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Principles of Allosteric Interactions in Cell Signaling

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ABSTRACT: Linking cell signaling events to the fundamental physicochemical basis of the conformational behavior of single molecules and ultimately to cellular function is a key challenge facing the life sciences. Here we outline the emerging principles of allosteric interactions in cell signaling, with emphasis on the following points. (1) Allosteric efficacy is not a function of the chemical composition of the allosteric pocket but reflects the extent of the population shift between the inactive and active states. That is, the allosteric effect is determined by the extent of preferred binding, not by the overall binding affinity. (2) Coupling between the allosteric and active sites does not decide the allosteric effect; however, it does define the propagation pathways, the allosteric binding sites, and key on-path residues. (3) Atoms of allosteric effectors can act as "driver" or "anchor" and create attractive "pulling" or repulsive "pushing" interactions. Deciphering, quantifying, and integrating the multiple co-occurring events present daunting challenges to our scientific community.

INTRODUCTION

Specific protein function is determined by the extent to which the protein populates a distinct active state.[1] Allostery, an inherent physical property of proteins, is a key factor governing the relative populations among accessible conformational states.[2] Allostery can be defined as the change in the distribution of the conformational ensemble through some perturbation, such as ligand binding or covalent post-translational modification (PTM)[3] or mutations taking place through directed evolution,[4,5] to alter the population of the active state.[6−20] Nature has co-evolved ligand−host protein interactions, optimizing them to tune the populations of the active (or inactive) states for function, either by stabilizing the active conformation and destabilizing the inactive conformations, or vice versa.[21,22] Here, we provide an overview of the fundamental underpinnings of allostery. We aim to delineate key challenging questions, such as, Can we predict *a priori*—and quantify—changes incurred by allosteric mutations or specific binding events to increase/decrease the population of the active or inactive state to up- or down-regulate the protein? When considered in large systems, on a cellular scale, with multiple events that form and/or disrupt non-covalent and covalent interactions, and with numerous mutations and different classes of molecules which are involved, these key questions stymie applications of the allosteric concept toward unveiling physiological signaling, deregulation in disease, and allosteric drug discovery. The extent of the stabilization, or population shift, toward the active (or inactive) state determines the efficacy of the mutation, PTM, or binding event. Below, we reason that our mechanistic understanding of the hallmarks of allostery already permits undertaking such challenges. We further formulate some guidelines toward such aims.

More and more data attest to the significance of allostery in cell life under physiological conditions[23−28] and in disease.[21,29,30] Allostery takes place across single molecules and large multimolecular assemblies, across the membrane, cytoplasm, and organelles, and across DNA and protein−DNA interactions.[14,18,31−44] Diseases often occur through allosteric mutations that shift the protein population from an OFF to a functional ON state and keep it there, with the ramifications propagating through cellular pathways, affecting the cell state.[21] Allostery is best described by a series of free energy landscape diagrams that map the conformational spread and the corresponding energy levels (Figure 1). Molecules exist as ensembles with certain conformational distributions under distinct conditions; allostery works by altering the distributions following some conformational trigger. They can be reflected by significant conformational changes at the active site and/or its dynamics between ordered and disordered states.[45] Allostery is the means through which the physicochemical basis of the conformational behavior of single molecules governs cellular behavior.[23] While not the only factor, it plays a decisive role in cellular response to changes in the environment, internal and external. Currently the mechanism of "how allostery works" on the molecular level is fairly well understood.[1] Linking fundamental mechanistic underpinnings, available data for proteins and pathways, and the dynamic spatial cellular organization[46] should allow us to address major bottlenecks in allostery-related research, such as quantification of the effects of mutations and identification of target proteins and sites, while accounting for regulatory feedback loops.[27,47] It should

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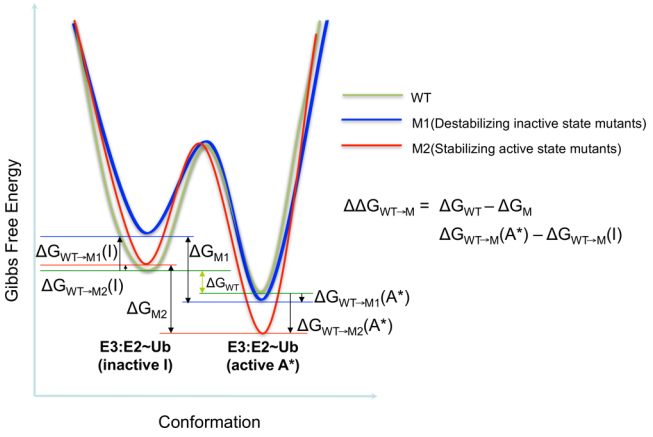

