Digested disorder
Quarterly intrinsic disorder digest (January/February/March, 2013)

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The current literature on intrinsically disordered proteins is blooming. A simple PubMed search for “intrinsically disordered protein OR natively unfolded protein” returns about 1,800 hits (as of June 17, 2013), with many papers published quite recently. To keep interested readers up to speed with this literature, we are starting a “Digested Disorder” project, which will encompass a series of reader’s digest type of publications aiming at the objective representation of the research papers and reviews on intrinsically disordered proteins. The only two criteria for inclusion in this digest are the publication date (a paper should be published within the covered time frame) and topic (a paper should be dedicated to any aspect of protein intrinsic disorder). The current digest covers papers published during the period of January, February and March of 2013. The papers are grouped hierarchically by topics they cover, and for each of the included paper a short description is given on its major findings.

Introduction

The protein intrinsic disorder phenomenon is becoming a popular research topic. This bold statement is illustrated by Figure 1 which represents the results of a very simple PubMed search for just two terms, “intrinsically disordered protein” OR “natively unfolded protein,” which returned almost 1,800 hits (as of June 17, 2013). Figure 1 shows that the number of protein disorder-related publications increases exponentially and that the number of papers published in 2012 only reaches 391 counts, clearly exceeding the one-paper-per-day limit. Obviously, the number of papers derived via this simple PubMed search represents a lower limit of protein disorder-related studies, since many more papers are dedicated to proteins whose intrinsically disordered nature is known but this fact is not mentioned in the corresponding study. For example, PubMed search for “(intrinsically disordered) OR (natively unfolded) AND synuclein” returned 172 hits, whereas there are 5,405 papers in PubMed which contain “synuclein” in their texts.

Since the field is developing fast and a number of important observations related to protein intrinsic disorder are now reported on a daily basis, a “Digested Disorder” project is started by the Intrinsically Disordered Proteins journal. The goal of this project is to keep the interested readers updated on the recent developments in the field by providing a reader’s digest-kind of articles which would represent objective overviews of the research papers and reviews on intrinsically disordered proteins published during the specified period. It is planned that the Digested Disorder articles will appear at the end of each published issue. No specific filtering will be used and any PubMed annotated paper dealing with the protein intrinsic disorder phenomenon and published during the period covered will be included in the digest. The digest article is structured hierarchically and papers are grouped in several sections: (1) structures of intrinsically disordered proteins (IDPs); (2) functions of IDPs; (3) methods for the IDP analysis; (4) proteomics of IDPs; (5) IDPs and diseases; and (6) IDPs/IDPRs as drugs or drug targets. One should keep in mind that the unambiguous classification of many papers is challenged by the intertwining of topics they cover.

Studies covered by the first paper of this series are research papers and reviews published during the period of January, February and March of 2013. The target papers were found by the PubMed search engine using the advanced search tool with the query containing the following terms “((intrinsically disordered protein) OR (natively unfolded protein)) AND (“2013/01/01” [Date - Publication]: “2013/03/31” [Date - Publication]).” The retrieved 114 papers were further analyzed to make sure that they are published within the specified period and that they are indeed dealing with the IDPs or IDP regions (IDPRs). Any publications that did not satisfy the chosen search criteria were removed.

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www.landesbioscience.com Intrinsically Disordered Proteins
Studies on Structural Properties of IDPs and IDPRs

IDPs/IDP regions are different from ordered proteins and domains not only at the level of their amino acid sequences, but at higher levels of structural organization too, possessing a wide array of characteristic and recognizable structural properties. For example, their conformational ensembles contain highly dynamic structures that interconvert on a number of timescales. Although in early studies it was emphasized that IDPs/IDP regions could be crudely grouped into two major classes, proteins with extended and compact disorder, it is now recognized that IDPs/IDPRs are characterized by extreme sequential, structural, and spatiotemporal heterogeneity of IDPs; the rough and relatively flat energy landscapes; the ability to undergo both induced folding and induced unfolding; the ability to interact specifically with structurally unrelated partners; the ability to gain different structures at binding to different partners; and the ability to keep essential amount of disorder even in the bound form. In fact, currently available data suggest that intrinsic disorder can have multiple faces, can affect different levels of protein structural organization, and whole proteins, or various protein regions can be disordered to a different degree. According to this classification, proteins can contain more or less of intrinsic disorder, and IDPs/IDPRs can be less or more compact, possess smaller or larger numbers of flexible secondary structure and contain smaller or larger numbers of tertiary contacts. Papers represented in this section are about various structural aspects and conformational behavior of IDPs and IDPRs.

Since neither IDPs nor IDPRs are able to fold autonomously into specific structures, their structural description requires conformational ensemble-based approaches. Mao et al. evaluated recent advances in quantifying sequence-ensemble relationships achieved through a four-way synergy between bioinformatics, biophysical experiments, computer simulations and polymer physics theories. The authors concluded that understanding of the quantitative relationships between information encoded in the amino acid sequences of disordered proteins and the ensemble of conformations they sample is crucial for the development of quantitative models able to unambiguously describe the sequence-ensemble relationships of intrinsically disordered proteins.

Tantos et al. performed a comprehensive structural characterization of an interesting highly phosphorylated IDP, human nucleolar phosphoprotein p140 (hNopp 140). The authors utilized a wide spectrum of techniques, such as anomalous electrophoretic mobility, protease sensitivity, heat stability, hydrodynamic behavior on size-exclusion chromatography, wide-line NMR analysis, 1H-NMR spectroscopy, differential scanning calorimetry, circular dichroism spectroscopic analysis under the variety of conditions, and partner binding. According to this multiparametric analysis, hNopp 140 a 699 residues-long protein that has ~20 phosphorylated residues and serves as protein inhibitor of casein kinase 2 (CK2), was shown to behave as a native pre-molten globule with a noticeable predisposition to become ordered in the presence of its binding partner.

