

Allosteric Regulation of Proteins

A Historical Perspective on the Development of Concepts and Techniques

Kabir H Biswas

Allostery is a mechanism by which the activity of a large number of proteins is regulated. It is manifested as a change in the activity, either ligand binding or catalysis of one site of a protein due to a ligand binding to another distinct site of the protein. The allosteric effect is transduced by a change in the structural properties of the protein. It has been traditionally understood using either the concerted MWC (Monod, Wyman and Changeux) model, or the sequential KNF (Koshland, Nemethy and Filmer) model of structural changes. However, allostery is fundamentally a thermodynamic process and requires an alteration in the enthalpy or entropy associated with the process.

Introduction

The regulation of essential biological processes such as metabolism, growth and reproduction is a hallmark of living systems. Cells – the fundamental units of life, employ a variety of mechanisms to achieve an exquisite control over these processes, and regulation of the activity of proteins is one of the means to fine-tune cellular processes. One of the ways of controlling the activity of proteins is to alter the absolute levels of the protein in a cell. This is achieved either by altering the levels of the mRNA coding for the protein, or by altering the stability of the protein in the cellular environment. Transcriptional regulation, along with the presence of microRNAs, is a common mechanism employed by cells to alter the levels of mRNA. Steady state amount of a protein in the cell is essentially determined by the balance of its rates of synthesis and degradation. The stability of a protein can be regulated by different mechanisms including degradation, molecular



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Keywords

Proteins, allostery, regulation, enthalpy, entropy.



The stability of a protein can be regulated by different mechanisms including degradation, molecular crowding, disulfide bond formation, and small molecule binding.

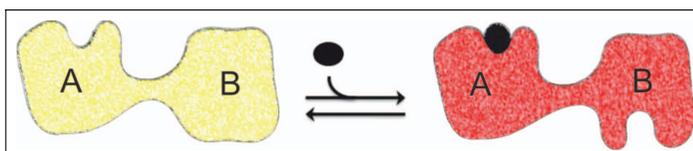
crowding, disulfide bond formation, and small molecule binding.

In addition to controlling the absolute levels of a protein, the activity of a protein can also be regulated by covalent modifications, interactions with other proteins, or binding of small molecules. Covalent modification of a protein involves the attachment of a functional group to the protein resulting in phosphorylation, glycosylation, or acetylation. A protein can also be covalently modified by the attachment of another protein such as ubiquitin, small ubiquitin-like modifier (SUMO), and prokaryotic ubiquitin-like protein (Pup) resulting in ubiquitination, sumoylation or pupylation, respectively. Protein–protein interactions also play a major role in regulating the activity of a large number of proteins and can include homo- or hetero-oligomerization of proteins. However, the most versatile mechanism of regulation of protein activity is the non-covalent binding of small molecules such as metabolites, signaling molecules and metal ions to proteins. This mechanism is generally referred to as allostery (*Figure 1*).

Allostery is defined as the phenomenon in which the function of a site in a protein is altered by a change in another distinct site known as the allosteric site [1–3]. In addition to binding of small molecules, factors such as pH, temperature, ionic strength, and binding of peptides or protein molecules can also give rise to allosteric effects. An allosteric effect is usually brought about by a change in the structure of the allosteric site followed by transmission of the signal to the functional site of the protein. Recent advances in structural biology techniques have revealed that allosteric effects can also be seen in the absence of a change in the mean structure of the protein [4, 5].

Although the mechanisms of regulation of a protein are often discussed disconnectedly, seldom is a cellular protein regulated

Figure 1. Schematic representation of allostery in a protein. Ligand binding to domain A in a two-domain protein induces a structural change in domain B, leading to an alteration in the activity of domain B.



by only one mechanism. Rather, the function of a protein present inside a cell is a cumulative result of all the possible forms of regulation that the protein can undergo at that instance. For example, while allosteric ligand binding may activate a protein, regulatory phosphorylation following allosteric ligand binding may stabilize the protein in the active form, thus allowing sustained activation of the protein.

