Sensing Domain Dynamics in Protein Kinase A-Ιαx Complexes by Solution X-ray Scattering*

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The catalytic (C) and regulatory (R) subunits of protein kinase A are exceptionally dynamic proteins. Interactions between the R- and C-subunits are regulated by cAMP binding to the two cyclic nucleotide-binding domains in the R-subunit. Mammalian cells express four different isoforms of the R-subunit (RIα, RIβ, RIα, and RIIB) that all interact with the C-subunit in different ways. Here, we investigate the dynamic behavior of protein complexes between RIα and C-subunits using small angle x-ray scattering. We show that a single point mutation in RIα, R333K (which alters the cAMP-binding properties of Domain B) results in a compact shape compared with the extended shape of the wild-type R-C complex. A double mutant complex that disrupts the interaction site between the C-subunit and Domain B in RIα, RαB/C helices (K285P) results in a broader Pr(r) curve that more closely resembles the Pr(r) profiles of wild-type complexes. These results together suggest that interactions between Rα Domain B and the C-subunit in the RαC complex involve large scale dynamics that can be disrupted by single point mutations in both proteins. In contrast to RIα-C complexes, Domain B in the RIIB/C heterodimer is not dynamic and is critical for both inhibition and complex formation. Our study highlights the functional differences of domain dynamics between protein kinase A isoforms, providing a framework for elucidating the global organization of each holoenzyme and the cross-talk between the R- and C-subunits.

The catalytic subunit (C) contains two cAMP-binding domains, and regulatory subunits (R) contain an N-terminal dimerization/docking domain and a C-terminal inhibitory domain. Binding of two cAMP molecules to each R-subunit releases two catalytically active C-subunits. Binding of two cAMP molecules to each R-subunit releases two catalytically active C-subunits.

There are four PKA R-subunit isoforms (RIα, RIβ, RIα, and RIIB), and all share the same domain organization. All of the R-subunits contain an N-terminal dimerization/docking domain that interacts with anchoring proteins, an inhibitor sequence that binds to the C-subunit active site, and two tandem cAMP-binding domains (designated Domains A and B). Despite these commonalities, each isoform differs in its cellular and tissue-specific distribution, abundance, sequence, structure, and biochemical properties. Moreover, each isoform is functionally nonredundant. Only RIα subunits are embryonically lethal (4, 5), and mutations in RIα are associated with Carney complex (6–8) and systemic lupus erythematosus (9, 10). Given the biological differences between the PKA isoforms and their relevance to disease, it is important to characterize their molecular properties more rigorously to better understand their functional differences.

The PKA-Iα holoenzyme crystal structure described by Kim et al. (11) illuminated the detailed interactions between the C- and RIα-subunits and elucidated a molecular mechanism for the ordered and cooperative activation of PKA by cAMP. Both the structure and mutational data highlighted the role of Domain B as the gatekeeper domain for Domain A. cAMP first binds to Domain B, triggering a conformational change that enables cAMP to bind to Domain A. This second cAMP binding step releases the active C-subunits (12). The first conformational change in RIα involves reorientation of the two cAMP-binding domains, induced by structural changes in the αB and αC helices connecting Domains A and B (Fig. 1). In the C-subunit-bound conformation, the αB and αC helices combine to form a single contiguous helix (αB/C), producing an overall dumbbell-shaped structure where the two domains are separated (Fig. 1A, left panel). In the cAMP-bound conformation, the αB/C helix is divided into three sections, producing an overall compact globular structure where the two domains share a common interface (Fig. 1A, right panel). Fig. 1B compares the changes in the αB and αC helices when RIα shuttles between the cAMP-bound and C-subunit-bound conformations. In the cAMP-bound conformation, the αB and αC helices form distinct segments with residues Gly230 and Tyr244 serving as two hinge points. In contrast, these same two residues are engaged to form the continuous helix in the C-subunit-bound conformation. These structural comparisons highlight the dynamic nature of the two domains within the Rα subunit and show that the conformational transition between the active (cAMP-
FIGURE 1. A, conformational change of RII upon binding to the C-subunit (left) and cAMP (right). The connecting αβ and αC helices are shown in red, and the two hinge points are highlighted with yellow spheres. Domain B is rendered in surface representation for clarity. B, left, overlay of αβ and αC helices in the C-subunit and cAMP-bound conformations. Right, the phosphate-binding cassette highlighting the role of a critical arginine (Arg209 for Domain A and Arg333 for Domain B) binding to the phosphate of cAMP. The phosphate-binding cassette is colored in yellow in A and B.

bound) and inactive (C-subunit-bound) states consists of large scale domain motions around two hinge points.

During efforts to crystallize the PKA-Iα holoenzyme complex, parallel crystallization trials were set up for three RII deletion mutants (RIIAB, RIIABR209K, and RIIABR333K, where RIIAB refers to the truncated form of RII containing both cAMP-binding domains) in complex with the C-subunit. Only the holoenzyme formed with RIIAB333K produced crystals that diffracted to 2.2 Å. Arg333 is essential for high affinity binding of RII to cAMP in Domain B (the corresponding residue in Domain A is Arg209). This residue is located within the phosphate-binding cassette of RII (residues 199–211 and 323–335 for Domains A and B, respectively), and its side chain forms a hydrogen bond with the cAMP phosphate (Fig. 1B, right panel). The R333K mutation was first identified in S49 mouse lymphoma cells (13) and was shown to diminish the ability of PKA to respond to cAMP (14). Our study addresses the effects of the R333K mutation on the global architecture of the RII-C complex in solution.

