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The changing landscape of protein allostery

Joanna F Swain and Lila M Gierasch

It is becoming increasingly clear that the fundamental capacity to undergo conformational change in response to ligand binding is intrinsic to proteins. This property confers on proteins the ability to be allosterically modulated in order to shift substrate binding affinities, alter enzymatic activity or regulate protein–protein interaction. How this allosteric modulation occurs — the pathways of communication, the shifting of conformational ensembles and the altered molecular dynamics — has received considerable attention during the past two years. Recent progress has helped outline the molecular origins of allostery in proteins as diverse as Hsp70 molecular chaperones and signal integrating proteins, such as WASP. In addition, allosteric properties have been successfully engineered into proteins for drug design or the development of novel biosensors. Methodological advances have provided exciting prospects for new insights and new biological roles of allosteric systems have been uncovered.

Addresses
Department of Biochemistry & Molecular Biology, Department of Chemistry, 710 North Pleasant Street, University of Massachusetts, Amherst, MA 01003-9305, USA

Corresponding author: Gierasch, Lila M (gierasch@biochem.umass.edu)

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Introduction

For more than four decades, scientists have been engaged in efforts to understand the mechanisms by which information is transmitted across long distances in proteins. Such signal transduction through protein structure is the basis of protein allostery, which can be broadly defined as the binding of a ligand at one site causing a change in affinity or catalytic efficiency at a distant site. Historically, allostery has been functionally attributed to homo-oligomeric proteins. It is now widely recognized that the modulation of protein–substrate, protein–ligand and protein–protein interactions by ligand binding distant from the interaction site is a property of many monomeric proteins. Indeed, it is a property that may be engineered into many proteins and exploited for a variety of purposes [1–3].

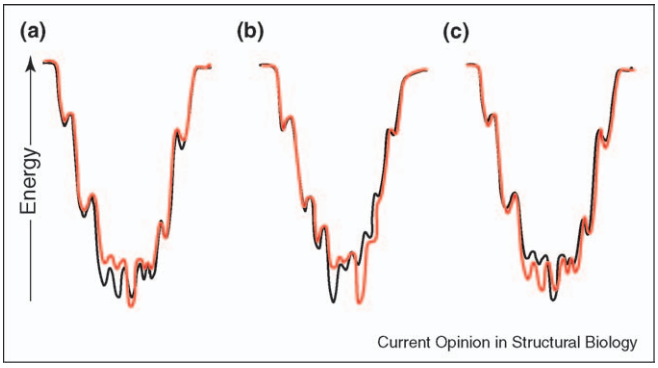
The current view of the molecular origins of allostery acknowledges the ensemble nature of protein native states and explains allostery as a ligand-induced shift in the population of states (Figure 1). This view must be contrasted with the notion that allosteric proteins have two alternate conformations in equilibrium and that allosteric effectors modulate this equilibrium. A deeper understanding of the molecular underpinnings of allostery requires methods that can investigate higher energy states of proteins that are only transiently visited and dynamic aspects of protein structure that may be influenced by the binding of allosteric ligands. Recently, several methodological advances have provided glimpses into the underlying molecular mechanisms of allostery. In addition, new biological examples of the role of allostery have been described, and engineered allostery has been applied to the development of biosensors and in drug design. In this review, we discuss work that has been reported during the past two years on these topics, and consider the implications of recent progress for future insights into protein allosteric properties and their manipulation.

New methods to explore allosteric networks and conformational ensembles

Understanding allosterism requires methodological innovation. Although X-ray crystallographic and NMR-based structure elucidation has provided us with exquisite atomic detail on the shapes and sizes of ligand-binding sites, the nature of protein interfaces and the architectural diversity of protein families, the nature of allostery is that it involves conformational changes throughout a protein's structure. No single structural view will reveal an allosteric mechanism. In fact, understanding allostery requires information on the linkages of substructures, exploration of ensembles of conformations and determination of detailed structures for rarely visited high-energy states.

