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The (still) underappreciated role of allostery in the cellular network

Ruth Nussinov^{1,2,*}, Chung-Jung Tsai¹, and Buyong Ma¹

¹Basic Research Program, SAIC-Frederick, Inc. Center for Cancer Research Nanobiology Program Frederick National Laboratory, NCI, Frederick, MD 21702

²Department of Human Genetics and Molecular Medicine Sackler School of Medicine, Sackler Inst. of Molecular Medicine, Tel Aviv University, Tel Aviv 69978, Israel

Abstract

Allosteric propagation results in communication between distinct sites in the protein structure; it also encodes specific effects on cellular pathways, and in this way it shapes cellular response. One example of long-range effects is binding of morphogens to cell-surface receptors, which initiates a cascade of protein interactions that leads to genome activation and specific cellular action.

Allosteric propagation results from combinations of multiple factors; takes place through dynamic shifts of conformational ensembles; and affects the equilibria of macromolecular interactions.

Here, we emphasize (1) the well-known, yet still underappreciated role of allostery in conveying *explicit* signals across large multimolecular assemblies and distances to specify cellular action; (2) the need for quantitation of the allosteric effects; and finally, (3) we propose that each specific combination of allosteric effectors along the pathway spells a distinct function. The challenges are colossal; the inspiring reward will be predicting function, malfunction, and outcomes of drug regimes.

Keywords

Allosteric; cellular pathways; regulation; conformational selection; induced fit; protein dynamics

Introduction

Cell signaling is a paramount requirement for function and has been an overwhelmingly important area of biological research (9; 14; 19; 34; 43; 77; 98; 125). The ability of cells to perceive and efficiently respond to their microenvironment underlies development, differentiation, motility, immunity and cell fate. Errors in signaling can lead to disease. Signals can propagate in multiple ways, e.g. by changes in pH/electrostatic gradients; diffusion; chemical reactions; or physical binding. Allosteric relay in signal transduction can be nanoscale, over hundreds of nanometers (69). From extracellular stimuli to responding nuclear events, signaling is mediated by allosteric events. However, surprisingly, the central role of allosteric propagation in conveying and modifying cellular signaling has been

*Corresponding author: Ruth Nussinov, NussinoR@helix.nih.gov.

underappreciated. In contrast, a large and rapidly increasing number of studies have been focusing on the allosteric effect on the single protein level (11; 12; 18; 20; 23; 27; 32; 35; 39; 50; 51; 61; 67; 71; 74; 82; 88; 92; 93; 103; 113; 119; 121; 123; 127; 129; 132; 133). These have led to a better understanding of how functional sites communicate following an allosteric event, and identified the pathways in the molecular structure through which communication may take place. Thus, while cellular response depends on coordination, which is largely the outcome of long range allosteric propagation, biochemists commonly study the allosteric effects on a single molecule level, largely overlooking their extensions to cellular processes such as genome activation, transcription ON/OFF states, and chromatin remodeling (54; 55; 72; 90; 91; 100; 119). Here we aim to increase the awareness to global allosteric effects, which communicate and translate incoming signals to cell actions. This is important because it may guide interpretations of the consequences of pathological allosteric events, such as binding, combinations of allosteric post-translational modifications (86), and mutations away from the binding site, not only with respect to the protein where they take place; but with respect to the cellular network. This may help in formulating testable hypotheses to relate between syndromes and physiological abnormalities in cells and in the organism as a whole, and dysfunctional, deleterious allosteric events which could be their origin. Tracing molecular events to systems outcome is of crucial importance in diagnosis and treatment. The growing availability of data such as pathway regulation, feedback loops, protein structures and mutational effects, and cellular responses in health and disease, may stimulate the immensely challenging development of methodologies to measure changes in allosteric propagation across proteins, and globally in pathway outputs, and make this a reality.

Signaling involves allosteric propagation; and propagation takes place across membranes; cells; organelles, compartments and large multi-molecular complexes; signaling also regulates and fine-tunes molecular machines (5; 15; 63; 87). Cells communicate with each other via direct contact over short distances; or indirectly, over large distance scales. Direct contact allows precise control of cell differentiation during embryonic development, where the choice of which cell continues to divide is controlled by competition of cell surface signals; indirect inter-cellular communication can take place by signals spreading through diffusion in the intercellular space to interact with cell surface receptors. Alternatively, small molecules may diffuse into the cell through the membrane, and bind intracellular receptors. With cell sizes on the scale of between 10 and 100 μm , allosteric propagation within cells can travel across long distances. Considering that signals can also move through complex and highly regulated feedback loops, the distances covered in intracellular communication can be very large (83). As the allosteric signal proceeds across the cell, it plays key functional roles in deciding which molecules would interact or dissociate. In turn, these events adjust the signals. The principles of communication within proteins and across pathways are similar; however, the increase in complexity is gigantic.