Previous small angle x-ray scattering (SAXS) data showcased differences in the overall shape of holoenzymes formed with truncated RI- and RII-subunits (15–17). In these R-subunit constructs, the N-terminal dimerization domains were removed so that only R-C heterodimers were monitored. The RII-C and RIIβ-C heterodimers formed compact, globular particles, whereas the RII-C heterodimer exhibited a more elongated shape that produced a P(r) curve with a broad shoulder in the high r region. Although the source of the extended tail has not been unequivocally determined, we hypothesize that it is due to extension of a highly dynamic Domain B in RII (18).

Here, we focus on four main questions: 1) In the RII P(r) curve, is the shoulder at the high r region due to Domain B? 2) Is mutagenesis, biochemical analysis, and small angle x-ray scattering to better understand the role of Domain B dynamics in PKA-Iα complexes. The SAXS results reported here show that heterodimers formed with the RIIABR333K mutant display a different SAXS profile from wild-type RIIAB and that these differences stem from Domain B. Our data also indicate that in RII, Domain B is dynamic even when complexed with the C-subunit. In contrast to RIIβ where Domain B is essential for C-subunit interactions, the interaction of Domain B from RII with the C-subunit is dispensable for holoenzyme formation.

EXPERIMENTAL PROCEDURES

Protein Preparation—The catalytic subunit was expressed and purified in Escherichia coli BL21 (DE3) cells (Novagen) as described previously (19). Expression and purification of the C-subunit mutant K285P followed the same protocol. All of the RII proteins (RIIA, RIIAB, RIIABR209K, and RIIABR333K, where RIIAB refers to constructs corresponding to residues 91–244 and 91–379, respectively) were expressed and purified as described previously (20).

Holoenzymes of RII mutants (RIIA, RIIAB, RIIABR209K, and RIIABR333K) were formed by mixing each R-subunit with wild-type C-subunit in a 1:1.2 molar ratio and then dialyzing overnight at 4 °C in 10 mM MOPS (pH 7.0), 50 mM NaCl, 2 mM MgCl2, 2 mM dithiothreitol, and 0.5 mM ATP. Each holoenzyme complex was separated from excess C-subunit by gel filtration chromatography. Care was taken to concentrate samples immediately prior to SAXS data collection because these complexes were extremely prone to aggregation at concentrations higher than 5 mg/ml.
**SAXS Data Collection**—Small angle x-ray scattering measurements were collected at the University of Utah with an Anton Paar SAXSess instrument with line collimation and an image plate detector. The protein samples were concentrated to 2–5 mg/ml and filtered (0.22 μm) immediately prior to data collection. Scattering data for protein samples and their respective solvent blanks were collected in a 1-mm diameter quartz capillary with a 10-mm beam slit at 12 °C.

**SAXS Data Analysis**—Normalized buffer subtraction and data reduction to \( I(q) \) versus \( q \) (where \( q = (4\pi\sin\theta)/\lambda, 2\theta \) is the scattering angle, and \( \lambda \) is the wavelength of radiation, 1.54 Å) were performed with the program SAXSquant1D (Anton-Paar, Austria). Radius of gyration \( (R_g) \) and zero angle scattering \( (I(0)) \) parameters were calculated using both GNOM (21) and by Guinier analysis with the program PRIMUS (22). Inverse Fourier transform calculations of \( I(q) \) to yield \( P(r) \) functions, \( I(0) \), \( R_g \), and the maximum dimension \( (D_{max}) \) were carried out using a \( q \) range of 0.013–0.17 1/Å. CRYSSOL was used to calculate theoretical scattering intensity from Protein Data Bank coordinates of R-C crystal structures (23). The figures were made in PyMOL (DeLano Scientific LLC, San Carlos, CA).

**Amino Acid Analysis**—Amino acid composition of each R-C complex was determined after SAXS data collection to accurately quantify protein concentrations. Amino acid analysis was performed by Dr. Dennis Winge at the University of Utah. The molecular weight (MW) of each complex was determined experimentally with the relation \( I(0) = \propto MW^{2c}, \) where \( I(0) \) is the scattering at zero angle, \( \propto \) is a proportionality constant, and \( c \) is the concentration as determined by amino acid analysis. \( I(0) \) was determined by GNOM, and the proportionality constant, \( \propto \), was determined using lysozyme as a protein standard.

**Structural Models**—Ab initio shape restoration with the program DAMMIF (24) was used to generate three-dimensional structures from the one-dimensional scattering data of all seven PKA complexes. No symmetry constraints were applied, and default parameters were used in each calculation. Ten independent models for each heterodimer complex were generated, then aligned, and averaged using the program DAMAVER (25). DAMMIF models for RIAαC and RIAαB-R333K-C complexes were aligned with their respective crystal structure coordinates (Protein Data Bank codes 3FHI and 2QCS, respectively) using the program SUPCOMB (26).

