Allostery in Its Many Disguises: From Theory to Applications

# Allostery in its many disguises: from theory to applications

*Shoshana J. Wodak[1*], Emanuele Paci[2], Nikolay V. Dokholyan[3a,3b], Igor N. Berezovsky[4], Amnon Horovitz[5], Jing Li[6], Vincent J. Hilser[6], Ivet Bahar[7], John Karanicolas[8], Gerhard Stock[9], Peter Hamm[10], Roland H. Stote[11], Jerome Eberhardt[11], Yassmine Chebaro[11], Annick Dejaegere[11], Marco Cecchini[12], Jean-Pierre Changeux[13], Peter G. Bolhuis[14], Jocelyne Vreede[14], Pietro Faccioli[15], Simone Orioli[15], Riccardo Ravasio[16] Le Yan[17], Carolina Brito[18], Matthieu Wyart[16], Paraskevi Gkeka[19], Ivan Rivalta[20], Giulia Palermo[21], J. Andrew McCammon[21], Joanna Panecka-Hofman[22], Rebecca C. Wade[23], Antonella Di Pizio[24], Masha Y. Niv[24], Ruth Nussinov[25a,25b], Chung-Jung Tsai[25a], Hyunbum Jang[25a], Dzmitry Padhorny[26], Dima Kozakov[26], Tom McLeish[27]*

[1]*VIB-VUB Center for Structural Biology, Brussels, Belgium*
[2]*Astbury Centre, University of Leeds, UK*
[3a]*Department of Biochemistry & Biophysics, University of North Carolina at Chapel Hill, NC, USA*
[3b]*Departments of Pharmacology and Biochemistry & Molecular Biology, Penn State Medical Center, Hershey, PA, USA*
[4]*Bioinformatics Institute (BII), Agency for Science, Technology and Research (A*STAR), and Department of Biological Sciences, National University of Singapore*
[5]*Department of Structural Biology, Weizmann Institute of Science, Rehovot, Israel*
[6]*Departments of Biology and T.C. Jenkins Department of Biophysics, Johns Hopkins University, Baltimore, USA*
[7]*School of Medicine, University of Pittsburgh, USA*
[8]*Fox Chase Cancer Center, Philadelphia, USA*
[9]*Biomolecular Dynamics, Institute of Physics, Albert Ludwigs University, Freiburg, Germany*
[10]*Department of Chemistry, University of Zurich, Switzerland*
[11]*Department of Integrative Structural Biology, Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Illkirch, France*
[12]*Institut de Chimie de Strasbourg, UMR7177 CNRS & Université de Strasbourg, France*
[13]*Institut Pasteur & Collège de France, Paris, France*
[14]*van 't Hoff Institute for Molecular Sciences (HIMS), University of Amsterdam, Netherlands*
[15]*Physics Department, Università di Trento and INFN-TIFPA*
[16]*Institute of Physics, Ecole Polytechnique Fédérale de Lausanne, Switzerland*
[17]*Kavli Institute for Theoretical Physics, University of California, Santa Barbara, CA 93106*
[18]*Instituto de Física, Universidade Federal do Rio Grande do Sul, 91501-970 Porto Alegre, RS, Brazil*
[19]*Structure Design and Informatics, Sanofi R&D, Chilly-Mazarin, France*
[20]*École Normale Supérieure de Lyon, Université de Lyon, CNRS, Université Claude Bernard Lyon 1, France*
[21]*Department of Chemistry and Biochemistry, University of California, San Diego, USA*
[22]*Centre of New Technologies, University of Warsaw, Poland*
[23]*Molecular and Cellular Modeling Group, Heidelberg Institute for Theoretical Studies (HITS) and Center for Molecular Biology (ZMBH), DKFZ-ZMBH Alliance, Heidelberg University, Germany; Interdisciplinary Center for Scientific Computing (IWR), Heidelberg University, Germany*
[24]*Institute of Biochemistry, Food Science and Nutrition, Robert H Smith Faculty of Agriculture Food and Environment, The Hebrew University, Israel*
[25a]*Frederick National Laboratory for Cancer Research, National Cancer Institute, Frederick, USA,*
[26b]*Sackler Inst. of Molecular Medicine Department of Human Genetics and Molecular Medicine Sackler School of Medicine, Tel Aviv University, Israel*

[26]*Department of Applied Mathematics and Statistics, Stony Brook University, Stony Brook, NY 11794*
[27]*Department of Physics, University of York, United Kingdom*

Corresponding Author:

Shoshana J. Wodak
Email: Shoshana.Wodak@gmail.com

**Abstract**

Allosteric regulation plays an important role in many biological processes, such as signal transduction, transcriptional regulation and metabolism. Allostery is rooted in the fundamental physical properties of macromolecular systems, but its underlying mechanisms are still poorly understood. A collection of contributions to a recent interdisciplinary CECAM (Centre Européen de Calcul Atomique et Moléculaire) workshop is used here to provide an overview of the progress and remaining limitations in the understanding of the mechanistic foundations of allostery gained from computational and experimental analyses of real proteins systems and model systems. The main conceptual frameworks instrumental in driving the field are discussed. We illustrate the role of these frameworks in illuminating molecular mechanisms and explaining cellular processes, and describe some of their promising practical applications in engineering molecular sensors and informing drug design efforts.

## Introduction

Allostery refers to processes whereby a binding event at one site of a biological macromolecule affects the binding activity at another distinct functional site, enabling the regulation of the corresponding function. Since its initial formulations over 50 years ago (Changeux, 1961, 2011; Koshland et al., 1966; Monod and Jacob, 1961; Monod et al., 1965), allosteric regulation has been recognized as playing a key role in many biological processes, most prominently in signal transduction (Changeux, 2012; Changeux and Edelstein, 2005; Falke and Piasta, 2014; Nussinov et al., 2013), molecular machine function (Saibil, 2013), transcriptional regulation (Li et al., 2017; Wright and Dyson, 2015), and metabolism (Link et al., 2014). Allostery is rooted in the fundamental physical properties of macromolecular systems, and probably of other materials as well. However, the detailed mechanisms whereby these physical properties underpin allostery are not fully understood. Furthermore, allosteric effects are modulated by the cellular context in both health and disease.

Computational approaches have all along played an important role in the investigation of allosteric mechanisms. They have provided insights into some of the underpinnings of allostery (Dokholyan, 2016; Guo and Zhou, 2016; Schueler-Furman and Wodak, 2016) and have recently shown great promise in various practical applications, such as engineering regulatory modules in proteins and identifying allosteric binding sites that can be targeted by specific drugs. Notable examples of the latter application include re-sensitizing resistant hepatitis C variants by a combination therapy that involves binding to the allosteric site of NS5A (Sun et al., 2015), allosteric inhibitors of HIV integrase (Hayouka et al., 2007), or the

discovery of allosteric drugs that inhibit PARP-1 without hampering its action in cancer-related DNA repair deficiencies (Steffen et al., 2014).

One should also mention various recent bioinformatics approaches, which analyse sequence information (patterns of sequence conservation or correlated mutations) with the goal of uncovering signals of evolutionary pressure that may either inform or validate mechanistic aspects of allosteric processes (Dima and Thirumalai, 2006; Kass and Horovitz, 2002; Livesay et al., 2012; Lockless and Ranganathan, 1999; May et al., 2007). Here, too, the vast increase in available data on protein sequences from different organisms and massive data on human polymorphism derived from next generation sequencing efforts (Clarke et al., 2016) is providing unprecedented (and still largely untapped) opportunities for investigating the role of evolution in shaping allosteric regulation.

A recent CECAM (Centre Européen de Calcul Atomique et Moléculaire) workshop brought together about 30 computational biophysicists, protein modellers and bioinformaticians as well as experimentalists for an inspiring 2.5 days of stimulating talks and discussions. Among the important topics addressed were the new insights gained into the mechanistic foundations of allostery from computational and experimental analyses of real protein systems, as well as from very simple *in silico* toy materials. Also presented were informative examples describing how allostery enables information processing in cellular signalling cascades. Real excitement was generated by reports on the rational design of allosteric systems that can be modulated to produce desired activity and cellular behaviour, or engineered to act as sensitive molecular sensors. Encouraging results were also

described on the rational discovery of allosteric drugs by combining computational and experimental approaches.