Despite these large distances and immense complexities, signaling must precisely specify molecular and cellular function. Quantitation of the key factors in signaling as they relate to the equilibria of macromolecular interactions under different cellular conditions is essential in order to understand signal propagation; predict the effect of mutations and reliably assess drug actions. This is an enormously challenging task. This review aims to bridge the gap

between allosteric communication in single molecules and across the cellular network; to highlight the mammoth complexity and some of the factors which are involved; and finally to propose possible quantitation venues. Figure 1 provides a schematic illustration of this immense complexity of signaling in the cell, through proteins, nucleic acids, and lipids, and their permanent and short-lived combinatorial associations. We start with an overview of the communication in the cell; explain the concept of allostery from the standpoint of the dynamic conformational ensembles and its propagation in single molecules and across intermolecular boundaries; how signaling may initiate or be altered by non-covalent binding and covalent post-translational modifications; and finally, illustrate these principles through detailed examples. Measurement of changes in allosteric output of signals as a function of events such as pathological mutations and drug regimens can help in reaching the ultimate aim of understanding how intact organisms work.

An overview of communication in the cell

Signaling can transverse single chain proteins; large multi-molecular complexes; and pathways; and through pathway crosstalk, travel across the entire cellular network. The binding of morphogens, for example, transforming growth factor β , sonic hedgehog, Wnt, epidermal growth factor, fibroblast growth factor and retinoic acid, to an extracellular domain of a cell-surface receptor can be a first step in transmitting extracellular information to the cell to activate downstream intracellular signaling events. Although the mechanisms are highly diverse, activation is often coupled to ligand-induced dimerization (as detailed for the receptor tyrosine kinases (62)). The information flows through a series of interactions, cascading through the cell. The binding of transcription factors (TFs) to particular DNA response element (RE) sequences can be viewed as a step in the long-range intracellular communication (40; 72; 76; 90; 91; 119). The DNA acts as allosteric effector; and the allosteric effect propagates from the RE-TF interface, via TF, protein cofactors, the huge 26-subunit Mediator complex, and RNA Polymerase II to initiate gene-specific transcription hundreds of Angstroms away. Crosstalk between signaling cascades generally takes place through shared proteins or through signaling molecules whose concentration is regulated that interact with both pathways. The MAPK/ERK pathway, that communicates a signal from a receptor on the cell surface, through the cellular membrane, a cascading series of protein-protein interactions and phosphorylation events in the cytoplasm, and finally through the nuclear membrane to the DNA can provide an example (36; 38; 101). The signal can initiate when the extracellular ligand (the epidermal growth factor, EGF) binds the cellular receptor (EGFR, epidermal growth factor receptor); this allows Ras (a GTPase) to exchange GDP for a GTP which leads to activation of MAP3K (e.g., Raf). MAP3K activates MAP2K, which activates MAPK. MAPK phosphorylates transcription factors, such as myc, and downstream kinases, such as MNK (MAP kinase-interacting serine/threonine kinase) which phosphorylates the CREB (cAMP response element-binding) protein. Eventually, the signal which initiated by interaction with the extracellular domain leads to changes in DNA expression; acting as an 'ON' or 'OFF' switch of the cell cycle (107).

The glucocorticoid receptor (GR) signaling can serve as another example (102). As detailed in Figure 1b, the pathway can initiate and proceed in multiple complex ways, for example through cytokines binding to cytokine receptors, to c-Jun N-terminal kinase (JNK) which

can communicate with cytoplasmic GR, which is activated upon ligand binding, crosses the nuclear membrane and regulates transcription; alternatively, the cytoplasmic signal can initiate through transforming growth factor β 1 (TGF β) binding to its receptor, and proceed through SMAD signaling. Noteworthy, crosstalk can also take place in the membrane, where interactions with the extracellular matrix (ECM) and neighboring cells can trigger responses within the cell, as well as cytoskeleton assembly and motility.

Signaling propagates in proteins; and through protein-protein interactions across pathways. DNA and RNA can similarly actively receive and transmit signals. Cellular and environmental (e.g. ions, light, irradiation, metabolites, drugs, morphogen concentration as a function of the distance from the morphogen source) factors play key roles in affecting and funneling the relevant information. Mutations can similarly modulate and re-direct the information. On the conformational level, signaling takes place through the allosteric effect. Allostery can be expressed by small or large conformational (enthalpic) and/or dynamic (entropic) changes (112); however, as we discuss below, switches in network states may be related to large scale, slow conformational changes. Thus, allostery takes place not only across single chain proteins and their complexes; it governs communication across membranes, compartments and cells. This leads us to propose that *in vivo*, each combination of allosteric effectors can specify a distinct function.