Marsini et al. used a small-angle X-ray scattering (SAXS)-based ensemble optimization method to analyze the effect of phosphorylation on structural properties of the regulatory domain (RD) of the cystic fibrosis transmembrane conductance regulator (CFTR), which acts as an anion channel activated by protein kinase A phosphorylation in the norm and which is compromised in cystic fibrosis. SAXS analysis revealed that phosphorylation at multiple sites induces noticeable compaction of this intrinsically disordered domain. These SAXS data were used in an ensemble optimization method to build the first experiment-based, low-resolution 3D-models of the native and the phosphorylated RD.

Based on the computational and experimental analyses, Elam et al. concluded that temperature and urea possess opposing impacts on polyproline II (PII) conformational bias. In fact, the predicted and experimentally observed PII propensity was shown to decrease with an increase in temperature, whereas urea promoted the PII conformation.

Mizuguchi and Okazawa reviewed currently available information on structural properties of the polyglutamine tract-binding protein 1 (PQBP1). PQBP1 is an intrinsically disordered nuclear protein regulating pre-mRNA splicing and transcription, mutations in which are associated with hereditary mental retardation. Although the 1H-15N HSQC NMR spectra of the polar domain and the C-terminal domain of PQBP1 are typical of highly disordered proteins, a continuous 23-residue segment of the C-terminal domain is used in binding to target protein U5–15 kD.
Analyzing Functions of IDPs and IDPRs

The extreme conformational plasticity of IDPs and IDPRs is crucial for their intricate and multifarious biological roles. Functions of IDPs and IDPRs complement functional repertoire of ordered proteins and domains, with disordered proteins being commonly involved in a wide range of intermolecular interactions.15-17 Furthermore, because sites within their polypeptide chains are highly accessible, IDPs can undergo extensive post-translational modifications (PTMs), such as phosphorylation, acetylation, and/or ubiquitination (sumoylation, etc.), allowing for modulation of their biological activity or function.18,24-26 Many IDPs and IDPRs are known to undergo complete or partial disorder-to-order transition as a result of interaction with specific binding partner(s).20,21 Some IDPRs possess remarkable binding promiscuity, being capable of specific interactions with many structurally unrelated partners.22 IDPRs are also able to gain different structures at binding to different partners,22,23 and many IDPs[IDPRs are characterized by the ability to keep essential amount of disorder even in their bound forms.8,24-26 Inside the cell, the abundance and functionality of IDPs, these crucial regulators of almost all cellular processes, are under tight surveillance.27,28 However, although many IDPs were shown to be less abundant than ordered proteins due to the lower rate of protein synthesis and shorter protein half-lives, some IDPs are known to be present in cells in large amounts or for long periods of time due to either specific PTMs or via interactions with other factors, which could promote changes in cellular localization of IDPs or protect them from the degradation machinery.4,19,29-31 Papers collected in this section are dedicated to the various functional aspects of IDPs and IDPRs.

An interesting discussion eliminating two opposite viewpoints on the phenomenon of protein intrinsic disorder was represented in published side-by-side papers by Uversky and Dunker32 and Janin and Sternberg.33 Uversky and Dunker argued that protein intrinsic disorder represents a novel and useful concept that helps better understanding protein functionality and correlation between protein structure and function.32 Janin and Sternberg took an opposing viewpoint, arguing that flexibility, not disorder, is an intrinsic property of proteins, and that most IDPs are in fact proteins waiting for a partner (PWP), which serve as parts of a multi-component complex that do not fold correctly in the absence of other components.33

Ubiquitination represents an important step in regulating functions of some proteins and in defining fate of other proteins. One of the best known applications of protein ubiquitination is the highly specific targeting of substrates for proteasomal degradation. Ubiquitination requires the participation of at least three different enzymes, a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3). To understand the determinants for the site-specific ubiquitination by E3 ligase, Landre et al. studied the roles of the E3 ligase docking site located within an intrinsically disordered Mf2 domain of the interferon regulatory factor-1 (IRF-1) in ubiquitination of this short-lived interferon γ-regulated transcription factor.34 The analysis revealed that the IRF-1 ubiquitination happened predominantly at the lysine residues located within loops protruding from the DNA-binding domain, whereas the highly disordered C-terminal half of the protein was not modified at all. Furthermore, the efficiency of the IRF-1 ubiquitination was shown to be modulated by binding of this transcription factor to DNA, suggesting the DNA-bound pool of IRF-1 is functionally protected from polyubiquitination and degradation.34

A phenomenological model was proposed by Das et al. to explain the N-type inactivation of potassium channels by their intrinsically disordered cytosolic loops.35 This model suggested the N-type inactivation through disordered regions, which is known to be stereospecific and dependent on the channel type, represents an illustrative example of the disorder-based function through conformational selection and not vs. induced fit. Importantly, this study also suggested that some subtle changes in the amino acid sequences of disordered regions represent crucial modulating factors defining binding efficiency and specificity in the absence of folding.35

A comprehensive review by Kovacsv et al. is dedicated to the analysis of various mechanisms of the binding-induced folding of IDPs and to the elucidation of the roles of IDPs in assisting folding of other proteins.20 Among subject covered in this important review are molecular mechanisms of IDP binding, such as induced folding and conformational selection; the use of short motifs and large disordered domains in recognition by IDPs; folding of IDPs[IDPRs before and after binding; binding of IDPs[IDPRs without folding (fuzziness).36 The authors also analyzed the interplay between protein disorder in chaperones and the assistance of folding of other protein. Among subjects covered there are molecular mechanisms of chaperone action, description of chaperone machines with IDPRs, fully disordered chaperones, late embryogenesis abundant proteins, RNA chaperones, and action of disordered chaperones via mutual induced folding transitions.36 Finally, the various roles of intrinsic disorder in protein quality control system were considered, with major focus being at the ubiquitin–proteasome system and its components, such as E3 ubiquitin ligases, as well as at the role of structural disorder in substrate recognition function of chaperones.36

