

Review

Renaissance of Allostery to Disrupt Protein Kinase Interactions

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Protein–protein interactions often regulate the activity of protein kinases by allosterically modulating the conformation of the ATP-binding site. Bidirectional allostery implies that reverse modulation (i.e., from the ATP-binding site to the interaction and regulatory sites) must also be possible. Here, we review both the allosteric regulation of protein kinases and recent work describing how compounds binding at the ATP-binding site can promote or inhibit protein kinase interactions at regulatory sites via the reverse mechanism. Notably, the pharmaceutical industry has been developing compounds that bind to the ATP-binding site of protein kinases and potentially disrupt protein–protein interactions between target protein kinases and their regulatory interacting partners. Learning to modulate allosteric processes will facilitate the development of protein–protein interaction modulators.

Modulating Protein–Protein Interactions That Involve Protein Kinases

The development of drugs that target protein–protein interactions is of great interest [1]. However, we have often heard over the past two decades that targeting protein–protein interactions for drug development is very difficult. Despite this difficulty, recent work shows that drugs directed to the ATP-binding site (see Glossary) of protein kinases can potentially inhibit the interactions between the target protein kinases and their regulatory interaction partners, thus disrupting protein kinase regulation. Hence, although it is difficult, potent small molecule inhibitors of interactions may have already been produced in abundance in projects where the protein–protein interactions were not directly targeted. Key to understanding this discrepancy is to view protein kinases as allosteric proteins. Also, we must grasp the concept that allostery works bidirectionally. Here, we first discuss the concepts of allostery and the terminology in the field of protein kinases. We then review work substantiating the theory that the activity of numerous protein kinases is allosterically modulated by interactions at their regulatory sites, which in turn affects their active site. Further, we review research that shows that small molecules binding at the ATP-binding site can exploit the allosteric system to regulate protein–protein interactions at the regulatory site (reverse allostery). The result is that many drugs developed to inhibit the activity of protein kinases by targeting their ATP-binding site are also potent inhibitors of protein kinase interactions.

Allosteric Regulation of Protein Kinases

Allostery is a fundamental principle where a signal is transmitted within a protein or through protein complexes. Allostery relies on the dynamics of the conformation of a population of molecules where a perturbation, such as protein–protein interactions, at a first site (often called allosteric or regulatory site) leads to a change in the distribution of the conformational states, with population shifts ultimately reflected by a change in the function of a second noncontiguous site (often the active site) [2,3]. For simplicity, bidirectional allostery can be presented as an equilibrium between two conformations (i.e., active and inactive populations), which can be modulated by proteins, protein domains, post-translational modifications, or compounds with positive or negative cooperativity. It is notable that in the protein kinase field, the allosteric terminology has often been used without a reference to an effect at a second distant site (Box 1). Although cell signaling usually uses allostery unidirectionally to transmit a signal, due to the thermodynamic relation between the different conformations, allostery is intrinsically bidirectional. In the context of protein kinases, the reverse allosteric mechanism is one in which a small molecule binds at its ATP-binding site in the active site and produces a conformational change at its regulatory site, influencing protein–protein interactions.

Highlights

Allostery is widespread in protein kinases.

Allostery is defined as bidirectional communication between the regulatory site and the active site in protein kinases.

Compounds binding at the ATP-binding site of protein kinases can enhance or disrupt protein–protein interactions.

Metabolites can bind to the ATP-binding site and can potentially modulate physiological protein kinase interactions.

Understanding allostery at the molecular level will enable the rational design of drugs with the desired allosteric effects.

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Box 1. Terminology

The appropriate use of the allosteric terminology requires defining two different binding sites on the target protein. The interaction of a partner at a first site is said to produce an allosteric effect if it produces a change at a second, distant, site. Unfortunately, the terminology has been misused in the protein kinase field. The allosteric inhibitor terminology is most often used when a relevant change at a second distant site on the target protein kinase is not described. Additionally, the term 'allosteric' is often used to define a binding site that is not the ATP-binding site. As extensively depicted in the main text, there are numerous examples of compounds that bind to the ATP-binding site and produce allosteric effects (i.e., disrupting protein kinase interactions mediated by the PIF-pocket). For clarity, the recommendation is to use the term 'allosteric' only when the system clearly defines two sites, a binding site and a second site that senses a change when a compound binds to the first site. Following the recommendations from the International Union of Basic and Clinical Pharmacology [70], an allosteric compound should be defined by stating the binding site, the identity of the second site being affected, and the kind of effect produced (enhancement of the interaction, inhibition of the interaction, etc.).

Protein Kinase Catalytic Domain: Regulatory and Active Sites

The protein kinase catalytic domain consists of a small lobe with a regulatory hotspot and a large lobe (Figure 1A) [4]. Between the two lobes is the active site, which contains an ATP-binding site and an activation loop containing the **DFG motif**. The bottom of the ATP-binding site corresponds to regions on the large lobe, while the ceiling includes the $\beta 1$ and $\beta 2$ strands of the small lobe and the Gly-rich loop that connects them. The activation or inhibition of the catalytic activity of protein kinases is frequently mediated by interactions of the regulatory hotspot with other domains in the protein kinase, by post-translational modifications, and by interactions with protein partners. Within the regulatory hotspot (region flanked by the β -sheet and αB and αC helices), the αC helix plays a key role in switching protein kinases from active to inactive states by adopting in or out conformations, respectively [5]. The αC helix directly contacts another element of the switch, the DFG motif. The DFG-in conformation is characterized by specific interactions between the Asp and Phe of the DFG motif that are required for catalysis and the DFG-out conformation is impaired for activity [5,6] (Figure 1B). The conformation of the DFG motif has been thoroughly studied due to its relevance in developing inhibitors that target the ATP-binding site [7].

Allosteric Regulation: From the Regulatory Hotspot to the ATP-Binding Site
The αC Helix: A Frequent Mediator of Allosteric Regulation in Protein Kinases

The key role of the αC helix in modulating the switch between active and inactive kinases was first described in cyclin-dependent protein kinases (CDKs) [8,9]. The first structure of CDKs (PDB ID 1HCL) unveiled an inactive structure where the activation loop was folded into the active site and the αC helix (termed PSTAIRE helix in CDKs) was bent outwards (Figure 1B) [8,10]. A subsequent structure of CDK2 in complex with its interacting partner, cyclin A (PDB ID 1JST), showed a picture of the active form (Figure 1B) [9]. Cyclin A binds to CDKs by occupying a broad surface that encompasses the region in between the αC helix and the β -sheet on the small lobe's regulatory hotspot and extends to the other side of the αC helix, interacting with the activation loop. The crystallization of a small viral cyclin (cyclin T1) in complex with CDK9 showed that the activation of a CDK could be due to a much reduced surface in the region between the αC helix and the β -sheet of CDK9 [11]. Further, in the activation process, the binding of cyclin A induced the rearrangement of the activation loop and the active position of the αC helix and promoted the formation of a salt bridge between the αC helix Glu51 and the active site Lys33 [9] (Figures 1B and 2A,B); formation of the equivalent salt bridge is a conserved characteristic of active protein kinases. While the active conformation of the catalytic domain is conserved, the conformation of the αC helix region can be very diverse in different inactive structures.

