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Advances in uncovering the mechanisms of macromolecular conformational entropy

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During protein folding, proteins transition from a disordered polymer into a globular structure, markedly decreasing their conformational degrees of freedom, leading to a substantial reduction in entropy. Nonetheless, folded proteins retain substantial entropy as they fluctuate between the conformations that make up their native state. This residual entropy contributes to crucial functions like binding and catalysis, supported by growing evidence primarily from NMR and simulation studies. Here, we propose three major ways that macromolecules use conformational entropy to perform their functions; first, prepaying entropic cost through ordering of the ground state; second, spatially redistributing entropy, in which a decrease in entropy in one area is reciprocated by an increase in entropy elsewhere; third, populating catalytically competent ensembles, in which conformational entropy within the enzymatic scaffold aids in lowering transition state barriers. We also provide our perspective on how solving the current challenge of structurally defining the ensembles encoding conformational entropy will lead to new possibilities for controlling binding, catalysis and allostery.

During protein folding, proteins go from a disordered linear polymer, which can access almost infinite states[1], to a globular, folded form, in which access to most conformations, especially in backbone atoms, is restricted by steric clashes[2,3] The hydrophobic collapse accompanying folding also liberates water molecules from ordering around side chains. This collapse is entropically favorable, as the water molecules acquire additional degrees of freedom[4]. However, the substantial reduction in conformational freedom of the protein that accompanies folding has a steep entropic cost. This entropic loss is governed by the Gibbs entropy equation ($(\sum p\ln(p))$; in which p denotes the number of states)[5]. Additional free energy favoring folding is due to attractive interactions (for example, hydrogen bonds), which must outweigh the cost of further reducing the number of accessible conformations that forming those interactions requires (Fig. 1).

The remaining conformations folded proteins access provide the residual entropy required for protein stability and activity. A strategy for maximizing the stability of the folded state is therefore to balance the attractive interactions (enthalpy) with as minimal a reduction in accessible conformations (entropy) as possible. In the conformational ensemble of the folded native state for most globular proteins, conformational heterogeneity is usually dominated by alternative conformations of side chains, with only minor influences from the backbone[6]. Perturbations, such as ligand binding, change the conformational ensemble, altering the residual conformational entropy alongside enthalpic interactions. Examining perturbations or alterations to a protein from a conformational ensemble perspective highlights the need to integrate enthalpic and entropic contributions, opening up the use of entropy prospectively to explain function, including allostery, binding and catalysis, or to drive down ligand binding affinity and functional protein design.

To concretely illustrate how the residual conformational entropy in the native state can influence the free energy of the system upon perturbation, consider a single leucine side chain near the active site of a protein kinase (Fig. 1). In the unfolded state, the leucine side chain can equally access all rotamers[7], and the backbone is only restricted by the neighboring residues on the chain (Fig. 1a). Upon folding, the backbone becomes heavily restricted by steric clashes with other residues and adopts a relatively unique conformation[2]. This loss of backbone

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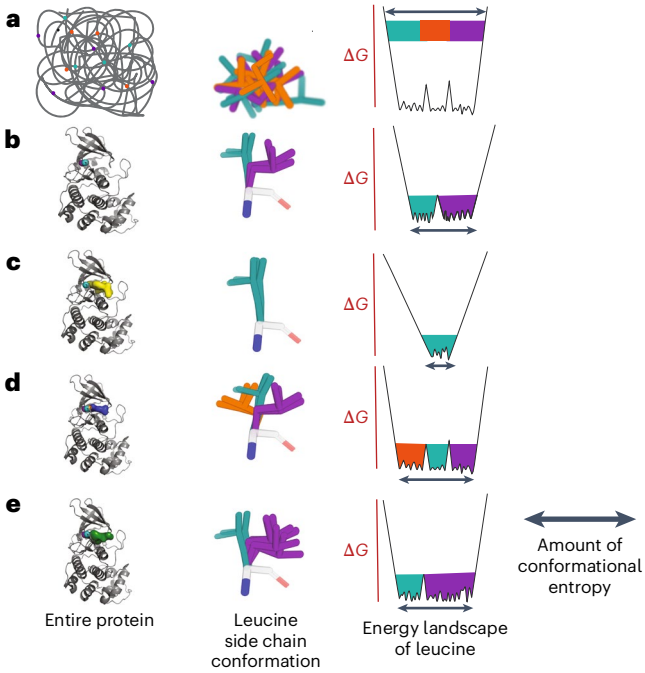

Fig. 1 | Examples of a structure and estimated conformational entropy for a leucine side chain. Left, the structural state of the entire protein with leucine depicted as colored spheres. Middle, the conformation(s) of leucine in each state. Right, the relative location of each conformation on the energy landscape of the leucine side chain. The width of the arrow represents the number of accessible conformations. **a**, In an unfolded state, the entire protein and the leucine side chain access many different conformations, with each conformation sitting at the top of a wide energy landscape. **b**, Upon folding, the majority of the protein is structured. This results in a large loss of entropy in the leucine side chain. The leucine can only access two rotamer states (teal and purple), separated by an energy barrier. One conformation (purple) has substantially more harmonic entropy, as shown by a wider energy well. **c**, When ligand 1 binds, the leucine loses access to an additional rotameric state, reducing its conformational entropy. **d**, When ligand 2 binds, the leucine gains conformational entropy compared to the unbound state. This would be observed as a decrease in NMR order parameters (representing increased disorder) but three alternative conformations in X-ray or cryo-EM data. **e**, When ligand 3 binds, the leucine has the same number of accessible rotamer states as in the unbound state (2); however, the harmonic motion in both rotamer states greatly increases. This would be observed as a decrease in NMR order parameters (representing increased disorder) but two alternative conformations with increased *B* factors in X-ray or cryo-EM data.

freedom incurs a substantial entropic penalty, with residual entropy contributed only by the harmonic motion about its mean position. Unlike the backbone, side chains can be much less restricted, and most side chains access multiple rotameric states, even within the protein core[8]. Without a ligand in the active site, the leucine side chain can access two of the five possible rotameric states, while steric clashes with other residues disfavor the remaining three. This scenario leads to a reduction in, but not the elimination of, side chain conformational entropy upon folding (Fig. 1b).

Perturbations, including ligand binding, can further alter the conformational ensemble with varying entropic consequences[9]. An active site ligand may form van der Waals contacts with the leucine side chain in a way that strongly prefers one of the apo state alternative conformations (Fig. 1c). The transition from two to one rotameric conformation further decreases conformational entropy. Because most structural models from X-ray crystallography or cryo-EM would only have one of the two conformations modeled in the apo state, whether ligand binding induces a 'conformational change' depends on which conformation is modeled in the apo state (usually, but not always, the more populated one). But, by considering the underlying ensemble, we can see that ligand binding induces a 'conformational redistribution' and can start to think about the entropic consequences for the free energy of binding.

