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Review

PKA: a portrait of protein kinase dynamics

S.S. Taylor*, J. Yang, J. Wu, N.M. Haste, E. Radzio-Andzelm, G. Anand

Howard Hughes Medical Institute, Bethesda, MD, USA
Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92014-0654, USA

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Abstract

Protein kinases play a critical role in the integration of signaling networks in eukaryotic cells. cAMP-dependent protein kinase (PKA) serves as a prototype for this large and highly diverse enzyme family. The catalytic subunit of PKA provides the best example of how a protein kinase recognizes its substrates, as well as inhibitors, and also show how the enzyme moves through the steps of catalysis. Many of the relevant conformational states associated with the catalytic cycle which have been captured in a crystal lattice are summarized here. From these structures, we can begin to appreciate the molecular events of catalysis as well as the intricate orchestration of critical residues in the catalytic subunit that contribute to catalysis. The entire molecule participates. To fully understand signaling by PKA, however, requires an understanding of a large set of related proteins, not just the catalytic subunit. This includes the regulatory subunits that serve as receptors for cAMP and the A kinase anchoring proteins (AKAPs) that serve as scaffolds for PKA. The AKAPs localize PKA to specific sites in the cell by docking to the N-terminus of the regulatory subunits, thus creating microenvironments for PKA signaling. To fully appreciate the diversity and integration of these molecules, one needs not only high-resolution structures but also an appreciation of how these molecules behave in solution. Thus, in addition to obtaining high-resolution structures by X-ray crystallography and NMR, we have used fluorescent tools and also hydrogen/deuterium exchange coupled with mass spectrometry to probe the dynamic properties of these proteins and how they interact with one another. The molecular features of these molecules are described. Finally, we describe a new recombinantly expressed PKA reporter that allows us to monitor PKA activity in living cells.
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Keywords: PKA; Dynamic; Kinase inhibitor; Balanol; Catalysis; AKAP

1. Introduction

Since approximately 2% of mammalian genomes encode for protein kinases [1], while 4% of plant genomes code for protein kinases [2], these enzymes constitute one of the largest gene families. The protein kinases regulate a myriad of cellular processes during growth and development, they are an integral part of the machinery that is activated in response to stress, they are essential for memory, and they are directly involved in orchestrating cell death. Because of their widespread involvement in the regulation of cellular events and because defects in protein kinase function are associated directly with so many diseases, these enzymes are primary targets for therapeutic intervention. The development of Gleevec, an adenosine triphosphate (ATP) analog with specificity for the Abl oncogene, the cause of myelogenous leukemia, demonstrated the remarkable specificity that such drugs are capable of displaying in spite of the highly conserved fold of the protein kinase core with its conserved ATP binding pocket [3].

One of the simplest members of the protein kinase family is cAMP-dependent protein kinase (PKA). Its kinetic properties have been well-defined, its crystal structure in the presence of nucleotide and peptide substrate, as well as the recently solved structures of the apoenzyme [4] and of an aluminum fluoride complex containing adenosine diphosphate (ADP) and substrate peptide [5], provide a detailed description of intermediates in the reaction pathway. These structures also allow us to appreciate the malleability of the protein kinase core as it binds its substrates and prepares to transfer the γ-phosphate of ATP to its protein substrate. This

Abbreviations: cAMP, cyclic-3′,5′-adenosine monophosphate; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; ATP, adenosine triphosphate; ADP, adenosine diphosphate; PKI, heat stable protein kinase A inhibitor; D/D domain, dimerization/docking domain; AKAP, A-kinase anchoring proteins

* Corresponding author. Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92014-0654, USA. Tel.: +1-858-534-3677; fax: +1-858-534-8193.
E-mail address: staylor@ucsd.edu (S.S. Taylor).

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malleability is likely a conserved feature of all protein kinases with PKA merely serving as a prototype.

The activity of the catalytic (C) subunit is regulated by a set of four different regulatory (R) subunit isoforms. Although each regulatory subunit has a conserved organization of subdomains with a dimerization/docking (D/D) domain at the amino terminus and two tandem cAMP binding domains at the carboxyl terminus, the R subunits play distinct roles and are not functionally redundant [6]. In addition to the regulatory subunits, specificity is also achieved by the scaffold proteins, the A kinase anchoring proteins (AKAPs), which target PKA through the regulatory subunits to different sites within the cell and in close proximity to specific substrates. All of these proteins contribute to the PKA signaling networks that permeate every mammalian cell. Each also contributes in novel ways to the dynamic features of this network.

To fully describe the molecular function of a protein kinase as well as the function of that kinase within the context of a cell requires a variety of techniques. In addition to crystallography which provides static high-resolution structures, it is necessary to use methods that allow us to appreciate the dynamics of these proteins in solution. In parallel, fluorescent techniques are being developed that allow us to follow kinase activation and localization in real time in living cells [7]. The malleability of the protein kinase provides for a wide range of conformational states that can be targeted for therapeutic intervention. In addition to the active site cleft, there are also multiple surface sites that can be targeted that will disrupt normal cellular function as well as disruption from the scaffolds. The static crystal structures provide clues as to the conformational versatility of the molecules; however, solution methods are required if we are to appreciate the dynamic behavior of these molecules as they move through the catalytic cycle and as they move between substrates, inhibitors, and scaffold proteins. To complement crystallographic structures, we have used fluorescence anisotropy [8,9], NMR [10], and, most recently, hydrogen/deuterium (H/D) exchange coupled with mass spectrometry [11,12] to probe the dynamical behavior of PKA and its scaffold proteins. H/D exchange, in particular, can be used to map domain boundaries, to map protein:protein interaction sites, and to map ligand-induced conformational changes. With these multiple techniques, we are beginning to assemble a description of the structure, function, and dynamical properties of PKA.

