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The ATCUN Domain as a Probe of Intermolecular Interactions: Application to Calmodulin−Peptide Complexes

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Structural studies of biomolecular complexes provide important insight into how each of the component molecules is regulated. In many cases an understanding of the mechanism of regulation can only be derived through comparison of a number of different complexes. In cases where structures of several complexes have already appeared, detailed and potentially time-consuming three-dimensional structural initiatives of additional complexes may not be required. In these cases, high-quality structures can be built by homology, guided by key pieces of experimental data.

One example where this is the case involves structural studies of the protein calmodulin (CaM) and its mechanism of interaction with target serine/threonine protein kinases (CaM kinases). CaM is comprised of two domains, each of which binds a pair of Ca^{2+} ions.[1] The conformational changes that occur upon metal binding enable the protein to bind to short helical segments in target kinases with high affinity ($K_d \approx 10^{-8}-10^{-9}$ M) leading to their activation.[2,3] Because of its key role in the Ca^{2+}-dependent signal transduction pathway in eukaryotes the structures of CaM in complex with a number of peptides, such as those derived from CaM kinase II (CaMKII),[4] mysoin light chain kinase (MLCK),[2,3] and CaM kinase kinase (CaMKK)[5] have been solved, establishing three distinct CaM binding modes. These three classes of structures are referred to as 1−10 (CaM−CaMKII), 1−14 (CaM−MLCK), and 1−16$^-$ (CaM−CaMKK), where the numbering denotes the positions of hydrophobic residues in the helical peptide that help anchor it to CaM.[6] In each mode, the structures of the domains are preserved, but their relative orientations differ.[2−5] In addition to the variability in the structures of CaM in different complexes, it has been found that peptide binding can be in one of two orientations.[2−5] Figure 1 shows X-ray-derived structures of complexes of CaM−CaMKII[4] and CaM−CaMKK,[5] illustrating the differences in both domain and peptide orientation.

There are over 180 distinct CaM binding targets that have been identified to date, and in some cases the nature of the binding interaction can be inferred from the structures in the database.[7] In other cases, there are several possibilities as to what the anchoring residues on the target sequence of the kinase might be, and it is thus not possible to come up with definitive models of the complex in the absence of experimental data. There is thus a need to develop the tools to rapidly characterize the binding mode of CaM with a query target peptide. We have recently shown that $^1H-^{15}N$ residual dipolar coupling data recorded on CaM in complex with such a target can be used to distinguish between the three binding modes described above.[8] However, the orientation of the helix remains undefined in this approach. Here we show that the orientation can be rapidly determined from a pair of $^1H-^{15}N$ correlation spectra of CaM in complex with a peptide construct containing the three-residue ATCUN (amino terminal $Cu^{2+}(Ni^{2+})$-binding) domain[9] recorded with and without Cu^{2+}.

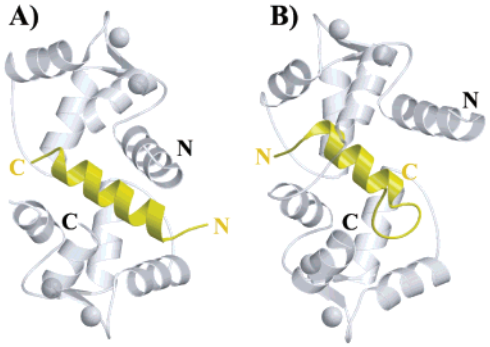

Figure 1. Ribbon representation of CaM−CaMKII[4] (A) and CaM−CaMKK[5] (B) complexes, with target peptides shown in yellow. Figures are generated using Molscript[16] and Raster3D.[17]

The utility of paramagnetic agents to study molecular complexes by NMR is well established,[10−12] and the ATCUN domain is a particularly useful probe in this regard because of its small size. The ATCUN motif, NH_2-X1-X2-His, coordinates either Cu^{2+} (paramagnetic) or Ni^{2+} (diamagnetic) metal with very high affinity ($K_d \approx 10^{-15}$ M) via the free NH_2 group from residue X1, the backbone amides of X2 and His along with the imidazole group of His.[9] Because the electronic relaxation time is long (nanoseconds) and the anisotropy of the **g**-tensor small, the ATCUN probe when bound with Cu^{2+} effectively broadens nuclear spins without substantial changes in chemical shifts.[13] This is illustrated in $^1H-^{15}N$ spectra[14] of ^{15}N-labeled CaM in complex with the ATCUN-MLCK peptide (primary sequence is shown at the top of Figure 2; ATCUN denoted by black ball) in the absence (A) and presence (B) of stoichiometric amounts of Cu^{2+}, Figure 2. Correlations that disappear in Figure 2B, indicated by the open red circles, can be mapped onto the structure of the CaM−MLCK complex (Figure 2C, red balls). These residues are located in helices I and VII, that are in proximity to the N-terminal of the MLCK peptide. The results are consistent with the binding orientation of the peptide from NMR and X-ray derived structures[2,3] and conclusively establish which of the two potential orientations of the helix (see Figure 1) is present in the complex. It is of interest that the N terminus of the peptide is more basic than the C terminus (blue residues in the sequence at the top of Figure 2). Placement of the peptide in the complex is determined not only by the hydrophobic anchoring residues (labeled 1 and 14) but also by the favorable juxtaposition of charge. Indeed, superimposing an electrostatic surface onto the structure of CaM