Figure 1. Extent of population shift, rather than binding affinity, determines allosteric efficacy. Here we use the distinct mutations of E3 ubiquitin ligase as an example. The population of the protein conformations is dominated by either the active form or the inactive form of the E3:E2∼Ub complex. The relative population of the inactive state (I state) and active state (A* state) depends on the relative energy of the two states. The free energy landscape is defined as $\Delta G = G(\mathrm{A}^*) - G(\mathrm{I})$. For the wild type (green curve), the dominant population is the active conformational state, allosterically facilitating the ubiquitin transfer process. Both destabilizing inactive-state mutants (blue curve) and stabilizing active-state mutants (red curve) can enhance the allosteric activity, here illustrated from the standpoint of population shift. The extent of enhancement is expressed by the free energy change due to the mutations, $\Delta\Delta G_{\mathrm{WT}\rightarrow\mathrm{M}}$. The destabilizing inactive-state mutants may destabilize the inactive state but stabilize the active state to shift population. The stabilizing active-state mutants stabilize both the inactive and active states, to different extents, resulting in a shift of the population. The binding affinity, or the mechanism of population shift, does not determine the allosteric efficacy. It is the extent of population shift, or the free energy change $\Delta\Delta G$, that determines the allosteric efficacy.

also allow us to design allosteric modifiers for specific outcome, agonist or antagonist.[22] The number of studies where observations are interpreted through allostery escalates rapidly, indicating that the community increasingly grasps its significance to the living cell.

Allostery initiates and propagates by breaking existing interactions in one state and gaining new interactions in the other. Through allosteric coupling, the distinct interactions formed at the allosteric site specify the outcome in the distal active site, and the pathway to get there.[1] Identifying the triggering ligand atoms at the allosteric site for the diverse ligand−host assemblies in the cell, the types of (attractive, repulsive) interactions that they form with the receptor atoms, and the allosteric consequences can provide the foundation for unraveling allosteric conformational control in cellular processes.[22] A receptor can bind tens of ligands or proteins at a given site; each can encode a distinct cellular outcome.[27] These may reflect different triggering "warheads", thrusting against the allosteric receptor site, and shaping the consequent allosteric modulation. The barcode that they encrypt is challenging, and in the cellular environment, at any given time, multiple allosteric triggers could act on a protein. Below, we provide the principles, aiming to map the landscape of current allosteric research and to focus on bottleneck questions. Within this framework, we underscore the challenge of identification of allosteric effectors—*anchors* and *drivers*—triggering interactions, where an anchor stabilizes the bound state and the driver fires its allosteric ramifications. We highlight the complexity of multiple drivers which may exist in protein−protein interfaces. We further draft an outline of how this problem could be construed and deciphered.

A UNIFIED MODEL OF THE ALLOSTERIC ACTIVATION (INACTIVATION) MECHANISM

Allostery reflects a change or a shift in the distribution of the conformational ensemble. Population shift between states takes place when the preceding state gets destabilized and/or the next state gets stabilized. Population shift takes place in proteins, nucleic acids,[48,49] and lipid assemblies,[50,51] including cholesterol[52] and phosphatidylinositol triphosphates. It takes place within molecules and across their interfaces. Population shift links protein behavior, cellular pathways, and regulation under normal physiological conditions and in disease. The concept of population shift that we suggested in the late 1990s[53−59] recognizes that all conformational states pre-exist, including the active, inactive, and rare high-energy transition states and substates in catalytic reactions.[58,60−67] It posits that rather than morphing one state into another, the ensemble shifts from the less stable to the more stable via sampling pre-existing conformations, and that this is the origin of the allosteric effect. The change in the relative stabilities between the states can take place either by destabilizing one state (e.g., the inactive) with respect to another (the active), or by stabilizing one state (e.g., the active) with respect to the other (inactive), or by both mechanisms. Two largely overlapping sets of residues are associated with the subtle conformational changes between the active and inactive conformations. Dynamic fluctuations of these same residues are responsible for the conformational switch between the two states.[68] The relative populations of the active and inactive states are largely determined by how much stabilization these two residue sets contribute to each.

To clarify the fundamental basis of the allosteric mechanism, we have recently described "how allostery works" from three different points of view.[1] The unified view considers allostery from the thermodynamic standpoint,[69,70] in terms of the energy landscape of population shift,[62,71] and from a simplified structural view of allostery, all with exactly the same allosteric descriptors. The unified view of allostery posits that allosteric efficacy is determined by the extent of the population shift. Allosteric coupling (or a communication pathway) does not determine the allosteric efficacy; however, it defines key residues that are critical for population shift. This substantiates the many works aimed at detecting allosteric propagation pathways—experimentally and computationally.[1,22] Both *driver* and critical *anchor* atoms exhibit specific interactions with their host protein, with the former mainly responsible for the allosteric efficacy and the latter for binding affinity (potency).

Allosteric Efficacy Is Determined by the Extent of Population Shift. Allosteric activation events can be portrayed by a double-well, two-state model. The protein dominantly populates one of the states, the inactive or the active. The states are separated by a sizable but surmountable free energy barrier.[71] The population of each state is determined by the free energy differences between the two states. Binding of the allosteric effectors shifts the population eliciting allosteric effects. This raises a question: Is the allosteric efficacy determined by the binding affinity of the allosteric effector to the host, or by the extent of population shift?

