Multi-state recognition pathway of the intrinsically disordered protein kinase inhibitor by protein kinase A

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Abstract In the nucleus, the spatiotemporal regulation of the catalytic subunit of cAMP-dependent protein kinase A (PKA-C) is orchestrated by an intrinsically disordered protein kinase inhibitor, PKI, which recruits the CRM1/RanGTP nuclear exporting complex. How the PKA-C/PKI complex assembles and recognizes CRM1/RanGTP is not well understood. Using NMR, SAXS, fluorescence, metadynamics, and Markov model analysis, we determined the multi-state recognition pathway for PKI. After a fast binding step in which PKA-C selects PKI’s most competent conformations, PKI folds upon binding through a slow conformational rearrangement within the enzyme’s binding pocket. The high-affinity and pseudo-substrate regions of PKI become more structured and the transient interactions with the kinase augment the helical content of the nuclear export sequence, which is then poised to recruit the CRM1/RanGTP complex for nuclear translocation. The multistate binding mechanism featured by PKA-C/PKI complex represents a paradigm on how disordered, ancillary proteins (or protein domains) are able to operate multiple functions such as inhibiting the kinase while recruiting other regulatory proteins for nuclear export.

Introduction

The cAMP-dependent protein kinase A (PKA) is a ubiquitous phosphoryl transferase that regulates numerous cellular signaling pathways (Taylor et al., 2012). As for other eukaryotic protein kinases, PKA regulome comprises globular domains as well as extensive disordered regions, which are essential for protein-protein interactions and signaling (Akimoto et al., 2013; Gógl et al., 2019; Pellicena and Kuriyan, 2006). In fact, the activity and localization of PKA-C within different cellular compartments are finely regulated by ancillary proteins such as the regulatory subunits (R), A-kinase-anchoring proteins (AKAPs) (Bauman and Scott, 2002; Johnson and Lewis, 2001), and the heat-stable protein kinase A inhibitor (PKI) (Dalton and Dewey, 2006). While R-subunits are primarily responsible for the regulation and localization of PKA-C in the cytoplasm through interaction with...
AKAPs (Taylor et al., 2012), PKI plays a key role in transcriptional regulation and the export of the enzyme from the nucleus to the cytoplasm (Fantozzi et al., 1992; Wiley et al., 1999).

PKI is expressed in different isoforms of 70–75 amino acids in length, which were first identified in skeletal muscle extracts (Dalton and Dewey, 2006). PKI isoforms contain several functional motifs: a high affinity region (HAR, residues 1–14), a pseudo-substrate recognition sequence (PSS, residues 15–22), a nuclear export signal (NES, residues 37–46), and a disordered C-terminal tail (residues 47–75) (Figure 1A; Johnson et al., 2001). The HAR is an accessory domain that increases the affinity of PKI for the enzyme, the PSS mimics the consensus sequence for the enzyme; while the NES motif mediates the recruitment by the CRM1/RanGTP complex for PKA-C translocation from the nucleus to the cytoplasm (Figure 1D; Wen et al., 1995). PKI binds PKA-C with nanomolar affinity (Whitehouse and Walsh, 1982), which is matched only by that of the R-subunits with whom it shares the same recognition/inhibitory sequence (Figure 1C; Hofmann, 1980). Unlike the R-subunits, PKI lacks any cAMP binding sites and its regulation of PKA-C is independent of the cyclic nucleotide-mediated signaling (Dalton and Dewey, 2006).

In humans, three different genes encode the corresponding PKI isoforms, PKIα, PKIβ, PKIγ (Dalton and Dewey, 2006). Among those, the alpha isoform (PKIα) has the highest level of

![Figure 1](image_url)

Figure 1. PKIα primary sequence, homology, and complex with PKA-C. (A) Primary sequence of PKIα, featuring the high affinity region (HAR), the pseudo-substrate region (PSS), and the nuclear export signal (NES). (B) X-ray structure of the PKA-C/ATP/PKIα ternary complex featuring only part of the HAR sequence and the PSS motif (PDB ID: 1ATP; Knighton et al., 1991a). PKA-C secondary structure elements are color-coded using Hanks and Hunter definition (Hanks et al., 1988). (C) Comparison of the substrate recognition sequences (SRS) for different PKA-C substrates, including PKIα, Kemptide, phospholamban (PLN), and the regulatory subunit RIIα and RIIβ. D. NES sequences from different proteins (Fung et al., 2015; Fung et al., 2017).
expression in various tissues and the highest inhibitory potency \((K_i \sim 0.22 \text{ nM})\), (Collins and Uhler, 1997; Gamm and Uhler, 1995). The initial crystal structures of PKA-C were obtained with truncated variants of PKI\(\alpha\), encompassing either residues 5–22 or 5–24 with part of the HAR and PSS sequences, lacking the NES as well as the C-terminal regions. (Figure 1B; Knighton et al., 1991a; Knighton et al., 1991b). More importantly, there are no reports on how PKI is recognized by the kinase and its binding mechanism.

Using a combination of NMR spectroscopy, small angle X-ray scattering (SAXS), stopped-flow fluorescence, replica-averaged metadynamics (RAM), and Markov model analysis, here we provide a comprehensive view of the molecular mechanism for recognition of PKI\(\alpha\) by PKA-C. We found that free PKI\(\alpha\) is intrinsically disordered, with incipient \(\alpha\)-helical segments spanning the HAR and NES regions. PKA-C recognizes and binds extended PKI\(\alpha\) conformations, rigidifying the PSS region and increasing the helicity of the HAR and NES motifs. Transient binding kinetics show that the recognition occurs via a multi-state pathway, with an initial fast binding step followed by a slow phase in which PKI\(\alpha\) undergoes a conformational rearrangement within the binding site. Although the C-terminal tail of PKI\(\alpha\) interacts transiently with the kinase’s C-lobe, it remains essentially disordered facilitating the recruitment by the CRM1/RanGTP complex for nuclear export. We propose that the multistate pathway for PKI\(\alpha\) recognition by PKA-C is preparatory for the recruitment by CRM1/RanGTP for nuclear export and regulation of gene expression.

**Results**

**PKI\(\alpha\) is an intrinsically disordered protein with transient secondary and short-lived tertiary interactions**

In agreement with Hauer et al. (Hauer et al., 1999a; Thomas et al., 1991), we found that the dichroic profile, the NMR fingerprint, and the dynamic NMR parameters display the typical signature of mixed \(\alpha\)-helix and random-coil structure for PKI\(\alpha\) (Figure 2, Figure 2—figure supplement 1A). Addition of urea to PKI\(\alpha\) only slightly changes the chemical shift of the amides, confirming the disordered nature of the protein and suggesting the presence of only transient secondary structures (Figure 2—figure supplement 1B; Felitsky et al., 2008; Marsh et al., 2007). We also probed the presence of long-range contacts using \(^1\text{H}_2\text{O}\) paramagnetic relaxation enhancement (\(^1\text{H}_2\text{O}\) PRE-\(\Gamma_2\)) experiments (Clore and Iwahara, 2009; Figure 2E–G). For these experiments, we conjugated a nitroxide spin label (MTSL) on engineered cysteine residues at three different positions: PKI\(\alpha\)\(^{V3C}\), PKI\(\alpha\)\(^{S28C}\), and PKI\(\alpha\)\(^{S59C}\) in analogy to earlier fluorescence studies (Hauer et al., 1999b). We then measured the peak intensities in the presence of paramagnetic (\(I_{\text{para}}\)) and diamagnetic (\(I_{\text{dia}}\)) labels to estimate the \(\Gamma_2\) relaxation rates (Clore and Iwahara, 2009). When the spin label was engineered at the N-terminus (PKI\(\alpha\)\(^{V3C}\)), we observed a reduction of the resonance intensities near the N-terminal region and gradual effects that culminate in the middle of the linker between the PSS and NES motifs and decrease toward the C-terminus (PRE values less than two standard deviations, 2\(\sigma\)). The C-terminal residues remained essentially unperturbed (Figure 2E). On the other hand, when the spin label was positioned between the PSS and NES motifs (PKI\(\alpha\)\(^{S28C}\)), we detected strong PREs near the spin label position (PRE greater than 3\(\sigma\)), at the N-terminus, and within the NES motif (Figure 2F). Finally, for the spin label at position 59, we found that approximately 75% of the residues display a reduced signal intensity, with marked effects in the PSS, linker region, NES, and C-terminus. In this case, we observed only marginal effects for the N-terminal residues (Figure 2G). Altogether, the CD and NMR data reveal that in absence of a binding partner PKI\(\alpha\) samples transient secondary structures encompassing the HAR and NES motifs with short-lived tertiary conformations.

