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Characterizing the magnetic susceptibility tensor of lanthanide-containing polymethylated-DOTA complexes

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Abstract

Lanthanide complexes based on the DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) cage are commonly used as phase contrast agents in magnetic resonance imaging, but can also be utilized in structural NMR applications due to their ability to induce either paramagnetic relaxation enhancement or a pseudo-contact shift (PCS) depending on the choice of the lanthanide. The size and sign of the PCS for any given atom is determined by its coordinates relative to the metal center, and the characteristics of the lanthanide's magnetic susceptibility tensor. Using a polymethylated DOTA tag (Ln-M8-SPy) conjugated to ubiquitin, we calculated the position of the metal center and characterized the susceptibility tensor for a number of lanthanides (dysprosium, thulium, and ytterbium) under a range of pH and temperature conditions. We found that there was a difference in temperature sensitivity for each of the complexes studied, which depended on the size of the lanthanide ion as well as the isomeric state of the cage. Using ¹⁷O-NMR, we confirmed that the temperature sensitivity of the compounds was enhanced by the presence of an apically bound water molecule. Since amide-containing lanthanide complexes are known to be pH sensitive and can be used as probes of physiological pH, we also investigated the effect of pH on the Ln-M8-SPy susceptibility tensor, but we found that the changes in this pH range (5.0–7.4) were not significant.

Keywords

Lanthanide; DOTA; Pseudocontact shift; PCS; Magnetic susceptibility tensor

Introduction

Paramagnetic lanthanide complexes have long been used in nuclear magnetic resonance (NMR) either in imaging applications as contrast agents (Aime et al. 2009; Caravan et al. 1999) or in spectroscopy to create pseudocontact shifts (PCS) that contain structural information (Bertini and Luchinat 1999; Mittag and Forman-Kay 2007; Pintacuda et al. 2007; Rodriguez-Castañeda et al. 2006). The most widely used complexes, valued for their stability and inertness, are formed by chelating the lanthanide ions to DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) (Baranyai et al. 2005; Cacheris et al. 1987, 1990; Kumar et al. 1993). In these complexes, the lanthanide metal ion is coordinated to four nitrogens and four oxygens of DOTA, while in the larger lanthanides an additional ninth coordination site is occupied by an apical water molecule. A number of modifications of the DOTA cage have been introduced to achieve unique physical characteristics suitable for specific applications. In paramagnetic chemical exchange saturation transfer (PARACEST) for MRI applications, the DOTA cage can be modified to maximize chemical exchange either by increasing the number of exchangeable protons on the complexes or by modulating the lifetime of the coordinated water (Aime et al. 2002; Krchová et al. 2013, 2016; Opina et al. 2011; Rudovský et al. 2005; Woods et al. 2003, 2005; Woods and Sherry 2003; Zhang et al. 2001a, b, 2003). Meanwhile, for NMR structural studies of proteins, a common chemical modification of the lanthanide tag is rigidification, which can result in larger observed PCS, either by methylation of the chelator (Häussinger et al. 2009; Müntener et al. 2016), two-site attachment (Keizers et al. 2007; Lee et al. 2016), or shortening of the attachment linker (Lee et al. 2015; Yang et al. 2016). Additionally, colored lanthanide complexes have been developed to aid sample preparation (Liu et al. 2012).

A number of studies have characterized the temperature and pH dependence of DOTA complexes with the goal of identifying mechanisms that dominate either relaxation or magnetization transfer from the complexes to the bulk water (Aime et al. 2002; Huang et al. 2015; Krchová et al. 2013, 2016; Rudovský et al. 2005; Woods et al. 2011). However, the analysis of DOTA complexes is complicated by the presence of two fast-exchanging DOTA diastereomers, square antiprism (SAP) and twisted square anti-prism (TSAP) (Aime et al. 1992, 1996; Hoeft and Roth 1993; Jacques and Desreux 1994). Rigidification of the tag by addition of bulky aromatics (C1) can trap DOTA in one isomeric conformation (Graham et al. 2011). Alternatively, an octamethylated DOTA cage (Ln-M8-SPy and Ln-M4DOTMA) provides increased rigidity, while still allowing the observation of both isomers (Häussinger et al. 2009; Opina et al. 2016; Ranganathan et al. 2002). The TSAP and SAP isomers of these compounds are distinguishable by RP-HPLC and NMR (Opina et al. 2016), making them amenable to independent but simultaneous pH and temperature analysis. At equilibrium, the two isomers exist in a characteristic ratio that is dependent on the ionic radius of the lanthanide center and are in slow exchange (Opina et al. 2016).

Previously, we studied the rigidified Ln-M8-SPy tag alone and covalently bound to ubiquitin-S57C (Ln-M8-Ub^{S57C}), and noted that protein PCS data was easier to collect and analyze than that of the free tag (Opina et al. 2016). The longer distance between the protein spins and the lanthanide center, in comparison to the protons in the free tag, results in a reduction in the size of the PCS and therefore, crucially, a large reduction in the necessary

spectral width (Opina et al. 2016). Additionally, the increase in the number of observable nuclei associated with protein attachment means that the axial and rhombic components of the magnetic susceptibility tensor can be more accurately determined (Häussinger et al. 2009). Since ubiquitin is stable over a wide range of buffer conditions, we have chosen this system (Ln-M8-Ub^{S57C}) to measure the effects of pH and temperature change on the DOTA tag and relate them directly to the magnitude and rhombicity of the magnetic susceptibility tensor for three lanthanide complexes (Yb-, Tm- and Dy-M8-Ub^{S57C}). We chose Dy-M8-Ub^{S57C} as it enabled us to study a system that forms both the TSAP and SAP isomers in near-equal populations (Opina et al. 2016), while the Tm- and Yb-M8-Ub^{S57C} complexes provide us with samples that are not expected to contain an apical water ligand (Benetollo et al. 2003), but differ in magnetic susceptibility tensor. The Dy-M8-Ub^{S57C} susceptibility tensor showed the highest temperature dependence, which we found was due to the presence of an inner sphere water molecule. An additional enhancement in tensor temperature sensitivity was observed for the SAP isomer of Dy-M8-Ub^{S57C} that we attributed to the shorter Ln–OH₂ bond length in comparison to TSAP (Opina et al. 2016), which corresponds to a more stable water coordination. In contrast, it appears that tag flexibility, which is isomer-independent, does not play a major part in reducing PCS at high temperatures. We also characterized the pH sensitivity of these compounds, but despite the presence of a titratable amide proton in the Ln-M8-SPy tag, we found that the effect of pH on the susceptibility tensor was minimal. Since the understanding of apical water coordination is vital for the development of phase contrast agents for use in MRI, our study provides a foundation for their analysis by the covalent attachment of protein scaffolds. Additionally, we have outlined the protocol for the simultaneous use of PCS from two isomers in Xplor-NIH, which has laid the groundwork for future use of the Ln-M8-SPy tag for high-quality structure refinement, elucidation, and docking.

Materials and methods

Preparation of Ln-M8-Ub^{S57C} samples

The expression and purification of ¹⁵N-Ub^{S57C}, the synthesis of Lu-M8-SPy, Yb-M8-SPy, Tm-M8-SPy, Dy-M8-SPy, and Tb-M8-SPy complexes, and the reaction of Ln-M8-SPy complexes with ¹⁵N-Ub^{S57C} were described previously (Opina et al. 2016). Briefly, the Ln-M8-SPy complex forms a disulfide bond with the cysteine residue of ubiquitin, using SPy as a leaving group. Each Lu-, Yb-, Tm-, and Dy-(SSSS)-(SSSS)-M8-tagged ¹⁵N-Ub^{S57C} complex (0.5 mM protein in 10 mM sodium phosphate buffer) was studied at pH 5.0, 6.2, and 7.4, using 1 M HCl or 1 M NaOH to alter the pH between experiments. Lu-M8-SPy was used as a diamagnetic reference for the Yb-, Tm-, and Dy-M8-SPy paramagnetic tags.

