Domain Organization of \( \text{D-AKAP}2 \) Revealed by Enhanced Deuterium Exchange-Mass Spectrometry (DXMS)

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Dual specific A-kinase anchoring protein 2 (\( \text{D-AKAP}2 \)) is a scaffold protein that coordinates cAMP-mediated signaling complexes by binding to type I and type II protein kinase A (PKA). While information is unfolding regarding specific binding motifs, very little is known about the overall structure and dynamics of these scaffold proteins. We have used deuterium exchange-mass spectrometry (DXMS) and limited proteolysis to probe the folded regions of \( \text{D-AKAP}2 \), providing for the first time insight into the intra-domain dynamics of a scaffold protein. Deuterium on-exchange revealed two regions of low deuterium exchange that were surrounded by regions of high exchange, suggestive of two distinctly folded regions, flanked by disordered or solvent accessible regions. Similar folded regions were detected by limited proteolysis. The first folded region contained a putative regulator of G-protein signaling (RGS) domain. A structural model of the RGS domain revealed that the more deuterated regions mapped onto loops and turns, whereas less deuterated regions mapped onto \( \alpha \)-helices, consistent with this region folding into an RGS domain. The second folded region contained a highly protected PKA binding site and a more solvent-accessible PDZ binding motif, which may serve as a potential targeting domain for \( \text{D-AKAP}2 \). DXMS has verified the multi-domain architecture of \( \text{D-AKAP}2 \) implied by sequence homology and has provided unique insight into the accessibility of the PKA binding site.

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Introduction

For a cell to communicate with its environment, a dynamic network of protein signaling molecules must transmit an external signal to the interior of the cell. The spatial and temporal organization of these protein assemblies is essential to ensure specificity in cell signaling. Over the past decade A-kinase anchoring proteins (AKAPs) have been recognized as integral in coordinating the specificity of signaling through cAMP-dependent protein kinase (PKA) by localizing the kinase near its substrate targets.\(^1,2\) AKAPs are multidomain proteins containing a PKA kinase binding (AKB) domain, which interacts with the regulatory subunit of PKA, and a targeting domain, which directs the AKAP to various subcellular locations. In addition to anchoring PKA, AKAPs have been shown to bind other signal transduction proteins, thus providing a scaffold for coordination of signaling complexes.\(^3\) Despite the important role that AKAPs play in localizing PKA activity, little is known about their overall domain organization or their ability to regulate signaling complexes.

Dual-specific A-kinase anchoring protein 2 (\( \text{D-AKAP}2 \)), initially identified from a genomic screen, binds to both type I and type II regulatory
subunit isoforms of PKA. This protein is expressed in nearly all tissues and localizes to mitochondria. The specific PKA signaling pathway that D-AKAP2 regulates is unknown; however, interaction partners are suggested by sequence homology (Figure 1(a)). There are two putative regulators of G protein signaling (RGS) domains, distinct from the PKA interaction site that could coordinate upstream G-protein signaling events with downstream PKA signaling.

There is a putative PDZ (PSD95/Dlg/ZO1) binding motif at the C terminus that could serve as a motif for targeting to membrane receptor complexes. To verify the multi-domain organization of D-AKAP2, we have used limited proteolysis and enhanced methods of deuterium exchange-mass spectrometry (DXMS) to determine the folded regions of this multi-domain protein in solution and to evaluate the role of the domain organization on PKA binding.

Amide hydrogen exchange has proven to be an increasingly powerful method by which protein dynamics, structure and function can be studied. These studies can provide information that greatly amplifies and refines inferences drawn from high-resolution structural studies, and can provide unique insights when reliable structural information is unavailable. Deuterium exchange methodologies coupled with liquid chromatography mass spectrometry (LCMS), developed over the past ten years, presently provide the most effective approach to study proteins larger than 30 kDa. Proteolytic and/or collision-induced dissociation (CID) fragmentation methods allow exchange behavior to be mapped to subregions of the protein. Amide hydrogen exchange studies are expected to play a central role in deciphering proteomic structure, function and dynamics over the next decade.

Previously, we reported the development of a number of improvements to the basic methodologies and experimental equipment first developed by Englander, Smith, and co-workers, which have significantly improved throughput, comprehensiveness, and resolution of such studies and have termed these collective enhancements deuterium exchange-mass spectrometry (DXMS).

The studies of D-AKAP2 contained in the present report employs many of these methodologies and represents the first application of this technology.

