APPLICATION OF OSMOTIC DEWATERING TO THE CONTROLLED CRYSTALLIZATION OF BIOLOGICAL MACROMOLECULES AND ORGANIC COMPOUNDS

Paul Todd, Subhas K. Sikdar and Cheryl Walker
National Institute of Standards and Technology 583.10,
325 Broadway, Boulder, CO 80303-3328. 303-497-5563.

Z. Richard Korszun, Division of Science, University of
Wisconsin-Parkside, Kenosha, WI 53141-2000. 414-553-2544.

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ABSTRACT

Several methods of crystallization of biological macromolecules depend upon the transport of water through the vapor phase -- a process that is sensitive to ambient conditions (temperature, relative humidity). Other methods depend on the transport of solute by diffusion or through a membrane. By regulating the solute concentration on the outside of a reverse-osmosis membrane it is possible to control the rate at which macromolecules and other solutes are concentrated inside a membrane-bound fluid. The effect of dewatering rate on lysozyme crystal quality and growth rate was assessed. A 3-fold increase in concentration over a 9 day period yielded tetragonal crystals 0.5 mm on a side with sharp edges and with ordering at least to 1.73 Å. Transparent crystals of triglycine sulfate were grown by osmotic dewatering; in this case crystal growth could be enhanced or reversed by manipulating the external solution.

INTRODUCTION

There are several unsolved problems in biological molecule crystallization, including the crystallization of certain cell-membrane proteins, glycoproteins and polysaccharides for structural studies and the growth of crystals sufficiently large to allow study of their electronic properties. Although there are many common methods for growing crystals, each has limited applicability. In this paper, we describe a new method for growing protein and organic crystals, osmotic dewatering, which appears to have potential for a wide variety of applications to crystal growth.

Crystal Growth Methods

Zeppezauer [1,2] used bulk dialysis and microdialysis as a means of concentrating protein and regulating salt concentration in protein crystal growth. In such methods solutes are transported mainly by diffusion. Salemme [3] used free-interface diffusion as a transport mechanism in the growth of protein crystals. Currently, vapor diffusion and batch crystallization are among the most popular methods of growing protein crystals [4,5]. At least three books and one database detail the variety of techniques that have been used to crystallize proteins and other biomolecules [4,5,6,7]. Most techniques lack direct control over the rate of

nucleation, which in turn controls the quality and final size of crystals.

A method was developed whereby the rate of change of concentrations of biological macromolecule and precipitant can be controlled exactly, thereby creating optimum concentrations at appropriate times throughout the processes of nucleation and growth of crystals. This method, "osmotic dewatering", in which only water is allowed to pass in and out of the mother liquor, has several advantages over the hanging-drop (vapor diffusion) method, which is also a dewatering method: the dewatering rate is more conveniently adjustable and controllable by the experimenter at all times; there are no constraints on the volume that can be used; and the solvent-transfer rate can be controlled independently of temperature (to name but a few advantages).

The principal methods of crystal growth from aqueous solutions (especially protein crystals) are listed in Table 1 and include batch crystallization, double diffusion, dialysis, gel permeation [8], and vapor diffusion [5,9]. The transfer of solutes and solvent through semi-permeable membranes [2,10,11] was found to provide the slowly varying conditions required for early crystal nucleation and for relatively undisturbed lattice formation over several days of crystal growth. Typically membranes with molecular-weight cut-off around 10,000 have been used. These are not useful for the crystallization of molecules of lower molecular

weight, as water and solutes travel through them together. In some (but perhaps not all) cases, it may be desirable to control solvent content independently of solute content. This approach has the advantage of concentrating both protein and precipitant to desired final concentrations at desired rates (similar to the advantages of the double diffusion method, but without zone effects and at a controllable rate). Controlled dewatering can be accomplished in two principal ways: vapor diffusion and reverse osmosis. The former method is most effective in a hanging drop, which is limited in volume (and is often applied using a sitting drop, which is less constrained in volume); the latter method has no volume constraints and can be used to grow crystals for applications (other than structure determination, for example) in which large crystals are required.

