Lanthanide Tags

DOI: 10.1002/anie.201007221

An Iminodiacetic Acid Based Lanthanide Binding Tag for Paramagnetic Exchange NMR Spectroscopy**

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When bound to proteins, paramagnetic lanthanide ions induce a range of effects that are observable by NMR spectroscopy, including pseudo-contact shifts (PCSs), paramagnetic relaxation enhancements (PREs), and residual dipolar couplings (RDCs).[1] These effects provide valuable constraints that can expedite protein structure refinement,[2] the analysis of protein–protein[3] and protein–ligand interactions,[4] and, potentially, the study of protein dynamics and lowly populated encounter states of protein complexes.[5] PCSs, measurable for nuclei beyond 60 Å away from some lanthanide ions, are especially useful for NMR structural analysis of multidomain proteins and multiprotein complexes.[6] These manifest as changes in chemical shifts between paramagnetic and diamagnetic samples, with the difference in shifts ($\Delta\delta^{PCS}$) dependent on the location of the nuclear (i.e., ^{15}N and ^{1H}N) spins with respect to the anisotropic magnetic susceptibility tensor ($\Delta\chi$) of the metal ion:

$$\Delta\delta^{PCS} = \frac{1}{12\pi r^3}\left[\Delta\chi_{ax}(3\cos^2\theta - 1) + \frac{3}{2}\Delta\chi_{rh}\sin^2\theta\cos 2\varphi\right] \quad (1)$$

where $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ are the axial and rhombic components of the $\Delta\chi$ tensor, r is the distance of the metal ion from the nuclear spin, and θ and ϕ are angles that describe the orientation the $\Delta\chi$ tensor with respect to the protein.[7] Assignment of PCSs provides access to a protein-anchored, metal-centered coordinate system that can be used as a reference frame to pinpoint the location of other nuclear spins by virtue of their PCSs.

Provided both a structural model and the assignment of a diamagnetic reference are available, assignment of paramagnetic NMR spectra can be achieved by an iterative procedure which involves minimization of the difference between observed and back-calculated PCSs.[8] An alternative strategy, developed by Otting and co-workers,[9–11] involves the recording of NMR spectra under conditions in which both paramagnetic and diamagnetic forms of the protein are present and interconvert through binding and dissociation of a paramagnetic lanthanide ion. If the exchange rate is sufficiently high within the slow-exchange NMR timescale, cross-peaks may be observed in exchange experiments performed with a mixture of paramagnetic and diamagnetic metal ions, greatly facilitating correlation of paramagnetic and diamagnetic resonances. The attractiveness of this approach is twofold. Firstly, it does not require prior knowledge of protein structure, making it a potentially much more broadly applicable method for assigning paramagnetic NMR spectra than the iterative procedure. Secondly, by taking advantage of the slowly relaxing heteronucleus and its insensitivity toward paramagnetic relaxation, ^{15}N longitudinal exchange spectroscopy can extend PCS detection into the usually PRE-broadened sphere close to the metal. While this region can be probed with weakly anisotropic metals, these yield few short-range PCSs, which makes the calculation of the magnetic susceptibility tensor difficult for an individual metal. Thus far, the exchange method has only been successfully demonstrated for a protein incorporating a natural metal ion-binding site (the ε186/θ complex of DNA polymerase III).[10,11] All reported synthetic lanthanide-binding tags bind metal ions too tightly to allow exchange between the bound and unbound states within the slow-exchange NMR regime.[1] Here we present the first example of a small synthetic lanthanide-binding tag for which the chemical exchange is sufficiently fast to produce definitive exchange cross-peaks, enabling the rapid assignment of both small and extraordinarily large PCSs by ^{15}N heteronuclear exchange spectroscopy, without recourse to a structural model.

