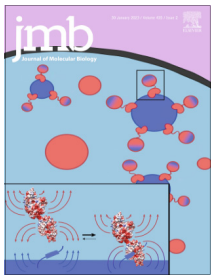

Allostery Frustrates the Experimentalist

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Abstract

Proteins interact with other proteins, with nucleic acids, lipids, carbohydrates and various small molecules in the living cell. These interactions have been quantified and structurally characterized in numerous studies such that we today have a comprehensive picture of protein structure and function. However, proteins are dynamic and even folded proteins are likely more heterogeneous than they appear in most descriptions. One property of proteins that relies on dynamics and heterogeneity is allostery, the ability of a protein to change structure and function upon ligand binding to an allosteric site. Over the last decades the concept of allostery was broadened to embrace all types of long-range interactions across a protein including purely entropic changes without a conformational change in single protein domains. But with this re-definition came a problem: How do we measure allostery? In this opinion, we discuss some caveats arising from the quantitative description of single-domain allostery from an experimental perspective and how the limitations cannot be separated from the definition of allostery *per se*. Furthermore, we attempt to tie together allostery with the concept of frustration in an effort to investigate the links between these two complex, and yet general, properties of proteins. We arrive at the conclusion that the sensitivity to perturbation of allosteric networks in single protein domains is too large for the networks to be of significant biological relevance.

Introduction

The interaction between protein and ligands is often regulated by changes in molecular conformation, arising from the structural complexity of macromolecules. These effects are commonly defined as 'allosteric', whereby other sites (from Greek 'allos-') may modulate the properties of the functional site of a given protein. By following this view, the molecular architecture of several proteins is endowed with well-defined structural changes and energetic communication between the functional and physically distinct sites.[1] Such effects are critical for many proteins to fulfill their physiological function.

But what is allostery? What does it imply? How can we measure it? Despite the fact that the concept of allostery is described in essentially all text books of biochemistry or molecular biology, the detailed description of allosteric effects from a thermodynamic and kinetic perspective still represents a major challenge and has been constantly attracting considerable attention.[2–4] The profound importance of protein allostery is highlighted by the observation that many disease-causing mutations are linked to a dysfunctional

allosteric regulation, as exemplified by the "first molecular disease" to be discovered, sickle-cell anemia,[5] as well as many cancer-causing mutations. Consequently, many novel drugs are designed to bind regulatory allosteric sites of target proteins.[6] It is argued that allosteric drugs often exhibit a higher specificity and lower toxicity when compared to orthosteric drugs, which bind to the functional site. Starting some 20 years ago, experiments and computational studies have led to a broadened definition stating that allostery might be at play not only when associated to an observable rearrangement in the quaternary or tertiary structure. In particular, a change in protein dynamics, even when the average structure is by-and-large unaffected, might contribute to changes in reactivity and/or affinity for the ligand, resulting in allosteric effects without detectable conformational changes.[7–9] Accordingly, the concept of allostery has been extended to simpler proteins, which are thought to contain subsets of residues involved in the propagation or distribution of energy through the protein ('allosteric networks').[10–12]

In this Perspective, we first recapitulate some of the key aspects concerning classic protein allostery. We then introduce the "new view" of allosteric effects in single protein domains and discuss how it might relate to the concept of frustration. Finally, we highlight major experimental challenges with probing allosteric effects in protein domains and implications for interpretation of data.

Protein allostery – Induced fit versus conformational selection

The classical models to describe allosteric effects in proteins involve conformational changes. In fact, by implying that the protein may explore two or more alternative conformations with different catalytic activities and/or affinities for a specific ligand, allosteric effects are ascribed to changes in the relative population of such conformational states. For example, in the case of hemoglobin, binding of the first oxygen molecule promotes a conformational switch of the protein from the T to the R state, which displays a different quaternary structure and higher oxygen-binding affinity, leading to the observed co-operative oxygen binding. The resulting sigmoidal binding curve is augmented by allosteric ligands promoting the T state. Analogously, to recall a prototypical textbook enzyme, the glycolytic enzyme phosphofructokinase-1 exists in two conformations, the active R state and the inactive T-state. If the allosteric ligand AMP binds to the allosteric site of this enzyme, there is a shift in the equilibrium between the states such that the R state is favored and the enzymatic activity increases. On the other hand, ATP promotes the T-state.

