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Improving protein crystal quality by decoupling nucleation and growth in vapor diffusion

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Abstract

A simple method for growing protein crystals in the metastable zone using the vapor diffusion technique is described. 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 that normally yield clear drops. Fewer, much larger and better diffracting crystals are obtained, compared with conventional crystallization at similar conditions. To our knowledge, this is the first report of a significant crystal improvement due to "backing off" from nucleation conditions, using the hanging drop method.

A correlation of the transfer time with published results for vapor diffusion equilibration of poly(ethylene glycol) solutions is also presented.

Keywords: crystallogenesis; equilibration kinetics; hanging drop; nucleation; protein crystallization; vapor diffusion

It is well known that to obtain spontaneous nucleation of protein crystals, it is not sufficient to set the crystallization trials at conditions of supersaturation. Indeed, at conditions just above the protein solubility curve, the supersaturation is not sufficient for spontaneous nucleation to take place. Within this zone of conditions, commonly called the "metastable zone," it is possible to sustain, but not to initiate, crystal growth (Mikol & Giege, 1992).

It has been argued, and to some extent shown, that the metastable zone is an optimum zone for crystal growth, because of the avoidance of excessive nucleation and the slow growth rate which it affords (Stura & Wilson, 1992). However, in order for crystals to be produced, crystal nuclei have to be somehow transferred to metastable conditions.

The most common method for doing this is to physically transfer "seeds" from labile (spontaneously nucleating) into metastable drops by streaking or pipetting (Stura, 1999). Another method involves diluting microbatch drops after incubating them for some hours at spontaneous nucleation conditions. It was shown that the optimum time for dilution was long before the appearance of the first visible microcrystals (Saridakis et al., 1994). Przybylska (1989) has described a double cell for changing reservoir concentrations during crystal growth to slow down the rate of evaporation of the droplet.

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This communication presents the results of a simple adaptation of the dilution method to the hanging-drop vapor diffusion technique, which is the most popular method of crystallizing proteins.

In this study, we focus on defatted human serum albumin (HSA) (solved to 2.8 Å by He & Carter, 1992) as a model protein. This is a more challenging protein to crystallize than standard model proteins like lysozyme or thaumatin.

Results

Preliminary conventional crystallization trials with HSA had shown that crystals, which could reach a maximum size of $0.5 \times 0.35 \times 0.12$ mm and maximum diffraction limit of 3.5 Å (but more typically between 4 and 7 Å), were growing in hanging drops over reservoirs consisting of 29 to 35% (w/v) PEG 3350 as precipitant. These conditions were obtained by extensive screening around conditions described by Carter et al. (1994). Crystals appeared four days after setup. Trials were run at both 18 and 4 °C: the best crystals were consistently obtained at 4 °C.

It was therefore decided to set the drops for the "dilution" experiment at 4 °C and 33% PEG 3350 reservoir concentration—a condition that, in the conventional trials, consistently gave fairly well-formed crystals of maximum sizes comparable to those mentioned above, as well as clusters of small crystals. The hanging-drop bearing coverslips were incubated for given times at these conditions. They were then transferred over wells containing 24% PEG 3350, a concentration that in conventional trials resulted in clear drops, even after arbitrarily long incubation times.

Additional experiments at 18 °C were also similarly run, with initial and final reservoir concentrations of 32 and 28% PEG, respectively.

Since nucleation can be initiated at any time from the moment at which the trial is set up, a wide spectrum of times was chosen, ranging from the first hours after setup to 72 h, over halfway into the time of the first appearance of crystals.

The times for the transfer of the drops were 2, 4, 24, 48, and 72 h after setup ($t = 0$). Controls, that is, drops set at the labile (high supersaturation controls) and at the metastable (low supersaturation controls) conditions at $t = 0$, and thereafter left undisturbed, were also set up. Further details of the procedures used are described in Materials and methods. Transfers performed at $t = 2$, 4, 24, and 48 h resulted in clear drops (as did the low supersaturation controls), showing either that equilibration of the drop had not proceeded past the nucleation zone boundary before the transfer, or that it had, but stable nuclei had not had time to form.

