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Abstract

Two-step crystallization experiments were conducted in low gravity employing a liquid–liquid diffusion method in an effort to eliminate problems associated with protein crystal growth under the supersaturating conditions required for nucleation. Experiments were performed in diffusion cells formed by the sliding of blocks on orbit. *Step gradient diffusion* experiments consisted of first exposing protein solutions in diffusion half-wells for brief periods to *initiating* buffer solutions of high precipitant concentrations to induce nucleation followed by exposure of the same protein solutions to solutions of lower precipitant concentration to promote growth of induced nuclei into crystals. To avoid convective disturbances that occur when solutions of discrepant densities are interfaced at normal gravity, crystallization of hen egg-white lysozyme and rabbit skeletal muscle aldolase by *step gradient diffusion* was investigated in low gravity on four NASA space shuttle flights. In general, the largest crystals of both proteins formed at the highest initiating precipitant concentration used, which is consistent with nuclei formation upon brief exposure to high precipitant concentration, and that these nuclei are competent for sustained growth at lower precipitant concentration. The two-step approach dissociates nucleation events from crystal growth allowing parameters affecting nucleation kinetics such as time, precipitant concentration and temperature of nucleation to be varied separately from conditions used for post-nucleation growth.

1. Introduction

Numerous experiments have shown that the critical supersaturations for promoting the aggregation of soluble protein into crystalline arrays exceeds that required for subsequent growth of the crystalline nuclei into macroscopic crystals [1–7]. Crystallization techniques have capitalized on this observation in order to optimize protein crystal growth [8]. Typically, methods have sought to identify supersaturating conditions that promote formation and growth of crystalline nuclei or seeds. These nuclei or seeds are subsequently added to solutions having concentrations that are typically lower than concentrations that induce formation of initial crystalline seeds [9–11]. Strategies of crystal growth optimization can thus be considered as separately optimizing the concentrations for initial seed or nuclei production and the concentrations for growth of these crystalline nuclei or seeds into large crystals. We have developed a

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technique wherein supersaturating concentrations that are conducive to nucleation and the concentrations that promote crystal growth can be controlled separately within a single procedure.

The approach chosen for independent simultaneous optimization of the supersaturation for nucleation and for growth makes use of temporal separation between the nucleation process and the growth of crystals from nuclei. The method involves diffusion of protein and precipitant across a liquid–liquid interface and consists of bringing into contact a slightly undersaturated protein solution containing low concentrations of precipitant (or none at all), with an *initiating precipitant solution* of sufficient concentration to induce nucleation. Brief exposure to the initiating precipitant solution is followed by contact of the protein solution with a subsequent solution of lower precipitant concentration that ensures growth of crystalline nuclei without further nucleation. Differences between concentrations inducing formation of nuclei and those ensuring preferential growth of crystalline nuclei can in some instances be relatively small [12].

The technique, as described here, requires bringing solutions into contact with temporal and spatial precision using free-boundary diffusion cells. A potential problem encountered in diffusion-cell methods is buoyancy-driven convective mixing across the freshly formed liquid–liquid interface between two solutions of differing density as revealed in classical studies [13,14]. This mixing will increase the precipitant concentration in the protein solution upon contact with the initiating precipitant and will compromise the final precipitant concentration required for preferential growth of crystalline nuclei in the subsequent growth step. In a low-gravity environment, natural convective mixing in diffusion cells is suppressed [15,16], and steep concentration gradients can be stably maintained for comparatively long periods of time. Equally significant, sedimentation of crystal nuclei out of the region of supersaturation is prevented in low gravity. Therefore, this initial investigation was carried out using a system of multiple diffusion cells [17,18] in low gravity aboard US space shuttle orbiters [19]. This approach guarantees diffusive transport of solutes between contacting solutions and allows any combination of solutions to be used, which was not the case in previous studies of the liquid–liquid diffusion method of protein crystal growth [20,21].

The extent of interfacial mixing *in low gravity* due to block sliding motion has been established on the basis of mixing-cup experiments on sounding rockets [17] and video records on KC-135 flights [22]. Approximately 0.4% of the volume of one well is found in the other well, even at Reynolds numbers below 10, and this transfer occurs during separation of the wells and not during contacting of the wells [20]. Diffusion calculations based on unsteady diffusion (Fick's second law) at a discontinuous boundary can therefore be used to describe interfacial transport at the nucleation step and growth phase.

Diffusive transport of protein is approximately 10 times slower than that of low molecular weight precipitants. Nuclei will be preferentially formed in the protein solution upon its contact with the initiating precipitant solution, and thus they will be transferred with the protein solution to the subsequent precipitant solution. This ensures growth of the induced nuclei without further nucleation. The principle of this technique, referred to as *step gradient diffusion*, is illustrated in Fig. 1 with numerical data corresponding to an experimental design (below). In this study, we investigated concentration and temporal parameters required for growth of large protein crystals by this technique.