Since its initial characterization, we now know of numerous examples where the αC helix participates both in the physiological activation of the kinase domain, by mechanisms that stabilize the active form of the kinase, and in the physiological inhibition of the activity, by stabilizing inactive forms (for reviews see [5,12]).

Glossary

AGC kinases: a group of evolutionary related protein kinases comprising over 60 members in humans. PKA, PKG, PKC, Akt/PKB, p70S6K (S6K), SGK, and p90RSK (RSK) are representatives of the 14 families in the group.

Allosteric: mechanism by which an interaction at one site of a protein (allosteric or regulatory site) produces a conformational change that affects the function of a second, distant site (i.e., the active site). The mechanism is bidirectional so binding at the active site can also generate a conformational change in the regulatory site.

ATP-binding site: a binding site for ATP-Mg present in all protein kinases. For catalysis to occur, ATP must bind to the site in a proper conformation so that the phosphates from ATP are adequately positioned to enable the transfer of phosphate to a protein substrate to occur. The site is a well-known 'druggable' pocket. Due to the conservation of the ATP-binding site, drugs developed to the site of one protein kinase often also bind and inhibit other protein kinases.

DFG motif: a conserved Asp-Phe-Gly (DFG) tripeptide motif at the proximal end of the activation loop of protein kinases that is part of the allosteric switch from the αC helix to the active site. Specifically, the DFG motif Phe is part of the hydrophobic regulatory spine that is assembled in every active kinase.

Docking interaction: a process where particular amino acid sequences outside from the catalytic core are recognized by some protein kinase families, including MAPKs and AGC kinases, through a specific docking site.

PIF-pocket: a regulatory site present in the small lobe of AGC kinases. The site was first depicted in the crystal structure of PKA, although in this kinase the C-terminal hydrophobic motif binds constitutively to the PIF-pocket and does not play a regulatory role.

Protein kinase: superfamily of enzymes that catalyze the transfer of the terminal phosphate from ATP to a protein substrate in a

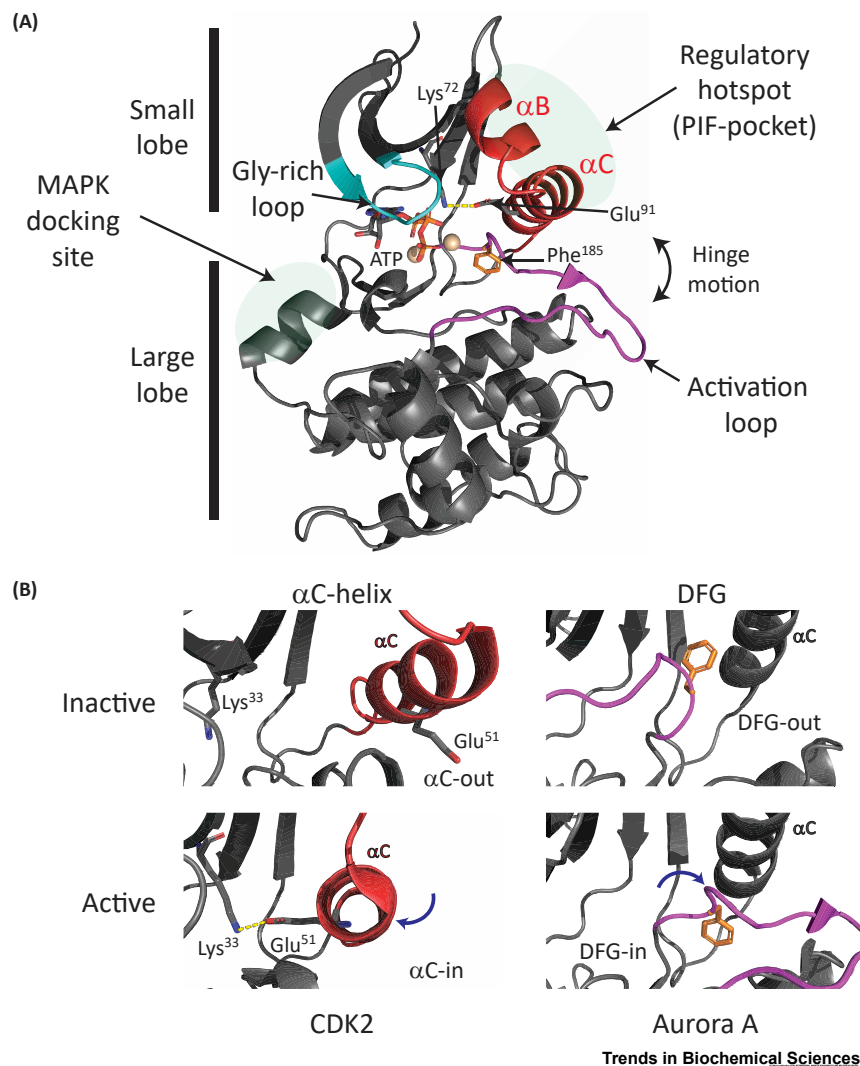


Figure 1. Structure and Regulatory Sites of Protein Kinases.

