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Dominic P. Byrne‡, Matthias Vonderach‡, Samantha Ferries, Philip J. Brownridge, Claire E. Eyers* and Patrick A. Eyers*

Department of Biochemistry, Institute of Integrative Biology, University of Liverpool, Crown Street, Liverpool, L69 7ZB, United Kingdom.

‡ These authors contributed equally to this work; *Co-corresponding authors.

ABSTRACT: cAMP-dependent protein kinase (PKA) is an archetypal biological signaling module and a model for understanding the regulation of protein kinases. In this study, we combine biochemistry with differential scanning fluorimetry (DSF) and ion mobility-mass spectrometry (IM-MS) to evaluate effects of phosphorylation and structure on the ligand binding, dynamics and stability of components of heteromeric PKA protein complexes in vitro. We uncover dynamic, conformationally-distinct populations of the PKA catalytic subunit with distinct structural stability and susceptibility to the physiological protein inhibitor PKI. Native MS of reconstituted PKA R2C2 holoenzymes reveals variable subunit stoichiometry and holoenzyme ablation by PKI binding. Finally, we find that although a ‘kinase-dead’ PKA catalytic domain cannot bind to ATP in solution, it interacts with several prominent chemical kinase inhibitors. These data demonstrate the combined power of IM-MS and DSF to probe PKA dynamics and regulation, techniques that can be employed to evaluate other complexes, with broad implications for cellular signaling.

Summary Statement
We present the first intact analysis of components of the PKA signaling complex. By combining Differential Scanning Fluorimetry with Ion Mobility Mass Spectrometry, we reveal new insights into heteromeric PKA complexes, their interactions with ligands and regulation by the heat-stable inhibitor protein PKI.

Short Title
MS-based analysis of PKA signaling complexes

Keywords
cAMP-dependent protein kinase (PKA); inhibitor; PKI; ATP;

Introduction
Protein phosphorylation is a reversible post-translational modification (PTM) catalyzed by the protein kinase-mediated enzymatic transfer of ATP gamma phosphate to an alcoholic side chain in a substrate [1]. A plethora of techniques, including X-ray crystallography, protein nuclear magnetic resonance (NMR), enzyme kinetics and (more recently) molecular dynamics, differential scanning fluorimetry (DSF) and mass spectrometry (MS), have been exploited to dissect enzymology, supramolecular structure, allosteric co-operativity and nucleotide/ligand binding propensity within protein kinase complexes [2-8]. Concomitantly, many members of the protein kinase superfamily have become important therapeutic targets, in part due to their inherent druggability [9], but also because they play rate-limiting roles in many human diseases, including inflammation, heart disease and cancer [10]. Protein kinases are ubiquitous and evolutionary related, possessing canonical amino acid motifs that are positioned at the catalytic interface, trapping divalent metal co-factors and positioning protein substrate directly adjacent to the ATP phosphoryl donor, which is thought to bind prior to substrate in the catalytic cycle [11, 12]. This conserved mode of ligand binding means that the development of specific probe compounds to interrogate protein kinase catalytic function is a challenge, and much remains to be learnt about the chemical and protein ligand sensitivity of ‘open’ and ‘closed’ (lying between ‘inactive’ and ‘active’) conformational states that exist within dynamic populations of kinase signaling complexes [13]. Although considerable progress has been made in the synthesis and exploitation of kinase-specific ligands, some of which employ allosteric (non-ATP) binding modes, the vast majority of clinically approved compounds interact with the nucleotide-binding site. Consequently, the cellular effects of ATP-competitive drugs have the potential for non-specific polypharmacology [14] caused by unpredictable interactions with different kinase conformers and a variety of ‘off-target’ proteins.