2. Experimental procedure

2.1. Crystallization hardware

The *step gradient diffusion* experiments were carried out in low gravity using the automated Materials Dispersion Apparatus ("MDA Minilab") developed by Instrumentation Technology Associates (ITA) [17–19]. A schematic description of the operation of the MDA is given in Fig. 2. It operates in the following manner: two blocks of inert thermoset plastic with a matching number of sample test wells are held together under pressure with a sealing mechanism in an aerospace aluminum housing. The thermoset plastic blocks and non-leaking silicone sealant were obtained from ITA (Exton, PA, USA). The test wells (up to about 100) are misaligned at launch, thus separating the fluids to be contacted.

After low gravity has been achieved, the blocks are moved into alignment by means of a motor-cam mechanism, allowing the fluids to contact. A typical cavity in the "top" block is filled with up to 135 μl of fluid and is slid into position so that it is continuous with a corresponding cavity containing 120 μl of another fluid in the opposite block. Thus opposing wells have three spatial relationships to one another: (a) launch, (b) initiating, and (c) growth positions, as shown in Fig. 2. The apparatus as a whole can function either in the laboratory or in space flight, and one of several functional equivalents was used for 1-g control experiments.

In orbital low-gravity experiments the minilab units were held at 1 atm ambient pressure and nominal 20°C temperature (temperatures actually achieved after launch were recorded). Variable timing was achieved by using four different minilabs with different temporal programs. Temperature control was achieved by installing the minilabs in a Commercial Refrigerator Incubator Module (CR/IM) [19]. In the four flights of the space shuttle on which experiments were performed, samples were stored in the CR/IM up to 60 h (typically 10 h) before launch, wells were opened less than 22 h into the low-gravity phase of flight. The period of solution contact included re-entry and de-integration on the later flights (STS-43, 52 and 56), but not the first flight (STS-37).

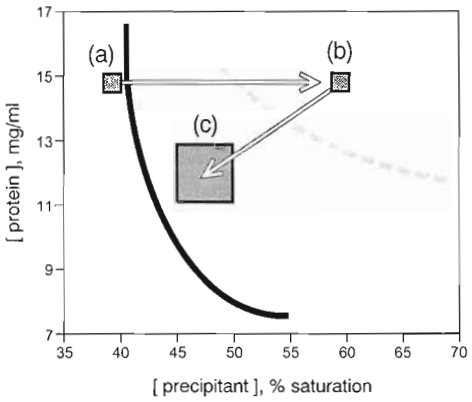

Fig. 1. The *step gradient diffusion* concept, as illustrated using a two-component phase (solubility) diagram. Launch condition (a) consists of a combined solution of 15 mg/ml rabbit muscle aldolase and 40% saturated ammonium sulfate – below the solubility line (solid curve). By exposing this solution to 60% saturating ammonium sulfate a rapid transition to the supersaturation region (beyond the dashed curve) is achieved (composition (b)) for enough time for nucleation to occur. Subsequent shifting to a contacting well with a lower salt concentration, a rapid transition into the crystal-growth region (position (c)) of the phase diagram occurs, and crystal growth continues at a suitable concentration, in this case 11–13 mg/ml protein and 45%–48% saturated salt, in the quiescent low-gravity environment. The downward shift in protein concentration is due to diffusion (see Fig. 3) and consumption of protein into growing crystals [3,7].

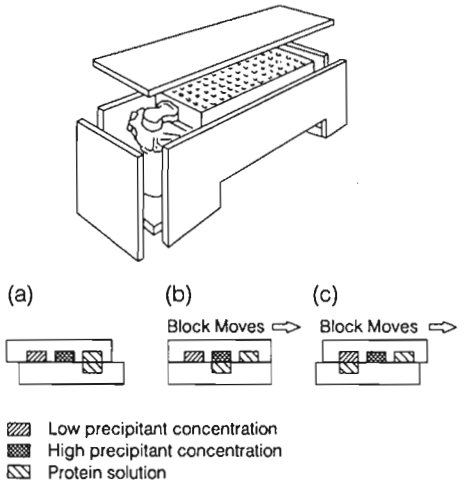

Fig. 2. Use of the Materials Dispersion Apparatus (MDA) to accomplish the step-diffusion method. Protein and low-salt solution in the bottom well is launched in contact with a small quantity of the same solution in the contacting upper well – position (a). After launch the blocks slide, and contact is made with an upper well containing a supersaturating salt concentration – position (b). Nucleation occurs at the interface between the two half-wells, and after a suitable time, such as 10 min, the blocks are slid to position (c) in response to a second electronic command, and the nuclei in the protein solution are in contact with an intermediate salt concentration that permits crystal growth to continue for the remainder of the flight. Top diagram is an exploded view of a self-contained MDA Minilab showing cam mechanism and sliding top block inside the aluminum housing.

