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High-resolution crystal structure of cAMP-dependent protein kinase from *Cricetulus griseus*

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Protein kinases (PKs) are dynamic regulators of numerous cellular processes. Their phosphorylation activity is determined by the conserved kinase core structure, which is maintained by the interaction and dynamics with associated domains or interacting proteins. The prototype enzyme for investigations to understand the activity and regulation of PKs is the catalytic subunit of cAMP-dependent protein kinase (PKAc). Major effects of functional regulation and ligand binding are driven by only minor structural modulations in protein–protein interactions. In order to resolve such minor structural differences, very high resolution structures are required. Here, the high-resolution X-ray structure of PKAc from *Cricetulus griseus* is reported.

1. Introduction

Protein kinases (PKs) catalyze chemical modifications of their interaction partners (peptides and proteins) mostly by transferring the γ -phosphates of nucleotide triphosphates (NTPs) to the free hydroxyl groups of the side chains of the amino acids serine, threonine and tyrosine (Hanks & Hunter, 1995). Hence, a change in the functional activity of the substrate is induced, resulting in changes in either enzymatic activity, cellular localization or interaction properties. The activity of PKs must be highly regulated, since their activity has profound effects on cell cycles. Dysregulation of kinases commonly results in severe diseases, including cancer, neurodegeneration and metabolic disorder (Noble *et al.*, 2004). PK activity can be modulated by autophosphorylation, interaction of activator or repressor proteins or the binding of small-molecule inhibitors to the catalytic protein kinase domain.

The catalytic subunit of the PK superfamily is highly conserved. Numerous homologous PK crystal structures have been reported in the Protein Data Bank (PDB; Berman *et al.*, 2000), but a number of these structures lack essential details of the kinase domain owing to high flexibility. Therefore, the crystallization of the catalytic domain still needs to be improved. Furthermore, key insight into the catalytic mechanism of kinases and their interaction with regulators, inhibitors and substrates has come from the availability of several kinase structures. New structures therefore continue to improve our understanding of the interactions between PKs and substrates at different steps of the reaction cycle (Adams, 2001).

The catalytic subunit of cAMP-dependent protein kinase (PKAc) has served as a model kinase as it can be expressed

in *Escherichia coli* owing to its autophosphorylation activity. Furthermore, PKAc is a surrogate kinase to study PKB/Akt kinase and also Rho-kinase, which are validated cancer targets and cannot be expressed in bacteria for structural studies. Structurally, PKAc is described by a single conserved catalytic core domain comprising a small amino-terminal N-lobe mainly characterized by β -sheets and a large carboxy-terminal C-lobe assembled from α -helices (Knighton *et al.*, 1991). The NTP-binding pocket is located in a cleft between the lobes. Nucleotide and substrate binding induce considerable changes in conformation and dynamics. These changes lead to movements of both lobes, resulting in an opening and closure of the active pocket, which is essential for the catalytic cycle (Taylor *et al.*, 2012). To date, 173 crystal structures of different PKAc homologues have been reported in the PDB with resolutions from 1.23 to 3.7 Å. Only two full-length ATP-bound crystal structures with a resolution below 1.5 Å have been described [PDB entry 1rdq (Yang *et al.*, 2004) and PDB entry 4dfx (Bastidas *et al.*, 2012)], providing more detailed information on the water network surrounding PKAc, which influences ligand binding and activity. Here, we report the full-length ligand-free crystal structure of PKAc from Chinese hamster (*Cricetulus griseus*; UniProt ID P25321) at a resolution of 1.14 Å.

2. Materials and methods

2.1. Protein production and crystallization

Full-length PKAc (residues 1–351) from *C. griseus* (UniProt ID P25321) was previously cloned into the NdeI/BamHI sites of a pET-TEV vector (a modified pET-16b vector; Novagen) with an N-terminal His₇ tag followed by a tobacco etch virus (TEV) protease cleavage site (Langer *et al.*, 2004; see Table 1). PKAc was recombinantly expressed in *E. coli* BL21 (DE3) cells (Life Technologies). The cells were grown at 310 K to an OD₆₀₀ of 0.6, stored on ice for 20 min, induced with 1 mM IPTG and grown for 18–20 h at 291 K. Cultures were centrifuged and microfluidized in a buffer containing 50 mM Tris pH 8.0, 5 mM β -mercaptoethanol, 5 mM imidazole, 500 mM NaCl and ultracentrifuged. PKAc was purified from the supernatant using immobilized metal-ion affinity chromatography (IMAC; HisTrap HP, GE Healthcare). The His₇ tag was removed from the protein by TEV protease digestion while dialyzing the protein against lysis buffer. After an additional IMAC run, the flowthrough protein fractions were dialyzed against a buffer containing 50 mM HEPES pH 7.4, 50 mM NaCl, 5 mM dithiothreitol (DTT). To separate the different phosphorylation states of recombinantly expressed PKAc, the protein was applied onto a strong cation-exchange column (Mono S, GE Healthcare) and eluted with a gently inclined linear salt gradient to 150 mM NaCl. Protein fractions eluted at the highest salt concentration were pooled and concentrated to 13–15 mg ml^{−1} in a buffer containing 25 mM MES/bis-tris buffer pH 6.4–6.6, 1 mM DTT, 0.1 mM EDTA, 75 mM LiCl. After the addition of 1.5 mM non-ionic detergent MEGA 8 (G-Biosciences) and 0.8 mM cAMP-dependent protein kinase inhibitor peptide PKI-tide (IAAGRTGRRQAIHDILVAA;

