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Measurement of One Bond Dipolar Couplings through Lanthanide-Induced Orientation of a Calcium-Binding Protein

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Current methods for protein structure determination by NMR spectroscopy are primarily based on parameters that yield short-range geometrical information, such as nuclear Overhauser effects and three bond coupling constants. In contrast, one-bond dipolar couplings depend on the angular coordinates of the corresponding bond vectors with respect to a common frame of reference, providing information on the relative orientation of bonds throughout a molecule independently of the distance between the bonds. Thus, measurement of one-bond dipolar couplings can considerably improve the quality of protein structures and define the relative orientations of distant domains within a protein or between two molecules in a multiprotein complex.[1,2]

Residual dipolar couplings can be measured in partially oriented proteins in which complete averaging of dipolar interactions is prevented. Residual N−H dipolar couplings were first measured in the paramagnetic protein cyanometmyoglobin.[3] Diamagnetic proteins can also be oriented provided that their diamagnetic susceptibility has some degree of anisotropy. However, the degree of alignment, even at the highest available magnetic fields, is very low[4] and the use of two magnetic fields introduces the need to correct for dynamic frequency shifts. Recently, considerable alignment has been obtained by using diluted liquid crystal systems based on bicelles[5] or filamentous viruses.[6]

Calcium-binding proteins constitute a major class of proteins with crucial roles in a wide variety of biological processes. Due to their similar ionic radii, some lanthanide ions can replace calcium in calcium-binding proteins with minimal structural changes.[7] Paramagnetic lanthanides, except Gd^{3+}, form complexes with anisotropic magnetic susceptibility that can be oriented in a magnetic field. Global orientation of a DNA fragment that forms a quadruplex in solution has been recently achieved by binding of two europium ions.[8] The possibility of using lanthanides to orient proteins has also been suggested by these authors and previously by Tjandra et al.,[1] but to our knowledge, it has not been yet demonstrated experimentally and it is unclear whether a sufficient degree of alignment can be achieved to obtain measurable dipolar couplings. In this communication we demonstrate that Yb^{3+} induces substantial orientation on a mutant of the C_2A domain of synaptotagmin I, yielding measurable one-bond HN dipolar couplings that provide information on the structure of the domain far from the metal-binding site. This information is different from that obtained with the classical studies of metal-binding sites using lanthanide-induced shifts or broadening that report on nuclei situated only at relatively short distances from the metal.

Synaptotagmin I is a synaptic vesicle protein that is believed to be the Ca^{2+} sensor in neurotransmitter release.[9] The C_2A domain of synaptotagmin I offered a good model system to test whether lanthanides can induce alignment of a Ca^{2+}-binding protein because its structure consists of a compact β-sandwich fold that exhibits very minimal and only local conformational changes upon Ca^{2+} binding,[10] similarly to the C_2 domain of PLC-δ1.[11] For the latter C_2 domain, lanthanides have been shown to substitute well for Ca^{2+}, again without structural perturbations.[11,12] Many C_2 domains bind phospholipids in a Ca^{2+}-dependent manner, which advises against the use of phospholipid-based diluted liquid crystalline phases to induce orientation in these systems. A potential complication with the use of C_2 domains for our study was the fact that they bind multiple Ca^{2+} ions. Thus, to simplify the study, we used a mutant of the C_2A domain of synaptotagmin I with an Asp232Asn substitution ($D232NC_2A$) that only binds Ca^{2+} at the highest affinity site.[13] Analysis of the Ca^{2+}-free and Ca^{2+}-bound forms of this mutant by 3D ^1H-^{15}N TOCSY−HSQC and NOESY−HSQC showed that the mutation causes minimal perturbations of chemical shifts with respect to the wild-type C_2A domain, with the exception of nuclei that are very close to the mutation (data not shown). Thus, the mutation has only very local structural consequences near residue 232.