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A recent study on E3 ubiquitin ligase sheds light on this question. Ubiquitination results in protein degradation and plays a critical role in nearly all cellular processes. A cascade of enzymes, including E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases, is involved in the ubiquitination process. It is known that binding of E3 ubiquitin ligases to E2s can allosterically bring substrates and E2s into proximity to facilitate substrate ubiquitination. But does the binding affinity of E3:E2 determine the allosteric efficacy of E3? A high-throughput "deep mutational scanning" method has been used to assess the effects of nearly 100 000 protein variants of the U-box domain of the murine E3 ligase ubiquitination factor E4B (Ube4b) on the ubiquitination activities.[72] Interestingly, two distinct classes of mutations were found to enhance activity. One class of mutations, L1107I and M1124V, increases E3:E2-binding affinity. We denote this type of mutations as "stabilizing active state mutants". The other class of mutations, D1139N and N1142T, do not change the binding affinity significantly, but exhibit the strongest induction of the active conformational states for ubiquitination, acting as "destabilizing inactive state mutations". We illustrate the mechanisms of these two types of mutations in Figure 1. To quantify the population shift from an inactive to an active state (or vice versa), the extent of preferential binding can be formulated by stabilization versus destabilization. In this case, the WT protein favors the active state. The stabilizing active state mutants stabilize the active conformation more than they destabilize the inactive conformation, resulting in population shift toward the active state and enhanced allosteric activity. The destabilizing inactive state mutants achieve the same goal probably by destabilizing the inactive conformation more than by stabilizing the active conformation. Both mechanisms can enhance allosteric activity, suggesting that binding affinity does not determine the allosteric efficacy. Instead, the allosteric efficacy is determined by the population shift from the inactive state to the active state, which is determined by the free energy change between the two states.

Free energy comprises of enthalpy and entropy. A binding event may have favorable enthalpy change but unfavorable entropy change. An allosteric effect could be either enthalpy-driven or entropy-driven, depending on which contributes more to the change of free energy. It is difficult to disentangle these two contributions to the change of free energy, partly because it is extremely hard to measure entropy, either experimentally or computationally. The difficulty of quantitatively measure entropy greatly hinders the understanding of allosteric efficacy, especially for entropy-driven allostery. Recently, NMR relaxation techniques have been successfully used to quantitatively measure conformational entropy.[73] Changes in conformational dynamics of fluctuating methyl groups in a protein between conformational states are used as a "dynamical proxy", which is an excellent proxy to quantitatively describe conformational entropy.[74] Molecular dynamics simulations[75] further validates the link between conformational dynamics and conformational entropy and provides the atomistic interpretation of employing "dynamical proxy" as the "entropy meter". These advances can permit further quantification of the allosteric efficacies.

Coupling between the Allosteric and Active Sites Defines Key Residues That Shift or Reverse Population Shift. Detection of coupling between residues has been successfully used to identify allosteric pathways, allosteric sites and key residues. However, without a perturbation, such as binding events or mutations, the coupling itself does not decide the allosteric effect. For example, a group of residues on the VHL protein are coupled; however, without a mutation, no allosteric effects are observed.[76] The dominant population of the WT VHL is in the inactive state. In this state, VHL interacts with the E3 ligase component elonginC to facilitate substrate ubiquitination. The inactive state does not lead to disease development. The disease-related mutation Y98N is located far away from the VHL interface with elonginC, but it allosterically disrupts the VHL interaction with elonginC. As a destabilizing-inactive-state mutant, Y98N shifts the population from the inactive to the active state, which leads to development of type 2B VHL diseases. The designed mutations of the key residues, G123F and D179N, far away from both Y98 and the VHL:elonginC interface, allosterically stabilize the VHL:elonginC interface of the Y98N mutant to rescue the protein−protein interactions. These mutations reverse the population shift from the disease-causing active state to the WT inactive state and allosterically rescue VHL's function. The population shift and the reversed population shift are illustrated in Figure 2.

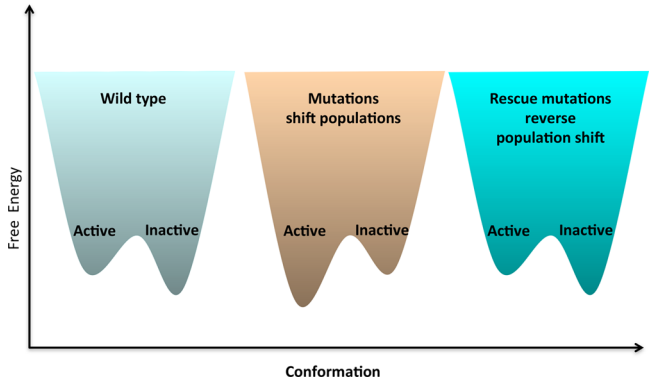

Figure 2. Key residue mutations may switch the population shift. The energy landscape diagram of the WT VHL protein is shown on the left. The key residue mutations (middle), such as VHL Y98N, elicit the allosteric event by shifting the free energy landscape. The rescue mutations (right), such as VHL G123F and D179N, may reverse the population shift to one similar to the WT.

The PDZ domain has been extensively studied to identify key-residues on allosteric pathways. Numerous experimental and computational methods have been developed to identify key residues on PDZ allosteric pathways, including NMR,[77] chemical shift covariance analysis (CHESCA),[78] statistical coupling analysis (SCA),[79] elastic network models (ENMs),[80−82] anisotropic thermal diffusion (ATD) MD simulation,[83] and pump−probe MD (PPMD) simulations.[84] Recently, a new method called "rigid residue scan" was developed to identify key residues for protein allostery using the PDZ domain as an example.[85] By systematically keeping each residue rigid during the MD simulations and comparing the correlated motions between the bound (active) and unbound (inactive) states, this method identified two groups of key residues in allosteric pathways. The degree of dynamics of one group of key residues, "wire residues", does not affect the protein residue-coupling upon effector binding. Considering that the energy flow along allosteric pathways may cause significant dynamic changes to on-path residues, these least disruptive residues upon effector binding appear ideal for

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carrying out the propagation of energy and may constitute key on-path residues. The dynamics of another group of key residues, called "switch residues", are important to differentiate the unbound (inactive) and bound (active) states. Therefore, mutations of these "switch residues" may have the potential to reverse the population shift caused by the binding events, as illustrated in Figure 2, from the active to the inactive states.