2. The catalytic subunit

2.1. Kinetic pathway for phosphoryl transfer

The classic work of Cook et al. [13] and Adams and Taylor [14] have described many features of the reaction pathway for the catalytic subunit. There is no obligatory order for binding of substrates; however, given the high concentration of ATP in the cell, it is assumed that ATP typically binds first. This preferred order pathway for binding of peptide substrate and ATP was first described by Walsh and Ashby [15]. The pre-steady state kinetics later revealed a very rapid (>500 s^{-1}) phosphoryl transfer step (k3) with a rate-limiting step (20 s^{-1}) that corresponds to release of the ADP product [16].

As summarized in Fig. 1, we have tried to capture these various steps along the reaction pathway in a crystal lattice and in so doing have defined a set of open and closed conformations. Parallel fluorescence anisotropy studies have provided a dynamic profile of these various states where the enzyme toggles between an ensemble of many conformational states [9]. In an effort to trap a transition state complex, we have also crystallized an ADP, substrate peptide complex with aluminum fluoride [5].

2.2. Open and closed conformations are essential parts of the catalytic cycle

Like all members of the protein kinase family, the catalytic subunit has a small amino terminal domain that is associated mostly with ATP binding and a larger carboxyl terminal domain that serves as a docking site for the protein substrate and also contains the conserved residues that direct the transfer of the phosphate. While the small lobe is dominated by β strands, the large lobe is mostly helical with two of the helices, the E and F helices, spanning the width of the enzyme and serving as the hydrophobic core of the large lobe. In addition to the conserved core that is shared by all members of the protein kinase family (residues 40–300 of the PKA catalytic subunit), the catalytic subunit has 40 additional residues, mostly a single helix, at the amino terminus and 50 residues at the carboxyl terminus that wind around the surface of the large and small lobes and then docks to a hydrophobic pocket on the surface of the small lobe.

The open and closed conformations described here most likely represent some of the physiological conformations of the active enzyme in solution as all of these proteins are fully phosphorylated on Thr197 in the activation loop and on Ser338. Overall, they reflect a highly dynamic protein that is a moving target for capture by substrates, by physiological inhibitors, and artificially by small molecule inhibitors. While the open and intermediate conformations are quite dynamic [17], we also have described two very stable conformational states. One is the inhibited state where the catalytic subunit is bound to ATP and a peptide derived from the heat-stable protein kinase inhibitor (PKI) (5–24) [18]. Unlike the transition state mimic with aluminum fluoride, the PKI:ATP complex is trapped in a transition state-like complex with no available acceptor for the γ-phosphate. A second very stable conformational state, discussed in more detail later, was observed when the catalytic subunit was crystallized with a natural product inhibitor, balanol [19].

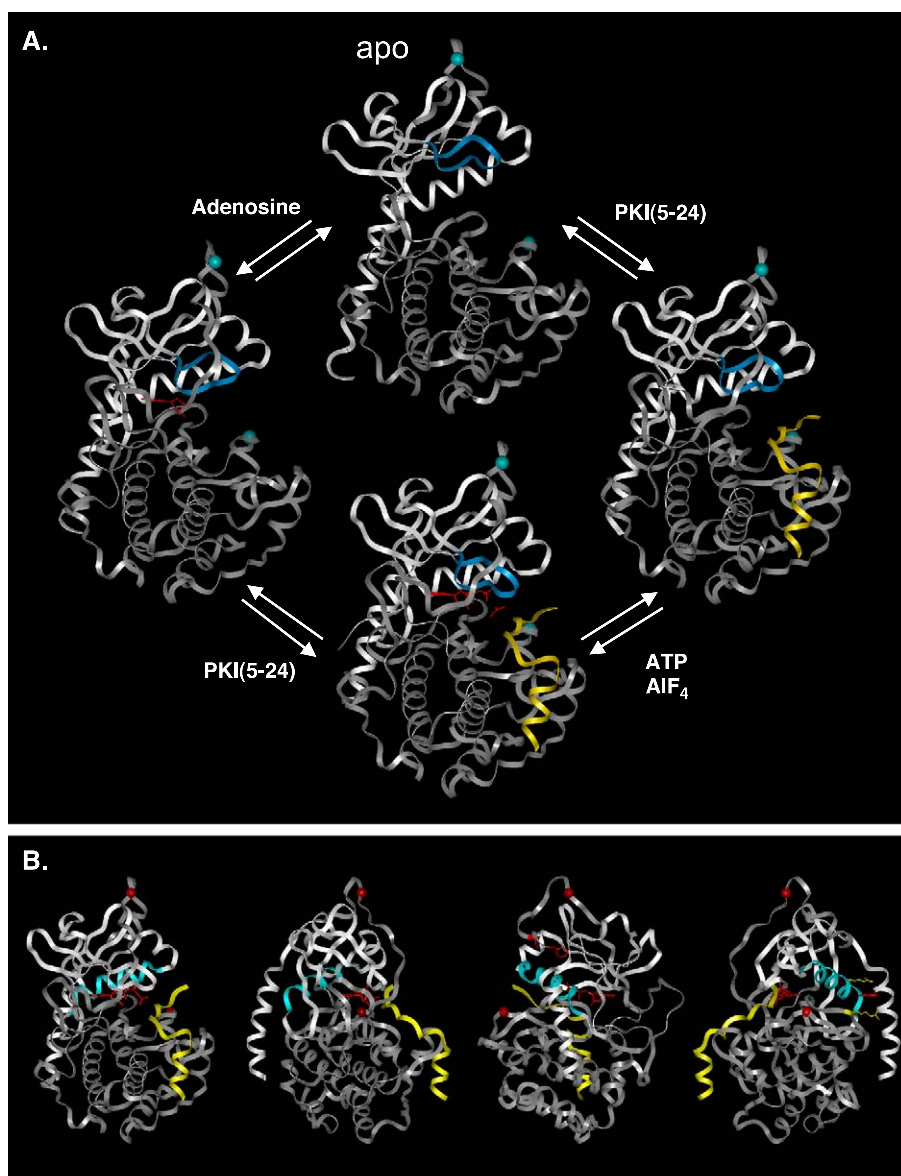

Fig. 1. Conformational states associated with catalysis. The catalytic subunit is capable of assuming an ensemble of open and closed conformational states as it goes through the various stages of catalysis. (A) Some of these conformational states have been captured in a crystal lattice while fluorescence anisotropy has demonstrated some of the dynamical properties of the enzyme as it toggles between these different states. The apoenzyme (PDB ID 1J3H), the adenosine binary complex (1BKX), the binary complex with a substrate peptide (1JLU), and a transition state complex with aluminum fluoride:ADP, and a substrate peptide (1L3R) are shown. The apoenzyme is the most open conformation while the two binary complexes represent intermediate stages of closing. The aluminum fluoride complex represents a fully closed conformation. Phosphorylation sites are shown as blue spheres, nucleotide is shown as red sticks and peptide is shown as yellow ribbon. The glycine-rich loop is highlighted in turquoise. (B) Rotation of the ternary complex containing ATP and PKI (5–24) shows how the N- and C-termini flank the core. Phosphorylation sites are red and the C helix is highlighted in turquoise.