The sequences of large families of allosteric proteins contain a record of conservation patterns that preserve allosteric functions. The statistical coupling analysis developed by Ranganathan and co-workers has enabled the mining of the evolutionary record of families of homologous proteins in order to discover networks of co-varying residues that have been maintained over many generations [4]. These methods have been applied to several allosteric proteins [5•,6,7]. Molecular dynamics simulations of protein dynamics and allostery, recently reviewed by Rousseau and Schymkowitz [8], offer the opportunity to explore mechanistic details that are difficult to observe experimentally. A novel non-equilibrium

Figure 1



Allosterically reshaping the energy landscape of a protein. In all panels, black represents the landscape in the absence of ligand and red represents the ligand-bound state. **(a)** Ligand binding to a native state with several energetically comparable states may stabilize one of those states at the expense of others, reducing conformational heterogeneity. **(b)** A native protein may have two major conformational states and ligand binding may perturb their relative energies, leading to a discrete conformational change. This landscape depicts the paradigmatic T↔R equilibrium of Monod-Wyman-Changeux allosteric theory. **(c)** A stable native state with a narrow conformational distribution may be perturbed by ligand binding in such a way that a more heterogeneous set of possible conformations becomes accessible. This occurs in proteins that are autoinhibited, such as many kinases and WASP (see text); allosteric ligand binding relieves the inhibiting interaction. These three scenarios are of course only a sampling of the growing array of possible allosteric mechanisms.

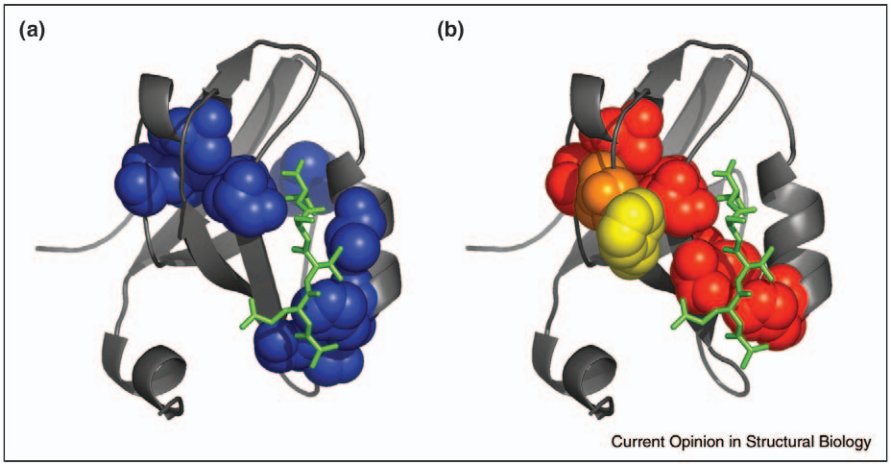
technique called anisotropic thermal diffusion has recently been reported that monitors the effect of an artificially large but local temperature fluctuation as it diffuses through a protein structure [9•]. Coarse-grained methods and normal mode analysis may be used to delineate the correlated conformational fluctuations of a protein structure in a model-independent manner [10,11]. To date, some of these methods have only been applied to model systems, but the promise is there. For example, Ming and Wall [12] conducted normal mode analysis before and after ligand binding to lysozyme to quantify changes in the distribution of conformations. The intriguing result that the largest changes in conformational distribution occurred when the ligand interacted with the active site relative to other randomly selected sites suggested a new computational means of searching for functional binding sites, allosteric or otherwise. An ensemble-based approach developed by Hilser and co-workers [13] has been applied to staphylococcal nuclease, and revealed a high degree of coupling between local structural fluctuations, ligand binding and global conformational changes.

New experimental methods also hold great promise for exploring allosteric systems, although they have not all been applied to such problems directly. Novel relaxation dispersion NMR methods developed by Kay and co-workers [14,15,16••,17] enable residue-by-residue mapping of conformational fluctuations, and report the fractional population of each state and the rates of interconversion between them, even when as few as 1% of protein molecules sample these conformations at any given time. NMR relaxation methods that report sidechain and backbone dynamics have been developed over many years [18], but have recently been applied to study the allosteric effects of ADP binding to an ABC transporter [19•]. X-ray crystallography with nanosecond to picosecond resolution has been applied to follow the conformational changes of photoactive yellow protein and myoglobin with exquisite structural detail, mapping the amplitudes, directions and timescales of conformational fluctuations [20,21]. These data are particularly powerful in combination with molecular dynamics simulations sampled on the same timescale [22•].

