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INVITED PAPER, SPECIAL SECTION IN HONOR OF MAX PERUTZ

Control of nucleation of protein crystals

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Abstract

Control of nucleation may be needed to obtain a reliable supply of large protein crystals, when standard techniques give many small or twinned crystals. Heterogeneous nucleation may be controlled by the use of fine filters, with the elimination of airborne contaminants by working under paraffin oil. The area of contact with the supporting vessel also has an important effect. A heterogeneous nucleant for lysozyme (identified earlier) has been shown to be effective for carboxypeptidase G_2. Control of homogeneous nucleation (previously demonstrated by dilutions of a nucleating sample after various times of incubation) may also be achieved by incubating a sample at 1 temperature, where nucleation can occur, and changing the temperature to conditions where there is growth but no nucleation.

Keywords: carboxypeptidase G_2; crystal growth; lysozyme; nucleation; protein crystallization; thaumatin

In 1954, Max Perutz published his discovery that the attachment of a heavy metal to a lattice site in a protein crystal could give useful information about the phases of its X-ray diffraction reflections (Green et al., 1954). Following directly from this discovery, we have now reached an era in which the molecular structure of most proteins for which suitable crystals can be obtained is readily determined. In 1954, many scientists considered any attempt to analyze protein structure by X-ray crystallography a quixotic undertaking, doomed to inevitable failure. Some imagined proteins themselves to be structureless, jelly-like globules. But for anyone who understood their message, the diffraction patterns of pepsin, produced 20 years earlier by Bernal and Crowfoot (1934), proved they were highly organized molecules forming tightly ordered structures.

Later in 1954, Max Perutz took his newest research student to the library and opened a profusely illustrated tome (Reichert & Brown, 1909). Looking through it together at the images of a large variety of hemoglobin crystals, they decided on a first task in protein crystallography for the student: he was to crystallize the hemoglobins of pig, dog, and rabbit, so defining the student's first research paper (Blow, 1958).

Many are surprised to learn that Reichert and Brown studied protein crystallography in the era before von Laue's discovery of X-ray diffraction. It adds more color to the famous story of Willstätter's refusal in 1928 to believe that Sumner had crystallized an enzyme (see, for example, Costa [1989]). Some hemoglobins crystallize very easily. The earliest account of protein crystallization is by Hünefeld (1840), who illustrates the tabular crystals that he obtained by adding a variety of salts to porcine and human blood and allowing the mixture to dry slowly. Hemoglobin had not yet been identified or named.

We still do not understand why many proteins stubbornly refuse to produce suitable crystals, in particular, crystals of suitable size. An insight is given by the observation that proteins that oligomerize into aggregates of differing sizes fail to crystallize (Zulauf & D'Arcy, 1992; Ferré-d'Amaré & Burley, 1994). This does not explain why hemoglobins, lysozymes, and xylose isomerases from a wide range of different species all crystallize easily, whereas other single-domain, monomeric, nonglycosylated, stable, soluble proteins have never been crystallized from any species. It is counterintuitive that myoglobins are more difficult to crystallize than hemoglobins. Because of its overriding importance for the structure analysis of moderate or large proteins, the science of protein crystallization has come back into its own, as first recognized by A. McPherson (1982), and now by many others.

Background

Homogeneous nucleation

Crystals may form from a solution if the system can achieve a lower energy by growing crystals. Because crystallization is normally carried out at constant temperature and pressure, the relevant energy is the Gibbs free energy. Under any conditions, the *solubility* of a solid form is the concentration of solute in equilibrium with the solid. Protein crystals require hydration for

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stability, so that, unlike most other crystalline materials, protein crystals at normal temperatures must be in contact with a solution, which at equilibrium must therefore be saturated. Most proteins can exist in an amorphous state (in which the intermolecular interactions do not form a regular pattern) and, under conditions where the amorphous material is less soluble than any crystalline form, no crystals can be stable. There is often more than 1 crystalline form of a protein, and these may have different solubilities (Fig. 1).