**Determination of Binding Kinetics by Surface Plasmon Resonance**—Binding experiments were performed on a Biacore 3000 instrument (GE Healthcare). Purified wild-type and K285P mutant C-subunits were diluted to 10–50 μg/ml in 10 mM potassium phosphate buffer (pH 6.0), 20 mM NaCl, 1 mM ATP, and 10 mM MgCl₂. These proteins were immobilized onto CM5 Sensor Chips (GE Healthcare) via amine coupling to a ligand density response of ~300 response units (RU). Dilutions of RIAαB, ranging from 0.5 to 250 nM were prepared in 10 mM HEPES (pH 7.4), 150 mM NaCl, 0.005% (v/v) surfactant P-20 (HBS-P buffer; GE Healthcare), 1 mM dithiothreitol, 1 mM ATP, and 10 mM MgCl₂. Each RIAα concentration was injected into the channels at a flow rate of 50 μl/min. The surface was regenerated with 200 μM CAMP and 2 mM EDTA in HBS-P buffer. Sensograms were analyzed using Bioevaluation software version 4.1 (GE Healthcare) to obtain kinetic parameters. Sensogram curves were fit to a 1.1 binding model.

**Inhibition of C-subunit by RIAα**—The inhibition assay was carried out as described earlier (27). Briefly, C-subunit wild-type or K285P mutant proteins were incubated with varying concentrations of RIAα for 30 min at 30 °C. Each reaction mix contained 0.5–1 mM of the substrate peptide Kemptide (LRRASLG), 1–2 nM C-subunit, and 0.5 mM ATP. R-subunit was serially diluted to final concentrations ranging from 0.5 to 500 nM. The reactions were initiated with a mixture of Kemptide, ATP, and \([γ-32P]ATP, \) allowed to proceed for 20 min at 30 °C, and then quenched with 30% acetic acid. Free \([γ-32P]ATP \) was separated from protein-bound radioactivity by ascending chromatography on phosphocellulose p81 Whatman paper as described previously (28). Phosphate incorporation into Kemptide was detected by Cerenkov counting on a Beckman LS 6000SC liquid scintillation system. Curve fitting and IC₅₀ calculations were performed using GraphPad Prism 5 software (GraphPad, San Diego, CA).

**RESULTS**

SAXS data were collected for seven PKA-1α holoenzyme complexes formed with a combination of four different Rα mutants (termed RIAα, RIAαB, RIAαBR333K, and RIAαBR209K) and two C-subunit proteins (wild type and K285P mutant; the RIAαBR209K-C(K285P) complex was not examined). To study the contribution of the two cAMP-binding domains on PKA-1α dynamics, we used RIA constructs RIAα91–244 and RIAα91–329, which lacked the N-terminal dimerization domain and the linker region. The minimal sequence required to form a high affinity complex with the C-subunit includes the inhibitor sequence region (residues 91–100) and Domain A (residues 120–244). The SAXS intensity plots of \( I(q) \) versus \( q \), Guinier plots for all samples were linear at the low \( q \) range, indicative of monodisperse solutions with no presence of nonspecific aggregation during data acquisition. Because each sample contained monodisperse particles, these data were suitable for structural analysis. The intensity curves were fit, and inverse Fourier transforms were performed using the program GNOM (21) to generate \( P(r) \) curves.

**RIA Heterodimers**—To assess the general shape characteristics of RIAαB-C heterodimers containing both Domains A and B, we measured the SAXS profiles of RIAαB and wild-type C-subunit complexes (Fig. 2A). The \( P(r) \) functions for RIAαB-C are similar to previous data from our laboratory (16), where a single peak with a maxima at 35 Å is followed by a long extended range. In the present experiments, the maximum dimension, \( D_{max} \) (105 Å), and \( R_g \) values (29.4 ± 0.1 and 32.4 ± 0.8 Å, from Guinier and GNOM analysis, respectively) are somewhat smaller than previously reported (\( D_{max} = 120–130 \) Å and \( R_g \) values = 36.4–38.6 Å) (16). These differences are most likely due to differences in sample handling or variations from one preparation to the next as was noted in our previous report (16). In the present study, we were careful to concentrate samples immediately prior to data collection to minimize possible aggregation.
Effect of Deleting Domain B—To assess the contribution of Domain B to the overall shape of the heterodimer, we measured the x-ray scattering of a complex formed with RI\textsubscript{H9251}\textsubscript{AB} containing only one of the two cAMP-binding domains (RI\textsubscript{H9251}\textsubscript{A}). This protein is the smallest fragment of RI\textsubscript{H9251}\textsubscript{AB} that still binds to both cAMP and the C-subunit with high affinity. The resulting \( P(r) \) function resembles a spherical, globular-shaped particle with a symmetrical Gaussian curve and no indication of the broad shoulder at the high \( r \) region detected in the RI\textsubscript{H9251}\textsubscript{AB} complexes (Fig. 2B). The \( D_{\text{max}} \) measured by SAXS was 70 Å, consistent with the maximum dimensions determined from the crystal structure solved with the same constructs (29). In addition to differences in the \( P(r) \) curve, both the \( D_{\text{max}} \) and \( R_g \) values for RI\textsubscript{A} are smaller than for RI\textsubscript{AB} (Table 1). Thus, not only does Domain B contribute to the overall size of the complex, it also contributes to the broad shoulder observed in the RI\textsubscript{AB} \( P(r) \) function.

