The role of dynamics in allosteric regulation

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The biomolecular conformational changes often associated with allostery are, by definition, dynamic processes. Recent publications have disclosed the role of pre-existing equilibria of conformational substates in this process. In addition, the role of dynamics as an entropic carrier of free energy of allostery has been investigated. Recent work thus shows that dynamics is pivotal to allostery, and that it constitutes much more than just the move from the 'T'-state to the 'R'-state. Emerging computational studies have described the actual pathways of allosteric change.

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Abbreviations

KNF	Koshland, Nemethy and Filmer
MD	molecular dynamics
MWC	Monod, Wyman and Changeux
PDB	Protein Data Bank
rms	root mean square

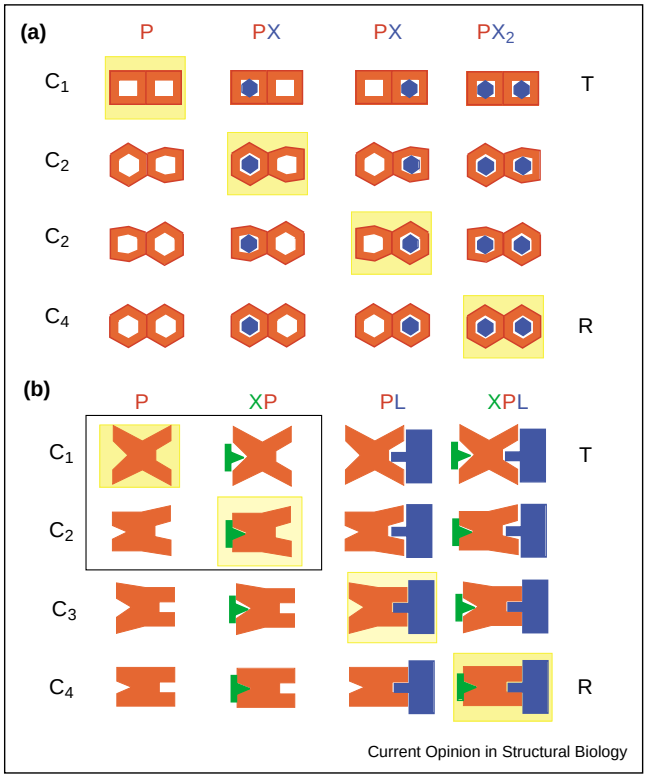
Introduction

Dynamic processes in biomolecules cover a large timescale regime, including very fast fluctuations of individual atoms on the picosecond timescale, loop and domain motions on the nanosecond timescale, conformational rearrangements on the millisecond timescale and breathing modes on a timescale slower than seconds. Several excellent reviews summarize the functional role of dynamics in catalysis [1,2]. Here, we review the role of dynamics in allostery. Allostery ('allo-steric = other-space') means that action in one part of the molecule causes an effect at another site. Allosteric processes are closely associated with ligand-induced conformational changes that propagate between the allosterically coupled binding sites [3]. The allosteric molecule thus changes its coordinates as a function of time, which constitutes dynamics. Hence, dynamics and allosteric processes are almost tautologically linked. In addition, changes in the dynamic properties of the different conformations of the allosteric protein may contribute to the free energy of allosteric coupling through entropic effects [4]. This review focuses on the different types of dynamics of allosteric proteins. Purists may associate only quasi-harmonic motions on the shortest timescales with the word dynamics. In this review, however, we will consider all motions — quasi-harmonic, diffusive rearrangements or fluctuations in populations over an ensemble of subconformations — as dynamics. The review ignores the role of allosteric proteins in systemic dynamic processes, such as the dynamic changes of gene expression and neuron proliferation.

It is often stated that allosteric systems are oligomeric and symmetric (e.g. [5]); for this review, we want to take a broader point of view. We define here as allosteric those systems in which the binding of one ligand affects the affinity of another ligand (Figure 1). This includes, in addition to the classical homotropic oligomeric systems such as hemoglobin and aspartate transcarbamylase, heterotropic monomeric systems such as Hsp70 chaperones, whereby allosteric coupling exists between ATP binding in one domain and protein substrate binding in another domain [6], and single-domain proteins, whereby phosphorylation at one site affects the structure of the protein in a remote area [7,8••]. Our definition also includes those proteins for which at least one of the ligands is a biological macromolecule; good examples are the Trp- and RNA-binding attenuation protein repressor, for which tryptophan binding affects RNA binding at a remote site [9••], and processes such as ligand-coupled oligomerization [10,11].

