Biophysical methods
Editorial overview

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Current Opinion in Structural Biology 2001, **11**:513–515

0959-440X/00/$ – see front matter

Abbreviations

EM	electron microscopy
EPR	electron paramagnetic resonance
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
NOE	nuclear Overhauser enhancement

New insights into biochemical processes are often the result of improved biophysical methodologies. The excitement created by the flood of atomic-resolution structures of proteins and other biomolecules from X-ray diffraction and NMR spectroscopy in the past decade or so has tended to overshadow the extremely important contributions that have been made — and are continuing to be made — by lower resolution techniques or techniques that focus on only a part of a biopolymer. Yet such contributions not only predate detailed structure determination, but also they frequently are the best follow-up to it. The questions raised by the structure of a macromolecule are usually answerable only by experiments that probe more deeply (or, in the case of electron microscopy, more broadly). In the past several years, a plethora of new experiments encompassing a wide range of different technologies have emerged. Spectroscopic techniques for characterizing structure and dynamics have been refined, increasingly more sensitive methods for probing the energetics of molecular interactions have been published and even more established approaches such as ultracentrifugation are undergoing exciting developments. In addition, it has become possible to study *in vivo* processes using fluorescence resonance energy transfer (FRET) between donor and acceptor sites. Thus, it is now possible to explore various aspects of molecular dynamics and structure in the living cell. These exciting advances foreshadow what will most certainly be a very productive period in biophysics and biomolecular structure studies. The reviews in this section are intended to provide the reader with a fresh perspective on what may sometimes seem to be 'old' techniques. They demonstrate that, for most biological questions, high-resolution structure determination will be only the beginning of what can, and should, be done.

The emerging field of pulsed infrared (IR) spectroscopy is reviewed by Zanni and Hochstrasser (pp 516–522). A major advance in this area has been the development of two-dimensional IR-based spectroscopy, which has, in some cases, rather striking analogies with multidimensional NMR. Two-dimensional spectra are obtained with diagonal peaks corresponding to vibrational modes in the molecule, with cross-peaks diagnostic of coupling between pairs of vibrational modes. Cross-peaks are broadened in ways that indicate interactions between the coupled regions of the molecule, as well as their relative motions. Cross-peaks can also be interpreted to obtain angular and spatial relations between vibrational displacements in the system, and to disentangle distributions of conformations that may exist. Structures of small peptides are beginning to emerge from the application of this exciting methodology, which has the great virtue of being applicable, in principle, to samples in a variety of physical states.

Other spectroscopies have also continued to develop over the past several years. Lakshmi and Brudvig (pp 523–531) describe contributions in the area of pulsed EPR (electron paramagnetic resonance) spectroscopy that enable measurement of distances between pairs of electrons up to 40–60 Å apart, and methods for measuring electron–nuclear interactions that provide shorter range distance information, on the order of 4–6 Å. EPR techniques are particularly important for cell biology because studies of large multisubunit molecules and molecular complexes can be performed readily. In addition, the use of powder samples obviates the requirement for soluble or crystalline materials, which is often a serious limitation in structural studies.

Structural studies of molecules by solution NMR techniques have traditionally depended on the use of nuclear Overhauser enhancement (NOE)-based methods by exploiting the distance dependence of the intensity of correlations in NOE spectra. However, because the correlations scale as r^{-6}, where r is the distance between the proximal spins, distance measurements are limited to interacting nuclei that are within 5–6 Å. Tolman (pp 532–539) describes the use of dipolar coupling technology, which addresses this critical limitation. Dipolar couplings are sensitive indicators of structure because they provide orientational information on internuclear vectors with respect to a common molecular frame. As such, these restraints are particularly powerful for determining the relative orientation of domains in multidomain proteins and for aligning structural elements in a molecule with respect to each other. Dipolar couplings have also been widely used, along with NOE and torsion angle restraints, in the refinement of protein and nucleic acid structures. Most importantly, this technology promises to greatly accelerate the speed at which structures can be obtained from NMR methods.

Advances in the spectroscopic techniques outlined above have had an important impact on structural studies of membrane proteins. In their review article on the different biophysical approaches to the determination of such structures, Arora and Tamm (pp 540–547) discuss the strengths and limitations of solution and solid-state NMR methods. They also suggest that it is likely that detailed structural studies will require more than a single approach. High-resolution structures of small domains determined by solution or solid-state NMR (or even by X-ray diffraction!) can be assembled into medium-resolution structures of the whole molecule determined by electron crystallography. Distance constraints from EPR spectroscopy can be used to assemble structures derived by NMR, X-ray crystallography or electron microscopy (EM) into lipid bilayer environments, and IR spectroscopy can provide information on the orientation of secondary structural elements of proteins in the bilayer.