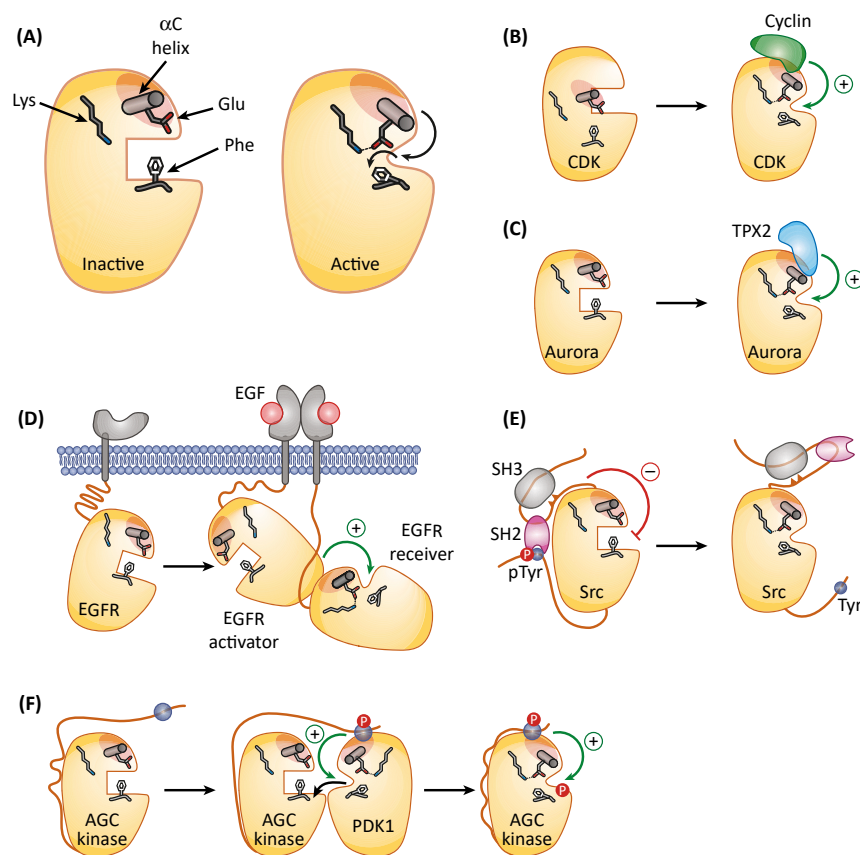
(A) Crystal structure of the catalytic domain of PKA (PDB ID 1ATP) in the active/closed conformation. The catalytic core consists of a small lobe and a large lobe; ATP binds between the two lobes in a cavity below the Gly-rich loop. A common feature of the catalytic core is the dynamic open–close hinge motion. The α B and α C helices (red) are key regulatory elements in a wide range of protein kinases and are part of a regulatory hotspot (PIF-pocket in AGC kinases). When the α C helix adopts an in/active conformation, it stabilizes the formation of a salt bridge between α C helix Glu (Glu91 in PKA) and a conserved Lys (Lys72 in PKA) that positions ATP for catalysis. Regulation often also involves the DFG motif (orange) on the activation loop (magenta) where the Phe residue can acquire in/active or out/inactive conformations. The indicated MAPK docking site is present in the MAPK family where it enables docking interactions. (B) Close-up of α C helix and DFG motif in inactive and active conformations of CDK2 (left) and Aurora A (right). The α C-helix (red) is swung outwards in the inactive structure of CDK2 (α C-out conformation; PDB ID 1HCL) and moves inwards upon activation (PDB ID 1JST), allowing the generation of the Glu-Lys salt bridge. In the inactive conformation of Aurora kinase, the DFG motif Phe (orange) is flipped (DFG-out conformation, PDB ID 3UOK), while in the active conformation (PDB ID 1OL5) the motif is in the DFG-in conformation and the activation loop (magenta) opens up to enable the binding of the peptide substrate. Abbreviations: MAPK, Mitogen-activated protein kinase; PIF-pocket, PDK1 interacting fragment-binding pocket.

process termed protein phosphorylation.

Protein kinase regulation: the activity of protein kinases is physiologically tightly regulated. The term refers to the distinct mechanisms present in different protein kinases to specifically control their catalytic activity. Evolutionary related protein kinases share general aspects of the regulatory mechanisms.

Residence time: the time a small compound remains bound to the target before dissociation. It is inversely proportional to the value of the off-rate ($1/k_{\text{off}}$).

Reverse allostery: in this context, a mechanism by which a ligand or small molecule interacts with the ATP-binding site of a protein kinase and produces a conformational change at a second, distant site (allosteric or regulatory site) that influences its protein–protein interactions.



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Figure 2. Allosteric Regulation of Protein Kinase Activity, from Regulatory Hotspot to Active Site.

(A) Schematic representation of the catalytic domain of protein kinases where key residues for the regulation of enzymatic activity (Glu residue on the α C helix, Lys residue on the β 3 sheet, and Phe from the DFG-motif) are designated. The inactive conformation, depicted with a square-shaped active site, has the α C helix out and DFG-out conformation, which blocks catalysis. The DFG motif and the α C helix are correctly positioned in the active DFG-in and α C helix-in conformation; the Glu from α C helix forms a salt-bridge bond (dashed line) with the Lys on the β 3 sheet and the Phe residue from the DFG-motif flips to allow catalysis. In both conformations, the regulatory hotspot is shown (orange). (B) Cyclin (green) binding to the regulatory hotspot of CDK pushes its α C helix into the correct position and stabilizes the active conformation. (C) TPX2 (light blue) binding to the regulatory hotspot activates Aurora A by stabilizing its active conformation. (D) In response to binding the epidermal growth factor (EGF, red) via its extracellular domain (gray), epidermal growth factor receptors (EGFRs) make head-to-tail dimers where one EGFR (activator) stabilizes the regulatory hotspot of the receiver EGFR and increases the receiver's phosphorylation activity. (E) The inactive form of Src kinase is characterized by intramolecular interactions of the SH2 domain (pink) with a C-terminal phospho-Tyr (pTyr, red) and by the SH3 domain (gray) and linker region (depicted with triangles) with the back regulatory hotspot. The release of the autoinhibitory interactions due to the dephosphorylation of the C-terminal Tyr (dark blue) is sufficient to achieve the active kinase conformation. (F) Members of the AGC protein kinases group possess a hydrophobic motif (dark blue) that, upon phosphorylation (red), mediates a docking interaction between the AGC kinase and the regulatory PDK1 interacting fragment-binding pocket of PDK1. This stabilizes the active form of PDK1, which subsequently phosphorylates the activation loop of the kinase substrate and, in combination with the tail hydrophobic motif binding intramolecularly to its own regulatory hotspot, activates the AGC kinase.

Activating Allosteric Mechanisms

Aurora kinases are activated by interactions of their regulatory hotspots with protein partners (TPX2 in Aurora A and INCEP in Aurora B) [13,14]. Like CDKs, the inactive structures have the α C helix turned

outwards and the Phe from the DFG motif in the DFG-out conformation (Figures 1B and 2C). TPX2 and INCEP regulatory proteins bind to the equivalent region that proved to be the regulatory hotspot in CDKs, located between the α B and α C helices and the β -sheet [13,15], and activate the kinase domain by moving the α C helix and the Phe from the DFG motif inwards.