The term "allosteric hotspots" has been used to describe residues on the protein surface that are important for allosteric regulation. Co-evolutionary analysis showed that residues that co-evolved comprise structurally contiguous networks called sectors,[86] and allosteric hotspots are connected to sectors.[87] From the ensemble point of view of allostery, a specific residue-by-residue pathway may not be necessary involved, especially for disordered proteins.[88] In these cases, allosteric hot spot residues are more important than others for population shift rather than energy propagation. Mutation of these residues will have the potential to alter the population distribution.

Why can single or multiple mutations alter the population distribution? We may explain this from the standpoint of protein folding. Protein folding is a process dominantly driven by the hydrophobic effect toward native conformations along a funnel-like free energy landscape.[89,90] When the folding process reaches the bottom of the funnel, it settles down to its native state by specific stabilizing interactions. Functioning as a node in the cellular circuit, a monomeric protein is usually required to switch its dominant population of conformations between active (ON) and inactive (OFF) states. This argues that a protein has been optimized by evolution to fold into several switchable conformational states at the bottom of the folding funnel. As individual conformations are stabilized by distinct residue−residue interactions, their relative populations rely on key interactions within these. Relaxation dispersion NMR spectroscopy[91] can detect a less-populated state so long as its relative population is above 0.5% and the different conformational states exchange on the millisecond time scale.[65] Although residues involved in the stabilization highly overlap among the conformational states, it is not surprising that single or multiple mutations could alter or even reverse the relative populations of the active and inactive states.[5,92]

THE CONCEPT OF CONJOINT *ANCHOR* AND *DRIVER* ATOMS

A key question in elucidating allosteric mechanisms in specific systems relates to what determines the direction of the population shift and how to explain or predict its consequences; that is, will the ligand be an agonist, inverse agonist, or antagonist.[22] To clarify this, we distinguish between two types of ligand atoms, *driver* and *anchor*. The ligand binds at an allosteric pocket. The pocket conformation with which an *anchor* atom interacts is unaffected during the transition from the inactive to the active state (or vice versa). The accurate positioning of the *anchor* in the allosteric pocket is critical since it provides the foundation that allows a *driver* atom to perform a "pull" and/or "push" action. This shifts the receptor population from the inactive to the active state (or vice versa) through a pre-existing communication pathway which has been optimized by evolution. The extent that pulling stabilizes the active and/or pushing destabilizes the inactive conformation, determines the shift to the active state. The mechanism of stabilization (or destabilization) differs between the *anchor* and *driver* atoms, and the agonism is determined by the presence or absence of a *driver* in an allosteric ligand. An *anchor* atom is likely to have the same interactions with the receptor in the active and inactive conformations. In contrast, a *driver* atom may form stabilizing or destabilizing interactions. A stabilizing attractive *driver* interaction such as a hydrogen bond or salt bridge which forms in the active but not in the inactive conformation can "pull" the inactive conformation into the active conformation. A destabilizing repulsive *driver* interaction in an allosteric pocket—in a position and orientation determined by the *anchor* atom—incurs steric hindrance in the inactive but not in the active conformation. Thus, the subtle conformational changes between the active and inactive conformations explain the type of *driver* atom interactions and its action. "Pulling" or "pushing" by even a single *driver* atom can favor a specific conformation unlike an *anchor* atom which favors both (active, inactive) states and does not provoke a population shift. This explains why even a slight change in ligand interactions, involving a mere substitution of a single atom may promote different—agonist or antagonist—consequences. Surprisingly, we observed that a combination of the global backbone displacement (GBD) and the local structural environment (LSE) change which reflects the extent of structural changes between active and inactive states, is able to identify and distinguish between *driver* types as well as identify coupled residues along the propagation pathway.[22]

Below, we provide few examples. The first relates to DNA acting as an allosteric effector, cooperatively mediating conformational changes in the dimerization and cofactor binding surface of nuclear receptors. Glucocorticoid receptor (GR) is one such case. GR consists of the N-terminal domain, DNA-binding domain (DBD), hinge region, ligand binding domain (LBD), and the C-terminal domain. Binding of agonists such as hormones to the LBD in the cytoplasm allosterically induces GR dimerization with consequent translocation into the nucleus where the DBD binds to DNA response elements (REs) to activate transcription initiation.[32] DNA binding induces a conformational change which alters the cofactor binding sites, shifting the GR population toward conformations which are complementary to the cofactor to modulate the glucocorticoid activity. The role of the RE as an allosteric effector of the steroid receptors has been well established.[39,93−96] Yamamoto and his colleagues have elegantly shown that GR binding to REs differing by a single base pair leads to differential effects in the GR conformations and activities.[32,97] Different RE sequences allosterically shift the ensemble of GR conformations, via a six-residue segment that connects helix H1 and the dimerization loop (the GR lever arm), with the population distribution further modified through interactions of the ligand binding receptor domain.[96] The cofactor then binds to a complementary conformation populated by the *driver* nucleotides, thereby initiating the cascading signaling pathway. Allosteric conformational changes can alter GR surfaces interacting with cofactors such as p160/SRC (steroid receptor coactivator) proteins which can interact with histone acetyltransferases, such as CBP (cAMP response element-binding protein (CREB)-binding protein) and p300).