In the following we summarize the highlights of the meeting. Further details are provided in the Supplementary Material.

**Mechanistic underpinnings of allostery: insights from computational and experimental approaches**.

The current understanding of allosteric systems has been increasingly influenced by the so-called ensemble model of allostery (Hilser et al., 2012; Motlagh et al., 2014), itself rooted in the seminal model of Monod Wyman and Changeux (MWC) (Monod et al., 1965), derived from studies on hemoglobin (Perutz, 1970), the 'ancestor' of all allosteric systems. According to the ensemble model, first described in the eighties (Cooper, 1984; Frauenfelder et al., 1988), the allosteric behavior of a macromolecular system arises from the properties of the native free energy landscape of the system, and how this landscape is remodeled by various 'perturbations', such as ligand binding, protonation, or interactions with other proteins (Dokholyan, 2016; Kern and Zuiderweg, 2003; Schueler-Furman and Wodak, 2016). The main parameters that determine the allosteric behavior are thus, 1) the relative stabilities (or populations) of all the states accessible to the system including those corresponding to active and inactive conformations (with respect to ligand binding for instance), 2) the time scales and energy barriers associated with the transitions between states, and 3) the binding affinities of the ligands/effectors or conditions, which may modify the set of dominant states, and thereby remodel the energy landscape of the system (Hilser et al., 2012; Motlagh et al., 2014). However, much remains unknown about these

important parameters. What is the role of thermodynamics, e.g. stabilizing/destabilizing different states of the system, versus the role of kinetics e.g. the time scales and energy barriers associated with the transitions between states? What are the relative contributions of entropy and enthalpy to the allosteric free energy? Is there a special role in allostery for protein intrinsic disorder? Are molecular machines a distinct category of allosteric systems? What can we learn about allostery from simple toy materials? These are some of the questions that the workshop set out to scrutinize.

**Bolhuis and Faccioli** reported progress in simulation algorithms for investigating and sampling rare events such as those associated with protein folding or unfolding, or with conformational transitions between active and inactive states in some allosteric systems. Such events may involve high free-energy barriers and long transition times that are not accessible by classical molecular dynamics (MD) simulations, even with the help of advanced high-performance computers, and therefore require the use of specialized sampling techniques involving various levels of approximations (Amaro et al., 2007; Markwick and McCammon, 2011; Pontiggia et al., 2015; Proctor et al., 2015).

The advantage of the enhanced sampling algorithms developed in the **Bolhuis** group is that they require no prior knowledge of the reaction coordinates (the main geometric parameters that change during the reaction process), which is usually not available. Using only information on the initial and final states, these algorithms generate the collection of trajectories that connect these two states, and employ the transition path sampling (TPS) algorithm (Bolhuis et al., 2002), which incorporates methods for selecting efficient moves along the energy landscape (Brotzakis and Bolhuis, 2016), to sample the shortest transition

paths across these trajectories. Those can then be scrutinized for pertinent reaction coordinates, and used to estimate the transition rates by evaluating the trajectory fluxes (Moroni et al., 2005). An example of the application of TPS to sample the light-induced conformational transition of the Photoactive Yellow Protein (PYP) (Vreede et al., 2010), a water-soluble blue-light photoreceptor from *H. halophila*, is illustrated in **Figure 1A**. All the path-finding methods developed by these authors are available in the OpenPathSampling software (Swenson et al., 2018).

The self-consistent path sampling (SCPS) method of **Faccioli** and collaborators affords further reductions in computational cost, but at the price of additional approximations, making it possible to simulate very slow conformational transitions of very large protein systems, using state-of-the-art atom-based force fields. Their method is based on a set of self-consistent stochastic equations of motion from which reaction pathways are generated by an iterative procedure (Orioli et al., 2017). The method also outputs a stochastic estimate of the reaction coordinates, and enables estimation of the potential of mean force of arbitrary collective coordinates. A variant of the SCPS methods was used to characterize the extremely slow conformational transition of the ~400 residues alpha1-antitrypsin of the serpin family (Cazzolli et al., 2014).

The two sampling methods, originally developed to model protein folding/unfolding reactions, represent important advances. But their potential to yield reliable mechanistic descriptions of the conformational transitions of allosteric systems still needs confirmation. Such confirmations could be obtained by applying the simulation procedures to systems for which the allosteric transition has been characterized experimentally, thereby enabling

direct comparison with the results of the simulations.

**Stock** and colleagues have done precisely that. Recent time-resolved infrared spectroscopy experiments on a photo switchable PDZ2 domain have indicated that the allosteric transition in this system occurs on multiple timescales (Buchli et al., 2013). Moreover, NMR relaxation experiments on the closely related PDZ3 domain revealed allosteric couplings between the binding pocket and the C-terminus (Petit et al., 2009). To gain insight into the underlying process, Stock employed exhaustive non-equilibrium molecular dynamics simulations to derive a time-dependent description of this transition (Buchenberg et al., 2017; Stock and Hamm, 2018). Results revealed that the structural and dynamic changes undergone by the system are highly non-linear and occur in a non-local fashion, in excellent agreement with the experimental data. This in turn led the authors to propose similarities with the process of downhill protein folding and to question the soundness of interpreting allosteric transitions in terms of well-defined pathways for propagating the conformational changes, as commonly done in the literature.

The experimental and modeling work by **Hilser** and colleagues on proteins with intrinsically disordered (ID) regions also undermines a strict pathway interpretation of allosteric transitions. Intrinsically disordered proteins represent a functional oddity because they lack stable tertiary structures, but represent nevertheless allosteric systems that play a central role in signaling processes (Ferreon et al., 2013; Garcia-Pino et al., 2010; Lum et al., 2012; Motlagh et al., 2014; Sevcsik et al., 2011). Investigating the mechanism of transcriptional regulation of the glucocorticoid receptor (GR), a protein involved in signaling whose functionally important N-terminal domain (NTD) is intrinsically disordered, the authors

showed that this protein is able to allosterically regulate function by simultaneously tuning transcriptional activation and repression (Li et al., 2017). This allosteric regulation is achieved by producing translational isoforms differing only in the length of the disordered domain and displaying different DNA-binding affinities and transcriptional activities that are uncorrelated to each other. Based on biophysical measurements analyzed in the framework of the ensemble model of allostery championed by the authors (Motlagh et al., 2014), compelling evidence was presented that this uncorrelated behavior is enabled through a mechanism of 'energetic frustration', whereby opposing energetic couplings between the structured domains and the disordered regions compete to modulate the overall response, as illustrated in **Figure 1B**.

**Bahar** reviewed approaches based on elastic network models (ENMs), which have demonstrated the significance of soft collective modes of motion in enabling allosteric regulation of protein systems (Bahar et al., 2007; Bahar et al., 2017). These approaches are in line with the ensemble view of allostery, but focus on conformational ensembles sampled by thermal fluctuations near the native state minimum of the energy landscape. The motions described by such ensembles can be evaluated by normal mode analysis (NMA) at full atomic detail (Go et al., 1983). However, the coarse-graining of the energy landscape with the help of ENMs permits sampling a relatively broad subspace of conformers and yields a unique analytical solution for the spectrum of modes for a given protein fold. The modes at the low frequency end of the spectrum (soft modes) are particularly relevant to allostery, as they are both highly cooperative and robustly defined by the overall architecture of the system.

Applying ENMs to several systems showed that the conformational changes of proteins elicited by ligand binding closely overlap with one or more of the soft modes accessible in the unbound form (Bahar et al., 2010; Tobi and Bahar, 2005), as illustrated for adenylate kinase (Temiz et al., 2004) (**Figure 1C).** The soft modes have therefore been described as 'paths' in conformational space enabling the allosteric transitions (Meireles et al., 2011), suggesting in turn that the ability to favour such soft modes may have played a role in the evolutionary selection of modules and domains that lend themselves to allosteric regulation.