Ligand binding can also, counterintuitively, increase the conformational entropy of side chains[10,11]. For example, a ligand binding at a different part of the active site may stabilize different residues in conformations that create a small void adjacent to the leucine. The void could permit populating an additional, third, leucine rotameric conformation compared to apo (Fig. 1d) or increase the thermal fluctuations around the two already populated rotameric conformations (Fig. 1e). Both of these scenarios increase conformational entropy and can, therefore, favor ligand binding.

While the entropic contribution of a single side chain may appear small, accumulating numerous conformational redistributions across the protein can lead to a substantial entropic impact and provide an allosteric source of free energy. Structural descriptions of allostery are frequently based on averaged single-conformer structures and described in a 'domino effect' of conformational changes through a well-defined pathway[12]. But, over 40 years ago, Cooper and Dryden[13] postulated that protein thermal fluctuations, without change in mean atomic positions, were sufficient to provide 'dynamic allostery' between sites. This model can be reconciled with the domino effect by examining allostery through a thermodynamic lens where any change in the conformational ensemble, whether large or small, can impact free energy and protein function through both entropy and enthalpy[9].

Despite the apparent energetic importance of conformational entropy, the ability to probe it experimentally is underdeveloped. Currently, the best experimental support comes from solution NMR relaxation techniques. By probing methyl or amide groups, NMR relaxation techniques obtain a site-specific measurement of the amount of disorder on the pico–nanosecond timescale, called an order parameter[14]. Changes in the average side chain order parameter for methyl-containing residues (like leucine) across a protein have been correlated with calorimetric measurements of conformational entropy[14–18]. Excitingly, these experiments reveal a great diversity of behaviors across systems: some binding events are entropically driven due to increased side chain disorder, whereas others are strongly entropically disfavored. Additionally, some side chains exhibit increased disorder even when the overall trend is toward unfavorable conformational entropy (and vice versa). Furthermore, different ligands bound to the same receptor can have drastically different impacts on conformational entropy[10,19,20]. While NMR order parameters provide the extent of the disorder, many equivalent side chain conformational ensembles can produce a given order parameter value, as seen in the packing void example[17] (Fig. 1d,e). This degeneracy makes it challenging to connect conformational entropy to structural models.

An alternative and readily available source to reveal the structural basis of conformational entropy is ensemble modeling of X-ray crystallography and cryo-EM data. These experiments collect ensemble-averaged data from tens of thousands to billions of individual molecules, capturing all conformations regardless of the exchange timescale. While the protein is in a solid state and, in the case of X-ray crystallography, a crystalline state, protein side chains, which contain most protein conformational entropy, are often not disruptive to diffraction or alignment. While careful attention to experimental noise and additional artifacts is needed[21,22], we have demonstrated that multiconformer modeling in high-resolution X-ray datasets can detect subtle and weak conformations, explaining statistically significant signals in the density map[23]. The leucine side chain would have a subtly different density surrounding it in each scenario. While a multiconformer model would help tease apart the contributions of different rotamers, a conventional single-state structure would almost certainly model all four examples the same way. A 'crystallographic order parameter' that agrees reasonably with NMR relaxation experiments can be obtained

by determining the distance between each alternative conformation and the weighted *B* factor, a refined parameter to account for harmonic disorder, of each conformer[24]. Importantly, it is not limited to methyl-containing side chains and, due to the time-averaged nature of diffraction or single-particle imaging, this metric extends to timescales beyond the pico–nanosecond timescale of NMR order parameters. Ideally, molecular dynamics (MD) simulations could directly observe the fluctuations of residue upon perturbation[25], but substantial protein dynamics happen on a timescale inaccessible to MD[26–29], necessitating hybrid approaches that bias agreement to experimentally observed order parameters[28,30]. Collectively, these techniques are beginning to quantify the structural basis of conformational entropy.

Our prevailing models for understanding proteins focus on explicitly representing and considering enthalpic interactions, emphasizing the precise orientation of 'functional' residues, with entropy relegated to a more implicit role in the analysis. However, we need to consider how a local narrowing of the conformational ensemble is opposed by entropy. This opens the question of what strategies proteins employ to optimize overall free energy by optimizing entropic contributions.

Here, we review evidence of how nature has evolved to harness protein conformational entropy and how new approaches in X-ray crystallography and cryo-EM could uncover the link between structure and conformational entropy. The first principle is most well known: prepaying entropic costs through ordering in the initial, or ground, state. The second phenomenon is spatial entropic redistribution: an increasing conformational entropy in one region of the protein or system in response to a decrease in conformational entropy elsewhere in the system. The third concept extends the concept of spatial redistribution to catalytically competent ensembles: conformational entropy in the enzymatic scaffold, distant from the catalytic site, contributes to lowering transition state barriers. Explorations of these mechanisms could profoundly impact our answers to long-standing questions in binding events, catalysis and cell signaling. After all, without explicitly considering conformational entropy, we ignore a large thermodynamic component driving free energy. Now is the time to apply a conformational entropy lens to our explanation of protein function and begin applying that lens prospectively to ligand and protein design.

Prepaying entropic penalties as a strategy for cooperativity

Binding affinity is largely dictated by surface complementarity and attractive interactions (for example, hydrogen bonds) between the ligand and the receptor. Like folding, both these features greatly constrain the potential conformations the receptor can adopt, incurring an entropic penalty. The loss of conformational entropy is countered largely by the enthalpy gained by these interactions. However, if the ground state conformational ensemble is restricted to a similar extent as that adopted in the bound state, the protein prepays the entropic penalty. The cost of the entropic penalty can be minimized or even rendered non-existent. This simple concept extends most interestingly to cooperativity in allostery.

Consider an allosteric system with two binding sites, each of which must be well ordered to stably bind a ligand. The first binding event substantially reduces the conformational freedom of side chains across the protein, paying a large entropic penalty[31]. This reduction in conformational heterogeneity can stabilize binding-competent conformations at the second binding site. In this scenario, the first event 'prepays' the entropic penalty of the second binding event and reduces the entropic organization penalty for stabilizing the binding-competent conformation. Compared to a scenario in which the second binding site remains dynamic, allosteric ordering lowers the energy gap for the second binding event, leading to an observation of 'positive cooperativity' (Fig. 2a).

A beautiful example of how entropy influences positive cooperativity is in the dimeric enzyme human thymidylate synthase (hTS)[19,32]. Careful calorimetric measurements at multiple protein concentrations demonstrated positive cooperativity between sites, with the second binding event requiring ~1.5 kcal mol^{-1} less energy than the first. This difference is driven primarily by entropy. Both crystallography and sensitive NMR measurements revealed no protein structural changes upon ligand binding. However, NMR measurements of side chain order parameters suggest that the first binding event greatly reduces conformational entropy throughout the protein, including at the second binding site. This ordering leads to virtually no change in protein conformational entropy after the second binding event, consistent with calorimetry indicating that the second binding event is more favored entropically (Fig. 2b). In addition, NMR relaxation dispersion and chemical exchange saturation transfer experiments revealed a reduction in the exchange of some lowly populated states (~1%) upon ligand saturation at both sites. However, this population change is insufficient to explain the large swing in entropy. The loss of slow dynamics and dramatic change in side chain fluctuations can be reconciled through population shuffling, where the fast dynamics undergo reorganization due to the influence of slower movements[19,33]. Remarkably, the entropy-driven positive cooperativity is highly specific to the hTS substrate dUMP. Even though the substrate (dUMP) and product (dTMP) differ by only a single methyl group, no positive cooperativity is observed by calorimetry for dTMP binding. Again, the NMR data are consistent with this observation, showing no difference in side chain order parameters between hTS–TMP and apo hTS. These observations link the thermodynamics of cooperativity to the pre-ordering of side chain conformations across the protein (Fig. 2c).

