Conformational ensembles of intrinsically disordered proteins and flexible multidomain proteins

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Abstract
Intrinsically disordered proteins (IDPs) and multidomain proteins with flexible linkers show a high level of structural heterogeneity and are best described by ensembles consisting of multiple conformations with associated thermodynamic weights. Determining conformational ensembles usually involves integration of biophysical experiments and computational models. In this review, we discuss current approaches to determining conformational ensembles of IDPs and multidomain proteins, including the choice of biophysical experiments, computational models used to sample protein conformations, models to calculate experimental observables from protein structure, and methods to refine ensembles against experimental data. We also provide examples of recent applications of integrative conformational ensemble determination to study IDPs and multidomain proteins and suggest future directions for research in the field.

Introduction
Understanding how proteins carry out their biological functions and what causes them to misfunction is important from the perspective of fundamental science and to develop new therapeutics and biotechnology. Protein dynamics and function are intimately related, and many proteins must modulate their shape to respond to environmental changes, accommodate binding partners, catalyze reactions, convey allosteric signals, and transport ligands (Teilum et al., 2009). Therefore, an important aim of structural biology is not only to determine static structures of proteins, but to characterize their conformational heterogeneity and its relationship to biological function.

Here we review the approaches used to determine conformational ensembles of intrinsically disordered proteins (IDPs) and flexible multidomain proteins, limiting ourselves to approaches that directly integrate experimental data in the generation of the ensemble. The focus will be on principles and examples, and this review is therefore best suited for readers who are looking for a brief, conceptual overview of the field. For a more technical introduction we refer the reader to previous literature (Hummer and Köfinger, 2015; Orioli et al., 2020).

Intrinsically disordered proteins and multidomain proteins
IDPs are proteins that do not fold into a well-defined structure, but rather interconvert between a large range of very different conformations. Based on disorder predictions, intrinsically disordered regions (IDRs) make up more than one third of eukaryotic proteins (Ward et al., 2004; Xue et al., 2012; Oates et al., 2013; Tunyasuvunakool et al., 2021). The conformational heterogeneity of IDPs allows for promiscuity in interaction partners, and IDPs are often involved in biological processes such as signaling, recognition, and regulation (Wright and Dyson, 2015; Bondos et al., 2021).
recent years, IDPs have received much attention for their role in the formation of a number of biomolecular condensates and membraneless organelles (Banani et al., 2017; Boeynaems et al., 2018; Peran and Mittag, 2020; Dignon et al., 2020; Choi et al., 2020; Martin and Holehouse, 2020; Borcherds et al., 2021). Condensate formation can be driven by interactions between a variety of different biomolecules, including IDPs, folded proteins, and RNA, and understanding the molecular details that underly condensate formation is an important motivation for structural characterization of IDPs and their interactions.

Many proteins are modular, consisting of folded domains connected by flexible linkers or IDRs (Apic et al., 2001; Ekman et al., 2005). Such multidomain proteins can display a high level of conformational heterogeneity, as the folded domains can rearrange with respect to each other (Wriggers et al., 2005; Roy et al., 2016; Delaforge et al., 2016). For this reason, many of the approaches used for structural characterization of IDPs also extend to flexible multidomain proteins. Here, we will use the term multidomain protein to refer to structurally dynamic multidomain proteins with flexible linkers.

Due to their structural heterogeneity, IDPs and multidomain proteins are best described by an ensemble of structures representing the conformations sampled by the system with associated thermodynamic weights. In this review, we will focus on the integrative approaches used to determine conformational ensembles of IDPs and multidomain proteins. Further, we will discuss applications of conformational ensemble determination using recent examples from the literature, and will suggest future directions for research in the field.

Figure 1. Conformational heterogeneity in proteins
Proteins do not exist as rigid structures in solution, but rather sample an ensemble of structures. Different proteins have variable levels of conformational heterogeneity. Stably folded proteins undergo relatively small conformational fluctuations around an average structure (left). Multidomain proteins consisting of folded domains connected by flexible linkers can display a higher level of conformational heterogeneity, as the folded domains can rearrange with respect to each other (middle). Intrinsically disordered proteins are characterized by a high level of conformational heterogeneity, as they do not fold into a well-defined structure, but rather interconvert between a range of conformations (right). The examples shown here are ubiquitin (folded protein) (Lindorff-Larsen et al., 2005), TIA-1 (flexible multidomain protein) (Larsen et al., 2020; Thomasen et al., 2021), and α-synuclein (intrinsically disordered protein) (Thomasen et al., 2021).
How do we determine conformational ensembles of IDPs and multidomain proteins?