NMR spectroscopy

For studies of PCS temperature dependence, ¹H/¹⁵N-HSQC NMR spectra of Ln-M8-Ub^{S57C} were obtained at pH 7.4, each with 16 scans at 288, 298, and 308 K. For pH dependence studies, ¹H/¹⁵N-HSQC NMR spectra were obtained at pH 5.0, 6.2, and 7.4, each with 16 scans at 298 K. Experiments were collected on a Bruker Avance 600 MHz spectrometer equipped with a cryoprobe. Spectra were processed using NMRPipe (Delaglio et al. 1995) and assigned using CCPN Analysis (Vranken et al. 2005). Lu-M8-Ub^{S57C} peaks were

assigned as previously described for Ub^{S57C} (Opina et al. 2016). For Tm- and Yb-M8-Ub^{S57C}, higher intensity peaks were assigned to the TSAP conformation, and lower intensity peaks to the SAP conformation (95 and 91 % TSAP for Yb and Tm, respectively), as previously described (Opina et al. 2016). Dy-M8-Ub^{S57C} was assigned in a similar manner, but instead with a majority (73 %) in the SAP form (Opina et al. 2016). Since PCS scales with the magnetic susceptibility tensor of the paramagnetic atom, all peaks (from Lu-, Yb-, Tm-, or Dy-M8-Ub^{S57C}) roughly fall on a diagonal line, which aided assignment (although it must be noted that atoms in close proximity to the metal center can deviate from the diagonal). Additionally, residues with small PCS, that are generally easier to assign, were used to locate the metal center in relation to the protein and therefore to obtain expected values for the remaining unassigned residues, those with larger PCS, which subsequently led to further assignments.

Structure calculations

Minimization of the TSAP and SAP lanthanide metal center positions, and calculation of the axial (χ_a) and rhombic (χ_r) components of the corresponding magnetic susceptibility tensors were carried out using Xplor-NIH (Schwieters et al. 2003, 2006) using observed proton and nitrogen TSAP and SAP PCS values as restraints. Although contributions from nitrogen residual chemical shift anisotropy should be taken into account at higher magnetic fields (John et al. 2005), we found that under the conditions used (600 MHz), the residual CSA contribution was within experimental error, and so no residual CSA correction was applied to the nitrogen PCS values. The ubiquitin structure [1D3Z, (Cornilescu et al. 1998)] was used for minimization, to which we added both the TSAP and SAP Yb-M8-SPy tag structures at residue C57, based on the previously calculated Yb-M4DOTMA structure (Opina et al. 2016). During minimization we allowed the tag linker atoms to move freely (from the C β atom of C57 to the amide nitrogen nearest to the lanthanide cage), while the entire lanthanide cage was grouped together (fixed in relation to each other, but allowed to move as a group in relation to the protein), and the ubiquitin structure was fixed throughout, including the remaining amino acid side-chains. The axial (χ_a) and rhombic (χ_r) components of the magnetic susceptibility tensor, as well as its direction, were calculated according to

$$PSC = \frac{1}{12\pi r^3} \left[\chi_a (3 \cos^2 \theta - 1) + \frac{3}{2} \chi_r \sin^2 \theta \cos 2\varphi \right]$$

where r is the distance between the paramagnetic ion and the nucleus of interest, θ and φ denote the angles between the lanthanide-nuclear vector and the principal axis of the χ tensor (Pintacuda et al. 2007). The principal axis orientation of the tensor is described by three Euler angles in the molecular frame. Proton PCS values were calculated as the difference between the paramagnetic (Yb(TSAP)-, Tm(TSAP)-, Dy(TSAP)-, or Dy(SAP)-M8-Ub^{S57C}) proton chemical shift and the corresponding diamagnetic (Lu-M8-Ub^{S57C}) proton chemical shift in ppm units ($H^{\text{para}} - H^{\text{dia}}$). Nitrogen PCS were calculated similarly ($N^{\text{para}} - N^{\text{dia}}$). It is important to note that in Xplor-NIH, the axial component of the tensor (χ_a) is calculated without prefactors, so the tensor was multiplied by 12π and divided by -10^6 , to add the prefactor and convert from ppm to Hz, respectively. The magnetic

susceptibility tensor and expected PCS values were back-calculated via singular value decomposition using the calcTensor helper program of Xplor-NIH and the minimized structure of Ln-M8-Ub^{S57C} (lowest energy structure from 20 calculations). Quality factors (Q), which compare expected and observed values, were calculated using the following equation: $Q = \text{rms}(PCS^{\text{obs}} - PCS^{\text{exp}})/\text{rms}(PCS^{\text{obs}})$, and multiplied by 100 for percentages (Cornilescu et al. 1998). All structure figures were rendered using PyMOL (Schrödinger 2010), with the exception of PCS isosurfaces, which were calculated using Xplor-NIH and rendered using UCSF Chimera (Pettersen et al. 2004).

Evaluation of errors in the magnetic susceptibility tensor

A measure of the uncertainty of the determined magnetic susceptibility tensor with regard to uncertainty in the measured PCS values was obtained by repeating structure minimization ten times, each time with 10 % of the data removed at random. Averages and standard deviations of the axial (χ_a) and rhombic (χ_r) components of the magnetic susceptibility tensor, and the average Euler angles were then calculated by comparing the results from each of the lowest energy structures generated by the ten sets of calculations.

Calculation of TSAP and SAP isomer populations

Peak volumes were used to calculate the relative population of TSAP and SAP isomers for Yb- and Dy-M8-Ub^{S57C} at all pH and temperature points in cases where both isomers could be assigned, excluding any weak or overlapped resonances.

Calculation of chemical shift perturbations (δ)

To check for structural differences between free Ub^{S57C} (in 2 mM DTT) and the tagged version (Lu-M8-Ub^{S57C}), chemical shift perturbations were calculated according to the following equation: $[(H_{\text{Lu}} - H_{\text{free}})^2 + (\alpha(N_{\text{Lu}} - N_{\text{free}}))^2]$, where the value of $\alpha = 0.11$ was calculated from free Ub^{S57C} chemical shifts using $\alpha = (H_{\text{max}} - H_{\text{min}})/(N_{\text{max}} - N_{\text{min}})$, as described in (Williamson 2013).

¹⁷O NMR studies of Dy- and Yb-M8-SPy

The hydration state, q , of Dy- and Yb-M8-SPy was determined by measuring the lanthanide induced chemical shift of the free water ¹⁷O signal of the two samples, using H₂¹⁷O as a reference (Djanashvili and Peters 2007), and using the equation $q = \delta_{\text{obs}} / (\langle S_z \rangle \cdot F)$, where $\delta_{\text{obs}} = \delta_{\text{obs}}/P_m$ (δ_{obs} is the difference in ¹⁷O chemical shifts of the water resonances and P_m is the mole fraction [Ln(III)]/[H₂O] = 0.00114). The $\langle S_z \rangle$ is the reduced value for the average spin polarization [28.591 and 2.591 for Dy and Yb at 333 K, respectively, calculated as in (Pinkerton et al. 1985)], and $F \approx (-2.40 \times 10^4)/T$, where F is given in ppm, and T is the absolute temperature. The experiments were conducted in water at 333 K with 19 % H₂¹⁷O (Cambridge Isotope Laboratories, Tewkesbury, MA) and 7 % D₂O, using 128 scans on a Bruker Avance 600 MHz spectrometer equipped with a room-temperature probe. Chemical shifts were also measured as a function of temperature for both compounds, using 8 scans for each data point with the deuterium providing the lock signal.

Luminescence studies of Tb-M8-SPy

The hydration state, q , of Tb-M8-SPy was calculated using the equation $q = A'(k_{\text{H}_2\text{O}} - k_{\text{D}_2\text{O}} - c)$, where A' is 5 ms for Tb, $k_{\text{H}_2\text{O}}$ and $k_{\text{D}_2\text{O}}$ are the luminescent decay rate constants in H_2O and D_2O (in ms^{-1}), respectively, and c is a correction factor of 0.06 ms^{-1} to account for the outer sphere water molecules for Tb^{3+} complexes (Beeby et al. 1999; Horrocks and Sudnick 1979). The decay rate constants of the excited states of Tb-M8-SPy were measured in H_2O and D_2O at 25°C with direct excitation of the terbium ions at 271 nm and monitoring at 544 nm using a Perkin-Elmer LS-55 filter LS-5. Tb-M8-SPy samples were prepared by repeated lyophilization (at least 3 times) either from H_2O or D_2O .

Results

PCS temperature dependence of Dy-, Tm-, and Yb- M8-Ub^{S57C}

Lanthanide complexes are known to have inherent temperature dependence (McGarvey 1979), but we were interested to see if complex coordination number could affect temperature sensitivity. DOTA complexes containing larger lanthanides, such as dysprosium, coordinate an apical water molecule, while complexes with smaller lanthanides, such as ytterbium, do not host water due to crowding of other ligands (Benetollo et al. 2003). To investigate the effect of water coordination on the temperature sensitivity of the lanthanide series, we chose three octamethylated DOTA complexes, Dy-, Tm-, and Yb-M8-SPy, with differing ionic radii (91.2, 88.0, and 86.8 pm, respectively). By conjugating each of the three tags to a ubiquitin cysteine mutant (Ub^{S57C}), we could observe their PCS, as described previously (Opina et al. 2016). Two-dimensional $^1\text{H}/^{15}\text{N}$ -HSQC NMR spectra were acquired at a range of temperatures and at constant pH. We used a diamagnetic reference (Lu-M8-Ub^{S57C}, 97 % of non-prolyl residues assigned) in order to account for the inherent temperature dependence of the chemical shift changes caused by the S57C mutation and tag attachment. However, it should be noted that structural changes were minimal, since we observed that the chemical shift perturbations for ubiquitin (δ) upon covalent attachment of the Lu-M8-SPy tag are small and homogeneous throughout all residues, with an average value of 0.013 ± 0.009 ppm.

