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Abstract

Protein flexibility spans a broad spectrum, from highly stable folded to intrinsically disordered states. In this review, we discuss how various techniques, including X-ray crystallography, nuclear magnetic resonance spectroscopy and ensemble-modeling strategies employing various experimental measurements, have enabled detailed structural and dynamic characterizations of proteins in their free and bound states. This has revealed a variety of possible binding scenarios in which flexibility can either decrease or increase upon binding. Furthermore, dynamic free-state ensembles have repeatedly been observed to contain transiently formed conformations that partially or completely resemble bound states. These results demonstrate an intimate connection between protein flexibility and protein interactions and illustrate the huge diversity of structure and dynamics in both free proteins and protein complexes.

Introduction

Proteins have highly diverse structures and functions. This diversity occurs not only between different proteins, but also at the level of individual proteins, which can experience a wide range of conformational dynamics. Proteins can have nearly rigid, compact folds, undergo small to large conformational rearrangements within the context of overall folded structures, or even traverse the vast conformational landscapes of intrinsically disordered proteins [1–3]. Structural flexibility has been associated with functional promiscuity [4]. Thus the traditional protein structure/function paradigm, which faced challenges from the observation of highly flexible but functional proteins [2], can be partially vindicated if the ability to form different conformational states is associated with functional variability. Nevertheless, flexibility itself remains intrinsically important for many biological processes and is therefore not merely a mechanism for facilitating multi-functionality.

The biological functions of most proteins involve interactions with other proteins and assembly into protein complexes [5]. While even highly flexible proteins have traditionally been assumed to be predominantly ordered when bound in protein complexes, in recent years it has been recognized that proteins can remain flexible in their bound states [6,7]. Recent methodological advances have tremendously aided our ability to characterize the structure and dynamics of free and bound states of proteins. This has revealed a diverse landscape of protein flexibility and binding, in which the structure and flexibility of both free and bound states are intimately related to the continuum of conformational and dynamical changes that occur upon binding.

Assessing flexibility and conformational changes upon binding from protein crystal structures

X-ray crystallography has long been the dominant tool of structural biology, providing numerous high-resolution models of protein structures. While the snapshot of a protein provided by an individual crystal structure often provides deep insights into protein function, it can also contribute to the perception of proteins as static, inflexible molecules. Moreover, the recent trend of X-ray diffraction at cryogenic temperatures strengthens this further [8]. Despite this, crystal structures themselves can provide much valuable information on protein dynamics, *e.g.* by comparing multiple structures determined under different conditions or in

free and bound states [9], integrating with molecular dynamics simulations [10] or normal mode analyses [11,12].

In a recent study, Dobbins *et al.* used an elastic network model to probe the relationship between flexibility and conformational changes upon binding for a number of proteins with crystal structures determined in both free and bound states [13]. They observed that the conformational changes upon binding are often closely related to low-frequency intrinsic motions that occur in isolation. Notably, the most flexible proteins were found to have a greater propensity for large binding-induced conformational changes. Other studies have found similar associations between intrinsic dynamics and the structural and dynamic changes that occur upon binding other proteins or small ligands [14–17].

An alternate strategy for probing the relationship between flexibility and conformational changes upon binding utilizes our recently introduced measure of relative solvent accessible surface area (A_{rel}) [18]. A_{rel} is simply the observed accessible surface area of a polypeptide chain, divided by the value expected for a folded protein of the same molecular weight [19]. Since proteins that bury less surface area are less able to overcome the conformational entropy of the unfolded state [20], A_{rel} functions as a good proxy for the intrinsic flexibility of free proteins. Furthermore, we found the A_{rel} values of both isolated and bound proteins to be highly predictive of the magnitude of conformational changes that occur upon binding [18]. This therefore demonstrates a general correspondence between intrinsic flexibility in the free state and conformational changes upon binding, as illustrated for several proteins in Figure 1.

Application of A_{rel} to a large set of protein complexes suggested that very large conformational changes upon binding are common: 27% of heteromeric subunits are predicted to have > 5 Å RMSD between free and bound states; and 58% > 2.5 Å [18]. This contrasts with other work comparing free and bound structures of proteins showing that large conformational changes upon binding are relatively rare [21]. The discrepancy is likely due to the greater difficulty of crystallizing flexible proteins: any analysis involving free- and bound-state crystal structures will be biased towards smaller conformational changes.