Most biophysical experiments used to study protein structure report on observables that are averaged over different conformations. Determining atomic resolution conformational ensembles of proteins from sparse, ensemble-averaged experimental data without further constraints is an ill-posed problem, as it involves fitting many more distributions of atomic positions than there are data-points available. Computational models and simulations can be used to sample conformational ensembles of proteins at atomic resolution. However, computational models have various limitations, especially for modeling IDPs, and are not always in agreement with experiments (Palazzesi et al., 2015; Rauscher et al., 2015; Henriques et al., 2015; Bottaro and Lindorff-Larsen, 2018; Robustelli et al., 2018). Thus, neither biophysical experiments nor computational modeling alone provide the optimal solution for conformational ensemble determination. However, combining both using integrative methods can provide conformational ensembles that are also in agreement with experimental observations. This process usually involves four central components: (1) a computational model for sampling protein conformations, (2) one or more biophysical experiments that report on protein structure, (3) corresponding models for calculating experimental observables from the conformational ensemble (so called forward models), and (4) a method for refining the ensemble based on experimental data. Depending on the approach, experimental data can either be integrated directly in the conformational sampling procedure or after sampling.

**Experimental methods**

For a biophysical technique to be useful in integrative ensemble determination of IDPs and multidomain proteins, it should report on protein structure under solution conditions where conformational heterogeneity is present. We write ‘report on’ because solution conditions are not a strict requirement; methods that use sample freezing, such as electron paramagnetic resonance (EPR), cryo-electron microscopy (cryo-EM and solid-state NMR), may capture the conformations present in solution if cooling is rapid (Fischer et al., 2010; Hu et al., 2010; Chen et al., 2019; Bock and Grubmüller, 2021; Klose et al., 2021). Additionally, for an experiment to be useful, an accurate forward model must be available to calculate the experimental observable from the ensemble.

Generally, the more different types of experiments that are integrated, the more trustworthy the resulting ensemble. Combining experiments that report on different structural features ensures that the ensemble is accurate at multiple structural levels and can prevent overfitting to a single experiment (Gomes et al., 2020; Naudi-Fabra et al., 2021). Another good approach to prevent overfitting is to use subsampling of the experimental data or to leave some data out of ensemble determination for later validation.

Small angle X-ray scattering (SAXS) experiments are often used in conformational ensemble determination. SAXS is a low-resolution technique that reports on the overall shape and size of the protein in solution. For IDPs and multidomain proteins, SAXS contains information on the global dimensions of the ensemble and can for example capture domain rearrangements in multidomain proteins (Yang et al., 2010; Różycki et al., 2011; Bernadó and Svergun, 2012; Tuukkanen et al., 2017).

Another commonly used technique is nuclear magnetic resonance (NMR) spectroscopy. NMR experiments can report on a variety of structural features. For example, chemical shifts depend on the local environment of nuclei and contain information on backbone conformations and secondary structure (Ozenne et al., 2012b; Kragelj et al., 2013), scalar J-couplings report on bond connectivity and dihedral angles, nuclear Overhauser effects (NOEs) report on short-range distances between nuclei, and residual dipolar couplings (RDCs) report on the alignment of bond vectors with respect to a global alignment tensor (Marion, 2013).

By chemically modifying the protein with a paramagnetic spin-label, transient long-range interactions between nuclei and the spin-label modification can be probed by NMR using paramagnetic
relaxation enhancement (PRE) experiments (Clore and Iwahara, 2009). Similarly, double electron-electron resonance (DEER) experiments probe long-range distances between two paramagnetic spin-labels using EPR (Pannier et al., 2000).

Other methods used for integrative conformational ensemble determination include single molecule Förster resonance energy transfer (smFRET), which reports on the distance between two sites in the protein chemically labeled with a fluorescence donor and acceptor dye (Metskas and Rhoades, 2020; Lerner et al., 2021; Alston et al., 2021), infrared (IR) spectroscopy, which is mostly used to report on protein secondary structure and local electrostatic environment based on amide I vibrational frequencies (Haris, 2013; Reppert and Tokmakoff, 2013), and cryo-EM, which can in principle resolve the conformational ensemble at atomic resolution, but where the electron density map can also be considered an ensemble-averaged observable (Cossio and Hummer, 2013; Bonomi et al., 2018; Bonomi and Vendruscolo, 2019).

