

Small-Scale Batch Crystallization of Proteins Revisited: An Underutilized Way to Grow Large Protein Crystals

Ways & Means

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Summary

Growth of high-quality crystals is a major obstacle in many structural investigations. In recent years, the techniques for screening crystals have improved dramatically, whereas the methods for obtaining large crystals have progressed more slowly. This is an important issue since, although many structures can be solved from small crystals with synchrotron radiation, it is far easier to solve and refine structures when strong data is recorded from large crystals. In an effort to improve the size of crystals, a strategy for a small-scale batch method has been developed that in many cases yields far larger crystals than attainable by vapor diffusion.

Introduction

In macromolecular crystallography, the success of a project ultimately resides in the characteristics of the crystals utilized for the study, since X-ray diffraction is an imaging technique and cannot extract more information from a lattice than is embedded within it. Much effort has been devoted toward maximizing the quantity and quality of the data that can be recorded from a crystal as manifested by the use of synchrotron radiation and cryofreezing techniques. Likewise, sophisticated numerical algorithms have evolved to optimize the amount of information from the resultant data. Consequently, the major hurdles now in a structural study are material preparation and crystal growth. The purpose of this short commentary is to stimulate interest in an alternative method for “batch” crystallization that can yield large crystals when other approaches prove problematic.

Historical Development of Crystallization

Methods

Given the obvious importance of crystals in a crystallographic study, considerable effort has been devoted toward improving this aspect of structural investigation, and there is an extensive literature describing the current methodologies and its history [1–4]. Many of the early crystals, in the distant ages of protein crystallography, were grown with large-batch techniques or bulk dialysis. In the former technique, as much as 1 ml of protein solution was mixed with ammonium sulfate to achieve saturation. Although these approaches generated some magnificent crystals that were essential for the development of this field, they were only suited to problems for which many hundreds of milligrams of material were

readily available. Consequently, more conservative approaches were developed to allow crystals to be grown while requiring considerably less material. Of these, the vapor diffusion methods for crystallization have proved to be the most successful.

Sitting drop methods, where the macromolecule is mixed with a precipitant and equilibrated against the same precipitant by vapor diffusion, were introduced by R.M. Bock in the late 1960s as a solution to the problem of growing crystals of tRNA [5], though evaporative techniques were well established by that time [6, 7]. This approach rapidly became the method of choice for growing large crystals [8]. Shortly thereafter (~1971), the hanging drop method was developed as a way to further reduce the size of the experiment by Bob Hartley at Cambridge while attempting to crystallize barnase with David Blow (D. Blow, personal communication). Since then, hanging drop crystallization has been the method of choice of screening crystallization conditions. At that time, the crystals obtained from this approach were usually too small for a structural determination. Consequently, considerable effort was devoted to improving the size of the crystals, usually by increasing the size of the experiment by switching to sitting drop techniques and by attempts to control the nucleation. Now, with the advent of powerful radiation sources, fast detectors, and the introduction of cryofreezing techniques, many structures are determined directly from crystals grown in hanging drops. This is possible because of the reduction of radiation damage by cryocooling and the enhanced signal-to-noise for the data recorded from a well-collimated and focused X-ray source. Indeed, structures are now routinely determined from crystals with dimensions as small as $50 \times 10 \times 100 \mu\text{m}$. Therefore, the absolute necessity for large crystals in many cases has been eliminated. Even so, there are benefits to be gained by continuing to strive for large crystals.

All other things being equal, better data will be recorded from a large crystal because the standard deviation of the data is controlled by counting statistics which are related to the square root of the total number of counts in the reflection and its background. Thus, a larger crystal scatters more X-rays relative to the background and generally has lower sigmas. This is important at the early stages of structural determination, since the sigmas control the quality of the small differences inherent in multiple wavelength measurements that are now used for most phasing experiments. Interestingly, the enthusiasm for single anomalous wavelength measurements to determine structures is a compromise designed to obtain one well-measured data set within the constraints of radiation damage, rather than several ill-determined data sets [9]. In the final stages of a structure determination, large crystals will often provide data that refine more easily, even if the merging R factors do

not appear radically different from those obtained from smaller crystals.