The correspondence between free-state flexibility and conformational changes upon binding naturally leads to questions about the structural and dynamic similarities between free and bound states of proteins. Much attention has been paid to the idea that binding mechanisms can involve induced fit, in which the free state is structurally dissimilar from the bound state [22], or conformational selection, in which binding proceeds via conformations that resemble the bound state [23,24]. In fact, rather than considering these mechanisms as distinct, recent work supports the idea of a spectrum of binding, in which varying degrees of structural similarity between free and bound states can play different roles in molecular recognition [25–30]. Detailed comparisons of the structure and dynamics of free and bound states at a level that goes beyond that possible using crystal structures alone are required to fully reveal the variety of these binding mechanisms.

NMR characterizations of flexibility in the free and bound states of folded proteins

Advances in protein nuclear magnetic resonance (NMR) spectroscopy have dramatically expanded our understanding of the conformational landscapes of proteins, allowing precise characterization of protein motions over a range of timescales [31]. Here we discuss some

recent applications of NMR to the free and bound states of folded proteins of varying flexibilities.

A fascinating example of the relationship between flexibility and binding in a folded protein involves the catabolite activator protein (CAP) [32]. When CAP binds to cyclic AMP (cAMP), it undergoes large conformational changes, adopting a conformation very similar to its DNA-bound state, thus explaining the increase in DNA-binding affinity in the presence of cAMP. However, the CAP mutant S62F hardly undergoes any conformational changes upon cAMP binding, yet surprisingly retains strong affinity for DNA. Relaxation dispersion NMR measurements revealed a minor 2% population in S62F:cAMP that strongly resembles the DNA-bound state. In addition, while wildtype-CAP undergoes extensive ordering upon DNA binding, S62F actually increases in flexibility. Thus there is a strongly favourable contribution from conformational entropy to binding, further explaining the tight binding of S62F. A recent paper expanded this analysis to several more non-interfacial CAP mutants, finding that the interplay between bound-state-like conformations at different populations and changes in flexibility upon binding can lead to a variety of thermodynamic binding scenarios [33]. These results demonstrate that not only can proteins retain significant conformational dynamics in complex, they can even increase in flexibility, and that this can be important for regulating binding activity. In addition, it illustrates the evolutionary plasticity of protein flexibility, in the sense that a single mutation can induce a large shift in the relative populations of conformers.

NMR relaxation dispersion methods were also used to characterize the binding of different sites on the KIX domain of CREB-binding protein (CBP) to two transcription factors: MLL and c-Myb [34]. Remarkably, in the KIX:MLL complex, the authors observed a ~7% populated alternate conformation with chemical shifts that showed remarkable similarity to the KIX:MLL:c-Myb ternary complex. Since this minor conformation was not observed in isolated KIX, the binding of MLL to one site of KIX appears to induce flexibility that facilitates the formation of the c-Myb binding-competent state. This result therefore rationalizes the previous observation that MLL binding to KIX cooperatively enhances binding to c-Myb [35].

A relationship between binding and a different type of protein dynamics, inter-domain flexibility, was recently demonstrated for RNA-binding protein U2AF65 [36]. The two tandem RNA recognition motif domains from U2AF65 were observed to exist in two very different conformations: an open form in which both domains contact the RNA, and a closed form in which inter-domain interactions occlude one of the RNA-binding surfaces. Using paramagnetic relaxation enhancement NMR, a minor population of the open state was seen in the free protein. Strikingly, RNA ligands of increasing affinity correlated with increased population of the open state, while mutants designed to promote or inhibit the open state also correspondingly increased or decreased the binding affinity. Thus, the considerable intrinsic inter-domain flexibility of this protein appears to allow fine-tuning of the interaction strength with different RNAs.

Recent work suggests that the interaction of the core domain of p53 as a client of the chaperone Hsp90 represents a new type of dynamic complex. The core domain of p53 is folded but fairly dynamic and unstable in isolation [37]. Upon binding to Hsp90, broadening occurred of NMR signals corresponding to residues within the folded core of p53 [38]. In combination with fluorescence and hydrogen-exchange measurements, this suggests that p53 exists in a dynamic molten globule-like state in the presence of Hsp90 [38]. Rather than binding to a single region on Hsp90, p53 transiently interacts in a highly dynamic manner

with a wide area of the Hsp90 surface [39]. Thus, the core domain of p53 appears to increase in flexibility upon Hsp90-binding, shifting further along the folded-to-disordered continuum.