**PKA-C binding rigidifies the PSS motif and stabilizes HAR and NES helices of PKI\(\alpha\)**

To map the fingerprints of both PKA-C and PKI\(\alpha\) in the PKA-C/ATP\(\gamma\)N/PKI\(\alpha\) complex, we used our previously developed method in which we combine asymmetric isotopic labeling for each binding partner with a spin-echo filter for \(^{13}\text{C}\)-linked amide protons in the heteronuclear single quantum coherence experiment (HSQC), \([^{1}\text{H},^{15}\text{N}]\)-carbonyl carbon label selective-HSQC or \([^{1}\text{H},^{15}\text{N}]\)-CCLS-HSQC (Tonelli et al., 2007; Larsen et al., 2018; Figure 3—figure supplement 1). This method makes it possible to monitor the fingerprints of two interacting proteins simultaneously using only
Upon binding to PKA-C, most of the PKI resonances in the \([^{1}\text{H},^{15}\text{N}]\)-HQSC spectrum show only minimal changes (Figure 3—figure supplement 1C). However, the HAR and PSS resonances display significant chemical shift changes (Figure 3A,B,E), suggesting that these regions of PKI undergo drastic structural rearrangements upon binding the kinase. This finding is also supported by the \(\delta^2\)D2 analysis (Camilloni et al., 2012) of the Ca and Cβ chemical shifts showing an increase in helical content of the HAR and rigidification of the PSS motif; while the rest of the peptide remain unaffected by the interaction with PKA-C (Figure 3B, Figure 3—figure supplement 2A). The analysis of the chemical shift difference between the ATPπN-bound form and the ternary complex mapped on PKA-C are reported in Figure 3 (panel C-D). As previously reported (Masterson et al., 2010; Masterson et al., 2008), the amide fingerprint of the kinase changes significantly upon nucleotide binding (Figure 3C). Additional changes are observed upon one sample preparation. Upon binding to PKA-C, most of the PKI resonances in the \([^{1}\text{H},^{15}\text{N}]\)-HQSC spectrum show only minimal changes (Figure 3—figure supplement 1C).

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PKIα adopts partial secondary structure upon interaction with PKA-C. (A) Chemical shift perturbation (CSP) of the amide fingerprint of PKIα upon binding PKA-C/ATPγN binary complex calculated using equation 1 (Williamson, 2013). The residues of PKIα that undergo significant CSPs are localized in the HAR and PSS, which are involved in strong electrostatic interaction within the binding site of PKA-C. (B) Chemical shift index (CSI) for Ca and Cβ of free (red) and bound (blue) PKIα. The PSS motif becomes more rigid upon interaction with PKA-C. The HAR and the NES adopt a transient α-helical conformation, which is enhanced upon binding the kinase. (C) CSP of PKA-C amide fingerprint upon binding ATPγN. (D) CSP of the PKA-C/ATPγN binary complex upon binding PKIα. The red lines in the histograms indicate one standard deviation from the average CSP: 0.38 ppm, 0.11 ppm, and 0.10 ppm for (A), (C) and (D), respectively. (E) CSPs for PKA-C and PKIα amide resonances mapped onto a selected conformer from the ensemble of the PKA-C/ATP/PKIα structures calculated from MD simulations (see Materials and methods).

The online version of this article includes the following figure supplement(s) for figure 3:

Figure supplement 1. Mapping PKA-C/PKIα interactions using [1H,15N] CCLS-HSQC experiments.

Figure 3 continued on next page.
binding PKIα, especially for αC, αF, αG helices as well as β6 loop. The map of the chemical shift changes on the PKA-C/ATPγN/PKIα complex for the two proteins is reported in Figure 3E. Interestingly, smaller changes are present for the NES region of PKIα, which are mirrored by higher values of heteronuclear nuclear Overhauser effects (NOE), indicating reduced motions of the backbone amides in the pico-to-nanosecond time scale (Figure 4A, Figure 3—figure supplement 2B; Dyson and Wright, 2004; Bah et al., 2015). The formation of the PKA-C/ATPγN/PKIα (ternary) complex is also reflected by the differences of longitudinal and transverse relaxation rates (ΔR1 and ΔR2) for both the HAR and PSS motifs upon forming the complex, which define a local rigidification of the PKIα backbone upon binding PKA-C, with the C-terminal portion (residues 47–75) remaining essentially unstructured (Figure 4B,C).

The T1/T2 ratios can be used as an estimate of the global correlation time for the PKA-C/PKI complex (Kay et al., 1989; Cavanagh et al., 2007a; Cavanagh et al., 2007b; Gaspari and Perczel, 2010). The PSS and HAR regions of PKIα, which show the most prominent chemical shift changes, adopt the overall correlation time of the kinase in agreement with previous fluorescence anisotropy measurements (Figure 3—figure supplement 2C; Hauer et al., 1999b). Interestingly, the ΔRex values obtained from the Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion experiments show the presence of μs-ms motions for residues in the HAR and PSS motifs, which gradually decrease toward the NES motif, suggesting the presence of conformational interconversion of these regions upon binding (Figure 4D, Figure 3—figure supplement 2D). Next, we analyzed the large amplitude motions of the kinase-bound PKIα by conjugating MTSL to cysteine 59 in the C-terminal region (PKIαS59C) and used the [1H,15N]-CCLS pulse sequence to simultaneously measure the PREs on both PKIα and PKA-C (Olivieri et al., 2018; Figure 5A,B). The most significant PREs for PKIα were detected for resonances of residues proximal to the MTSL label, with a gradual reduction moving away from residue 59. Interestingly, we detected some long-range effects for specific residues located in the HAR and PSS motifs and the intervening linker. This implies that the C-terminal

Figure 4. Changes in the NMR dynamic parameters of PKIα upon formation of the ternary complex with PKA-C and ATPγN. (A) Histogram of the changes in heteronuclear NOE (ΔHX-NOE) values of PKIα free and bound and map of the ΔHX-NOE values onto a selected conformer from the ensemble of the PKA-C/ATP/PKIα structures calculated from MD simulations (see Materials and methods). (B-D) Corresponding ΔR1, ΔR2, and ΔRex plots for PKIα free and bound forms. The sample concentration for free form of [2H/15N]PKIα was 0.2 mM; while for PKIα bound to PKA-C/ATPγN was 0.3 mM. All the experiments were recorded at 27˚C.
domain of PKI\(\alpha\) undergoes large amplitude motions and transiently interacts with the N-terminus, N-lobe (\(\beta_1-\beta_2\) loop), C- lobe (G- and H- helices) as well as the peptide-positioning loop of PKA-C. Taken together, the relaxation data show that PKA-C binding to PKI\(\alpha\) causes differential effects on the various regions of the inhibitor. While disorder-to-order transitions occur for the HAR, PSS, and NES motifs, the C-terminal tail of the PKI\(\alpha\) remains disordered. (Figure 5C).

The PKA-C/PKI\(\alpha\) complex relaxes through two structurally and kinetically distinct states to form the complex

To determine the mechanism of binding PKA-C and PKI\(\alpha\), we utilized stopped-flow rapid mixing fluorescence resonance energy transfer (FRET) and analyzed transient and total changes in donor fluorescence during the binding reaction. This procedure enables the interpretation of the kinetic mechanism and structural changes occurring during a binding reaction (Muretta et al., 2015; Nesmelov et al., 2011). To determine the kinetics of binding, we engineered a double mutant of PKA-C (PKA-C\(\text{C}^{199A,S325C}\)) and labeled the single reactive S325C with Alexa Fluor 488 (PKA-C\(\text{DONOR}\)). We also expressed and purified three different mutants of PKI\(\alpha\) (PKI\(\text{V}^3\), PKI\(\text{S}^{28}\), and PKI\(\text{S}^{59}\)) and labeled each cysteine with tetramethylrhodamine-5-maleimide (PKI\(\text{ACCEPTOR}-3\), PKI\(\text{ACCEPTOR}-28\), and PKI\(\text{ACCEPTOR}-59\)). For the first set of experiments, we mixed PKA-C\(\text{DONOR}\) with increasing concentrations of each acceptor (PKI\(\text{ACCEPTOR}-3\), PKI\(\text{ACCEPTOR}-28\), PKI\(\text{ACCEPTOR}-59\), (Figure 6A–D). As expected
for the formation of the PKA-C/PKI\(\alpha\) complex, we observed a gradual decrease in the total fluorescence of the donor probe. In a second set of experiments, we tested the time-dependence of long-range rearrangements of PKI\(\alpha\) as suggested by PRE measurements (Figure 6A, Figure 6—figure supplement 1A). To this extent, we utilized the PKA-C\(^{C199A,S325C}\) mutant without fluorescent label and engineered a double mutant of PKI\(\alpha\) with two cysteines at residue 3 and 59 (PKI\(\alpha^{V3C,S59C}\)). The PKI\(\alpha^{V3C,S59C}\) mutant was then labeled with both Alexa-488 (donor) and TMR (acceptor) at positions 3 and 59, respectively (PKI\(\alpha^{DONOR-ACCEPTOR}\)). When a fixed concentration of PKI\(\alpha^{DONOR-ACCEPTOR}\) was titrated with increasing concentrations of unlabeled PKA-C\(^{C199A,S325C}\), we detected an increase of the total fluorescence (Figure 6E, Figure 6—figure supplement 1B), indicating that the distance between the two probes of PKI\(\alpha\) increases upon formation of the complex, that is the conformational ensemble of PKI\(\alpha\) becomes on average more extended during the binding reaction in agreement with the PRE data. From an initial analysis of the binding curve, we found that the total fluorescence of the Alexa-488 donor emission from the PKA-C\(^{DONOR}\)/PKI\(\alpha^{ACCEPTOR}\) changed bi-exponentially through the course of the binding reactions (Figure 6A–D). The bi-exponential behavior suggests the presence of an initial fast binding step followed by a slow structural rearrangement (Table 1; Gianni et al., 2014).