In a slightly different manner, the membrane anchored epidermal growth factor receptor (EGFR) kinase is activated by a head-to-tail asymmetric dimer after the receptors bind epidermal growth factor (EGF; Figure 2D). Briefly, one EGFR (the activator) stabilizes the active conformation of a second EGFR (the receiver) by interacting with its regulatory hotspot [16]. The regulatory site is also equivalent to that found in CDKs, again demonstrating conservation between allosteric mechanisms in protein kinases. Further, B-Raf kinases are activated by the formation of side-to-side dimers, where specific interactions between the regulatory hotspot of both protomers destabilize the inactive monomer by pushing the α C helix into a productive orientation [17,18]. Finally, RET kinases do not form dimers, but are activated by interactions of the phosphorylated juxtamembrane region with a hydrophobic pocket, equivalent to the CDK's regulatory hotspot, which stabilizes the α C helix [19].

Inhibiting Allosteric Mechanisms

Multiple examples have shown that the α C helix also participates in stabilizing the inhibition of protein kinases, such as Src kinases [20], Bruton's tyrosine kinase (BTK) [21], Zeta Chain-associated protein of 70 kDa (ZAP-70) [22,23], c-Fes [24], and Csk [25]. In all examples, the displacement of the α C helix stabilizes conformations that are not catalytically competent (Figure 2E). Additionally, in the Ser/Thr protein kinase Akt/PKB, the N-terminal PH domain was shown to directly interact with and stabilize an unfolded form of α C helix to maintain an inactive kinase domain. The release of this autoinhibition and the intramolecular interaction of the Akt C-terminal phosphorylated hydrophobic motif with the regulatory hotspot stabilizes the α C helix in an active conformation, mediating the activation of the kinase domain [26].

Phosphoinositide-Dependent Protein Kinase 1, an Extensively Studied Model of Allostery

The above are examples from numerous cases in which the regulation of the protein kinase activity has been investigated and structural information is available, depicting the active and inactive structures of the catalytic domain. In most cases, we only observe two snapshots of the kinases (the active or inactive structures) but the dynamics between the conformations is missing. Abundant information on the allosteric process was obtained for phosphoinositide-dependent protein kinase 1 (PDK1), a member of a group termed **AGC kinases** (named after its original members, PKA, PKG, and PKC), which also includes Akt/PKB.

As depicted above for Akt/PKB, as well as for other AGC kinases (excluding PDK1), phosphorylation of a hydrophobic motif, which is located C-terminal to the catalytic domain, triggers its intramolecular interaction with a hydrophobic cavity between the α C helix and the β -sheet at the site equivalent to the regulatory hotspot found in CDKs, Aurora kinases, EGFR, RET, and others. In AGC kinases, this site is termed the PDK1 interacting fragment-binding pocket (**PIF-pocket**) [27–29]. Intramolecular binding of the hydrophobic motif to the PIF-pocket allosterically increases the kinase activity of protein kinases from the AGC group [30]. In the case of PDK1, the PIF-pocket is essential for the **docking interaction** mechanism that allows PDK1 to recognize its substrates (for a recent review see [31]). Briefly, because PDK1 does not have a hydrophobic motif, it intermolecularly interacts with the phosphorylated hydrophobic motif of another AGC kinase (substrate kinase); this promotes allosteric effects on the active site of PDK1, increasing its specific activity and enabling the phosphorylation of the substrate at the activation loop (Figure 2F).

This mechanism was thoroughly validated with the development of small compounds [32–39] that precisely bind to the PIF-pocket of PDK1 [33,39]. In solution hydrogen/deuterium (H/D) exchange studies of PDK1 showed that the α B and α C helices are extremely mobile. Incubation of PDK1 with

small molecules that bind the PIF-pocket and stabilize its active conformation increased the protection of the PIF-pocket helices and showed changes in the H/D exchange patterns of peptides from the Gly-rich loop and DGF motif, supporting the model of an allosteric communication from the regulatory hotspot to the active site [33]. Allosteric between the PIF-pocket and the ATP-binding site was also determined using a fluorescent ATP analog (TNP-ATP) as a probe for the conformation of the ATP-binding site. The binding of activating compounds at the PIF-pocket produces a specific change in TNP-ATP fluorescence, validating that the compounds produce an allosteric change in the conformation of the ATP-binding site [33]. Further, crystal structures of PDK1 in complex with an activating small compound showed changes at the ATP-binding site, resulting in the active/closed conformation of the catalytic domain of PDK1. In this structure, an ATP-Mg sat in the active site ready for catalysis (PDB ID 4AW1) [37], as depicted originally in the structure of the closed/active PKA in complex with ATP and the pseudosubstrate inhibitor PKI (PDB ID 1ATP) [40]. Together, the studies on PDK1 mechanism of regulation are strong evidence of an allosteric communication that links the binding of activators at the PIF-pocket with the stabilization of the α C helix and a conformational change at the ATP-binding site.

Reverse Allosteric Regulation: From the ATP-Binding Site to the Regulatory Hotspot

Allosteric Modulation of Protein Kinase Intramolecular Interactions by Compounds Binding to the ATP-Binding Site

Throughout the 25 years of drug development directed to the ATP-binding site of protein kinases, there have been numerous reports of inhibitors that, in cells, actually enhance the level of phosphorylation of the target kinase. This increased phosphorylation sometimes increased the signaling downstream of that kinase. The paradoxical activation of the kinase upon treatment with its inhibitors could be due to the inhibition of a negative feedback loop, which ultimately enhanced the signaling. However, the findings could have also been due to reverse allosteric effects that affect the regulatory sites.

For example, the binding of an ATP-competitive inhibitor (A-443654) at the ATP pocket of Akt/PKB potentiates the binding of the Akt PH domain to phosphatidylinositol (3,4,5)-triphosphate and increases the phosphorylation levels of the activation loop (Thr308) and the hydrophobic motif (Ser473), which are known to promote the activity of the Akt/PKB [41]. The structure of the inactive full length Akt/PKB provides a simple explanation: the binding of A-443654 promotes a reverse allosteric effect on the regulatory hotspot (on the PIF-pocket) by stabilizing the α C helix in the active conformation and disrupting the intramolecular inhibitory interaction of the regulatory hotspot with the PH domain (PDB ID 3O96) [26] (Figure 3A).

