Discovery of Allostery in PKA Signaling

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REVIEW

Discovery of allostery in PKA signaling

Ping Zhang[1] · **Alexandr P. Kornev**[1] · **Jian Wu**[1] · **Susan S. Taylor**[1,2]

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Abstract Cyclic AMP (cAMP)-dependent protein kinase (PKA) was the second protein kinase to be identified, and the PKA catalytic (C)-subunit serves as a prototype for the large protein kinase superfamily that contains over 500 gene products. The protein kinases regulate many biological functions in eukaryotic cells and are now also a major therapeutic target. The discovery of PKA nearly 50 years ago was quickly followed by the identification of the regulatory subunits that bind cAMP and release the catalytic activity from the holoenzyme. Thus in PKA we see the convergence of two major signaling mechanisms—protein phosphorylation and second messenger signaling through cAMP. Crystallography provides a foundation for understanding function, and detailed knowledge of the structure of the isolated regulatory (R)- and catalytic (C)-subunits has been extremely informative. Yet it is the R_2C_2 holoenzyme that predominates in cells, and the allosteric features of PKA signaling can only be fully appreciated by seeing the full-length protein. The symmetry and the quaternary constraints that one R:C heterodimer exerts on the other in the holoenzyme simply are not present in the isolated subunits or even in the R:C heterodimer.

Keywords Cyclic AMP · cAMP-dependent protein kinase · PKA catalytic subunit · PKA regulatory subunit · Allostery

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While protein phosphorylation was being discovered as a regulatory mechanism for biological systems through the pioneering studies of Krebs and Fischer in 1959 (Krebs et al. 1959), the fundamental principles of allostery were being elucidated by Changeux and colleagues (Monod et al. 1965). Independently, Sutherland identified cyclic AMP (cAMP) as a second messenger for hormone signaling (Rall and Sutherland 1958). The second protein kinase to be discovered, in 1968, was cAMP-dependent protein kinase (PKA) (Walsh et al. 1968). The discovery that the regulatory (R)-subunits of PKA were the major receptors for cAMP (Gill and Garren 1970; Tao et al. 1970; Brostrom et al. 1971) brought together two major regulatory mechanisms, phosphorylation and second messenger signaling, and also introduced the concept of oligomerization and allostery into PKA signaling.

Discovery of PKA holoenzymes and their allosteric regulation

The PKA catalytic (C)-subunit, initially identified as the enzyme responsible for phosphorylating and activating glycogen phosphorylase kinase, was named phosphorylase kinase kinase by Walsh et al. (1968). Knowledge of PKA thus originally introduced the concept of cascades in kinase signaling. Only later when its regulatory mechanism was elucidated was it renamed cAMP-dependent protein kinase. PKA is distinct from phosphorylase kinase in several important ways; most importantly, phosphorylase kinase is part of a large oligomeric complex that does not dissociate ($\alpha_4\beta_4$, γ_4, δ_4), whereas PKA subunits can readily be isolated as free and soluble proteins, which has greatly facilitated the biochemical and biophysical characterization of PKA. The discovery of the R-subunits resulted in PKA being defined as an oligomeric protein that contains an R-subunit dimer and two C-subunits (Gill and

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Garren 1970; Tao et al. 1970; Brostrom et al. 1971). The C-subunit contains the catalytic activity while the R-subunits have high-affinity binding sites for cAMP. It was only with the identification of holoenzymes that researchers came to appreciate that activation of PKA is also highly cooperative with Hill coefficients of >1. Understanding the molecular mechanism for allosteric activation, however, has taken over four decades, and the mechanistic details are still being elucidated. The need to understand PKA allostery emphasizes the importance of biological complexity and oligomerization and also demonstrates why it is essential to reach across scales of time and space and use a range of interdisciplinary techniques.

Our enormous advances in X-ray crystallography also began in the 1950s with the pioneering work of Perutz and Kendrew on myoglobin and hemoglobin (Kendrew et al. 1960; Perutz et al. 1960). The fundamental importance of oligomers for allostery was recognized immediately by Changeux and his group even though at that time hemoglobin was the only oligomeric protein for which a structure was available. Describing proteins at atomic level resolution has been a driving force for understanding biological processes ever since. Enormous advances have been made in the kinase signaling community beginning with the structure of the PKA C-subunit (Knighton et al. 1991), but it is now essential that researchers further their understanding of the large macromolecular signaling complexes. This will require both high- and low-resolution data, as well as the computational tools to understand the dynamics. Understanding the higher levels of complexity in signaling systems is often challenging not only because of the increased size of the complex but also because of the inherent dynamic properties of signaling proteins in contrast to the stable properties of the hemoglobin tetramer and the stable phosphorylase kinase oligomer. The fundamental target of studies on PKA is to gain an understanding—at atomic level resolution—of how the binding of a ligand leads to the observed allosteric activation. Newly developed structural methods, combined with advanced computational and kinetic studies, now make such an approach feasible. Clearly with PKA one could never fully appreciate the allostery without seeing the oligomers. Visualizing these oligomers and appreciating the fundamental differences between the isoforms defines for the first time the challenge that we face.

PKA R-subunits

In the absence of cAMP the dimeric R-subunits bind to and inhibit two C-subunits. Activation is mediated by the binding of cAMP to the R-subunits, and this process is clearly allosteric based on Hill coefficients for activation of 1.4–1.8. Two classes of R-subunits were initially identified, referred to as I and II, based on the order in which they eluted from an ion exchange column (Hofmann et al. 1975; Rosen et al. 1975). Tissue differences were also recognized early on by Rubin and colleagues who found that the form of RII present in the brain was distinctly different from that in the heart (Erlichman et al. 1980). These two RII isoforms were designated as RIIα (heart) and RIIβ (brain). In the intervening decades the sequence, structure, and isoform diversity of the four PKA R-subunits were elucidated; however, the mechanism for allosteric regulation remained obscure. Much was learned from the structures of the free C- and R-subunits and from truncated R:C heterodimers; however, the fundamental mechanisms for allosteric activation remained to be elucidated. For this we needed to see the full-length R_2C_2 holoenzyme structures. Only then did we appreciate the symmetry and beauty of PKA signaling where binding of a small molecule, distal from the R:C interface, unleashes the catalytic activity that is trapped in the R_2C_2 holoenzyme complex. In this review, we use RIIβ as a prototype for defining these concepts of allostery since high-resolution structures of some of the different states are available.

