein at a total ligand concentration, T. F_r is the measured fluorescence in the absence of protein at a total ligand concentration T. B is the concentration of bound ligand at any point in the titration. F_B is the specific molar fluorescence intensity of bound fluorophore, and F_F is the specific molar fluorescence intensity of free fluorophore.

Then, at any given point in the titration:

$$F_m = (F_B)(B) + F_F(T - B) \quad \text{eq. 11}$$

$$F_m = (F_B)(B) + (F_F)(T) - (F_F)(B) \quad \text{eq. 12}$$

Since $F_r = (F_F)(T)$, we can substitute:

$$F_m = (F_B)(B) + (F_r) - (F_F)(B) \quad \text{eq. 13}$$

Thus:

$$\frac{F_m}{F_r} = \frac{F_B}{F_r}(B) + 1 - \frac{F_F}{F_r}(B) \quad \text{eq. 14}$$

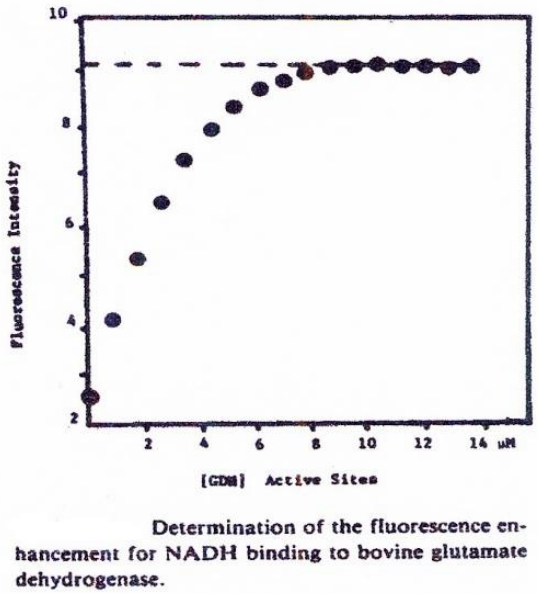
And rearranging yields:

$$\frac{F_m}{F_r} - 1 = \left(\frac{F_B}{F_r} - \frac{F_F}{F_r}\right)(B) \quad \text{eq. 15}$$

Thus:

$$B = \frac{\left(\frac{F_m}{F_r} - 1\right)}{\left(\frac{F_B}{F_r} - \frac{F_F}{F_r}\right)} \quad \text{eq. 16}$$

Substituting back $(F_F)(T)$ for F_r in the denominator, yields:



Determination of the fluorescence enhancement for NADH binding to bovine glutamate dehydrogenase.

Figure 5. Fluorescence enhancement of NADH when bound to glutamate dehydrogenase.

$$B = \frac{\left(\frac{F_m}{F_r} - 1\right)}{\left(\frac{F_B}{(F_F)(T)} - \frac{F_F}{(F_F)(T)}\right)}$$

eq. 17

Multiplying top and bottom by T yields:

$$B = \frac{\left(\frac{F_m}{F_r} - 1\right)T}{\left(\frac{F_B}{(F_F)} - 1\right)}$$

eq. 18

Defining a fluorescence enhancement, F.E., as F_B/F_F yields:

$$B = \frac{\left(\frac{F_m}{F_r} - 1\right)T}{\left(F.E. - 1\right)}$$

eq. 19

Hence, we have an equation for the concentration of bound fluorophore (B) in terms of T, the total ligand concentration, F_m and F_r the fluorescence measured at a given ligand concentration in the presence and absence of protein, respectively, and a parameter F.E., which by definition is the fluorescence of the bound ligand relative to that of the free ligand. This last parameter must be determined independently. Experimentally, the fluorescence enhancement is determined by using a fixed concentration of ligand, and varying the concentration of the protein. Such an experiment is shown for glutamate dehydrogenase in Figure 5.

As the protein concentration is increased, more of the ligand is bound. At sufficiently high concentrations of protein, no further increase in fluorescence will be observed, indicating that all of the ligand is bound. At this point, the fluorescence enhancement can be calculated from the initial fluorescence at zero protein concentration, and the final fluorescence attained at limiting protein concentrations. Experimentally, this situation can only be achieved if one can use protein concentrations significantly higher than the dissociation constant for the ligand. Since this is not always possible, the limiting fluorescence can be determined by varying the protein concentration over a limited range and extrapolating in a double reciprocal plot to limiting protein concentrations. Once a value for the fluorescence enhancement is obtained, binding parameters for ligand binding to the protein can be determined. It should be noted that the fluorescence enhancement for a given ligand binding to protein may be affected by other ligands binding at other sites to the protein. This is shown for glutamate dehydrogenase in Table 1. As can be seen, the fluorescence enhancement in various ternary and quaternary complexes is very different from that observed in the binary enzyme-NADH complex. Unless fluorescence enhancement values are determined for the particular situation being studied, very strange artifacts may be obtained.

Table 1. Fluorescence enhancement for NADH binding to glutamate dehydrogenase

Other ligands	*Fluorescence Enhancement*
At pH 7	
None	2.1
Glutamate	3.47
Glutamate + ADP	3.6
Glutamate + GTP	4.2
At pH 8	
None	3.6
Glutamate	3.47
Glutamate + ADP	3.13
Glutamate + GTP	4.2

It might be noted that arguments similar to those presented here may be applied to the situation where a decrease in ligand fluorescence is observed upon binding to protein. A very similar equation to that derived above holds for this situation:

$$B = \frac{T\left(1 - \left(\frac{F_m}{F_r}\right)\right)}{(1 - F.E.)}$$

eq. 20

Where of course, F.E. is no longer a fluorescence enhancement, but a quenching coefficient.

Frequently, when ligand binds to protein, a decrease in the fluorescence of the protein is observed. This quenching of protein fluorescence may also be used to determine ligand binding. There are two types of experimental situations that can occur, depending on the range of dissociation constants to be measured and protein concentrations that can be used. When a very low dissociation constant is to be determined a very different experimental approach applies, and can yield information with fewer uncertainties than if weaker binding is to be studied. In studies on rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, it was observed that at relatively high protein concentrations quenching of protein fluorescence was linear with coenzyme concentration up to a ratio of coenzyme to protein of about 2. Beyond this point the quenching was no longer linear. From this linear region of quenching the extent of quenching induced by the binding of each coenzyme molecule could be determined. Once this parameter is obtained, much lower protein concentrations can be used where ligand binding is not stoichiometric with ligand concentration. Such titrations can then be used to obtain binding data for coenzyme binding to glyceraldehyde3-phosphate dehydrogenase, since the amount of quenching produced by each molecule of coenzyme binding has been determined. However, in many instances, where weak binding is being observed, it is difficult to attain experimentally a situation where quenching of protein fluorescence is stoichiometric with ligand added. Hence, the ligand-dependent parameter described above cannot be obtained. However, ligand binding can still be observed and followed in such a situation with several limiting assumptions.

