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Biophysical methods: doing more with less

Editorial overview

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Keith's research focuses on the development of synchrotron-based, time-resolved X-ray crystallographic techniques and their application to biological problems such as the molecular bases of light-driven signal transduction in photoreceptors.

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Experimental developments in modern structural biology are largely methods driven, as examination of the structural and computational reviews in any other results-oriented issue of *Current Opinion in Structural Biology* will confirm. This particular section has historically focused on the methods themselves, both to highlight emerging new experimental approaches and to re-examine the status of old favorites (and 'old' in this context may mean no more than of a few years standing). We have selected topics that are having (or promise to have) an increasingly important impact on structural biology studies. Unsurprisingly, assembly of the set of independently completed topics reveals linkages between some of them; a pregnant phrase in one points to a paragraph in another.

Many new methods 'do more with less', as in our title. They may require much less sample or less manual intervention; extend the use of current instrumental approaches to new areas; offer enhanced signal-to-noise; or offer the ability to handle larger data sets, or to cross-compare data sets and extract unanticipated correlations. Best of all, they may reveal new biological and biophysical phenomena that no prior method has detected. The topics in this section display, to a greater or lesser extent, these attributes.

Crystallization of biological macromolecules and structure determination by X-ray scattering techniques remain the foundation for much of structural biology. However, there are two major limitations: the necessity for well-ordered crystals in the first place and the unavoidable fact that, in almost all cases, the structure is determined in a quasi-static state. The latter limitation is beginning to be overcome by explicitly time-resolved crystallographic approaches that complement and extend the more widely used trapping techniques. Two major articles that apply an extended form of this technique to myoglobin have just appeared [1,2], together with a commentary on one of them [3]. A highly novel strategy aimed at addressing the first limitation is discussed in the review by Hansen and Quake, who apply developments in microfluidics to macromolecular crystallization, as an example of the effective manipulation of tiny volumes of solution. Mass transport via diffusion or convection in mixing experiments is sensitive to such physical parameters as density, viscosity and inertia; it is intriguing that this sensitivity differs markedly on the microscale considered here from the more conventional macroscale. This is both a strength and a weakness. One strength is displayed by the unusual effectiveness of free interface diffusion on the microscale, but a weakness is that crystallization conditions established on the microscale may not readily be extrapolated to the macroscale. So, what could be done with the microcrystals themselves? Could they be automatically bathed in a cryosolvent, transferred to an X-ray-transparent chamber in the microfluidics apparatus, frozen and examined *in situ*?

If so, examination of these microcrystals is limited by radiation damage and falls squarely into the province of cryocrystallography, the topic of the article by Garman. As Figure 1 of this review illustrates, the vast majority (80–90%) of today's structure determinations are conducted on crystals flash-frozen to temperatures below 150K; this approach has completely swept the field in the past ten years. The invention of the loop mount for cryocrystallography by Teng in 1990 [4] and of open-flow cryostats made the approach experimentally facile, so much so that the fiddly craft of mounting crystals at room temperature in thin-walled glass capillaries is now dying out. The chief advantage of cryocrystallography is that secondary radiation damage (arising largely from diffusion of highly reactive species generated by primary radiation damage) is greatly minimized. However, primary radiation damage cannot be eliminated because it depends on the X-ray absorption cross-section and, as Garman clearly indicates, there is renewed understanding and appreciation of the magnitude of radiation damage at cryotemperatures. When the user is presented with a brilliant, tightly focused synchrotron beam, there is a strong temptation to acquire as much data from a single crystal as possible, though hindsight would suggest that many crystals have inadvertently been over-irradiated, perhaps undergoing specific structural and electronic damage. But, troubles can be good for you. Specific destruction by radiation damage of the ordered structural environment of heavy-atom sites used for phasing (including intrinsic sites such as the sulfurs in methionine, cystine and cysteine) leads to the novel strategy of radiation-induced phasing.

The diffusion of reactive species in cryocrystallography is limited precisely because a dynamical transition occurs in the macromolecule–solvent complex in the crystal (and in solution) at around 150K, as reviewed by Parak. Biological molecules below that temperature are essentially inactive. Thus, we have the paradox that many of our elaborate explanations for biological function are ultimately based on cryocrystallographic structure determination of profoundly inactive molecules. The structures are undeniably precise — but are they accurate? What role do dynamical fluctuations play in function? In this regard, phonon-assisted Mössbauer spectroscopy has been added to the arsenal of spectroscopic, X-ray and neutron scattering approaches that probe fluctuations. This novel technique yields the density of phonon states that couple to a suitable Mössbauer nucleus, such as the iron in myoglobin, and samples a rather different range of timescales.