Osmotic Dewatering

The osmosis method ("osmotic dewatering") utilizes a membrane through which ideally only water molecules can pass, such as a reverse osmosis (RO) membrane. Protein and precipitant are dissolved in a volume of water that is separated by an RO membrane from an external solution of salt at a higher concentration. Water, and only water, diffuses down its chemical potential gradient (resulting from the difference in osmotic pressure) at a rate that is determined by two completely controllable factors: the relative heights

(gravity head) of liquid inside and outside the membrane and the relative concentrations of all solutes inside and outside the membrane. The relative roles of these two driving forces (the gravity head and the van't Hoff osmotic pressure for ideal solutions) are specified by

$$P = \rho g \Delta h + RT \Delta c \qquad (1)$$

A typical initial osmotic pressure is around 2 MPa, while that due to the difference in fluid heights is around 20 Pa, so the gravity head can usually be neglected. The rate of dewatering, ignoring gravitational flow, is

$$J(H_2O) = D_{eff}H([H_2O]_i - [H_2O]_o) \qquad (2)$$

where D_{eff} is the effective diffusion coefficient of water in the membrane (in free liquid $D = 1.7 \times 10^{-9}\ m^2s^{-1}$), $D_{eff}H$ is membrane permeability (ms^{-1}), and $J(H_2O)$ is water flux in moles $m^{-2}s^{-1}$. The dewatering rate can be controlled on the basis of net flux of water across the membrane by varying the difference in solute concentration, Δc, and the membrane permeability, $D_{eff}H$. Varying the fluid-level difference, Δh, has very little effect on rate of dewatering.

Such adjustments make it possible, for example, to allow nucleation to occur very slowly [19] while the rate of lattice formation can be increased at later times by increasing the rate of solvent removal after the danger of excess nucleation has passed.

To use this procedure, a protein is dissolved at or below supersaturation in a buffered solution of low salt

concentration and is separated from a high salt concentration by a RO membrane highly selective for the passage of water molecules only. The drop in osmotic pressure across the membrane forces water into the highly concentrated external solution. Dewatering of the solution containing the protein raises the concentration of the protein and causes nucleation. The rate of osmotic dewatering, which can be controlled exactly, allows control over the rate at which crystals form and grow. Reducing the osmotic pressure will concentrate the protein at a reduced rate, thereby leading to conditions thought to favor more accurate lattice formation and a lower frequency of amorphous nucleation.

Choice of test materials

Ever since the successful crystallization of several lysozyme salts by Alderton and Fevold in 1946 [20] and the x-ray analysis of lysozyme crystals by D. Phillips in 1964, this material has become a standard for the testing of novel protein crystallization methods. Its diffraction pattern is well known, and several conditions for its crystallization have been characterized [4,5,7]. Its low cost makes it suitable for nucleation kinetics and lattice formation studies [21,22]. Excellent crystals of hen egg-white lysozyme have been grown in microgravity by the double diffusion method [23] and by the vapor diffusion method [24]. It was therefore chosen as the standard test material in

osmotic dewatering experiments.

To demonstrate the osmotic dewatering method, an osmotic gradient was set up using a high salt concentration (such as 25% NaCl) on one side of the membrane and a low salt concentration (2% NaCl) on the other in a subsaturated solution of lysozyme. Fluid volumes as well as the nucleation and growth of crystals were monitored. The lysozyme crystals that were grown as the result of osmotic dewatering diffracted x rays to a resolution of 1.73 Å. Additional aspects of the method were tested using triglycine sulfate (TGS) in water with a seed crystal in the inner membrane vessel and various high concentrations of salt on the other side of the membrane.

