COMMENTARY

When worlds colloid

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Macromolecular crystallography is the pre-eminent experimental technique for the determination of accurate and precise protein structures, and these structures provide one of the bases for modern molecular biology. These structures provide highly-detailed input for the design of experiments to probe molecular function, as well as (in some cases) serving as the input for structure-based lead compound discovery. Of course, a suitable sample is required in order for all of this scientific bounty to spill forth; namely, the earnest (or even semi-earnest) structural biologist requires crystals in which a sufficient number of molecules pack with sufficient long-range order for diffraction of incident X-rays to occur with a resolution sufficient for structure determination. Therein lies one of the challenges of the field: The molecules whose structures we wish to solve do not always form crystals sufficient for structure determination. This challenge is especially acute for those targets at the frontiers of structural biology: proteins possessing multiple domains, protein complexes, and membrane proteins.

To the community of structural biologists seeking to solve interesting and important protein structures by macromolecular crystallography, crystallization is largely an empirical method that is guided (even if implicitly) by a number of sensible and generally successful principles. These principles have evolved, influenced by the ease of many molecular biology protocols, the simplicity of setting up crystallization experiments and the decreasing amount of sample required for each experiment (due to miniaturization and automation), the availability of commercial crystallization screens, the explosion of synchrotron beamlines for screening and shooting crystals, and, recently, the results of structural genomics efforts where thousands of proteins have been subjected to crystallization. To paraphrase a previous U.S. president, "It's the

protein, stupid." That which matters the most in the crystallization experiment is the protein that is being crystallized. A few hundred crystallization conditions are probably sufficient to try with a given protein construct. If it does not crystallize, or if optimized crystals still do not diffract well, then move on to making another variant of the protein. Trim it, ortholog it, surface-mutate it, co-crystallize it, and modify it (somehow), and success will likely be yours (eventually). Inclusion of solution properties (precrySTALLIZATION screening) can also be useful. An elegant presentation of this approach, denoted "variational crystallization," describes its application to crystallization of the HIV gp120 surface glycoprotein (Kwong et al. 1999). Crystallization screening is de-emphasized in this approach.

An alternative view emphasizes crystallization. The crystallization of macromolecules is a physical process, subject to the same rules of physics and chemistry as everything else. A more detailed understanding of crystallization will reduce the seemingly ad hoc black-art empiricism of the structural biologist; hundreds or thousands of crystallization experiments may not need to be set up, if enough is understood of the physical chemistry of macromolecular crystallization. It is not too much of an exaggeration to say that the group of scientists working upon the physical chemistry of crystallization and the group of scientists working on solving new protein structures are, with rare exception, two disjoint sets. Moreover, the substantial corpus of work in the physics and physical chemistry of crystallization has had very little specific impact upon the manner in which structural biologists go about crystallizing new proteins. There are a variety of possible reasons for this, but the most sensible or fair is the notion that each group is interested in a different question. To the structural biologist, the "burden of proof" is to obtain good crystals (and the subsequent structure) of a new protein by the direct application of some new physical-chemical insight. Get crystals where none were gotten before, or perhaps get them faster and more easily, and the utility of the physical chemistry of crystallization will gain much traction in structural biology. To the physical chemist or physicist, realize that

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variational crystallization is a successful tool, molecular biology is easy, crystallization screening is fast, and, critically, that the metric for the structural biologist is structures.

The gap between these two worlds decreases in the significant paper of Berger et al. (2006) in this issue of *Protein Science*. Membrane proteins present significant current challenges in expression and crystallization. Although comprising 20%–30% of the ORFs of a genome (Wallin and von Heijne 1998), and being the target of the majority of current and future drugs, only a small fraction of the entries in the PDB are integral membrane proteins (refer to http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). One complication is the requirement for detergents to maintain the solubility of the membrane protein by shielding the hydrophobic transmembrane region from the aqueous milieu. Thus, the entity crystallized is the protein–detergent complex (PDC); the variation of the crystallization behavior of the same protein in different detergents adds another dimension (and a large number of additional experiments) into crystallization screening. Given current substantial technical challenges in integral membrane protein structure determination, the development of improved methods would be significant; toward this end, methods development for membrane protein structural biology is an NIH Roadmap Initiative.