Figure 1. (a) The predicted domain organization of human D-AKAP2. D-AKAP2 contains two putative regulators of G protein signaling (RGS) domains, a PKA kinase binding (AKB) domain, and a PDZ binding motif at the C terminus (TKL). A consensus PKA phosphorylation site is indicated between RGS B and the AKB domains. The bold, black line indicates the region of D-AKAP2 used in the current study. (b) A Clustal W sequence alignment of RGS4, RGS A and RGS B of D-AKAP2. An asterisk (*) indicates the identical residues and the residues comprising the hydrophobic core are in red. The secondary structure corresponding to RGS4 is indicated above the alignment.

to a protein whose structure is unknown. We have used DXMS to obtain 99% sequence coverage of mouse D-AKAP2 by exchange-assessable peptide fragments, which has allowed us to identify and resolve this protein’s domain organization in solution. We have generated a structural model of the RGS domain of D-AKAP2 and have shown that regions of higher deuteron incorporation were located in loops and turns, whereas regions of lower incorporation were located on the \( \alpha \)-helices. DXMS has validated our structural model of the RGS domain and has provided valuable insights into the domain organization of D-AKAP2 and its role on PKA binding.

Results

Computational domain analysis

Human D-AKAP2 was used as the query sequence to perform a RPS-BLAST\(^ {14} \) search of the NCBI CDD database (version 1.54). Two domains that matched Smart 000315 and Pfam PF00615 models were identified as RGS domains (Figure 1(a)). The domain closer to the N terminus (RGS A) was split in two parts, corresponding to 37% and 63% of the model length. A 124 amino acid-residue insert separated the two regions. The first region of RGS A (Smart \( E \)-value \( = 1 \times 10^{-24} \)) encompassed residues 125–168, whereas the second region (Smart \( E \)-value \( = 7 \times 10^{-12} \)) included residues 292–368. The second RGS domain (RGS B) included residues 380–505 (Smart \( E \)-value \( = 1 \times 10^{-18} \)). \( E \)-values and boundaries reported by Pfam were consistent with those obtained from Smart. To determine the structural location of the RGS A insert, the region including the first RGS domain of D-AKAP2 was aligned with the sequence corresponding to the crystal structure of rat RGS4 (PDB 1AGR, chain 2).\(^ {15} \) On the basis of the alignment, the insertion was predicted to lie between \( \alpha \)-helices 4 and 5 of the RGS domain (Figure 1(b)). The hydrophobic residues that comprise the core of the RGS domain appeared to be conserved for both D-AKAP2 RGS domains.

Protein expression

Although attempts to purify the full-length, human protein from a bacterial expression system were unsuccessful, the initial mouse D-AKAP2 described by Huang \( et \) \( al \),\(^ {4} \) which corresponded to residues 291–662 of the human clone, was expressed in a soluble form and used as a model system (Figure 1(a)).

The folded state of truncated mouse D-AKAP2

To evaluate the folded state of truncated, soluble D-AKAP2 (375 amino acid residues), the protein was characterized using circular dichroism (CD) spectroscopy and limited proteolysis. The CD spectrum of D-AKAP2 contained two local minima, at 209 nm and 223 nm, indicative of a mostly \( \alpha \)-helical protein (data not shown). In addition to forming stable secondary structure, limited proteolysis revealed two protease-resistant domains that were inaccessible to both trypsin and Glu-C proteases. Domain boundaries were mapped using mass spectrometry after one hour and after 24 hours of digestion (Table 1). After 24 hours of digestion, both trypsin and Glu-C further cleaved the N terminus of the larger domain, leaving a stable core containing the putative RGS domain (residues 93–214). The smaller domain contained the AKB domain (residues 336–360) and was not detected after 24 hours of digestion with either protease.

Table 1. Peptide fragments from limited proteolysis identified by mass spectrometry

<table>
<thead>
<tr>
<th>Proteolysis time (h)</th>
<th>Large domain</th>
<th>Small domain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trypsin</td>
<td>Glu-C</td>
</tr>
<tr>
<td>24</td>
<td>75–265</td>
<td>85–276</td>
</tr>
</tbody>
</table>

Binding of D-AKAP2 to the regulatory subunit of PKA

To evaluate the functional properties of this protein, binding of D-AKAP2 to the RII\( \alpha \) isoform of PKA was examined using surface plasmon resonance.\(^ {16} \) RII\( \alpha \) (cAMP free) was immobilized to cAMP that was attached covalently to a sensor chip. D-AKAP2 was injected over the surface at varying concentrations and the affinity determined by fitting the curves to a 1:1 binding model.