A case study: dissecting allosteric networks in a signal transduction module

PDZ domains are small protein interaction modules that bind short C-terminal or sometimes internal sequences of target proteins to mediate the assembly of large signaling complexes [23]. Several years ago, Lockless and Ranganathan [4] used statistical coupling analysis of the PDZ family to demonstrate that residues important for peptide recognition co-varied with residues at a site distal to the binding pocket, suggesting a pathway for allosteric communication, although the physiological relevance was unclear at the time. Several recent computational and experimental methods have now identified similar pathways (Figure 2). An NMR comparison of the protein dynamics of the human tyrosine phosphatase 1E PDZ domain with and without bound peptide revealed two distal sites with ligand-induced changes in sidechain dynamics; one of these sites coincides with the site identified by Ranganathan and co-workers [24]. The fact that the change in dynamics occurs in the absence of significant structural change argues that, in some cases, dynamics alone may convey allosteric information. Ota

Figure 2



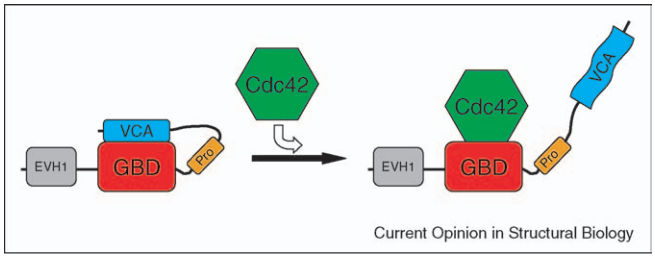
Multiple methods identify a similar pathway of allostery in PDZ domains. **(a)** A pathway of evolutionary coupling (represented by blue spheres) between a binding site residue and a distal patch, as identified by the statistical coupling analysis of Lockless *et al*. [4]. **(b)** A similar but more direct pathway (red and orange spheres) identified using anisotropic thermal diffusion leads to the same distal site [9•]. Residues with significant changes in sidechain dynamics upon ligand binding, based on NMR data [24], are shown as yellow and orange spheres. Residues are represented on the structure of the third PDZ domain of PSD-95 (PDB code 1BE9), with the bound C-terminal peptide from the CRIPT protein indicated in green stick representation. Figure prepared using PyMOL (http://www.pymol.org).

and Agard [9•] applied anisotropic thermal diffusion to follow the spread of kinetic energy away from a ligand-binding site residue in the third PDZ domain of PSD-95. Interestingly, nearly the same pathway was delineated as in the statistical coupling analysis. Peterson *et al*. [25] explored the effects of the binding of the Rho family GTPase Cdc42 to the PDZ domain of the cell polarity protein Par-6 at a site near the distal site identified by Lockless *et al*. [4]. They found that Cdc42 enhances the binding of Par-6 to archetypal PDZ-domain-binding ligands by changing the orientation of a helix via a long-range allosteric mechanism. Thus, both experimental and computational studies point to a similar coupling between distant locations in the PDZ fold. Future work should focus on how these conformational and dynamic linkages specify the timing with which PDZ-containing proteins participate in intracellular signaling networks [26].

