Catalytic Independent Functions of a Protein Kinase as Revealed by a Kinase-dead Mutant: Study of the Lys72His Mutant of cAMP-dependent Kinase

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A highly conserved lysine in subdomain II is required for high catalytic activity among the protein kinases. This lysine interacts directly with ATP and mutation of this residue leads to a classical “kinase-dead” mutant. This study describes the biophysical and functional properties of a kinase-dead mutant of cAMP-dependent kinase where Lys72 was replaced with His. Although the mutant protein is less stable than the wild-type catalytic subunit, it is fully capable of binding ATP. The results highlight the effect of the mutation on stability and overall organization of the protein, especially the small lobe. Phosphorylation of the activation loop by a heterologous kinase, 3-phosphoinositide-dependent protein kinase-1 (PDK-1) also contributes dramatically to the global organization of the entire active site region. Deuterium-exchange mass spectrometry (DXMS) indicates a concerted stabilization of the entire active site following the addition of this single phosphate to the activation loop. Furthermore the mutant C-subunit is capable of binding both the type I and II regulatory subunits, but only after phosphorylation of the activation loop. This highlights the role of the large lobe as a scaffold for the regulatory subunits independent of catalytic competency and suggests that kinase dead members of the protein kinase superfamily may still have other important biological roles although they lack catalytic activity.

Introduction

The catalytic subunit (C-subunit) of protein kinase A (PKA) consists mainly of the kinase core, conserved in all eukaryotic kinases.¹ ² This core has a bilobal structure with a small lobe involved in nucleotide binding and a large lobe primarily involved in Mg²⁺ binding, substrate binding and phosphoryl transfer.³ There are several conserved structural motifs in each of the domains such as the glycine-rich loop and the C-helix in the small lobe, and the activation loop, catalytic loop and magnesium-positioning loop in the large lobe. In addition to these structural motifs, there are several conserved residues that are essential for optimal kinase activity.² Generally, mutation of these key residues results in proteins that lack the ability to autophosphorylate or catalyze phosphorylation of substrates.⁴

Mutation of one particular residue, Lys72, results in a “kinase-dead” mutant.⁵ This residue, located in β-strand 3 (β3) in the small lobe, is conserved in most kinases (Figure 1).⁶ Typically inactivation of kinases by mutation of the equivalent lysine has been used repeatedly to confirm kinase activity of novel gene products predicted to be kinases.⁷ Lys72 chelates the α and β phosphate groups of ATP and was first found to be important for kinase function of the C-subunit using an ATP affinity analog, p-fluorosulfonylbenzoyl 5'-adenosine (FSBA).⁸,⁹ Treatment with a hydrophobic carbodiimide, dicyclohexylcarbodiimide (DCCD), in the absence of MgATP irreversibly inhibits the C-subunit due to cross-linking of Lys72 to Asp184, another conserved residue.¹⁰ By interacting with Glu91 in the C-helix,
Lys72 also serves to stabilize the small lobe in an active conformation. Comparison of the active and inactive structures of other kinases such as cyclin-dependent kinase indicates an important rearrangement of the C-helix upon binding to cyclin. In the absence of cyclin the C-helix is twisted such that the equivalent of Glu91 faces the active site cleft where it interacts with the equivalent of Arg165 in PKA. The rearrangement on cyclin binding brings the conserved Glu in the C-helix in close proximity to the Lys to form the essential lysine–glutamic acid dyad (Figure 1) and leaves the activation loop poised for phosphorylation by a heterologous protein kinase.

While it is known that Lys72 is critical for activity of the C-subunit and mutation of the equivalent residue typically generates a dominant negative mutant, it is unclear what the functional and structural consequences of the mutation are. The first evidence for the essential role of the lysine in phosphoryl transfer but not ATP binding was provided by Carrera et al. Studies with the equivalent lysine in ERK2 also demonstrated that mutation of K52 created a non-productive binding mode for ATP and suggested that K52 is essential for orienting ATP for catalysis. Here, the effect of this mutation on biophysical properties of PKA was investigated, using fluorescence and circular dichroism. The structural consequences of the mutation were also characterized using deuterium exchange coupled to mass spectrometry (DXMS). This technique is advantageous for monitoring changes in protein conformation observed upon protein phosphorylation, protein folding and mapping protein interfaces. It has been successfully used to validate changes that are observed in crystal structures of the different states of the protein or identify regions that exhibit dynamic behavior in solution where such dynamics are not obvious from the crystal structure.