In mammals there are four functionally non-redundant R-subunits (RIα, RIβ, RIIα and RIIβ) (Brandon et al. 1997), and all have the same general domain organization (Fig. 1). At the N-terminus is a four-helix bundle that mediates dimerization as well as binding to PKA scaffold proteins that are referred to as A Kinases Anchoring Proteins or AKAPs. Each AKAP contains a signature amphipathic helix that docks with high affinity onto the surface of the dimerization/docking (D/D) domain, and it is this mechanism which allows PKA to be localized to discrete sites in the cell where it is committed to a specific function, such as phosphorylation of the tail of a channel (Wong and Scott 2004; Taylor et al. 2012). The D/D domain is joined by a flexible linker to two tandem cyclic nucleotide binding (CNB) domains at the C-terminus of each R-subunit (CNB-A and CNB-B). Embedded within the flexible linker is an Inhibitor Site (IS) that resembles a PKA substrate and docks into the active site cleft of the C-subunit in the absence of cAMP. The RI-subunits have a pseudo-substrate IS (RRXG/AX) where the P-site residue is either glycine or alanine, while the RII-subunits are substrates as well as inhibitors since the P-site residue is serine. This is a fundamental difference between the RI- and RII-subunits which has significant functional consequences (Herberg et al. 1999; Martin et al. 2007).

Although the IS is the most common way to distinguish RI- and RII-subunits, there are also structural differences that can clearly be demonstrated even at low resolution. In this regard small-angle X-ray and neutron scattering (SAXS/SANS) are especially informative (Fig. 1). What is most striking about the SAXS data is that each of the four R-dimers and holoenzymes is different (Heller et al. 2004; Vigil et al. 2004, 2006).

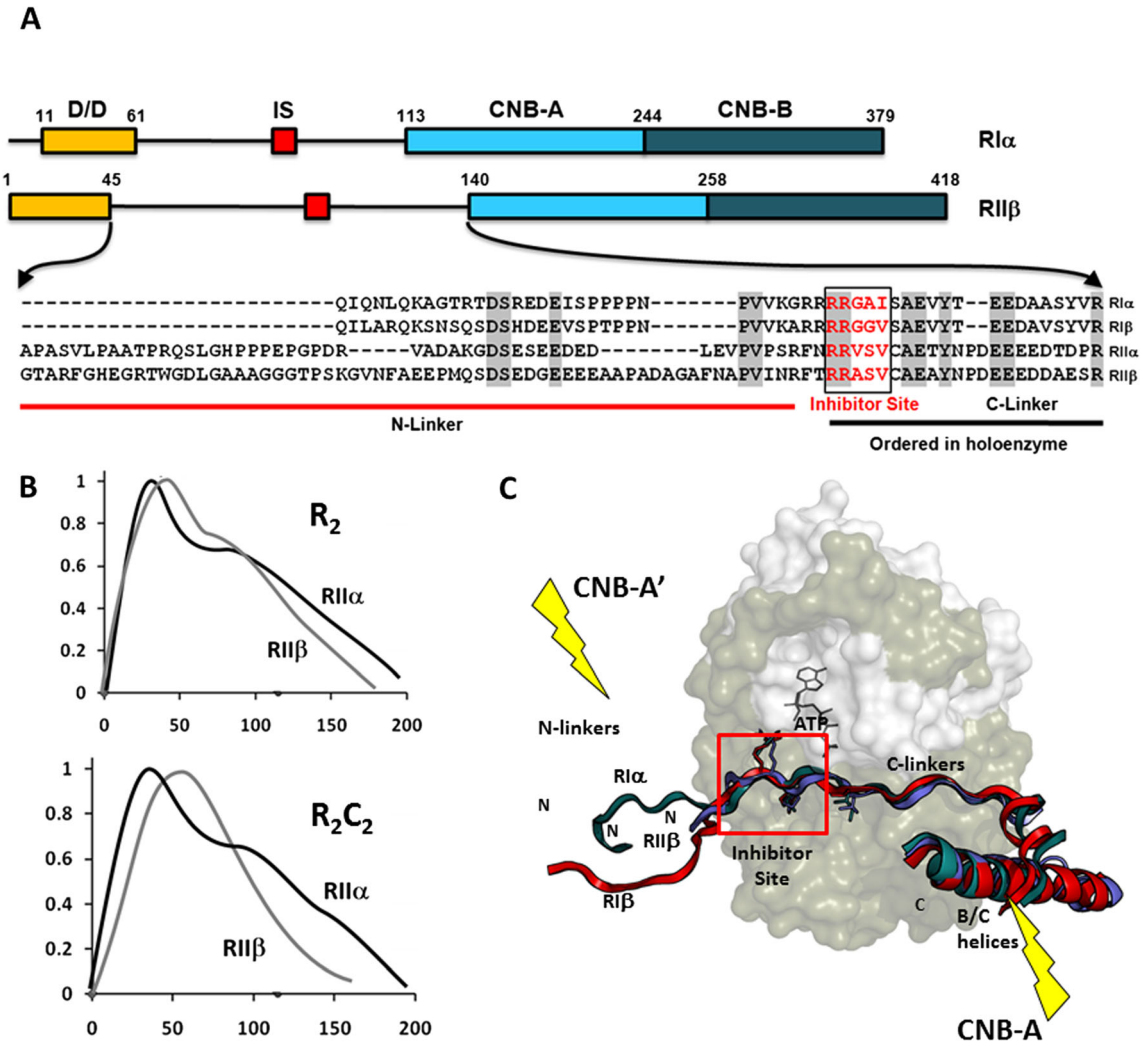

Fig. 1 Organization of the cAMP-dependent protein kinase (PKA) R-subunits. **a** The organization of the RIα and RIIβ regulatory (R)-subunits and the sequence of the linker regions for all four isoforms are shown. **b** Small-angle X-ray scattering (SAXS) profiles of the PKA RII-homodimers and holoenzymes show the isoform differences. **c** Following binding to the catalytic (C)-subunit, the Inhibitor Site docks at the active site cleft of the C-subunit (*red box*) and the C-linker becomes ordered. The N-linker plays an important role in defining the quaternary structure of each holoenzyme and is ordered differently in the RIα-, RIβ- and RIIβ-subunits following their binding to the C-subunit. This isoform-specific positioning of the N-linker contributes in unique ways to the organization of each tetrameric holoenzyme. *Highlighted* are the positions of the two symmetry related cyclic nucleotide binding (CNB)-A domains in the same holoenzyme. Each CNB-A domain also allosterically regulates the adjacent C-subunit in the holoenzyme