The discovery of cAMP-dependent protein kinase (PKA) in 1968 established the importance of second-messenger regulated signaling complexes in signaling [15]. The founding members of the ‘AGC’ family of Ser/Thr protein kinases are the three human PKA catalytic (C) subunits (PKAc), two cGMP-regulated kinases (PKG) and nine PKC family members, all of which share common structural features [16, 17] that have...
been employed for design and profiling of protein kinase inhibitors [18, 19]. However, PKAc is generally regarded as a drug ‘anti-target’ due to its central role in human physiology. This model protein kinase has also been central to X-ray (and later NMR) studies that have established it as a structural and enzymatic paradigm for benchmarking the entire human kinase [20]. This came about in part because components of the PKA complex can be overexpressed, (de)phosphorylated, myristoylated and affinity purified for experimental exploitation in vivo [21-23], and assembled into multivalent signaling complexes for analysis in vitro [24]. The regulation of PKA is unusually complex, involving a variety of PTMs. In common with other AGC kinases, the catalytic activity of PKAc is dynamically regulated in cis through flanking elements of the polypeptide and enzymatic post-translational (auto)phosphorylation at multiple conserved sites [25, 26]. In addition, N-terminal PKAc myristoylation has been demonstrated in human cells [27], where this modification is reported to drive PKA holoenzyme complexes to the plasma membrane [28]. This in addition to well-characterised effects on intrinsic stability (but not catalysis) in vitro [29]. PKAc enzyme activity is controlled via a plethora of partner proteins, which make up complex physiological signaling complexes [3, 30]. In the absence of cAMP, catalytic activity and subcellular location of PKAc are tightly constrained by binding to functionally non-redundant regulatory (R) subunits, which generate physiologically combinations of R and C assemblies. The ‘textbook’ (catalytically inactive) R2C2 PKA holoenzyme originally purified from tissues [31, 32] is also thought to represent a physiologically-relevant complex in human cells [22]. PKA is activated by localized fluxes in cAMP concentration mediated through opposing effects of families of adenylate cyclases and nucleotide phosphodiesterases [16]. Co-operative cAMP binding at two sites per R subunit unleashes PKAc, facilitating the phosphorylation of physiological substrates containing a minimal RXXS PKA consensus motif [33]. In the PKA signaling module, further layers of regulation are encoded by intracellular targeting through A-kinase Anchoring Proteins (AKAPs), which interact mainly via RII-subunits [30]. PKA is further regulated through potent pseudosubstrate inhibition of PKAc by three isoforms of the heat-stable inhibitor protein of PKA, termed PKI [34-36], whose physiological roles are slowly being uncovered in vertebrates [37, 38]. Inhibitory subnanomolar-affinity binding of PKI to PKAc is also thought to expose a canonical nuclear export signal (NES) in PKI, which may redistribute the (inhibited) complex between different intracellular compartments [39]. Whether RII and PKI binding to PKAc is mutually exclusive remains unknown. An appreciation that protein kinases are inefficient enzymes has strengthened the idea that they act in cells as regulated macromolecular switches, whose structural dynamics and cross-domain communication enable transition between low and high activity conformations. X-ray analysis confirms that PKA holoenzymes are subject to allostery. Moreover, different C and R-subunits can co-assemble to generate distinct quaternary complexes, although their composition and stoichiometry are potentially susceptible to artefacts of crystallisation. Importantly, intrinsic cooperativity within the C-subunit exists [40], so that ligand binding is sensed conformationally, potentially coupling ATP site occupancy with substrate dynamics during the catalytic cycle [41]. Classes of small molecule or protein ligands might therefore exhibit differential effects on PKA signaling, potentially through distinct binding modes, as noted for several kinase and inhibitor combinations [42, 43]. For PKAc, the discovery of regulatory hydrophobic ‘spines’, whose assembly and disassembly enables communication within the bi-lobal kinase domain [44], enables ligand binding to have far-reaching consequences, including allosteric coupling across networks or communities of amino acids [45, 46]. In this study, we exploit the complementary analytical approaches of solution differential scanning fluorimetry (DSF) [47] and gas-phase ion mobility-mass spectrometry (IM-MS) [48] to characterize highly purified and re-assembled components of the PKA complex and explore the effects of ligand binding. TSA assays, such as DSF, have recently demonstrated great promise in quantifying the (de)stabilizing effects of protein-ligand interactions both in vitro [49] and in cellulo [50], while ‘native’ MS and IM-MS are increasingly being exploited to interrogate the structure and dynamics of protein complexes [48], and analysis of different classes of small molecule ligands [7, 51, 52].

### Experimental Section

#### Protein Expression and Purification

All proteins analyzed were produced in BL21 (DE3) pLysS E. coli cells (Novagen) with expression induced with 0.5 mM IPTG for 18 h at 18°C. Murine PKI (alpha isoform) and PKAcx1 (non-myristoylatable) were cloned into the pET-30 Ek/LIC vector (Novagen) and purified as N-terminal His6-tag fusion proteins by immobilized metal affinity chromatography (IMAC) and size exclusion chromatography using a HiLoad 16/600 Superdex 200 column (GE Healthcare) equilibrated in 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 10 % (v/v) glycerol and 1 mM DTT. PKAc K72H and R133A point mutants were generated by PCR-site directed mutagenesis [21], expressed and purified across networks or communities of amino acids [45, 46].

#### Phosphopeptide enrichment and LC-MS/MS

Protein samples were reduced and alkylated prior to overnight digestion with trypsin (2% w/w) and subsequent phosphopeptide enrichment using Titansphere Phos-TiO2 spin tips
(GLSciences) with minor modification of manufacturer’s instructions (see Supplementary information). Tryptic peptides were analysed by LC-MS/MS before and after TiO2-based phosphopeptide enrichment. nLC-ESI-MS/MS analysis was performed using either an Orbitrap Fusion Tribrid mass spectrometer (ThermoScientific) attached to a Ultimate 3000 nanoLC system (Dionex), or an AmaZon ETD ion trap (Bruker Daltonics) arranged in-line with a nanoAcquity u-HPLC system (Waters). .mgf files were searched using MASCOT (version 2.1) against the E. coli IPI database (downloaded 24th March 2015) with the complete sequence of 6His-tagged PKAc included. Carbamidomethylation of Cys was set as a fixed modification; phosphorylation of Ser and Thr, oxidation of Met and deamidation of Asn and Gln were set as variable modifications. The tandem mass spectra for all identified phosphopeptides were interrogated manually.

**Diphosphorylation of PKAc with GST-lambda protein phosphatase (APP) and PKA kinase assay**

60 µg of PKA (~13 µM, 100 µl final volume in 50 mM NH4OAc buffer) was incubated at 37 °C with 100 ng of 2πP in the presence of 1 mM MnOAc₂, for 2 hours. The reaction was stopped by buffer exchange into 50 mM NH₄OAc. For kinase assays, 1 ng of purified PKAc, or R133A PKAc was assayed in the presence and absence of recombinant full-length 6His-tagged PKI using a Caliper EZ Reader II enzyme assay platform in 25 mM Hepes pH 7.4, 1 mM ATP, 5 mM MgCl₂, 1 mM DTT, and 5 µM fluorescent 5-Fam-Leu-Arg-Arg-Ala-Ser-Leu-Gly-CONH₂ (Kemptide). Activity (kinetic mode) was reported as % peptide phosphorylation after ~10 cycles.