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Early Observations of Allostery

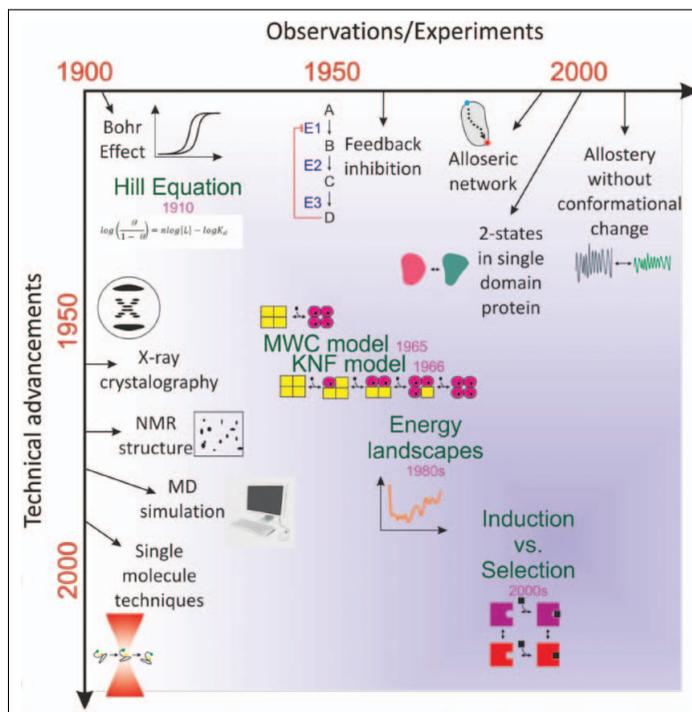
The observation of a shift in the sigmoidal oxygen-binding curve of haemoglobin with changing pH, in the early 20th century, marked the beginning of the history of allostery (*Figure 2*). This shift in the oxygen-binding curve is called the Bohr effect, named after its discoverer [6]. Shortly afterwards came the mathematical equation to explain the reversible association of oxygen to haemoglobin; derived by Hill in 1910. Popularly called the Hill equation, it allowed description of the fraction of a protein saturated by a ligand as a function of the ligand concentration. Accounting for the oligomerization of haemoglobin in the Hill equation allowed the development of the idea of the Hill coefficient, which provided an estimate of the effect of ligand (oxygen) binding to one of the monomers of haemoglobin on the successive binding of the ligand to other monomers in the tetramer. In other words, it allowed the determination of the degree of cooperativity in oxygen binding to haemoglobin.

The ‘Regulatory’ Site

The observation of feedback inhibition of metabolic enzymes [7, 8] in the middle of the 20th century led to a phenomenal progress in the field of allostery (*Figure 2*). The concept of feedback inhibition was proposed based on the observation that isoleucine inhibits the initial step in the isoleucine biosynthesis pathway [7]. Negative feedback is a mechanism that allows for a control over the levels of metabolites in a cell that are generated via natural biosynthetic pathways (‘cellular economy’). Multi-



Figure 2. Evolution of prominent observations and technical advancements in the field of protein allostery. Development of major concepts is depicted on the diagonal.



ple enzyme-catalysed steps constitute biosynthetic pathways, and the rate of each of these steps can potentially determine the flux through the pathway. Inhibition of the enzyme catalysing the first step by the end product of the pathway, however, is a commonly used mechanism to control the flux through the pathway. Intriguingly, the substrate or the product of the inhibited enzyme can be structurally different from the inhibitor. For example, while aspartate transcarbamoylase (ATCase) uses aspartate and carbamoyl phosphate to generate N-carbamoyl-L-aspartate and inorganic phosphate (Pi), it is inhibited by the structurally different cytidine triphosphate (CTP). The inability to explain this phenomenon within the framework of competitive inhibition led to the idea of a regulatory site in the enzyme which is distinct from the catalytic site. Activation of ATCase in the presence of adenosine triphosphate (ATP), in contrast to the inhibition seen in the presence of CTP, further indicates that these molecules bind to a site that is distinct from the catalytic site. In addition, incu-

bation of ATCase at elevated temperatures results in specific loss of the regulatory activity without any loss in the catalytic activity. Thus, the activity of an enzyme is modulated through ligand binding to a distinct ‘regulatory’ site.

The Structure–Function Paradigm

The concept of distinct catalytic and regulatory sites broadened the scope of the type of interactions that are required for the regulation of protein functions. Monod and Jacob renamed the regulation of proteins in this fashion as ‘allosteric’ in the year 1961 [9]. The word allostery originated from the Greek words *allos* meaning ‘other’ and *stereos* meaning ‘solid’ or ‘object’ or ‘site’, referring to the regulatory site of these proteins. Following this, the revolutionary, Monod, Wyman, and Changeux (MWC) model was proposed in the year 1965 to describe the allosteric regulation of proteins. The model postulated that allosteric proteins are oligomers, with monomers in a symmetric arrangement. An allosteric effect is produced by binding of a ligand that leads to a change in the quaternary structure of the protein. This occurs by a rotation of the monomers about an axis such that the symmetry of the oligomer is preserved. The model is also called the ‘concerted’ model of allostery [10]. Immediately following this, the Koshland, Nemethy, and Filmer (KNF) or ‘sequential’ model of allostery was proposed in the year 1966. In this model, the subunits of the oligomeric enzyme need not be in the same conformational state and therefore, need not be symmetric. Ligand binding to one of the subunits causes a change in the structure of that subunit to produce an ‘induced-fit’. This change in the structure of the ligand-bound subunit subsequently alters the ligand binding properties of other subunits [11].

