nature chemical biology

© 2008 Nature Publishing Group http://www.nature.com/naturechemicalbiology

Allosteric regulation and catalysis emerge via a common route

Nina M Goodey[1] & Stephen J Benkovic[2]

Allosteric regulation of protein function is a mechanism by which an event in one place of a protein structure causes an effect at another site, much like the behavior of a telecommunications network in which a collection of transmitters, receivers and transceivers communicate with each other across long distances. For example, ligand binding or an amino acid mutation at an allosteric site can alter enzymatic activity or binding affinity in a distal region such as the active site or a second binding site. The mechanism of this site-to-site communication is of great interest, especially since allosteric effects must be considered in drug design and protein engineering. In this review, conformational mobility as the common route between allosteric regulation and catalysis is discussed. We summarize recent experimental data and the resulting insights into allostery within proteins, and we discuss the nature of future studies and the new applications that may result from increased understanding of this regulatory mechanism.

Allostery can be defined as the regulation of protein function, structure and/or flexibility induced by the binding of a ligand or another protein, termed an effector, at a site away from the active site, also referred to as the allosteric site. Those effectors that increase a particular protein function, for example, ligand affinity or catalytic rate, are named allosteric activators; those that decrease the function are known as allosteric inhibitors. Nonligand sources of allosteric perturbations include mutations and covalent modifications at an allosteric site. Allostery, by this definition, is an intrinsic property of all proteins. All protein surfaces are then potential allosteric sites subject to ligand binding or to single or multiple mutations that might introduce the property of allostery. The exceptions are fibrous, structural proteins with uniform and stable conformations; these proteins are not likely to show allosteric behavior due to their lack of flexibility.

Historically, allostery was defined as the regulation of a protein through a change in its quaternary structure. Allostery was typically referred to as "cooperativity," a term associated with allostery in quaternary proteins, in which there are four binding sites and the binding of a ligand at site A impacts the affinity for the same ligand at the other sites (homotropic allostery). Classical examples of cooperative proteins include aspartate transcarbamoylase and hemoglobin (**Box 1** and **Fig. 1**)[1]. Both of these proteins consist of four subunits and exhibit cooperative effects upon binding of a ligand, caused by subtle changes in their quaternary structures. In the past, examples of cooperativity and allostery were limited to such multimeric proteins, whereas today allostery is recognized as a property in monomeric proteins as well. The principles that define allosteric behavior in proteins are similar for monomeric and quaternary proteins, although it will be interesting to learn whether the timescales of conformational changes or changes in flexibility are different depending on whether a signal is being transmitted within a monomer or through subunit interfaces.

In this review, we focus on recent experimental data and interpretations that suggest that a common mechanism, that of conformational mobility, underlies allostery and catalysis. In principle, allostery and catalysis result from a common property of proteins: flexibility, which gives rise to populations of conformers that interconvert on various timescales. The binding of an allosteric effector can result in the redistribution of protein conformational ensembles and can alter the rates of their interconversion, thereby modulating active site or binding site geometries. Both enzymes and noncatalytic proteins can be regulated by allosteric means[2]. In this review we discuss examples of both and highlight the strong interdependence between views of enzymatic catalysis and allosteric regulation.

We discuss how protein conformational states, their rates of interconversion and the existence of amino acid networks that enable communication between distant sites are linked to catalysis, and how such conformational equilibria in a protein structure may be altered as a result of an allosteric event. Important issues include the mechanism by which an allosteric effect is transmitted via amino acid networks, how the distribution of protein conformations is altered, and the timescales at which these conformational redistributions take place. Generally, many well-designed experiments indicate that enzymatic catalysis is closely connected to protein motion and that the rate of conformational change on the millisecond timescale often presents a bottleneck to the conversion of substrate to product[3–6]. These dynamics can lead to changes in the relative populations of protein conformations, some of which have greater affinity for certain ligands or are more catalytically competent than others. The timescales of these redistributions of protein conformations vary. Though the time regimes cannot be firmly distinguished, they range from the approximate timescales of linked networks of amino acids within the protein (millisecond to microsecond) to

[1]Montclair State University, Department of Chemistry and Biochemistry, 1 Normal Avenue, Montclair, New Jersey 07043, USA. [2]The Pennsylvania State University, Department of Chemistry, 414 Wartik Laboratory, College Park, Pennsylvania 16802, USA. Correspondence should be addressed to S.J.B. (sjb1@psu.edu).

Published online 18 July 2008; doi:10.1038/nchembio.98

Petitioner Merck, Ex. 1205, p. 474
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00004

those of domain motion (microsecond to nanosecond) to the very fast timescales of atom fluctuations (nanosecond to picosecond)[7].

It follows that altering the rates or extent of these conformational changes can have a profound impact on ligand binding and/or catalysis. The emerging view of allosteric regulation in proteins examines the ability of an effector to cause redistribution in the relative populations of protein conformations and the mechanism by which this shift in populations can cause changes in catalytic or ligand binding competence. An allosteric effect might be limited to the rates of interchange between different populations and may manifest as increased flexibility or rigidity in the global or local structure[8].

The difficulty of 'seeing' an allosteric signaling mechanism in a protein structure emphasizes the need to develop methods to answer such questions. Recent computational analyses have uncovered amino acid residue networks that are responsible for function and for communication within proteins[9,10]. Changes imposed on these residue networks by allosteric effects can alter important protein dynamics and conformational equilibria, and ultimately protein function[11]. Furthermore, it is increasingly clear that there are proteins in which allosteric effects on function are transmitted through the protein as a result of changes in dynamics in the absence of detectable structural changes, so that energetic coupling between sites must be considered (**Fig. 2**)[12–14].

This view of allostery elicits many questions. What are the structural and energetic bases that underlie allostery? How can the residues that participate in the critical residue networks be identified, and what percent of residues in a protein are involved in allosteric interactions? What are the physical mechanisms by which residues involved in allostery interact? On what timescales do dynamics that are altered by allosteric effects occur? We discuss these questions, methods for addressing them experimentally and computationally, and the importance of understanding the mechanism of allosteric interactions for protein engineering and drug design.

Protein motion and catalysis

The modern concept of catalysis is convincingly connected to protein motion[9,15–23]. The relationship between motion and catalysis has been demonstrated in several studies, and in many enzyme catalytic cycles conformational changes and not active site chemistry are rate limiting for the conversion of substrates to products[5,24]. Understanding that such changes sometimes limit the efficiency of catalysis is critical for the reengineering and design of new enzymes. Although establishing a definitive connection between protein motion and a step in the catalytic cycle is experimentally challenging, there are several informative examples in the literature where such a link has been forged[3].

For example, Cole *et al.* formed RNase A complexes with stable active site ligands that mimic the different complexes along the reaction coordinate and studied these complexes via NMR[17]. NMR relaxation dispersion experiments on the apo enzyme revealed a conformational exchange process at multiple unconnected sites throughout the enzyme, with a rate constant that coincides with k_{cat} and k_{off}, the rate of product release. The authors found that ligand binding had little influence on the kinetics of this motion. These results couple the conformational change

Box 1 A timeline showing evolution of allostery as a concept

The timeline (**Fig. 1**) includes some of the key experiments and realizations of the field and some insight into how the mentality of the field has shifted.

1903—The Bohr effect (sigmoidal binding curve of hemoglobin to O_2 was observed).

1910—A. Hill formulates the Hill equation to describe the sigmoidal binding of O_2 to hemoglobin.

1958—First X-ray structure (sperm whale myoglobin) solved by M. Perutz and Sir J. Cowdery Kendrew[78].

1950s—Repression of gene expression, covalent modification of enzyme activity, and feedback inhibition of enzymes are discovered[79].