In Src-family kinases, compounds targeting the ATP-binding site can both enhance or reduce the ability of the SH3 and SH2 domains to engage in intramolecular inhibitory interactions [42] and the ability of the kinase–inhibitor complex to undergo post-translational modification by effector enzymes [43]. Similarly, drugs developed to bind to the ATP-binding site of Plk1 (i.e., BI6727 and FM00204) also release the intramolecular interaction of the polo-box domain (PBD) with Plk1, thereby enhancing the active conformation of the kinase domain. The exposure of the PBD in turn enhances its protein–protein interactions [44] (Figure 3B). Akt/PKB, Src, and Plk1 exemplified above are physiologically regulated by the intramolecular interaction of regulatory domains with the catalytic domain. From the perspective of this review, the effects produced by compounds binding at the ATP-binding site reflect the reverse of the physiological allosteric regulation.

Allosteric Enhancement of Protein Kinase Intermolecular Interactions by Compounds Binding to the ATP-Binding Site

The potential of ATP-competitive drugs to enhance protein kinase interactions were originally documented for the dimerization of B-Raf [45,46] (reviewed in [17]). The activation of RAF kinases requires a shift from an autoinhibited monomer to a catalytically active state characterized by adoption of a side-to-side dimer. RAF inhibitors that bind the ATP-binding site, originally designed to inhibit B-RAF and

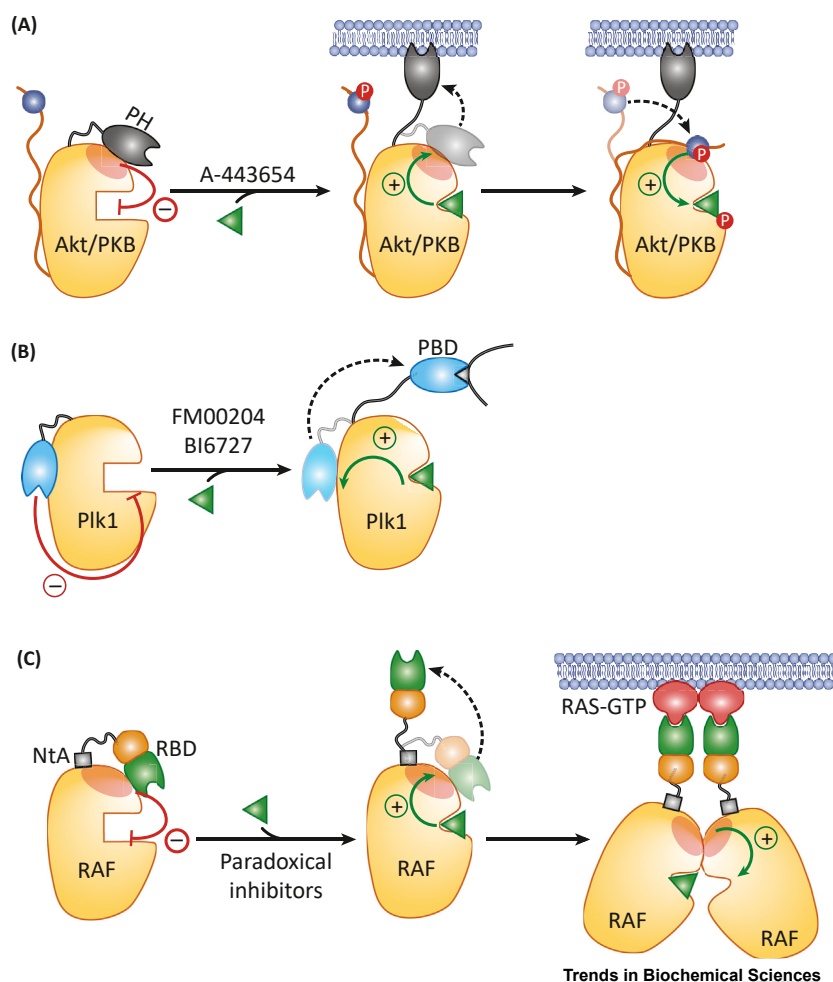


Figure 3. Reverse Allosteric Enhancement of Protein Kinase-Protein Interactions by Compounds Targeting the ATP-Binding Site.

(A) Akt/PKB kinase activity is inhibited by its own PH domain (gray) binding its regulatory hotspot (orange) and stabilizing a form with disrupted/inactive α C helix. The active conformation of Akt/PKB is stabilized by binding the compound A-443654 (green triangle) in its ATP-binding pocket, which stabilizes the α C helix [PDK1 interacting fragment (PIF)-binding pocket], enhances the binding of Akt/PKB to membranes via the released PH domain, and increases phosphorylation of Akt/PKB at T308 (activation loop) and S473 (hydrophobic motif) regulatory sites. The phosphorylated hydrophobic motif can intramolecularly bind to the PIF-pocket, further promoting the active state. (B) Binding small molecules FM00204 and BI6727 compounds (green triangle) in the ATP-binding site relieve the autoinhibition of Plk1 by its own PBD domain (light blue) and enhance its binding to specific interacting partners (black). (C) Paradoxical RAF inhibitors (green triangle) relieve the autoinhibition of the RAS binding domain (RBD, orange and green), which binds its regulatory hotspot (orange). Binding of paradoxical RAF inhibitors results in the formation of RAF dimers where the inhibited RAF molecule allosterically activates the noninhibited partner by interacting with its regulatory hotspot. The increased exposure of the RBD promotes its binding to RAS-GTP at the plasma membrane. The allosteric activation requires the participation of an N-terminal acidic motif (NtA).

its oncogenic signaling, showed paradoxical enhancement of RAF enzymatic activity, increasing ERK signaling, and as a result increasing cellular proliferation. These counter-intuitive results are due to the increase in dimer formation that is promoted by some compounds that bind at the ATP-binding site of RAF (Figure 3C) [45,46], although the dimerization alone does not fully explain the paradoxical

activation [47]. A similar mechanism was later identified for other protein kinases. For example, APY29 derivatives targeting the ATP-binding site of PERK paradoxically enhance phosphorylation activity by transactivation within a dimer [48]. Additionally, APY29 derivatives targeting the ATP-binding site of the kinase domain of IRE-1 α increase the oligomerization and the activity of its RNase domain [49].

Paradoxical effects of inhibitors binding to the ATP-binding site were also described for pseudokinases [50]; for example, in the EGFR family, the binding of lapatinib and bosutinib to the pseudokinase HER3 promotes HER2–HER3 heterodimerization and ligand-independent proliferation of HER2+ breast cancer cells [51]. Recent studies further showed that inhibitors that stabilize the active conformation of EGFR (i.e., erlotinib) increase the affinity of EGFR for its extracellular ligand, while inhibitors that stabilize the inactive conformation (i.e., lapatinib) decrease the receptor binding affinity for EGF [52]. Both inhibitors block EGFR kinase activity but show differential potency against different tumor types, which might be due to their differential reverse allosteric effects on the affinity for EGF. In these cases, the drugs binding to the ATP-binding site appear to modulate the reverse allosteric process across the cell membrane.