Although one might have predicted that having one tetrameric structure would allow all four to be modeled, the SAXS data say differently. The RIα and RIβ homodimers have a similar relatively compact Y-shaped geometry, while the two RIIα and RIIβ homodimers are much more extended and rod-like (Taylor et al. 2012). SAXS also showed that there are very substantial differences between RIIα and RIIβ. When the C-subunits bind to RIIα, for example, the structure remains extended and rod-like, and this was confirmed in recent electron microscopy studies of RIIα (Smith et al. 2013). In contrast, the RIIβ holoenzyme is very compact and almost globular. Linker swaps between RIIα and RIIβ showed that the linker region is responsible for the remarkably different quaternary structures (Vigil et al. 2006). The RIIβ holoenzyme has the smallest maximum diameter (D_{max}) of the four holoenzymes. For this reason, as well as its compactness and globular nature based on SAXS, we focused initially on the RIIβ holoenzyme as a candidate for further structural studies.

As mentioned, the four R-subunits are also functionally non-redundant. Only RIα is embryonically lethal when removed (Amieux and McKnight 2002), and most of the known diseases that are associated with PKA signaling are linked to RIα (Horvath et al. 2010). RIα undergoes nonsense-mediated decay, and the resulting haplo-insufficiency, as well as a number of single point mutations, leads to Carney Complex Disease (CNC) which is associated with numerous cardiac

and endocrine disorders. CNC is characterized by unregulated PKA and high basal levels of PKA activity. Another disease associated with RIα is acrodysostosis (Linglart et al. 2011), an autosomal dominant genetic syndrome. The activity of PKA is inhibited in patients with acrodysostosis, with severe consequences, including dwarfism and learning disorders. Genetic knockouts of RIβ, in contrast, lead to learning defects and defects in long-term potentiation (Brandon et al. 1995; Huang et al. 1995). A recent study shows that a point mutation in the RIβ D/D domain causes severe neurological defects (Wong et al. 2014). RIIβ(−/−) mice have a lean phenotype: they do not become obese with a high fat diet nor do they become insulin resistant (Cummings et al. 1996; Schreyer et al. 2001). A prevailing challenge is to understand how or whether the structure and/or localization of the specific holoenzymes contribute to their functional diversity.

Intrinsically disordered linkers

It is important to appreciate that PKA in cells exists as an R_2C_2 holoenzyme due to the presence of the D/D domain at the N-terminus. Although the entire linker is completely disordered in the free R-subunits, the portion of the linker that extends from the inhibitory site to the CNB-A domain becomes partially ordered following binding of the C-subunit (Fig. 1). The remaining part of the linker, referred to as the N-linker, however, remains mostly disordered, as will be discussed later, and is mostly missing from the initial PKA holoenzyme structures. The N-linkers exhibit the highest sequence variability among the four isoforms of the R-subunits (Fig. 1) and are major determinants for the organization of the oligomeric (R_2C_2) holoenzymes.

Much biological information is embedded within the flexible linkers, including phosphorylation sites and PEST sequences that target the R-subunits for proteolytic degradation (Fig. 1). Such flexible or intrinsically disordered regions (IDRs) allow for highly dynamic interactions between the various domains and subunits and are characteristic features of many eukaryotic signaling proteins; however, they introduce major challenges for trying to understand at high resolution how such complexes are regulated, as IDRs typically interfere with crystallization by favoring a more dynamic state. Thus, the tendency in structural studies is to simply delete these regions, even though IDRs are extremely important for biological function and regulation. While elucidating structures of the component parts is a logical initial strategy, ultimately it is the full-length proteins that reflect the physiological state of PKA in cells, and the essential information for mediating allosteric activation by cAMP can only be seen in the R_2C_2 holoenzymes.

CNB domains: mediators of the allosteric transitions

The CNB domain has been conserved throughout biology from bacteria to man as the major receptor for cAMP (Berman et al. 2005), and nature has coupled this CNB domain to many signaling nodes (Kannan et al. 2007b). Prokaryotes possess many catabolite gene activator proteins that regulate gene transcription, while in eukaryotes the major CNB domains are coupled to ion channels, guanine nucleotide exchange factors and kinases [PKA and cGMP-dependent protein kinase (PKG)]. Here we focus on PKA where the cAMP-binding R-subunits are encoded by genes that are distinct from the C-subunit; this is in contrast to PKG where the CNB domains are fused directly to the kinase domain as a single gene product (Francis and Corbin 1999). The sequences of the PKA CNB domains are compared in Fig. 2. Each CNB domain has a helical subdomain that flanks an 8-stranded β sandwich. Located between β strands 6 and 7 is a short loop containing a small helix. This is the signature motif of all CNB domains and is referred to as the phosphate binding cassette (PBC). A highly conserved arginine in the PBC of every cAMP-binding CNB domain binds to the phosphate of cAMP, while a conserved glutamate at the beginning of the PBC binds to the ribose OH (Fig. 2). The β sandwich is flanked by two helical motifs. The N3A motif at the N-terminus precedes β1, while the B/C helix follows β8. The three helical domains toggle between two different states—the cAMP bound state (B state or Active state) and the holoenzyme state (H state or Inactive state). A general summary of the correlated motions of these motifs is given in Fig. 2 (Kornev et al. 2008). Here, we first summarize the dynamic and allosteric features of the CNB domains, then we describe both the Active state captured in the cAMP-bound R-subunit and the Inactive state captured in the R:C complex.