Crystals grow as molecules adhere to their faces, forming a regular pattern of interactions. The growth rate depends on the relative "on" and "off" rates in this process, which depend in turn on the excess of concentration over the solubility (at which, by definition, "on" and "off" have equal rates). If the growth rate is too high, or if the intermolecular interactions are not specific, an incorrectly oriented molecule on the crystal surface may be surrounded by other molecules before it dissociates from the crystal, leading to imperfect crystal growth, frequently with phase boundaries and multiple interpenetrating crystallites.

The early stages of crystal formation are energetically unfavorable because, as the molecule associates only with 1 or 2 others, it makes a smaller number of favorable interactions. Under conditions where the growth of large crystals will be slow enough to give adequate crystal perfection, the association of the first few molecules is energetically unfavorable, and these small aggregates will rapidly dissociate again. The formation of an aggregate large enough to be stable constitutes an energy barrier to crystallization that may be overcome by random fluctuations. In practice, this aggregate is likely to require 10–100 molecules. This nucleation process has been thoroughly analyzed by Feher and Kam (1985).

At low levels of oversaturation, the formation of stable nuclei will occur at a slow rate. If this rate is less than 10^{-6} per mL per s, no crystals will be observed in a sample of practical size in a matter of weeks, and it may be said that crystals do not grow spontaneously. In this range of oversaturation, the solution is called "metastable" because small crystals will grow larger, but no nuclei form (Mikol & Giegé, 1992). As oversaturation increases, nucleation becomes rapid. The other extreme occurs when the nucleation rate exceeds the rate of diffusion to the nucleation site (rates in excess of about 10^{12} per mL per s). Under these conditions, the protein will precipitate. These requirements define a "nucleation zone" on a multidimensional phase diagram (Ataka, 1993) (Fig. 1).

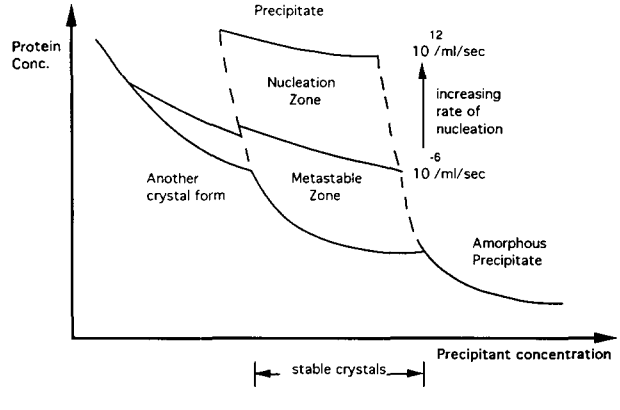

Fig. 1. Schematic solubility diagram for a protein in the presence of a precipitant. The solubility curve represents the concentrations at which there is a true equilibrium between crystals and solution. Above it lies the "metastable zone" (Mikol & Giegé, 1992) within which no nuclei can form at a measurable rate. Within the nucleation zone, nucleation rate increases as protein concentration increases. The possible existence of precipitant concentrations in which another crystal form is stable, or in which only amorphous precipitates can be obtained, is indicated.

Many workers have used light-scattering techniques to study nucleation (see Feher & Kam, 1985; Malkin et al., 1993), but this technique can only detect nuclei when thousands are present. In our experiments, nucleation has been monitored by counting the numbers of crystals after incubation under growth conditions.

Traditional methods of vapor diffusion or microdialysis to obtain protein crystals gradually increase protein and/or precipitant concentration until a significant amount of nucleation occurs. Crystal growth then reduces the protein concentration, taking the solution out of the nucleation zone before too many nuclei form. The dynamic nature of diffusion or dialysis makes the conditions less easy to control or reproduce. In the experiments to be described, a microbatch method is used in which a droplet of solution is maintained under oil (Chayen et al., 1990, 1992), providing conditions that are much more static.

Heterogeneous nucleation

An alternative type of nucleation event is initiated by some other kind of solid material in the crystallization medium, on which the growing crystal forms. This may happen on the wall of the containing vessel, on a crystalline surface (McPherson & Schlichta, 1988a, 1988b), on a foreign particle (Malkin et al., 1993), or on a biological contaminant (Chayen et al., 1993). This type of nucleation can happen at oversaturations, where the homogeneous nucleation rate is negligible. It is fairly common to find that cleaner technique fails to give crystals under the same conditions that first gave them.