**McLeish** presented work focusing on allosteric control enabled solely through the modulation of thermal fluctuations and the resulting entropy changes, induced by ligand binding. A feature of this mechanism, first formalized by Cooper & Dryden (1984) (Cooper and Dryden, 1984) and termed *thermal fluctuations allo*stery by the author or '*dynamic allostery'* elsewhere (Guo and Zhou, 2016; Kern and Zuiderweg, 2003; Schueler-Furman and Wodak, 2016), is that soft global modes of motion rather than more local ones are recruited to enable allosteric cooperativity. As seen above, such soft modes may be readily described by coarse-grained models like those of Bahar and co-workers (Bahar et al., 2010) and others (Hawkins and McLeish, 2004; Zhu et al., 2011). To further investigate the implications of 'fluctuation allostery' McLeish uses the coarsest possible toy model of a protein, consisting of just one (harmonic) internal degree of freedom. This simple unit, termed *allosteron,* of which a real example was described in the eighties (Onan et al., 1983), features one or more ligand binding sites and can also oligomerize. Crucially, it undergoes internal fluctuations modified by the binding of each ligand (**Figure 2A**). The author demonstrates that using the classical approximation to the harmonic-oscillator

partition function yields reasonable estimates of the allosteric free energy between two ligands bound to such a system, which contain no enthalpic terms. Extensions of the allosteron model have been helpful in identifying the physical origin of associated phenomena, such as the coupling of global and local vibrational modes in dynamic allostery of proteins (Hawkins and McLeish, 2006), the negative cooperativity of the CAP (Catabolite Activator Protein) homodimer (Toncrova and McLeish, 2010), or the sequence of effector-binding events in allosteric multi-protein assemblies (McLeish et al., 2018).

The important role of protein dynamics in enabling allosteric regulation was further highlighted by the computational studies of **Palermo and McCammon,** performed on the large multi-domain CRISPR-Cas9 system (Palermo et al., 2016, 2017a; Palermo et al., 2017b), the centerpiece of a recently emerged transformative genome editing technology (Chen and Doudna, 2017). In this multi-domain system, the endonuclease Cas9 associates with single-guide RNAs to site-specifically recognize and cleave any DNA sequence bearing a Protospacer Adjacent Motif (PAM) sequence. RNA-mediated binding to this sequence initiates DNA association and cleavage, with the latter performed by two spatially distant domains of the protein, HNH and RuvC, via a concerted mechanism. From MD trajectories of the CRISPR-Cas9 complex bound to PAM and for its analogue crystallized without PAM (Palermo et al., 2017b), the authors computed the generalized correlations (GC), capturing both linear and non-linear correlated motions of the system. Using the GC coefficients as edge weights, a residue dynamic network was built from each trajectory. Analyzing these networks revealed tighter communication (and increased correlated motions) between the HNH and RuvC domains in the presence of PAM (**Figure 1E**). This led the authors to conclude that PAM binding to CRISPR-Cas9 plays a key role in

triggering the interdependent conformational dynamics of HNH and RuvC, likely enabling the concerted cleavage of the DNA strands. It furthermore allowed the identification of residues responsible for the information relay. Mutating two of these residues (K775A and R905A) was shown to decrease off-target cleavage of partially complementary DNAs (Chen and Doudna, 2017), opening an avenue for modulating the activity of CRISPR-Cas9 systems.

In their contribution to this workshop review, **Nussinov and colleagues** adhere to the ensemble model of allostery, and view allosteric regulation as resulting from perturbations of the inactive (or active) conformational ensembles leading to activation (or inactivation) via a 'population shift' (Gunasekaran et al., 2004; Tsai and Nussinov, 2014). They also acknowledge the role of dynamics, but argue against the concept of *dynamic allostery* discussed above, which involves no changes between distinct conformational states (see also (Kern and Zuiderweg, 2003)). In Nussinov's view, only distinct states, corresponding to local minima of the native free energy landscape, can contribute to functional allostery, because specific functions are performed by distinct protein conformations (Nussinov and Tsai, 2015) as exemplified in the supplementary **Figure S1.** In support of their view they enumerate reasons for failing to observe conformational changes in some prominent allosteric systems. These reasons include crystal-packing effects, non-native crystallization conditions, which may stabilize the inactive state or destabilize the effector-bound active conformation and therefore trap a state exhibiting no conformational change. Also mentioned are, inadequate accounting for disordered regions, ignoring synergistic effects between allosteric effectors, and too short molecular dynamics simulations.

Novel mechanistic insights into the allosteric transitions of large multi-subunit molecular machines were derived from the experimental work of **Horovitz.** The efficiency of molecular machines is path-dependent. Understanding how these machines work therefore requires characterizing the intermediate and transition states of the allosteric switch reaction. In the case of ATP-consuming bio-molecular machines, which are often multimeric proteins, a key issue is whether they undergo concerted (Monod et al., 1965), sequential (Koshland et al., 1966), or probabilistic conformational changes. Horovitz showed how recent advances in single-molecule techniques and native mass-spectrometry finally made it possible to distinguishing between these models. Using these techniques enables quantification of the populations of co-existing states with different numbers of bound ligand molecules, giving rise to a particular degree of fractional saturation (**Figure 1D**). Given these populations, it is possible to determine the ligand binding constants for a multimeric protein and thus, to infer its allosteric mechanism (Gruber and Horovitz, 2018). Results showed that the ATP-promoted allosteric transitions of the homo-heptameric rings of GroEL are concerted (Dyachenko et al., 2013). Phi-value analysis, shown to be useful for studying protein folding reactions, revealed two parallel pathways for the allosteric transition of this protein (Gruber and Horovitz, 2016). A different approach based on an Arrhenius analysis of ATP hydrolysis by the group II chaperonin CCT/TRiC, the eukaryotic homolog of GroEL, revealed that the intra-ring conformational changes in this protein associated with ATP hydrolysis are sequential (Gruber et al., 2017). Structural features and possible evolutionary pressure that may underlie these intriguing differences between the two chaperonins were briefly discussed.

Thought-provoking investigations of the architectural principles and properties of allosteric materials were presented by **Wyart.** Considering allostery as the process whereby ligand binding at one site of a protein transmits a signal to a distant functional site, the authors investigate this process from a purely physical perspective. Among the questions that they set out to answer were how materials can be designed to carry mechanical information over long distances, or what allosteric pathways may be optimized for? The approach consists in using *in silico* 'evolution' schemes to optimize elastic toy materials, two-and three-dimensional spring networks, for carrying out a specific 'function' (Flechsig, 2017; Rocks et al., 2017; Yan et al., 2017a; Yan et al., 2017b, 2018). A surprising result from these *in silico* experiments is that the type of 'function' greatly affects the resulting architectures. Optimizing the networks for a geometric task, by selecting network structures where binding a ligand leads to a defined displacement on the other side of the network ('active site'), yields networks displaying a powerful lever at the active site, where the signal is required (Flechsig, 2017; Rocks et al., 2017; Yan et al., 2017a; Yan et al., 2017b). This lever has distinctive structural properties (between those of a solid and liquid) and may represent a potential candidate mechanism for allosteric proteins in which motion such as that for opening or closing a channel is required (**Figure 2B**). Completely different architectures evolve when the networks are optimized for cooperative binding energy between the allosteric and active site (Yan et al., 2017a). These evolved architectures feature a very soft elastic mode that extends throughout the structure. In addition, most of the response tends to be captured by a single normal mode, as observed in some allosteric proteins. Crucially, it was found that to induce cooperativity, the frequency of this mode must adopt moderate values, with the predicted optimal frequency depending on the linear size of the system. Despite the simplicity of the investigated materials, one is left with the

impression that these *in silico* evolution approaches should be very useful for formulating key questions about real allosteric systems that may be addressed experimentally.

**Allostery and signalling**

The allosteric behavior of proteins and protein assemblies plays a key role in signaling processes. Unraveling the mechanistic underpinning of this behavior should therefore lead to improved understanding of how signaling events are relayed and regulated, and enable their modulation with promising pharmaceutical avenues for targeting human disease (Dokholyan, 2016)

**Stote and Dejaegere** reported findings on the mechanism of allosteric regulation of retinoic acid receptors (RARs), members of the nuclear receptor (NR) superfamily implicated in the transcriptional cascades underlying many physiological phenomena, such as cell differentiation and growth (Brelivet et al., 2012; Helsen and Claessens, 2014). Although retinoic acid has been considered the primary regulator of RARs, phosphorylation of the ligand-binding domain (LBD) has been shown to modulate downstream nuclear signaling by phosphorylation of the regulatory N-terminal domain (NTD) (**Supplementary Figure S2**). Crystallographic studies of phospho-mimetic mutations of RAR$\gamma$ (S371E) and molecular dynamics simulations showed that phosphorylation of the RAR$\gamma$ (and RAR$\alpha$) receptors of this family leads to subtle changes in the dynamic properties of the protein without producing significant conformational rearrangements (Chebaro et al., 2013; Chebaro et al., 2017). It was furthermore proposed that a conserved long alpha-helix plays a key role in mediating the allosteric communication between sites in these receptors and

likely in other members of the nuclear receptor superfamily where the long helix in question is well conserved.