The tagging agent, **1** (Figure 1),[12] is a hybrid of the well-known tridentate chelator iminodiacetic acid (IDA)[13] and L-cysteine. The thiol group permits ready attachment of the tag to a protein through disulfide bond formation with a surface-exposed cysteine residue. Although IDA is small and flexible, we reasoned that the stereocenter within the ligand, com-

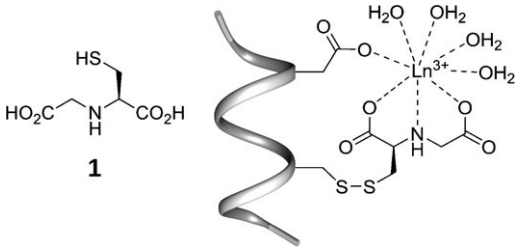

Figure 1. Left: Structure of the IDA-based tagging agent **1**. Right: Representation of rigid lanthanide ion chelation by the Cys-linked IDA ligand in combination with an Asp residue within an α helix. Aquo ligands are expected to occupy the remaining coordination sites about the lanthanide ion.

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[**] We thank Prof. Gottfried Otting for stimulating discussions and recognizing the potential of **1** to be used for NMR exchange spectroscopy, and Dr. Marcello Tellioni for coding the ZZ exchange pulse sequences.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201007221.

bined with judicious positioning of the tag near an additional metal ion-coordinating residue, might be sufficient to achieve rigid complexation of a lanthanide ion in a single isomeric form (or fast exchange between a limited number of isomers), a prerequisite for the generation of useful PCS data. As detailed below, attachment of the tag to a regular α-helix motif in human ubiquitin containing an aspartic acid residue at the $i+4$ position (Figure 1) led to observation of PCSs for 90% of the protein residues. PCSs beyond 8 ppm in magnitude, and for nuclei as close as 8 Å to the same metal center, were measured using a series of different lanthanide ions.

The IDA-based tag was chemically attached to an Ala28Cys mutant of human ubiquitin (UbiqA28C-IDA), pre-activated with Ellman's reagent.[14] Cys28 is situated on the solvent-exposed face of the α helix and is located between two acidic residues, Glu24 and Asp32, which provide two potentially additional coordination sites. Titration with the diamagnetic lanthanide ion La^{3+} showed slow exchange on the NMR timescale, and a single set of resonances in the ^{15}N HSQC NMR spectrum after addition of excess metal, consistent with a single-metal-bound isomer (Supporting Information, Figure S1). Whilst significant resonance perturbations were observed, these were for residues proximal to the tag, particularly on the solvent exposed face of the helix (Figure S1). Moreover, ^{15}N-edited NOESY data showed that the secondary structure is well maintained along the helix, indicating that attachment of the tag and metal coordination does not disrupt the native structure of the helix.

Addition of Dy^{3+} to UbiqA28C–IDA produced large PCSs in slow exchange. This, combined with weak metal binding from the La^{3+} titration ($K_d \approx 10$ μM), prompted us to carry out a series of $^{15}N_Z$-exchange experiments to optimize the single-mixing time period for exchange-based assignments.[10] For these, aliquots of an equimolar mixture of diamagnetic and paramagnetic lanthanide ions were added to UbiqA28C–IDA (ca. 70 μM) until the intensity of the resonances of the diamagnetic and paramagnetic species were approximately equal in the ^{15}N HSQC spectra (approximately 130 μM of each). This process was performed for a series of different paramagnetic lanthanide ions (Tb^{3+}, Dy^{3+}, Ho^{3+}, Er^{3+}, Tm^{3+}, and Yb^{3+}), using La^{3+} as the diamagnetic reference (Figures S2–S6).

Outstanding quality data was obtained using mixing times of 200–300 ms, within a short data collection time (6–12 h) (Figure 2). From this data, an exchange rate of approximately 15 s^{-1} was estimated (Figure S7), which is almost twenty times higher than that found for ε186,[10] and consistent with the three-fold shorter mixing time employed herein. Even using a 1:1 metal-to-protein ratio, good quality $^{15}N_z$ exchange data were recorded using mixing times of 300–400 ms (Figures S8 and S9).