The dynamic CNB domains function as regulators of PKA activity (Kornev et al. 2008). The correlated motions of the three helical motifs in the CNB domain propagate signals that are initiated by the binding and release of cAMP and are sensed by both the flanking CNB domains and in the holoenzyme by the C-subunit even though cAMP per se does not directly contact the C-subunit. The dynamic features of the CNB domain are best described in the extensive studies of the CNB-A of RIα (Das et al. 2006; Akimoto et al. 2013), which serves as a prototypical CNB domain (Fig. 2). Although extensive nuclear magnetic resonance (NMR) studies on the RIIβ-subunits have not yet been performed, NMR studies of RIα suggest that the two states (Active and Inactive) are highly populated even in the absence of cAMP and the C-subunit and that the energy barrier between the two states is low (Akimoto et al. 2013). These findings support a conformational selection model for allostery where the equilibrium between the two states is altered as a consequence of ligand binding or C-subunit binding. These NMR predictions

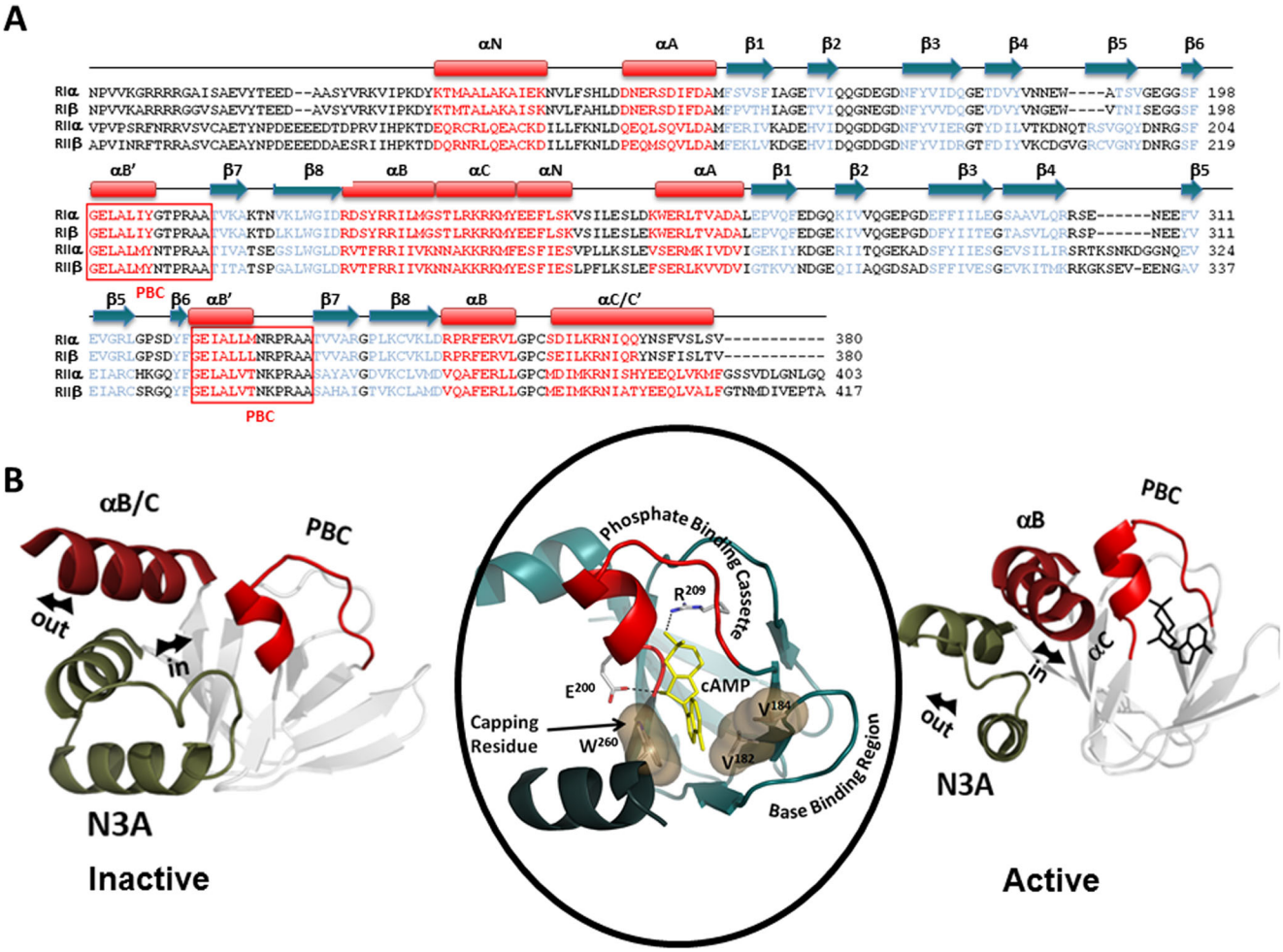

Fig. 2 CNB domains of R-subunits. **a** Sequence alignments of the R-subunits C-linker and CNB domains (*red* helices, *blue* strands). **b** The CNB-A domain of RIα is used as a prototype for this highly conserved structural domain. C-subunit-bound Inactive (H conformation) and cAMP-bound Active (B conformation) (PDB ID 1CX4) conformations are shown. In the absence of cyclic AMP (*cAMP*) (*left*), the B/C helix moves 'out', while the N3A motif moves 'in.' Upon cAMP binding (*right*), the phosphate binding cassette (*PBC*) moves towards the cAMP phosphate, moving the B/C-helix 'in' and causing the N3A motif to move 'out.' *Middle panel* shows how the cyclic nucleotide is bound to the PBC, which is the signature motif for this domain

are also reflected in recent Markov models of the RIα CNB-A domain (Malmstrom et al. 2015) and for activation of the RIα holoenzyme (Boras et al. 2014).

There are two contiguous CNB domains in PKA, and in the presence of cAMP these two CNB domains interact with each other to form a network of allosteric contacts. There are thus two protein interfaces that are sensed by the CNB-A domain: the interface with the CNB-B domain and the interface with the C-subunit in the holoenzyme. The cAMP-bound conformation represents the Active conformation, and this state is captured in many different isoforms (Su et al. 1995; Diller et al. 2001; Rinaldi et al. 2010) (Fig. 3a, b). Although the folds for each cAMP-bound CNB domain can be easily superimposed, the orientation of the two domains relative to each other is actually quite distinct for each R-subunit. In this Active conformation, the B/C helix of the CNB-A domain moves "in" towards the cAMP-bound PBC while the N3A motif of CNB-A moves "out." These same motions are seen in the CNB-B domain. In addition to the internal motions of each CNB domain, there is a hydrophobic residue that caps the adenine ring of cAMP, and this residue typically comes from outside the CNB domain. In all PKA isoforms the CNB-B domain provides the hydrophobic capping residue for cAMP that is bound to the CNB-A domain, although the location of the capping residue is isoform specific (Berman et al. 2005). In RIα, the N3A motif of the CNB-B domain, which is contiguous with the B/C helix of CNB-A, caps the cAMP in CNB-A, thereby creating a major interface between CNB-A and CNB-B. In RIIβ, the capping residue also comes from the CNB-B domain, but in this case it is located in the B Helix. The interface between the CNB-A and CNB-B domains is thus very different in RIα and RIIβ (Fig. 3). The recent structure solution of bcy1, the yeast homolog of the PKA R-subunit, shows yet another variation on this theme (Rinaldi et al.