Figure 2. Binding of D-AKAP2 to the regulatory subunit (RII\( \alpha \)) of PKA using surface plasmon resonance. A total of 450 response units of RII\( \alpha \) was immobilized to a cAMP sensor chip. Serial dilutions (250–2 nM) of D-AKAP2 were injected over the surface. Both the on rate (\( k_{on} \)) and the off rate (\( k_{off} \)) were fit globally using the concentration series. A 1:1 binding model fits the data well and is illustrated as a bold line.
Figure 2. D-AKAP2 bound tightly to RIIα with on and off rates of $1.3 \times 10^6 \text{M}^{-1} \text{s}^{-1}$ and $18 \times 10^2 \text{s}^{-1}$, respectively, resulting in a $K_d$ of 14 ($\pm 2$) nM. In contrast, the dissociation constant for the AKB domain alone was much lower at 2 nM (our unpublished results), suggesting that the AKB domain was not fully accessible in the intact, soluble protein.

Tuning of D-AKAP2 proteolytic fragmentation

Prior to studying the hydrogen-exchanged samples, digestion conditions that produced D-AKAP2 fragments of optimal size and distribution for exchange analysis were established by varying the concentration of GuHCl and flow rate over the pepsin column. Minimal back exchange and optimal pepsin digestion for D-AKAP2 (4.7 μM) were obtained by diluting one part of the deuterated sample with one and a half parts of quench solution (3.2 M GuHCl in 0.8% (v/v) formic acid, pH 2.0). The quenched sample was then run over immobilized pepsin (66 μl bed volume) at a flow-rate of 200 μl/minute, resulting in a digestion duration of 20 seconds. These conditions generated 119 peptides covering the entire amino acid sequence of D-AKAP2 (Figure 3). Forty of the identified peptides, representing 99% of the entire sequence (373 out of 375 amino acid residues), were of good quality for DXMS analysis (black lines in Figure 3). Since both the amino group of the first amino acid residue and the amide hydrogen atom of the second amino acid residue exchange too rapidly to retain deuterons during the experiment, the total number of amide hydrogen atoms followed by DXMS was 302 out of 362 non-proline residues (83%).

Deuterium on-exchange of D-AKAP2 at 0 °C

D-AKAP2 was incubated in deuterated buffer for 10–3000 seconds at 0 °C and quenched with a low-pH buffer to slow the back-exchange rate during analysis. The extent of deuterium incorporation was determined by mass spectrometry and reported as percentage deuteration, as described in Materials and Methods (Figure 4). The deuterium exchange profile divided the protein into four main regions. The first region (G1–C52) was fast exchanging, with all three peptides heavily deuterated. This region contained part of the RGS A domain and was most likely disordered due to the N-terminal truncation of this protein used in this study. The second region (A56–D220) included the rest of the RGS A domain and the...
entire RGS B domain. This region showed the greatest area of protection and was presumably the most structured region of the protein. Computational modeling was applied to further investigate the structure of this region (see below). The third region (H223–M327) was heavily exchanged and included a putative PKA phosphorylation site. The final region (Q330–L375), located at the C terminus, was exchanging relatively slowly and included the AKB and PDZ domains.

The overall exchange pattern of D-AKAP2 was consistent with the limited proteolysis results. Seven cleavage sites (black arrows in Figure 4) were identified after one hour of limited proteolysis and included a putative PKA phosphorylation site. The final region (Q330–L375), located at the C terminus, was exchanging relatively slowly and included the AKB and PDZ domains.

**Modeled RGS domain**

In order to provide some structural insight into the deuteration levels observed, the sequence for the RGS B domain of D-AKAP2 was threaded onto the crystal structure of RGS4. Residues L93-Y214 were identified as the RGS domain boundaries for D-AKAP2 and were 22% identical (41% similar) to RGS4 (Figure 1(b)). Helices 2 through 9 of RGS4 were used as a template for structural modeling. The region corresponding to helix 1 did not share significant homology between the two sequences and therefore was not modeled. The residues comprising the hydrophobic core of the RGS domain as described by Tesmer et al. were highly conserved in D-AKAP2, suggesting a potential to fold into an RGS domain (Figure 1(b)). The majority of the residues in the modeled RGS domain occupied the most favored regions of the Ramachandran plot (data not shown). Only 6.6% of the residues were in the disallowed region, similar to the initial RGS4 template, which had 6.4% in the disallowed region. The structural alignment for the C-carbon atoms of the RGS4 template and the model RGS domain of D-AKAP2 were within 0.7 Å, with a Z-score of 6.0, indicating a high level of structural similarity between the RGS4 template and the modeled RGS domain (Figure 5(a)). The structure of the modeled RGS domain deviated the most...
from RGS4 in the loop region between helices 4–5 and 6–7 of the four-helix bundle of this domain. For the modeled RGS domain, there was a seven residue insertion between helices 4 and 5 (Figure 1(b)), which extended the loop, making this region difficult to model.

