

Summary and Perspectives

The DLS experiment is quick (a few minutes), is nondestructive, and requires a minimum of purified material (typically <1 mg). Macromolecular samples can be assayed routinely for monodispersity as a function of solvent conditions, the presence of ligands, inhibitors, or cofactors, or following posttranslational modifications or partial proteolysis, etc., greatly enhancing, we believe, the likelihood of successful crystallization trials. The DLS experiment can be used as the screening step in a crystallization strategy that involves the use of protein engineering to generate a number of related recombinant constructs of a macromolecule or macromolecular complex. This approach may also find some application in the challenging arena of membrane protein crystallization, where it could be used to screen different recombinant constructs and monitor various detergent solubilization strategies. Sample monodispersity is also critical for other biophysical methods, including nuclear magnetic resonance spectroscopy and small-angle X-ray and neutron scattering. DLS should be a useful screening step for samples prepared for these solution-based techniques. Finally, DLS may represent an important method for quality control during the manufacture of recombinant proteins for therapeutic use.

[11] Two-Dimensional Protein Crystals in Aid of Three-Dimensional Protein Crystal Growth

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A general method of forming single-layer-thick, or two-dimensional (2-D), protein crystals has been devised, based on adsorption of proteins to lipid layers.¹⁻³ Adsorbed proteins are constrained in 2-D but retain lateral mobility, due to the rapid lateral diffusion of lipids,⁴ which enables crystallization. Adsorption may be accomplished in two ways: proteins are bound specifically to ligands attached to the polar head groups of lipids

¹ E. E. Uzgiris and R. D. Kornberg, *Nature* **301**, 125 (1983).

² R. D. Kornberg and H. O. Ribi, in "Protein Structure, Folding, and Designeditor" (D. L. Oxender, ed.), p. 175. Alan R. Liss, Inc., New York, 1987.

³ R. D. Kornberg and S. A. Darst, *Curr. Opin. Struct. Biol.* **1**, 642 (1991).

⁴ R. D. Kornberg and H. M. McConnell, *Proc. Natl. Acad. Sci. USA* **68**, 2564 (1971).

(e.g., nucleotide-lipid,⁵ drug-lipid,⁶ Ni²⁺-lipid for binding hexahistidine-tagged proteins,^{7,8}) which allows the proteins to adopt a unique orientation at the lipid/water interface; or proteins are bound nonspecifically through electrostatic interactions with a charged lipid layer, allowing rotational as well as translational diffusion of the bound molecules. In either case, a high affinity of the proteins for the lipid monolayer ensures a high concentration of proteins at the lipid/water interface (500–1000 mg/ml), and this high concentration drives the crystallization process.

The lipid layer crystallization approach has been successfully applied to dozens of proteins, including antibodies, enzymes, and polypeptide toxins,³ and the following benefits have emerged. First, the method is useful for proteins that have resisted attempts at the formation of 3-D crystals. One reason may be that 2-D crystals form rapidly, often within minutes, before denaturation of labile proteins can occur. Second, the formation of 2-D crystals requires only microgram quantities of material, much less than is usually needed for the growth of 3-D crystals. Third, in all cases tested, the formation of 2-D crystals occurred under a wide range of solution conditions, including physiologic conditions. This permits structural analysis and other applications of 2-D crystals to proteins in their native states, in particular conformational states, or in fragile complexes with other molecules. Fourth, the exposed face of a 2-D crystal may be exploited for interaction with additional molecules in solution. These additional molecules may be of various types, forming multiprotein complexes, or they may be identical to the proteins adsorbed to the lipid layer, resulting in epitaxial crystal growth.

The main purpose of 2-D protein crystallization to date has been for structure determination by electron microscopy. Electron diffraction is employed for most accurate measurement of structure factor amplitudes, while Fourier transformation of electron microscope images yields phase information.⁹ While 2-D crystals formed on lipid layers may diffract to atomic resolution, as in the case of streptavidin crystallized on layers of bioinylated lipid,¹⁰ structure determination beyond 5–10 Å resolution is limited by loss of image contrast, and X-ray diffraction remains the method of choice for revealing atomic detail.

The formation and analysis of 2-D crystals can facilitate structure determination by X-ray diffraction in a number of ways. First, because of the

⁵ H. O. Ribi, P. Reichard, and R. D. Kornberg, *Biochemistry* **26**, 7974 (1987).

⁶ L. Lebeau, C. Mioskowski, and P. Oudet, *Biochim. Biophys. Acta* **939**, 417 (1988).