Entropy can also contribute to cooperativity in monomeric proteins with multiple binding sites, as observed in protein kinases[34–36]. For example, in protein kinase A, cooperativity is observed between the nucleotide and substrate (peptide) binding sites[34]. As in hTS, this cooperativity has an entropic origin with prepaying entropic costs and pre-organization as dominant molecular mechanisms. Inhibitors that resemble the natural substrate, ATP, lead to ordering at the substrate binding site and exhibit greater apparent cooperativity[34]. This example highlights how evolution (and, perhaps unwittingly, medicinal chemistry efforts) have manipulated conformational entropy to achieve positive cooperativity.

In contrast to the positive cooperativity discussed above, entropy can also drive negative cooperativity. In the homotetramer transthyretin (TTR), the affinity for small molecules to the second site is weaker. However, the structure of each subunit, including the binding sites, looks structurally similar, posing the question of why there is a difference in affinity. Cryo-EM revealed an inherent asymmetry in the ensemble of conformations of each dimer and that the first binding event does not lead to ordering the second binding site. On the contrary, the subsequent binding event requires more extensive structural rearrangements, including loop ordering, which comes at a higher entropic cost[37]. Entropically driven negative cooperativity is also observed with NMR order parameter measurements in the bacterial transcription factor CzrA, which has a zinc-binding site more than 15 Å away from a DNA-binding site. Without zinc, DNA binding increases side chain flexibility throughout the core of CrzA, increasing DNA-binding affinity through favorable entropy. However, upon binding zinc, side chain flexibility (and thus conformational entropy) strongly decreases, reducing DNA-binding affinity[38] (Fig. 2c).

These examples show how both negative and positive cooperativity can result from changes in the conformational distributions that accompany initial binding events. These variations in conformational distributions arise not only from substantial shifts in their dominant conformation (conformational change) but also, crucially, from fluctuations. Prepaying (or, in the case of negative cooperativity, exacerbating) entropic penalties is a key way proteins allosterically control function. A greater structural understanding of the entropic and reorganization penalties accompanying these binding events may be especially therapeutically relevant to double-drugging strategies[39,40].

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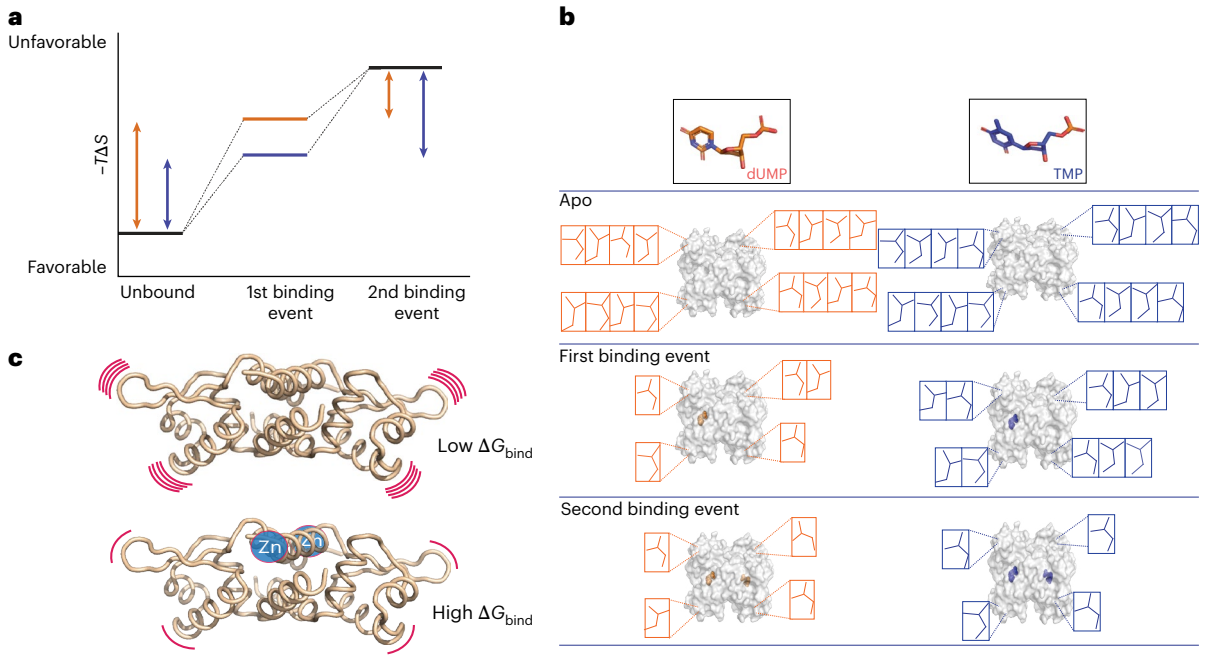

Fig. 2 | Entropy can be used as a way for proteins to modulate cooperativity with the first binding event, either raising or lowering the protein conformational entropy, thereby leading to cooperativity through increasing (negative cooperativity) or reducing (positive cooperativity) the entropic penalty required for subsequent binding events. a, In entropically driven positive cooperativity (orange), a substantial entropic penalty occurs upon the initial binding event (likely leading to some pre-ordering in the second binding site), allowing the second binding event to cause a smaller entropy reduction, which increases the binding affinity, leading to positive cooperativity. By contrast (blue), the initial binding event only pays the entropic penalty of the first binding event, leading to the second binding event paying the same entropic penalty, preventing entropically driven positive cooperativity. **b**, hTS displays entropically driven positive cooperativity when binding its substrate dUMP (orange). The entropic contribution of the first dUMP binding hurts the binding affinity (+2.5 kcal mol^{-1}) and corresponds to a large reduction in side chain flexibility (represented by the number of side chain conformation boxes). However, the entropic contribution of the second dUMP helps the binding affinity (−2.5 kcal mol^{-1}). When hTS binds to its product dTMP (blue), no positive cooperativity is observed. Both binding events have similar binding affinities, with the entropy decreasing upon both binding events, and there are a similar number of reductions in side chains in both events. **c**, In the bacterial transcription factor CzrA, when zinc is not bound, there is an increase in side-chain flexibility, leading to improved DNA-binding affinity due to favorable conformational entropy. Zinc binding (blue sphere) reduces side chain flexibility, diminishing the favorable protein conformational entropy and lowering DNA-binding affinity.

