Distinct interaction modes of an AKAP bound to two regulatory subunit isoforms of protein kinase A revealed by amide hydrogen/deuterium exchange


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Distinct interaction modes of an AKAP bound to two regulatory subunit isoforms of protein kinase A revealed by amide hydrogen/deuterium exchange

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Abstract

The structure of an AKAP docked to the dimerization/docking (D/D) domain of the type II (RIα) isoform of protein kinase A (PKA) has been well characterized, but there currently is no detailed structural information of an AKAP docked to the type I (RIα) isoform. Dual-specific AKAP2 (D-AKAP2) binds in the nanomolar range to both isoforms and provided us with an opportunity to characterize the isoform-selective nature of AKAP binding using a common docked ligand. Hydrogen/deuterium (H/D) exchange combined with mass spectrometry (DXMS) was used to probe backbone structural changes of an α-helical A-kinase binding (AKB) motif from D-AKAP2 docked to both RIα and RIIα D/D domains. The region of protection upon complex formation and the magnitude of protection from H/D exchange were determined for both interacting partners in each complex. The backbone of the AKB ligand was more protected when bound to RIα compared to RIIα, suggesting an increased helical stabilization of the docked AKB ligand. This combined with a broader region of backbone protection induced by the AKAP on the docking surface of RIα indicated that there were more binding constraints for the AKB ligand when bound to RIα. This was in contrast to RIIα, which has a preformed, localized binding surface. These distinct modes of AKAP binding may contribute to the more discriminating nature of the RIα AKAP-docking surface. DXMS provides valuable structural information for understanding binding specificity in the absence of a high-resolution structure, and can readily be applied to other protein–ligand and protein–protein interactions.

Keywords: DXMS; PKA; D-AKAP2; isoform diversity; hydrogen/deuterium exchange; mass spectrometry

Supplemental material: see www.proteinscience.org

Abbreviations: PKA, protein kinase A; AKAP, A kinase anchoring protein; DAKAP, dual specific A kinase anchoring protein; AKB, A kinase binding; R, regulatory subunit of protein kinase A; D/D, dimerization/docking domain of regulatory subunit; RGS, regulators of G-protein signaling; DXMS, deuterium exchange mass spectrometry; GdnHCl, guanidine hydrochloride; TFA, trifluoroacetic acid.

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Signal transduction is a combinatorial assembly of protein interaction networks that typically involves interaction modules containing a sequence motif that binds to discrete protein interaction domains (i.e., Src homology 2 [SH2] domains, Pleckstrin homology [PH] domains, and Phox homology [PX] domains) (Pawson and Scott 1997; Pawson et al. 2002). Identifying and characterizing these critical interaction modules is essential for understanding signaling specificity and affinity and for developing methods to selectively interfere with specific signaling pathways.

cAMP-dependent protein kinase (PKA) phosphorylates a diverse set of proteins involved in numerous biological signaling pathways. A major way of achieving signal specificity for PKA is through subcellular localization via A-kinase anchoring proteins (AKAPs), a family of proteins which binds to PKA and targets it to various intracellular compartments (Colledge and Scott 1999; Edwards and Scott 2000). AKAPs tether PKA through a critical interaction motif, which we have termed the A-kinase binding (AKB) domain. For most AKAPs this consists of a 15–20 amino acid amphipathic, helical motif that binds to the dimerization/docking (D/D) domain of the regulatory subunit homodimer. This interaction is essential for intracellular compartmentation of PKA and for integration of PKA signaling in the cell.

The regulatory (R) subunit homodimer of PKA is a modular and highly dynamic protein. Each R subunit monomer consists of an N-terminal D/D domain, two cAMP binding domains, and a disordered interconnecting hinge region (Taylor et al. 1990). It regulates the catalytic activity of the kinase directly through cAMP binding and indirectly through subcellular localization by AKAPs. The D/D domain is responsible for homodimerization and, once dimerized, provides a binding surface for the AKB helix. Four regulatory subunit isoforms of PKA (R\(\alpha\), R\(\beta\), R\(\gamma\), and R\(\delta\)) serve to diversify cAMP-mediated activation of the kinase (Skalhegg and Tasken 2000; Feliciello et al. 2001). These isoforms have a similar domain organization but differ in tissue distribution, subcellular localization, and cAMP sensitivity. In general, PKA-RI isoforms bind fewer AKAPs than PKA-RII and have a reduced affinity. Another important difference in the biology of these isoforms is their sensitivity to cAMP activation. PKA-RI isoforms are more sensitive to cAMP concentrations compared with PKA-RII, and this can result in a differential cAMP response (Doskeland et al. 1993; Skalhegg and Tasken 2000; Feliciello et al. 2001). Localization through AKAPs and cAMP sensitivity are two functions of the regulatory subunit that seem to have evolved to diversify PKA signaling.