The microbatch method has a great advantage here because, after filtration, the solutions are protected by the oil from biological and inorganic contaminants present in the air. Modern filtration methods remove extremely small particles from small volumes of solution with very little loss and are now more convenient than centrifugation techniques.

Results

Control of heterogeneous protein crystal nucleation

The multiplication of a nucleant susceptible to fungicides and capable of passing through a standard 0.22-μm filter, which can control crystal numbers in lysozyme solutions, has already been described (Chayen et al., 1993). Though it has not yet been characterized, the effect of the same nucleant on the growth of carboxypeptidase G_2 (CG_2) crystals has been tested by adding a small amount of the nucleant solution into crystallization samples of CG_2 that had been filtered through a 300-kDa filter (Fig. 2), showing clearly that it affects crystal nucleation.

To define further the particle size of the nucleant, the number and size of crystals obtained from an aged solution of lysozyme filtered through filters of different pore sizes were observed. An unfiltered solution typically yielded thousands of tiny crystals. Filtration through a 0.22-μm filter resulted in the growth of 50–70 crystals (150 μm in their largest dimension). Using a

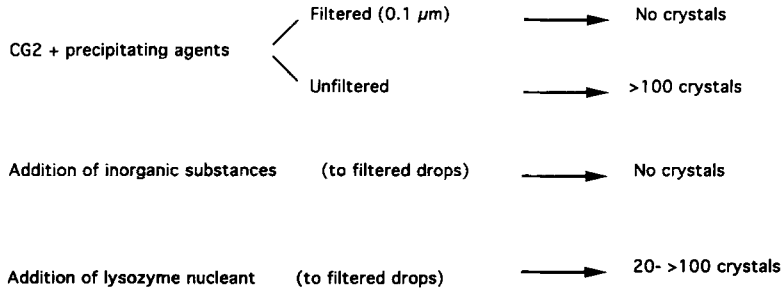

Fig. 2. Summary of experiments on heterogeneous nucleation of CG_2.

0.1-μm filter, 1–14 crystals (dimension 600 μm) were obtained, similar to the number of crystals grown from a fresh solution. No crystals were obtained from the solution filtered through 0.02-μm and 300-kDa filters. These results suggest a particle size between 0.1 μm and 0.02 μm. Because fungal cells and spores are larger than 0.1 μm, it appears that the nucleant is not the fungus itself but may be a fungal product.

Attempts to control homogeneous protein crystal nucleation indicate that a heterogeneous element also exists

Experiments have recently been described in which a solution of CG_2 was incubated under nucleation conditions for various periods of time, and was then diluted with buffer to leave it oversaturated but incapable of further nucleation (Saridakis et al., 1994). This method allowed useful control of the number of crystals obtained in each sample, with a corresponding control of crystal size, but a proportion of experiments gave no crystals at all, even after relatively long incubation under nucleation conditions, suggesting a further uncontrolled factor in the nucleation experiments. It has now been shown that some control can be achieved by filtration.

Depending on the concentrations of polyethylene glycol (PEG) and CG_2 in the trials, conditions were found where no crystals were obtained if the crystallization samples were filtered through 0.1-μm or 300-kDa filters immediately after mixing of the crystallization solution. When unfiltered, the same solutions gave rise to more than 100 crystals, whereas a 0.22-μm filter gave an intermediate result.

When the concentration of either PEG or CG_2 was raised, crystals would grow after filtration, but these were fewer and larger in size compared with unfiltered samples at the same conditions.

The nucleation observed in the original experiments (Saridakis et al., 1994) evidently depended on the presence of a filtrable nucleant that was often present but occasionally absent. There cannot be 1 nucleant particle for each crystal because, after long incubation, there are either dozens of crystals or none. More likely, the same nucleant particle is capable of initiating a number of crystals, depending on the time of incubation.