Results

Purification and phosphorylation state

To probe the function of Lys72 more closely, it was replaced with histidine (K72H). The mutant protein was purified by the addition of a poly(His) tag at the N terminus followed by affinity chromatography. Binding of the lysates to Talon resin (BD Biosciences) was followed by elution with increasing concentrations of imidazole (50 mM, 100 mM, 200 mM and 500 mM). The elutions were combined and further purified on an S75 gel-filtration column (Pharmacia) (Figure 2). An identical protocol was followed for the purification of K72H co-expressed with PDK-1.

Phosphospecific antibodies were used to probe the phosphorylation state of the mutant as before. The K72H mutant expressed in Escherichia coli was not phosphorylated at Thr197 with histidine (K72H). The mutant protein was purified by the addition of a poly(His) tag at the N terminus followed by affinity chromatography. Binding of the lysates to Talon resin (BD Biosciences) was followed by elution with increasing concentrations of imidazole (50 mM, 100 mM, 200 mM and 500 mM). The elutions were combined and further purified on an S75 gel-filtration column (Pharmacia) (Figure 2). An identical protocol was followed for the purification of K72H co-expressed with PDK-1.

Phosphospecific antibodies were used to probe the phosphorylation state of the mutant as before. The K72H mutant expressed in Escherichia coli was not phosphorylated at Thr197 on the activation loop. K72H treated with PDK-1 or co-expressed with PDK-1 was phosphorylated at Thr197 and this mutant is henceforth referred as pK72H. Neither the K72H nor the pK72H mutant was...
phosphorylated at Ser338 (Figure 2(d)). These results were also confirmed by mass spectrometry (data not shown). Activity of the mutants was tested using the PepTag assay and under these conditions neither K72H nor pK72H had detectable activity. Three other mutants, K72R, K72M and K72A, were purified in a similar way. They were also inactive and were not characterized further.

**Stability**

Circular dichroism (CD) was used to investigate the global conformational state of the phosphorylated and unphosphorylated mutant proteins. The mutant K72H is less stable \( T_m = 37.8 \, ^\circ \text{C} \) than the wild-type C-subunit \( T_m = 48.5 \, ^\circ \text{C} \). Phosphorylation of the mutant increases the thermostability \( T_m = 40.3 \, ^\circ \text{C} \) (Figure 3(a)). Urea-induced unfolding monitored by fluorescence reveals a pattern consistent with that observed in thermal denaturation. The unphosphorylated K72H has a lower transition point (2 M urea) compared to the wild-type C-subunit (3 M urea). Phosphorylation of the mutant increases the stability of the mutant (2.5 M urea) consistent with thermal unfolding (Figure 3(b)).

**Ligand binding**

While it is known that K72H is catalytically deficient, it is not known how this mutation affects ATP and/or substrate binding. To test for nucleotide binding, a fluorescent analog of ATP, the methyllanthraniloyl derivative of ATP (mant-ATP) was used. The binding of the mant nucleotides leads to a large increase in fluorescence energy transfer at 450 nm with a decrease in protein fluorescence, allowing direct measurements of nucleotide binding. The fluorescent energy transfer from protein to nucleotide that takes place on ligand binding was measured as a function of mant-ATP concentration. The data were fitted to a hyperbolic function to obtain a \( K_d \) of 36(±5) \( \mu \text{M} \) for mant-ATP for the wild-type C-subunit (Figure 4(a)) while the K72H (32(±6) \( \mu \text{M} \)) and pK72H (34(±3) \( \mu \text{M} \)) mutant proteins also had similar \( K_d \) values. The stability of the mutants, monitored by thermal unfolding and urea unfolding, was also increased on binding to ATP similar to the wild-type C-subunit. Stability of the mutant C-subunits was also enhanced in the presence of a kinase.
inhibitor (H-89) and this was also comparable to the stabilization observed in the wild-type C-subunit Table 2.