Magnetic susceptibility anisotropy results from ligand field effects that remove the spherical symmetry around the metal ion. The principal components of the susceptibility tensor are proportional to Bleaney's C_J values[14,15] characteristic for each lanthanide, and both the contact interactions and the Curie-spin relaxation broadening[16] are proportional to the spin expectation value $\langle S_z \rangle$, which is also characteristic for each lanthanide, and that are tabulated.[17] Ytterbium was selected as the paramagnetic agent because the ratio $C_J/\langle S_z \rangle$ is one of the largest.[18]

Yb^{3+} binding to ^{15}N-labeled $D232NC_2A$ was followed using HSQC spectra obtained after the addition of microliter aliquots of a 16.35 mM $YbCl_3$ solution adjusted to the same pH (4.98) of a sample of 128 μM apo-$D232NC_2A$ in 40 mM NaAcO, 100 mM NaCl. The dissociation constant was estimated to be ≤ 7 μM from the dependence of the cross-peak volumes with the Yb^{3+} concentration. This affinity is at least 1 order of magnitude higher

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(18) The lanthanide with the largest $C_J/\langle Sz \rangle$ ratio is samarium, but its C_J value is only −0.7 as compared to 22 for ytterbium. Curie-spin relaxation should be proportional to $\langle Sz \rangle^2$. The ratio $C_J/\langle Sz \rangle^2$ is larger for cerium than for ytterbium, but its C_J value is only −6.3. Considering the degree of orientation obtained in the present work using ytterbium, cerium should be an acceptable alternative.

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than the Ca^{2+} affinity (~70 μM). Lanthanides often have much higher affinities for calcium-binding proteins than calcium itself.[19] Most of the backbone resonances of the Yb^{3+}-bound D232NC_2A domain were assigned using 3D ^{1}H-^{15}N TOCSY−HSQC and NOESY−HSQC experiments, except for nuclei within ~10 Å of the bound Yb^{3+} ion, which were broadened beyond detection.

One-bond HN couplings for the apo-, Ca^{2+}-bound, and Yb^{3+}-bound forms of D232NC_2A were measured on a Varian Inova-600 spectrometer as described by Tjandra et al.[4] The $^1J_{HN}$ values measured for residues outside of the binding site in the apo and Ca^{2+}-bound forms were identical within the experimental error estimated to be <0.2 Hz.[20] This correlates with the previous observation that metal binding does not cause any conformational changes far from the binding site in the wild-type C_2A domain.[10] $^1J_{HN}$ values for Yb-D232NC_2A ($^1J_{HN}$(Yb)) are significantly different from the ones measured in the two diamagnetic proteins, indicating that residual dipolar couplings are being observed in the paramagnetic protein. The residual dipolar couplings resulting from the Yb^{3+} -induced orientation of the molecules were obtained by subtracting the $^1J_{HN}$(Ca) values from the $^1J_{HN}$(Yb) values measured at the same magnetic field. To compare measured with predicted residual dipolar couplings, we extracted the orientations of the HN bonds from the crystal structure of the wild-type C_2A domain at 1.8 Å resolution.[21] The validity of this structure as a model was confirmed by the good agreement between the observed and predicted dipolar couplings (see below).

The magnetic susceptibility tensor and its orientation with respect to the molecule-fixed reference frame can be obtained by nonlinear fitting of observed residual dipolar couplings to eq 1.

$$D_{AB} = -\frac{1}{4\pi}\frac{B_o^2}{15kT}\frac{\gamma_A\gamma_B h}{4\pi^2 r_{HN}^3} \times S\left[\Delta\chi_{ax}(3\cos^2\theta - 1) + \frac{3}{2}\Delta\chi_{rh}(\sin^2\theta\cos 2\phi)\right] \quad (1)$$

where $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ are the axial and rhombic components of the susceptibility tensor and θ and ϕ are polar coordinates describing the orientation of the N−H bond vector with respect to the principal axes of the susceptibility tensor. The order parameter S is used to correct, as a first approximation, for internal motions and was set to one. Residues close to the binding site were severely broadened and were not included in the calculations. H254, which shows very different conformations in the NMR and X-ray structures, was also excluded. All calculations were done using Mathematica (Wolfram Inc., Champaign, IL).