Recent studies have advanced our understanding of the molecular basis for the R–AKAP interaction (Angelo and Rubin 1998, 2000; Banky et al. 1998, 2000, 2003; Miki and Eddy 1998, 1999; Newlon et al. 1999; Herberg et al. 2000; Newlon et al. 2001; Burns et al. 2003). Despite a similar X-type four-helix bundle comprising the D/D domain of these isoforms, there are distinct structural differences (Banky et al. 2003). The R\(\alpha\) D/D domain contains an extended N-terminal helix, helix N-1, which gives a more rugged surface topology to the AKAP binding groove. A small hinge segment allows for significant variation in the positioning of the N-1 helix. The R\(\beta\) D/D domain contains a shorter, \(\beta\)-strand-like N termini, which creates a more accessible AKAP binding surface (Newlon et al. 1999). The charge distribution is also very different for these isoforms. The R\(\alpha\) D/D domain contains more acidic and basic residues that line the surface of the AKAP binding groove, whereas the AKAP binding surface of R\(\beta\) contains primarily hydrophobic residues (Newlon et al. 1999, 2001; Banky et al. 2000, 2003; Burns et al. 2003).

Dual-specific AKAPs (D-AKAPs) can bind to both R\(\alpha\)s and R\(\beta\), and represent an interesting subfamily of AKAPs, which can potentially recruit both PKA-RI and PKA-RII to a given intracellular location. D-AKAP2 is a multisubunit protein containing two putative RGS domains and a 40 amino acid C-terminal domain containing the AKB helix and a PDZ binding motif. To better understand how the differences in surface topology contribute to isoform specific binding, we have examined the binding of a peptide corresponding to the AKB domain of D-AKAP2 to the D/D domains of both R\(\alpha\)s and R\(\beta\) using amide hydrogen/deuterium (H/D) exchange combined with mass spectrometry (DXMS) (Woody and Hamuro 2001). Amide H/D exchange has proven to be an invaluable tool for studying protein structure (Zhang and Smith 1993; Resing et al. 1999; Hamuro et al. 2002a; Yan et al. 2002), protein dynamics (Neubert et al. 1997; Engen and Smith 2001; Koobnagle et al. 2001), protein–ligand interactions (Engen et al. 1999; Andersen et al. 2001; Hamuro et al. 2002b), and protein–protein interactions (Ehring 1999; Mandell et al. 2001; Anand et al. 2002; Hamuro et al. 2003). Using this technique we have shown that the backbone of the AKB ligand was more stabilized when bound to R\(\alpha\), and that the peptide ligand induced a broader region of protection on the surface of R\(\beta\). We suggest that these differences represent distinct modes of AKAP binding to the regulatory subunit isoforms, and may contribute to the more discriminating nature of the R\(\beta\) binding surface.

**Results**

**H/D exchange of the free AKB ligand and in complex with R\(\alpha\)s and R\(\beta\) D/D domains**

**Digestion of the AKB ligand**

To identify the regions of deuteron uptake in the backbone, it was necessary to digest the AKB ligand.
See Materials and Methods for calculations. A₀ and B₀ are the original concentration in the exchange solution (after the addition of deuterated buffer). In the exchange solution, each peptide exists as either monomer or complex (A₀ = A + AB or B₀ = B + AB). % A-bound is the molar fraction of analyte peptide in the bound form (AB/A₀).

