

crystallized so that every subunit is in an identical environment. Electron microscopy has been one of the main techniques used to study such polymers, but resolution has been limited by disorder and heterogeneity, and in many cases three-dimensional reconstructions have not even been possible due to very weak scattering. An iterative single-particle type approach to 3D reconstruction has proven to be remarkably successful in studying actin filaments, protein-DNA filaments, bacterial pili, filamentous bacteriophage, etc. A number of examples will be shown, and some general conclusions drawn. One is that subunits in these filaments frequently have the ability to switch among multiple different states. The notion that there is a single 'structure' for a polymer may thus be misleading. Another conclusion is that small changes in sequence can lead to large changes in the quaternary structure of these polymers, and this may be an important mechanism in the evolutionary divergence of organisms.

Keywords: helical polymers, three-dimensional reconstruction, electron cryo-microscopy

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Structural and functional significance of the N-terminus of Cx26 gap junction channels

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Investigations of gap junction channels have used varying experimental approaches to elucidate the gating mechanism of these intercellular conduits. Here, we report two structures of mutant connexin26 channels by electron cryo-crystallography. Full length connexin26 (Cx26M34A) and an N-terminal deletion mutant (Cx26M34Adel2-7) were overexpressed using Sf9 insect cells. Purified proteins were reconstituted into the lipid bilayers that formed two dimensional crystals. Three dimensional maps of the two Cx26 isotypes were obtained at 10 Å resolution and revealed that the crystals from both constructs were comprised of three lipid bilayers with the channels forming a P22₁2₁ lattice. The structure of Cx26M34A clearly showed a prominent density we refer to as a "plug", which resides in each hemichannel pore and contacts the innermost helices of surrounding subunits at the bottom of the vestibule. These channels showed no dye transfer and dramatically reduced conductance, although the voltage gating characteristics of the residual conductance was normal. In the 3D structure of Cx26M34Adel2-7, the density in the pore was significantly reduced along with a partially reduced density of the cytoplasmic domain which bridges the adjacent four helix bundles. These results demonstrate that the N-terminus of Cx26 contributes to the structure and/or organization of pore plug and cytoplasmic domains. However, while the N-terminal deletion mutant was expressed on the cell surface, it failed to form functional channels, suggesting that the positioning of the plug inside the channel may also play a role in stabilization of open gap junction channels in situ. While the pore plug may contribute to the closure of the Cx26M34A mutant, this may represent one of multiple gating configurations.

Keywords: membrane protein channels, electron microscopy, two-dimensional protein crystals

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Merging data from Cryo-EM and X-ray crystallography to reveal biomolecular function

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We have developed a novel method to flexibly fit atomic structures into EM maps using molecular dynamics simulations. The simulations incorporate the EM data as an external potential defined on a grid that is added to the molecular dynamics force field, allowing all internal features present in the EM map to be used in the fitting process, while the model remains fully flexible and stereochemically correct; harmonic restraints are also applied to enforce secondary structure and to prevent overfitting. Validation for the method using available crystal structures of protein and RNA in different conformations, as well as measures to assess and monitor the fitting process, will be introduced. I will present example applications, including the the ribosome and poliovirus. Emphasis will be placed on E. coli ribosome structures obtained in various functional states imaged by cryo-EM, including a structure derived from a 6.7-Å resolution EM map that reveals the interaction between the ribosome and a bound ternary complex at unprecedented detail.

Keywords: flexible fitting, electron microscopy, ribosome structure and function

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The search for good crystals: How far have we come?

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Crystallization is acknowledged to be a rate-limiting step in structure determination of biological macromolecules. This lecture will give an overview of the past decade's developments to examine how far the field has come and how far it has yet to go in solving the crystallization problem. Two significant developments have been the miniaturization and automation of the crystallization trials. Although automation has become affordable even for small laboratories, the cost of screening has nevertheless increased because more conditions are tested. The pros and cons of this will be discussed. Automation also generates an enormous number of crystallization experiments. A vast pool of data is available, but has this led to more rational strategies for crystallization? Bioinformatics can be used to analyze crystallization feasibility. Ideally, one would like to be able to predict if a protein can crystallize simply by looking at the amino acid sequence or some easily measurable physico-chemical property. How successful are the current prediction programs? Biophysical diagnostic tools will also be reviewed. How well does the information from methods like mass spectrometry, dynamic light scattering, Thermofluor, microcalorimetry, etc., correlate with the crystallization results? Finally, microfluidics, diagnostic tools to detect the onset

of nucleation and control it, and in situ X-ray diffraction of crystals will be examined in this overview lecture. Conclusion: There is no universal solution in the search for good crystals. If anything, the search has become more difficult as more challenging targets are attempted. A multi-faceted approach is therefore required.

