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

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 assess 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.
MEMBRANE PROTEIN CRYSTALLIZATION USING CUBIC LIPID PHASES, BICELLES AND VAPOR DIFFUSION

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The Cubic Lipid Phase (CLP) method for membrane protein crystallization has been refined to allow large-scale screening of membrane proteins. Various parameters (CLP lipid, water content, bilayer lipid additive, pH, ionic strength, precipitating agent etc.) can be varied. Several distinct seven-transmembrane proteins were crystallized and their high-resolution structures determined. In cases where the CLP method fails, the bicelle method or detergent-based methods were employed to crystallize other membrane proteins.

Bacteriorhodopsin (BR): High-resolution maps from X-ray diffraction of bacteriorhodopsin crystal obtained in CLP and some of its photointermediates have yielded insights to how the isomerization of the bound retinal drives ion transport. Although some important mechanistic details are still undecided, the events of the photochemical cycle are now understood to reflect changes in specific hydrogen bonds of protein groups and bound water molecules in response to motions of the retinal chain. A nearly complete lipid bilayer is also part of the X-ray-derived atomic model. Surprisingly, we were unable to use the CLP method to obtain crystals of the A215T mutant of BR, only the bicelle method provided results.

Anabaena SR (ASR): The structure of a sensory rhodopsin from the cyanobacterium Anabaena has been determined to 1.9Å resolution using the CLP method. This represents the first eubacterial rhodopsin structure. In comparison to the archaeal rhodopsins BR and SR there are many striking rearrangements and shifts in hydrogen bonding patterns on both the extracellular and the cytoplasmic half of the receptor. Also, the cytoplasmic face, which is thought to interact with its soluble transducer (ASRT), is structurally well defined and very different from that of the archaeal rhodopsins. More recently, we determined the structure of a single-site mutant (D217E) that pumps proton in the opposite direction in a different spacegroup using the CLP approach. The structure of ASRT has also been determined: it forms a C4 tetramer with a new all-beta fold.

Xanthorhodopsin (XR): a light-driven ion pump from the halophilic endosymbiont Salinibacter ruber found in salt-tern crystallizer ponds of Spain, contains a blue-absorbing carotenoid that functions as a light-harvesting antenna for its retinal chromophore. This protein only crystallized from bicelles. In addition to the adaptations to bind and accurately position the carotenoid antenna for efficient excited-state energy transfer to the retinal, XR exhibits major structural differences to the previously studied microbial pumps and photoreceptors. We also determined the ring structures of a pentameric (C5) and a hexameric (C6) proteorhodopsin, from bicelles and CLP, respectively.

Lastly, I will present the results of structural & functional studies on a system responsible for acid tolerance in certain pathogenic bacteria. The system is able to maintain a periplasmic pH of ~6 even when the medium has a pH of 2. The buffering system involves the enzyme urease that readily hydrolyzes urea into NH3 and CO2, which in turn act as a proton sink to reduce [H+] by four orders of magnitude. This membrane protein crystallizes as a C6 hexameric using vapor diffusion after many rounds of optimization, including a detergent mix and E. coli polar lipids.

MYTHS AND REALITIES ABOUT THE INFLUENCE OF ELECTRIC AND MAGNETIC FIELDS ON PROTEIN CRYSTALLIZATION AND PROTEIN CRYSTAL GROWTH

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Single crystal X-ray crystallography is a powerful technique for determining the 3D structure of biological macromolecules at very high resolution, when optimal crystals are obtained by classic or advanced methods of protein crystal growth. Although, this is not the only way to obtain the three dimensional structure of many biological macromolecules existing in living organisms. There are four additional ways to achieve this purpose: 1) Powder X-ray diffraction, 2) NMR techniques (experiments done in solution), 3) Modeling by means of using the PDB, and 4) combined methods of reconstruction by Cryo-SEM and SAXS. This contribution will show different strategies and methodologies by means of using temperature-control and high-pressure for protein crystallization that might help to increase the success rate of obtaining protein crystals, different polymorphs as well as to get high quality single crystals for crystallographic research. This paper will also present new approaches, where sophisticated methods are
used not only to grow protein crystals, but also to control the size and the orientation by applying electromagnetic fields of different intensities. Finally, some case studies from the author’s lab will show the advantages and disadvantages of using these non-conventional approaches for high resolution X-ray crystallography.