p53 provides another example of RE-specific signaling pathway: binding of the p53 DNA binding domain (DBD) to the p53 REs initiates signaling that propagates to the p53 activation domain (p53AD) which in turn binds Mediator to activate or initiate transcription by RNA Polymerase II (Pol II) at the promoter.[40] Different REs have slightly different atomic contacts with the DBDs. These result in different pathways which transmit the DNA sequence specificity to the activation

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domain to initiate or activate an initiated-and-stalled Pol II.[40] These examples suggest that specific interactions of the DNA act as *driver* for GR and p53 to elicit sequence-specific effects. To verify and pinpoint the assignments, a detailed analysis of the crystal structures is needed. The multiple structures of GR with mutated REs and the functional consequences provide rich data for such analysis.

The third example relates to Akt1 kinase.[98−100] The activation of Akt kinase is regulated by the phosphorylation state of two residues in the activation loop (T308 in Akt1) by PDK1 and in the carboxyl-terminal tail (S473 in Akt1). Phosphorylation of these regulatory sites can take place following conformational changes induced by releasing its regulatory pleckstrin homology domain to dock to membrane lipid products. PDK-dependent Akt1 phosphorylation is reversed by protein phosphatase 2A (PP2A) which dephosphorylates pT308 and, to a lesser extent, pS473. In the phosphorylated state Akt is active; dephosphorylation by the phosphatase deactivates it. ATP, bound at the catalytic site between the two Akt lobes, reduces the sensitivity of phosphorylated Akt to be dephosphorylated by protein phosphatase 2A. The binding of ATP stabilizes the closed conformational state which has a strong structural coupling with the phosphorylated T308 in the activation loop, shielding it from phosphatase access. Following hydrolysis, with the ADP in the catalytic pocket, Akt1 relaxes into its open conformation, pT308 is exposed and dephosphorylated. This mechanism may account for ATP/ADP acting as ON/OFF switches in Akt1 catalysis. The difference between the actions of ADP versus ATP argues that the γ phosphate group of the allosteric ATP acts as a *driver*. The sugar moiety may act as an *anchor*. However, the verification of *anchor* and *driver* via a simple structural analysis[22] is only feasible when the structure of ADP bound Akt1 is available.

ATP/ADP acting as ON/OFF switches is also observed in the allosteric mechanism in the chaperone protein hsp70. Hsp70 has two domains: an N-terminal nucleotide-binding domain (NBD) connected to a C-terminal substrate-binding domain (SBD) through a linker about 10−12 residues in length. Hsp70 has closed and open states that are important to its function.[101] In its open state, as shown on the left panel in Figure 3A, hsp70 binds to ATP at NBD and displays low binding affinity to the substrate at SBD. Therefore, substrates can easily dissociate from SBD and cannot be refolded in the open state of hsp70s. Following ATP hydrolysis at the NBD, hsp70 switches to its closed state, which has strong binding affinity to the substrate. This new state, in which ADP is now bound to the NBD (Figure 3A, right), is the state in which hsp70 performs its main tasks as a chaperone protein to either refold or assist in degrading misfolded peptides. ATP hydrolysis has an integral role in the allosteric mechanism of hsp70. Structural analysis, as shown in Figure 3B, indicates a "pulling effect" by the γ-phosphate, whereas the conformation around the sugar moiety is relatively unchanged. Therefore, similar to the Akt1 kinases, the difference between ATP and ADP suggests assigning a *driver* role to the γ-phosphate group and an *anchor* role to the rest of the ATP molecule.

Amino acid synthesis can provide another striking example.[102] 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAH7PS) catalyzes the first step in the shikimate pathway, which is responsible for the biosynthesis of the aromatic amino acids Trp, Phe, and Tyr. *Mycobacterium tuberculosis* expresses a single DAH7PS enzyme which is controlled by combinations of