1963—J. Monod renames regulatory sites 'allosteric sites.'

1965—J. Monod, J. Wyman and J.-P. Changeux propose a theoretical model of concerted allosteric transitions (MWC model)[1].

1966—D. Koshland, G. Nemethy and R. Filmer propose the sequential model for allosteric transitions (KNF model)[80].

1984—Allosteric regulation in the absence of conformational change is proposed[12].

1980s—Protein folding studies lead to the concept that proteins exist in different conformations in an "energy landscape"[81].

1990s—Mutations, covalent modifications and changes in conditions such as pH are included as allosteric effectors.

1999—Allosteric networks in the PDZ domain proposed by R. Ranganathan[50].

2006—Negative allostericity reported in the absence of conformational changes[54].

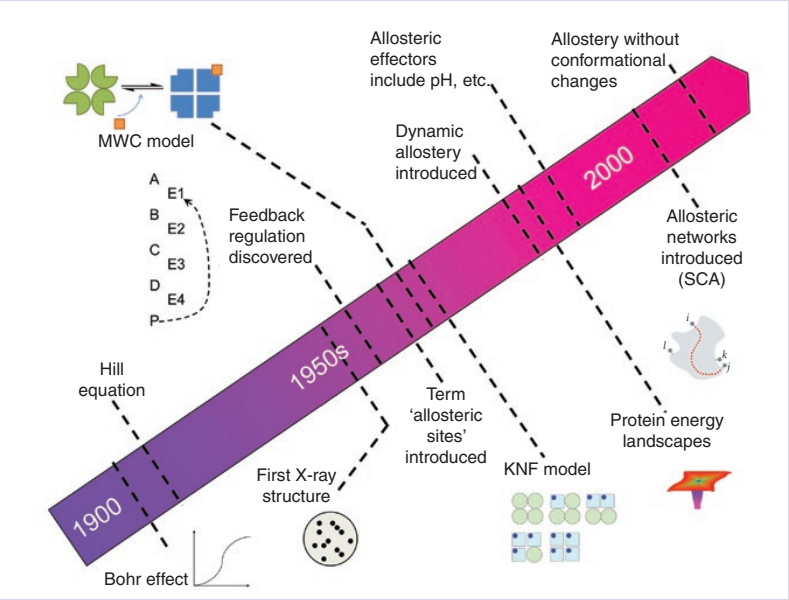

Figure 1 Timeline showing the discovery and progression of the concept of allosteric regulation in proteins.

© 2008 Nature Publishing Group http://www.nature.com/naturechemicalbiology

Petitioner Merck, Ex. 1205, p. 475
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00004

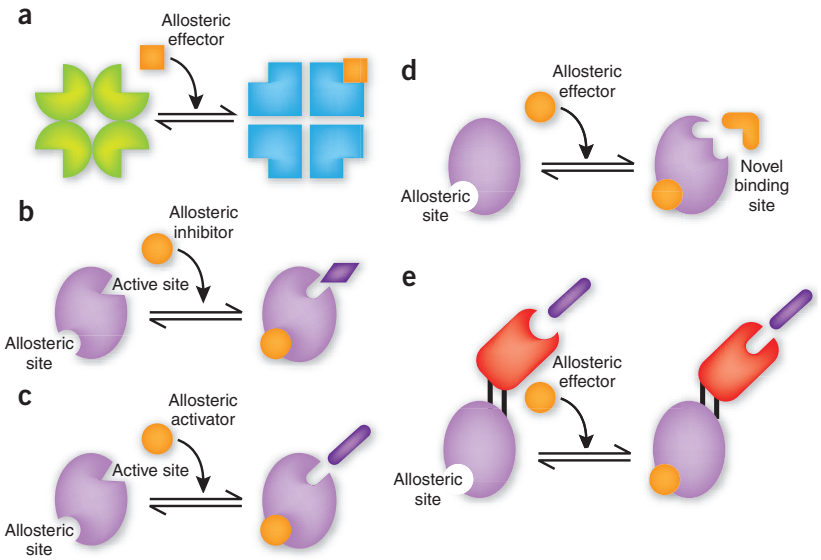

Figure 2 Different modes of allosteric behavior. (**a**) Cooperativity: a cartoon representation of the Monod-Wyman-Changeux (MWC) model of allosteric transitions. A symmetric, multimeric protein can exist in one of two different conformational states—the active and inactive conformations. Each subunit has a binding site for an allosteric effector as well as an active site or binding site. (**b**) A monomeric, allosterically inhibited protein. The binding of an allosteric inhibitor alters the active site or binding site geometry in an unfavorable way, thereby decreasing affinity or catalytic efficiency. (**c**) A monomeric, allosterically activated protein. The binding of an allosteric activator results in increased affinity or activity in the second site. (**d**) The binding of an allosteric effector might introduce a new binding site to a protein. Binding of a ligand to this new binding site could lead to changes in active site geometry, providing an indirect mechanism of allosteric control. This type of effect is of great interest in the design of allosteric drugs and can be considered a subset of the example shown in **c**. (**e**) The fusion of an enzyme (maroon) to a protein under allosteric control. This type of construct can act as an allosteric switch because the activity of the enzyme is indirectly under allosteric control via the bound protein with an allosteric site. Such constructs are both present in nature and the target of protein engineering studies.

© 2008 Nature Publishing Group http://www.nature.com/naturechemicalbiology

to the rate-limiting product release step in RNase A and show that the conformational change is not ligand induced. Rather, the role of ligand binding is to stabilize a pre-existing conformation, selecting this conformation in the population of protein conformers. This study highlights the unique suitability of NMR spectroscopy for studying protein dynamics: NMR relaxation dispersion experiments can both quantitate the rates of kinetic processes and estimate conformer populations. Moreover, the determination of the chemical shift difference ($\Delta\omega$) between exchanging conformations allows the study of sparsely populated species that may be functionally important[25–27].

In a parallel study, Boehr *et al.* found that the dynamics of transitions between different enzyme conformations limit the rate of catalysis by dihydrofolate reductase (DHFR) from *Escherichia coli*[3]. Similar NMR experiments revealed that five conformations of DHFR that constitute the key species in the enzyme's catalytic cycle interconvert at rates that are equal to those previously determined by kinetic experiments (**Fig. 3**). The NMR results revealed that each enzyme intermediate consists of an ensemble of conformations, including minor populations of conformations that resemble the predominant species featured in the preceding and following kinetic steps. This suggests that, as substrates bind, release and become chemically converted to other compounds, the populations of conformations in a protein ensemble adjust and act to drive catalysis along the preferred pathway. The correlation between rate-limiting steps of catalysis and rates of conformational change found in both studies highlights the role that such changes play in guiding catalysis. These phenomena fit our definition of the kinetic parameters of catalysis.

Changes in distributions of protein conformations

In the past, proteins in their native state were viewed as existing in equilibrium between stable discrete conformations. Moreover, conformational change events were often thought of as interconversions between two different protein conformations. This classical view is being replaced by a more comprehensive idea that proteins exist as complex statistical ensembles of conformers and that proteins unfold and fold continuously in localized regions[28,29]. These local unfolding events result in a large number of conformations that may differ from each other only slightly, and this collection of conformers is considered the native-state ensemble. This view relies on the idea that there are regions of varying stability in a given protein[30]. For a catalytic cycle, there are multiple sequential protein species that occur along the reaction coordinate, and each of these intermediates is associated with an ensemble of conformations, enabling parallel reaction paths (**Fig. 4a**).