**Conformational sampling**

One must choose a computational method to sample or generate protein conformations. A common approach is to use molecular dynamics (MD) or Monte Carlo (MC) simulations (Hollingsworth and Dror, 2018; Vitalis and Pappu, 2009; Braun et al., 2019), where conformations are sampled based on the energy specified by a force field, a potential energy function describing bonded and non-bonded interactions between all atoms. There are two central limitations to such simulations: (1) force fields contain inaccuracies due to inherent approximations and parameterization (2) full sampling, meaning that all conformations have been sampled with the correct weights, can be computationally expensive or infeasible (Bottaro and Lindorff-Larsen, 2018). In recent years, popular force fields for proteins have been modified to improve accuracy for IDPs (Best et al., 2014; Piana et al., 2015; Huang et al., 2017; Robustelli et al., 2018; Zerze et al., 2019; Thomasen et al., 2021).

Coarse-grained (CG) models, where system complexity is reduced by mapping groups of atoms to single particles, can alleviate problems with sampling in biomolecular simulations, but come at the cost of accuracy and resolution (Ingólfsson et al., 2014; Kmiecik et al., 2016). However, because the generated ensembles are optimized to be in accordance with experimental data, CG simulations are a useful tool for integrative ensemble determination. Since most observables are a function of atomic positions, forward models must be developed specifically for the CG model or CG ensembles must be back-mapped to all-atom structures in order to use conventional forward models, partly undermining the gained computational efficiency. A popular CG model for biomolecular systems is Martini (Marrink et al., 2007; Monticelli et al., 2008; Souza et al., 2021), which has previously been used to simulate multidomain proteins and IDPs with slight modifications to the force field (Berg et al., 2018; Berg and Peter, 2019; Larsen et al., 2020; Jussupow et al., 2020; Martin et al., 2021; Kassem et al., 2021; Benayad et al., 2021; Thomasen et al., 2021). Additionally, numerous CG models have been developed specifically to study IDPs (Dignon et al., 2018; Regy et al., 2021; Tesei et al., 2021b; Wu et al., 2018; Cragnell et al., 2016, 2018; Vitalis and Pappu, 2009; Das et al., 2018a,b; Choi and Pappu, 2019; Mioduszewski and Cieplak, 2018; Rutter et al., 2015).

There are also less computationally demanding methods to generate conformational ensembles. For example, Flexible-Meccano generates IDP conformations by sampling backbone dihedrals using information from non-secondary structural elements of existing protein structures (Ozenne et al., 2012a). Conformational propensities such as transient secondary structure and long-range interactions can be included based on prior knowledge. In a similar approach to Flexible-Meccano, IDP ensembles can also be constructed from a fragment library sampled by MD simulations (Pietrek et al., 2020). For multidomain proteins, conformations can be generated with Pre_bunch, a part of the BUNCH program (Petoukhov and Svergun, 2005; Bernadó et al., 2007). Here conformations are generated by sampling Cα-Cα dihedrals in linkers, while folded domains are treated as rigid bodies.
Calculating experimental observables

Linking experiments with an underlying conformational ensemble requires forward models to calculate experimental observables from the ensemble. In most cases, the observable is calculated from each static structure of the ensemble and subsequently averaged. Thus, effects of dynamics (i.e. the timescales of interconversion) on the observable are neglected if these are not implicitly included in the forward model, and one must keep in mind that this is an approximation for time-dependent processes such as several types of NMR and fluorescence measurements.

Some experimental observables have accurate forward models available. A good example is the calculation of SAXS intensities, which are a function of all interatomic distances, and are well-described by the Debye scattering equation, although SAXS forward models often use approximations of the Debye equation to increase computational efficiency (Hub, 2018; Svergun et al., 1995; Petoukhov et al., 2012; Schneidman-Duhovny et al., 2010, 2013; Gumerov et al., 2012; Grudinin et al., 2017).