From a mechanistic perspective, there are two different models, which have been introduced to approach quantitatively the mechanism of binding of an allosteric protein. These alternative scenarios postulate a different order of events along the reaction pathway. In particular, a binding event whereby ligand binding induces a conformational change represents the induced fit model (i.e. binding precedes structural rearrangements, the classic Koshland-Némethy-Filmer model).[13] Alternatively, the structural conformations are in equilibrium in the absence of the ligand, and ligand binding induces a shift in population toward the ligand-bound state(s). This is the conformational selection model, which is formally similar to the Monod-Wyman-Changeaux concerted model.[14]

There is an interest in elucidating whether a protein–ligand interaction follows an induced fit or conformational selection scenario.[15] In the latter case, the allosteric behavior can be described by studying the structural heterogeneity, *i.e.*, different conformational states of a protein, in the absence of ligand. Experimental techniques such as NMR could be used to describe poorly populated states, which might harbor potentially druggable allosteric sites. We and others have previously described an experimental strategy to discriminate the two mechanisms, based on the careful analysis of binding kinetics.[16–19] However, it may prove futile to attempt to distinguish the two scenarios as they likely co-occur in many cases.

Allostery involving interactions between protein interaction domains

The concept that small molecule allosteric effectors can work as drugs has attracted considerable attention. A prototypical example is represented by the protein tyrosine phosphatase SHP2, which displays a structural transition between closed and open conformations with very different phosphatase activities.[20] Dysregulation of SHP2-related signaling pathways is associated with a range of diseases including Juvenile myelomonocytic leukemia, Noonan syndrome, LEOPARD syndrome, myelodysplastic syndromes, acute lymphoblastic leukemia, acute myeloid leukemia, chronic myelomonocytic leukemia, breast cancer, and lung cancer.[21–23] SHP2 contains two protein domains in one polypeptide, a catalytic phosphatase domain and a regulatory SH2 domain. The activity of the phosphatase domain is regulated by an intra-molecular interaction with the SH2 domain, which exerts an auto-inhibitory role in the absence of bound ligands,[24] whereas it is displaced from the phosphatase domain upon binding of its physiological ligands. Since the interface of interaction between the phosphatase and SH2 domain of SHP2 is well defined, several groups have successfully targeted this surface, with the aim of designing allosteric inhibitors with a possible therapeutic role.

Some of these molecules are very promising and currently in clinical trials, highlighting the importance and potential value of allosteric inhibitors.[20]

Allosteric networks within protein interaction domains – Different experimental approaches and protein frustration

Over the years, the collaborative efforts of experimentalists and theoreticians have contributed to the development of a wide array of different approaches to unveil the molecular determinants of protein allostery. The allosteric modulation of enzymes via effectors is generally studied by considering inter-subunit (phosphofructokinase-1) or inter-domain communication (SHP2). On the one hand, from a thermodynamic perspective, in the case of multimeric co-operative binding, as exemplified by the case of hemoglobin, this has been classically championed by the analysis of the so-called Hill coefficient, which reports the degree of communication between distinct binding sites within a single protein.[25–26] On the other hand, from a structural perspective, allostery may be captured by monitoring the changes in structure and/or dynamics of the relevant protein of interest, by using for example NMR, native mass spectrometry and single-molecule techniques.[27–29] All these methods have been extensively reviewed elsewhere (see for example Wodak et al.[3] and references therein) and, especially in the case of multimeric or multidomain proteins contributed an exquisite advance to our current knowledge of protein allostery. Yet, in the case of single domain systems, allosteric networks may be subtle and therefore difficult to analyze.

How can we understand and describe the allosteric communication that occurs within a single protein domain? This question is particularly relevant for so-called protein–protein interaction domains, such as SH2, SH3, PDZ or WW, which are highly abundant in the proteome. Any particular type of interaction domain typically displays specific interactions with short amino acid sequences despite a highly conserved overall tertiary structure. In fact, since binding pockets in interaction domains often appear conserved within the family, specificity has been proposed to be achieved by allosteric long-range communication between physically distant sites.[12] Given the advances in deep scanning mutagenesis methods, it is of interest to consider these approaches when applied to single-domain protein allostery. For example, in a recent issue of Nature, Faure et al.[30] describe an impressive high-throughput method, named doubledeep protein fragment complementation (ddPCA), to address the folding and function of proteins. By fusing the proteins of interest with fragments of a reporter enzyme (dihydrofolate reductase, DHFR) while performing deep scanning mutagenesis, the authors could explore simultaneously the stability (as probed by protein abundance) and binding properties of two very common types of interaction domains: a PDZ domain and an SH3 domain. The analysis was performed using a large mutational space and characterizing the 19 alternatives to the wild-type residue for each single position within the two domains. Thus, the analysis described the structural distribution of amino acid mutations that affect the stability and affinity of the two domains.