Figure 1 shows a plot of experimental vs calculated values, which, in general, are in excellent agreement. A total of 64 dipolar couplings, measured from cross-peaks that do not exhibit overlap in either the Yb^{3+}- or the Ca^{2+}-bound forms, were included in the calculations. This represents ~70% of the assigned HN pairs. The largest deviations between observed and calculated values (≤0.75 Hz) correspond to residues K192 and S217, which are in loops and may have some disorder. Similar results were obtained using the apoprotein as reference. The calculated axial component of the paramagnetic susceptibility is 5.40×10^{-32} m^3 and the rhombic component is -1.13×10^{-32} m^3. Both components are larger than the ones reported for the paramagnetic contribution in cytochrome b5[22] (2.8×10^{-32} m^3 and -1.1×10^{-32}, respectively).[23] The ytterbium-induced anisotropy is also larger in absolute value than the diamagnetic susceptibility anisotropy observed in the GATA−DNA complex ($\Delta\chi_{ax} = -3.01 \times 10^{-32}$ m^3).[24]

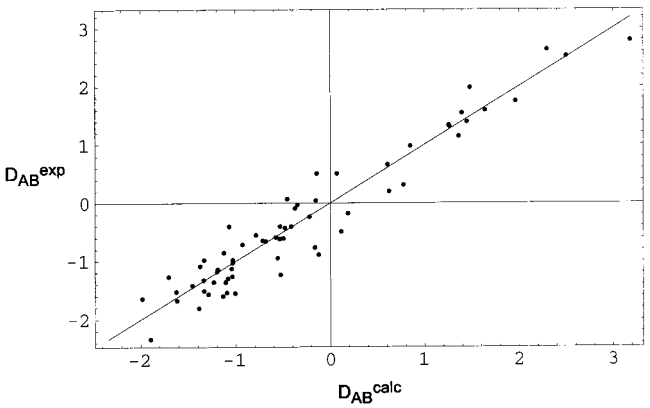

Figure 1. Experimental ($^1J_{HN}$(Yb) − $^1J_{HN}$(Ca)) versus calculated residual one-bond NH dipolar couplings of D232NC_2A domain of synaptotagmin I using the geometry obtained from the 1.8 Å crystal structure of wild-type C_2A domain.

Lanthanide replacement in calcium-binding proteins provides residual dipolar couplings without the need to use two magnetic fields or liquid crystal phases. The method relies on structural conservation upon metal substitution, at least far from the binding site, and on acquiring the data for the Ca^{2+}- and lanthanide-bound forms under identical conditions. This approach avoids the need to correct for the effect of dynamic frequency shifts[25] arising from dipole−dipole CSA interference.[26] In the paramagnetic proteins studied thus far, the diamagnetic anisotropy of the haem group partially cancels the paramagnetic contribution from the metal ion. In our example, the diamagnetic contribution from the protein does not have to be taken into consideration, as it will contribute equally to the susceptibilities of calcium and ytterbium complexes. The use of different lanthanide ions could provide different orientations that would help to resolve the degeneracy of the spherical angles of the NH bond vectors. Additionally lanthanide-induced orientation provides a way of selectively orienting metal-binding proteins in complex mixtures. This opens the possibility of studying induced orientation by protein−protein interactions that should provide information on their relative orientation in the complex.

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Supporting Information Available: Experimental $^1J_{HN}$ values for free, Yb^{3+}-bound, and Ca^{2+}-bound D232NC_2A domain of synaptotagmin I. This material is available free of charge via the Internet at http://pubs.acs.org.

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