2.3. Apoenzyme reveals a "preformed" active site

The structure of the apoenzyme revealed an open conformation where the small lobe is displaced due to both a hinging motion and a sliding motion of the small lobe relative to the large lobe [4]. Several features of this enzyme had not been fully appreciated previously. For example, it is in the apoenzyme structure that one can begin to fully appreciate the different dynamic properties of the two lobes. Most of the small lobe, in the absence of ligands, appears to be quite dynamic based on the temperature factors and the difficulty in tracing the chain fully in regions such as the tip of the glycine-rich loop. The portion of the carboxyl terminal tail which eventually will clamp down onto the small lobe when ATP and peptide are bound is also quite disordered.

In contrast to the small lobe, the large lobe is quite stable. Indeed most of the active site where phosphoryl transfer takes place is already formed in the apoenzyme [4]. There does not appear to be a requirement for significant induced

fit to accommodate binding of substrate. The stability of the large lobe is due to the solid hydrophobic core which anchors the catalytic loop and the magnesium-positioning loop at the active site cleft so that they are poised for action. Although these two loops, each containing essential residues, are positioned between β strands 6 and 7 and 8 and 9, respectively, each loop is anchored firmly through hydrophobic side chains to the hydrophobic core of the domain. This extended hydrophobic core accounts for the remarkable stability of this domain. The only portion of the large lobe at the active site that is not firmly anchored by hydrophobic interactions to the core is the activation loop. In contrast, this loop is oriented in its active conformation by the phosphorylation of Thr197. Through multiple hydrogen bonding and ionic interactions, nucleated by this single phosphate, the enzyme is locked into a stable conformational state and ensures that the active site is optimally configured for catalysis [20,21].

In Fig. 2, one can better appreciate how the catalytic loop is specifically anchored to the F helix. This is one of two helices that permeate the core, even though, in general, it is quite unusual to have hydrophobic helices like this. Typically, helices in proteins are amphipathic with the hydrophobic surface facing the core and the hydrophilic surface facing the solvent. The hydrophobic properties of this helix are a conserved feature of all protein kinases. The two ends of the F helix are hydrophilic and also carry out specific conserved roles. At the amino terminus is Asp220, con-