Appropriate functioning of a protein requires dynamic structural changes involving both rigid as well as flexible parts of the protein. These structural components show movements on a wide range of timescales ranging from picoseconds to seconds, including atomic vibrations to global motions of the molecule. The

Protein function requires structural flexibility.



¹See Hanudatta S Atreya, *Resonance*, Structures of biomolecules by NMR spectroscopy, Vol.20, No.11, pp.1033–1039, 2015.

²See Ramanathan Natesh, *Crystallography beyond crystals: PX and SPCryoEM*, *Resonance*, Vol.19, No.12, pp.1177–1196, 2014.

allosteric motions take place in the slower timescales and are usually global in the spatial scale. As is true for any scientific endeavor, progress in the field of allostery was driven by methodological advances such as X-ray crystallography, Nuclear Magnetic Resonance (NMR)¹, molecular dynamic simulations, and single molecule experiments². For example, the structural changes that allowed for allosteric regulation of haemoglobin were revealed through structural elucidation of the protein in free and oxygen-bound forms by X-ray crystallography. Following this, X-ray crystallography has been utilized to study a variety of allosteric proteins including ATCase. These studies have not only revealed large changes in the organisation of regulatory and catalytic domains of the protein, but also the detailed structural reorganisation of specific residues in the catalytic domain that is required for allosteric regulation of the enzyme.

While X-ray crystallography provided snapshots of a protein under different conditions, development of NMR allowed appreciation of the dynamics associated with protein structure, revealing a spectrum of conformations displayed by proteins in solution. Meanwhile, development of various computational tools provided insights into protein dynamics *in silico*. Single molecule studies of proteins further allowed detection of dynamic fluctuations in activity, transient intermediates in enzyme-catalysed reactions, and multiple, metastable configurations of proteins.

Energy Landscapes

The revelations of the inherent dynamics of proteins led to the development of the concept of protein ‘energy landscape’ [12, 13]. This allowed mapping of the free energy of a protein against all of its possible conformations. For example, in the process of folding, a linear chain of amino acids goes through different arrangements of the polypeptide chain to achieve the native tertiary structure. Further, a folded protein can visit conformational substates that are described by the energy landscape in the solution. These substates are in dynamic equilibrium,

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and follow statistical thermodynamic distributions. Thus, a protein can sample multiple conformations in solution, and each of these conformations carries out its function via a specific path. For instance, NMR studies of dihydrofolate reductase (DHFR), an enzyme that carries out the multistep reduction of dihydrofolic acid into tetrahydrofolic acid using reduced nicotinamide adenine dinucleotide monophosphate (NADPH) as an electron donor, revealed that multiple conformations of the enzyme perform catalysis via their own reaction coordinates that display distinct thermodynamic parameters. Thus, proteins exist as an ensemble of conformers in a solution with each conformer possessing specific structural and functional properties.

Mechanism of Allostery

The structure–function paradigm presupposes a change in the structure of a protein for allosteric effects to occur. This change in the structural state leads to an altered function of the protein, such as a change in the affinity for its ligand. For example, the low affinity tensed (T) state of haemoglobin has to shift towards the high affinity relaxed (R) state to display allostery. Such structural states have been observed experimentally for a variety of proteins. For instance, haemoglobin tetramers with distinct oligomeric structures or protein kinases with distinct orientation of the N- and C-lobes have been revealed by X-ray crystallography.

The concept of energy landscapes of proteins implies that a protein in a solution samples multiple conformations that are in thermal equilibrium and distributed in a wide range of free energy regimes. The frequency of encountering a specific conformation depends on its free energy content. Thus, there will be certain conformations that are readily explored in biologically relevant timescales. However, certain other conformational states of the proteins are not visited in biologically relevant timescales. The transition between the readily explored conformations requires very less activation energy. In contrast, the transition from the readily explored conformations to the ones that are not frequently

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visited would require passing through a high activation energy barrier.

