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Evaporative microdialysis: an effective improvement in an established method of protein crystallization

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Evaporative dialysis is a simple variant of conventional microdialysis in which the reservoir solution is allowed to evaporate slowly. The slow increase in precipitant concentration allows crystals to grow without increasing nucleation. The method is useful for proteins that have a very narrow metastable zone (the range of solution conditions under which crystals grow but nuclei do not form at an appreciable rate). The method is demonstrated with the coat protein of potato virus X.

1. Introduction

Crystallizing macromolecules is essentially a search for conditions under which crystal growth occurs orders of magnitude more rapidly than nucleation. Most methods use diffusion as a means of slowly approaching equilibrium, searching for a level of supersaturation that is just sufficient to nucleate crystals. Methods may be based on vapor diffusion, for example the popular hanging- and sitting-drop techniques, or on liquid diffusion, usually utilizing dialysis in some way, often in commercially available microdialysis buttons (Ducruix & Giegé, 1992).

There are numerous examples of methods for slowing the approach to equilibrium, thus minimizing overshooting the desired level of supersaturation: in vapor diffusion, the reservoir may be covered by a layer of oil (Chayen, 1997); in both liquid and vapor diffusion, diffusion within a more or less structurally complex reservoir may be used to modify the reservoir solution conditions very slowly (Weber & Goodkin, 1970; Thomas *et al.*, 1989; Luft *et al.*, 1994; Carter *et al.*, 1999). Most of these methods are, however, expensive (sometimes prohibitively), labor-intensive or both.

Many years ago (Stubbs, 1972), we grew crystals of hemerythrin by dialyzing 0.5 ml aliquots of hemerythrin against large volumes of potassium phosphate solution, typically at phosphate concentrations of about 2.2 *M*. Allowing the solutions outside the dialysis bags to evaporate slowly routinely produced crystals having dimensions of about 0.3 × 0.4 × 0.8 mm, about 50 times the volume of crystals grown by the more customary procedure of incrementing the phosphate concentration by 0.05 *M* every few days. We observed at that time that the metastable zone (Riès-Kautt & Ducruix, 1992) for hemerythrin in phosphate was extremely small. Those experiments provided the stimulus for the experiments described here.

We demonstrate here that the simple process of allowing the reservoir solution in a microdialysis experiment to evaporate at a controlled rate can greatly increase the size of protein crystals in cases where the metastable zone (the range of conditions under which crystal growth can proceed but the rate of nucleation is vanishingly small) is exceptionally small.

2. Materials and methods

2.1. Reagents

Chicken egg-white lysozyme (L-6876) and all reagents were purchased from Sigma.

Potato virus X coat protein (PVX-CP) was prepared by a procedure modified from the method of Baulcombe *et al.* (1984) for PVX and the LiCl method of Goodman (1975) for the coat protein. The principal modifications were the addition of protease inhibitors and reducing agents at all stages (Ferrell, 1998).

2.2. Crystallization

PVX-CP solutions were approximately 10 mg ml^{-1} in protein, 10 m*M* Tris–HCl buffer pH 8.0, 5 m*M* EDTA, 5 m*M* DTT and 0.2% in a protease-inhibitor cocktail consisting of 0.1 *M* benzamidine, 0.5 *M* ε-amino-*n*-caproic acid and 0.1 mg ml^{-1} leupeptin in water and 1 mg ml^{-1} pepstatin A in methanol. Crystallizing solutions for PVX-CP were mixtures of dipotassium hydrogen phosphate and sodium dihydrogen phosphate pH 6.5, 0.5% in β-mercaptoethanol.

Lysozyme solutions were 20 mg ml^{-1} in protein, 50 m*M* sodium acetate buffer pH 4.5,

0.15 *M* NaCl. Crystallizing solutions for lysozyme were NaCl, 50 m*M* sodium acetate buffer pH 4.5.

Microdialysis buttons (Cambridge Repetition Parts, Cambridge, England) with 50 µl cavities were filled with protein solution and placed in vials containing 10 ml volumes of crystallization solution. The vials, which were approximately 2.5 cm in diameter and 5 cm in height, were either capped or covered with Parafilm. For evaporation experiments, the initial concentration of the crystallizing solution was chosen to be just below the concentration required for crystal growth in conventional microdialysis. Holes were cut in the Parafilm and the vials were left undisturbed at room temperature for about two weeks.

3. Results

3.1. Potato virus X coat protein

PVX-CP crystallized reproducibly by conventional microdialysis using phosphate concentrations between 1.0 and 1.1 *M*, varied in increments of 0.01 *M*. The largest crystals obtained were 50 × 50 × 120 µm. PVX-CP crystals grown by evaporative microdialysis with initial crystallization solutions at 0.95 *M* phosphate concentration and using holes about 1 cm in diameter were typically 135 × 135 × 170 µm, about ten times the volume of those grown by conventional microdialysis (Fig. 1).

