INSTRUMENTAL ANALYSIS OF INTRINSICALLY DISORDERED PROTEINS
WILEY SERIES ON PROTEIN AND PEPTIDE SCIENCE

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INSTRUMENTAL ANALYSIS OF INTRINSICALLY DISORDERED PROTEINS

Assessing Structure and Conformation

Edited by

VLADIMIR N. UVERSKY AND SONIA LONGHI
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The notion that protein function relies on a precise three-dimensional (3D) structure constitutes one of the central paradigms of biochemistry. According to this concept, a protein can perform its biological function(s) only after folding into a unique 3D structure, and all the information necessary for a protein to fold into this unique 3D structure is encoded in the amino acid sequence. Only recently has the validity of this structure–function paradigm been seriously challenged, primarily through the wealth of counterexamples that have gradually accumulated over the past 20–25 years. These counterexamples demonstrated that many functional proteins or protein parts exist in an entirely or partly disordered state. Intrinsically disordered proteins (IDPs), also referred to as natively unfolded proteins, lack a unique, stable 3D structure in solution, existing instead as a dynamic ensemble of conformations. Many IDPs function without a prerequisite stably folded structure. The protein flexibility that is inherent to disorder confers functional advantages. Their increased plasticity (1) enables the binding of or to numerous structurally distinct targets; (2) provides the ability to overcome steric restrictions by enabling larger surfaces of interaction in protein complexes than those obtained with rigid partners; and (3) allows protein interactions to occur with both high specificity and low affinity.

IDPs possess peculiar sequence properties that allow them to be distinguished from globular proteins. In particular, (1) they are generally enriched in amino acids preferred at the surface of globular proteins (i.e., A, R, G, Q, S, P, E, and K) and depleted in core-forming residues (W, C, F, I, Y, V, L, and N); (2) they possess a distinct combination of a high content of charged residues and of a low content of hydrophobic residues; (3) they typically possess a low predicted secondary structure content; (4) they tend to have a low sequence complexity (i.e., they make use of fewer types of amino acids);
and (5) they often have a high sequence variability. The peculiar sequence features of IDPs have led to the development of various disorder predictions, which have allowed an estimation of the occurrence of disorder in biological systems. Computational studies have shown that the frequency and length of disordered regions increases with increasing an organism’s complexity. For example, long intrinsically disordered regions have been predicted to occur in 33% of eukaryotic proteins, with 12% of these latter being fully disordered. Furthermore, viruses and eukaryota were predicted to have 10 times more conserved disorder (roughly 1%) than archaea and bacteria (0.1%). Beyond these computational studies, an increasing amount of experimental evidence has been gathered in the last years pointing out the large abundance of intrinsic disorder within the living world: More than 523 proteins containing 1195 disordered regions have been annotated so far in the Disprot database (http://www.disprot.org).

Regions lacking specific 3D structure have been so far associated with a number of distinct biological functions, including nucleic acid and protein binding, interaction with small molecules, display of posttranslational modification sites, display of proteolysis sites, and prevention of interactions by means of excluded volume effects. Most IDPs are involved in functions that imply multiple partner interactions (e.g., one-to-many and many-to-one binding scenarios), such as molecular recognition, molecular assembly (and amyloidogenesis), cell cycle regulation, signal transduction, and transcription. As such, IDPs are implicated in the development of several pathological conditions, including cancer and cardiovascular diseases. They have been shown to be promising targets for drug development.

Intrinsic disorder is a distinctive and common feature of “hub” proteins, with disorder serving as a determinant of protein promiscuity. A recent study indicates that the size of macromolecular assemblies is related to the abundance of disorder of the protein components. Intrinsic disorder also serves as a determinant of the transient nature of the interactions that IDPs can establish, by virtue of the presumed rather low affinity that typifies interactions involving IDPs. Indeed, the ability of “social hubs” to establish transient interactions has been associated to the overall flexibility of the hub proteins. The relationship between structural disorder and regulation provides a plausible explanation for the prevalence of disorder in higher organisms, which have more complex signaling and regulatory pathways. However, the abundance of disorder within viruses likely reflects the need for genetic compaction, where a single disordered protein can establish multiple interactions and hence exert multiple concomitant biological effects. In addition, structural disorder might endow viral proteins with broader ability to interact with the components of the host and may also be related to high adaptability levels and mutation rates observed in viruses, thus representing a unique strategy for buffering the deleterious effects of mutations.