Benefits and Disadvantages of Vapor Diffusion

As noted above, vapor diffusion is the most popular method for screening and growing crystals of macromolecules and can yield perfectly adequate materials for structural analyses. It has the benefit of requiring very little material for each experiment and of being easy to apply to a new protein through the use of crystallization screens [10]. Even so, it is widely recognized in the field of crystal growth that vapor diffusion contains inherent problems when trying to grow large crystals. The reasons for this are quite simple. First, the precipitant concentration required for crystal growth is lower than that necessary for nucleation, which often results in many small crystals that grow quickly. Second, the crystallization conditions change during the equilibration process. In a typical vapor diffusion experiment, a few microliters of protein are mixed with an equal volume of precipitant and then equilibrated against the original precipitant concentration. During equilibration, the vapor pressure of the solution rises as the protein crystallizes (the protein in solution lowers the water activity), whereupon the water evaporates to maintain equilibrium, which causes the precipitant concentration to rise. Thus, if the crystal growth is sensitive to the precipitant concentration, vapor diffusion can rapidly force the mixture to unstable conditions where growth and nucleation are too rapid.

Many imaginative strategies have been adopted to control nucleation in hanging drops, such as varying the size of the droplets, altering the conditions of the equilibrant, or incorporating additives that retard crystal growth. In some cases this is successful, but since vapor diffusion is an inherently unstable way of growing crystals, it often leads to frustratingly small crystals. Under such circumstances, alternative crystallization strategies must be tried, which include dialysis, free interface diffusion, or batch. Each of these strategies can yield large crystals on occasions where vapor diffusion proves to be difficult [1]. For example, dialysis is the method of choice where the macromolecule crystallizes at low ionic strength. In other cases, direct mixing (batch) has particular advantages for growing large crystals and, as noted by McPherson, this technique deserves more attention [1].

Batch Techniques in Crystallography

Traditional batch techniques (from the 1930s) involved mixing large volumes of protein (upward of 1 ml) with a precipitant until a slight opalescence was observed. Thereafter, the solutions were either clarified by centrifugation or placed directly in vials and allowed to crystallize unattended. The goal of these experiments was to achieve supersaturation without reaching the point of spontaneous nucleation. Clearly, the drawback to this approach is the amount of material required and therefore it fell out of favor for many years. More recently, direct mixing techniques have experienced a renaissance through the microbatch technique, in which small volumes of protein and precipitant are mixed under oil

[11]. Microbatch is utilized by one of the popular robotic systems for protein crystallization [12] and has been developed into a successful high-throughput method for rapid screening of massive numbers of crystallization conditions that requires exceedingly little protein [13]. The use of oil reduces the evaporation of the small volumes, though in many cases where the drops are small, desiccation of the droplet is an important parameter in achieving the crystallization condition. Oil also eliminates many of the effects of surfaces and walls of the crystallization containers. The benefits of microbatch have been well documented [3, 11, 14]; however, it works best for small volumes of protein and precipitant. The latter restriction is an inevitable consequence of the manner in which the protein and precipitant are mixed.

Small-Scale Batch without Oil

There is a simple alternative method for growing crystals by direct mixing without the use of oil which has been used at the University of Wisconsin for many years (though no claim is made for its originality). This strategy has the advantage of providing fine control of the mixing of the protein with the precipitant and subsequent nucleation, both of which are critical components of a successful crystallization experiment. More than 70% of the time in the author's laboratory, better crystals are obtained with this technique than with vapor diffusion methods.

The small-scale batch procedure is very simple. It consists of slowly and thoroughly mixing the precipitant with the protein and then placing the mixture in a well-sealed container (Figure 1). Because of the time invested in each crystallization droplet, this approach requires knowledge of an initial set of crystallization conditions. If vapor diffusion techniques were utilized for the initial screen, the actual conditions at which crystal growth occurs would be unknown, since the crystallization mixture does not achieve the concentration of the original precipitant. This occurs because the protein, buffer, and salts associated with the crystallization mixture all contribute to lowering its water activity and reduce the evaporation of the droplet.

