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Editorial overview: Biophysical methods: Multiple structures of proteins underpin their biological functions

Irina Gutsche and Gaetano T. Montelione



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Irina Gutsche, graduated from the Paris Saclay University where she obtained a Ph.D. in biophysics. She then turned to cryo-electron microscopy and performed her postdoctoral training at the Max-Planck Institute for Biochemistry with Prof. Baumeister. Back in France, she first worked with Drs. Felix Rey and Jean Lepault in the Molecular and Structural Virology Laboratory in Gif-sur-Yvette, before joining the Unit of Virus Host Cell Interactions in Grenoble. Since 2015, she is a PI in the Institute of Structural Biology in Grenoble, and in 2023 she was appointed Associate Visiting Professor at the University of Umeå.

Biomolecules sample conformational energy landscapes shaped by their evolution, allowing for various degrees of both internal and intermolecular structural variability. These dynamics underlie their biological function. They span a wide range of timescales, from picoseconds to hours or longer, and amplitudes, with structural variations ranging from vibrations of covalent bonds, to librational backbone motions and sidechain reorientations, to interdomain motions, fold switching, and alternative subunit or interprotein orientations. Conformational changes of proteins depend on their environment, and may be induced by pH or temperature changes, ligand binding, partner interactions, or posttranslational modifications. The various biophysical techniques available for characterizing these dynamic changes each have their own timescale window(s), and exhibit significant complementarity both in the kinds of conformational heterogeneity they monitor and the timescales of motions they are tuned to.

In this issue of Current Opinions in Structural Biology, we have collected a diverse range of reviews highlighting recent advances in biophysical methods for studies of the structural dynamics of proteins. The focus of the collection is on “hybrid” or “integrated” approaches that utilize multiple experimental methods and/or combine experimental and computational techniques, for characterizing conformational distributions of biomolecular structures. Hence, the collection does not cover the full range of significant advances in computational methods, including molecular dynamics simulations (MD) and normal mode analysis.

One of the most important emerging experimental methods for characterizing the multiple conformational states of biomolecules is cryogenic electron microscopy (cryo-EM), where class-averaged 3D image reconstructions often identify a wide range of conformational states in a single sample. Cryo-EM, and even more so cryo-electron tomography (cryo-ET), are also powerful tools for the analysis of continuous conformational variability. These methods are often enhanced through deep learning approaches and integration with molecular dynamics simulations, normal mode analysis, or both. Time-resolved cryo-EM is also gathering

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Gaetano T. Montelione, Gaetano Montelione graduated from Cornell University, Ithaca, NY, with a B.S. in Biochemistry and a Ph.D., under the supervision of Profs. H.A. Scheraga and K. Wüthrich, in Physical Chemistry. He completed postdoctoral studies at The University of Michigan with Prof. G. Wagner, and then joined the faculty at Rutgers, The State University of New Jersey in 1989, where he rose to the rank of Distinguished Professor and inaugural Jerome and Lorraine Aresty Chair in Cancer Biology. In 2019, Montelione was invested as Constellation Endowed Chair in Structural Bioinformatics at Rensselaer Polytechnic Institute, and appointed Professor of Chemistry and Chemical Biology.

pace. However, as a companion issue of COSB focused on cryo-EM methods was in progress at the same time this compendium was developed, these important advances are not covered in this issue.

Representative of the theme of this collection is the review of [Perez and MacCallum](#) focused on hybrid/integrative methods that combine sparse experimental data together with models generated by MD. The authors discuss recent developments, including improved force fields and sampling techniques, that provide more accurate MD-based models of multiple conformational states of proteins and synergize with advances in experimental methods. They outline the critical concepts of *informed sampling*, in which the data is used to guide the modeling, vs *uninformed sampling*, where the modeling is based purely on theoretical principles and experimental data is used to select the model(s) that best fit the data, or to reweight the conformational ensemble distributions. This paradigm is then used to describe various challenges and approaches for hybrid/integrative modeling of protein structural diversity.

