



CRYSTALLIZATION OF BIOLOGICAL MACROMOLECULES

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Abstract

All of the macromolecules are polymers consist of amino acids, ribonucleotides and deoxyribonucleotides, sugars of various sorts, fatty acids, etc. Crystallization of macromolecules is complex process based on finding individual conditions and parameters leading to formation of crystal. Experience and reproducibility are guides in making crystallization experiments.

All of the macromolecules are polymers of one of the precursor classes that include the amino acids, the ribonucleotides and deoxyribonucleotides, sugars of various sorts, fatty acids, etc. These small molecules are linked together in a sequence by complicated series of chemical reactions in the cell to form the macromolecules such as proteins, nucleic acids (RNA and DNA), polysaccharides and lipids. The structural complexity and physiological role of macromolecules are a function of the diversity of the precursors, the sequence in which they are joined together, the number of precursors in the polymer, and finally, the 3D form after polymer synthesis. Macromolecules assume 3D structures that sequester and pack hydrophobic groups in their interior and leave hydrophilic groups exposed to solvent. Solvent molecules form solvent layers around macromolecules. In this abstract, we are concerned mainly with properties and crystal structures of proteins.

Protein crystals contain network of solvent filled channels that make up 40-80% of their volume. Due to the high solvent content as well as the limited number of weak interactions that hold the crystal together, the environment of the molecules in the crystal resembles that of a very concentrated solution.

The first step in the crystallization of macromolecules is their **purification** and **characterization**. Some proteins form precipitates at low salt concentration, others only from highly concentrated salt solutions, and in some cases, only when salt is removed from solution. Because proteins precipitate at different salt concentrations, this "salting-out" effect provides a method for selectively precipitating and purifying unique proteins from mixture. In recent years, polymeric precipitating agents (Peg in variety of polymer lengths) as well as organic solvents (ethanol, acetone, MPD) have been used to selectively precipitate macromolecules. The most common buffers are intended to be effective in the pH range of 6.0 to 8.0, because the most physiologically important reactions occur near neutral pH. For specific pH ranges, biological buffers extending from pH 2 to 13 are used. The similar conditions as for

purification and isolation of proteins are used to provide following crystallization experiments.

The solubility of proteins in water depends on properties such as temperature, pH, and the presence of other solution components as well as amino acid composition. When the concentration of a protein solution is brought above its solubility limit [see Figure 1], the solution becomes supersaturated. At this point, the protein begins to aggregate. Aggregation occurs in two stages as a nucleation and as a growth. During nucleation, protein molecules associate into a stable complex as an amorphous precipitate or as a microcrystals. Amorphous precipitates tend to predominate when the protein concentration is well above saturation. In addition, crystals grow much slower than amorphous precipitates do, so when the concentration of a protein is brought above its saturation point too quickly, precipitation will again predominate. In the metastable region, if a few nuclei are present, they will continue to grow, but without spontaneous formation of new nuclei. As the crystals grow, the solution will be depleted of nutrient and supersaturation will fall. Growth will be slow and orderly and will produce the fewest and largest single crystals. Thus, the most *general crystallization strategy is to bring the protein to the point only slightly above its saturation point as slowly as possible.*

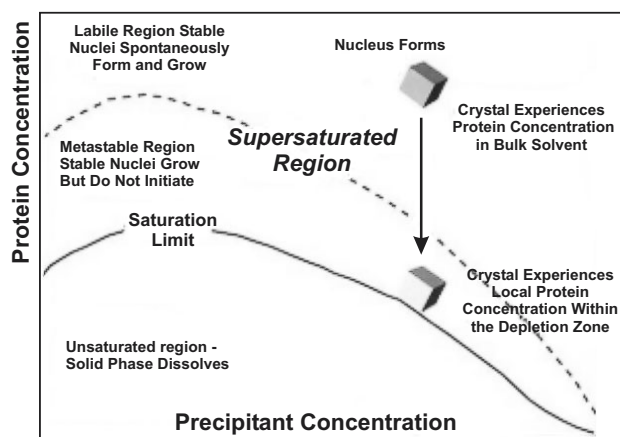


Figure 1. The phase diagram for the description of protein crystallization (from McPherson, 1999 [1])

Generally, the **protein crystallization experiments** proceed in two steps. First step is test screening of the protein solubility considering the precipitants (precipitating agents) and other solution components. Usually at this step, insoluble protein is observed as an amorphous precipitate, what means that the precipitation conditions are severe to allow crystal growth. In the second optimization step, conditions which gave rise to precipitates in the first step are modified systematically to allow the approach to insolubility that is required for the formation of crystal nuclei.