**Differential scanning fluorimetry (DSF) assays**

Thermal-shift assays were performed with a StepOnePlus Real-Time PCR machine (Life Technologies) using Sypro-Orange dye (Invitrogen) and thermal ramping (0.3 °C per minute between 25 and 94°C). All proteins were diluted to 5 µM in 50 mM Tris/HCl, pH 7.4 and 100 mM NaCl in the presence or absence of the indicated concentrations of ligand (final DMSO concentration 4 % v/v). Nucleotide, PKI peptide (amino acids 5-24) or kinase inhibitors (diluted from 10 mM DMSO stocks) were assayed in the presence or absence of the appropriate divalent cations, as described. Data were processed using the Bolzmann equation to generate sigmoidal curves, and average Tₘ/ΔTₘ values calculated as previously described[47] using GraphPad Prism software.

**Detection of PKAc and PKA Rllo binding by SEC**

Equimolar concentrations (~2.5 µM) of catalytic (C; WT or K72H) and Rllo (regulatory) PKA subunits were pre-incubated at 4°C for 2 h and loaded onto a Superdex 200 10/300 GL column (GE Healthcare) equilibrated in 50 mM Tris/HCl, pH 7.4, 100 mM NaCl. Eluted fractions (0.5 ml) were collected and complex formation analysed by SDS-PAGE and Coomassie staining. Binding was confirmed by co-elution of the C and Rllo subunits from the column.

**Ion Mobility-MS and Collisional Activation**

IM-MS analysis was performed on a Waters Synapt G2-Si instrument. All proteins were buffer exchanged into 50 mM NH₄OAc (LC grade, Sigma) employing Amicon spin filter columns (10 kDa molecular cutoff). Typically, 1-3 µl of 2-5 µM sample was analyzed using borosilicate emitters (Thermo ES 387). Spraying voltage was adjusted to 1.1 -1.8 kV, sampling cone was 20 V. Pressure in the travelling wave (T-wave) ion mobility cell was 2.78 mbar (nitrogen), wave height was kept at 30 V, wave velocity at 750 m/s. For collision-induced unfolding (CIU) experiments, a single charge state was quadrupole isolated and subjected to collisional activation by applying CID activation in the ion trap of the instrument. The activation voltage was varied between 25 and 41 V. Ion mobility wave height was 40 V, wave velocity 650 m/s. Data was processed with MassLynx 4.1 and OriginPro 9.0 and used to generate contour plots of the intensity of ion populations as a function of collision cross section and collision voltage. Non-covalent interactively were studied by mixing the correspond- ing components in 200 mM NH₄OAc and equilibrating for at least 10 minutes at room temperature prior to MS analysis.

**CCS Calibration and Theoretical Calculations**

In order to experimentally determine collision cross section (CCS), drift time through the T-wave mobility cell was performed using β-lactoglobulin A (Sigma L7880), avidin (Sigma A9275), transhyretin (Sigma P1742), concanavalin A (Sigma C2010) and serum albumin (Sigma P7626) according to standard protocols [54]. The exact hard sphere scattering (EHSS) model implemented in the Mobsca software was used to calculate CCS values on the basis of X-ray structures [55].

**Results and Discussion**

**Analysis of purified recombinant proteins**

We expressed and purified to homogeneity a panel of proteins that can be used to reconstitute multimeric or holoenzyme (R:C3) PKA signaling complexes in vitro. We also generated mutations in the catalytic subunit that have previously been shown to selectively prevent autophosphorylation and abrogate catalytic activity [56] (‘kinase-dead’ K72H PKAc mutant) or that specifically reduce binding to the Rllo subunit and the heat-stable inhibitor of PKA (PKI), whilst sparing autophosphorylation and catalytic activity [21, 57] (R133A PKAc mutant). As shown in Figure 1, these proteins were pure, and size exclusion chromatography and DSF (see below) suggested that all proteins were folded and monomeric. Circular Dichroism (CD) also confirmed similar secondary structures in both WT and K72H PKAc proteins when analyzed in the presence and absence of ATP and Mg²⁺ ions, the latter inducing no major changes in the spectra. A key goal of our study was to determine the effects of phosphorylation on PKA complex formation in vitro. Using peptide-based tandem MS analysis, we initially determined the sites of phosphorylation on PKAc proteins, confirming that it was highly phosphorylated on several known sites, including Ser10, the key T-loop (Thr197) and C-terminal tail motif (Ser338) sites, these being required to generate a stable, catalytically active PKAc [26, 58, 59]. Interestingly, our comprehensive sequence coverage revealed a total of 11 sites of autophosphorylation in recombinant WT and 11 phosphosites on R133A PKAc, all of which were absent in K72H PKAc preparations (Table 1, Supplementary information), confirming they are autophosphorylation sites.

**Phosphorylation regulates PKA conformation**

To assess effects of phosphorylation on the structure and intrinsic stability of the PKA catalytic subunit, recombinant
intact autophosphorylated PKAc was analyzed by MS before and after treatment with λ, protein phosphatase (λPP) (Figure 1). We observed an average of ~7 autophosphorylation sites in WT PKAc (Fig. 1A), out of a total of 13 sites of phosphorylation that had been mapped (Table 1, Supplementary Tables 1-3, Supplementary Figure 1); these identified sites of modification include three non-physiological autophosphorylated residues found within the 6His affinity tag, whose non-specific modification by basophilic kinases was noted in previous work [60]. Importantly, of the 11 phosphosites observed in the core PKAc polypeptide, we mapped phosphorylation at Ser34, only previously reported in a single high-throughput screen, and PKAc R133A. Peaks are annotated with the number of modified residues identified (Table 1), and the likelihood of non-stoichiometric phosphosite occupancy.