For each of the lanthanide complexes, two peaks were observed for each residue, one for each enantiomer of the tag, TSAP and SAP (Fig. 1a, b). While the dysprosium complex (Dy-M8-Ub^{S57C}) had a high enough population of both isomers (73 % SAP, 27 % TSAP, Supp. Fig. 1a) to make an assignment and analysis for both enantiomers, the overwhelming majority of enantiomer for Tm- and Yb-M8- Ub^{S57C} was TSAP (91 and 95 %, respectively, Supp.Figs. 2a, 3a). Lanthanide complexes are notorious for causing peak broadening, due to their ability to induce paramagnetic relaxation enhancement (PRE). However, this contribution is small for ytterbium and so we could assign 89 % of Yb-M8-Ub^{S57C} at all three temperatures. In addition to the residues that are broadened in wild-type ubiquitin (E24 and G53) only three residues were missing (T9, R74, G75) or overlapped (L8, D58, N60) in the paramagnetic or diamagnetic HSQC spectra at one or more temperatures. Thulium has a higher magnetic susceptibility tensor than ytterbium, so induces larger PCS, resulting in three peaks coincident with the water line (T22, T55, I61), along with additional weak (S20, Q62) and missing (A46, C57, Q59, K63) peaks, resulting in a 79 % assignment rate. For Dy-

M8-Ub^{S57C}, despite the presence of two sets of peaks in the spectrum, we could assign 71 % of residues for each of the two isomers. As expected, we found additional overlapped peaks in comparison to Tm-M8-Ub^{S57C} caused by the peak doubling (T22, D52, T55, D58, E64, S65), along with additional weak or missing peaks due to the stronger PRE.

The size and sign of the PCS is directly related to the magnitude and sign of the magnetic susceptibility tensor, as well as the orientation and coordinates of the metal center with respect to the atom of interest. Interestingly, for most residues the TSAP and SAP isomers of Dy-M8-Ub^{S57C} induced PCS of opposite sign, indicating a difference in the positioning of the metal atom with respect to the protein (Fig. 1c–e). Additionally, the two isomers appear to have different temperature sensitivities, with the magnitude of the PCS decreasing for both, but to different extents over the same temperature range (Fig. 1c–e). It is important to note that the population ratio essentially stays within the range of 21–24 % (TSAP) at all temperatures, as calculated from peak integrals.

For the TSAP isomer of Tm-M8-Ub^{S57C}, the profile of the residue-specific PCS (Supp. Fig. 4a) shows a similar magnitude and the same overall trend as Dy-M8-Ub^{S57C} TSAP (Fig. 1e), but with opposite sign due to its negative susceptibility tensor. The fact that the profile is similar indicates that the positioning of the metal center with respect to the protein must be quite similar for the two samples, which is also the case for ytterbium (Supp. Fig. 5a). However, the PCS values for ytterbium are much smaller than thulium and dysprosium, due to the smaller magnetic susceptibility tensor. The PCS values for Yb-M8-Ub^{S57C} were also less sensitive to temperature than those for Tm- and Dy-M8-Ub^{S57C}. Additionally, no assigned resonances near to the Yb-M8 tag were visibly broadened, indicating a smaller contribution from Curie-spin relaxation in ytterbium in comparison to dysprosium and thulium, where visible broadening could be observed up to 13.0 (T55) and 13.2 Å (Q62) from the metal centers, respectively. Proton and nitrogen PCS values for all four samples are summarized in Supp. Table 1.

The differences between the PCS profiles for the TSAP and SAP isomers of Dy-M8-Ub^{S57C} indicate a difference in metal orientation or position with respect to ubiquitin. In order to confirm this, we refined the position of the M8 tag using the observed PCS. This also allowed us to back-calculate the expected PCS and characterize the magnetic susceptibility tensors at each of the three temperatures. In Xplor-NIH, PCS values can be fit using either the PARAREstraints module (Banci et al. 2004) or the variable tensor module varTensor, of which we chose the latter (Schwieters et al. 2003, 2006). In the fitting procedure the orientation of the M8 tag was allowed to vary through rotations of chemical bonds in the linking arm as well as the S–C bond of the cysteine that it is conjugated to, while the protein and the Ln-M8 cage were fixed. Note that if the measurements were carried out in a very high magnetic field, a significant contribution from the residual chemical shift anisotropy to the measured nitrogen PCS may be present and must be corrected (John et al. 2005). However, we carried out the measurements at 600 MHz, which did not require a correction.

The correlation between experimentally measured and back-calculated PCS is shown for the two isomers of Dy-M8-Ub^{S57C} at the three different temperatures in Fig. 2a, calculated using a high-resolution NMR structure of ubiquitin, PDB ID: 1D3Z (Cornilescu et al. 1998).

The agreement between the measured and calculated proton PCS are excellent, with Q-factors of 3.1–4.5 %, where a lower number indicates a better agreement. Interestingly, there is no significant difference in PCS Q-factors for each of the two isomers (Table 1), indicating that neither isomer causes structural changes in the protein. As expected, the coordinates for the metal center of the TSAP and SAP isomers are quite different, but do not change as a function of temperature (Fig. 2b). The optimized susceptibility tensor parameters including the axial component χ_a , rhombic component χ_r , and the relative difference in the principal axis for the SAP and TSAP isomers at the three temperatures are listed in Table 1. For both isomers of Dy-M8-Ub^{S57C}, the axial components of the tensors (χ_a) were positive, as expected, with the magnitude 6 % smaller for the SAP isomer compared to the TSAP isomer at 288 K. Interestingly, when the temperature changed from 288 to 308 K the tensor magnitude and rhombicity of the SAP isomer decreased significantly (by 30 and 52 %, respectively), while the same values for TSAP (10 and 19 %) were much smaller. These results show that the enantiomeric state of DOTA complexes can have a large effect on their temperature sensitivity.

Isosurfaces can be useful to visualize and compare the effects of susceptibility tensors on the PCS of any given protein. In Fig. 2c, the isosurfaces for the TSAP and SAP isomers of Dy-M8-Ub^{S57C} are compared. Positive lobes, shown in blue, correspond to a PCS value of +5 ppm, where the surface of the lobe intersects an atom. Analogously, the surfaces of the negative lobes (in red) signify –5 ppm. The TSAP isosurface is slightly larger, due to the larger magnitude of its magnetic susceptibility tensor, and it is clear that there is a rotation between the TSAP and SAP isomers.

The tensor parameters for Tm- and Yb-M8-Ub^{S57C} are also displayed in Table 1, with correlation plots shown in Supp. Figs. 4b and 5b, respectively. Parameters from only one isomer, TSAP, are reported since the population of the SAP isomer was too low to provide reliable PCS values. Similar to the Dy-M8-Ub^{S57C} fit, the positions of the lanthanide ion and the orientation of the tensor do not change as a function of temperature (Supp. Figs. 4c, 5c; Table 1). For both Tm- and Yb-M8-Ub^{S57C}, the axial components of the susceptibility tensors are negative, and are 18 and 83 % smaller in magnitude, respectively, in comparison to the TSAP isomer of Dy-M8-Ub^{S57C} at 288 K (Table 1). The temperature dependence of Tm-M8-Ub^{S57C} is similar to the TSAP isomer of Dy-M8-Ub^{S57C}, with a decrease in magnitude of 11 % between 288 and 308 K. In contrast, Yb-M8-Ub^{S57C} displays no temperature dependence. However, unlike dysprosium, the tensor rhombicity (χ_r) for both samples shows no significant change within the temperature range studied. The PCS isosurfaces of Tm-M8-Ub^{S57C} (± 5 ppm) and Yb-M8-Ub^{S57C} (± 1 ppm) at 288 K are shown in Supp. Figs. 4d and 5d, respectively. Put together, our results show that the lanthanide complexes each possess distinct temperature sensitivity.

pH dependence of Dy-, Tm- and Yb-M8-Ub^{S57C}

Lanthanide DOTA complexes containing tetraamide ligands are known to have strong pH dependence and as such have been utilized as pH probes in MRI applications (Aime et al. 2002). To investigate the pH dependence of the Ln-M8-Ub^{S57C} complexes, we measured amide pseudo-contact shifts at a range of pH values and at a constant temperature (Supp.