Principles of allostery in proteins

The phenomena where conformational perturbations at one location in a biomacromolecule affect others have been called ‘allostery’. The cooperative behavior between sites reflects the existence of molecules as ensembles of conformational states. The ensemble has a certain distribution which can be described by the free energy landscape (10; 22; 33).

Environmental changes, for example in temperature, pH; non-covalent binding events (e.g. of proteins, DNA, RNA, cofactors, and small molecule drugs), and covalent (mutational events and post-translational modifications), alter the distribution of the states (58; 70; 114; 115; 117). The redistribution, which we called ‘population shift’ (41), takes place because the relative stabilities of the conformations change. For example, an ‘open’, unbound state conformation might be unstable on its own. However, binding stabilizes it, and the equilibrium shifts towards this conformer. We proposed that the free energy landscape of biological macromolecules in their cellular environment is dynamic; and this dynamism is the essence of function (68; 70; 114; 115; 117). Static free energy landscapes capture a certain distribution under a particular set of conditions, such as that of a crystal structure snapshot. However, association/dissociation events; phosphorylation; acetylation; methylation; oligomerization; interaction with DNA; etc. alter the relative stabilities of the states, and this alteration is accompanied by an equilibrium shift (41; 86; 112; 113). We suggested that this concept is fundamental to the understanding of the allosteric effect and to its propagation across the cellular network (48); and as such to function. Figure 2 presents a schematic description of the shift in the free energy landscape following allosteric events.

Similar to protein folding (22), allosteric perturbation propagates in the structure via *multiple*, major and minor, pathways (20); thus, we argued that a change in conditions will alter not only the distribution of the conformational states, but also the relative distributions

of the pathways. Because function requires efficient communication between the allosteric and the active (or functional) sites, mutations in the protein occurring on major allosteric pathways which are likely to affect the distribution, frequently cause disease. Aside from mutations at the active site which directly impair function, allosteric mutations are major causes of functional disorders. Examples include prevention of an essential conformational change by a tumor-associated mutation (126) and gate-keeper cancer-related mutations in tyrosine kinases (2); ABL and EGFR kinases (23; 128).

Prior allosteric event in one site may lead a second site to prefer one partner over another

In vivo, signaling often initiates, or is altered by, non-covalent binding and covalent post-translational modifications; and most, if not all, proteins interact with more than one partner. Some of the interactions are ‘permanent’, i.e. long-lived; others transient (89). The number of partners varies: mostly only few; however, the number can be very large, in the tens and even in the hundreds. The only way a single protein can accommodate that many partners is by partners sharing the same site. Shared-site interactions are transient. Transient interactions are the hallmark of dynamic regulation in the cell (131). Permanent protein-protein interfaces are typically extensive ($\sim 1000\text{--}4000\text{ \AA}^2$), tightly packed, and largely hydrophobic (118); transient interfaces are often much smaller, more hydrophilic/charged, and mediated by water. Intriguingly, the partners may or may not be structurally similar (53). Proteins with multi-partnered sites raise two key questions: (1) how can proteins bind *specifically* more than one partner at the same site; and (2) what are the functional consequences to the cellular network. These questions are important because specific binding events at specific times determine the eventual cellular response.

How can proteins bind specifically more than one partner at the same site?—

Evolution appears to have adopted several strategies (116). Among these, (1) all partners sharing a binding site have the same, *specific* motif; (2) even if the surfaces of the partners differ, they often share conserved interactions; (3) each distinct conformations of the binding region ensemble can select its most favored partner (7; 17; 68; 114; 115); (4) a single binding site conformation can interact with different proteins through combinatorial domain linkage to a common binding motif; and (5) protein disorder; disordered proteins have broad, (almost) equally-populated states, with varied binding site conformations which preferentially select the most complementary partner (117). The multiple conformations of the free receptor permit conformational selection events to a broad range of ligands. The specific selected ligand molecule largely depends on two factors: first, the relative local concentration; the higher the concentration the higher the likelihood; and second, prior allosteric events. Prior allosteric events can be a key factor in deciding which partner will bind: they lead to a population shift, which may result in a change in the preferred conformation of the second site, and thus affect partner selection.