Allosteric signaling via modulation of a folding-unfolding equilibrium

Wiskott–Aldrich syndrome protein, or WASP, integrates multiple cellular inputs to regulate actin polymerization [27]. Among these inputs are Cdc42•GTP, phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] and phosphorylation by Src kinases, all of which act synergistically to relieve autoinhibition of WASP. This allows productive interaction with the Arp2/3 complex, which nucleates actin polymerization. Cdc42 has been modeled as altering the equilibrium between a well-folded autoinhibited conformation of WASP (the T state) and a largely unfolded R state that can bind Arp2/3 (Figure 3) [28••]. Cdc42•GTP has a stronger preference for binding the R state compared to Cdc42•GDP and thus can cause a greater shift to the active conformation of WASP [29]. Cdc42•GTP binding is required for tyrosine phosphorylation, which activates WASP and provides a form of molecular memory [30]. Although the exact mechanism of allosteric stimulation by PtdIns(4,5)P_2 is as yet unclear, it was recently shown that multivalent binding of PtdIns(4,5)P_2 to the polybasic region of the neuronal homolog N-WASP underlies highly cooperative activation by PtdIns(4,5)P_2 above a certain threshold and that even small amounts of Cdc42•GTP significantly decrease the threshold for activation of N-WASP by PtdIns(4,5)P_2 [31]. Intriguingly, Peterson *et al*. [32] screened for small molecules that inhibited actin polymerization in *Xenopus* oocyte extracts and found that one of the most potent inhibitors, wiskostatin, acts by binding and stabilizing the

Figure 3



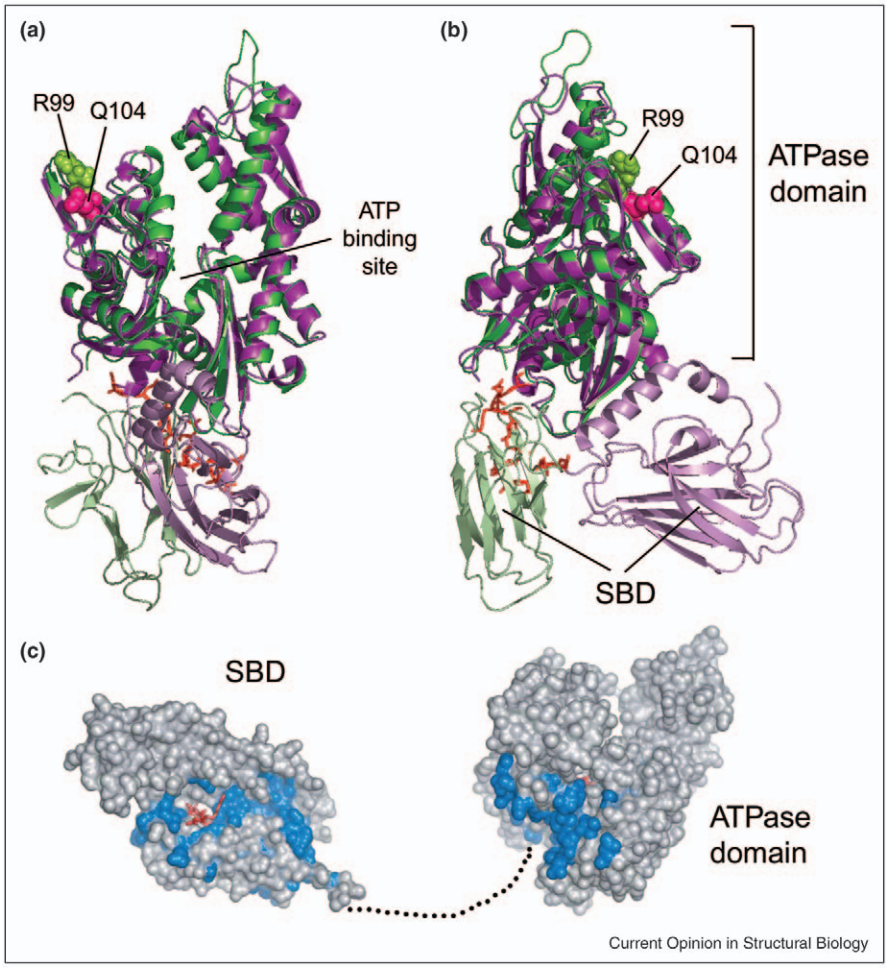
Cdc42 activates WASP by destabilizing the inactive autoinhibited conformation. In the resting state, the VCA region of WASP is repressed by binding to the GBD. Cdc42•GTP displaces VCA from the GBD, allowing VCA to productively interact with Arp2/3.

autoinhibited conformation of WASP. Thus, the pre-existing allosteric equilibrium of WASP corresponds to a folding-unfolding equilibrium. One might speculate that different inputs act by either stabilizing the unfolded state or destabilizing the folded inactive (autoinhibited) one. WASP activity inside the cell would then result from the integration of all possible influences on this equilibrium.