Several techniques such as sitting drop vapor diffusion, hanging drop vapor diffusion, sandwich drop, batch,

microbatch under oil, microdialysis, and free interface diffusion could be used for setting up crystallization experiments. The most frequently used crystallization method is the *vapor diffusion technique*. The difference in concentration between the drop (protein, buffer, salt and precipitant) and the reservoir (buffer, salt and precipitant) drives the system toward equilibrium by diffusion through the vapor phase. The protein becomes supersaturated and crystals start to form when the drop and reservoir are at or close to equilibrium. *Free interface diffusion* is one of the methods used by NASA in microgravity crystallization trials. Using this method the sample is in liquid contact with the precipitant. Over time the sample and precipitant diffuse into one another and crystallization may occur at the interface. *Batch crystallization* is method where the sample is mixed with the precipitant and additives creating a homogenous crystallization medium. This technique is popular with small molecule crystallographers. In *microbatch under oil* a small drop of the sample combined with the crystallization agent is pipetted under a layer of oil (paraffin, silicon oils). Such oils allow water vapor to permeate from the drop and allow sample and reagent concentration. Unless the drop is equilibrated with a reservoir, water will leave the drop until those only solids remain. *Dialysis crystallization* involves placing the sample in a Dialysis Button, which is sealed with a dialysis membrane. The Dialysis Button is placed into a container with crystallization medium. Water and some precipitants are then allowed to exchange while retaining the sample in the cell.

For the **examination** of the crystallization trials a stereomicroscope is used. Crystals are usually easy to distinguish from amorphous precipitate. Diffractable crystals are typically single, transparent, they have definite form characterized by planar faces and they are free of cracks and defects. Crystals are often birefringent, so that they ap-

pear dark and bright as they are rotated under crossed polarizers in the stereomicroscope. Several methods are available to test whether crystals are protein or salt. These are crush test, dehydration test, dye binding test, gel electrophoresis and X-ray diffraction. In the case of getting microcrystals, the seeding techniques could be used to grow the crystal. The seeds (microcrystals) are transferred to a new protein-precipitant drop using a streak seeding wand or a crystal transfer syringe, respectively. Seeds provide a template on which further molecules can assemble, and given the proper environment, time, and patience, the seed will enlarge into a crystal.

The crystallization of membrane proteins proceeds in the same manner as crystallization of soluble proteins, except for the addition of detergents in the crystallization conditions. Selection of proper detergent is the most critical parameter for this kind of crystallization.

Experience and reproducibility are guides in making crystallization experiments.

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CRYOCRYSTALLOGRAPHY OF BIOLOGICAL MACROMOLECULES

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Abstract

Crystals of biological macromolecules at near to room temperature are sensitive to X-rays and frequently suffer from radiation damage, especially when X-ray experiments are carried out on highly intense synchrotron beamlines. Performing such experiments at cryogenic temperatures greatly reduce, or eliminate radiation damage and thus produce higher quality diffraction data. Since about 1985, the cryocrystallography methods have become widely used and well established technique. A brief discussion of the most important experimental aspects and advantages of data collection at low temperature is given. Reviews on cryogenic techniques in macromolecular crystallography can be found in: [1, 2, 3].

Radiation damage

Radiation damage of biocrystals appears to be related to the formation of free radicals. Although the photochemical processes producing free radicals (primary radiation damage) are localized event; subsequent chemical reactions can be induced at relatively remote sites due to the propagation of free radicals in the solvent regions of a protein crystal via diffusion (secondary radiation damage) [4]. Damage is spread and leads to crystal decay, typically accompanied by changes in reflection profiles and cell dimensions.

It was noted as early as 1970 [5] that performing diffraction experiments on protein crystals cooled to near liquid-N₂ temperature leads to significant reduction in radiation damage. This effect is due to facts that by lowering the temperature diffusional processes and therefore propagation of highly reactive free radicals within the crystals is slowed down.