🗨 **COMMENTARY**

Bringing disorder and dynamics in protein allostery into focus

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The intricacies of protein-mediated signaling continue to be revealed in exquisite detail. It therefore seems fitting to return to the fundamental and almost magical feature of protein molecules that makes it all possible: allostery, or action at a distance. This long-held concept arose and was refined during the explosive expansion of the structural biology of proteins. The resulting models of allostery through discrete two-state structural transitions have held sway for over half a century. However, they are incomplete. Even for the paradigm allosteric protein hemoglobin, alternate functionally relevant structures that are dynamically averaged (1) force a broader ensemble description of the thermodynamics of allostery (2). Furthermore, the view seen through the lens of structure is a very enthalpic one that focuses analysis on the details of specific interactions within and between proteins and their ligands. However, it is free energy that guides the allosteric response. Entropy, which is what makes the energy free, has rarely been examined experimentally in the same microscopic detail. Although it was known decades ago that protein molecules should (3, 4) and do (5–7) dynamically fluctuate, it remains uncertain to what degree conformational entropy (S_{conf}) actually does contribute to the free energy of ligand binding and allostery. This uncertainty is because S_{conf} has largely resisted experimental measurement. Obviously, motion between various states that a protein visits reports, albeit indirectly, on its S_{conf}. Developments in solution NMR spectroscopy have recently converged to allow detailed analysis of protein internal motion on many time scales (8), and thus open the door to the elusive thermodynamic variable. In an elegant and unusually comprehensive study reported in PNAS, Capdevila et al. (9) illuminate the dynamical aspects of ligand binding and the contributions of ΔS_{conf} to allostery in a transcription factor that is negatively regulated by zinc. Their results are not comprehensible within the confines of interconversions between static structures and require a context of an ensemble of states. How they achieved this insight requires some explanation.

With the development of advanced multidimensional heteronuclear NMR spectroscopy has come the capability to measure NMR relaxation phenomena in
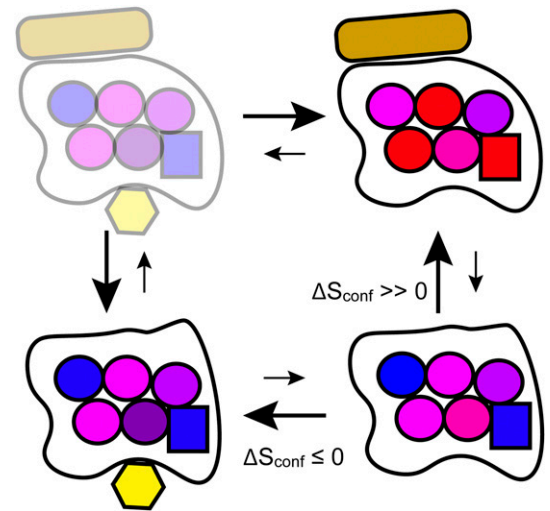
Fig. 1. Schematic illustration of the basic features of an allosteric mechanism regulated by S_{conf}. S_{conf} directs binding by a protein (black envelope) of a ligand (rectangles), which is suppressed by the binding of a negative heterotropic allosteric regulator (hexagons). Detailed dynamical studies by Capdevila et al. (9) support this model for the binding of DNA to the CzrA transcription factor and its allosteric regulation by zinc. In the zinc-bound states, side-chain (circles) motion is, on average, suppressed (more blue), whereas in the DNA-bound states, it is, on average, elevated (more red). A number of residues in CzrA show extraordinary influence on the overall the entropic response (squares). The slightly transparent ternary complex, which is strongly disfavored, was not directly examined in this way. For graphical simplicity, the $CzrA_2$ and $Crz_2{:}Zn_2$ species are represented as the monomer and 1:1 complexes, respectively.

proteins in a site-resolved way (8). NMR relaxation is fundamentally linked to motion, and a wide range of times scales is accessible. Important here are the fast picosecond-nanosecond and slower microsecond-millisecond motions that are sampled at the backbone amide N-H and the methyl groups of side chains. Relatively simple experiments using cross-correlated relaxation in methyl groups lead to a particularly direct analytical path for fast motion (10). This advance has