Deuterium exchange at 3000 seconds and 0°C was mapped onto the modeled RGS domain. The most protected regions mapped to the α-helices and the more exchanged regions mapped to the connecting loops and turns (Figure 5(b) and (c)). Seven peptides in the RGS B domain remained less than 30% deuterated after 3000 seconds on-exchange at 0°C and were mapped to α-helices 2 (peptide 93–97), 3 (104–108), 4 (122–128), 5 (150–155), 7 (189–200), 8 (201–213) and the C terminus of the RGS domain near helix 9 (214–220). Consistent with this, all but two (L164 and F166) of the hydrophobic core residues that were conserved throughout the RGS family were located within or near these highly protected regions (Figure 5(c)). Peptides 168–172 in helix 6 showed deuteration levels of about 50%, indicating that this helix was more flexible and/or solvent-accessible than the other helices of the RGS domain. The turn between helices 2 and 3 and the extended loop between helices 4 and 5, which contained the seven residue insertion, were approximately 70% and 80% deuterated, respectively. The most exchanged peptide covered the loop between helices 5 and 6 with over 90% deuteration. The Ga interaction surface for RGS domains includes this highly solvent-accessible region at the base of the four-helix bundle.

**AKB domain**

In addition to the putative RGS domain, the AKB domain was well protected from deuterium exchange at 0°C. A 27 residue peptide (box in Figure 4) binds to the regulatory subunit of PKA with high affinity (our unpublished results). A high level of protection from exchange was observed within this region from L349 to M354. To further sub-localize the slowly exchanging regions near the predicted amphipathic helix (L347–M360), the deuteration levels of overlapping peptides were compared. First, the deuteration
percentages of peptides 355–375 and 359–375 were converted to the numbers of deuterons incorporated by multiplying the deuteration percentage by the maximum deuterium incorporation (maxD, see Materials and Methods). The deuteron difference of the two peptides was divided by maxD of residues S357–M360 to give the deuteration percentage of the residues. The results indicated that residues S357–M360 were not deuterated in the time-window examined, extending the protected region to residues W349–M360 (Figure 6). Similar analysis for peptide 328–346 and peptide 334–346 revealed a well-protected region upstream of the PKA binding site.

The high level of protection of the AKB domain was somewhat unexpected, since this surface interacts with the regulatory subunit of PKA. To investigate if the protection was due to secondary or tertiary structural effects, a peptide containing only the C-terminal 40 residues was examined. After deuteration for ten seconds at 22 °C the entire peptide was fully deuterated (data not shown), even though the CD spectrum of this peptide indicated that the peptide could form secondary structure under the conditions used for the deuterium exchange experiments (data not shown). This suggests that, in the context of the larger d-AKAP2 fragment, the AKB domain is further stabilized by tertiary interactions.

The comparison of deuteration levels at 0 °C and 22 °C

It was important to establish that the results at 0 °C did not differ significantly from those obtained at 22 °C, where most of the binding studies were performed. According to the Arrhenius equation, the exchange rate would increase by a factor of approximately 10 at 22 °C from that at 0 °C \( k(T) = A \exp(-E_a/RT); \ E_a \) for the base-catalyzed amide hydrogen exchange reaction is 17.4 kcal mol \(^{-1}\); 1 cal = 4.184 J.\(^{17}\) There was no evidence of a major conformational change of the protein in going from 0 °C to 22 °C. All peptides that were highly protected at 0 °C remained highly protected at 22 °C; all peptides that were heavily exchanged remained heavily exchanged, and all moderately exchanged peptides increased their deuteration levels continuously at longer exchange times. Only two (87–93 and 98–103) of the 15 moderately exchanging peptides showed 10% or more increase in deuteration levels over what would be predicted due to the higher temperature (Table 2), indicating that the conformational change due to temperature was very small.\(^{18}\)

Table 2. Deuteration level increase from 0 °C to 22 °C

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Increase (%)</th>
</tr>
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<tbody>
<tr>
<td>87–93</td>
<td>13</td>
</tr>
<tr>
<td>93–97</td>
<td>7</td>
</tr>
<tr>
<td>98–103</td>
<td>10</td>
</tr>
<tr>
<td>109–117</td>
<td>9</td>
</tr>
<tr>
<td>129–148</td>
<td>8</td>
</tr>
<tr>
<td>156–166</td>
<td>2</td>
</tr>
<tr>
<td>168–172</td>
<td>9</td>
</tr>
<tr>
<td>173–188</td>
<td>9</td>
</tr>
<tr>
<td>189–200</td>
<td>1</td>
</tr>
<tr>
<td>201–213</td>
<td>3</td>
</tr>
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</tr>
<tr>
<td>291–301</td>
<td>2</td>
</tr>
<tr>
<td>310–327</td>
<td>–1</td>
</tr>
<tr>
<td>328–346</td>
<td>3</td>
</tr>
<tr>
<td>355–375</td>
<td>7</td>
</tr>
</tbody>
</table>