Substrate/inhibitor binding

To test for the ability to form holoenzyme, the mutant proteins were incubated with RII regulatory subunit (in the presence of \(\text{Mg}^{2+}/\text{ATP}\)) and RIIβ regulatory (in the absence of \(\text{Mg}^{2+}/\text{ATP}\)) and run on a non-denaturing Tris-glycine gel. While the RIIx is a pseudo substrate the RIIβ subunit with serine at the P-site is also a substrate. As shown in Figure 4(b), the K72H mutant did not bind to either the RIIx or RIIβ subunit. In contrast the phosphorylated pK72H C-subunit mutant formed a complex with both the RIIx and RIIβ subunit (Figure 4(b)). The bands run slightly higher than the wild-type C-subunit holoenzyme because the mutant C-subunit has a poly(His) tag. Similar results were seen for RIIx binding when the mutants were incubated and purified on an S75 gel-filtration column (data not shown).

To quantify the interaction between mutant C-subunit and R-subunit, surface plasmon resonance (SPR) was used (Figure 5). The apparent association (\(k_{\text{assoc}}\)) and dissociation (\(k_{\text{dissoc}}\)) constants for binding to the wild-type RIIx were determined in the presence of \(\text{MgCl}_2\) and ATP. As seen in Table 1, the ability of the unphosphorylated K72H to bind to the regulatory subunit is severely compromised. No binding was detected even at concentrations of 100 nM RIIx. In contrast, for the phosphorylated K72H mutant, the \(K_d\) was only tenfold higher than the wild-type C-subunit. The increase in \(K_d\) was mainly due to a higher dissociation rate (6.93 × 10^{-4} s^{-1} versus 2.3 × 10^{-5} s^{-1} for the wild-type C-subunit). Thus, phosphorylation at the
activation loop plays an essential role in ordering the loop such that productive binding with the regulatory subunit can be achieved. Even when the enzyme is inactive, the large lobe is still functional as a scaffold for the R-subunit, but its ability to serve as a scaffold is dependent on phosphorylation of the activation loop. To ascertain if the absence of phosphorylation at the C-terminal tail (Ser338) was affecting the dissociation rate, mutation of Ser to Glu (S338E) was carried out in the K72H background (K72HS338E). The binding of pK72HS338E was similar to pK72H, with the association and dissociation rates of the same order of magnitude. The $K_d$ values were also in the same range, with the pK72HS338E showing a slight increase (15 nM) compared to the pK72H (3.2 nM), indicating that the higher dissociation rate is an effect of the mutation and not the absence of the phosphate at the C-terminal tail.

Hydrogen–deuterium exchange

To evaluate the effect of the mutation on the local and global environment of the protein in solution, we used DXMS. K72H and pK72H were compared to the wild-type C-subunit.

Catalytic subunit fragmentation pattern

Digestion conditions were optimized to give maximum coverage of the wild-type catalytic subunit. These same conditions were applied to the unphosphorylated and phosphorylated states of K72H. A total of 144 distinct peptides were generated for the wild-type protein, which covered 84% of the protein, while 118 peptides were obtained for the K72H mutant (85%) and 135 peptides were obtained for the pK72H mutant (75%). Comparison of the three different states yielded 53 peptides that were common among all three different proteins. The peptides map different subdomains from the small (residues 20–138) and large lobe (residues 140–310) of the protein. Each peptide was numbered and discussed in terms of the amide residues with exchange phenomenon that could theoretically be followed (i.e. the length of the peptide minus the first second amides and any proline residues thereafter).$^{23}$ The percentage deuteration of each peptide at the different time-points for each protein is indicated in Figure 6.