Ensemble modeling of intrinsically disordered proteins using various experimental measurements

Intrinsic disorder represents an extreme case of protein flexibility in which entire proteins or regions of proteins do not form stable, folded structures. This phenomenon is intimately related to their primary amino acid sequence, with disordered regions being rich in small polar and charged residues and deficient in large hydrophobic residues [40,41], thus accounting for their inability to bury sufficient surface area to stabilize their tertiary folds and facilitating sequence-based disorder predictions [42]. Much recent work has demonstrated the variety of important biological roles played by intrinsically disordered proteins [43–47].

Due to their extreme conformational heterogeneity, the structural properties of intrinsically disordered proteins are generally much more difficult to characterize than folded proteins. While long-timescale molecular dynamics simulations seem to hold much future potential [48–50], most of our detailed structural knowledge still comes from experimental measurements. In recent years, a number of approaches have emerged using various experimentally derived restraints to obtain detailed structural characterizations of disordered states [51–58]. Although variable in their implementations, these different approaches have converged upon a similar fundamental framework of ensemble modeling, in which multiple conformations are simultaneously fit to experimental measurements, resulting in an ensemble of structures that are collectively consistent with the experimental data. Although ensemble-modeling strategies face significant challenges in terms of conformational sampling, energy functions for converting experimental measurements into structural restraints and the potential for overfitting, considerable work using cross validation or synthetic restraints now supports the general accuracy of ensemble models calculated with different methods [52–54,56,59].

Our ENSEMBLE software (http://abragam.med.utoronto.ca/~JFKlab) is unique among ensemble-modeling strategies for its ability to combine large amounts of diverse experimental data, including several types of NMR measurements and small-angle X-ray scattering (SAXS) [59–62]. ENSEMBLE is also flexible in its method of conformational sampling, allowing characterizations of diverse conformational states such as partially folded proteins [63] and dynamic complexes [64,65].

Protein phosphatase 1 regulators exhibit structural and binding diversity

The assorted regulatory functions performed by protein phosphatase 1 (PP1) are controlled by interactions with >200 different proteins that can function as targeting subunits, substrates or inhibitors, many of which are intrinsically disordered [66]. ENSEMBLE was recently used to characterize the disordered free states of three non-homologous proteins that bind to and regulate PP1 and have been crystallized in complex with PP1 [63,64].

In isolation, the PP1-binding domain of spinophilin, which functions by targeting PP1 to the postsynaptic density [67], is highly disordered, although significantly more compact than expected for a protein of its sequence [64,68]. Ensemble modeling revealed a ~25% populated α-helix that perfectly corresponds to the lone helix observed in the PP1-bound crystal structure and a peak in the β-strand region of Ramachandran space consistent with one of the two bound-state β-strands [69]. Overall, the transition from highly disordered to fully

ordered upon binding (Figure 2a) represents the classic "folding-upon-binding" scenario, However, it is interesting to note that a subsequent study found that the adjacent spinophilin PDZ domain, which does not directly interact with PP1, retains considerable inter-domain flexibility in complex with PP1 [70].

The PP1-binding region of the myosin phosphatase targeting subunit (MYPT1) contains both a fully folded ankyrin-repeat domain, and an intrinsically disordered N-terminal segment [63]. Similar to spinophilin, a ~25% populated α-helix corresponds to the lone helix observed in the bound state of the N-terminal segment [71]. The folded region appears to undergo very little conformational change upon binding, while the intrinsically disordered region undergoes a large folding transition, becoming fully ordered upon binding, albeit mostly lacking regular secondary structure (Figure 2b).

The 159 residue PP1 inhibitor-2 (I-2) is one of the largest intrinsically disordered proteins to have been characterized by ensemble modeling [64]. Free I-2 has a ~70% populated α-helix near its C terminus that is also observed in the complex crystal structure, demonstrating that intrinsically disordered proteins can have highly populated secondary structural elements within an overall disordered context. Intriguingly, the crystal structure of the bound state shows that I-2 is still largely disordered upon binding [72]. By combining SAXS and NMR measurements, an ensemble model of the partially disordered PP1:I-2 complex was generated [64]. Therefore, I-2 demonstrates a scenario in which some pre-formed structure is important for interaction, but the highly disordered free state only partially folds upon binding (Figure 2c). Notably, a recent study found that the dynamic PP1-bound state of I-2 actually increases in flexibility upon formation of the ternary complex with spinophilin, due to a partial displacement of I-2 [73].