Test-case calculations representing the evolution of the protein and salt concentration gradient across the liquid–liquid interface as a function of time and distance from the interface following superposition of two MDA half wells are shown in Fig. 3. The calculations assume unsteady diffusion (Fick's second law), i.e. existence only of diffusive transport (absence of convection), across an initial discontinuous boundary [23,24]. The initial boundary conditions used in the calculations corresponded to solute concentration, C_s, constant in one half-well and C_s

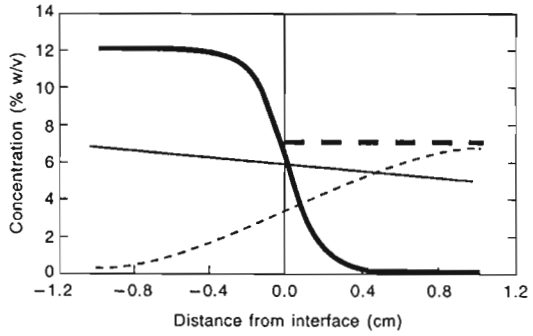

Fig. 3. Lysozyme and salt concentration profiles calculated from unsteady diffusion equations 10 min after contact between a 7% protein solution (thick dashed line) and 12% NaCl solution (thick line) and after 72 h of contact between the same protein solution (thin dashed line) and NaCl solution (thin line). The vertical line at the center represents the starting interface formed between two half-wells with lysozyme solution on the right and NaCl solution on the left.

$= 0$ beyond the interface in the contacting half-well. The spatial and temporal distribution of solute concentration, C, is then given by

$$C_x - C = C_s \operatorname{erf}\{x/2(Dt)^{1/2}\},$$

where x is the distance from the interface, t is the time elapsed after contact of the diffusion half-wells, D is the diffusivity of the solute and erf is the error function. Diffusivities of $D = 6 \times 10^{-7}$ cm^2/s and $D = 2 \times 10^{-5}$ cm^2/s were used for lysozyme and NaCl respectively in the calculations. It is noteworthy that even over the duration of a typical 7–9 days orbital mission, a protein concentration gradient will still be present in the crystallization well (result not shown), while the precipitant has diffused, as shown in Fig. 3, to nearly equilibrium by the third day.

2.2. Protein and precipitant solutions

Hen egg-white lysozyme, E.C. 3.2.1.17, which digests bacterial cell walls, is one of the most extensively studied molecules by crystallographers and crystal growers. Lysozyme was purchased from Sigma Chemical Company, St. Louis, MO, dissolved without further purification in 0.1M NaAc buffer, pH 4.0 at 20–80 mg/ml and exposed to graded concentrations of NaCl in the same buffer at 12% to 25% (w/v) for 10 min in low gravity. The same lysozyme solution was subsequently exposed to 4%–7% (w/v) solutions of NaCl for 5–8 days. Glycolytic enzyme fructose 1,6-bisphosphate aldolase was purified from rabbit muscle using published procedures [25] and dissolved at 15 mg/ml in 40% saturated ammonium sulfate made up in 100mM Tris HCl pH 7.4 buffer and exposed to concentrations of 50% to 60% saturating ammonium sulfate for 10 min. The aldolase solution was subsequently exposed to 45% or 48% saturating ammonium sulfate for 5–8 days. The concentrations of ammonium sulfate were chosen on the basis of their ability to preferentially dissolve amorphous precipitates but not crystalline aggregates or protein crystals (see Fig. 1).

Preliminary tests using lysozyme on the commercial sounding rocket CONSORT 5 suggested that exposing the protein solution for 4–5 min to the initiating precipitant concentrations was sufficient to induce nucleation, as about a dozen crystals, 50–200 μm in length, were produced. Preliminary tests using aldolase on space shuttle flight STS-37 suggested that exposing the protein solution for 15 s to the initiating precipitant concentrations was insufficient to induce nucleation. Exposure times in excess of 8 min under identical conditions, however, resulted in the subsequent growth of large aldolase crystals. For subsequent shuttle flights, exposure time was therefore set to 10 min.

Protein concentrations were quantitated by measuring absorption at 280 nm and using extinction coefficients of 9.1 and 26.5 for 1% (w/v) solutions of rabbit aldolase and lysozyme, respectively. Protein content was also measured using the Pierce BCA protein assay. Low concentrations of rabbit muscle aldolase were quantitated by enzymatic activity assayed in presence of 1mM fructose 1,6-bisphosphate [25]. A specific activity of 9.8 U/mg [25] was used to calculate the amount of aldolase present. One unit (U) of enzymic activity corresponds to 1 μmol of substrate cleaved per minute at 22°C.