The two membrane-anchored hub protein families Eph receptor tyrosine kinases and their ephrin ligands can serve as examples of proteins with shared binding sites (59; 84; 97). Ten EphA and six EphB receptors evolved to interact with six ephrin-A and three ephrin-B ligands, respectively, maximizing the number of combinations of Eph-ephrin interactions; however still maintaining specificity, a principle often encountered in evolution. While the

interactions between the Eph receptors and ephrins of the same subclass are promiscuous, cross-subclass binding is observed only for two receptors: EphA3 can bind ephrin-B2, and EphA4 interacts with all nine ephrin ligands. Each of those nine has a different function. Figure 3 shows the crystal structures of those nine EphA4 ligand binding domain (LBD) conformations in complex with the ligands. Recently, two new crystal structures of EphA4 revealed eight additional unique conformations in each crystal structure (97). These multiple conformations of the free EphA4 LBD illustrate how conformational dynamics of EphA4 and the Eph-ephrin can facilitate cross-subclass ephrin signaling.

What are the roles of the allosteric effects and the functional consequences to the cellular network?—As illustrated schematically in Figure 4, the transient binding of each ephrin ligand leads to (slightly) different preferred conformation of the second site and/or altered binding site dynamics. These may lead to selecting different partners and thus pathways and functional outcomes. Below, we focus on the propagation across the cellular network. The combination of short-lived binding events which initiate or staple pathways in response to cell cycle events and environmental cues, and permanent pre-organized multimolecular complexes is a powerful theme in evolution.

Allosteric effects propagate across protein-protein interfaces

Evolution aims at *efficient*, *specific* and *targeted* cellular response; and the phenomena harnessed to achieve these aims are allosteric propagation. Population shift takes place not only across proteins (60); it continues, and spans their interactions with other proteins (6; 7; 60; 67; 73; 79; 96; 130); nucleic acids (35); and lipids; along the pathways (1; 85), and throughout the cell. Propagation takes place across permanent and transient, short lived interactions, and can be helped by molecular disorder.

The cellular network is also dynamic

Signaling pathways consist of series of molecular interactions. Cellular networks are composites of interconnected pathways; and network dynamics are changes in these series of interactions, which may affect the interconnectivity of the pathways. A drug often blocks a specific major pathway; drug resistant mutations may overcome drug effects by changing the interconnectivity. Pathway and network dynamics reflect interplay between the changes in the distributions of the conformational ensembles of the proteins composing the pathway, which in turn are also affected by the changes in the network. Figure 5 provides an overview of how signaling pathways and the cellular network can change following allosteric population shifts in the protein; and how changes in the pathways can affect the conformational distributions in the protein. Collectively, these can lead to major alterations in cellular behavior.

A good example of the dynamic changes and their consequences is provided by the effect of mutations in cancer, where each type can have its mutational make-up. Since different cancers may have different sets of mutations, the signaling pathways are shifted in altered ways, which may be observed in the differing chances of the patient survivability. These distinct sets of effects were recently observed to be reflected in ‘signatures’ embedded in these networks (8), with cancers that have more complex molecular pathways being more

refractory than those with less complex molecular pathway. Another large scale study illustrated that somatic mutations and copy number alterations resulted in alteration of the receptor tyrosine kinases (RTKs) and RAS/MAPK pathway (RTK/RAS) across most cancer types, and the JNK pathway to be significantly altered in HR1 breast cancer (47). Mutations can also affect network dynamics by turning a protein into a constitutively ON (or OFF) state, as in the case of the signal transducer and activator of transcription 3 gene (STAT3) (56). The SH2 dimerization and activation domain of STAT3 is frequently mutated in patients with large granular lymphocytic leukemia. Structural and functional data suggest that the mutations result in STAT3 hyperactivation which apparently confers ligand-independent signaling. Dynamic changes in the network can take place in response to changes in allosteric post-translational modifications, metabolic conditions, and changes in the environment, such as protein and ligand concentrations, irradiation and light (111).

That the cellular network is dynamic and sensitive to its surroundings can also be strikingly seen in the varied responses of different cell lines to different stimuli, which may also help in identifying key network hubs that act as signal amplifiers. Recently such an example was published. It focused on the function of a non-receptor guanine nucleotide exchange factor (GEF) of GIV (Gα-interacting, vesicle-associated protein, Girdin) (37). *In vivo* signaling assays of mutants with different Gαi-binding and -activating properties showed the impact of changes in GEF function on cellular responses. The experiments revealed a threshold effect of GEF activity for the activation of phosphoinositide 3 kinase (PI3K)-Akt signaling in different cell lines, and by different stimuli: Akt signaling was minimal at low levels of GEF activity and sharply increased to reach a maximum above a threshold of GEF activity. This suggested that GIV is a critical signal amplifier and that activation of Akt is ultrasensitive to changes in GEF activity. Further, similar threshold dependence was observed for other biological functions promoted by GIV, such as remodeling of the actin cytoskeleton and cell migration. Thus, while the number of GIV copies may determine the global amplitude of the response, GIV's GEF function acts as a critical regulatory element (37).