The MWC model requires that the protein explores both the T and the R states in thermal equilibrium in the absence of ligand. However, the R state is visited less frequently due to its higher free energy. Ligand binding to the R state fixes the protein in that state, thus shifting the equilibrium towards the R state. In other words, a particular conformation is selected from the preexisting conformational equilibrium in the free energy landscape of the protein. The MWC model is a very plausible model of allosteric transition, and has been experimentally verified in numerous proteins. A number of proteins have been shown to display distinct conformations in the absence of ligands. For instance, ultrafast two-dimensional infrared vibrational echo chemical-exchange spectroscopy of myoglobin shows two well-defined conformational substates of the protein, and the protein switches between these two substates in fast timescales (MS) [14]. X-ray crystallographic analysis of monomeric cytochrome P450 from *Saccharopolyspora erythraea* showed two different structures of the protein in the ligand-free state. NMR studies show that the active and inactive conformations of response regulator Spo0B exist prior to phosphorylation-induced activation. Distinct conformations of importin beta – a transport protein, are observed in the same crystallographic asymmetric unit when bound to snurportin suggesting that two distinct conformations of the protein exist in the solution, and one of the conformations has been selected by the interacting protein.

Not only *in vitro*, but such equilibrium has been observed in intact cells as well. Fatty acid-binding protein 4 (FABP4) is involved in the nuclear transport of interacting proteins. The activity of the protein is dependent on the conformational change that exposes the nuclear localization signals and is induced by the binding of specific ligands. However, a fraction of FABP4 localizes to the nucleus even in the absence of ligands, indicating that the active conformation is explored by FABP4 in the absence of ligands. Moreover, molecular dynamic simulation of



protein tyrosine kinase Abl shows that binding of the inhibitor imatinib, takes place by the conformational selection mechanism. An NMR study of the structure of *Bacillus subtilis* acylphosphatase shows that multiple conformations are explored in acidic pH, one of which binds with the ligand and shifts the equilibrium of conformations towards the ligand-bound state. Molecular dynamic simulation studies performed recently on bovine pancreatic trypsin inhibitor for a long period (1 ms) shows multiple, well-defined potential energy ‘basins’ where the protein resides, one of which resembles the conformer found in the crystalline state (solved by X-ray crystallography). Further analysis of the simulation showed that the protein exchanges between the basins are in the timescales of 10 ms, while exchanges within the basins are several orders of magnitude faster.

Besides the selection of a conformation, allosteric structural transition in proteins can take place via the induced-fit mechanism (KNF model). This model is plausible in cases where the ligand-bound conformational state R, is not visited by the protein in the absence of the ligand. Thermal fluctuations in the T state may not be enough to allow crossing the high-energy barrier to reach the ligand-bound conformational state. However, ligand binding provides enough energy that allows the protein to cross the high-energy barrier, thus inducing the protein to acquire the ligand-bound conformational state.

A number of proteins have been shown to utilize the induced-fit mechanism for the allosteric structural transition. For example, structural analysis of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase showed that the closing of the ‘lid’ on the active site of the enzyme is induced by ligand binding. Conformational changes are also induced by ligand binding in the thioesterase – DynE7, an enzyme in the dynemicin biosynthetic pathway in *Micromonospora* species. Based on cryo-electron microscopy images of the actin–myosin complex, it was proposed that the binding of these two proteins takes place by an induced-fit mechanism. Molecular dynamic simulation of binding of the neurotoxin fasciculin–2 to acetylcholinesterase revealed that the

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final complex formation passes through rapid conformational rearrangements to achieve an intermediate state, followed by conversion to the final complex. Pre-steady-state kinetic analysis of DNA polymerase η , which is involved in DNA repair and shows low DNA synthetic fidelity, revealed its selective incorporation of the correct nucleotide using the induced-fit mechanism. Several crystal structures of the methionyl-tRNA synthetase in the free and ligand-bound states show a large rearrangement of aromatic residues around the ligand-binding pocket on ligand binding. Prolylendopeptidase (PEP) is a serine protease that cleaves peptides at internal proline residues. The open state conformation of the protein observed in the absence of the ligand can be induced to undergo a large conformational change on incubation of the crystals with the ligand.

Although the two mechanisms could satisfactorily explain the allosteric transitions in some proteins, a growing body of literature suggests that many proteins employ both mechanisms to achieve allostery [15, 16]. For example, the initial selection of a conformer resembling the structure of the protein when bound to the ligand is followed by induction of structural changes in the protein leading to the final ligand-bound structure. For instance, molecular dynamic simulation of glutamine binding protein shows that strong and long-ranged interactions favour the induced-fit mechanism while weak and short-ranged interactions favour the conformational selection mechanism. In another example, some of structural changes associated with ligand binding, observed by X-ray crystallography, are accessed in the simulation timescales, implying a preexisting equilibrium of the conformers. However, certain other changes that are observed by X-ray crystallography in the structure are induced by ligand binding. Analysis of a large number of free and interacting partner-bound structures of ubiquitin, determined by both X-ray crystallography and NMR, clearly show that the binding pocket undergoes a significant conformational change once a conformer is selected by a specific interacting partner.