### Calculated molar fraction of bound-form analyte after D₂O addition

<table>
<thead>
<tr>
<th>Analyte (A)</th>
<th>Binding partner (B)</th>
<th>A₀ (µM)</th>
<th>B₀ (µM)</th>
<th>Kᵦ (µM)</th>
<th>% A-bound (AB / A₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKB</td>
<td>RIα D/D</td>
<td>20.3</td>
<td>30.3</td>
<td>0.100</td>
<td>99.0%</td>
</tr>
<tr>
<td>AKB</td>
<td>RIIα D/D</td>
<td>28.7</td>
<td>43.2</td>
<td>0.004</td>
<td>99.97%</td>
</tr>
<tr>
<td>RIα D/D</td>
<td>AKB</td>
<td>16.8</td>
<td>24.9</td>
<td>0.100</td>
<td>98.8%</td>
</tr>
<tr>
<td>RIIα D/D</td>
<td>AKB</td>
<td>26.3</td>
<td>55.0</td>
<td>0.004</td>
<td>99.99%</td>
</tr>
</tbody>
</table>

### Table 1.

Binding of both RIα D/D and RIIα D/D domains to the AKB ligand resulted in a significant slowing of deuteron incorporation into the backbone amides of the AKB ligand. However, the extent of the deuterium incorporation was different for the two isoforms. Figure 2 illustrates an example of a peptide isotopic envelope from the AKB ligand (residue 14–19, indicated by asterisk in Fig. 1A) in the non-deuterated state (Fig. 2A), after 300 sec on-exchange when complexed with RIIα D/D (Fig. 2B), with RIIα D/D (Fig. 2C) or in the free state (Fig. 2D). Fewer deuterons were incorporated into this region of the AKB ligand when bound to RIα with an isotopic envelope that more closely resembles the non-deuterated state. In contrast, the isotopic envelope for RIIα was shifted further to the right indicating more deuteron uptake and less protection in this region when RIIα was bound.

Nine contiguous and overlapping peptides were evaluated for deuteron uptake as a function of deuteration time in the free and RIα and RIIα-bound states (Fig. 3). The data is illustrated in two different formats. In Figure 3A, a color-coded grid makes it easy to qualitatively visualize the regions in the AKB ligand that are protected in the complexes. Regions highlighted under the amino acid sequence represent the peptide analyzed minus the first two residues of the peptide. This designation was used since it has been shown that the N-terminal amino group of the peptide and the amide hydrogen of the second amino acid exchange too rapidly to retain deuterons during the experiment (Bai et al. 1993). For example, exchange data for residues 14–19 corresponded to peptide 12–25. Residues 12–25 showed varying degrees of protection from exchange in both the free and RIα and RIIα D/D bound states (solid line in Fig. 1A).

**H/D exchange of the AKB ligand in the free state**

The backbone amide hydrogens of the AKB ligand in the free state were fully exchanged within 10 sec at pH 6.9, suggesting that the peptide contained little ordered secondary structure under these conditions. This was not surprising for a peptide of this size, and was consistent with circular dichroism measurements (Burns et al. 2003).

**H/D exchange of the AKB ligand in complex with the RIα and RIIα D/D domain**

H/D exchange experiments were performed with the AKB ligand in complex with either the RIα D/D or RIIα D/D domain at pH 6.9. Using the dissociation constants, maximal complex formation was calculated using the concentrations of analyte and binding partner (see Table 1 and Material and Methods). Approximately 99% of the analyte was complexed for both RIα and RIIα.

 Nine contiguous and overlapping peptides were evaluated for deuteron uptake as a function of deuteration time in the free and RIα and RIIα-bound states (Fig. 3). The data is illustrated in two different formats. In Figure 3A, a color-coded grid makes it easy to qualitatively visualize the regions in the AKB ligand that are protected in the complexes. Regions highlighted under the amino acid sequence represent the peptide analyzed minus the first two residues of the peptide. This designation was used since it has been shown that the N-terminal amino group of the peptide and the amide hydrogen of the second amino acid exchange too rapidly to retain deuterons during the experiment (Bai et al. 1993). For example, exchange data for residues 14–19 corresponded to peptide 12–25. Residues 12–25 showed varying degrees of protection from exchange in both the RIα and RIIα complexes, surrounded by unprotected regions (Fig. 3A). This 14 amino acid stretch when modeled using a helical wheel projection (data not shown) includes four turns of an amphipathic helix that place
hydrophobic residues (underlined in Fig. 3A) on one face of the helix. These residues have previously been shown to be essential for high affinity binding to the D/D domain (Burns-Hamuro et al. 2003). The magnitude of RIα-induced protection was focused over the length of the helix (12–25), while that of RIIα showed greater protection at the C terminus of the helix (Fig. 3A).