Large icosahedral viruses have long attracted structural biologists. The range of sizes and structural complexities they exhibit has necessitated the use of both X-ray and electron scattering and imaging techniques; tying these techniques together into a coherent whole is a more recent undertaking, as Lee and Johnson indicate. Cryo-electron microscopy is yielding images at higher and higher resolution, with quality sufficient, in the most favorable cases, to identify secondary structural elements with confidence and, in other cases, to allow an X-ray-derived electron density distribution to be located and oriented in a larger complex. Even here, dynamics is of key importance; these viruses undergo elaborate structural transitions involving remodeling of their capsids, and often insertion or extrusion of their nucleic acid at key stages of their life cycle. What intermediate structural states are involved? How are the elaborate quaternary structural changes initiated and coordinated over large distances?

NMR spectroscopy has the capacity to probe aspects of protein structure that are complementary to those revealed by X-ray crystallography; at the same time, it does not require the production of protein crystals. Thus, NMR spectroscopy can give unique atomic-resolution insights into dynamic aspects of proteins, the organization of domains within multidomain proteins and details of intermolecular interactions in protein complexes. Until recently, these applications have been restricted to entities with molecular weights that are generally <30 kDa. The introduction of transverse relaxation-optimized spectroscopy (TROSY) in combination with various isotope-labeling techniques (discussed by Fernández and Wider) is opening up avenues to the study of very large protein complexes, which, for assemblies with symmetrically repeated structures, can approach 1,000,000 Da. An immediate challenge is to extend these methods to the study of large heterogeneous complexes. For this purpose, the application of segmental labeling of targeted portions of proteins or protein complexes (as reviewed by Casi and Hilvert) holds great promise. Fernández and Wider discuss progress on several intriguing applications of TROSY, including the study of membrane proteins in detergent/lipid micelles, and the definition of the interfaces of protein–protein and protein–ligand interactions.

For interactions between proteins and small often flexible ligands (e.g. peptides), it can be challenging to define of the structure of the bound ligand. Post reviews another NMR spectroscopic technique, termed exchange-transferred NOE (et-NOE), which is proving of increasing value for this purpose. After defining the experimental requirements for such experiments, Post discusses a series of applications involving peptide–protein complexes, peptide–lipid complexes, ternary complexes involving the binding of two ligands to a protein, and drug screening.

It has long been the dream of structural biologists to manipulate and alter primary protein structures at will (down to a given individual atom), and to produce these modified polypeptides in amounts sufficiently large for detailed structural and functional studies. Such methods would allow the biologist ''exacting control over covalent

structure and the ability to explore structural realms outside nature's normal scope''. Casi and Hilvert review a set of convergent protein synthetic technologies that in essence bring this dream to life. In the early 1990s, Kent and co-workers blazed the trail with the development of chemical ligation [5] and then native chemical ligation [6] for the production of synthetic proteins. Today, it is even becoming possible to carry out related semisynthesis reactions *in vivo*. Together with a newly developed technology from Schultz and co-workers [7] for the addition of unnatural amino acids to the genetic code of organisms, protein semisynthesis *in vivo* should provide unprecedented control over proteins within living organisms. The impact of these techniques promises to be truly profound, especially if they become available to the biological community as a whole.

The identification of proteins isolated from organisms has been revolutionized by the development of rapid, sensitive mass spectrometric techniques used in combination with sequence database searching. This method requires the availability of extensive protein, cDNA or genomic sequences from the organism of interest (or at least from a closely related species). If such sequence information is not available, as remains the case for the vast majority of extant organisms, it is desirable to utilize '*de novo*' peptide sequencing to identify and obtain information about their protein(s). Although *de novo* peptide sequencing by mass spectrometry has shown great promise for many years, it has been a somewhat challenging procedure. Standing reviews the current status of *de novo* peptide sequencing by mass spectrometry. The combination of electrospray ionization or matrix-assisted laser desorption/ionization with new generations of powerful mass analyzers has greatly improved the ease/speed of the technique, and may even allow the possibility of automatic spectrum interpretation for *de novo* sequencing.

As noted by Heraclitus (~500 BC), ''nature is wont to hide herself''. To uncover her secrets, we need to develop tools that are adequate to the task. This section demonstrates that the quest for new and improved structural biology tools continues at a healthy rate.

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