EQUILIBRIUM MICRO-DIALYSIS

Introduction:

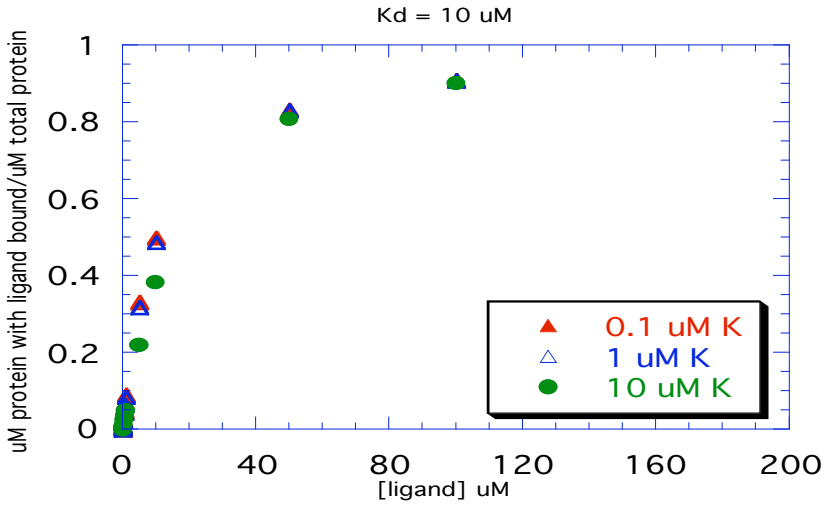
This technique uses a microdialysis apparatus (50 µl cell volume) to measure the fraction of a labeled ligand bound to a macromolecule under equilibrium conditions. The fraction bound as a function of ligand concentration enables one to determine the dissociation constant between the ligand and the macromolecule (K_d).

$$K_d = ([\text{Protein}]_f [\text{Ligand}]_f)/[\text{Protein-Ligand Complex}]$$

A working familiarity with ligand binding equations is critical to appropriate experimental design and data analysis. This topic is discussed in a wide variety of textbooks, for example Cantor and Shimmel vol III chapter 15.

General information and experimental design:

First, it is necessary to decide what range of ligand concentration you want to test. It is good to keep the range within 2-3 orders of magnitude for each experiment. You must also decide what concentration of protein to use. This will depend on the K_d and the ligand concentration range you want to test. Of course initially these are unknown values, so it is best to make guess about the K_d and then design the experiments to test that estimate. It is helpful to do some simulations first to know for a given K_d, what concentration of ligand is necessary to reach saturation.



Example:

Just for instruction we will estimate that the K_d is 10 µM. If you use a protein concentration that is lower than the K_d, you should observe 50% of the protein to contain bound ligand at about 10mM ligand. Under these conditions the amount of total

ligand bound is a very small portion of the total ligand concentration. See plot above which is a simulation for K_d = 10 μM if the experiment were done at 3 different concentrations of protein, 0.1, 1, and 10 mM. You want a range of ligand concentration below and above the 50% value, in order to get a full curve, so a ligand concentration range of 1μM to 200 μM would be a place to start. Based on this, choose the protein concentration such that you can expect to detect a reasonable amount bound. Specifically, given the 10 μM Kd, you expect to be close to saturation at the 200μM point. If your protein is at 10 μM and is completely filled with ligand (10 μM) then you will be trying to detect 10 μM bound / 200 μM total or 5%. This value (5%) should be relatively easy to detect.

In contrast, if you had used 0.1μM protein, then you would be trying to detect 0.05% of the signal. Keep in mind that the other points will be at similar concentrations and you need to see a curve of values, so if another data point is at 50 μM ligand and you expect 80% saturation :

O.1 μM protein * 0.8 = 0.08 μM ligand bound

You are looking for 0.08/50 = 0.16 %. It is not realistic to expect to be able to detect the difference between 0.2% and 0 .05%. In sum for the microdialysis experiments, if you keep the protein concentration near or above the estimated K_d, detecting the amount bound will be easier.