Deuteration versus time plots allowed for more complete comparison of the individual peptide segments (Fig. 3B). There was almost no protection in the AKB ligand for either complex at the far N- and C-terminal [see Fig. 3B, residues VQGNTE (1–7) and QQAHHDQPLEKSTKL (26–40)]. Beginning with the residues “LA” (12–13), the AKB ligand was more protected when bound to RIα. Since this difference was nearly 2 deuterons at 10 sec, this suggested that both of these residues are protected to a greater extent in the RIα complex. It was clear from the data that RIα induced more protection throughout this region (residues 14–25) in the AKB ligand. The residues “SD” (22–23) were protected for both the RIα and RIIα complex. Since these residues map to the opposite side of the interacting amphipathic helix, this suggested to us that protection along the length of the helix was most likely due to a direct stabilization of the backbone of the helix rather than specific contacting sites along the helix.

H/D exchange of the D/D domain isoforms in the free and bound state

Digestion of RIα and RIIα D/D domains

The same digestion and analysis method used for the AKB domain digestion above identified 15 and 17 peptides for the RIα and RIIα D/D domains, respectively (Fig. 1B,C). After complexation and deuteration, a subset of these peptides was used to monitor amide H/D exchange patterns (highlighted bold in Fig. 1B,C).

H/D exchange of RIα and RIIα D/D domains in the free state

RIα and RIIα D/D domains were incubated in 75% deuterated water at pH 6.9 for various time periods in the absence of the AKB domain (10–10,000 sec = ~2.8 h for RIα and 10–300,000 sec = ~83 h for RIIα). The extent of deuteration of nine pepsin-generated peptides from the RIα D/D domain and 10 peptides from the RIIα D/D domain was evaluated (Fig. 4 [RIIα] and Fig. 5 [RIα]). The slow exchanged regions of the free state for both RIα and RIIα D/D domains were centered around helix I and helix II, and were consistent with secondary structure assignments by NMR as well as H/D exchange by NMR (Newlon et al. 1997, 1999; Banky et al. 2000, 2003; Fayos et al. 2003).

H/D exchange of RIIα in complex with the AKB ligand

Analogous exchange experiments were carried out for the D/D domains in the presence of excess AKB ligand to ensure over 99% complexation (Table 1; Material and Methods). Figure 4 illustrates the color-coded data format and plots of individual segments for the RIIα-AKB complex. Helix I and helix II showed the greatest protection by the AKB ligand. AKB-induced protection in helix I was consistent with this region being the direct interaction site of the AKB ligand as seen by NMR (Newlon et al. 2001). Since there was no evidence by NMR that helix II directly interacts with the ligand, protection in this region was likely due to indirect effects propagated through the binding surface. The AKB ligand induced little if any changes at the N terminus, the turn between helix I and II and the C terminus (Fig. 4).
H/D exchange of RIα in complex with the AKB ligand

The AKB ligand induced similar regions of protection in RIα with some notable differences (Fig. 5). Helix I and Helix II were protected, suggesting that the docking surface for RIα was the same as for RIIα. However, the AKB-induced protection of RIα extended more into the N terminus when compared with RIIα. At least four deuterons were protected from exchange after 10 sec on-exchange. This was in contrast to RIIα, in which little if any AKB-induced protection was observed in the region leading into helix I (Fig. 4, see segment GLTEL [12–16]). To compare the differences in AKB-induced protection between RIα and RIIα, the percent deuteration difference between free and bound states was calculated and plotted versus the sequence for each isoform (Fig. 6). In the bar-graph format it was clear that the region leading into helix I of RIIα was more protected by the AKB ligand compared with a similar region in RIα. When the protected regions were mapped onto the structures of RIα and RIIα, the enhanced protection leading into helix I of RIα translated into a broader protected region on the AKAP docking surface (Fig. 7).