When ESI-MS was performed under non-denaturing (‘native’ solution) conditions, autophosphorylated PKAc was observed over an extremely narrow charge state distribution, predominantly yielding 13+ and 14+ ions (Fig. 2A), indicative of highly ordered tertiary protein structure [63]. IM-MS was subsequently used to determine the collision cross (CCS) section distributions and, following calibration of the T-wave ion mobility cell, a \(^{70}\text{CCS}_{62-136}\) value of 29.4 nm\(^2\) for the 12+/13+/14+ charge states of hyperphosphorylated PKAc was determined. As expected, charge-mediated unfolding of the 15+ species yielded multiple conformations with higher CCS values (Supplementary Figure 2). Comparison of CCS for PKAc before and after phosphatase treatment (Figure 2B, C) revealed phosphorylation-dependent effects on both the absolute \(^{70}\text{CCS}_{62-136}\) value and the half-height width of the CCS distribution (CCSD); CCS (\(Ω\)) increased by 1.5% following treatment with λPP, while CCSD demonstrably reduced from 2.2 nm\(^2\) (28.4 – 30.6 nm\(^2\)) to 1.8 nm\(^2\) (28.8 - 30.6 nm\(^2\)). Autophosphorylation thus promotes compaction of PKAc while increasing conformational dynamics. Interestingly, hyperphosphorylated PKAc also exhibited a noticeable asymmetry in the CCSD, not apparent upon phosphatase treatment. This phosphorylation-dependent increase in conformational space is perhaps unsurprising given the number of modified residues identified (Table 1), and the likelihood of non-stoichiometric phosphosite occupancy.

![Figure 1](image)

**Figure 1.** Analysis of purified recombinant proteins that make up the PKA signaling complex. (A) Coomassie blue staining of purified recombinant PKA proteins: 1.5 μg of PKA catalytic (wild-type (WT), K72H or R133A proteins) and regulatory (RIα subunits, and 0.6 μg of PKI protein were analyzed by SDS-PAGE and proteins stained with Coomassie Blue. Coomassie staining of PKI is weak due to its small size and amino acid composition. (B) Circular dichroism (CD) spectra demonstrating similar secondary structures of WT and K72H PKAc in the absence of presence of 1 mM ATP and 10 mM Mg\(^{2+}\) ions. The mean average of three replicate spectra of 0.6 mg/ml WT (red) and K72H (blue) PKAc in 10 mM sodium phosphate (pH 7.4), and 25 mM NaF, recorded in a 0.1 cm cell are shown. (C) Electrospray ionization (ESI) mass spectra of WT PKAc with Mn\(^{2+}\) - λ protein phosphatase. The K72H and R133A variants of PKAc were employed as comparators for further structural investigation of the PKA.
complex. Of particular interest for understanding the role of phosphorylation on PKAc conformation, stability and ligand binding capabilities is the K72H PKAc mutant, given its inability to autophosphorylate (Table 1, Figure 1). Although the potential folding status of this purified mutant cannot be assessed as a function of catalytic output, the near-identical CD spectra obtained for WT and K72H PKAc (Fig. 1B), suggests similar secondary elements of structure in both proteins.

Gaussian fitting of the CCS distributions determined by native IM-MS revealed two primary conformers for WT PKAc, with CCS values of 29.0 and 30.0 nm², the larger of which overlaid with the predominant conformer apparent following phosphatase treatment (Figure 2B). To better understand the conformational stability of these PKAc variants, we performed a series of collision-induced unfolding (CIU) experiments evaluating the effect on CCSD. By elevating the collision energy (CE) applied to the native-solution derived conformers (but maintaining it below that required for covalent bond dissociation), it is possible to gradually unfold a protein and thereby gain an understanding of its (gas-phase) stability and the structure of partially unfolded intermediates [64]. The CIU profile of hyperphosphorylated PKAc was markedly different from that of the phosphatase treated form of PKAc (Figure 2C, Supplementary Figure 3). WT untreated phospho-PKAc is significantly more stable, requiring higher CE to initiate unfolding. While λPP-treated PKA starts to undergo conformational change as low as ~31 V, WT PKAc is conformationally stable under these conditions, not undergoing significant unfolding until ~36 V.

Akin to data obtained at low CE (Figure 2), the conformational space (CCSD) adopted by hyperphosphorylated PKAc at high CE (>40 V) is larger than the phosphatase-treated form. Interestingly, both K72H and R133A PKAc are more similar to λPP-treated than untreated PKAc in their native state, with CCS values of 29.6 and 29.8 nm² respectively. Furthermore, both exhibit CIU profiles more typical of the less stable, more open conformer of λPP-treated WT PKAc. That both PKA variants exhibit similar CCS values of lower conformational stability, suggests that these structural effects are not primarily dependent on the extent of phosphorylation. In particular, the fact that the conformation of R133A PKAc overlays with the more elongated (open) conformer of WT PKAc, led us to hypothesize differential roles for these two predominant PKAc configurations (see below).

In agreement with CIU profiles, WT PKAc was observed to have greater thermostability, as determined by DSF, than either R133A or K72H (Figure 3A). Although the Tm values at 50 % were comparable for WT and R133A PKA (43-44 °C), the thermal unfolding profiles were different, with R133A exhibiting a biphasic response and a 20 % Tm value of ~37°C (compared to ~43°C for WT PKAc), suggesting rapid thermal unfolding of R133A to a more stable intermediate. In contrast, K72H PKAc was even less thermostable (Tm = 39 °C), in agreement with previous literature findings [56].

Figure 3. PKA binding of nucleotides is detectable by DSF. (A) Thermal stability assay (TSA) of PKA WT, K72H and R133A (5 μM) in the presence of 10 mM MgCl₂ and 1 mM (blue), 2 mM (green) or 4 mM (orange) ATP; buffer control is in red. (B) ΔTm for WT, K72H and R133A PKA upon nucleotide binding, as measured by DSF. Mean ΔTm values ± SD (n=2) were calculated by subtracting the control Tm value (buffer, no nucleotide) from the measured Tm value.