Figs. 1b, 2b, 3b). In this study, it was especially important to use the reference sample Lu-M8-Ub^{S57C}, since large changes could be observed upon changing pH that were unrelated to PCS (Fig. 3a, b). In contrast to the temperature dependence studies of Dy-M8-Ub^{S57C}, where the overall magnitude of the PCS values changed, the observed pH-dependent PCS values showed a different trend. Interestingly, the pH affected both isomers of Dy-M8-Ub^{S57C} to the same extent, with proton PCS values becoming more positive with increased pH, with a stronger effect for those residues that have a larger magnitude of PCS (Fig. 3c, d). For Tm- and Yb-M8-Ub^{S57C}, however, proton PCS values became more negative, due to their negative susceptibility tensors (Supp. Figs. 6a, 7a). All pH-dependent PCS values are summarized in Supp. Table 2.

To have an idea of the influence of pH on the characteristics of the susceptibility tensor and metal ion position, the measured PCS values for all Dy-, Tm-, and Yb-M8-Ub^{S57C} complexes were fit to the ubiquitin NMR structure. The agreement between measured and calculated proton PCS values is excellent at all pH values (Fig. 4a, Supp. Figs. 6b, 7b; Table 2), indicating that there is no structural change in ubiquitin for any of the samples. Similar to the temperature study, the position of the lanthanide ion and tensor direction doesn't seem to be sensitive to pH (Fig. 4b, Supp. Figs. 6c, 7c; Table 2). Surprisingly, any changes in the magnetic susceptibility tensor as a function of pH are comparable to the standard deviation, indicating that these complexes are relatively impervious to pH changes in this range. Approximate positioning of the TSAP and SAP tags are shown for reference (Fig. 4c, Supp. Figs. 6d, 7d). Axial and rhombic components of the magnetic susceptibility tensors for all pH and temperature measurements are summarized in Fig. 5.

¹⁷O NMR studies of Dy- and Yb-M8-SPy

In this study, we observed a marked difference in the temperature dependence of the magnetic susceptibility tensor between Dy- and Yb-M8-Ub^{S57C}. Additionally, the TSAP and SAP isomers of Dy-M8-Ub^{S57C} also displayed differences in their sensitivity to temperature. One possible mechanism that might explain these observations could be a difference in apical water coordination, which changes depending on the size of the lanthanide (Benetollo et al. 2003) and the enantiomeric state of the cage (Meyer et al. 1998; Rudovský et al. 2005). We employed ¹⁷O NMR spectroscopy to determine the hydration states of the Dy- and Yb-M8-SPy^{S57C} complexes (in the absence of ubiquitin) and probed their temperature dependence. Assuming fast exchange between the bulk solvent and the apical water, the induced ¹⁷O chemical shift of the bulk water can be used to determine the lanthanide hydration state, *q*, to within an error of approximately ± 0.2 water molecules, where *q* = 1 indicates the binding of one water (Djanashvili and Peters 2007; Manus et al. 2012). We measured the chemical shift (δ_{obs}) for Dy- and Yb-M8-SPy in 19 % H₂¹⁷O, using 19 % H₂¹⁷O alone as a reference. To induce fast exchange we used a high temperature (333 K), and to ensure a measurable chemical shift, we used a high concentration of ligand (63 mM). Dy-M8-SPy was shown to have 0.92 inner-sphere water molecules ($\delta_{\text{obs}} = -2.1752$ ppm), whereas Yb-M8-SPy was determined to have zero ($\delta_{\text{obs}} = 0.0007$ ppm). From this data, although we cannot make an assumption about the SAP isomer of Yb-M8-SPy, since it constitutes only 5 % of the sample, we can expect that the ytterbium TSAP isomer has a coordination number of eight, hosting no apical water, while both isomers of the dysprosium

complex must have a coordination number of nine, each binding to one apical water. A lower coordination number for either isomer would result in a reduced value of q . We also measured the temperature dependence of the bulk water ^{17}O chemical shift to give an indication of the influence of water coordination on temperature sensitivity. For Dy-M8-SPy, the magnitude of δ_{obs} decreased significantly with increasing temperature, which we attributed to an increase in the length of the Ln–O bond (Supp. Fig. 8a). In contrast, increasing the temperature for Yb-M8-SPy did not produce any change in δ_{obs} , confirming the absence of the bound water.

Luminescence studies of Tb-M8-SPy

Luminescence can also be used to probe the hydration state of lanthanide complexes. The terbium complex (Tb-M8-SPy), which exists as 80 % SAP isomer (Opina et al. 2016), is most amenable for these studies due to its long luminescence lifetime. The emission spectrum of the Tb-M8-SPy complex is shown in Supp. Fig. 8b. Using an adaptation of the Horrocks' method (Beeby et al. 1999; Horrocks and Sudnick 1979) and the measured rate constants for depopulation of the excited states in H_2O and D_2O ($k_{\text{H}_2\text{O}} = 0.604 \text{ ms}^{-1}$,

$k_{\text{D}_2\text{O}} = 0.427 \text{ ms}^{-1}$), the hydration state (q) of Tb-M8-SPy was found to be 0.58, which is

below the number expected for the coordination of one water molecule ($q = 1$). A low q value (0.62) was also reported for Tb-*SSSS-RRRR*-M4DOTMA, a derivative of Tb-M8-SPy that has two opposite chiralities between the ring (SSSS) and the arm (RRRR) (Ranganathan et al. 2002). This has been ascribed to an elongation of the Ln-OH₂ bond due to the crowding brought about by the polymethylated substituents. It has been established that the inner sphere water in the TSAP isomer lies further from the lanthanide ion than in the SAP isomer, and this elongation of the Ln-OH₂ is sufficient enough to bring the bound water molecule out of the first coordination sphere. It should be noted that an elongation of the Ln-OH₂ bond causes a reduction in the effective q value as a result of the $1/r^6$ relationship for the efficiency of the vibrational energy transfer (Beeby et al. 1999).

Discussion and conclusions

In this study, we have observed that the temperature sensitivity of Ln-M8-Ub^{S57C} complexes changes across the lanthanide series, and is also distinct between the tag isomers, TSAP and SAP. The magnetic susceptibility tensor decreases with increased temperature for Tm- and Dy-M8-Ub^{S57C}, while ytterbium is completely insensitive to the same change in conditions. Furthermore, the SAP isomer is three times more sensitive to temperature change than the TSAP isomer, indicating that a physical or chemical characteristic of the enantiomeric state may be responsible for these observations. We also measured the ^{17}O chemical shift of the bulk water in samples containing Yb- and Dy-M8-SPy. Using these measurements we calculated the hydration state of ytterbium and dysprosium to be equal to zero and 0.92, respectively. This indicates that while dysprosium is capable of binding to an apical water molecule in this context, ytterbium is not. We also observed a change in ^{17}O chemical shift of the bulk water in the presence of Dy-M8-SPy as a function of temperature and concluded that this was a result of a change in the length of the Ln-OH₂ bond. In contrast, ytterbium

saw no change. Put together, we propose that the difference in water coordination across the lanthanide DOTA series also affects the temperature sensitivity of these compounds.

However, there are several other possible contributions to the temperature sensitivity observed here. One obvious possibility is the increased flexibility of the connecting linker between the M8 cage and ubiquitin as the temperature increases (Häussinger et al. 2009). If this was the only contributing factor, one would see the same degree of reduction in the magnitude of the susceptibility tensor for both SAP and TSAP isomers, since both have the same connecting linker configuration. However, this was not the case (Fig. 5a, d), with the SAP isomer being much more sensitive to temperature changes. Similarly, the effect of temperature on the axial and rhombic components of the susceptibility tensor for Yb-M8-Ub^{S57C} is negligible (Fig. 5c, f), even though it has the same connecting linker as the TSAP and SAP isomers of Dy-M8-Ub^{S57C}. These observations indicate that a different mechanism dominates the temperature-dependent behavior of the susceptibility tensor.

Another contribution to temperature sensitivity might be a change in interconversion rate between the two isomers at higher temperatures. Previously, a pronounced interconversion was observed at much higher (333 K) temperatures than were investigated here (Opina et al. 2016). However, in our case the population ratio of the SAP and TSAP isomers of the Ln-M8-SPy tag did not change significantly (less than 1 % for Yb-M8-Ub^{S57C} and less than 3 % for Dy-M8-Ub^{S57C}) within the temperature ranges in this study. It might also be suggested that at particularly high isomer interconversion rates, an exchange equilibrium between the two different tensors could exist, which would result in significant peak broadening. However, Häussinger et al. (2009) showed that there was virtually no broadening of the proton resonances of Lu-M8-SPy as the temperature was increased from 280 to 333 K, indicating that any change in the interconversion rate of the two isomers as the temperature is increased has no significant contribution to the temperature effects that we observed. Similarly, there was no significant broadening observed in the ¹H-¹⁵N resonances of Ln-M8-Ub^{S57C} complexes as the temperature was increased from 288 to 308 K in our study. This indicates that the intrinsic temperature dependence of Curie-spin relaxation does not significantly change the line widths in the temperature range measured.