Many experiments report on distances, which are straightforward to calculate from ensembles. For example, NOEs are often calculated simply as an $r^{-6}$-weighted ensemble-average of interatomic distances $r$, with the approximation that dynamics do not contribute to the NOE intensity (Brüschweiler et al., 1992; Peter et al., 2001; Smith et al., 2020). Distance-based experiments that require chemical labeling, such as smFRET, PRE, and DEER, introduce an additional challenge, as label dynamics must be taken into account in the forward model (Steinhoff and Hubbell, 1996; Tombolato et al., 2006a,b; Salmon et al., 2010; Polyhach et al., 2011; Sindert et al., 2011; Kalinin et al., 2012; Reichel et al., 2018; Borgia et al., 2018; Tesel et al., 2021a; Klose et al., 2021).

For other experiments, the relationship between structure and observable is less straightforward. For example, the relationship between NMR chemical shifts and the local environment of nuclei is complex, so forward models are usually empirically optimized against data on many proteins (Kohlhoff et al., 2009; Shen and Bax, 2010; Han et al., 2011). In some cases, the lack of an accurate forward model is the main limitation in the use of experimental data. For example, circular dichroism (CD) spectra contain useful structural information, but are usually interpreted as a sum of basis spectra determined for folded proteins, making CD difficult to use for IDPs (Nagy et al., 2019; Jephthah et al., 2021). When studying IDPs, it is a general problem that empirically-derived forward models are often trained on folded proteins, and may not capture e.g. solvation properties of IDPs (Piana et al., 2015; Henriques et al., 2018; Pesce and Lindorff-Larsen, 2021). However, it is challenging to obtain training sets of accurate IDP ensembles with experimental data available that are independent of the forward model one wishes to optimize (Lindorff-Larsen and Kragelund, 2021).

Forward models often require system-specific setting of parameters and care must be taken to avoid overfitting to experimental data. One example is the calculation of SAXS profiles from protein structures, which often involves implicit modeling of the solvation shell. This presents a dilemma, as fitting solvation shell parameters against experimental data individually for each conformation likely leads to overfitting, but a global fit for all conformations may not capture conformation-dependent changes in the hydration shell (Pesce and Lindorff-Larsen, 2021). Another example is the calculation of RDCs, where fitting the alignment tensor against experimental data individually for each frame may result in overfitting and there may be parameter correlation between the axial component of the alignment tensor and the order parameter that describes the dynamics of the internuclear vector (Zweckstetter, 2008).

Integrating the modeled ensemble and experimental data

Integrative ensemble determination is usually based on refining the modeled ensemble to improve agreement with experimental data. This can either be done by biasing the conformational sampling procedure or by reweighting the probability distribution of conformations after sampling.

Two distinct approaches are commonly used to modify the initial ensemble based on experimental data; maximum-entropy (MaxEnt) and related Bayesian methods minimally perturb the ini-
Reweighting and biasing are the two central approaches used to integrate experimental data and computational models in conformational ensemble determination. In reweighting, unbiased conformational sampling is performed initially. Subsequently, experimental observables are calculated from the ensemble using forward models, and conformations are reweighted to improve agreement with experimental data. The optimization of the weights is usually regularized by a maximum-entropy constraint to minimally perturb the initial ensemble. In biasing, experimental data is integrated directly in the conformational sampling by biasing the force field. Experimental observables are calculated iteratively while running the simulation, and agreement with experimental data is imposed either through simple restraints over multiple simulation replicas or by on-the-fly modification of the force field, usually in accordance with the principle of maximum-entropy.

In MaxEnt approaches, the minimal perturbation of the initial ensemble is achieved by optimizing the relative Shannon entropy (or negative Kullback-Leibler divergence) between the initial ensemble and the refined ensemble (Shannon, 1948; Kullback and Leibler, 1951). In MaxEnt reweighting, the MaxEnt regularization is included as a constraint in the optimization of the ensemble weights after generating the initial ensemble (Różycki et al., 2011). In MaxEnt biasing methods, sampling and MaxEnt ensemble refinement are performed simultaneously by modifying the forces on the fly to optimize agreement between ensemble and experiment. Alternatively, MaxEnt biasing can be achieved by simulating multiple replicas and restraining the average over the replicas to agree with experiment (Pitera and Chodera, 2012; Roux and Weare, 2013; Cavalli et al., 2013; Jaynes, 1957), whereas maximum-parsimony (MaxPars) methods select the fewest number of conformations required for agreement with experimental data. As ensembles of IDPs and multidomain proteins are not expected to be captured well by few conformations, MaxEnt methods are most suitable for these cases.
Reweighting and biasing methods are commonly expressed in the framework of Bayesian statistics to explicitly include sources of error and noise. Both MaxEnt and MaxPars approaches can be expressed in Bayesian formalisms (Fischer et al., 2010; Olsson et al., 2013; Hummer and Köfinger, 2015), but the Bayesian frameworks also opens up for the possibility to use other priors and likelihood functions than those given by MaxEnt and MaxPars (Orioli et al., 2020). A plethora of different reweighting and biasing methods exist, applying different combinations of MaxEnt, MaxPars, and Bayesian statistics. For an extensive list, see Bonomi et al. (2017).