In the above cases, the kinases are activated by dimerization and the compounds binding to the ATP-binding site in the active conformation produce a reverse allosteric effect at the dimerization interface and stimulate the dimer interaction. Interestingly, from the different forms of allosteric effects of compounds binding at the ATP-binding site, the first ones to be described were those that produced a paradoxical activation of protein kinases. Less notorious were the other available compounds that bound to the ATP-binding site and disrupted protein kinase interactions.

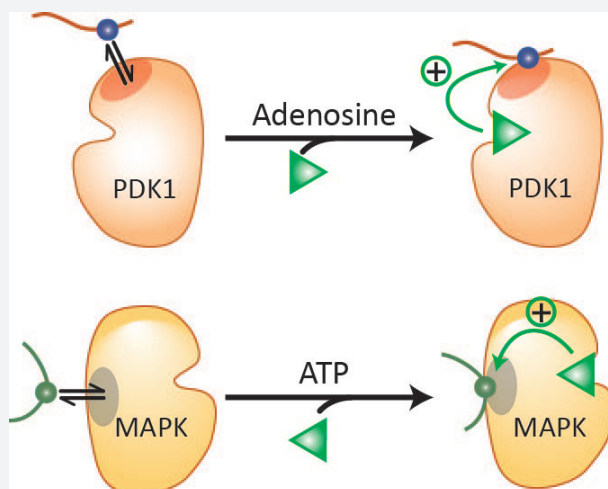
Allosteric Inhibition of Protein Kinase Interactions by Compounds Binding to the ATP-Binding Site

Above, we described how compounds binding to the PIF-pocket regulatory hotspot on PDK1 allosterically affected the ATP-binding site. The reverse allosteric effects were also studied in the model kinase PDK1 and it was observed that the hit small compound PS653 disrupted interactions at the PIF-pocket by binding to the ATP-binding site [53]. Other molecules that bound to the ATP-binding site did not affect the PIF-pocket (i.e., ATP, ADP, AMP, adenine). However, adenosine enhanced interactions of a hydrophobic motif-containing polypeptide at the PIF-pocket (Box 2). The crystal structures of PDK1-PS653 and PDK1-adenosine complexes showed that the compounds bind to the ATP-binding site, confirming the allosteric effects of PS653 and adenosine at the PIF-pocket site [53] (Figure 4A). However, crystallography did not unveil the mechanisms for the observed effect. Molecular dynamics (MD) simulations suggested that PS653 stabilized an inactive conformation of the kinase domain, with well-formed stable helices at the PIF-pocket [53]. By contrast, adenosine enhanced the protein interactions at the PIF-pocket, by promoting the deformation of the PIF-pocket characterized by an extension of the α B helix length [53].

When other commercially available ATP-competitive inhibitors of PDK1, GSK2334470, and UCN01, were tested, only GSK2334470 potentially disrupted interactions at the PIF-pocket regulatory site, as observed for the PS653 compound. As discussed above, PDK1 phosphorylates different protein kinase substrates and, except for Akt/PKB, these events are mediated by intermolecular interactions of the PIF-pocket of PDK1 with its substrates. Interestingly, when used in cells, GSK2334470 more potently inhibits phosphorylation of S6K by PDK1 compared with Akt/PKB [54]. It is conceivable that the substrate-selective effect of GSK2334470 may be due to a double action: inhibition of the activity of PDK1 and by disrupting PDK1–S6K complexes by reverse allostery. A recent patent filed by Sunesis Pharmaceuticals describes a series of compounds targeting the PDK1 active site that also strongly disrupt interactions at the PIF-pocket of PDK1 and are described as potent inhibitors of the activation of RSK [55]. Together with the information on the allosteric activation from the PIF-pocket to the ATP-binding site, the studies on PDK1 represent a model example of bidirectional allosteric communication between the regulatory site and the ATP-binding site in a protein kinase.

Box 2. Metabolites

Are protein kinase interactions regulated by metabolites? A recent work identified that adenosine binding at the ATP-binding site, but not adenine, AMP, ADP, or ATP, allosterically enhanced *in vitro* interactions of PDK1 with the PIF-pocket docking site [53]. Adenosine exploits the reverse path of the allosteric regulatory mechanisms described in the main text and could also be the physiological mediator of reverse allosterity identified in drugs binding to the ATP-binding site (Figure I). Could adenosine regulate the formation of protein complexes with PDK1 in any physiological situation? This is not known. However, this finding opens the intriguing and unexplored possibility that metabolites could physiologically regulate the formation of protein kinase complexes modulating intra- and inter-molecular interactions, the localization of protein kinases, the generation of multiprotein complexes, etc. If it existed, such a mechanism could link the metabolic state of cells to the regulation of cell signaling hubs. Interestingly, since their discovery more than 50 years ago [71], numerous allosteric systems are known to be modulated by metabolites and are continuously being identified [72,73]. Adenosine was identified *in vitro* by testing fragments of ATP. The screening of unbiased libraries of metabolites for their interactions with different protein kinases could short-list possible allosteric regulatory candidates. A combination of unbiased screening data with metabolomics studies on relevant pathways could then unveil the possible physiological metabolic modulators.

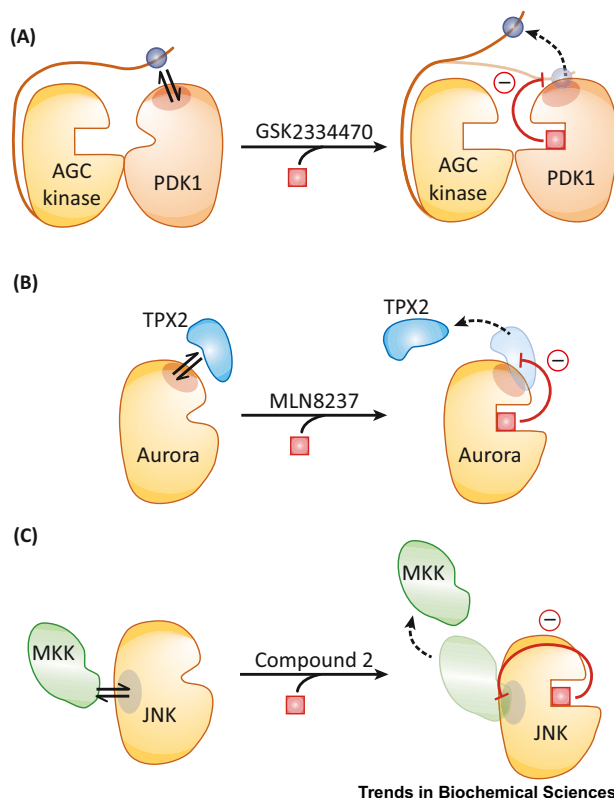


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Figure I. Allosteric Modulation of Protein–Protein Interactions by Metabolites