Spatial entropic redistribution can counter local ordering at binding sites

While there are examples of 'fuzzy' ligand–protein binding events in which both the protein and the ligand adopt many conformations upon binding[41], most binding events involve a local reduction in conformational dynamics at the binding site[10]. As in folding, this creates an entropic cost that must be countered by the new attractive interactions between ligand and receptor. As stated above, prepaying this entropic cost using a ground state with a relatively less dynamic binding site is an attractive strategy. However, such well-packed binding sites are at odds with the fact that most binding sites have large voids that can be filled by alternative conformations of binding site residues in the unbound form.

Although binding often comes with a local entropic cost, not all binding events have large overall entropic penalties. A substantial contributor to counter the cost is surely the liberation of ordered water molecules in the bound state; however, even after accounting for solvation changes, the contributions of protein conformational entropy can still favor binding, even outweighing the contribution of solvent in some systems[42,43]. This finding raises the hypothesis that nature counters the entropic penalty incurred at the binding site by modulating entropy elsewhere in the protein. Substantial evidence suggests that proteins have evolved to redistribute entropy from the binding site upon ligand interaction. This phenomenon, which we term spatial entropic redistribution, involves countering local ordering, manifested as a loss of entropy at the binding site, with an increase in disorder at more distant sites, reducing the overall change in conformational entropy (Fig. 3a). Importantly, this is not a 'zero sum' compensation, as in classic entropy–enthalpy compensation[44], but rather spatial entropy redistribution. This redistribution is an important mechanism for reducing the impact of unfavorable local entropic changes and, therefore, improving the overall free energy of binding. While prepaying entropic penalties result from ordering a specific binding site in the ground state, spatial entropic redistribution results from distal sites increasing entropy in response to a local reduction at the binding site. The change in entropy from distal sites can, therefore, partially contribute to overcoming the entropy reduction in the binding site or even exceed it, leading to a favorable change in the conformational entropy of binding.

The most intuitive way of thinking about spatial redistribution of entropy is through portions of a protein acting as 'entropic reservoirs' that are poised to greatly change their conformational ensemble upon perturbation. Intrinsically disordered regions (IDRs) are prime candidates to serve as these entropic reservoirs[45]. In human UDP-α-D-glucose-6-dehydrogenase (UGDH), the deletion of the C-terminal tail IDR reduces the affinity for the allosteric metabolite UDP-xylose by approximately tenfold, despite making no direct contact with the ligand[46]. The ligand-bound state stabilizes altered contacts near the IDR, which allows the IDR to adopt an even more expanded conformational ensemble relative to the apo state. Thus, a major driving force for binding is increased disorder and entropy in an already high-entropy IDR. To test this model, they showed that binding affinity lacked sequence dependence in the IDR region, but that the length of the IDR was strongly predictive of affinity. These results are consistent

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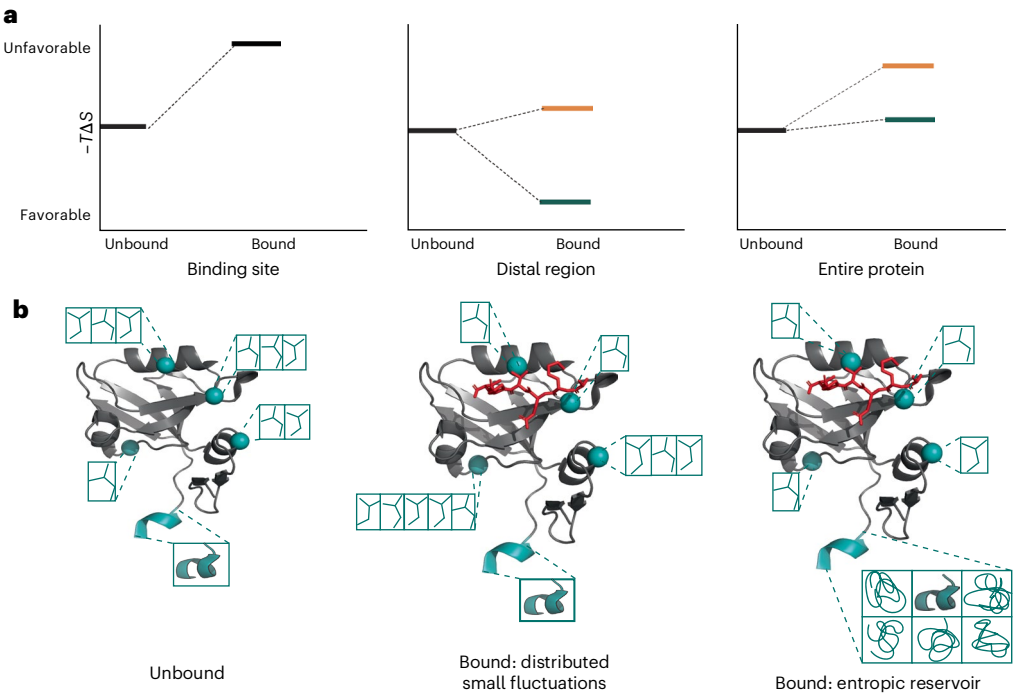

Fig. 3 | Proteins have evolved to counter entropy loss at the binding site by increasing disorder in distant sites. a, Upon binding, the binding site tends to lose conformational entropy; however, entropy can be redistributed to distal regions, which could favor binding (teal). By contrast, if distal regions do not increase their entropy, the protein will play a larger entropic penalty that disfavors binding (orange). **b**, Entropic redistribution upon binding can occur in many different ways. As represented by the PDZ domain, upon binding the peptide (red), entropy (represented by the number of conformational boxes) can be redistributed from binding site residues to distal regions. This can occur through entropic reservoirs, like a transition from ordered (α-helix) to disordered (IDR). It can also be distributed throughout the protein, observed by residues accessing new rotameric states or increased harmonic disorder.

with an entropic force where the IDR can impact the overall entropy through its entropic reservoirs but also its impact on the globular protein conformation and entropy, as observed in other proteins[47,48]. A similar interplay likely modulates allostery in the hTS example discussed above. In hTS, the interaction between an N-terminal IDR and the globular domains may influence the side chain fluctuations underlying positive cooperativity. Intriguingly, this IDR segment is absent in the *Escherichia coli* homolog of TS, which is also dimeric but is noncooperative[49]. In addition to IDRs, local unfolding, leading to a substantial increase in residue conformational entropy, provides another potential 'entropic reservoir'. In adenylate kinase, mutations that affect the amount of local unfolding in lid subdomains are important for different aspects of catalysis, with an underlying entropic mechanism driving the changes in free energy[50]. These examples suggest that the modulation of thermodynamics by the entropy of unfolded or intrinsically disordered segments influences the dynamics of folded regions and can, therefore, tune the free energy of binding and allostery.