EXPERIMENTAL

Materials

Lysozyme Grade I from chicken egg white was purchased and used with no further treatment. All other chemicals were reagent grade. Seed crystals of triglycine sulfate were provided by Roger L. Kroes of Marshall Space Flight Center [25]. Reverse osmosis membrane was purchased from Osmonics, Inc, Minnetonka, MN 55343, "Sepa" Part No. MS03-42406[1*]. Some experiments were also performed with Part No. MS05-43715, MS03-42405 and MS02-42405, which are stated to have the same water-transport qualities but differing breaking pressures.

Methods

In the large-volume experiments membranes were shaped into cylinders closed at one end, using polyurethane bond glue. The membrane cylinders were positioned vertically in the center of a round fingerbowl, with a typical starting solution on the outside consisting of 100 ml of 25% NaCl. The cylindrical membrane was filled with salt and protein solution (concentrations specified below) to a level below or

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[1*] (Disclaimer) The National Institute of Standards and Technology, an agency of the U.S. Government, does not endorse any brand of product or company. Trade names are used only for the clarification of experimental procedures. Lysozyme was purchased from Sigma Chemical Co., St. Louis, MO, and triglycine sulfate was purchased from Quantum Technologies, 2620 Iroquois Ave., Sanford, FL 32773.

equal to that of the outside solution. A parafilm lid was placed over the fingerbowl to prevent evaporation. The bowl was then placed onto a vibrationless table and maintained (typically) at a temperature of 17 - 18°C by a thermoelectric cooler (see Figure 1).

Various concentrations of salt and protein were used. Table 2 lists, as examples, three solutions that produced diffraction-quality crystals and the procedures that were used. In the case of solutions B and C, after seven days the outside concentration was altered by removing 20 percent of its volume and replacing it with a higher concentration of salt ("yes" in 3rd column). This increased the osmotic pressure, allowing further dewatering of the inner solution. Table 2 also outlines the experimental parameters for each solution. The lysozyme-salt solutions were prepared in a 0.1M sodium acetate buffer at pH 4.0. The depths of the inner and outer solutions were measured daily, and concentrations were calculated by volumetric analysis. Where indicated, protein concentration was measured independently by the Coomassie method [26].

Crystals were evaluated by microscopic observation and measured using an ocular micrometer with a calibrated grid.

Transport of Water

The radial transport of water from the inner cylinder to the outer dewatering solution was calculated on the basis of the volume change in the two compartments and the least-wetted membrane area. The protein and salt concentration rates could be determined from the same data, and an estimated membrane permeability ($D_{eff}H$) in this configuration was calculated using equation (2) [27].

Diffraction Studies

Still and precession photographs were made with a Picker* sealed tube generator operated at 40 kV, 30 mA, and a Hueber precession camera. A 15° precession photograph of the H0L zone was taken at a 6 cm crystal-to-film distance, giving 3.0 Å resolution at the pattern border.

RESULTS

High-quality crystals were obtained from this procedure using the range of solute concentrations shown in Table 2. On the basis of these results and those from less successful experiments, the ideal final protein-to-liquid ratio is between 7.5% and 8.0% (w/v), including protein in crystals. The final salt concentration in solution A, for example, was higher than necessary and resulted in larger numbers of smaller crystals. The quality of crystals was assessed by x-ray diffraction studies. In the precession pattern of a

crystal grown in solution B, for example, all reflections were sharp single spots, indicating a single crystal without intergrowth or dislocations. Still diffraction pattern of crystals showed strong reflections about 79 mm from the film center, corresponding to a resolution of 1.73 Å, the limit of the camera.

In the example designated Experiment A the external salt concentration was not controlled but was allowed to equilibrate on its own. In experiments B and C the initial internal salt and protein concentrations were changed, as shown in Table 2. Once nucleation had begun, the external salt concentration was increased to produce a greater osmotic gradient enabling faster dewatering of the crystallizing solution.

