Systematic Improvement of Protein Crystals by Determining the Supersolubility Curves of Phase Diagrams

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ABSTRACT A systematic approach for improving protein crystals by growing them in the metastable zone using the vapor diffusion technique is described. This is a simple technique for optimization of crystallization conditions. Screening around known conditions is performed to establish a working phase diagram for the crystallization of the protein. Dilutions of the crystallization drops across the supersolubility curve into the metastable zone are then carried out as follows: the coverslips holding the hanging drops are transferred, after being incubated for some time at conditions normally giving many small crystals, over reservoirs at concentrations which normally yield clear drops. Fewer, much larger crystals are obtained when the incubation times are optimized, compared with conventional crystallization at similar conditions. This systematic approach has led to the structure determination of the light-harvesting protein C-phycocyanin to the highest-ever resolution of 1.45 Å.

INTRODUCTION

The detailed understanding of three-dimensional protein structure is important in the design of new drugs and for engineering proteins with improved properties for industrial applications. X-ray diffraction is the most powerful method to determine the structure of such large molecules but can be applied only when suitable crystals are obtained.

Crystallization has always been a difficult step, often referred to as the ''bottleneck'' to structure determination. The urgent need to develop efficient crystallization methods is further highlighted in this era of structural genomics, where production of diffraction-quality crystals is crucial to the success of the next step of the genome project.

Biocrystallization, like any other crystallization, is a process involving the steps of nucleation and growth, where molecules have to be brought into a supersaturated, dynamically unstable state. However, the supersaturation level required for the nucleation step is higher than that required for the growth of existing postcritical nuclei. Nucleation is a prerequisite and the first step to crystal growth, yet excess nucleation causes the production of a large number of small crystals instead of a smaller number of useful ones (Feher and Kam, 1985; Chayen, 1997a). The optimum conditions for crystal growth are thought to be in a metastable zone of conditions, which allows growth without the production of further nuclei (Ataka, 1993; McPherson, 1999; Ducruix and Giegé, 1992; Stura and Wilson, 1992).

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The aim is to control crystal growth by separating the phases of nucleation and growth, i.e., to start the process at conditions that induce nucleation and then transfer the nuclei to metastable conditions. The most widely used method for separation is seeding, that is, transferring whole crystals, crushed crystals or precipitate from the nucleating drops into metastable drops (Stura, 1999). Other methods involve changing of the temperature (Rosenberger et al., 1993; Haire Lloyd, 1996) or diluting microbatch drops after incubating them for a given time at spontaneous nucleation conditions (Saridakis et al., 1994). In both cases, the optimum time for intervention was long before the appearance of the first visible crystals. A method for slowing down the rate of evaporation of a droplet was designed by applying a double cell for changing reservoir concentrations during crystal growth. This was used successfully in the crystallization of the *Brucella abortus*-specific Fab (Przybylska, 1989). A simpler way of achieving the same is by placing an oil barrier over the reservoirs of vapor diffusion trials (Chayen, 1997b). This technique has enabled the successful crystallization of a titin kinase domain after years of failure using standard crystallization methods (Mayans et al., 1998).

The area of conditions called the ''metastable zone'' lies between the solubility and supersolubility curves on a crystallization phase diagram of a macromolecule. The supersolubility curve is defined as the line separating conditions where spontaneous nucleation (or phase separation, precipitation) occurs from those where the crystallization solution remains supersaturated without the formation of a solid phase for an indefinite length of time if it is left undisturbed (Ducruix and Giegé, 1992).

The supersolubility curve is less well defined than the solubility curve but experimentally, it is found to a reasonable approximation much more easily. To construct it, one must set up many crystallization trials, varying at least two conditions (one of which is typically the protein concentration) and plot their outcomes, after 3–4 weeks, on a two- or

many-dimensional parameter grid. These experiments may be called ''static,'' by contrast to the ''dynamic'' experiments involving seeding or some process of nucleation inducement. The static experiments will show where on the grid (i.e., for which conditions) the crystallization solution remains clear and where it spontaneously nucleates or precipitates. The line dividing the two regions is the supersolubility curve. The automated microbatch technique (Chayen et al., 1990; 1992) is perfectly suited for such experiments.