* Increase was the average of deuteration level increases from 0 °C to 22 °C at four equivalent exchange times (100 seconds at 0 °C versus ten seconds at 22 °C, 300 seconds at 0 °C versus 30 seconds at 22 °C, 1000 seconds at 0 °C versus 100 seconds at 22 °C, and 3000 seconds at 0 °C versus 300 seconds at 22 °C).
interacting with the regulatory (R) subunit isoforms of PKA. After subsequent cloning of the full-length gene (coding for 662 amino acid residues), the protein was predicted to have multiple domains, including two putative RGS domains, a PKA binding site and a PDZ binding motif. We have been able to express a truncation of D-AKAP2 (residues 291–662) and have shown that this protein bound tightly to the type II regulatory subunit of PKA. The domain boundaries were mapped using traditional limited proteolysis and further refined using DXMS, providing a higher resolution of detail and an insight into the intradomain dynamics and function of D-AKAP2.

The two tandem RGS domains suggested the potential of D-AKAP2 to coordinate upstream heterotrimeric G proteins signaling with downstream PKA signaling. The evidence presented here suggested that RGS B does assume an RGS-like fold. The sequence containing this region modeled well as an RGS domain. The CD spectrum of D-AKAP2 was suggestive of a mostly α-helical protein. Moreover, hydrophobic residues that comprised the core of the fold were conserved in D-AKAP2 and mapped to the highly protected, helical regions of RGS B. In addition, the flexible and/or solvent-accessible regions, determined by DXMS analysis, mapped to the loops and turns, consistent with the structural model. The turn between helix 5 and 6 was the most solvent-accessible region of RGS B. Interestingly, this region in RGS4 makes contacts with the switch regions of Gα, stabilizing the transition state and enhancing the GTPase activity of Gα.

Despite the evidence that this region folds into an RGS domain, no Gα binding partner has been identified. Lack of a traditional Gα binding partner may be explained by several notable differences between the RGS domains of D-AKAP2 and other RGS proteins. In a phylogenetic classification of mammalian RGS proteins, D-AKAP2 did not contain significant homology to other known members in the family. The residues important for interaction with the switch regions of Gα subunit are not conserved in D-AKAP2. In addition, D-AKAP2 is the only mammalian protein identified that contains two RGS domains one of which is a “split” RGS domain. The presence of two RGS domains and the split nature of the N-terminal RGS domain are properties recognized only by lower eukaryotes such as Caenorhabditis elegans and Saccharomyces cerevisiae. Sst2 from S. cerevisiae, the first RGS domain identified, contains large insertions of amino acid residues between helices 1 and 2; 4 and 5; and 6 and 7 of the RGS domain. It is unclear how the presence of the tandem RGS domains and the large 124 residue insert between helices 4 and 5 of the RGS A domain of D-AKAP2 would affect the RGS fold or the specificity of its interaction partners. The extended region may provide additional interaction sites for binding non-traditional partners.

Lack of a Gα binding partner for an RGS domain is not unusual. Axin, a negative regulator of the Wnt growth-factor signaling pathway, contains an RGS domain and has not been shown to interact with any Gα subunits. However, it does use its RGS domain to interact directly with adenomatous polyposis coli (APC), a tumor suppressor protein. D-AKAP2 may use similar alternative binding surfaces.

In addition to providing a scaffold for binding other proteins, the multi-domain organization of D-AKAP2 altered the binding affinity to PKA. The low level of protection of the AKB domain alone compared with the intact protein suggested that, in the absence of PKA, this region was stabilized by intramolecular interactions. Residues 347–360 comprise the PKA binding surface and included the most protected region at the C terminus of the intact protein.

The reduced binding affinity observed for the binding of the intact protein to the regulatory subunit compared with the AKB domain alone was consistent with the model that a conformational change was required to form a tight complex with PKA. In vitro studies have shown that PKA can phosphorylate D-AKAP2 at S267 (data not shown), which is located in a highly solvent-accessible region between the putative RGS domain and the AKB domain. The effects of phosphorylation on the binding affinity to PKA and the domain organization are currently being determined, but it is possible that a phosphorylation feedback mechanism may be important to modulate the accessibility of this region to PKA.