Effect of R333K on RI\textsubscript{A} Heterodimers—In contrast to the extended \( P(r) \) curve observed with the wild-type RI\textsubscript{AB}\textsubscript{C} heterodimer, the \( P(r) \) function for the RI\textsubscript{AB}R333K-C heterodimer displays a Gaussian distribution typical of a globular, spherical particle (Fig. 3A). GNOM analysis reveals a \( P(r) \) function with a peak maximum at 35 Å that goes to zero at a \( D_{\text{max}} \) of 83 Å and an \( R_g \) value of 28.4 ± 0.4 Å (the \( R_g \) was estimated to be 28.3 ± 0.9 Å by Guinier analysis). The extended tail at the high \( r \) region in the RI\textsubscript{AB}R333K-C heterodimer data is not detected in RI\textsubscript{AB}R333K-C. Both the \( D_{\text{max}} \) and \( R_g \) values are smaller for RI\textsubscript{AB}R333K-C complexes compared with RI\textsubscript{AB} heterodimers without the R333K mutation. Moreover, PKA heterodimers formed with RI\textsubscript{AB}R333K
resemble the more compact shapes observed with RII heterodimers (16).

The crystal structure of the PKA-\(\alpha\) complex was solved using the R333K mutant, R\(\alpha\)A,R333K-C. We hypothesized that the R333K mutation trapped the R-subunit into a compact conformation enabling favorable crystal packing necessary to achieve crystals that diffract to \(\sim\)3 Å. To assess the validity of the crystallographically observed conformation in light of our solution scattering studies, we calculated the theoretical scattering intensities from atomic coordinates of the R\(\alpha\)A,R333K-C structure (Protein Data Bank code 2QCS) using the program CRYSOL (23) and generated a \(P(r)\) function based on this theoretical scattering data. The experimental and calculated \(P(r)\) curves are both very symmetrical without any significant tail at long \(r\) values (Fig. 4). Furthermore, both \(D_{\text{max}}\) and \(R_g\) values are in excellent agreement between the two data sets. Clearly, the experimental and calculated SAXS data closely overlap, confirming that the overall shape of the R\(\alpha\)A,R333K-C complex measured in solution is very consistent with what is observed in the crystal structure.

Based on the SAXS data of wild-type and R\(\alpha\)A,R333K-C heterodimers, the overall shape of these complexes are undoubtedly distinct (Fig. 3A). Although the \(P(r)\) functions of wild-type R\(\alpha\)A\C heterodimers have a broad tail at long vector lengths, both experimental and calculated \(P(r)\) functions of R\(\alpha\)A,R333K-C heterodimers do not. The shoulder originates from Domain B because removal of this entire domain in R\(\alpha\)A\C complexes yield symmetrical \(P(r)\) curves. Clearly, the overall shape of the wild-type heterodimer in solution as observed by SAXS is not consistent with the static image presented in the R\(\alpha\)A,R333K-C crystal structure. In solution, the wild-type R\(\alpha\)A\C complex likely exists as an ensemble of conformational states ranging from compact to extended. The conformation observed in the crystal structure represents only one of many possible states available for the wild-type R\(\alpha\)A\C heterodimer in solution. We propose that when bound to the C-subunit, the R333K mutation pushes the equilibrium state of R\(\alpha\) toward a dumbbell shape (Fig. 1A, left panel), resulting in a very compact R-C complex. The SAXS data suggest that in the absence of the R333K mutation, R\(\alpha\) is more dynamic and spends a significant fraction of time in an extended conformation.

**Effect of R209K on R\(\alpha\) Heterodimers**—The R333K mutation impairs cAMP binding to Domain B, and the corresponding mutation for Domain A is R209K. To test whether this mutation also affects the dynamics of the R- and C-subunit interaction, we measured the x-ray scattering of R-C heterodimers formed with R209K. Unlike the R333K mutation, the \(P(r)\) function for R209K-C shows little difference in the overall shape compared with the wild-type R-C heterodimer (Fig. 3B). The \(P(r)\) function has a maximum peak at 34.7 Å, a \(D_{\text{max}}\) of 105 Å, and an \(R_g\) value of 34.6 \(\pm\) 0.4 Å (33.5 \(\pm\) 0.9 Å from Guinier analysis; Table 1). Clearly, the compaction of the R-C complex is specific only to the R333K mutation in Domain B.