greatly accelerated the pace of detailed dynamical studies of protein motion and is used extensively by Capdevila et al. (9). Relaxation due to fast internal protein motions is usually interpreted using the popular Lipari–Szabo "model-free" formalism (11) that captures the disorder of some "interaction vector" in terms of the squared generalized order parameter (S^2). The S^2 is related to the populations of the various states that are visited by the NMR probe during equilibrium fluctuations of the protein (11). Hence, in principle, changes in motion are related to changes in S_{conf}. Indeed, as long ago as the mid-1990s, it was pointed out that if one assumed a specific energy potential describing motion of an NMR-accessible vector (e.g., the N-H bond, the symmetry axis of a methyl group), then one could solve the parametric relationship between S^2 and the associated entropy (12–14). These model-dependent interpretations gave comfort to the idea that "more motion means more entropy" and vice versa. Early studies of calmodulin binding of various regulatory domains found a significant redistribution and reduction of side motion (15) that correlated with the total binding entropy (ΔS_{tot}) (16). This finding suggested that ΔS_{conf} was a major component of ΔS_{tot}. Unfortunately, several problems complicate making this view quantitative through a local model-dependent interpretation (17, 18). A breakthrough was to establish an empirical relationship between changes of motion and changes in S_{conf} that avoided the main problems with a model-based interpretation (18, 19). The resulting NMR-based dynamical proxy or "entropy meter" approach provides measures of ΔS_{conf} for the entire protein molecule. Capdevila et al. (9) use the entropy meter to probe the thermodynamic origins of the allosteric response in Zn-induced allosteric inhibition of DNA binding by the homodimeric repressor CzrA.

The role of S_{conf} in CzrA function was examined by comparing fast backbone and methyl side-chain motion in three functional states: $CzrA_2$, $CzrA_2$:DNA, and $CzrA_2$:Zn_2. The backbone of the protein was found to be largely unresponsive to the binding of operator DNA, whereas the methyl side-chain motion was broadly influenced and, on average, increased upon forming the complex. The decoupling of backbone and side-chain motion is not unprecedented and warns strongly that both need to be examined (17). The net increase in side-chain dynamics on forming the CzrA:DNA complex was found to correspond to nearly two-thirds of the large and favorable ΔS_{tot}. This determination set the stage for understanding how negative allosteric regulation by zinc is achieved. Binding of zinc to the CzrA causes only localized diminishment of methyl side-chain motion, corresponding to a small unfavorable change in ΔS_{conf}. Importantly, binding of zinc appears to abolish the ability of the protein to unlock the large favorable change of S_{conf} necessary to bind the operator DNA with high affinity. This observation broadly explains the thermodynamic origins of the negative allostery (Fig. 1).

To reveal how the allosteric signal is coordinated by the protein, Capdevila et al. (9) use the temperature dependence of methyl side-chain dynamics to highlight residues with unusual motion. Using an analytical strategy introduced by Massi and Palmer (20), they find a remarkable degree of motional coupling

in the core of CzrA. They then take advantage of a pioneering effort by Tzeng and Kalodimos (21), who showed that seemingly innocuous point mutations in the catabolite activator protein often profoundly influenced its dynamics and the thermodynamics of DNA binding. Introduction of cavity-creating mutations in CzrA illuminated a strong correlation between binding affinity and dynamic (entropic) contributions. Through these two approaches, a large network of residues was found to provide a reservoir of S_{conf} that is controlled through a smaller number of critical side chains. The influence of Zn binding on these "hot spot" residues is the key to locking away the contribution of ΔS_{conf} to the binding of DNA. Importantly, this work also clearly

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reaffirms that the seductive concept of restricted pathways of dynamic connectivity need not be operative for allostery involving S_{conf} (17).

Capdevila et al. (9) also address the mechanism of molecular recognition and binding of DNA to CzrA. Structural transitions are very often critical to the recognition and binding of a ligand by a protein. Kinetic aspects of molecular recognition give rise to "conformational selection" and "induced fit" ligand-binding mechanisms (22), which can lead to fundamental insights of both biological and medical importance (23). Dispersion relaxation NMR techniques (8) were used to show that, in the absence of ligands, CzrA fluctuates on the millisecond time scale to a minor conformation that is likely structurally compatible with DNA binding. Zn binding quenches this motion, suggesting that conformational selection is the operative mechanism for recognition and binding of operator DNA and is intimately connected to CzrA allostery.

In closing, it is interesting to note that only a few dozen examples of comprehensive NMR relaxation studies of the dynamical effects of ligand binding and allostery have been reported in the literature. The contribution by Capdevila et al. (9) exemplifies the richness of the dynamical-entropy axis in protein function that has been exposed by this small number of examples. It seems likely that there are many more arenas in which internal motion, and the S_{conf} that it reflects, will prove to be critical elements of the physical basis of protein function. Indeed, as molecular dynamics reaches reliable accuracy (24) and crystallography more fully adopts the notion of conformational heterogeneity (25), the combination of NMR spectroscopy, crystallography, and simulation will dance across the landscape of relationships between protein internal motion, structure, and function.

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