Figure 2. (A) ESI mass spectrum of PKAc obtained under non-denaturing ‘native’ conditions; (B) 100 CCSₙ₂₋₄₄⁺ for the [M+13H]₁₃⁺ form of untreated wild-type PKAc (PKA), PKAc following treatment with Mn²⁺-λ protein phosphatase (PKA λPP), and the K72H and R133A variants. Two overlapping conformations of PKA are indicated, the more extended of which (green) matches the CCSD of the other PKA species; (C) (top to bottom) CIU profiles of PKAc WT, λPP-treated, K72H and R133A.

In agreement with CIU profiles, WT PKAc was observed to have greater thermostability, as determined by DSF, than either R133A or K72H (Figure 3A). Although the Tm values at 50 % were comparable for WT and R133A PKA (43-44 °C), the thermal unfolding profiles were different, with R133A exhibiting a biphasic response and a 20 % Tm value of ~37°C (compared to ~43°C for WT PKAc), suggesting rapid thermal unfolding of R133A to a more stable intermediate. In contrast, K72H PKAc was even less thermostable (Tm = 39 °C), in agreement with previous literature findings [56].
Small molecule and PKI binding to PKA is conformation-specific

In contrast to MS analysis, where electrospray ionization is suppressed by metal ions, a major strength of DSF is its ability to report nucleotide and metal interactions amongst active and inactive conformers present in a variety of (pseudo)kinase domain preparations [47]. We therefore exploited DSF to investigate the binding of nucleotides to PKAc. As expected[65, 66], thermostabilization was readily observed for WT PKAc using a broad range of nucleotides (particularly ATP and ADP) in the presence of divalent cations (Figure 3B), and this was recapitulated with the R133A variant. In contrast to the increase in $T_m$ observed for WT PKAc ($\Delta T_m = 4.24 \pm 0.09 \, ^\circ C$) and R133A ($\Delta T_m = 3.49 \pm 0.16 \, ^\circ C$) with 1 mM ATP and Mg$^2+$, incubation of K72H PKAc (or a less stable K72R PKAc mutant, data not shown) with any combination of nucleotides or cations failed to promote any stabilization under these conditions ($\Delta T_m$ for K72H $= -0.49 \pm 0.04 \, ^\circ C$ upon incubation with ATP/Mg$^2+$ ions), even at very high concentrations (up to 4 mM) of ATP. We therefore believe that our assay reveals either a loss of nucleotide binding in the K72H mutant, or an inability to detect thermal stabilization upon nucleotide binding. These findings contrast with previous work, which employed fluorescent ATP displacement (mant-ATP) and CD-based thermal unfolding in urea, to demonstrate that K72H C-subunit could bind to ATP and the small molecule inhibitor H89, despite a detectable lack of phosphorylation or catalytic activity [56].

We next studied the effects of exogenous protein binding to PKAc, taking advantage of the resistance of PKI to denaturation at temperatures above 70°C, at which temperature PKAc is fully unfolded (Figure 3A). Binding of purified PKI inhibitor protein to all three PKAc proteins was assessed both by DSF and (IM-)MS. The high-affinity interaction of PKAc with PKI measured by DSF was confirmed by native MS with observation of the PKA:PKI heterodimer over multiple (15'-17') charge states (Figure 4A). A $\Delta T_m$ value of $+1.97 \pm 0.04 \, ^\circ C$ was determined for WT PKAc upon PKI binding (Figure 4B), whilst the IC$_{50}$ value for inhibition of PKAc activity was in the sub nM range (Supplementary Figure 4), consistent with previous data measuring a Kd$_{app}$ of $<0.2 \, nM$ [57]. However, neither K72H nor R133A formed a stable interaction with the inhibitor protein under the relatively mild conditions used for the MS analysis (Figure 4A), and both had negligible shifts in thermal stability as determined by DSF: 0.29 ± 0.37 °C and -0.36 ± 0.01 °C upon PKI addition for K72H and R133A variants respectively (Figure 4B). Furthermore, R133A exhibited a nearly 200 fold higher IC$_{50}$ value for inhibition by PKI (Supplementary Figure 4), confirming loss of enzyme inhibition despite unchanged autophasphorylation and ATP affinity. Interestingly, the binding of both PKI (pseudosubstrate inhibitor) and ATP/Mg$^2+$ (nucleotide), to the WT C-subunit was additive, or even synergistic, over a range of ATP concentrations (Figure 4B), confirming that DSF detects co-operative binding at two separate sites on PKAc, consistent with recent work [41].

In order to assess PKAc conformational dependence of PKI binding by IM-MS, it was necessary to look indirectly, by comparing the CCS ($\Omega$) of the non-PKI bound form of PKAc remaining in the presence of excess PKI ($\Omega_{\text{WT}(PKI)}$) to $\Omega$ of PKAc in the absence of PKI ($\Omega_{\text{WT}}$). The direct effects of PKI binding on the structure of PKAc could not be evaluated due to the innate presence of ligand and the resultant increase in complex size. IM-MS analysis of the non-PKI binding form of PKAc revealed a small, but highly reproducible 1.4% increase in $\Omega$, with a $\Omega_{\text{WT}(PKI)}$ value of 29.8 nm$^2$ compared with $\Omega_{\text{WT}}$ of 29.4 nm$^2$ (Figure 4). Interestingly, $\Omega_{\text{WT}(PKI)}$, i.e. the non PKI-bound form of PKAc, was essentially identical to that of the more elongated form of PKAc, suggesting conformation specific binding of PKI to the more compact PKAc species. Our model of conformation specific binding of PKI to PKAc is also supported by IM-MS analysis of PKA mutants; both K72H and R133A lack the ability to bind PKI (Figure 4) and both exhibit similar $\Omega$ values to WT(PKI).