These two phases can be identified for all the PKI labeled species, irrespective of the fluorescence acceptor position. The rate constants for the fast phase increases linearly with PKI\(\alpha\) concentration, with an average second-order rate constant of 1.63 \(\times\) 10\(^7\) M\(^{-1}\) s\(^{-1}\) and an extrapolated average dissociation rate constant of 7.0 s\(^{-1}\). Comparatively, the observed rate constant for the slow phase increased hyperbolically with an average \(K_{0.5}\) of 3.1 \(\mu\)M and a maximum value \(k_{\text{slow}}\) of 24 \(\pm\) 1.8 s\(^{-1}\).
Table 1. Rate constants obtained from the fluorescence decay of PKI\textsuperscript{ACCEPTOR3}, PKI\textsuperscript{ACCEPTOR28}, and PKI\textsuperscript{ACCEPTOR59} upon mixing with unlabeled PKA-C\textsuperscript{C199A,S325C} at 25°C.

|                  | PKI\textsuperscript{ACCEPTOR3} | PKI\textsuperscript{ACCEPTOR28} | PKI\textsuperscript{ACCEPTOR59} | PKI\textsuperscript{DONOR-ACCEPTOR} |
|------------------|-------------------------------|----------------------------------|----------------------------------|-----------------------------------|
| **Fast phase**   |                               |                                  |                                  |                                   |
| \(k_{on} (M^{-1} \text{s}^{-1})\) | 1.88 ± 0.03 × 10^7            | 1.40 ± 0.02 × 10^7               | 1.62 ± 0.11 × 10^7               | 0.85 ± 0.04 × 10^7               |
| \(k_{off} (s^{-1})\)     | 13 ± 3                       | 5 ± 1                           | 2 ± 10                           | 6 ± 7                            |
| \(K_{d,\text{app}} (nM)\)       | 700 ± 170                    | 390 ± 110                       | 150 ± 620                        | 700 ± 800                        |
| **Slow phase**    |                               |                                  |                                  |                                   |
| \(k_{slow} (s^{-1})\)       | 24 ± 2                       | ND                              | ND                               | 37 ± 2                           |
| \(K_{0.5} (\mu M)\)        | 2.4 ± 0.4                    | 3.0 ± 0.4                       | 3.8 ± 0.5                        | 17 ± 3                           |

ND: Non determined.

We also observed two distinct binding phases for the second set of experiments with unlabeled PKA-C\textsuperscript{C199A,S325C} and PKI\textsuperscript{DONOR-ACCEPTOR}. Indeed, the kinetic constants obtained for these experiments are very similar to those found in the first set of the experiments, underscoring the same binding mechanisms. The pre-exponential amplitudes for the fast phases increased hyperbolically, while the slow phase decreased hyperbolically (Figure 6D–H). Taken together, the behavior of the rate constants and the pre-exponential amplitudes for the fast and slow phases are consistent with a multi-pathway mechanism in which there is a fast binding phase of PKI\(\alpha\) ensembles competent for binding and a subsequent structural rearrangement of these conformers upon binding (Dogan et al., 2014; Gianni et al., 2014).

Conformational landscape and kinetics of conformational transitions of PKI\(\alpha\)

Using chemical shift (CS) and residual dipolar couplings (RDC) (Figure 2D) as structural restraints, we performed replica-averaged metadynamics (RAM) to define the energy landscapes of free and kinase-bound PKI\(\alpha\). During the simulations, the HAR and NES motifs of free PKI\(\alpha\) undergo multiple coil-to-helix transitions (Figure 8). We identified two major basins, featuring a compact ensemble \(R_g <1.5\ \text{nm}\), with three relative minima with helical structures for the HAR and NES motifs; and a more extended ensemble \(R_g >3.0\ \text{nm}\), where the protein is essentially disordered. The HAR motif populates mostly a random coil conformation, with a sparsely populated helical conformation, whereas the NES motif populates both helical and coil conformations (Figure 7A,B). To validate the RAM-generated ensembles, we carried out small angle X-ray scattering (SAXS) experiments and back-calculated the SAXS profiles from the conformers sampled in the RAM trajectories. Figure 7C shows the scattering intensity plots (log \(I(q)\) vs \(q\)) and the corresponding Kratky plot for free PKI\(\alpha\). These profiles show the typical signatures of intrinsically disordered proteins, with the Kratky plot featuring a plateau at high \(q\) values as well as the absence of a bell-shaped curve (Figure 7C, bottom panel). In addition, the \(P(r)\) curve shows that PKI\(\alpha\) adopts a highly extended conformational ensemble, with an abnormally large \(D_{\text{max}} (\sim 110\ \text{Å})\) and \(R_g (\sim 30\ \text{Å})\) values for a protein of its size, both parameters are indicative of an extended, intrinsically disordered protein (Kikhney and Svergun, 2015; Figure 8D). In fact, the back-calculated SAXS profiles from the RAM-generated ensembles are in excellent agreement with the experimental SAXS scattering profiles (\(\chi^2 = 0.86\) for \(q < 0.2\)) (Figure 7C). In the bound state, the HAR motif of PKI\(\alpha\) is more ordered and adopts a more stable helical conformation; whereas the NES motif is considerably more dynamic and adopts a transient helical conformation (Figure 7B).

In this case, the SAXS data are less informative as the Kratky plot for the PKA-C/ATP\(\gamma\)N/PKI\(\alpha\) complex is dominated by the bell-shaped curve of the globular enzyme, with a well-defined maximum in the low \(q\) region \((\sim 0.08\ \text{Å}^{-1})\) that decays to near zero at high \(q\) values. To determine the rates of conformational exchange and the populations of PKI\(\alpha\) free and bound, we performed unbiased molecular dynamics (MD) simulations and analyzed the conformational transitions building a Markov model. To simplify and visualize the conformational space of PKI\(\alpha\), we reduced the high-dimensionality space of the residue-residue contacts into a 6-dimensional space using time-lagged
independent component analysis (tICA). We then projected the free energy landscape along the main components, tIC1 and tIC2. In this reference frame, free PK1α occupies six local minima ($\Delta G < 1$ kcal/mol) and a higher energy state ($\Delta G \sim 2.5$ kcal/mol) (Figure 8). We then clustered the conformers into four major ensembles based on the secondary structure content of the HAR, PSS, and NES motifs: HLH (helix-loop-helix), CLC (coil-loop-coil), HLC (helix-loop-coil), and CLH (coil-loop-helix) (Figure 8—figure supplement 1). For free PK1α, the CLH ensemble is dominant, with a mean population of 51%, followed by CLC (32%), HLC (15%), and HLH (~2%) (Figure 8A). The kinetics of the conformational transitions were calculated using the mean-first-passage-time (MFPT) among these states. We found that all the conformational transitions take place on a $\mu$s time scale, with the slowest transitions from the HLC, CLH, and CLC ensembles to the HLH ensemble; while the transitions from CLC, HLH, and HLC to the CLH ensemble are more rapid (Figure 8, Figure 8—figure supplement 1). For PK1α bound to PKA-C, the conformational space spanned becomes more restricted (Figure 8D). Specifically, the HLC, CLC, and HLH minima become more populated, with 72, 22, and 6% (Figure 8B). In addition, the transition kinetics from CLC and HLC to the HLH ensemble is estimated to occur approximately 10 times faster (Figure 8, Figure 8—figure supplement 1).
Proposed mechanistic model

Based on NMR, SAXS, fluorescence, RAM simulations as well as Markov model analysis, we propose that PKIα binding to PKA-C occurs via a multistate recognition pathway. We envision that free PKIα spans a broad conformational space with incipient helical elements for the HAR and NES motifs. These conformations are in fast exchange in the NMR time scale. We propose that the initial step (fast) of recognition by PKA-C is driven by electrostatic interactions between the conformations of PKIα featuring an exposed PSS motif (CLC and HLC) and PKA-C (Tsigelny et al., 1996).
encounter complexes undergo slower conformational rearrangements in which a) the HAR motif folds upon binding in a helical conformation, b) the PSS motif binds within the active cleft in an extended conformation, and c) the transient helix of the NES motif adopts a more defined conformation. To validate our hypothesized mechanism, we fitted the fluorescence curves using a set of equations detailed in the supporting information (Scheme 1 and Scheme 2). Under these constraints, we obtained a kinetic constant of $1.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for the formation of the PKA-C*/PKI* encounter complex and a slow relaxation of the complex with a constant of $2400 \text{ s}^{-1}$. These kinetics parameters are in the same order of magnitude of the association rates measured for binding of other intrinsically disordered proteins (Mollica et al., 2016).