Should one choose reweighting or biasing methods for ensemble optimization? An advantage of the reweighting approach is that new data can be included to improve the ensemble at any time after generating the initial ensemble. Additionally, one can use initial ensembles from other sources than molecular simulations, such as Flexible-Meccano (Ozenne et al., 2012a). A disadvantage of the reweighting approach is that all structures in the refined ensemble must already be present in the initial ensemble. Therefore, reweighting is only reliable when the initial ensemble is reasonably accurate, and the biasing approach is preferable if the force field is known to be inaccurate or the sampling is poor. However, biasing approaches for MD sampling require that forward models are differentiable and that the forward model calculations are fast as they are executed at (almost) every integration step in the simulations. Finally, it is possible to combine experimentally-biased sampling and post-simulation integration of experimental data (Shen et al., 2008; Boomsma et al., 2014b; Hummer and Köfinger, 2015; Rangan et al., 2018; Stelzl et al., 2021).

What can conformational ensembles tell us?
Integrative conformational ensemble determination can be viewed as a way of combining our general knowledge about the chemistry and physics of proteins, encoded for example in the force field or other conformational sampling model, with our interpretation of the experimental data. Thus, conformational ensembles can be used to examine detailed properties of the system that are not directly available from the experimental data, but which are likely accurate given the data and our prior knowledge about proteins. In some cases, conformational ensemble determination can provide a consistent structural interpretation of seemingly inconsistent experimental results, illustrating the strength of the approach (Fuertes et al., 2017; Gomes et al., 2020).

While much of the research in integrative conformational ensemble determination has been focused on developing new methods and proof-of-concept, the field has advanced to a point where it can address biological questions from the ensemble perspective. Here, we provide some recent examples where conformational ensembles have been applied to study IDPs and multidomain proteins.

Multidomain proteins
Conformational ensembles have been used to study flexibility and domain interactions in multidomain proteins. For example, Weber et al. (2018) used MD simulations biased using RDC data from NMR using metadynamics metainference (Bonomi et al., 2016), to generate conformational ensembles of the antibody light chain, a multidomain protein with two folded domains connected by a flexible linker. They showed that a mutation in the linker increases flexibility and decreases interdomain contacts, illustrating the importance of the linker sequence in maintaining correct domain orientations and reducing amyloidogenicity.

In another example, Martin et al. (2021) determined conformational ensembles of the multidomain protein hnRNPA1 using the CG MD model Martini (Marrink et al., 2007; Monticelli et al., 2008), SAXS and a Bayesian MaxEnt (BME) reweighting approach (Bottaro et al., 2020) to study interactions between folded domains and an IDR as a function of ionic strength. Based on the correlation between folded domain-IDR contacts and phase separation propensity, they proposed a model for how the folded domains of hnRNPA1 modulate its ability to form biomolecular condensates.
Jussupow et al. (2020) used Martini, SAXS, and metainference to study linear polyubiquitin and its binding to NEMO, a regulator of inflammation, which preferentially binds to long polyubiquitin chains. They determined conformational ensembles of two, three, and four-ubiquitin chains in their NEMO-bound and unbound states. Their results suggest that linear polyubiquitin behaves as a self-avoiding polymer, that NEMO binding greatly restricts conformational flexibility, and that the likelihood of finding a NEMO binding site in a bound-like conformation increases with chain length, in line with the preferential binding to longer chains.

Multidomain protein-nucleic acid complexes
Integrative conformational ensemble determination is not limited to isolated multidomain proteins; the approach has recently been applied to study complexes between multidomain proteins and nucleic acids. Kooshapur et al. (2018) used SAXS data and metainference MD simulations to study the complex between the RNA-binding domains of hnRNPA1 and the micro-RNA pri-mir-18a, which is processed by hnRNPA1. The conformational ensemble was consistent with recognition of two UAG-motifs in the terminal loop of pri-mir-18a and revealed that the upper stem of pri-mir-18a is partially melted in the complex.