The theoretical derivation of the magnetic susceptibility for the lanthanide series has long been established (Bleaney 1972). The susceptibility is proportional to the crystal field parameters that depend on the coordination and geometry of the lanthanide. The PCS resulting from the susceptibility can be expressed in a series of T^{-n} terms, and it has been shown that only the T^{-2} term is non-vanishing. This intrinsic temperature dependence of the PCS was further expanded to the T^{-3} term and shown that the T^{-2} term alone was accurate enough to predict the PCS within 10–20 % accuracy at room temperature (McGarvey 1979). Measurements on small molecules to evaluate the PCS temperature dependence show that for a proton four bonds away from the lanthanide, praseodymium, its PCS decreased by around 12 % when the temperature was increased from 281 to 310 K (de Boer et al. 1977). The lanthanide coordination in the small molecule studied is quite different than in our complex. Nevertheless we observed a similar reduction in the axial component of the magnetic susceptibility tensors for the TSAP isomers of Dy- and Tm-M8-Ub^{S57C} complexes (10 and 11 %, respectively) when the temperature was increased from 288 to 308 K.

However, for the SAP isomer of Dy-M8-Ub^{S57C}, the reduction was 30 %, indicating that the crystal field parameters for the SAP isomer must change as a function of temperature, presumably due to the altered water coordination. More recently Bertini et al. (2001) compared the axial component of the susceptibility tensors (χ_a) for a series of lanthanides bound directly to a protein calbindin with their theoretical values at 300 K (Bleaney 1972). They showed a very good agreement between experimental and theoretical χ_a for the lanthanide series, with observed values of 3.47×10^{-31} , -2.19×10^{-31} , and -0.83×10^{-31} m³ for Dy, Tm, and Yb, respectively. Interestingly, their χ_a values are in agreement with our experimental values at 298 K (Table 1).

Previous studies on lanthanide DOTA complexes in MRI suggested that the temperature dependence of saturation transfer was related to the change in the rate of exchange of the apically bound water ligand, or an amide proton on the pendant arm (Krchová et al. 2013; Rudovský et al. 2005; Woods et al. 2003). These studies were carried out with lanthanide-DOTA complexes free in solution, and simultaneous observation of the different contributions from SAP or TSAP isomers was not possible. For Dy-M8-Ub^{S57C}, the temperature dependence of the PCS induced by the SAP isomer is greater than that of TSAP. The TSAP structure is less compact than the SAP isomer, with a longer Ln–O bond between the lanthanide and the apically bound water (Meyer et al. 1998; Rudovský et al. 2005). Therefore it is expected that the coordinated water will exchange more rapidly in the TSAP isomer than SAP. In fact, a difference upwards of 50 times faster has been measured for the water exchange rate in TSAP versus SAP (Aime et al. 1997, 1998, 1999). In summary, this would indicate that the largest contribution to changes in the susceptibility tensor as a function of temperature is the extent of water coordination. One would expect the TSAP susceptibility tensor to have less temperature dependence than the SAP susceptibility tensor, which is precisely what was observed. Furthermore, the temperature sensitivity of the Yb-M8-Ub^{S57C} TSAP isomers was negligible, due to the absence of the apical water as confirmed by variable temperature ¹⁷O NMR spectroscopy.

The saturation transfer of DOTA complexes can have a marked pH sensitivity when all four carboxylic acid groups are replaced by amides (Woods et al. 2011; Zhang et al. 2002), and these complexes can be used as pH-sensitive probes within a physiological range (Aime et al. 2002; Opina et al. 2011). Ln-M8-SPy, however, contains only one amide group so is expected to have lower pH sensitivity. Despite observing small changes in the PCS as a function of pH, any alterations in the characteristics of the magnetic susceptibility tensor were comparable to the standard deviation. As such, we conclude that the effect of pH on these Ln-M8-SPy complexes is minimal within the range studied. Furthermore, in cases where the apical water of the lanthanide complex is affected by pH change, for example due to direct hydrogen bonding to a nearby titratable histidine, a much larger difference is observed in the PCS values and the axial component of the susceptibility tensor (Liu et al. 2012). In our case, the observed pH insensitivity precludes this possibility.

In summary, the main mechanism for temperature dependency of the magnetic susceptibility tensor in the Ln-M8-Ub^{S57C} complexes is the presence of an inner sphere water molecule that is directly bound to the lanthanide. We can also suggest that the differences observed between the TSAP and SAP isomers might be due to the difference in Ln-OH₂ bond length

(Opina et al. 2016), with a shorter bond conferring a more stable coordination to the water in the SAP isomer (Aime et al. 1997; Woods et al. 2003, 2005). A dynamic contribution from the linker or cage to the observed PCS must also exist, however its contribution must be smaller than the changes in the tensor that were observed for the TSAP isomer of Dy-M8-Ub^{S57C} as a function of temperature. We therefore conclude, that for applications where temperature insensitivity is required, M8 complexes should be chosen that lack an apical water molecule, such as Yb-M8-SPy. Similarly, any of the Yb-, Tm-, and Dy-M8-SPy complexes can be used in situations that require a consistent magnetic susceptibility tensor over a range of pH values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Aime S, Botta M, Ermondi G (1992) NMR study of solution structures and dynamics of lanthanide(III) complexes of DOTA. *Inorg Chem* 31:4291–4299. doi:10.1021/ic00047a016
- Aime S, Botta M, Ermondi G, Terreno E, Anelli PL, Fedeli F, Uggeri F (1996) Relaxometric, structural, and dynamic NMR studies of DOTA-like Ln(III) complexes (Ln = La, Gd, Ho, Yb) containing a p-nitrophenyl substituent. *Inorg Chem* 35:2726–2736. doi:10.1021/ic950981u
- Aime S, Barge A, Botta M, Parker D, de Sousa AS (1997) Prototropic vs whole water exchange contributions to the solvent relaxation enhancement in the aqueous solution of a cationic Gd³⁺ macrocyclic complex. *J Am Chem Soc* 119:4767–4768. doi:10.1021/ja963743m
- Aime S, Barge A, Botta M, De Sousa AS, Parker D (1998) Direct NMR spectroscopic observation of a lanthanide-coordinated water molecule whose exchange rate is dependent on the conformation of the complexes. *Angew Chem Int Ed Engl* 37:2673–2675. doi:10.1002/(SICI)1521-3773(19981016)37:19<2673:AID-ANIE2673>3.0.CO;2-# [PubMed: 29711615]
- Aime S et al. (1999) NMR, relaxometric, and structural studies of the hydration and exchange dynamics of cationic lanthanide complexes of macrocyclic tetraamide ligands. *J Am Chem Soc* 121:5762–5771. doi:10.1021/ja990225d
- Aime S, Barge A, delli Castelli D, Fedeli F, Mortillaro A, Nielsen FU, Terreno E (2002) Paramagnetic lanthanide(III) complexes as pH-sensitive chemical exchange saturation transfer (CEST) contrast agents for MRI applications. *Magn Reson Med* 47:639–648. doi:10.1002/mrm.10106 [PubMed: 11948724]
- Aime S, delli Castelli D, Crich SG, Gianolio E, Terreno E (2009) Pushing the sensitivity envelope of lanthanide-based magnetic resonance imaging (MRI) contrast agents for molecular imaging applications. *Acc Chem Res* 42:822–831. doi:10.1021/ar800192p [PubMed: 19534516]
- Banci L, Bertini I, Cavallaro G, Giachetti A, Luchinat C, Parigi G (2004) Paramagnetism-based restraints for Xplor-NIH. *J Biomol NMR* 28:249–261. doi:10.1023/B:JNMR.0000013703.30623.f7 [PubMed: 14752258]
- Baranyai Z, Brücher T, Iványi T, Király R, Lázár I, Zékány L (2005) Complexation properties of N,N',N'',N'''-[1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayltetra(1-oxoethane-2-yl-diyl)]tetrakis[glycine](H4dotagI). Equilibrium, kinetic, and relaxation behavior of the lanthanide(III) complexes. *Helv Chim Acta* 88:604–617. doi:10.1002/hlca.200590042