Discussion

Amide H/D exchange was used to investigate the molecular basis for AKAP binding selectivity to the regulatory subunit isoforms of PKA. Distinct differences in the magnitude and extend of ligand-induced backbone protection were observed by monitoring changes in H/D exchange perturbations for each pair of interacting partners. This has given us insight into the backbone changes accompanying AKAP binding to each isoform.

Backbone of the AKB ligand is stabilized to a greater extent when bound to RIα

The AKB ligand spanning residues 12–25 was protected upon binding RIα and RIIα. The more localized region of AKB protection when bound to RIIα was consistent with recent peptide array analysis in which each residue in the AKB peptide was replaced with the other 19 amino acids and binding to both RIα and RIIα characterized (Burns-Hamuro et al. 2003). The critical contact areas mapped out by the peptide substitution array extended the length of the AKB helix for RIα binding.
(residues 12–25), but were more localized to the C-terminal residues of the AKB helix for RIIα binding (residues 16–25) (Burns-Hamuro et al. 2003).

Interestingly, the magnitude of protection for the AKAP peptide was different when bound to each isoform, suggesting differences in helical stabilization of the docked AKAP. The binding affinity for the AKAP peptide is 25-fold weaker for RIα than RIIα (Burns et al. 2003), yet was protected to a greater extent when docked to the RIα isoform, suggesting an increase in helical stabilization of the AKB ligand. This at first interpretation seemed counterintuitive, but H/D exchange measures only backbone contributions to the free energy change for an EX2 mechanism (Englander and Kallenbach 1984) and solvent effects and side-chain contributions to the binding energy are not measured directly by this technique. Likewise, binding affinities that are dominated by side chain or solvent effects may have lower than expected protection levels. Weaker protection of the RIα-bound AKB ligand suggested that the ligand backbone was less stabilized and perhaps more dynamic when bound, even though the overall affinity is higher relative to RIα. Support of this interpretation came from NMR experiments, where modest H/D exchange protection factors were found for residues contacting the AKAP along with increases in backbone flexibility for selected regions of the RIα docking surface when the AKAP is bound (Fayos et al. 2003). This type of observation may be a consequence of the predominately, hydrophobic binding surface of RIα and increased backbone dynamics may further contribute to a favorable binding entropy, leading to enhanced AKAP binding affinity for this isoform. Further experiments that characterize the thermodynamic contributions to binding of each isoform will be important to verify this hypothesis.

**Similar regions of exchange for both RIα and RIIα isoforms in the unbound state**

The H/D exchange patterns for the isolated D/D domains are similar for the two isoforms, reflecting the fact that both domains have similar four helix bundle structures. Concentrated regions of protection map to helix I and helix II for both of these isoforms. Helix I and helix I' form the AKAP docking surface and helix II and helix II' form the primary dimerization contacts for this domain. The slow exchanged regions of the native state were consistent with previous H/D exchange experiments by NMR, which map slowly, exchanging amide hydrogens to similar regions (Newlon et al. 1997; Banky et al. 2000).
The D/D domain is quite stable for its size, and can function as an isolated unit as indicated by similar AKAP binding affinities for this domain compared with full-length protein (Newlon et al. 1999; Burns et al. 2003). Previous studies have shown that the RIα D/D domain is highly thermostable with interchain disulfide bonds that are resistant to high concentrations of reducing agent (Leon et al. 1997). The disulfide bonds, although not necessary for dimerization, are unusually stabilized by the X-type helical motif (Banky et al. 2003).

Backbone perturbations in the N-1 helix upon AKAP-binding to the RIα isoform

Two primary regions of the D/D domain were protected in the AKAP–R complex for each isoform: helix I and helix I' and helix II and helix II'. Segments covering 51% of the backbone hydrogens of RIα were protected significantly from exchange upon AKAP binding, compared with only 29% for RIIα. The additional protected residues in RIα primarily map to the N-1 helix. Although there is no structural information for an AKAP docked to the RIα D/D domain, the data presented here predicts that the N-1 helical region undergoes backbone structural rearrangements to accommodate the AKAP. It cannot be distinguished by this technique alone if the N-1 helix is directly contacting the peptide, but mutagenesis experiments have shown that residues within this helix are important for AKAP binding. Mutating tyrosine at position 19 with an alanine significantly interferes with binding of the D-AKAP2, AKB peptide to RIα (L.L. Burns-Hamuro, unpubl.). In addition, valine at position 20 is also important for binding the AKAP domain from D-AKAP1 (Banky et al. 1998).