Using N_z-exchange spectroscopy, both small and large paramagnetically shifted resonances were easy to assign unambiguously. Typically 80–90% of the PCS data obtained from a single exchange spectrum were accurately measured prior to first round tensor calculations within the program Numbat,[15] using the high-resolution RDC-refined structure of human ubiquitin.[16] A PCS of 4.3 ppm was measured in the ^{15}N dimension that was otherwise broadened beyond detection in the standard ^{15}N HSQC spectrum (Figure 2). The high quality of the PCS data (Table S1), combined with facile assignments and large magnetic anisotropies, meant that both the metal position and the $\Delta\chi$ tensor were accurately determined from a single exchange experiment for each of the metals Tb^{3+}, Dy^{3+} and Ho^{3+}, without recourse to additional metal-to-coordinating group distance restraints or estimation of the position from the center of the alignment tensor (Figures S10–S12).[17] The smaller magnetic anisotro-

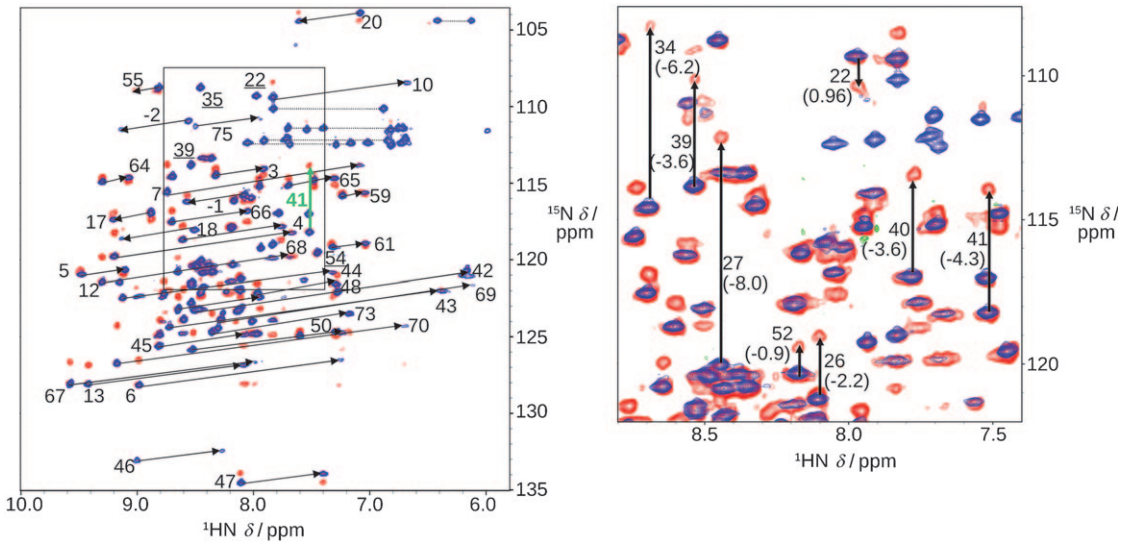

Figure 2. $^{15}N_z$-exchange spectra for UbiqA28C–IDA. Left panel: Superposition of the ^{15}N fast-HSQC spectrum (blue) of a mixture of 70 μM ^{15}N-labeled UbiqA28C–IDA and 130/130 μM La^{3+}/Dy^{3+} with the 300 ms single-mixing $^{15}N_z$-exchange spectrum (red). Arrows highlight the extent and direction of the observed PCS. The PCS for Gln41 is observed only in the ^{15}N dimension. Right panel: Expanded region (boxed in left panel) of the 150 ms two-mixing $^{15}N_z$-exchange spectrum (red). Arrows highlight PCSs (values shown in parentheses) that are only observed in the ^{15}N dimension and are otherwise not observed in the single-mixing $^{15}N_z$ experiment.

pies of Tm^{3+}, Yb^{3+}, and Er^{3+} (and thus smaller PCSs) led to deviations from this position and instability in the $\Delta\chi$ tensor calculation. The metal position and $\Delta\chi$ tensor for these lanthanides were therefore each refined in turn, using simultaneous refinement with the PCS data from Dy^{3+} within Numbat.[17] Final axial and rhombic components, Euler angles, and metal position are presented in Table S2, while correlations of the observed vs. calculated PCSs for all tested metals are shown in Figure 3.