- Beeby A et al. (1999) Non-radiative deactivation of the excited states of europium, terbium and ytterbium complexes by proximate energy-matched OH, NH and CH oscillators: an improved luminescence method for establishing solution hydration states. *J Chem Soc Perk Trans* 2:493–503. doi:10.1039/a808692c
- Benetollo F, Bombieri G, Calabi L, Aime S, Botta M (2003) Structural variations across the lanthanide series of macrocyclic DOTA complexes: insights into the design of contrast agents for magnetic resonance imaging. *Inorg Chem* 42:148–157. doi:10.1021/ic025790n [PubMed: 12513089]
- Bertini I, Luchinat C (1999) New applications of paramagnetic NMR in chemical biology. *Curr Opin Chem Biol* 3:145–151. doi:10.1016/S1367-5931(99)80026-X [PubMed: 10226044]
- Bertini I, Janik MBL, Lee YM, Luchinat C, Rosato A (2001) Magnetic susceptibility tensor anisotropies for a lanthanide ion series in a fixed protein matrix. *J Am Chem Soc* 123:4181–4188. doi:10.1021/ja0028626 [PubMed: 11457182]
- Bleaney B (1972) Nuclear magnetic-resonance shifts in solution due to lanthanide ions. *J Magn Reson* 8:91–100. doi:10.1016/0022-2364(72)90027-3
- Cacheris WP, Nickle SK, Sherry AD (1987) Thermodynamic study of lanthanide complexes of 1,4,7-triazacyclononane-N,N',N''-triacetic acid and 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid. *Inorg Chem* 26:958–960. doi:10.1021/ic00253a038
- Cacheris WP, Quay SC, Rocklage SM (1990) The relationship between thermodynamics and the toxicity of gadolinium complexes. *Magn Reson Imaging* 8:467–481. doi:10.1016/0730-725X(90)90055-7 [PubMed: 2118207]
- Caravan P, Ellison JJ, McMurry TJ, Lauffer RB (1999) Gadolinium(III) chelates as MRI contrast agents: structure, dynamics, and applications. *Chem Rev* 99:2293–2352. doi:10.1021/cr980440x [PubMed: 11749483]
- Cornilescu G, Marquardt JL, Ottiger M, Bax A (1998) Validation of protein structure from anisotropic carbonyl chemical shifts in a dilute liquid crystalline phase. *J Am Chem Soc* 120:6836–6837. doi:10.1021/Ja9812610
- de Boer JWM, Sakkars PJD, Hilbers CW, de Boer E (1977) Lanthanide shift-reagents. II. Shift mechanisms. *J Magn Reson* 25:455–476. doi:10.1016/0022-2364(77)90209-8
- Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* 6:277–293. doi:10.1007/BF00197809 [PubMed: 8520220]
- Djanashvili K, Peters JA (2007) How to determine the number of inner-sphere water molecules in lanthanide(III) complexes by ¹⁷O NMR spectroscopy. A technical note. *Contrast Media Mol Imaging* 2:67–71. doi:10.1002/cmmi.132 [PubMed: 17451189]
- Graham B et al. (2011) DOTA-amide lanthanide tag for reliable generation of pseudocontact shifts in protein NMR spectra. *Bioconjug Chem* 22:2118–2125. doi:10.1021/bc200353c [PubMed: 21877751]
- Häussinger D, Huang JR, Grzesiek S (2009) DOTA-M8: an extremely rigid, high-affinity lanthanide chelating tag for PCS NMR spectroscopy. *J Am Chem Soc* 131:14761–14767. doi:10.1021/ja903233w [PubMed: 19785413]
- Hoeft S, Roth K (1993) Struktur und dynamik von lanthanoid-tetraazacyclododecantetraacetat-(DOTA-)komplexen in Lösung. *Chem Ber* 126:869–873. doi:10.1002/cber.19931260404
- Horrocks WD, Sudnick DR (1979) Lanthanide ion probes of structure in biology—laser-induced luminescence decay constants provide a direct measure of the number of metal-coordinated water-molecules. *J Am Chem Soc* 101:334–340. doi:10.1021/ja00496a010
- Huang Y, Coman D, Ali MM, Hyder F (2015) Lanthanide ion (III) complexes of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraaminophosphonate for dual biosensing of pH with chemical exchange saturation transfer (CEST) and biosensor imaging of redundant deviation in shifts (BIRDS). *Contrast Media Mol Imaging* 10:51–58. doi:10.1002/cmmi.1604 [PubMed: 24801742]
- Jacques V, Desreux JF (1994) Quantitative two-dimensional EXSY spectroscopy and dynamic behavior of a paramagnetic lanthanide macrocyclic chelate: YbDOTA (DOTA=1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid. *Inorg Chem* 33:4048–4053. doi:10.1021/ic00096a033

- John M, Park AY, Pintacuda G, Dixon NE, Otting G (2005) Weak alignment of paramagnetic proteins warrants correction for residual CSA effects in measurements of pseudocontact shifts. *J Am Chem Soc* 127:17190–17191. doi:10.1021/ja0564259 [PubMed: 16332059]
- Keizers PH, Desreux JF, Overhand M, Ubbink M (2007) Increased paramagnetic effect of a lanthanide protein probe by two-point attachment. *J Am Chem Soc* 129:9292–9293. doi:10.1021/ja0725201 [PubMed: 17608481]
- Krchová T, Kotek J, Jiráček D, Havlíková J, Čísarová I, Hermann P (2013) Lanthanide(III) complexes of aminoethyl-DO3A as PARACEST contrast agents based on decoordination of the weakly bound amino group. *Dalton Trans* 42:15735–15747. doi:10.1039/c3dt52031e [PubMed: 24051547]
- Krchová T, Gálisová A, Jiráček D, Hermann P, Kotek J (2016) Ln(III)-complexes of a DOTA analogue with an ethylenediamine pendant arm as pH-responsive PARACEST contrast agents. *Dalton Trans* 45:3486–3496. doi:10.1039/c5dt04443j [PubMed: 26795214]
- Kumar K, Chang CA, Tweedle MF (1993) Equilibrium and kinetic studies of lanthanide complexes of macrocyclic polyamino carboxylates. *Inorg Chem* 32:587–593. doi:10.1021/ic00057a017
- Lee MD et al. (2015) Compact, hydrophilic, lanthanide-binding tags for paramagnetic NMR spectroscopy. *Chem Sci* 6:2614–2624. doi:10.1039/c4sc03892d [PubMed: 29560247]
- Lee MD, Dennis ML, Swarbrick JD, Graham B (2016) Enantiomeric two-armed lanthanide-binding tags for complementary effects in paramagnetic NMR spectroscopy. *Chem Commun (Camb)* 52:7597–7954. doi:10.1039/c6cc02325h
- Liu WM et al. (2012) A pH-sensitive, colorful, lanthanide-chelating paramagnetic NMR probe. *J Am Chem Soc* 134:17306–17313. doi:10.1021/ja307824e [PubMed: 22994925]
- Manus LM, Strauch RC, Hung AH, Eckermann AL, Meade TJ (2012) Analytical methods for characterizing magnetic resonance probes. *Anal Chem* 84:6278–6287. doi:10.1021/ac300527z [PubMed: 22624599]
- McGarvey BR (1979) Temperature-dependence of the pseudo-contact shift in lanthanide shift-reagents. *J Magn Reson* 33:445–455. doi:10.1016/0022-2364(79)90261-0
- Meyer M, Dahaoui-Gindrey V, Lecomte C, Guillard L (1998) Conformations and coordination schemes of carboxylate and carbamoyl derivatives of the tetraazamacrocycles cyclen and cyclam and the relation to their protonation states. *Coord Chem Rev* 178:1313–1405. doi:10.1016/S0010-8545(98)00169-6
- Mittag T, Forman-Kay JD (2007) Atomic-level characterization of disordered protein ensembles. *Curr Opin Struct Biol* 17:3–14. doi:10.1016/j.sbi.2007.01.009 [PubMed: 17250999]
- Müntener T, Häussinger D, Selenko P, Theillet FX (2016) In-cell protein structures from 2D NMR experiments. *J Phys Chem Lett* 7:2821–2825. doi:10.1021/acs.jpclett.6b01074 [PubMed: 27379949]
- Opina ACL, Wu Y, Zhao P, Kiefer G, Sherry AD (2011) The pH sensitivity of –NH exchange in LnDOTA-tetraamide complexes varies with amide substituent. *Contrast Media Mol Imaging* 6:459–464. doi:10.1002/cmmi.445 [PubMed: 22144023]
- Opina AC, Strickland M, Lee YS, Tjandra N, Byrd AR, Swenson RE, Vaslatiy O (2016) Analysis of the isomer ratios of polymethylated-DOTA complexes and the implications on protein structural studies. *Dalton Trans* 45:4673–4687. doi:10.1039/c5dt03210e [PubMed: 26857249]
- Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE (2004) UCSF Chimera—a visualization system for exploratory research and analysis. *J Comput Chem* 25:1605–1612. doi:10.1002/jcc.20084 [PubMed: 15264254]
- Pinkerton AA, Rossier M, Spiliadis S (1985) Lanthanide-induced contact shifts—the average electron-spin polarization. *Theory Exp J Magn Reson* 64:420–425. doi:10.1016/0022-2364(85)90104-0
- Pintacuda G, John M, Su XC, Otting G (2007) NMR structure determination of protein-ligand complexes by lanthanide labeling. *Acc Chem Res* 40:206–212. doi:10.1021/ar050087z [PubMed: 17370992]
- Ranganathan RS, Raju N, Fan H, Zhang X, Tweedle MF, Desreux JF, Jacques V (2002) Polymethylated DOTA ligands. 2. Synthesis of rigidified lanthanide chelates and studies on the effect of alkyl substitution on conformational mobility and relaxivity. *Inorg Chem* 41:6856–6866. doi:10.1021/ic025695e [PubMed: 12470084]