The topics of the intrinsic disorder-based protein interactions and various modes of their modulation, regulation and control are continued in a review by Uversky.37 Here, various interaction modes of IDPs[IDPRs are compared with the interaction mechanisms of ordered proteins and domains. Considered binding models of ordered proteins include a classic “lock-and-key” model, “induced fit” hypothesis, “conformational selection” mechanism, extended “conformational selection” model (where conformational selection is followed by conformational adjustments), and the “game theory” model.37 On the other hand, intrinsic disorder-based interactions can result in the formation of various static and dynamic (fuzzy) complexes via several mechanisms, such as binding-induced folding, conformational preference, fly-casting, and game theory for static complexes, and
analysis revealed that prothymosin α, and Kelch domain of Kelch-like ECH-associated protein 1 (Keap1). This interaction was characterized by nuclear magnetic resonance spectroscopy, isothermal titration calorimetry, peptide array analysis, site-directed mutagenesis, and molecular dynamic simulations. The analysis revealed that prothymosin α retains a high level of flexibility, even in the bound state with Kelch. Furthermore, based on the mutational analysis of prothymosin α, guided by peptide array data and isothermal titration calorimetry, the prothymosin α region NANEENG was shown as a primary binding site for the Kelch domain of Keap1.

Huang et al. investigated regulation of the interactions between the cytoplasmic poly(A)-binding protein C1 (PABPC1) and the PAM2 motif-containing proteins. In silico analysis revealed that PAM2 motifs are embedded within IDPRs next to next to cluster(s) of potential phosphorylation sites, suggesting that phosphorylation at these sites is needed for regulation of the interactions between PAM2-containing proteins and PABPC1. This hypothesis was validated via the analysis of the effect of variable phosphorylation on the PABPC1 binding affinity of three PAM2-containing proteins (Tob2, Pan3, and Tnrc6c).

An interesting mechanism of phosphorylation-induced regulation of a hybrid protein containing ordered and intrinsically disordered domains was described by Chen et al. These authors analyzed an F-actin binding and bundling protein domain (band 4.9) and showed that phosphorylation of the well folded C-terminal villin-type headpiece of this protein increased the affinity of this structured module to the intrinsically disordered N-terminal core domain of dematin leading to the formation of a compact structure that sterically eliminated one of the F-actin binding sites, thereby reducing the F-actin bundling activity of this important regulatory protein.

Kjaergaard et al. analyzed one illustrative example of morphing MoRFs; i.e., disorder-based binding regions with
remarkable adaptability that can fold into different conformations depending on the binding partner. The authors analyzed the millisecond dynamics of the nuclear co-activator binding domain of CBP (NCBD) using relaxation dispersion NMR spectroscopy and showed that the energy landscape of this domain resembles the energy landscapes of the fold-switching proteins that have two coexisting native states. Such a configuration of the energy landscape may serve as an explanation for binding via conformational selection.47

Hayward et al. analyzed the functional roles of IDPR and ordered domain in the product of the mitochondrial genome maintenance gene, MGM101.48 To this end, they studied the chimeric Mgm101p proteins from the yeast Saccharomyces cerevisiae and the coral Acropora millepora with switched ordered core regions and disordered N-terminal domains. This analysis revealed that the ordered domain of A. millepora can functionally replace the yeast core region, whereas lost functions of the disordered yeast N-terminal domain cannot be replaced by its coral counterpart.49

A new family of IDPs involved in stabilization of the bacterial carbon storage granules was described by Maestro et al.50 One of these newly discovered IDPs is PhaP phosphorylase from Pseudomonas putida KT2440. The intrinsically disorder nature of this protein was validated by a series of hydrodynamic, spectroscopic and thermodynamic experiments, whereas computational tools suggested that other members of the phasin family are intrinsically disordered in the absence of its ligands.51

The roles of IDPRs in function of small- and intermediate-conductance Ca2+-activated potassium (SK/IK) channels were investigated by Zhang et al.50 The authors showed that although a fragment of SK channels that connects the transmembrane segment S6 and the claudin-binding domain is disordered (being invisible in the protein crystal structure), this IDPR becomes readily identifiable in the presence of the specific compound that potentiates the channel activities.50 Furthermore, this compound-stabilized IDPR increases the channel open probability at a given Ca2+ concentration, emphasizing crucial biological role of this fragment in coupling Ca2+ sensing by calmodulin and mechanical opening of SK channels.50

Methods for the IDP/IDPR Analysis

Computational approaches for the analysis of intrinsic disorder. The fact that amino acid sequences of IDPs and IDPRs possess several recognizable features is well-established,2,4,8,33–34 and various computational tools for finding intrinsic disorder in proteins are being developed with an amazing pace.55–62 It was emphasized that the existence of numerous computational tools that give prediction of disorder far above that expected by chance provides direct support for the hypothesis that intrinsic disorder is encoded in a protein’s amino acid sequence.1 Papers below are dedicated to various computational tools elaborated for the analysis of IDPs and IDPRs.

Predictors of intrinsic disorder. Huang et al. proposed to use the Nearest Neighbor based on mRMR (maximum Relevancy Minimum Redundancy). Since this new method was reasonably accurate, the authors concluded that sequence conservation and secondary structure might play various important roles in IDPs/IDPRs.63

Jin and Lui dedicated their study to the finding of the inherent relationships among different biophysics-based predictors of intrinsic disorder.64 Based on the correlation analysis approach of realistic data sets, they found correlations among some physical-chemical properties (charge-hydrophopyt plot, packing density, pair-wise energy) typically used for protein disorder predictions. This allowed determination of a projected direction to discriminate ordered and disordered proteins.64

Molecular dynamics simulations. Molecular dynamics (MD) simulations are ideally suited to investigate protein and peptide plasticity and flexibility simultaneously at high spatial (atomic) and high time resolution.65 Despite limitations determined by the force field accuracy and by the maximum simulation time that can be routinely achieved in current MD simulations, this technique provides a first principle-based description of the conformational behavior of proteins.