It is of particular interest to compare the ddPCA methodology in light of the concept of protein frustration, introduced by Frauenfelder and co-workers.[31–32] Frustration is a condition arising from the perceived incapacity to accomplish a goal. Physical systems may be defined as frustrated when it is impossible to simultaneously optimize all the possible interactions. Experiments and theory demonstrated that folded proteins tend to be minimally frustrated, such that there is a strong energy bias towards the native conformation.[33] One key implication of this finding is that the folding free energy landscape of proteins is generally funneled and mutations tend to destabilize the native state.[34] In other words, a protein sequence is by-and-large optimized for its corresponding tertiary structure and folding is cooperative, *i.e.*, it usually appears as an all-or-none two-state process where only the denatured and native states are significantly populated. However, since proteins have evolved to function rather than to fold, functional sites in proteins are often frustrated, representing a signature of the contrasting demands between folding and function.[35–36] Furthermore, since allostery is a hallmark of function, it is conceivable that frustrated sites that are not directly involved in function may be associated with allosteric networks.[35]

By considering the concept of frustration, it could be argued that ddPCA probes experimentally the frustration patterns for binding and stability, thereby depicting the level of native bias with respect to the energy landscape of the reaction of interest. Indeed, we note that the rationale of ddPCA parallels the theoretical work by Ferreiro et al.[35–37] who developed a theoretical framework in which each amino acid residue is mutated (*in silico*) to every other of the 19 residues. Usually, most substitutions would lead to lower stability (or affinity) than the wild-type residue. The position is then defined as minimally frustrated. On the other hand, if any, or several, of the 19 substitutions result in greater stability or higher affinity the position is not minimally frustrated. Obviously, *in silico* there is no need to involve chimeric fusion proteins to obtain a readout.

So, could ddPCA function as an experimental benchmark of frustration? Alas, subjecting the PDZ and SH3 domain to the computational frustration analysis, the patterns appear different from the experimental ones, displaying some similarities but also some obvious discrepancies (Figure 1). One explanation for the discrepancy is

that while minimal frustration is usually observed among interactions that stabilize the fold, and functional sites (allosteric or orthosteric) are expected to be frustrated,[38] frustration may still occur in all parts of a protein, not just in functional sites.

Double mutant cycles and the malleable nature of allosteric networks

We and others have previously assessed the potential presence of allosteric networks of different protein domains, including the PDZ and SH3 domains used in Faure et al., using different experimental approaches. In our work we define and quantify allosteric communication by the coupling free energy $\Delta\Delta\Delta G_c$.[12,39] The advantage with this parameter is that it measures the interaction energy between two side chains in a double mutant cycle such that side-effects of the single mutations are cancelled out. This is arguably the most stringent way to experimentally measure the interaction energy between structural elements in proteins since, at least in theory, it could account for unwanted changes in folding and stability.[40] In other cases, such as in Faure et al.,[30] allosteric interactions are measured by changes in stability or affinity from a single mutation, giving $\Delta\Delta G$. This parameter suffers to a larger extent than $\Delta\Delta\Delta G_c$ from ground state effects like structural changes. Interestingly, a comparison between our data and those from Faure et al. shows a good correlation for $\Delta\Delta G$ (the effect of point mutation) but not for $\Delta\Delta\Delta G_c$ (the interaction energy between two side chains). This is not surprising. In fact, $\Delta\Delta G$ would measure the effect of mutation on binding of the entire peptide ligand used in the experiment, whereas $\Delta\Delta\Delta G_c$ is the interaction energy between two side chains, one in the protein and one in the ligand. Furthermore, large differences in the structural distribution of allosteric networks obtained by different methods is a recurrent theme in intradomain allostery.[41] Our experimental observations have unambiguously shown that intradomain allosteric networks are highly sensitive to the structural context. For example, PDZ3 is part of a three-domain structural unit, a supramodule, together with one SH3 and one GK-like domain.[42] We obtained distinct allosteric networks (as probed by $\Delta\Delta\Delta G_c$) with the isolated PDZ3 domain as compared to PDZ3 in the supramodule.[43] This result is particularly important with respect to the data obtained by Faure et al., who used a PDZ3 construct lacking the third alpha helix (Figure 1). Helix three is important for the stability and folding of PDZ3[44] as well as affinity toward the ligand