When the supersolubility curve has been determined, the metastable zone can be accessed for the growth of nuclei into crystals by dilution experiments such as the ones described here, or by seeding, etc. Knowledge of the supersolubility curve means that informed decisions can be made as to which conditions are metastable.

We have shown improvement of human serum albumin crystals in hanging drops by diluting the reagents after a certain incubation time (Saridakis and Chayen, 2000). Conditions were at the time selected merely by trial and error. The present communication presents the use of supersolubility curves as a systematic approach for growing protein crystals in the metastable zone utilizing the hanging drop technique. The method does not involve handling of the crystals.

MATERIALS AND METHODS

Pure, crystallized trypsin from porcine pancreas was purchased from Sigma (Steinheim, Germany, Cat. No. T-0134). It was dissolved to various stock concentrations with deionized water. Solutions of C-phycocyanin were prepared as described by Nield (1997) to a stock concentration of 70 mg/ml. Trizma base, MES, and ammonium sulfate were purchased from Sigma.

Determination of the supersolubility curve

Using the automated microbatch method (IMPAX), which consists of a computer-controlled robot that rapidly dispenses numerous small-volume crystallization trials, two-dimensional phase diagrams were obtained for two proteins, trypsin and C-phycocyanin, by varying the concentrations of protein and precipitating agent until the supersolubility curve could be determined. All trials were performed at 20°C. Trypsin was crystallized from ammonium sulfate and 100 mM Tris, pH 8.4 (Christopher et al., 1998). C-phycocyanin was crystallized from ammonium sulfate and 40 mM MES, pH 6.1.

Additional trials were performed to adjust the phase diagrams to vapor diffusion conditions (see Results and Discussion). These were performed in hanging drops as described below.

Separation of the nucleation and growth phases

The separation between the nucleation and growth stages was performed as follows. First, the hanging drops were incubated for some time at conditions normally giving many small crystals. The coverslips were then transferred over reservoirs containing precipitant concentrations that normally yield clear drops.

The wells of Linbro and VDX crystallization plates contained 1 ml of the reservoir solutions. The drops were dispensed on silanized glass coverslips that were inverted above the wells at the initial conditions (see Results and Discussion) and sealed with Apiezon C oil (M&I Materials, Manchester, UK). The initial drops consisted of 1 μl of protein stock mixed with 1 μl of well solution. The dilution experiments consisted in transferring each coverslip, after incubation, over a well containing a lower concentration of precipitating agent (ammonium sulfate). The rim of the new well had already been covered with Apiezon C oil, to form a new seal. Each transfer of a coverslip between the wells was carried out in 1–2 s. All the transfer trials were performed in duplicate for each incubation time in the case of C-phycocyanin, and in triplicate for trypsin. Controls set initially at the nucleation (high supersaturation) and at the metastable (low supersaturation) conditions and left undisturbed were also set up for every experiment, in triplicate. An experiment was considered successful if the low supersaturation controls (considered then to belong to the metastable zone) stayed clear for at least 3 weeks, inasmuch as good crystals appeared in the transfer trials before that time. Successful experiments, therefore, were those where single crystals appeared in the drop because it was diluted to true metastable conditions, rather than just to the low side of the spontaneous nucleation zone, where crystals would appear in any case.

At least a dozen repeat experiments were conducted over several months for each protein to ensure reproducibility of the reported results. The crystals were x-rayed at room temperature, using an Enraf-Nonius GX21 rotating anode $CuK\alpha$ source with MAR image plate detector.

