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COMMUNICATION

Engineering of a bis-chelator motif into a protein α-helix for rigid lanthanide binding and paramagnetic NMR spectroscopy†

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Attachment of two nitrilotriacetic acid-based ligands to a protein α-helix in an *i*, *i* + 4 configuration produces an octadentate chelating motif that is able to bind paramagnetic lanthanide ions rigidly and with high affinity, leading to large pseudocontact shifts and residual dipolar couplings in the NMR spectrum.

The tagging of proteins with paramagnetic lanthanide ions (Ln^{3+}) produces large effects that are observable in NMR spectra, including pseudocontact shifts (PCSs), paramagnetic relaxation enhancements (PREs) and residual dipolar couplings (RDCs).[1,2] These effects provide extremely valuable structural restraints to expedite protein structure determination[3] and facilitate structure analysis of protein–protein[4] and protein–ligand interactions.[5] Consequently, there is increasing interest in methods for attaching lanthanides to proteins in a site-specific manner.[2,6,7]

Whilst some proteins feature natural metal ion binding sites that can bind lanthanides,[8] most require these ions to be attached using specially designed 'tags'.[9] Tag design is a challenging task, as stringent stereochemical and structural requirements have to be met in order to exploit the full paramagnetic potential of the metal. Firstly, the position of the lanthanide ion must be well-defined with respect to the protein to avoid averaging of paramagnetic effects and, hence, reduction of the size of observed PCSs and RDCs.[6,10,11] Secondly, the bound lanthanide complex should form a single stereoisomer, so that multiple NMR signals are not observed for each nucleus in the protein, rendering assignment more difficult (or intractable).[11,12] Thus far, the most common approaches to lanthanide tagging have involved either the introduction of metal-binding peptides into fusion proteins,[13] or attachment of synthetic metal chelates to single surface-exposed cysteine residues.[2,6,7,10] In addition, lanthanide cyclen derivatives with two thiol reactive groups have been made for rigid anchoring to proteins displaying two cysteine residues.[14] Here we describe a related tagging strategy, but which employs a very small lanthanide-binding ligand that is much easier to synthesize, far less expensive, and, despite a very high metal affinity, allows exchange of lanthanide ions *via* treatment with EDTA. Attachment of a *pair* of these ligands to the *i*, *i* + 4 positions of an α-helix immobilizes single lanthanide ions in the form of a bis-chelate complex.

The tagging agent, **1**, is a hybrid of the well-known tetradentate chelator nitrilotriacetic acid (NTA)[15] and L-cysteine, and can be prepared in just three steps from commercially-available *S*-trityl-L-cysteine.[16] The thiol group within the structure permits ready attachment of the tag to a surface-exposed cysteine residue *via* formation of a disulfide bond mediated by Ellman's reagent.[17] Although NTA is highly flexible, we reasoned that the stereocentre within the ligand, combined with either positioning of the NTA ligand near an additional coordinating residue of the protein or the use of *two* proximal NTA ligands, might be sufficient to bind lanthanide ions in a single isomeric form and lead to the generation of useful PCS data (Fig. 1).

To explore the utility of the tag, we first attached it to two proteins bearing single surface-exposed cysteines—the Ala28-Cys mutant of human ubiquitin (UbiqA28C) and the N-terminal domain of the *E. coli* arginine repressor (ArgN)[18]—and recorded ^{15}N-HSQC spectra in the presence of different lanthanide ions (Dy^{3+}, Tb^{3+}, Tm^{3+}, and Yb^{3+}, using Y^{3+}

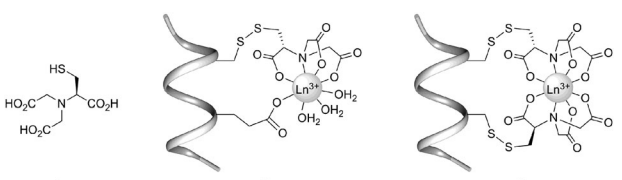

Fig. 1 Structure of the NTA-based tagging agent, **1**, and schematic representations of La^{3+} ion chelation by one (A) or two NTA ligands (B) attached to a protein α-helix *via* disulfide bridges to cysteine residues.