We want to mention a few developments that have made many of the exciting recent discoveries in allosteric dynamics feasible. Improvements in mutagenesis and site-specific labeling techniques extended the applicability of fluorescence lifetime methods beyond naturally available tryptophan residues [12]. Ultrafast laser technologies can resolve many time steps in dynamic processes [13]. The measurement of site-resolved quasi-harmonic dynamics on the picosecond to nanosecond timescale by NMR relaxation methods is feasible for both protein backbone and protein sidechains [14–16]. Fluctuations between conformational substates on the millisecond to microsecond timescale can now be measured quantitatively and site resolved by NMR as well [17]. Importantly, NMR dynamic measurements can now be combined with TROSY detection methods [18], which extends its application to larger systems, such as a 91 kDa

Figure 1



Generalized models of **(a)** homotropic and **(b)** heterotropic allosteric interactions between two ligands involving conformational change. The models emphasize that the protein can dynamically adopt all conformations in all ligation states; the process of ligand binding merely shifts the population of the conformational states in the dynamic ensemble, as first formulated by Weber [31]. Both diagrams contain the MWC model ('T' and 'R' conformations in the first and fourth rows of both diagrams, respectively) and the KNF model (conformational states on yellow backgrounds). Diagram (b) emphasizes that allosteric proteins can be monomeric and that ligands can be other (downstream) protein(s). The intermediate states (conformations C_2 and C_3) were chosen such that the unoccupied binding site changes partially towards the bound conformation (C_4). This is a plausible (but not unique) mechanical model of positive cooperativity for sequences P → XP → XPL and P → PL → XPL. The four species in the box in (b) illustrate the pre-existing equilibrium as described by Volkman *et al.* [8••] (see also Figure 2).

allosteric protein [9••]. The NMR measurement of residual dipolar couplings in solution has recently been applied to characterize dynamic process as well [19]. The commercial availability of precise microcalorimeters allows a quantitative assessment of entropic effects, which, in some cases, can be related to changes in dynamic processes [20]. A new, unsuspected source of dynamic information is ultra-high resolution low-temperature X-ray crystallography; anisotropic B-factors allow motional models for atomic and collective motions to be developed, and the observation of multiple conformations is suggestive of fluctuations over conformational substates [21•]. Comprehensive synthetic approaches make it possible to design small allosteric systems [22•]. Last but not least, the steady increase in computational power and the development of new computational approaches for the study of protein dynamics push simulations into time regimes that have previously not been accessible [23]. This permits the comparison of simulated and experimental dynamics, ultimately allowing predictions of allosteric dynamic pathways.

To the best of our knowledge, this subject has been reviewed systematically only once before [24]. That work is an excellent source for an overview of movements in allosteric proteins as inferred from static structures, NMR methods for the detection of picosecond to nanosecond backbone dynamics, and the dynamic properties of the allosteric Trp repressor system. In the following, we selected several representative examples from the enormous volume of literature that has been reported since then and have organized the information in four sections.

Allosteric dynamics as inferred from static limit structures

To date, the vast majority of allosteric dynamic processes are still investigated by comparing the structures of allosteric molecules in their limiting states. Classical examples include hemoglobin and aspartate transcarbamylase, in which an up to 20° subunit reorientation occurs upon ligation [25,26], leading to changes in the atomic coordinates of up to 10 Å. An extensive body of literature has been devoted to describing the dynamic pathway of such conformational changes; one of the best known is the Perutz mechanism, which suggests a set of consecutive motional moves leading hemoglobin from its T-state (tensed) to its R-state (relaxed) [25,27]. We will not consider these 'inferred' motions further in this review, as they have been well described and reviewed (e.g. [24]). We do note the recent development of sophisticated Web-based 'morphing' programs that illustrate the minimally required motions for the allosteric conformational change [28]. The identity of the intermediate structures in these instructive movies is of course hypothetical and it is assumed that the path of conformational change is the shortest one.

Experimental observation of motions relevant to allosteric conformational change

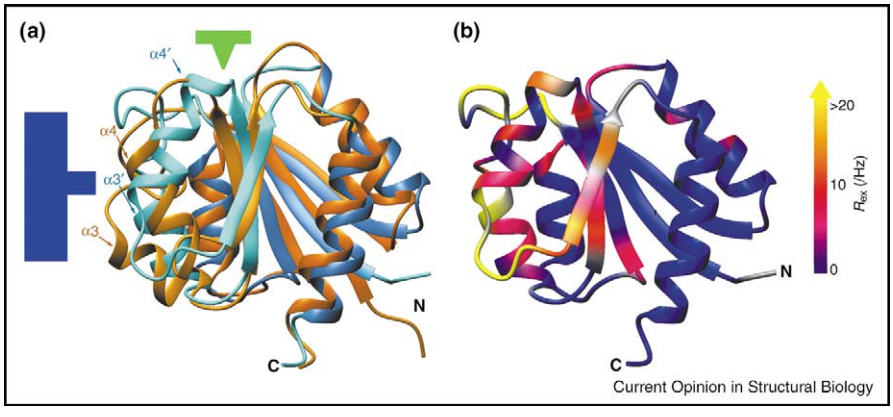
Evidence is mounting that allosteric motions take place not only when the allosteric conformational change is induced upon ligand binding but also in the absence of this process, confirming the early realization that proteins should be described as a dynamic ensemble of conformational substates [29]. A mechanical realization of a thermodynamic allosteric model is depicted for homotropic oligomeric (Figure 1a) and heterotropic monomeric (Figure 1b) systems. This is a generalization of the Monod, Wyman and Changeux (MWC) [3], and Koshland, Nemethy and Filmer (KNF) [30] allosteric models, as first put forth by Weber [31]. In this model, the protein