Allostery and “all-or-none” network cellular response switches

Above, GIV's GEF function serves as the critical regulatory element in the network that shapes cellular response, and as such was described by the authors as an “all-or-none” switch. Thus, a drug that targets GEF's function might be therapeutically relevant for the treatment of cancer metastasis by abolishing the enhanced PI3K-Akt signaling and cell migration (37). Networks have often been described as being in bi-stable switch-states; and disease as transitioning the network from a healthy to a disease state. Persistence of a disease is described as implying that the cellular network is ‘locked’ in this state. While in reality cellular response is graded, from the functional standpoint, bi-stable networks can indeed be viewed as toggling between two, ON/OFF states (49; 110; 120). Surprisingly, while there has been abundant literature on bi-stable network switches and their relevance to disease and drug regime strategies, the implications of these from the mechanistic molecular standpoint have never been considered. This is probably because network topology and conformational free energy landscapes of proteins relate to different scientific disciplines.

Like GEF, a key regulatory motif can be viewed as a switch that converts a continuous input signal into a discontinuous ON/OFF response. Additional examples include the mitogen-activated protein kinase (MAPK) cascade (3; 4; 28–31; 75; 108; 124), the sonic hedgehog network in stem-cell differentiation (75), cell-cycle regulatory circuits (16; 94; 104), and the rapid lateral propagation of receptor tyrosine kinase activation (99). Switches may be simple, composed of a single protein which dominates the network; or a complex, composed of a group of proteins which form a network core. A recent large scale analysis of the network of human lung, liver and hepatocellular carcinomas was able to identify genes that control the cancers' bi-stable states and follow their effects in the malignant transition, and as such can potentially serve as drug targets (109). One example related to induced (by Oct3/4 and Sox2 with either Klf4 and Nanog or Lin28) pluripotent stem cells which led to a switch from mutual activation or inhibition.

Even though proteins consist of conformational ensembles with a broad range of states, from the functional standpoint we may *observe* bi-stable states. This reflects a threshold effect: we may observe function only above a certain level. Because all conformations pre-exist, including the native functional state, function will always be performed at some level. However, from the biological standpoint, that level may be too low to be useful to the cell. Non-covalent binding, or post-translational modifications of the critical control elements in the network, may induce a relatively large change in the protein conformational distribution, and as such to an *apparent* “all-or-none” network switch.

Large conformational changes may play a role in network ON/OFF switches

Large conformational change usually creates a large free energy gap between the two states. The shift between the two states can be triggered by some allosteric event, such as noncovalent binding (as long as the concentration is sufficiently high to populate the binding site); or a covalent event (in which case, the signal can last longer). In an ultra-sensitive protein such as in our GIV's GEF example above, even a small change in the concentration can be sufficient to shift the relative distribution of the ensemble, driving it to this second state. If the free energy gap is larger, as it may be in the case of a large conformational change, the shift between the states will be sharper. When we consider the cellular network, this sharp shift in the protein at the top level of the pathway can switch it between the ON/OFF states; and in a simple scenario, this will lead to an ON/OFF switch of the network in the bottom, output level.

Network switches control cellular network ON/OFF states by switching between conformational states. From the free energy landscape standpoint, a ‘network stable’ state is when a conformational state is trapped. To be trapped, there should be a high barrier between the states, each presenting a distinct minimum on the free energy landscape (Figure 2). A protein which has a flat free energy landscape is unlikely to be a switch. Because the time scales of conformational transitions relate to the barrier heights (7), high barrier may imply trapped conformation. Switching suggests conformationally distinct states, with equilibrium that favors one or the other. In disease, higher ligand concentration or mutations away from the binding site allosterically change the thermodynamic equilibrium of the open versus the closed states. An allosteric drug can similarly shift the equilibrium. However,

while the conformational (or oligomerization) changes can transduce a binding event into a specific output signal which is sensed by another molecule (122), to ensure a lasting switch, nature chose covalent allosteric PTMs.

Network switches may be composed of a single protein; however, a multi-molecular assembly is likely to be more common. Since switches are intrinsic control points where an allosteric event induces a relatively large change in the protein conformational distribution, they may be expected to involve large conformational changes. Large conformational changes may entail high barriers and long (microsecond/millisecond) timescales. Domains connected by linkers and large loop/linker movements may provide good examples, as in the E3 ligase system (66), adenylate kinase (95; 106), L to T transition in the multidrug transporter (26), 'lid' opening/closing, as in the HSP90, and helix movement (which is similar to a large loop) in the estrogen receptor ligand-binding domain (44). Because hinge bending motions may take place over long time scales, in the absence of the allosteric effector the conformation appears locked. There are numerous examples, including the cAMP-induced conformational change in the intracellular cAMP sensor Epac2 activation where the hinge-bending flip is further hindered by an ionic latch (64).