Figure 6 illustrates a typical experimental situation. As ligand concentration is increased at a fixed protein concentration, a gradual decrease in fluorescence is observed. From such data, it is not possible to calculate either how much quenching is produced per ligand bound, or how much of the fluorescence is quenched at saturating concentrations of ligand. The latter parameter can be obtained from a double reciprocal plot of $1/\Delta F$ vs. $1/[L]$ if it is assumed that each molecule of

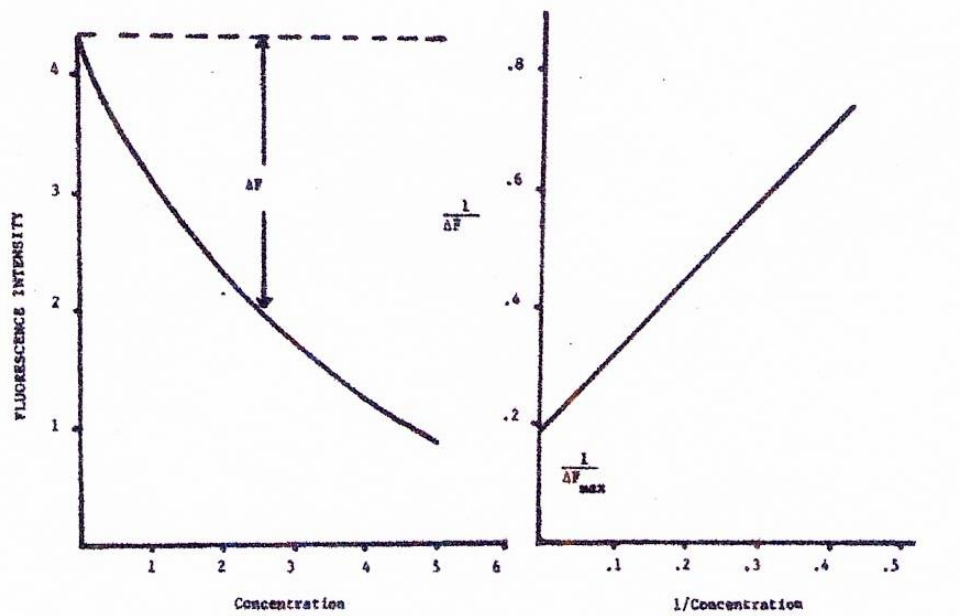


Figure 6. A representation of ligand produced fluorescence quenching in a "low affinity situation." A. Fluorescence vs. ligand concentration. B. Double reciprocal plot of "data" from A. showing determination of maximum quenching.

ligand bound produces an equal amount of quenching. Once this parameter is obtained, the amount of ligand bound at any point in the titration can be calculated if it is assumed that at maximum quenching all of the protein sites contain ligand. Hence, one must assume a number of binding sites per protein molecule, and no information about this parameter can be obtained from the binding study. However, information about affinity is obtained.

In the absence of protein, fluorescence intensity is linearly related to the ligand concentration. In the presence of protein, the fluorescence intensity rapidly increases, but eventually becomes parallel to that obtained in the absence of protein, refer back to Figure 4. The maximum change in fluorescence is defined as ΔFmax. If experimentally the two titrations do not become parallel, a value for ΔFmax can be obtained from a double reciprocal plot of l/Δ versus 1/[ligand], Figure 7.

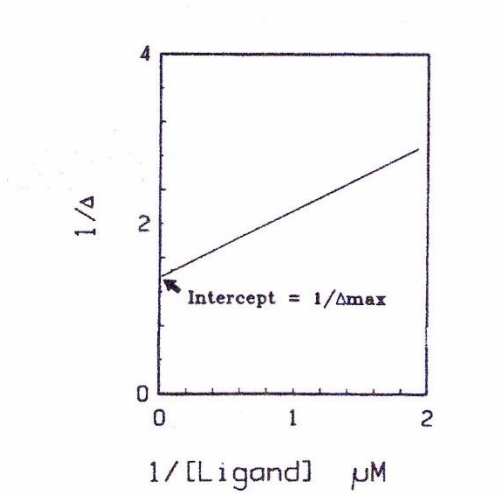


Figure 7. Experimental determination of Δ_{max} from double reciprocal plots.

If it is assumed that ΔFmax occurs when all the protein sites for the ligand are occupied, one can calculate the amount of ligand bound (L_b, L_{bound}) at any point in the titration curve if the initial concentration of binding sites (which is Bmax) is known, from

$$L_b = \left(\frac{\Delta F}{\Delta F_{max}}\right) B_{max}$$

eq. 21

Knowing the total ligand concentration at any point in the titration allows calculation of L_f and subsequently a value for K_D. *This approach depends on prior knowledge of* B_{max}.

These two ways of approaching essentially the same type of experimental data illustrate an important point: ΔFmax is a *protein-dependent parameter*, while FE is a *ligand-dependent parameter.* The experimental determination of ligand-dependent parameters allows B_{max} to be determined from indirect methods. However, if only a protein-dependent parameter can be followed, then only information about K_D can be obtained since B_{max} must be assumed.

Ligand-dependent parameters such as FE must be independently determined from an experiment of the type illustrated above. FE is determined by fluorescence titrations at a fixed ligand concentration with varied protein concentrations. Control titrations omit the ligand to allow the contribution (if any) of the protein to the fluorescence. σ_0 is the fluorescence of the chosen ligand concentration in the absence of protein. σ_i is the measured fluorescence at various protein concentrations (minus the protein fluorescence) and σ_{max} is obtained when the experimental and control titrations become parallel. At σ_{max}**,** all the ligand is bound by protein and hence FE is simply given by

$$FE = \left(\frac{\sigma_{max}}{\sigma_0}\right)$$

eq. 22

If σ_{max} is not experimentally obtained from the titration, a double reciprocal plot of $1/\sigma$ versus 1/[protein] gives an intercept of $1/\sigma_{max}$. This example involves a fluorescent ligand that undergoes a fluorescence enhancement on binding to a protein. In some instances the ligand fluorescence may be quenched, and a titration of the type shown in Figure 8 is obtained. As with the case of enhanced fluorescence, equations for use in the calculation of the bound ligand concentrations at any point in the titration can be derived using either the protein-dependent parameter, Δmax, or a ligand-dependent parameter, Q_C, defined by:

$$Q_c = \left(\frac{F_B}{F_F}\right)$$

eq. 23

where F_B is the specific molar fluorescence intensity of bound fluorophore, and F_F is the specific molar fluorescence intensity of free fluorophore. The equation for the protein-dependent parameter is as before, while the equation using Q_C is:

$$L_B = \frac{T\left(1 - \left(\frac{F_m}{F_r}\right)\right)}{(1 - Q_c)}$$

eq. 24

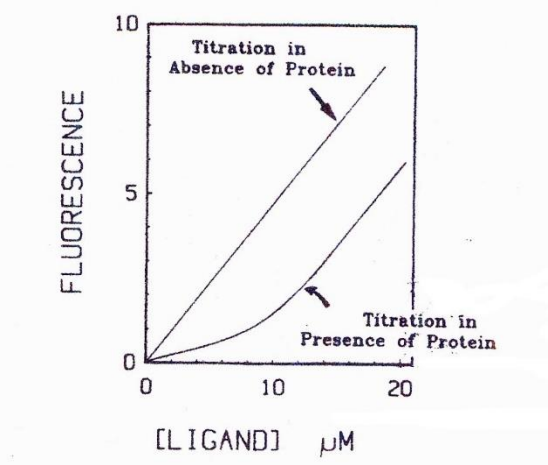


Figure 8. Fluorescence titration data obtained when the ligand fluorescence is quenched upon binding to the protein.