continuously samples a range of allosteric (sub) conformations. Ligands bind preferentially to one of the sampled conformations, causing a change in its free energy and hence shifting the equilibrium. Modern concepts of protein folding funnels and energy landscapes pioneered the idea of ensembles of folded conformational substates. A similar model of protein conformational dynamics for biological function was recently reviewed by the Freire [32,33•] and Nussinov groups [34]. The latter group equates the rough landscape of conformational substates at the 'bottom' of the protein folding funnel with the range of allosterically accessible conformations.

An early indication of 'pre-sampling' of the conformational change, aside from arguments of principle, came from molecular dynamics (MD) calculations on myoglobin, which showed the largest conformational fluctuations for areas that experience the largest conformational difference between the limiting static X-ray structures [29]. More recently, a combined X-ray and fluorescence study of the allosteric protein fructose-1,6-bisphosphatase [35] showed that the structural loop putatively responsible for allosteric regulation of catalysis is disordered in the T-state and becomes ordered in the R-state. Trp fluorescence experiments indicated that the R-state is part of the conformational family in the T-state. Hence, the conformational transition T → R already occurs transiently in the T-state ensemble.

A particularly clear example of this principle was recently uncovered by Volkman *et al.* [8••], who used NMR spectroscopy to demonstrate a strong correlation between the phosphorylation-driven activation of the signaling protein NtrC and microsecond timescale backbone dynamics in an area remote from the phosphorylation site (Figure 2). They characterized the solution structures and the motions of the regulatory domain of NtrC in three functional states: unphosphorylated (inactive), phosphorylated (active) and a partially active mutant. The NMR-detected dynamics in the unphosphorylated state occur exactly at the area of conformational change upon activation, indicative of an equilibrium of states (as highlighted in Figure 1b). Chemical shift analysis of NtrC states with different activity revealed that the dynamics detected by NMR relaxation experiments represent an exchange between inactive and active states in unphosphorylated NtrC. The relative amount of shift is a direct measure of the equilibrium constant between the two states (the C_1 and C_2 apo conformational states in Figure 1b). Phosphorylation shifts the equilibrium toward the active state (C_2 single-ligand conformation in Figure 1b). Additional studies have indicated that the area involved in dynamic exchange is responsible for downstream signal transduction, the activation of ATPase activity in the central domain of NtrC and consequently activation of transcription. Hence, the NtrC protein is an example of a single-domain heterotropic allosteric system, in which one of the allosteric ligands is the downstream protein interaction partner (species on the right in Figure 1b). Such a general mechanism for phosphorylation-driven allosteric signal transduction dynamics was proposed earlier [7].

Figure 2



Experimental evidence of the existence of a pre-existing equilibrium encompassing both allosteric conformational states in the signaling protein NtrC [8••]. **(a)** The NMR structures of the switch domain of NtrC in the inactive (cyan/blue, PDB code 1DC7) and active (yellow/orange, PDB code 1DC8) states are superimposed using the residues indicated in darker colors. The structural differences between the two allosteric states are highlighted in lighter colors. Phosphorylation of the active site D54 (by ligand X, shown in green) shifts the equilibrium towards the active state, dramatically increasing the affinity of the remote site for the downstream target (ligand L in Figure 1b, illustrated in blue), that is, the transcriptional activation domain of NtrC. **(b)** Direct detection of the dynamics of interconversion between the two allosteric states in the nonphosphorylated form of NtrC. NMR backbone relaxation techniques were applied to detect microsecond timescale motion in exactly the area that undergoes conformational changes due to ligand binding. These dynamic residues were identified by the NMR exchange term R_{ex} (shown as a continuous color scale), which indicates conformational exchange between states that sense different chemical environments. This NMR study provides an example of positive heterotropic allostery in a single-domain protein. Figure modified from [8••].

Structural comparison of the apo form of glutamate dehydrogenase with crystal structures determined with bound substrate, cofactor or allosteric effectors revealed that the electron density of the NAD-binding domain is more disordered in the ligand-free enzyme [36•]. The flexibility of this domain was found to distort the remote GTP-binding site, such that it can no longer bind GTP. These results support both the notion that the structural change from the apo form to the form with bound coenzyme and substrate is already encoded in the dynamics of the NAD-binding domain, and an active role of dynamics in the allosteric process itself, as will be discussed extensively below.