The N-1 helical extensions are the least well defined in the ensemble of NMR structures of the free RIα D/D domain due to the apparent rotation of these helices with respect to the core of the domain (Banky et al. 2003). Residues His23 and Asn24 have been proposed as a hinge, allowing the N-1 helix to adopt multiple conformations (Banky et al. 2003). These residues are contained within the AKAP-induced protected region, and consistent with the model that the N-1 helices rearrange to accommodate the AKB ligand (Banky et al. 2003).

The increased protection of the AKB peptide backbone when bound to RIα relative to when bound to RIIα, suggested that the AKB peptide formed a more stabilized hydrogen-bonded network with RIα. The N-1 helices may wrap around the docked peptide, extending the interaction surface and further stabilizing the hydrogen-bonding network of the bound peptide. Whether this is through direct interaction of the AKB
ligand in other domains of the regulatory subunit.

there are long-range protections induced by the AKB with a full-length regulatory subunit will determine if the multidomain regulatory subunit. Future experiments important for signal transmittance to other regions of the domain upon AKAP binding may be

tion networks within the domain. Propagation of a sig-

longer-range effects potentially highlight communica-

tions in this region. This area of protection was consistent with recent H/D exchange experiments by NMR which showed a similar protection pattern for helix II and helix II’ when the AKAP peptide, HT31, was bound to RI\(\alpha\), indicating that protection in helix II is not specific to a given AKAP ligand (Fayos et al. 2003). Protection in these helices was presumably due to longer-range effects that were propagated through the helix I and II interface, as there was no evidence to suggest from mutagenesis or structural experiments that helix II interacts directly with the AKAP. These longer-range effects potentially highlight communication networks within the domain. Propagation of a signal through the domain upon AKAP binding may be important for signal transmittance to other regions of the multidomain regulatory subunit. Future experiments with a full-length regulatory subunit will determine if there are long-range protections induced by the AKB ligand in other domains of the regulatory subunit.

Figure 6. Average difference in deuteration levels for individual segments in the free and bound states for RII\(a\) (A) and RII\(\alpha\) (B). For the RII\(\alpha\) isoform, over half of the residues (51%) show a significant level of ligand-induced protection (>10%) with an extended area of protection mapping to helix N-1. For the RII isoform only 29% of the residues are protected from exchange by the ligand.

Intradomain communication within the X-type four-helix bundle motif

Helix II and helix II’, which directly oppose the AKAP binding surface, were also protected when the AKB peptide was bound to each isoform (Fig. 6). The magnitude of protection was similar for both AKB–R complexes, suggesting a similar level of backbone communication in this region. This area of protection was consistent with recent H/D exchange experiments by NMR which showed a similar protection pattern for helix II and helix II’ when the AKAP peptide, HT31, was bound to RII\(\alpha\), indicating that protection in helix II is not specific to a given AKAP ligand (Fayos et al. 2003). Protection in these helices was presumably due to longer-range effects that were propagated through the helix I and II interface, as there was no evidence to suggest from mutagenesis or structural experiments that helix II interacts directly with the AKAP. These longer-range effects potentially highlight communication networks within the domain. Propagation of a signal through the domain upon AKAP binding may be important for signal transmittance to other regions of the multidomain regulatory subunit. Future experiments with a full-length regulatory subunit will determine if there are long-range protections induced by the AKB ligand in other domains of the regulatory subunit.