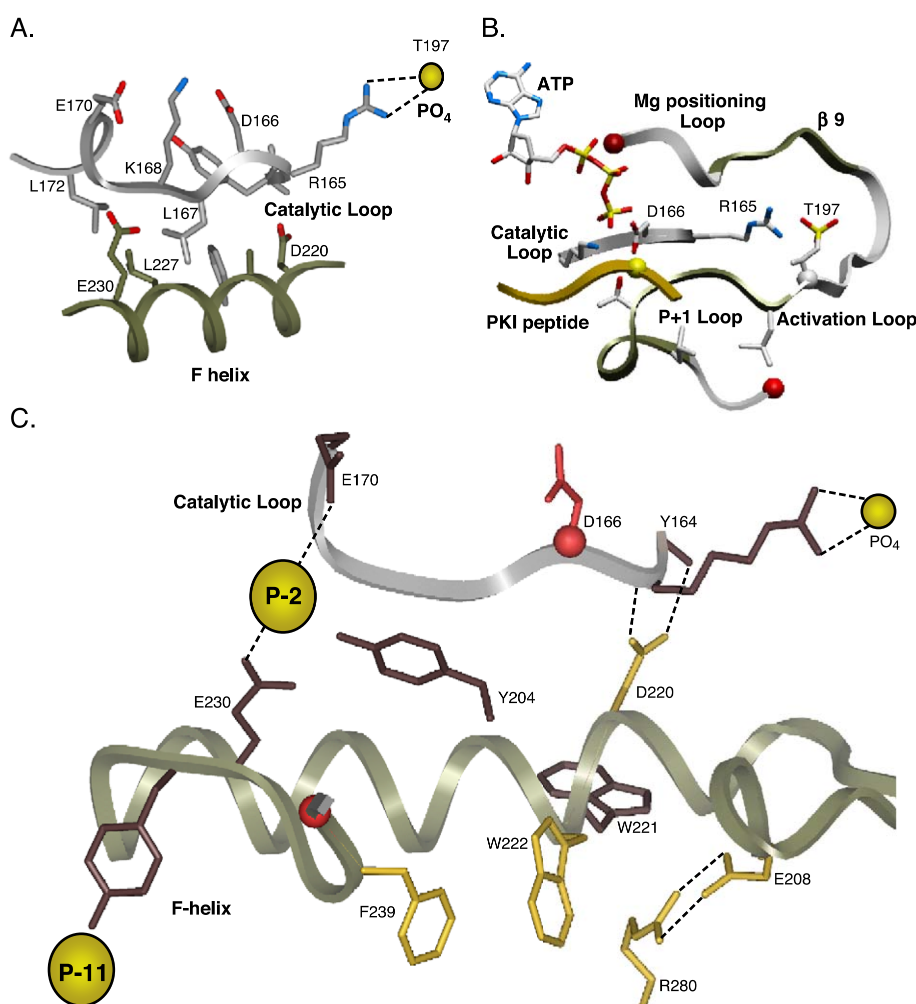

Fig. 2. The catalytic loop is docked by hydrophobic interactions to the large lobe and is mostly preformed prior to the binding of nucleotide and substrates. The structure of the apoenzyme revealed that the large lobe, including the catalytic loop and the magnesium-positioning loop, is mostly preformed prior to the binding of ATP and substrates. It is anchored to the large lobe primarily by hydrophobic interactions shown here in panel A. The F helix is a very hydrophobic helix that is buried in the large lobe. The hydrophilic residues of the catalytic loop face into the active site cleft where they are poised to participate in catalysis while the intervening hydrophobic residues point inward to the hydrophobic surface of the F helix. Panel B shows how the activation segment, residues 184–208, integrates the entire molecule through the phosphorylation of Thr197 and its interaction with Arg165. The two ends of the F helix (Panel C) are hydrophilic and are exposed on the surface at opposite ends of the large lobe. Glu230 contributes directly to the P-2 Arginine recognition site of the peptide. Thus the P-2 Arg is anchored to the C-terminus of the F helix and to Glu170 in the catalytic loop. The other end of the F helix is Asp220, one of the invariant residues in all protein kinases. The side chain of Asp220 helps to anchor the catalytic loop by hydrogen bonding to the backbone amides of 17 Tyr164 and Arg165 that immediately precede the catalytic loop. The side chain of Tyr164 also anchors the catalytic loop to the magnesium-positioning loop by interacting with the backbone carbonyl oxygen of Asp184.

served in all protein kinases. It serves to anchor the catalytic loop through interactions of its side chain with the backbone carbonyl moieties of Tyr164 and Arg165 which just precede the catalytic loop. Arg165 is an essential anchor to the phosphate moiety on Thr197, and Asp166 is the catalytic base that starts the catalytic loop. At the carboxyl terminus of the F helix is Glu230 which contributes to recognition of the P-2 arginine in the peptide substrate. This P-2 arginine is anchored on the other side by Glu170, which is also part of the catalytic loop. Just beyond the F helix are residues 235–239 that form the hydrophobic pocket where the P-11 side chain of PKI (5–24) docks. As seen in Fig. 2B, the catalytic loop, the magnesium positioning loop, and the F helix are all integrated by phosphorylation of Thr197 in the activation loop through its interaction with Arg165. As shown in Fig. 2C, the end of the activation segment, Asp208 is anchored to the C helix through its interactions with Trp222 and Arg280. Fig. 2 allows us to appreciate the extensive interactions that permeate the entire molecule.

As seen in Fig. 1, and also in Fig. 3, the apoenzyme structure represents one of the most open conformations of the catalytic subunit that we have observed so far. It probably reflects the major conformation state in solution that the enzyme adopts prior to any ligand binding. In this state, the small lobe rotates and slides away from the large lobe, the glycine-rich loop is highly dynamic and is away from the active site. The C-terminal segment (318–326), described previously as a "gate" controlling the access to the nucleotide binding site by covering the front of the active site cleft, is largely disordered [4]. The position of the glycine-rich loop relative to the catalytic loop is an indicator of the "openness" of the conformation, and in this apoenzyme, the tip of the glycine-rich loop is far from the catalytic loop (Figs. 1 and 3). Fluorescence anisotropy where selected cysteines were labeled with a fluorescent probe also confirmed that the conformation of the apoenzyme was likely to be very open and highly dynamic [9]. All these features ensure the active site of the apoenzyme has maximum accessibility to the nucleotide substrate.

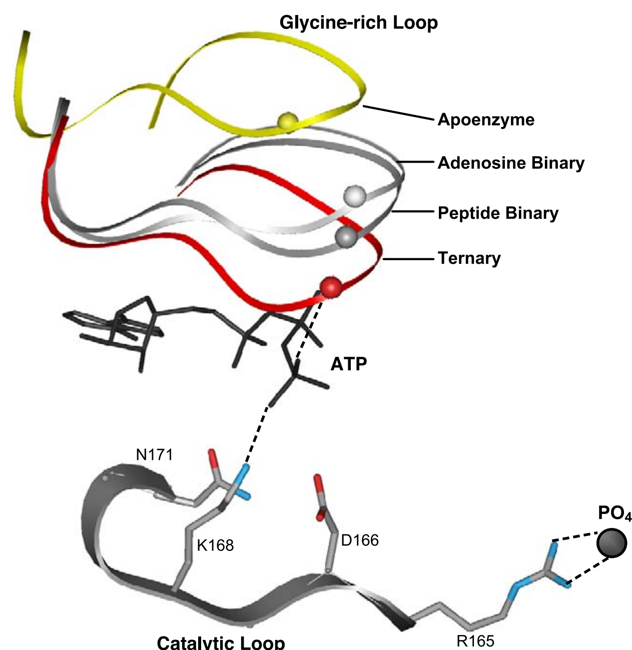

Fig. 3. Opening and closing of the glycine-rich loop at the active site cleft. The catalytic loop is locked into a stable conformation even when nothing is bound to the active site cleft, whereas the glycine-rich loop is positioned for catalysis only when the enzyme assumes a fully closed conformation. It is the most mobile element in the conserved catalytic core. The position of the glycine-rich loop relative to the catalytic loop is shown here for the conformational states represented in Fig. 1. The α-carbon of Ser53 is rendered as a sphere.

2.4. Binary complexes reflect an intermediate conformational state

In the presence of either an ATP analog or a peptide substrate or inhibitor the enzyme assumes a more closed conformation, but the enzyme remains quite dynamic. As seen in Figs. 1 and 3, the glycine-rich loop is closer to the catalytic loop but the tip of the loop is still quite dynamic and is not anchored firmly. The fluorescence anisotropy studies are consistent with this model of a highly dynamical state that is poised for transfer of the γ-phosphate. Until the tip of the loop, specifically the backbone amide of Ser53, is bound to the γ-phosphate of ATP, the enzyme is in a dynamical state. When these structures are compared to the structure of the apoenzyme, it is found that a few additional residues, most importantly Lys72, become oriented in their catalytically competent state as a consequence of binding the nucleotide (described below). The critical step for catalysis is engaging the tip of the glycine-rich loop, and neither of these structures is capable of doing that.