Recent advances in deep learning artificial intelligence (DL) for computing protein structures are having transformative impact in the field of structural biology. [Sala, Meiler et al.](#) review the potential of AlphaFold2 and its derivatives in modeling conformational landscapes of proteins. [Bhardwaj et al.](#) describe the complementary problem of using conformational energy potentials and DL methods for designing cyclic polypeptides that flip between structures with polar vs non-polar surfaces, providing an approach for designing membrane permeable polypeptide drugs. While still in their infancy, these applications of generative artificial intelligence for understanding conformational dynamics and *de novo* protein design are emerging research areas, with the potential of overcoming the ergodic limitations of MD in sampling the phase space of alternative conformational states.

Nuclear magnetic resonance spectroscopy (NMR) provides a wide range of experimental methods, each sensitive to dynamic windows of specific timescales, ranging from fast sub-nanosecond torsional librations to slow conformational isomerizations with time constants of seconds or longer. It would be easy to devote an entire issue of COSB to these evolving NMR methods and their applications for characterizing dynamic protein structures and conformational distributions. Here we focused on four particularly exciting areas. [Napoli, Becker, and Shanda](#) review recent technological developments in Magic Angle Spinning (MAS) solid-state NMR (ssNMR) allowing accurate measurements of $R_{1\rho}$ relaxation dispersion. These experiments are sensitive to microsecond-to-millisecond motions. The authors highlight the novel information and applications accessible by ssNMR compared to solution-state NMR relaxation studies, and the unique capabilities of ssNMR for investigating key biologically-important systems. [Necelis, Columbus et al.](#) summarize their experience and related recent literature applying NMR to studies of the internal dynamics of integral membrane proteins (IMPs). As sample preparation of IMPs remains a key challenge to biophysical studies, the authors also review isotope-labeling strategies and various approaches for generating systems amenable to NMR studies of proteins in membrane-mimicking environments, including micelles, bicelles, and nanodiscs. They also review approaches for studying IMP interactions with non-covalent ligands and small molecule screening, which are important for drug development. The complementary pair of reviews by [Parigi et al.](#) and by [Valafar, Abdollahi, and Prestegard](#) describe innovative experimental and computational technologies combining Residual Dipolar Coupling (RDC)

and paramagnetic NMR data, including measurements of Paramagnetic Relaxation Enhancements (PREs) and Pseudo Contact Shift (PCSs), to characterize protein structure rearrangements and to identify low-population conformational states. Several examples are described using these kinds of data, in some cases together with other biophysical data, to model conformational ensemble distributions of proteins in solution. These NMR technologies are poised to make major contributions to our understanding of the relationships between dynamic structures and protein function.

G protein-coupled receptors (GPCRs), the largest family of human transmembrane proteins, are important drug targets. The GPCR signaling cascade has evolved to respond to a multitude of signals, reflected in the diversity of GPCRs and their specific ligands, whereas on the intracellular side, the cascade involves only a restricted number of transducers. In their review, [Junker et al.](#) explore the thermodynamics and evolutionary aspects of the ligand-GPCR-G protein system. They discuss how spectroscopic techniques, including ^1H , ^{13}C , ^{15}N -triple resonance NMR, ^{19}F NMR, and electron paramagnetic resonance (EPR), have been combined with MD simulations and modern DL structure prediction tools to reveal that GPCRs exist in a dynamic ensemble of conformational states, and explain how the formation of the ternary complex reduces the conformational flexibility, incurring an entropic cost.

Other powerful biophysical methods for characterizing conformational dynamics of proteins include fluorescence spectroscopy, particularly Förster Resonance Energy Transfer (FRET), chemical cross-linking, and small angle X-ray scattering (SAXS). In this collection of reviews, [Vedel, Milles, and co-workers](#) describe studies combining NMR and fluorescence spectroscopy to characterize protein dynamics. They illustrate the complementary time- and length scales characterized by various NMR experiments and single-molecule FRET (smFRET) studies, and describe applications of these combined measurements to studies of multiple conformational states, interdomain dynamics, and intrinsically disordered regions (IDRs) of proteins, demonstrating the value of combining multiple spectroscopic measurements to elucidate an accurate picture of the structural dynamics. [Chen and Rappsilber](#) describe chemical cross-linking using mass spectrometry (CCL-MS) technologies for identifying alternative conformational states of proteins in dynamic equilibrium. A unique advantage of CCL-MS is that it can be applied to proteins *in situ* – i.e., even inside of cells. The authors point out that a key challenge in interpreting cross-link data is classifying the short distances detected with respect to which of the multiple conformations they arise from. The authors illustrate various experimental approaches for addressing this challenge, and propose

that the full power of CCL-MS will be realized by combining it with other experimental data such as single-particle cryo-EM or electron tomography, and/or with advanced molecular modeling methods, such as the DL methods outlined in some of the other reviews.

