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Quantitative Biophysical Characterization of Intrinsically Disordered Proteins

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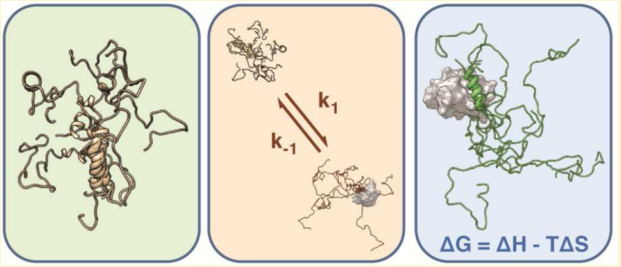
ABSTRACT: Intrinsically disordered proteins (IDPs) are broadly defined as protein regions that do not cooperatively fold into a spatially or temporally stable structure. Recent research strongly supports the hypothesis that a conserved functional role for structural disorder renders IDPs uniquely capable of functioning in biological processes such as cellular signaling and transcription. Recently, the frequency of application of rigorous mechanistic biochemistry and quantitative biophysics to disordered systems has increased dramatically. For example, the launch of the Protein Ensemble Database (pE-DB) demonstrates that the potential now exists to refine models for the native state structure of IDPs using experimental data. However, rigorous assessment of which observables place the strongest and least biased constraints on those ensembles is now needed. Most importantly, the past few years have seen strong growth in the number of biochemical and biophysical studies attempting to connect structural disorder with function. From the perspective of equilibrium thermodynamics, there is a clear need to assess the relative significance of hydrophobic versus electrostatic forces in IDP interactions, if it is possible to generalize at all. Finally, kinetic mechanisms that invoke conformational selection and/or induced fit are often used to characterize coupled IDP folding and binding, although application of these models is typically built upon thermodynamic observations. Recently, the reaction rates and kinetic mechanisms of more intrinsically disordered systems have been tested through rigorous kinetic experiments. Motivated by these exciting advances, here we provide a review and prospectus for the quantitative study of IDP structure, thermodynamics, and kinetics.



The folding funnel hypothesis established a new perspective on the widely accepted view that the biological function of a protein is determined by its "native state". Pioneering investigations of heme protein dynamics and function, much of it published in this journal,[1,2] revealed many decades ago that the native state of proteins must be characterized by multiple conformational substates to accommodate observed functions. In the modern view, we recognize that the natively folded state exists as an ensemble of conformations sampled from an energy landscape in which dynamic fluctuations between closely related conformers facilitate catalysis, macromolecular association, and other biological functions of cooperatively folded proteins.[3] Intrinsically disordered proteins (IDPs) represent the most extreme examples of this ensemble view, because they lack cooperatively folded structure under native conditions and are best described by highly dynamic and heterogeneous conformational ensembles yet retain function. This leads to a broader paradigm, affirming that the native state determines biological function, regardless of whether folding occurs. Our awareness that function can arise from native protein disorder suggests a pressing need for quantitative biochemical and biophysical characterization of the mechanisms linking structure and function in this exciting class of proteins.

The relatively recent expansion of the interest in disordered proteins among biochemists contrasts with their high prevalence in nature, particularly in eukaryotes. Within the human proteome, ~50% of all proteins are predicted to contain long disordered segments (≥30 residues)[4,5] with enrichment of ≤70% of all polypeptide sequence among transcription factors and signaling proteins.[6] In this context, structural plasticity facilitates multiple protein−protein interactions, placing IDPs at the "nodes" of large interaction networks.[7] Upon binding, IDPs often experience disorder-to-order transitions that tend toward desolvation and burial of a surface area larger than what an equivalent cooperatively folded protein would bury, while requiring shorter amino acid sequences to do so.[8] These properties support the hypothesis that retaining disorder is an evolutionary strategy that facilitates complex function within a compact genome.[9] In contrast, while disorder may support complex function, it can also promote complex pathology.[10] In cells, the abundance and turnover of IDPs are under tight control,[11] and aberrant IDP regulation has been implicated in cancer pathways and neurodegenerative disease,[10] completing the rationale for seeking to understand the molecular properties of disordered proteins.

For the reasons reviewed above, there is extraordinary current interest in elucidating the unique physicochemical properties and biological functions of IDPs. Presumably, unbound IDPs sample a diverse ensemble of conformations along a rugged free energy surface that lacks a significant bias

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toward any particular equilibrium structure (Figure 1, top surface).[4,12] Conversely, IDP interactions appear to be

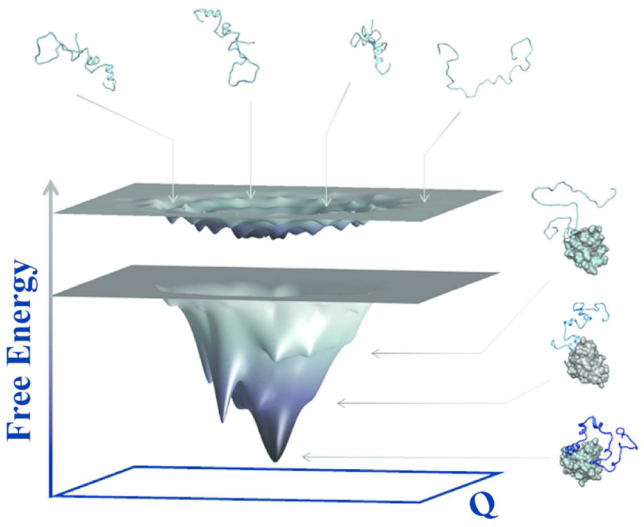

Figure 1. Free energy landscape hypothesis that can be generalized to describe intrinsically disordered proteins. The free energy surface for an IDP in the unbound state can be characterized by a rugged but relatively flat landscape, while IDP binding events funnel the free energy landscape, yielding a well-defined minimum.

governed by a funneled free energy surface that guides them toward a bound, folded structure (Figure 1, bottom surface), drawing strong parallels with the current view describing cooperatively folding proteins.[13,14] Despite the appeal of mechanistic proposals built from these broad observations, remarkably few experimental studies have quantified the ensembles IDPs adopt in solution, the thermodynamics that govern their interactions, or the kinetics that describe their transitions between biologically relevant states. Improving our understanding of the native disordered state will require quantitative descriptions of IDP conformational ensembles and interaction mechanisms, both of which are fertile grounds for modern biophysics. In the following, we review recent, ever intensifying efforts toward filling this gap and conclude with our perspectives on the future of reconciling pervasive conformational disorder with quantitative mechanistic insight for intrinsically disordered systems.