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**TABLE 1**

Summary of SAXS data for PKA-\(\alpha\) heterodimers

<table>
<thead>
<tr>
<th>R-subunit</th>
<th>C-subunit</th>
<th>(R_g) Derived*</th>
<th>(R_g) Calculated†</th>
<th>(D_{\text{max}}) ‡</th>
<th>(V^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R(\alpha)A</td>
<td>C</td>
<td>29.4 (\pm) 0.1</td>
<td>32.4 (\pm) 0.8</td>
<td>105</td>
<td>157 (\times) 10²</td>
</tr>
<tr>
<td>R(\alpha)A</td>
<td>C(K285P)</td>
<td>32.8 (\pm) 0.04</td>
<td>34.1 (\pm) 0.2</td>
<td>106</td>
<td>175 (\times) 10²</td>
</tr>
<tr>
<td>R(\alpha)A</td>
<td>C</td>
<td>26.5 (\pm) 0.7</td>
<td>25.7 (\pm) 0.2</td>
<td>70</td>
<td>92 (\times) 10²</td>
</tr>
<tr>
<td>R(\alpha)A</td>
<td>C(K285P)</td>
<td>27.3 (\pm) 0.6</td>
<td>26.4 (\pm) 0.2</td>
<td>72</td>
<td>116 (\times) 10²</td>
</tr>
<tr>
<td>R(\alpha)A,R333K (experimental)</td>
<td>C</td>
<td>28.3 (\pm) 0.9</td>
<td>28.4 (\pm) 0.4</td>
<td>83</td>
<td>133 (\times) 10²</td>
</tr>
<tr>
<td>R(\alpha)A,R333K (calculated)</td>
<td>C</td>
<td>28.8 (\pm) 0.008</td>
<td>28.8</td>
<td>83</td>
<td>NA*</td>
</tr>
<tr>
<td>R(\alpha)A,R333K</td>
<td>C(K285P)</td>
<td>32.6 (\pm) 0.6</td>
<td>31.44 (\pm) 0.3</td>
<td>105</td>
<td>158 (\times) 10²</td>
</tr>
<tr>
<td>R(\alpha)A,R209K</td>
<td>C</td>
<td>33.5 (\pm) 0.9</td>
<td>34.6 (\pm) 0.4</td>
<td>105</td>
<td>155 (\times) 10²</td>
</tr>
</tbody>
</table>

* Derived using Guinier approximation.
† Calculated using the program GNOM.
‡ \(V^d\) is the molecular volumes calculated from DAMMIF. The expected dry volumes are calculated based on molecular mass and a partial specific volume of 0.073 cm³/g. Hydration layer effects are expected to give rise to increases in the experimentally derived volumes.

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**FIGURE 4.** Calculated scattering intensity from the R\(\alpha\)A,R333K-C heterodimer crystal structure. A, calculated scattering intensity data using Protein Data Bank code 2QCS coordinates with the program CRYSOL (gray). The experimental data are shown in black. B, \(P(r)\) functions of the experimental (solid squares) and calculated (open diamonds) data of R\(\alpha\)A,R333K-C heterodimers.
Effect of the K285P Mutation on Rα Heterodimers—The PKA-α holoenzyme crystal structure defined a novel interaction site between Domain B of Rα and a short S-shaped loop (αH-αl loop) in the C-subunit (see Fig. 6, A and B) (11). The surface of each protein precisely complements the other at this site. Specifically, Domain B docks onto the C-subunit through side chain interactions between Asp276C/Arg352R and Thr278C/Arg355R in addition to backbone interactions between Lys285C and both Arg355R and Leu357R. Mutation of Lys285 to proline reduced the ability of RIIβ to inhibit C-subunit activity (27), providing biochemical evidence that Domain B (in the R-subunit) and αH-αl loop (in the C-subunit) interaction site is important for at least the RIIβ isoform. Bioinformatics analysis has also pinpointed a short segment within the αH-αl loop (Gly282–Gly286) to be a conserved AGC kinase-specific insert (Fig. 6B, left panel) whose function has yet to be fully elucidated (31).

To specifically block Domain B in Rα from interacting with the C-subunit, we utilized the C-subunit mutation, K285P. To assess the effect of the K285P mutation on the solution structure of the RαABC heterodimer complex, we compared the x-ray scattering profiles of complexes formed with wild-type and mutant K285P C-subunits. Indeed, the P(r) functions of both RαABC and RαABC(K285P) complexes show very similar profiles where a single peak is followed by a long extended tail at the high r region (Fig. 2A). The maximum dimension of each complex was roughly the same (105 and 106 Å for wild-type and K285P C-subunits, respectively). Furthermore, the Rg values were similar for both proteins (32.4 and 34.1 Å for wild type and K285P, respectively).

We predicted that the x-ray scattering of R-C heterodimers formed with the RαC protein and the K285P mutant would be identical to wild-type C-subunit because the absence of Domain B in RαC eliminates the R-subunit interaction site with Lys285 in the C-subunit. Indeed, the P(r) functions are indistinguishable for RαC complexes formed with wild-type and K285P C-subunits (Fig. 2B).

For the RαABR333K-C heterodimer, we investigated whether the compact nature of the complex would be disrupted by interfering with the interaction site between Domain B and the large lobe of the C-subunit using the C(K285P) mutant. Indeed, the RαABR333K-C(K285P) double mutant complex no longer exhibits the compact symmetric P(r) curve observed with RαABR333K-C (Fig. 3A). Instead, the P(r) curve exhibits an extended tail at the high r region, similar to but not as pronounced as in the wild-type R-C heterodimer. Comparison of Dmax and Rg parameters between wild-type and K285P heterodimers show remarkable overlap. Both the double mutant complex and wild-type heterodimer have a Dmax of ∼105 Å, and the Rg of the double mutant is 31.4 ± 0.3 Å compared with 32.4 ± 0.8 Å for RαABC. SAXS scattering parameters for all wild-type and mutant R-C heterodimer complexes are summarized in Table 1. The inability of the K285P C-subunit mutant to fully recapitulate the solution structure of the wild-type R-C heterodimer probably indicates that the RαABR333K mutant still retains some effect on R-C interactions that prevents Domain B from exploring the full conformational space accessible to the wild-type RαABC complexes.