Traditionally, electron cyro-microscopy has been a method of choice for obtaining structural information on membrane proteins. Typically, low-resolution structures at 6–8 Å can often be obtained within several months once suitable crystals are obtained, whereas structures at approximately 4 Å resolution require significantly longer periods of time. However, advances in cryo-techniques, instrumentation and data acquisition have been forthcoming and, in his review of this exciting area, Unger (pp 548–554) suggests that the generation of suitable biological specimens, rather than data acquisition and analysis, will soon be the limiting factor. Advances have also been made in the study of soluble proteins using lipid monolayers at the water/air interface as crystallization templates. Of special interest, it is possible to obtain information about the function of molecular machines through the use of time-resolved cryo-EM, whereby sample conditions are manipulated on millisecond timescales.

In addition to static structural information, which is available through spectroscopic, X-ray and EM studies of biomolecules, it is also important to obtain insight into how structure changes in time. Over the past decade, a significant effort has been expended in developing NMR-based methods for measuring picosecond to nanosecond dynamics in molecules and, subsequently, in translating the dynamic parameters obtained from experiment into measures of entropy on a per site basis. Spyracopoulos and Sykes (pp 555–559) summarize the state of the art in this area. It must be kept in mind that the NMR experiment senses only the contribution that rapid timescale motions make to entropy and that solvent effects are not explicitly measured. Nevertheless, site-specific information that complements calorimetric-based approaches is available from this approach.

A direct measure of the thermodynamic parameters associated with a binding event can be obtained using isothermal titration calorimetry, as discussed by Leavitt and Freire (pp 560–566). Because this technique allows the dissection of enthalpic and entropic contributions to binding, it is possible to obtain structural information about the binding process, which can be of importance in the design of pharmaceuticals. In this regard, improved experiments have been developed that allow the characterization of very high affinity interactions, which was previously difficult; this is again of significance in drug design. Calorimetry can, in principle, be adapted to screening libraries of compounds for binding to a desired target, so the revival of interest in this technique is expected to continue.

Many biophysical methods measure properties averaged over whole ensembles of molecules. Atomic force microscopy, as reviewed by Yip (pp 567–572), allows one to carry out single-molecule imaging and force spectroscopy. This field has benefited from significant advances in tip design, along with new methods for scanning molecular surfaces. Kinetic phenomena can be studied by real-time imaging, providing insight into the details of protein assembly and function. Forces between pairs of molecules in the piconewton range can be measured, although the forces obtained cannot be simply related to binding affinities. Nevertheless, parameters such as the number of binding sites and the contact area between the partners can be obtained, together with the relative contributions of particular sites on a given receptor to the binding energy. Single-molecule studies represent one of the fastest-growing areas of biophysics and promise to transform the way we think about the stochastic behavior of biopolymers.

Biophysical techniques allowing the observation of dynamic molecular events *in vivo* are also under development. Truong and Ikura (pp 573–578) describe advances in FRET methods which allow one to localize interactions in the cell and to follow the time course of these interactions. Energy transfer is measured between two green fluorescent protein (GFP) variants that can be fused to the same molecule, so that intramolecular FRET is observed, or to different molecules (intermolecular FRET). Over the past several years, a variety of new GFP mutants have been engineered to maximize the sensitivity of the method and a large number of very elegant applications demonstrating molecular interactions *in vivo* have appeared. The importance of knowing the location of a given gene product in a time-dependent manner cannot be overemphasized if one is concerned, as all of us increasingly must be, about function *in vivo*.

Ultracentrifugation is the most widely used hydrodynamic technique for the analysis of macromolecular properties. In the past decade, the technique has become increasingly important as a probe of molecular aggregation state before other studies are carried out, such as those involving NMR spectroscopy, for example. Laue (pp 579–583) points out in his review that significant advances in the past few years, in particular in data analysis, have led to rapid growth in this field. For example, using sedimentation velocity measurements on a heterogeneous solution, it is possible to determine the stoichiometry of components in a

high-affinity complex, as well as the stoichiometry and thermodynamics of complexes that are more weakly associated. This has important applications in the area of proteomics research, for which it is frequently necessary to establish whether molecules bind and, if so, to obtain the correct stoichiometry of the interaction.

In summary, these reviews show that the study of biomolecules by physical methods continues to grow in scope and power. In the future, the greatest advances in our understanding of how these wonderful molecular machines function will come from the application of many such techniques, guided by detailed structural information.