It was also observed that the number of CG_2 crystals appeared to depend on the area of contact between the crystallization droplet and the containing vessel. Identical samples were tested and the area of contact was varied by changing the dispensing procedure. In normal practice, the aqueous crystallization mixture is dispensed into an oil-filled well and forms a rounded droplet resting on the bottom ("after oil"). In the changed procedure, the crystallization mixture was dispensed directly onto a clean surface and then covered by a layer of oil, forming a flattened droplet with a larger area of contact ("before oil") (Fig. 3).

Table 1 shows the results of CG_2 crystallization trials (2-μL drops) comparing samples that were set up on a variety of different contact surfaces, "before" and "after oil." On all surfaces except perhaps siliconized glass, a larger number of crystals was observed in the flattened drops set "before oil" compared with the rounded drops with little surface contact set up "after oil" (Fig. 3). The greatest increase of nucleation appeared to be in the Terazaki plates. The results from siliconized glass may reflect that the aqueous solution does not spread on this surface.

Table 1. *Effects of surface contact on growth of carboxypeptidase G_2 crystals*[a]

Terazaki plates: polystyrene		Linbro wells: polystyrene		Microtiter plates: polyvinyl chloride		Coverslips: glass		Coverslips: siliconized glass		Parafilm: hydrocarbon	
Before oil	After oil	Before oil	After oil	Before oil	After oil	Before oil	After oil	Before oil	After oil	Before oil	After oil
1,000s	80–100	60–400	16–20	12–50	0–12						
100s	20–40			60–80	25–60						
1,000s	100s	ca. 100	6–25							40–50	16–20
		130	70			130	35	45	50		

[a] Numbers of crystals of CG_2 obtained on various surfaces, using the "before oil" (large contact area) and "after oil" (small contact area) techniques described in the text. Each row represents a separate experiment, in which identical samples were used for trials in triplicate or more.