RESULTS AND DISCUSSION

Two phase diagrams are presented in Fig. 1: trypsin (Fig. 1 *a*) and C-phycocyanin (Fig. 1 *b*). These are, of course, not complete, true phase diagrams, because the solubility curve has not been determined; we therefore call them ''working phase diagrams,'' because they are sufficient for our purpose of separating the phases of nucleation and growth.

The dilution experiments described here involve the method of vapor diffusion in hanging drops. It has been shown (Chayen, 1998) that some adaptation of the batch conditions to the vapor diffusion method is necessary; therefore, some more experiments were done, with vapor diffusion, to establish a correspondence of the working phase diagrams with the vapor diffusion situation. The agreement was perfect in the case of C-phycocyanin, and only minor adjustments had to be made to the trypsin diagram to fit the vapor diffusion results (the diagram presented here is the one adjusted to vapor diffusion).

The arrows connect initial and final conditions of successful dilution experiments. It can be seen that the successful starting (spontaneous nucleation) conditions are well beyond the supersolubility curve. The endpoints of the arrows show the conditions that sustained crystal growth when drops which had been incubated at nucleation conditions were transferred to them. Drops which were set at these conditions without previous incubation remained clear for at least 3 weeks, showing that they belong to the metastable zone.

For trypsin, incubation times of 0, 2.5, 4.5, 6, 7, and 9 h were tried for the various conditions in the nucleation zone shown in Fig. 1 *a*. The chosen initial conditions mostly yielded crystals overnight when left undisturbed, and therefore our chosen incubation times span a range of

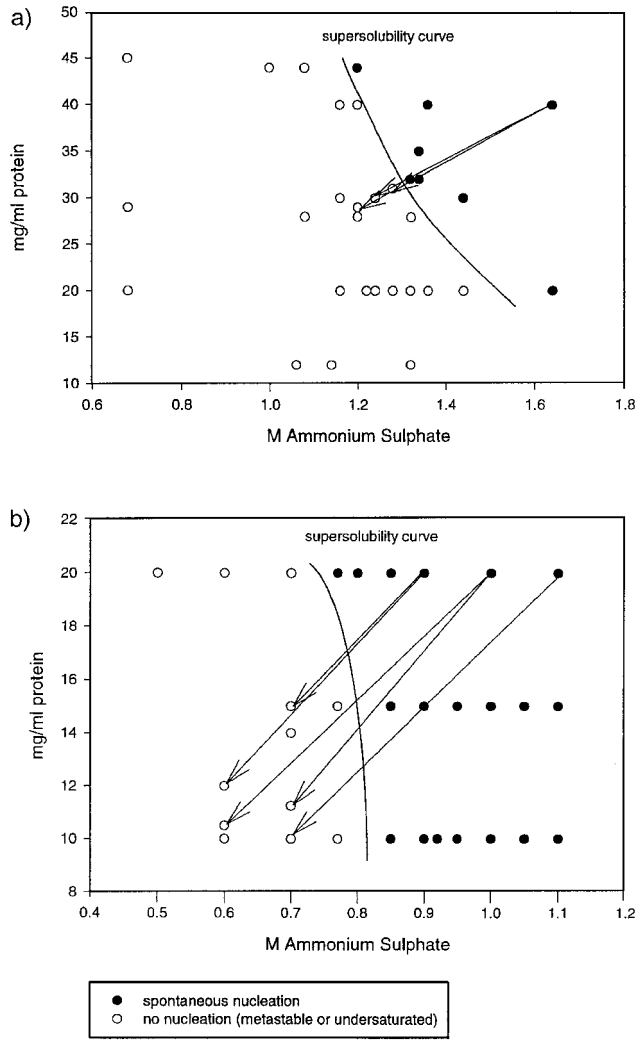

FIGURE 1 Working phase diagram and supersolubility curve (*a*) for trypsin and (*b*) for C-phycocyanin, established for the vapor diffusion technique, around conditions described in the text. The arrows correspond to dilution experiments yielding enhanced crystals in the metastable zone.