■ DEFINING STRUCTURE AMID DISORDER

Certain three-dimensional (3D) folds are repeated in proteins with specific functions; similarly, some biological functions are optimally performed by IDPs.[15−20] The unique functional properties of IDPs present a compelling reason for their study and suggest there is insight encoded in their structural properties, just as is the case for cooperatively folding domains.[6] Rigorous testing of this hypothesis requires that quantitative structural studies of IDPs be pursued. Of special interest, many recent studies have investigated the remarkable disorder-to-order transition that couples IDP folding to ligand binding,[21−27] suggesting a further biological rationale for IDP structure assessment. Among the many structure assessment tools available to the protein chemistry community, NMR, small-angle X-ray scattering (SAXS), and single-molecule Förster resonance energy transfer (smFRET) have emerged as the clear leaders for IDP applications. We recognize the successful application of these methods to studies of pathogenic IDPs, but for the sake of brevity, we have focused our discussion to IDPs that support regulation of normal cellular function; we direct interested readers to several in-depth reviews of pathological IDP misfolding and aggregation.[28−30] Finally, we note that a variety of purely computational approaches to this problem have also generated significant advances in our field,[31−34] but we have elected to focus solely on experimental techniques for the sake of constraining the scope of this review. There is one necessary exception to this decision; computational models are required to evaluate the meaning of averaged experimental observables, because of the ensemble nature of IDP structure.[35] Therefore, a brief discussion of computational methods for IDP conformer generation will close this section.

NMR Spectroscopy. Solution NMR spectroscopy is by far the most widely applied method for studying the dynamic structural ensembles of IDPs.[36,37] Despite the enormous potential seen in the examples we will review here, the general study of IDPs by NMR remains limited by the extremely poor ^{1}H-amide chemical shift dispersion typically observed in their spectra. To date, this has represented the most substantial barrier to broad applications in this field, because investigators are limited to working with those few IDPs whose spectra do show sufficient peak dispersion. Clearly, more systematically successful methods for NMR applications to IDPs are needed, given the biological imperative to better understand IDP structure−function relationships.

Within the protein NMR community, the ^{1}H−^{15}N heteronuclear single-quantum correlation (HSQC) experiment has emerged as the detection platform of choice for routine applications. The principal advantages for choosing the ^{1}H−^{15}N HSQC are the low costs of sample preparation and this spectrum's simple structure, with one two-dimensional (2D) resonance per amino acid residue, except for proline. In principle, protein NMR strategies could be built around any 2D spectrum meeting these minimal requirements, as evidenced by the adoption of transverse relaxation-optimized (TROSY) methods for large systems. Recently, ^{13}C direct detection spectroscopy has re-emerged as a viable tool for studying proteins in solution.[38] For IDPs, the ^{15}N,^{13}C-CON spectrum, correlating the ^{13}C carbonyl with the ^{15}N amide of each peptide plane, is an especially effective choice of 2D platform.[39−44] As illustrated for the disordered C-terminal tail of the phosphatase FCP1 in panels A and B of Figure 2, the overwhelming peak overlap observed in the ^{1}H−^{15}N HSQC is almost completely relieved through ^{15}N,^{13}C-CON detection. Perhaps even more significantly for IDPs, which tend to be enriched 1.7−1.8-fold with proline compared to cooperatively folding proteins,[45] the ^{15}N,^{13}C-CON spectrum contains a resonance for each peptide bond involving a proline nitrogen. The significance of this advantage is clearly seen for the C-terminal tail of the transcription factor Pdx1 (Figure 2C,D), for which 21% of the residues in the construct studied in our laboratory are prolines.[43]

The number of NMR studies published for IDPs has grown tremendously in recent years.[46] Intriguingly, chemical shifts, which represent one of the most direct NMR observables, have emerged as one of the most effective of the sparse structure constraints available for the refinement of disordered protein ensembles.[47] This is largely true because chemical shifts are indispensable probes for local secondary structure.[48] More importantly, regions of (partially ordered) secondary structure in the native IDP ensemble are strong candidates for

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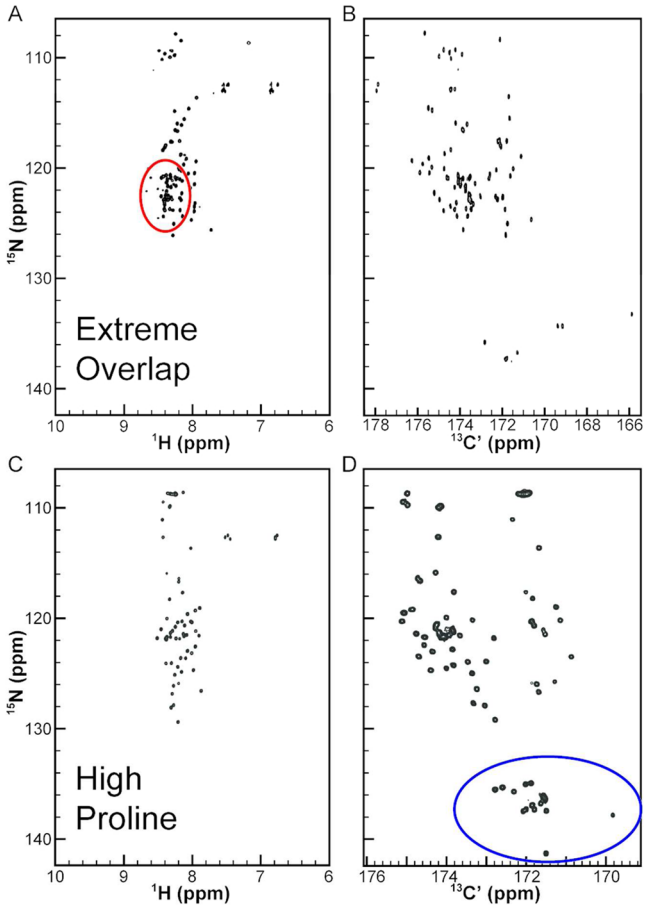

Figure 2. ^{1}H−^{15}N HSQC spectra of IDPs generally suffer from poor chemical shift dispersion, which is typically relieved in the ^{15}N,^{13}C-CON spectrum, as demonstrated for the C-terminus of FCP1 (A and B, respectively). Additionally, the C-terminus of Pdx-1 (C and D) displays the power of the ^{15}N,^{13}C-CON for proline-rich disordered proteins.

establishing connections between the fine details of IDP structure and their biological functions. The binding elements of IDPs that undergo disorder-to-order transitions upon forming interactions are often partially or completely preformed in the apo state, leading to their categorization as molecular recognition fragments (MoRFs).[49−51] When validated, these nonrandom structural features lend direct support to the hypothesis that native disorder provides a molecular pathway to biological fitness.