**Cecchini & Changeux** presented a strategy for modeling allosteric transitions in proteins. This strategy involves adding or removing an agonist from the binding site of an allosteric protein and using unbiased MD simulations to capture the spontaneous transition/relaxation of the system to a distinct physiological state **(Supplementary Figure S3)**. The approach was applied to the pentameric ligand-gated ion channels (pLGICs), representing typical allosteric membrane proteins that serve as signal transducers in neurotransmitter-mediated intercellular communication. In these systems, the activation/relaxation MD protocol was used to explore the pore-closing transition or *un-gating* of the prokaryotic proton-gated channel GLIC (Nury et al., 2010). Similarly, MD relaxation of the open form of the eukaryotic glutamate-gated ion channel, (GluCl) upon removal of the positive allosteric modulator ivermectin, was shown to promote partial closure of the ion pore through a complex quaternary mechanism involving global receptor *twisting* and a radial expansion (*blooming)* of the extracellular domain (Calimet et al., 2013). A more extended relaxation of the same channel in the absence of ivermectin captured the full closing motion that is consistent with the ligand-free GluCl x-ray structure (Martin et al., 2017). Using the same approach, the gating mechanism of pLGICs was explored also in the forward direction (from resting to active), revealing a correlation between orthosteric agonist binding and ion-pore opening (Yoluk et al., 2015; Yuan et al., 2016). The MD-based activation/relaxation protocol thus appears as a useful approach for exploring the allosteric transitions at atomic resolution in these large important systems,

despite its high computational costs and the fact that it collects only a limited number of transition events.

**Rational design of allosteric systems and identification of allosteric sites.**

Several approaches for the rational design of allosteric systems, allosteric switches and allosteric sensors, were described by Dokholyan, Berezovsky, Karanicolas, and Plaxco.

**Dokholyan** described new optogenetic and chemogenetic tools for controlling individual proteins and signaling cascades in living cells (Dagliyan et al., 2017; Dagliyan et al., 2013; Dagliyan et al., 2016). The approach consists of using computational procedures to identify solvent accessible allosteric sites (Proctor et al., 2015) on a target protein and physically engineering naturally occurring light-sensitive or ligand-sensitive domains into these sites. Light or a ligand are then used to modulate structural disorder in these domains, which, in turn, affects the active site of the target protein, switching it between inactive (increased disorder) and active (less disorder) states. In the illustrated examples (**Figure 3A**) the small naturally-occurring light-sensitive LOV2 domain, and the rapamycin-responsive uniRapR domain, were respectively engineered into several kinases involved in cell motility (Dagliyan et al., 2013). Light and rapamycin were then used to respectively, inactivate and activate the target proteins, with the resulting effects on cell motility directly monitored by imaging techniques.

The computational approach of **Berezovsky** quantifies the configurational work exerted in different parts of a protein as a result of ligand binding to a known or putative allosteric site and can be used to infer allosteric sites, ultimately enabling the design of effector

molecules (Guarnera and Berezovsky, 2016a, b). In this approach, an approximation similar to those described by Bahar and McLeish is used to model the protein native state dynamics. The protein force field is represented by a simple Cα-based harmonic potential, and the presence of a ligand at the allosteric site is modeled by locally restraining residue pairs at the binding site. Next, the dynamics of the ligand-free and ligand-bound proteins are described using normal mode analysis, from which a set of relevant normal modes is derived. These modes are then used to evaluate the so-called 'allosteric potential', defined as the mean work exerted on a residue as a result of the local motion of its neighbors. Lastly a per-residue "allosteric free energy" is computed from the difference between the ligand-free and ligand-bound conformational ensembles sampled by the relevant modes. Extension of the method to identify the effect of allosteric mutations and its application to the regulation of the activity of the insulin-degrading enzyme, (Guarnera and Berezovsky, 2016b; Kurochkin et al., 2017), were also mentioned. The extended method is implemented in the AlloSigMA, (http://allosigma.bii.a-star.edu.sg/home/) web-server (Guarnera et al., 2017), which can be used as a first approach for investigating allosteric effects on protein activity elicited by ligands or mutations, or for identifying potential new allosteric sites and candidates for allosteric mutations (Tee et al., 2018).

**Karanicolas,** on the other hand, described a method for building molecular switches, which involves the chemical rescue of the active conformation of a protein. In this procedure, a disruptive mutation (often of a hydrophobic residue important for protein stability) introduced into the protein is rescued by addition of a small molecule that complements the deleted atoms. Proof-of-concept for this approach was demonstrated by

introducing a (deactivating) tryptophan-to-glycine mutation into an enzyme, then showing that activity could be restored by adding indole to complement the resulting cavity (Deckert et al., 2012). The generality of this approach for building allosteric control into proteins other than enzymes was then explored by developing a cell-based reporter assay. This allowed for screening of many W→G mutations to determine which would attenuate protein activity, and then for testing which of these mutants could subsequently be rescued using indole. A suite of computational and experimental methods, collectively led to the insight that protein structure and function were most frequently modulated *indirectly* through control of protein stability (Xia et al., 2013). Addition of indole in these allosteric cases served not to revert a discrete conformational change, but rather as an allosteric ligand that rescues activity by inducing the protein to refold to its original conformation (Budiardjo et al., 2016), thereby representing an excellent illustration of the ensemble model of allostery.

**Plaxco** described how allostery and cooperativity may be leveraged to engineer a wide range of artificial optical, biochemical and electrochemical biosensors. Among the examples used to illustrate the approach was the rational design and engineering of a synthetic DNA-based nanodevice containing up to four interacting binding sites that can load and release a cargo over narrow concentration ranges, and whose affinity could be finely controlled via both allosteric effectors and environmental cues like pH and temperature (Mariottini et al., 2017). In another example, catalytic DNAzyme sequences (e.g. peroxidase-like DNAzymes) were combined with the consensus sequence recognized by specific transcription factors (either TATA binding protein or the microphthalmia-associated transcription factor). The resulting constructs exhibited, respectively, a more stable catalytically inactive conformation unable to bind the cognate transcription factor,

and a less stable conformation competent to bind it. The presence of the transcription factor pushes the equilibrium between these states towards the catalytically active one, in a manner that can be finely controlled further by optimizing the original design (Adornetto et al., 2015).

**Kozakov** presented an approach for identifying allosteric binding sites (also denoted as cryptic sites) in ligand-free protein structures, and predicting their drug binding potential**.** The method involves the identification of binding hotspots on the protein surface. These hotspots represent clusters of low energy binding poses for small organic molecular probes of various shapes, sizes and polarity, generated by their FTsite computational procedure (Ngan et al., 2012). Applying FTsite to protein structures with known allosteric sites (Cimermancic et al., 2016) it was found that the ligand-free apo structures generally feature binding hotspots for the tested small molecular probes that are in close proximity to the known allosteric sites (**Supplementary Figure S4**). Of those, the more highly populated hotspot clusters (≥16 low energy poses) were deemed druggable, e.g. can be targeted by ligands with sufficient affinity (Kozakov et al., 2015). The authors also reported that regions of protein structures close to cryptic binding sites are significantly more flexible than regions surrounding any other potential binding hotspots detected by their procedure (Beglov et al., 2018). This increased flexibility seems to be linked to missing loops or side chains of less reliably modeled regions of the corresponding X-ray structures, suggesting that such regions may be good cryptic binding site candidates.