Recently, it was shown that the CO-ligated state of the prototypical allosteric system hemoglobin is actually a dynamic ensemble [37••]. Using the new NMR method of measuring residual dipolar couplings in solution, the relative orientation of the α1/β2 dimers in the tetramer could be determined with very high precision. It was found that the solution conformation is exactly halfway between the two different crystal structures observed for this ligation state. Extensive microsecond dynamic perturbations of the resonances belonging to residues in the dimer interface strongly suggest that the solution structure represents a dynamic average of the two X-ray structures. This study does not exactly provide proof of the pre-existence of the T ↔ R equilibrium; nevertheless, it clearly shows that different (crystallographic) states can be in dynamic equilibrium in solution.

X-ray diffraction data for single stranded DNA binding proteins showed that the DNA-binding domains adopted multiple orientations in the absence of DNA, in contrast to a unique structure found for the complex with single-stranded DNA. Analysis of intermolecular contacts suggested the possibility that other proteins, such as the tumor suppressor p53, could compete with DNA for the same binding site. The authors propose a dynamic model for the DNA-binding mechanism [38].

Matoba *et al.* [21•] deconvoluted the B-factors of a 1.05 Å resolution X-ray structure of a prokaryotic phospholipase A2 (PLA2) into global (rigid-body motions in terms of translation, libration and screw motions) and local dynamic contributions. Regions exhibiting larger internal motion are associated with calcium binding, phospholipid binding and the allosteric interfacial activation areas.

As a last example of the existence of an allosteric pre-equilibrium, we discuss a paper on the dynamics of a HIV TAR RNA fragment in the presence and absence of the binding protein TAT. As shown through a variety of techniques, the free state of TAR contains bulge nucleotides, which lead to a pronounced kink between the two stems. Binding to basic peptides derived from TAT results in co-axial stacking of the stems and extrusion of bulge nucleotides. Using residual dipolar couplings, Al-Hashimi *et al.* [39••] were able to demonstrate that the protein-free form of TAR is in dynamic equilibrium between a fully linear and a 90° bent structure, again indicating the existence of a pre-equilibrium; the ligand (protein) binds selectively to the fully extended conformation.

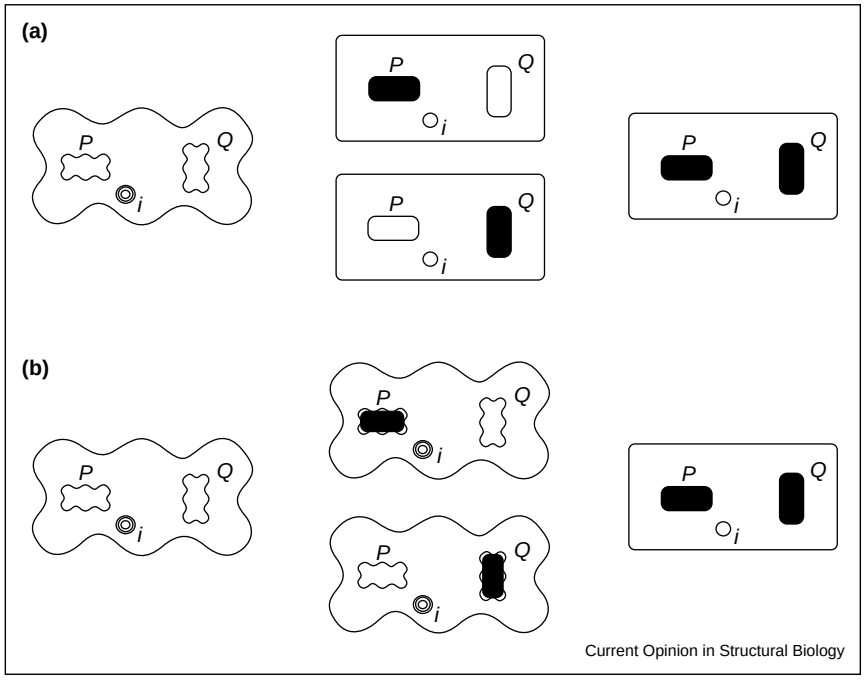
In contrast to the described examples of existing positive or negative allostery in nature, efforts to design and synthesize artificial molecular and ion recognition systems with allosteric guest responses have been of great interest [22•]. The authors of this paper describe how intrinsic flexibility is needed in the design to allow for the four different kinds of allosteric effects: positive and negative, homotropic and heterotropic.

