Supporting Online Material for

Structure and Allostery of the PKA RIIβ Tetrameric Holoenzyme

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Structure and Allostery of the PKA RIIβ Tetrameric Holoenzyme

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Supporting Online Material

Materials and Methods

Protein Purification. Since a major challenge for obtaining crystals of full length tetrameric PKA holoenzymes is likely due to the dynamic nature of the different domains, we generated two mutants of RIIβ. Specifically we replaced the essential Arg in each PBC with Lys. We then set up crystallization trials for RIIβ holoenzymes formed with each of these mutant RIIβ-subunits as well as with wild type RIIβ. Although the wild type construct and the B domain mutant were set up in parallel for crystallization, the R230K mutant was the only one that yielded well-diffracting crystals. The RIIβ(R230K) and RIIβ(R359K) mutants are generated by Quick change site-directed mutagenesis according to the Stratagene protocol. Small angle X-ray scattering showed no significant differences between the wild-type and mutant holoenzymes.

All RIIβ mutants were expressed in E. coli BL21 (DE3) (Novagen) and purified as described previously (19, 20) using cAMP resin and cGMP elution. RIIα(90-400), RIIβ(108-402), and RIIα dimers were expressed and purified according to the same protocol.

The C-subunit was expressed and purified as previously described (39). Peak I, which contains four phosphorylated residues (Ser\textsuperscript{10}, Ser\textsuperscript{139}, Thr\textsuperscript{197}, and Ser\textsuperscript{338}), was used for forming the holoenzymes.

Complex Formation. RIIβ and wild type C-subunit were mixed in a 1:1.2 molar ratio and spin dialyzed at 4°C into holoenzyme buffer containing 20mM MES, pH 5.8, 50mM NaCl and 1mM TCEP-HCl. The complex was then purified on Superdex 75 in the same buffer to remove excess C-subunit. The holoenzyme was concentrated to approximately 12 mg/ml for crystallization.

Crystallization. The RIIβ(R230K)\textsubscript{2}:C\textsubscript{2} holoenzyme complex was crystallized at room temperature in 10% PEG8000, 8% ethylene glycol, pH7.5 HEPES buffer by using vapor diffusion at a 1:1 ratio of protein to crystallization solution. Crystal quality was improved by maro-seeding. Crystals were flash frozen in cryo-protectant solution with mother liquor and
20% PEG8000. To generate the nucleotide bound holoenzyme crystals, the apo holoenzyme crystals were soaked in 10% PEG8000, 8% ethylene glycol, pH7.5 HEPES, and 10mM MgCl₂ and 5mM ATP solution overnight.

**Data collection and processing.** The best diffracting crystals (2.3Å) for the RIIβ(R230K)₂:C₂ holoenzyme were grown at room from macro-seeding and crystallization solution containing 30mM CH₂Cl₂ as additives. The best RIIβ(R230K)₂:C₂:(Mg₂:ADP)₂ holoenzyme crystal diffracted to 3.1Å. Datasets were collected at the Advanced Light Source beamline 8.2.2 and then processed using HKL2000 program. Initial phases were obtained using only the C subunit (PDB:1ATP) as a search model by the CCP4 program PHASER. The remainder of the model was manually built based on the density maps using Coot. The refinement was done using the REFMAC5 program in CCP4 and the simulated annealing process in PHENIX. Water molecules were added to the model at the final step of refinement. Final models were evaluated by PROCHECK.

**Small Angle Scattering Evaluation.** Evaluation of the Xray solution scattering curves was made by CRYSOL (40). Previously published SAXS data (24) were used for the space filling model for fig. S2 using GASBOR (41).

**Assay for cAMP Activation Constants (Ka).** Holoenzyme (10nM) was incubated for 2 min at room temperature in assay buffer that contained 1mM ATP and 10mM MgCl₂ as described by Cook et al.(42) with varying concentrations of cAMP ranging from 1nM to 100µM. The reaction was initiated by adding Kemptide (100µM), and the activity of the free C-subunit was followed using the coupled spectrophotometric assay.