PDK1 possesses a PDK1 interacting fragment-binding pocket (PIF-pocket; orange) to which a hydrophobic motif (dark blue) of AGC kinases binds. Adenosine (green triangle) binding to the ATP-binding site enhances *in vitro* interactions of the PIF-pocket with the hydrophobic motif of AGC kinases. Similarly, MAP kinase (MAPK) interacts with MAPK kinase (MKK, green) via the MAPK docking site (gray). This interaction is enhanced by binding ATP (green triangle) to the ATP-binding site of MAPK. Abbreviations: MAPK, Mitogen-activated protein kinase; PDK1, phosphoinositide-dependent protein kinase 1.

Similar to the activation of PDK1 by interactions with hydrophobic motif-containing polypeptides, TPX2 also binds at the regulatory hotspot of Aurora A to activate it [56] and its activity can be modulated by compounds binding to the regulatory hotspot [57]. Using a similar mechanism to TPX2 binding, Aurora A binds and is activated by the oncogene N-myc and the interaction stabilizes N-myc [58]. Reverse allosterity has also been observed for Aurora A. Gustafson *et al.* found that the Aurora kinase ATP-binding site inhibitors CD532 and, to a lesser extent, MLN8237 (currently in clinical trials), produced a conformational change that disrupts the interaction between the regulatory hotspot of Aurora A with TPX2 (Figure 4B) and destabilizes the oncogene N-myc [59]. Other compounds, such as VX680, do not disrupt these interactions [53,60], which shows that the selection of lead candidates can be based on the strength of the reverse allosteric effect on the destabilization of protein kinase complexes. TPX2 also provides anchorage at specific locations for Aurora A during the cell



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Figure 4. Reverse Allosteric Disruption of Protein Kinase-Protein Interactions by Compounds Targeting the ATP-Binding Site.

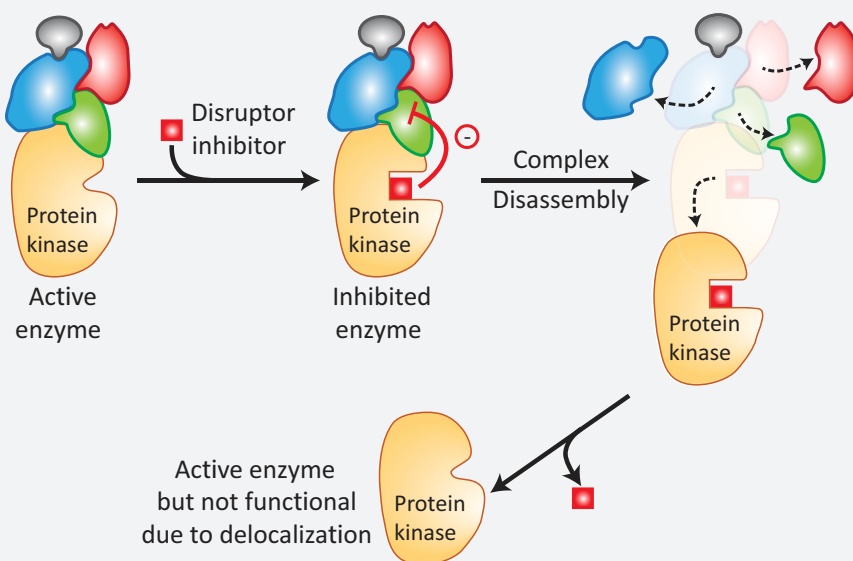
(A) The hydrophobic motif (dark blue) of AGC kinases mediates docking interactions between the AGC kinase and the PDK1 interacting fragment (PIF)-binding pocket of PDK1. GSK2334470 compound (red square) binding at the ATP-binding site of PDK1 allosterically affects its PIF-pocket and disrupts docking interactions, inhibiting docking of the two enzymes and subsequent activation of AGC kinases. (B) MLN8237 (red square) binds at the ATP-binding site and potentially disrupts the interaction of Aurora A with its binding partner, TPX2 (light blue), by stabilizing the inactive conformation of the α C helix. (C) The mitogen-activated protein kinase (MAPK) JNK interacts with the MAPK kinase (MKK, green) at the MAPK docking site (gray). Compound 2 [61] (red square) binding at the ATP-binding site allosterically blocks the binding of MKK to the MAPK docking site.

cycle. It was shown that small compounds that stabilize DFG-in have a positive cooperativity with TPX2 (i.e., ENMD-2076), while compounds stabilizing DFG-out conformation (i.e., MK-8745) potentially disrupt the interaction with TPX2 (negative cooperativity) [60]. In such an example, the disruption of protein-protein interactions could add a second level of inhibition to Aurora A signaling because the disruption of the interaction with TPX2 will alter the colocalization of the Aurora A with substrates that are part of the TPX2-multiprotein complex [53] (Box 3). However, depending on the dynamics of the TPX2 interaction with Aurora A, compounds binding to the DFG-out conformation may struggle to inhibit the pool of kinases that are stabilized in DFG-in conformation [60].

Mitogen-activated protein kinases (MAP kinases; JNK, ERK, and p38) also possess docking mechanisms of interactions that are used by their upstream kinase, their substrates, and the phosphatase that inhibits the MAP kinases. Unlike PDK1, the docking site is located at the back of the kinase, behind the hinge region (PDB IDs 4E73 and 4UX9). Maly's group recently described the reverse allosteric modulation of the MAP kinase JNK docking site with ATP-competitive drugs. Compounds that target the ATP-binding site stabilize different conformations of the protein, enhancing and

Box 3. Mislocalization

It can be argued that the allosteric effects of inhibitors may be irrelevant for drugs that achieve full inhibition of the kinase. However, drugs should be expected to have different effects in cells if they enhance or disrupt multiprotein complexes where they are located. Besides the independent effects that could be produced by the disruption of multiprotein complexes, we expect that drugs that disrupt protein kinase interactions will delocalize the kinase from the multiprotein complexes where they physiologically function and where their substrates are located. The possible effect of delocalization of a protein kinase can be better evaluated in the cases where the kinases are known to function at very particular locations (i.e., Aurora kinases and Plk1, which are required at the spindle and centrosomes during cell division) [74]. The delocalization of such kinases during the cell cycle may turn the kinase functionally inactive, even after the drug may have been released from the active site (Figure I). As a consequence of such a model, one must expect that the longer the residence time of the drug, the lower the probability that the protein kinase will return to its proper location. A further theoretical consequence of such a model is that the compounds that affect the localization of the target may be released from a kinase molecule, leave it delocalized/inactive, and may be free to bind, delocalize, and functionally inhibit more protein kinase molecules. Under that scenario, particular inhibitors that bind to the ATP-binding site could produce cellular effects at considerably lower concentrations on the target kinases that are mislocalized. Interestingly, in our own work, we found that compounds that affected the conformation of Plk1 and its interaction with protein partners were exceptionally good inhibitors of Plk1 signaling, even though they were weak inhibitors of the activity of Plk1 *in vitro* and in cultured cells [44].