Although this discussion has centered on fluorescent ligands, any spectral property of the system (i.e., protein or ligand) that changes upon complex formation can be used to study ligand binding. Some of these are summarized in the Table 2.

In addition to the question of whether a protein-dependent or a ligand-dependent parameter can be determined, spectral methods of following ligand binding suffer from a fundamental limitation that can be resolved only through independently determining B_{max} using a direct method. Implicit throughout all of this is the assumption that in a multisite-per-molecule system (either two or more sites for the same ligand per polypeptide chain or a multi-subunit situation) all molecules of bound ligand contribute equally to the followed parameter. If one or more sites in a multisite system are spectrally unobservable, only binding of ligand molecules that do contribute to the signal are observed, and the true B_{max} cannot be determined, even using a ligand-dependent parameter.

Other Indirect Methods. Any property of a protein that changes upon the binding of a ligand can be used to study that ligand binding process. All such approaches depend on the experimental determination of a parameter that is equivalent to the Δmax discussed previously. As a result, these methods must assume a value for B_{max}, and can only allow determination of K_D. They are, however, quite useful, as they are often experimentally easy. Some of the experimental properties that have been used to study ligand binding include: Susceptibility to proteolysis, Denaturation by solvents, Heat stability or Chemical modification. In all instances the presence of ligand in a protein-ligand complex may either increase or decrease susceptibility to the approach; in either the case the dependence of the change on ligand concentration can give binding information.

Table 2. Spectral properties used to study ligand binding

Parameter	***Dependence***	***Comments***
Protein Fluorescence	Usually P	Usually quenching of protein fluorescence observed
Polarization of fluorescence	L	Bound ligand has higher polarization than free ligand
Absorbance	P or L	Can present problems if the ligand absorbs in the region of 260-280 nm since protein absorbance changes may mask ligand absorbance
ESR	P	useful with paramagnetic metal binding (e.g. MN): otherwise must introduce a spin label to ligand
NMR	P	In theory almost universally applicable; in practice is limited by experimental considerations such as protein concentration needed.
NOE	L	Useful with weakly bound ligands

EXPERIMENT 2

Cofactor binding to glutamate dehydrogenase: Monitoring ligand fluorescence

NADH is fluorescent if excited at 340 nm with an emission maxima between 400 and 500 nm.
When bound to a protein, the emission spectra, intensity and maxima, may be altered by the protein. As discussed in the lab manual, the ligand fluorescence may be enhanced or quenched when captured in a protein:ligand complex. In this

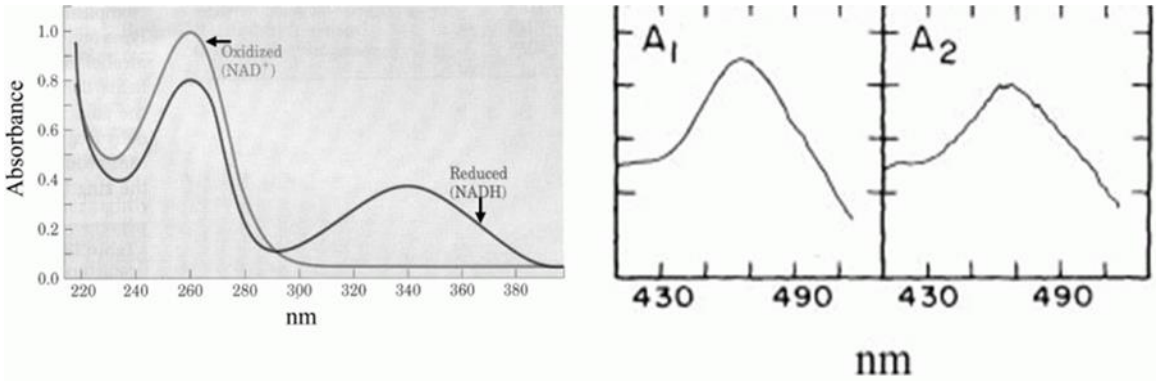


Figure 9. Excitation and emission spectra of NADH. NADH is maximally excited at 340 nm (left) and emits maximally between 460 – 480 nm (right).

experiment, you will examine NADH binding to GDH by directly exciting the ligand at 340 nm and monitoring its emission spectra from 400-500 nm.

Set Up of the Experiment that You Will Perform

In the experiment that you will perform, you are provided with the following solutions:
2 mg/mL GDH in 0.1 M Phosphate, pH 8, measure exact concentration via A_{280nm}, ε_{280nm} = 0.93 $(mg/ml)^{-1}cm^{-1}$
~420 μM NADH in Buffer, measure exact concentration via A_{340nm}, ε_{340nm} = 6.22 $(mM\ cm)^{-1}$
0.1 M Phosphate Buffer, pH 8
50 mM ADP
10 mM GTP
1 M Glutarate

And equipment:
Fluorometer
3 ml Quartz fluorometer cuvette

Experiment 2.1
Determine the dissociation constant for NADH binding to glutamate dehydrogenase

2/11 Purple group: Complete study at pH 8 + 0.5 mM ADP
2/18 Blue group: Complete study at pH 8 + 0.1 mM GTP
2/23 Green group: Complete study at pH 8 + 10 mM Glutarate

Enzyme concentration calculated:_____

NADH concentration calculated _____

You will be titrating NADH into a cuvette containing buffer (control experiment) or your enzyme (and effector, depending upon your experiment). At each concentration of NADH, you will collect an emission spectra. You

will examine NADH emission spectra from 0 - 20 µM NADH. Complete the Experimental Setup Tables 2.1-2 to guide your procedure and complete calculations necessary for data analyses.

NOTE: Use the same transfer pipette throughout the series.

NOTE: If you are testing an effector, reduce the volume of buffer commensurate with the volume of effector to be added to reach desired final concentration.

NOTE: If you are using the PTI fluorometer, you will need two cuvettes: Cuvette 1 ("The blank") will be the 0 NADH cuvette; Cuvette 2 will be the cuvette into which you titrate the NADH.

Table 2.1 Experimental Setup: Free NADH

Measurement	Titration	Conc. NADH
#	***To cuvette – add in sequence***	***µM***
1	Fill cuvette with 3 ml buffer, measure spectrum: BUFFER BLANK	0
2	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
3	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
4	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
5	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
6	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
7	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
8	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
9	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
10	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
11	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
12	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
13	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
14	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
15	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
16	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	

NOTE: Use the same transfer pipette throughout the series.