Each crystal solution was analyzed under a light microscope. Amorphous crystals were detected in cases in which dewatering was too rapid at the beginning of crystal growth (Figure 2). Under certain dewatering conditions crystals would fuse together in polycrystalline clusters (Figure 2). If the rate of nucleation was increased amorphous crystals formed at elevated frequencies. As described in Table 3, not only did amorphous nucleation increase, but also fragmentation and polycrystalline clusters were observed when the dewatering rate was increased at inappropriate times (while dissolved protein concentration was still high) during the course of crystal growth. Under

optimized conditions, single tetragonal crystals grew with very flat faces and straight, sharp edges as indicated in the micrograph of Figure 2C.

Final crystal size was influenced not only by dewatering rate but also by initial conditions that established the approach to supersaturation. Figure 3 is a two-dimensional plot from a partial factorial experiment in which initial salt and lysozyme concentrations were varied and maximum crystal size was measured. It suggests that larger crystals result when initial concentrations are lowest: 2% (w/v) salt and 2% (w/v) protein, so that supersaturation is approached from subsaturation protein concentration, where saturation occurs at about 3.2% lysozyme at the temperature of these experiments, 17°C [22,28].

A set of dewatering curves is shown in Figure 4 for a system consisting of 1.9% (w/v) salt and 2% (w/v) protein in 15 ml inside and 25% (w/v) salt in 100 ml outside an RO membrane cylinder. The dashed saturation line, derived from Feher and Kam [22], indicates that supersaturation was reached during the first day of water removal, which was coincident with the appearance of the first crystals in this case. The initial slope of the dewatering curve gives a value of $D_{eff}H$ of 1.6×10^{-6} cm/s. The osmotic pressure was calculated from equation (1) using a buffer concentration of 0.1 mol dm^{-3}. In the case of unchanged solutions, osmotic equilibrium is not reached after 10 days. These conditions

produced 0.5 mm crystals in 10 days.

Triglycine sulfate crystals were grown from a 91% saturated solution (1.09 mol dm^{-3}) in distilled water [25] with 1.0 mol dm^{-3} Na_2SO_4 at 22 $\pm$ 2 °C as dewatering solution. A 2 mm seed crystal, hanging on a sting, grew to 6 mm in 18 hr. Upon increasing the external Na_2SO_4 to 1.5 mol dm^{-3} the same crystal grew to 12 mm in an additional 23 hr, while crystal size remained constant when external solution was not modified. Conversely, when the external solution consisted of pure water, 5 ml of water was introduced by osmosis into the 12.7 ml crystallizing solution, and the resulting dilution of internal TGS reduced the size of the seed crystal and eventually dissolved it in 4 days, thereby demonstrating the reversibility of lattice formation in this system.

DISCUSSION

Summary of results

The primary difference between the osmotic dewatering method and methods customarily used for growing protein crystals is the control exerted on crystal growth by altering both the external and internal salt concentration and the internal protein concentration to maintain a steadily conducive environment for controlled crystal growth.

Two standard crystal types have been grown under a variety of conditions in partial factorial experiments [29] with highly constrained search conditions. In the case of

lysozyme this meant holding pH constant at 4.2 and varying only NaCl concentration, dewatering rate, and temperature. Lysozyme crystals of high optical quality were grown to 1.2 mm on a side in NaCl, and the tetragonal habit was the same as reported when other methods were used [19]. Conditions were established that minimized early nucleation, amorphous crystal growth and crystal fusion under dewatering conditions. Diffraction studies showed that a 0.6 mm lysozyme crystal grown by osmotic dewatering produced a diffraction pattern that equalled or exceeded the resolution of the still camera that was used, namely 1.65 Å, and spots produced at the corresponding Bragg angle were sharp and bright. Triglycine sulfate crystals of better visual quality than the seed crystals used were also grown. It was found possible to reverse the crystallization process at will by controlling the concentration of salt exterior to the membrane in which the seed crystal was hung on a sting. The flat, completely transparent rhombohedral morphology was maintained at all sizes of crystal from approximately 1 to 15 mm. These results indicate that the osmotic dewatering method is superior to several methods currently used for the growth of protein and organic crystals.

