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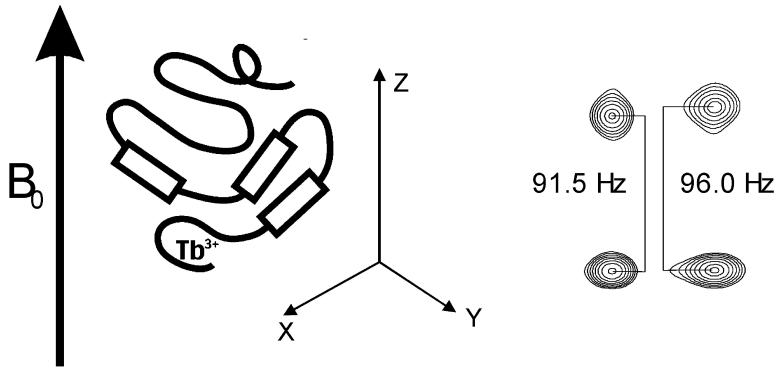

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Protein Alignment by a Coexpressed Lanthanide-Binding Tag for the Measurement of Residual Dipolar Couplings

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Long-range structural restraints derived from residual dipolar couplings (RDCs) have become exceptionally useful parameters for improving the speed and accuracy of protein structure determination by NMR.[1] Since molecular tumbling averages out dipolar couplings in solution, several strategies have been developed to observe RDCs by weakly aligning molecules in a magnetic field. Effective alignment media include liquid crystals,[2] polyacrylamide gels,[3] phage,[4] and purple membrane fragments.[5] Alignment is thought to result from steric and charge interactions with the media, and consideration must be taken with respect to possible influences of the aligning media on the target protein.

An alternative approach to induce alignment is the use of paramagnetic metal ions with anisotropic magnetic susceptibilities.[6] This strategy has been exploited for iron-containing metalloproteins such as myoglobin[7] and cytochromes,[6,8] and for lanthanide-substituted calcium-binding proteins.[9] Extending this approach to proteins lacking native metal-binding sites is less straightforward and currently lacks a generalizable approach. Synthetic metal chelates have been attached via chemical modification of proteins,[10] and native metal-binding protein domains such as zinc-finger or EF-hand motifs[11] have been incorporated into fusion proteins. However, these approaches suffer from a limited degree of alignment and from the small size of the measured dipolar couplings due to flexibility of the linker region and/or the limited affinity of the metal-binding site requiring excess metal ions with deleterious consequences, which include nonspecific binding, additional line broadening and overall relaxation. Fusion of a calmodulin-binding peptide allows a target protein to form a large complex with Tb^{3+}-substituted calmodulin,[12] whereas fusion with a Cu^{2+}-binding peptide motif provides relaxation enhancement although the isotropic **g** tensor of Cu^{2+} prevents molecular alignment.[13]

Recent efforts in the design, combinatorial synthesis, and screening of short peptides that bind lanthanide ions have provided lanthanide-binding tags (LBTs) with significantly improved properties compared to those of conventional EF-hand motifs.[14] LBTs are easily incorporated into fusion proteins, allowing facile overexpression of a protein containing a minimally invasive, versatile protein tag. The luminescent properties of Tb^{3+}-loaded LBT−ubiquitin have been demonstrated.[14] Here, we report a novel application of the LBT in its utility to achieve significant alignment of a fusion protein in a magnetic field, without the need for an external alignment medium but in the presence of stoichiometric lanthanide ion.

The LBT chosen for this study has the sequence YIDTNNDGWYEGDELLA,[14] which was appended to the N-terminus of human ubiquitin by standard cloning techniques.[15] The peptide's affinity for trivalent lanthanide ions follows a parabolic relationship dependent on the ionic radii across the series, with the tightest apparent dissociation constant occurring for Tb^{3+} ($K_D = 57$ nM).[14] The affinity is reduced moving to the largest (La^{3+}, $K_D = 4\ \mu$M) and smallest (Lu^{3+}, $K_D = 130$ nM) lanthanide (unpublished results). The K_D of the fusion protein for Tb^{3+} was determined to be 130 nM by fluorescence titration, affirming that the LBT retains lanthanide affinity in the context of a protein fusion.[15]

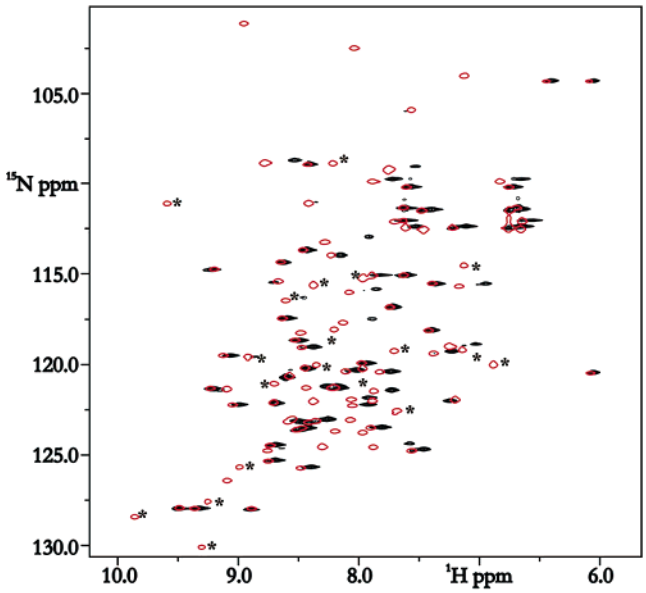

Figure 1. Two-dimensional ^{1}H,^{15}N HSQC spectra showing the backbone amide resonances of the LBT-tagged protein human ubiquitin in the presence of equimolar concentrations of diamagnetic Lu^{3+} (single red contours) and paramagnetic Tb^{3+} (multiple black contours). Additional cross-peaks observable from the LBT tag are marked by an asterisk (*).