Table 1

Protein-production information.

Source organism	<i>C. griseus</i>
Expression vector	Modified pET-16b
Expression host	<i>E. coli</i>
Complete amino-acid sequence of the construct produced	
cAMP-dependent protein kinase catalytic subunit $\alpha^{\dagger}$	MGHHHHHHHASENLYFQGHMGNAAAAKKGSEQES- VKEFLAKAKEEFLKKWESPSQNTAQLDHFDR- KTLGTGSFGRVMLVKHKETGNHYAMKILDKQK- VVKLKQIEHTLNEKRILQAVNFPFLVKLEFSF- KDNSNLYMVEYVPGGEMFSLRRIGRFSEPH- ARFYAAQIVLTFEYLHSLDLIYRDLKPENLLI- DQGGYIQVTDGFAKRVKGRWTLCGTPEYLA- PEIILSKGYNAVDWWALGVLIYEMAAGYPPF- FADQPIQIYEKIVSGKVRFPFSSDLKDLLR- NLLQVDLTRKFGNLKNGVNDIKNHKWFATTDW- IAIYQRKVEAPFIPKFKPGDTSFDDYEEEE- IRVSINEKCGKEFTEF
cAMP-dependent protein kinase inhibitor PKI-tide	IAAGRTGRRQAIHDILVAA

[†] The cleaved sequence is underlined.

Table 2

Crystallization.

Method	Vapour diffusion
Plate type	Cryschem plate
Temperature (K)	277
Protein concentration (mg ml ^{−1})	13–15
Buffer composition of protein solution	25 mM MES/bis-tris buffer pH 6.4–6.6, 1 mM DTT, 0.1 mM EDTA, 75 mM LiCl, 1.5 mM MEGA 8, 0.8 mM PKI-tide
Composition of reservoir solution	20–25% methanol
Volume of drop (μl)	3
Volume of reservoir (μl)	400

Sigma–Aldrich), crystals were grown by the vapour-diffusion method at 277 K in 3 μl hanging drops against freshly prepared reservoir solution (400 μl) containing 16–23% (v/v) methanol (see Table 2).

2.2. Data collection, structure solution and refinement

Rod-shaped crystals (Fig. 1a) were harvested using mounted cryoloops (Hampton Research), transferred into 30% (v/v) MPD and flash-cooled in pucks immersed in liquid nitrogen for storage until X-ray diffraction data collection. Diffraction data were collected on BL14.1 operated by the Joint Berlin MX-Laboratory at the BESSY II electron-storage ring (Berlin-Adlershof, Germany; Mueller *et al.*, 2012). The best data set was processed using *XDSAPP* (Krug *et al.*, 2012). Data-collection statistics are shown in Table 3. The structure was determined by molecular replacement with *Phaser* (McCoy *et al.*, 2007) using the crystal structure of bovine PKAc (PDB entry 1yds; Engh *et al.*, 1996) as a search model. A final translation-function Z-score (TFZ) of 8.8 indicated successful structure solution. Model building was performed using *Coot* (Emsley & Cowtan, 2004) and the structure was refined using *PHENIX* (Afonine *et al.*, 2012). Figures containing molecular graphics were prepared using *PyMOL* (Schrödinger). Structure-solution and refinement statistics can be found in Table 4.