While it might appear that simple evaporation under controlled conditions might accomplish the same goals, it is important to remember that evaporation from a surface results in locally high concentrations of solute near the vapor

in locally high concentrations of solute near the vapor interface of the crystallization volume; the highly concentrated solutes then convect to the bottom of the volume creating convection currents that may interfere with lattice formation. This is one source of convection that presumably can be eliminated by using the osmotic dewatering method in the presence of gravity. If the membrane is placed at the bottom of the vessel, dewatering will be more rapid there, thereby increasing the density at the bottom and preventing convection. Additionally, some precipitants are volatile and will not be concentrated during evaporation, nor can their vapor pressure be controlled independently of that of water.

It is possible to draw the following conclusions about the osmotic dewatering method:

1. High quality crystals can be grown in salt solutions using osmotic dewatering.

2. Crystal growth procedures can be initiated below saturation, and supersaturation can be approached at a controlled rate.

3. The rate of dewatering, and hence the rate at which protein is concentrated, can be regulated by controlling the concentration of water outside the membrane.

4. Crystals can be grown in large volumes of mother liquor using the same principle (water removal) as in the hanging-drop or sitting-drop vapor diffusion methods.

Potential Applications

The investigation of crystal growth methods that provide an opportunity to grow large (~1 cm), perfect crystals of interesting organics or biochemicals (for example, beta-carotene, cytochrome c) has not been carried out. Some of the interesting electronic properties of cytochrome c have been investigated in molecular interaction studies based on its high-resolution three-dimensional structure [30]. The large volumes available by the osmotic dewatering method should facilitate the production of large crystals of biomolecules for the study of their electronic and biological properties and for neutron studies.

Beta-carotene, the phototransducer molecule in the optoelectronic system of the vertebrate eye, has been grown to very large crystals (3-4 cm) by a dialysis method ("diffusion zone process") similar to the osmotic dewatering method [10,11], in which one organic solvent is replaced by another in which beta-carotene is less soluble. An RO membrane could be used for solvent exchange in this process. DC conductance along 2 or more crystal axes and clock-like resonance behavior in conductance [31], for example, can be sought in large organic crystals.

Gravity-Related Issues

The osmotic dewatering method solves several of the problems associated with the successful growth of crystals

from aqueous solutions. However, it cannot directly solve some gravity-related problems, such as container-wall effects and local convection. High-density crystals (typically 1.2 or greater [7]) still settle to the bottom of the vessel or membrane and may undergo lattice dislocations as a result of growth while contacting a surface [24]. Convective flows may be reduced, but they are probably not absent [32,33]. Thus a test of the method in the near absence of gravity is appropriate.

Future Research

Important remaining research includes successful scaling down of the osmotic dewatering method with successful management of the accompanying fluid volume changes. Two systems have undergone preliminary testing: a cuvette method modeled after the method of Zeppezauer [2] having 300 μl capacity, and (2) a pervaporation (permeation + evaporation) method modeled after the hanging drop [18] and an automated dual-well apparatus [34,35]. Sketches of these are shown in Figures 5A and 5B, respectively. In preliminary experiments lysozyme crystals have been successfully grown in these devices.

Further research is needed to determine the suitability of this method for growing a wider variety of crystals, the possibility of growing "difficult" crystals, the volume range over which it is applicable, the potential roles of

convection in the process, and the effects of vessel-wall contact in the case of crystals that sediment. The expected results include a determination of the utility of the micropervaporation method, as defined in Fig 5, and an evaluation of the significance of convection and vessel-wall contact in crystal growth under dewatering conditions.

Light-scattering methods can be used to characterize the kinetics of nucleation and early crystal growth. Methods available include two-angle light scattering, which detects the appearance of submicron particles in suspension [36], quasi-elastic light scattering [19,21,22], laser extinction/scattering [37], and double-exposure holography. Schlieren and holographic data can confirm the absence of convective plumes and indicate crystal growth rate, respectively. Signals from such optical devices would be useful in controlling the regulation of the concentration of water outside the membrane.

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