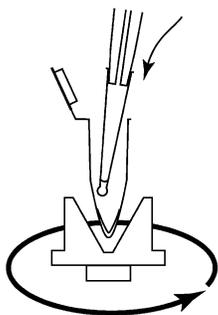
The search for batch crystallization conditions is done in two stages. The first experiments are designed to discover what concentration of precipitant is required to obtain spontaneous crystal growth by direct mixing. This is followed by a search for conditions that support crystal growth with controlled nucleation and yield optimal crystal morphology and size.

Preliminary Small-Scale Batch

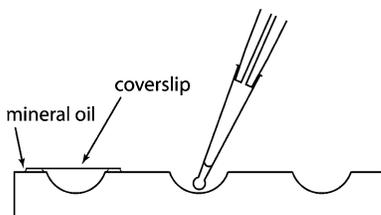
From experience, crystallization by direct mixing occurs at about 60%–80% of the concentration of the precipitant required in a hanging drop experiment [15]. Thus, crystals obtained from a hanging drop experiment in which buffered 20% PEG containing 200 mM NaCl is mixed 1:1 with protein most likely grow from a solution that contains 12%–16% PEG and 120–160 mM NaCl. Although the exact point of crystallization is unknown, the contents of the drop during the crystallization trajectory can be predicted from the starting components. Thus, it is possible to design a simple batch experiment



1. Transfer protein to eppendorf tube.



2. Vortex gently (5-10 rotations/second) while adding the precipitant, then an additional 1-4 seconds.



3. Transfer to a greased batch plate and seal with a coverslip and mineral oil.

Figure 1. Overview of the Small-Scale Batch Procedure

to determine the real conditions that support crystal growth.

Although a series of accurately prepared solutions of PEG of varying concentration might seem to be required for a batch experiment, preparation of a single precipitant solution at twice the concentration of the midrange of the expected final conditions is often sufficient. By varying the ratio of the volumes of precipitant to protein, it is possible to cover the predicted crystallization point (Table 1). In the example above, a solution might be constructed that contains 28% PEG and 280 mM NaCl with an appropriate buffer. This has the advantage of eliminating the errors involved in preparing solutions of slightly differing precipitant concentrations. In addition, since accurate preparation of solutions is the slowest part of this procedure, preparation of a single solution saves considerable time. It should be stressed that batch crystallization is exceedingly sensitive to precipi-

Table 1. An Example of a Simple Mixing Experiment

Ratio of Mixture Protein:PEG	Final [PEG] %	Final [Protein] mg/ml
5:3.5	11.5	8.8
5:4	12.4	8.3
5:4.5	13.3	7.9
5:5	14	7.5
5:5.5	14.7	7.1
5:6.0	15.3	6.8
5:6.5	15.8	6.5

Final concentrations of PEG and protein were obtained by mixing varying amounts of 28% PEG with 5 μ l of protein at 15 mg/ml. The seven combinations listed here would typically be tried in an initial batch experiment.

tant concentration. Often, variations of less than 0.5% in the concentration of PEG have a profound effect on crystal size and nucleation.

The one apparent disadvantage of the one-solution strategy is the variation in protein, buffer, and salt concentration. In general this is not a problem, since crystallization is usually insensitive to a 10%–20% variation in protein concentration. Likewise, although the presence of salts or other additives is often essential for crystallization, the exact concentration is rarely critical for the observation of crystals when a range of precipitant concentrations is examined. These apparent drawbacks are more than compensated by the systematic differences between adjacent crystallization experiments and by the ability to reproduce the conditions exactly at a later date.