Mittal et al. performed replica exchange molecular dynamics simulation of an intrinsically disordered 15-residue wild-type p53 fragment from the TAD domain and its mutant (TAD-P27L) using an optimized (fully atomistic, explicit solvent) protein model and the experimental validation of the simulation results.66 This analysis revealed that although this peptide is characterized by the relatively flat conformational free-energy landscape, its conformational ensemble contains significant fraction of solution structures resembling the MDM2-bound form.66

McDowell et al. performed extensive atomistic simulations of the S100B bound conformation of p53 negative regulatory domain (NRD) in explicit solvent (with 1.0 μs total effective sampling).67 In agreement with earlier NMR studies, this analysis revealed that p53-NRD preserves significant flexibility when bound to S100Bβ, providing an atomistic description of this important fuzzy complex.67

Computational analysis of IDP structures and functions. One of the important features of disordered protein binders is their ability to fold differently as a result of interaction with different binding partners. Originally, this concept of binding diversity and divergent binding-induced folding was introduced based on the example provided by the C-terminal regulatory domain of p53, the same short segment of which interacts with several structurally unrelated partners adopting different conformations (α-helix, a β-sheet, and differently laid irregular structures) when bound to the different partners.11 Hsu et al. continued analysis of this interesting phenomenon of the MoRF-based one-to-many protein-protein interactions, where MoRFs represent short IDRPs that bind to partners via disorder-to-order transitions.23 To this end, a set of multispecific MoRFs (i.e., MoRFs that were shown to bind to 2–9 structurally dissimilar...
Experimental approaches for the analysis of intrinsic disorder. Obviously, structural characterization of highly heterogeneous ensembles of IDPs/IDPRs requires rather specialized approaches. In fact, the determination of a unique high-resolution structure is not possible for an isolated IDP, and rather complex methods have to be used to obtain experimental constraints on the ensemble of states that is sampled by the intrinsically disordered polypeptide chain. Therefore, IDP-related structural studies typically rely on a host of biophysical methods that can provide information on the overall compactness of IDPs, their conformational stability, shape, residual secondary structure, transient long-range contacts, regions of restricted or enhanced mobility, etc.1 Currently, there are more than 70 experimental approaches for structural/conformational characterization of IDPs/IDPRs.27-27 Papers below introduce various experimental approaches and their applications for the analysis of structures and functions of IDPs and IDPRs.

NMR. Ota et al. proposed a unique computational method to assign IDPRs based on NMR structures.78 The tool was developed based on the comparison of missing residues of X-ray structures with residue-wise deviations of NMR structures for identical proteins. Based on this analysis, a threshold deviation of 3.2 Å was derived for the best correlation of ordered and disordered regions of both structures and then was applied for the analysis of proteins whose structures were only determined by NMR. The authors believe that this tool can significantly extend the current pool of proteins with experimentally determined IDPRs.78

Nyarko et al. proposed a new approach to identify active recognition motifs based on NMR-detected β-sheet propensities.79 Furthermore, these authors analyzed the intrinsically disordered Dyn2 binding domain of Nup159 by solution NMR and isostructural titration calorimetry and showed that binding of one equivalent of Dyn2 dimer aligns two Nup159 chains along the full Dyn2 binding domain to form a bivalent scaffold that promotes binding of other Dyn2 dimers.79

An effective method for generation of the small bicelles with a uniform confined size that display a series of gangliosides was proposed by Yamaguchi et al.80 These functionalized bicelles represent nanoscale standardized membrane mimics suitable for NMR characterization of weak encounter complexes formed between ganglioside clusters and amyloidogenic IDPs, such as α-synuclein.80

Prestegard et al. reported 1H and 15N chemical shifts for a set of 20 alanine-based pentapeptides, with the central residue of each being varied among the 20 amino common acids, under the low pH urea denaturing conditions.81 This new set of chemical shifts is then used in empirical formula to predict chemical shifts of unfolded proteins, which is a crucial starting point in the process of the cross peak assignment in 1H-15N HSQC spectra of sparsely labeled proteins (including IDPs). The proposed in this study set of chemical shifts is more appropriate for sparse label assignments, since the authors showed a small, but significant, improvement in shift predictions for unfolded ubiquitin.81

Since the quality of NMR spectra of IDPs is known to be lower than that of ordered proteins, being characterized by low chemical shift dispersion and efficient broadening (often beyond detection) of amide proton resonances, Bermel et al. discussed some key aspects that need to be taken into account when new NMR experiments optimized for the study of IDPs are designed and proposed one of such new experiments based on direct detection of 13Cα.82 Here, the authors emphasized that in the NMR-based analysis of IDPs it is important to exploit heteronuclear, since a prominent increase in the chemical shift dispersion was evident passing from 1H to the directly bound heteronucleus, both for 13Cα as well as for 15N. Also, the correlation of 15N with the attached carbonyl carbon (13C) through the 2D CON spectrum represents another useful trick to further improve the cross peak dispersion and to detect signals deriving from proline residues.82

The topic of the improvement of NMR experiments for the analysis of IDPs was continued in the article by Kim et al. who investigated the effect of fast hydrogen exchange (HX) of unprotected amide protons with protons from the solvent on the measurement of the 15N transverse
relaxation rate ($R_2$), which is crucial for the analysis of the protein backbone dynamics. The authors used $^{15}N$ backbone transverse relaxation experiments ($R_2$) using the CPMG (Carr–Purcell–Meiboom–Gill) pulse train ($^{15}N\ R_2^{\mathrm{CPMG}}$) to investigate dynamics of solvent exposed backbone amides in $\alpha$-synuclein. This analysis revealed that although the $R_2^{\mathrm{CPMG}}$ rates are modulated by fast HX rates, the HX effect on $R_2^{\mathrm{CPMG}}$ can be extracted by the derived equation and therefore can be corrected quantitatively.