Berger et al. (2006) unify a number of previous observations in membrane protein crystallization with results from the physical chemistry of crystallization in a way that is comprehensive and that points the way toward a real possibility of satisfying the structural biologist's "burden of proof" utility claim for crystallization physics. The detergents used in solubilization and crystallization of integral membrane proteins possess a rich and varied phase behavior, in addition to the monomer-to-micelle transition that these amphipathic molecules undergo. Aqueous solutions of detergents can possess lower (or upper) consolute phase boundaries. At a given concentration (above the CMC [critical micelle concentration]), the detergent is in equilibrium between monomers and micelles within a single phase. Above a specific temperature, the solution separates into two phases, one of higher detergent concentration and one of lower. The lowest temperature at which this occurs in the temperature-composition phase diagram is a critical point; at and near this point the solution becomes cloudy ("the cloud point"), and a phase boundary between one- and two-phase regions exists. Previous investigators have observed correlations between this phase boundary and crystallization; specifically, some membrane proteins crystallize in a single-phase region close to the consolute boundary (Garavito and Picot 1990; Zulauf 1991). Although the specific molecular mechanism of the phase transition is not known, a reasonable assumption is that surfactant

interactions increase in the vicinity of this phase transition, and that this attractive interaction may increase the likelihood of crystal nucleation and growth. Also, amphipathic additives have been used with success in membrane protein crystallization, and in some cases these additives have changed detergent phase behavior by shifting the consolute boundary.

Is there a way to measure the interactions between PDCs? Osmotic virial coefficients describe solute–solute, solute–solvent, and solvent–solvent interactions. For a binary mixture, such as a soluble protein in aqueous buffer, the second virial coefficient B_{22} describes protein–protein interactions in that solvent. George and Wilson (1994) demonstrated that a number of soluble proteins crystallized under conditions where their B_{22} values corresponded to a weakly attractive protein–protein interaction; the range of values defined a "crystallization slot" which could (in principle) be used for the rational design of crystallization conditions that are within that slot. However, the standard method for measuring B_{22} , static light-scattering, is sufficiently time-consuming and specialized so as to preclude its widespread use for the design of crystallization experiments (also, recall that for soluble proteins, commercial screens plus variational crystallization have worked rather well). An advance that may reduce the energy barrier for making B_{22} measurements was the publication of several papers (primarily from the group of A.M. Lenhoff, a coauthor of Berger et al. 2006) showing how self-interaction chromatography (SIC) could be used to measure B_{22} (for example, see Tessier et al. 2002). Although not yet common practice, this method—which is faster, requires much less sample, and which can likely be miniaturized and automated—may make the larger-scale acquisition of B_{22} data feasible.

Berger et al. (2006) characterize the phase behavior (consolute boundary and cloud point), micelle size, and second virial coefficient B_{22} (PDC–PDC interaction) of the detergent β -octylglucoside (β -OG) and the light-driven proton pump bacteriorhodopsin (bR). This characterization is done in the presence of salts, PEGs, and additives (such as heptane-triol). The trenchant results of these studies include the following: The "crystallization slot" observed by George and Wilson for soluble proteins (and by Hitscherich et al. [2000] for the β -barrel membrane protein OmpF) often occurs close to the cloud point, particularly in the presence of PEGs; additives (and protein) change B_{22} values and shift the cloud point; "successful" additives and precipitant combinations move the cloud point and crystallization slot B_{22} values to an experimentally accessible condition; a relation exists between B_{22} and cloud point temperature; additives reduce micelle size and permit attractive interactions over a wider range of conditions. These results bring together previous observations of crystallization

“close” to detergent phase boundaries and provide an operational answer to the question of what does “close” to the boundary mean—it means that part of the single phase region that possesses B_{22} values in the crystallization slot. In a stunning practical application, Berger et al. (2006) construct a B_{22} -based crystallization screen for bR in β -OG and obtain crystals that diffract to ~ 4 Å resolution.

So, what might this work portend? Clearly, further studies need to be performed; one detergent and one protein do not make a generalized method. Measurement of B_{22} values by SIC and comparison to cloud points for other detergents and other proteins will indicate the range of applicability of the observations of Berger et al. Crystallization screens informed by this type of data will be constructed. As useful as such screens themselves may be to indicate which crystallization conditions to set up, it is their potential application to the exclusion of hundreds or thousands of conditions (per protein per detergent) that may be especially valuable to workers in the field. So much can be tried; to know what not to try would be a significant advance. And, although “rationally-designed” crystallization screens may enhance the probability of obtaining crystals, the protein itself still counts. Variational crystallization, making multiple protein variants, will remain critical. Precrystallization screening methods, for example, fluorescence detection (Kawate and Gouaux 2006) or static light-scattering (Hayashi et al. 1989) coupled to gel filtration, may be useful to select those proteins suitable for crystallization. Another recent paper from Berger et al. (2005) describes a detergent “stability slot” for a G-protein-coupled-receptor; this stability slot is described by a range of values of a detergent property (the hydrophilic–lipophilic balance [HLB]) in which the protein is stable. Therefore, one can perhaps envisage a future where the number of different detergents that have to be examined for a specific protein and the number

of crystallization conditions that need to be set up per protein can both be reduced. The combination of this reduced space of crystallization with an informed use of variational crystallization holds the possibility of increasing the throughput of membrane protein structure determination and, critically, perhaps bringing this endeavor within the reach of more (and more modestly funded) investigators. If so, we structural biologists should thank the physical chemists for sharing their world with us.

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