Experimental Considerations

In a typical initial batch crystallization experiment, 5 μ l of protein is mixed carefully with varying amounts of a precipitant solution and placed in a sealed container to prevent evaporation. It is important to note that the way in which the protein and precipitant are mixed is a critical feature of this method, since the goal is to achieve supersaturation without triggering nucleation. This is accomplished by mixing the protein and precipitant in a conical eppendorf tube while *gently* vortexing and then transferring the mixture to a batch plate (an example of a simple batch plate is shown in Figure 2). The order in which the proteins and precipitant are mixed is also very important. The protein must be placed at the bottom of the eppendorf tube *first*. Then, while vortexing the solution gently (\sim 5–10 rotations/s), the precipitant is slowly added to the protein and mixed for a further 2–5 s. Adding the precipitant to the protein allows its concentration to rise slowly in the mixture and avoids local high concentrations of precipitant that have the potential to trigger nucleation or precipitation of the protein. Once mixed, the solutions are transferred to the batch plate and sealed. Mixing the solutions by aspiration with a pipettor does not work, since it is difficult to attain a homogenous mixture by drawing solutions in and out of a pipette tip.

Expectations from the Initial Batch Experiment

The purpose of the initial batch experiments is to determine where the protein crystallizes spontaneously. For

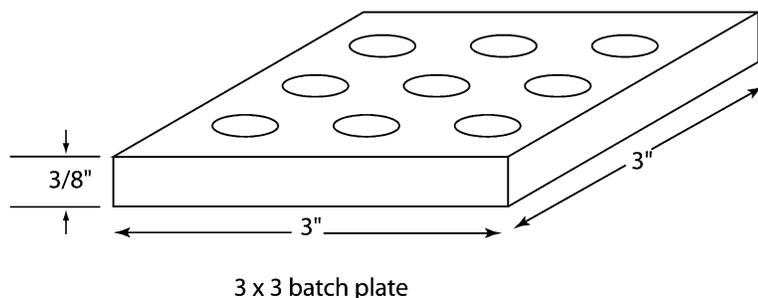


Figure 2. Dimensions of a Low-Budget Batch Plate

The plates may be constructed from any 3/8 inch clear plastic sheet. Usually these are made from acrylic, but PETG and clear PVC have significantly lower levels of water absorption. The depressions may be cut with a 1/2 inch diameter ball-end mill to a depth of 0.10 to 0.17 inches, depending on the preferred size of well.

crystals that grow rapidly in hanging drop experiments, either at room temperature or in the cold room, evidence for crystallization should be observed within 24 to 72 hr. If the crystallization conditions cover the range from below to above the spontaneous nucleation point, a clear trend should be observed in the droplets. These initial trials should suggest the nature of the next series of experiments. Under ideal circumstances, no crystals will be observed in the droplets that have low precipitant concentration, with increasing numbers at higher concentrations. If this is observed, those droplets without crystals should be nucleated with streak seeding to check for supersaturation [16]. If crystallization can be induced by seeding, growth of large crystals should be simply a matter of increasing the drop size and controlling the number of seeds (macro or micro) introduced into the mixture [17, 18]. In this case, when the experiment is repeated, the drops should be seeded immediately after transfer to the batch plates.

If crystals are observed in every drop, the precipitant or protein concentration was probably too high. This can be remedied by lowering the concentration of either or both until conditions that yield supersaturation or satisfactory spontaneous nucleation are located. Alternatively, the protein and precipitant might need to be mixed at a lower temperature to avoid nucleation.

The absence of crystals is more problematic and may

be caused by lack of nucleation, low precipitation concentration, or fundamental differences between the reagents in the initial crystallization screen and those used to prepare the solutions for batch crystallization. Careful examination of each of these scenarios should reveal the cause of the problem and suggest a way to solve them. If the components of the batch experiment are identical to those in the original crystallization screen it should always be possible to grow crystals in batch.

In those cases where the presence of crystals across a series is nonsystematic, the cause is probably due to inconsistent mixing of the solutions or errors in pipetting. Both of these can be remedied by attention to the details of the technique. In some cases, the solutions might need to be clarified by a short spin in a microfuge to remove nuclei or amorphous material, although in most instances this is not required.