The relaxed landscape paradigm for ligand binding incorporates the aforementioned concept. It features individual protein molecules in an ensemble that undergo small and large changes in conformation[6,13,31,32]. The interconverting conformations have similar energies, and the ensemble itself can be described as the 'native state.' The binding of an allosteric effector can lead to a change in the distribution of protein molecules that reside in the same or different conformational states (**Fig. 4b**)[33]. This change may or may not be observable by X-ray or NMR methods because it may be subtle in terms of impact on the average structure. However, in the case of an allosteric effect, the new distribution of protein conformational states is by definition functionally different. If such states are sparsely populated in the native state, even a small change in the distribution (a change that may not be visible via X-ray or NMR spectroscopy) can result in a large effect on function.

Allosteric networks in proteins

Allostery by definition involves the propagation of signals between sites in a protein structure. A change in function could be brought about by the binding of a ligand, covalent modification or mutation at a distant site. The idea that there is a network of physically interconnected and/or thermodynamically linked residues along which signals are communicated is intuitively very appealing, and there are many studies that provide evidence for this concept[34–37]. The mechanism of information transfer in such a network has been discussed in the literature[34]. An attractive concept is that the mechanisms can be divided into enthalpic and entropic factors[9,38,39]. The percentage of residues in a protein that

Petitioner Merck, Ex. 1205, p. 476
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00004

participate in these networks is of interest, as is the possible evolutionary conservation of these communication networks. The sensitivity of these networks to a single amino acid mutation further emphasizes the interdependence between the network residues[40].

Gaining insights into the functioning of allosteric networks

The changes in protein structure that result from binding of an allosteric effector are, by definition, intimately linked to protein motion. But how does the binding of an allosteric effector at one site influence the properties at another site? How do the two sites, often removed by a great distance (even 20–30 Å), communicate? An amino acid that is far removed from the active site with no obvious direct physical linkage can in some cases have a profound impact on catalysis or binding[41]. The long-range interactions between amino acids in different parts of a protein are remarkable, and the effects of binding, covalent modification or mutation can be felt through great distances. Does the effect transmit via subsequent interactions between residues, like dominos, following a channel in a protein? Answers to these questions can in some cases be illuminated by X-ray crystallography from comparison of static structures in their free and bound states[42,43]. Additional binding and thermodynamic studies via isothermal titration calorimetry (ITC), electron paramagnetic resonance and fluorescence quenching (steady state and time resolved) have been the mainstay of studies of allosteric proteins[44,45].

An experimental approach to implicate the interaction between two or three amino acid residues is through measurements of a double or triple mutant cycle. Such an approach provides an avenue for estimating the energetic interactions (independent or dependent) between residues and allows one to infer the degree to which residues in different sites of a protein are functionally coupled[46]. For example, Piper *et al.* performed a double mutant analysis on the voltage-sensing module of the hERG1 channel, which is a transmembrane channel specific for K^+ that senses voltage changes in the cell's membrane potential. They investigated the potential coupling between a key residue (Arg531) in the "S4 domain" of the hERG1 channel and negatively charged residues Asp411, Asp456, Asp460, Asp466 and Asp509 in domains S1–S3 to test the independence or dependence of two mutations[47]. Their hypothesis was that activation (opening) and inactivation (closing) of the channel are functionally coupled to movement of the α-helix S4, on which Arg531 resides, and that interactions between Arg531 and negatively charged residues result in the different voltage dependencies of activation and inactivation that have been observed. Arg531 was energetically coupled to aspartate residues 411, 456, 460, 466 and 509 during activation but coupled only to residues 456, 460 and 509 near the extracellular portion of S2 and S3 during inactivation. These findings suggest that hERG1 activation involves an allosteric conformational change involving residues in many parts of the voltage-sensing module.

A drawback of double or multiple mutant cycle analyses is that, due to their labor-intensive nature, they can be performed only on a limited number of residues. Also, though they yield interesting information, it is difficult to probe the existence of an entire 'channel' or network of amino acids through which allosteric signals are communicated. An alternative approach developed by Rama Ranganathan and colleagues uses a sequence-based statistical analysis (SCA) method for estimating the architecture of functional couplings in proteins[11,48–51]. The principle behind this approach is that if two sites (amino acids) in a protein are functionally coupled, they should have coevolved and this coevolution should be evident from a statistical comparison of homologous protein sequences. The coevolution could result from either structural or functional factors; in both cases mutation at one residue would statistically favor variants in which the coupled amino acid accompanies a complementary mutation. In other words, the mutual dependence of the amino acid identities at two sites reflects the functional coupling between these residues. This analysis requires the availability of many homologous sequences for the protein of interest. It does not reveal whether two sites are coupled for structural or functional reasons. As shown by Chi *et al.*, statistical coupling can be a result of either thermodynamic or functional coupling between residues; however, it can sometimes be explained by a distance rather than a sequence relationship[52]. Double mutant cycles using functional, structural and folding analyses can give insights into the reasons behind the coevolution of two residues. Such combined studies might parse the network of coupled residues into those that have coevolved principally for function.

Using SCA, Ranganathan and colleagues identified a network of coevolving residues that structurally connects two distant but

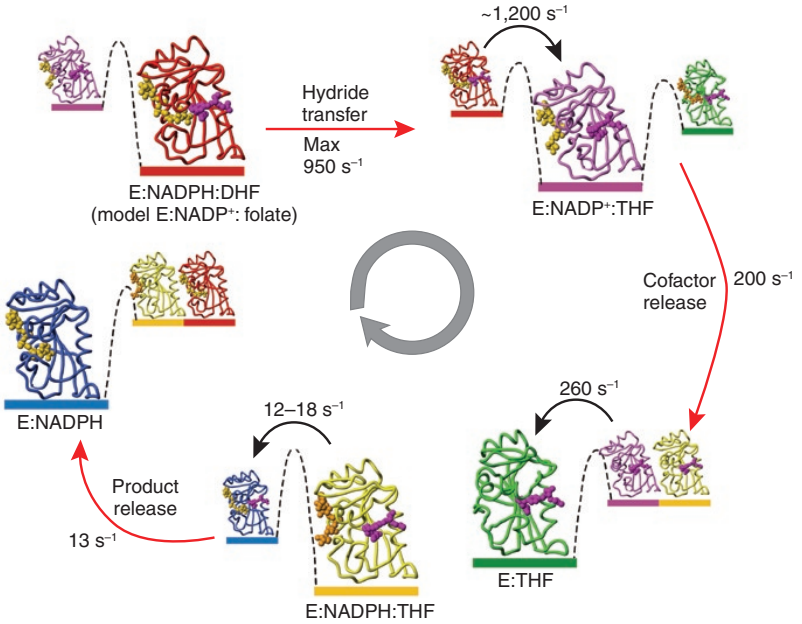

Figure 3 The kinetic pathway of DHFR catalysis shown together with DHFR conformational ensembles identified by NMR relaxation dispersion measurements. The larger structures represent the more stable ground-state conformations, and the smaller structures represent the higher energy structures of each intermediate in the catalytic cycle. As represented by the color scheme in the figure, for each intermediate the higher energy conformations resemble the ground-state conformations of adjacent intermediates. The red arrows show the rate constants determined previously by kinetic experiments, and the black arrows show the conformational interconversion rates obtained from NMR relaxation dispersion measurements (from ref. 3, reprinted with permission from the American Association for the Advancement of Science).