Sic1 binds Cdc4 in a highly dynamic complex

The intrinsically disordered cyclin-dependent kinase inhibitor Sic1 contains several different Cdc4-binding sites. When phosphorylated, these sites engage in a dynamic equilibrium with Cdc4, with the individual sites transiently binding the single Cdc4 binding pocket [74]. ENSEMBLE was used to characterize free Sic1 in both its phosphorylated and non-phosphorylated states [65], as well as in complex with Cdc4. These calculations reveal the presence of significant local transient structure around the Cdc4-binding sites, which are modulated in electrostatic potential upon phosphorylation, thus promoting complex formation. Given that Sic1 remains highly disordered in its bound state, the free and bound states of Sic1 can be considered to be very similar, with only slight local ordering of the transiently associated binding sites in complex.

Calmodulin represents an intermediate on the continuum of protein dynamics

Calmodulin represents an interesting case of an intermediate on the continuum between folded and disordered states. While crystal structures of Ca^{2+}-bound calmodulin have been determined, they are characterized by high A_{rel} values (Figure 1d), and NMR measurements suggest that the N-terminal and C-terminal domains are highly flexible with respect to each other in solution [75]. Upon binding to various partner proteins, Ca^{2+}-bound calmodulin becomes largely ordered, although it can adopt different bound-state conformations [76]. However, it has been observed that calmodulin can vary in flexibility when bound to different peptides, which appears to be closely related to its binding entropy [77,78].

Ensemble modeling was used to characterize the conformational dynamics of calmodulin in its free state (although bound to Ca^{2+}) and to a peptide from myosin light chain kinase [79]. Interestingly, the free state was observed to be in a dynamic equilibrium with a number of

bound-state-like conformations. Furthermore, it is likely that this intrinsic flexibility leads to transient populations resembling other bound states, facilitating its interactions with different binding partners. An alternate approach was recently used to characterize calmodulin in complex with a peptide from intrinsically disordered myelin basic protein [80]. This revealed substantial conformational heterogeneity within the complex, and suggested great variability for both the structure and dynamics of calmodulin in complex with different partners.

Conclusions

Proteins across the continuum of flexibility, from folded to disordered, can undergo widely varying changes in their structure and dynamics upon binding. For proteins that are fully ordered and can be crystallized in their bound states, a strong correlation exists between free-state flexibility and conformational changes upon binding [13,18]. However, many proteins retain considerable flexibility, or can even increase in flexibility, upon complex formation. In Figure 3, we have attempted to show this by plotting the relative free- and bound-state flexibilities for the various proteins discussed in this review. This emphasizes the diversity of possible binding scenarios available to proteins in order to carry out their various functions.

At present, our knowledge of free-state flexibility and conformational changes that occur upon binding is much greater than for bound-state flexibility. This is largely due to the fact that binding-induced conformational changes can be assessed from the vast number of published crystal structures, and because experimental characterizations of the free states of flexible proteins using NMR and other techniques are generally much easier than for protein complexes. Thus, our understanding of the frequency and nature of dynamic complexes remains limited. However, given the increasing number of examples that have been identified in the literature in recent years, it is likely that significant flexibility in the bound state is very common. The rapid advancement of computational and experimental methods for characterizing both free and bound states means that our understanding of binding mechanisms at a molecular level and the general recognition of the importance of protein flexibility will continue to grow.