Deuterium incorporation into the small lobe of the catalytic subunit

Two peptides map to subdomain I that corresponds to the glycine-rich loop, a segment that plays a critical role in catalysis. Peptide (45–54) maps to the glycine-rich loop in subdomain I, specifically to $\beta_1$ while peptide (55–59) maps to the $\beta_1$-$\beta_2$ loop and $\beta_2$. Each peptide showed enhanced incorporation of deuterons in the K72H mutant, when it is compared to the wild-type C-subunit. At

Table 1. Binding kinetics of the K72H protein to RIz

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<th>$k_a$ (1/M s)</th>
<th>$k_d$ (1/s)</th>
<th>$K_D$ (M)</th>
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<tr>
<td>Wt*</td>
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<td>2.3×10$^{-5}$</td>
<td>1.9×10$^{-10}$</td>
</tr>
<tr>
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<td>Not measurable</td>
<td>NA</td>
</tr>
<tr>
<td>pK72H</td>
<td>3.6×10$^3$±0.3</td>
<td>1.1×10$^{-3}$±0.6</td>
<td>3.2×10$^{-9}$±1.3</td>
</tr>
<tr>
<td>pK72H/S338E</td>
<td>0.7×10$^3$±0.2</td>
<td>1.0×10$^{-3}$±0.5</td>
<td>15.7×10$^{-9}$±5.7</td>
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The experiments were performed in the presence of Mg/ATP as indicated in Materials and Methods. All proteins were used directly and not treated with urea.

* The values were obtained for the R-subunits which was stripped of cAMP by urea treatment.
every point deuteration is significantly higher in the K72H mutants than for the wild-type. Phosphorylation has a significant effect on this subdomain. Upon phosphorylation the pattern of deuteration becomes almost biphasic on a logarithmic scale. The initial deuteron incorporation for both peptides in the phosphorylated mutant (pK72H) closely resembles the wild-type protein at early time (ten seconds) but is identical with the unphosphorylated K72H at later time-points (1000 seconds). The transition point for both peptides is 164 seconds and 238 seconds, respectively, suggesting that the solvent is coordinated (Figure 7).

Two peptides were obtained in subdomain II. Peptide (60–71) maps to the β3 strand, which includes the region of the Lys72 mutation, while peptide (92–103) maps part of the C-helix and the loop that docks to the large lobe. Similar to subdomain I, these peptides also showed enhanced deuteration for the mutants compared to the wild-type protein. Likewise, these peptides displayed a biphasic behavior on a logarithmic scale upon phosphorylation. At the early time-points they resembled the wild-type C-subunit whereas at the later times they resembled the unphosphorylated mutant. The difference in behavior for the peptide (60–71) for the unphosphorylated and phosphorylated mutant indicates an effect independent of the mutation at Lys72; it is only sensing phosphorylation. In part, the mid-point for the β3 strand peptide (154 seconds) is close to β1 and β2 above, suggesting a coordinated effect on the entire β-sheet. The nature of the exchange also points to the presence of faster exchanging protons in the pK72H peptides compared to the wild-type.

**Deuteration at the catalytic loop of the catalytic subunit**

The peptides covering the catalytic loop and the β-sheet region of the large lobe also showed enhanced deuteration in the mutants compared to the wild-type in all the peptides covering this region, i.e. peptides (154–162), (163–172), and (173–178). However the effect of the phosphorylation is clearly sensed in the mutant. In each of these peptides that span the β-strand of the catalytic loop, peptides from the pK72H mutant showed rates of hydrogen exchange that were intermediate between the K72H and WT enzyme. The mid-point of exchange for the catalytic loop is similar to β strands in the small lobe. Thus the entire active site is sensing the phosphorylation of Thr197 (Figure 8).

**Deuteration in the large lobe of the catalytic subunit**

In contrast to the small lobe, the hydrophobic core of the large lobe much more closely resembles the wild-type protein overall. This part of the protein is not sensitive to phosphorylation of the activation loop. Most of the peptides obtained in the large lobe...
showed similar deuteration incorporation at the various time-points. Some of the peptides that did exhibit a difference in behavior are illustrated in Figure 9. These peptides map part of the F-helix (212–221), part of the G-helix (247–264) and I-helix (277–296) and the level of deuteration is typically lower for the wild-type than the mutants for all these peptides. As can be seen for Figure 9, the effects of phosphorylation are localized and not as striking as in the small lobe (Figure 7).