While the chemical shifts of backbone nuclei are powerful constraints on secondary structure, they are generally not sufficient to fully constrain the ensemble in the absence of other data. Measurement of residual dipolar couplings (RDCs) provides an effective means of complementing chemical shifts, particularly for the characterization of MoRFs. For example, the C-terminal region of the Sendai virus nucleoprotein was demonstrated to be α-helical through systematic RDC measurement.[52] More importantly, RDCs offer orthogonal constraints to chemical shifts because they are sensitive to long-range interactions.[47] On a cautionary note, dipolar couplings are sensitive to dynamic averaging, up to the millisecond time scale, and therefore are subject to limitations when employed in isolation as a long-range structural constraint for highly flexible systems.

One effective means of complementing RDC constraints on global order in IDP ensembles is to measure the paramagnetic relaxation enhancement (PRE) generated through deliberate attachment of paramagnetic species to the polypeptide chain. PRE measurements are powerful reporters of transient tertiary interactions within the ensemble.[53−56] While incorporating PRE constraints is relatively straightforward for cooperatively folding domains, caution is needed for IDP applications because backbone and probe dynamics both heavily influence the intensity of induced effects, causing complications. Also, as discussed for RDC measurements, the convolution of effects from dynamics and low-population conformational states can cause interpretation of PRE data to be ambiguous. Fortunately, certainty in the interpretation of PRE data can be improved through concurrent analysis of backbone spin relaxation, which helps to define the amplitude of local conformational dynamics in the ensemble.

Small-Angle Scattering Methods. Small-angle X-ray scattering (SAXS) provides low-resolution structural information for biomolecules in solution, which is often used synergistically with NMR constraints.[57−61] This is demonstrated by three remarkable examples from the intrinsically disordered segments in the Sendai virus phosphoprotein,[62] tumor suppressor p53,[63] and cyclin-dependent kinase inhibitor Sic1.[64] SAXS curves directly provide information about the oligomeric state, size, and overall shape of a molecule. Using the Guinier approximation, the radius of gyration (R_g) can be extracted from SAXS curves,[58] while conformational flexibility can be assessed both qualitatively and quantitatively through Kratky analysis and application of the Porod−Debye law.[65] SAXS data can also be used as input for various computational procedures to quantitatively describe the distribution of conformers within the IDP's structural ensemble,[58] yielding valuable information about the 3D shape,[66] and the global level of compaction present.[67]

In addition, small-angle neutron scattering (SANS) has emerged as a complementary method for the structural characterization of IDPs.[68−70] SANS is similar to SAXS in that it provides information about the overall shape of a molecule in solution. When SANS is combined with contrast variation (CV) or selective deuteration, the benefits for disordered proteins, especially those in complex, can be clearly seen. For example, CV-SANS has been used to show that the histone tails within the canonical H2A nucleosome intertwine with the surrounding DNA, while those within the H2A.B variant, which features outstretched DNA, turn inward toward the core, thus rationalizing the differences in the observed stabilities of the two forms.[71] Future studies may exploit CV-SANS to obtain useful distance restraints as input for ensemble generation of IDPs in the bound state. This would be especially advantageous for systems that are large by the standards of NMR spectroscopy or for highly dynamic complexes that elude detection, because of chemical exchange on the time scale of chemical shift evolution.

Single-Molecule FRET. The final experimental technique we will review is single-molecule FRET, which has also contributed substantially to our understanding of the conformational landscapes of disordered proteins.[72−74] Several notable studies have used smFRET-derived distances to explore how the unique physicochemical properties of IDPs affect their dimensions in solution. For example, it was found that despite convergence in the dimensions of both IDPs and cooperatively folded proteins at high GdmCl concentrations, the polymer

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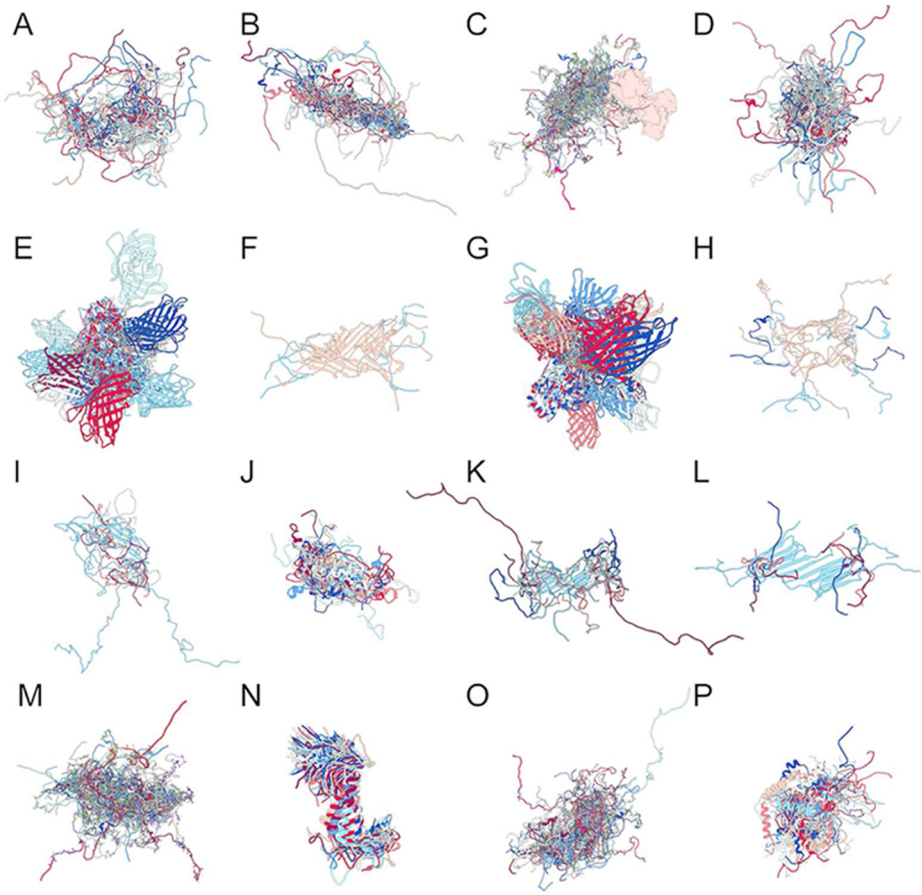