**Discussion**

When unleashed from the R-subunit, the C-subunit of PKA is in a constitutively active state, where binding of nucleotide engages the N- and C-lobes priming the kinase for substrate recognition and catalysis. R-subunits and PKI share small linear motifs (SLiMs) that bind the substrate binding site with high affinity and trap PKA-C in an inactive state. SLiMs are intrinsically disordered regions that are part of the allosteric machinery of kinases and control the cross-talk between different kinase signaling pathways (Akimoto et al., 2014; Akimoto et al., 2013; Delaforge et al., 2018; Gógl et al., 2019; Kragelj et al., 2015; Wang et al., 2011; Wright and Dyson, 2015; Guiley et al., 2019). PKI can be portrayed as a prototypical SLiM that is able to bind and inhibit the kinase and recruit the CRM1/RanGTP complex to export the enzyme out of the nucleus, thereby regulating cell proliferation. Previous structural studies focused on the 5–24 region of PKIx that encompasses both the HAR and PSS motifs, which has been instrumental in trapping the ternary complex of the kinase and obtain the first crystal structure of a kinase (Knighton et al., 1991a). Subsequent spectroscopic studies provided evidence for the dynamic nature of the PKIx peptide, suggesting the presence of minimal secondary structural elements such as the HAR and NES regions, with a C-terminal tail completely disordered (Padilla et al., 1997; Hauer et al., 1999a). The HAR is extremely important for binding/anchoring to the catalytic subunit as well as positioning the PSS within the catalytic cleft of the enzyme (Walsh et al., 1971; Cheng et al., 1986). However, most PKA target substrates do not possess this motif and the only substrate recognition requirement is the highly positively charged consensus sequence in the linear PSS motif. In fact, in most cases this recognition motif is located in highly dynamic or intrinsically disordered regions as it molds into the binding groove of the kinase situated at the interface between the small and large lobes. Our studies bring new insights into the dynamic nature of the PKIx recognition mechanism. After the initial binding of ATP in the pocket between the two lobes that primes the substrate binding site (Chu et al., 2017; Hyeon et al., 2009; Masterson et al., 2010), this highly disordered polypeptide, with incipient helices at both the HAR and NES motifs, is driven toward the kinase via positively charged Arg residues in the PSS sequence. Deletion of one of the Arg residues in the PSS provokes a dramatic reduction of the phosphorylation kinetics and has been linked to the progression of dilated cardiomyopathy (Kim et al., 2015; Haghighi et al., 2006). On the other hand, mutations in the active site of the kinase that change electrostatic interactions such as L205R dramatically influence the

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**Scheme 1.** The proposed Kinetic Model of PKA-C/PKIX binding. Highlighted pathway is used in the numerical fitting of Stopped Flow rapid mixing FRET data using MATLAB.
thermodynamics and kinetics of substrate binding, leading to the development of adrenocortical adenomas and the resultant Cushing’s syndrome (Calebiro et al., 2014; Cheung et al., 2015; Walker et al., 2019). More importantly, our studies reveal a significant ordering of the PKIα structure upon interaction with the kinase, particularly for the HAR, PSS, and NES motifs. This ordering occurs in the second, slower binding step as revealed by FRET measurements, while the C-terminal tail of PKIα remains essentially disordered, undergoing large amplitude motions with transient interactions with the C-lobe of the kinase. Interestingly, when PKIα is bound to PKA-C, the NES region undergoes a structural rearrangement, showing a higher degree of helicity as supported by NMR and MD simulations. The NES motif comprises Leu 37, Leu 41, and Leu 44 that are essential for high binding affinity to the nuclear export machinery CRM1/RanGTP (Göttler et al., 2010; Fu et al., 2018). The CRM1 binding pocket is a relatively rigid cavity and NES sequences must possess significant plasticity to mold into the binding pocket (Göttler et al., 2010; Fu et al., 2018). This observation could explain why the NES assumes a defined conformation only upon interaction with CRM1. Based on our data, it is possible to speculate that the interactions of PKIα with PKA-C preambles the formation of the complex and that the helix-coil equilibrium of the NES domain is critical for recruiting the CRM1/RanGTP nuclear export complex (Fu et al., 2018; Fung et al., 2017; Göttler et al., 2010). This complex binding mechanism shows how SLiMs can orchestrate multiple functions and regulate kinases through disordered ancillary proteins or protein domains.
# Materials and methods

## Key resources table

| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|-------------|------------------------|
| Gene (Oryctolagus cuniculus)      | PKIA or PK\(\alpha\) | Uniprot ID P61926   |             |                        |
| Gene (Mus musculus)               | PKA-CA or PKA-C | Uniprot ID Q9DBC7   |             |                        |
| Strain, strain background (Escherichia coli) | BL21(DE3) | New England Biotech (NEB) | C2527I | Chemically competent cells |
| Recombinant DNA reagent           | pT7-7 PK\(\alpha\) | Dr. Herberg (Universität Kassel, Germany) |             | DOI: 10.1042/BJ20071665 |
| Recombinant DNA reagent           | PK\(\alpha\)\(^{V3C}\) | This study |             | Single Cys mutant of PK\(\alpha\) |
| Recombinant DNA reagent           | PK\(\alpha\)\(^{S28C}\) | This study |             | Single Cys mutant of PK\(\alpha\) |
| Recombinant DNA reagent           | PK\(\alpha\)\(^{S59C}\) | This study |             | Single Cys mutant of PK\(\alpha\) |
| Recombinant DNA reagent           | PK\(\alpha\)\(^{V3C, S59C}\) | This study |             | Double Cys mutant of PK\(\alpha\) |
| Recombinant DNA reagent           | PKA-C\(^{\alpha}\) | Prof. Taylor S.S. (USCD, CA, USA) |             | DOI: 10.1006/abio.1997.9952 |
| Recombinant DNA reagent           | (His\(^{60}\))-PKA-Rl\(\alpha\)\(^{S213K}\) | Prof. Taylor S.S. (USCD, CA, USA) |             | DOI: 10.1006/abio.1997.9952 |
| Sequence-based reagent            | PK\(\alpha\)\(^{V3C}\) | This study |             | PCR primer (Forward): aaggagatatacat atgggaactgattgcg aaactacttatgccgatttta |
| Sequence-based reagent            | PK\(\alpha\)\(^{S28C}\) | This study |             | PCR primer (Forward): ccatccagatatc ctggtctgcagtgcttccgg |
| Sequence-based reagent            | PK\(\alpha\)\(^{S59C}\) | This study |             | PCR primer (Forward): aggagatgtctaaaagatctt gcactgaacaatccggagaag |
| Sequence-based reagent            | PK\(\alpha\)\(^{V3C, S59C}\) | This study |             | PCR primer (Forward): 1)aaggagatatacatatgcg aactgattgcgaaa ctacttatgccgatttta 2)aggagagatctaaaagatctt gcactgaacaatccggagaag |
| Chemical compound, drug           | MTS\(L\) | Toronto Research Chemical | O875000 | Spin label |
| Chemical compound, drug           | dMTSL | Toronto Research Chemical | A188600 | Spin label |
| Chemical compound, drug           | Alexa Fluor 488 C5 Meleimide | Thermo Fisher Scientific | A10254 | FRET acceptor |
| Chemical compound, drug           | Tetramethylrhodamine-5-maleimide (TMR) | Life Technologies | T6027 | FRET donor |
| Chemical compound, drug           | AMP-PNP or ATP\(\gamma\)N | Roche Applied Science | 10102547001 | ATP analogous |
| Commercial assay or kit           | QuikChange Lightning Multi Mutagenesis Kit | Agilent genomics | 210519 | Commercial mutagenesis kit |

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| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|-------------|------------------------|
| **Commercial assay or kit**       | PepTag Assay, Non-Radioactive Detection of PKA | Promega | VS340 | Commercial assay kit |
| **Software, algorithm**           | TopSpin 3.0 | Bruker Inc |             | https://www.bruker.com/ |
| **Software, algorithm**           | NMRPipe | Delaglio F, NIH (Delaglio et al., 1995) |             | https://www.ibbr.umd.edu/nmrpipe/install.html |
| **Software, algorithm**           | PyMol | Schrödinger, LLC |             | https://pymol.org |
| **Software, algorithm**           | MatLab2019b | MathWorks |             | https://www.mathworks.com/products/matlab.html |
| **Software, algorithm**           | GraphPad Prism 8 | GraphPad Software Inc |             | https://www.graphpad.com/ |
| **Software, algorithm**           | Origin 8 | OriginLab |             | https://www.originlab.com/ |
| **Software, algorithm**           | SAXSQuant software suit | Anton Paar | N/A |          |
| **Software, algorithm**           | Primus | ATSAS 2.8.3 software |             | https://www.emb-l-hamburg.de/biosaxs/download.html |
| **Software, algorithm**           | MultiFoXS server | (Schneidman-Duhovny et al., 2013). |             | https://modbase.compbio.ucsf.edu/multfoxs/DOI: 10.1016/j.bpj.2013.07.020 |
| **Software, algorithm**           | GROMACS 4.6 | (Hess et al., 2008) |             | http://www.gromacs.org/ |
| **Software, algorithm**           | PLUMED 2.1 | (Bonomi et al., 2009) |             | http://www.plumed.org/doc/v2.5/user-doc/html/_c_h_a_n_g_e_s-2-1.html |
| **Software, algorithm**           | ALMOST 2.1 | (Fu et al., 2014) |             | https://sourceforge.net/projects/almost/ |
| **Software, algorithm**           | MSMBuilder 3.5 | (Harrigan et al., 2017) |             | http://msmbuilder.org/3.5.0/index.html |
| **Software, algorithm**           | δ2D | (Camilloni et al., 2012) |             | http://www-mvssoftware.ch.cam.ac.uk/index.php/d2D |