Although there are IDPs that carry out their function while remaining disordered all the time (e.g., entropic chains), many of them undergo a disor-
order-to-order transition upon binding to their physiological partner(s), a process termed induced folding. IDPs show an extremely wide diversity in their structural properties: They can attain extended conformations (random coil like) or remain globally collapsed (molten globule like), where the latter possess regions of fluctuating secondary structure. Conformational and spectroscopic analyses showed that random coil-like IDPs can further be divided into two major groups. While the first group consists of proteins with extended maximum dimensions typical of random coils with no (or little) secondary structure, the second group comprises the so-called premolten globules, which are more compact (but still less compact than globular or molten globule proteins) and contain some residual secondary structure. The residual intramolecular interactions that typify the premolten globule state may enable a more efficient start of the folding process induced by a partner.

Many IDPs bind to their target(s) through “molecular recognition elements” (MoREs) or “molecular recognition features” (MoRFs). MoRFs are interaction-prone short segments with an increased foldability, which are embedded within long disordered regions and which become ordered upon binding to a specific partner. The conformation of MoRFs in isolation can be either disordered or partially preformed, thus reflecting an inherent conformational preference. In this latter case, a transiently populated folded state would exist even in the absence of the partner, thus implying that the folding induced by the partner would rely on conformer selection (i.e., selection by the partner of a preexisting conformation) rather than on a “fly-casting” mechanism. It has been proposed that the restriction in the conformational space of MoRFs in the unbound state could reduce the entropic cost of binding thereby enhancing affinity. IDPs can bind their target(s) with a high extent of conformational polymorphism, with binding generally involving larger normalized interface areas than those found between rigid partners, with protein interfaces being enriched in hydrophobic residues. Thus, protein–protein interactions established by IDPs rely more on hydrophobic–hydrophobic than on polar–polar contacts.

Although a large body of experimental evidence of the abundance (and biological relevance) of disorder in the living world has been gathered in the last decade, the notion of a tight dependence of protein function on a precise 3D structure is still deeply anchored in many structuralists’ mind. The reasons for this lack of awareness or even “resistance” to the concept of protein intrinsic disorder are multiple. First, the growing numbers of protein structures determined by X-ray crystallography and by NMR in the last three decades has shifted the attention of structuralists away from the numerous examples of IDPs. Second, IDPs have been long unnoticed because researchers encountering examples of structural disorder mainly ascribed it to errors and artifacts, and as such, purged them from papers and reports. Third, structural disorder is hard to conceive and classify. Fourth, IDPs have been neglected because of the perception that a limited amount of mechanistic data could be derived from their study.
The evidence that IDPs exist both in vitro and in vivo is compelling and justifies considering them as a separate class within the protein realm. Furthermore, their distinguishing interaction abilities justify designating the protein–protein interactions they establish as “close encounters of the third kind.” Key questions that have to be addressed to unravel the way IDPs exert their biological functions concern how they can be correctly recognized by their partner proteins in the absence of a folded, stable structure, and assessing the extent to which these proteins are structurally preconfigured prior to partner protein binding.

Answering these questions requires appropriate methods for the description of conformational ensembles in the free, pre-recognition states, as well as in complex with their partner proteins. IDPs inherently escape structural characterization by conventional high-resolution techniques. In fact, since IDPs exist as a dynamic continuum of conformations, their crystallization is generally precluded, and in those rare cases where it is successful, it only leads to a snapshot of poorly representative conformations. As such, the structural characterization of IDPs requires the use of combined, complementary physicochemical approaches. In this book, a thorough description of the principal experimental approaches that can be used to assess structural disorder and induced folding is provided. We highlight the type of information that can be derived by the different approaches and point out how information can be broadened by using different methods.

The book has seven sections dedicated to (1) assessment of IDPs in the living cell (chapters 1 and 2), (2) spectroscopic techniques for the analysis of IDPs (chapters 3–12), (3) single-molecule techniques applied to the study of IDPs (chapters 13 and 14), (4) assessment of IDP size and shape (chapters 15–18), (5) tools for the analysis of IDP conformational stability (chapters 19 and 20), (6) mass spectrometry (chapter 21), and (7) approaches for expression and purification of IDPs (chapters 22–24). A brief outline of the corresponding chapters is presented below.

