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Targeting disordered proteins with small molecules using entropy

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The human proteome includes many disordered proteins. Although these proteins are closely linked with a range of human diseases, no clinically approved drug targets them in their monomeric forms. This situation arises, at least in part, from the current lack of understanding of the mechanisms by which small molecules bind proteins that do not fold into well-defined conformations. To explore possible solutions to this problem, we discuss quite generally how an overall decrease in the free energy associated with intermolecular binding can originate from different combinations of enthalpic and entropic contributions. We then consider more specifically a mechanism of binding by which small molecules can affect the conformational space of a disordered protein by creating an entropic expansion in which more conformations of the protein become populated.

Disordered proteins and disease

Disease-modifying proteins involved in cancer, neurodegeneration, cardiovascular diseases and diabetes comprise about one-third of those encoded by the human genome (Figure 1A). Of these proteins, only approximately 22% are currently considered 'druggable', as they are known or predicted to interact with drugs (Figure 1A) [1–3]. Moreover, all clinically approved small-molecule therapeutics target structured domains [2,3], despite the fact that intrinsically disordered proteins or intrinsically disordered regions (see Glossary) of otherwise ordered proteins are also commonly involved in disease [4–7] (Figure 1A). These disordered proteins, which lack a well-defined stable structure, exist in a dynamic equilibrium of conformationally distinct states.

Proteins with more than 40 consecutive disordered residues have been reported to comprise one-third to one-half of the human proteome [8,9]. These proteins exhibit widely varying degrees of disorder, and this disorder is rather evenly distributed. An analysis using the s2D method [10] indicated that disordered proteins correspond to approximately 40% of the protein-coding human genome (Figure 1). This result was obtained by defining disordered proteins as those that contain more than 40% of their residues in regions of at least 40 consecutive disordered amino acids, consistently with similar previous conventions [8,9].

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Following an initial surprise after its discovery, it is now increasingly recognised that disorder serves a biological role, because conformational heterogeneity granted by disordered regions enables proteins to exert diverse functions in response to stimuli. Unlike structured proteins, which are essential for catalysis and transport, disordered proteins appear crucial for regulation and signalling, acting as network hubs interacting with a wide range of biomolecules [4,5,11–16].

Given the variety of their functions, dysregulation of disordered proteins can give rise to a variety of diseases including cardiovascular disorders, diabetes, cancer, and neurodegeneration [4,6,7]. However, there is an underrepresentation of disordered proteins among those encoded by the current 'druggable genome' (Figure 1C). Even in cases in which proteins with disordered regions are targeted, most drugs are directed towards the structured domains of these proteins. Overall, despite their high prevalence in disease, disordered proteins are not targeted by clinically available drugs. Here, we discuss possible strategies to modify this situation to identify opportunities to exploit this untapped potential.

Small molecules binding to disordered proteins

Major advances have been recently made in understanding the molecular roles of disordered proteins in disease [4–9,11–16]. However, the development of therapeutics that target disordered proteins is still in its infancy, in part because the highly dynamic nature of these proteins renders it difficult to study them experimentally. For example, in the case of Alzheimer's disease, despite the enormous efforts over the past two decades to develop drugs capable of inhibiting the aggregation process of the disordered amyloid β peptide, currently no compound that effectively does so

Glossary

Binding Database (BindingDB): an online database of measured binding affinities (http://www.bindingdb.org). Entries are mainly proteins considered to be targets of small drug-like molecules.

Disordered proteins or disordered regions: proteins or protein regions that, under native conditions, do not populate a well-defined conformation, but rather a heterogeneous ensemble of states.

Entropic expansion: an increase of the size of the conformational space of a disordered protein upon the introduction of a ligand, whereby the bound protein populates even more states than the unbound protein.

Isothermal titration calorimetry (ITC): an experimental technique that can be used to determine thermodynamic parameters for a binding interaction.

s2D method: a computational method to simultaneously predict disordered regions and secondary-structure populations of proteins from their amino acid sequences.