Figure 3. Ensemble models currently deposited in the Protein Ensemble Database (pE-DB): (A) 1AAA, (B) 9AAA, (C) 5AAC, (D) 6AAA, (E) 3AAA, (F) 8AAA, (G) 4AAA, (H) 3AAB, (I) 7AAA, (J) 8AAC, (K) 2AAB, (L) 1AAB, (M) 6AAC, (N) 2AAA, (O) 7AAC, and (P) 5AAA.

scaling laws of IDPs diverged significantly from those of folded proteins when the denaturant concentration was reduced.[75] The clearest interpretation of these results is that, under native conditions, IDPs adopt ensembles fundamentally different from the unfolded state of cooperatively folding proteins, because of differences in solvation of the relatively hydrophilic and charged amino acid sequences found in natively disordered proteins.[75] The prior result was achieved using the charged denaturant guanidinium hydrochloride; when the neutral denaturant urea is used instead, this result is not reproduced, highlighting the importance of repulsive electrostatic forces for establishing the dimensions of IDP ensembles and confirming predictions from polyampholyte theory.[76] For Sic1, polyampholyte behavior manifests with a similar dependence on denaturant and ionic strength upon scaling. Interestingly, investigation of Sic1 revealed three distinct subpopulations, each of which exhibited different sensitivities to electrostatic screening, thus highlighting the utility of single-molecule techniques for resolving conformational subpopulations otherwise obscured by ensemble averaged measurements.[77] In addition, intramolecular distances derived from smFRET have been used to elucidate structure–function relationships for IDPs. For example, compaction of the N-terminus of PAGE4, a stress-response protein overexpressed in prostate cancer cells, was observed following phosphorylation by HIPK1. This modification weakened the affinity of PAGE4 for c-Jun, suggesting a possible regulatory role for expression of c-Jun target genes.[78] As a final note, smFRET-based intramolecular distances have not yet been broadly applied in ensemble modeling schemes, but a recent demonstration of their utility for modeling flexible single-stranded DNA[79] suggests smFRET distances will prove to be increasingly valuable for IDP ensemble generation in the future.

Ensemble Generation. Spectroscopic data suggest that IDPs may adopt anything from a heterogeneous, but relatively compact, ensemble of structures to a denatured state highly enriched with nativelike secondary structural features, reminiscent of molten globules.[80] Physically, IDPs are best described by ensemble states in which the protein is able to interconvert, on some time scale(s), between multiple conformations on a rugged potential energy landscape (Figure 1). To rigorously assess the relationship between this landscape and function requires quantitative evaluation of the IDP conformers generated through application of experimental data as constraints. Particularly for the case of molecular recognition fragments, which are often enriched with secondary structure, examples now abound lending support to the hypothesis that the details of protein structure offer insights into function and that this is especially true for IDPs, for which our mechanistic understanding is still largely incomplete.

Since its inception in 1971, the Protein Data Bank (www.pdb.org)[81] has provided an irreplaceable resource for the research community, aiming to make the products of structural biology freely accessible to all potential beneficiaries. Unfortunately, for those who study disordered proteins, the conformational ensembles we generate are very often inappropriate for inclusion in the PDB. While it is now routine to refine IDP ensembles capable of reproducing average structural observables (e.g., NMR chemical shifts and SAXS

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radii of gyration), it generally is not the case that the individual conformers contributing to the structure sets are themselves unique. On the other hand, the sets of conformers generated in these efforts often lead to unique and important hypotheses, as was the case for Sic1,[64] creating an imperative to distribute the conformer sets generated both broadly and freely. Recently, a consortium of investigators has launched the Protein Ensemble Database, which seeks to facilitate biological data-mining and structural methods development through the broad distribution of structural coordinates and primary data for disordered protein ensembles.[82]

At the time of this writing, there were 16 entries in the pE-DB, each of which is presented graphically in Figure 3. As can be seen, a holistic view of the deposited IDP ensembles often reveals unique structural properties, despite the often degenerate nature of individual conformers. For example, the bent pSic1 ensemble promotes ultrasensitive binding to CDC4 (Figure 3A), whereas the rodlike Sic1 ensemble (Figure 3B) does not. Finally, the entries highlight the diversity of experimental techniques currently applied to ensemble generation, for example, p15PAF (Figure 3D), generated from NMR and SAXS, or the unbound p27 KID domain (Figure 3N), generated through molecular dynamics (MD) simulation. Although the pE-DB is still in its infancy, its establishment sets an important milestone for the study of structural disorder.

Ensemble generation can be broadly separated into *de novo* strategies (purely computational) or strategies built upon experimental constraints. Most experimental approaches rely on generating a large pool of starting structures, from which experimental parameters are back calculated and compared to experimental data, resulting in conformer rejection or refinement based on some statistic, for example, minimization of χ^2. Because of the sparse nature of ensemble averaged data, IDP ensembles can be sensitive to the structures used as input. Accordingly, starting pool generation has been accomplished by various techniques. Molecular dynamics simulation provides an attractive option for input conformer generation.[83,84] However, there is still reason to be concerned that classical MD is not well suited for rigorous sampling of IDP potential energy surfaces and extremely long trajectories are likely required to reach convergence, if possible.[85] In this regard, advanced sampling techniques, including replica exchange molecular dynamics (REMD)[33,86] and accelerated molecular dynamics (AMD),[63,87] may be advantageous. Monte Carlo (MC) simulations also provide an attractive option for generating large conformational ensembles.[88] For example, the ABSINTH implicit solvent force field, developed specifically for MC simulation of IDPs,[89] efficiently and accurately samples the conformational space spanned by highly disordered proteins.

