# Protein Crystallography

## Part I. Crystal Growth BBMB 334

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**Part I. Crystal growth.**

**Purpose:** To grow crystals of a protein molecule and explore the phase space around the region in which the protein crystallizes.

**Background:**

Once you have determined that hemoglobin binds oxygen cooperatively you might like to know how this happens at an atomic level. Similarly, once you know how the stability of a protein changes with some particular mutation, it would be helpful to know the structure of the protein with and without the mutation. In other systems you may be interested in how an enzyme binds its substrate, or the structure of a previously undetermined protein. These questions can be addressed by determining the 3D structure of the macromolecule.

There are two methods used for determining high-resolution 3-dimensional structures of macromolecules: nuclear magnetic resonance (nmr) and crystallography. Here we will be concerned with the first steps of crystallography. In particular we will study protein crystallization.

The first requirement for growing crystals of proteins or nucleic acids is to have pure material. Protein purification is covered in the biochemistry lab, and will not be discussed in much detail in biophysics. We will start with proteins that have been purified by someone else and hope that they are pure enough for our purpose – which is to grow crystals!

Crystal growth can be thought of as occurring in two steps: 1. nucleation and 2. growth. During nucleation, a few molecules in solution come together to form the first nanocrystal, to which other molecules can add during the growth process. Usually, nucleation is the hardest part and represents an activation barrier to overcome to get to the growth phase.

To grow crystals of a salt or a small molecule, the typical procedure is to dissolve the substance of interest in water or some other solvent, then let the solution sit. As the solvent evaporates away, the material of interest becomes more and more concentrated until nucleation occurs and crystals start to grow. Typically this is near the solubility limit of the material – usually somewhat higher than this limit, so the crystals initially start to grow when the solution is supersaturated.

The procedure for growing crystals of macromolecules is similar in concept to growing salt crystals, with some added complications. First, the range of solution conditions over which crystals will form is typically smaller than with salt crystals. Second, there are many additional factors that can determine whether or not crystals will form, such as pH, temperature, buffer identity, type of precipitant (see below) and the presence or absence salts. Finally, different macromolecules can have different factors that are important. This means that it is nearly impossible to predict exactly under what conditions any given protein will form crystals. Often many, many different combinations of factors need to be explored in order to find the right

conditions for crystal growth.  If the right combination of factors is not used, then the protein remains in solution without crystallizing, or the protein comes out of solution, but as an amorphous precipitate rather than a crystal.  Macromolecular crystal growth is very much a black art, with great room and need for basic research to uncover general principles.

Protein crystallization can be considered in terms of a crystallization phase space. One small part of a crystallization phase space is illustrated schematically below (Figure 1). The job of the experimenter is to maneuver the protein solution into the nucleation zone. At that point, it will take some time for nucleation to occur, after which the crystal will grow.