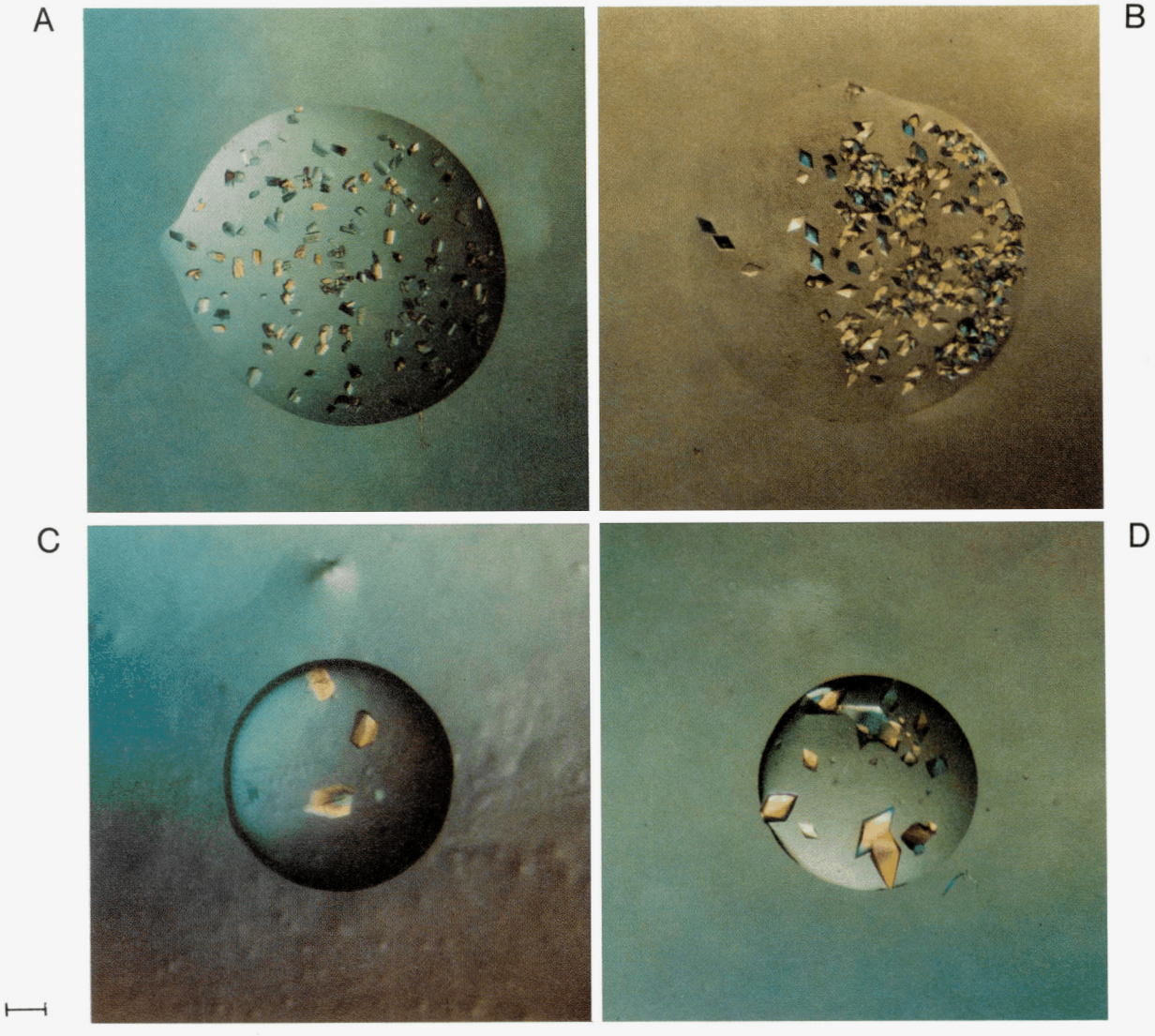

Fig. 3. Effect of contact with containing vessel on the crystallization of CG_2 and thaumatin under oil. **A:** CG_2 crystals grown "after oil." **B:** CG_2 crystals grown from an identical sample delivered "before oil." **C,D:** "After" and "before oil" crystallizations of thaumatin. Scale bar = 200 μm.

Identical results were obtained using water-saturated oil, confirming that the smaller number of crystals obtained after oil was due to contact with the surface and not due to any evaporation. The experiments were repeated up to 10 times, to ensure that the variations were not random fluctuations. Numbers of crystals varied, but all experiments showed the same trends.

The surface contact experiments in Linbro plates were conducted with a variety of precipitating agents and proteins. In the case of glucose isomerase, a difference between the numbers of crystals before and after oil was observed after several hours. However, within 24 h, the numbers of crystals were the same in both preparations, indicating that, in this case, a time-dependent bulk nucleation dominates the result.

Lysozyme showed no difference in the number of crystals before and after oil. This was not surprising because the control of nucleation of lysozyme is relatively easy. Thaumatin showed a difference before and after oil but not as reproducibly as CG_2 (Fig. 3). Indications of the same phenomenon have also been obtained with lysyl-tRNA synthetase (lysU) and photosystem I (data not shown).

Control of crystal nucleation by temperature change

Many proteins are more soluble at higher temperatures, though this is not an inflexible rule. The variation of solubility with temperature provides a means of incubation under nucleation conditions, followed by warming to metastable conditions where existing nuclei will grow, but no new nuclei will form. This method is attractive because the change of conditions is achieved without adding anything to the drop; its main difficulty is that the available change in solubility is often small, so that only small quantities of crystalline material are formed. The solubility of lysozyme has been studied in detail as a function of NaCl concentration, pH, and temperature (Howard et al., 1988; Cacioppo & Pusey, 1991).

The approach described here has also been suggested by Rosenberger et al. (1993), who used a light-scattering method to monitor the onset of crystal nucleation but could only observe very large numbers of nuclei by this technique.