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† Electronic supplementary information (ESI) available: Synthetic details and analytical data for **1**; expression and purification of ubiquitin mutants; protocols for ligation of **1** to proteins; ^{15}N-HSQC spectra of ArgN-NTA, UbiqA28C-NTA and UbiqE24C/A28C-NTA_2 with Dy^{3+}, Tb^{3+}, Tm^{3+}, and Yb^{3+}; $\Delta\chi$ tensors; correlation plots of experimental and calculated PCSs and RDCs; tables of PCSs and RDCs; modeling of ArgN-NTA-Ln^{3+} and UbiqE24C/A28C-NTA_2-Ln^{3+}. See DOI: 10.1039/c1cc11893e

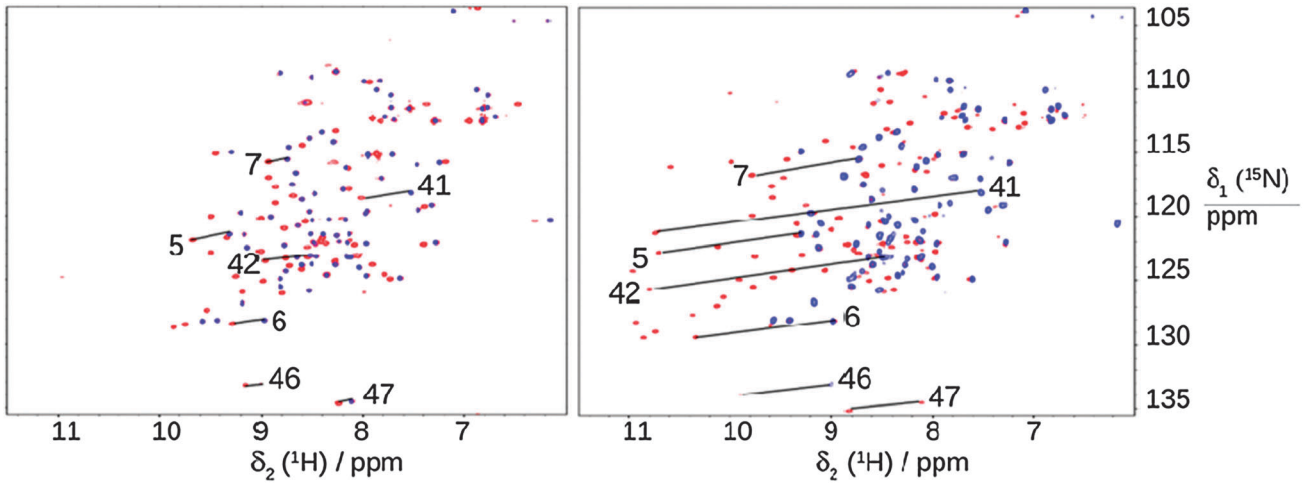

Fig. 2 ^{15}N-HSQC spectra of ~100 μM solutions of uniformly ^{15}N-labeled UbiqA28C-NTA (*left panel*) and UbiqE24C/A28C-NTA$_2$ (*right panel*) in the presence of a 1 : 1 mixture of 60 μM La^{3+} and 60 μM Tm^{3+} (red), or in the presence of 500 μM La^{3+} (blue). Lines illustrate PCSs of selected backbone amides (residue numbers indicated). The spectra were recorded at 25 °C and pH 7 at a ^{1}H NMR frequency of 600 MHz.

or La^{3+} as the diamagnetic reference). In each case, single sets of resonances were observed, consistent with a single stereoisomer. Paramagnetic and diamagnetic cross-peaks were observed simultaneously in samples prepared with a mixture of paramagnetic and diamagnetic ions (indicating slow metal ion exchange), which was used to measure the PCSs from single spectra (Fig. S1 and S2†). Together with the NMR structures of the proteins,[19,20] the PCSs were then used to determine the position, magnitude and orientation of the magnetic susceptibility anisotropy (Δχ) tensors associated with the metal ions (Tables S1 and S2, Fig. S5†).[20]