Comparing the adenosine binary structure [22] with the apo form, we see that upon binding of adenosine the C-terminal "gate" is now traceable, albeit still quite dynamic. Tyr330 in this segment will become part of the P-3 peptide recognition site. Lys72 is also ordered even though the triphosphate moiety of ATP is not present. Although the substrate binding site on the large lobe is largely preformed, binding of nucleotide also seems to further engage or orient some of the residues involved in peptide binding. This communication from the nucleotide binding at the active site to the distal peptide binding on the large lobe is probably mediated through the hydrophobic core [4]. The intermediate conformation captured by the binary complex is very likely predominant when ATP binds to the active site at the initial stage of the catalytic cycle.

2.5. Aluminum fluoride:ADP:substrate complex reflects a transition state complex

In an effort to capture the catalytic subunit in a state that accurately reflects the transition state, the catalytic subunit was co-crystallized with ATP, a substrate peptide, and aluminum fluoride (Fig. 4). The peptide docks onto the

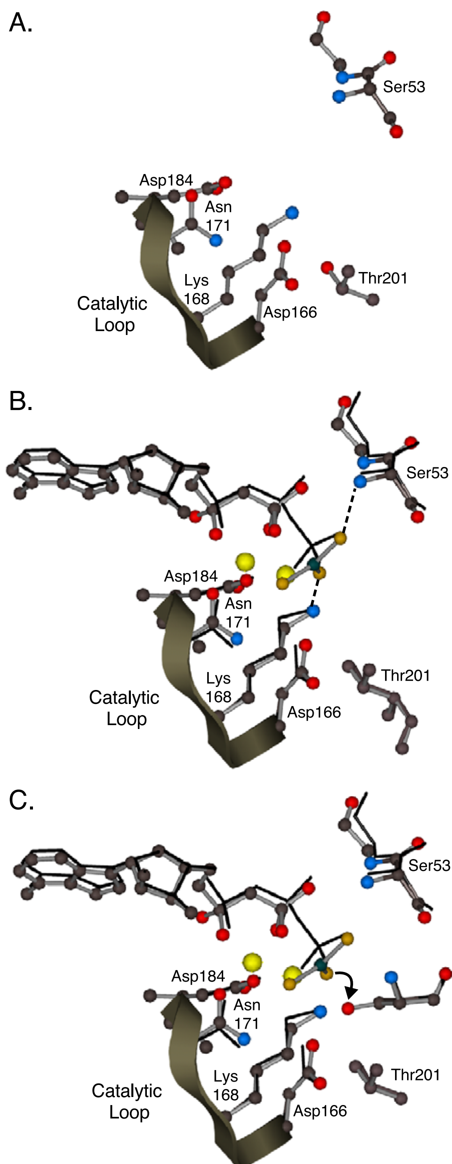

Fig. 4. An aluminum fluoride complex mimics docking of ATP and peptide to the active site cleft. The ATP:PKI (5–24) ternary complex resembles a transition state complex but is missing an acceptor moiety for the phosphate. The ternary complex with ATP and PKI (5–24) is shown here superimposed with the structure of the AlF3:ADP:PKS (5–24) complex. Shown in panel A is the position of the catalytic loop and the glycine-rich loop (Ser53) in their putative active and fully closed conformations. In panel B, the position of the ATP is shown with the γ-phosphate moiety locked between the catalytic loop and the glycine-rich loop through its interactions with Lys168 and the backbone amide of Ser53. The position of the ADP:AlF3 is superimposed. In panel C, the positioning of the peptide is shown with the P site residue poised for catalysis. Thr201 from the P+1 loop which plays a critical role in positioning both the Lys168 and Asp166 in the catalytic loop is also shown.

large lobe and makes further interactions with the small lobe, including the glycine-rich loop, to bring it even closer to the active site. The planar aluminum fluoride lies equidistant between the β–γ bridging oxygen of ADP and the phosphoacceptor hydroxyl. The resulting structure does indeed appear to resemble a transition state that is poised to transfer the γ-phosphate of ATP. In this structure, as in the adenosine binary complex, the adenine binding pocket is occupied. What is essential, however, is for the glycine-rich loop to grab onto the γ-phosphate of ATP and orient it for transfer to the protein substrate. This is mediated by the backbone amide of Ser53 hydrogen bonding to the γ-phosphate. In this structure the γ-phosphate is clamped between the side chain of Lys168 in the catalytic loop and the backbone amide of Ser53. The distance between the aluminum and the γ-phosphorus of ADP and the oxygen at the P site of the substrate peptide is equidistant (approximately 2.3 Å). It is poised for a direct in-line transfer as predicted earlier [5].

One can see that the active site takes conformations from open to intermediate to closed, as the catalysis proceeds. The open conformation allows maximum access of ATP to the active site. Binding of nucleotide synergistically orchestrates the binding of peptide. The closed conformation excludes water from the active site, mediated by the side chains of Phe54 and Phe187, and brings the phosphate donor and acceptor close together, to ensure that phosphoryl transfer takes place efficiently. The open and closing of the active site provide the structural environment for the catalysis to proceed and indeed constitutes an integral part of the catalysis (Fig. 4). A recent quantum mechanical calculation of catalysis using the essential atoms poised in the transition state indicate that catalysis proceeds rapidly [23]. The energy barrier is low, approximately 11 kcal. This calculation also reveals that Asp166, positioned to serve as a catalytic base, actually functions as a proton trap by accepting the proton from the peptide substrate after phosphoryl transfer has occurred.