In another example, Saad et al. (2021) used SAXS data and metainference MD simulations to investigate the heterodimeric transcription factor E2F1/DP1 bound to DNA. Their results revealed that the complex is highly dynamic and that flexibility in DP1 may be important for stabilizing interactions with DNA, contextualizing cancer-related mutations found in flexible regions of the protein.

IDRs and membranes
Recently, integrative conformational ensemble determination has been used to study IDRs in the context of lipid membranes. Pond et al. (2020) integrated neutron reflectometry data and MD simulations using MaxEnt biasing to study interactions between a lipid-bilayer and the intrinsically disordered SH4-U domain of Hck kinase, which anchors Hck kinase to the membrane. Their results suggest that SH4-U preferentially interacts with anionic lipids and can insert itself into the lipid-bilayer through interactions between charged residues and lipid head-groups.

In another example, Kassem et al. (2021) used Martini, SAXS, and BME reweighting to investigate the conformational ensemble of the human growth hormone receptor (hGHR) in a nanodisc. hGHR is a single-pass transmembrane protein with a folded extracellular domain and a disordered intracellular domain. The conformational ensemble of hGHR revealed that the intracellular domain does not interact strongly with the membrane, but instead extends into the cytosol, giving it a large capture radius for interaction with its many binding partners.

Multimodal conformational ensembles
Conformational ensembles are especially useful for investigating systems that populate several distinct populations and systems with interesting subpopulations, as information on these properties are inherently not contained in ensemble-averaged observables. For example, Reppert et al. (2016) determined conformational ensembles of fragments from the IDP Elastin using MD simulations and MaxEnt reweighting against amide I IR spectroscopic data. These fragments each consisted of different repeated sequence-motifs thought to be important for the elastic properties of Elastin. For all different motifs, the ensembles revealed a bimodal distribution of conformations centered around a collapsed kinked state and an extended state, with the population of each state determined by small differences in the sequence of the motifs. The authors proposed that the elastic properties of Elastin are related to the bimodality of these motifs and that the motif-composition of Elastin is evolutionarily tuned to maintain disorder and elastic properties.

In another example, Huang et al. (2020) investigated the structural effect of linker mutations that increase the kinase activity of the multidomain protein Hck kinase. Using as a starting point nine conformations of Hck kinase previously determined with CG simulations (Yang et al., 2010),
they applied the reweighting method BSS-SAXS, which optimizes the weights of a small conformational ensemble without MaxEnt regularization or MaxPars selection (Yang et al., 2010), to reweight the conformations against SAXS data on both the wild-type and mutant protein. This revealed a population shift from the “assembled”, inhibited state of the protein to the “disassembled”, active state of the protein, demonstrating the importance of linker regions in tuning kinase activity.

**Small-molecule binding to IDPs**

It has recently been demonstrated that integrative ensemble determination can be used to study small-molecule binding to IDPs. The important role of IDPs in signalling and regulation and their propensity to form disease-related aggregates makes them interesting targets for drug development (Metallo, 2010; Dunker and Uversky, 2010; Heller et al., 2015, 2018). Heller et al. (2017) investigated the binding of a small-molecule to the IDP c-Myc using metadynamic metainference MD simulations (Bonomi et al., 2016) biased against NMR chemical shifts. They found that the small-molecule does not bind to a specific site on c-Myc, but rather diffuses across the protein with increased affinity for certain residues, which were in agreement with experimental mutational studies.

In a related study, Heller et al. (2020) investigated the binding of a small-molecule shown to inhibit amyloid aggregation to amyloid-β. Again they used metadynamic metainference simulations with NMR chemical shifts. Their results showed that small-molecule binding increases the conformational heterogeneity of amyloid-β, suggesting that binding may be driven by entropic expansion of the IDP. In these two studies, the ensemble perspective shed light on the mechanisms underlying small-molecule binding to IDPs, and the results suggest general principles for designing drugs to target IDPs.

**Future perspectives**

In this section, we will highlight five areas of research which we think will be important for the future of integrative conformational ensemble determination.