Table 2.2 Experimental Setup: Enzyme + NADH

Measurement	Titration	Conc. NADH
#	***To cuvette – add in sequence***	***µM***
1	Fill cuvette with 2.25 ml buffer + 0.75 ml GDH, measure spectrum: ENZYME BLANK	0
2	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
3	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
4	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
5	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
6	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
7	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
8	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
9	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
10	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
11	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
12	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	

13	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
14	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
15	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
16	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	

NOTE: Use the same transfer pipette throughout the series.

NOTE: If you are testing an effector, reduce the volume of buffer commensurate with the volume of effector to be added to reach desired final concentration.

NOTE: If you are using the PTI fluorometer, you will need two cuvettes: Cuvette 1 ("The blank") will be the 0 NADH cuvette; Cuvette 2 will be the cuvette into which you titrate the NADH.

Table 2.3 Experimental Setup: Free NADH + ligand

Measurement	Titration	Conc. NADH
#	***To cuvette – add in sequence***	***µM***
1	Fill cuvette with 2.XX ml buffer + 0.XX mL ligand = 3 mL, measure spectrum: BUFFER BLANK	0
2	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
3	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
4	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
5	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
6	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
7	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
8	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
9	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
10	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
11	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
12	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
13	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
14	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
15	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
16	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	

NOTE: Use the same transfer pipette throughout the series.

Table 2.4 Experimental Setup: Enzyme + ligand + NADH

Measurement	Titration	Conc. NADH
#	***To cuvette – add in sequence***	***µM***
1	Fill cuvette with 2.25 ml buffer + 0.75 ml GDH + ligand, measure spectrum: ENZYME BLANK	0
2	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
3	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
4	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
5	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
6	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
7	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
8	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
9	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
10	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
11	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	

12	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
13	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
14	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
15	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	
16	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	

Fluorometer setup: **You will be collecting emission spectra.** Excite your solution at 340 nm (NADH excitation maxima) and collect the emission spectrum from 400 – 500 nm.

Analyses: Subtract your buffer or enzyme blank from all other spectra in the series. Plot the spectra. Select a single emission wavelength to follow across the series. Plot a graph of Fluorescence Intensity @ your chosen wavelength versus NADH concentration in the presence and absence of protein. Calculate the ΔF between NADH in buffer and NADH in the presence of protein. Plot ΔF versus [NADH]. Fit data to saturation function to obtain K_D and ΔF_{max}. Alternatively, plot 1/ΔF versus 1/NADH to derive parameters.

EXPERIMENT 3

Cofactor binding to glutamate dehydrogenase: Monitoring protein fluorescence

Proteins are most conveniently excited at the wavelengths that correspond to the excitation wavelengths of their aromatic amino acids, phenylalanine, tyrosine and tryptophan. An excitation scan (220 – 320 nm) with an emission wavelength at 340 nm will reveal the λ_{max} of excitation as it varies with Phe, Tyr and Trp content. Typically, proteins emission maxima is between 300 – 400 nm with a λ_{max} of emission centered at 350 nm. Note: NADH has a λ_{max} of excitation at 340 nm which overlaps the emission spectra of proteins. When the emission spectrum of a protein overlaps with its ligand's excitation spectrum, the potential exist for resonance energy transfer from protein to ligand.

Resonance energy transfer describes the mechanism by which energy transfer occurs between two chromophores, like a protein and NADH. A donor chromophore (protein), in an electronic excited state, may transfer energy to an acceptor chromophore (NADH) through nonradiative dipole–dipole coupling. The efficiency of this energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor, making FRET extremely sensitive to small changes in distance.

In this experiment, you will excite the protein component of the protein:ligand complex and monitor both protein and ligand emission spectra. As discussed in the lab manual, the ligand fluorescence may be enhanced, in this case by RET, or quenched when captured in a protein:ligand complex.

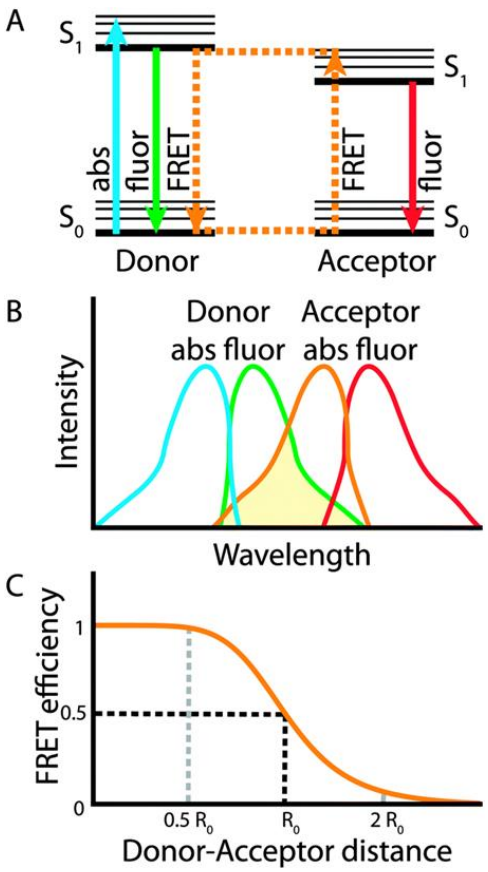


Figure 10. Overview of resonance energy transfer. A) Donor is excited and can return to ground state emitting fluorescence or through a non-radiative dipole-dipole coupling transfer the energy of its excited state to an acceptor placing the acceptor in an excited state that can then emit fluorescence as it returns to the groundstate. B) For the donor to "transfer" energy to an acceptor, the emission spectrum of the donor must overlap the excitation spectrum of the acceptor. C) The efficiency of the energy transfer between donor and acceptor is distance ($1/r^6$) and orientation dependent.

Set Up of the Experiment that You Will Perform

In the experiment that you will perform, you are provided with the following solutions:

2 mg/mL GDH in 0.1 M Phosphate, pH 8, measure exact concentration via A_{280nm}, $\varepsilon_{280nm} = 0.93\ (mg/ml)^{-1}cm^{-1}$

3.1 mg/ml lysozyme in 0.1 M Phosphate, pH 8, measure exact concentration via A_{280nm}, $\varepsilon_{280nm} = 36\ (mM\ cm)^{-1}$

~420 μM NADH in Buffer, measure exact concentration via A_{340nm}, $\varepsilon_{340nm} = 6.22\ (mM\ cm)^{-1}$

0.1 M Phosphate Buffer, pH 8

50 mM ADP

10 mM GTP

10 mM Glutarate

And equipment:

Fluorometer

3 ml Quartz fluorometer cuvette

Experiment 3.1

Determine the dissociation constant for NADH binding to glutamate dehydrogenase

2/11 Blue group: Complete study at pH 8 + 0.1 mM GTP

2/16 Green group: Complete study at pH 8 + 10 mM Glutarate

2/23 Purple group: Complete study at pH 8 + 0.5 mM ADP

Enzyme concentration calculated:_____

NADH concentration calculated _____

You will be titrating NADH into a cuvette containing a lysozyme (control experiment) or your enzyme (and effector, depending upon your experiment). At each concentration of NADH, you will collect an emission spectra. You will examine NADH emission spectra from 0 - 20 μM NADH. Complete the Experimental Setup Tables 2.1-2 to guide your procedure and complete calculations necessary for data analyses.