Table 2 shows numbers of crystals obtained in duplicate experiments on lysozyme crystallization, under conditions defined in the Materials and methods section, with nucleation at 10 °C followed by incubation at 18 °C. The results suggest nucleation rates at 10 °C increasing from 0.09 mL^{-1} s^{-1} at 12 mg/mL to 0.9 mL^{-1} s^{-1} at 30 mg/mL. In a control experiment, it was shown that incubation of solutions up to 30 mg/mL under these conditions at 18 °C gave no crystals after 72 h (nucleation rate less than 8×10^{-4} mL^{-1} s^{-1}). The nucleation rates thus vary by a factor of at least 1,000 over an 8° temperature change.

Conclusions

There has always been an element of randomness in the results of crystallization trials, and there have been many examples of crystals that could not be reproduced. A number of technical improvements offer more reproducible results.

1) Methods of protein purification are now very reliable, especially when the starting material comes from recombinant systems. There can, however, be difficulties with highly labile sites for proteolysis.

2) Use of batch, rather than diffusion, methods eliminates dynamic changes of conditions, which are difficult to control. There can be dynamic difficulties during the mixing of batch samples ("shock nucleation"), but by dispensing the mixture simultaneously through channels whose delivery points are within 100 μm, as in the Impax microtip, this problem appears to be eliminated.

3) Dispensing from an enclosed capillary directly under the surface of an oil essentially eliminates airborne contamination of the sample.

In this paper other methods to improve reproducibility are investigated:

4) Filtration through fine filters removes small particles on the order of 100 nm in diameter, with a significant change in the numbers of crystals formed. There seems to be no noticeable contamination from these filters. There is little evidence of any further reduction when 300-kDa filters are used (pores estimated as 20 nm or less).

Table 2. *Nucleation of lysozyme crystallization at 10 °C followed by incubation at 18 °C*

Time of nucleation (h)	Numbers of crystals in drops[a]			
	Lysozyme 12 mg/mL	Lysozyme 15 mg/mL	Lysozyme 18 mg/mL	Lysozyme 30 mg/mL
0.25				1–7 (mean 4)[b]
1	None	4, 6	8, 5	
2	7, 5	8, 8	14, 15	
3	4, 4	8, 7	20, 20	
4	6, 4	14, 16	40, 30	
6	10, 12	21, 14	50, 20	

[a] Results for duplicate drops in each experiment.
[b] Result of 13 trials.

5) The area of contact between the solution and any solid support should be minimized, to reduce surface nucleation. Glass causes less nucleation than polystyrene.

6) Temperature change provides, in principle, a technique to arrest nucleation after a sufficient number of nuclei have formed, by transferring the preparation from a "nucleation" part of the phase diagram to a "metastable" part. Though a small change of solubility is achievable in practice, nucleation rates may be altered by a factor of more than 1,000. The advantage over the dilution technique (Saridakis et al., 1994) is that there is no need to change the composition of the crystallization sample, with the attendant mixing problems.

7) A preparation of nucleant particles effective for lysozyme, which has not yet been adequately characterized (Chayen et al., 1993), has been shown to be effective in nucleating another protein (CG_2). Use of a heterogeneous nucleant might provide a direct control of the number of crystals grown.

The main practical reason for control of nucleation is to control the number of crystals grown in a sample. If this number is kept small, there is a prospect of increasing crystal size reliably. It may also be possible to control the appearance of clusters of needles or plates around a central nucleation point, an effect that often makes the growth of large single crystals very unreliable, if not impossible.

Controlled protein crystallization is becoming possible but requires precision, cleanliness, and excellent purity of the protein sample. With the more sophisticated tools now available to search for conditions where some crystalline material may be obtained (Carter, 1990; Shaw-Stewart & Khimasia, 1994), the labor required to obtain large crystals of a new protein is being considerably reduced.

Materials and methods

Proteins

Hen egg white lysozyme (L-6876 and L-2879) and thaumatin (T-7638) were obtained in the form of freeze-dried powders from Sigma. Lysozyme was desalted on a Sephadex G25M PD-10 column (Pharmacia). CG_2 in 0.05 M Tris-HCl, 0.1 M NaCl, and 0.1 mM $ZnCl_2$, pH 7.4, was supplied by Dr. R. Sherwood of the Public Health Laboratory Service, Porton Down, UK. *Arthrobacter* glucose isomerase (freeze-dried powder) was supplied by the Institute for Biochemistry and Protein Research, Budapest, Hungary.