The diversity of software packages available to implement experimentally constrained ensemble refinement strategies has also grown in recent years. For example, the Flexible-Meccano algorithm, and its more recent evolution into the ASTEROIDS package for ensemble refinement, has proven to be highly successful for modeling IDPs.[47] Although not formally intended for NMR applications during its initial development, the TRaDES package provides an alternative for generating initial trial sets of structures.[90] In this capacity, TRaDES has been bundled with a set of algorithms collectively named ENSEMBLE that has also proven to be highly effective for IDP ensemble generation and refinement.[91] Finally, ensemble generation using Bayesian Statistics also provides an estimate of the uncertainty in the weights of each conformer.[86] Regardless of which method for ensemble refinement is applied, it is clear that our capacity to generate IDP ensembles and use them for rigorous hypothesis testing has rapidly matured. As their use grows and rigorous validation methods are established for quality control, there is reason to hope that quantitative structural biology of intrinsically disordered systems will become an expected tool in comprehensive biochemical investigations, just as it has for highly ordered systems.

THERMODYNAMIC ANALYSIS OF IDP INTERACTIONS

Structural and dynamic descriptions of disordered states and bound complexes are necessary, but not sufficient, to understand the functional behavior of IDPs. Accordingly, there is considerable interest in quantifying the thermodynamic forces that govern IDP interactions. For example, NMR titrations have been employed to extract residue specific equilibrium dissociation constants (K_d) when multiple binding sites on an IDP are present.[92−94] In addition, various florescence-based techniques, including tryptophan quenching,[95] fluorescence anisotropy,[94,96] smFRET,[7] and fluorescence correlation spectroscopy (FCS),[97−99] have been applied to study IDP interactions. Fluorescence-based methods are useful for accurately measuring K_d when binding is extremely tight[7] or when one or more of the interacting species is prone to aggregation.[7,94] Importantly, fluorescence-based techniques are not restricted by any apparent limitation on the size of the IDP under investigation.[98] In a recent example, smFRET was used to study the interactions between the adenovirus E1A oncoprotein, an intrinsically disordered hub, and two of its partners, the Taz2 domain CBP and pRb.[7] This study demonstrated that the system can be driven to either positive or negative cooperatively, depending on the availability of E1A binding sites. Thus, E1A demonstrates that allosteric modulation of a disordered protein hub, through binary or ternary interactions, can influence population distributions and functional outcomes.

Isothermal Titration Calorimetry. When applicable, isothermal titration calorimetry (ITC) is by far one of the most robust methods available for thermodynamic studies.[100] ITC provides a direct measurement of the binding enthalpy (ΔH) and equilibrium association constant (K_a), allowing the calculation of the Gibbs free energy (ΔG) through the known temperature and parametric determination of the binding entropy (ΔS). Significantly, when ΔH is determined by ITC in a temperature series, the constant-pressure heat capacity change (ΔC_p) associated with the binding process is also experimentally accessible.

For many IDPs, binding is accompanied by a disorder-to-order transition, leading to the hypothesis that the unfavorable entropy loss incurred by conformational restriction of the IDP in the bound state must be offset by a favorable gain in enthalpy. It has been proposed that this entropy penalty may be mitigated in "fuzzy" IDP complexes, which retain some extent of disorder in the bound state (Figure 4).[101] Although the potential functional advantages available to fuzzy complexes are intriguing, the broader picture implicit in the hypothesis is that entropy losses incurred upon IDP binding promote reversibility. While it seems likely that the conformational entropy of the IDP chain will decrease upon binding, this point of view does not account for the role of solvent in mediating association of IDP with binding partners. In other words, as

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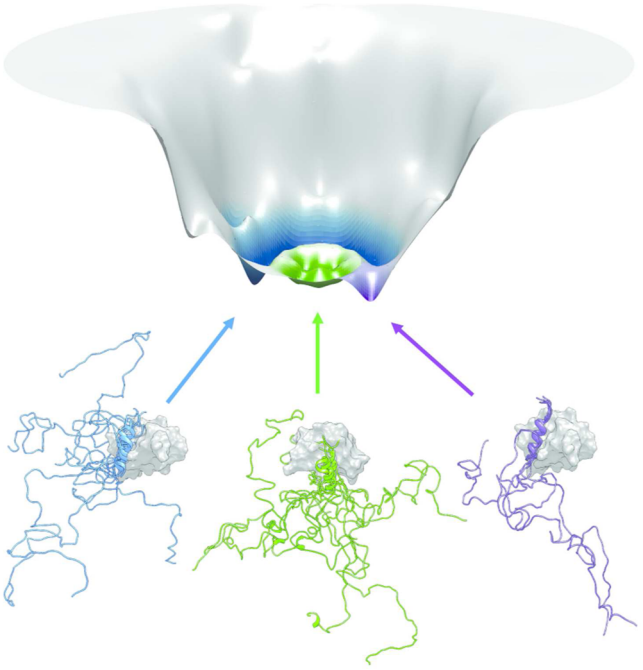

Figure 4. Free energy surface of a "fuzzy complex" depicted as a funneled yet wide free energy surface, where multiple distinct IDP conformations are sampled in the bound state.

with many other favorable protein folding phenomena, a favorable change in solvent entropy near room temperature is likely to overwhelm the loss of chain entropy, making the functional value of any marginal stabilization caused by "fuzziness" unclear. Fortunately, the detailed thermodynamic information conveyed through temperature-dependent ITC measurements provides exactly the experimental data needed to rigorously evaluate the energetics of coupled IDP folding and binding, even if the entropy estimates generated are indirect.

Structural analysis of bound IDPs has revealed significant hydrophobic character at many IDP binding interfaces, suggesting that hydrophobic forces play an important role in coupled folding and binding.[8,102] One striking example is the Gcn4:Gal11/Med14 interaction, which is stabilized exclusively by three hydrophobic contacts.[103] Variable-temperature ITC performed on this system revealed the signatures of apolar desolvation through strong temperature dependence in the observed binding enthalpy, resulting in a large negative heat capacity.[103] Far from being unique, this trend is also seen in the FCP1−Rap74 interaction where binding is endothermic at low temperatures but becomes exothermic above room temperature.[104] Significantly, the calculated change in system entropy made a favorable contribution to FCP1 binding at all temperatures, indicating that the FCP1−Rap74 interaction is at least partially under entropic control, despite being accompanied by an increase in the extent of FCP1 folding.

In macromolecular assembly, structural disorder finds a delicate balance between conformational adaptability and induced stabilization. In fact, recent work has shown that IDPs often utilize cooperativity to assemble large macromolecular complexes.[105,106] One prominent example is Nup159, an intrinsically disordered hub involved in nucleocytoplasmic transport that assembles with Dyn2 to support the central pore.[25] The binding of one Dyn2 dimer to two Nup159 molecules creates a bivalent scaffold. This structure, albeit weakly assembled, lowers the conformational entropy of the system, thus allowing two more Dyn2 dimers to bind in a cooperative manner, characterized by increasingly favorable enthalpic and entropic contributions. However, after the third Dyn2 dimer binds, binding becomes an enthalpically driven process, because of the entropic cost of increased rigidity. Interestingly, although there are six Dyn2 binding sites on each Nup159 molecule, only five become occupied in the fully bound complex. Presumably, this feature is an evolutionary compromise that allows Nup159 to balance the unfavorable entropy of binding successive Dyn2 dimers with moderate affinity and the stability required for its biochemical function.