**Interface between the two heterodimers.** The two interaction interfaces between the two heterodimers are strictly 2-fold rotational symmetry related. The β4-β5 loop’ interacts with two primary contact sites. It contacts the FDDY motif (figs. S3, A and B), which is part of the AST site in the C-tail of the symmetry-related C-subunit. This interface is formed by three
hydrogen bonds and extensive hydrophobic interactions between FDDY motif residues Asp$^{328C}$, Asp$^{329C}$, Tyr$^{330C}$ and β4-β5 loop residues Tyr$^{306R'}$, Val$^{306R'}$, Gly$^{207R'}$, Arg$^{208R'}$. In addition to its contacts with the C-subunit, the side chains of Arg$^{208R'}$, Val$^{210R'}$ and Phe$^{107R}$ contribute prominently to the hydrophobic interface between the β4-β5 loop and the N-linker of R (fig. S3C). There is also a hydrogen bond between Arg$^{208R'}$ and Phe$^{107R}$. N-linker residues Arg$^{106R}$ and Arg$^{110R}$ form network interactions with the conserved Arg$^{133C}$-Glu$^{230C}$ salt bridge (Fig. f3D). Replacing Arg$^{133C}$ with Ala decreases affinity of the C-subunit for RII (9).

PKA R subunits have two argine residues upstream of the Phosphorylation site (RRXSΦ). In the RIα and RIIα heterodimers, the side chain of the conserved P-3 site residue Arg$^{109R}$ faces towards the active site and interacts with Glu$^{127C}$, however, in the tetramer, it flips about 180° away from the active site and now interacts not only with Glu$^{127C}$ but also forms hydrogen bonds with the hydroxyl group of Tyr$^{330C}$ and salt bridges with Asp$^{328C}$ in the FDDY motif (fig. S4). These interactions likely facilitate the C subunit forming a closed conformation.
### Table S1: Data Collection and Refinement Statistics

<table>
<thead>
<tr>
<th>Data Set</th>
<th>RIIβ(^{2+}):C(_2)</th>
<th>RIIβ(^{2+}):C(_2):(Mg(_2)ADP)(_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data collection</strong></td>
<td>ALS beamline 8.2.2</td>
<td>ALS beamline 8.2.2</td>
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<tr>
<td>Space group</td>
<td>C2</td>
<td>C2</td>
</tr>
<tr>
<td>No. of molecules in one asymmetric unit</td>
<td>1</td>
<td>1/2</td>
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<tr>
<td>Cell constants</td>
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<td></td>
</tr>
<tr>
<td>a (Å)</td>
<td>151.96</td>
<td>153.82</td>
</tr>
<tr>
<td>b (Å)</td>
<td>213.27</td>
<td>212.75</td>
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<tr>
<td>c (Å)</td>
<td>61.63</td>
<td>61.95</td>
</tr>
<tr>
<td>α (°)</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>β (°)</td>
<td>90.4</td>
<td>90</td>
</tr>
<tr>
<td>γ (°)</td>
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<td>90</td>
</tr>
<tr>
<td>Average Redundancy</td>
<td>2.8</td>
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<tr>
<td>No. of unique reflections</td>
<td>82530</td>
<td>16119</td>
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<tr>
<td>Resolution (Å)</td>
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<td>3.1</td>
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<tr>
<td>(R_{\text{sym}})</td>
<td>0.062 (0.31)(^{1})</td>
<td>0.085 (0.49)(^{1})</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>95.4 (93.6)(^{2})</td>
<td>84.2 (86.9)(^{2})</td>
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<tr>
<td>I/(σ)</td>
<td>15.9 (3.4)(^{1})</td>
<td>19.8 (3.3)(^{1})</td>
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<tr>
<td><strong>Refinement</strong></td>
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<tr>
<td>Resolution (Å)</td>
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<td>40.0-3.1</td>
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<tr>
<td>(R_{\text{work}} \lor R_{\text{free}}) (%)</td>
<td>22.6/23.5</td>
<td>23.1/27.5</td>
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<tr>
<td>No. of protein atoms</td>
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<td>4899</td>
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<td>ligand and ion</td>
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<td>Mg(_2)ADP</td>
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<tr>
<td>No. of water</td>
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<td>14</td>
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<td>R.m.s. deviations</td>
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<tr>
<td>Bond lengths (Å)</td>
<td>0.010</td>
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<tr>
<td>Bond angles (°)</td>
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<tr>
<td>Average B-factor</td>
<td>50.6</td>
<td>56.21</td>
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<td><strong>Ramachandran angles (%)</strong></td>
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<tr>
<td>most favored (%)</td>
<td>95.5</td>
<td>88.9</td>
</tr>
<tr>
<td>disallowed</td>
<td>none</td>
<td>none</td>
</tr>
</tbody>
</table>

\(^{1}\)\(R_{\text{sym}} = \sum_\delta \sum_\ell |I(h) - \langle |I(h)| \rangle|/\sum_\delta \sum_\ell |I(h)|\), where \(|I(h)|\) is the mean intensity after rejections.