Lastly, among the notable poster presentations, three reported analyses of the dynamics and allosteric regulations in important multi-subunit enzymes from various origins. **Rivalta**

and colleagues used classical MD simulations and a community network analysis (Sethi et al., 2009), not unlike that of Palermo & McCammon, to elucidate the allosteric regulation in the imidazole glycerol phosphate synthase (IGPS) from *T. maritima* (Rivalta et al., 2012). This analysis stimulated single site mutagenesis experiments and allosteric inhibitor design (Rivalta et al., 2012) (**Supplementary Figure S5** for details). **Gkeka** and collaborators described potentially important findings from combined experimental and computational analyses on the allosteric modulation of the lipid phosphoinositide 3-kinase alpha, PI3Kα , which plays a pivotal role in cell proliferation and is a target for anti-cancer drug development (see **Supplementary Figure S6** for details]. They discovered a ligand-binding site distinct from the enzyme active site capable of inhibiting a cancer-associated PI3Kα mutant responsible for enzyme □□□□□□□□□□□□□□□. Ligand binding to this site was found to modulate the membrane binding domain of the protein, and not the active site, opening the avenue for designing selective inhibitors of protein-membrane interactions in this and other systems (Gkeka et al., 2014; Gkeka et al., 2015). **Panecka-Hofman and Wade** reported preliminary results on the dynamic allosteric coupling between distant residues of Pteridine reductase 1 (PTR1), a folate pathway enzyme unique to trypanosomatid parasites (Panecka-Hofman et al., 2017) (**Supplementary Figure S7**). The 4$^{th}$ poster reported progress towards gaining insight into the allosteric regulation of taste GPCRs (Di Pizio et al., 2016) (**Supplementary Figure S8**).

## Concluding remarks

In this collection of contributions presented at the CECAM workshop, we endeavored to provide an overview of the current understanding of allosteric processes and its perceived

limitations. We also described how this still incomplete understanding is exploited more or less successfully to illuminate the underlying molecular mechanisms, explain cellular processes, design molecular sensors and inform drug design efforts.

The concept of allostery has evolved significantly since the first allosteric proteins have been characterized (Motlagh et al., 2014; Schueler-Furman and Wodak, 2016). We now have a better grasp of the important functional role of protein dynamics and, in particular, the role of protein intrinsic disorder. We also have more powerful computational and experimental tools for sampling significantly populated states of complex protein systems.

Notwithstanding these advances, current computational methods are still unable to chart the free energy landscape of allosteric systems in an unbiased way, e.g. without prior knowledge of some significantly populated states of the system. Even when such knowledge is available, computational procedures employ various levels of approximations to sample the conformational transition paths between these states, as illustrated by the contributions of Bulhuis, Faccioli, and Stock, and work of groups employing Markov State Models (MSM) (Chodera and Noe, 2014; Pande et al., 2010). Specific approaches depend moreover on the size and complexity of the systems under study, making it difficult to evaluate the information they provide about the identified transition paths. To enable such evaluation it would be useful to come up with a few allosteric protein systems with well-characterized active and inactive states, to which different computational methods for charting the allosteric transition paths could be applied, results compared, and eventually evaluated against experimental data.

Particularly useful would be data derived from phi-value type analyses. Such analyses measure the changes in the activation energy of unfolding and the free energy of unfolding brought about by mutations, and those are used to characterize the transition states and intermediates of protein folding reactions (Fersht et al., 1992). Employing similar analyses to characterize the transition state of an allosteric pathway was suggested during the meeting, but not further elaborated on, although there have indeed been insightful precedents. Eaton et al. (Eaton et al., 1991) were the first to apply such analyses, generally referred to as rate-equilibrium linear free-energy relationships (LFERs), to allostery. Using pH and ligand states instead of mutations to perturb the kinetics and thermodynamics of the allosteric transition in hemoglobin, they showed that the transition state of the R<->T quaternary conformational change had closer thermodynamic properties to those of the R than the T conformations, validating a much earlier computational study, based on a crude analysis of the surface areas buried between the subunits (Janin and Wodak, 1985). A subsequent study of Yifrach and Horovitz (Yifrach and Horovitz, 1998) employed a genuine phi-value analysis, involving a limited number of mutations, to map the transition state of the allosteric pathway of GroEL. A LFER derived from perturbations, notably by a series of site-specific mutations, was used to map or the transition state of the gating reaction pathway of the muscle acetylcholine receptor (Grosman et al., 2000), yielding detailed information on the gating mechanism, described as involving a wave-like conformational change.

Computational approaches to the seemingly more tractable problem of identifying paths that mediate allosteric ‘communication’ between sites in a protein would also benefit from a more objective benchmarking. Although fundamentally different from allosteric transition

paths on the free energy landscape, identifying communication paths also involves sampling the free energy landscape, but only in the vicinity of the stable 'end' states, and then quantifying the correlated motions of the corresponding conformational ensembles. But here too, computational procedures and the set of investigated systems tend to differ significantly between authors. Assessing the agreement between communication paths identified by different methods in the same set of allosteric systems should therefore be very informative. Since even in a highly structured protein 'communication' between sites is likely mediated by multiple paths (Guo et al., 2015; Taylor et al., 2016), the questions of whether a given path can be rigorously validated against experimental data, or whether its specific role can be rationalized, need to be critically evaluated.

Two distinct but complementary conceptual frameworks for probing the mechanism of allosteric regulation, highlighted in this review, deserve special mention. One considers allosteric regulation as enabled by the so-called 'soft' modes of collective motions sampled by thermal fluctuations near the native state minimum, usually of highly structured protein systems. These soft modes are estimated computationally from experimental structures, using coarse-grained elastic network models (ENM), which strongly depend on the reference structure (usually the experimentally determined one). With skeptics, wary of such coarse-grained models, one would argue that ENM and the underlying conceptual framework have been quite instrumental not only in capturing the conformational transitions associated with the allosteric regulation of complex protein systems, but also in modeling the entropic contributions to the allosteric free energy, and potentially for predicting allosteric binding sites in protein systems, as reported by several contributions to this review and references therein.

The other conceptual framework refers to the so-called ensemble model of allostery, which focuses entirely on the thermodynamic analysis of the energy landscape of allosteric systems, including those featuring intrinsic disorder. It is thus of very broad applicability. As already mentioned, the main task of such analysis is quantifying the relative populations (stabilities) of all the states accessible to the system and how this population landscape is modified by ligand/effector binding, or disorder-to order transitions. Focusing on these thermodynamic properties is amply justified. In many systems, the rate limiting step of the allosteric transition elicited by effector binding, may indeed be governed by the concentration (population) of the pre-existing ligand-binding competent state of a protein, rather than by the free energy barrier of the conformational transition it needs to undergo to adopt this state. As illustrated here by a number of contributions, fine-tuning the relative populations of the active and inactive states of protein or nucleic acid systems and the binding affinities of allosteric effectors, are very effective ways, by nature or in the laboratory, to design systems undergoing allosteric regulation of different levels of complexity and versatility.

Clearly, allosteric regulation still needs to deliver many of its secrets. An advantage of allosteric regulation over regulation involving gene expression is its shorter response time to changing conditions. One may therefore wonder if this may determine the set of properties of allosteric systems, such as the existence of soft collective motions or population levels of relevant states, that evolution tends to select. Are all proteins allosteric, as some have suggested (Gunasekaran et al., 2004)? Are molecular machines a special category of allosteric systems? And lastly, how much can we learn about the very

fundamental requirements of allostery from simple toy materials? These are only some of the many intriguing questions to address, going forward.

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## Author contributions

SJW wrote the manuscript on the basis of extended abstracts contributed by all authors. EP NVD and AH, helped proofreading and editing. NVD, INB, AH, JL, VJH, IB, JK, GS, PH, RHS, JE, YC, AD, MC, SO, RR, LY, CB, MW, PG, IE, GP, JAM, JPH, RCW, ADP, MYN, RN, CJT, HJ, DP, DK, TML, contributed extended abstract and Figures.