Table 3.1 Experimental Setup: Correction for absorption of excitation or emission by ligand.

Measurement	**Titration**	**Conc. NADH**
#	***To cuvette – add in sequence***	***μM***
1	Fill cuvette with 2.25 ml buffer + 0.75 ml lysozyme, measure spectrum: Ctl Protein	0
2	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
3	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
4	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
5	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
6	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
7	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
8	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
9	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
10	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
11	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
12	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
13	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
14	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
15	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
16	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	

NOTE: Use the same transfer pipette throughout the series.

NOTE: Set up a second cuvette with buffer only to serve as your blank for this experiment.

Fluorometer setup: You will be collecting emission spectra. Excite your solution at 287 nm (GDH excitation maxima) and collect the emission spectrum from 300 – 500 nm.

Table 3.2 Experimental Setup: Protein fluorescence & cofactor RET fluorescence.

Measurement	Titration	Conc. NADH
#	*To cuvette – add in sequence*	*μM*
1	Fill cuvette with 2.25 ml buffer + 0.75 ml GDH, measure spectrum: Ctl. ENZYME	0
2	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
3	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
4	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
5	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
6	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
7	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
8	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
9	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
10	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
11	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
12	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
13	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
14	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
15	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
16	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	

NOTE: Use the same transfer pipette throughout the series.
NOTE: Set up a second cuvette with buffer only to serve as your blank for this experiment.
NOTE: If you are testing an effector, reduce the volume of buffer commiserate with the volume of effector to be added to reach desired final concentration.

Fluorometer setup: You will be collecting emission spectra. Excite your solution at 287 nm (GDH excitation maxima) and collect the emission spectrum from 300 – 500 nm.

Table 3.3 Experimental Setup: Protein fluorescence & cofactor RET fluorescence.

Measurement	Titration	Conc. NADH
#	*To cuvette – add in sequence*	*μM*
1	Fill cuvette with 2.25 ml buffer + 0.75 ml GDH + ligand, measure spectrum: Ctl. ENZYME	0
2	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
3	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
4	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
5	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
6	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
7	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
8	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
9	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
10	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
11	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
12	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
13	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
14	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	
15	Add 10 μl NADH, Gently mix with disposable transfer pipette, measure spectrum	

16	Add 10 µl NADH, Gently mix with disposable transfer pipette, measure spectrum	

Analyses: Which GDH sample gives you the maximum donor emission spectrum? Plot the fluorescence spectra obtained with lysozyme at the different concentrations of NADH. Select a wavelength for correction factor calculation: the emission maximum of glutamate dehydrogenase. Calculate the correction factor as outlined in Table 3.4. What does the correction factor "correct?"

Table 3.4 Calculating correction factor for GDH:NADH binding.

NADH Added	[NADH]	Measured Fluorescence	Measured Fluorescence	Correction Factor (From the Lysozyme Experiment)	Correction Factor
µl	*µM*	*Form*	*Arbitrary units*	*Form*	*Calculated*
0		F1		F1/F1	
10		F2		F1/F2	
20		F3		F1/F3	
30		F4		F1/F4	
40		F5		F1/F5	
50		F6		F1/F6	
60		F7		F1/F7	
70		F8		F1/F8	
80		F9		F1/F9	
90		F10		F1/F10	
100		F11		F1/F11	
110		F12		F1/F12	
120		F13		F1/F13	
130		F14		F1/F14	
140		F15		F1/F15	
150		F16		F1/F16	

Now apply this correction factor to the GDH:NADH data: Plot the protein fluorescence spectra obtained with GDH at different concentration of NADH. Use the fluorescence intensity at the "peak" wavelength observed in the 300-400 nm region of the spectrum. Apply the correction to your data as outlined in Table 3.5.

Table 3.5 Correcting the experimental NADH Quenching of Protein Fluorescence data.

NADH Added	[NADH]	Measured Fluorescence	Measured Fluorescence	Correction Factor	Correction Factor	Correction Obs
µl	*µM*	*Form*	*Arbitrary units*	*Form*	*Calculated*	*Obs#(F1/F#)*
0		Obs1		F1/F1		
10		Obs2		F1/F2		
20		Obs3		F1/F3		
30		Obs4		F1/F4		
40		Obs5		F1/F5		
50		Obs6		F1/F6		
60		Obs7		F1/F7		
70		Obs8		F1/F8		
80		Obs9		F1/F9		

90		Obs10		F1/F10		
100		Obs11		F1/F11		
110		Obs12		F1/F12		
120		Obs13		F1/F13		
130		Obs14		F1/F14		
140		Obs15		F1/F15		
150		Obs16		F1/F16		

Calculate ΔF: corrected Obs2-corrected Obs1, corrected Obs3 – corrected Obs1, etc. Plot ΔF versus [NADH]. Fit to saturation function to obtain K_D and ΔF_{max}. Alternatively, plot 1/ΔF versus 1/NADH to derive parameters.

Using the Sensitized (FRET) NADH Fluorescence Data to Follow Binding

When you excite the protein and Resonance Energy Transfer to the bound ligand can occur, you can follow binding using sensitized NADH fluorescence observed in the usual NADH emission range (400-500nm). To accurately estimate the amount of sensitized fluorescence you must again correct for the absorption by the added NADH of the exciting light. This is the same correction factor you calculated and used above, but this time applied to the peak of the sensitized NADH fluorescence. Use Table 3.6 to do your calculations.

Table 3.6 Correcting the experimental NADH Sensitized (FRET) Fluorescence data.

NADH Added	**[NADH]**	**Measured Fluorescence**	**Measured Fluorescence**	**Correction Factor**	**Correction Factor**	**Correction Obs**
μl	*μM*	*Form*	*Arbitrary units*	*Form*	*Calculated*	*Obs#(F1/F#)*
0		Obs1		F1/F1		
10		Obs2		F1/F2		
20		Obs3		F1/F3		
30		Obs4		F1/F4		
40		Obs5		F1/F5		
50		Obs6		F1/F6		
60		Obs7		F1/F7		
70		Obs8		F1/F8		
80		Obs9		F1/F9		
90		Obs10		F1/F10		
100		Obs11		F1/F11		
110		Obs12		F1/F12		
120		Obs13		F1/F13		
130		Obs14		F1/F14		
140		Obs15		F1/F15		
150		Obs16		F1/F16		

Calculate ΔF: corrected Obs2-corrected Obs1, corrected Obs3 – corrected Obs1, etc. Plot ΔF versus [NADH]. Fit to saturation function to obtain K_D and ΔF_{max}. Alternatively, plot 1/ΔF versus 1/NADH to derive parameters.