In addition to solvation/desolvation effects, IDPs often rely on finely tuned electrostatic interactions to achieve high specificity.[107] In these cases, ITC can aid in revealing the molecular origins of these phenomena, particularly in conjunction with site-directed mutagenesis of mechanistically significant charged residues.[108−111] For example, in studies of the tumor suppressor p53, ITC has been combined with site-directed mutagenesis to characterize the effects of oncogenic

Table 1. Thermodynamic Characterization of Interactions of IDP with Folded Partners by ITC[a]

folded	IDP	K_d (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔC_p (cal mol^{-1} K^{-1})	ref
CcdB	CcdA	3.66 × 10^{-6}[d]	−15.6	−35.5	−19.9	−630	26
eIF4E	4E-BP2	0.0032	−11.42[e]	−8.81	2.61		134
Keap1	NRF2	0.023	−10.4	−16.96	−6.56		113
Cdk2-cyclin A	p27-KID	0.035	−11.6	−40.2	−28.6	−872	117
SBDS[c]	EFL1	0.0787	−9.86	−14	−4.09		135
RPP29[b,f]	RPP21	0.105	−9.35	5.95	15.29	−1115	27
Keap1	WTX	0.25	−9.01	18.04	−9.03		113
IκBα[b]	Relα NLS	0.371	−8.6	−4.2	4.4	−400	136
Keap1	WTX pS286	1.5	−7.95	−10.83	−2.88		113
RAP74	FCP1	1.91	−7.797	−1.337	6.46	−240	104
NudE	IC (1−143)	2.2	−7.8	−4.2	3.6		137
Keap1	ProTα	2.6	−7.61	−14.8	−7.19		111
SEC3	mVβ8.2	12	−6.70	−4.88	1.82	−136	138
Sem-5 C-SH3	SosY	39.22	−6	−8.3	−2.3	−166	139
Pcf11 CID[b]	RNAPII pSer2CTD	180	−5.094[d]	−9.394[e]	−4.3		140

[a]All parameters except those indicated are from data collected at 25 °C. [b]Reported parameters are from data collected at 20 °C. [c]Reported parameters are from data collected at 30 °C. [d]$\Delta G = -RT \ln K_a$. [e]$\Delta G = \Delta H - T\Delta S$. [f]Both interacting molecules are IDPs.

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mutations on ASPP2 binding.[112] Similarly, post-translational modifications can lead to changes in electrostatics, and ITC has been used to address the role of phosphorylation in binding mechanisms,[94,113] as highlighted for binding to p300 Taz2.[114] These and other studies have generally shown that electrostatics contribute a modest enhancement to hydrophobic interactions, but extreme cases have been reported where binding is dominated by direct enthalpic contributions. For example, charge−charge complementarity is almost exclusively responsible for the interaction between the Kelch domain of Keap1, a hub protein involved in oxidative stress response, and the intrinsically disordered oncoprotein ProTα.[111] Systems that rely primarily on direct enthalpic contributions for complex formation tend to remain highly disordered in the bound state,[77,94,111,115] underpinning the role of electrostatics in fuzzy complex formation and the order-to-disorder transitions observed for some IDPs upon binding.[115]

While hub proteins have generated significant attention, the thermodynamic profiles for a broad set of IDP interactions, determined from ITC, highlight the functional diversity available to this class of proteins (Table 1). The wide range of binding affinities observed parallels the multifaceted roles of IDPs in the cell. First, the extremely high binding affinity that supports inhibition of the gyrase poison CcdB by the antitoxin CcdA[26] demonstrates that not all IDP interactions are weak and easily reversible. It is especially instructive to compare the varying affinities of ProTα, NRF2, and WTX for the hub protein Keap1, as discussed above.[113] Finally, the delicate balance between entropy and enthalpy that facilitates the coupled folding and binding of IDPs is often masked in single-temperature titrations. Therefore, it appears that the most prudent course for investigators is to perform titrations over a range of temperatures, to avoid generalizations regarding entropic penalties to binding that may lead to incorrect modeling of the data, or the generation of uninstructive hypotheses.

KINETICS OF IDP INTERACTIONS

Elucidating the mechanisms of IDP interactions, and distinguishing between competing kinetic mechanisms, requires knowledge of kinetic rate constants. To this end, NMR relaxation dispersion,[116] surface plasmon resonance,[117,118] and stopped-flow spectroscopy[119−122] have all been applied. As seen in many of these studies, IDP binding is often described well by an apparent two-state kinetic model, which features a linear dependence of k_{obs} on protein concentration (Figure 5A).[23,123] However, more complex multiphasic kinetics (three-state and higher), which display a nonlinear dependence of k_{obs} on protein concentration, have also been observed.[119,124] While this behavior reveals that a conformational change takes place along the reaction coordinate, the nature of this transition is widely debated. Several theories have emerged, the dominant two being the conformational selection model, wherein a preformed bound state-like conformation is required for ligand recognition and binding (Figure 5B), and the induced fit model, wherein ligand recognition occurs in the disordered state prompting IDP folding (Figure 5C). As confirmation of our expectation that not all IDPs behave equivalently, there is abundant evidence in support of both of these limiting models among the set of proteins studied.[22,116,125] In contrast, direct experimental evidence to exclusively support either pathway, through rejection of the other, is often difficult to obtain. The similarity of these models and experimental designs and those

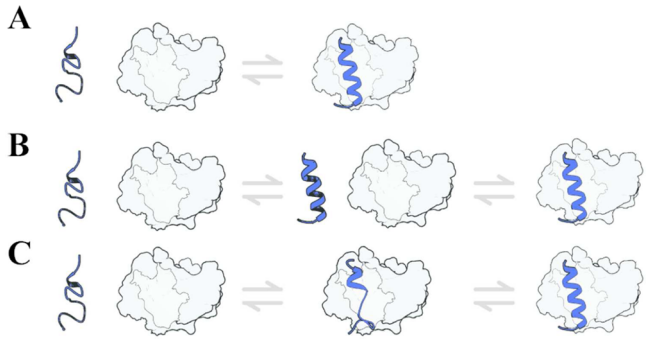

Figure 5. Three proposed IDP binding mechanisms include (A) one-step binding (apparent two-state) and two-step binding schemes (apparent three-state), where (B) conformational change precedes binding (conformational selection) and (C) conformational change follows binding (induced fit).

used routinely in the protein folding and enzymology communities is no accident. However, IDPs do possess distinct physicochemical properties, so adaptation of methods and models is almost certain to be required to accurately represent the kinetic behavior of disordered proteins.