The structures of two well-characterized AKB domains from AKAP79, an AKAP involved in neuronal signaling, and human thyroid anchoring protein HT31 have been solved by NMR in complex with the D/D domain of RIIα. Both AKBs bind similarly to the regulatory subunit with the hydrophobic face of the helix interacting with a hydrophobic groove on the surface of the regulatory subunit D/D domain. This raises the issue of how binding specificity is achieved for AKAPs involved in very different signaling pathways, and suggests that domains outside of the AKB could modify PKA binding affinity.

In contrast to the high level of protection of the AKB domain, the adjacent C-terminal PDZ-binding motif was located in a very solvent-accessible region. The accessibility of this region may be important for recognition by PDZ domain-containing proteins, thereby serving as a targeting domain for D-AKAP2. It will be important to identify interacting PDZ domains and to determine if binding of PDZ domains influences PKA binding and the accessibility of this region.

We have previously reported our efforts to systematically improve and automate amide hydrogen exchange liquid chromatography-mass spectroscopic methods with the aim of developing them into a high-throughput, high-resolution tool.
well suited to the study of isolated proteins as well as protein–protein binding interactions.\textsuperscript{13}

Many of the improvements that have been developed are focused on the protein chemistry employed, such as improved quench-compatible methods using denaturants and immobilized pepsin in tandem with mass spectrometry. The combination of these improvements has produced comprehensive amide hydrogen coverage of \(D\)-AKAP2 and has allowed us to map the domain organization of this protein without any prior knowledge of its structure. In this study of \(D\)-AKAP2, 99% of the protein sequence was covered by pepsin-generated peptide fragments of high quality and 83% of the peptide linkage (including proline residues) could be followed. This high degree of coverage enabled us to investigate the overall structure of \(D\)-AKAP2 (Figure 4). Sub-localization of deuterons incorporated was demonstrated at the C-terminal AKB domain. The subtraction of the deuterium incorporations of analogous peptides expanded the highly protected region (Figure 6), indicating that the accuracy of the current study is sufficient to obtain higher-resolution information via subtraction of two peptides. The principal challenge to large-scale proteomic analysis is to devise high-throughput techniques to gain information on protein structure and function. Several global initiatives have focused on structural determination by high-resolution techniques such as NMR and X-ray crystallography.\textsuperscript{22} While these methods are invaluable in understanding the 3D structure of a protein, they need to be complemented with techniques that examine the dynamic nature of proteins in solution and the assembly and disassembly of proteins into large multi-functional complexes. Amide hydrogen exchange analysis is uniquely valuable in this regard. Here, we have shown that enhanced amide hydrogen exchange methodologies, DXMS, can be used to rapidly gain high-resolution and comprehensive information on the folded regions of a protein. This approach, in combination with sequence and structural modeling, can provide unique insights into the structure and function of the protein.

Materials and Methods

**Protein expression and purification**

The sequence of mouse \(D\)-AKAP2 (Genbank AF021833) was sub-cloned into pET-15b (Invitrogen), using NdeI and XhoI restriction sites after mutating an internal Ndel site. As a result of cloning, three non-native amino acid residues were attached to the N terminus of the protein after thrombin cleavage. The plasmid was transformed into BL21 (DE3) cells (Novagen) and grown in LB medium with 100 \(\mu\)g/ml of ampicillin at 37 \(^\circ\)C, 300 rpm. The cells were induced at 0.8 \((a\cdot b)/c\) with 0.5 mM IPTG and the protein expressed for five hours at 24 \(^\circ\)C. Six liters of culture were pelleted and lysed in 100 ml of lysis buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 5 mM benzamidine) using a French press (1000 psi: 1 psi = 6.9 kPa). The lysate was centrifuged at 17,000 \(g\) for 30 minutes at 4 \(^\circ\)C. The protein was purified from the supernatant using Talon resin (Clontech) and the His tag cleaved with 3 mg of thrombin (Sigma). The protein was dialyzed into gel-filtration buffer (20 mM Mops (pH 7.0), 200 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM DTT) and purified further using an S200 gel-filtration column (Pharmacia). The protein concentration was determined by measuring the absorbance at 280 nm and using an extinction coefficient of 0.99 ml mg\(^{-1}\) cm\(^{-1}\), calculated by the method of Pace et al.\textsuperscript{23}