In addition to the large changes in entropic reservoirs, small fluctuations within folded domains can contribute substantially to spatial entropic redistribution (Fig. 3b). The interplay between the backbone and side chains in entropic spatial redistribution plays out in the relationship of peptide binding in PDZ domains. The third PDZ domain from the postsynaptic scaffolding proteins PSD-95 or PDZ3 (also known as SAP90) shows a connection between a non-essential distal α-helix and binding affinity. Truncation of α-helix 3 (α_3) reduces peptide binding affinity by 21-fold, even though it is over 6 Å away from the binding site. The lack of change in chemical shifts shows that this change is not due to a major structural perturbation. Rather, the dynamics of the unbound state are altered by the truncation. In the wild-type protein, the side chains are much more rigid relative to the truncated variant. While, in the peptide-bound state, both wild type and the truncated variant have similar side-chain fluctuations. The change in binding affinity therefore results in increased entropic penalty in the receptor. In this context, the α_3 truncation is artificial, but it is a site where other domains are commonly linked to PDZ domains and may highlight a mechanism of interdomain allosteric communication[51]. This type of interaction is reminiscent of the 'entropic reservoirs' discussed above; however, in this case, the dominant entropic contribution is on the side chain fluctuations of the folded domain, not on the backbone of the terminal segment. The dominance of side chain fluctuations in dictating the entropic contributions also played out in a study in the homologous second PDZ domain of human tyrosine phosphatase 1E (PTPN1). This PDZ domain demonstrates clear spatial redistribution: side chains around the binding site lose entropy, while specific distal residues increase entropy[52]. As in the PDZ3 example, there is no evidence from chemical shifts that suggests a major conformational change. This result suggests that sub-ångström changes in positions can be amplified nonlinearly to enable alternative conformations of side chains at neighboring residues, greatly contributing to the number of states the protein populates. This is a provocative mechanism for a protein to have evolved ways to 'transfer' areas of higher entropy (that is, more dynamics) through creating new voids and changes in rotamer states[8].

The most extensive evidence for this mechanism emerges from correlations between changes in NMR order parameters and calorimetry measurements. This correlation has been most extensively studied by Wand and colleagues in calmodulin, which binds various peptides with roughly similar overall binding affinity, but with distinct entropic and enthalpic profiles. For example, upon binding a peptide derived from smooth muscle myosin light-chain kinase peptide (smMLCKp), the receptor loses side chain conformational entropy, especially around the smMLCKp binding site[53]. This result agrees with the direction and magnitude of the estimate of residual conformational entropy from calorimetry. In a series of papers, the authors extended

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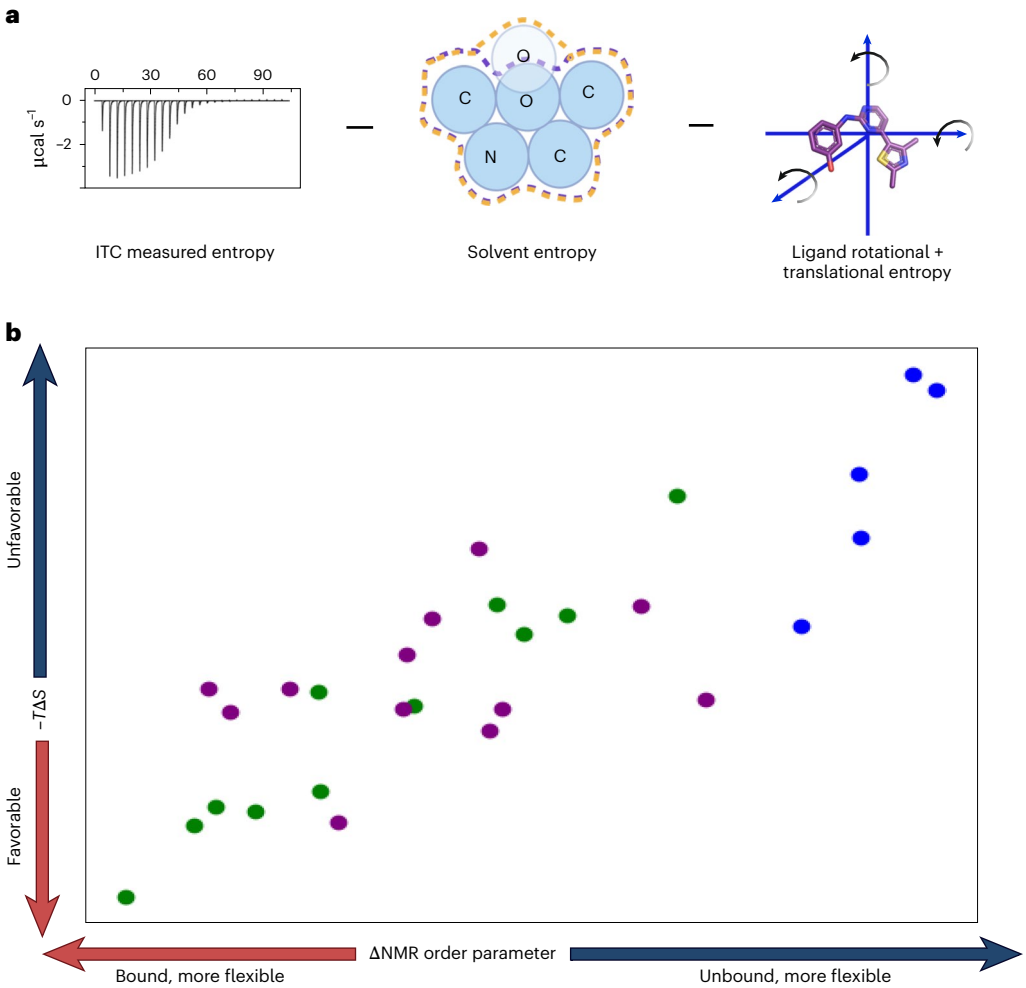

Fig. 4 | Graphical representation of the establishment of the entropy meter that relates NMR-based measurements of conformational entropy to a biophysical measurement of binding entropy from ITC. a, The experimental biophysical estimate of protein conformational entropy is determined by taking the entropy measurement from ITC and subtracting estimates of solvent entropy (based on differences in solvent accessible surface area) and estimates of ligand rotational and translational entropy. **b**, *x* axis, ΔNMR order parameter of protein and ligands between the bound and unbound states. *y* axis, the estimated protein $-T\Delta S$ (in which T denotes temperature, and ΔS denotes entropy) from experimental calorimetry. Each point represents a unique protein–ligand complex, and each color represents a different protein. Caro et al.[42] established a linear relationship between the average ΔNMR order parameter and calorimetric binding entropy. Proteins plotted: calmodulin (green data points), CAP (blue data points), galectin, hen egg white lysozyme (HEWL), PDZ3, PDZ2, dihydrofolate reductase (DHFR) and SAP (purple data points). Data are from ref. 43.

this work to six peptides, each with similar affinity to calmodulin[20]. The contribution of conformational entropy to the binding affinity of these peptides varies widely, ranging from highly favorable to highly unfavorable. In each case, the average change in side-chain NMR order parameters correlated with the estimates from calorimetry, leading to the concept of the 'entropy meter'[42] (Fig. 4).