\(^{2}\)Numbers in parentheses correspond to the highest resolution shell of data.

\(|R_{\text{work}} = \sum_\delta |F_{\text{obs}}(h)| - |F_{\text{calc}}(h)|/\sum_\delta |F_{\text{obs}}(h)|\); no \(I/σ\) cutoff was used during refinement.

\(^{5.1}\)% of the truncated data set was excluded from refinement to calculate \(R_{\text{free}}\).
Table S2: Averaged B-factors for the N-and C-lobe of the C-subunit in different holoenzymes

<table>
<thead>
<tr>
<th></th>
<th>$B_N^*$</th>
<th>$B_C^*$</th>
<th>Ratio of $B_N$: $B_C$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIα(91–379):C: Mg$_2$AMPPNP</td>
<td>40.57</td>
<td>32.71</td>
<td>1.24:1</td>
</tr>
<tr>
<td>RIIα(90–400):C</td>
<td>76.83</td>
<td>34.81</td>
<td>2.21:1</td>
</tr>
<tr>
<td>RIIβ$_2^*$:C$_2$</td>
<td>52.12</td>
<td>41.33</td>
<td>1.26:1</td>
</tr>
<tr>
<td>RIIβ$_2^p$:C$_2$:(Mg$_2$ADP)$_2$</td>
<td>54.84</td>
<td>44.73</td>
<td>1.23:1</td>
</tr>
</tbody>
</table>

* $B_N$: Averaged B-factors of C-subunit small N-lobe (residues 40-126)
  $B_C$: Averaged B-factors of C-subunit large C-lobe (residues 127-300)
Figure Legends

Fig. S1. Stereoview of part of the RIIβ∗2:C2 structure in the 2.3 Å resolution 2Fo - Fc map.

Fig. S2. SAXS profiles of RIIβ2:C2 Tetrameric Holoenzyme. (A) The log(I) versus q plot. Data calculated from the RIIβ2:C2 tetrameric complex shown on the Fig. 1B is represented by continuous line. Black dots (●) represent experimental data (Materials and methods) for RIIβ2:C2 holoenzyme. (B) P(r) curves calculated from Xray scattering data (●) are compared with the data calculated from the crystal structure shown in black line.

Fig. S3. Interactions of the two RC heterodimers in RIIβ∗2:C2. (A) Sequence alignment of β4-β5 loops from different R subunits. (B) and (C) Interactions of the R' subunit to the neighboring RC heterodimer. (D) P-2 and P-6 Arginines form network interactions with Arg133C-Glu230C salt bridge.

Fig. S4. Interactions of P-3 Arg109R with FDDY motif of C-subunit. (A) In RIIβ∗2:C2 holoenzyme, P-3 Arg109R interacts with residues in FDDY motif of C-subunit. (B) and (C) in RIIα and RIIβ heterodimers, the side chain of P-3 Arg faces towards the active site.

Fig. S5. Overlay of RIIβP2:C2:(Mg2ADP)2 (black) and apo RIIβ∗2:C2 (gray). The Gly-rich loop in the phosphorylated structure (yellow) shifts about 5Å away from the P-site Ser112R.

Fig. S6. Multivalent membrane binding sites of RIIβ holoenzyme. Possible position of the D/D domain (red) bound to the AKAP (cyan) is illustrated using 3IM4 structure. The myristyl groups (yellow) are on the same side as the AKAP -binding surface of the D/D domain.

Fig. S7. Isoform-specific quaternary structures of RIIα2:C2 model (3PVB) and RIIβ∗2:C2 structure. The right-side RC heterodimers of these two holoenzymes are aligned; however, the overall quaternary structures are different.
Figure S1
Figure S2
Figure S3
Figure S4
Figure S5
Figure S6
Figure S7

RIα Holoenzyme

RIIβ Holoenzyme
References


25. W. T. Heller et al., C subunits binding to the protein kinase A RI alpha dimer induce a large conformational change. J. Biol. Chem. 279, 19084 (2004). doi:10.1074/jbc.M313405200 Medline