The ^{1}H,^{15}N HSQC spectrum of LBT−ubiquitin loaded with diamagnetic Lu^{3+} is shown in Figure 1.[16] Notably, the ubiquitin signals can be assigned by comparison with known assignments,[17] indicating that the presence of the LBT does not significantly alter the protein structure. In addition, the only chemical shift changes upon addition of Lu^{3+} to metal-free LBT−ubiquitin occur for resonances of the ubiquitin N-terminus and of the tag itself, suggesting an absence of nonspecific metal binding despite the high negative charge of ubiquitin. In contrast, early generation LBT−ubiquitin constructs with weaker lanthanide affinities were plagued by metal-induced precipitation at concentrations necessary for NMR. Clearly, employing an optimized LBT with a high-affinity lanthanide site is critical for the success of this strategy and its future broad applicability.

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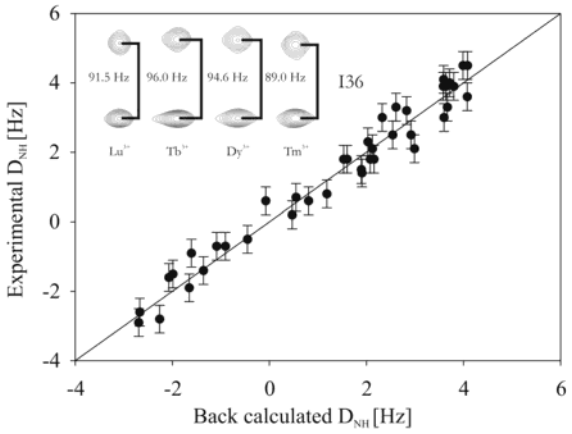

Figure 2. Correlation of the experimental residual $^1D_{NH}$ couplings for Tm^{3+} to values back-calculated on the basis of the ubiquitin X-ray structure[20] using the program MODULE.[21] (Insert) Peak patterns in the ω_1-coupled $^1H,^{15}N$ HSQC spectra and the measured $^1J_{HN}$ couplings for residue I36 in the presence of Lu^{3+}, Tb^{3+}, Dy^{3+}, and Tm^{3+}.

The $^1H,^{15}N$ HSQC spectrum of the protein bound to Tb^{3+} is also shown in Figure 1. Despite paramagnetic relaxation caused by the presence of the paramagnetic metal, 48 out of 72 expected backbone amide signals of ubiquitin are observable. A number of peaks are shifted due to Tb^{3+}-induced pseudocontact shifts, but a large fraction of the signals can still be assigned without any further experiments. Similar quality spectra were obtained for the Dy^{3+}- and Tm^{3+}-bound protein (62/72 and 61/72 backbone peaks for Dy^{3+} and Tm^{3+}, respectively).[15]

$^1D_{NH}$ RDCs were measured in the presence of Tb^{3+}, Dy^{3+}, and Tm^{3+} relative to the diamagnetic Lu^{3+} reference in ω_1-coupled $^1H,^{15}N$-HSQC and IPAP spectra[18] for well separated and reliably assigned amide signals.[15] Tb^{3+}, Dy^{3+}, and Tm^{3+} generate residual dipolar couplings of different sizes (Figure 2). $^1D_{NH}$ values range from -7.6 to 5.5 Hz for Tb^{3+}, -6.6 to 6.1 Hz for Dy^{3+}, and 4.5 to -2.9 Hz for Tm^{3+}.[15] As predicted,[19] couplings of opposite sign were observed with Tm^{3+} compared to those with Tb^{3+} and Dy^{3+}.[15] The measured $^1D_{NH}$ values were compared with theoretical couplings back-calculated on the basis of the X-ray structure of ubiquitin[20] with the program MODULE.[21] The correlation for Tm^{3+} (Figure 2) where 40 RDCs were measured after confirming the assignments for the pseudocontact shifted residues yields an R^2 value of 0.96. Tb^{3+} and Dy^{3+} induced highly collinear alignment frames;[15] however, the difference in the alignment frames induced by Dy^{3+} and Tm^{3+} is similar in size to those induced by DMPC/DHPC vs. DMPC/DHPC/CTAB,[22] a pair of alignment media proposed specifically for resolving the degeneracy of RDC-derived orientational information.[21,22]

In conclusion, we have shown that a high-affinity LBT fused to the N-terminus of ubiquitin can effectively align the protein in solution upon addition of paramagnetic lanthanide ions. The alignment induced due to the specific binding of lanthanides to the LBT provides residual dipolar couplings of a magnitude that can be accurately measured by simple methods. Since the LBT residues contribute only a limited number of additional signals, the presence of the tag does not increase signal overlap. In addition to RDCs, the affinity of the tag for different lanthanide ions can be used to measure pseudocontact shifts and/or paramagnetic relaxation enhancements that provide additional sources of long-range distance constraints.[23] Taken together with the facile incorporation of the LBT into the target protein[15] by the overexpression of a fusion construct, the LBT strategy should provide a complementary tool which can be used with currently available methods for macromolecular structure determination.

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Supporting Information Available: $^1H,^{15}N$ HSQC spectra of LBT−ubiquitin in the presence of Dy^{3+} and Tm^{3+}, correlation plots of measured versus theoretical RDCs for Tb^{3+} and Dy^{3+}, pairwise correlation plots of the experimental RDCs, titration of LBT−ubiquitin with Tb^{3+}, a table of measured RDCs and the Euler angles and the axial and rhombic components of the alignment tensors, a table of Tb^{3+} affinities for tags fused to different proteins (PDF). This material is available free of charge via the Internet at www.pubs.acs.org.

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