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Figures

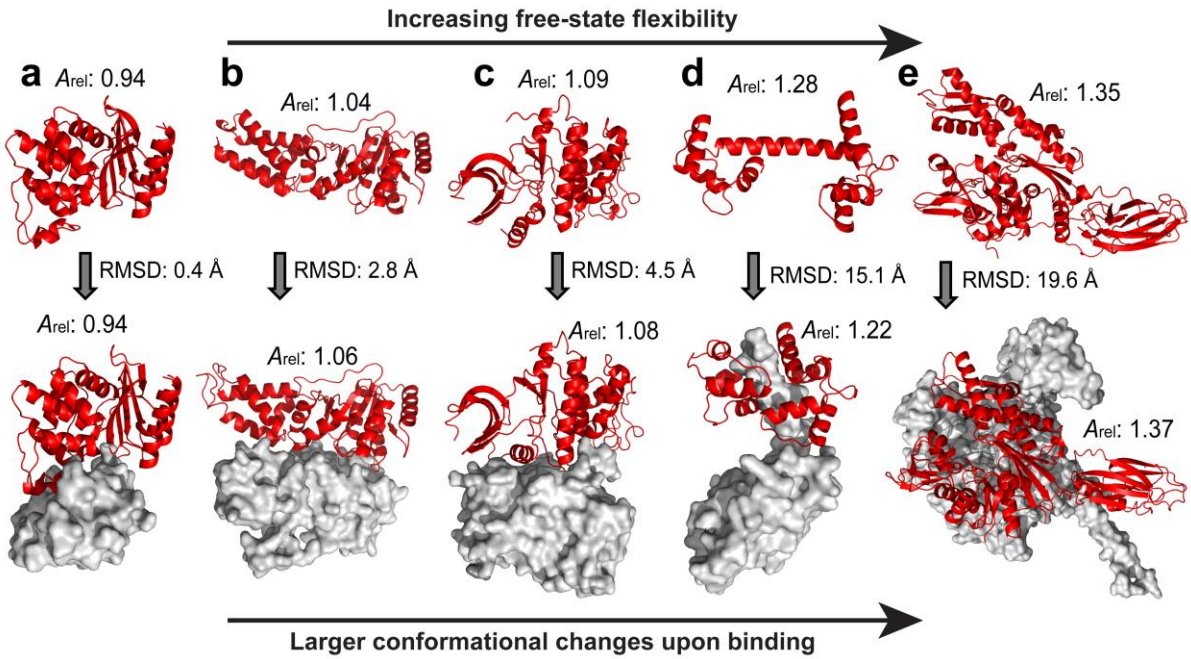

Figure 1. Comparison of free and bound states for proteins of increasing intrinsic flexibility and conformational changes upon binding. **(a)** TEM-1 β-lactamase (PDB ID: 1M40) binding to β-lactamase inhibitor protein-II (PDB ID: 1JTG); **(b)** NG domain of Ffh (PDB ID: 1LS1) binding to FtsY (PDB ID: 1RJ9); **(c)** cyclin-dependent kinase 2 (PDB ID: 2R3I) binding to cyclin A (PDB ID: 2CCH); **(d)** calmodulin (PDB ID: 3CLN) binding to inducible nitric oxide synthase (PDB ID: 3HR4); **(e)** Hsc70 (PDB ID: 1YUW) binding to Sse1 (PDB ID: 3C7N).