EXPERIMENT 4

Fluorescence-based thermal shift assay (FTS) assay to assess ligand binding

As proteins unfold, the hydrophobic residues typically buried within the interior become exposed to the aqueous environment. To monitor the exposure of these hydrophobic residues, the binding of a hydrophobic fluorescent dye (SYPRO Orange) is quantitated as a function of the protein unfolding. The SYPRO Orange dye is highly fluorescent upon binding to hydrophobic sites on unfolded proteins. Typically in a Fluor-based thermal shift assay, the thermal denaturation of a protein is marked by a change (increase) in fluorescence intensity as the protein unfolds, generating a sigmoidal curve that can be described by a two-state transition.

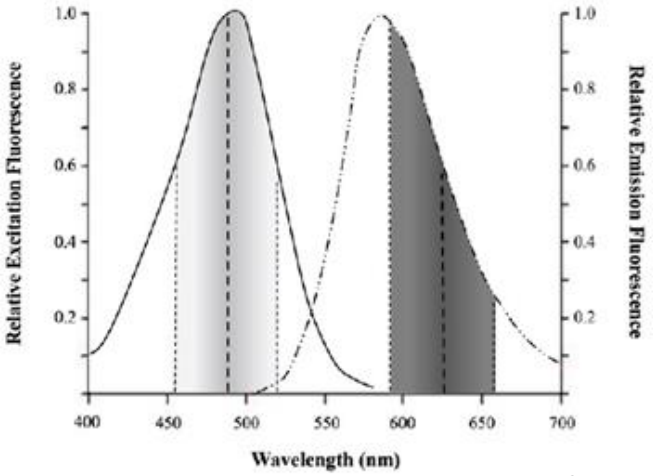


Figure 11. Excitation (solid) and emission (dashed) spectra of SYPRO orange fluorescent dye in BSA. Shaded areas represent the relative range of light transmitted through the BioRad 485 ± 30 nm excitation and 625 ± 30 nm emission filters used in this experiment. This figure is modified from Invitrogen Life Technologies product information (Cat. No. S-6650) and copied from Biotechniques (2012) 53:231-238.

Once this thermal shift is defined for a protein, this characterization can be used to monitor alterations to a protein's structure, such as ligand interactions, that would modulate the thermal melt behavior of the protein.

Experiment 4.1
Fluorescent thermal shift assay to assess glutamate dehydrogenase stability

You are provided with the following solutions:
1 mg/ml Glutamate dehydrogenase in 5 mM phosphate buffer, pH 8 (subunit MW of 56,000)
100 mM L-leucine in 1 M HCl
200 mM L-glutamate in 1 M HCl
1 mM NADPH in 0.01 M NaOH
5 mM diethylstilbestrol in methanol
1 mM palmitoyl CoA in water
200X stock of SYPRO Orange in DMSO
The following ligands dissolved in 5 mM phosphate buffer, pH 8:
1 mM NADH
1 mM NADPH
1 mM NAD+
5 mM ADP
1 mM GTP
1 mM Zn Acetate
150 mM malate
200 mM glutamate

And equipment:
BioRad thermocycler with CFX96 Reaction Module
96 well plate with sealing tape

Ligands will be used at final concentrations of
10 mM L-leucine
20 mM L-glutamate

100 μM NAD+
100 μM NADH
100 μM NADPH
500 μM ADP
100 μM GTP
100 μM Zn Acetate
15 mM malate
1.5 μM palmitoyl CoA
50 μM diethylstilbestrol

You will examine the denaturation of glutamate dehydrogenase:ligand complexes by fluorescence-based thermal shift (FTS) assay using SYPRO Orange fluorescence as your readout. You should set up your rxns in microcentrifuge tubes on ice and then transfer to a 96 well plate on ice. Your final enzyme concentration in the reaction will be 4 μM. Each reaction will occur in a total volume of 25 μl. *You should complete each reaction in triplicate.* For each ligand, you will need a control (no GDH) condition to examine the SYPRO Orange reaction with ligand. ***Fill out the experimental setup worksheet below to aid in mapping out your reaction setup. Assume that you are preparing enough sample for 3.5 volumes –that is for a total volume of 87.5 μl. This will allow for any pipetting errors – human or instrument!*** For these reactions you will use p2 and p10 pipettes as well as the pipettes in your lab drawer. Setup your reactions such that you do not pipette any volumes less than 1 μl.

Experimental Setup Worksheet 4.1

Reaction	Tube	GDH	SYPRO	Buffer	Ligand
	#	4 μM, μl	20X	5 mM, μl	μl
Ctl. No GDH	**1**	0			0
Ctl. GDH std. conditions	**2**				0
Leucine	**3**				
Glutamate	**4**				
NAD+	**5**				
NADH	**6**				
NADPH	**7**				
ADP	**8**				
GTP	**9**				
Zn Ac	**10**				
Malate + Palmitoyl CoA	**11**				
Diethylstilbestrol	**12**				
Leucine	**13**	0			
Glutamate	**14**	0			
NAD+	**15**	0			
NADH	**16**	0			
NADPH	**17**	0			
ADP	**18**	0			
GTP	**19**	0			
Zn Ac	**20**	0			
Malate + Palmitoyl CoA	**21**	0			
Diethylstilbestrol	**22**	0			

The thermal scans will be performed from 25 to 95°C at 0.5°C increase/minute and fluorescence scans collected every 30 s. Return the temperature to 25°C and record a final fluorescence intensity. Calculate the average of triplicates. Subtract average "No GDH" FI control from the average of your samples. Make plots of the average, corrected fluorescence intensity versus temperature. From these plots, calculate the Tm. Compare the Tm for your different samples. What conclusions can you draw with respect to the protein:ligand complex and protein stability? Does stability correlate to the function of the ligand: substrate, activator or inhibitor? Using a minimum of 5 points within the transition between the folded and unfolded state, calculate the K_{eq} for the folded to unfolded process. Create a van't Hoff plot from this analysis and derive the thermodynamic parameters. Are these analyses valid?