**Discussion**

Since the first crystal structure was solved in 1993, the structure of the catalytic subunit of PKA has served as a model for the protein kinase family. An important challenge for understanding this enzyme family is to elucidate the specific role that is played by key residues and to better understand the functional consequences of phosphorylation at the activation loop. Lysine 72, located
in β-strand 3 of the small lobe of the C-subunit, is one of the essential residues at the active site of all protein kinases. It has at least two apparent roles: it interacts with the α and β phosphate groups of ATP, and it also interacts with another conserved residue, Glu91 in the C-helix. Lys72 also forms a catalytic triad with Glu91 and Asp184, and is thus important for maintaining the catalytically active conformation of the small lobe. Although this residue has been mutated in almost every kinase and typically leads to a dominant negative phenotype when expressed in mammalian cells, we know little about the biophysical consequences of this mutation in PKA. Is it more stable or less stable than the wild-type protein? Can it bind substrate or inhibitors? Does phosphorylation change its properties? To answer these questions we characterized a kinase-dead mutant C-subunit where Lys72 was replaced with His and explored in detail the effects of this mutation on the structure and function of the C-subunit.

The mutant C-subunit shows no catalytic activity and is not phosphorylated when expressed in E. coli. It is also less stable than the wild-type C-subunit based on CD and endogenous fluorescence. In contrast to the wild-type C-subunit, unfolding in the presence of denaturant is also not cooperative. Surprisingly, the mutant

Figure 8. Exchange of peptides around the catalytic loop region. The peptides obtained are color-coded for different regions: black (154–162), white (163–172), black (173–178) and white (179–184). Peptide (185–197), which could not be obtained, is shown in grey.
The C-subunit was fully capable of binding ATP as monitored by the fluorescent ATP analog, mant-ATP (Figure 4(a)). Thus, although we know from the crystal structure that K72 binds to the γ-phosphate groups of ATP, it does not contribute to the $K_m$ (ATP). Most likely this is because the energy gained by interacting with the phosphate groups is negated by the energy that is lost by desolvation of the lysine side-chain. Mutation of this residue (K52) in Erk2 also leads to a non-productive binding mode where the mutation of this residue has a negligible effect on $K_m$ for ATP but has a marked effect on $k_{cat}$.14

Our results further support the conclusion that interactions between ATP and Lys72 are important for generating the transition state structure or facilitating conformational changes needed to promote the phosphoryl transfer reaction. It is also significant that Lys72 is the only active site residue that is disordered in the apoenzyme structure whereas Lys168 in the large lobe is in position to bind the γ-phosphate.27 In the K72H mutant the tip of the glycine-rich loop must not bind the γ-phosphate correctly and position it for phosphoryl transfer. The other role of Lys72 is to optimally position the C-helix for catalysis. This function depends on a constructive ion pair between Glu91 and Lys72. This mutant is incapable of making that bridge; the His presumably cannot reach far enough. Ala and Arg mutants behave in a similar way to K72H. In the absence of a positive charge in β strand 5, it is likely that Glu91 interacts with Arg165 that precedes the catalytic loop in the large lobe. This positioning is seen in the inactive conformation of cdk211 and hck,28 and leads to a distortion of the position of the C-helix. Correct position of this triad (Lys72, Glu91 and Asp184) is essential for catalysis, and we suggest that this is the most important role of Lys72. In the case of cdk2 it is binding of cyclin that pushes the C-helix into place and restores the Lys72-Glu91 ion pair.11,29 while in hck it is phosphorylation of the activation loop.28,30

To determine whether phosphorylation of the activation loop could play a comparable role in this mutant, we phosphorylated Thr197 with a heterologous kinase PDK-1. Stability of the phosphorylated mutant is greater than the unphosphorylated mutant but still less than the wild-type protein. This could be due to lack of phosphorylation at the C-terminal site, Ser338. However, it is more likely due to the inability of His72 to compete Glu91 away from Arg165. An analysis of the mutants of Glu91 of the C-subunit will be needed to confirm this prediction.

DXMS was also used to monitor the local and global effects of this point mutation. Results indicate quite different profiles for the large and the small lobe. In general, the large lobe in the mutant appeared to be folded in a manner that more closely resembled that of the wild-type C-subunit. In contrast the small lobe was much more solvent accessible than the wild-type C-subunit (Figure 6). This general profile is also reflected in the crystal structure of the apoenzyme, where the large lobe is well folded with the active site preformed while parts of the small lobe are disordered.27 Mutation of Lys72 destabilizes the small lobe even further.