2.6. The ternary complex containing ATP:PKI represents a closed conformation and resembles a transition state complex

When the structure of the aluminum fluoride complex is compared to the ATP:PKI (5–24) complex, the similarities are remarkable (Fig. 4). The side chains of the conserved catalytic residues in the catalytic loop (Fig. 4A) are positioned to accept ATP or in the case of the ATP mimic, ADP:AlF3. Upon binding the nucleotide, the γ-phosphate of ATP is clamped between Lys168 in the catalytic loop and the backbone amide of Ser53 in the glycine-rich loop (Fig. 4B). The addition of peptide (Fig. 4C) completes the assembly of substrates at the active site cleft. PKI (5–24) is an inhibitory peptide derived from the heat-stable PKI [15]. The single missing feature, compared to the AlF3 complex which has a substrate peptide, is that there is no acceptor for the phosphate. The peptide, PKI (5–24), is a pseudosubstrate where the P site Ser is replaced with an Ala; thus there is no acceptor for the phosphate. In both structures, the aromatic side chains of Phe54 and Phe187 very effectively shield the

active site from the water solvent. Both the inhibitor peptide and ATP bind to the catalytic subunit with high affinity (2 nM for PKI (5–24) and 60 nM for ATP). This high affinity binding is synergistic [24]. It is not clear whether this mechanism is unique for PKA or whether other inhibitor proteins are dependent on the presence of ATP to achieve tight binding. In the case of PKA, this inhibitor complex does appear to be a good mimic of the transition state.

2.7. ATP inhibitors

To date, most small molecule kinase inhibitors are competitive inhibitors of ATP. These analogs that are mimics of ATP can lock the enzyme into either an open or closed conformation. Because of the inherent malleability of the protein kinase core, in principle a small molecule inhibitor could select any number of conformational states to bind. Most small molecule inhibitors are competitive inhibitors of ATP and dock to the adenine binding pocket in a way that mimics ATP. Typically, these inhibitors form at least one hydrogen bond to the backbone of the linker segment that joins the small and larger lobe (residues 120–127 in PKA). Although this adenine binding pocket is quite conserved, the ATP analog inhibitors have been able to achieve remarkable specificity. Identification of a "gate-keeper" residue at the adenine binding pocket provides some explanation for the selectivity of these analogs [25,26].

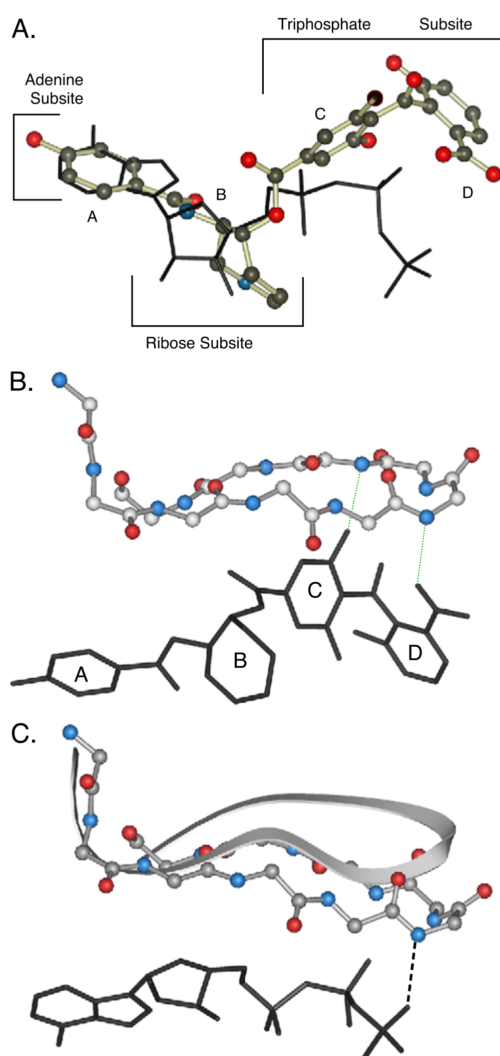

Fig. 5. Balanol locks the catalytic subunit into a stable but open conformation. (A) Structure of balanol (ball and stick), superimposed with ATP (black stick). (B) Balanol is anchored to the active site cleft by hydrophobic and hydrophilic interactions with conserved residues. The entire glycine rich loop (residues 47–38), is engaged with the inhibitor. (C) Superimposition of glycine rich loop when balanol (grey ribbon) and ATP (ball and stick) are bound to the catalytic subunit. ATP is taken from the ternary complex (1ATP).

Several inhibitors have been co-crystallized with the catalytic subunit including H-89 series [27] and staurosporin [28]. One of the high affinity inhibitors that has equivalent 10 nM affinity for cGMP-dependent protein kinase (PKG) and PKC is Balanol, a natural product inhibitor synthesized by the fungus *Verticillium balanoides* [29]. Balanol contains four rings, and one of these, the A ring, fits into the adenine binding pocket (Fig. 5A,B). The other three rings complement the ribose binding pocket (B ring) and the glycine-rich loop (rings C and D) with multiple contact sites. The balanol:catalytic subunit complex is also quite thermostable. Surprisingly, the position of the glycine-rich loop in the balanol complex is quite open (Fig. 5C). Instead of bringing the tip of the glycine-rich loop into close proximity with the catalytic loop, it is pointing away from the active site cleft. Nevertheless, this open conformation is quite stable based on crystallographic temperature factors and on thermostability [19,25].

3. The regulatory subunits

3.1. Organization and isoform diversity

The regulatory subunits are modular, highly dynamic proteins that have multiple functions. Not only do they capture the catalytic subunit and inhibit its catalytic activity, they also serve to target the holoenzyme to the AKAP scaffold proteins. They are also the primary receptor in eukaryotic cells for cAMP. cAMP is an ancient signaling molecule, and the cAMP binding module found in the regulatory subunits is an ancient signaling motif that allows cells to translate an extracellular signal into a biological response. Docking to the AKAPs is mediated by the stable D/D domain that lies at the amino terminus while the cAMP binding modules and the regions that bind to the catalytic subunit are localized to the linker region and to the two tandem cAMP binding domains at the carboxyl terminus (Fig. 5). The stable domains are linked by a highly mobile and variable linker segment. The