**Expression and purification of Full-length PKIα**

Gene coding for full-length PKIα (Oryctolagus cuniculus, PKIA PRKACN1) subcloned into pT7-7 vector was a gift to us by Dr. Friedrich Herberg (Universität Kassel, Germany). For the fluorescence and PRE-experiments, three different single-site (PKIαV3C, PKIαS28C and PKIαS59C) and a double-site (PKIαV3C, S59C) mutants were generated by QuikChange Lightning mutagenesis kit (Agilent genomics) using PKIα as template. The choice of the sites of mutation was based on previous work (Hauer et al., 1999b). Recombinant full-length PKIα was expressed in E. coli BL21 (DE3) cells at 30˚C and the purification was performed as previously reported (Hauer et al., 1999a). Briefly, transformed cells were grown in Luria-Bertani (LB) medium for fluorescence while uniformly labeled 15N, 13C/15N, and 2H/13C/15N PKIα was expressed in minimal medium (M9) for NMR experiments. Protein expression was induced by the addition of 0.4 mM of IPTG to a culture with optical density of ~1.3–1.4 at 30°C and was performed for 5 hr. The cell pellet was resuspended in 20 mM MOPS (pH 7.5) and lysed by sonication. The lysate was then centrifuged at 20,000 rpm for 30 min. The supernatant was then heated in a boiling water bath at 95°C for 5 min to precipitate out most of the undesired proteins. The protein suspension was centrifuged a second time at 20,000 rpm for 30 min. The resulting supernatant was dialyzed against 20 mM TRIS-HCl (pH 7.0) buffer overnight. A second purification step with anion exchange chromatography was carried out using HiTrap Q HP column (GE Healthcare Biosciences Corp) in 20 mM TRIS at pH 7.0 where a NaCl gradient was used to elute the
protein. A reversed phase HPLC purification step was also performed to obtain a higher grade of purity using C18 column (HiCHROM, UK) using water as buffer A and isopropanol as buffer B with 0.1% trifluoroacetic acid in both buffers. The purified peptide was concentrated, lyophilized, and stored at −20°C. The final product was accessed using SDS-PAGE with a final purity of >97%. The molecular weight and the quantity of the peptide were verified by amino acid analysis (Protein chemistry laboratory at Texas A and M University, TX, USA). All the cysteine mutants of PKIα were purified following the same protocol used for the wild-type protein with additional supplement of reducing agent (5 mM β-mercaptoethanol) in all the dialysis buffers.

**Expression and purification of PKA-C**

Recombinant catalytic subunit of PKA-C (*Mus musculus* gene) was expressed in *E. coli* BL21 (DE3) cells at 24°C in M9 minimal medium. The purification was performed using the His6-Rlal(R213K) subunit as described previously ([Hemmer et al., 1997](#)). A subsequent second purification step was performed using a HiTrap SP HP cation exchange column (GE Healthcare Biosciences Corp) to separate out the three isoforms of PKA-C that differ in their phosphorylation profiles ([Yonemoto et al., 1997](#)). The purified peptide was then stored in phosphate buffer supplied of 10 mM DTT, 10 mM MgCl₂, and 1.0 mM NaN₃, at 4°C. For isotopically ²H-labeled protein, the cells were growth 80% D₂O M9 minimal medium in a bench fermenter (2.0 L). The most abundant isoform of PKA-C, corresponding to phosphorylation at S338, T197, and S10 residues (isoform II) ([Walsh and Ashby, 1973](#)), was used for all experiments. The purity was assessed using SDS-PAGE electrophoresis and the final purity was >97%. The kinase activity was tested with a gel-shift assay from Promega (Fitchburg, Wisconsin) and quantified using A₂₈₀ = 52,060 M⁻¹ cm⁻¹.

The C199A S325C mutant of the catalytic subunit of PKA (PKA-C⁰¹⁹⁹A, S₃₂₅C) was recombinantly expressed by growing transformed *E. coli* BL21(DE3) cells in LB medium and induced by addition of IPTG at 24°C as described previously. The mutant PKA-C was purified using the previously described protocol ([Masterson et al., 2009](#)). Briefly, cells were resuspended in lysis buffer (30 mM MES, 1 mM EDTA, 50 mM KCl, 5 mM β-mercaptoethanol, 0.15 mg/mL lysozyme, 1 mM PMSF, pH 6.5), and lysed using French press at 1000 psi. The supernatant after pelleting the cell debris was batch-bound with P11 phosphocellulose resin in lysis buffer at pH 6.5. PKA-C was purified by eluting over a gradient of 0–500 mM KH₂PO₄. The protein solution was then buffer exchanged into 20 mM KH₂PO₄, pH 7.0. The sample was concentrated and divided into two equal aliquots to perform two separate spin labeling reactions in parallel: one using the paramagnetic spin label MTSL (1-oxyl-2,2,5,5-tetramethyl-δ-3-pyrroline-3-methyl)methanethiosulfonate, Toronto Research Chemicals, Inc), the second using the diamagnetic form, dMTSL (1-acetyl-2,2,5,5-tetramethyl-δ-3-pyrroline-3-methyl)methanethiosulfonate, Toronto Research Chemicals, Inc). The reactions were carried out at 4°C overnight with ten-fold excess of labeling compounds that was then removed by passing the reaction solution through a 5 ml HiTrap Desalting column (GE Healthcare Biosciences Corp.) equilibrated with 20 mM KH₂PO₄, 90 mM KCl, 10 mM MgCl₂, 1 mM NaN₃ at pH 6.5. Upon purification, wild-type or mutant proteins were supplied with 10 mM MgCl₂, 10 mM DTT and 1 mM NaN₃ and stored at 4°C.

**Spin labeling of proteins**

Lyophilized single site ¹⁵N PKIα mutants were first dissolved in 1 mL of buffer containing 20 mM KH₂PO₄, pH 7.0, and incubated for about an hour in the presence of 1 mM TCEP. The sample was concentrated to 0.6 mM. The protein solution was then buffer exchanged into 20 mM KH₂PO₄, 90 mM KCl, 10 mM MgCl₂, 1 mM NaN₃ pH 7.0. The sample was concentrated and divided into two equal aliquots to perform two separate spin labeling reactions in parallel: one using the paramagnetic spin label MTSL (1-oxyl-2,2,5,5-tetramethyl-δ-3-pyrroline-3-methyl)methanethiosulfonate, Toronto Research Chemicals, Inc), and the second using the diamagnetic form, dMTSL (1-acetyl-2,2,5,5-tetramethyl-δ-3-pyrroline-3-methyl)methanethiosulfonate, Toronto Research Chemicals, Inc). The reactions were carried out at 4°C overnight with ten-fold excess of labeling compounds that was then removed by passing the reaction solution through a 5 ml HiTrap Desalting column (GE Healthcare Biosciences Corp.) equilibrated with 20 mM KH₂PO₄, 90 mM KCl, 10 mM MgCl₂, 1 mM NaN₃, pH 6.5. The extent of labeling was assessed by ESI TOF mass spectrometry (Mass Spectrometry Laboratory, Department of Chemistry, University of Minnesota), and was found to be >99%.

**NMR sample preparation**

The NMR samples used to study the free form of PKIα were composed primarily by ¹⁵N and ¹³C/¹⁵N labeled protein expressed in M9 media. The final concentration of samples ranged from 0.5 mM to 0.8 mM in 20 mM KH₂PO₄, 90 mM KCl, 10 mM DTT, 10 mM MgCl₂, 1 mM NaN₃ at pH 6.5. Samples for the assignment of the PKIα in complex with PKA-C were performed using uniformly ²H/¹³C/¹⁵N and ²H/¹⁵N labeled PKIα and uniformly ²H/¹⁵N PKA-C. The ternary complex between PKIα and
PKA-C and ATP-analogues (AMP-PNP) were reconstituted in 20 mM KH$_2$PO$_4$, 90 mM KCl, 12 mM of ATP$\gamma$N, 10 mM DTT, 10 mM MgCl$_2$, 1 mM Na$_2$S, at pH 6.5. A molar ratio of 1:1.2 (PKI$\alpha$:PKA-C) to saturate the complex with a concentration of 0.150 mM of PKI$\alpha$. All experiments were performed at 27°C. The denaturation experiments were carried out on 0.3 mM sample of uniformly $^{15}$N PKI$\alpha$ resuspended in 20 mM KH$_2$PO$_4$, 90 mM KCl, 10 mM DTT, 10 mM MgCl$_2$, 1 mM Na$_2$S, 8 M Urea at pH 6.5. The $^1$H$_2$O-T2 relaxation experiments were performed on 0.25 mM $^{15}$N labeled single mutants of PKI$\alpha$ for the free form and uniformly $^2$H/$^{13}$C/$^{15}$N protein for the bound form. In both preparations the buffer used for the analysis was 20 mM KH$_2$PO$_4$, 90 mM KCl, 10 mM MgCl$_2$, 1 mM Na$_2$S at pH 6.5. For all the NMR experiment carried out on the PKA-C/ATP$\gamma$/PKI$\alpha$ complex, a low protein concentration sample was prepared to due to the high tendency to aggregate.