Photo-crosslinking. An article by Phan et al. describes the use of various photo-crosslinking approaches for mapping the interactome networks inside the living cells. At the first stage of a new generation of photo-crosslinking methods, the photo-crosslinking analogs of amino acids or sugars are incorporated into cellular biomolecules via the metabolic engineering or genetic code expansion. Then, the crosslinked complexes and related interactomes are analyzed by mass spectrometry and immunological techniques. This analysis, being performed under the conditions of living cell, provides a unique possibility of gaining information on the context-dependent interactions. Photo-crosslinking is well-suited for mapping interaction interfaces and determining the interactome dynamics. These techniques can be also used for finding and characterization of transient interactions typical for IDPs. Some illustrative examples of the successful use of cell-based photo-crosslinking are given, where photo-crosslinking methods were applied to gain information on chaperone-assisted protein folding, transcription, nucleo-cytoplasmic transport, membrane protein dynamics, and immune cell signaling.

Neira represented a comprehensive review of recent advances in the application of NMR for the structural analysis of viruses. The author emphasized that NMR can be used for the determination of solution structures of viral proteins and their isolated domains (including viral IDPs and IDPRs), for the description of their conformational changes and complex dynamic equilibria, for building of pseudo-atomic models of entire virus capsids, and for the identification of conformational changes in intact viral capsids accompanying their insertion to the host membranes.

EPR. Martinho et al. looked at the induced folding of the intrinsically disordered C-terminal domain of the nucleoproteins (N$_{\mathrm{T\!A\!I\!L}}$) from the Nipah and Hendra viruses (which are two recently emerged pathogens gathered within the Henipavirus genus) by site-directed spin labeling coupled to electron paramagnetic resonance (EPR) spectroscopy (SDSL EPR). This approach, that relies on the introduction of a paramagnetic spin label through covalent modification of a unique sulfhydryl group (e.g., of the strategically introduced cysteine residue) using a selective nitrooxide reagent, and on the subsequent analysis of the EPR spectra whose shape reflects the mobility of the spin label, represents a very useful addition to the existing arsenal of the biophysical techniques for the structural characterization of IDPs/IDPRs. Since cysteine residues can be introduced at any part of the protein sequence, SDSL EPR can, in principle, provide information at the residue level, while introducing minimal perturbation of the system. The authors showed that this technique can be used to establish structural differences between the homologous proteins. For example, the portions of the N$_{\mathrm{T\!A\!I\!L}}$ proteins from the Nipah and Hendra viruses that are responsible for the interaction with the C-terminal X domain of the phosphoprotein (P$_{\mathrm{X\!D}}$), possess noticeable structural differentiation, with the Nipah virus N$_{\mathrm{T\!A\!I\!L}}$ protein being characterized by the conformational heterogeneity of the partly pre-configured $\alpha$-helical segments.

High pressure studies. Somkuti et al. broadened the range of experimental conditions traditionally used for structural and conformational analysis of proteins by considering the effects of a wide range of pressure and temperature on the ordered and disordered domains of titin. This was done using Fourier transform infrared (FTIR) and fluorescence spectroscopy combined with a diamond anvil cell that allowed investigation of protein secondary structure and fluorescent parameters across the broad range of pressure (0–16 kbar), temperature (0–100°C), pH (3–10.5), and different solvent conditions. As targets proteins, they used an ordered Ig domain (I27) and a 171-residue-long fragment (polyE) of the disordered PEVK domain derived from titin, a giant elastic protein (canonical form of human titin consists of 34,350 residues) responsible for striated-muscle elasticity, which is known to possess a lot of disorder. This analysis revealed that the PolyE domain preserved its disordered characteristics in the whole range of conditions studied, whereas structured I27 possessed an intricate conformational response to changing conditions that can only be described by the complex temperature-pressure phase diagram.

Mass spectrometry-based methods. The use of various mass spectrometry methods for the analysis of IDPs and IDPRs was covered by Beveridge et al. in a comprehensive review. Here, the authors emphasized that the last decade witnessed revival of mass spectrometry as a structural tool, and this explosion in the use of various mass spectrometry methods was provoked by the intensive use of this powerful biophysical technique for obtaining unique insights into the structure and dynamics of IDPs and IDPRs. Mass spectrometry-based methods can provide a wide spectrum of structural characteristics, ranging from the evaluation of conformational heterogeneity of a given IDP in solution to the provision of structural description based on the rotationally averaged collision cross-sections of molecular ions, to delineation of other structural features of a protein molecule, to identification of oligomer distributions, etc.

In line with this aforementioned review, Pagel et al. provided a detailed ion mobility mass spectrometry (IM-MS)-based structural description of the intrinsically disordered tumor suppressor protein p53 and a series of constructs, where the specific IDPRs (flexible linker, N and C termini) were systematically introduced to the to the 2-folded domains of this protein. IM-MS is one of the mass spectrometry techniques that are frequently used to study the topology of proteins and their complexes. This study revealed that p53 and constructs comprising of its ordered domains and disordered regions are collapsed in the gas phase. The authors concluded that the majority of the rearrangement of p53 in the gas phase occurs...
at the flexible linker and the disordered termini, which behave as unfolded protein chains that collapse in the gas phase.92