Murine RII\(\alpha\) was expressed in BL21 (DE3) cells and lysed in lysis buffer (20 mM Mes (pH 6.5), 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM DTT, 5 mM benzamidine) as described above. RII\(\alpha\) was precipitated from the supernatant by adding 45% \((w/v)\) ammonium sulfate and incubating for one hour. The precipitated protein was solubilized in lysis buffer and added to a cAMP affinity resin (Sigma). After binding to the resin overnight at 4 \(^\circ\)C, the protein was washed with lysis buffer containing 1 M NaCl followed by lysis buffer. RII\(\alpha\) was eluted for 30 minutes at room temperature using 25 mM cGMP (Sigma) in lysis buffer (pH 5.1). The protein was dialyzed overnight at 4 \(^\circ\)C in gel-filtration buffer (50 mM Mes (pH 5.8), 200 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM DTT) and purified further using an S200 gel-filtration column (Pharmacia). The protein concentration was determined using an extinction coefficient of 0.69 ml mg\(^{-1}\) cm\(^{-1}\), on the basis of amino acid analysis.

**Circular dichroism**

The spectrum was obtained using an AVIV 202 spectropolarimeter in a 0.1 cm rectangular, quartz cuvette (AVIV). The time constant for data collection was 100 ms with a four seconds averaging time. Three acquisitions were averaged. The buffer (20 mM NaPO\(_4\), 150 mM NaCl, pH 7.0) spectrum was subtracted from the sample spectrum. The protein concentration was 2 \(\mu\)M. The mean residual ellipticity (MRE) was calculated according to the following equation:

\[
\text{MRE} = \theta_{obs}(M/100\text{Ir})
\]

where \(\theta_{obs}\) is the buffer corrected ellipticity, \(M\) is the molecular mass (42,800 Da) of \(D\)-AKAP2, \(l\) is the pathlength, \(c\) is the concentration (in molarity) and \(n\) is the number of amino acid residues in the protein.

**Limited proteolysis and boundary mapping**

\(D\)-AKAP2 (0.85 mg/ml) was digested separately with both trypsin (Worthington Biochemical Corporation) and endoproteinase Glu-C (Boehringer Mannheim) in a 1:100 \((w/w)\) ratio. At various time-points, aliquots were taken and quenched with 10% \((v/v)\) glacial acetic acid for the trypsin digests or quick-frozen on solid CO\(_2\) for the endoproteinase digests. The samples were analyzed by SDS-PAGE and by LCMS. For each of the various digest time-points, 5.0 \(\mu\)g of total digested protein was loaded onto a Michrom BioResources Magic 2002 micro-bore HPLC system (Auburn, CA) equipped with a 1.0 mm \(\times\) 150 mm Vydac C\(_4\) column (5 \(\mu\)m, 300 A\(\text{\AA}\)), equilibrated at a flow-rate of 50 ml/minute and a column temperature of 35 \(^\circ\)C. A gradient from 10% to 80%
solvent B over 60 minutes was then initiated (solvent A, 2% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid (TFA); solvent B, 90% acetonitrile, 0.095% TFA). The eluent from the UV detector of the HPLC was plumbed without splitting into a PE Scieix/Applied Biosystems QSTAR quadruple-TOF mass spectrometer (Foster City, CA). Spectra from 400 to 2000 m/z were recorded continuously over the entire gradient with the CAD gas set to 3 and the following source parameters: ionspray gas = 60, ionspray voltage = 5 kV, and curtain gas = 20.

**Surface plasmon resonance**

Binding of RIIs to d-AKAP2 was performed using a BIAcore 3000 (BIAcore). The regulatory subunit of PKA was immobilized to a CM5 chip (BIAcore) as described. Using this method, 80 response units (RU) of a cAMP analog (8-(6-aminohexyl) aminoadenosine-3',5'-cyclic monophosphate (8-AHA-cAMP) (BioLog) were covalently immobilized to flow channel 1 (FC1) and flow channel 2 (FC2) of an activated CM5 chip (BIAcore) using the amine coupling kit from BIAcore. RIIs (cAMP free) was injected over FC2 at a flow-rate of 50 μl/minute for one minute, resulting in 450 RU of bound RIIs. Serial dilutions (250–2 nM) of d-AKAP2 were injected over FC1 and FC2 at 50 μl/minute for two minutes with a four minute dissociation time using the kinetic injection mode. The surface was regenerated by injecting 0.2% (w/v) SDS and running buffer (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.005% (w/v) P20 (HBS-EP, BIAcore)) for 30 seconds at 50 μl/minute. Three replicates were obtained using the automated Kinetic Analysis module provided in the BIAcore instrument software. Each replicate was fit separately to a 1:1 binding model using BIAevaluation software V. 3.0 (BIAcore). The rates and dissociation constants from each fit were averaged.