Preparation of fluorescent labeled proteins

Lyophilized single-site PKI$\alpha$ mutants were first dissolved in 1 mL of buffer containing 20 mM KH$_2$PO$_4$, pH 7.0, and incubated for about an hour in the presence of 1 mM TCEP. About four-fold excess of fluorescent dye (tetramethylrhodamine-5-maleimide, TMR) was added to the sample and incubated for about an hour at room temperature with stirring and protected from light. The reaction mixture was then passed through two 5 mL HiTrap Desalting columns (GE Healthcare Biosciences Corp.) equilibrated with 20 mM KH$_2$PO$_4$, 90 mM KCl, 10 mM MgCl$_2$, 1 mM Na$_2$S pH 6.5 to remove the unreacted dye. The extent of labeling was also verified using mass spectrometry. For the double-mutant PKI$\alpha$, the procedure described above was used. In this case, Alexa-488 was added to the solution in 1:1 stoichiometric ratio with PKI$\alpha$ and the reaction was allowed to proceed for one hour at room temperature with stirring and protected from light. Since Alexa Fluor 488 carries a charge, the single-labeled PKI$\alpha$ was separated using anion exchange chromatography with HiTrap Q HP column using an NaCl gradient in 20 mM TRIS pH 7.0. The fraction containing the single Alexa-labeled PKI$\alpha$ was buffer exchanged using a PD-10 MidiTrap column equilibrated with 20 mM KH$_2$PO$_4$, 90 mM KCl, 10 mM MgCl$_2$, 1 mM Na$_2$S, pH 7.0. Five-fold excess of tetramethylrhodamine-5-maleimide (TMR) was added and the reaction mixture was incubated for about an hour with stirring and protected from light. The asymmetrically double-labeled PKI$\alpha$ was separated from the unreacted dye by passing the reaction through two 5 mL HiTrap Desalting columns (GE Healthcare Biosciences Corp.) equilibrated with 20 mM KH$_2$PO$_4$, 90 mM KCl, 10 mM MgCl$_2$, 1 mM Na$_2$S pH 6.5. The extent of labeling in each step was determined using mass spectrometry. For the labeling of PKA-CC$_{199}$A, S$_{325}$C mutant, the protein was buffer-exchanged by passing through a PD-10 MidiTrap column (GE Healthcare Bio-Sciences Corp.) equilibrated with 20 mM KH$_2$PO$_4$, 90 mM KCl, 10 mM MgCl$_2$, 1 mM Na$_2$S, pH 7.0 at room temperature. The fractions containing PKA-C were pooled and the concentration was adjusted to ~5–6 μM. ATP at a final concentration of 8 mM was added to protect the C343 site from being labeled as shown previously (Nelson and Taylor, 1981). The reaction was initiated by adding a five-fold molar excess of Alexa Fluor 488 and allowed to proceed for about one hour at room temperature with stirring and protected from light. The sample was then buffer-exchanged with the same buffer composition as before, with 10 mM DTT and pH 6.5. The extent of labeling was assessed using mass spectrometry. The protein sample was then concentrated to ~20 μM.

NMR experiments

Backbone resonance assignment of PKI$\alpha$ bound to PKA-C was achieved using standard triple-resonance 3D NMR experiments (Ikura et al., 1990; Kay et al., 2011). For the assignment of the free form, all the NMR experiments were acquired on a Varian Inova 600 MHz spectrometer equipped with an HCN Cold Probe and on a Bruker 700-MHz Avance spectrometer equipped with a 5 mm triple resonance cryoprobe. The $^1$H-$^{15}$N HSQC (Bodenhausen and Ruben, 1980) experiments were acquired with 16 scans, 2048 ($^1$H) and 100 ($^{15}$N) complex points, before and after each triple-resonance experiment. The HNCACB and CBCA(CO)NH (Grzesiek and Bax, 1992) experiments were collected with 64 scans, 1643 ($^1$H), 108 ($^{15}$N), and 128 ($^{13}$C) complex points. The HNCO (Kay et al., 2011; Yang and Kay, 1999) experiments were acquired with 32 scans, 1024 ($^1$H), 40 ($^{15}$N), and 54 ($^{13}$C) complex points. Standard $^{15}$N-edited TOCSY-HSQC and NOESY-HSQC (Marion et al., 1989) experiments were acquired with 64 scans, 1024 ($^1$H) and 120 ($^{15}$N) complex points. The CC(CO)NH-TOCSY (Cavanagh et al., 2011) experiment was recorded with 1024 ($^1$H), 30 ($^{15}$N), and 70 ($^{13}$C) points.
complex points, with 32 scans on a Bruker 700 MHz Advance III spectrometer equipped with a 1.7 mm TCI MicroCryoProbe. All data was processed using NMRPipe (Delaglio et al., 1995) and visualized using NMRFAM-Sparky (Goddard and Kneller, 2004; Lee et al., 2015).

All experiments for the backbone assignment of the PKA-C/ATPγN/PKια ternary complex were performed on a Bruker Avance III 850 MHz spectrometer with a TCI cryoprobe. TROSY-based (Salzmann et al., 1998; Salzmann et al., 1999) HNCA and HN(CO)CA (Kay et al., 2011) experiments were collected with a minimum of 32 scans, 1024 (\(^{1}\)H), 32 (\(^{15}\)N), and 64 (\(^{13}\)C) complex points. The TROSY-based HNCACB experiment was collected with 32 scans, 1024 (\(^{1}\)H), 35 (\(^{15}\)N) and 50 (\(^{13}\)C) complex points were performed to measure the \(^{13}\)Ca and \(^{13}\)Cb correlations. The HNCO experiments were acquired with a minimum of 16 scans, 1024 (\(^{1}\)H), 30 (\(^{15}\)N), and 40 (\(^{13}\)C) complex points. Before and after each triple resonance experiments a \(^{1}\)H-\(^{15}\)N CLEAN-TROSY-HSQC (Schulte-Herbrüggen and Sorensen, 2000) spectrum was acquired with 1024 (\(^{1}\)H) and a minimum of 64 (\(^{15}\)N) complex points. All data were processed using NMRPipe (Delaglio et al., 1995) and visualized using Sparky (Lee et al., 2015; Goddard and Kneller, 2004). The CSI for Ca, Cβ, C’ and Hα were derived with respect to the reference calculated by Schwarzinger et al. (Schwarzinger et al., 2000; Schwarzinger et al., 2001) and plotted using the GraphPad Prism six software package (GraphPad Software, Inc). The CSP plot (Williamson, 2013) was calculated using the amide chemical shifts according to the following equation:

\[
\Delta \delta = \sqrt{\Delta \delta_{HN}^2 + (0.154 \Delta \delta_N)^2}
\]

(1)

Where \(\Delta \delta\) is the compounded chemical shift perturbation, \(\Delta \delta_{HN}\) is the chemical shift perturbation of the amide proton, \(\Delta \delta_N\) is the chemical shift perturbation of nitrogen, and 0.154 is the scaling factor for nitrogen (Mulder et al., 1999).

NMR relaxation experiments

The heteronuclear [\(^{1}\)H-\(^{15}\)N]-NOE spectra were acquired using standard pulse sequences (Farrow et al., 1994a) on a Bruker 850 MHz and 900 MHz Advance spectrometer III equipped with TCI cryoprobes at 27°C. The heteronuclear [\(^{1}\)H,\(^{15}\)N]-NOE values were calculated from the ratio of the peak intensities with and without proton saturation. The errors were estimated by evaluating the standard deviation of the NOE values (\(\sigma_{\text{NOE}}\)):

\[
\frac{\sigma_{\text{NOE}}}{\text{NOE}} \sqrt{\left(\frac{\sigma_{\text{sat}}}{I_{\text{sat}}}\right)^2 + \left(\frac{\sigma_{\text{unsat}}}{I_{\text{unsat}}}\right)^2}
\]

(2)

where \(\sigma_{\text{sat}}\) and \(\sigma_{\text{unsat}}\) are the root-mean-square noise of the spectra and \(I_{\text{sat}}\) and \(I_{\text{unsat}}\) are the intensities of the resonance with and without proton saturation (Farrow et al., 1994b).