Summarizing this section, there is now a preponderance of evidence that dynamic transitions observed in allosteric processes can be described by an equilibrium shift of pre-existing populations, which dynamically interchange also in the absence of the allosteric ligand. Clearly, there are large differences in the extent of the areas involved in these processes; sometimes entire proteins are involved in the equilibrium, but it is more common that smaller areas have multiple conformations. As pointed out by Freire and co-workers [33•], it is more rule than exception that proteins have areas of different dynamic stability, making them naturally primed for the kinds of allosteric transitions described.

Dynamics as an intrinsic carrier of allosteric free energy

Proteins are generally tightly packed, except around ligand-binding sites, which may be viewed as packing deficiencies. These packing deficiencies allow mobility of their perimeters, which propagates through other parts of the protein because they are so tightly packed. Ligand binding 'repairs' the packing defect, leading to global protein rigidification with concomitant entropy loss. In many of the above-described studies, such rigidification upon ligand binding was experimentally detected. When an allosteric macromolecule contains two (or more) binding sites, each would cause partial rigidification upon individual ligation. Consequently, much of the entropy loss associated with global rigidification would already be partially accomplished by either of the ligand-binding steps, automatically resulting in positive cooperativity for the binding of the next ligand as less entropy cost is incurred. This situation is sketched in Figure 3a. The presence of an entropic component to positive cooperativity should be the rule, rather than the exception. This is formally expressed as an entropic component to the allosteric interaction free energy. Here, motion not only serves to move the macromolecule from 'T' to 'R', but also is an integral part of the allosteric energetics.

Figure 3



Illustrations of cooperativity as caused by changes in dynamical processes, first described by Cooper and Dryden [4]. **(a)** Positive cooperativity. The unliganded state exists as a dynamic ensemble, as indicated by a wavy outline of the protein at the left. Ligand binding to either site *P* or site *Q* (mostly) immobilizes the protein with concomitant loss of motion and thus entropy at atom *i*, symbolized by the changing circles (middle). Binding of the second ligand does not incur (or incurs less) further immobilization, with no (less) loss of entropy at atom *i* (right). A protein contains many different atoms *i*, which can all experience such effects, together contributing a significant entropic component to the free energy of cooperativity. See text for more details. This case has been observed experimentally and is described in [9••,40]. **(b)** Negative cooperativity. The unliganded state (left) also exists as a dynamic ensemble. However, binding of the first ligand does not quench (all) motions (middle). The major loss of configurational entropy occurs upon the second binding event (right). This case has been observed experimentally and is described in [47•].

The issue was recognized and formalized by Cooper and Dryden in 1984 [4] (c.f. Monod *et al.* [3]). Cooper and Dryden illustrate the extreme case of no conformational change (i.e. there would be no enthalpic term to the allosteric free energy). The authors separate the motions into a vibrational (normal mode) effect and a conformational effect. For the vibrational (nanosecond to picosecond) aspect, they show that free energies of a few kcal/mol can easily be derived from only a slight stiffening of a few of the many global dynamic modes of motion available to a protein. The (slower) averaging over conformational substates is described as uncorrelated Gaussian rms fluctuations of the atom coordinates. Ligand binding at site *P* of a heterotropic allosteric protein (see Figure 3a) causes a fractional decrease, δ_i^P, in the rms coordinate fluctuation of atom *i* compared to the fluctuations in the ligand-free state; ligand binding at site *Q* causes a fractional decrease δ_i^Q. When ligand binding at site *P* and site *Q* both affect the coordinate fluctuations of atom *i*, the contribution to the allosteric free energy per atom *i*, $\Delta\Delta G_i$, becomes (adapted from [4]):

$$\Delta\Delta G_i \approx -RT \cdot \delta_i^P \cdot \delta_i^Q$$

Accordingly, minute but global rigidification in rms conformational flexibility of only 2% upon binding of either ligand yields an allosteric free coupling energy of 1.5 kcal/mol at room temperature for a heterotropic monomeric protein of 400 residues (about 6400 atoms) or for a dimeric homotropic protein of 200 residues. We note that a decrease in atomic coordinate fluctuations of 2% will barely be detectable, even with the best experimental methods. Consequently, one may encounter cases for which current experimental methods may not be able to show any difference in either structure or dynamics between the 'T'-state and 'R'-state, even though a substantial allosteric interaction free energy may exist.