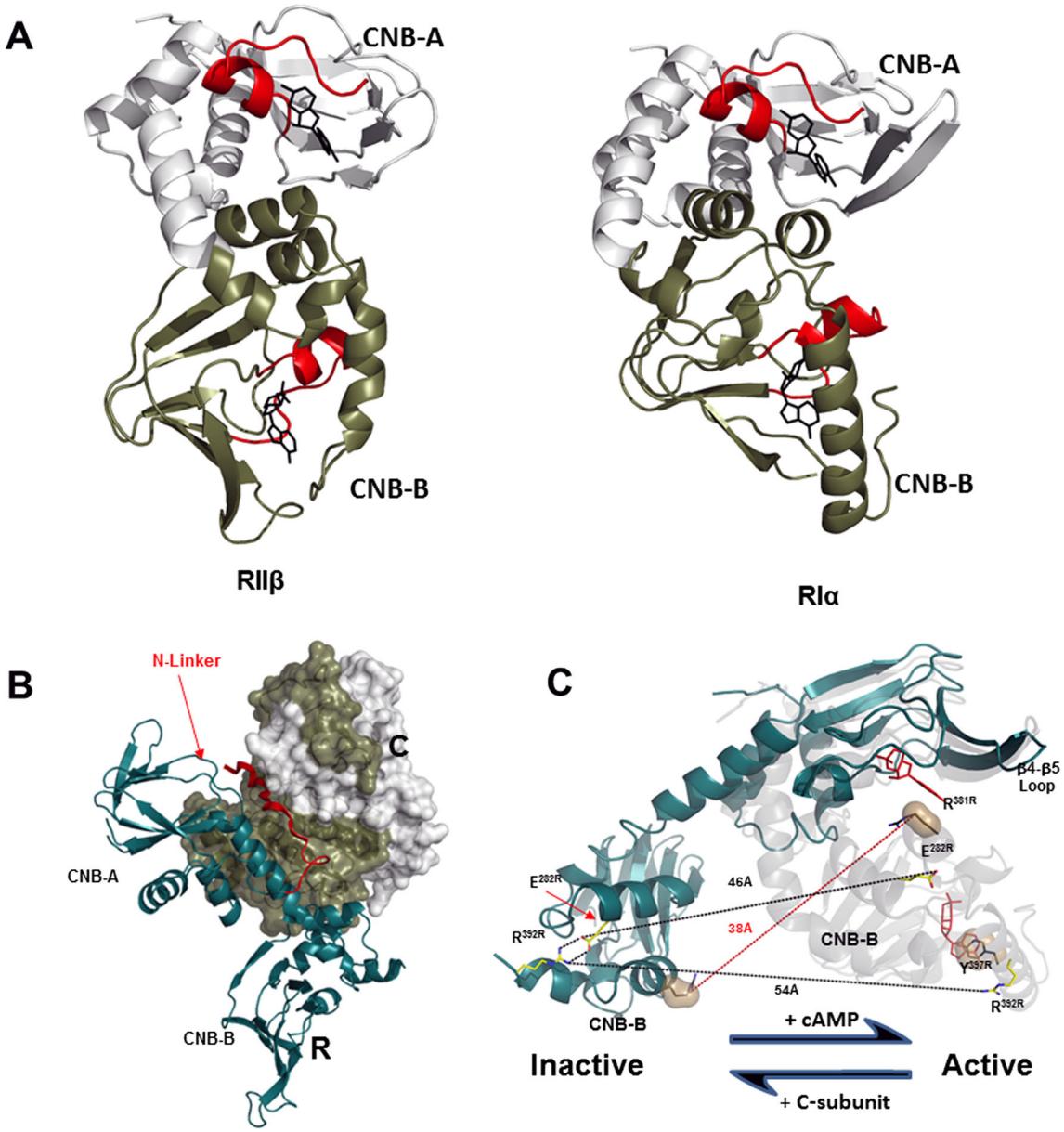

Fig. 3 Flexibility of the CNBs in the PKA R-subunits. **a** Different cAMP-bound R-subunits. Although each CNB domain is highly conserved, the two domains of RIIβ (Diller et al. 2001) (PDB ID 1CX4) and RIα (Su et al. 1995) (PDB ID 1RGS) are oriented in distinctly different ways. To emphasize this, the two structures have their CNB-A domains oriented the same position (*white* CNB-A, *olive* CNB-B, *red* PBCs). **b** The R:C conformation of an RIIβ-subunit complexed with a C-subunit in the holoenzyme complex (*white* N-lobe, *olive* C-lobe, *teal* R-subunit). **c** The conformational changes in the RIIβ in cAMP-bound (Active) and holoenzyme (Inactive) states. The B/C-helix in CNB-A is kinked in the cAMP-bound state and extends into a single long helix in the holoenzyme. In the presence of cAMP, RIIβ has a compact configuration with two CNB domains packed against each other. In the cAMP-free form RIIβ unfolds and forms an extensive interface with the C-subunit of PKA (Zhang et al. 2012)

2010) and demonstrates why it is essential to solve each structure separately. Knowing the structure of the CNB domains of RIα is not sufficient to model the interfaces between the two CNB domains in RIIβ, and bcy1.

The essence of the conformational switch between the active and inactive conformations is captured in the structure of the R:C heterodimer. The major conformational change is seen in the A domain where the B/C helix is extended and moves "out" away from the PBC. Folded on top of the extended B/C helix is the C-terminal portion of the linker that is now locked into place in the holoenzyme by the docking of the IS to the active site cleft of the C-subunit (Fig. 3c).