### COMPUTATIONAL TOOLS TO AID CRYSTALLIZATION

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Many of the techniques used for, or in association with, crystallization produce such large amounts of (often unreadable) data that it is difficult to extract an appropriate interpretation without over-interpretation. Systematic (automated) data analyses are crucial to obtaining consistent interpretation of results, particularly for techniques which a researcher only uses intermittently. Current computational analysis tools are sparse due to the complex nature of most output data. Single results that consist of multiple points (for example, a visible spectrum) require functional analysis to make curve-to-curve rather than point-to-point comparisons.

In the Collaborative Crystallisation Centre (C3), the creation of computer software which considers data as functional sets of points has allowed us to simplify and speed up numerous data analyses. We have created automated analyses of both thermal melt experiments [1, 2] and pH experiments that produce spectral output. By allowing easier understanding of machine output, different techniques are made more useful and accessible to the end users of the analyses.


### HOW LIGHT SCATTERING CAN CONTRIBUTE TO PURIFICATION, CHARACTERIZATION AND CRYSTALLIZATION OF PROTEINS

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The advent of proteins used in many fields like diagnostics or as therapeutic agents brought the necessity to monitor all steps of synthesis and processing as well as highly efficient and reliable quality control procedures before administration, e.g. in human disease. Moreover, isolation or synthesis of numerous structural and membrane proteins in life sciences require powerful techniques to determine various parameters such as molecular weight, radius, oligomeric state or aggregate formation to assess the quality of a preparation. For many samples, their crystallization behavior also has to be evaluated and predicted, if possible. The choice of an appropriate separation method such as Size Exclusion Chromatography (SEC) or Field Flow Fractionation (FFF) can also play an important role in the process. In our presentation we will address the basics of light scattering technology and show application examples to give an insight into the usefulness of the Wyatt light scattering toolkit for macromolecular characterization.
Session IX - Scoring Methods

Tuesday, July 5 - afternoon

S9-L1

WHAT'S IN A DROP? MOVING FROM IMAGES TO OUTCOMES
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State of the art protein crystallization is a numbers game: as it is unlikely that the conditions under which any given macromolecule will crystallize can be deduced a priori, conditions must instead be found by experimentation.

Crystallization is a time-dependent trial and error sampling of the extremely large space of possible crystallization conditions: large number of conditions are tested, and each experiment is observed (often by imaging) at several time points. The ultimate goal is to have a consistent machine generated score for each image describing the outcome and then to correlate image similarity with condition similarity, building up an accurate picture of the phase diagram for any system. This would enable conditions for crystallization to be located, even if the initial set of experiments did not sample the appropriate set of experimental conditions in the space of all possible conditions.

Currently, automation is used routinely to miniaturize the experiments and to capture their results, but not to interpret the results of the experiments. We are interested in different approaches to using machine learning to interpret the results of crystallization experiments – what tools have already been developed, and how can they be best implemented in a practical and timely way? We will discuss progress of implementation, and compare and contrast existing approaches to automation of scoring. Finally, we will discuss the steps we are taking to find relationships between the experimental conditions and the outcomes of those experiments.

S9-L2

AUTOMATED SCORING OF CRYSTALLISATION EXPERIMENTS USING MULTIPLE IMAGES
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Finding the conditions that will produce diffraction quality crystals can require many crystallization experiments. The use of robots has increased the number of experiments performed in most laboratories and, in structural genomics centres, tens of thousands of experiments can be produced each day. Visual inspection is becoming increasingly impractical and automated imaging systems are now used routinely to record the results of these experiments. Image analysis software has been developed a number of research groups [1-4] to provide scores, allowing the images from crystallization trials to be examined in order of merit and reducing the number that need to be examined by eye. However, scoring individual images does not take advantage of the fact that each experiment is assessed regularly over a period of time. As each new image is produced, further information about the experiment becomes available and changes between images can be encoded as additional features for classification. The more information that can be obtained, the greater the likelihood of correct classification and, in addition to analysis of the time-course images as a sequence, the information gained from UV imaging is considered. For example, although the drop in figure 1 is easily identified in the greyscale gradient magnitudes, the drop in figure 2 cannot be found. However, the additional information from the UV image taken at the same time, allows a mask to be found so that further processing is restricted to the crystallisation drop.