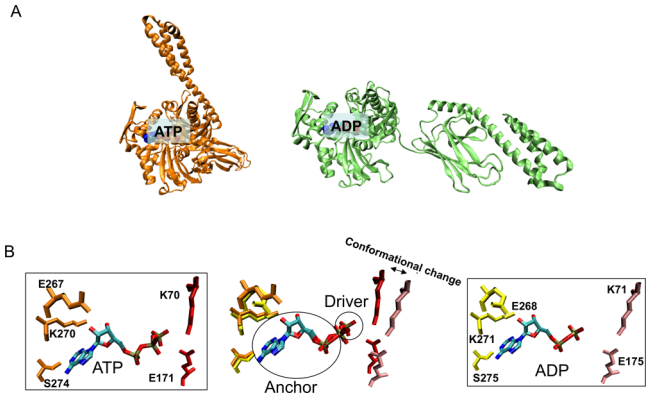

Figure 3. Schematic illustration of the definition of *anchor* and *driver* in hsp70 allosteric activation. (A) A structural comparison of *Escherichia coli* hsp70 DnaK in the ATP-bound form (left panel, orange) (PDB code: 4B9Q[130]) and ADP-bound form (right panel, green) (PDB code: 2KHO[131]). (B) Comparison of ATP and ADP at the catalytic center of hsp70. The left and right panels show the hsp70 catalytic center residues coordinated with the ATP structure (left, DnaK.ATP complex, PDB code: 4B9Q) and ADP structure (right, Hsc70.ADP.Pi complex, PDB code: 1HPM[132]). The middle panel is the superimposition of the left and right panels. The circled *driver* atoms (γ-phosphate) of ATP "pull" the Lys70 and Glu171 by more than 2 Å in the low-substrate-affinity conformation. The circled *anchor* atoms induced little conformational change in hsp70 residues in both states.

these residues. The tetrameric enzyme has three allosteric sites. Site 1 is at the tetramer interface and is occupied dominantly by Trp. Site 2 is at the dimer interface and is occupied dominantly by Phe. Site 3 is Tyr-selective occupied in only two of the four subunits. In addition to site 2, Phe also binds to site 3 at high concentrations. When only one type of amino acid is present, the enzyme is unnoticeably inhibited. Allosteric synergistic inhibition is observed by a combination of two amino acids, Trp+Phe or Trp+Tyr. However, maximal inhibition requires the involvement of all three amino acids, presumably with binding of Phe in site 2 and Tyr in site 3.

This fine-tuning of enzyme activity by three different allosteric effectors with three distinct binding sites provides an important example of complex allosteric regulation revealing a network of three synergistic allosteric sites on one enzyme. The inhibition data has three clear allosteric implications. First, the hydroxyl group of Tyr bound at site 3 is the *driver* while the aromatic rings in Phe and Tyr are *anchors*. Second, an individual amino acid binds alone at its corresponding site, acting as an allosteric modulator but not as an allosteric effector, which by itself, is able to reverse the population of the active and inhibited states. Third, two additive allosteric modulators become an allosteric effector. Although various crystal structures of apo as well as single and duo amino acids occupancy are available in the PDB, a systematic structural analysis via 3-D superposition among them revealed that the overall structure and particularly the catalytic triads at the active site show no noticeable change. The results imply that the population shift from the active to the inactive state due to allosteric amino acids binding has been compensated by crystal packing effects (or crystallization conditions). A recent study with microseconds molecular dynamic simulation[5] has provided direct support for this suggested reasoning.

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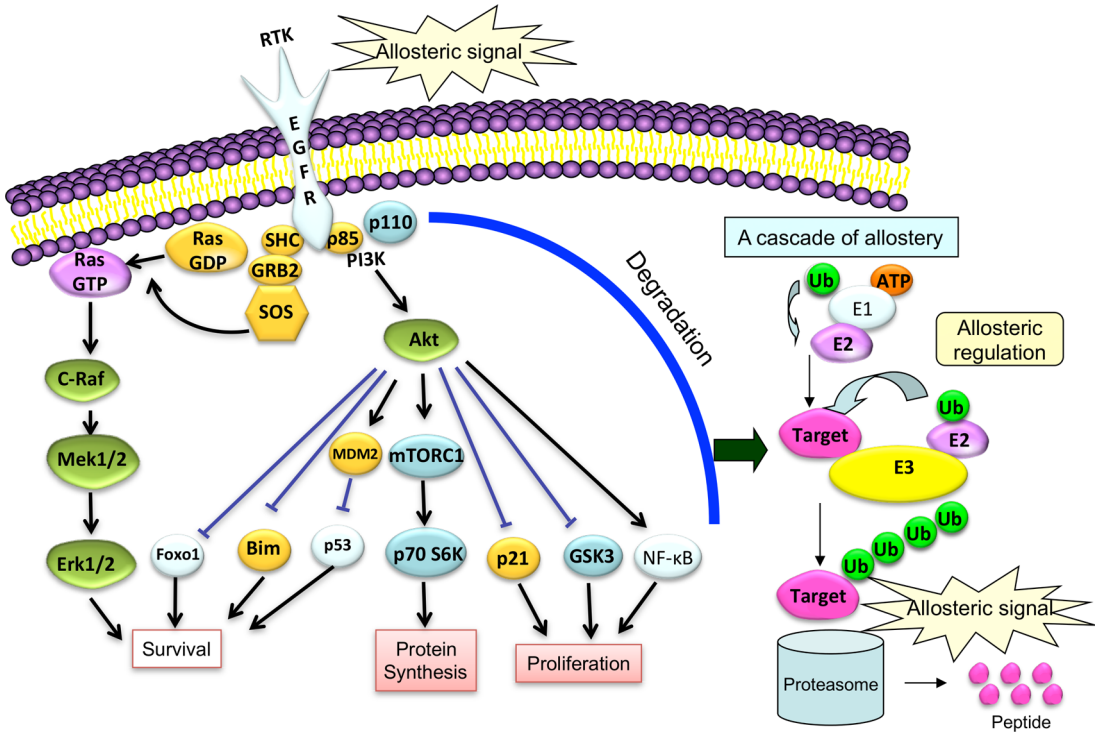

Figure 4. Cell signaling pathways are allosterically elicited by the activated receptor tyrosine kinase (RTK). The illustration includes the two major signaling pathways, Ras-RAF-MEK-ERK and PI3K-AKT, for protein synthesis, cell proliferation, and cell survival, and the ubiquitin-proteasome pathway for allosteric regulation and signaling of protein degradation.