⁷ E. W. Kubalek, S. F. J. LeGrice, and P. O. Brown, *J. Struct. Biol.* **113**, 117 (1994).

⁸ C. Dietrich, L. Schmitt, and R. Tampe, *Proc. Natl. Acad. Sci. USA* **92**, 9014 (1995).

⁹ P. N. T. Unwin and R. Henderson, *J. Mol. Biol.* **94**, 425 (1975).

¹⁰ E. W. Kubalek, R. D. Kornberg, and S. A. Darst, *Ultramicroscopy* **35**, 295 (1991).

ease, rapidity, and small amount of material required for 2-D crystallization, it may be used as a structural "assay" to guide the preparation of protein and obtain material forming the largest, best-ordered crystalline arrays. Second, 2-D crystals can seed the growth of large single crystals for X-ray analysis. Third, electron crystallography can provide or assist in obtaining phase information for X-ray structure determination; for example, molecular envelopes may be employed in solvent flattening and are potentially of value for determining heavy atom positions from difference Patterson maps.

The use of 2-D crystallization as a guide for protein preparation is illustrated by studies of yeast RNA polymerase II. This large multiprotein complex (~600 kDa) initially formed small, poorly ordered crystals on positively charged lipid layers, giving diffraction to about 30 Å resolution.¹¹ Experiments with many different protein preparations revealed a correlation of crystal quality with the amount of two small subunits of the polymerase present at a substoichiometric level relative to the other subunits. The consequent heterogeneity of the protein preparation was corrected by removal of the two small subunits, leading to the formation of large 2-D crystals ordered to at least 10 Å resolution.^{12,13} The improved protein preparation was then employed in 3-D crystallization trials, which were almost immediately successful.¹¹ Subsequent experiments confirmed the importance of removing the two small subunits for 3-D crystallization (D. A. Bushnell and R. D. Kornberg, unpublished).

The possibility of 2-D crystals serving as seeds for 3-D crystal growth was originally suggested by electron micrographs showing multilayered crystals of antihaptenic antibodies formed on lipid-hapten (J. Reidler and R. D. Kornberg, unpublished) and of RNA polymerase II formed on charged lipids as mentioned previously.¹² This apparent tendency toward epitaxial crystal growth with dilute protein solutions (50–100 µg/ml) in the absence of a precipitant was pursued by the use of conditions more conducive to 3-D crystallization. The approach has so far been tried and has proved successful with three protein and lipid combinations, streptavidin with biotinylated lipids, RNA polymerase II with charged lipids, and Epstein-Barr virus nuclear antigen-1 (EBNA1) with charged lipids.^{11,14} In the case of streptavidin, a thorough study was performed, demonstrating the formation of 3-D crystals of a size and quality suitable for X-ray diffraction

¹¹ A. M. Edwards, S. A. Darst, S. A. Hemming, Y. Li, and R. D. Kornberg, *Nature Struct. Biol.* **1**, 195 (1994).

¹² S. A. Darst, E. W. Kubalek, A. M. Edwards, and R. D. Kornberg, *J. Mol. Biol.* **221**, 347 (1991).

¹³ F. J. Asturias and R. D. Kornberg, *J. Struct. Biol.* **114**, 60 (1995).

¹⁴ S. A. Darst and A. M. Edwards, *Curr. Opin. Struct. Biol.* **5**, 640 (1995).

analysis and establishing that these crystals arose by epitaxial growth from 2-D crystals.¹⁵ Such epitaxial growth occurred at very low protein concentrations and yielded 3-D crystals in space groups corresponding to those of the 2-D crystals from which they were derived, rather than those normally obtained under the ionic conditions used.

Three procedures for epitaxial growth of 3-D crystals are described here. The first (procedure A) illustrates the use of lipid layers without preformed 2-D crystals in the conventional hanging drop procedure. The second and third procedures (B and C) utilize preformed 2-D crystals to nucleate 3-D crystal growth. Procedure A is perhaps most straightforward, since it presumes nothing about the rate of formation or stability of 2-D crystals. It may be employed as an adjunct to any set of crystallization trials with little additional effort beyond the preparation of a lipid solution. Procedures B and C are particularly useful if 2-D crystals have previously been characterized.

Materials

The following materials are needed for application of the procedures to the particular proteins described below. They are merely illustrative of the materials required for other applications. In particular, the use of a biotinylated lipid is appropriate for studies of streptavidin but would not, in general, be employed for other purposes.