R:C heterodimers

While studies of the free R- and C-subunits have enabled us to understand how cAMP binds to the R-subunits and how the

Table 1 Isoform-specific allosteric activation of cAMP-dependent protein kinase

Complex	K_a	Hill coefficient
$RI\alpha_2:C_2$	101 nM	1.7
$RI\beta_2:C_2$	29 nM	1.4
$RII\alpha_2:C_2$	137 nM	1.5
$RII\beta_2:C_2$	584 nM	1.8
RIIβ(108–402):C	65 nM	0.82
$RII\beta(1–280)_2:C_2$	60 nM	1.2
$RII\beta(R230K)_2:C_2$	12.9 μM	0.99
$RII\beta(R359K)_2:C_2$	490 nM	0.62
RIIα(90–400):C	40 nM	0.81

The values of the K_a (activation coefficient) and Hill coefficient are from Zawadzki and Taylor (2004), Ilouz et al. (2012) and Zhang et al. (2012)

C-subunit functions as a catalyst, they did not reveal how the C-subunit is inhibited by the R-subunit nor did they explain how catalytic activity is unleashed by cAMP. Such knowledge required structures of R:C heterodimers that were engineered with monomeric forms of the R-subunits that include the IS, and it was these structures which allowed us to appreciate for the first time the inherent conformational flexibility of the CNB domain and the domain reorganization of the two CNB domains. The organization of part of the linker can also be observed in these R:C heterodimers (Kim et al. 2005, 2007; Wu et al. 2007). As predicted, the IS, which is disordered in free R-subunits, is locked into the active site cleft of the C-subunit so that it sterically blocks the binding of other substrates. The C-linker region is wrapped around the C-lobe of the C-subunit where is covers the B/C helix of CNB-A. The two CNB domains are then docked onto the C-lobe of the C-subunit. The heterodimers revealed, in particular, the flexibility of the CNB domains as they extend across the C-lobe. Binding of cAMP to the CNB-A domain thus stabilizes a new protein:protein interface between CNB-A and the C-subunit and alters the previous interface between the CNB-A and CNB-B domains. This extended conformation seen in the inhibited R:C heterodimers is very similar for all four isoforms, which is in striking contrast to the active conformations that are seen in the dissociated cAMP-bound R-subunits.

Full-length R_2C_2 holoenzyme

Although the R:C heterodimer structures contain all of the information required for high-affinity binding of the C-subunit, the Hill coefficients for activation of the R:C

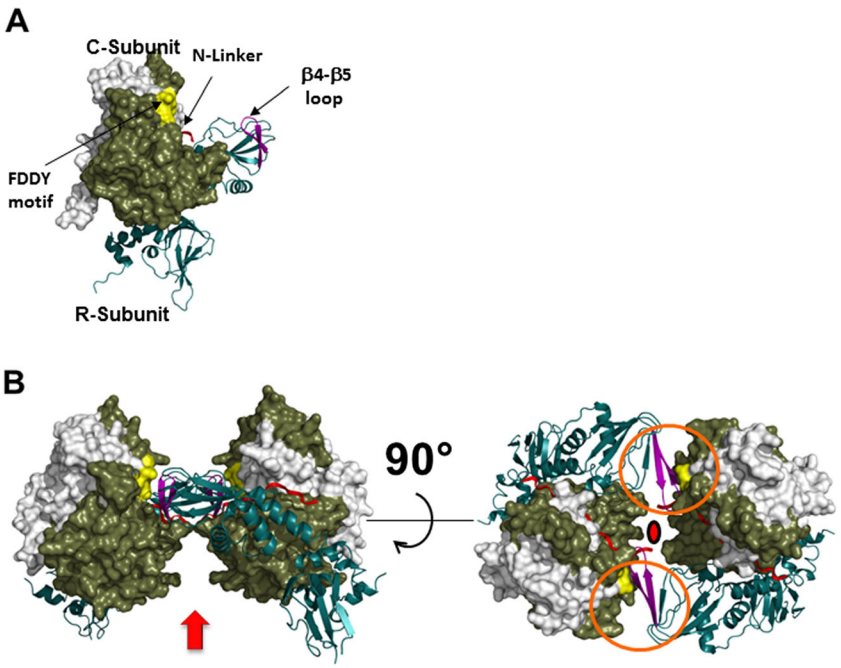

Fig. 4 Structure of the R_2C_2 RIIβ holoenzyme. **a** Structure of the RIIβ:C heterodimer. The RIIβ heterodimer is shown as a ribbon, and the C-subunit is shown as a space-filling model with the N-terminal residues (*white* residues 14–121, *tan* C-terminal residues 12–350). Two motifs and the N-linker of the RIIβ-subunit (*red*) are exposed to solvent in the R heterodimers, but have important roles in the assembly of the holoenzyme. The β4–β5 loop (*purple*) in the RIIβ holoenzyme is located within the CNB-A domain of the R-subunits. The Phe-Asp-Asp-Tyr (FDDY) motif (*yellow*) resides in the C-tail of the C-subunit and is an integral part of the ATP-binding site. **b** Quaternary structure of the R_2C_2 RIIβ holoenzyme. *Arrow* Possible position of the D/D domain, *red* linkers. Rotation allows one to appreciate the twofold symmetry that is found in the holoenzyme and also how the two motifs, the β4–β5 loops and the FDDY motifs (enclosed in *orange circles*) contribute to the assembly of the holoenyzme. *Filled red circle* Twofold axis of symmetry

heterodimers is still close to 1.0. These structures thus do not capture the allostery that is embedded within the full-length holoenzyme where the Hill coefficients are 1.4–1.8 (Table 1). The structure of the R_2C_2 RIIβ holoenzyme allowed us for the first time to appreciate not only the symmetry of the holoenzyme but also the extensive crosstalk between the two heterodimers and the importance of this crosstalk for mediating allosteric activation (Zhang et al. 2012). The domain organization of the RIIβ holoenzyme and the detailed interactions between the two RC heterodimers (R:C and R':C') are shown in Fig. 4. Although the dimerization domain is present in the RIIβ construct, we do not see density for the D/D domain, most likely because it is flexible. The oligomeric constraints of one heterodimer on the other defines this holoenzyme complex clearly as a dimer of dimers.