Distinct modes of AKAP interaction with the regulatory subunit isoforms

The RII\(\alpha\) isoform can bind many different amphipathic AKAP binding motifs, which contain primarily branched chain amino acids along one face of the AKAP helix. The RII\(\alpha\) surface is more discriminating, and has only been shown to bind a few AKAPs (Huang et al. 1997a,b; Angelo and Rubin 1998; Miki and Eddy 1998; Li et al. 2001). The structural features that may contribute to reduced AKAP binding affinity and higher binding selectivity of the RII\(\alpha\) surface have recently been revealed with the solution NMR structure of the RII\(\alpha\) D/D domain. Although, the overall structures of the RII\(\alpha\) and RII\(\alpha\) D/D domains are similar, there are distinct differences in the surfaces that they present to the AKAP (Banky et al. 2003). RII\(\alpha\) has a central, deep cleft created by the isoform-specific N-terminal exten-
sions (helix N-1). These helical extensions are tethered through a disulfide bridge between C16 (helix N-1) and C37 (helix 1) of the adjacent protomer, which most likely place conformational restrictions on this segment of the docking surface. The RII\(\alpha\) surface is more extended and preformed for AKAP binding (Fig. 6). The N-terminal extensions in RII\(\alpha\) also contain several charged residues that most likely play a role in AKAP binding, whereas the surface of RII\(\alpha\) is primarily hydrophobic (Burns et al. 2003).

The H/D exchange data presented here expand our current understanding of the isoform-selective nature to AKAP binding. The broader region of backbone protection induced by the AKAP on the RII\(\alpha\) surface and the more enhanced backbone stabilization of the AKAP helix when bound to RII\(\alpha\) versus RII\(\alpha\) suggest that the AKAP helix forms a more intimate contact with the hydrogen-bonded network of the RII\(\alpha\) core. In this model for AKAP binding to RII\(\alpha\), the AKAP preferen-
tially interacts with an N-1 helical conformation on the RII\(\alpha\) surface that allows for optimized helical stabilization of the AKAP backbone. Molecular details of this complex await structural determination of the RI–AKAP complex, but this model is in stark contrast to the RII\(\alpha\) D/D surface, which presents as a preformed docking surface with only minor changes in conformation of the N-terminal region between the free and bound forms (Newlon et al. 2001). The data presented here also suggests that the higher affinity AKAP docking surface of RII\(\alpha\) is more localized than RII\(\alpha\). The AKAP induced a narrower region of backbone protection on the surface of RII\(\alpha\) and contained decreased backbone stabilization relative to when docked to RII\(\alpha\). The localized, preformed and primarily hydrophobic binding surface of RII\(\alpha\) may enable this surface to recognize a multitude of binding partners with fewer sequence constraints on AKAP binding. Presumably, water exclusion from the interacting hydrophobic surfaces and the preformed
nature of the RIIα binding surface contribute to the enhanced affinity of this isoform for AKAPs. Although in general the RIα surface binds AKAPs weaker than RIIα, AKAP peptides that contain appropriately spaced aromatic groups (Phe or Trp) dramatically enhance the affinity and confer selective binding to RIα (Angelo and Rubin 2000; Burns-Hamuro et al. 2003). This demonstrates that while the RIα surface is selective in binding, it can accommodate the appropriate side chains for high affinity binding, perhaps by filling the deep cleft on the docking surface. A future challenge will be to design cell-permeable, isoform-specific AKAP disruptors of PKA localization to enable a better understand of the functional importance of isoform-selective anchoring of PKA in cells.

We have used hydrogen/deuterium exchange mass spectrometry to map the binding of an AKAP helical peptide to the D/D domain of RIα and RIIα subunits of PKA. The results reveal a common docking surface but show differences in ligand stabilization and surface interactions, which have enabled us to propose distinct interaction modes for the docked ligand. This technique is applicable to any set of binding partners, and can be used to map both local and long-range perturbations in the backbone upon complex formation.

Materials and methods

Protein expression and purification

The AKB domain from mouse D-AKAP2 was subcloned, expressed, and purified as previously described (Burns et al. 2003). This protein fragment contains 40 amino acids from the C terminus of mouse D-AKAP2 containing the AKB domain and an additional 15 N-terminal amino acids introduced by the vector (GSPGISGGGGGILLS). These additional residues have little effect on binding to the regulatory subunit D/D domains (Burns et al. 2003). The expression and purification of RIIα D/D and RIα D/D was previously described (Banky et al. 2000; Burns et al. 2003). The protein concentrations were determined at OD280 using an extinction coefficient of 5499 M⁻¹ cm⁻¹, 5960 M⁻¹ cm⁻¹, and 6210 M⁻¹ cm⁻¹ for the AKB domain, RIIα D/D and RIα D/D, respectively (Pace et al. 1995; Burns et al. 2003).