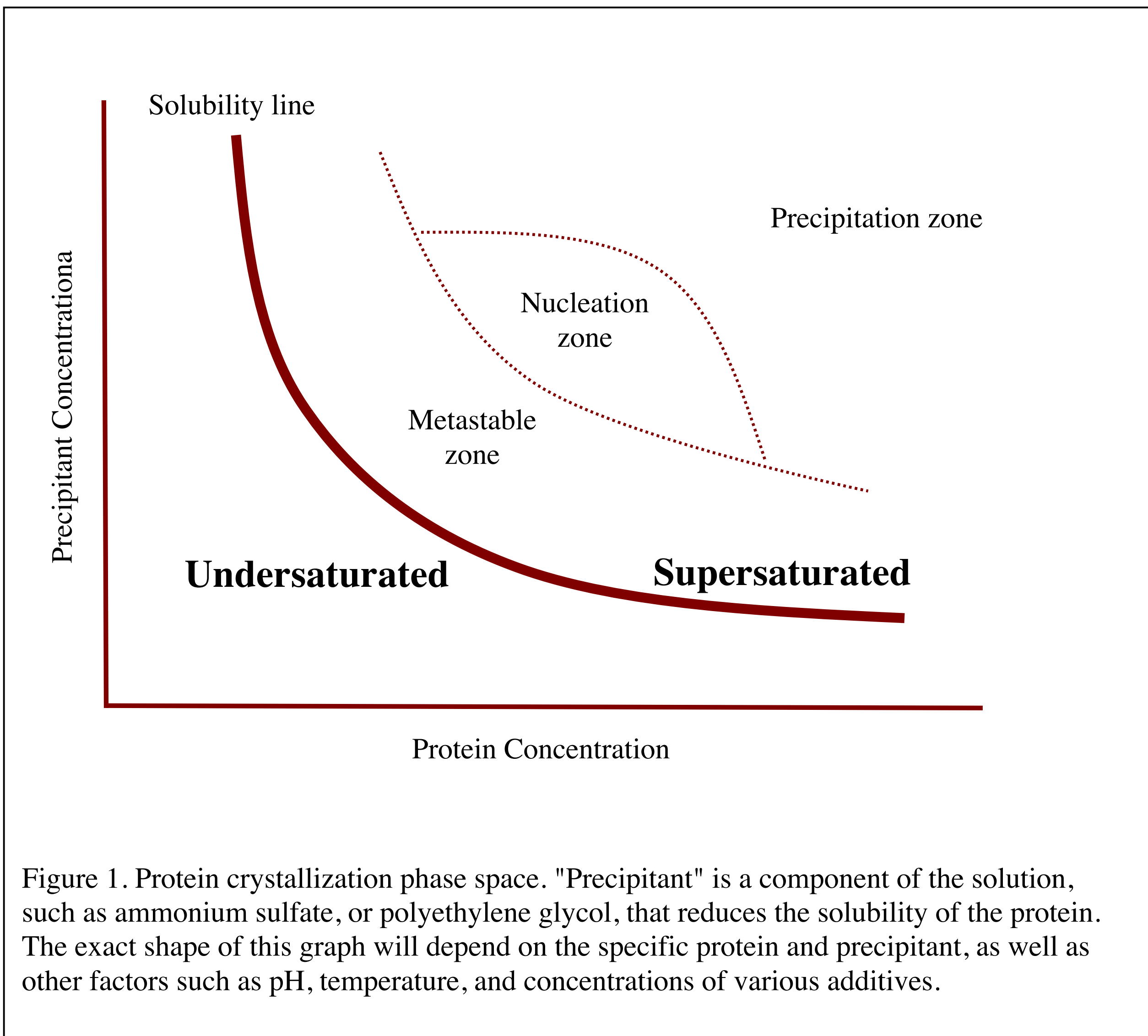


Figure 1. Protein crystallization phase space. "Precipitant" is a component of the solution, such as ammonium sulfate, or polyethylene glycol, that reduces the solubility of the protein. The exact shape of this graph will depend on the specific protein and precipitant, as well as other factors such as pH, temperature, and concentrations of various additives.

To accomplish this, several different methods have been used.  The most common method, and the one we will use in this lab, is called vapor diffusion. The basic setup is shown in the figure below.  First, the protein is dissolved, in a buffer at some pH. Then, the well is filled with a solution, called the reservoir solution (about 1 mL). Then equal volumes of the protein solution and the reservoir solution (about 3 $\mu$L of each) are mixed together and this is placed on a glass coverslip. The coverslip is inverted over the well (sealed with grease).

Usually the reservoir solution contains one additive at fairly high concentration, which has a significant effect on the solubility of the macromolecule, and is consequently called the precipitant. Once the drop is prepared, there is a higher concentration of the precipitant in the well than in the drop. Consequently, water diffused out of the drop and into the well. As the water leaves the drop, the concentrations of the components of the drop changes protein increases until (hopefully!) crystals form.