Transfers performed at $t = 72$ h resulted in fewer, larger single crystals, which appeared within 7 days from the transfer (i.e., 10 days from setup). One of the drops set at 4 °C yielded a single crystal of size $1 \times 0.7 \times 0.25$ mm (Fig. 1A), which is approximately eight times larger than the largest crystals grown conventionally in our laboratory (twice as large in each dimension) and approximately 70 times larger than single crystals in our controls (see below). This crystal diffracted to 3.1 Å (as compared to 3.5 Å for the largest and best-looking crystal obtained by us by conventional crystallization). Another drop at 4 °C yielded a single crystal of size $0.5 \times 0.4 \times 0.12$ mm. All high supersaturation control drops (three at each temperature) resulted in small single crystals (maximum size $0.2 \times 0.2 \times 0.06$ mm), but mainly crystal clusters. A typical high supersaturation control drop at 4 °C is shown in Figure 1B. Improvement of crystals grown from the drops diluted at $t = 72$ h was also evident at the 18 °C conditions, but the best crystals, and most marked differences between the controls and the transferred drops, were always obtained at 4 °C.

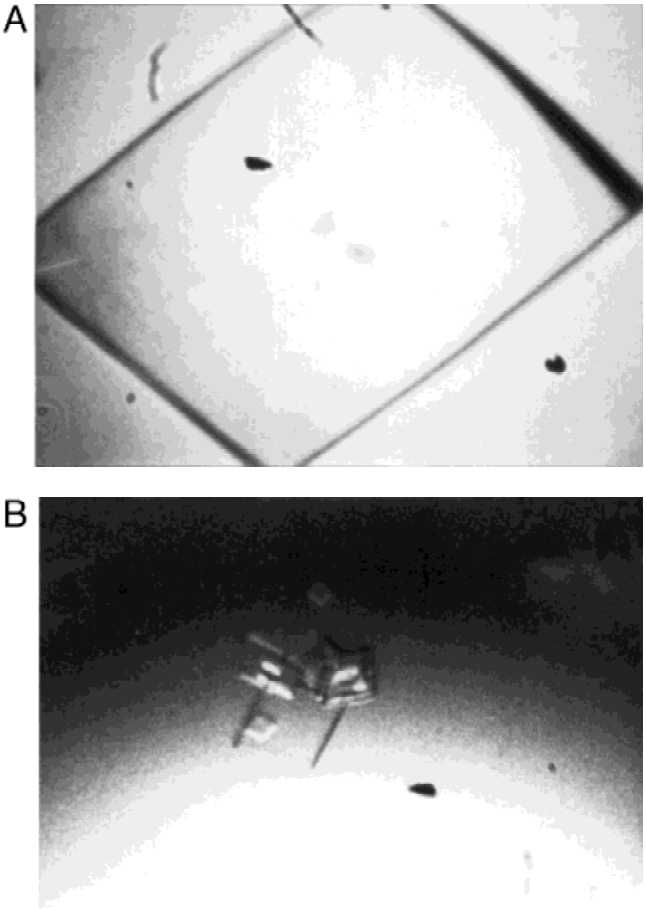

Fig. 1. A: HSA crystal grown in a drop transferred to low supersaturation (24% PEG) at $t = 72$ h. Crystal size: $1 \times 0.7 \times 0.25$ mm. **B:** HSA crystals grown in the high supersaturation (33% PEG) controls. Note: Both pictures were photographed at the same magnification.

Although these crystals were not used for structural studies, this is to our knowledge, the first reported significant enlargement of macromolecular crystals due to dilution of hanging drops.

Dilution experiments performed with lysozyme and a phycobiliprotein, consistently showed better results (higher reproducibility of large crystals) than conventional experiments set up at either the higher or the lower supersaturation conditions.

Discussion

The following discussion focuses on the results obtained for HSA at the 4 °C conditions.