### TABLE 2

<table>
<thead>
<tr>
<th>R-subunit</th>
<th>C-subunit</th>
<th>Kd</th>
<th>Km</th>
<th>Kd</th>
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</thead>
<tbody>
<tr>
<td>RαAB</td>
<td>C</td>
<td>91.9 x 10^-7</td>
<td>8.44 x 10^-4</td>
<td>0.9</td>
</tr>
<tr>
<td>RαAB</td>
<td>C(K285P)</td>
<td>90.6 x 10^-7</td>
<td>4.43 x 10^-4</td>
<td>4.9</td>
</tr>
<tr>
<td>RIIβAB</td>
<td>C</td>
<td>11 x 10^-7</td>
<td>3.16 x 10^-4</td>
<td>2.9</td>
</tr>
<tr>
<td>RIIβAB</td>
<td>C(K285P)</td>
<td>11 x 10^-7</td>
<td>123 x 10^-4</td>
<td>111</td>
</tr>
</tbody>
</table>

Biochemical Analysis of Rα and RIIβ Heterodimers—The previous data show that the K285P mutant played a pivotal role in RIIβ inhibition of the C-subunit; however, the R-C binding constants were not measured (27). Given the differences in SAXS profiles between RαABC and RIIβABC heterodimers, we postulated that the K285P mutation would not affect either the inhibition or binding affinities between Rα and the C-subunit.

Surface plasmon resonance was used to measure the binding constants between the R- and C-subunits. C-subunits were immobilized to CM5 chips by amine coupling, and RαAB was flowed across the chip surface. Compared with wild-type C-subunit, the K285P mutant exhibited a modest 5-fold decrease in binding affinity between the RαAB and C-subunits. The Kd was 4.9 nm for the K285P complex compared with 0.9 nm for the wild-type R-C complex (Table 2). In contrast, the K285P mutation caused a 38-fold decrease in binding affinity for the RIIβC heterodimer, where the Kd was 2.9 and 111 nm between RIIβ and wild-type C-subunit and C(K285P) mutant, respectively. The difference between the binding constants is solely attributed to differences in the dissociation rate.

We also used a 32P incorporation protein kinase assay to measure the ability of Rα to inhibit both wild-type and mutant C-subunits. There was no statistically significant difference between wild-type and K285P C-subunit inhibition by Rα, where the IC50 was 2 ± 1 and 3 ± 1 nm, respectively (Fig. 5).

### DISCUSSION

Proteins are in continuous motion, constantly sampling different conformations. Although crystallography provides high resolution information, this technique does not guarantee that the conformational state of the crystallized protein is representative of the predominant conformational state under physiological conditions and can be occasionally plagued by artificial conformations induced by crystal packing. To fully understand the intrinsic behavior of Rα-C complexes, solution techniques must also be utilized. In this study, we analyzed PKA-α heterodimers using a combination of SAXS and biochemical analysis to investigate domain dynamics within the protein complexes. SAXS is highly advantageous in that it provides low resolution structural information under a variety of solution-based physiological conditions and can be used to obtain size and shape information of large, highly dynamic proteins and protein complexes. Here, we compare our results to previous high resolution crystallographic data to obtain a more complete understanding of the large scale conformational dynamics of PKA. To explore the dynamics of Domain B in PKA-α holoenzyme complexes, we measured the equilibrium
Domain Dynamics of PKA-Iα

![Graph](image)

**FIGURE 5. Inhibition of wild-type and K285P C-subunits by RIα.** Purified wild-type and K285P C-subunits were incubated with various concentrations of RIα-subunits. Catalytic activity was determined by measuring Kemptide phosphorylation via radioisotope [γ-32P]ATP labeling. The data for wild-type C-subunit are shown as solid squares, and K285P mutant C-subunits are shown as open squares. The experiments were done in triplicate. The data for RIβ are taken from Ref. 27 (asterisks).

R-C dissociation constants, the inhibition of the C-subunit by the R-subunit, and the overall shape of various mutant RIα-C complexes by small angle x-ray scattering. The most pronounced result of our study is that a single point mutation, K285P, alters the solution structural properties of PKA-Iα holoenzymes, presumably through effects on large scale domain motions of Domain B. The calculated x-ray scattering curve of the RIαABR333K-C crystal structure is very consistent with our experimental SAXS results, indicating that the solution conformation of the RIαABR333K-C heterodimer is very similar to what is observed in the RIαABR333K-C crystal structure. Our SAXS data clearly indicate a large difference in the overall molecular shape between wild-type RIαABR333K and RIαABR333K-C heterodimers. Differences in these solution structures likely arise from variations in domain dynamics within the two complexes, particularly with respect to Domain B. We postulate that Domain B in the RIαABR333K-C heterodimer forms a stable interaction with the C-subunit via the αH-α loop, but in wild-type RIα-C, Domain B interactions with the C-subunit are more transient, giving rise to the shoulder observed in the P(r) profiles.

The RIαABR333K-C crystal structure revealed a previously unidentified single contact point between the αB helix in Domain B of RIα and the αH-α loop in the C-subunit (Fig. 6A). We examined whether the interaction between the C-subunit αH-α loop and the RIα Domain B observed crystallographically remains stable in solution or whether Domain B might have a more dynamic behavior in the PKA-Iα complex. Lys285 in the C-subunit αH-α loop operates as a helix cap for the R-subunit αB helix (Fig. 6B, right panel), so we utilized the C-subunit mutation K285P as a strategy for disrupting the R-C interaction at the Domain B interface. K285P was first identified in a yeast genetic screen (32) and was shown to reduce the ability of RIβ-β subunits to inhibit C-subunit catalytic activity (27). If the interaction between Domain B (in RIα) and αH-α loop (in the C-subunit) interface is stable as observed in the crystal structure, the K285P mutation would be expected to affect the overall shape characteristics in the RIαABR333K-C complex. In contrast, if the Domain B interaction with the C-subunit in wild-type RIαABR333K complexes is not stable, the K285P should not affect the binding affinities or inhibition. Indeed for RIαABR333K(K285P) complexes, we found that K285P did not affect the general size or shape characteristics (Fig. 2) or inhibition by RIα (Fig. 5) and had only modest effects on the binding affinity (Table 2).