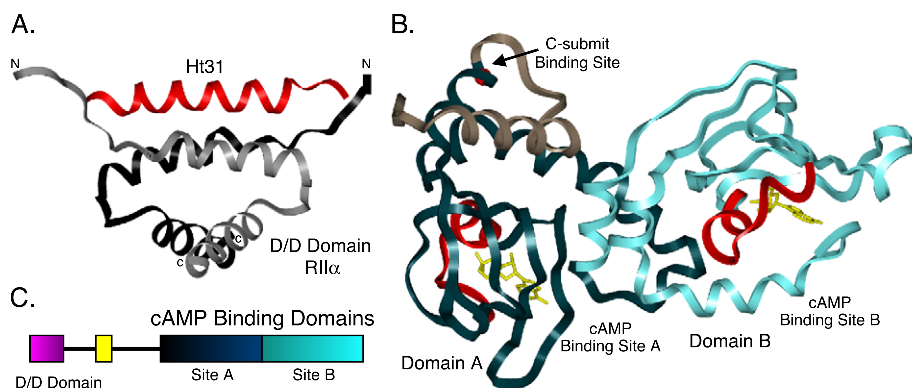

Fig. 6. Structure domains of the regulatory subunit. (A) The D/D domain of RIIα (residues 1–45) docked to an AKAP peptide (red), Ht31 (PDB ID 1L6E). The two chains of the R subunit are shown in black and gray. (B) The cAMP binding domains of RIα (residues 113–376). Domain A is shown in dark green, domain B in turquoise, and the N-terminal segment preceding domain A in tan. The phosphate binding cassettes (residues 199–211 for domain A and residues 323–335 for domain B are in red). cAMP is shown in yellow. (C) General domain organization of the regulatory subunits.

dynamic properties of this linker were demonstrated by fluorescence anisotropy by engineering unique cysteines into various sites in the RIα regulatory subunit and by the endogenous fluorescence of the RIIβ subunit where one of its two tryptophanes lies in the linker region [30]. The isoform diversity of the regulatory subunits is revealed by structures of the D/D domains of RIα [31] and RIIα [32] (Fig. 5) by NMR spectroscopy and by the cAMP binding domains of RIα [33] (Fig. 5) and RIIβ [34] solved by X-ray crystallography.

3.2. Docking of the regulatory and catalytic subunits

An additional approach that we have used to map the intersubunit interface of PKA and the RIα subunit is amide H/D exchange coupled to mass spectrometry. High affinity binding of the regulatory and catalytic subunits is mediated by two spatially distinct sites of interaction. The primary site of interaction is mediated by the pseudosubstrate site in the linker region of the regulatory subunit that fills the active site cleft of the catalytic subunit. A secondary site of interaction between the catalytic and regulatory subunits, referred to as a peripheral docking site, is comprised of residues in cAMP binding domain A. Together these two low-affinity sites contribute to a high affinity binding site for the R–C complex (K_d = 0.2 nM) [35]. In the Type I holoenzyme, amide H/D exchange has enabled identification of the surfaces of RIα and the catalytic subunit that are protected from solvent upon complexation [11]. In addition, this method has allowed an understanding of the opposite effects of cAMP and catalytic subunit binding on RIα. The results from the amide H/D exchange studies were combined with computational docking experiments using the rigid body docking program DOT [36] to generate models of the Type I holoenzyme [37]. Application of suitable distance filters retained a limited number of solutions of the Type I holoenzyme that satisfied the amide H/D exchange data. The top solutions from this analysis were also found to be most consistent with previous mutagenesis and predict an intersubunit interface of 1400 Å^2 for the second site of interaction between RIα and the catalytic subunit (Fig. 7). The top solution also predicts a role for the activation loop of the catalytic subunit for mediating interactions with the cAMP binding domain A of the regulatory subunit. It also demonstrates the importance of phosphorylation of the conserved Thr in the activation loop not only for catalysis but also for arranging the primary and secondary sites of interaction with the regulatory subunit (Fig. 7).

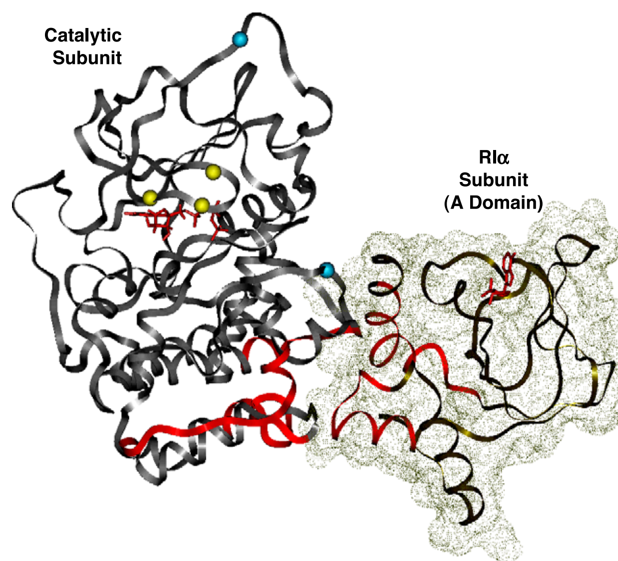

Fig. 7. Docking of the RIα subunit to the catalytic subunit. The model is based on the docking program DOT, coupled with H/D exchange data. Peptides that show protection upon interaction are shown in red. The yellow balls are the α-carbons of Gly 50, 52, and 55 in the glycine-rich loop. The blue balls indicate the phosphorylation sites Thr197 and Ser338. The ribbon diagram (gray) of the crystal structure of the C subunit is the ternary complex with bound ATP. The region 113–224 of RIα is shown as ribbon. cAMP and ATP are in red.

4. A kinase anchoring proteins

4.1. A kinase anchoring proteins contribute to localization

In addition to the catalytic and regulatory subunits, PKA is targeted to specific sites in the cell by AKAPs. These multidomain proteins are capable of sequestering a number of signaling molecules such as phosphatases, kinases, and phosphodiesterases in close proximity to target substrates [38,39]. This sequestration provides a mechanism for generating microdomains for localized signaling. This adds further to the complexity of the signaling networks and opens up the possibility that signaling by cAMP could take place in one location and might not require the diffusion of cAMP across the cell. Indeed there is significant data in the literature to suggest that such sequestration must occur [40]. The AKAPs provide a mechanism for achieving this.