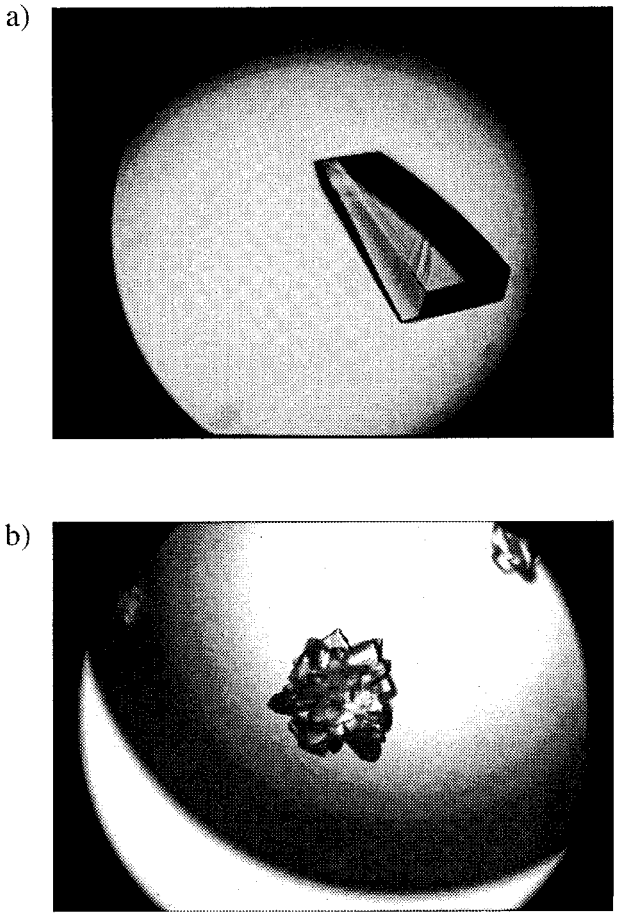

FIGURE 2 Trypsin crystals grown (*a*) after transfer of the drop to metastable conditions (incubation time $t = 6$ h), largest dimension of crystal 0.6 mm; and (*b*) at the nucleation conditions at which growth of the crystals in Fig. 2 *a* was initiated, but not transferred to metastable conditions (same magnification as Fig. 2 *a*).

possible nucleation induction times within that interim. The best results were obtained by transferring the drops from reservoirs containing 1.7 M ammonium sulfate (40 mg/ml stock protein, 100 mM Tris, pH 8.4) to metastable conditions ranging from 1.2 to 1.3 M ammonium sulfate. The largest crystals (single crystals of sizes up to $0.6 \times 0.3 \times 0.2$ mm diffracting to 1.6 Å—see Fig. 2 *a*) were obtained in drops incubated for 6 or 7 h at the nucleation conditions and then transferred over wells at 1.3 M ammonium sulfate (same buffer). Transfers performed after shorter incubation times resulted in clear drops. The high supersaturation controls yielded mostly clusters (Fig. 2 *b*) or very small single crystals (typically up to 20 μm in each dimension) which did not diffract at all.

For C-phycocyanin, incubation times of 0, 30 min, 1 h, 2 h, 5 h, and 7 h were tried for the various conditions in the nucleation zone shown in Fig. 1 *b*. Again, small crystals grew overnight when left undisturbed at these chosen conditions. By transferring the drops from reservoirs containing concentrations of 1–1.4 M ammonium sulfate (20 mg/ml stock protein, 40 mM MES, pH 6.1) to metastable conditions ranging from 0.6 to 0.7 M ammonium sulfate (final protein concentrations from 10 to 14 mg/ml) large single crystals were obtained. Although equally good crystals could eventually also be obtained in the low controls after 6–8 weeks, the transfers resulted in the formation of such crystals ($>250 \times 250 \times 150$ μm, diffracting to 1.9 Å on a table top x-ray generator; see Materials and Methods) within one week (Fig. 3 *a*). This was achieved by incubating the drops for 7 h at 1.1 M ammonium sulfate (20 mg/ml stock protein, 40 mM MES, pH 6.1) and then transferring them over wells at 0.6 M ammonium sulfate (same buffer). The high supersaturation controls yielded clusters of very small crystals, typically 40–50 μm in each dimension (Fig. 3 *b*). This systematic approach has led to the crystallization

