Session VII - Chemistry of Crystallization

Tuesday, July 5 - morning

S7-L1

STRATEGIES AND STORIES FOR THE SMALL-SCALE CRYSTALLIZATION LABORATORY

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Automation has made it possible to generate massive amounts of crystallization data. How can academic laboratories benefit from this to establish a best-practice procedure for small-scale throughput facilities?

The crystallization facility at Uppsala University processes 800 SBS-format plates per year, of which 73% are for the in-house structural biology groups. Our automation consists of two crystallization robots (Mosquito, Oryx), a desktop imaging system (CrystalMation), and a liquid-handling robot (Scorpion). Most of our groups work on structure-based drug design, which means dealing with (often insoluble) compounds intended for cocrystallization or soaking. In this lecture I will share experiences from our crystallization facility regarding:

- recommendations for how many and which screens to stock for initial screening
- examples of false positives and negatives with UV detection of protein crystals
- how to recognize leads worth optimizing
- matrix microseeding as a first-choice optimization method
- real-life horror stories and successes

Our experiences may be useful for other small-scale facilities hoping to gain the most crystallization information about their targets for the least amount of materials, time and effort.

Microseed matrix screening for optimization in protein crystallization: what have we learned?, A. DArcy, T. Bergfors, S. W. Cowan-Jacobs & M. Marsh (2014). *Acta Cryst.* F**70**, 1117-1126.

S7-L2

THERMODYNAMIC STABILISATION, ENTROPY, AND CRYSTALLISATION OF PROTEINS

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When molecules previously in solution form a crystalline phase, their entropy is reduced. The crystallization driving force must then come either from the enthalpy of the intermolecular interactions in the crystal, or from a gain of entropy of the solvent. There is indeed often an entropy gain upon crystallisation, since a large proportion of the water molecules previously forming ordered hydration shells around the macromolecules are excluded from the crystal. The importance of both entropic and enthalpic effects for crystallisation suggests that changes in these parameters in the pre-crystallisation solution, brought about by the precipitant and buffer, will be crucial to the outcome.

High-accuracy Differential Scanning Microcalorimetry was used to measure the thermodynamic parameters of temperature-driven unfolding of two globular proteins, lysozyme and ribonuclease A, in various salt solutions. The salts were categorised into those that were conducive to crystallisation of the protein and those that were not. Both the Free Energy (G) of unfolding at different temperatures and salt concentrations and its breakdown into entropic and enthalpic contributions were obtained.

It was found that even fairly low -by crystallisation standards- salt concentrations had very large effects on thermodynamic parameters. High concentrations of salts conducive to crystallisation stabilised the native folded forms of proteins, whereas high concentrations of salts that did not crystallise them tended to destabilise them. Considering the H and T S contributions to the G of unfolding separately, high concentrations of crystallising salts were found to enthalpically stabilise and entropically destabilise the protein, and vice-versa for the non-crystallising salts (Fig. 1).

These observations suggest an explanation, in terms of protein stability and entropy of hydration, of why some salts are good crystallisation agents for a given protein and others are not. This in turn provides theoretical insight into the process of protein crystallisation, possibly suggesting ways of predicting and controlling it.

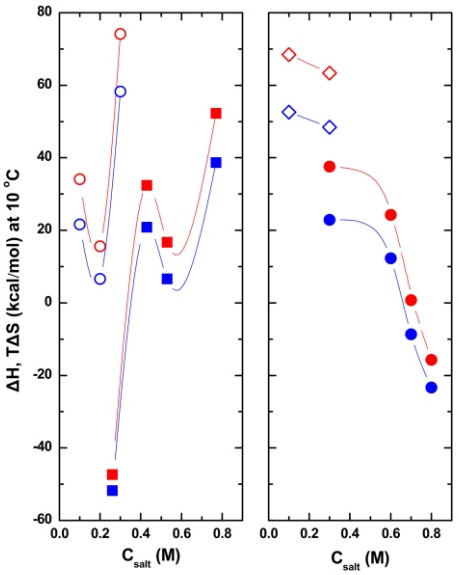

Figure 1. Enthalpic (H, red) and entropic (T S, blue) contributions to the thermodynamic stability of lysozyme in 50 mM sodium acetate buffer (pH 4.5) at 10 °C. Left panel: crystallising salts NaCl (full squares) and Na_2SO_4 (empty circles). Right panel: non-crystallising salts $(NH_4)_2HPO_4$ (full circles) and Li_2SO_4 (empty diamonds).