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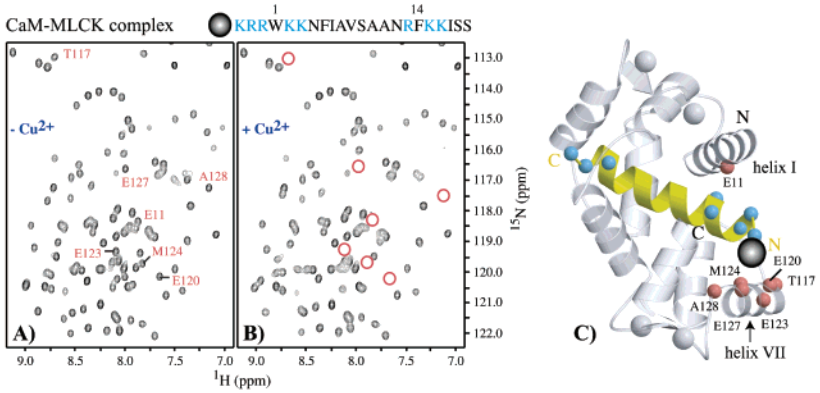

Figure 2. $^{1}H-^{15}N$ HSQC spectra of the CaM−MLCK complex without (A) and with (B) stoichiometric amounts of Cu^{2+}. Correlations that are broadened beyond detection by the addition of Cu^{2+} are identified in red (A, assignments; B open circles). The ATCUN-MLCK peptide sequence is indicated at the top of the spectra, with basic residues in blue, and the ATCUN domain (Gly-Ser-His) is denoted by the black ball. Residues whose correlations are broadened are mapped onto the X-ray structure[3] of the complex in C (red balls). All spectra were recorded on a 600-MHz Varian Inova spectrometer at 32 °C, with samples comprised of 0.9 mM ^{15}N-labeled protein, 10 mM Bis-Tris, 100 mM KCl, 6 mM $CaCl_2$, pH 6.8. Details of sample production are as described previously.[18] ATCUN-derived peptides were generated by solid-phase peptide synthesis.

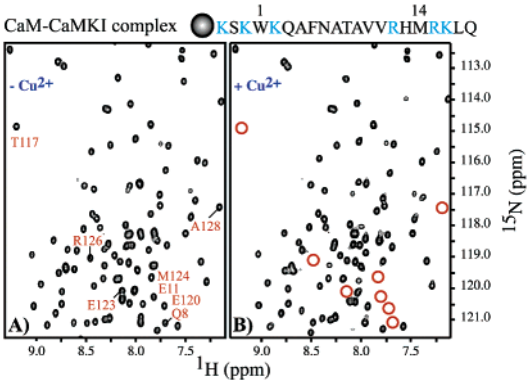

Figure 3. $^{1}H-^{15}N$ HSQC spectra of the CaM−CaMKI complex without (A) and with (B) stoichiometric amounts of Cu^{2+}. Correlations indicated in (A) are those that are completely broadened upon addition of Cu^{2+}, highlighted by open red circles in (B).

confirms that this orientation is favored over the case where the peptide is reversed (as in CaMKK, Figure 1B; see also Supporting Information).

Figure 3 shows $^{1}H-^{15}N$ spectra of ^{15}N-labeled CaM bound to its target peptide from CaM kinase I, CaMKI, along with the sequence of the peptide. A structure is not available for this complex. Both N and C termini of the peptide have similar numbers of basic residues, and the orientation of the peptide cannot, therefore, be inferred on the basis of charge complementarity with the protein. The HSQC spectra show a pattern of disappearance of cross-peaks that is consistent only with a peptide orientation similar to that found in the CaM−CaMKII (Figure 1A) and CaM−MLCK complexes (Figure 2C) and not the orientation observed for CaM−CaMKK (Figure 1B). As with the CaM−MLCK complex (Figure 2) residues that disappear are localized to helices I and VII. This result, in combination with dipolar coupling data[8] establishing that the mode of binding is 1−14, strongly suggests that the CaM−MLCK structure is an excellent model for the CaM−CaMKI interaction.

In summary, we have shown that a comparison of HSQC spectra of an ATCUN-tagged peptide with and without Cu^{2+} can be used to rapidly define peptide-binding orientations in a biologically important class of complexes. The small size of the ATCUN domain ensures that its introduction leads to only very modest changes in both peptide−CaM affinity and in the structure of the resulting complex. For example, in the case of CaM bound to the MLCK peptide, introduction of the ATCUN tag changes the binding constant by less than a factor of 1.5. Root-mean-squared differences in ^{1}H and ^{15}N shift values of 0.03 and 0.16 ppm, respectively, are observed in a comparison of chemical shifts between complexes with (−Cu^{2+}) and without the ATCUN tag, with shifts of residues proximal to the tag changing by no more than 0.08 ppm (^{1}H) and 0.42 ppm (^{15}N). Finally, it is noteworthy that bi-directional modes of recognition have also been found in other systems, such as in SH3 domain interactions with target proline-rich peptides.[15] It is very likely that this approach will be useful, therefore, to rapidly survey a wide array of intermolecular interactions.

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Supporting Information Available: Figure showing $^{1}H-^{15}N$ correlation spectra recorded on a complex of CaM and ATCUN−CaMKK with and without Cu^{2+} (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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