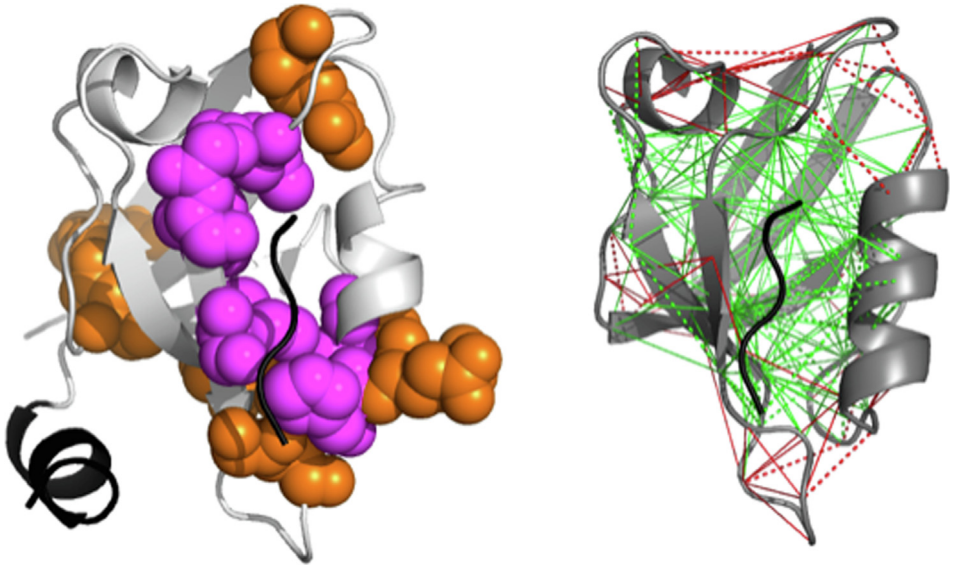

Figure 1. ddPCA, frustration and allostery. As discussed in the text, the ddPCA methodology represents an experimental realization of the computational frustratometer method designed by Ferreiro, Wolynes and co-workers[36]. Left, major allosteric (orange) and orthosteric residues (magenta) identified by ddPCA; adapted from Faure et al.[30] Note that helix 3 (black lower left) was not present in the construct used in the ddPCA study. Right, frustrated (red) and non-frustrated (green) interactions identified by the frustratometer. It is evident that, whilst some of the important residues identified by ddPCA superpose with those identified by the frustratometer, the agreement is only partial and does not allow identifying an allosteric network in a univocal manner. The disagreement is likely a reflection of the sensitivity of energetic connectivity between amino acid residues suggesting that technical limitations of the approaches preclude characterization of any potentially existing functional allosteric network. The bound peptide (YKQTSV) is depicted as a black stick. The structural models and frustration analysis (without helix 3) were made using protein data bank entry 1BE9.[54]