The existence of such an allosteric dynamic entropy effect, in combination with a more conventional change in conformation, was recently detected for the two-domain protein calbindin D9k, a member of the EF-hand family of Ca^{2+}-binding proteins [40]. The protein binds two Ca^{2+} ions with positive cooperativity. The structural and dynamic properties of the apo, half-ligated and fully liganded forms were studied by NMR methods (the half-ligated state was created by blocking one of the sites

through mutagenesis). Large differences exist for the structures of both domains between the apo and fully liganded forms. In the half-ligated form, the conformations of both the ligated and unligated domains resemble the fully ligated conformation. Hence, an induced fit, propagated from one site to the other, would account for a favorable enthalpic contribution to the allosteric free energy. Using NMR relaxation methods that probe the dynamics of the backbone N–H vectors on the picosecond to nanosecond timescale, Maler *et al.* [40] detected dynamic disorder in both domains of the apo form, whereas both domains are rigid in the fully liganded form. Significantly, both domains of the half-ligated form are rigidified. Thus, the entropic penalty of rigidification is already paid, to large extent, by the first ligation step and the free energy of the second binding step is less encumbered by entropy loss (see Figure 3a). Interpretation of these changes in the NMR order parameters in terms of entropy using empirical relations [41,42] yields values of up to 1.5 kcal/mol for the allosteric free energy at room temperature, indicating that the effects can be substantial. Just this entropic component would account for a 12-fold decrease in K_D for the second binding site. The authors correctly note that ''it must be stressed that these values are providing only limited insight into overall changes in conformational entropy, because the relaxation measurements characterize motions of backbone N–H vectors only and because the time scale of motion being probed (ps–ns) is narrow. Moreover, the values reported are estimates because this treatment ignores the effects of correlated motions and contributions from backbone motions not accurately characterized by the model-free treatment'' [40].

Similar measurements for the protein calmodulin reveal very dramatic changes in sidechain dynamics upon complexation with a myosin-derived peptide. This is interpreted as an overall change in conformational entropy of about –35 kcal/mol at 35°C [43•]. By contrast, the dynamic character of the mainchain, as measured by N–H NMR relaxation, was largely unaffected by peptide binding. This work indicates that very large dynamic/entropic effects can occur upon binding of larger ligands such as peptides. Moreover, it indicates that the routinely applied backbone amide relaxation methods often underestimate global dynamic events and that most dynamics may be hidden in the sidechains. In fact, the standard N–H dynamics even systematically underestimate the motion/entropy of the polypeptide backbone itself [44,45]. Most likely, the dynamic NMR community is just beginning to uncover the tip of the entropic iceberg.

Cooperativity between ligand binding and oligomerization, as observed in many biochemical systems, is generally attributed to changes in structure as well (e.g. [10,11]). In glycopeptide antibiotics such as vancomycin, however, positive cooperative processes are not accompanied by significant structural changes at the monomer level. Calorimetry shows that the enthalpy of allostery is likewise zero, suggesting that the free energy of positive cooperativity is dominated by entropy. Conformational entropy contributions were calculated based on fully solvated long-term MD simulations of vancomycin, in the absence and presence of ligand, and in the monomeric and dimeric state [46••]. Both dimerization and ligand binding were found to shift the internal vibrational modes of vancomycin to higher frequencies with less amplitude. Thus, both processes have a loss of entropy in common, leading to positive cooperativity. Quantitative analysis of the entropic content of the vibrational eigen modes in each state shows that this mechanism could be completely sufficient to explain the positive allostery, verifying the Cooper–Dryden model [4].

McElroy *et al.* [9••] used TROSY techniques to characterize the conformational exchange line broadening of the ^{15}N and ^{1}H NMR resonances of the 91 kDa 11-mer Trp- and RNA-binding attenuation protein in the absence and presence of tryptophan. In the absence of tryptophan, resonances mapping to the tryptophan-binding site and the remote RNA-binding site are severely broadened, indicating an averaging process over conformational substates on the millisecond to microsecond timescale. Upon tryptophan binding, the NMR line broadening is quenched at both sites, indicating that both sites become more rigid. The NMR data are supported by the results of proteolytic sensitivity measurements. This clearly constitutes a case of a heterotropic allosteric process with a strong dynamic contribution (Figure 3a), partially explaining the experimental positive cooperativity between tryptophan and RNA binding to this protein. Regretfully, it is currently not possible to even estimate the entropy contained in the millisecond motions, as disclosed by NMR conformational exchange broadening.

The direct detection of a contribution to negative cooperativity by dynamics has recently been provided by backbone amide NMR relaxation experiments performed for the anti-cooperative binding of CTP to the dimeric enzyme CTP:glycerol-3-phosphate cytidylyltransferase [47•]. The protein is characterized by very extensive and global conformational dynamics on the millisecond timescale for both protein monomers in the apo and single-ligand bound states. The conformational dynamics is quenched, in both monomers, upon binding the second ligand (see Figure 3b). Hence, dynamic effects, as sensed by the backbone, are not affected by the binding of the first ligand and the entropy contained in those motions is lost only during the second step, which contributes to the experimental negative cooperativity. The effect is exactly opposite the situation encountered for calbindin, discussed above [40]. One may mechanically visualize dynamic negative cooperativity if the dynamics-permitting 'packing deficiencies' in the ligand-free state are so large

and close together that occupying one site may not be sufficient to rigidify the entire protein, especially if it is not very rigid to begin with. Only when the second ligand comes in is the packing deficit removed, with a concomitant loss of motion and entropy.

