Derivation of Binding Constants by Inhibition Equilibrium Dialysis

Preparing Reagents:

Buffer: Use 1 x PBS with 0.5% gelatin and 0.02% azide. Filter through a 0.2 μm filter and store at 4^oC.

Peptides: Dissolve the inhibitor peptides in water or PBS. If the peptide contains tyrosine, run a scan on the spectrophotometer from 310 nm to 210 nm. Determine the molar concentration by dividing the A_{274} by 1400. If the peptide does not contain tyrosine, the concentration will have to be determined by BCA assay. A good range for the peptide concentration is between 4 and 8 μM. Store the peptides at -20^oC. See either my or Peggy's "Peptides" notebook for typical peptide scans.

^{3}H peptide: This is purified on the peptide RPC column on the FPLC. Refer to Peggy's or my "Peptides" notebook for purification guidelines, or use the spec sheets from Pharmacia on this column. Store this peptide at -70^oC.

Empty K^d protein: Refer to "Jennie's Book of Purified Proteins," the section on $K^d/\beta_2 m$ on how to make and store this.

Dialysis membranes: These are bought from Hoeffer Scientific (part # EMD103). Prepare them by boiling in 5% Na_2CO_3/50 mM EDTA for 5 minutes, washing twice in distilled water, and storing at 4^oC in 50% ethanol.

The apparatus: There are three Plexiglas discs labeled with an "A," "B," or "C," and one white Teflon disc. The Plexiglas discs should be paired with their matching letters to minimize leakage. There are small orange o-rings that fit into the sample wells. Grease these o-rings lightly with vacuum grease and place them into the wells shortly before using the apparatus. Never use more than three sets of discs at a time, or the apparatus will leak from all the wells. Use microcapillary pipette tips to load sample into the wells (order from VWR, part # 53503-167).

<u>Running the Experiment in Triplicate:</u>

1. Prepare 2.4 ml of empty K^d protein at a final concentration of 2 μM (0.12 mg/ml). Use the buffer described above for the dilution.

2. Prepare 480 μl of ^{3}H-labeled peptide at a final concentration of 10 μM. Use the buffer described above for the dilution.

3. Prepare seven eppendorf tubes with varying concentrations of inhibitor peptide (refer to my "Inhibition Data" notebook for typical ranges). Use the buffer described above for the dilution; final volume of each tube should be 240 μl.

4. Prepare one eppendorf tube with 240 μl buffer only. This will be the tube with labeled peptide alone, and is necessary for step number 2 in the calculations.

5. Add 60 μl of the diluted ^{3}H-labeled peptide to each of the eight eppendorf tubes. This makes the concentration of the ^{3}H-labeled peptide 2 μM. Spin down all the tubes prior to loading them into the dialysis apparatus.

6. Soak three membranes in PBS for 10 minutes before putting them into the discs. Place the discs + membranes onto the shaft and **tighten** the nut to prevent leakage. [Don't be surprised if some of the wells leak anyway.]

7. Load the protein into the back half of the chamber, and the peptide(s) into the front. Only load 95 μl into each well, since pipetting error will prevent you from getting all 300 μl into the three wells. Also, only load 95 μl of protein into the back wells.

8. After loading two chambers on a disc, place scotch tape over the loading holes to prevent leakage. Repeat 3 more times to fill all the wells, plug in the apparatus, and let it spin overnight.

9. The next day, unplug the apparatus, remove 50 μl from a well, place it into a scintillation vial, and add 10 ml scintillation fluid (order from Research Products International. I have always used Safety-Solve; however, the company has come out with a new scintillation fluid that is bio-degradable). **The paired wells must not be interchanged, or the data are meaningless!** Count the samples in the Imperiali lab scintillation counter (room 109 Church [ask permission the first time you use it]). Use program 4 on their Beckman counter.

<u>Calculating the K_D</u> (ref: Methods in Enzymology, vol. 92, pp. 589-601 and
Fahnestock et. al., Biochemistry, vol. 33, pp. 8149-8158):

1. Subtract the CPM value of the front well from the back well for all the wells. This will give you the CPM of the peptide bound to the protein (the front well is the CPM of the free peptide).

2. For the wells with labeled peptide alone, calculate the percent peptide bound. This number is called "b" in the final equation:

$$b = CPM_{bound}/CPM_{total} = (CPM_{back} - CPM_{front})/CPM_{back}$$

    The values I typically get range from .7-.8. Take the average of the three points for the final calculations.

3. Calculate the % inhibition for the seven inhibitor peptide concentrations (use the averaged value CPM_{bound} for the three wells with no inhibitor):

$$\%I = 100 \times (1 - [CPM_{bound\ with\ inhibitor}/CPM_{bound\ without\ inhibitor}]).$$

4. Plot % inhibition versus peptide concentration. **Remember** that the concentration value plotted for the inhibitor peptide is 1/2 the concentration used in setting up the experiment since the inhibitor peptide has diffused into the back well. The relationship between concentration and inhibition is logarithmic between 10% and 90% inhibition. Do not plot points whose inhibition is below 10% or above 90%. From the graph, extrapolate the concentration of peptide that gives 50% inhibition. This concentration will be called $[I_t]$ in the final equation.

5. The last number you need is the concentration of labeled peptide. This is 1/2 the initial concentration of peptide since it has diffused into the back well. For the setup I described above, the final concentration of labeled peptide ($[T_t]$ in the final equation) is 1 μM. [Note: if the value of $[I_t]$ is $\leq [T_t]$, use the second equation to calculate K_D].

$$K_D = ([I_t] - [T_t])(1 - 1.5b + 0.5b^2)$$

$$K_D = \frac{[I_t]}{(1 + [T_t]/K_P)}$$

where K_P is the K_D of the labeled peptide determined from Scatchard analysis (see following page and ref: FEBS Letters, vol. 317, pp. 49-52).