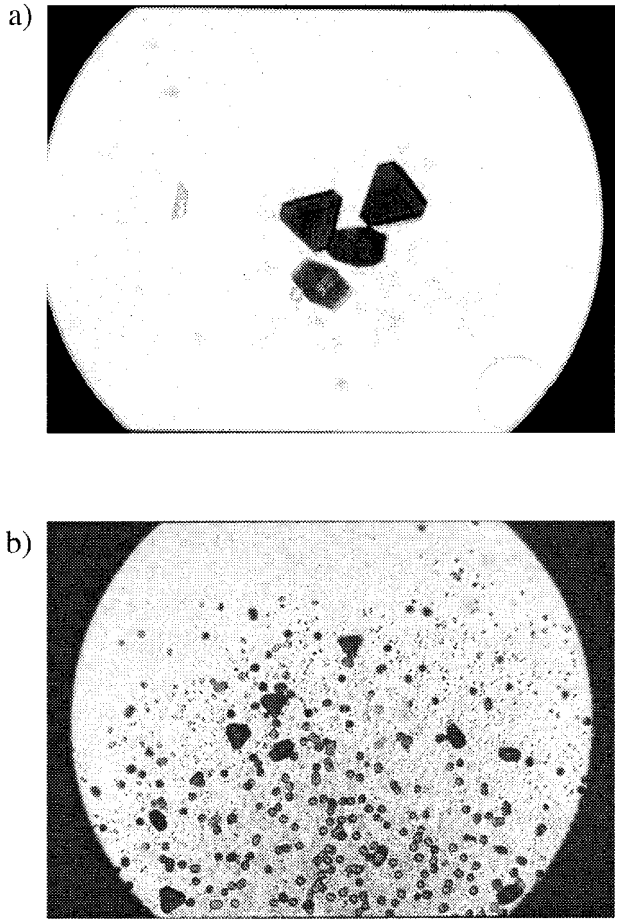

FIGURE 3 C-phycocyanin crystals grown (*a*) after transfer of the drop to metastable conditions (incubation time $t = 6$ h), dimensions of largest crystal $0.25 \times 0.25 \times 0.15$ mm; and (*b*) at the nucleation conditions at which growth of the crystals in Fig. 3 *a* was initiated, but not transferred to metastable conditions (same magnification as Fig. 3 *a*).

conditions used for the determination of the x-ray structure of C-phycocyanin to 1.4 Å at the Daresbury synchrotron (manuscript in preparation). These are the highest resolution crystals ever obtained for this protein (compared to the best published diffraction limit of 2.0 Å of Toriumi et al., 2001).