A CASCADE OF ALLOSTERY IN CELL SIGNALING

Signaling takes place through single-chain proteins and pathways, and through pathway crosstalk, ultimately across the cellular network down to gene activation or transcriptional regulation in the nucleus. Pathways are often activated by external stimuli or by metabolic messengers. Binding of hormones, peptides or small molecules to an extracellular domain of a cell-surface receptor transmits extracellular information to the cell to activate downstream intracellular signaling events. Although the mechanisms are diverse, activation is often coupled to ligand-induced dimerization that results in intracellular dimerization, as in the case of epidermal growth factor receptor (EGFR), a receptor tyrosine kinase (RTK).[103] Signals propagate through protein−protein interactions (or second messengers), cascading in the cell to initiate or repress gene-specific transcription. Crosstalk between signaling pathways generally takes place through shared proteins or signaling molecules whose concentration is regulated by both pathways. The two major cellular signaling pathways downstream of the activated RTK and G protein-coupled receptor (GPCR), Ras-RAF-MEK-ERK and PI3K-AKT, as illustrated in Figure 4, provide good examples. The pathways transduce signals received at the cell surface to give rise to protein synthesis, cell proliferation and survival. In the under-regulated MAPK/ERK pathway[104−106] the signal initiates when an extra-cellular ligand (the epidermal growth factor, EGF) binds EGFR. A resultant large conformational change in the ectodomain then facilitates EGFR dimerization. In turn, the increased proximity results in intracellular kinase activation through an asymmetric dimerization mechanism. The SH2 domain of the adaptor protein GRB2 binds to the phosphotyrosine residues of the activated EGFR and recruits the guanine nucleotide exchange factor (GEF) SOS through two SH3 domains. SOS becomes activated and promotes Ras (a GTPase, often existing in its H-Ras or K-Ras isoforms) to exchange GDP for GTP by destabilizing the Ras-GDP interaction. The activated GTP-bound Ras then leads to activation of Raf (MAP3K). The EGFR-Ras-Raf-MEK-ERK pathway, summarized in Figure 4, is involved in phosphorylation of transcription factors, such as myc, and downstream kinases, such as MNK (MAP kinase-interacting serine/threonine kinase) which subsequently phosphorylates the CREB (cAMP response element-binding) protein. Thus, a signal which initiated by interaction with the extracellular domain of a membrane receptor leads to changes in DNA expression, acting as an ON or OFF switch of the cell cycle.[107] Evolutionary mechanisms of proteins shared among pathways remain unclear. However, allosteric residues at protein−protein interfaces coevolve.[108] Combined mutations by directed evolution have been reported to evolve a robust signal transduction pathway from weak cross-talk.[109] Therefore, it is possible that a number of residues of the shared protein coevolve to merge pathways. Such mutations may be viewed as *latent drivers* in the evolution of cancer.[110]

Ubiquitination signals protein degradation. A sequential cascade initiates with covalent binding of ubiquitin to E1, transfer to E2, and finally to substrates via E3 ligases. The spatial conformational arrangement in each step requires an allosteric cascade in the ubiquitination process. The activation of ubiquitin requires ATP-catalyzed adenylation and thioester bond formation between ubiquitin and an E1 cysteine residue. Spatially, there exists a ∼35 Å gap between the E1 adenylation site and the catalytic cysteine. There is also ∼20 Å gap between the catalytic cysteine sites of E1 and E2.[111] Crystallographic data[111,112] suggested a "thioester switch" mechanism, in which the thioester bond serves as a *driver* to induce a large

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conformational change and shift the population from the inactive to the active state to transfer ubiquitin from E1 to E2. Binding of E3 (gp78) to E2,[113] rather than the overall binding affinity, allosterically induces conformational changes in the active sites, shifting the population to signal the end of the ubiquitin transfer from E1 to E2 and the start of the ubiquitin transfer step from E2 to the substrate. Couplings have been observed between residues of E3 ligases,[114−119] which determine key residues on the allosteric pathways, but not the allosteric efficacy. It is the protein−protein interactions, rather than binding affinity, that shift the populations to the active state to position E2 and the substrate in proximity to conclude the ubiquitination process.

SOME GUIDELINES TOWARD DELINEATING THE ALLOSTERIC EFFICACY

As long as the conformational states of the *active* and major *inactive* structures are available, the structural mechanism of the allosteric agonism can be assessed by following the guidelines provided in the unified view of "how allostery works".[1] The efficacy of allosteric activation is proportional to the stabilization of the *active* state plus destabilization of the prevailing *inactive* state and the activation event can be either an allosteric ligand binding or mutations. If structural comparison of the *active* and *inactive* conformations illustrates changes at the active and allosteric ligand binding sites, a straightforward structural analysis[22] may be sufficient to verify the *driver* and *anchor*. In principle, the parts of an agonist (*driver*) that stabilize the *active* or destabilize the *inactive* state can be distinguished from the (*anchor*) parts that are responsible for the binding. It is the degree of preferential binding to the *active* (or *inactive*) state but not the overall ligand binding potency that determines the allosteric efficacy of activation (or inhibition).