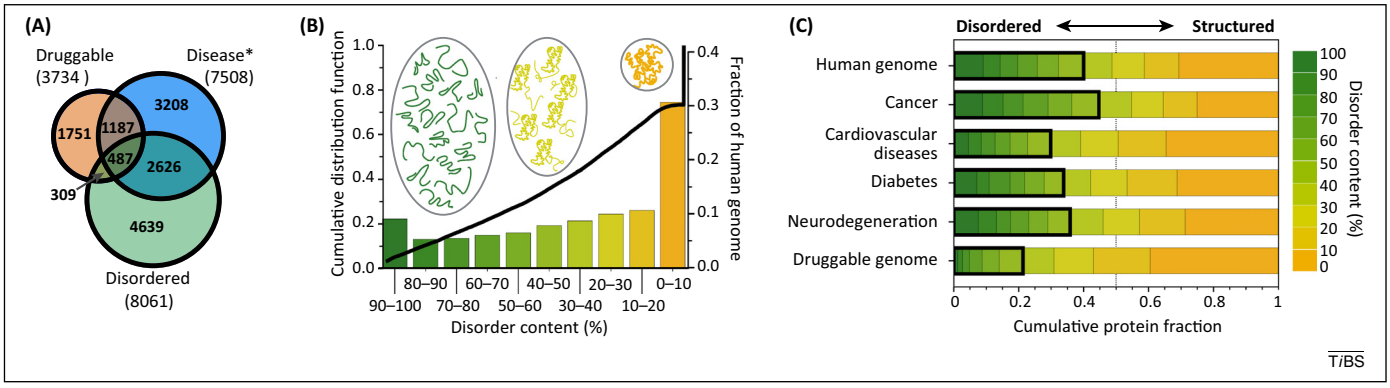

Figure 1. Prevalence of protein disorder in some common human diseases. **(A)** Venn diagram of three subsets of the human proteome. Proteins are defined as 'disordered' if they contain more than 40% of their residues in regions of at least 40 consecutive disordered amino acids, as 'druggable' if they are known or predicted to interact with drugs [1], and as 'disease-related' or 'disease-modifying' (disease*) if they are involved in cancer, diabetes, neurodegeneration, or cardiovascular diseases (proteins in these groups were determined with a keyword method adapted from [55,56]). **(B)** Fraction of proteins encoded by the human genome (right axis) binned according to their content of structural disorder (x-axis). Green bins represent highly disordered proteins, and orange bins structured ones. The black line is the cumulative distribution function (left axis). Cartoons illustrate ensembles of three proteins with varying disorder content. **(C)** Comparison of the amount of protein disorder encoded by the human genome, by the druggable genome, and in disease-related proteins. Proteins are binned horizontally by disordered content (colour bar). Black boxes represent the fraction of disordered proteins as defined in (A). The analysis of disorder was performed using the s2D method [10]; an individual residue was considered disordered if its α-helical and β-strand populations are smaller than 0.5.

has entered clinical use [17–19]. A recently proposed approach to obtain drugs targeting disordered regions relies on the computational docking of small-molecule fragments against an ensemble of representative conformations of the protein of interest [20]. Its application to α-synuclein, a disordered protein involved in Parkinson's disease, identified a compound that inhibits the aggregation of α-synuclein [20]. However, it is still poorly understood whether this compound binds more preferentially the monomeric protein than its aggregated species. A clearer example of direct targeting of monomeric disordered proteins is the case of the oncoprotein c-Myc [21–23]. A recent high-throughput screening yielded a series of compounds, which interact with its disordered regions and prevent binding to its partner, Max. However, the mechanism of these drug-binding interactions remains unclear and these compounds have not yet entered clinical use [21–24].

Disordered proteins populate ensembles of many conformations, each with its own occupation probability. The behaviour of disordered proteins is governed by these ensembles and can be drastically different from that of any individual conformation. Upon interacting with other molecules, such as protein-binding partners, disordered proteins may pay an entropic cost because their conformation space is restricted in the bound form, which can be compensated by an enthalpic gain [11,25]. Conversely, in an alternative scenario, a change in the behaviour of disordered proteins may be achieved through the use of small molecules, such that the conformational space of a disordered protein is not restricted, but rather entropically expanded by new, transiently bound states. In the following, we discuss these and other potential mechanisms through which small molecules could be effective at targeting monomeric disordered proteins.

Thermodynamics of protein-ligand binding

The binding of two molecules occurs spontaneously when it is associated with an overall decrease in free energy ($\Delta G < 0$), where ΔG indicates the difference between the free energy G of the final state and that of the initial state. This difference can be expressed as the sum of enthalpic and entropic contributions (Equation 1):

$$\Delta G = \Delta H - T\Delta S \quad [1]$$

where the change in enthalpy (ΔH) is determined by a variety of interatomic forces, including electrostatic, van der Waals, and hydrogen-bonding interactions, and the entropic contribution ΔS represents the change in the size of the conformational space available to the overall system, including the protein, ligand, and solvent molecules.