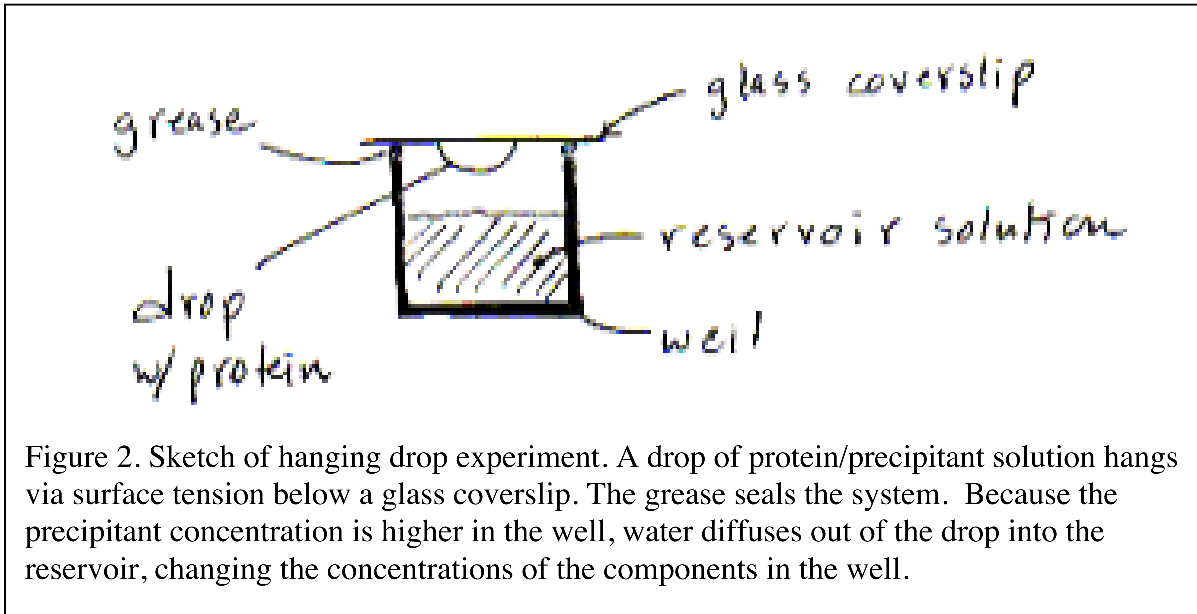


Figure 2. Sketch of hanging drop experiment. A drop of protein/precipitant solution hangs via surface tension below a glass coverslip. The grease seals the system. Because the precipitant concentration is higher in the well, water diffuses out of the drop into the reservoir, changing the concentrations of the components in the well.

In this lab (Crystallography, part I) you will grow crystals of trypsin. Trypsin is a serine protease that catalyzes peptide bond hydrolysis. The crystallization solution will include an inhibitor of trypsin, called benzamidine, which binds in the active site region of the protein. In subsequent labs, you will collect X-ray diffraction data from one of your crystals and determine the 3D atomic structure of the inibitor bound to the active site of the enzyme. However, this first part of the lab is concerned mainly with crystal growth.

**Prelab Questions**

Suppose you prepare a vapor diffusion experiment as described above. Initially the drop composition puts it in the undersaturated zone. Assuming that the only volatile component of the system is water, plot the progress of the drop in the phase space for the following scenarios.

1. After a time, precipitation occurs.
2. After a time, protein crystals start growing, increase in size, and then cease growth.
3. After a time, precipitation occurs. Later, crystals start growing, increase in size, and then cease growth.

**General Procedure and Resources:**

There are two goals for this lab:

1. Grow crystals of trypsin suitable for diffraction experiments.
2. Find a region of phase space that changes the crystals in some way. Some possible changes would be:
    a. crystal shape
    b. crystal size
    c. number of crystals in the drop
    d. absence of crystals

You therefore need to decide which part of phase space you want to explore to look for changes in your crystals. Some of the parameters you might vary include:

a. pH
b. temperature
c. precipitant concentration
d. protein concentration
e. drop ratios
f. salt identity (for instance, if NaCl is used in your solutions, you could try substituting other chloride salts)
g. salt concentration
h. presence or absence of gravity (a difficult parameter to explore on earth)

You will set up the crystallization experiments using plates with 24 wells. Each plate contain an array of 4x6 wells. Set up two to four plates, exploring a region of phase space nearby the conditions for your crystals by varying the solution conditions for each well.

Setting up the Crystals

1. Making stock solutions

Once you have decided on which region of phase space to explore, make up the required stock solutions of buffer, salts, and preciptants that will permit you to set up your crystals and explore the region of interest in phase space. These should be bottles or tubes of concentrated solutions of each component of your reservoir solution.

(Some of these items can be done in parallel by different members of your lab group. Multi-tasking is an important skill.)

1. Make up the solution into which your protein and store this on ice.
2. Put grease around the edges of each of the 24 wells on the crystallization tray. (Ask for a demonstration of this before you start.)
3. Add different amount of stock solutions to the wells, resulting in a tray that covers some region of phase space.
4. Mix the protein and well solution and place on a cover slip.
5. Invert the cover slip over the well.

Monitoring the Experiment

Since we want to characterize the growth process as much as possible, you should make observations of the drops in your crystal trays. For the first 3 days record your observations each day, and every 2nd day thereafter. Come up with a way of describing what's going on in each drop and how it is changing. Some possible things to record are the presence or absence of crystals or precipitate, the quality and extent of the precipitate, the number and size of the crystals etc.

The Crystallization Conditions

We will be working with the orthorhombic form of bovine trypsin. Two well solutions for crystallizing orthorhombic trypsin are:

1. 23-25 % PEG 8000 (w/v)
   0.2 M AmSO4
   0.1 M benzamidine HCl
   0.1 M tris pH 8.0

2. 3 M TMAO (trimethylamine N-oxide)
   0.1 M AmSO4
   0.05 M benzamidine HCl
   0.1 M tris pH 8.0

In both cases, protein at 40 mg/mL and drops of 3 $\mu$L protein/3 $\mu$L well solution are good places to start.

The exact trypsin purchased has an effect on crystallizability. We have found that Sigma-Aldrich T8003 can be easily crystallized. (We have not, however, been able to crystallize T9201.) Note also that occasionally, trigonal crystals also appear, sometimes in the same drop as the orthorhombic crystals. Both crystal forms should provide high quality data.