The only differences between K285P and wild-type C-subunit were seen in complexes formed with the RIαABR333K mutant. The P(r) curve for the RIαABR333K-C curve for the RIαABR333K-C shows that it assumes the most compact shape of all the RIα complexes, which is presumably why crystallization of this complex (and not the wild type) was successful. SAXS analysis of RIαABR333K-C(K285P) reveals a P(r) curve with a slightly extended tail at the high r region, but not to the large extent observed in RIαABR333K-C complexes. In other words, although the K285P mutation partially disrupted the interaction between Domain B and the C-subunit, it did not completely restore the full dynamic range of the wild-type heterodimer, suggesting that the average conformational state of Domain B in the RIαABR333K-C(K285P) complex is intermediate between the fully compact form observed in the RIαABR333K-C crystal and solution structures and the more extended RIαABR333K-C heterodimer solution structure.

The RIαABR333K-C crystal structure highlights two additional ion pairs at the Domain B site: Asp278C:Arg355R and Thr278C:Arg355R (Fig. 6B, right panel). These two interactions may partially compensate for the release of the Lys285C/Arg355R interactions, thereby allowing for transient stabilization of Domain B against the C-subunit. The shoulder at 90–105 Å observed in the RIαABR333K-C P(r) curve likely stems from Domain B because the P(r) function becomes completely symmetrical upon removal of Domain B entirely in the RIαAB333K-C complex (Fig. 2B). Given that the shoulder in the P(r) curve is only a small component, and the first peak at ~35 Å is the dominant component, the RIαABR333K-C complex is most likely a dynamic ensemble of conformational states where Domain B is mostly associated with the C-subunit but is detached from the C-subunit in a small fraction of complexes at any given time. This hypothesis is consistent with hydrogen-deuterium exchange mass spectrometry data where only 1 of 11 amides are protected in the αH-α loop of the RIαABR333K-C complex (33).

Our SAXS results indicate that Domain B in RIα explores a large conformational space when bound to the C-subunit. Although the precise motion of Domain B within RIα-C complexes is not clear, we speculate that the flexibility stems from a hinge motion in some portion of the αB/C helix that connects the two CAMP-binding domains (Fig. 1). Analysis of various crystal structures demonstrates that RIα can adopt a large range of conformations. When RIα is bound to

<table>
<thead>
<tr>
<th>R-subunit</th>
<th>C-subunit</th>
<th>IC50 (nM)</th>
</tr>
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<tbody>
<tr>
<td>RIαAB</td>
<td>C</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>RIαAB</td>
<td>C (K285P)</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>RIβAB</td>
<td>C</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>RIβAB</td>
<td>C (K285P)</td>
<td>16 ± 1</td>
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- **R-subunit** refers to the regulatory subunit.
- **C-subunit** refers to the catalytic subunit.
- **IC50** is the inhibitory concentration at 50% effect.

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the C-subunit, the αB and αC helices are extended into one contiguous helix. When Rlα is bound to CAMP, the same
αB/αC helix is divided into three distinct helices separated
by two bends at Gly235R and Tyr244R. There is a 22 Å differ-
ence in the maximum dimension between the most compact
(RlαABR333K/C) and the most extended (RlαAB/C) het-
erodimer, most likely because of hinge motions at the
Gly235R position. Gly235R forms a hinge point at the C termi-
nus of the αB helix (residues 226–235). This αB helix forms
a hydrophobic interface with the C-subunit through Leu233R
and Met234R in the R-subunit, and mutation of either residue
permits activation of holoenzymes at lower CAMP concen-

FIGURE 6. Model of Domain B motions in the PKA-Iα complex. A, crystal structure of the RlαABR333K/C complex highlighting the interaction between Rlα Domain B and the C-subunit (gray box). The small lobe of the C-subunit is in white, and the large lobe is in brown. The R-subunit Domain A is in dark teal, Domain B is in light teal, and the αB/C helix is depicted as a red cylinder in the right panel. B, left, the region between the αH-αI loop of the C-subunit (residues 282–287) is an insert specific to AGC kinases. Right, detailed illustration of the interaction between Rlα Domain B and the C-subunit αH-αI loop. C, left, modeled movement of Domain B within the RlαABR333K/C complex. Domain B was rotated manually using residues Gly235 and Tyr244 as the hinge points. Right, simulated P(r)
curves for the four different models. Theoretical scattering curves of each model were calculated using CRYSTAL, and their corresponding P(r) curves were
generated using GNOM. The experimental data for RlαABR333K/C (red) and RlαAB/C (gray) are shown for comparison. D, averaged and filtered DAMMIF models
for the seven PKA-Iα heterodimers. The DAMMIF models for RlαABR333K/C and RlαABR333K/C complexes are superimposed onto the crystal structure models (Protein
Data Bank accession numbers 3FHI and 2QCS, respectively). The experimental scattering data (colors) were fit to the scattering curve calculated for the DAMMIF
structure (black) in the graph in the bottom right panel. Wild-type (WT) C-subunit complexes are shown as solid symbols, and K285P C-subunit mutants are
shown as open symbols. The colors for each curve are identical to colors for the DAMMIF models.
trations, suggesting that this region is strongly anchored to the C-subunit in wild-type proteins.