Rey et al. proposed to modify hydrogen/deuterium exchange mass spectrometry (HDX-MS) approach by substituting pepsin, which was almost exclusively used in these experiments for fast digestion of target proteins at pH 2–3 to retain deuterium label, by nepenthesin from the secretions of the pitcher plant Nepenthes.94 The authors showed that nepenthesin is at least 1,400-fold more efficient than pepsin under HDX-competent conditions. Furthermore, a selectivity profile of nepenthesin is partially similar to that of pepsin, but also includes efficient cleavage C-terminal to “forbidden” residues K, R, H, and P, which promotes higher coverage of disordered regions. Therefore, nepenthesin represents a logical and highly efficient alternative to pepsin in all HDX-MS applications for the IDP/IDPR analysis.94

Saikusa et al. introduced a novel method for structural characterization of IDPs, IDPRs, and their complexes based on the combination of electrospray ionization ion mobility mass spectrometry (ESI-IM-MS) and SAXS.95 Here, ESI-IM-MS is used to derive experimental collision cross-section (CCS) values, whereas SAXS serves as a source of theoretical CCS values evaluated based on the SAXS low-resolution model. The authors applied this approach for the characterization of the Schizosaccharomyces pombe S. Swi5-Sfr1 complex in which the N-terminal portion of Sfr1 is a long IDPR.95

Single-molecule spectroscopy. Schuler and Hofmann provided a comprehensive overview of single-molecule spectroscopy as an important method for probing protein structure and dynamics in structurally heterogeneous systems, such as IDPs.96 It is emphasized that single-molecule Förster resonance energy transfer (FRET) and photo-induced electron transfer (PET) are uniquely positioned to investigate a wide span of timescales, and therefore provide unprecedented information on the dynamic behavior of one molecule at a time. The single-molecule spectroscopy-based techniques are used for the description of the structure and dynamics of unfolded and intrinsically disordered proteins and for the analysis of coupled folding and binding events.

Rahman et al. used single-molecule force spectroscopy, compression studies and ellipsometry to investigate the interaction modes of the intrinsically disordered acidic dehydrin TsDHN-1 and the basic dehydrin TsDHN-2 derived from the crucifer Thellungiella saluginea with membranes.97 These analyses revealed that dehydrins can stabilize lipid monolayers, induce an increase in monolayer thickness, and promote temperature-dependent phase transitions and domain formation in the supported lipid bilayers, suggesting that dehydrins interact with and potentially stabilize plant outer mitochondrial membranes in conditions of cold stress. Furthermore, single-molecule force spectroscopy analysis revealed that that binding of the dehydrins to membranes induced formation of tertiary conformations in both proteins.97

An application of solid-state nanopores for the single-molecule analysis of IDPs was introduced in a research paper by Japrung et al.98 and the various aspects of generation and use of solid-state nanopores was covered in a tutorial review by the same group.99 Here, single-molecule experiments were performed by translocating IDPs through a nanopore embedded within a thin dielectric membrane. The advantage of the approach is in the fact that the single-molecule statistics can be generated without the need of fluorescent labels or other modification groups.98 Application of this methodology to two IDPs, a binding domain from activator of thyroid hormone and retinoid receptors (ACTR) and the nuclear coactivator binding domain of CREB-binding protein (NCBD), and to their bimolecular complex suggested conformational heterogeneity of intrinsically disordered ACTR and NCBD within the nanopore, whereas the folded ACTR-NCBD complex exhibited only one conformation when translocating through the nanopore.98

In cell analysis of IDPs. Atkinson et al. argued that local geometry constrains the orientational organization of the intrinsically disordered phenylalanine-glycine (FG) domains of nucleoporins (nups).100 This conclusion was based on the use of the polarized fluorescence microscopy to characterize behavior of the FG nucleoporins in vivo. Here, the fluorescent analysis of nucleoporins tagged with green fluorescent protein along their FG domains revealed that some of these proteins were ordered, suggesting the existence of some orientational organization within the nuclear pore complex (NPC).100

Miscellaneous. Rogers et al. applied stopped-flow techniques to systematically analyze coupled folding and binding process in a model system where the intrinsically disordered ‘BH3 region’ of PUMA forms a single, contiguous α-helix upon binding the folded protein Mcl-1.101 Comparison of the association rate constant (k’) under a variety of solvent conditions and temperatures clearly showed that although binding was fast, the analyzed system was not ‘diffusion-limited’. The authors also made a very important conclusion that standard experimental test developed for the analysis of protein-protein interactions between ordered proteins fail to provide an appropriate description of the folding/binding reactions where one protein is disordered.101

Proteomics of IDPs

As the number of IDPs and IDPRs in various proteomes is very large (e.g., for mammals, ~75% of their signaling proteins are predicted to contain long disordered regions (> 30 residues), about half of their total proteins are predicted to contain such long disordered regions, and ~25% of their proteins are predicted to be fully disordered), and because IDPs and IDPRs have amazing structural variability and possess a very wide variety of functions, the unfolding and unfoldedomics concepts were introduced.102-104 Papers below cover different aspects of the large scale analysis (both computational and experimental) of IDPs and IDPRs.

Oldfield et al. provided a detailed analysis of the effects of intrinsic disorder on the structure determination process and the usefulness of disorder prediction in selecting and improving proteins for structural characterization.105 This analysis revealed that although intrinsic disorder is tolerated to some extent in crystal structures, long IDPRs are rather infrequent in solved structures. The authors also concluded that intrinsic disorder predictions represent a useful approach that
helps increasing the proportion of ordered targets in the structure determination pipeline. Surprisingly, increased success in purification was shown to be correlated with a higher proportion of predicted disordered residues in a target protein. Therefore, prediction of intrinsic disorder represents an effective tool for tailoring proteins for structure determination.