Although the K72 mutant was inactive even when it was phosphorylated, DXMS showed that the mutant did sense the addition of the phosphate over long distance. The most striking effect was on the small lobe where at the early time-points (ten seconds) the phosphorylated K72 mutant was nearly identical with the wild-type protein but at the later time-points it resembles the unphosphorylated mutant. There is a clear phosphorylation-dependent transition in the deuteration profile and this is a direct consequence of phosphorylation. This same pattern was seen in the β-sheet region of the large lobe that includes the catalytic loop. This is the first time that we have seen a clear demonstration of the global

**Figure 9.** Exchange of peptides mapping part of helix F (212–221), helix G (247–264) and helix I (277–296).
and well-coordinated effect of phosphorylation on the organization of the active site of the C-subunit. The effect of phosphorylation on the large lobe is less striking. Overall, the large lobe is not as sensitive to the mutations or to phosphorylation. Phosphorylation of Thr197 does, however, restore an important function. When Thr197 is phosphorylated it can now bind reasonably with the RIz and RIIb subunits (Figure 4(b)). Although the C-subunit requires ATP to form a tight complex with RIz, it apparently can manage without Lys72. The affinity is only tenfold weaker for the mutant, due mostly to an increased off-rate, and is at least orders of magnitude better than the unphosphorylated protein, which could not be measured (Table 1). The large lobe therefore serves as a phosphorylation-dependent scaffold for the R-subunits even in the absence of catalysis.

Analysis of the K72H mutant leads to some important conclusions. Mutations in the active site can have a global effect on stability and the overall organization of the C-subunit. While the small lobe is especially sensitive to mutation of Lys72, phosphorylation on the activation loop can also be sensed across the entire active site. This is perhaps the first specific description of the long-range effects of phosphorylation even though such effects had been demonstrated kinetically. Even when the enzyme is not catalytically active, phosphorylation contributes dramatically to the global organization of the entire active site region. In addition to structural organization of the active site, phosphorylation of the activation loop restores the capacity of the C-subunit to bind to both RI and RII regulatory subunits and this role as a scaffold for R is independent of catalytic competency. Retention of this interaction could be an extremely important function for several protein kinases in the human genome that are predicted to be dead kinases due to mutation of the critical active site residues such as Lys72.

Materials and Methods

Materials

The reagents used were the pET15b expression vector (Invitrogen), E. coli strain BL21(DE3) (Novagen, Madison, WI), Quick-Change mutagenesis kit (Strategene), horse-radish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences), SuperSignal West Pico chemiluminescent substrate detection kit (Pierce), oligonucleotides (Sigma), mant-ATP (Molecular Probes), H-89 (LC Labs, Woburn, MA) and the PepTag PKA activity assay kit (Promega, Madison, WI). Antibodies against the catalytic subunit of PKA and the Ser338 phosphorylation site were described previously, while antibodies that specifically recognize the phosphorylated activation loop of protein kinase C (PKC), referred to as α-Thr197-P, were a gift from A. Newton (University of California, San Diego). DNA sequencing was performed with the ABI PRISM 310 genetic analyzer from Perkin Elmer Life Sciences. All other materials were reagent grade from standard commercial sources.