Published crystal structures of PKAc lacking (PDB 3OWP) or containing a PKI-derived peptide (PKI 5-22, PDB 2CPK and 2ATP) [67-69] allowed us to compare our experimentally observed differences in PKAc conformation with those from published X-ray structures. The exact hard spheres scattering (EHSS) model, implemented in Mobcal, was used to calculate $\Omega_{\text{EHSS}}$ for PKAc from PDB files. In agreement with our MS data, a decrease in PKAc $\Omega$ was observed upon PKI binding: $\Omega_{\text{EHSS}}$ (PKA 15-350) = 32.8 nm$^2$; $\Omega_{\text{EHSS}}$ (PKAc 15-350 with PKI) = 31.9 nm$^2$. Although $\Omega$ cannot be compared directly between our studies and the computed $\Omega_{\text{EHSS}}$ values from X-ray structures, primarily because the core PKA sequence is different in these two sets of experiments, the reduction in conformational space adopted by PKAc upon PKI binding is entirely consistent with our IM-MS data.

![Figure 4](image-url)
shown: (C) $^{13}$CCS$^{54-196}$ for [M+13H]$^{13+}$ and [M+14H]$^{14+}$ forms of WT PKAc in the absence (top) or presence (bottom) of PKI. CCS distribution of non-PKI bound form of PKAc is presented (PKA(PKI)); (D, E) CIU profiles of PKAc upon addition of PKI: (D) PKI-bound PKA (PKA(PKI)), (E) non-PKI bound PKAc.

Interestingly, CIU profiles of the heterodimeric PKA/PKI complex (Figure 4D) revealed distinct partially unfolded intermediates, unlike the gradual elongation observed for the non-PKI bound species. In contrast to the complex formed between PKAc and the nM kinase inhibitor staurosporine, the PKA/PKI complex remained stable up to a CE of 50 V, confirming the strength of the inhibitory (non-covalent) PKA/PKI interaction, and consistent with the picomolar affinity of PKI for PKA [34, 35, 57].

The PKI binding conformer of PKAc has enhanced structural stability

The preferential binding of PKI to the more compact PKAc conformer led us to question whether there was a difference in the structural stability of these species. The non-PKI bound PKAc remaining in the presence of excess PKI, PKA(PKI), was thus subjected to CIU (Figure 4) and compared with the unfolding profiles of WT, R133A and K72H PKAc (Figure 2). The CIU profile of PKA(PKI) was very similar to that observed for R133A; both demonstrate a significant reduction in gas-phase stability compared to WT PKAc, with collision-mediated unfolding starting at ~32 V. These data suggest that it is the conformation, rather than phosphorylation status, of PKAc that regulates its structural stability.

PKI disrupts hetero-oligomers of PKA catalytic and regulatory subunits

To examine the disruptive effects of PKI on the physiological (catalytically inactive) hetero-tetrameric $R_2C_2$ complex of PKAc and PKA RII$\alpha$ regulatory subunits, we undertook a series of novel MS and biochemical analyses. As shown in Figure 5, under native ESI conditions we clearly observe monomers and homodimers of PKAc, monomers, homodimers, trimers and tetramers of RII$\alpha$, and various combinations of multimers after reconstitution of the PKA holoenzyme from individually purified components at a 1:1 ratio. Importantly, we can readily detect the physiological $R_2C_2$ holoenzyme complex. Dramatically, titration of equimolar PKI completely ablates observation of the $R_2C_2$ complex, additionally disrupting $R_2C$, $CR$ and $C_2$ complexes, with preferential formation of the PKAc:PKI heterodimer. Disruption of PKAc, RII$\alpha$ complexes by PKI, and formation of a PKAc:PKI heterodimer, was also confirmed by size exclusion chromatography, the standard biochemical method for PKA complex analysis (Supplementary Figure 5). Consistent with previously published data with the RI subunit [62], there was no evidence for holoenzyme complex formation and RII$\alpha$ binding with either the inactive K72H or the catalytically active R133A[57] variants either by size exclusion chromatography (Supplementary Figure 5) or native MS analysis.

Figure 5. Native ESI mass spectra of WT PKAc (C, blue dots), PKA regulatory subunit RII$\alpha$ (R, green squares), and equimolar ratios of the two (C+R) in the absence or presence of PKI proteins (red triangles).

Binding of small molecules to PKAc decreases conformational flexibility

The reversible binding of small molecule ligands has revolutionized the study of protein kinases, and is a powerful strategy for inhibiting (or activating) the catalytic activity of protein kinases in vitro and in vivo [10]. Using DSF, we assessed the effect of a panel of small molecule PKA inhibitors on PKAc thermostability (Figure 6). We also evaluated compound binding to the catalytically inactive K72H variant, which we demonstrated (Figures 3, 4) could not interact with nucleotides. Remarkably, a number of ATP mimetics known to target the PKAc ATP site markedly increased thermostability of K72H PKAc. Staurosporine, an indolocarbazole that is a pan-protein kinase inhibitor, stabilizes PKAc WT and K72H equally well, as do the closely-related (but more specific) compounds N-benzoyl staurosporine and K-252a. In contrast, and in a similar fashion to the pseudosubstrate PKI full-length protein or PKI (5-23) peptide, the AGC kinase sulfonylsoquinoline inhibitors H89, HA-100 and the oral pan AGC inhibitor AT13148 bind only to WT and R133A PKAc under these conditions, and not the catalytically inactive (unphosphorylated) K72H PKAc, suggesting conformer-specific selection by specific ligand classes. This raises the question as to whether catalytic activity is a useful indication of the potential ability of kinase domains to bind ATP-competitive molecules in vitro and in cells and whether the physiological effects of inhibitors may be mediated in a compound-specific manner through interactions with ‘inactive’ conformers found in both canonical protein kinases and the multiple different classes of protein pseudokinase [70].
Figure 6. Binding of small molecule kinase inhibitors to PKAc as determined by DSF. Thermal stability of WT (A), K72H (B) or R133A (C) PKAc (5 μM) was assessed as a function of inhibitor binding at the indicated concentrations. Mean ΔTm values ± SD (n=2) are shown.