Refinement of Batch Conditions and Future Prospects

Once the initial conditions that yield crystals in batch have been defined, refinement of the crystallization protocol usually involves adjusting the precipitant concentration, pH, salt components, and additives, and optimizing the protein concentration. This is similar to the way in which conditions are optimized in vapor diffusion experiments, except in this case it is easier to use larger

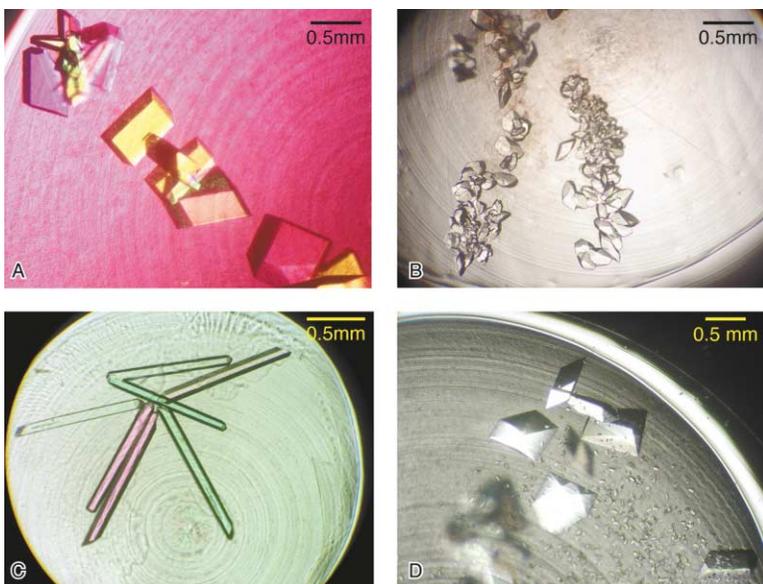


Figure 3. Collage of Crystallization Results from Small-Scale Batch Experiments

(A) Crystals of adenosylcobinamide kinase/adenosylcobinamide phosphate guanylyltransferase (CobU) from *Salmonella enterica* [20].

(B) Small crystals of 4-hydroxybenzoyl CoA thioesterase from *Pseudomonas* sp. obtained by streak seeding a saturated solution (J. Thoden and H. Holden, personal communication).

(C) Crystals of L-Ala-D/L-Glu epimerase from *B. subtilis* complexed with L-Ala-D/L-Glu (V.A. Klenchin and I.R., unpublished data).

(D) Crystals of glycylamide ribonucleotide transformylase (PurT-encoded) from *E. coli* [21]. In all cases, crystallization was induced with macro- or streak seeding and yielded crystals 3–4 times the size grown by vapor diffusion.

crystallization volumes (up to 100 μ l) at a lower protein concentration to inhibit spontaneous nucleation. In many cases, it is possible to control the nucleation by either micro- or macroseeding [17, 18] and obtain substantial crystals (Figure 3).

The concept of mixing solutions carefully to obtain a supersaturated solution could readily be incorporated into a robotic approach to crystallization. This might provide a systematic tool for growing large crystals, which would profoundly benefit structural genomics. The strategy could be applied equally well to crystallization in small depressions sealed with glass or tape where the evaporative loss can be reduced to a minimum. Alternatively, carefully premixing solutions and injecting them under oil would work equally well. This latter approach was used successfully in the structural determination of telokin [19]. Efforts to incorporate careful mixing of protein with precipitant into a system for robotic crystallization are in progress.

Summary

The goal of most crystallographic studies is to maximize the quantity and quality of the data that is recorded from a given sample. Given the time and effort involved, material preparation, and structural determinations, it would seem worthwhile to devote more time to optimizing the crystalline material, since ultimately it is the quality of the crystals that controls how much is learned. The small-scale batch technique described here is worth a try, though it should be emphasized that its use always follows an initial characterization of crystallization conditions by hanging drop or microbatch under oil. In many cases, careful application of this approach will yield much larger crystals. Even in those cases where batch methods prove to be difficult, a greater understanding of the factors that control crystallization will be achieved. As a final note, most students trained today never experience the beauty of a crystal that can be seen without the need for a microscope. The excitement induced by the growth of large crystals is something to be encouraged.

A full description of this approach to crystallization and construction of the batch plates may be found at www.biochem.wisc.edu/holdenrayment/ or www.uwstructuralgenomics.org.

Acknowledgments

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