Enthalpic and entropic factors can either contribute favourably or unfavourably to ΔG, resulting in the four possible modes: (i) $\Delta H>0$, $\Delta S<0$; (ii) $\Delta H<0$, $\Delta S<0$; (iii) $\Delta H<0$, $\Delta S>0$; and (iv) $\Delta H>0$, $\Delta S>0$. Only modes (ii–iv) yield negative ΔG values, thus lead to binding. Protein-ligand binding systems can be characterised experimentally into one of these four modes using, for example, isothermal titration calorimetry (ITC) [26]. ITC experiments allow direct, in-solution, label-free determination of both ΔG and ΔH for a protein-ligand binding system, including contributions from the solvent. The difference of these observed values can be used to calculate $-T\Delta S$ using Equation 1 [26–28]. While many protein-ligand binding events are driven by enthalpic factors, in some cases entropy can contribute favourably towards a negative change in free energy and, thus, result in binding.

To better understand the role of entropy in protein-ligand binding interactions, we reviewed all entries in the Binding Database (BindingDB) for which there are thermodynamic data (139 unique, non-mutant entries). We categorised these entries according to the magnitude of the entropic contributions [27,29–32] (Figure 2A). Some enthalpically favourable interactions come at an entropic cost (black points in Figure 2A). This compromise is commonly referred to as enthalpy–entropy compensation.

In rational drug design, it is possible to optimise enthalpic contributions to promote binding to a target, and occasionally the entropy is also optimised. This emphasis is reflected by the distribution of the entropic contributions to binding across the BindingDB (Figure 2B). We note that