Interdomain allostery in Hsp70 molecular chaperones

Hsp70 molecular chaperones harness ATP binding and hydrolysis by an N-terminal ATPase domain to control the interaction of a C-terminal substrate-binding domain (SBD) with unfolded or misfolded polypeptide substrates [33]. This interdomain allostery is bi-directional; ATP binding reduces affinity for substrates and, conversely, substrate binding increases the ATPase rate. Until very recently, structures were only available of the isolated domains. The ATPase domain has structural homology to actin and sugar kinases, with a bi-lobed structure enclosing the active site. The SBD comprises a β subdomain, which binds substrates in an extended conformation using the loops between strands, and a C-terminal helical lid subdomain, which covers the binding pocket. The pathway of allosteric communication is unknown, although several

Figure 4



New models of interdomain interaction within Hsp70 proteins indicate potential allosteric docking sites. **(a,b)** A truncated *T. thermophilus* DnaK (corresponding to residues 1–501 with A423E, ΔT422, L501G mutations and a C-terminal His tag) derived from NMR data [37•] is shown in green and a truncated bovine Hsc70 (corresponding to residues 1–554 with E213A and D214A mutations, PDB code 1YUW) derived from X-ray diffraction data [36•] is shown in purple. The ATPase domains are indicated by dark colors and the SBDs as light colors. The solvent-exposed interdomain linker in the Hsc70 structure is shown in red stick representation and the residues corresponding to *E. coli* W102 (R99 and Q104 in *T. thermophilus* DnaK and bovine Hsc70, respectively) are indicated in space fill. The view in (b) is rotated 90° about the vertical axis relative to (a). **(c)** Surface representation of the evolutionarily coupled residues (blue) identified in the Hsp70 family by statistical coupling analysis [7]. This reveals a surface patch on each domain of *E. coli* DnaK (PDB codes 1DKX and 1DKG) that is proposed to reflect the site of interdomain docking (RG Smock, JF Swain, WP Russ, R Ranganathan, LM Gierasch, unpublished). The peptide ligand in the SBD is in red stick representation and the interdomain linker is shown by a dashed line. The orientation of the ATPase domain is similar to that in (a). Figure prepared using PyMOL (http://www.pymol.org).

allosterically defective mutants have been identified. It is clear that ATP binding causes a more intimate arrangement of the two domains, with substantial destabilization of the SBD [34,35]. Two recent papers have described a docked conformation of the two domains [36•,37•], but surprisingly the docking site and the relative orientation of the domains differ between the two. In fact, it is possible that neither may correspond to an allosterically functional arrangement.

Zuiderweg and co-workers [37•] used NMR chemical shift mapping and residual dipolar couplings to orient the two domains of a truncated version of a bacterial Hsp70, DnaK from *Thermus thermophilus*, in the ADP-bound state (Figure 4). In this model, the region of the β subdomain near the entry point of the interdomain linker interacts with a cleft in the ATPase domain on the outside of the hinge between the two lobes. A new crystal structure of a truncated version of the bovine Hsp70 family member, Hsc70, in the absence of nucleotide shows a different packing arrangement, in which a portion of the helical lid of the SBD (which is missing in the *T. thermophilus* DnaK construct) interacts with the same ATPase domain cleft, but at an adjacent site (Figure 4) [36•]. These structures illustrate the difficulty of determining a mechanism of allostery based on structural information alone. Furthermore, neither structure is fully consistent with previous data. In the case of the *T. thermophilus* NMR model, several mutations, one deletion and a substantial truncation are present in the construct, which could all affect function. Indeed, biochemical data suggest that its functionality is impaired; ATP binding causes a fivefold drop in substrate affinity (a 100-fold drop is typically measured for wild-type Hsp70) and shifts just five NMR resonances in the SBD, and no data are presented to show that peptide binding stimulates the ATPase rate. In the Hsc70 crystal structure, the central involvement of a portion of the SBD helical lid at the interdomain interface is inconsistent with the fact that a truncated *E. coli* DnaK lacking the entire helical lid is allosterically functional [38]. In addition, highly conserved hydrophobic residues of the interdomain linker (^{391}LLLL^{394}) are fully solvated and do not interact with either domain, which seems to conflict with the fact that mutations of these residues cause a complete allosteric defect in *E. coli* DnaK [39]. Finally, neither of these structures accounts for the reported ATP-dependent burial of W102 from the ATPase domain of *E. coli* DnaK at the interdomain interface ([40]; R Sivendran, PhD thesis, University of Massachusetts, 2004) (Figure 4). Additionally, they are inconsistent with our own NMR results on a fully functional truncated *E. coli* DnaK, which demonstrate that docking of the two domains occurs only when ATP is bound (JF Swain and LM Gierasch, unpublished).