Resolving Induced Fit from Conformational Selection.

In principle, kinetic measurements can be used to distinguish between the two limiting scenarios of conformational selection and induced fit. For example, Gianni et al. argue that by performing experiments in which the concentrations of both the protein and ligand are varied separately, the induced fit mechanism will manifest itself as a hyperbolic dependence of k_{obs} on the concentration of both species.[124] However, if a fast conformational change precedes binding, k_{obs} will display hyperbolic behavior only when the species that undergoes conformational change is held constant; linear behavior will manifest when the concentration of the species undergoing a conformational change is increased. While such an experimental design is theoretically sound, it is often difficult to implement in practice, as high concentrations of both interacting species are required. Also, kinetic methods with unusually fast time resolution may be needed to detect fast folding of preformed structural elements, such as α-helices. Another type of decisive experiment involves determining whether a binding reaction is diffusion-limited. As Rogers et al. argue, reaching the diffusion limit would require all molecular collisions between cognate partners to result in binding, regardless of the particular conformation of the IDP (i.e., binding would proceed through induced fit).[126] Detailed kinetic investigation of the Bcl-1−PUMA interaction showed that the criteria necessary to define the diffusion-limited reaction for folded proteins, the predictable dependence of k_a on solvent viscosity and temperature, may not be sufficient for disordered proteins, because of geometrical considerations.[126]

Synergistic models, which combine aspects of both mechanisms, have also been reported. For example, the extended conformational capture model builds upon a framework similar to the folding funnel hypothesis discussed above and highlights how the energy surfaces of both the IDP and the folded partner can influence each other and bias the IDP's binding trajectory.[14] In a natural extension of the induced fit/conformational capture dichotomy discussed earlier in this section, flux-based models acknowledge that conformational selection and induced fit pathways may both be present as limiting behaviors for most systems. From this new point of

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view, the flux of the reaction can be biased by both intrinsic and extrinsic factors, explaining why some systems have produced evidence in support of both models (Figure 6). For example,

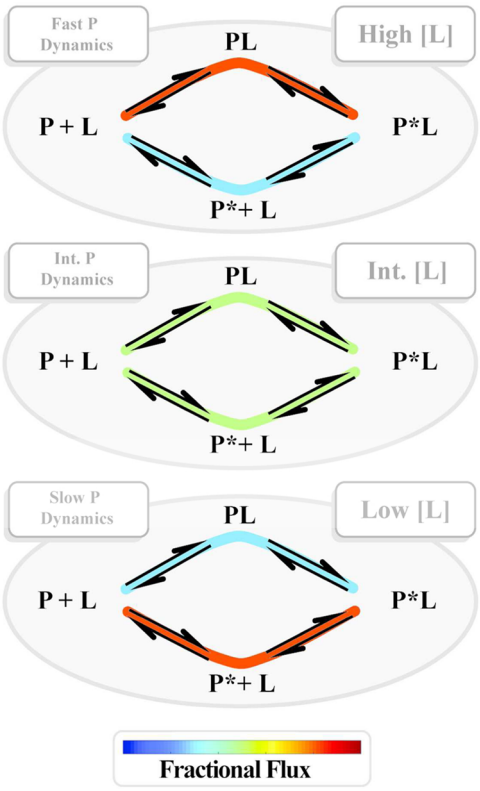

Figure 6. Generalized reaction scheme where flux is kinetically partitioned between the conformational selection and induced fit pathways, based on IDP conformational dynamics and ligand concentration.

Greives et al. suggest that intrachain dynamics within the IDP ensemble can shift the binding mechanism, with slower conformational transitions favoring conformational selection and fast interconversion rates favoring induced fit pathways.[127] In addition, Hammes et al. have suggested ligand concentration is an important determinant to increase flux through a particular pathway.[128] As an example of these mechanistic nuances, Daniels et al. showed that the *Bacillus subtilis* RNase P protein experienced varying levels of folding through either mechanism, based on the concentration of PP_i, with lower and higher concentrations of ligand favoring conformational selection and induced fit pathways, respectively.[122] Thus, from a biological perspective, flux models provide an attractive rationale for kinetic control of signaling in response to environmental stimuli.

Transition States. In the field of kinetic enzymology, identification of transition states and their description via the substrate or product structure has been the key to broad progress, often leading to rational design of inhibitors and motivating the search for drug candidates. To this end, several groups have applied a protein engineering method known as Φ-value analysis, which has also seen some success in describing the kinetics of IDP binding.[23,24,120,129,130] In Φ-value analysis, atomic-level structural information about the transition state is inferred by comparing the binding kinetics of the wild-type protein to those of a series of single-point mutants to obtain the "Φ-value", which is calculated as the ratio of the change in activation energy for folding upon mutation ($\Delta\Delta G^{\dagger\dagger}$) to the change in equilibrium free energy upon mutation ($\Delta\Delta G^{eq}$).[131] For IDP interactions, it is common to introduce alanine-glycine substitutions or nondisruptive mutations to reduce the size of side chains, to gain insight into the secondary or tertiary structure of the transition state, respectively.