Expression and purification of the PKA catalytic subunit

(His)6-tagged K72H mutants in pET15b vector (amp+) were expressed in E. coli [BL21 (DE3)] and purified using the Talon resin from Clontech. Mid-exponential cultures of BL21 cells were induced with IPTG and grown for another five hours at 24 °C prior to harvesting at 6000 rpm. Following resuspension in lysis buffer (50 mM KH2PO4, 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 5 mM β-mercaptoethanol (β-ME)), cells were lysed using a French press at 1000 PSI. Cells were clarified by centrifugation at 17,000 rpm at 4 °C in a Beckman JA20 rotor for 50 minutes and the supernatant was incubated with Talon resin and pre-equilibrated in the same buffer for one hour at 4 °C. The resin was then spun down at 3000 rpm and the supernatant removed. Two wash steps with the lysis buffer were followed by two wash steps with Wash buffer (WB; 50 mM KH2PO4, 20 mM Tris–HCl (pH 7), 100 mM NaCl, 10 mM imidazole, 5 mM β-ME). Each wash was done by treating the resin with the buffer followed by gentle end-over-end mixing for five minutes at 4 °C. An imidazole elution buffer using four different imidazole concentrations (viz. 50 mM, 100 mM, 200 mM and 500 mM) in WB was used to elute the His-tagged protein. Samples that had the most amount of protein were then dialyzed into gel-filtration buffer (20 mM Mops (pH 8), 100 mM KCl, 5 mM β-ME) and purified on a Sephacryl S75 gel-filtration column (Pharmacia). To obtain the phosphorylated form of K72H, cells were co-expressed with PDK-1 enzyme cloned in PGEX and phosphorylated K72H (pK72H) was purified using the same approach as K72H.

The activity of the mutants was assessed using the PepTag activity assay. This assay uses a fluorescent-tagged Kemptide substrate, where a change in net charge occurs upon phosphorylation. This change is detected by a shift in its direction of mobility when run on an agarose gel.

Deuterium exchange experiments

Digestion conditions were optimized to yield fragments that were of high quality and optimal for exchange analysis. Preparation of deuterated samples and subsequent DXMS analysis was carried out as described. Briefly, the deuterium exchange reaction was started by combining 5 μl of protein sample (5 mg/ml, or 0.125 mM, mutant or wild-type C-subunit) with 15 μl of 20 mM Mops (pH 7.4), 50 mM NaCl, 1 mM dithiothreitol in 2H2O (deuteration buffer). After incubation at room temperature for various times of 10, 30, 100, 300, 1000, or 3000 seconds, the reaction was quenched with 30 μl of 0.8% formic acid, 1.6 M guanidine–HCl in 2H2O, pH 2.3–2.5 at 0 °C. Samples were immediately passed through a solid-phase pepsim column (66 μl bed volume, Upchurch Scientific) coupled to an Aspergillus saitoi fungal protease type XIII (EC 3.4.23.6, Sigma).17 Proteolytic peptides were collected by a C18 column (Vydac), and were subsequently developed with a linear gradient of 10 ml of 8%-40% acetonitrile in 0.05% TFA, at 0.2 ml/minute. Mass spectrometric analyses were carried out with a Finnigan LCQ mass spectrometer as described. Fully deuterated samples were prepared by incubating the protein in 0.5% formic acid in 95% 2H2O for 24 hours.
Recoverd peptide identification and analysis were carried out using software specialized in processing DXMS data. DXMS analysis was used to confirm sequence identity and track time-dependent deuterium buildup for all proteolyzed peptides. In brief, the SEQUEST program (Thermo Electron Corporation) was used to identify the likely amino acid sequence of peptide fragments and analyzed as described. DXMS analysis was performed using SEQUEST output MS1 and MS2 data (i.e. the parent and daughter tandem mass spectrometric data). SEQUEST identifications were confirmed with DXMS by comparing the predicted isotopic envelopes for the proteolyzed peptides generated from SEQUEST against MS1 data acquired from the Q-TOF mass spectrometer. DXMS data reduction was used to track deuterium buildup for each peptide fragment over time. Selected peptides passing this automated quality-control step were manually checked for correct fit and mass identification.

Circular dichroism

CD spectra in the far-UV range (190–250 nm) were recorded on a computer-controlled AVIV CD spectropolarimeter using a 1 mm path-length cuvette. Samples were dialyzed in 20 mM KPO4, 20 mM KCl (pH 6.5) (with and without 5 mM MgCl2, 1 mM ATP) and were heated in a jacketed cell at a rate of 60 deg.C/hour over the 24–60 °C range, using a Neslab RT-11 programmable water-bath. Protein stability in the presence of ligand was monitored by incubating the protein with 5 mM MgCl2, 1 mM ATP and were all background-corrected and smoothed using AVIV a jacketed cell at a rate of 60 deg.C/hour over the 24–60