In chapter 1, Yosef Shaul, Peter Tsvetkov, and Nina Reuven represent an overview of the mechanisms underlining the process of intracellular IDP degradation and describe experimental techniques used for the analysis of IDP degradation in vivo. The authors also describe various mechanisms used by IDPs for escaping degradation by default and show that several human diseases, including certain types of cancers and neurological disorders, can result from aberrant protein degradation.

In chapter 2, Philipp Selenko introduces a set of in-cell NMR methods that are used to extend the structural understanding of IDPs in a cellular environment. It is emphasized that these techniques are comparable to an atomic resolution microscope and, therefore, can provide high-resolution structural information on proteins, including IDPs, inside prokaryotic and eukaryotic cells.

Chapter 3, authored by Frans A. A. Mulder, Martin Lundqvist, and Ruud M. Scheek, is dedicated to the description of the peculiarities of IDP
analysis by NMR spectroscopy, which is believed to be the most suitable tool to investigate the behavior of these highly dynamic proteins. The authors emphasize that many NMR parameters, including chemical shifts, line widths, spin-spin multiplet patterns, relaxation rates, and residual dipolar couplings, are atom specific and harbor information about the local conformations that polypeptide chains adopt, including record of their dynamic behavior. As such, they provide unique information on the peculiarities of IDP structure.

In chapter 4, Martin Blackledge, Pau Bernadó, and Malene Ringkjøbing Jensen show how IDP ensembles and local structural propensity in IDPs can be characterized at the atomic level using NMR residual dipolar couplings that report on time- and ensemble-averaged conformations up to the millisecond timescale. The authors conclude that the combination of appropriate ensemble descriptions allows the extraction of unique and important information on the conformational propensities of IDPs.

In chapter 5, Gary W. Daughdrill describes approaches for the determination of realistic structural ensembles of IDPs and emphasizes that this task requires the development of suitable experimental and computational methods. Very well-suited experimental techniques are small-angle X-ray scattering (SAXS), which can be used for the determination of a Boltzmann-weighted distribution of gyration radii, and NMR spectroscopy, which provides ensemble-averaged angular and interatomic distance information. The usefulness of this approach is illustrated in the case of the intrinsically disordered transactivation domain of the human tumor suppressor, p53.

Valérie Belle, Sabrina Rouger, Stéphanie Costanzo, Sonia Longhi, and André Fournel, in chapter 6, introduce site-directed spin labeling electron paramagnetic resonance (EPR) spectroscopy as a useful and unique tool for the structural analysis of IDPs. The theoretical principles of this approach are introduced and an illustrative example is provided highlighting the adequacy of this method to investigate the structural properties and the induced folding of IDPs.

In chapter 7, Reinhard Schweitzer-Stenner, Thomas J. Measey, Andrew M. Hagarman, and Isabelle C. Dragomir consider the application of vibrational spectroscopy in the structural analysis of IDPs and unfolded peptides. This chapter introduces the basic physical concepts and reviews utilization of UV resonance Raman, visible nonresonance Raman, vibrational circular dichroism (CD), Raman optical activity, and electronic CD spectroscopies for the exploration of IDPs and disordered peptides.

Chapter 8, by Antonino Natalello and Silvia Maria Doglia, is focused on applications of Fourier transform infrared spectroscopy in the analysis of IDPs and induced folding. This chapter represents a general survey of the standard experimental methods to obtain the infrared absorption spectrum of a protein, together with the data analysis that enables evaluation of the secondary structure content. The potential of infrared spectroscopy to document induced folding is illustrated through examples of IDPs that either fold in the presence
of different effectors, such as DNA, partner proteins, and osmolytes, or undergo amyloid aggregation.

Chapter 9 by Natalya I. Topilina, Vitali Sikirzhytski, Seiichiro Higashiya, Vladimir V. Ermolenkov, John T. Welch, and Igor K. Lednev describes the analysis of IDP structure and aggregation by deep UV resonance Raman spectroscopy (DUVRR). DUVRR is a novel method for acquisition of quantitative information on the peptide backbone conformation. In particular, the authors focused on the use of genetic engineering in the study of the mechanism of fibrillation of large biopolymers that are excellent models for IDPs. They believe that the selective design of the polypeptide sequence by genetic engineering represents a great tool for studies of the relationship between the sequence and the cross-β-sheet structure.