This entropy meter represents the transformation of the dynamical indicator of conformational entropy into a quantitative metric. The entropy meter concept has now generalized to 28 protein–ligand complexes from eight different protein receptors[42]. Many of these examples do not have a clear spatial relationship of changes in entropy upon binding. However, it is difficult to discern whether this is due to the sporadic methyl side chain probes used to determine the protein conformational entropy or the lack of spatial entropic redistribution in these systems. A spatial pattern is observed in the catabolite activator protein (CAP), a dimeric protein that binds DNA in a cyclic AMP-dependent manner[54,55]. After cyclic AMP binding, CAP undergoes a large conformational change to a state compatible with DNA binding. A series of mutants exhibit similar overall binding affinities, but entropic contributions swing by almost 30 kcal mol^{-1}. Across these mutants, the ones with entropically driven binding have increased side chain fluctuations in side chains distal from the DNA-binding site, while the residues near to DNA experience a loss of flexibility. This spatial redistribution of entropy has also been demonstrated in protein–protein interactions, such as the activation of TGF-β[56]. The concept of how entropic contributions cluster in space has also been examined as a function of pressure in ubiquitin and for protein–protein interactions in barnase–barstar[57,58]. These studies also demonstrate clustering of side chains that increase or decrease flexibility in a correlated manner, suggesting that the redistribution of side chain fluctuations may be an evolved feature of proteins to modulate the free energy of folding and binding.

To assess the general trends of entropic spatial redistribution after ligand binding, we identified ~700 pairs of high-resolution X-ray crystal structures in which the only difference was the presence of a small-molecule ligand. We then rebuilt multiconformer models containing well-supported alternative conformations into the experimental data. These models allow us to estimate crystallographic order parameters[24] for each model and compare the conformational entropy change upon ligand binding[10]. A striking finding emerges:

the conformational entropy lost at the binding sites is correlated with entropy gained at distal sites. We also observed diversity in the amount of entropy redistributed based on both the protein and the ligand involved. Even for a single receptor, different ligands result in distinct patterns of entropy redistribution. Two ligands that cause equivalent reductions in entropy in the active site can differ by ~±1–2 kcal in the response at distal sites. This difference can occur because the ligands will engage different parts of the binding site in specific interactions that induce distinct patterns of spatial entropic redistribution. For example, in the kinase CDK2, the patterns of entropy in type I inhibitors, which bind to the active-like conformation, are more similar to other type I inhibitors than those in type II inhibitors, which bind to an inactive-like conformation. Similarly, type II inhibitors were more similar to each other than they were to type I inhibitors. However, even within the type I inhibitors, ligands still showed unique patterns of conformational entropy, highlighting the possibility of designing for these features. The potential for tuning ligand properties to redistribute entropy also is evident across the entire dataset. Ligands with more specific and oriented interactions (for example, hydrogen bonds) lead to greater local reductions in entropy, and those binding events driven by apolar ligands lead to lower local reductions in entropy. Critically, these observations of spatial entropic redistribution are due to small, distributed changes in side chain alternative conformations and harmonic motions (*B* factors), which are less well captured by traditional, single-state structural models. This example highlights the critical importance of modeling the conformational ensemble to move toward a predictive understanding of the contributions of conformational entropy to ligand binding.

The importance of side chain entropy and the relevance of spatial redistribution to ligand binding extends beyond soluble proteins to membrane proteins. Many membrane proteins have high side chain conformational entropy in their apo state, which may offset the entropic penalty of sitting in the membrane[59]. The modulation of these dynamics upon ligand binding can influence binding and allostery[60]. For example, some GPCRs exhibit similar binding affinities for agonists and antagonists[61]. Several studies have identified how subtle changes in side chain conformational dynamics in the presence of either agonists or antagonists can influence the relative stability of specific conformations compatible with distinct binding downstream partners[62,63]. These results suggest a role for conformational entropy, located in specific distal regions of the protein, in controlling the free energy of binding and the output of allosteric signaling.

The concept of spatial entropic redistribution extends our thinking of the contributors to the free energy of binding to the rest of the scaffold. This strategic transfer of entropy is likely a fundamental aspect of dynamic allostery, where proteins distribute their inherent disorder in a controlled manner to facilitate allosteric regulation. There are multiple mechanisms that nature can harness to optimize the entropic contribution from distal regions. These include 'entropic reservoirs', such as IDRs and local unfolding, and subtle conformational changes to well-packed regions poised to increase side chain fluctuations. This mechanistic diversity emphasizes how the optimization of free energy can come from unexpected places and calls attention to the opportunity for considering entropy to predictably tune binding affinity.

Catalytically competent ensembles formed by enzyme scaffolds can stabilize the transition state

Enzymes catalyze chemical reactions through a high-energy transition state, in which active site residues are precisely arranged to orient specific chemical groups responsible for catalysis. It follows that the positioning requirements will lead to an extreme reduction of conformational entropy for active site residues. How can this penalty be offset? One solution is having these residues pre-organized in the apo or substrate-bound states. Another is the Circe effect, in which the substrate binds in such a constrained, almost unique conformation that is entropically disfavored[64]. A third potential strategy is for the enzyme to allow a much broader set of transition states, reducing the entropic penalty required in the binding site[65]. Finally, it is also possible to extend the concept of spatial redistribution for equilibrium ligand binding to catalysis. The capacity of residues in the rest of the enzyme ('the scaffold') to adopt many states compatible with the positioning of catalytic residues throughout the catalytic cycle[66,67] can contribute favorably to reducing the entropic penalty of active site positioning. In extreme cases, if the scaffold conformational entropy is higher in the transition state than in the ground state, it can reduce the height of the barrier and entropically favor catalysis. This concept extends beyond the chemical step, which in most cases has a narrow transition state ensemble, to other steps of the catalytic cycle, each of which can be rate limiting (for example, substrate binding, product release, conformational change, etc.). We call the ensemble of states compatible with the rate-limiting step of catalysis the catalytically competent ensemble (Fig. 5a).

Strong evidence for the importance of scaffold conformational entropy in catalysis emerges from a series of time-resolved crystallographic, NMR and computational studies of the homodimeric enzyme fluoroacetate dehalogenase (FAcD)[68,69]. Although the enzyme is dimeric, upon substrate binding at one active site, the other protomer does not bind substrate and can be considered an extended part of the scaffold. With clever mutations and substrate analogs, these studies show how solvent and protein scaffold entropy support the active site organization responsible for the chemical step of catalysis. This change to the scaffold occurs without any major backbone changes in any part of the catalytic cycle but is evident in changes in *B* factors, alternative conformations of side chains, the number of bound waters and NMR measurements of dynamics. Importantly, it is the contributions of distal residues and distal water molecules, not just water molecules displaced by the small molecules bound in the active site, that dominate this entropic contribution. In addition, an analysis of MD trajectories using rigidity theory identified interprotomer allosteric pathways that suggest the mechanism of how these changes occur. Collectively, these studies reveal how entropy can favor conformations responsible for rate-limiting steps of the catalytic cycle via increased conformational multiplicity of the scaffold and the release of solvent. The interplay between solvent and scaffold calls attention to the importance of holistically modeling the conformational dynamics of the entire system, not just the active site.