To corroborate our DSF assays, binding of the PKA inhibitor H89, the oral multi-AGC-kinase inhibitor AT13148 (which is in phase I clinical trials) [71] and staurosporine [72], was confirmed by native ESI-MS (Figure 7, Supplementary Figure 6).

Given our discovery of preferential binding of PKI to a specific PKA conformer, we also assessed the effect of these small molecules on PKAc conformation by IM-MS. Interestingly, there was a reduction in the charge states observed upon incubation with all three inhibitors, and a slight reduction in CSD potentially suggesting a more ordered structure upon inhibitor binding. Inhibitor-dependent changes in CCSD for the same charge state could not thus be compared directly. Average Ω could be analyzed between the lowest two charge states for vehicle control (12+, 13+) and inhibitor bound (11+, 12+), as these were deemed to be most representative of the stable folded PKAc structures. All the inhibitor-bound forms of PKAc exhibited reduced conformational flexibility, with CCS decreasing over 20% from 2.1 nm² to less than 1.6 nm². CCS also decreased upon inhibitor binding (in agreement with the reduction in CCSD), suggesting preferential adoption of a more compact PKAc conformer. Unlike PKI binding to PKAc, interaction with these inhibitors does not appear to be conformation-dependent, although binding did promote structural compaction with reduced conformational flexibility.

Given the indiscriminate binding of staurosporine and K252a to both WT and K72H PKAc, and formation of selective complexes between AT13148 and WT PKAc, we reasoned that there must be a difference in the binding mode of these small molecules ligands that could potentially regulate interaction between members of the PKA signaling module. We therefore evaluated whether pre-incubation with a small molecule influenced PKI:PKA binding or PKI disruption of the PKA holoenzyme. Unlike PKI, staurosporine exhibited conformer-independent binding to PKAc, since no unbound catalytic subunit was observed (Supplementary Figure 7), consistent with our solution DSF data. Increasing the CE applied to the PKA:small molecule:PKI complex preferentially resulted in dissociation of the small molecule (~55 V), leaving the stable PKA:PKI heterodimer. Interestingly, there was no discernible effect of PKI disruption of PKA holoenzyme when it was pre-formed in the presence of any ATP-competitive inhibitors, demonstrating independent binding mechanisms (Figure 7), which are entirely consistent with DSF analysis (Figure 4B).

Figure 7. Binding of small molecule inhibitors to PKAc and effect on disruption of PKA holoenzyme by PKI. Native ESI mass spectra (A) and ^19CCSN2 → He distributions (B) acquired in the presence of DMSO vehicle or with 10-fold molar excess of staurosporine (STS), H89 or AT13148. CCSD are presented.
for $[\text{M+11H}]^{11+}$ (red dotted line), $[\text{M+12H}]^{12+}$ (blue line), $[\text{M+13H}]^{13+}$ (black line). (C) Equimolar ratios of WT PKAc (C, blue dots) and PKA RIIa (R, green squares), were pre-incubated with vehicle control, staurosporine, K252a or AT13148, prior to addition of equimolar PKI (red triangles). Native MS spectra are annotated with identified complexes.

CONCLUSIONS

The complementary techniques of TSA and IM-MS are exploited here to examine structure, stability and binding of proteins and inhibitors to PKA signaling components in vitro. Even though PKA is used as a benchmark for studying other protein kinases, MS-based structural evaluation of the PKA complexes has not previously been reported, to the best of our knowledge. MS studies previously evaluated ligand and inhibitor sensitivity of type I cGMP-dependent protein kinase (PKG) subunits [73, 74]. However, PKG differs from PKA in that its regulation involves sensing of cyclic GMP nucleotide within a single polypeptide. In this paper, we report the first MS-based characterization of the PKA holoenzyme, and explore heteromeric PKA complexes using native MS and IM-MS. By building on previous studies of PKA autophosphorylation after isolation from bacteria [25, 26], we identify three novel sites of C-subunit autophosphorylation. Future studies will evaluate whether these sites are also phosphorylated in cells and in vivo, and whether they contribute to catalytic activity, the formation or stability of PKA protein:protein complexes or both. We provide new evidence by IM-MS that PKAc exists in two primary conformations that are differentially permissive for ligand binding at the PKI (protein pseudosubstrate) site on the surface of the protein. Unfortunately, low MS resolution using ESI under native conditions prevents us from categorically stating that the phosphorylation status of the two C-subunit conformers are different. However, PKAc that has reduced (λPase treated) or completely lacks (K72H) phosphorylation preferentially exhibits an elongated conformation, similar to that of the elongated WT PKAc structure that does not bind to PKI. This predicts that these structural differences might, in part, be driven by altered phosphorylation status, similar to previous data that uncovered marked differences in structural stability between myristoylated and non-myristoylated PKAc proteins [29, 75]. However, our finding that the CCS value of normally phosphorylated R133A PKAc is identical to that of the larger (more elongated) WT PKAc conformer, and similar to that of unphosphorylated K72H, confirms that the structural diversity of PKAc is driven by factors in addition to the extent of phosphorylation. Modeling of R133A PKAc predicts an altered conformation, caused by ablation of the positive charge at Arg133 and loss of an electrostatic interaction with Glu230 [57]. Based on our work with R133A PKAc, we propose that this mutation can drive the phosphorylation-independent conformational differences observed by IM-MS. In this context, in the future it will be interesting to compare these structural effects with those of stoichiometrically myristoylated PKAc, and a panel of PKAc phosphorylation site mutants, including the 3 novel sites identified in our in vitro study, whose cellular function is currently unknown.