In chapter 10, Robert W. Woody describes application of CD to analysis of IDPs. As IDPs are readily recognized by CD, a large number of IDPs have been identified and characterized using this technique. Complex (e.g., heterogeneous) IDPs containing folded domains interspersed with extensive unordered regions are more difficult to diagnose. These IDPs can be analyzed by combining limited proteolysis with CD, where CD analysis of the individual domains identified by proteolysis can be used to decipher their modular organization.

Fluorescence spectroscopy of IDPs is discussed by Eugene A. Permyakov and Vladimir N. Uversky in chapter 11. As IDPs are typically characterized by surface location of tryptophan residues, their intrinsic fluorescence is red-shifted and is readily quenched by external fluorescence quenchers. However, IDP interactions with binding partners transfer tryptophan residues into a more hydrophobic or more rigid environment, resulting in a measurable blue-shift of the fluorescence maximum position and a noticeable decrease in the quencher accessibility. Furthermore, spectral probes and labels are widely used for IDP analysis.

Chapter 12 (by Kálmán Tompa, Monika Bokor, and Peter Tompa) is dedicated to the analysis of structural and dynamical properties of interfacial water at the protein surface by wide-line NMR spectroscopy and nuclear relaxation time measurements. The authors provide a detailed description of the theoretical background and practice of this approach, followed by the description of its implementation on ordered and disordered proteins.

Benjamin Schuler in chapter 13 introduces methods for single-molecule spectroscopy of IDPs. Recent years have seen a remarkable development of single-molecule spectroscopy, especially single-molecule Förster resonance energy transfer (FRET), which has evolved into a versatile method to probe distances, distance distributions, and dynamics of unfolded proteins, including IDPs.

The theme of single-molecule spectroscopy is further addressed in chapter 14, where Massimo Sandal, Marco Brucale, and Bruno Samorì describe the technique of single-molecule force spectroscopy (SMFS), a description of the various SMFS experimental approaches applicable for protein conformational
analysis, and application of these techniques for the evaluation of the conformational equilibria and structural diversity of IDPs.

Chapter 15 starts the section dedicated to various hydrodynamic techniques. This chapter, authored by Florence Manon and Christine Ebel, introduces analytical ultracentrifugation (AUC) and describes how this technique can be used to probe and characterize the size and oligomeric state of IDPs. The authors provide basic information on theory of AUC and describe the AUC instrumentation. They show how the sedimentation phenomenon can be used to evaluate various hydrodynamic parameters of IDPs and how two usual types of AUC experiments, sedimentation equilibrium (SE) and velocity (SV), are used for IDP characterization.

Pau Bernadó and Dmitri I. Svergun in chapter 16 describe the type of information on IDPs that can be extracted by SAXS, which is one of the very few techniques yielding low-resolution structural information about flexible macromolecules. This chapter introduces the technical and experimental details of SAXS, including classical approaches based on the analysis of overall parameters and a recent development, the ensemble optimization method. The latter approach, in combination with other biophysical techniques such as NMR, FRET, and molecular simulations, provides unique insights into the analysis of IDP structure and structural perturbations induced by the environmental changes or binding to biological partners.

Chapter 17, authored by Klaus Gast, describes dynamic and static light scattering (SLS) and shows how these techniques can be used to evaluate various molecular parameters of IDPs, such as size, molar mass, and intermolecular interactions. The physical bases of light scattering, experimental techniques, sample treatment, and data evaluation schemes are outlined, with special emphasis on studies on IDPs. It is also emphasized that since SLS and dynamic light scattering (DLS) yield different physical quantities of macromolecules, the combined application of these techniques improves the accuracy of the molar mass evaluation and is essential when changes in the molecular dimensions and molecular association/dissociation take place simultaneously.

Chapter 18 by Vladimir N. Uversky is focused on size exclusion chromatography (SEC) and its applications for the analysis of IDPs, allowing estimation of the hydrodynamic dimensions, evaluation of the association state, analysis of IDP interactions with binding partners, and assessment of induced folding events. As SEC can physically separate IDP conformers based on their hydrodynamic dimensions, this method provides a unique possibility for the independent analysis of the physicochemical properties of these conformers.