4.2. Molecular basis for AKAP binding to PKA

The AKAPs bind to the regulatory subunits through an amphipathic helix [41]. The RII subunits typically bind to AKAPs in the low nM range [42]. Recently, RIα has also been shown to bind to AKAPs although usually the affinity is less (50–100 nM). Several dual specific AKAPs that bind to both RI and RII have now been identified [43,44]. Amphipathic helices with selectivity for both RI and RII have also now been identified using spot array analysis [41,45]. The amphipathic helix in the AKAP binds to the amino terminal D/D domain of the regulatory subunit. With the structures of the RIα and RIIα D/D domains, recently solved by NMR spectroscopy [10], and the structure of RIIα [32] docked to an AKAP peptide, Ht31 (see Fig. 6A) [46], we are beginning to unravel the molecular basis for targeting of PKA to AKAPs. This small module, shown in Fig. 8, leads to the generation of an extended network that brings the kinase close to the substrates that it modulates.

The AKAPs themselves have been less well studied. In an effort to determine whether the AKAPs contain functional domains as well as significant regions of disorder, DAKAP2, a dual specific AKAP was characterized using H/D exchange coupled with mass spectrometry. DAKAP2 contains near its amino terminus a putative RGS domain and at is carboxyl terminal end the A kinase binding (AKB) domain. The linker region contains a putative PKA phosphorylation site, but no known domain maps to this region. Based on the exchangeability of the backbone amides to deuterium, the RGS domain appeared to be folded in a manner that was consistent with it having a conformation that resembles other RGS domains [12]. The carboxyl terminal helix was also shielded due to interaction with another part of the molecule or possibly to dimerization. In contrast, the linker region appeared to be quite unstructured. Within 10 s, all of the backbone amides in the linker region had fully exchanged. This initial profile suggests that the AKAPs will also contribute significantly to the dynamic properties of this signaling complex.

The carboxyl terminal 40 residues contain the AKB motif and the docking of this motif to the RIα and RIIα regulatory subunits has been mapped by H/D exchange (manuscript submitted). In addition, the three terminal residues are predicted to be a PDZ binding motif. Recently a binding partner was identified for this motif. Gisler et al. [47] identified PDZ-KI and PDZ-K2 as PDZ binding proteins in kidney proximal tubules that bind to the carboxyl terminus of DAKAP2. PDZ-K1 also binds to the Na^+ phosphate exchanger that mediates uptake of phosphate. This process of phosphate uptake is regulated by PKA through a mechanism that involves internalization of the exchanger. This poses a novel PKA signaling complex where the details of this process are being unraveled.

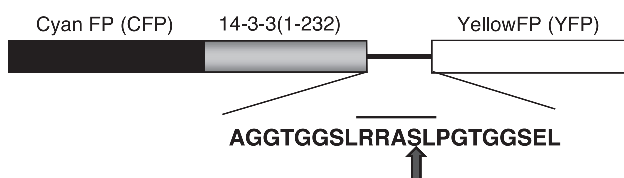

Fig. 8. Design of a recombinant AKAR. The recombinant AKAR shown here provides a FRET response when the PKA-specific peptide is phosphorylated in cells. CFP and YFP refer to cyan fluorescent protein and yellow fluorescent protein, respectively. The peptide linking the 14–3–3 domain and the YPF contains the PKA-specific sequence underlined where the arrow indicates the site of phosphorylation.

5. Monitoring of PKA activity in living cells

While we can monitor kinase activity and measure kinetic properties in vitro, it is ultimately the activity of the enzyme in cells that is important to monitor. A recent advance in the development of a recombinant probe, A Kinase Activity Reporter (AKAR), to measure PKA activity has made this possible [7]. AKAR was engineered by fusing a cyan fluorescent protein (CFP) to a peptide that could be phosphorylated by PKA (Fig. 8). This was followed by a phosphate binding protein, 14–3–3, and then a yellow fluorescent protein (YFP). Following phosphorylation, this construct shows enhanced fluorescence resonance transfer (FRET) as measured by emission at 527 nm. The response is seen both in vitro and in vivo. In addition, Zhang et al. [7] was able to show delayed response to forskolin stimulation when the AKAR was localized to the nucleus. She also demonstrated that the signal was more rapid when the kinase was directly associated with the AKAR, either by fusing an AKAP peptide to AKAR and thereby recruiting endogenous PKA or by fusing a regulatory subunit directly to the YFP. The AKAR can now be used to more comprehensively evaluate the importance of targeting for PKA signaling in cells.

6. Conclusions and perspective

The integration of signaling by PKA is complex and involves a variety of primary and auxiliary positions. While the primary signaling molecules for PKA are the regulatory and catalytic subunits, there are also auxiliary proteins that contribute to building an extended network that brings PKA in close proximity to its substrates. In addition, there are multiple isoforms of the regulatory and catalytic subunits and numerous splice variants of the catalytic subunit as well as splice variants of the AKAPs that compound the complexity of these signaling networks. It is this entire assembly that constitutes the physiological signaling complex, and it is capable of creating microdomains within a single cell. Thus one can disrupt the network not only by generating inhibitors to the active catalytic subunit, but also by stabilizing the inhibited complex and also by disrupting targeting. Each strategy is capable of disrupting function.

As indicated in this review, in order to fully appreciate the complexity of protein kinase structure and function requires a variety of different techniques. It requires not only high-resolution structures of the participating molecules but also complementary solution methods that allow us to appreciate the dynamics of the molecules in a more physiological environment. Ultimately, we must be able to monitor the interactions and functioning of these molecules in living cells. This demands a broad spectrum of scientific expertise. Our challenge is to build interdisciplinary teams that can address such questions. To meet this challenge, we have used a variety of approaches, in addition to crystallography and NMR, to probe the structure and function of PKA. We have used fluorescence anisotropy to probe local motions and FRET to monitor PKA activity in living cells. We also have used H/D exchange coupled with mass spectrometry to probe domain organization, ligand- and protein-induced conformational changes, and to define protein:protein interfaces. In this way, we are beginning to better understand the molecular features and complexity of these signaling networks.

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