For both proteins, the metal positions were found approximately 4 Å from the cysteine sulphur atom, consistent with binding to the NTA tag. In the case of ArgN-NTA, the metal was 4.7 Å from the carboxyl group of the nearest acidic residue (Glu21), and no other groups were involved in metal binding. Nonetheless, the axial components of the fitted Δχ tensors were as large, or larger, than those observed previously for ArgN with a dipicolinic acid (DPA)-based tag, where the lanthanide was located near the carboxyl group of Glu21.[17] For UbiqA28C-NTA, considerably smaller PCSs and tensors were found, and RDCs were not measurable, indicating a high degree of tag mobility despite the presence of potential metal binding groups either side of the tag (Glu24 and Asp32). These observations reflect the greater solvent exposure of the tag in UbiqA28C-NTA, and suggest that the neutral Ln^{3+}-NTA complex has only low affinity for proximal acidic residues that might otherwise help to restrict movements of the lanthanide with respect to the protein. Recently we reported a singly-tethered iminodiacetic acid (IDA) tag attached to UbiqA28C that exhibited large Δχ tensors and RDCs, yet showed fast metal exchange kinetics suitable for convenient structure-independent PCS assignments *via*^{15} N ZZ exchange specroscopy.[21] Rigidity was inferred from a preferential coordination to an ancillary aspartate residue in the *i* + *4* position. As the uncharged NTA-lanthanide tag in UbiqA28C shows much smaller Δχ tensors, the positive charge of the IDA-lanthanide tag seems to be a critical property for coordination to the aspartate group.

The above findings led us to hypothesize that more rigid metal binding might be achievable using a *pair* of proximal NTA ligands. Highly stable bis-NTA lanthanide complexes ($\log\beta_2$ = 18–20) are well-known, with the larger lanthanides forming 9-coordinate pseudo-monocapped square antiprismatic (SAP) species, $[Ln(NTA)_2(H_2O)]^{3-}$, and the smaller lanthanides forming 8-coordinate SAP species, $[Ln(NTA)_2]^{3-}$.[15,22] Modelling studies indicated that attachment of two copies of **1** to the *i*, *i* + *4* positions of an α-helix would permit formation of such species. To test our hypothesis, we engineered a bis-NTA motif into the α-helix of human ubiquitin *via* the double-cysteine mutant UbiqE24C/A28C. Addition of lanthanide ions to the doubly-tagged protein, UbiqE24C/A28C-NTA$_2$, produced single sets of resonances, as for UbiqA28C-NTA. The observed PCSs (Fig. 2 and Table S6†) and calculated Δχ tensors (Table S3†), however, were considerably larger in magnitude. For example, the Tm^{3+}-loaded protein yielded $\Delta\chi_{ax} = 28 \times 10^{-32}$ m^3, some seven times greater than for the single NTA case. This would be expected to produce well-detectable PCSs (≥ 0.08 ppm) at distances up to 55 Å in the axial direction of the Δχ tensor. The metal ion was positioned between the two tagged cysteine residues

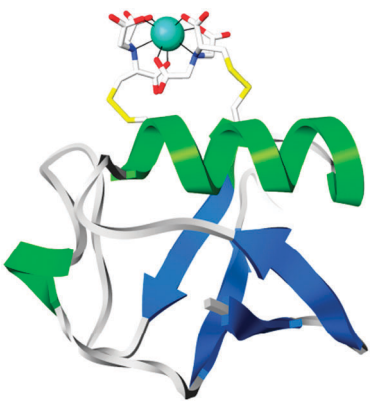

Fig. 3 Model of human ubiquitin with an Ln^{3+}-NTA$_2$ complex attached. The protein is shown as a ribbon, the side chains of Cys24 and Cys28 with the attached NTA tags as sticks, and the lanthanide as a ball.

(Fig. 3), and $^{1}D_{HN}$ RDCs up to ~10 Hz were measured for the La^{3+}/Tm^{3+}-loaded protein at 600 MHz (Fig. S10, S11†). Back-calculation of the RDCs from the calculated $\Delta\chi$ tensors, assuming an order parameter of 0.9, revealed that the experimentally observed RDCs were, on average, only 10% smaller (Fig. S11†), indicating high rigidity of the lanthanide tag. High rigidity was also indicated by the fact that the principal axes of the RDC-derived alignment tensor aligned closely with those of the $\Delta\chi$ tensor (see Fig. S12;† the Z and XY components deviated by only ~8 and ~13° from one another, respectively), and that the magnitude of the axial component of the RDC-derived alignment tensor was 74% of that calculated from the $\Delta\chi$ tensor (Fig. S12†).[14]

In summary, **1** is a very attractive tool for attaching lanthanide ions close to protein surfaces with high affinity. Two copies of **1** coupled to cysteines in the *i* and *i* + *4* positions of an α-helix creates a bis-chelator motif that binds lanthanide ions rigidly, leading to significant PCSs and RDCs. As α-helices are abundant motifs that are easily identifiable from backbone chemical shifts and secondary structure prediction, the novel tagging strategy described here is highly suitable for the general study of proteins of unknown 3D structure by paramagnetic NMR.

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