Slow transition rates allow conformation selection while fast transition rates allow induced-fit mechanism.

To distinguish induced-fit and conformational selection mech-



anisms, binding of NADPH to DHFR was analysed mathematically by determining flux via the two mechanisms. Such an analysis showed that flux occurs via the conformational selection pathway at low concentrations of the ligand, while the trend shifts to induced-fit at higher concentrations of the ligand. However, a wide range of ligand concentration shows fluxes through both the pathways. Further, mathematical analysis of receptor-ligand binding suggests that the mechanism of allostery depends on the rate of structural transitions. Slow transition rates allow conformation selection while fast transition rates allow induced-fit mechanism. Thus, a continuum of the two mechanisms is employed by the proteins for binding ligands.

Enthalpy vs. Entropy

Initial efforts by numerous laboratories directed towards understanding the conformational selection and induced-fit mechanisms of protein allostery made it appear that structural changes are essential for the allosteric transition to take place in a protein. However, ligand binding affinity of a protein is fundamentally dependent on the free energy change associated with the event. Free energy change is a composite of enthalpy and entropy changes. A change in either enthalpy or entropy, or both, can give rise to allosteric changes in the activity of a protein. While the enthalpy change of the protein-ligand complex is realized by a change in the mean structure of the protein involving backbone and side chain atoms of the protein, a change in entropy of the complex takes place by a change in the dynamics of the complex. Such an idea was proposed three decades ago by Cooper and Dryden [4] but experimental testing of the idea had to wait till the determination of relaxation rates of magnetized atomic nuclei in a protein was made possible by NMR. Allostery based on entropy changes was observed for the first time in the cyclic adenosine monophosphate (cAMP) responsive protein, CRP [17, 18]. Negatively cooperative cAMP binding to the two N-terminal cyclic nucleotide binding (CNB) domains in a dimer of CRP allosterically increased the affinity of the DNA binding domain for spe-

Free energy change is a composite of enthalpy and entropy changes.



cific DNA sequences present in gene promoters, thereby regulating transcription. Using NMR and Isothermal Titration Calorimetry (ITC), the effect of sequential cAMP binding on the structure of the two CRP subunits, and changes in the thermodynamic parameters in the binding process were determined. Cyclic AMP binding to the first subunit altered the conformational entropy without substantially changing the mean structure of the second subunit. Thus, the less favourable entropy change during the binding of the second cAMP molecule was the cause for the negative cooperativity observed in this protein.

Entropy-based allosteric regulation has also been documented in the cyclic guanosine monophosphate (cGMP) hydrolysing enzyme phosphodiesterase (PDE) 5 in living cells using Bioluminescence Resonance Energy Transfer (BRET) assays [19, 20]. PDE5 is a multi-domain protein consisting of N-terminal regulatory GAF domains (GAFa & GAFb) and a C-terminal catalytic domain. Cyclic GMP binding to the regulatory GAFa domain results in the activation of divalent metal ion-mediated cGMP hydrolytic activity of the catalytic domain. Structural studies using a BRET-based conformational sensor revealed that while divalent metal ion binding to the catalytic domain do not result in a change in the average structure of the protein, it distinctly regulates structural changes induced by other ligands. While divalent metal ion binding negatively affected regulatory GAFa domain-mediated structural changes, it positively modulated the catalytic domain-mediated structural changes. These suggest that divalent metal ion binding to the catalytic domain largely involves entropic changes in the protein that modulate structural changes induced by a ligand which in turn, causes enthalpy changes in the protein. Importantly, the BRET-based assays allowed observation of this allosteric effect in living cells.

Conclusion

Observing allosteric regulation of proteins in living cells still remains a major challenge.

The field of allosteric regulation of proteins has seen substantial progress starting from the initial observations of alteration



in the ligand-binding behaviour of proteins to the detailed illustration of the mechanism by which allosteric effects are achieved in some cases. This has resulted in a major shift in the way allostery is understood today. However, observing allosteric regulation of proteins in living cells still remains a major challenge, and has only been demonstrated for some proteins at low resolutions mainly using resonance energy transfer-based techniques. Thus, it appears that a detailed illustration of the process in the living cells would require a substantial improvement in the experimental techniques used for these kind of studies. Given the way techniques are evolving today, such as live cell NMR, it appears that the field of allostery will witness the uncovering of many other details of the process that has remained undetected.

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