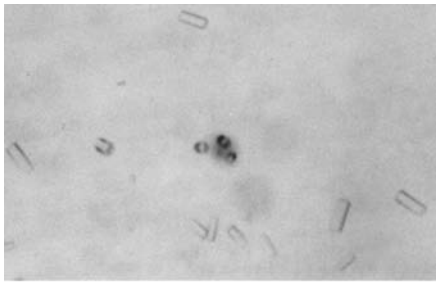
Although we did not find it necessary to slow down the evaporation rate for PVX-CP, we found that the evaporation rate could readily be controlled using various numbers of smaller holes. Rates of evaporation could be predicted by means of a few simple control experiments without buttons, following changes in the weights of the vials or the volumes of the solutions.

3.2. Lysozyme

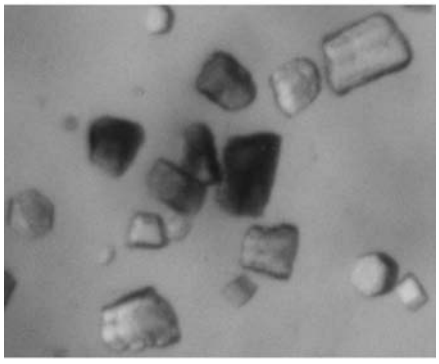
Lysozyme crystals of about 500–1000 µm in all dimensions were easily grown from crystallization solutions at various NaCl concentrations above 0.7 *M*. Evaporative microdialysis sometimes gave slightly larger crystals, but the effect was much less than that observed for PVX-CP.

4. Discussion

The reasons for the effectiveness of evaporative microdialysis may be perceived by looking at a solubility diagram of the type made popular by Riès-Kautt & Ducruix (1992) (Fig. 2). Immersion of the button in the reservoir causes an initial increase in precipitant concentration. While luck or perfect judgement may limit this increase so that the resulting conditions lie just inside the nucleation zone as desired, it is rather more likely that the metastable–nucleation zone boundary will be overshot, resulting in excess nucleation and smaller crystals. Even if the initial conditions are optimal, the subsequent decrease in macromolecular concentration at constant precipitant concentration may take the conditions into the solubility zone so rapidly that crystal growth is severely limited. Of course, with a protein such as lysozyme for which the metastable zone is very wide, neither of these events is likely. However, with proteins such as PVX-CP and hemerythrin, they are very likely. In evaporative microdialysis, the decrease in macromolecular concentration owing to crystallization is accompanied by a slow increase in precipitant concentration, effectively 'riding the solubility curve'.



(*a*)



(*b*)

Figure 1
Crystals of potato virus X coat protein (*a*) grown by conventional microdialysis, (*b*) grown by evaporative microdialysis. (*a*) and (*b*) are on the same scale; crystals in (*b*) are approximately ten times the volume of crystals in (*a*).

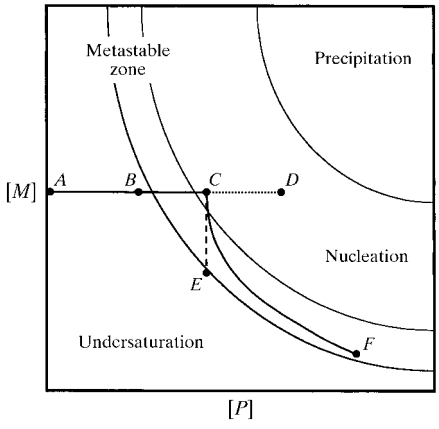


Figure 2
Solubility diagram comparing crystallization by conventional and evaporative microdialysis. [*M*], concentration of macromolecule. [*P*], concentration of precipitant. The solution in the microdialysis button is initially at *A*. In conventional microdialysis, the reservoir is ideally at *C*, just sufficient for nucleation. As the crystals grow, [*M*] decreases until *E* is reached. Crystal growth then ceases. If the metastable zone is very narrow, condition *C* may be difficult to determine; the reservoir is more likely to be at a condition such as *D*, causing rapid nucleation and reduced crystal growth. In evaporative microdialysis, the reservoir should initially be at *B*, but it may be anywhere along *AC* in the undersaturation or metastable zones. As evaporation proceeds, conditions change until nucleation begins at *C*. Simultaneous crystal growth and increase in precipitant concentration cause the conditions to follow the line *CF*. Both the overshoot to *D* and the incomplete crystal growth terminating at *E* are avoided.

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