S7-L3

GLYCEROL ALTERS SUBSTRATE BINDING IN PTPLP:IP COMPLEXES

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Protein tyrosine phosphatase-like *myo*-inositol phosphatases (PTPLPs or phytases) follow an ordered, sequential dephosphorylation pathway that utilizes the abundant *myo*-inositol-1,2,3,4,5,6hexa*kis*phosphate ($InsP_6$ or phytate; approximate charge of -6 to -9 at physiological pH) to produce lessphosphorylated *myo*-inositol phosphates (IPs) containing between one and five phosphoryl groups [1]. Ultimately, we aim to engineer PTPLPs by rational design to manipulate the substrate specificity and produce alternate IP products [2]. The engineered PTPLPs can then be used for large-scale production of IPs. To rationally design an enzyme, clear understanding of how the structure affects function is required. Therefore, the goal is to understand PTPLP substrate specificity at atomic resolution in order to identify and understand the structural determinants that govern substrate specificity. To this end, multiple crystal structures of different IP ligands in complex with IPases are necessary. This work focuses on two PTPLPs which have high activity towards $InsP_6$ and divergent hydrolysis pathways: Phytase A from *Mitsuokella multacida* (PhyAmm; a tandem repeat) and *Selenomonas ruminantium* (PhyAsr) [1, 3].

Glycerol is commonly used to protect proteins while stored frozen, for stabilizing and enhancing protein solubility, in cryocrystallography, and is also used during protein crystallization [4-5]. PhyAmm can be crystallized in the presence or absence of glycerol at concentrations suitable for cryoprotection. The presence of glycerol during crystallization produces larger crystals with increased stability than those grown in the absence of glycerol. However, when soaked or co-crystallized with the highly-charged IP substrates, the resulting structures have the IPs bound to the active site in a catalytically incompetent manner, with inorganic phosphate bound to the phosphate-binding loop (P-loop) and the IP bound above the inorganic phosphate. When the concentration of glycerol is decreased before the PhyAmm crystal is soaked with an IP the structure results in the IP bound in a catalytically competent manner. In the case of PhyAsr, glycerol is used strictly as a cryoprotectant. When PhyAsr is soaked with an IP solution which contains glycerol, the IP binds to PhyAsr in a manner inconsistent with the known pathway or in a catalytically incompetent manner. When PhyAsr is first soaked with the IP and then glycerol added as the cryosolvent, an alternate substrate binding mode is observed.

This work demonstrates that glycerol can have a significant effect on protein-substrate interactions when involving high-charge density substrates. In the case of PhyAmm, the substrate is incapable of binding the P-loop, while the PhyAsr substrate binds the P-loop with alternate binding modes.

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S7-L4

CONTROLLING THE GRYSTAL GROWTH. WHY IS POLY(ETHYLENEGLYCOL) THE MOST EFFECTIVE PRECIPITANT FOR PROTEIN CRYSTALLIZATION?

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The lecture introduces a special approach to controlled crystallization based on modification of protein-protein adhesion properties using additives modifying properties of adhesive patches on protein surface.

Stability of protein crystal depends on stability of the 3D network of identical **protein-protein interfaces (PPI)** ensuring the correct position and orientation of the molecules in highly concentrated solution. Details on the **PPI** can change with crystallization solution and water content in the crystal. However, an inspection of **protein crystal architecture (PCA)** can easily reveal a **set of adhesion modes** characteristic for each crystal form. The strength of adhesion mode depends on adhesion forces between pairs of mutually attractive **adhesive patches** on the opposite protein surfaces. We can identify a set of dominant **protein-protein adhesion modes (PPAM)** that is responsible for formation of the respective crystal polymorph.

Classical model of crystallization process supposes that only the dominant **PPAM** between naked protein molecules is responsible for correct stacking of protein molecules into the growing crystal. New dynamic concept of the **protein surface-shielding agents (PSSA)** introduces more complex crystallization model based on competition between the **protein-protein interactions** and the **PSSA-protein interactions.**

Principal advantage of this approach is that using selective **PSSA**, we can rationally suppress the original dominant adhesion mode and initiate the crystallization process according to the new dominant **PPAM**.

Protein molecules stacked into the crystal by contacts of adhesive patches are always in dynamic equilibrium with solution. The optimal solvent content in crystal, corresponding to the best diffraction ability, depends largely on the **protein crystal architecture (PCA)** and on the physico-chemical composition of the crystallization solution. With increasing osmotic pressure and higher water content, the crystals lose stability. With lower water content, the regular crystal lattice brakes. In both cases, the crystal loses its diffraction ability. Experience shows that there we are not able stabilize protein crystals with solvent content over 85 % or bellow 25 %.