Experiments were performed with C-phycocyanin and another target protein (α-crustacyanin), to assess the effect of the drop size and of the reservoir/drop volume ratio on the time of appearance of the first visible crystals and on their numbers and sizes. The drop volumes were typical of those that are routinely used in crystallography laboratories, mostly working with proteins which are limited in supply. Thus, 2–6 μl drops were set up at three different ammonium sulfate concentrations in the nucleation zone, at reservoir volumes ranging from 150 μl to 1 ml (volume ratios in the range 25–500). A difference in the size and number of crystals obtained was observed only for the trials set at the lowest ratio, 25 (6 μl drops over 150 μl reservoirs), leading to a larger number of smaller crystals. (Note that 150 μl does not fully cover the bottom of the Linbro plate). No differences were observed from the next ratio, 67 (6 μl drops over 400 μl reservoirs), upwards. At ratios $\geq$67, drop size did not have an effect, except for increasing the overall numbers (but not the sizes) of crystals in proportion to the increase in drop volume. The number of crystals per unit volume is thus kept roughly constant. No differences in the time of appearance of the crystals were observed at any ratio within the range studied. A wider range of drop volumes and volume ratios was tested with lysozyme in sodium chloride buffered solution, where 1, 2, 3, 4, 5, and 10 μl drops were equilibrated against 300 μl and 1 ml reservoirs. The 1 and 10 μl drops displayed atypical behavior for both reservoir volumes: the 1-μl drops yielded excessive nucleation and small crystals, whereas in the 10-μl drops crystals appeared more slowly, but were neither fewer nor larger. Intermediate size drops showed no differences in sizes or time of appearance of the crystals, as long as the reservoir/drop volume ratio was above 60 (lower ratios slowed down the appearance of crystals). The constant number per unit volume rule was respected for all but the 1 μl drops. The geometry of the vessels and the solution composition are important parameters in the equilibration process (Luft et al., 1994; Luft and DeTitta, 1995). We find that when using Linbro plates and high ionic strength solutions, as was the case here, only extreme volume ratios, or drop volumes that lie outside the 2–6 μl range, may make a difference to the final result and therefore to the timing and efficiency of the dilution procedure. This is probably due to the fact that the drops equilibrate quickly at the conditions used and therefore the nucleation induction time, rather than the equilibration time, becomes the limiting factor in the process.

Vapor diffusion systems involving solutions with high concentrations of high molecular weight polyethylene glycols (PEG) and low ionic strengths are very sensitive to drop volume and take a longer time to equilibrate (Mikol et al., 1990; Luft and DeTitta, 1995). The transfers therefore need to be carried out after a longer incubation over initial conditions reservoirs. Human serum albumin crystals, obtained from 29–35% (w/v) PEG 3350 (Bhattacharya et al., 2000), took 4 days to appear in 2 μl hanging drops. Dilutions, by transfer to lower PEG concentrations, were effective only 72 h after setup (Saridakis and Chayen, 2000).

Ignorance of the solubility curve may, of course, result in some of the trials being transferred to undersaturated, rather than metastable, conditions. This is more likely to occur if it is attempted to transfer them to conditions at the lowest possible supersaturation that is assumed to be metastable. Such experiments, ending as ''clear drops,'' can possibly be reset at nucleation conditions and transferred again to reach true metastability, without loss of sample.

If needed, it is also possible to establish an approximate solubility curve by transferring crystallization drops to various conditions below the supersolubility curve. The solubility curve is the boundary below which crystals will

not appear after transfer, even after long initial incubations of the transferred drops at nucleation conditions. In this case it is desirable to leave the trials to incubate at nucleation conditions for a longer time than would be most effective for crystal optimization, in order not to overestimate the solubility conditions. However, leaving them long enough for large crystals to form risks leading to an underestimate of the solubility conditions, because kinetic factors prevent a crystal from dissolving easily even when surrounded by unsaturated solution. The most reliable transfer time in this case is just before the appearance of the first visible crystals, that time being estimated from conventional trials set at the nucleation conditions. A microbatch version of this method has already been used to determine a phase diagram for the crystallization of carboxypeptidase G_2 (Saridakis et al., 1994). The use of vapor diffusion is probably more reliable, because ''shock nucleation'' is less likely.

However, and contrary to the common consensus that the determination of the solubility curve is imperative for successful rational nucleation-growth decoupling experiments, we show here that, for practical purposes, it is sufficient to obtain the supersolubility curve. The advantage of this is that the experiments take a far shorter time compared to solubility determinations which can take weeks (or even months) and consume much larger quantities of materials than those required for the working phase diagrams.

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