The allosteric properties of the RIIβ holoenzyme are distinct from those of the other three PKA isoforms (Zhang et al. 2012). The R_2C_2 holoenzyme, for example, has a very high activation coefficient (K_a) for activation by cAMP (584 nM) in contrast to the heterodimer (K_a=65 nM) (Table 1). The K_a for the RIIβ tetrameric holoenzyme is also significantly higher than that for other holoenzymes (101 nM in RIα , 29 nM in RIβ and 137 nM in RIIα tetrameric holoenzymes). The Hill coefficient for the holoenzyme is also 1.8, in contrast to 0.82 for the heterodimer. The increased K_a for the RIIβ tetrameric holoenzyme can be explained by the extensive interfaces between the two heterodimers where the PBC docking site for cAMP in CNB-A is juxta-positioned directly against the ATP binding site in the N-lobe of the C-subunit in the opposite heterodimer (Fig. 4). One also needs to recruit the cAMP capping residue from approximately 40 Å away to form a cAMP-bound R-dimer (Fig. 3). Hence, the RIIβ holoenzyme is more resistant to cAMP activation. The considerable crosstalk between the two CNB domains, as well as between the two heterodimers, is also reflected by several mutations. When the CNB-B domain is deleted entirely, for example, the K_a for activation is 60 nM and the Hill coefficient is reduced to 1.2, indicating that there is still significant allosteric crosstalk between the two CNB-A domains (Zhang et al. 2012). When the cAMP-binding site in the CNB-A domain is destroyed by mutating Arg230 to lysine, the K_a for activation is very high, 13 μM, confirming that cAMP binding to CNB-A is essential for activation. However, when the same mutation is introduced into the CNB-B domain, the enzyme still has a similar K_a for activation (490 nM) but all cooperativity is lost. The molecular basis for this complex allostery can simply not be appreciated in the absence of the holoenzyme structure.

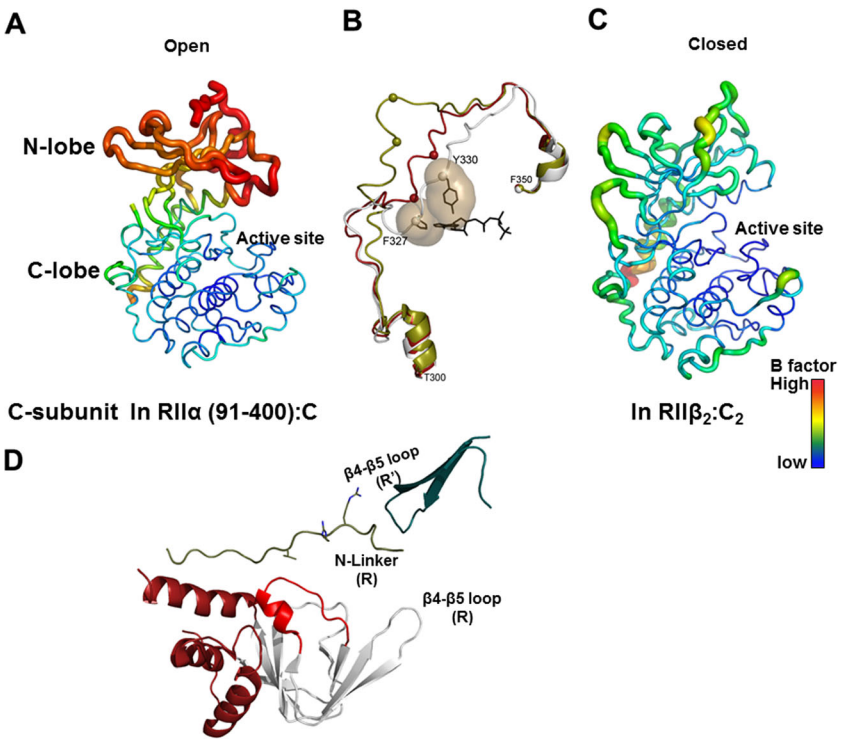

Fig. 5 The conformation and B-factor analysis of the C-subunit in the RII:C heterodimer and the RIIβ$_2$:C$_2$ holoenzyme structures. **a** In the RIIα:C heterodimer (Wu et al. 2007; PDB ID: 2QVS), the C-subunit is dynamic with high B factors and has the active site open in the absence of ATP. **b** The dynamic nature of the C-tail. The intermediate state (PDB ID:4NTT; *red*) adopts a conformation that does not conform to the open state (PDB ID: 4NTS; *olive*) or the closed state (PDB ID: 1ATP; *gray*). The residues Phe327 and Tyr330 are displayed in surface representation which are important for ATP binding (*black*). **c** In RIIβ$_2$:C$_2$ holoenzyme (PDB ID: 3TNP), the C-subunit is more stable with relatively low B factors and has the active site closed even in the absence of ATP. **d** Allosteric interactions of the two RIIβ-subunits in the holoenzyme

The major interface between the two heterodimers in the holoenzyme is between the N-lobe of the C-subunit and the CNB-A domain of the R′-subunit (Fig. 4). Two motifs form this interface: the FDDY motif in the C-terminal tail of the C-subunit (Kannan et al. 2007a; Romano et al. 2009) and the β4-β5 loop in the R′-subunit. In the RIIα:C heterodimer this portion of the C-tail is disordered, and the temperature factors for the entire N-lobe of the C-subunit are very high (Fig. 5a) In contrast, in the R_2C_2 holoenzyme, these two motifs are now engaged by the opposite R:C heterodimer, and the temperature factors are very low (Fig. 5c). This quaternary constraint can not be seen in the R:C heterodimer. The consequence of the β4-β5 loop of the R′-subunit being buttressed up against the C-tail of the C-subunit is that the C-tail is now pushed into a closed conformation even though there is no bound nucleotide. The C-tail, and in particular the FDDY motif, is typically disordered in the nucleotide-free C-subunit (Bastidas et al. 2015) (Fig. 5b), and it has also been found to be highly disordered in the RIIα:C heterodimer (Wu et al. 2007). Here it is ordered independent of the nucleotide. The β4-β5 loop from the RIIβ′-subunit engages most of the ATP-binding machinery of the N-lobe in the opposite C-subunit and assembles it into a closed conformation. Based on previous structures of the ligand-free C-subunit, ATP is required to bring these elements together (Fig. 5b). The apo C-subunit has an open conformation, and the FDDY motif in the C-tail is disordered. In