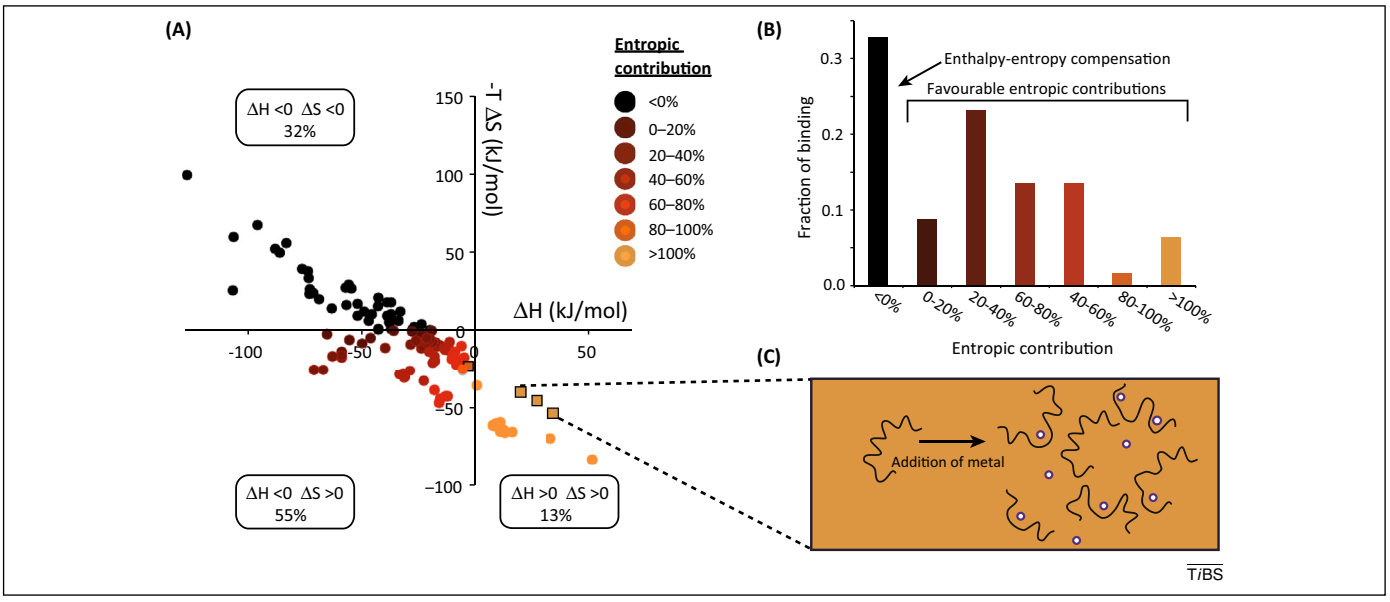

Figure 2. Entropic contributions to protein-ligand binding in the Binding Database (Binding DB). **(A)** Entries from the BindingDB are plotted according to their enthalpic (ΔH, x-axis) and entropic (–TΔS, y-axis) contributions. Data points are representative of all unique, non-mutant entries in the BindingDB for which there were thermodynamic data available and are coloured according to their entropic contributions. Squares represent entries in which the release of solvent molecules is not considered to be the cause of the change in entropy, which is instead mostly associated with the many conformations of the backbone itself. These cases include: Ca^{2+} binding to phospholipase D βC2, Ca^{2+} binding to phospholipase D αC2 [33], Zn^{2+} binding to conantokin-G, and Zn^{2+} binding to conantokin-T [34]. **(B)** Distribution of entropic contributions to ΔG. **(C)** Illustration of a possible entropy-driven binding of Zn^{2+} to conantokins, where the free energy decreases in the presence of Zn^{2+}. This entropy may arise from the exposure of the backbone to the solvent, thereby introducing new states in the bound form.

strong entropic contributions to protein–ligand binding are highly underrepresented in the BindingDB. Furthermore, most of the entropically favourable interactions in Figure 2 are hydrophobic in nature, because binding originates from a large positive solvation entropy [26]. Before interacting, the protein and ligand are each solvated separately. Upon binding, which in this context usually involves the burial of hydrophobic surfaces, many water molecules from the separate hydration shells surrounding the protein and ligand are freed into bulk solvent. This process increases the number of conformational states of the water molecules and, thus, of the system as a whole, overcoming the loss of entropy due to restraining the molecule and binding site [26,27].

Interestingly, however, cases have been reported (highlighted as squares in Figure 2A) of entropy-driven binding where solvation effects may not fully explain the increase in entropy upon binding. The first of these cases is the binding of a Ca^{2+} ion to phospholipase D βC2, or to phospholipase D αC2 [33]. The second is the binding of a Zn^{2+} ion to conantokin-G or to conantokin-T [34]. These changes in entropy seem to arise from conformational changes in the proteins themselves, rather than from released solvent molecules. More specifically, the binding of metal ions may expose hydrophobic groups to water, causing the backbones to behave in a disordered manner [33,34]. The increased conformational flexibility enables the protein target to occupy significanlty more states, thereby increasing the entropy of the system (Figure 2C).

Such induced disorder-upon-binding mechanisms have been proposed and validated for other classes of proteins. For example, one ligand, PD173955, has been optimised to promote disorder of the Bcr-Abl kinase activation loop and, therefore, increases the overall binding affinity compared with its parent compound, imatinib [35]. Along similar lines, variable differences in the conformational entropy between free and bound states of galectin-3 have been observed upon binding to different carbohydrate ligands. The extent of this induced conformational entropy has been shown to control the affinity [36]. These studies, among others [37–40], support the validity of ligand binding through entropic expansion for disordered proteins.

In contrast to traditional binding scenarios, in which a drug locks a protein in an inactive state, the entropy-driven binding scenario may appear unconventional due to the multiplicity of low populated conformations in which the protein is weakly interacting with the ligand (Figure 2C). This mechanism of interaction suggests a more general definition of 'binding' than one in which two molecules form a stable association through strong intermolecular interactions. Within this framework, any interaction in which a ligand significantly affects the conformational space of a protein may be considered binding.

This view raises two issues (Box 1): first, by this definition, solutes affecting protein hydration, high salt concentrations, and crowding agents that affect the conformational space of a protein should be considered as 'ligands' even though they are not traditionally considered as such. Although discussing binding in this way may be unconventional, we are prompted here to consider it because it is the natural consequence of entropic expansion.

A second concern is how these interactions can be specific, as binding interactions governed by a network of weaker processes to induce favourable entropic changes may not be localised to a single binding region. However, we propose that a combination of entropically and enthalpically favourable interactions may be used to optimise specific interactions that can alter the behaviour of disordered regions. In other words, one way in which ligands may bind disordered regions consists of using relatively

weak enthalpic interactions to ensure specificity, but relying on entropic factors for making the binding free energy more favourable. Mutagenesis studies have confirmed that drugs found to bind to the disordered region of c-Myc have specific binding regions 11–29 residues long within the 84-residue long disordered basic helix-loop-helix (bHLH)-Zip domain [41], thus suggesting that specificity is achievable when binding to disordered regions. However, it is possible that these small molecules may also interact with other proteins as some of their constituent scaffolds have been shown to bind a large number of targets with weak or moderate affinity [42].