Although the changes in dynamics/rigidity are directly associated with changes in entropy, the reverse is not necessarily true. Entropy gain upon ligand binding can also be caused by solvent release from the protein. When different amounts of solvent are released during the different ligand-binding steps, an entropic component of the allosteric free energy exists. Hence, dynamic and solvent contributions to system entropy may be difficult to separate if only the net change in entropy is measured. In the following, we will review a few studies in which the entropy of allostery was directly measured by thermodynamic methods. We focus on those papers for which dynamic, rather than solvent, effects appear to dominate the entropy of allostery.

Measurements of the variation of the allosteric coupling free energy between ligand and substrate with temperature disclosed a key role of entropy in both positive and negative allosteric regulation [20]. For phosphofructokinase with the allosteric inhibitor MgATP and isocitrate dehydrogenase with the allosteric activator ADP, both coupling enthalpy and entropy are negative. However, for phosphofructokinase, entropy dominates the allosteric coupling free energy, resulting in inhibition by MgATP, whereas for isocitrate dehydrogenase, enthalpy dominates, leading to an overall positive allosteric effect by ADP. The authors hypothesize that ''the negative coupling entropy arises from the constraints placed upon the internal dynamic properties of the enzyme by the simultaneous binding of both allosteric and substrate ligands'' and ''the different protein species must themselves differ in entropy content, and hence, the ability to interconvert between various configurational states or 'substates' implying that the dynamic properties of these protein species are germane to the establishment of the allosteric effect''.

Using similar techniques, enthalpic and entropic contributions to the coupling energy for the activation (by ornithine) or inhibition (by UMP) of MgATP binding to carbamoyl phosphate synthetase were measured [48]. MgADP binds with a large negative change in heat capacity, whereas no detectable change in heat capacity is associated with the thermodynamic coupling between binding of the substrate (MgADP) and binding of either the allosteric activator (ornithine) or inhibitor (UMP). This indicates that the observed allosteric effect arises from a change in entropy of the system. The coupling enthalpy even opposes the allosteric phenomenology, supporting the suggestion that a positive or negative change in coupling entropy is solely responsible for the positive and negative cooperativity of ornithine and UMP, respectively. Current experimental data for this system are, however, not sufficient to explain these interesting observations in molecular detail.

Another potential mechanism of entropy contribution to allostery is remote entropy compensation. Ligand binding typically accomplishes a rigidification (enthalpy/entropy compensation). However, if a concomitant conformational change allows another area in the protein to become less densely packed, that area may gain flexibility and thus compensate for the entropy loss upon binding. Some data are indicating the existence of such compensatory processes in allostery. For instance, X-ray data indicate that the allosteric induction of the Tet repressor should carry a high entropic penalty due to a decrease in mobility of a helix adjacent to the inducer-binding site [49]. However, fluorescence studies showed that disorder increases in another loop that is adjacent to the binding site, by measuring the fluorescence depolarization lifetimes of single tryptophan mutants at nine sites in that loop [12]. The fluorescence data show that, on the timescale <30 ns, only limited motion exists in the absence of inducer (order parameters >0.8), whereas in the presence of inducer, increased motion occurs, leading to an order parameter of 0.5. The increased mobility of the loop is possibly an entropy-compensating mechanism essential to the function of the allosteric protein.

Description and modeling of the dynamics of allosteric conformational change

It appears to be generally assumed that the timescale of global allosteric conformational change is relatively slow. Hammes [50], reviewing the role of conformational change in enzyme catalysis, mentions that the rate of allosteric conformational change in aspartate transcarbamylase is a millisecond process. NMR studies indicate that the interconversion of allosteric subconformations takes place on the millisecond to microsecond timescale [8••,9••,47•]. Allosteric transitions in hemoglobin triggered by flash photolysis of CO-ligated hemoglobin were detected by the binding of a fluorescence-quenching DPG analogue. The allosteric rates obtained are $3.4 \times 10^3\ s^{-1}$ for the R to T transition and $2.1 \times 10^4\ s^{-1}$ for the T to R transition for the triply ligated species [51]. Hu *et al.* [52] used resonance Raman spectroscopy to detect vibrational modes in hemoglobin induced upon photolysis of CO. A sequence of three events associated with an $R \rightarrow T$ transition are resolved at 50, 500 and 20 000 ns. The 20 000 ns (20 μs) step is rate determining for the quaternary transition. At present, it is not known how fast the actual movements from one substate to the other are or how much of the measured timescale of conformational change is due to much faster low-probability fluctuations. The difference between the timescales reported in [51,52] may be indicative of the latter. Single-molecule studies should allow this key question to be addressed in more detail.