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Figure I. Disruption of Protein Complexes by 'Reverse Allostery'

Binding of an inhibitor (red square) to the ATP-binding site of a protein kinase may allosterically block its interactions within multiprotein complexes, promoting complex disassembly and rendering the kinase inactive. Even after the kinase releases the inhibitor, the enzyme may be active but not functional due to its mislocalization.

disrupting docking interactions [61] (Figure 4C). Studies on the MAP-kinases ERK2 and p38 also demonstrated that ATP-competitive inhibitors can positively or negatively influence the interactions of the kinases with their physiological partners [62]. Complementing the results obtained with inhibitors, recent NMR and MD studies showed that ATP affects the docking site on p38 [63,64].

Additional examples of reverse allostery inhibiting protein–protein interactions have been observed. An ATP-competitive small molecule (APS-2-79) stabilizes a conformation of the pseudokinase KSR2 that it is not able to interact with RAF, reducing RAF-mediated phosphorylation of MEK [65]. Additionally, compounds targeting the ATP-binding site of IRE-1 α (i.e., compound 3 in [49]) and the pseudokinase RNase L (i.e., sunitinib) inhibit oligomerization and the activity of their RNase domains [49,66].

Altogether, there is substantial evidence that protein kinases are regulated allosterically, affecting the active site, and that the binding of compounds to the ATP-binding site can allosterically affect the conformation of the regulatory sites, stabilizing or disrupting protein kinase interactions.

The Mechanism of Bidirectional Allostery

The systems summarized above rely on the protein kinases having an equilibrium with an allosteric coupling between the active site and regulatory sites (i.e., α C helix-in/active or -out/inactive and DFG-in/active or -out/inactive). A small conformational change in the regulatory site can increase the probability of a large conformational change at the active site, pushing the inactive conformation into the active [67]. As a consequence of the equilibrium and allosteric coupling, the reverse path is also possible. Binding of small molecules at the ATP-binding site can allosterically modulate the protein–protein interactions at the regulatory sites. At a molecular level, allosteric transitions between active and inactive conformations rely on dynamic organization–reorganization of key hydrophobic residues, that, in the active conformation, forms a continuous spine transversing the kinase core from the large lobe to the small lobe (regulatory spine) [68]. MD simulations were employed to understand the mechanism of allosteric coupling between the PIF-pocket and the ATP-binding site in PDK1 [53]. Without binding partners or small molecules, the simulations showed the existence of populations of conformations, where the most notable changes were the dynamic opening–closing between the two lobes and the organization of the PIF-pocket. In the PIF-pocket, for example, an equilibrium was found between populations with well-formed α B helices (active state) and disrupted PIF-pockets (inactive state).

The MD simulations demonstrated that transmission of the structural changes along the regulatory spine required contacts between the lobes, as only the active/closed conformations have populations with disrupted α B helices [53]. For example, in simulations which included allosteric activators binding to the PIF-pocket, the α B helix was stabilized and the population of molecules with the active/closed conformation was enriched. In this conformation, it was observed that interactions were made between the large lobe and residues from the regulatory hotspot, the DGF motif, and the Gly-rich loop. In the reverse pathway, simulations showed that binding of adenosine to the ATP-binding site enhanced protein–protein interactions at the PIF-pocket by stabilizing a population of molecules with an active/closed conformation and partial destabilization of α B helix. The increased flexibility of the PIF-pocket is thought to enhance the probability of the generation of proper contacts between the PIF-pocket and the interacting proteins.

A specific analysis of the residues involved in the conformational transitions showed that when PDK1 adopted an active/closed state, a salt-bridge between α C helix Asp138 and Lys199 was disrupted and a salt-bridge between Asp138 and Lys144 was formed, stabilizing the active conformation [53]. Mutation of Lys144 severely decreased the protein–protein interactions at the PIF-pocket and stabilized inactive/open conformations in the MD simulations, suggesting that the transmission of information between the active site and PIF-pocket is disrupted. Notably, this mutation also changed the allosteric effects of PDK1; in its wild-type state, PDK1 protein–protein interactions are not influenced by adenine but in Lys144 mutants, adenine allosterically enhanced these interactions.

Together, the mechanism of allosteric transition from the inactive to active state relies on the dynamics of a conserved hydrophobic regulatory spine and an allosteric coupling that, in PDK1, is mediated by salt bridges at the surface of the kinase, which are modulated by interactions at the PIF-pocket and ATP-binding sites. Variations on a common theme are expected to mediate the

mechanism of allostery in different kinases, possibly involving other surface salt bridges, or, as described for Aurora A, a water-mediated allosteric network dependent on a Gln residue instead of a hydrophobic residue in the regulatory spine [69].

Concluding Remarks

Recent studies have shown that compounds binding to the ATP-binding site can be potent inhibitors of protein kinase interactions. The allosteric nature of protein kinase regulation is key to understanding this phenomenon. Due to the bidirectional properties of allostery, it should have been expected, rather than surprising, that small compounds and drugs binding to the ATP-binding site can allosterically enhance or disrupt interactions at regulatory sites. We foresee that *in silico* tools will enable the rational design of drugs with desired allosteric effects, modulating the stability of protein kinase interactions, protein complexes, the subcellular localization of kinases, etc. (see Outstanding Questions). Similarly, the experience with protein kinases could be applied to other proteins that transduce intracellular signals using allostery as the central language for receiving and transmitting signals.

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Outstanding Questions

Can we routinely model protein kinase allosteric systems?
Can we design small compounds with the desired allosteric effects?
Are metabolites involved in the regulation of protein kinase complexes?
What is the effect of the mislocalization of protein kinases or of enhancing or disrupting protein complexes?

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