To test our hypothesis that hinge motions are responsible for the extended structures seen in the wild-type RIα-AB-C heterodimer, we took a computational approach and artificially introduced kinks at the Gly235R and Tyr244R hinge points of the RIαAB-R333K-C crystal structure and computed their scattering intensities and corresponding $P(r)$ functions (Fig. 6C). Movement of Domain B away from the C-subunit resulted in larger $R_b$ values, larger particle dimensions, and the presence of a shoulder at higher $r$ values. Although the extended tail in these modeled $P(r)$ functions is present in each curve, they are not as pronounced as the experimental data for the wild-type RIαAB-C complex. Previous modeling of scattering data with the programs DAMMIN (34) and CONTRAST (35) do show that Domain B is positioned far from the C-subunit (16). Our efforts in computing ab initio three-dimensional shapes for each of the seven PKA-αo mutant complexes resulted in an array of molecular structures (Fig. 6D). The DAMMIF results for the RIαAB-C complex resulted in the most globular shape and superimposed very well with the crystal structure model (Protein Data Bank code 3FHI). The RIαAB-R333K-C complex with the additional Domain B resulted in a compact structure as well and also superimposed very well with the crystal structure (Protein Data Bank code 2QCS). In contrast, the DAMMIF envelope predictions for the remaining complexes (RIαAB-C, RIαAB-C(K285P), RIαAB-R333K-C(K285P), and RIαAB-R209K-C) all exhibited elongated shapes. These results corroborate our hypothesis that in the wild-type R-C complex, Domain B in RIα is mobile in solution and samples an assortment of conformational states.

In light of data from other studies, it is apparent that the function of Domain B in RIα is not to provide high affinity binding to the C-subunit but to facilitate cAMP-dependent activation of the holoenzyme. Deletion of Domain B from RIα results in 500-fold higher concentrations of cAMP required to activate the holoenzyme and does not impair the ability of the remaining Rα structure to bind the C-subunit with high affinity (36). Similarly, preventing cAMP access to Domain B with the R333K mutation increases the activation constant for cAMP from 166 to >1500 nM (12). Collectively, these data are in agreement with the notion that Domain B is dispensable for stable Rα-C complex formation. Instead, the role of Domain B in RIα is to enhance activation of the Rα-C complex in response to cAMP.

From our data and others, it is unmistakable that the role of Domain B differs between RI- and RII-subunits. First, the activation of Rα-C is a stepwise process where cAMP must bind to Domain B before Domain A (12). For RIIβ, sequential binding of cAMP is not required for activation, where cAMP binding to either domain is sufficient to release catalytically active C-subunits (37). Second, Domain B contributes to high affinity binding between RIIβAB and the C-subunit, but not between RIIβAB and the C-subunit. Disruption of the interaction interface by the K285P mutation in the C-subunit resulted in a 38-fold decrease in $K_D$ for RIIβ, versus a modest 5-fold decrease in $K_D$ for RIα, suggesting that K285P is not critical for RII-C complex formation but is significant for RIIβ-C formation. Third, previous studies demonstrated that the K285P mutation reduced the ability of RIIβ to inhibit the C-subunit and was sufficient to completely abolish BCY1 (the yeast homolog of mammalian RII subunits) inhibition of the C-subunit (27). Fourth, based on data from hydrogen/deuterium exchange-mass spectroscopy experiments, the αH-α loop is well protected in the RIIβ-C complex (33, 37), whereas minimal protection is seen in the RIα-C complex (30). Lastly, SAXS analysis of the RIIβ-C complex shows a $P(r)$ curve that reflects a compact particle (16), corroborating the view that Domain B forms a tight interaction with the C-subunit.

In this study, SAXS, in conjunction with mutagenesis and solution-based biochemical techniques, provides insights into domain dynamics within PKA complexes that are not feasible with x-ray crystallography alone. Taken together, our data suggest different roles of Domain B in RIα and RIIβ. For RIIβ, not only does Domain B interact tightly with the C-subunit, it is also necessary for inhibition of the C-subunit. In contrast, for RIα, Domain B is highly mobile, and its interaction with the C-subunit αH-α loop is not necessary for inhibition, but Domain B is essential for the highly cooperative process of cAMP-dependent activation. Given the sequence and overall structural similarity between the four isolated R-subunit PKA isoforms, it is surprising that their dynamic behaviors differ in the context of holoenzyme complexes, giving rise to unique and sophisticated modes of cAMP-mediated activation. RI holoenzymes are finely tunable multimeric complexes that require a sequential series of events, unlike RII holoenzymes. Our investigation of protein dynamics in RI-C and RII-C heterodimers provides yet another layer of complexity that distinguishes the molecular features between PKA isoforms. From a global perspective, these structural distinctions give rise to the functionally non-redundant roles each isoform performs within the cell.

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