None of the studies discussed so far have addressed what actually happens during the dynamics of allosteric transition. Many attempts have made at hypothesizing what gears and levers are pulled, and in what order, to traverse from one to the other static state; such descriptions are plentiful, some even famous [25] and many were reviewed in 1996 [24]. The identity of the intermediate structures in such descriptions is hypothetical and it is assumed that the path of conformational change is the shortest one; an allosteric conformational change could theoretically proceed through a completely unfolded protein as an intermediate. Regretfully, experimental evidence showing the intermediates through which an allosteric protein travels has not been forthcoming to our knowledge. Currently, one is still confined to mapping the dynamic pathway by studying structurally artificial intermediates [40,53].

Fully unrestrained computational MD simulations of allosteric processes are not yet entirely feasible because the simulation times are still too short (approaching microseconds — tens to hundreds of milliseconds are probably needed). Below, we review a few computational approaches to describing the allosteric dynamic pathway that artificially bypass these time restrictions.

The GroEL–GroES chaperone system (840 kDa) is a fascinating allosteric molecular machine consisting of two seven-membered rings of identical monomers [54]. The chaperone sequesters misfolded protein substrates in a hydrophobic cavity formed by one of the rings and further unfolds them. ATP binding to one ring causes very large conformational changes allowing the substrate protein to refold. This step constitutes positive ATP-binding homotropic cooperativity between the seven ATP-binding sites, combined with heterotropic negative cooperativity between ATP and substrate binding. Subsequent ATP binding to the opposite ring causes opening of the cavity to release the refolded protein; this is a negative heterotropic allosteric effect. Because the system is too large for unrestrained MD simulations, the following different computational approaches were used to obtain high-resolution dynamic models. In 1998, Ma and Karplus [55] carried out a normal mode analysis of the individual subunits. The predicted lowest-frequency eigen mode (~30 ps) contains motions similar to the global conformational changes described above and predicts hinge motions similar to those deduced from X-ray data comparing the limiting states. This is again an indication that a dynamic equilibrium encompassing (part of) the allosteric conformational change already exists in the absence of ligands. De Groot *et al.* [56] used a method to generate different protein conformations based on distance restrictions from the X-ray coordinates of complete GroEL and GroEL–GroES assemblies. Covariance analysis of the artificial conformational scatter data gives insight into the possible low-frequency collective fluctuations. For single rings, ATP-induced structural changes were found to be uncoupled from changes occurring upon GroES binding. By contrast, for double rings, coupling was found between ATP-induced conformational changes and GroES-induced transitions, rationalizing one of the reasons for the existence of the double-ring architecture. Ma *et al.* [57] carried out a targeted/steered MD simulation of the double ring, in which the coordinates of the closed form are pulled towards the GroES-bound open conformation. The MD simulation during this forced transition ensures that the system remains as close to equilibrium as possible and follows a low-energy transition path. This method forces the structures of intermediates to lie between the coordinates of the beginning and end states. It is observed that the intermediate domain moves before the apical domain (indicating the presence of a floppy hinge), that all seven subunits need to make the transitions in a concerted fashion as clashes would otherwise occur and that the simultaneous presence of nucleotide in both rings would cause steric clash, thus accounting for the *trans* anti-cooperativity.

Hemoglobin is also still too large (64 kDa) for an unrestrained MD simulation that would help elucidate the quaternary structural change dynamics (which occur on the microsecond to millisecond timescale [51]). Therefore, a targeted MD method was used that forces an artificially rapid T to R transition [58•]. Interestingly, these authors compute a ''natural propensity for dimer rotation'' in the T-state, which is hampered by steric hindrance. Binding of ligands to the α-units removes this hindrance, allowing completion of the dimer rotation towards the R-state and increasing the accessibility of the β-hemes.

Normal mode calculations on the T-state and R-state of aspartate transcarbamylase identified large-amplitude motions that may be responsible for the inter-trimer elongation and rotation that occur during the cooperative transition [59]. This is an indication that a dynamic equilibrium encompassing the allosteric conformational change already exists in both of the limiting states and drives them towards each other.

Conclusions

Many outstanding publications now demonstrate the central role of a dynamic equilibrium between conformational substates in the allosteric process. Moreover, it has become clear that changes in entropy upon ligand binding are a common, not often recognized, contribution to allosteric free energy. Consequently, dynamics constitutes not only the move from 'T' to 'R', but also an integral part of the allosteric system in all of its states. We anticipate that this awareness will inspire new studies to quantitate and model the relevant motions. Progress towards the description of the actual pathway of allosteric change has been limited. We expect that single-molecule studies will shed light onto the question of the timescales

of the actual process of allosteric change and that longer-term unrestrained MD simulations will, in the near future, become feasible for the modeling of the pathways.

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