© 2008 Nature Publishing Group http://www.nature.com/naturechemicalbiology

Petitioner Merck, Ex. 1205, p. 477
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00004

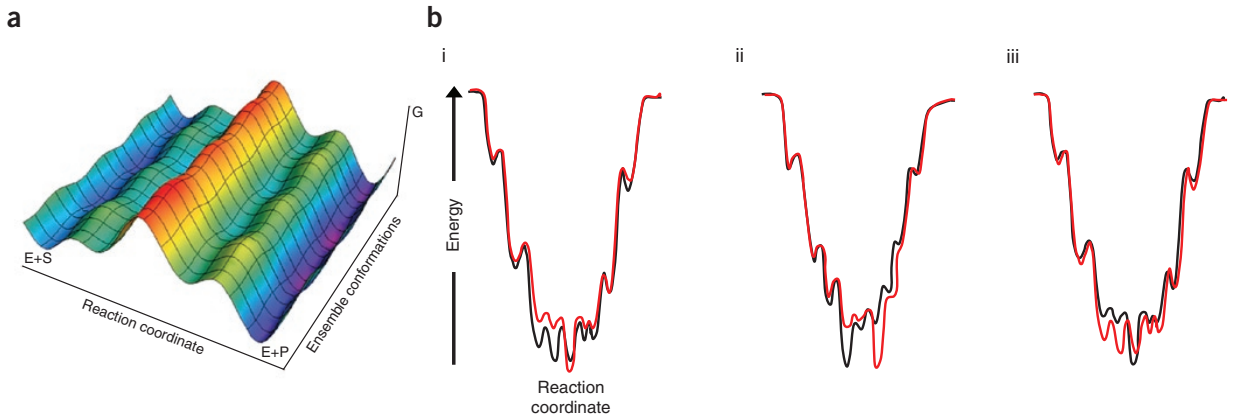

Figure 4 Changing ensembles of protein conformation are manifested in catalysis and allostery. (**a**) Schematic representation of the free energy landscape for an enzyme reaction. Conformational changes occur along both axes. The conformational changes occurring along the reaction coordinate axis correspond to the reorganization that facilitates the chemical reaction. In contrast, the conformational changes occurring along the ensemble conformations axis represent the ensembles of configurations existing at all stages along the reaction coordinate, leading to a large number of parallel catalytic pathways. This figure illustrates multiple populations of conformations, intermediates and transition states. For actual enzymes, the number of maxima and minima along the coordinates is expected to be greater than shown. The dominant catalytic pathways can be altered by external conditions and allosteric effectors. Reprinted with permission from ref. 22, copyright 2008 American Chemical Society. (**b**) Three possibilities (i, ii and iii) for the impact of an allosteric effect on the protein energy landscape. The black lines represent the landscape in the absence of an allosteric effector, and the red lines represent the effector-bound state. (i) The unbound state has several energetically comparable states. Effector binding stabilizes one state at the expense of others, leading to a conformation with a majority population. (ii) Effector binding leads to a discrete conformational change. (iii) The unbound state has a single low-energy conformation. Effector binding results in a more heterogeneous set of possible conformations (reprinted from ref. 8 with permission from Elsevier).

© 2008 Nature Publishing Group http://www.nature.com/naturechemicalbiology

functionally connected sites in FecA, a membrane transporter protein that actively pumps ferric citrate into Gram-negative cells and has a role in transcriptional activation. FecA belongs to the family of TonB-dependent receptors, which are signal-transmitting β-barrel proteins on the outer membrane of Gram-negative bacetia, and the interaction between the periplasmic TonB protein and FecA is allosterically regulated[53]. The binding of TonB to the N-terminal sequence of FecA, called the TonB box, at the periplasmic surface is dependent on the binding of ferric citrate at the extracellular surface. The binding of ferric citrate is followed by conformational changes in FecA that drive the import of ferric citrate. FecA consists of a 22-stranded β-barrel, an internal 'plug' that is located inside the barrel, and the TonB box. Comparison of free and ligand-bound FecA crystal structures showed that ferric citrate binding triggers several local conformational changes in the extracellular and apical loops of the barrel and the periplasmic pocket of the TonB box similar to those observed in other TonB-dependent transporters, such as FhuA and BtuB, which suggests that the allosteric effects are conserved across the protein family. As so often is the case, inspection of crystal structures does not reveal structural changes that would physically link the regions of conformational change. It is possible that the propagated conformational changes are so small that they cannot be seen in crystal structures or that they exclusively involve changes in dynamics. Consequently it is not clear what the mechanism is for allosteric communication between the iron binding site on the surface of FecA (the allosteric site) and its affinity for TonB in the intracellular side.

Ranganathan and colleagues addressed this question by performing an SCA analysis on multiple sequence alignments of a large group of TonB-dependent transporters to identify evolutionary interactions between residues. They found that in the barrel and the plug, coevolving residues form a contiguous network of van der Waals interactions that begin at the cell surface running adjacent to the ferric citrate binding site and end in the periplasmic pocket near the TonB box, physically linking the TonB box to the ferric ion binding site (**Fig. 5**). Only 17% of the residues form most of this physically connected network. It is interesting to note that the network that emerged from the SCA analysis is conserved among TonB-dependent transporters even though different transporters have widely diverse extracellular binding sites (**Fig. 5**). A very nice aspect of this study is that the authors followed the statistical analysis by functional analyses of the network residues through single amino acid mutagenesis and deletions of loops that are part of the network to confirm the involvement of these residues in the mechanism that regulates transcriptional activation[48].

The results from the SCA analysis on the FecA transporter highlight the ability to dissect residues in an allosteric pathway even when discrete, linked conformational changes cannot be seen in crystal structures. These observations point to the complex nature of allosteric communication—messages between two distant sites may be mediated not only by changes in conformation but also by changes in protein motions. In other words, both enthalpic and entropic factors have a role[12–14]. As a demonstration of this, Popovych *et al.* used NMR relaxation measurements, determination of H/D exchange rates and ITC experiments to demonstrate that allostery can take place in the absence of detectable conformational changes and be exclusively mediated by transmitted changes in protein motions in the dimeric catabolite activator protein (CAP)[54]. The binding of a single molecule of cAMP to CAP lowers its affinity for a second molecule of cAMP but has no effect on the conformation of the second subunit. The dynamics, on the other hand, are changed; binding of the first cAMP enhances motions within CAP on the micro- to millisecond timescale, whereas binding of the second cAMP decreases these motions as well as the faster motions on the pico- to nanosecond timescale (**Fig. 6**). Moreover, the flexibility of CAP as measured by H/D exchange rate decreases upon binding of the second molecule of cAMP. The enhancement in motion that is subsequently quenched by the second molecule of cAMP results in an entropic penalty for the binding of the second cAMP, and this price was shown to be responsible for the observed allostery. It is noteworthy that the authors found that motions of residues located at distant regions from the binding sites are affected in the absence of a visible connectivity pathway.

Petitioner Merck, Ex. 1205, p. 478
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00004

This is a result similar to that of FecA. In the case of FecA, the binding effect was assumed to propagate through van der Waal's interactions between residues in the physically connected network. The results seen for CAP are analogous in the sense that changes in fluctuations take place in regions linked by cooperative interactions. This study, among others, highlights the usefulness of NMR for studies of interconverting conformational ensembles and protein motion[55–58].

Computational studies present a complementary method to NMR for studying the link between the changes in protein dynamics and long-range propagation of allosteric signals[59]. This was illustrated by Zhuravleva *et al.* in their study of the changes in chemical shifts and dynamics of the RNase barnase upon binding to its natural inhibitor barstar[60]. X-ray and NMR structures showed that the free and bound structures of barnase are very similar. Even so, the authors were able to use chemical shift perturbation analysis and methyl ^{2}H relaxation measurements to show changes in the dynamics of side chains and an extended β-sheet that is located far from the binding interface upon binding of barstar. They complemented their findings by performing a normal mode analysis that revealed rigid domains that are separated by the previously mentioned extended β-sheet. Molecular dynamics simulations rationalized the observed changes in NMR parameters upon ligation in terms of changes in interdomain dynamics and in populations of exchanging states. The combined experimental NMR evidence and computational theory supports propagation of a molecular response to ligand binding across a protein based on changes in protein dynamics alone.