Whereas cryo-EM literally freezes the protein molecules in different states of their dynamic action, high-speed atomic force microscopy HS-AFM allows real-time observation of single molecules during their functioning, delivering both structural and dynamical information simultaneously. [Fleshig and Ando](#) discuss the complementarity of temporal and spatial resolution of HS-AFM and time-resolved cryo-EM. While AFM imaging is inherently a topology imaging technique with limited resolution, the authors review cutting-edge methods inferring higher-resolution information from AFM imaging, again drawing a parallel to cryo-EM and various fitting algorithms. They summarize tools that leverage existing, predicted, or simulated 3D structures to derive atomic-scale 3D conformations from topographic AFM data, and describe direct methods designed to compute higher-resolution maps through improved quantitative analysis of measured topographies.

[Pounot, Schirò, and, Levantino](#) review how information on biological processes is obtained from time-resolved X-ray solution scattering (tr-XSS). Here, large-scale structural changes are probed in transient kinetic studies in solution. While many applications may be envisioned, the representative examples provided include light-activation of intrinsically photosensitive proteins, including structural rearrangements around a targeted protein chromophore, photoactivation of photocaged compounds, such as photocaged-ATP, and thermally-induced (T-jump) studies of protein unfolding. The authors also review recent studies using tr-XSS data to guide and/or interpret MD modeling studies.

With the advent of X-ray free electron lasers (XFEL) and serial femtosecond X-ray crystallography (SFX), time-resolved X-ray crystallography (TR-XRD) is gaining new momentum. The review of [Bessaw and Miller](#) underscores the necessity to carefully choose the appropriate illumination conditions in order not to compromise the biological significance of the results and to generate the desired photoexcited state and relevant structural dynamics. The authors put forth a set of guidelines for future time-resolved structural biology experiments conducted at ultrabright sources. A second review on TR-XRD at synchrotron and XFEL sources by [Makita et al.](#) explains the benefits of a systematic integration of TR-XRD with concurrent spectroscopic experiments. Such a tandem approach empowers the interpretation of structural data regarding protein dynamics, informing about intermediates of enzymatic reactions and their associated

kinetics. Additionally, it provides a means to assess radiation-induced damage and sample quality *in situ* and in real-time. The authors substantiate their arguments through multiple biological case studies, and emphasize the importance of integrating suitable spectrometers at the X-ray beamlines.

Royer *et al.* broaden the scope of protein dynamics, shifting from a focus solely on a protein's dynamic conformational changes to encompassing the dynamics of protein populations within cells. They delve into quantitative fluorescence imaging and spectroscopy methods designed to precisely determine the quantity and concentration of specific biomolecules in individual cells or intracellular regions of interest. The review particularly highlights the utilization of scanning number and brightness (sN&B) analysis and its derivative, Raster scanning image correlation spectroscopy (RICS), which leverage stochastic noise within small measurement volumes to accurately measure protein abundance, local concentration, stoichiometry, and dynamics *in situ*.

Finally, this issue includes a commentary from the laboratory of one of the editors, Ramelot, Tejero, and Montelione, on the urgent need to consistently represent, share, and archive information about the structures and dynamics of the multiple conformational states of proteins determined by these various biophysical methods. While some conventions are already established, such as the *Altloc* tags utilized to represent multiple atomic occupancies of some X-ray crystal structures, for many experimental methods the data, atomic coordinates, and position uncertainties generated from studies of multiple conformational states are not adequately supported in modeling software and structural databases. The authors highlight this problem for the particular case of solution NMR protein structures. Intentionally, a specific solution to this problem is not proposed, as addressing this challenge will require community-wide discussions and consensus.

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