- Rodriguez-Castañeda F, Haberz P, Leonov A, Griesinger C (2006) Paramagnetic tagging of diamagnetic proteins for solution NMR. *Magn Reson Chem* 44:S10–S16. doi:10.1002/mrc.1811 [PubMed: 16921533]
- Rudovský J et al. (2005) Lanthanide(III) complexes of a mono(methylphosphonate) analogue of H4dota: the influence of protonation of the phosphonate moiety on the TSAP/SAP isomer ratio and the water exchange rate. *Chemistry* 11:2373–2384. doi:10.1002/chem.200400367 [PubMed: 15685711]
- Schrödinger L (2010) The PyMOL molecular graphics system Version 1.5. Schrödinger LLC, New York
- Schwieters CD, Kuszewski JJ, Tjandra N, Clore GM (2003) The Xplor-NIH NMR molecular structure determination package. *J Magn Reson* 160:65–73. doi:10.1016/S1090-7807(02)00014-9 [PubMed: 12565051]
- Schwieters CD, Kuszewski JJ, Clore GM (2006) Using Xplor-NIH for NMR molecular structure determination. *Prog Nucl Magn Res Spectrosc* 48:47–62. doi:10.1016/j.pnmrs.2005.10.001
- Vranken WF et al. (2005) The CCPN data model for NMR spectroscopy: development of a software pipeline. *Proteins Struct Funct Bioinform* 59:687–696. doi:10.1002/prot.20449
- Williamson MP (2013) Using chemical shift perturbation to characterise ligand binding. *Prog Nucl Magn Reson Spectrosc* 73:1–16. doi:10.1016/j.pnmrs.2013.02.001 [PubMed: 23962882]
- Woods M, Sherry AD (2003) Synthesis and luminescence studies of aryl substituted tetraamide complexes of europium(III): a new approach to pH responsive luminescent europium probes. *Inorg Chem* 42:4401–4408. doi:10.1021/ic0300823 [PubMed: 12844313]
- Woods M, Kovacs Z, Zhang S, Sherry AD (2003) Towards the rational design of magnetic resonance imaging contrast agents: isolation of the two coordination isomers of lanthanide DOTA-type complexes. *Angew Chem Int Ed Engl* 42:5889–5892. doi:10.1002/anie.200352234 [PubMed: 14673928]
- Woods M, Botta M, Avedano S, Wang J, Sherry AD (2005) Towards the rational design of MRI contrast agents: a practical approach to the synthesis of gadolinium complexes that exhibit optimal water exchange. *Dalton Trans* 24:3829–3837. doi:10.1039/b510778d
- Woods M et al. (2011) Investigations into whole water, prototropic and amide proton exchange in lanthanide(III) DOTA-tetraamide chelates. *Dalton Trans* 40:6759–6764. doi:10.1039/c1dt10616c [PubMed: 21625687]
- Yang F, Wang X, Pan B-B, Su XC (2016) Single-armed phenylsulfonated pyridine derivative of DOTA is a rigid and stable paramagnetic tag in protein analysis. *Chem Commun (Camb)*. doi:10.1039/C6CC06114A
- Zhang S, Winter P, Wu K, Sherry AD (2001a) A novel europium(III)-based MRI contrast agent. *J Am Chem Soc* 123:1517–1518. doi:10.1021/ja005820q [PubMed: 11456734]
- Zhang S, Wu K, Biewer MC, Sherry AD (2001b) ^1H and ^{17}O NMR detection of a lanthanide-bound water molecule at ambient temperatures in pure water as solvent. *Inorg Chem* 40:4284–4290. doi:10.1021/ic0003877 [PubMed: 11487334]
- Zhang S, Michaudet S, Burgess S, Sherry AD (2002) The amide protons of an ytterbium(III) dota tetraamide complex act as efficient antennae for transfer of magnetization to bulk water. *Angew Chem Int Ed Engl* 41:1919–1921. doi:10.1002/1521-3773(20020603)41:11<1919:AID-ANIE1919>3.0.CO;2-Q [PubMed: 19750633]
- Zhang S, Merritt M, Woessner DE, Lenkinski RE, Sherry AD (2003) PARACEST agents: modulating MRI contrast via water proton exchange. *Acc Chem Res* 36:783–790. doi:10.1021/ar020228m [PubMed: 14567712]

Fig. 1. PCS temperature dependence for Dy-M8-Ub^{S57C}. **a** Ln-M8-SPy, a lanthanide-containing tag that induces PCS, is present in two NMR-observable forms; twisted square antiprism (TSAP) and square antiprism (SAP). The two structures interconvert on a slow time scale and are related via rotation of the carboxylate and amide pendant arms. The two isomers are displayed in **(b)**, using the Pr-M8-SPy structure calculated in (Opina et al. 2016), in sticks (PyMol) with carbon (*grey*), nitrogen (*blue*), oxygen (*red*), praseodymium (*green*), polar hydrogens (*white*), and sulfur (*yellow*). Non-polar hydrogens are not displayed. **c** A small section of the ¹H/¹⁵N-HSQC spectrum for Lu- and Dy-M8-Ub^{S57C} showing the PCS for residue I36 at a range of temperatures. At 288 K, Lu-M8-Ub^{S57C}, the diamagnetic version of the complex, is shown in dark red, and the paramagnetic version, Dy-M8-Ub^{S57C} is shown in purple. Two peaks are observed for Dy-M8-Ub^{S57C} since each of the two isomers induces different PCS (TSAP, left, minor isomer; SAP, right, major isomer). The PCS is measured as the difference in chemical shift (ppm) between the paramagnetic and diamagnetic peaks. As

the temperature increases to 298 K, the PCS value decreases for both isomers (dark orange, Lu- and dark blue, Dy-M8-Ub^{S57C}). This trend is continued at 308 K (light orange, Lu- and cyan, Dy-M8-Ub^{S57C}). Proton PCS (ppm) of Dy-M8-Ub^{S57C} are shown on a per-residue basis at three temperatures (288 K, purple; 298 K dark blue; 308 K cyan) for d the SAP isomer and e the TSAP isomer. Note that the PCS values of the SAP isomer are more sensitive to temperature

Fig. 2. Temperature dependence of the lanthanide metal center position for Dy-M8-Ub^{S57C}. **a** Observed vs calculated PCS for the SAP isomer at 288 K (*cyan*), 298 K (*dark blue*), and 308 K (*purple*) and the TSAP isomer at 288 K (*yellow*), 298 K (*orange*), and 308 K (*red*). Minimization of the metal center position using PCS values as restraints was carried out using Xplor-NIH for the TSAP and SAP isomers simultaneously. The lowest energy structure was used to back-calculate expected values. **b** Minimized metal center positions for each of the isomers are shown in spheres at the three temperatures [color system as in (**a**)], with ubiquitin shown in green cartoon. **c** Isosurfaces of SAP and TSAP isomer-induced PCS values where the blue and red lobes signify +5 ppm and -5 ppm, respectively, for Dy-M8-Ub^{S57C} at pH 7.4, 298 K

Fig. 3.