The \(^{15}\)N spin relaxation rates (\(T_1\) and \(T_2\)) for PKια free and bound to PKA-C/ATPγN complex were measured as reported by Barbato et al. (1992) by acquiring a series of \(2D\) [\(^{1}\)H-\(^{15}\)N]-HSQC spectra at different relaxation delays. The relaxation rates for each residue were obtained by fitting the intensities of each peaks with an exponential decay function. 16 scans, 1024 (\(^{1}\)H), 60 (\(^{15}\)N) complex points were used to record \(T_1\)-relaxation experiment. The \(T_2\) measurements were recorded using 32 scans, 1024 (\(^{1}\)H), 64 (\(^{15}\)N) complex points for both PKια free and bound. For \(T_1\), the relaxation delays were: (20), 100, 200, 300, 400, 600, 800, and 1000 ms. For \(T_2\), the relaxation delays were (16.6), 83.1, 166, 249, (332), 498, 664, 830 ms. The numbers in parenthesis indicate the experiments that were repeated for error estimation. All the experiments were recorded using a Bruker 900 MHz Advance spectrometer III equipped with TCI cryoprobes at 27°C. All experiments on the free form of PKια was performed using a 0.2 mM sample of uniformly \(^{2}\)H\(^{15}\)N labeled protein. To measure PKια relaxation in the ternary complex, the final sample concentration was 0.180 mM \(^{2}\)H\(^{15}\)N labeled PKια and 0.220 mM of U\(^{-}\)labeled PKA-C. All samples were prepared in aqueous buffer consisting of 20 mM KH\(_2\)PO\(_4\), 90 mM KCl, 60 mM MgCl\(_2\), 10 mM DTT, and 1 mM Na\(_2\)SO\(_4\) at pH 6.5.

The \(\mu\)-ms timescale conformational dynamics of backbone amides in PKια free form and the PKA-C/ATPγN/PKια complex were measured using the relaxation-compensated version of the Carr-Purcell-Meiboom Gill (CPMG) relaxation dispersion experiments (Baldwin and Kay, 2009; Long et al., 2008; Palmer et al., 2001). The experiments were performed in an interleaved mode with a \(\nu_{\text{CPMG}}\) values of 0, 12.5 and 1000 Hz. Effective transverse relaxation rate constant, \(R_2\), were
determined at each $v_{CPMG}$ value using the peak intensities with and without relaxation period according to the equation (Mulder et al., 2001):

$$R_{2,\text{eff}} = \frac{\ln(\frac{I}{I_0})}{T}$$

where $I_0$ and $I$ are the peak intensities of the resonances in the 2D spectra acquired in the absence and presence of a relaxation period, respectively, and $T$ is the total CPMG time (Mulder et al., 2001) which was 40 ms. All the experiments were acquired on Bruker 900 MHz spectrometer using 16 scans with 1024 (1'H) and 64 (15N) complex points with a recycle delay of 4.5 seconds.

All the spectra were processed using NMRPipe (Delaglio et al., 1995) and visualized using NMRFAM-Sparky (Goddard and Kneller, 2004; Lee et al., 2015). (T.D. Goddard and D.G. Kneller, UCSF).

Paramagnetic relaxation measurements

The intra-molecular $^1H$ PRE-$\Gamma_2$ relaxation measurements on the free form of PKI$\alpha$ mutants were carried out using the pulse sequence by Iwahara et al. (2007) on a Bruker Advance 700 MHz spectrometer at 27°C. All experiments were performed using 160 scans with 2048 (1'H) and 128 (15N) complex points. A two-time point measurement was performed using a relaxation time of 4 and 14 ms in an interleaved fashion. The $^1H$ PRE-$\Gamma_2$ values were calculated using the equation (Iwahara et al., 2007):

$$\Gamma_2 = \frac{1}{T_b - T_a} \ln \left( \frac{I_{\text{dia}}(T_b)/I_{\text{para}}(T_a)}{I_{\text{dia}}(T_a)/I_{\text{para}}(T_b)} \right)$$

where $\Gamma_2$ is the PRE-relaxation rate, the time points are $T_a$ and $T_b$, $I_{\text{para}}$ is the corresponding intensity with a spin label and $I_{\text{dia}}$ is the corresponding intensity with a reduced spin label:

$$\sigma(\Gamma_2) = \frac{1}{T_b - T_a} \sqrt{\left( \frac{\sigma_{\text{dia}}(T_a)}{I_{\text{dia}}(T_a)} \right)^2 + \left( \frac{\sigma_{\text{dia}}(T_b)}{I_{\text{dia}}(T_b)} \right)^2 + \left( \frac{\sigma_{\text{para}}(T_a)}{I_{\text{para}}(T_a)} \right)^2 + \left( \frac{\sigma_{\text{para}}(T_b)}{I_{\text{para}}(T_b)} \right)^2}$$

Where $\sigma_{\text{dia}}$ and $\sigma_{\text{para}}$ are the roots mean square noise of the respective spectra. For the simultaneous detection of inter- and intra-molecular PREs of PKI$\alpha$ bound to PKA-C/ATP$\gamma$N, we used a TROSY-based CCLS variation of the standard PRE pulse sequence (Olivieri et al., 2018). For both paramagnetic and diamagnetic experiment, the complex sample was prepared with a total protein concentration of 140 $\mu$M using a 1:1 PKA-C/PKI$\alpha$ molar ratio.

Residual dipolar coupling measurements

Backbone amide $^1D_{\text{NH}}$ RDCs of $^{15}N$ PKI$\alpha$ were measured at 27°C by taking the difference in $^1J_{\text{NH}}$ scalar coupling in aligned and isotropic media (Tjandra and Bax, 1997). The aligned medium was constituted by DMPC/D7PC bicelles (q = 3.5) and $^1J_{\text{NH}}$ couplings were measured using the in-phase anti-phase $[^1H,^{15}N]$ HSQC sequence (Ottinger et al., 1998). To get an orthogonal set of $^1D_{\text{NH}}$ RDC values, we used stretched polyacrylamide gel (Ishii et al., 2001). All the experiments were carried out on a Bruker 850 MHz Avance III spectrometer equipped with a 5 mm triple resonance cryoprobe, using 320 scans with 1024 (1'H dimension) and 60 (15N dimension) complex points.

Circular dichroism (CD) spectra acquisition and data analysis

The CD spectra in the far-UV of free PKI$\alpha$ in native conditions were recorded using a JASCO J-815 spectropolarimeter (Chemes et al., 2012). The spectra were acquired at 25°C scanning from 180 to 260 nm and recording at 20 nm/min scan speed with a bandwidth of 5 nm and a pitch of 0.1 nm. 100 $\mu$M PKI$\alpha$ was solubilized in 20 mM PIPES buffer (pH 7.0) with 150 mM NaCl. To record the baseline, a blank sample with buffer alone was used. The data were analyzed using the BeSTSel web server (http://bestsel.elte.hu/; Micsonai et al., 2018).

Small angle X-ray scattering (SAXS) experiment and data analysis

SAXS data were collected at the University of Utah using an Anton Paar SAXess instrument with line collimation and solid-state detector. In both PKI$\alpha$ free and the PKA-C/PKI$\alpha$ complex, the proteins were equilibrated by dialysis in buffer containing 20 mM MOPS, 90 mM KCl, 60 mM MgCl$_2$, 10 mM
DTT, 1 mM NaN\textsubscript{3} (pH 6.5), and 12 mM of ATPγN. For measurements of the PK\textalpha free form, a protein concentration of 1 mM was used, and for the PKA-C/PK\textalpha complex sample, a final protein concentration 0.2 mM protein with PKA-C/PK\textalpha in 1:1 molar ratio. SAXS data were reduced and desmeared using the SASQQuant (Anton Paar) software suite and further analyzed using PRIMUS in the ATSAS 2.8.3 software suite (Franke et al., 2017). The fitting of the MD structural ensembles to the experimental scattering data was performed using the MultiFoXS server (Schneidman-Duhovny et al., 2013). 100 random snapshots from MD simulations with different radius of gyration (R\textsubscript{g}) were chosen as the conformational ensembles, and the fitting was done for the q range (0.01 to approximately 0.18 Å\textsuperscript{-1}). The top 10 structures with the lowest \( \chi \) values were selected as the final structure ensembles.

Modeling of PK\textalpha ensembles using replica-averaged metadynamics (RAM) simulations

For the free form, we used the extended conformation of the full-length PK\textalpha as a template. The protein was solvated in a rhombohedral solvent box with TIP3P water molecule layer extended approximately 10 Å away from the protein’s surface. Counter ions (K\textsuperscript{+} and Cl\textsuperscript{−}) were added to ensure electrostatic neutrality corresponding to an ionic concentration of \( \sim 150 \text{mM} \). All protein covalent bonds were constrained with the LINCS (Hess et al., 1997) algorithm and long-range electrostatic interactions were calculated using the particle-mesh Ewald method with a real-space cutoff of 10 Å (Best et al., 2012; Darden et al., 1993). Parallel simulations of 130 ns were performed simultaneously using GROMACS 4.6 (Hess et al., 2008) with CHARMM36a1 force field (Best et al., 2012). As a starting configuration for the bound form, we used the ternary complex of PKA-C/ATP/PK\textalpha\textsubscript{5-24} (PDB: 1ATP) and built the reminder amino acids of PK\textalpha using PyMOL (pymol.com). The remaining parameters were set using the same protocol for the free form.