## Figure captions

**Figure 1: Mechanistic underpinning of allostery: insights from computations and experiments**

**(A)** Artist rendering of the conformational transitions network of the Photoactive Yellow Protein (PYP), the 125-residue water-soluble blue-light photoreceptor from *H. halophile*, mapped onto the energy landscape of the system using the simulation procedures of Bolhuis and collaborators**.**

**(B)** Frustration-based allostery in the human glucocorticoid receptor (GR), an intrinsically disordered transcription factor analysed by Li and Hilser (Li et al., 2017).
**(I)** Domain organization of the constitutively active GR constructs for translational isoforms, wherein the intrinsically disordered N terminal domain (NTD) varies in length. Residues 1-97 (red) are labeled R (for Regulatory) and residues 98-420 (grey) are labeled F (for Functional). Also labeled are residues corresponding to the activation function 1 core (AF1 core) region, which is required for transcriptional activity. (**II)** Competing thermodynamic coupling in GR produces frustration. Schematic view of the thermodynamic configuration of GR. According to the displayed convention, the positive (+) signs between the DBD and F-domain, and the DBD and R-domain signify they are positively coupled; stabilization of one domain stabilizes the other. The negative (-) sign between the R- and the F- domains indicate they are negatively coupled; stabilization of one domain destabilizes the other.

**(C)** The closed conformation adenylate kinase observed upon ligand binding is sampled by the open form apo-structure, illustrating the work of Bahar**. (I)** Two experimentally resolved structures, unbound (left) and ligand-bound (right). **(II)** Conformer predicted by ENM

(Elastic Network Model) analysis to be accessible via a soft mode to the unbound structure. Blue and green refer to different domains. The substrate is shown in orange spheres. (Adapted from (Temiz et al., 2004)).

**(D)** Distributions of GroEL molecules with different numbers of bound ATP molecules at different ATP concentrations from the work of Horovitz and co-workers.

**(E)** Allosteric regulation in CRISPR-Cas9, by Palermo and McCammon. **(I)** Dynamical network model of CRISPR-Cas9, identifying groups (or "communities") of closely correlated residues and the strength of correlation between them before (top) and upon (bottom) PAM binding. **(II)**. The allosteric path between the spatially distance HNH and RuvC domains of the Cas9 protein.

**Figure 2. Allosteric toy models and allosteric materials**

**(A):** Schematics of the allosteron model of McLeish, in binding (**a**) and self-assembly (**b**) illustrating local changes to spring constants $\kappa$, and the introduction of coupling springs between allosteron units $\kappa_C$.

**(B)** Illustration of the work of Wyart and collaborators: (**I)** Response (black) arrows to a stimulus (purple arrows) in random spring network decays rapidly with distance, i.e. there is little action at a distance. **(II)** Networks can be evolved in which there is specific action at a distance. Note that the response is amplified near the active site (blue arrows), indicating the presence of a lever in the structure. **(III)** Example of hinge architecture obtained while optimizing cooperativity, in which two parts of the material rotate around a hinge located at the center of the system. **(IV-VI)** Illustration of the cooperative architectures found: hinge (clothespin), shear (mint box) and twist (Rubik's cube).

**Figure 3. Rational design of allosteric systems and identification of allosteric sites.**

**(A)** Schematic diagrams illustrating the work of Doholyan and colleagues on optogenetic and chemogenetic control of target proteins using allostery and protein order-disorder transition, reprinted from (Dagliyan et al., 2016).

**(B)** Illustration of the approach by involving the chemical rescue of the active conformation of a protein. The example shows how mutation of a buried tryptophan to glycine leads to a structural disruption – either through a discrete conformational change or through loss of protein stability – that leads to loss of protein function. Adding exogenous indole can then complement the cavity caused by the deleted sidechain, restoring the original protein conformation and, thus, its function.

**(C)** Principle of the rational design and engineering of a synthetic DNA-based nanodevice described by Plaxco. Top: the designed cooperative DNA-nanodevice comprises the recognition element consisting of a triplex forming DNA sequence, which behaves like a "clamp" that binds a specific 9-base DNA ligand via the formation of both Watson-Crick and Hoogsteen base-pair interactions. The cooperative DNA-nanodevice is obtained by joining together two sequential copies of one half of such recognition element linked via a flexible 22-base, single-stranded loop (grey portion) to two sequential copies of its other half. Binding of the ligand to the first receptor decreases the entropic cost associated with the binding to the second receptor (and thus improves its affinity for the ligand). As a result, this nanodevice shows a Hill-type cooperative response, with a Hill coefficient $n_H = 2.1 \pm 0.1$. Figure reproduced from (Mariottini et al., 2017).

**(D)** Binding hotspots of small chemical probes to flexible regions of the protein tend to correspond to cryptic binding sites**.** Example from the work of Kozakov**,** showing the mapping of hotspots identified by FTsite in the unbound structure of the catalytic subunit of the cAMP dependent protein kinase PKA (PDB ID code 2GFC, chain A) displayed in tan. Three hot spots, obtained after domain splitting, are shown as clusters of molecular probes: a cluster of 18 probes (cyan); cluster of 16 probes (magenta); cluster of 13 probes (gray). An inhibitor (yellow) is superimposed for reference.

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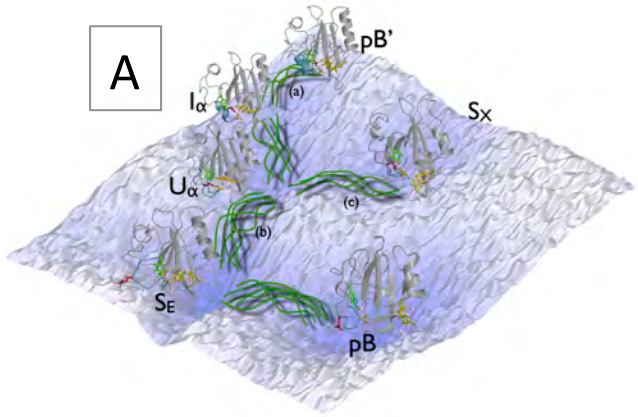
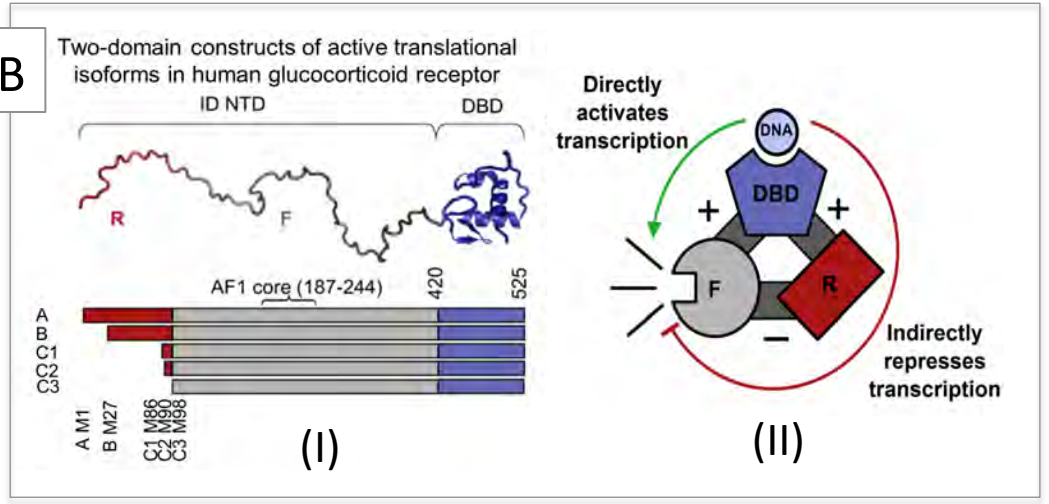
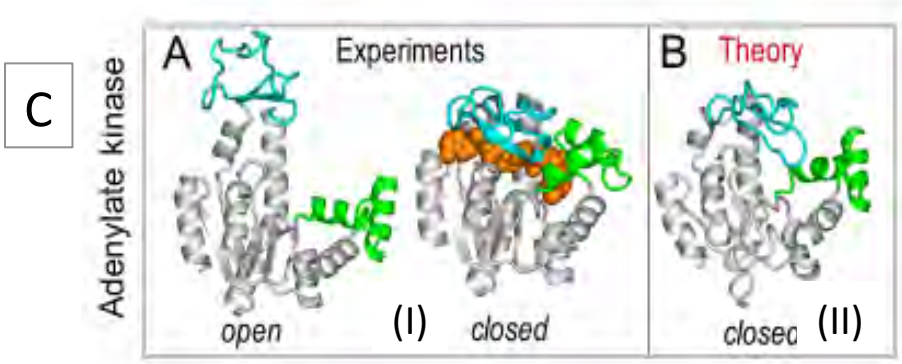
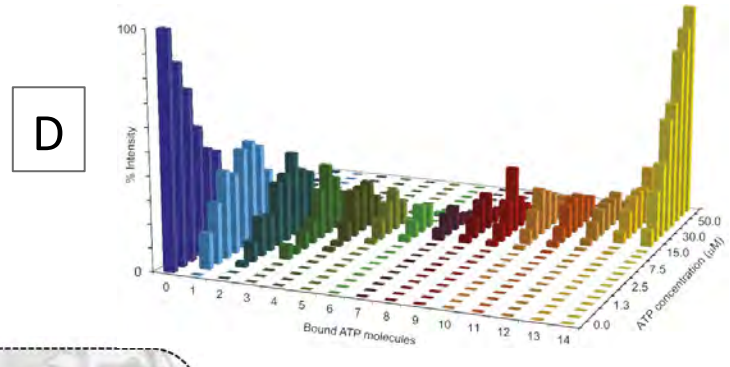
Figure1