The catalytically competent ensemble model can also be expanded to understand the operating principles of large macromolecular machines. For example, the ribosome undergoes many large structural rearrangements during the catalytic steps involved from initiation to elongation. These rearrangements involve the making and breaking of many interactions that are relatively straightforward to interpret enthalpically. An elegant series of single-molecule fluorescence resonance energy transfer experiments leveraged temperature to probe the relative importance of enthalpy and entropy when two different charged tRNAs are bound to the ribosome. They revealed that both the equilibrium and the rate of interconversion between the two conformational states of the ribosome, GS1 (associated with initiation) and GS2 (associated with elongation), have distinct entropic contributions depending on the identity of the tRNA present. The model that emerges is that $tRNA^{Met}$, which is critically important for initiation, and $tRNA^{Phe}$, which only plays a role in elongation, differ in how they affect ribosome conformational dynamics. The energetic basis of this difference is driven by entropy favoring the corresponding states and barriers, indicating that the ribosome has evolved to manipulate entropy in response to specific tRNA-binding events that bias toward functionally relevant states (Fig. 5b). How exactly this entropic difference is partitioned between broadening the transition state itself or increasing the conformational entropy of the scaffold (including bound metals and water) remains an open question. This and other studies

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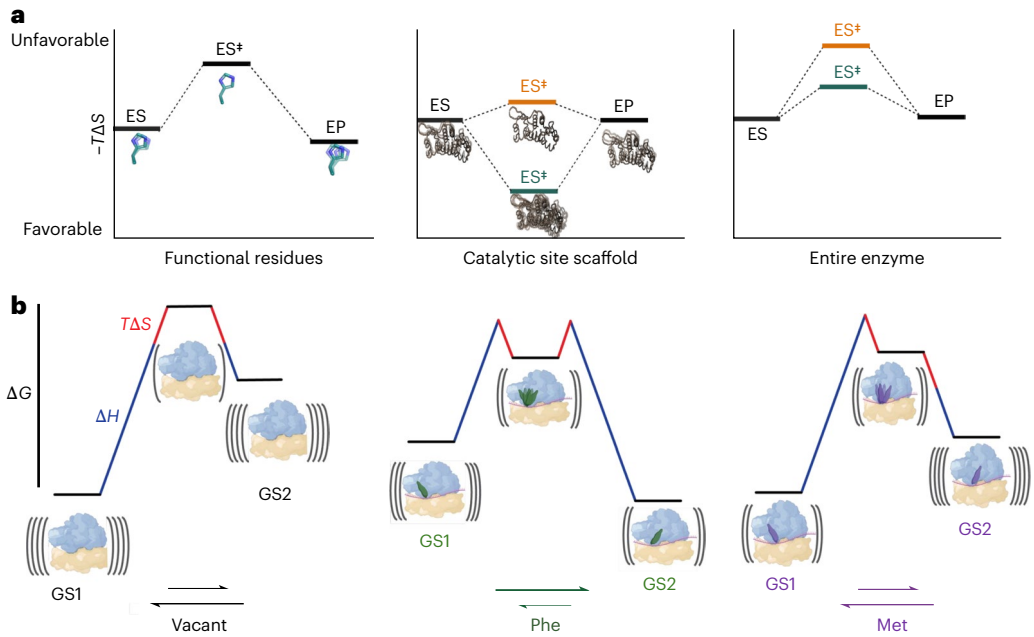

Fig. 5 | In a transition state, specific residues must be precisely arranged while the rest of the protein can adopt multiple states, accelerating the rate-limiting step. a, As the enzyme moves through the transition state (‡), catalytic residues must undergo extreme entropic loss to allow for efficient chemistry. However, the scaffold surrounding the catalytic site can still access many states, reducing the entropic loss across the entire enzyme (teal). This contrasts with the scenario in which the enzyme scaffold also loses entropy, increasing entropic loss across the entire enzyme (orange). ES, enzyme–substrate complex; EP, enzyme–product complex. **b**, The ribosome (large subunit, blue; small subunit, yellow; Phe mRNA, green; Met mRNA, purple) goes through large conformation changes between the initiation state (GS1) and the elongation state (GS2). The transition between these states is contributed enthalpically (blue) and entropically (red). The direction of the entropy (favorable or unfavorable) leads to different preferred states, with Met being favored for the GS1 state and Phe being favored for the GS2 state.

demonstrating how the ribosome harnesses entropy during peptide bond formation[70] suggest a complex interplay of different entropic forces during its conformational and catalytic cycles of translation[71].

The catalytically competent ensemble model also provides an energetic grounding for how conformational dynamics can be correlated with the evolvability of proteins. The relationship between increased conformational dynamics and evolvability is often discussed as altering the probability of active site conformational states[72], but it can extend to the energetic stabilization contributed by distal entropy in a catalytically competent ensemble. Promiscuous reactions are the first step toward evolutionary novelty. A broader conformational ensemble, locally in the active site and in distal residues that increase the overall conformational entropy, can promote such promiscuous reactions. Indeed, there is evidence for an increased error rate for $tRNA^{Met}$ on the ribosome[73], which suggests that the entropically favored transition state may also be broader at the active site. This model also provides a hypothesis for contributing to some distal mutations accumulated in directed evolution experiments, especially for enzymes that change flexibility in regions beyond the active site[74,75]. With this framework, the challenge remains to quantify how conformational heterogeneity in the protein scaffold (or, in the case of the ribosome, the ribosomal RNA scaffold), substrate and solvent molecules alters the conformational ensemble in a way that affects both the equilibrium and the transition rates between different functional steps of a catalytic cycle.

Future directions in exploring conformational entropy

Failing to explicitly account for conformational entropy in structural biology skews our understanding of thermodynamics, hindering our ability to harness this free energy source. Conformational entropy modulates free energy through three main mechanisms: entropic prepayment, spatial entropic redistribution and the catalytically competent ensemble. However, the lack of widespread structural ensemble representations limits our ability to explicitly explain the contributions of entropy and leverage this driving force for the prospective design of function[76]. Conformational ensembles, grounded in experimental data, will allow entropy to be integrated with probabilistic accounting of enthalpic-dominated interactions such as hydrogen bonds, salt bridges and hydrophobic contacts to ground the complex interplay between structure, dynamics and function in thermodynamics.