Allostery in drug discovery and protein design

Increased understanding of the mechanism of allostericity is a factor in pharmacological drug design. Because the binding of an allosteric effector can influence protein function, allosteric binding sites can be targets for new drugs. In some cases, allosteric inhibitors may be more selective across species because, while the active sites may be relatively conserved, the evolutionary pressure on allosteric sites has been less severe, resulting in less conformity[61,62]. Species-specific inhibitors serve useful purposes in the design of antibiotics and as probes in chemical biology. Allosteric inhibitors have been extensively investigated for G protein–coupled receptors, kinases and ligand-gated ion channels[62–66].

Extending allosteric inhibition to other important target proteins requires the ability to identify allosteric sites[67]. Such sites have been identified by high-throughput screening. The mechanism of inhibition for a target hit can be determined by kinetic experiments and might indicate an allosteric binding site. If allostery is implicated, the binding site for such a compound should be further experimentally determined by methods such as X-ray crystallography, NMR, fluorescence studies or cross-linking experiments[67]. Nussinov and Liu have proposed a general strategy for identification of allosteric sites that involves finding large correlated motions between binding sites through examination of covariance matrix maps. Comparison of covariance matrix maps for a ligand bound to an unbound protein can reveal which residue–residue correlated motions change upon ligand binding and hence can suggest new allosteric sites.

As a test case for this method, the authors studied the von Hippel-Lindau tumor suppressor protein (pVHL), which has been found to be mutated in von Hippel-Lindau cancer predisposition syndrome characterized by the appearance of tumors in the central nervous system, kidney, retina, pancreas and adrenal glands. Many tumor suppressor proteins are unstable, a property linked to cancer development, and pVHL is one of these proteins. pVHL, which has two domains (α and β), is a molten globule in its unbound form but is stabilized upon binding of the α-domain to elongin C. The β-domain of pVHL interacts with hypoxia-inducible factor (HIF), which is regulated by the pVHL–elongin C complex (**Fig. 7**). The mutation Y98N is one of the carcinogenic mutations in pVHL. It leads to further loss of pVHL stability and consequently HIF accumulation, thereby promoting the tumor growth and vascularization that is associated with renal cell carcinoma[68]. Molecular dynamics simulations showed that the interface between the α-domain and β-domain of pVHL is the least stable region in the unbound protein. Five residues whose motions were strongly correlated with the unstable interface in pVHL were identified by simulations. These residues were mutated *in silico* and found to stabilize the α-β interface in an allosteric fashion. Two of those mutations, G123F and D179N, also stabilized the Y98N cancer-related mutant, which demonstrates that these mutations can rescue Y98N pVHL stability. The authors suggest that drugs mimicking the allosteric effects of these mutations might rescue pVHL function in von Hippel-Lindau disease mutants.

The identification of allosteric sites in this protein is a first step toward discovering drugs that mimic these effects. A fragment tethering strategy could perhaps be used to investigate the potential binding of inhibitors at these newly discovered sites by introducing a cysteine residue in the vicinity of the site followed by the screening of a library of thiol-containing small-molecule ligands. Ligands that bind in the vicinity of these critical positions would possibly be covalently linked to the enzyme by a disulfide bond and could be detected by mass spectrometry. This approach has been reviewed by Erlanson *et al.*[69]. Fragments

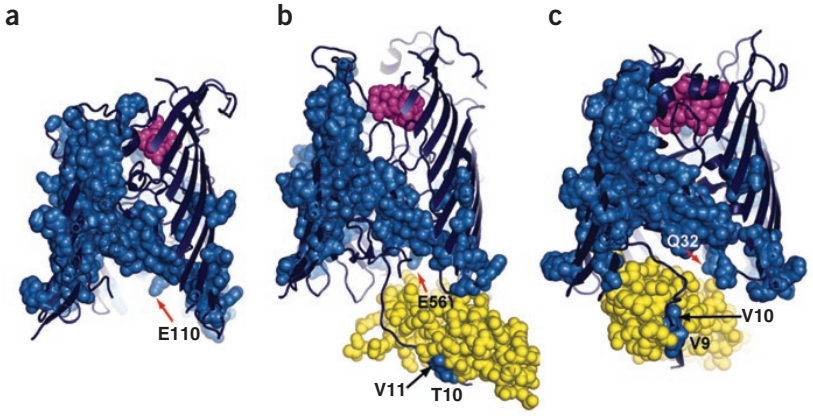

Figure 5 Signal transduction network for TonB-dependent transporters. Network residues are shown as blue spheres. (**a**–**c**) In ribbon diagrams, three different TonB-dependent transporters—FecA (**a**), FhuA (**b**) and BtuB (**c**)—in complex with the periplasmic domain of TonB (yellow) are shown. The chelated iron compounds (ferric citrate for **a**, ferrichrome for **b** and vitamin B12 for **c**) that are pumped into the cell by these transporters are shown as purple spheres. The network of residues responsible for allostery is conserved among these three transporters and can be seen to extend from the binding site for the chelated iron compounds to the periplasmic pocket for TonB, shown in yellow (from ref. 48). SCA network residues E110, E56 and Q32 in FecA, FhuA and BtuB, respectively, are deemed responsible for a specific polar interaction with TonB. SCA network residues V11 and T10 in FhuA and V9 and V10 in BtuB are located in the respective TonB boxes that likely physically link the coevolving network of the barrel and plug to the signaling domain.

© 2008 Nature Publishing Group http://www.nature.com/naturechemicalbiology

Petitioner Merck, Ex. 1205, p. 479
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00004

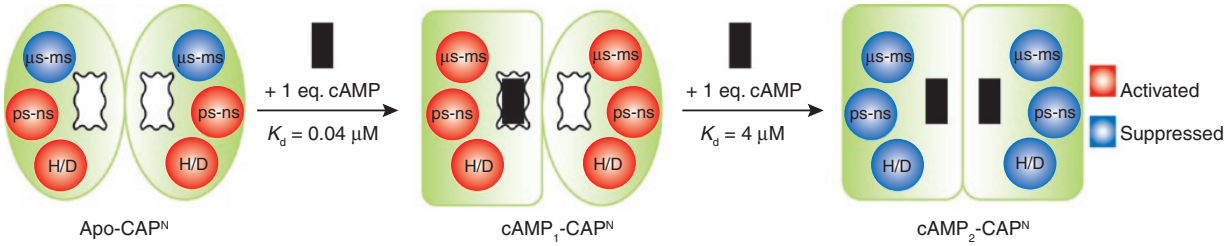

Figure 6 Summary of the overall effect of binding of cAMP on the dynamics of CAP at various timescales. The red spheres indicate activated motions, and the blue spheres indicate suppressed motions. In the apo state, CAP shows low stability (as indicated by the red circle around H/D) and significant motions in the pico- to nanosecond timescale (as indicated by the red circle around ps-ns).Binding of the first cAMP activates motion in the micro- to millisecond time domain, as indicated by the red sphere around the µs-ms label. Binding of the second cAMP suppresses both the H/D exchange and motions in the micro- to millisecond and pico- to nanosecond timescales, as indicated by the blue spheres (from ref. 54).

© 2008 Nature Publishing Group http://www.nature.com/naturechemicalbiology

that act as allosteric effectors to stabilize the α-β interface of pVHL could be incorporated into compound design. However, fragment-based drug design, like multiple protein mutagenesis to alter protein specificity, presupposes the additivity of individual effects. Given the likely coupling between subsites, additivity is not an assured outcome. In other systems, knowledge about allosteric sites has provided direction for rational synthetic efforts in medicinal chemistry. For example, Larson and colleagues identified, via structure-based design, a novel set of p38 map kinase inhibitors that bind to both an allosteric site and an ATP binding site[70,71].