**DXMS 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 GuHCl (the final pH was measured on a non-deuterated mock solution at room temperature using a pH meter (model 250; Denver Scientific Co., Arvada, Colorado)). At 0 °C, the quenched solution was immediately passed over a column (66 μl bed volume; Upchurch Scientific, cat no. C.130) filled with porcine pepsin (Sigma, cat no. P6887) immobilized on Poros 20 AL medium at 30 mg/ml following the manufacturer’s instructions, with 0.05% TFA (200 μl/minute) for two minutes with contemporaneous collection of proteolytic products by a C18 column (Vydac cat no. 218MS5105). Inline filters (Upchurch cat no. A.430) were placed on each side of the pepsin column, and just before the C18 column (Vydac prefilter, cat no. CPF 10) to minimize column fouling. Subsequently, the C18 column was eluted with a linear gradient of 10%–50% solvent B over ten minutes (solvent A was 0.05% TFA in water, and solvent B was 80% acetonitrile, 20% water, 0.01% TFA). Mass spectrometric analyses were carried out with a Finnigan LCQ mass spectrometer with capillary temperature at 200 °C.

**Sequence identification of pepsin-generated peptides**

To quickly identify pepsin-generated peptides for each digestion condition employed, spectral data were acquired in “triple play” mode. The triple play data set was then analyzed employing the Sequest software program (Finnigan Inc) to identify the sequence of the dynamically selected parent peptide ions. This tentative peptide identification was verified by visual confirmation of the parent ion charge state presumed by the Sequest program for each peptide.

**Deuterium exchange experiments**

Deuterated samples were prepared (both at 0 °C and at 22 °C) by diluting 1 μl of d-AKAP2 stock solution with 19 μl of deuterated buffer (10 mM Hepes (pD 7.4), 150 mM NaCl), followed by “on-exchange” incubation for varying times (10–3000 seconds) prior to quenching in 30 μl of 0.8% formic acid, 3.2 M GuHCl, 0 °C. These functionally deuterated samples were then subjected to DXMS processing as above, along with control samples of non-deuterated and fully deuterated d-AKAP2 (incubated in 0.5% formic acid in 95% H2O for 24 hours at 22 °C). The centroids of probe peptide isotopic envelopes were measured using the Magtran program provided by Zhongqi Zhang. The corrections for back-exchange were made employing the methods of Zhang & Smith:

\[
\text{Deuteration level(%) = } \frac{m(P) - m(N)}{m(F) - m(N)} \times 100
\]

where \(m(P)\), \(m(N)\), and \(m(F)\) are the centroid value of partially deuterated peptide, non-deuterated peptide, and fully deuterated peptide, respectively; maxD is the maximum deuterium incorporation calculated by subtracting the number of proline residues in the third or later amino acid residue and two from the number of amino acid residues in the peptide of interest (assuming the first two amino acid residues cannot retain deuterons\(^8\)). Typically deuteron recovery of fully deuterated sample \((m(F) - m(N))/\text{maxD}\) was 80–90%.

**Computational analysis and three-dimensional structure homology modeling**

Sequence alignments were done using CLUSTALX, a Windows-ported version of CLUSTALW. Domain searches against the Pfam domain database\(^{25}\) were performed using HMMER 2.1.\(^{19}\) A three-dimensional model of the RGS domain B of mouse d-AKAP2 was created using the SwissModel server.\(^{23}\) A suitable template was identified by performing a BLAST search\(^{26}\) of RCSB Protein Data Bank (PDB) using the sequence of the RGS B domain of d-AKAP2 as query. The coordinates of chain E from the X-ray crystal structure of the complex of Alf4-Activated Gi-Alphai-1 with RGS4 from rat (PDB ID = 1AGR_E) were used as template. Initial structural alignments were created using the first approach mode of the SwissModel server and the final mode of the three-dimensional structure was calculated on the basis of this preliminary model, utilizing the optimize mode of the software.

The model was subjected to additional energy minimization using the GROMOS 43B1 force-field as implemented in the DeepBlue SwissPDB Viewer\(^{27}\) using
5000 cycles of steepest descent followed by 5000 cycles of conjugate gradients. All computations were done in vacuo, without reaction field. Harmonic constraints of 50 C-factors and 2500 C-factors were used for each method.

The final model was validated using the Biotech Validation Suite for Protein Structures, which includes the packages PROCHECK V3.5, PROVE V2.3, and WHAT IF V4.99. Structure models were visualized and images of structures were rendered with WebLab Pro v4.0 (Accelrys). Structural sequence alignments were performed using the CE (Combinatorial Extension) method.25

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† http://biotech.ebi.ac.uk:8400

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