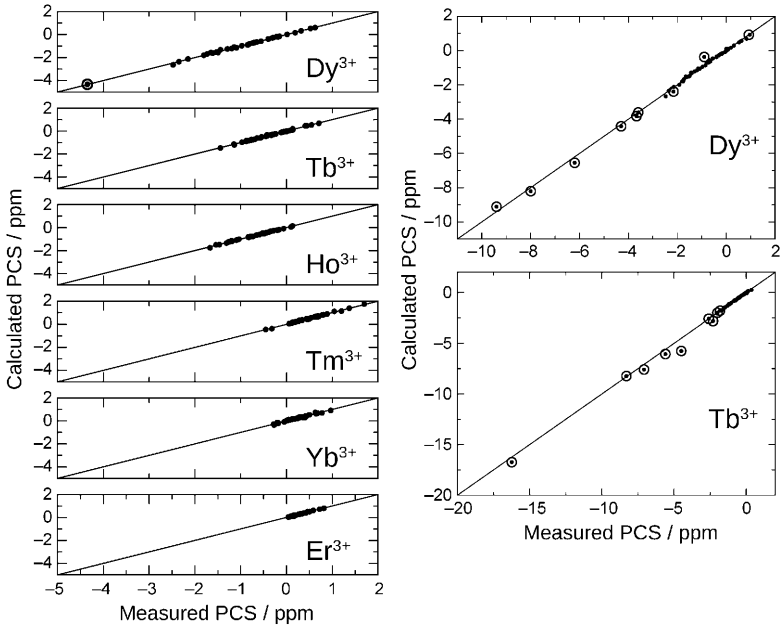

Figure 3. Left panels: Correlations between calculated ^{1}HN PCSs and those measured in a single-mixing $^{15}N_z$-exchange experiment for the backbone amides of UbiqA28C–IDA in the presence of an equimolar mixture of the specified paramagnetic lanthanide ion and La^{3+}. Right panels: Correlations between calculated PCSs and those measured in a two-mixing, out-and-back $^{15}N_z$-exchange experiment for the amides of UbiqA28C–IDA in the presence of equimolar La^{3+}/Dy^{3+} or La^{3+}/Tb^{3+}. Additional close-range ^{15}N PCSs that were not observed in ^{15}N HSQC experiments are circled.

Given the success of the single-mixing exchange experiment for rapid assignments, we conducted a two-mixing period "out-and-back" $^{15}N_z$ exchange experiment[11] for the La^{3+}/Dy^{3+}- and La^{3+}/Tb^{3+}-coordinated UbiqA28C–IDA, to enable the detection of substantially PRE-broadened nuclear spins located close to the metal centers. Additional PCSs from amides as close as 8 Å to the strongly paramagnetic Dy^{3+} and Tb^{3+} ions were measured in the ^{15}N dimension (Figures 2 and S13). These were in excess of 8 ppm, with the largest being $\delta =$ 16.2 ppm for a PCS detected just above the noise threshold for Tb^{3+}. With the inclusion of these large PCSs, the correlation between the measured PCSs and those back-calculated from the alignment tensor remained very good (Figure 3). The calculated metal position was unchanged, and only a minor change (ca. 10%) in the magnitude of the $\Delta\chi$ tensor was observed (Table S2). This indicates that the structure of the helix is essentially unperturbed by the addition of the tag and/or metal, in agreement with the previously collected 3D ^{15}N NOESY data. In contrast, Otting and co-workers reported significant deviation for the lanthanide-binding ε186/θ complex,[11] attributing this in part to the presence of multiple metal-binding sites.

The $\Delta\chi$ tensors show that the lanthanide ion is located approximately 2.0 Å from the side-chain δO of Asp24 (Figure S12). This Ln–O distance is within the range typically observed for lanthanide ions complexed by proteins or organic ligands,[18] and is suggestive of a preference for additional coordination of the ions by Asp32 over Glu24, both located four residues away from the **1**-tagged Cys residue.