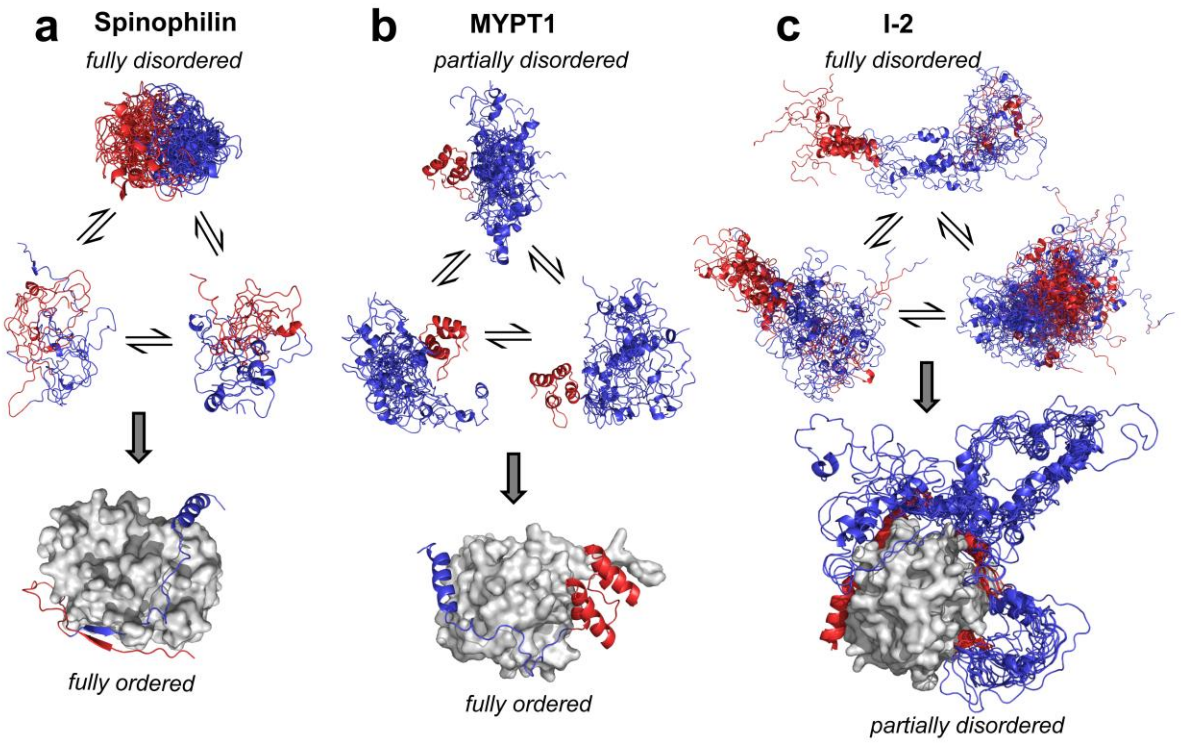

Figure 2. Interaction of protein phosphatase 1 (PP1) with intrinsically disordered regulatory proteins: **(a)** spinophilin; **(b)** MYTP1; **(c)** I-2. The intrinsically disordered free states are represented by the three most highly populated clusters from their calculated ensemble models [63,64] while PP1 is shown as a surface representation. Structures are coloured as follows: spinophlin: residues 1-34 red, 35-78 blue; MYPT1: residues 1-39 (intrinsically disordered region) blue, 40-98 (ankyrin-repeat domain) red; I-2: residues 6-11, 37-49, 123-159 (regions observed in bound crystal structure) red, everything else blue.

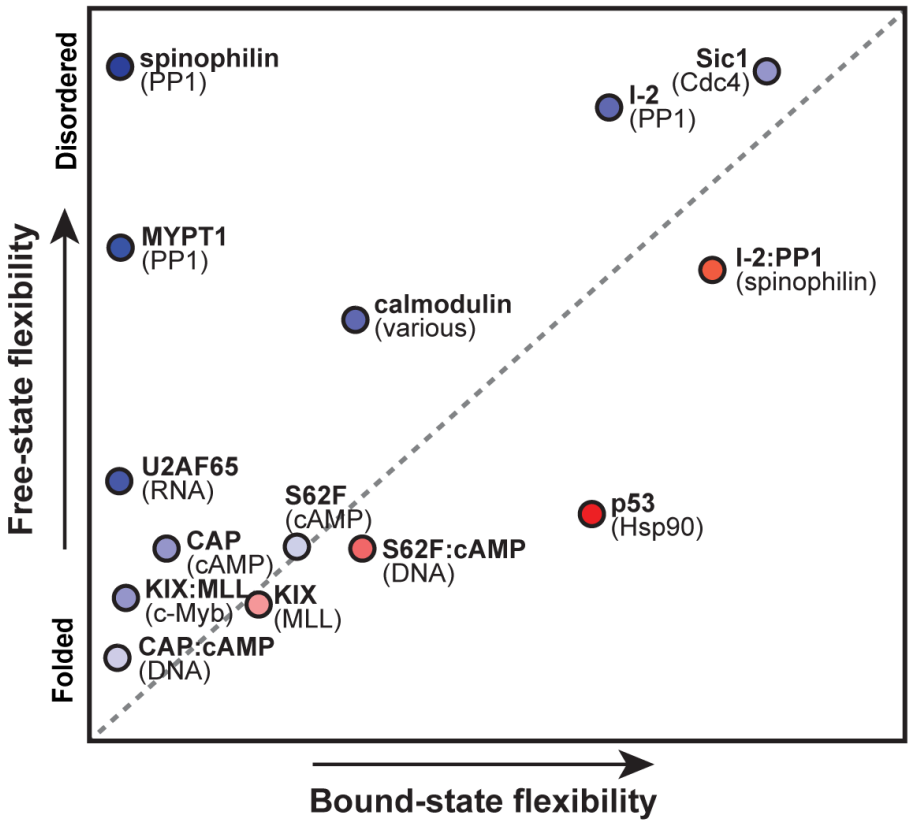

Figure 3. Comparison of free-state and bound-state flexibility. Binding partners are shown in parentheses. Proteins above the diagonal (blue) become more ordered upon binding, while those below the diagonal (red) increase in flexibility. The degree of colour represents the magnitude of the change in flexibility. Since these systems were characterized with different methods, these values are estimates meant to highlight the diverse relative flexibilities of the various proteins discussed in this review, and are not meant to quantitatively represent the relative flexibilities of different proteins.