Fluorescence spectroscopy

Urea unfolding

Amberlite MB-150 (Sigma, St. Louis, MO) mixed-bed exchanger was added to 8.5 M urea solution and stirred for one hour to remove ionic urea degradatation products. The urea solution was filtered and frozen at −20 °C. Samples (1 μM) were dialyzed in 20 mM KPO4, 20 mM KCl (pH 6.5) (with and without 5 mM MgCl2, 1 mM ATP) and were heated in a jacketed cell at a rate of 60 deg.C/hour over the 24–60 °C range, using a Neslab RT-11 programmable water-bath. Protein stability in the presence of ligand was monitored by incubating the protein with 5 mM MgCl2, 1 mM ATP or 150 μM H-89 at 4 °C prior to unfolding. The spectra were all background-corrected and smoothed using AVIV noise reduction software. Changes in the structure of PKA due to unfolding were followed at 222 nm every 1 deg.C with protein concentration at 0.3 mg/ml (8 μM) (Table 2).

Table 2. Thermostability as an indicator of protein stability and ligand binding

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<th>+H-89</th>
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<td>K72H</td>
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<td>pK72H</td>
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mamt-ATP binding

The fluorescence spectra of the mant-ATP in the absence and presence of PKA were measured using a Fluoromax spectrofluorimeter (FluroMax2, Jvyn Yovich) equipped with a 200 μl cuvette and holder. The assay was carried out in 20 mM KPO4, 20 mM KCl (pH 6.5). Spectra of the nucleotides, PKA, and a mixture of the nucleotides and PKA were measured either by exciting the samples at 295 nm and collecting the emission intensity between 270 nm and 550 nm or by exciting the samples at 340 nm and collecting the emission intensity between 380 nm and 550 nm. Excitation and emission slit-width of 5 nm were used in all experiments.

Regulatory subunit binding

The ability of the catalytic subunit mutants to bind to the regulatory subunits was tested by incubating the regulatory subunits (RIz and RIIβ) with the catalytic subunit and its mutants (K72H and pK72H) in 20 mM Mops, (pH 7) 150 mM KCl. To test binding with RIIβ, 6 μg of the catalytic subunit (wild-type, K72H and pK72H) were incubated with 5 μg of RIIβ on ice for 60 minutes and run on an 8% (w/v) Tris–glycine gel (Invitrogen) at 85 V for four hours. For RIz binding, 6 μg of the catalytic subunits were pre-incubated with 5 mM MgCl2, 1 mM ATP on ice for 30 minutes, followed by addition of 5 μg of RIz and further incubation on ice for 60 minutes. Samples were run on an 8% Tris–glycine gel (Invitrogen) as before. Aliquots of the above reactions were also run on SDS-denaturing gels to confirm the presence of the proteins. The interaction can also be tested by incubating 1 mg of the H1-K72H with 0.8 mg of RIz regulatory subunit in the presence of 5 mM MgCl2 and 0.2 mM ATP. Loading the mixture onto a Sephacryl 200 gel-filtration column (Pharmacia) gave two peaks with the first peak correponding to the holoenzyme and the second peak correponding to the excess catalytic subunit. The holoenzyme peak was obtained only when phosphorylated K72H mutant was used (data not shown).

Surface plasmon resonance

Surface plasmon resonance (SPR) was used to study the interaction between the C-subunit and R-subunit of PKA using a BIACore instrument (Pharmacia/Biosensor). A sensor chip surface was prepared by direct coupling of the C-subunit by primary amines to the CM dextran (Biosensor Amine Coupling Kit) as described. To determine non-specific binding, blank runs were performed with 100 nM wild-type R-subunit using a non-activated surface. About 100 RU of the K72H mutants were immobilized. All runs were performed using 2 mM ATP and 5 mM MgCl2 in 20 mM Mops, 20 mM KCl buffer at pH 7.0 during the association and dissociation phase; regeneration of the surface was achieved by injecting 10 μl of 100 μM cAMP and 5 mM EDTA in buffer. The first ten seconds of every sensogram was subtracted, correcting for bulk refractive index changes. The observed data were also checked for mass-transport limitations by evaluating the binding at different flow rates.

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Supplementary Data

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