Nonetheless, barriers are passable, linkers can be encoded for faster time scales, and the states will be sampled even in the absence of the allosteric effectors, albeit to a lesser extent. This is clearly seen in molecular dynamics simulations (24; 25): if the simulations time scales are sufficiently long, barriers will be overcome and the 'open' conformation will be sampled. The conformation is effectively locked in the activated (or deactivated) state only so long as the allosteric effector is there.

Within this framework it behooves us to note that large, multi-unit molecular machines typically have distinct functional ON/OFF states; and they undergo large conformational changes accompanied by large changes in the distributions of their ensembles. It will be interesting to carry out a large scale structural analysis of network control proteins to see if, or to what extent, they present large conformational changes.

How to quantify allosteric effects?

Allosteric effects can be usefully measured; and some studies have already been carried out primarily aiming to assess the effects of designed allosteric modulators. Traditional methods focused on measuring the affinity using radiolabeled ligands. Subsequent improvements aimed to have more homogeneous biochemical fluorescent assays to characterize orthosteric ligand affinity and dissociation rates that are sensitive to the presence of the allosteric modulator (45). Modulation of a receptor was also evaluated using kinetic experiments which allowed determining the delay of radioligand dissociation caused by allosteric modulation (57); and allosteric effects were quantified by comparisons of dose-dependent curves obtained by varying the concentrations of allosteric modulators with model-based predictions. Several approaches to quantify an allosteric interaction based on formation of a ternary complex that describes an allosteric interaction between two ligands binding concomitantly to topographically distinct binding sites on the same receptor protein, were reviewed (13), focusing on the behavior of allosteric modulators at both ligand-gated ion channels and GPCRs. A comprehensive overview of the quantification of allosteric effects as

related to allosteric modulators can be found in a recent textbook (52). Kenakin has also pointed out that allosteric modulators may also have direct effects, for example by stabilizing the active state independent of the natural endogenous agonist binding, resulting in an additive effect.

Differing from all above strategies, Ming and Wall (78) developed theoretical tools to quantify allosteric effects based on a theory of allosteric regulation that explicitly considers changes in the protein conformational distribution.

Thus, steps toward quantification of the outcomes of single allosteric effectors have been taken, and some of these have been proven useful in evaluation of allosteric modulators. Nonetheless, the aim of quantification of the collective allosteric effects taking place *in vivo* is immensely more complex. The large number of potential binding partners and their combinations which may associate simultaneously, and their possible dissociation events which similarly perturb the protein conformations; and coupled with these the gigantic number of (orthosteric and allosteric) post-translational modification types and combinatorial co-occurring linkage and cleavage events, presents what currently seems an insurmountable challenge. Moreover, the effects will vary in different protein environments. For completeness, these should also be combined with allosteric mutations.

To address this challenge, we propose the following steps: First, quantify the allosteric effects of the most common single post-translational modifications, such as phosphorylation, ubiquitination, and acetylation (21) in highly interacting protein domains. Candidates can include 14-3-3, MH1; FF; Sh2; FHA; TRAF; Bromodomain; SH3 (46). In all, phosphorylation and acetylation are common modifications, although frequently in the binding sites. Protein recognition domains recognize certain motifs, which could help in benchmarking the measurements. Second, quantify the allosteric effects of single binding events of these domains, taking advantage of their common recognition motifs. Starting from single allosteric events is similar to quantification of allosteric modulators. Third, test some combinations of such events. Fourth, quantify the outcomes for highly homologous family members; this can be helpful in providing certain variability scales.

Why quantify allosteric effects? Quantification of effects in single proteins is important; however, the overriding aim is to quantify the allosteric effects in the network, that is, to quantify the collective cellular outcome. Quantification can help in the understanding of how the network works, and it may account for pathological mutations, and in this way allow predictions. The goal of measuring changes in allosteric propagation in pathway outputs is immensely challenging; while currently it is not feasible to quantify the allosteric functional codes, obtaining some quantitative parameters for several different common occurrences in the cellular network is already expected to provide some clues into pathway outcomes.

Conclusions

Cellular signaling, key proteins that control it and how changes in these relate to disease, is a major focus of paramount importance in the biological sciences. At the same time, ample literature spanning tens of years documents allosteric effects in proteins; their functional

roles, and their theoretical underpinning. Experiments (60) and theory support the view that allosteric effects propagate across the boundaries of interacting biomacromolecules, and that they can have profound functional effects. Despite this, allostery is still largely perceived as acting on single proteins and their allosteric effectors, for example, the allosteric effects induced by the binding of DNA recognition elements on the glucocorticoid receptor (40; 72; 76).