Tyanova et al. took a focused look at the correlation between the dynamic properties of phosphoproteome and protein structural features. To this end, they studied how the variation of the amount of phosphorylation correlates with the protein structure in the vicinity of the modified site. The new twist in this study was that the analysis was done not in a static, non-quantitative way, but at six time points of the cell division cycle. The authors showed that variability of phosphorylation at a given site generally followed the degree of disorderliness in the vicinity of the modified site, with IDPRs containing dynamically varying phosphorylation levels, and with ordered regions predominantly keeping more constant phosphorylation levels. This study suggested that the dynamics of phosphorylation is defined by the structural organization of the region in which a phosphorylation site resides.

Sun et al. provided a comprehensive overview of various functional aspects pertaining to plant IDPs. The authors emphasize the importance of IDPs in plant biology and illustrate this point by providing description of the multifarious roles of intrinsic disorder in five different types of plant protein families experimentally confirmed as IDPs. Functions ascribed to plant IDPs possess broad impact on many areas of plant biology, such as transcriptional regulation, light perception, abiotic stress, and plant development.

In agreement with this work, study by Pietrosemoli et al. revealed that IDPs and proteins with IDPRs are rather common in Arabidopsis thaliana, the most widely used model organism in plant biology. This analysis also revealed that many Arabidopsis proteins related to environmental response were significantly enriched in disorder. Based on these observations the authors proposed that the increased level of intrinsic disorder in the environmental response-related proteins in plants represents a useful defense mechanism that allows plants to quickly adapt and respond to challenging environmental conditions.

Also in line with this work, Wood et al. showed that Actinidia DRM1 (Dormancy Associated Gene 1), a plant-specific IDP, represents a robust dormancy marker whose mRNA transcript expression exhibits a strong inverse correlation with the onset of growth following periods of physiological dormancy. The intrinsically disordered nature of this protein was suggested by several bioinformatics tools, and several DRM1 homologs, which align into two distinct Actinidia-specific families, Type I and Type II, have been identified.

Costantini et al. investigated the distribution of the structural features of N- and C-terminal segments of sirtuins in all known organisms to understand their evolutionary histories by taking into account average length of terminal segments, amino acid composition, intrinsic disorder, presence of charged stretches, presence of putative phosphorylation sites, flexibility, and GC content of genes. This study revealed that this ancient family of proteins contain multiple functionally crucial IDPRs.

Coelho Ribeiro et al. used a wide array of bioinformatics methods to analyze the abundance and functional roles of intrinsic disorder in proteins involved in the formation of the yeast spliceosome. The spliceosome is a multimegadalton ribonucleoprotein machine catalyzing the excision of introns from eukaryotic pre-mRNAs, which, in yeast, consists of five small nuclear RNAs (U1, U2, U4, U5, and U6) and a range of associated proteins. The analysis revealed that intrinsic disorder is abundant in all spliceosome-related proteins, being found both in proteins constituting cores of the ribonucleoproteins (snRNPs) and in numerous auxiliary proteins.

**Looking at IDPs/IDPRs in Diseases**

Because IDPs play crucial roles in numerous biological processes, being involved in control and regulation of almost all crucial processes inside the cell, many of these proteins are implicated in human disease. This conclusion is based on analysis of the pathological roles of several individual IDPs and extensive computational/bioinformatics studies addressing the abundance of IDPs in various pathological conditions, including cancer and neurodegenerative diseases.

**IDPs in cancer**. Mahmoudabadi et al. created and analyzed a model of a protein network with the topological characteristics of a cancer protein network. The cornerstone idea of this model are the protein intrinsic disorder-based concepts of conformational noise and transcriptional (genetic) noise that define activation of hidden and dormant pathways some of which are responsible for cellular transformation in cancer and other state-switching events. Conformational noise characterizes the stochastic interactions between IDPs and their partners and defines an ensemble of protein network configurations, from which the most suitable can be explored in response to perturbations, conferring protein networks with remarkable flexibility and resilience. Transcriptional noise on the other hand is defined by the disorderedness of transcription factors and hub proteins. Therefore, IDPs serve as master controllers and effectors of transcriptional and conformational noise, playing crucial roles in rewiring protein networks and unmasking dormant interactions in response to perturbations.

Analysis of mixed lineage leukemia (MLL) fusion proteins by Leach et al. revealed that these IDPs play a number of important roles in normal hematopoiesis and in acute leukemia. The authors studied the ANCl homology domain (AHD) of one of the most common MLL fusion proteins, AF9, that promotes oncogenic transformation of hematopoietic cells by constitutive recruitment of elongation factors to HOX promoters, resulting in overexpression of target genes. AF9 AHD was shown to undergo coupled folding and binding as a result of interaction with multiple transcription factors. Importantly, AF9 AHD is engaged in the formation of fuzzy complexes, were it retains significant dynamic behavior in the bound form which may facilitate exchange between disordered partners.
Wang et al. provided a comprehensive overview of the current state of the art in the field of the structural and functional characterization of highly conserved coactivators CREB-binding protein (CBP) and its paralog, E1A-binding protein (p300). CBP/p300 is one of the important coactivators that promote transcription by connecting transcription factors to the basal transcriptional machinery. This protein has several functional domains (e.g., four separate transactivation domains (TADs) that interact with the TADs of a number of DNA-binding transcription activators and general transcription factors, a catalytic histone acetyltransferase (HAT) domain and several IDPRs) and is involved in multivalent interactions with the various components of the transcription machinery. Because of the multitude of its transcriptional and epigenetic functions, CBP/p300 is involved in a set of complex physiological and pathological processes (such as proliferation or apoptosis), and dysregulation of this protein is associated with leukemia and other types of cancer.

IDPs in neurodegenerative diseases. Work of Ariesandi et al. unraveled the role of pre-existing oligomeric species in promoting α-synuclein fibrillation. These authors established that the heat pretreatment depleted the amount of rare pre-existing α-synuclein oligomers leading to the dramatic inhibition of this protein fibrillation under the ambient temperatures.