As explained above, the assumption in these experiments is that the protein incubates for some time in the spontaneous nucleation zone before being transferred to metastable conditions. In the conventional vapor diffusion experiments, the drops remained clear (i.e., no nucleation) at final (reservoir) PEG concentrations below 28% (w/v). This means that the transferred drops, which at $t = 0$ had a PEG concentration of 16.5% (half of the 33% original reservoir concentration), had reached before $t = 72$ h, a PEG concentration above 28%; that is, the equilibration process had reached at least 70% completion, since $(28 - 16.5)/(33 - 16.5) \approx 70\%$. Their transfer over a reservoir at 24% (w/v) PEG therefore reversed the osmotic pressure gradient, leading to an increase of drop volume and concomitant reduction of the concentrations.

These observations and assumptions were compared with Mikol et al.'s (1990) results concerning equilibration of PEG solutions in vapor diffusion experiments.

Mikol et al. (1990) propose an empirical model describing the volume change in the hanging drop as

$$\left.\frac{-dV}{dt}\right|_{t=0} = (1 - \delta)\,\frac{V_0}{\tau} \tag{1}$$

$$\tau = \gamma\,\frac{V_0^{\varepsilon}}{p^0} \tag{2}$$

where V_0 is the initial drop volume, p^0 the water vapor pressure, δ the dilution factor between drop and reservoir, and τ a time constant; ε and γ are constants, empirically determined at $\varepsilon = 0.81 \pm 0.06$ and γ (20% PEG in reservoir) $= 149 \pm 28$, irrespective of the PEG mean molecular weight. However, γ is dependent on the reservoir PEG concentration and an inverse proportionality is suggested. Our γ is therefore two-thirds that of Mikol et al. (1990), having therefore a value of about 100. p^0 (at 4 °C) ≈ 6 Torr.

In our setup, $V_0 = 2\ \mu$L and $\delta = 0.5$. The units given above for p^0 and V_0 correspond to the values given for the dimensionless

constants and give a result in mL/h. Substituting (2) in (1) and replacing the above numerical values:

$$\frac{\Delta V}{\Delta t} \approx 0.034\ \mu\text{L/h} \tag{3}$$

assuming a linear volume decrease, which, according to Mikol et al. (1990), is the case until equilibration comes close to completion.

From the above, equilibration is complete after ~29 h, a time that should be shortened somewhat to account for the presence of salt (Luft & DeTitta, 1995).

This result supports our assumption that the drops had stayed for a considerable amount of time at conditions of spontaneous nucleation before being transferred. By that time, invisible crystal nuclei had formed, which later grew at metastable conditions.

This empirical model may provide an indication of dilution times to try in future experiments, with different proteins and initial conditions, by inputting the relevant experimental parameters.

Materials and methods

Purified recombinant HSA, produced in yeast, was supplied by Delta Biotechnology Ltd. (Nottingham, UK), and purified further, as described in Curry et al. (1998).

Poly(ethylene glycol) of mean Molecular Weight 3350 (PEG 3350) was purchased from Sigma (St. Louis, Missouri).

The wells of Linbro-type (VDX, Hampton Research, Laguna Niguel, California) crystallization plates contained 1 mL of the reservoir solutions. The drops were dispensed on silanized glass coverslips (Hampton Research) that were inverted above the initial wells and sealed with Apiezon C oil (D. Bewhay Ltd., Borehamwood, Hertfordshire, UK). In the case of HSA, the initial drops consisted of 1 μL of the 40 mg/mL protein stock mixed with 1 μL of reservoir solution. All the transfer trials were performed in duplicate for each incubation time, and the controls in triplicate for each condition. Trials were set up at 18 and 4 °C. At 18 °C, transfers were performed from wells containing 33% (high supersaturation-labile) to wells containing 24% (low supersaturation-metastable) PEG 3350. At 4 °C, the respective well concentrations were 32 and 28% PEG 3350. All reservoirs had 100 mM phosphate buffer (pH 7.0). Each transfer lasted for 1–2 s.

The drops were observed throughout the experiment and at various later times, up to 3 months later. Up to the time of the last transfer (t = 72 h), all the drops, including the controls at high supersaturation, were still clear.

The crystals were X-rayed at room temperature, using an Enraf-Nonius GX21 rotating anode CuKα source with MAR image plate detector.

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