A complementary approach to discovering new 'targetable' areas by predicting allosteric sites is engineering new allosteric sites into proteins. Bishop and Zhang used scanning insertional mutagenesis to introduce new allosteric inhibition sites into the protein tyrosine phosphatase (PTP)[72]. They first created a library of PTP mutants with insertions and screened it for those mutants whose activities can be allosterically modulated by a cell-permeable fluorescein arsenical hairpin binder (FlAsH). To create the library, the FlAsH-binding peptide motif (CCPGCC) was inserted in the catalytic domain of PTP at various loop positions. A large fraction of the resulting mutants expressed as soluble enzymes and had catalytic efficiencies in the absence of FlAsH similar to those of the wild-type PTP. Conversely, when incubated with FlAsH, the catalytic efficiencies of many PTP mutants with CCPGCC insertions were reduced. In one PTP variant, in which the insertion position was 17 Å removed from the catalytic cysteine, FlAsH-induced inhibition was 12-fold. In another mutant, in which the replacement was located more than 20 Å from the catalytic cysteine, the inhibition was found to be three-fold. From inspection of the X-ray structure, the authors suggest that in the former case allostery may emerge because the bound FlAsH may impede proper closure of the activation loop in PTP. This study revealed allosteric sites in PTP, perhaps making it possible to design PTP-specific small-molecule inhibitors that can be used as chemical tools for targeting specific phosphatases.

Knowledge about the mechanism of allostery can be used to design useful proteins that exhibit switch-like behavior[73–75]. A new switch can be created by covalently connecting an enzyme to a ligand binding domain that binds a small molecule. In these switches, the mechanism of function between the enzyme (the output domain) and the ligand binding domain (input domain) is complex and cannot necessarily be predicted (**Fig. 2e**). Switching might depend on a conformational or entropy change in the input domain that is transmitted to the output domain. Guntas *et al.* constructed allosteric switches that responded to maltose binding by combining *E. coli* maltose binding protein (the input domain) to TEM1 β-lactamase (the output domain). A combinatorial strategy using iteratively alternated random domain insertion and random circular permutation of the gene encoding maltose binding protein provided a library that was then screened for optimal switches[76]. It is of great interest to design ultrasensitive switches that can be built from modular components and that display complex signal processing behavior. Such switches could potentially be used as tools to study cellular function, to sense cellular conditions and to perform functions (such as modulating a signal transduction pathway) in response to the cellular conditions.

Future studies and applications

The concept of allostery has matured from that of a multimeric protein with homotropic cooperative binding sites to a more inclusive concept of long-range communication in protein structures. As new methods (including relaxation dispersion NMR methods and X-ray crystallography with nano- to picosecond time resolution) are further developed to understand the stunning catalytic power of enzymes, the field of allostery is also likely to benefit[77]. Allostery and catalysis are, after all,

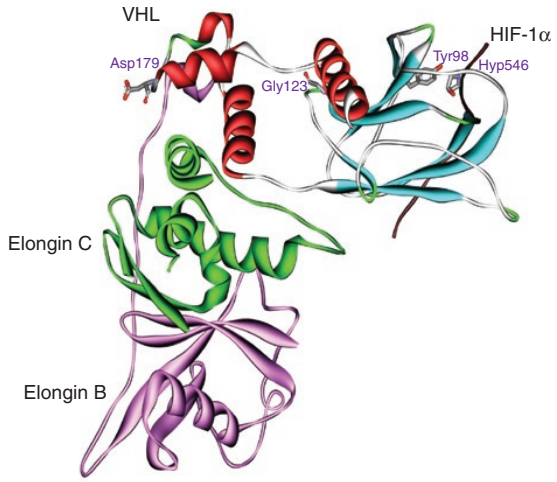

Figure 7 The crystal structure of a tumor suppressor protein complex. The structure of the HIF-pVHL (red and cyan)–elongin C (green)–elongin B (purple) complex (Protein Data Bank code 1LM8) is shown. The mutated positions and the cancer-related mutation Tyr98 are noted in purple (from ref. 68).

Petitioner Merck, Ex. 1205, p. 480
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00004

intimately linked since allosteric effects impact one or more of the features of catalysis: ligand binding, ligand release and the chemical steps in between. Whether allostery is always transmitted by channels such as those identified by SCA and how involved a subset of amino acids is in a protein are both challenging issues. Identification and prediction of amino acid networks that support allosteric effects is an important challenge, and SCA and other methods will likely be the basis for the next significant advances. The timescales of the dynamics implicated in allostery and in catalysis need to be subjects of expanded investigations to determine differences and similarities as well. For example, in the work described above on cAMP binding to CAP, only the motions on the micro- to millisecond timescale become suppressed as the first cAMP is bound, but upon binding of the second molecule of cAMP, both the micro- to millisecond and the pico- to nanosecond motions are suppressed. We expect that both the micro- to millisecond and the pico- to nanosecond motions will be found essential for allostericity within proteins.

As discussed in this review, we have learned much about the basis for and pathways of allostery, but much work remains to be done to fully understand and harness these principles. As more allosteric sites are identified and studied and the mechanisms of communication between an allosteric site and a remote site are elucidated, we anticipate that it should become increasingly feasible to computationally predict new allosteric sites in proteins. The advances in understanding allostery are also being used in the engineering of sensitive modular switches in which an input domain is connected to an output domain. These switches may prove useful in sensing a cellular state and conditionally executing functions such as drug delivery in response to that state. Understanding the transmission of allosteric signals across subunits will allow the building of increasingly complex and sensitive systems for multiple uses.

ACKNOWLEDGMENTS
We thank S. Hammes-Schiffer for helpful discussions.