Singh et al. showed that the polyphenolic ingredient of Asian food, curcumin, reduces toxicity of α-synuclein aggregates by binding to preformed oligomers and fibrils and modifying the exposure degree of their hydrophobic surfaces. Fluorescence and 2D-NMR analysis revealed that there is a specific binding of curcumin to the oligomeric intermediates, whereas monomeric protein does not interact with this polyphenol. Importantly, although the addition of curcumin resulted in the accelerated aggregation α-synuclein, the population of toxic oligomeric intermediates of this protein was noticeably reduced.

Maltsev et al. investigated site-specific interaction between α-synuclein and membranes using the analysis of the NMR-detected methionine oxidation rates. In this work, the ability of membrane containing a small fraction of peroxidized lipids to rapidly oxidize the N-terminal methionine residues in α-synuclein (Met1 and Met5) was utilized. This study showed that the oxidation rates of Met1 and Met5 were mutually reduces as a result of decreased membrane affinity of the partially oxidized protein.

Among three point mutations of α-synuclein associated with the familial cases of the early onset Parkinson disease, the E46K genetic missense mutation is the most recent addition. Wise-Scira et al. analyzed the effect of this mutation (which was originally identified in a family of Spanish origin with hereditary Parkinson disease) on the structural properties, conformations, and free energy landscape of α-synuclein using the extensive parallel tempering molecular dynamics simulations along with thermodynamic calculations. The authors also used the recently developed theoretical strategy for calculation of the free energy change values associated with the transitions between two different secondary structure components for each residue of the wild-type and E46K mutant of α-synuclein. This study revealed that in addition to obvious local changes, the E46K mutation induced noticeable long-range alterations in the structural properties of α-synuclein. In particular, E46K mutation promoted a significant decrease in helical content accompanied by a large increase in β-sheet structure propensity. Furthermore, E46K mutation was shown to increase the existing long-range intramolecular interactions between the C-terminal region and the N-terminal and NAC regions of α-synuclein, leading to the formation of more compact structures.

In another study by the same group, the effects of another familial mutation (A30P) on structure, dynamic properties, and free energy landscapes of this interesting protein were investigated by the analogous theoretical approach. This analysis showed that helical content of Ala18-Gly31 region of the mutant α-synuclein was less prominent in comparison to the wild-type protein. Also, mutation affected the long-range intramolecular protein interactions making them less abundant, especially between the NAC and C-terminal regions, leading to the less compact and less stable structures of the A30P mutant.

Rabe et al. reported that α-synuclein can spontaneously aggregate at concentrations as low as 1 nM and below (i.e., far lower than physiological concentrations of this protein) in the presence of both hydrophilic glass surfaces and cell membrane mimicking supported lipid bilayers. The authors used three-dimensional supercritical angle fluorescence (3D-SAF) microscopy to follow the α-synuclein aggregation process in situ. This analysis revealed the heterogeneous nature of the aggregation process, where two different types of α-synuclein aggregates were formed. The first type was completely adsorbed to the surface and grew along the surface plane, whereas the second type of extended was tethered with one end to the surface being mobile at the other end. The growing mechanisms of these two amyloid-like structures were significantly different.

Gury et al. investigated the aggregation behavior of an α-synuclein construct containing a 10-residue N-terminal extension. Data from NMR chemical shifts and residual dipolar couplings were used to generate the conformational ensemble of this construct. Analysis of this conformational ensemble showed that a disordered monomer was the dominant state of this ensemble, complemented by a small fraction of α-helical and β-structural trimers and tetramers.

Fantini et al. delved into the analysis of the molecular mechanisms underlying the ganglioside- and cholesterol-controlled interaction of α-synuclein with neural membranes. The mechanistic model is proposed where α-synuclein first interacts with a cell surface glycosphingolipid. This primary binding to ganglioside GM3 in astrocytes or GM1 in neurons induces partial folding of an α-helical domain that contains a high affinity binding motif (67–78 peptide) for cholesterol. At the last stage of the insertion process, an oligomeric channel is formed.

Viral IDPs. Schulze-Gahmen et al. investigated the molecular mechanisms of the selective recruitment of the human
positive transcription elongation factor b (P-TEFb) that phosphorylates RNA polymerase II and regulatory proteins to trigger elongation of many gene transcripts by the HIV-1 Tat protein. The authors solved the structure a tripartite super elongation complex (SEC) containing P-TEFb, flexible AFF4 scaffold and HIV Tat. This study revealed that AFF4 is involved in the direct interaction with HIV Tat and that Tat enhanced P-TEFb affinity for AFF4.

IDPs/IDPRs as Drugs or Drug Targets

Since many proteins associated with various human diseases are either completely disordered or contain long disordered regions and since some of these disease-related IDPs/IDPRs are involved in recognition, regulation and signaling, these proteins/regions clearly represent novel potential drug targets. There are at least two potential approaches for the inhibition of the disorder-based interactions, where small molecule either bind to the binding site of the ordered partner to outcompete the IDPs/IDPRs or interacts directly with the IDP/IDPR. Importantly, small molecules can inhibit disorder-based protein-protein interactions via induction of the dysfunctional ordered structures in targeted IDPR; i.e., via the drug-induced misfolding.

Rawat et al. used NMR to investigate the backbone dynamics and conformational properties of drug peptide salmon calcitonin (sCT) in aqueous solution. This analysis revealed that sCT being mostly unfolded in solution, contained noticeable residual structure organized in multiple conformational states exchanging slowly on the NMR timescale (in a range of 10^2–10^3 s^-1). More detailed analysis suggested that sCT is structurally heterogeneous and contains highly flexible C-terminal region (residues Thr25-Thr31) that is needed for the identification of specific target receptors, and more structured N-terminal region (residues Cys1-Gln20) possessing significant amount of conformational plasticity and strong bias toward biologically active α-helical structure that is responsible for favorable functional adaptation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest disclosed.

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