Interestingly, although the details of the interactions differ between these structures, some general aspects of the interfaces are consistent with the results of statistical coupling analysis of the Hsp70 superfamily, which has identified surface patches on each domain containing residues that are proposed to participate in interdomain coupling (Figure 4) (RG Smock, JF Swain, WP Russ, R Ranganathan, LM Gierasch, unpublished). The surface patches identified in this study overlap to some degree with each of the interaction sites identified in the experimental structures. In particular, the cleft in the ATPase domain has been implicated in protein–protein interactions in the structurally homologous actin and glucokinase families [41]. Mutation of the same cleft in human glucokinase or binding of an allosteric inhibitor affect the rate of glucose phosphorylation [42,43], suggesting that a general mechanism may exist for this family, whereby structural alterations of the cleft enhance the rate of catalysis at the active site. Clearly, delineating the allosteric mechanism of Hsp70 family proteins will require more than simple static structures.

Conclusions and future prospects

The methodological advances of the past few years have enabled substantial progress in teasing out the molecular details of allostery for many systems. It is clear that one must consider the full range of accessible conformations of a protein fold and how the relative energies of each might be influenced by ligand binding. What are the next trends? As we begin to understand how allostery is encoded in a protein, we gain the ability to predict allosteric sites and the influence of allosteric modulators on the conformational ensemble, opening new doors to engineering allostery. We are now faced with the exciting possibility of taking what has been learned and applying it to the rational design of new allosteric proteins for use as biosensor tools, or taking advantage of previously unknown allosteric sites for the development of new therapeutics.

The benefit of utilizing allosteric sites in drug design is primarily one of specificity. Active sites are under enormous evolutionary pressure to maintain function and, for some enzymes (e.g. kinases), it is very difficult to develop inhibitors that bind to one family member but not others. Allosteric sites are typically not as highly conserved and thus present an opportunity for drug discovery. Methods of finding allosteric sites have just been reviewed [3,44••]. Amstutz *et al.* [45••] recently used rational protein design coupled with ribosome display to select for an ankyrin repeat protein that inhibits a bacterial aminoglycoside phosphotransferase with high affinity. Further structural analysis has shown that the ankyrin repeat stabilizes an inactive conformation [46]. Once such allosteric sites are identified, the standard methods of drug design can be applied to find small molecules that stabilize the same conformation.

Similarly, rational design has led to the development of new allosteric switches, such as protein-based sensors that

can detect low amounts of small molecules, such as sugars, amino acids or even other proteins [47]. For instance, Dattelbaum *et al.* [48] engineered a maltose sensor by labeling maltose-binding protein with a fluorophore; mutational analysis suggests that the alteration in fluorescence upon maltose binding results from the loss of hydrogen bonding to the fluorophore in the closed state. Deuschle *et al.* [49] have designed glucose and glutamate sensors that rely on the efficiency of fluorescence energy transfer between cyan and yellow fluorescent proteins inserted internally or at the termini of the glucose/galactose- and glutamate/aspartate-binding proteins MglB and YbeJ. These novel approaches to finding new small-molecule inhibitors and creating new allosteric switches will then be useful in interrogating cellular signal transduction networks.

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