Using entropy to target disordered proteins

Enthalpy-focussed rational drug design, which is widely used for targeting globular proteins [26–28], has not yet led to major advances for disordered proteins. While focussing on favourable enthalpic contributions may be a good strategy for targeting structured proteins, we suggest that exploiting entropically favourable binding provides novel opportunities for obtaining drugs to target monomeric disordered proteins. We anticipate that many drugs targeting disordered proteins will populate the bottom quadrants of Figure 2C by similar mechanisms to those described above for metal-binding proteins. Much like phospholipase D and conantokins, the conformational space available to a polypeptide chain may be susceptible to entropic expansion upon the introduction of a ligand, thus resulting in an increase in the number of states effectively accessible to the disordered polypeptide chain (Figure 3). Such an increase of entropy may result in favourable protein–ligand interactions, and be modulated by hydrophobic effects, electrostatics, π-effects, and van der Waals forces.

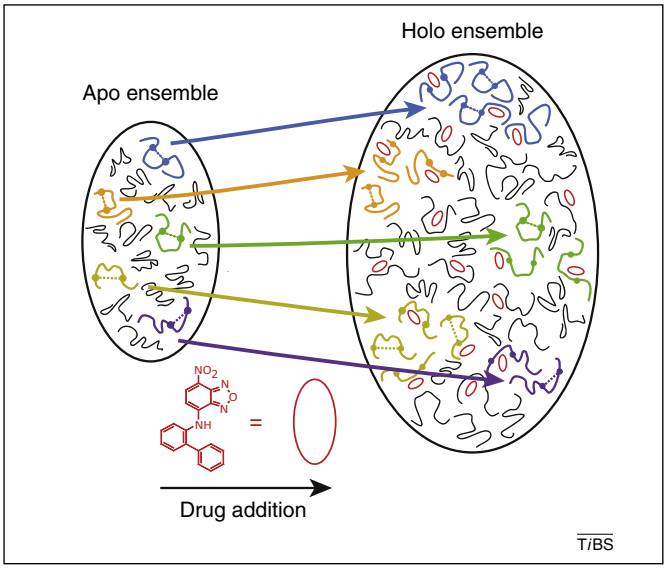

Figure 3. Entropic expansion of the conformational space of a disordered protein upon binding a small molecule. A small molecule can interfere with the intramolecular interactions within a disordered protein, thereby increasing its entropy by changing the statistical weights of the conformations that it populates, in some cases by effectively increasing their number. The ensemble on the left represents the conformational space of an unbound disordered protein, and the one on the right represents the remodelling of this conformational space upon binding a drug (shown in red). Different colours represent how various states may be affected upon drug binding.

This type of entropically favourable binding may be more effective than targeting disordered proteins with strong enthalpic or solvent-mediated entropic interactions that stabilise a single conformation. Such types of interaction indeed occur for disordered proteins, such as the many disorder-to-order transitions reported in the literature [9,12,43]. However, these interactions tend to involve large interfaces, which currently can be disrupted by binding the structured partner to block the binding region of the disordered protein [11]. We propose that small molecules can also be developed to directly target monomeric disordered proteins to alter their behaviour via entropic expansion. We illustrate this concept in Figure 3, which represents the effective conformational space of an unbound disordered protein being expanded upon the introduction of a drug. The mechanisms by which this type of binding can happen include the disruption or creation of contacts yielding perhaps more extended or more structured conformations, thus expanding the conformational space of the bound protein with a corresponding overall increase in entropy. Given that new states of the protein are introduced upon the addition of a drug, the statistical weights of existing states in the unbound ensemble may decrease, thus providing a potential avenue to significantly alter the average behaviour of the disordered target.

This concept may be used to rationalise simulations performed recently on a peptide fragment of c-Myc and its binding partner, the small molecule 10058-F4. The c-Myc peptide was observed to occupy an increasingly large number of low populated states upon binding 10058-F4 compared with the unbound form [44]. We interpret this observation as an example of the extended conformational space of a bound disordered protein and thereby a potential increase in the overall entropy of the system. Similar analyses on another c-Myc peptide and its binding partner, 10074-A4, further suggest that drug binding to c-Myc occurs weakly at many binding sites along the binding region [45]. These reports are consistent with the hypothesis of an entropy-increasing mechanism, as described above. Further studies will be necessary to confirm these possibilities.