Published online at http://www.nature.com/naturechemicalbiology/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Monod, J., Wyman, J. & Changeux, J.P. On the nature of allosteric transitions: a plausible model. *J. Mol. Biol.* **12**, 88–118 (1965).
2. Kuriyan, J. & Eisenberg, D. The origin of protein interactions and allostery in colocalization. *Nature* **450**, 983–990 (2007).
3. Boehr, D.D., McElheny, D., Dyson, H.J. & Wright, P.E. The dynamic energy landscape of dihydrofolate reductase catalysis. *Science* **313**, 1638–1642 (2006).
4. Loria, J.P., Berlow, R.B. & Watt, E.D. Characterization of enzyme motions by solution NMR relaxation dispersion. *Acc. Chem. Res.* **41**, 214–221 (2008).
5. Watt, E.D., Shimada, H., Kovrigin, E.L. & Loria, J.P. The mechanism of rate-limiting motions in enzyme function. *Proc. Natl. Acad. Sci. USA* **104**, 11981–11986 (2007).
6. Henzler-Wildman, K. & Kern, D. Dynamic personalities of proteins. *Nature* **450**, 964–972 (2007).
7. Henzler-Wildman, K.A. *et al.* A hierarchy of timescales in protein dynamics is linked to enzyme catalysis. *Nature* **450**, 913–916 (2007).
8. Swain, J.F. & Gierasch, L.M. The changing landscape of protein allostery. *Curr. Opin. Struct. Biol.* **16**, 102–108 (2006).
9. Agarwal, P.K., Billeter, S.R., Rajagopalan, P.T.R., Benkovic, S.J. & Hammes-Schiffer, S. Network of coupled promoting motions in enzyme catalysis. *Proc. Natl. Acad. Sci. USA* **99**, 2794–2799 (2002).
10. Böde, C. *et al.* Network analysis of protein dynamics. *FEBS Lett.* **581**, 2776–2782 (2007).
11. Süel, G.M., Lockless, S.W., Wall, M.A. & Ranganathan, R. Evolutionarily conserved networks of residues mediate allosteric communication in proteins. *Nat. Struct. Biol.* **10**, 59–69 (2003).
12. Cooper, A. & Dryden, D.T.F. Allostery without conformational change. A plausible model. *Eur. Biophys. J.* **11**, 103–109 (1984).
13. Gunasekaran, K., Ma, B. & Nussinov, R. Is allostery an intrinsic property of all dynamic proteins? *Proteins* **57**, 433–443 (2004).
14. Kern, D. & Zuiderweg, E.R.P. The role of dynamics in allosteric regulation. *Curr. Opin. Struct. Biol.* **13**, 748–757 (2003).
15. Bosco, D.A. & Kern, D. Catalysis and binding of cyclophilin A with different HIV-1 capsid constructs. *Biochemistry* **43**, 6110–6119 (2004).
16. Bosco, D.A., Eisenmesser, E.Z., Pochapsky, S., Sundquist, W.I. & Kern, D. Catalysis of cis/trans isomerization in native HIV-1 capsid by human cyclophilin A. *Proc. Natl. Acad. Sci. USA* **99**, 5247–5252 (2002).
17. Cole, R. & Loria, J.P. Evidence for flexibility in the function of ribonuclease A. *Biochemistry* **41**, 6072–6081 (2002).
18. Louis, J.M., Ishima, R., Torchia, D.A. & Weber, I.T. HIV-1 protease: structure, dynamics, and inhibition. *Adv. Pharmacol.* **55**, 261–298 (2007).
19. Katoh, E. *et al.* A solution NMR study of the binding kinetics and the internal dynamics of an HIV-1 protease-substrate complex. *Protein Sci.* **12**, 1376–1385 (2003).
20. Freedberg, D.I. *et al.* Rapid structural fluctuations of the free HIV protease flaps in solution: relationship to crystal structures and comparison with predictions of dynamics calculations. *Protein Sci.* **11**, 221–232 (2002).
21. Massi, F., Wang, C. & Palmer, A.G. III. Solution NMR and computer simulation studies of active site loop motion in triosephosphate isomerase. *Biochemistry* **45**, 10787–10794 (2006).
22. Benkovic, S.J., Hammes, G.G. & Hammes-Schiffer, S. Free-energy landscape of enzyme catalysis. *Biochemistry* **47**, 3317–3321 (2008).
23. Osborne, M.J., Schnell, J., Benkovic, S.J., Dyson, H.J. & Wright, P.E. Backbone dynamics in dihydrofolate reductase complexes: role of loop flexibility in the catalytic mechanism. *Biochemistry* **40**, 9846–9859 (2001).
24. Henzler-Wildman, K.A. *et al.* Intrinsic motions along an enzymatic reaction trajectory. *Nature* **450**, 838–844 (2007).
25. Brath, U., Akke, M., Yang, D., Kay, L.E. & Mulder, F.A. Functional dynamics of human FKBP12 revealed by methyl 13C rotating frame relaxation dispersion NMR spectroscopy. *J. Am. Chem. Soc.* **128**, 5718–5727 (2006).
26. Mulder, F.A., Hon, B., Mittermaier, A., Dahlquist, F.W. & Kay, L.E. Slow internal dynamics in proteins: application of NMR relaxation dispersion spectroscopy to methyl groups in a cavity mutant of T4 lysozyme. *J. Am. Chem. Soc.* **124**, 1443–1451 (2002).
27. Choy, W.Y. *et al.* Distribution of molecular size within an unfolded state ensemble using small-angle X-ray scattering and pulse field gradient NMR techniques. *J. Mol. Biol.* **316**, 101–112 (2002).
28. Hilser, V.J. & Thompson, E.B. Intrinsic disorder as a mechanism to optimize allosteric coupling in proteins. *Proc. Natl. Acad. Sci. USA* **104**, 8311–8315 (2007).
29. Luque, I., Leavitt, S.A. & Freire, E. The linkage between protein folding and functional cooperativity: two sides of the same coin? *Annu. Rev. Biophys. Biomol. Struct.* **31**, 235–256 (2002).
30. Daily, M.D. & Gray, J.J. Local motions in a benchmark of allosteric proteins. *Proteins* **67**, 385–399 (2007).
31. Weber, G. Ligand binding and internal equilibria in proteins. *Biochemistry* **11**, 864–878 (1972).
32. Freire, E. Can allosteric regulation be predicted from structure? *Proc. Natl. Acad. Sci. USA* **97**, 11680–11682 (2000).
33. Chennubhotla, C., Yang, Z. & Bahar, I. Coupling between global dynamics and signal transduction pathways: a mechanism of allostery for chaperonin GroEL. *Mol. Biosyst.* **4**, 287–292 (2008).
34. Amaro, R.E., Sethi, A., Myers, R.S., Davisson, V.J. & Luthey-Schulten, Z.A. A network of conserved interactions regulates the allosteric signal in a glutamine amidotransferase. *Biochemistry* **46**, 2156–2173 (2007).
35. Masterson, L.R., Mascioni, A., Traaseth, N.J., Taylor, S.S. & Veglia, G. Allosteric cooperativity in protein kinase A. *Proc. Natl. Acad. Sci. USA* **105**, 506–511 (2008).
36. Rakauskaite, R. & Dinman, J.D. rRNA mutants in the yeast peptidyltransferase center reveal allosteric information networks and mechanisms of drug resistance. *Nucleic Acids Res.* **36**, 1497–1507 (2008).
37. Torres, M., Fernandez-Fuentes, N., Fiser, A. & Casadevall, A. Exchanging murine and human immunoglobulin constant chains affects the kinetics and thermodynamics of antigen binding and chimeric antibody autoreactivity. *PLoS ONE* **2**, E1310 (2007).
38. Daily, M.D., Upadhyaya, T.J. & Gray, J.J. Contact rearrangements form coupled networks from local motions in allosteric proteins. *Proteins* **71**, 455–466 (2008).
39. Niu, X. *et al.* Interesting structural and dynamical behaviors exhibited by the AF-6 PDZ domain upon Bcr peptide binding. *Biochemistry* **46**, 15042–15053 (2007).
40. Ricketson, D., Hostick, U., Fang, L., Yamamoto, K.R. & Darimont, B.D. A conformational switch in the ligand-binding domain regulates the dependence of the glucocorticoid receptor on Hsp90. *J. Mol. Biol.* **368**, 729–741 (2007).
41. Cameron, C.E. & Benkovic, S.J. Evidence for a functional role of the dynamics of glycine-121 of *Escherichia coli* dihydrofolate reductase obtained from kinetic analysis of a site-directed mutant. *Biochemistry* **36**, 15792–15800 (1997).
42. Gandhi, P.S., Chen, Z., Mathews, F.S. & Di Cera, E. Structural identification of the pathway of long-range communication in an allosteric enzyme. *Proc. Natl. Acad. Sci. USA* **105**, 1832–1837 (2008).
43. Lee, M., Chan, C.W., Mitchell Guss, J., Christopherson, R.I. & Maher, M.J. Dihydroorotase from *Escherichia coli*: loop movement and cooperativity between subunits. *J. Mol. Biol.* **348**, 523–533 (2005).
44. Heddle, J.G. *et al.* Dynamic allostery in the ring protein TRAP. *J. Mol. Biol.* **371**, 154–167 (2007).
45. Yu, P., Lasagna, M., Pawlyk, A.C., Reinhart, G.D. & Pettigrew, D.W. IIAGlc inhibition of glycerol kinase: a communications network tunes protein motions at the allosteric site. *Biochemistry* **46**, 12355–12365 (2007).
46. Sadovsky, E. & Yifrach, O. Principles underlying energetic coupling along an allosteric communication trajectory of a voltage-activated K+ channel. *Proc. Natl. Acad. Sci. USA* **104**, 19813–19818 (2007).
47. Piper, D.R., Rupp, J., Sachse, F.B., Sanguinetti, M.C. & Tristani-Firouzi, M. Cooperative interactionis between R531 and acidic residues in the voltage sensing module of hERG1 channels. *Cell. Physiol. Biochem.* **21**, 37–46 (2008).