First, the development of accurate and transferable force fields is an important element in advancing integrative ensemble determination. We note that force fields are generally improving (Lindorff-Larsen et al., 2012; Beauchamp et al., 2012), and as mentioned previously, specific attention has been given to improving force fields for IDPs (Best et al., 2014; Piana et al., 2015; Huang et al., 2017; Robustelli et al., 2018; Zerze et al., 2019; Thomasen et al., 2021), and importantly developing force fields that work well for both folded proteins and IDPs (Robustelli et al., 2018). Applications of machine learning, Bayesian inference, maximum likelihood and reweighting approaches to automate force field optimization based on quantum mechanical calculations and biophysical experiments show potential for the development of more accurate force fields (Norgaard et al., 2008; Li and Brüschweiler, 2010; Wang et al., 2014; Köfinger and Hummer, 2021; Tesei et al., 2021b; Noé et al., 2020; Gkeka et al., 2020; Lindorff-Larsen and Kragelund, 2021; Unke et al., 2021; Yang et al., 2021).

Second, an important area of future research is the development of forward models to calculate observables from structure. Focus should be given to developing forward models that are transferable between folded proteins and IDPs. One of the challenges lies in choosing which data to train such forward models on. Ideally, models should be trained on IDP ensembles that are already well determined, but this quickly becomes a “chicken or the egg”-type problem. One possibility may be to optimize the forward model and IDP conformational ensemble in a self-consistent manner using reweighting approaches.

Third, a future prospect is to exploit recent advances in protein structure prediction. All of the conformational sampling methods discussed in this review require that the structures of the folded domains are known initially. Previously, this meant that an experimentally determined structure of each folded domain in the protein must be available. Substantial improvements in the accuracy
of protein structure prediction (Jumper et al., 2021) open up for the possibility to perform high-throughput ensemble determination of multidomain proteins with experimental data available using for example CG simulations.

Fourth, a potential development in the field is to determine conformational ensembles based on time-resolved experiments or time-dependent data that depend on the time-scales on which motions occur. The experiments discussed here all report on static observables that are simply an average over all conformations of the ensemble. However, for certain types of biophysical experiments, time-dependent dynamics must be taken into account. For example, NMR relaxation experiments report on molecular motions which can only be calculated from a time-series of structures or models of the dynamics, such as the Lipari-Szabo model-free approach (Lipari and Szabo, 1982; Brüschweiler et al., 1992; Peter et al., 2001; Salvi et al., 2016; Smith et al., 2020; Kümmerer et al., 2021). Additionally, many biological processes do not happen at equilibrium, but rather involve transitions from one state to another. Such transitions can be measured by many of the biophysical techniques mentioned in this review. For example, time-resolved SAXS and NMR experiments can be used to measure kinetic processes such as conformational changes and binding (Rennella and Brutscher, 2013; Tuukkanen et al., 2017; Cho et al., 2021). While approaches have already been developed to interpret NMR relaxation data using conformational ensembles (Salvi et al., 2016; Kümmerer et al., 2021), future research could involve the development of methods exploiting time-resolved data to resolve biologically relevant transitions.

Fifth, in order to move to more complex systems we need to be able to study conformational ensembles of heterogeneous or polydisperse mixtures of proteins. The integrative modeling approaches discussed in this review are based on calculating averaged observables from ensembles of isolated protein species and refining ensembles against experimental data measured on conformationally heterogenous but chemically homogeneous samples of pure proteins. Future research in the field could involve the development of methods that use experimental data not only averaged over conformations but also over a mixture of different molecular species. This would be useful in cases where specific species cannot be isolated, such as for polydisperse oligomers. Such methods would involve the extraction of even more information from sparse experimental data, so care will have to be taken to prevent overfitting of ensembles.

Conclusion

In this review, we have discussed the steps that go into determining conformational ensembles of IDPs and multidomain proteins using integrative approaches. Choosing the right combination of experiments, computational modeling, forward models, and integration approach will ensure that the resulting ensemble is accurate with regards to different structural features. We have also discussed recent applications of integrative conformational ensemble determination, showing that the field is ripe for addressing real biological questions from the ensemble perspective. Finally, we have proposed future directions for the field, including the development of more accurate and transferable force fields and better forward models for IDPs. We have also discussed the potential of interpreting time-resolved experiments and heterogenous protein samples using conformational ensembles. These advances can bring the field beyond static thermodynamic ensembles of simple systems and towards modeling of dynamic biological processes and complex polydisperse mixtures.

Acknowledgments

We acknowledge numerous fruitful discussions with members of the Lindorff-Larsen group on integrative modelling. Our work in this area is supported by the Lundbeck Foundation BRAINSTRUC initiative (R155-2015-2666 to KL-L).
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