The most prominent link between conformational entropy and structural ensembles has been inferred from NMR order parameters. However, there is ambiguity in the structural elements that give rise to a set of order parameters, as outlined in Fig. 1. One way to resolve this ambiguity is to use experimental data as a bias in MD simulations[28,30]. This approach has provided insights into how mutations, counterintuitively, stabilize chymotrypsin inhibitor 2 through increased side chain fluctuations in the hydrophobic core[30]. The use of experimental biases in simulation is currently distinct from improved modeling and refinement methods that create structural ensemble models in X-ray crystallography or cryo-EM[10,23,77–83]. Once refined, these models can simultaneously extract conformational entropy and structural interpretations[10], unhindered by the timescale constraints in NMR and MD simulations. Integrating multiple datasets simultaneously, particularly time-resolved[84] or fragment-soaking experiments[85], can further augment conformational entropy–structure discovery. The interface of experimental data, AlphaFold-type machine learning approaches and MD simulations will surely catalyze developments in quantifying conformational entropy[86,87]. Machine learning already realizes some of this immense potential for ensemble discovery and model generation across length scales in cryo-EM[88,89]. Understanding the relationship between latent spaces leveraged by these machine learning techniques and the underlying thermodynamics of the system may help guide the generative ensemble process to be even more predictive biologically. These methodological advancements also necessitate better data structures than static Protein Data Bank files

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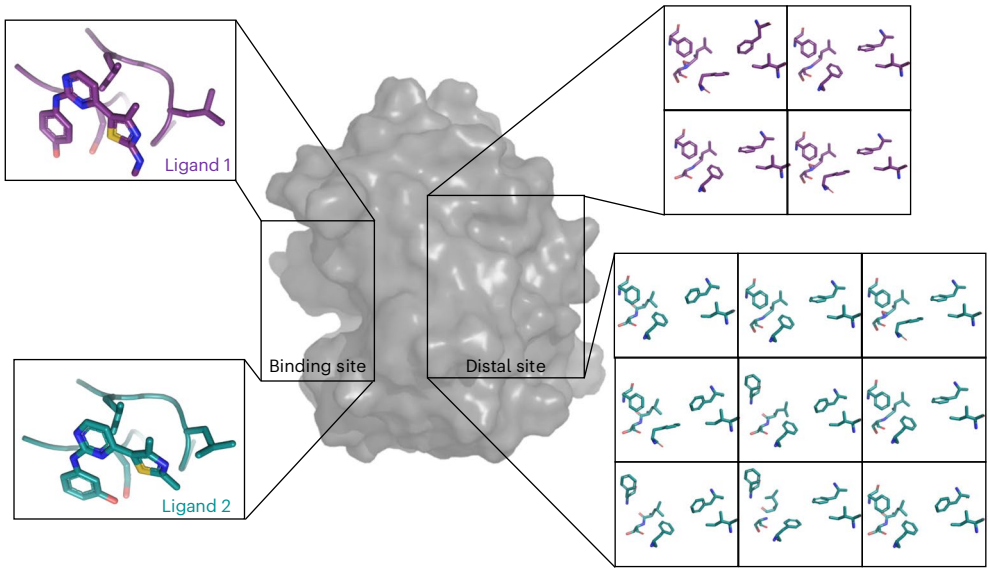

Fig. 6 | We propose the idea that spatial entropic redistribution be leveraged in ligand design. Upon binding of two highly related ligands (teal and purple), a single binding site leucine changes rotamer states. Simultaneously, the conformational entropy of distal regions (represented by the number of unique conformations) substantially differs between the two ligands. This leads the teal ligand-bound state to have more conformational entropy in the distal region, which could increase the binding affinity of the teal ligand compared to that of the purple ligand.

to represent ensemble-level features[76]. Advancing the modeling and encoding of data to better account for protein dynamics will facilitate breakthroughs in structural ensemble predictions, enabling a more facile interpretation of the conformational ensemble and how it relates to conformational entropy and protein function.

As we improve representations of macromolecules, it will be increasingly important to consider the cellular context, which can provide other sources of entropy, including solvent and ligands. Much attention has been focused on the behavior at the binding site, with the reduction of the ligand ensemble into a single state being countered by the displacement of ordered water molecules. However, in high-resolution X-ray crystallography data, multiple conformations of ligands are often detected in binding sites, suggesting that many protein–ligand complexes have multiple binding states[90–92] and indicating that ligand binding results in less entropic loss than previously believed. Additionally, as seen in the FAcD model, solvent plays a critical role beyond the binding site. Furthermore, solvent and ligand entropy changes can lead to entropy–entropy compensation as observed in galectin-3C[93]. To gain a deeper insight into the entropic implications of solvent interactions, it is crucial to refine our modeling and thermodynamic interpretation of first and second shell water molecules, particularly those in exchange with the bulk solvent and thus 'partially occupied', as revealed by MD simulations[94]. Creating a more atomistic model of the entropic contributions of solvent may reconcile outlier observations in the entropy meter, which links side chain fluctuations to experimental isothermal titration calorimetry (ITC) data and moves us closer to predicting the entropic impact of a perturbation.

Integrating structural and entropic features from conformational ensemble models will open up the use of a powerful lens to drive down ligand binding affinity and stabilize protein conformational states in design. Traditional drug design emphasizes optimizing the complementarity of a small molecule with the binding site. While this can improve enthalpy, it almost completely ignores entropy (except for consideration of the entropy gained by a displaced solvent at the binding site). However, ligand properties, such as the number of hydrogen bonds, are associated with a difference in conformational entropy[10]. Furthermore, minor chemical changes can substantially affect entropies and affinities, as seen in the hTS case where a single methyl group difference leads to vastly different entropies[19]. How minor ligand changes propagate to protein conformational entropy differences remains a mystery (Fig. 6). Structural perturbations do not necessarily propagate through the structure via a series of dramatic 'domino' conformational changes from one binding site to the distal site. Instead, distributed and nonlinear changes throughout the ensemble lead to changes in distal dynamics (entropy) or the dominant conformational state (enthalpy). Examining these through a free energy landscape perspective, any conformational changes modify the system's free energy landscape and thus function, highlighting the need to combine rather than isolate these features. Likewise, protein design, optimized for protein packing and stability, now faces the next frontier: designing improved binders and enzymes[95,96]. While prepaying entropic cost by establishing order in the ground state has been explored[97], spatial entropic redistribution, achieved by increasing conformational entropy in functionally distal regions, is harder to design[98]. Integrating structural and entropic changes from natural versus designed enzymes could lead to innovative ways to design functional proteins.

Collectively, the examples discussed here reveal distinct strategies discovered by nature to optimize the free energy of functionally important states. Although it is easier to explicitly represent and discuss specific and enthalpically directed interactions like hydrogen bonds, improved ensemble representations of macromolecular structure should allow us to integrate structural and entropic information for the prospective design of function. After all, nature cares about free energy, regardless of whether the source is easy to describe. Even if the energetic impact of conformational entropy is relatively small in some cases, the move from ensemble-averaged singular representations of protein structure to ensemble views could parallel the revolution catalyzed by single-cell methods in genomics. In single-cell work, the diversity of cell types and behaviors masked by bulk methods leads to new insights into the properties of systems[99]. Indeed, the configurational entropy of cell types may also have a role in tissue organization, connecting the importance of considering statistical mechanical concepts in decoding function across scales[100]. In the case of macromolecules, we look forward to future work highlighting how rare states contribute to specific functions and leveraging conformational entropy as part of the toolkit to solve design, catalysis and cellular signaling challenges.

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Author contributions

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Competing interests

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