The subsequent RAM simulations were started from the random snapshots of the production trajectory (the last 100 ns). As for previous RAM simulations (Roux and Weare, 2013), we chose 10 replicas: five replicas initiated from partially folded conformers, and the others from extended conformers. We set two protocols imposing different restraints: a) RAM with CS restraints (RAM\_CS) for both free and bound forms in which the predicted backbone chemical shifts were back-calculated by Camshift method implemented in ALMOST 2.1 (Fu et al., 2014); and b) RAM with CS and RDC restraints (RAM\_CSRDC) for the free form only in which the predicted RDCs were back-calculated using the tensor-free method (Camilloni and Vendruscolo, 2015) implemented in PLUMED 2.1 (Bonomi et al., 2009). Four collective variables (CVs) were chosen to monitor the conformational plasticity and secondary structure of PK\textalpha: a) psi angles for all residues (backbone and dihedral); b) radius of gyration for all backbone Ca atoms (radius of gyration); c) helical content of residues 4–22 (helical content of the N-terminus); and d) helical content of residue 32–50 (helical content of the NES motif). Gaussian deposition rate was performed with an initial rate of 0.125 kJ/mol/ps, where the \( \sigma \) values were set to 0.5, 0.02 nm, 0.1, and 0.1 for the four CVs. Of the 10 replicas, each of the four CVs were imposed on two replicas, and the remaining two replicas were left neutral. The RAM simulations were carried out using GROMACS 4.6 in conjunction with PLUMED 2.1 (Bonomi et al., 2009) and ALMOST 2.1 (Fu et al., 2014), and continued for \( \sim 250 \text{ns} \) for each replica with exchange trails every 0.5 ps. The free energy along the four CVs converged after 150 ns, with the standard deviation below one kcal/mol. The last 100 ns of each replica constituted the production trajectory for a total simulation time of 1.0 μs and used for analysis.

Adaptive sampling of pk\textalpha in free and bound forms and Markov model analysis

For the free form, the adaptive sampling was started from randomly chosen 320 snapshots of PK\textalpha from the RAM simulations. The initial velocities were randomly generated to satisfy the Maxwell distribution at 300K. A \( \sim 200 \text{ns} \) simulation was initiated from each starting configuration. Therefore, a total of 60 μs trajectories and 240,000 snapshots (250 ps per frame) were collected for subsequent analysis. For the bound form, the adaptive sampling was started from 50 snapshots of the RAM simulations, and \( \sim 20 \text{μs} \) trajectories have been collected. More samplings are undertaken.
Markov model and time-lagged independent component analyses (tICA)
The Markov model analysis was carried out using MSMBuild 3.5 (Harrigan et al., 2017). The contact distances between residue pairs that are separated by at least four residues, were chosen as the metrics to characterize the conformational transition of PKIα. The representation in this metric space was further reduced to 6-dimension vectors using time-lagged independent component analysis (tICA) at a lag time of 15 ns. The 240000 snapshots were clustered into 50 microstates with minibatch K-Mean clustering and Markov model was built upon the transition counts between these microstates. The same tICA analysis was applied to both free and bound forms.

Statistical analysis of equilibrium population and MFPT
To obtain the statistical distribution and errors on the equilibrium population and MFPT along the given tICA projections, 150 rounds of bootstrapping with replacement were performed. Specifically, for each round, new clustering and Markov model is built, and the microstates are categorized into four major states based on the tICA projections. The equilibrium population and MFPT are subsequently computed. The same MFPT analysis was applied to both free and bound forms.

Stopped-flow TR-FRET experiments and kinetic binding model
The kinetics of binding between PKA-C and PKIα were measured using home-built stopped-flow spectrofluorometer (Muretta et al., 2015). Equal volumes of each component were mixed, the samples were then excited at 475 nm, and fluorescence emission was measured using a 520 nm filter. For Alexa-labeled PKA-C<sup>C<sub>3</sub>199A, S523C</sup> and TMR-labeled PKIα, the final concentration of the kinase was fixed at 100 nM, while the concentration of PKIα was varied from 0 to 250 times the concentration of PKA-C. For the doubly-labeled PKI<sup>V3C, S59C</sup> and unlabeled PKA-C, the concentration of the peptide was fixed at 100 nM final concentration while the concentration of the kinase was varied from 0 to 800 times the concentration of PKIα<sup>V3C, S59C</sup>. The buffer used in both setups contains 20 mM KH<sub>2</sub>PO<sub>4</sub>, 90 mM KCl, 10 mM MgCl<sub>2</sub>, 16 mM ATP, 10 mM DTT, 1 mM NaN<sub>3</sub>, pH 6.5 at 25°C. The total fluorescence was fit into equations describing either single, double or triple exponential decays. Data analyses were done using Origin (OriginLab) software.

Since we saturated PKA-C with an excess of nucleotide, we neglected the equilibrium between open (apo PKA-C) and intermediate (ATP-bound, PKA-C*) state in the fitting procedure. In the model, PKI* represents the ensemble of conformations that is recognized by PKA-C*. The proposed model of binding is reported in Scheme 1.

The differential equations describing the kinetic model of Scheme 1 are:

\[
\frac{d[P^*]}{dt} = +k_{-b}[P^*I^*] - k_b[P^*][I^*]
\]

(6)

\[
\frac{d[I]}{dt} = k_1[I^*] - k_1[I]
\]

(7)

\[
\frac{d[I^*]}{dt} = k_1[I] - k_{-1}[I^*] + k_{-b}[P^*I^*] - k_b[P^*][I^*]
\]

(8)

\[
\frac{d[P^*I^*]}{dt} = +k_b[I^*][P^*] - k_{-b}[P^*I^*] - k_0[P^*][I^*] + k_{-1}[(PI)^*]
\]

(9)

\[
\frac{d[(PI)^*]}{dt} = +k_b[P^*I^*] - k_{-1}[(PI)^*]
\]

(10)

For simplicity, PKA-C is denoted as P and PKI as I. The instantaneous fluorescence intensity FI was calculated using the formula,

\[
FI = \frac{(c[PKA C^* PKI^*]) + c''[(PKA C^* PKI^*)]}{(c[PKA C^* PKI^*]eq + c''[(PKA C^* PKI^*)]eq)} \times SC + \text{Baseline}
\]

(11)

Where \((c[PKA C^* PKI^*]) + c''[(PKA C^* PKI^*)]eq\) is the normalized intensity, ‘SC’ is a scaling factor, ‘Baseline’
is the basal fluorescence, c and c* are the FRET efficiency parameters. The differential equations 1-5 were solved numerically using MATLAB ode solver ‘ode23s’, which was incorporated in an optimization protocol to evaluate the kinetic parameters. The optimization protocol included an initial minimization using MATLAB’s nonlinear programming solver (fminsearch), which was ran for 10^4 steps and followed by Genetic Algorithm optimization with a population size of 50 and 10^4 generations. All the six rate constants (k₁, k⁻¹, kₐ, k⁻ₐ, kᵣ, k⁻ᵣ) were collectively fit for all data points. The FRET efficiency parameters (c and c*) were fit for each specific labelling scheme. The parameters ‘SC’ and ‘Baseline’ were fit for each FRET experiment to correct for the experimental errors.

The fitting resulted in an on rate binding constant (kₐ) of 1.5 \times 10^7 \text{M}^{-1} \text{s}^{-1} and a dissociation rate constant (k⁻ₐ) of 0.5 s⁻¹. For the slow phase, we found a forward rate of 2400 s⁻¹ and reverse rate of 720 s⁻¹. The ‘on’ and ‘off’ conformational exchange rates (k₁ and kᵣ) of free PKIα were 1.9 \times 10^4 s⁻¹ and 1.5 \times 10^4 s⁻¹.

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Additional files

Supplementary files
  • Transparent reporting form

Data availability

All NMR data are deposited as NMR-Star File in the BMRB (BMRB entries 50238, 50243).

The following datasets were generated:

| Author(s) | Year | Dataset title | Dataset URL | Database and Identifier |
|-----------|------|---------------|-------------|-------------------------|
| Veglia G, Olivieri C, Li GC, Muretta J | 2020 | Analysis of the kinetics of binding of Protein Kinase A Inhibitor alpha (PKIa) to cAMP-dependent protein kinase a catalytic subunit (PKA-C) | https://conservancy.umn.edu/handle/11299/212383 | University of Minnesota repository, 212383 |
| Olivieri C, VS M, Veglia G | 2020 | Backbone (1H, 13C and 15N) Chemical Shift Assignments and 15N Relaxation Parameters for protein kinase Inhibitor alpha (PKIa) free state | http://www.bmrb.wisc.edu/data_library/summary/index.php?bmrbId=50238 | Biological Magnetic Resonance Data Bank, 50238 |
| Olivieri C, VS M, Veglia G | 2020 | Backbone (1H, 13C and 15N) Chemical Shift Assignments and 15N Relaxation Parameters for protein kinase Inhibitor alpha (PKIa) bound to cAMP-dependent protein kinase A | http://www.bmrb.wisc.edu/data_library/summary/index.php?bmrbId=50243 | Biological Magnetic Resonance Data Bank, 50243 |

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