3. Results and discussion

3.1. Protein expression and crystallization

Previous studies reporting the NMR backbone assignment of PKAc (Langer *et al.*, 2004) required extensive long-term protein stability investigations on different PKAc homologues. These studies revealed that the cAMP-dependent protein kinase from *C. griseus* can be expressed in prokaryotic hosts with high yields (>10 mg per litre of culture). Furthermore, the protein is phosphorylated and catalytically active. The most important advantage of choosing this particular homologue for *in vitro* experiments is its good stability at high protein concentrations at 298 K. These features allow a systematic transfer from NMR studies to different biophysical methods requiring good protein stability at high concentration

(including X-ray crystallography and ITC measurements). Initial crystallization attempts indicated that the homogeneity of the phosphorylation state of the sample is a crucial requirement to obtain highly diffracting crystals. Only by strong cation-exchange chromatography (Mono S) was it possible to separate the different phosphorylation states properly. Generally, the UV₂₈₀ chromatogram of a typical Mono S run is characterized by two well separated peaks of approximately equal heights at different elution volumes. Homogeneously threefold-phosphorylated PKAc eluted at a higher NaCl concentration as a second UV₂₈₀ peak and yielded very well diffracting crystals, while attempts to produce good diffracting crystals from higher phosphorylated protein samples of the first peak did not succeed.

Figure 1

(a) PKAc protein crystals. (b) The high-quality $2F_{\text{obs}} - F_{\text{calc}}$ electron-density map reveals the existence of phosphorylated residues Ser11 (i), Thr198 (ii) and Ser339 (iii) and alternative conformations, for example His143 (iv). (c) Interaction network of PKAc and PKI-tide (generated with *PDBsum*). Hydrogen bonds are marked in blue and nonbonded contacts are shown as orange dashed lines. (d) Stereo cartoon representation (wall-eyed) of the PKAc-PKI-tide complex. PKI-tide (red) binding introduces a closed conformation of the N-lobe (green) and C-lobe (blue). The N-terminal linker helix (light green) and the C-terminal loop region (dark blue) are fully visible in the electron density.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL14.1, BESSY
Wavelength (Å)	0.918409
Temperature (K)	100
Detector	Pilatus 6M
Crystal-to-detector distance (mm)	180
Rotation range per image (°)	0.1
Total rotation range (°)	180
Exposure time per image (s)	0.1
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	58.28, 72.95, 109.35
α , β , γ (°)	90, 90, 90
Mosaicity (°)	0.064
ISa†	25.51
Resolution range (Å)	45.53–1.139 (1.21–1.139)
Total No. of reflections	1101841
No. of unique reflections	169712
Completeness (%)	99.7 (98.7)
Multiplicity	6.49 (6.00)
$\langle I/\sigma(I) \rangle$	17.59 (2.3)
$R_{\text{meas}}^{\ddagger}$	0.054 (0.76)
Overall <i>B</i> factor from Wilson plot (Å ²)	12.5

† ISa = $\langle I/\sigma(I) \rangle^{\text{asymptotic}}$ represents the highest possible signal-to-noise ratio of a data set (Diederichs, 2010). Data sets with ISa values of 25 or greater are considered to be very good and should allow straightforward structure determination. $\ddagger R_{\text{meas}} = \sum_{hkl} [N(hkl)/[N(hkl) - 1]]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$ (Diederichs & Karplus, 1997; Weiss & Hilgenfeld, 1997).

Table 4

Structure solution and refinement.

Values in parentheses are for the outer shell.

PDB code	4wih
Resolution range (Å)	39.874–1.139 (1.1520–1.1391)
Completeness (%)	99.7
No. of reflections, working set	161224 (5043)
No. of reflections, test set	8485 (265)
Final R_{cryst}	0.177 (0.2510)
Final R_{free}	0.191 (0.2633)
No. of non-H atoms	
Protein	2978
Ligand	114
Solvent	585
R.m.s. deviations	
Bonds (Å)	0.006
Angles (°)	1.013
Average <i>B</i> factors (Å ²)	
Protein	25.0
Water	36.9
Ramachandran plot	
Most favoured (%)	92.8
Allowed (%)	7.2

3.2. Overall structure of *C. griseus* PKAc

The ternary structure of the PKAc–PKI-tide complex in the absence of nucleotides or small molecules bound to the active site was solved at 1.14 Å resolution with a crystallographic *R* factor of 17.7% and an R_{free} of 19.1% (Table 4). Although the quality of the electron-density map was not completely homogeneous, all expected residues could be placed without ambiguity. Three phosphorylation sites important for membrane association (Ser11), kinase activation (Thr198) and facilitating protein–protein interaction (Ser339) could be clearly mapped (Fig. 1*b*, i–iii; Taylor *et al.*, 2012). Several side

chains with alternative conformations were found [Glu26, Met72, Gln97, Met121, His143 (Fig. 1*b*, iv), Ser160, Thr184, Lys218 and Asn287]. Additionally, four C-terminal residues of the PKI-tide (LVAA) were missing, but the core binding motif (RTGRRQAI) is fully visible. Overall, 17 strong hydrogen-bond interactions mainly mediated by Arg8 and Arg9 stabilize PKI-tide binding. Additionally, 148 nonpolar contacts increase the affinity substantially (Fig. 1*c*). In the PKI-tide bound state the N- and C-lobes are in close proximity. The catalytic core domain adopts a closed conformation, as reported for all PKAc crystal structures with bound inhibitor peptides (Fig. 1*d*). The ATP-binding site is not accessible to substrates. The globular heterodimer consisting of 351 PKAc and 15 PKI-tide residues is surrounded by an extensive water network consisting of 585 water molecules.