In allosteric regulation, the various extents of structural changes at the active site provide a direct explanation of protein function-switching. However, frequently there are no noticeable structural changes between a liganded *active* (or *inactive*) complex and an unliganded *inactive* (or *active*) protein.[120] In such cases, a simple structural analysis is clearly insufficient to unveil the allosteric efficacy. Three possible situations can account for cases that lack significant structural changes. First, the functional switch is mediated by a disorder-to-order population shift. In this case, only one structure is captured, which is the ordered state. Second, a ligand acting as an allosteric modulator instead of an agonist or inverse agonist is not expected to confer noticeable conformational changes unless an orthosteric ligand or an allosteric agonist are bound at the respective sites. Third, crystal packing or crystallization conditions stabilize the opposite state or destabilize the state promoted by the allosteric action, resulting in limited conformational change. Microseconds molecular dynamic simulations[5] might be able to sample the other prevailing state and overcome such a single state problem.

CONCLUSIONS

The current understanding of the allosteric phenomena is based on molecular conformational ensembles rather than only two, T (tense) and R (relaxed) states. This leads to viewing allostery in terms of population statistics, conformational sampling and probabilities. Allostery works by shifting a populated conformation from one state to another. Because each distinct state has a distinct function, the shift alters the specific function executed by the molecule. Allosteric shift between states takes place through changes in the interactions, which alter the relative stabilities of the states. Allostery takes place in proteins, RNA, DNA and lipids; it propagates through their interactions, and the effects control cell signaling. Allostery can be expressed by small or large conformational (enthalpic) and/or dynamic (entropic) changes.[45] From the evolutionary standpoint, pre-existing optimized states facilitate the emergence of new functions; on the down side, it also leads to "allosteric diseases", driven through pathological interactions, covalent (e.g., mutations) and non-covalent (pathogen proteins), which transform physiological signaling, keeping it in a constitutively ON (or OFF) state. Even though allostery takes place in single molecules, its consequences propagate through their interactions, which may eventually span the cell.[46] Since allostery reflects the behavior of the ensemble, allostery is a statistical effect, and this holds across the structural spectrum, from stable, structured proteins to highly disordered protein states.[57] The probabilistic nature of allostery can be seen from the behavior of inter-domain linkers and loops. Rather than sampling conformational space homogeneously, an allosteric event in one domain can result in biased sampling of space of the other. Evolution has pre-encoded successive conformational states along major allosteric propagation pathways in linker sequences, with each state encoded by the previous one. The lower barriers between hierarchically populated states result in faster time scales even for large conformational changes.[18]

Here, we outlined the principles of allosteric interactions in cell signaling and provided an overview of the mechanisms through which they operate. We distinguished between allosteric efficacy and potency; efficacy relates to the stabilizing/destabilizing effect of the effector on the active/inactive conformational substates of the protein and potency relates to binding affinity. We emphasized that an allosteric propagation pathway does not determine the allosteric efficacy; however, it defines what makes a binding site allosteric. Allosteric propagation consists of residues with coupled behavior. We suggested that two, largely overlapping, sets of residues are responsible for stabilizing the active and destabilizing the inactive states (or vice versa), and that these same residues are also responsible for dynamic fluctuation of the conformations. Finally, we emphasized that to reveal the allosteric mechanism availability of the structures of the active and inactive states is essential. The changes of the local environment and global backbone movement between the two states may provide clues. Conformational changes are likely to propagate through the backbone, whereas the perturbation upon effector interaction at the allosteric site may settle and be accommodated by local conformational changes of side chains.[121] When considering ligand design, it behooves us to recall that the change of affinity for an agonist at the allosteric site does not determine the capacity of the protein to acquire a specific allosteric effect. Instead, it is the relative stabilization (or destabilization) which determines the population shift from one state to the other. Collectively, these provide the underpinnings of allostery.

The overarching challenge is to quantify the allosteric efficacies and integrate the results across proteins and pathways in the living cell. To quantify the allosteric efficacies, a big challenge is to determine the structures of low-populated yet functionally important states, which may be accessible by relaxation dispersion NMR.[75,122,123] How to characterize the dynamics of the allosteric mechanism is yet another challenge

facing experiment and computation. To identify atoms of allosteric effectors that can act as *driver* or *anchor*, which is critical for gaining insight in drug discovery, ultra-high-resolution X-ray structures at subatomic level will be invaluable. How to integrate the current knowledge of allostery at the molecular level across proteins and pathways in the living cell requires collaborative efforts of chemists, biophysics, and systems biologists, for example, to build allo-networks, which depict allostery at the cellular level. Lastly, we behooves us to mention that the concept of allostery has been used beyond the living cell by chemists, including the allosteric modulation of supramolecular chirality in an artificial self-assembled system,[124] biomimetic molecular allosteric analogues in chemosensors to amplify signaling,[125] to incorporate allosteric regulation in organometallic catalysts,[126] as well as in non-cooperative receptors,[127] to design allosterically tunable switches by heavy metals,[128] and to narrow the dynamic range of aptamer-based sensors.[129]

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Notes

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