Quantitative analysis of ligand binding data

For the simple equilibrium

$$P + L \leftrightarrow PL$$

a dissociation constant, K_D: can be defined

$$K_D = \frac{[P][L]}{[PL]} \quad \text{eq. 25}$$

The total concentration of protein, $P_t = [PL] + [P]$, and the concentration of PL may be expressed as a function of [L] if [P] is eliminated from the equilibrium equation describing the dissociation constant, as shown by:

$$[PL] = \frac{[P_t]}{\left(1 + \frac{K_D}{[L]}\right)} \quad \text{eq. 26}$$

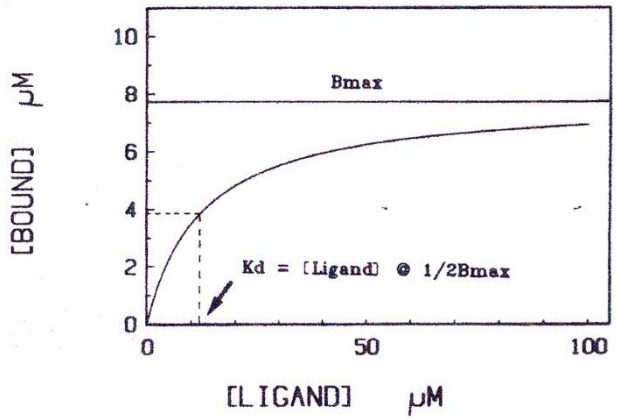


Figure 12. Saturation curves for ligand binding to protein.

If P_t is constant, as is usually the case, a plot of [PL] versus [L] is a rectangular hyperbola and, analogous to the Michaelis-Menten equation, the concentration of [L] at which $[PL] = 1/2[P_t]$ is equal to K_D**.** This is summarized in the Figure 12. As with enzyme kinetics, several linearized versions of this equation are used, most notably the Klotz equation:

$$\frac{1}{[PL]} = \frac{1}{[P_t]} + \frac{K_D}{P_t}\frac{1}{[L]} \quad \text{eq. 27}$$

and the Scatchard equation:

$$[PL] = [P_t] - \frac{(K_D)([PL])}{[L]} \quad \text{eq. 28}$$

The equivalent linear plots, the Klotz and the Scatchard plot, are illustrated in Fig. 13. All of these equations refer to a case where a single protein molecule has a single binding site for ligand.

Identical, Independent Binding Sites

Many oligomeric proteins contain more than one binding site per molecule for a particular ligand, and in cases where the sites are independent and have the same microscopic dissociation constant, the-interactions of the protein (P) with the

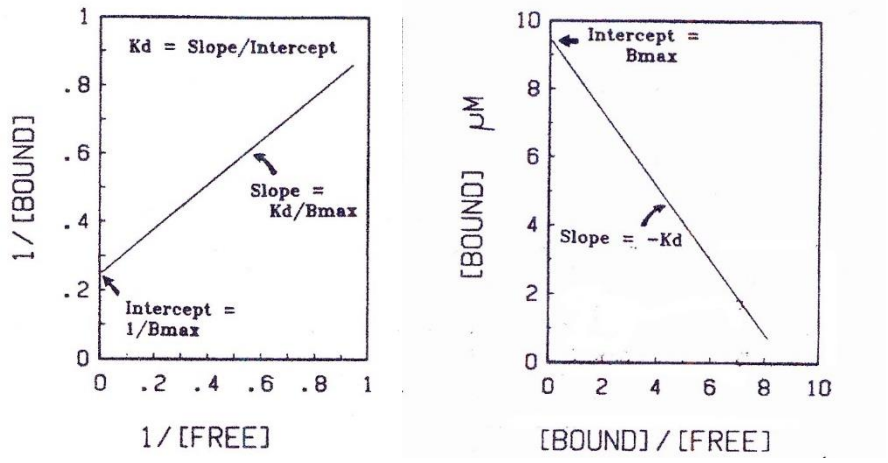


Figure 13. Klotz (left) and Scatchard (right) plots of equilibrium binding data.

ligand (L) can be characterized by the following equilibria:

$$P_0 + L \leftrightarrow P_1$$

$$P_1 + L \leftrightarrow P_2$$

$$P_{n-1} + L \leftrightarrow P_n$$

where the number indicates the number of ligand molecules that are bound to the protein. Each site has the same microscopic dissociation constant, designated by ${}^{m}K$. *The macroscopic dissociation constant* $\mathbf{K_D}$**,** however, depends on the level of occupancy of the molecule, as indicated by

$$K_{D1} = \frac{[P_0][L_f]}{[PL_1]} \quad \text{eq. 29}$$

$$K_{D2} = \frac{[P_1][L_f]}{[PL_2]} \quad \text{eq. 30}$$

$$K_{Dn} = \frac{[P_{n-1}][L_f]}{[PL_n]} \quad \text{eq. 31}$$

The macroscopic dissociation constant at any degree of saturation, K_{Di} is related to the microscopic dissociation constants by:

$$K_{Di} = \frac{\Sigma n_{i-1}}{(\Sigma n_i)^{m}K} \quad \text{eq. 32}$$

Where all of the microscopic dissociation constants are equal:

$$[PL] = n[P_t] - \frac{({}^{m}K)([PL])}{[L]} \quad \text{eq. 33}$$

which is similar to the Scatchard equation derived above for a single site except that the y intercept now indicates $n[P_t]$ allowing n to be obtained if $[P_t]$ is known.

Multiple Classes of Independent Sites

A frequently encountered situation is the case where a Scatchard plot is nonlinear, as illustrated in Figure 14. There are n_i independent sites with intrinsic microscopic dissociation constants ${}^{m}K_{Di}$, we can write

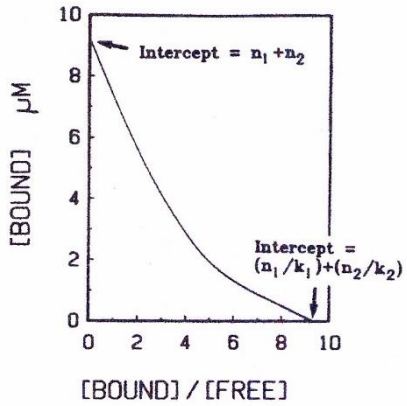


Figure 14. Nonlinear Scatchard plot. This nonlinear character might arise from two independent but non-identical binding sites.

$$[L_B] = \frac{\Sigma\left(\frac{n_i[L]}{{}^mK_{Di}}\right)}{\left(1 + \frac{[L]}{{}^mK_{Di}}\right)}$$

eq. 34

which gives (following the same process as previously):

$$[PL] = \Sigma n[P_t] - \Sigma {}^mK\frac{[PL]}{[L]}$$

eq. 35

In the Scatchard plot, the intercept on the [PL] axis is, for the case shown (which involves two classes of sites) nj + n2, and the intercept on the [PL]/[L] axis is $n_1/{}^mK_{D1} + n_2/{}^mK_{D2}$. The most realistic values of n_1 n_2, ${}^mK_{D1}$, and ${}^mK_{D2}$ are obtained by an iterative process. Assuming initially that the x-axis intercept is dominated by the smaller ${}^mK$ value (i.e., ${}^mK_{D1}$) a tangent to the curve at regions approaching [PL] = 0 gives an intercept of $n_1/{}^mK_{D1}$ and on the y-axis an initial value for n_1. With initial estimates of n_1 and ${}^mK_{D1}$, we can subtract the contribution of the high-affinity sites from the data obtained at higher degrees of saturation, which can then be plotted to give initial estimates of n_2 and ${}^mK_{D2}$. Once these estimates of n_2 and ${}^mK_{D2}$ have been obtained, the contribution of the low-affinity sites to the data at low degrees of saturation can be subtracted and new estimates of n_1 and ${}^mK_{D1}$ obtained. Throughout the procedure $n_1 + n_2$ must equal the observed [PL] intercept, and the iterations are continued until $n_1/{}^mK_{D1} + n_2/{}^mK_{D2}$ equals the x-axis intercept.