Keywords: protein crystallization strategy, automation, screening

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Photochemically induced nucleation of protein

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An important area of post-genomic research is the determination of 3-D protein structures. The main technique used for this purpose is X-ray crystallography. Protein crystallization experiments are carried out in the presence of crystallization agents, e.g., inorganic salts, non-adsorbing polymers, and alcohols, which reduce protein solubility and increase intermolecular interactions to form cluster, nucleus and crystal. In these experiments, account needs to be taken of the protein concentration, nature and concentration of the crystallization agent, pH, buffer constitution, and temperature. Here, we demonstrate photochemically-induced crystallization of metastable protein solutions by weak UV irradiation for several ten seconds. Intermediates, neutral radicals at tryptophan or tyrosine residual produced by one photon absorption, enhance nucleation. The radical forms protein dimer that is detected by an SDS-PAGE electrophoresis experiment. An addition of polyethylene glycol (PEG) greatly enhances light-induced nucleation. PEG affects to shorten the intermediate radical lifetime, which suggests that PEG assists to form dimer. We consider that the photochemical dimer behaves as smallest cluster to grow critical nucleus. The smallest cluster formation is the rate determining step in classical nucleation theory due to surface energy disadvantage. The photochemical dimer is formed by a covalent bond, and the nucleation is initiated from stable dimer. The nucleation enhancement is reasonably explained. The present researches results point out the development of a new method for controlling nucleation and growth that could be applied structural genomics and pharmaceutical industry for instance.

Keywords: photochemistry, protein, crystal growth

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Applications of designed ankyrin repeat proteins as chaperones in structural biology

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Repeat proteins are ubiquitous protein-protein interaction molecules in biology [1]. We made use of this feature in vitro and designed ankyrin repeat proteins (DARPin) which consist of repeat modules with fixed framework residues and randomized surface residues suitable for target binding. The random assembly of such modules yields combinatorial libraries of DARPins of varying length and large

diversities. DARPins are very stable, soluble and produced in large amounts by bacterial expression. By using ribosome display highly specific binders against different protein targets with low nanomolar affinity can be selected. This opens the possibility to crystallize a target protein in complex with a variety of DARPins and therefore enhances the chance of obtaining structures of target proteins that are difficult to crystallize. We selected DARPins having high affinity and specificity for proteases, kinases and membrane proteins and used them for cocrystallization of the target protein. The methodology and the X-ray structures of a DARPin-MBP- [2], a DARPin-kinase- [3], a DARPin-caspase-2- [4] and a DARPin-membrane protein-complex [5] have been published. This illustrates the usefulness of this novel technology in structural biology. It opens a new avenue in macromolecular crystallization and is an attractive alternative to antibodies for the crystallization of membrane proteins. In addition, highly specific DARPin binders and/or inhibitors of target proteins allow the analysis of the role of this protein in signalling pathways.

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Keywords: crystallization, designed proteins, membrane proteins

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A simple method to introduce anomalous scatterers in a wide number of proteins

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A recent Protein Data Bank survey indicated that more than 5500 X-ray crystal structures contain at least one Sulfate ion and the total number of X-ray crystal structures is approximately 43000 therefore approximately 13 % of the total structures contain Sulfate. Since Sulfate ions are predominantly introduced during the crystallization step, we have taken advantage of a simple substitution in the crystallization reagent. Sulfate (SO₄) was substituted with Selenate (SeO₄) during the crystallization of two model proteins known to crystallize in SO₄. Crystals were obtained in similar conditions and diffracted to similar resolution. Their SAD structure were determined solely relying on anomalous scattering from SeO₄. One structure was determined from the peak energy and the second from the high energy remote.

Keywords: protein, phasing, selenium

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Dynamic light scattering in protein crystallization: Analysis and optimization

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