Allosteric propagation is not the sole element responsible for signaling in cells. Factors such as relative concentrations, cell metabolism, organism development, diffusion through the membrane play critical roles. However, allostery is a major player. Allostery harnesses the fundamental property of biomacromolecules: the fact that they exist as broad and dynamic conformational ensembles. Evolution exploited this property which allows selection of (complementary) ligands, followed by re-distributions of the conformations. In this way the dynamic shift of the conformational ensemble affects the equilibria of macromolecular interactions. Because multiple varied allosteric events (always) take place simultaneously, the shape of the second binding site is a composite of all; each combination of allosteric events specifies distinct function.

Here we emphasize the need to piece together allosteric events in molecules into the key role of allostery in signaling pathways and collectively in the cellular networks. We believe that this will help in clarifying processes and how they are regulated. Further, for in depth understanding, and in particular for prediction of the consequences to the cell, there is a need to classify the combinatorial effects; that is, the distinct function spelt by a specific combination of allosteric effectors. We emphasize the need for quantitation of the allosteric effects which would allow measuring changes in allosteric propagation in pathway outputs. Currently, allosteric effects are largely provided in qualitative, descriptive terms. The challenges are huge; however, experiment and theory support the view that allostery is a fundamental *cellular* phenomenon, and as such it cannot be overlooked.

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A mini-glossary

Allostery

Allostery is the phenomena where perturbation at one site in the molecule affects an active site far away

Propagation pathways

Propagation of the perturbation from one site in the protein structure to another can proceed via multiple distinct pathways

Allosteric effector

An allosteric effector is a physical phenomenon, such as binding of a molecule, or a change in temperature or pH, which perturbs the protein structure

Conformational ensemble

The conformational ensemble is the collection of all conformational states of the protein molecule and its complexes

Conformational distribution

The distribution of the conformers in the ensemble is dynamic, and changes when the structure is perturbed

Population shift

The change in the distribution of the conformation in the ensemble

Cellular pathways

The signal propagates in the cell through regulated proteinprotein interactions, which collectively define a cellular pathway

Cellular network

The composite of all interconnected pathways intersecting through shared proteins

Intra-cellular communication

the transfer of the signal across the protein structure and across the cellular pathway and network

A list containing all important acronyms

TF	transcription factor
RE	DNA response element
MAPK	mitogen-activated protein
ERK	Extracellular signal-regulated kinases
EGFR	epidermal growth factor receptor
Ras	small GTPases involved in transmitting signals in cells
CREB	cAMP response element-binding protein
GR	glucocorticoid receptor
SMAD	intracellular proteins that transducer extracellular signals from transforming growth factor β ligands

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A summary list highlighting the central points

1. Allostery harnesses the fundamental property of biomacromolecules: the fact that they exist as broad and dynamic conformational ensembles. Evolution exploited this property which allows selection of ligands, followed by re-distributions of the conformations.
2. Allostery conveys *explicit* signals across single proteins; large multimolecular assemblies and long-range distances in the cell to specify cellular action.
3. There is a need to *bridge* the gap between allosteric communication in single molecules and across the cellular network.
4. *The dynamic shift of the* conformational ensemble affects the equilibria of macromolecular interactions. Because multiple *varied* allosteric events (always) take place simultaneously, the shape of the active (or functional) site is a composite of all.
5. *Each specific combination* of allosteric effectors along the pathway spells a distinct cellular function.
6. *There is a need for quantitation* of the allosteric effects and their combinations which would allow measuring changes *in allosteric* propagation in cellular pathway outputs.
7. *The complexity of the allosteric* code in the cell has a mammoth complexity.
8. *The role of allostery in* global cellular *functions* has been underappreciated

Figure 1.

The complexity of signaling in the cell. (A) The figure illustrates extracellular stimuli transmitted through the membrane, cytoplasm to the nucleus, and cellular response via turning genes on/off. Signaling is (often) mediated by allosteric events through proteins, nucleic acids, and lipids, and their permanent and short-lived combinatorial associations. (B) An outline of the estrogen receptor (ER) signaling pathways as an example of the complexity of signaling in the cell. Estrogen receptors (Era and ERP) can be selectively activated by ligand binding (80). ER activation is controlled by extracellular signals, hormone and co-factor binding events (105). Extracellular signals lead to phosphorylation of the ER monomer. Examples of the extracellular signals are: (i) binding of dopamine and cAMP to GPCR can activate PKA; (ii) growth factors (GFs) activate their receptors with subsequent activation of the RAS- RAF-ERK pathway; and (iii) non-genomic action of ER in the membrane activates the PI3K-Akt pathway. Both antagonist and agonist ligands can prompt ER dimerization with different allosteric consequences for the helix H12 position. The shift in helix H12 triggered by antagonist ligands blocks subsequent co-factor binding, while agonist ligands allosterically change the ER conformations to allow co-factor recruitment. Cofactors (42) like the nuclear receptor corepressor (NCoR) and the repressor of the estrogen receptor activity (REA) lead to repression of ER response elements (ERE).