Streptavidin Crystallization on Biotinylated Lipids

Streptavidin from Boehringer Mannheim, dissolved in water at 10 mg/ml

Dioleoylphosphatidylcholine (DOPC) from Avanti Polar Lipids (Birmingham, AL)

N-{6[(biotinoyl)amino]hexanoyl}-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (PE-biotin) from Molecular Probes (Eugene, OR)

Protein Crystallization on Charged Lipids

Dioleoylphosphatidylcholine (DOPC) from Avanti Polar Lipids
Stearylamine and sphingosine from Sigma (St. Louis, MO)

¹⁵ S. A. Hemming, A. Bochkarev, S. A. Darst, R. D. Kornberg, P. Ala, D. S. C. Yang, and A. M. Edwards, *J. Mol. Biol.* **246**, 308 (1995).

Procedure A

Lipids in organic solvent solution are applied to a silanized glass coverslip and allowed to dry, and a hanging drop is assembled on the lipid patch. The lipids are presumed to form a layer with polar head groups oriented toward the aqueous solution, supporting the growth of 2-D and 3-D crystals.

1. Apply 1–2 μl of lipid in chloroform/hexane (1/1, v/v) in a spot on a glass coverslip silanized with a solution of 2% dichlorodimethylsilane in 1,1,1-trichloroethane. Lipid solutions: for streptavidin, 0.45 mg/ml DOPC, 0.05 mg/ml PE-biotin; for RNA polymerase II, 0.45 mg/ml DOPC, 0.05 mg/ml stearylamine; for EBNA1, 0.45 mg/ml DOPC, 0.05 mg/ml sphingosine. Lipid solution for proteins adhering to negatively charged surfaces may be 0.45 mg/ml DOPC, 0.05 mg/ml stearic acid.
2. Withdraw lipid solution and wash the spot by repeated application and removal of a 10- μl volume of mother liquor [e.g., 30% (w/v) ammonium sulfate, 200 mM NaCl, 50 mM potassium acetate, pH 4.5 for growth of streptavidin crystals in space group *PI*].
3. Assemble hanging drop on lipid spot.

Procedure B

Preformed 2-D crystals are drawn up into a pipet and applied to a silanized glass coverslip, and a hanging drop is assembled at the same location on the coverslip. In all likelihood, lipid layers bearing 2-D crystals vesicularize during pipetting. Vesicles adhering to the coverslip nucleate 3-D crystal growth.

1. Form 2-D crystals as previously described.^{13,16} For example, apply 1.5 μl of lipid solution to the surface of a 15- μl drop of protein solution in a well 6 mm in diameter and 0.7 mm deep in a Teflon or nylon block. (Blocks are custom-made by drilling. Nylon has the advantage of giving a flatter meniscus.) Incubate under argon at room temperature or at 4°C for 1–12 hours.
2. Draw the contents of the well with 2-D crystals into a pipette and spot on a silanized glass coverslip.
3. Withdraw solution from the coverslip and wash the spot with 10 μl of reservoir solution.
4. Assemble hanging drop on spot.

¹⁶ S. A. Darst, H. O. Ribi, D. W. Pierce, and R. D. Kornberg, *J. Mol. Biol.* **203**, 269 (1988).

Procedure C

The approach is the same as in procedure B, but a different method of harvesting 2-D crystals is used, allowing direct transfer to the surface of protein drops, with minimal perturbation of the solution conditions.

1. Form 2-D crystals (as in procedure B).
2. Harvest 2-D crystals from the air/water interface with a loop, about 3.5 mm in diameter, formed from 0.75-mm diameter Pt/Pd wire as described.¹³
3. Bring loop in contact with the surface of a preformed and equilibrated hanging drop to transfer 2-D crystals to the drop. This step may be repeated with a series of drops to effect serial dilution of the 2-D crystals. In experiments with RNA polymerase II, in which protein and salt concentrations were far below those required for nucleation of 3-D crystal growth, and hanging drops were equilibrated for a week or more without visible crystal growth, introduction of 2-D crystals by this procedure resulted in the appearance overnight of a shower of needles, 50–150 μm in length, which could be used for further seeding experiments.

Conclusion

In view of the ready availability of streptavidin and biotinylated lipid and the novelty of these procedures, we recommend that epitaxial growth of streptavidin crystals be reproduced before other systems are investigated. The first procedure (A) should suffice for such a preliminary study. Ultimately, experience in many other systems is needed to determine the generality and utility of the approach.

[12] Reductive Alkylation of Lysine Residues to Alter Crystallization Properties of Proteins

By IVAN RAYMENT

Introduction

Chemical modification has played an essential role in the development of our understanding of protein function. Although in many respects it has been superseded by site-directed mutagenesis, it is still a valid approach when it can be established that the result of the chemical reaction is unique