A
pB'
Iα
(a)
Sx
Uα
(c)
(b)
SE
pB


B
Two-domain constructs of active translational isoforms in human glucocorticoid receptor
ID NTD
DBD
R
F
AF1 core (187-244)
420
525
A
B
C1
C2
C3
A M1
B M27
C1 M86
C2 M90
C3 M98
(I)
Directly activates transcription
DNA
DBD
+
+
F
R
−
Indirectly represses transcription
(II)


C
Adenylate kinase
A
Experiments
B
Theory
open
(I)
closed
closed
(II)


D
100
% Intensity
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Bound ATP molecules
50.0
30.0
15.0
7.5
2.5
1.3
0.0
ATP concentration (uM)

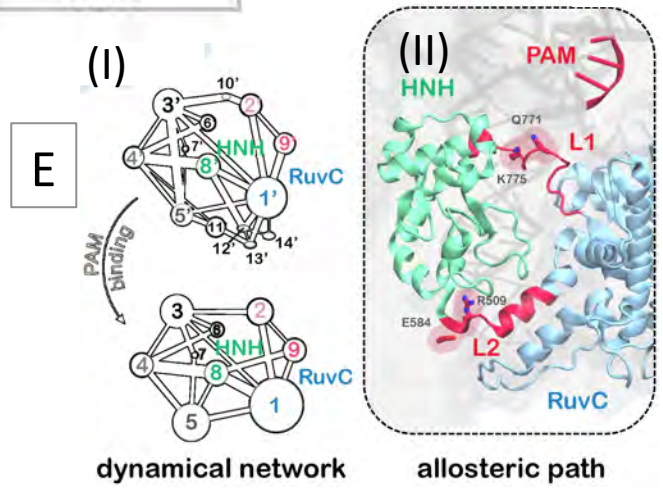
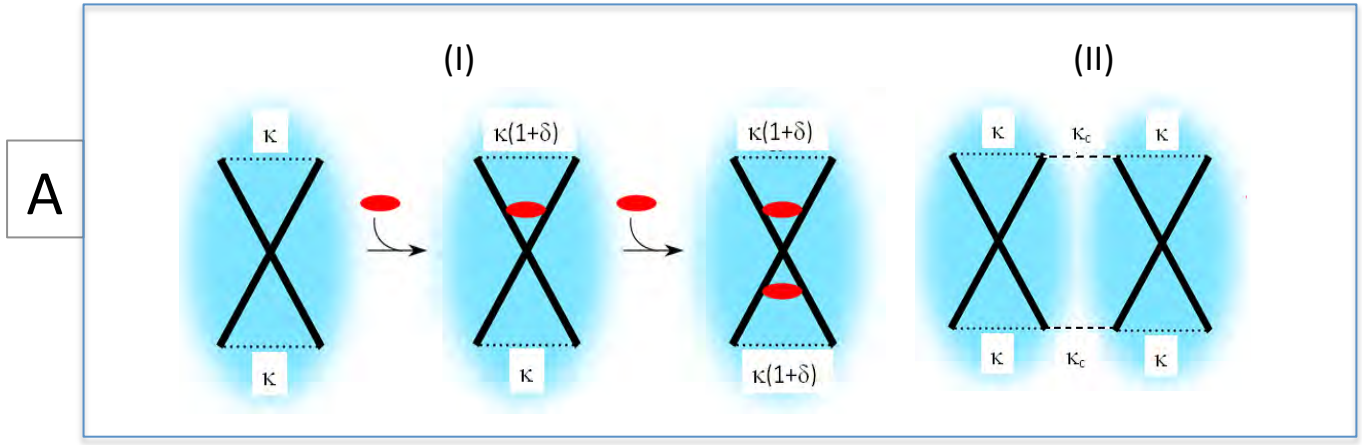

E
(I)
10'
3'
2
6
5'
4
7
HNH
9
8
RuvC
1'
11
12'
13'
14'
PAM binding
3
2
6
4
7
HNH
9
8
RuvC
1
5
dynamical network
(II)
HNH
PAM
Q771
L1
K775
R509
E584
L2
RuvC
allosteric path