Set up microdialyzer:

The dialyzer (Model EMD 101B Hoefer Scientific Instruments) consists of two Teflon disks, each containing 8 chambers. A circular dialysis membrane is sandwiched between the two disks. The dialysis membranes that we have in the lab have a 12 kD cut off, so any ligand that is ≥ this size cannot be used. The dialysis membranes are prepared by boiling for 5 minutes in 5% $NaCO_3$, 50 mM EDTA (1.86 gr/100 ml) and stored in 50% ethanol/H20 at 4 ^oC. Wash off the ethanol and soak the membrane in your buffer before use. One of the primary experimental difficulties of this technique is nonspecific adsorption of the ligand to the walls of the microdialyzer or dialysis membrane. The manual recommends siliconizing the dialyzer with Siliclad. It is also recommended to apply a thin coat of silicon grease to the rubber gaskets, but if the ligand sticks to the grease, it can be omitted.

To assemble the dialyzer:

1. put the red rubber gaskets in all of the grooves on the 2 disks (16 total)
2. put the membrane between the two disks
3. slide the whole thing onto the arm of the rotation unit, secure it with the plastic bolt.

The discs should be placed as far as possible from the motor because the motor heats up over time and could heat the samples if they are adjacent. The samples will be added to the chambers after the device is assembled through either of the two small holes/chamber on the edge of the disks.

Set up the aliquots of protein and ligand:

The dialyzer can accommodate up to 8 samples at a time. The ligand can theoretically go in either side or both sides of a chamber (because it should equilibrate). I found it convenient to add the ligand (at 2x concentration) to the protein and put that in one chamber, and put buffer in the other chamber. This may over-estimate the fraction bound (all non-specific binding occurs on the protein side) but I felt the alternatives that I tested (negative % bound, multiple pipetting of ligand) were less desirable.

Again to reduce the amount of sample sticking to the eppendorf tubes, I bought siliconized eppendorfs. Be sure that the sample is hot enough that half of it can be accurately measured above the noise of the counting method you are using. In the end you are going to be looking for a significant difference between two numbers that is different from the noise (for ^{125}I it was good to have at least 30,000 counts going into the dialyzer, and in general the higher the better). A stock hot ligand solution must be made first, so it is necessary to know the concentration of the pure labeled ligand. Either be sure that the amount of hot ligand added to the cold ligand is insignificant or calculate the new total ligand concentration of the stock solution you are using. Make up 60 µl aliquots at the protein and ligand concentrations you want to test. Remember that the final experimental ligand concentration will be what ever you add in a final volume of 100 µl (both sides of the chamber). Always make up a control sample without protein to test if the ligand can equilibrate under the conditions you are using. Count the amount of radioactivity in each aliquot going into the dialyzer. This can be used to determine if you pipetted accurately and to calculate how much hot ligand sticks to the machine. If most of it sticks to the machine, then the numbers you are getting out probably are not meaningful. Typically, I recovered about 70-80% of the total counts.

Load the microdialyzer:

Using a blunt ended Hamilton syringe, load 50 µl of the hot sample into one side of a chamber. Rinse the Hamilton, then load 50 µl of buffer to the adjacent side of the same chamber. Seal each chamber with the vinyl dots and label them. After all 8 chambers are loaded, wrap parafilm around the circumference of the disk, to prevent evaporation of your samples. Plug in the dialyzer to start rotation. We have found that for peptide samples 1.5 kD, at least 20 hours at room temperature is required to reach equilibrium. After 20 hours or more, take out an aliquot from each chamber (40 µl) and measure the counts.

Data analysis:

For each concentration of ligand you have the number of counts on the protein side ($\#_m$) and the number on the buffer side ($\#_b$). The counts on the buffer side should be representative of the concentration of free ligand.

calculate: $\#_m - \#_b$ = # bound counts

$\#_m + \#_b$ = # total counts = [total ligand] you put in each aliquot

(# bound counts/# total counts) * total ligand concentration = [bound ligand]

[bound ligand]/[protein] = fraction of protein containing ligand (y axis)

plot:

(y axis) fraction of protein containing bound ligand vs. (x axis) [total ligand]

Estimate K_d by noting the total ligand concentration at which the protein is 50% bound. Determine Kd by fitting the data to a binding model for your system.