The concept of the selective **PSSA** provides rational approach to protein crystallization has important practical implications and allows us:

1 Rational change of crystallization solution allows us a design of more suitable polymorph for our purposes (e.g. polymorph with active site open for ligand soaking, polymorph with biological oligomerization, polymorph with the best diffraction accuracy of structure determination, etc.)
2 The weakening of the dominant $\mathbf{P_1P_1AM}$ and the strengthening the $\mathbf{P_1P_2AM}$ allows us to grow the required co-crystals of several proteins (multicomponent crystals).

PEG (poly(ethyleneglycol) has, in addition to its precipitating ability, also a large scale of special adhesive modes to protein surface. One can easily regulate the PEG activity on various patches on protein surface almost of all types – binding to positively charged residues, binding to hydrogen donor or acceptor clefts, hydrophilic and also hydrophobic areas. The review of more than 3000 PEG-protein interactions published in [2,3] shows the way how one can skip between different PEG-protein adhesion modes and thus change the preferences of dominant adhesion modes driving the crystallization process. It explains why PEG is so convenient tool for a control of crystal growth.

Practical part shows the examples clarifying activity of PEG on the protein surface.

A. Protein-protein interfaces **(PPAM)** of (1) biological relevance, (2) crystal oligomerization, (3) crystal contacts [4]
B. PSSA-protein interfaces (1) blocking the crystal contacts, (2) build in the crystal lattice [2], etc.
C. Review of PEG-protein interfaces (1) in presence of different salts, (2) build in the crystal lattice [3], etc.

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The project was supported by BIOCEV CZ.1.05/1.1.00/02.0109 from ERDF, RVO 86652036, and Czech Science Foundation 15-15181S.

S7-L5

EXPERIMENTS ON THE DIFFUSION OF DYES AND IONS INTO PROTEIN CRYSTALS

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Experiments have been carried out on 15 different protein crystals, most in the range of 0.5 to 1.5 mm dimensions, to measure the rates of diffusion of dye molecules into crystal interiors. Measurements have also been made of the diffusion of dyes through protein solutions of 50 mg/ml to 400 mg/ml concentration for comparison. We found in the course of our study that for most protein crystals, once saturated with dye (as indicated by their rich colors), the dye is retained in the crystals for at least six months, perhaps indefinitely, after the crystals are submerged in large volumes of clear, dye free mother liquor. This indicates strong association of the dyes with the interior of the crystals. Dialysis experiments further indicate a strong association between the dyes and the protein molecules even in solution. X-ray diffraction experiments on over 25 dye-saturated lysozyme, thaumatin and trypsin crystals, however, failed to reveal (with a few noteworthy exceptions) any difference electron density indicative of ordered binding. This raises the question of how high affinity between proteins and small molecules can arise from completely disordered interactions.

Some of the dyes we use are pH sensitive (pH indicator dyes) and change color as a function of H+ concentration. We exploited the color change of numerous dye-saturated crystals to measure the rates of H+ movement into and out of crystals as the pH of the mother liquor was changed. Some other dyes are sensitive to reduction by, among others, bisulfite or dithionite. Again, color changes within the dye-saturated crystals were used to measure the rates of flow of reductants into the crystals and the rates of subsequent reoxidation of the crystal bound dyes by ambient oxygen. We were further able to saturate protein crystals simultaneously with pH sensitive dyes and redox sensitive dyes and then produce a sequence of color changes in protein crystals by addition of reductants followed by changes in pH of the mother liquor.

Finally, we made the observation that, in general, crystals grown from PEG or other polymers of similar characteristics, unlike those grown from salt, MPD, low ionic strength, etc., can not be stained using any of the more than 30 dyes we investigated. Dyes appear to be barred from entering these crystals. We will discuss possible implications of this observation for crystals grown from PEG, and the mechanism by which PEG promotes crystallization.

Session VIII - Complementary Methods

Tuesday, July 5 - morning

S8-L1

ITC-ASSISTED CRYSTALLIZATION OF PROTEIN-LIGAND COMPLEXES

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Co-crystallization of protein/ligands complexes is frequently problematic, even on established biological systems. Much time, efforts and samples can be spared using preliminary biophysical methods in order to asses a correct formation of the complex in conditions that are optimal for co-crystallization. Among these approaches, Isothermal Titration Calorimetry (ITC) is well-suited for analysis of nucleic acids interactions. A major advantage of ITC over other equivalent biophysical approaches is that no labeling of the sample is needed. In addition, ITC is not subjected to molecular weight limitations and can be used virtually in any buffers. As a consequence, it can be directly used for crystallization experiments, allowing the formation of the complex in a more controlled way and with the additional advantage of providing thermodynamic and binding information 'for free'.

Here we will show through various examples how to implement ITC as a guide to improve co-crystallization of protein-ligand complexes.