3. Results

Crystallization experiments using hen egg lysozyme and rabbit skeletal muscle aldolase as

benchmark proteins were conducted to evaluate the *step gradient diffusion* technique. The growth of aldolase crystals was evaluated as a function of initiating precipitant concentration and final well precipitant concentrations. Results obtained on the STS-52 and STS-56 orbital missions are shown in Table 1. Aldolase crystals grown in 6 days using the step gradient diffusion method were typically of about 1/2 the dimensions of crystals grown in 3–6 weeks by batch crystallization. Crystals initiated by the step gradient diffusion method thus appeared to display better growth characteristics, having grown to 1/2 the size in less than 1/3 the time.

A similar experimental matrix design was used to evaluate lysozyme crystallization, and the initial protein concentration was also varied. Results obtained on STS-43 and STS-52 orbital missions are shown in Table 2.

In most cases the largest protein crystals were grown following exposure to the highest initiating precipitant concentration. A slight exception was observed (Table 1, column 2) in the case of aldolase solutions in which the initial precipitant concentration in the protein solution corresponded to the threshold concentration at which aldolase crystals do not dissolve, namely 42% of saturation at 20°C. Conceivably, in this case, the final precipitant concentration after exposure to the initiating precipitant solution was higher than necessary and yielded nuclei in excessive numbers, suggesting that crystal growth is sensitive to the exact final precipitant concentration. An alternative explanation is that initial conditions may have induced some pre-flight nucleation. A similar shift is noted in Table 2 for the lysozyme data at high protein concentration (~ 7%).

The test-case calculations shown in Fig. 3 indicate negligible transfer of lysozyme into the initiating precipitant half-well. Recovery of the protein content in the initiating precipitant half-well revealed a protein residuum corresponding to less than 3% mass transfer of lysozyme across the liquid–liquid interface during the 10 min contact. The residuum was considerably less (< 1%) in the case of aldolase, presumably due to the lower diffusion of the aldolase tetramer with respect to that of lysozyme. This small amount of initial transfer is not inconsistent with previous observations [17] and suggests that unavoidable mixing by shear flow during block motion is not a significant factor in low gravity under our experimental conditions.

Table 1
Crystallization of rabbit skeletal muscle aldosale[a] on NASA shuttle flights STS-52 and STS-56 by step gradient diffusion

Precipitant concentration of initiating half-well (% saturating $(NH_4)_2SO_4$)	Precipitant concentrations of half-wells corresponding respectively to initial protein solution and to final growth step		
	40 → 48	42 → 46	40 → 45
50	–	< 0.2 mm[b]	–
55	< 0.2 mm[b]	< 0.2 mm[b]	–
60	0.15 × 0.4 × 0.8 mm[c]	–	< 0.2 mm[b]

[a]Rabbit skeletal muscle aldolase, 15 g/ml, was made up in 0.1M Tris HCl pH 7.4, 0.1mM EDTA. Batch crystallization yielded aldolase crystals after 3–6 weeks having typical dimensions of 0.25 × 1 × 2 mm.
[b]Dimension of largest crystallites obtained.
[c]Average size based on sample of several crystals.

Table 2
Maximum crystal size following crystallization of hen egg-white lysozyme on NASA shuttle flights STS-43 and 52 using the step gradient diffusion technique with various concentrations (% w/v) of initiating precipitant, growth precipitant and protein

Lysozyme	Initiating [NaCl]	Growth [NaCl]	Maximum crystal size (μm)
4	20	4	100[a]
5	20	5	750
5	20	4	60
6	12	6	800
6	20	6	850
7	12	7	800
7	20	7	550

[a]In the first experiment listed above, 3% NaCl was present in original lysozyme solutions; in all other cases there was no NaCl in the protein well.

4. Conclusions

Protein crystallization using a *step gradient diffusion* procedure has been successfully tested in low gravity. The design of the experiment hardware has allowed the separate optimization of nucleation and crystal growth and the evaluation of a large number of initial crystallization conditions. Optimum concen-

trations for inducing nucleation appear to correspond to high precipitant concentrations which includes the concentration range at which the protein normally precipitates. Optimization of subsequent growth of crystalline nuclei requires lower concentrations typically corresponding to those required to dissolve an amorphous protein precipitate but high enough to prevent crystal dissolution. A matrix of initial conditions derived from these considerations and using this technique should guarantee success in the growth of protein crystals by the liquid–liquid diffusion technique in future low-gravity experiments.

Low gravity is crucial for the success of the method; however, low gravity has also been shown to afford secondary benefits in protein crystal growth such as improved crystallinity resulting in higher data resolution as well as novel crystal habits [26–29].

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