PCS pH dependence for Dy-M8-Ub^{S57C}. Zoom of the $^1\text{H}/^{15}\text{N}$ -HSQC spectrum for **a** Lu- and **b** Dy-M8-Ub^{S57C} showing the PCS for residue K6 at pH 5.0 and 6.2. In (**b**), a large difference is observed between the peak position of the Dy-M8-Ub^{S57C} at pH 5.0 and 6.2, induced by the SAP isomer, which could be mistaken as entirely caused by paramagnetism. Since there is also a large change in the peak position of the diamagnetic version **a** in this pH range, this is an entirely diamagnetic effect, and underlines the importance of using a reference compound in calculating PCS. Proton PCS values (ppm) of Dy-M8-Ub^{S57C} are shown on a per-residue basis at three pH values (pH 5.0, purple; pH 6.2, dark blue; pH 7.4, cyan) for **c** the SAP isomer and **d** the TSAP isomer

Fig. 4. pH dependence of the lanthanide metal center position for Dy-M8-Ub^{S57C}. **a** Observed vs calculated PCS for the SAP isomer at pH 5.0 (*cyan*), pH 6.2 (*dark blue*), and pH 7.4 (*purple*) and the TSAP isomer at pH 5.0 (*yellow*), pH 6.2 (*orange*), and pH 7.4 (*red*). Minimization of the metal center position using PCS values as restraints was carried out using Xplor-NIH for the TSAP and SAP isomers simultaneously. The lowest energy structure was used to back-calculate expected values. **b** Minimized metal center positions for each of the isomers are shown in spheres at the three pH values [color system as in (a)], with ubiquitin shown in green cartoon. **c** Stick representations of the TSAP (*red*) and SAP (*cyan*) isomers of the Dy-M8 tag are shown, with the corresponding metal centers displayed using spheres. It should be noted that although the metal positions are calculated using PCS restraints and can therefore be considered accurate, other atoms in the tag are not calculated using experimental restraints and should therefore not be treated as reliable, but are simply shown as an estimate of their approximate distance from the ubiquitin surface

Fig. 5.

Temperature and pH dependence of the axial (χ_a) and rhombic (χ_r) components of the magnetic susceptibility tensor for Dy-, Tm-, and Yb-M8-Ub^{S57C}. Mean and standard deviation are calculated as described in Table 1. The SAP isomer is shown in red and the TSAP isomer is in black, throughout. **a–c** The axial (χ_a) component of the susceptibility tensors for the SAP and TSAP isomers of Dy-M8-Ub^{S57C} and the TSAP isomer of Tm-M8-Ub^{S57C} decrease in magnitude with increased temperature, with the strongest effect for the SAP isomer of Dy-M8-Ub^{S57C}. No change is observed for the TSAP isomer of Yb-M8-Ub^{S57C}. **d–f** The rhombic (χ_r) component of the susceptibility tensors for the SAP and TSAP isomers of Dy-M8-Ub^{S57C}, and the TSAP isomer of Tm- and Yb-M8-Ub^{S57C} as a function of temperature. **g–i** The axial (χ_a) component of the susceptibility tensors as a function of pH. **j–l** The rhombic (χ_r) component of the susceptibility tensors as a function of pH

Table 1
Temperature dependence of the parameters associated with the magnetic susceptibility tensor

Metal	Isomer	T/K	$\chi_a/10^{-3} \text{ m}^3$	$\chi_r/10^{-3} \text{ m}^3$	Metal coordinates			Difference in principal axis between 288 and 308 K			$Q_H/\%$	$Q_N/\%$
					X	Y	Z	X(°)	Y(°)	Z(°)		
Dy	SAP	288	3.12 ± 0.06	1.68 ± 0.15	57.3	-79.4	18.2	7.5	4.7	0	3.6	5.8
		298	2.63 ± 0.05	1.18 ± 0.09	57.2	-80.2	18.0				3.7	5.7
		308	2.18 ± 0.06	0.81 ± 0.10	57.1	-80.3	17.8				4.2	5.9
Tm	TSAP	288	3.32 ± 0.07	1.59 ± 0.03	64.6	-81.1	16.3	5.6	5.9	2.2	3.1	6.0
		298	3.18 ± 0.08	1.42 ± 0.02	64.7	-81.1	16.0				3.6	6.4
		308	2.98 ± 0.10	1.28 ± 0.04	65.1	-81.4	15.6				4.5	8.7
Yb	TSAP	288	-2.72 ± 0.04	-0.76 ± 0.04	63.4	-80.2	14.9	0	4.9	2.4	2.7	3.5
		298	-2.59 ± 0.06	-0.76 ± 0.06	63.1	-80.2	15.0				2.5	3.3
		308	-2.42 ± 0.03	-0.69 ± 0.03	62.9	-80.4	15.1				2.5	3.4
Yb	TSAP	288	-0.55 ± 0.02	-0.16 ± 0.01	63.4	-80.3	16.6	5.4	5.2	2.7	4.4	8.8
		298	-0.56 ± 0.01	-0.17 ± 0.01	63.2	-80.1	16.7				4.3	8.1
		308	-0.53 ± 0.02	-0.16 ± 0.01	63.0	-80.3	16.8				4.6	8.8

Values shown are the axial (χ_a) and rhombic (χ_r) components of the magnetic susceptibility tensor for the TSAP and SAP isomers of Dy-M8-UbS57C, and the TSAP isomer of Tm- and Yb-M8-UbS57C, calculated using Xplor-NIH and PCS measured at 288, 298, and 308 K (pH 7.4). The average and standard deviation were calculated using 10 replicates where 10 % of the PCS values were removed at random. The difference between the calculated principal axis at 288 and 308 K are shown for the X, Y, and Z components of the magnetic susceptibility tensor, using the calculated average of the ten replicates. The metal coordinates correspond to the position of the metal center with respect to the ubiquitin structure (PDB ID: 1D3Z). The proton (Q_H) and nitrogen (Q_N) Q-factors measure the difference between observed and expected PCS (lowest energy structure of 20 calculated with all data), using the formula in the "Materials and methods" section. Lower Q-factors indicate a better fit

Table 2
pH dependence of the parameters associated with the magnetic susceptibility tensor

Metal	Isomer	pH	$\chi_a/10^{-31} \text{ m}^3$	$\chi_r/10^{-31} \text{ m}^3$	Metal coordinates			Difference in principal axis between pH 5.0 and 7.4			$Q_H/\%$	$Q_N/\%$
					X	Y	Z	X(°)	Y(°)	Z(°)		
Dy	SAP	5.0	2.79 ± 0.02	1.30 ± 0.03	57.3	-80.0	17.9	1.6	0	1.0	3.5	4.9
		6.2	2.73 ± 0.04	1.27 ± 0.05	57.4	-79.7	18.0				3.7	6.3
		7.4	2.62 ± 0.05	1.18 ± 0.09	57.2	-80.2	18.0				3.7	5.7
Tm	TSAP	5.0	3.12 ± 0.06	1.34 ± 0.02	65.1	-81.6	15.6	1.8	0	1.8	5.1	5.6
		6.2	3.31 ± 0.12	1.53 ± 0.04	64.8	-81.1	16.1				4.0	7.3
		7.4	3.18 ± 0.08	1.42 ± 0.02	64.7	-81.1	16.0				3.6	6.4
Yb	TSAP	5.0	-2.52 ± 0.05	-0.67 ± 0.05	62.9	-80.1	15.1	9.5	10.2	5.5	3.0	5.0
		6.2	-2.61 ± 0.03	-0.69 ± 0.02	63.1	-80.2	15.0				2.4	3.6
		7.4	-2.59 ± 0.06	-0.76 ± 0.06	63.1	-80.2	15.0				2.5	3.3
Yb	TSAP	5.0	-0.56 ± 0.02	-0.17 ± 0.01	63.2	-80.1	16.6	7.1	7.5	2.6	5.3	8.2
		6.2	-0.60 ± 0.03	-0.17 ± 0.01	63.0	-80.1	17.0				5.7	11.6
		7.4	-0.56 ± 0.01	-0.17 ± 0.01	63.2	-80.1	16.7				4.3	8.1

Values shown are the axial (χ_a) and rhombic (χ_r) components of the magnetic susceptibility tensor for the TSAP and SAP isomers of Dy-M8-UbS57C, and the TSAP isomer of Tm- and Yb-M8-UbS57C, calculated using Xplor-NIH and PCS measured at pH 5.0, 6.2, and 7.4 (298 K). The average and standard deviation were calculated using 10 replicates where 10 % of the PCS values were removed at random. The difference between the calculated principal axis at pH 5.0 and 7.4 are shown for the X, Y, and Z components of the magnetic susceptibility tensor, using the calculated average of the ten replicates. The metal coordinates correspond to the position of the metal center with respect to the ubiquitin structure (PDB ID: 1D3Z). The proton (Q_H) and nitrogen (Q_N) Q-factors measure the difference between observed and expected PCS (lowest energy structure of 20 calculated with all data), using the formula in the "Materials and methods" section. Lower Q-factors indicate a better fit