Additionally, a second relevant example is the MDM2 protein, which contains a large disordered lid region. Upon binding some classes of p53/MDM2 antagonists the base of the lid region becomes ordered via contacts with the ligand, with an accompanying increase in flexibility of the remainder of the disordered region compared to the unbound disordered state [46,47]. In current attempts to curtail the toxic behaviour of disordered proteins, likely milestones may include the fine-tuning of flexible disordered regions, such as the flexible lid of MDM2.

Therapeutic potential of the entropic expansion mechanism of binding

Conformational ensembles of disordered proteins are extremely sensitive to external factors. Post-translational modifications [48], point mutations [49], and pH changes [50] can significantly reweight the states within an ensemble, including introducing nontrivial states and significantly decreasing the weights of existing ones. Small changes within ensembles can have profound physiological effects.

In the case of mutational variants of α-synuclein, it was reported that relatively small differences in the ensembles and, more specifically, in the populations of β-strands [49] and polyproline II [51] secondary structure elements, correspond to large differences in the aggregation rates, thus affecting the formation of neurotoxic species.

Small molecules can have a similar potential to change the ensembles of disordered proteins from those that are disease promoting to those that may be disease preventing, thereby enabling small molecules to have a therapeutic effect. Earlier in this article, we described drug development opportunities aimed at entropically expanding the ensemble, although reweighting, restricting, or shifting the ensemble are also candidate strategies to change the behaviour of disordered proteins and eliminate disease-promoting conformations.

As is the case with c-Myc described earlier, drugs that target disordered regions have shown promising initial results both *in vitro* and *in vivo*, and more metabolically stable analogues are currently in development [6,22,52]. An ability to modify the behaviour of disordered proteins via the use of small molecules may have profound implications for disease. For instance, major neurotoxic agents associated with Alzheimer's disease are soluble oligomeric species formed by the aggregation of the amyloid β peptide [19,53,54]. We believe that particularly effective therapeutic agents against aggregation may inhibit the formation of oligomeric assemblies via entropic expansion of monomeric disordered peptides or proteins. Given that many of the toxic oligomeric species involved in neurodegeneration largely comprise disordered proteins, reweighting the monomeric ensembles of these disordered proteins may slow down the formation of toxic oligomeric species. As new states become more populated, populations of existing, potentially disease-promoting states will decrease, and such an expansion of the ensemble can be achieved by the introduction of certain small molecules that increase the entropy of the overall system.

Concluding remarks

In most cases, the binding of ligands to structured proteins is either driven by enthalpic factors or by solvent-mediated entropic interactions, such as hydrophobic attraction. However, in the case of disordered proteins, we expect that favourable entropic contributions arising from the target proteins themselves rather than from the solvent may represent an alternative drug-discovery strategy, which can be then further tuneable using enthalpic contributions. Disordered proteins have largely been considered as 'untargetable' because they do not readily lend themselves to enthalpy-driven binding, as no tight-binding small molecules have been identified to interact with them (Box 1). However, they may be more amenable to binding via entropic expansion, which may only require weak enthalpic interactions between proteins and ligands. We anticipate that, by exploiting recent developments in experimental measurements and molecular dynamics simulations, it will become possible to obtain increasingly accurate estimates of entropic contributions of disordered protein–ligand systems, which will hopefully facilitate the rational development of small molecules targeting disordered proteins.

Box 1. Outstanding questions

- What are the interaction mechanisms of disordered proteins and small molecules? It is particularly challenging to address this question because of the highly dynamic nature of disordered proteins. Advances in experimental and computational techniques are now offering novel opportunities for providing possible answers.
- Can novel entropically favourable binding mechanisms be identified? It may be possible to find small molecules that bind disordered proteins by increasing the overall entropy, including perhaps an entropic expansion of the proteins themselves.
- Can targeting disordered proteins using entropy have therapeutic effects? Given that relatively small perturbations such as point mutations and post-translational modifications can significantly alter functional and dysfunctional behaviours of disordered proteins, we anticipate that small molecules can be found with analogous effects.
- What is the most useful definition of binding between a small molecule and a disordered protein? Since the binding of small molecules to disordered proteins may be weak and having major contributions from entropic terms, the line between specific ligands and generic solutes may become blurred.

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