Interestingly, we find that the smaller (less elongated) of the two PKAc conformers preferentially binds the pseudosubstrate inhibitor protein PKI, a pM affinity regulatory subunit with complex biological roles [37]. Consistent with this observation, the smaller conformer was absent upon IM-MS analysis of the K72H and R133A PKAc mutants, which are significantly impaired in their ability to bind PKI [21]. Distinct MS-unfolding profiles were observed for the non-PKI binding form of PKA compared with the mixed conformer species. Of particular interest, the conformer incapable of binding PKI exhibited a markedly lower structural stability than the inhibitor-sensitive C-subunit population. Native MS analysis also reveals ternary formation of tetrameric PKA holoenzyme R$_2$C$_2$ complexes, as well as mixed R$_C$ and RC multimers in vitro. Furthermore, we provide unambiguous experimental evidence that PKI directly disrupts the formation of all possible complexes (likely at the R:C interface), confirming the exclusive binding of PKI to structurally-permissive PKAc subunits, even in the presence of RIIa. The resultant (non-covalent) PKA:PKI complex is extremely stable, exhibiting a multi-stage unfolding profile with populations of discrete stable (partially unfolded) intermediate structures. It will be interesting to translate our findings to the cellular environment, where subcellular PKAc complexes with differential affinity for PKI could exist, with inhibitor affinity regulated as a function of PKAc phosphorylation or heterodimerisation. In this regard, the analysis of PKI binding to non-phosphorylated, myristoylated or mutated PKAc, or PKAc complexes disrupted by cell-permeable AKAP ligands [76], will be of particular interest.

Based upon careful MS analysis, kinase-dead (K72H) PKAc does not autophosphorylate when expressed in bacteria, likely due to an inability to bind nucleotides (either in the presence or absence of divalent metal cations), as confirmed using a DSF assay previously validated to detect binding of a variety of ligands to a panel of catalytically-inactive and ATP binding-deficient pseudokinases [47]. Interestingly, the K72H mutant behaves as a potent ‘dominant negative’ in vivo when injected into oocytes [21], and retains the ability to bind to the ATP-competitive small molecule inhibitors staurosporine and K-252a, but not isoquiolinesulfonl PKAc inhibitors [77] or the clinical candidate AT13148. The ability of the type I inhibitor staurosporine to stabilise an ‘open’ (inactive) conformation of phosphorylated PKI-bound PKAc is known from X-ray analysis, and a potential induced van der Waals interaction with Lys 72 has been postulated [78]. However, our DSF analysis confirms that K72H PKAc still binds with high affinity to staurosporines. Interestingly, the preferential targeting of ‘type II’ kinase inhibitor classes to inactive kinase conformers of tyrosine kinases is well known, and clinically relevant [43, 51]. However, the propensity of inactive Ser/Thr kinases, exemplified by K72H PKAc (this study) or multiple pseudokinases [47], to bind to different classes of kinase inhibitor is an important new area of research focus [70, 79, 80]. Our work demonstrates that for an unphosphorylated K72H PKAc mutant, an inability to bind to ATP does not preclude binding to bisindole-based ATP-competitive inhibitors whose lack of specificity precludes their rational exploitation as chemical biology probe compounds [19]. In the absence of a crystal structure, it is challenging to explain why we are unable to detect K72H nucleotide binding using standard procedures [65], especially given previous findings [56, 62]. However, Lys72 of PKA is reported to interact with the α and β phosphates of ATP and a critical C-helix Glu residue in the closed, active PKA structure [69]; mutation of the equivalent residue
in (pseudo)kinases can ablate ATP binding that can be quantified by solution DSF [47, 81]. We therefore speculate that should ATP still bind to K72H PKAc, its conformation must be markedly different to that of WT and R133A PKAc, but nonetheless remain responsive to thermal stabilization by some kinase inhibitors. All the kinase inhibitors evaluated in this study are formally ATP-competitive (type I) kinase inhibitors, and our work has ramifications for studying cellular small molecule ligand effects on phosphorylated or inactive kinases, predicting that for PKAc, phosphorylation might also be able to indirectly control kinase:small molecule interactions.

We conclude that analytical DSF complements IM-MS and CIU for interrogating ligand binding and ligand-mediated conformational dynamics of PKA signaling components. These strategies are quantitative, permit evaluation of specific and non-specific interactions and enhance our understanding of the structural effects pertaining to ligand:protein interactions in vitro. Indeed, we provide new insight into the PKA holoenzyme complex, a signaling system that continues to inspire, almost fifty years after its discovery.

ASSOCIATED CONTENT
Supplementary Information. Contains methods describing phosphopeptide analysis, tables and figures detailing phosphosite identification, annotated mass spectra, protein purification, size exclusion chromatography, PKAc kinase assays and CIU IM-MS profiles.

Author Contributions
The manuscript was written with the contributions of all authors. All authors have given approval to the final version of the manuscript. ‡ D. P. B. and M. V. contributed equally to this work.

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ABBREVIATIONS
CE, collision energy; CCS, collisional cross section; CCSD, CCS distribution; CSD, charge state distribution; DSF, differential scanning fluorimetry; ESI, electrospray ionization; IM, ion mobility; JPP, lambda protein phosphatase; MS, mass spectrometry; PKA, cAMP-dependent protein kinase; PKAc, PKA catalytic subunit; PKI, heat stable PKA inhibitor protein; SEC, size-exclusion chromatography; TSA, thermostability assay.

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