PCSs for the same residues measured with different lanthanide ions lay along straight lines in superimposed ^{15}N HSQC spectra (Figure S14) and displayed either good correlation (Tb^{3+}/Dy^{3+}) or good anticorrelation (either Dy^{3+} or Tb^{3+} with Tm^{3+}, data not shown), indicating an equivalent coordination geometry for each of these lanthanides. The principal axes of the susceptibility tensors were similarly oriented (Figures S10) and in all cases, the axial component of the anisotropy tensor, $\Delta\chi_{ax}$, lay approximately perpendicular to the helix axis (Figure S11). As most of the protein lay within a single lobe, the PCSs for each lanthanide tended to shift in one direction (Figure 3). For La^{3+}/Dy^{3+}-loaded UbiqA28C–IDA, $\Delta\chi_{ax}$ was determined to be 32.4×10^{-32} m^3, which would be expected to yield well-detectable PCSs ($\geq$0.08 ppm) at distances in excess of 60 Å in the axial direction.

The measured and calculated ^{1}HN PCSs correlated very well for spins close to the lanthanide centers (Figure 3) and indicated that large-amplitude tag motion was probably minimal in Ln^{3+}-loaded UbiqA28C–IDA (for a mobile/flexible tag, PCSs for proximal spins are much more sensitive to erroneous PCS fitting due to an r^{-3} averaging effect). This observation encouraged us to measure $^1D_{HN}$ RDC data to evaluate the tag motion in more detail. At 18.8 T, RDC values up to 20 Hz were measured for La^{3+}/Tb^{3+} bound to UbiqA28C–IDA (Figure S15). Back-calculation of the RDCs from the $\Delta\chi$ tensors,[14,19,20] assuming an order parameter of 0.9, revealed that the observed RDCs were, on average, 80% of the size of those calculated (Figure S16). The orientations of the principal axes of alignment and the $\Delta\chi$ tensor were in very close agreement (Figure S17) as expected for rigid metal coordination. Furthermore, the RDC-derived axial and rhombic components of the $\Delta\chi$ tensor for Tb^{3+}-loaded UbiqA28C–IDA (Table S2) were 80% and 87% of the PCS-derived values, respectively, also indicative of highly rigid lanthanide coordination. These values are comparable to those obtained by Grzesiek,[19] Ubbink[20] and co-workers using extremely tight metal-binding DOTA-based tags (DOTA = 1,4,7,10-tetraazacyclodecane-1,4,7,10-tetraacetic acid). The larger anisotropies observed in these latter systems are therefore most likely attributable in part to coordination geometry and ligand field effects, rather than

just differences in rigidity. Our results clearly demonstrate that tight binding is not an essential requisite for rigid lanthanide tagging of proteins.

In conclusion, an IDA-based lanthanide binding tag with exceptional properties for rapid PCS determination has been developed. Attachment of this tag to an α helix containing an Asp residue at the $i+4$ position creates a metal binding site that complexes lanthanide ions in a rigid, yet kinetically labile, fashion. Significant PCSs are observed for nuclei located 8–60 Å from single, strongly paramagnetic ions, which are easily assignable with the aid of heteronuclear exchange spectroscopy. The notion of a small, highly flexible ligand that becomes rigid in situ upon complexation of a metal ion represents a novel concept as compared to the traditional approach of employing rigid, tight binding ligands for immobilization of lanthanide ions. Given that α helices are extremely ubiquitous and easily identified from backbone chemical shifts alone,[21] we envisage that the methodology described herein may potentially be transferable to the study of other proteins. Moreover, the ability to generate PCS and RDC data in the absence of a prior structural model could see the use of tags such as **1** integrated with the recently reported methodology of Baker,[22] Bax[23] and co-workers for rapid NMR structure determination of proteins from backbone-only data.

Received: November 17, 2010
Revised: January 31, 2011
Published online: April 7, 2011

Keywords: lanthanides · NMR spectroscopy · protein structure · pseudo-contact shifts · residual dipolar couplings

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