The conformational behavior of IDPs is characterized by the low cooperativity of the denaturant-induced unfolding, lack of the measurable excess heat absorption peak(s) in calorimetric analysis, “turned out” response to heat and changes in pH, the ability to gain structure in the presence of various counterions, and the unique response to macromolecular crowding. Chapter 19 (by Vladimir N. Uversky) describes these unique features of the conformational behavior of IDPs.
Angelo Fontana, Patrizia Polverino de Laureto, Barbara Spolaore, Erica Frare, and Marcello Zambonin in chapter 20 show how limited proteolysis can be used to analyze protein structure and dynamics, and to identify disordered sites or regions within otherwise folded globular proteins. Often, sites of limited proteolysis coincide with sites of enhanced flexibility of the polypeptide chain, that is, with regions characterized by high values of the crystallographic temperature factor (B-factor) or with regions of missing electron density. As limited proteolysis can be used to probe protein structure and dynamics, and to detect sites of disorder in proteins, this technique complements other physicochemical and computational approaches.

Mária Šamalíková, Carlo Santambrogio, and Rita Grandori dedicate chapter 21 to the description of mass spectrometry tools, which recently became central to structural biology, and their applications for the investigation of the structure and the dynamics of protein conformations and protein assemblies. The authors focus on methods based on maintenance of noncovalent interactions under electrospray conditions, such as charge-state distribution analysis and ion mobility, and show that these methods offer useful structural information, which are complementary to that gained by other biophysical techniques.

Dmitri Tolkatchev, Josee Plamondon, Richard Gingras, Zhengding Su, and Feng Ni dedicate chapter 22 to the description of a procedure for recombinant expression of IDPs in *Escherichia coli*, which leads to yields comparable to and even higher than those typically obtained for well-folded proteins. The method is based on the high-level expression of IDPs fused to a carrier protein derived from the N-terminal oligonucleotide binding domain of staphylococcal nuclease, leading to the accumulation of inclusion bodies in the cell.

In chapter 23, Vladimir N. Uversky, Marc S. Cortese, Peter Tompa, Veronika Csizmok, and A. Keith Dunker argue that the unique conformational behavior of IDPs (such as their high temperature and acid stability, and apparent insensitivity to chemical denaturation) can be exploited for the large-scale identification of IDPs and isolation of these proteins from cell extracts. Aviv Paz, Tzviya Zeev-Ben-Mordehai, Joel L. Sussman, and Israel Silman continue the theme of IDP purification in chapter 24. The authors survey some of the specific procedures reported in the literature for purifying recombinant IDPs that are sensitive to the degradation machinery of the host cell in which they are being overexpressed. They also describe approaches elaborated to stabilize IDPs in the course of purification.

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INTRODUCTION TO THE WILEY SERIES ON PROTEIN AND PEPTIDE SCIENCE

Proteins and peptides are the major functional components of the living cell. They are involved in all aspects of the maintenance of life. Their structural and functional repertoires are endless. They may act alone or in conjunction with other proteins, peptides, nucleic acids, membranes, small molecules and ions during various stages of life. Dysfunction of proteins and peptides may result in the development of various pathological conditions and diseases. Therefore, the protein/peptide structure-function relationship is a key scientific problem lying at the junction point of modern biochemistry, biophysics, genetics, physiology, molecular and cellular biology, proteomics, and medicine.

The *Wiley Series on Protein and Peptide Science* is designed to supply a complementary perspective from current publications by focusing each volume on a specific protein- or peptide-associated question and endowing it with the broadest possible context and outlook. The volumes in this series should be considered required reading for biochemists, biophysicists, molecular biologists, geneticists, cell biologists and physiologists as well as those specialists in drug design and development, proteomics and molecular medicine with an interest in proteins and peptides. I hope that each reader will find in the volumes within this book series interesting and useful information.

First and foremost I would like to acknowledge the assistance of Anita Lekhwani of John Wiley & Sons, Inc. throughout this project. She has guided me through countless difficulties in the preparation of this book series and her enthusiasm, input, suggestions and efforts were indispensable in bringing the *Wiley Series on Protein and Peptide Science* into existence. I would like to take this opportunity to thank everybody whose contribution in one way or another has helped and supported this project. Finally, special thank you goes to my wife, sons and mother for their constant support, invaluable assistance, and continuous encouragement.
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