Derivation of Binding Constants by Scatchard Analysis

Preparing Reagents:

These reagents are prepared in the same way as for inhibition equilibrium dialysis. Refer to this protocol for instructions. Note: as this experiment does not require inhibitor peptides, ignore this section in the protocol.

Running the Experiment:

1. Prepare 2.4 ml of empty K^d protein at a final concentration of 2 µM (0.12 mg / ml). Use PBS / 0.5% gelatin / 0.02% azide to dilute the protein.

2. Prepare eight eppendorf tubes with ^3H-labeled peptide at varying concentrations from 0.25 µM to 3 µM. The final volume in each tube should be 300 µl. Use the buffer described above to dilute the peptide.

3. 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.]

4. Load the protein into the back half of the chamber, and the peptide 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.

5. 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.

6. 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.

Calculating the K_D:

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. Calculate the concentration of bound peptide and free peptide by using the CPM / µmol value written on the ^{3}H-labeled peptide tube. This number varies slightly from batch to batch of purified peptide, so check the actual tube you use. A typical calculation:

$$(1.61 \times 10^9 \text{ CPM} / \mu\text{mol})(50 \times 10^{-6} \text{ L}) = 80500 \text{ CPM} / \mu\text{M}$$

$$[\text{Free}] = \frac{\text{CPM}_{\text{free}}}{80500 \text{ CPM} / \mu\text{M}}$$

3. Plot the [bound peptide] vs. [bound peptide] / [free peptide]. (The [bound peptide] is the x axis; [bound peptide] / [free peptide] is the y axis).

4. Calculate (-1 / slope) from the graph. This is the K_D for the peptide.

Note: Scatchard analysis is not extremely reliable. It is not uncommon for the plots to be highly scattered, and the values for K_D typically have a high margin of error ($\pm$ 50% error is normal). If possible, K_D should be determined by the inhibition method, as these numbers tend to be cleaner and more reproducible.