Materials

Sodium chloride, sodium hydroxide, citric acid, potassium sodium tartrate, PEG 4000, and paraffin oil were obtained from BDH Chemicals Ltd., UK. Magnesium chloride, ammonium sulfate, cacodylic acid, zinc acetate, and PIPES were supplied by Sigma.

Crystallization

Crystallization experiments were set up as microbatch trials of 2 or 5 μL drops. Final concentrations for the homogeneous and heterogeneous nucleation trials were: for lysozyme, 20 mg/mL protein in 10 mM sodium citrate buffer, pH 4.6, and 60 mg/mL sodium chloride; for CG_2, 9 mg/mL protein in 110 mg/mL (11%

w/v) PEG 4000, 0.2 M zinc acetate, and 0.1 M sodium cacodylate, pH 6.3; for thaumatin, 30 mg/mL protein in 0.1 M PIPES, pH 6.6, and 0.5 M NaK tartrate; for glucose isomerase, 6 mg/mL protein in 50 mM Tris, pH 7.0, 10 mM $MgCl_2$, and 1.5 M ammonium sulfate. Crystallization of lysozyme, glucose isomerase, and thaumatin took place at 18 °C; CG_2 was crystallized at 4 °C. At least 3 identical drops were set up in each case. Nucleation was assessed by counting the total number of crystals in a drop visible under a microscope after 2 days, unless stated otherwise.

Filtration

Precipitant solutions were filtered through 0.22-μm filters. Crystallization samples (protein plus precipitating agents) were mixed on a vortex mixer, and a comparison was made between crystallization results from unfiltered samples and from aliquots of the same solution that were filtered immediately after mixing, through filters of different exclusion sizes: 0.1-μm, 0.22-μm filters (Millipore UK, Ltd.), 0.02-μm (Anopore, Whatman Laboratories, UK), and 300-kDa molecular weight cut-off filters (ultrafree-MC 300-kDa NMWL Polysulfone, Millipore UK, Ltd.). Protein concentrations measured after filtration by absorption at 280 nm showed no loss of protein.

Application of the nucleant formed in lysozyme solutions to crystallization trials of CG_2

Freeze-dried lysozyme (20 mg) was dissolved in 500 μL of 10 mM sodium citrate buffer, pH 4.6, aged at least 4 days at 4 °C, and then centrifuged for 30 min at 9,000 × *g*. Four-hundred microliters of supernatant was removed, the remaining 100 μL was mixed with 400 μL of deionized water and centrifuged for 30 min at 9,000 × *g*. The upper 430 μL of supernatant was removed and the remaining 70 μL was defined as the nucleant solution; 0.5 μL of nucleant solution was added to 2-μL drops of CG_2 (protein plus precipitating agents) that had been filtered through a 300-kDa filter. Identical drops acting as controls were either untouched or had 0.5 μL of water added.

Experiments on contact with the crystallization vessels

Crystallization samples were mixed in Eppendorf tubes and set up unfiltered, as microbatch trials of 2 μL in Linbro plates (Flow Laboratories, USA), Terazaki plates (Sterilin, UK), glass vials, on parafilm, and on glass coverslips (siliconized and unsiliconized).

Some drops were dispensed onto the floor of the vessel and immediately covered with 1 mL of paraffin oil ("before oil"), whereas other, identical drops were dispensed into 1 mL of paraffin oil and allowed to sink ("after oil"). Experiments acting as controls were set up under water-saturated paraffin oil made by mixing equal volumes of water and paraffin, shaking overnight, allowing to separate, and using the top fraction of the emulsion.

Control of crystal nucleation by temperature change

Droplets (5 μL) containing 30 mg/mL NaCl, 50 mM Na acetate, pH 4.5, and varying concentrations of lysozyme were filtered through a 0.22-μm filter, dispensed under oil, incubated at 10 °C for different times, and transferred to 18 °C for growth overnight. Controls were set up at fixed temperatures, 10 °C and 18 °C.

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