© 2008 Nature Publishing Group http://www.nature.com/naturechemicalbiology

Petitioner Merck, Ex. 1205, p. 481
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00004

48. Ferguson, A.D. *et al.* Signal transduction pathway of TonB-dependent transporters. *Proc. Natl. Acad. Sci. USA* **104**, 513–518 (2007).
49. Hatley, M.E., Lockless, S.W., Gibson, S.K., Gilman, A.G. & Ranganathan, R. Allosteric determinants in guanine nucleotide-binding proteins. *Proc. Natl. Acad. Sci. USA* **100**, 14445–14450 (2003).
50. Lockless, S.W. & Ranganathan, R. Evolutionarily conserved pathways of energetic connectivity in protein families. *Science* **286**, 295–299 (1999).
51. Shulman, A.I., Larson, C., Mangelsdorf, D.J. & Ranganathan, R. Structural determinants of allosteric ligand activation in RXR heterodimers. *Cell* **116**, 417–429 (2004).
52. Chi, C.N. *et al.* Reassessing a sparse energetic network within a single protein domain. *Proc. Natl. Acad. Sci. USA* **105**, 4679–4684 (2008).
53. Ferguson, A.D. & Deisenhofer, J. Metal import through microbial membranes. *Cell* **116**, 15–24 (2004).
54. Popovych, N., Sun, S., Ebright, R.H. & Kalodimos, C.G. Dynamically driven protein allostery. *Nat. Struct. Mol. Biol.* **13**, 831–838 (2006).
55. Alfano, C. *et al.* Structural analysis of cooperative RNA binding by the La motif and central RRM domain of human La protein. *Nat. Struct. Mol. Biol.* **11**, 323–329 (2004).
56. Bonaccio, M., Ghaderi, N., Borchardt, D. & Dunn, M.F. Insulin allosteric behavior: detection, identification, and quantification of allosteric states via 19F NMR. *Biochemistry* **44**, 7656–7668 (2005).
57. Li, Z. *et al.* A mutation in the S-switch region of the Runt domain alters the dynamics of an allosteric network responsible for CBFbeta regulation. *J. Mol. Biol.* **364**, 1073–1083 (2006).
58. Abu-Abed, M., Das, R., Wang, L. & Melacini, G. Definition of an electrostatic relay switch critical for the cAMP-dependent activation of protein kinase A as revealed by the D170A mutant of RIalpha. *Proteins* **69**, 112–124 (2007).
59. Budiman, M.E., Knaggs, M.H., Fetrow, J.S. & Alexander, R.W. Using molecular dynamics to map interaction networks in an aminoacyl-tRNA synthetase. *Proteins* **68**, 670–689 (2007).
60. Zhuravleva, A. *et al.* Propagation of dynamic changes in barnase upon binding of barstar: an NMR and computational study. *J. Mol. Biol.* **367**, 1079–1092 (2007).
61. Salvesen, G.S. & Riedl, S.J. Caspase inhibition, specifically. *Structure* **15**, 513–514 (2007).
62. Schweizer, A. *et al.* Inhibition of caspase-2 by a designed ankyrin repeat protein: specificity, structure, and inhibition mechanism. *Structure* **15**, 625–636 (2007).
63. Bertrand, D. & Gopalakrishnan, M. Allosteric modulation of nicotinic acetylcholine receptors. *Biochem. Pharmacol.* **74**, 1155–1163 (2007).
64. Raddatz, R., Schaffhauser, H. & Marino, M.J. Allosteric approaches to the targeting of G-protein-coupled receptors for novel drug discovery: a critical assessment. *Biochem. Pharmacol.* **74**, 383–391 (2007).
65. Ross, R.A. Allosterism and cannabinoid CB1 receptors: the shape of things to come. *Trends Pharmacol. Sci.* **28**, 567–572 (2007).
66. Shi, Z., Resing, K.A. & Ahn, N.G. Networks for the allosteric control of protein kinases. *Curr. Opin. Struct. Biol.* **16**, 686–692 (2006).
67. Hardy, J.A. & Wells, J.A. Searching for new allosteric sites in enzymes. *Curr. Opin. Struct. Biol.* **14**, 706–715 (2004).
68. Liu, J. & Nussinov, R. Allosteric effects in the marginally stable von Hippel-Lindau tumor suppressor protein and allostery-based rescue mutant design. *Proc. Natl. Acad. Sci. USA* **105**, 901–906 (2008).
69. Erlanson, D.A., Wells, J.A. & Braisted, A.C. Tethering: fragment-based drug discovery. *Annu. Rev. Biophys. Biomol. Struct.* **33**, 199–223 (2004).
70. Pargellis, C. *et al.* Inhibition of p38 MAP kinase by utilizing a novel allosteric binding site. *Nat. Struct. Biol.* **9**, 268–272 (2002).
71. Montalban, A.G. *et al.* The design and synthesis of novel alpha-ketoamide-based p38 MAP kinase inhibitors. *Bioorg. Med. Chem. Lett.* **18**, 1772–1777 (2008).
72. Zhang, X.-Y. & Bishop, A.C. Site-specific incorporation of allosteric-inhibition sites in a protein tyrosine phosphatase. *J. Am. Chem. Soc.* **129**, 3812–3813 (2007).
73. Ostermeier, M. Engineering allosteric protein switches by domain insertion. *Protein Eng. Des. Sel.* **18**, 359–364 (2005).
74. Dueber, J.E., Mirsky, E.A. & Lim, W.A. Engineering synthetic signaling proteins with ultrasensitive input/output control. *Nat. Biotechnol.* **25**, 660–662 (2007).
75. Dueber, J.E., Yeh, B.J., Chak, K. & Lim, W.A. Reprogramming control of an allosteric signaling switch through modular recombination. *Science* **301**, 1904–1908 (2003).
76. Guntas, G., Mitchell, S.F. & Ostermeier, M. A molecular switch created by in vitro recombination of nonhomologous genes. *Chem. Biol.* **11**, 1483–1487 (2004).
77. Sathyapriya, R. & Vishveshwara, S. Structure networks of *E. coli* glutaminyl-tRNA synthetase: effects of ligand binding. *Proteins* **68**, 541–550 (2007).
78. Kendrew, J.C. *et al.* A three-dimensional model of the myoglobin molecule obtained by x-ray analysis. *Nature* **181**, 662–666 (1958).
79. Pardee, A.B. & Reddy, G.P. Beginnings of feedback inhibition, allostery, and multiprotein complexes. *Gene* **321**, 17–23 (2003).
80. Koshland, D.E. Jr., Nemethy, G. & Filmer, D. Comparison of experimental binding data and theoretical models in proteins containing subunits. *Biochemistry* **5**, 365–385 (1966).
81. Bryngelson, J.D. & Wolynes, P.G. Spin glasses and the statistical mechanics of protein folding. *Proc. Natl. Acad. Sci. USA* **84**, 7524–7528 (1987).

© 2008 Nature Publishing Group http://www.nature.com/naturechemicalbiology

Petitioner Merck, Ex. 1205, p. 482
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00004