3.3. Structural comparison of PKAc homologues

Sequence-based alignments of PKAc from different vertebrates demonstrate their high homology (Fig. 2*a*). PKAc from *C. griseus* (KAPCA_CRIGR) shows the following sequence identities to its homologues: mouse (*Mus musculus*; KAPCA_MOUSE), 98.6%; human (*Homo sapiens*; KAPCA_HUMAN), 98.3%; cattle (*Bos taurus*; KAPCA_BOVIN), 97.7%. Less surprisingly, the overall structure of PKAc reported here (PDB entry 4wih) was very similar to that of the nucleotide-free protein ternary complexes from mouse (PDB entry 4o22; Gerlits *et al.*, 2014) and human (PDB entry 3nx8; Behnen *et al.*, 2012). Both structure backbones align very well with hamster PKAc, with a root-mean-square deviation of 0.5 Å (Fig. 2*b*). Nevertheless, several structural differences can be observed. Besides the fully exposed N-terminal helix carrying a phosphorylation site (Ser11), hamster PKAc (Fig. 2*b*, blue) shows a slightly more closed conformation than human PKAc (Fig. 2*b*, green), while mouse PKAc (Fig. 2*b*, red) represents the most open conformation. The C^α–C^α distance between Leu83 of α -helix 2 and Pro244 of α -helix 8 (hamster, 15.5 Å; human, 16.8 Å; mouse, 17.9 Å) indicate movement of the N- and C-lobes. Further differences are located in the loop connecting α 1 and β 1. The course of the protein backbone of hamster PKAc does not align well with the others. Twofold to threefold increased *B* factors and a weak electron density suggest increased mobility of this region. The glycine-rich loop of human PKAc is tilted into the active site as a result of the binding of a phenol molecule in the ATP pocket. In the ligand-free structures from mouse and hamster the glycine-rich loops have a straight orientation (Fig. 2*b*, right). Hence, protein crystallization of hamster PKAc was performed with a truncated protein kinase inhibitor peptide in which the N-terminal PKI-tide α -helix was missing. Residues 1–4 interact differently with PKAc, leading to a different orientation of the PKI-tide N-terminus. This did not affect the kinase inhibition. With few exceptions, all residues of the further PKI-tide core binding motif align very well. Interestingly, the side chain of Arg8 interacts *via* hydrogen-bond contacts with the glycine-rich loop of human and mouse PKAc

Figure 2

(a) Amino-acid sequence alignment between PKAc from *C. griseus* (KAPCA_CRIGR), *M. musculus* (KAPCA_MOUSE), *H. sapiens* (KAPCA_HUMAN) and *B. taurus* (KAPCA_BOVIN) with secondary-structure annotation from PDB entry 4wih reprocessed with *ESPrpt* (Robert & Gouet, 2014). Identical residues are boxed in red and differences are marked in white. (b) Comparison of PKAc–PKI-tide complexes from *C. griseus* (PDB entry 4wih; PKAc, blue; PKI, cyan), *M. musculus* (PDB entry 4o22; PKAc, red; PKI, orange) and *H. sapiens* (PDB entry 3nx8; PKAc, green; PKI, yellow). The positioning of the phosphate-anchoring ‘glycine-rich loops’ is magnified in the circle. Structural alignments were performed with *PyMOL* (Schrödinger).

but not hamster PKAc. Here, Arg8 contacts the C-terminal tail loop.

4. Conclusion

The reported crystal structure of PKAc from *C. griseus* represents the current limit for high resolution of all available cAMP-dependent protein kinase crystal structures. In the context of further structural studies on small-molecule inhi-

bitor binding, we provide a prototype protein kinase model offering the highest possible resolution and the most detailed information about the water network. PKAc represents a tool kinase for future studies investigating the selectivity and affinity of kinase inhibitors by various biophysical techniques (crystallography, NMR, ITC and SPR). For a wide analysis of structure, solvation patterns, mobility and energetic aspects of ligand binding, the reported crystal structure of PKAc from *C. griseus* represents a very good starting point.

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