Amide hydrogen/deuterium exchange analysis

General operation procedure

A 20-μL hydrogen-exchanged protein solution was quenched by shifting to pH 2.2–2.5, 0°C with 30 μL of 0.8% formic acid with various concentrations of GdnHCl (final pH was measured on a nondeuterated mock solution at room temperature using a Model 250 pH meter; Denver Instrument Co.). At 0°C, the quenched solution was immediately passed
over a column (66 μL bed volume; Upchurch Scientific) filled with porcine pepsin (Sigma) immobilized on Poros 20 AL media at 30 mg/mL per the manufacturer's instructions, with 0.05% TFA (200 μL/min) for 2 min with contemporaneous collection of proteolytic products using a C18 column (Vydac). Inline filters (Upchurch) were placed on each side of the pepsin column, and just before the C18 column (Vydac prefilter) to minimize column fouling. Subsequently, the C18 column was eluted with a linear gradient of 10% to 50% solvent B over 10 min (solvent A was 0.05% TFA in water, and solvent B was 80% acetonitrile, 20% water, 0.01% TFA). Mass spectrometric analysis was carried out with a Finnigan LCQ mass spectrometer with a capillary temperature of 200°C.

**Sequence identification of pepsin-generated peptides**

To identify pepsin-generated peptides for each digestion condition employed, spectral data was acquired in “data-dependent MS/MS” mode. The “data-dependent MS/MS” data set was then analyzed using Sequest (Finnigan, Inc.) to identify the sequence of the dynamically selected parent peptide ions.

**Hydrogen/deuterium exchange experiments**

Deuterated samples were prepared by diluting 5 μL of protein solution (either analyte alone or with excess binding partner) with 15 μL of deuterated buffer, followed by “on-exchange” incubation at room temperature (23 ± 1°C) for varying times (10–300,000 sec) prior to quenching in 30 μL of 0.8% formic acid with GdnHCl at 0°C. For pH 7 experiments, the deuterated buffer was 10 mM HEPES, 150 mM NaCl, pHread = 6.90, and the pHred of exchange solution was 6.88 ± 0.01. These functionally deuterated samples were subjected to DXMS processing as described above, along with control samples of nondeuterated and fully deuterated protein. The centroids of probe peptide isotopic envelopes were measured using the DXMS software provided by Sierra Analytics. The corrections for back-exchange were made employing the methods of Zhang and Smith (1993),

Deuteration Level (%) = \( \frac{m(P) - m(N)}{m(F) - m(N)} \times 100 \)

Deuterium Incorporation (‰) = \( \frac{m(P) - m(N)}{m(F) - m(N)} \times \text{MaxD} \)

where m(P), m(N), and m(F) are the centroid value of partially deuterated peptide, nondeuterated peptide, and fully deuterated peptide, respectively. MaxD is the maximum deuterium incorporation calculated by subtracting the first two residues of the peptide, which have been shown to be fully exchanged due to end effects (Bai et al. 1993) and by subtracting the number of prolines from the total number of amide hydrogens in the peptide. The experimentally determined deuteron recovery of the fully deuterated sample was on average 90% (i.e., [m(F)–m(N)]/MaxD) 0.75, 0.75 is the deuteron content in the exchange buffer).

**Sublocalization of deuteriums**

The deuterium levels of the peptides were further sublocalized using overlapping peptides. For example, the deuterium incorporation of segment 10–11 was obtained by the subtrac-

**Complex formation**

The proteins were mixed in the ratios indicated (Table 1) and the fraction of analyte bound was calculated using the following equations (Manandell et al. 2001):

\[ f_{\text{bound}} = \frac{A_0 + B_0 + K_D \pm \sqrt{(A_0 + B_0 + K_D)^2 - 4A_0B_0}}{2A_0} \]

% Complexation = \( f_{\text{bound}} \times 100 \)

where \( A_0 \) is the original concentration of the analyte in the exchange reaction, \( B_0 \) is that of the binding partner, and \( K_D \) is the dissociation constant.

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**Conflict of interest**

V.L.W., D.D.S., and Y.H. have financial interests in ExSAR Corporation.

**References**


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