Dependent Binding Sites

In a situation where two identical ligand molecules bind to a protein molecule (either one subunit with two sites or two subunits, each with a binding site), it may be that binding the ligand to the first binding site alters the affinity of ligand binding to the second site. Two cases are possible: In the first, ligand binding at the first site *increases* the affinity of the second site, while in the second case, ligand binding at the first site decreases the affinity of the second site. In either instance nonlinear Scatchard or Klotz plots results, Figure 15.

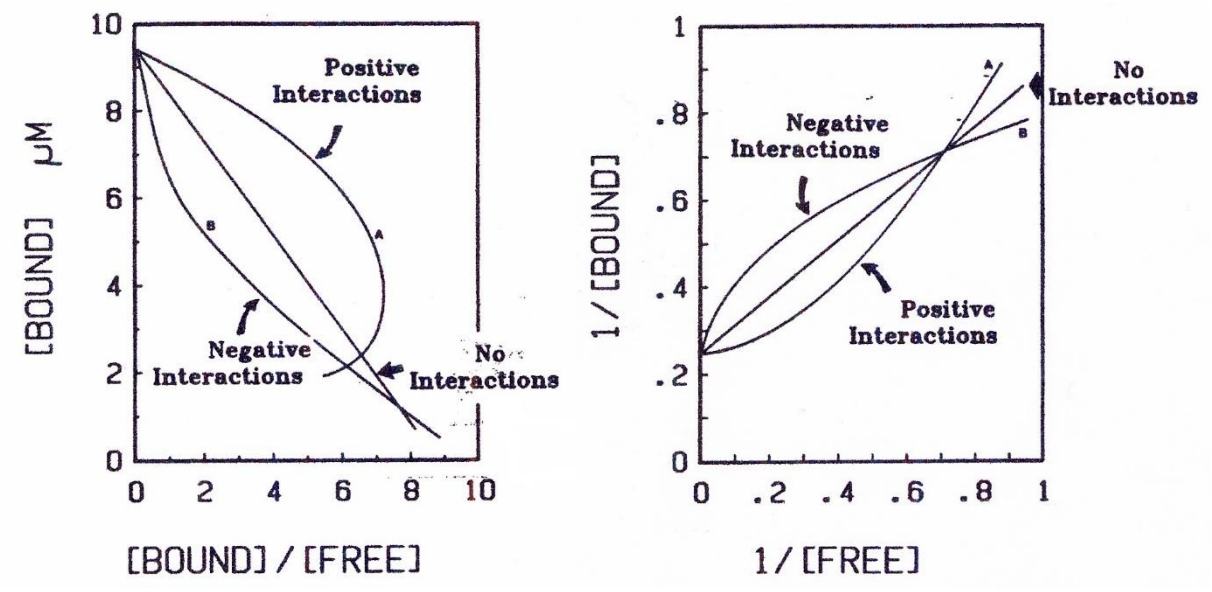


Figure 15. Scatchard (left) and Klotz (right) plots for dependent binding sites.

It is not possible to distinguish cases of independent *non-identical* sites from cases where the first ligand decreases the affinity of the second ligand. Mechanistically, such binding can result from allosteric interactions giving negative cooperativity, or from direct steric interaction of the bound ligands. In contrast to this situation, Scatchard or Klotz plots indicating that the first ligand molecule to bind increases the affinity of the second ligand molecule can be explained only by allosteric models.

The Hill Equation is often used to determine the Hill Constant, n, for ligands that do not obey "normal" behavior when binding to a protein.

$$\log\left(\frac{F}{(1-F)}\right) = n\log[L] \; - \; n\log K_D^{Ligand}$$

eq. 36

Where: $F = \Delta_{Parameter}/\Delta max$, and is the fractional saturation of the protein with the ligand. The Hill Coefficient is n; n=1 for a non-cooperative system, n<1 for a negatively cooperative system, and n>1 for a positively cooperative system. A value significantly different from 1 would indicate that the ligand molecule bound to the protein with some type of cooperativity. In cases of extreme positive cooperativity n approaches the number of interacting sites in the system. An example treated in biochemistry classes is the saturation of hemoglobin with oxygen, Figure 16.

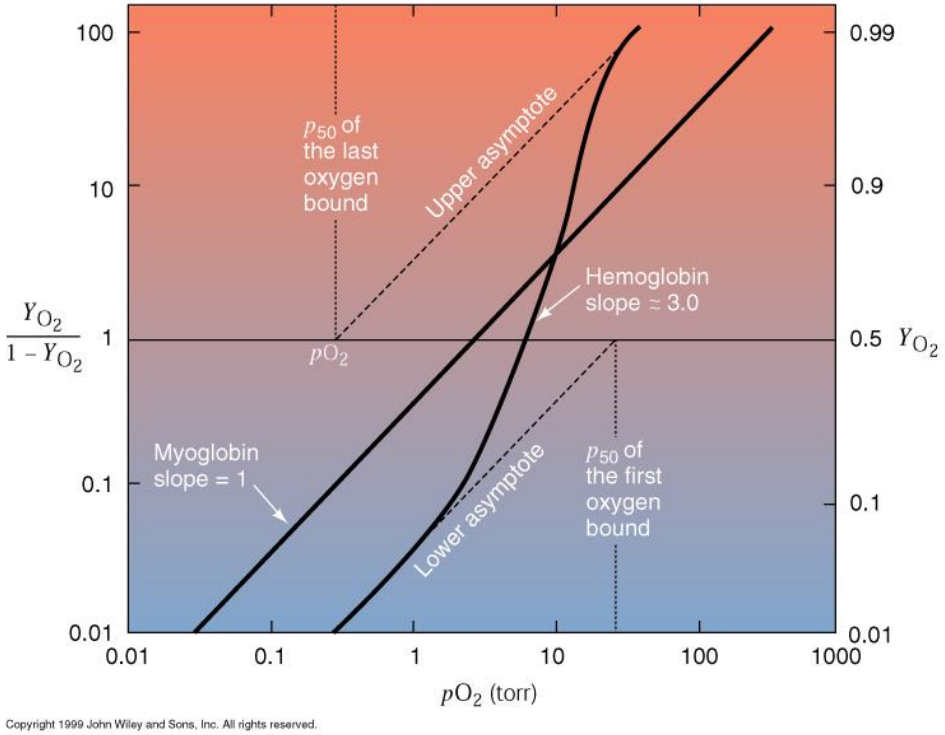


Figure 16. Hill plot of oxygen binding to hemoglobin. Copyright 1999 John Wiley and Sons.