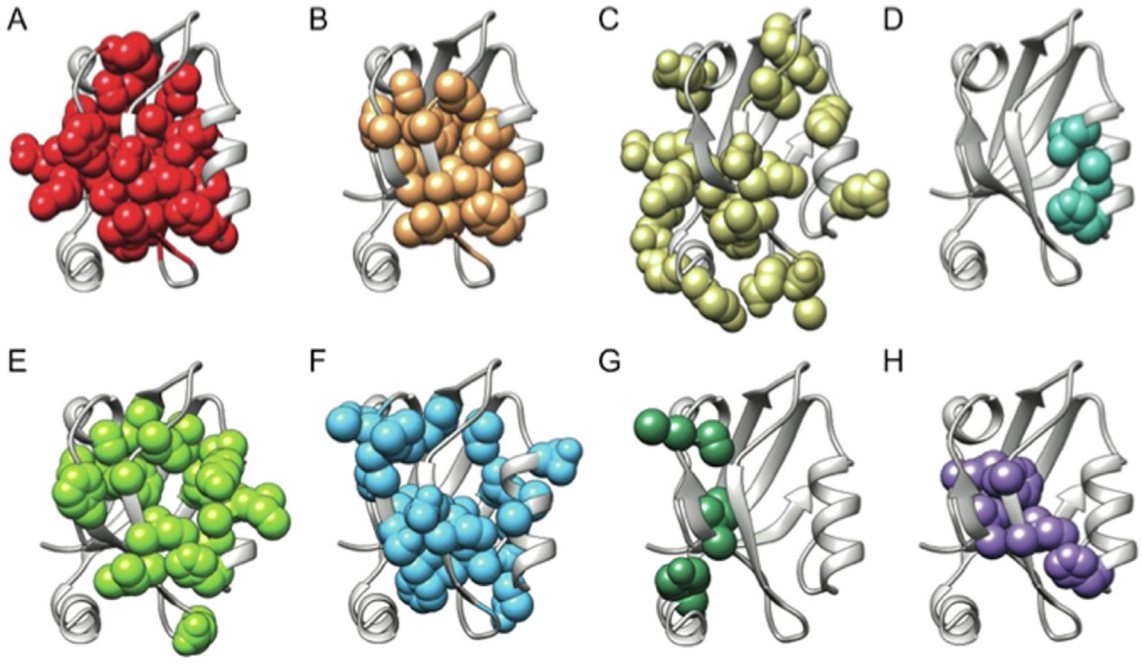

Figure 2. The elusive nature of allosteric networks in PSD-95 PDZ3. Residues underlying long range allosteric networks identified by different methods. The methods used were: (A) Perturbation response scanning[55], (B) Statistical coupling analysis (SCA)[11], (C) Molecular dynamics simulation[56], (D) Deep coupling scan (DCS)[57], (E) Thermodynamic double mutant cycle (TDMC)[12], (F) Conservation mutation correlation analysis (CMCA)[58], (G) Rigid-residue scan (RRS)[59], and (H) Monte Carlo path (MCPath)[60]. Each panel report the key allosteric residues identified by each respective study. Different colors were merely used in the different panels to highlight the different distribution of allosteric sites in each study. The Figure is adapted from Gautier et al.[41] and was drawn by Louise Laursen.

CRIPT.[45–46] Furthermore, presence/absence of helix three also completely redefines the allosteric network as defined by another property, side-chain dynamics derived from NMR relaxation experiments.[45] However, as recapitulated below, by taking the average $\Delta\Delta G$ of 19 mutations, ddPCA assesses the possibility of allostery (rather than presence) at the respective site.

The highly malleable nature of intra-domain allosteric networks is likely a result of the relatively weak energetic connectivity and complexity of the interactions involved. At variance with protein folding, which is typically robust to perturbation due to the highly funneled nature of the energy landscape, allosteric networks are grafted on slightly frustrated tertiary interactions and are therefore plastic and prone to remodeling, as is indeed exemplified by PDZ3 from PSD-95.[43] In another example, deep mutational scanning was applied to the DNA-binding domain of steroid receptors to assess how allosteric networks (epistasis) evolve over time.[47] The data showed that mutation at one site often influenced the effect of a second mutation. Because of this documented sensitivity of allostery to context in small protein domains,[43,47] it is clear that any perturbation of the system risks changing the allosteric properties, whether it is the fusion of the protein of interest to a reporter enzyme or even a point mutation. In the case of PDZ3 from PSD-95, the elusive nature of allosteric networks is further emphasized by the remarkable differences in the results obtained when using different experimental or theoretical approaches (Figure 2). Thus, whilst we do not dispute the value of high-throughput methods, including ddPCA, to address stability and binding, we caution on making conclusions regarding presence of allostery based on any experimental approach that perturbs the native state. Indeed, allostery is sensitive to small changes in structure.[48–51] Therefore, the smaller the perturbation, the more likely is the experimental result and its interpretation correct. However, even conservative point mutations may change the structure and the allosteric network.

So where do we go from here? We argue that ddPCA or other mutational scanning experiments on protein domains may be valuable tools to assess the *potential* for allostery, *i.e.* the possibility that an allosteric network may be at play or that it could evolve within a protein domain. However, in the absence of the identification of a proper allosteric effector, we argue that the allosteric networks that might be identified are of little, if any, biological relevance. In this context, we think it is fair to conclude that the study of protein allostery in single domain systems should rather follow a more classical approach based on i) identification of the allosteric

pocket and its potential ligand(s) and ii) experimental evaluation of the role of this pocket by performing experiments with the allosteric and orthosteric ligands to infer the molecular mechanisms.

Concluding remarks - the elephant in the room

To close, in analogy to Heisenberg's uncertainty principle stating that position and momentum of an electron cannot be determined at the same time, we argue that the perturbations needed to probe allosteric networks in protein domains may jeopardize the result. Furthermore, it appears that the evolutionary constraints on sequence conservation that are linked to the structural architecture of a given protein domain are much more pronounced than the subtle details dictating protein allostery,[52] an observation that leads to the apparent complexity and redundancy of allosteric networks in small protein domains.[53] In fact, one overarching and unanswered question is whether the allosteric networks of small domains, such as PDZ[11,41] have any functional relevance if they are so sensitive to the smallest of perturbations? Based on available experimental data we are inclined to conclude that they do not.

CRediT authorship contribution statement

Stefano Gianni: Conceptualization, Funding acquisition, Visualization, Writing – original draft, Writing – review & editing. **Per Jemth:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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