Recently, Hill et al. used a detailed Φ-value analysis to characterize the formation of spectrin repeat domains (Figure 7A,D).[23] The analysis invoked a model wherein the preformed

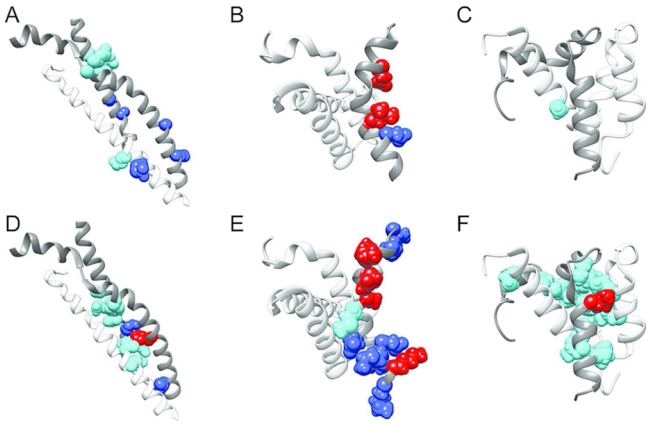

Figure 7. Secondary (A–C) and tertiary (D–F) Φ-values classified as weak ($0 < \Phi < 0.3$; colored cyan), medium ($0.3 < \Phi < 0.7$; colored blue), and strong ($0.7 < \Phi < 1$; colored red). Φ-values are mapped onto PDB structures for (A and D) the erythroid α-spectrin (white)–β-spectrin (gray) complex (PDB entry 3LBX), (B and E) the pwtKIX (white)–c-MYB* (gray) complex (PDB entry 1SB0), and (C and F) the ACTR (gray)–NCBD (white) complex (PDB entry 1KBH).

C-helix from α-spectrin acts as a template that guides the ensemble of transiently formed secondary structures in the helices A and B of β-spectrin toward the bound state, which is a fully folded triple-helical domain. This "templating mechanism" is an interesting example of synergistic binding, where both the presence of preformed structural elements and structural adaptation act in concert to accomplish folding and binding. In the coupled folding and binding of c-Myb to KIX (Figure 7B,E), Φ-values suggest c-Myb possesses a high degree of nativelike structure in the transition state.[24] In contrast, Φ-value analysis of the ACTR–NCBD interaction (Figure 7C,F) suggested the presence of substantially more disorder in the transition state.[120] It is also interesting to consider that there is a positive correlation between preformed structure and binding kinetics for ACTR–NCBD interaction,[125] while residual structure has little effect on the binding kinetics of c-Myb–KIX interaction.[24] This suggests that, for IDPs possessing a large degree of bound state structure in the transition state, prestabilization of these conformations can lower the energetic barrier of the rate-limiting step for association. Importantly, these Φ-value analyses highlight the general applicability of methods traditionally used to study catalysis, ligand binding, or protein folding to the study of coupled folding and binding involving IDPs.

The most general conclusion to be drawn from the kinetic investigations conducted thus far is that IDPs are not monolithic as a class of proteins. Rather, these studies suggest that disordered proteins rely on a range of mechanisms to bind

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their partners, just as cooperatively folding proteins do. It is becoming increasingly apparent that IDPs may possess various pathways toward the bound state, which are often influenced by extrinsic factors, such as local ligand concentration.[119,122] Furthermore, detailed structural knowledge can facilitate the design of experiments aimed at discriminating between limiting pathways, because the dynamics of conformational fluctuations within the ensemble often couple to kinetic outcomes.[127] Of significant biological interest, the kinetic mechanism describing IDP interactions is often influenced by the presence or absence of post-translational modifications.[132,133] Clearly, this is a rich area of future growth for biochemists to explore.

CONCLUSION

In the past few decades, the field of intrinsically disordered proteins has grown dramatically, yielding several general conclusions to be drawn from the studies presented here. Most importantly, IDPs have much in common with their cooperatively folded counterparts. Experimental methods and computational procedures for ensemble generation now allow routine modeling of disordered systems, facilitating hypothesis testing as in any other field of structural biology. Of pressing need now is a formal framework for ensemble validation. Rigorous assessment is needed to define input constraint combinations that cost-effectively produce the most unique ensembles, while also minimizing overfitting. More significantly, standardized reporting practices would benefit the community. For example, the PDB has established data-reporting criteria for model deposition, and the community has agreed to helpful norms regarding, e.g., the number of models to be included in NMR structure bundles. Similar guidelines for reporting IDP ensembles in the pE-DB or other databases would help investigators assess model quality for themselves.

Also of great significance, equilibrium thermodynamics experiments have helped to dispel the common misconception that structural disorder constrains interactions to a narrow range of affinities by imposing entropic penalties to binding. Indeed, IDPs interact over a broad range of affinities, utilize cooperativity to enhance stability, and rely on hydrophobic effects for coupled folding and binding, in strong analogy to protein folding. Most notably, recent applications of isothermal titration calorimetry have provided insight into the hydrophobic impetus for coupled folding and binding. The continued application of variable-temperature ITC will deepen our understanding of this process and may help to improve our understanding of the functional advantages of bound state-induced disorder and dynamic fuzziness.

Finally, kinetic studies have demonstrated that IDPs rely on a broad range of mechanisms to accomplish biological function. Although models for coupled folding and binding are typically built upon thermodynamic observations, mechanistic insights into these processes require detailed kinetic analyses. Recent studies have demonstrated that many of the experimental techniques used in enzymology or protein folding can be directly transferred to disordered systems or require modest adaptation to the physicochemical norms of IDPs. Continued kinetic investigation will be necessary to elucidate the ways in which IDP interactions can be tuned to support intricate biochemical pathways. The prevalence of protein nonfolding as an important regulatory mechanism in biology is well-established, yielding a rich new class of proteins for biochemists to characterize quantitatively in the laboratory.

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ABBREVIATIONS

IDP, intrinsically disordered protein; NMR, nuclear magnetic resonance; SAXS, small-angle X-ray scattering; smFRET, single-molecule Förster resonance energy transfer; HSQC, heteronuclear single-quantum correlation; TROSY, transverse relaxation-optimized spectroscopy; MoRFs, molecular recognition fragments; RDC, residual dipolar coupling; PRE, paramagnetic relaxation enhancement; R_g, radius of gyration; SANS, small-angle neutron scattering; CV, contrast variation; CV-SANS, contrast variation small-angle neutron scattering; GdmCl, guanidinium hydrochloride; pE-DB, Protein Ensemble Database; MD, molecular dynamics; REMD, replica exchange molecular dynamics; AMD, accelerated molecular dynamics; MC, Monte Carlo; K_d, dissociation constant; K_a, association constant; FCS, fluorescence correlation spectroscopy; ITC, isothermal titration calorimetry; ΔH, change in binding enthalpy; ΔG, change in Gibbs free energy; ΔS, change in binding entropy; ΔC_p, constant-pressure heat capacity change; k_{obs}, observed rate constant; PP_i, diphosphate; $\Delta\Delta G^{\dagger\dagger}$, change in activation energy for folding upon mutation; $\Delta\Delta G^{eq}$, change in equilibrium free energy upon mutation.

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