Figure2



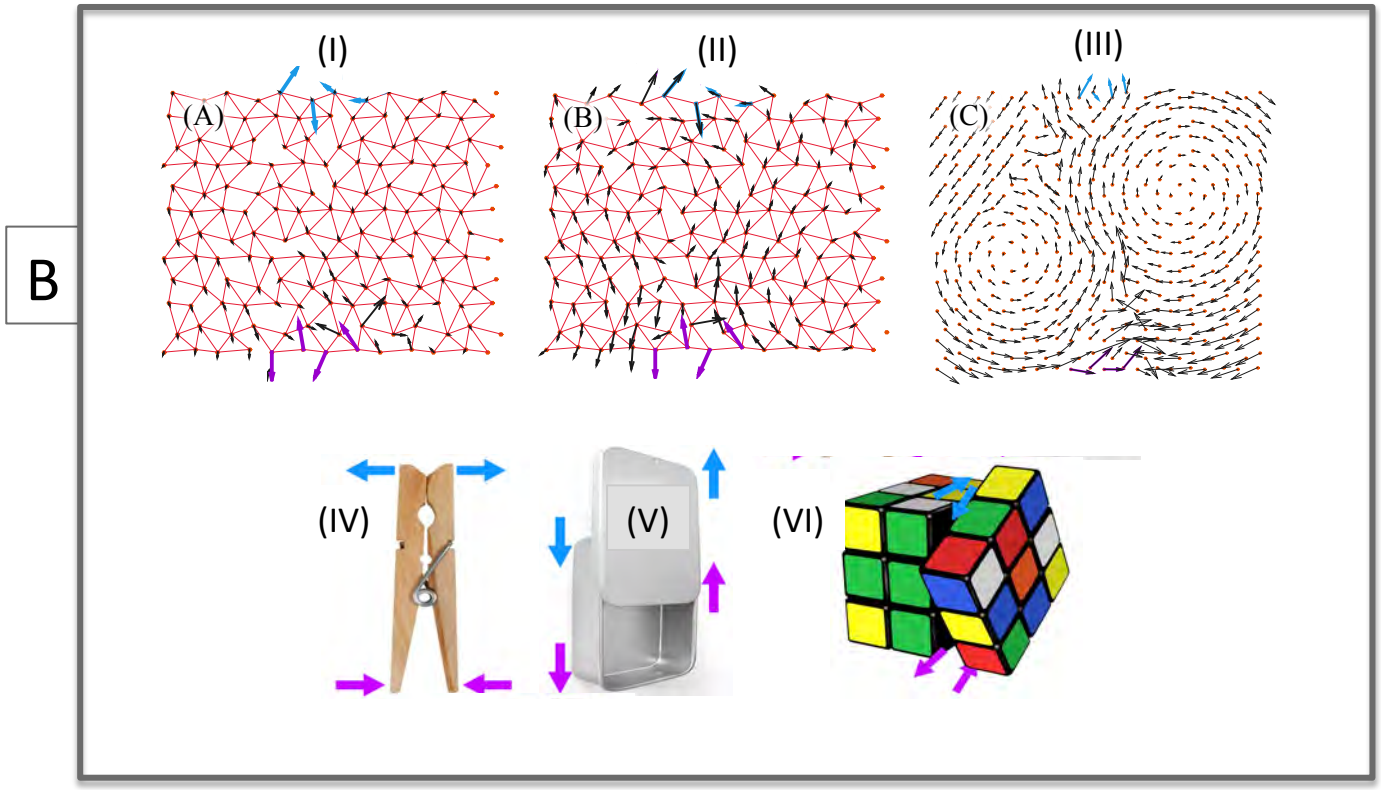
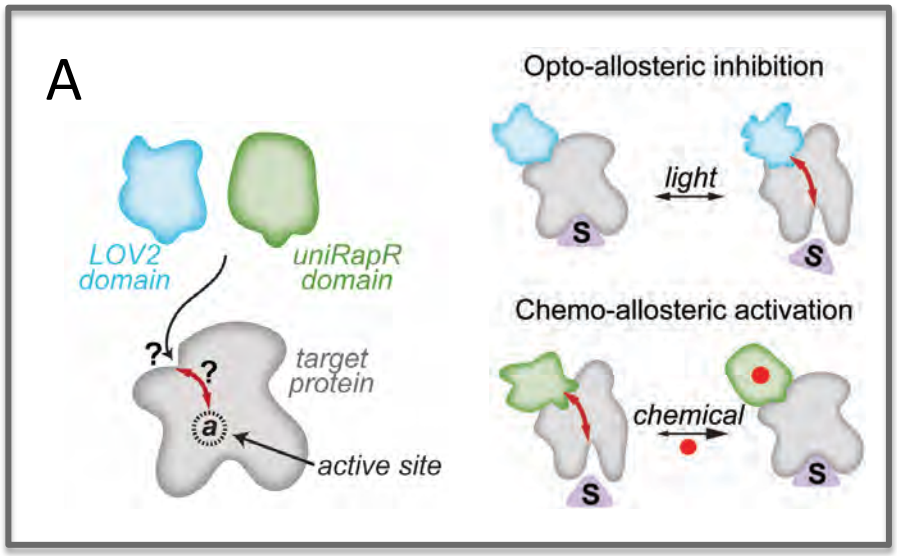
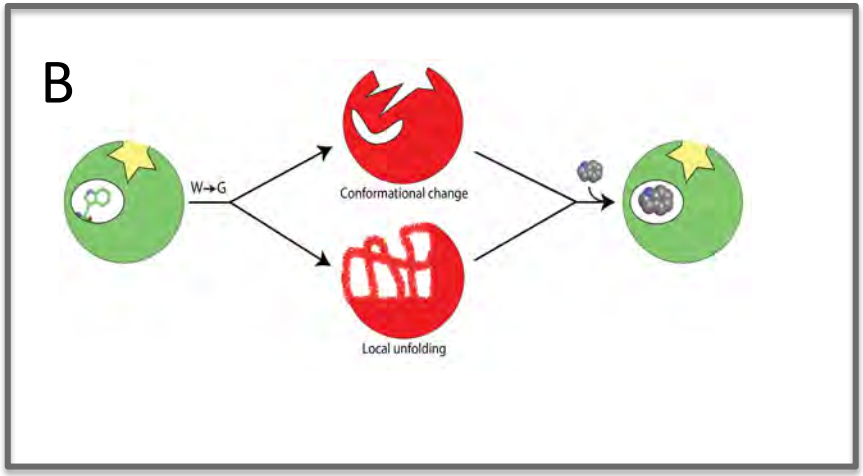
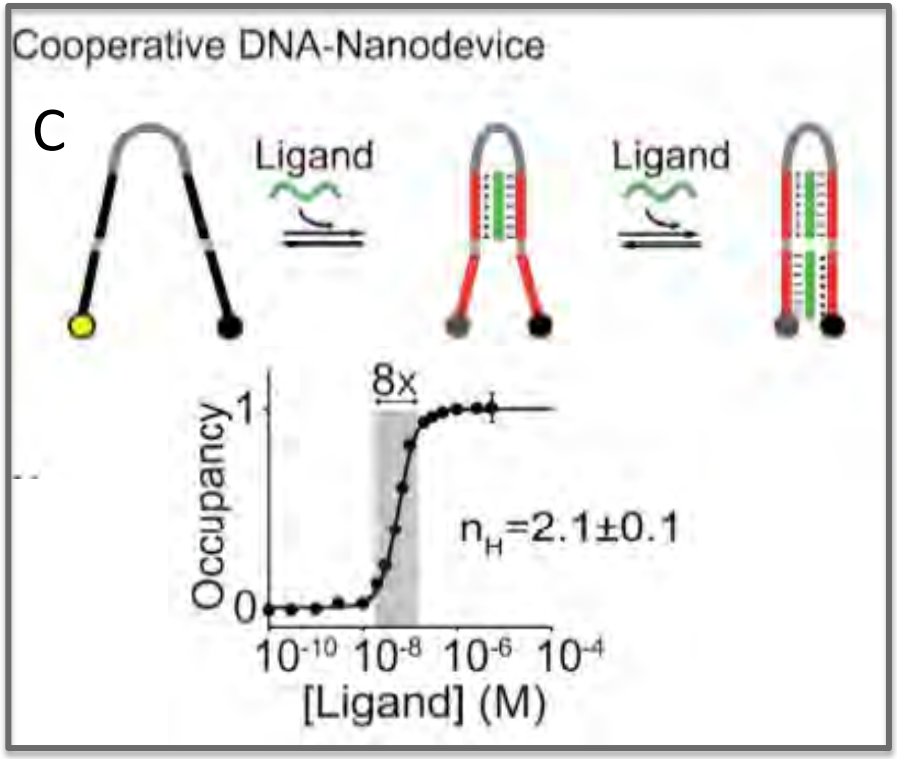
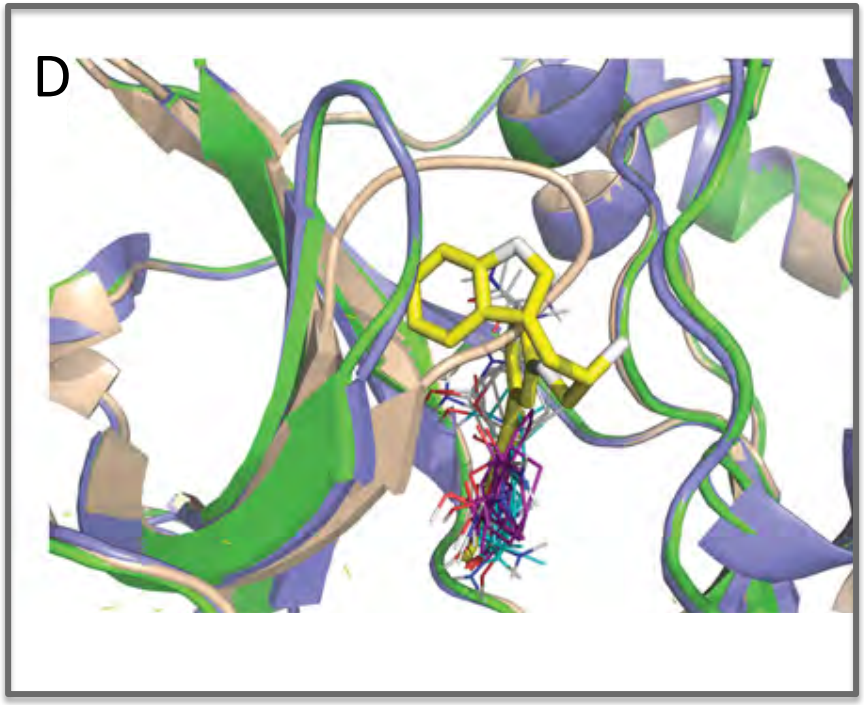



Figure3


A
Opto-allosteric inhibition
light
LOV2 domain
uniRapR domain
target protein
active site
Chemo-allosteric activation
chemical


B
W→G
Conformational change
Local unfolding


Cooperative DNA-Nanodevice
C
Ligand
Ligand
8x
Occupancy
1
0
$n_H$=2.1±0.1
$10^{-10}$ $10^{-8}$ $10^{-6}$ $10^{-4}$
[Ligand] (M)


D