Examples of direct activators are the thyroid hormone receptor (TRAP), steroid receptor activator (SRA), and steroid receptor co-activators (SRCs). Secondary co-activators (like CoCoA and PRMT) also bind ERS indirectly through association with SRCs. Thus, the network diagram provides an overview of the binding events.

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Conformational Switch via a Binding Event

Figure 2.

Schematic description of the shift in the free energy landscape following allosteric binding events. The activation of cyclin-dependent kinase 6 (CDK6) is controlled by the regulatory subunits of various cyclins. On the left in Figure 2A, the free energy shifts from the favorable inactive conformation of apo CDK6 (green) to active conformation (red) following binding to a virus-encoded cyclin (v-Cyclin). The conformational change of CDK6 due to the allosteric binding of v-Cyclin (orange ribbon) is highlighted in dark color on the right by superimposing the two CDK6s with the apo inactive CDK6 (PDB 3nux) depicted by a green ribbon and the cyclin-bound active form (PDB 1jow) in a red ribbon. In Figure 2B, the energy landscape adversely shifts from the active state (red) back to the favorable inactive state (green) after the p18 (yellow ribbon) binds to the CDK6 (green ribbon) which is in complex with k-Cyclin (yellow ribbon). As shown in the dark color part of the two superimposed CDK6s, the hallmarks of activated kinase conformation (red) were lost due to the conformational changes (green) which were caused by the allosteric p18 binding.

Figure 3.

The extracellular Eph region is a long distance away from the intracellular region; and the signals are transmitted over these distances to control cell movement. The Eph contains a conserved N-terminal ligand-binding domain (LBD), an adjacent cysteine-rich domain (CRD), followed by two fibronectin repeats (FN3) in the extracellular Eph region; it is connected through trans-membrane domain (TM), and linked to a kinase domain, and a SAM domain (not shown). The LBD domain has multiple open and closed conformations recognized by different ligands. On the right hand-side, nine EphA4 ligand binding domain (LBD) conformations are in complex with the ligands. Recently, two new crystal structures of EphA4 revealed eight additional unique conformations in each crystal structure (97). These multiple conformations of the free EphA4 LBD illustrate how conformational dynamics of EphA4 and the Eph-ephrin can facilitate cross-subclass ephrin signaling. The crystal structures are taken from the PDB, codes: 3MX0.

Figure 4.

The transient binding of each ephrin ligand A1, A2, B1, B2 (α , β , γ , δ) to different conformations of the Eph receptor (A, B, C, D) leads to (slightly) different preferred conformation of the second site and/or altered binding site dynamics. These may lead to selecting different partners (A α , B β , C γ , D δ) and thus pathways (I, II, III, IV) and functional outcomes (59; 84; 97)

Figure 5.

An illustration of how diversified signaling pathways are achieved via allostery in G protein-coupled receptors (GPCR), also known as seven-transmembrane spanning receptors (7TMR). The first step of the diversified GPCR signaling involves a process of conformational selection among the multiple active states of a single GPCR. The population shift toward one of the active GPCR conformations elicited by the bound ligand leads to two distinctive pathways, the agonist (G protein-dependent) pathway and the biased agonist (arrestin-dependent) pathway. The binding of an agonist referred as ‘the classical model’ results in the activation of heterotrimeric G proteins and promotes the consequent signaling of second messenger such as cyclic AMP. However, the activated conformations of GPCRs also bind and activate GPCR kinases (GRKs) to phosphorylate Ser/Thr in the cytoplasmic loops and tail of the GPCR. In turn, the phosphorylation enables the recruitment of β -arrestins to mediate receptor desensitization and internalization. The biased agonist pathway emphasizes that a distinct active GPCR conformation activates a different set of GRKs to create phosphorylation patterns (‘barcode’) on GPCR. It is the barcode that imparts distinct conformations (illustrated in different colors in the Figure) to the recruited arrestin either through orthosteric (at the binding site) or allosteric phosphorylation events, which in turn

mediate different signaling pathways such as the ERK 1/2 activation. Note that the detailed cell/tissue- specific variability of regulated signaling pathways as well as those related to isoforms of GPCRs, GRKs, and arrestins, is not included in the Figure (65; 81).

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