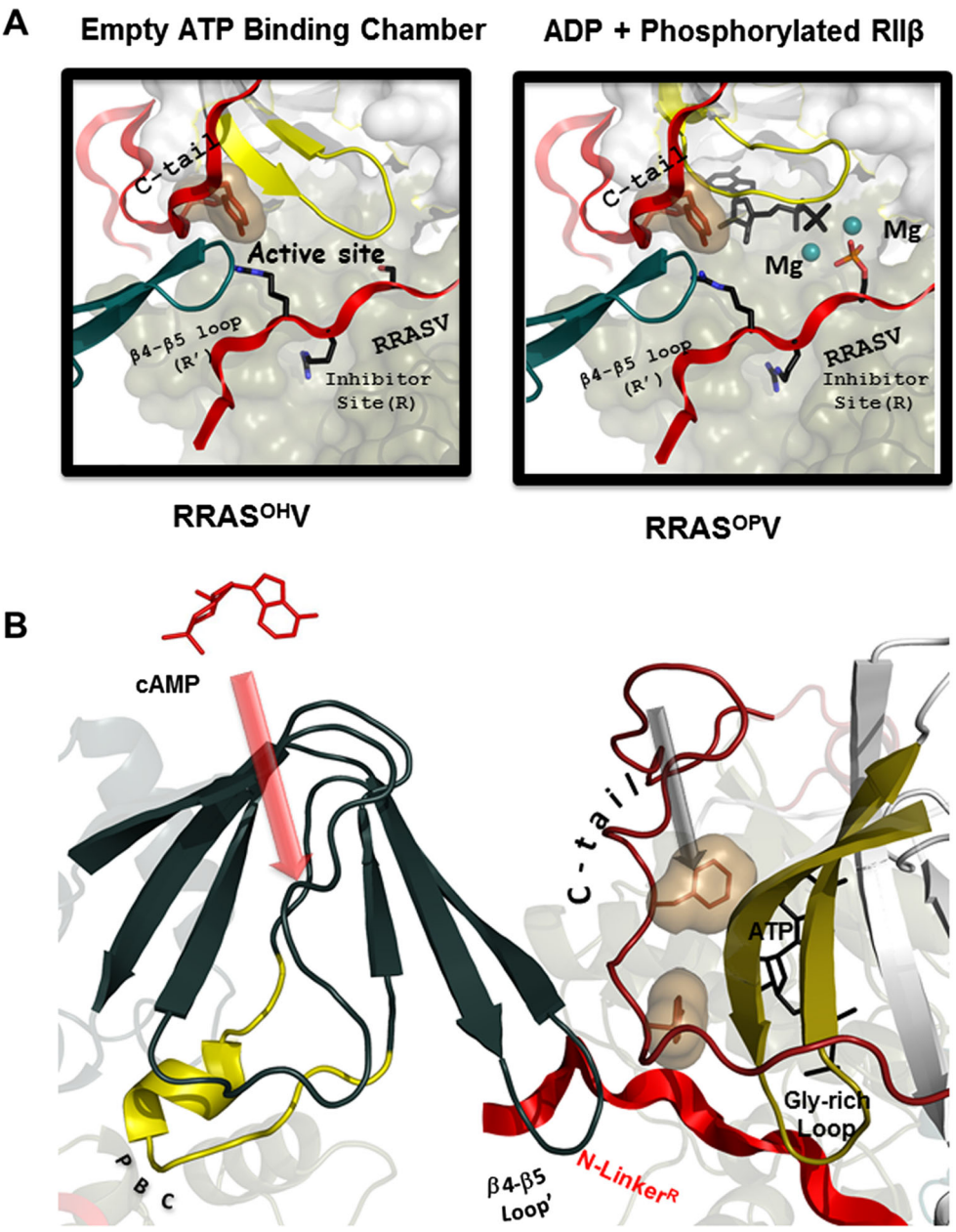

Fig. 6 Allosteric interactions in the RIIβ holoenzyme allow for single turnover phosphorylation of RIIβ. **a** *Left* Active site of the C-subunit in the $RII\beta_2:C_2$ tetrameric holoenzyme is fully closed in the absence of ATP, *right* by diffusing MgATP into the apo-crystals, the reaction products [ADP, (P)-RIIβ and two Mg^{2+} ions] are trapped. **b** Binding site for cAMP to CNB-A′ in the R′C′ heterodimer (*left arrow*) is juxtapositioned against the ATP-binding site in the N-lobe of the C-subunit in the symmetry-related RC dimer. *Red arrow* Trajectory for binding of cAMP to the PBC

the R_2C_2 holoenzyme, the β4-β5 loop of the R′ heterodimer accomplishes the same thing: rather than assembling the ATP-binding pocket by ATP, the β4-β5 loop pushes the C-tail into a closed conformation. The holoenzyme provides a stunning view of how allostery may be achieved by the cooperative interaction of the R- and C-domains in the tetrameric oligomer. cAMP bound to one domain not only influences the interaction of that domain with the C-linker—and in turn the active site of its own C-subunit—but at the same time contacts the C-tail of the C′-subunit through its β4-β5 loop. The cAMP does not directly interact with the IS of its own C-subunit nor does it directly contact the C-tail of the C-subunit, yet binding of this small molecule to the CNB-A domain has a major effect on disrupting the protein interfaces. These interactions from a heterodimer that contains only one R-and one C-subunit cannot be appreciated (Fig. 5d).

The initial holoenzyme was crystallized in the absence of ATP. However, seeing that the ATP binding chamber was perfectly assembled we asked whether ATP could be added to the crystals and trapped in the preformed site. Surprisingly, ATP was not trapped, rather both ADP and the phosphorylated R-subunit were trapped (Fig. 6). In other words, we were able to carry out a full catalytic cycle in this crystal. The temperature factors were not high for the phosphorylated IS, suggesting that product release is not significantly influenced by phosphorylation of the IS Ser. This was the first experiment in which both products were trapped in the crystal lattice of a protein kinase. The structure of the ADP-bound holoenzyme shows clearly how these two nucleotides, ADP and cAMP, compete for the two subunits of the holoenzyme (Fig. 6). The essence of the allosteric regulation of the RIIβ holoenzyme is embedded within this interface. While we have not yet determined the structure of the cAMP-bound holoenzyme, we know that the RR/AA mutant, which cannot bind to the active site, still forms a compact holoenzyme that is no longer inhibited (Wang et al. 1991). Our challenge is to trap these different conformational states of the holoenzyme even at low resolution.

Future challenges

While much has been learned from high-resolution structures of the PKA RIIβ- and C-subunits, these structures do not explain how the activity of PKA is inhibited by the R-subunit nor do they explain cAMP-mediated activation. They also do not shed light on the allosteric mechanism; neither do the R:C heterdimers. It was only the full-length R_2C_2 holoenzyme that allowed us to at least begin to understand symmetry and allostery, although many questions remain unanswered. We still do not understand, for example, how the flexible linkers communicate with the folded D/D domains and the folded CNB domains and the C-subunit. Most importantly, we do not understand the dynamic intermediate states that result from cAMP binding to the holoenzyme. Without this we will never have a full mechanistic understanding of how the allosteric activation of PKA is achieved.

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Compliance with Ethical Standards

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Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human or animal subjects performed by the author, with the exception of those carried out on electric fish.

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