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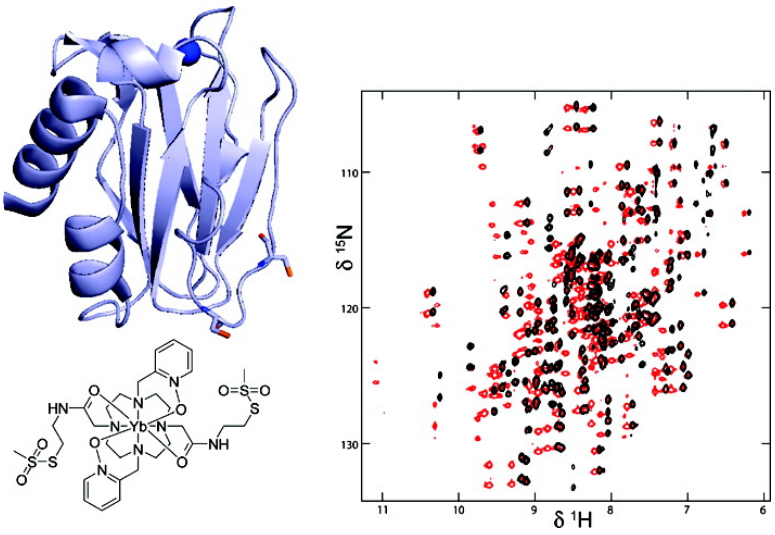

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Increased Paramagnetic Effect of a Lanthanide Protein Probe by Two-Point Attachment

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An unpaired electron of a paramagnetic metal or stable radical can influence the NMR spectrum of macromolecules such as proteins, causing shifts or broadening of the resonances. These effects can be converted into distance and angular restraints for structure determination, provided the paramagnetic center is fixed at a specific location on the protein.[1−3] Lanthanides (Ln) are particularly suited as paramagnetic metals because their magnetic properties vary from diamagnetic to strongly paramagnetic, while their coordination chemistry is similar.[4,5] Ln ions can substitute endogenous cofactors such as Ca^{2+} or Mg^{2+},[6,7] but in a more general approach, an artificial Ln-containing probe molecule is advantageous.[8−10] Such a probe should have sufficiently high affinity for Ln ions without giving rise to multiple or averaged shifts due to multiple conformations of the probe. Given the preference of lanthanide ions for coordination by eight or nine ligands, designing a rigid probe is not trivial.[11]

In this study, we have used the reported favorable conformational properties of the metal chelating 1,4,7,10-tetraazacyclododecane-1-[*N*-oxidopyridine-2-yl)methyl]-4,7,10-triacetic acid (H_3DO3A-pyNox)[12] to develop novel caged lanthanide NMR probes (CLaNPs). H_3DO3A-pyNox was reported to exclusively exist in a SA conformation. The metal complexing ligand was modified with a second pyridine *N*-oxide arm for symmetry reasons, and a 2-(aminoethyl)methanethiosulfonate moiety was included to allow the attachment to cysteine thiolates engineered into the target protein. To determine whether a two-point attachment is advantageous over a single-point attachment, both a one-armed and a two-armed probe (**1** and **2** respectively, Figure 1) were synthesized. The small copper-containing electron transporter protein pseudoazurin (Paz) from *Alcaligenes faecalis* S-6 was used as a model protein to validate the probes.[11]

The probes were synthesized by alkylating commercially available 1,4,7,10-tetraazacyclododecane-1,7-bis(*tert*-butyl acetate) with 2-(chloromethyl)pyridine *N*-oxide in the presence of K_2CO_3. Removal of the *tert*-butyl ester groups, transformation into the active ester, and condensation using the appropriate equivalents of 2-(aminoethyl)methanethiosulfonate provided the precursors of the compounds **1** and **2**. Metals were ligated at neutral pH by adding 1.1 equiv of $Lu(OAc)_3$ or $Yb(NO_3)_3$, over 24 h at room temperature. Products were purified by reversed phase HPLC.

The probes containing paramagnetic Yb or diamagnetic Lu were attached to ^{15}N-labeled Paz E51C (for **1**) and E51C/E54C (for **2**) as described previously.[13] In the [^{15}N−^{1}H] HSQC spectra, no significant differences were observed between the proteins with and without the Lu probes, except for the loss of the signals of several residues near the attachment site of the probe. The disappearance of these resonances has been observed before and

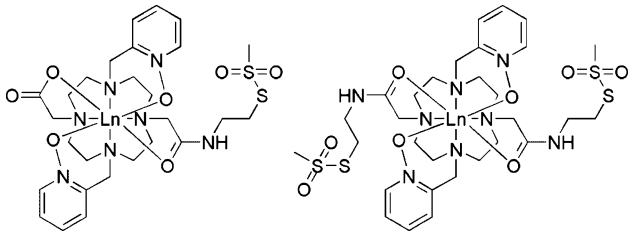

Figure 1. Structures of probes CLaNP-5.1 (**1**) and CLaNP-5.2 (**2**).

was attributed to exchange, due to a slow motion in the loop carrying C51 and C54 caused by the attachment of the probe.[11]

Attachment of the paramagnetic Yb probes to the Paz mutants led to significant pseudocontact shifts (PCS) of many resonances compared to the diamagnetic Lu controls (Supporting Information, Figure S1). The shifts were similar in size in the ^{1}H and ^{15}N dimensions on the parts per million scale as expected for PCS. For both proteins carrying probes **1** and **2**, only single resonances were observed. The probes can therefore be considered a significant improvement over the previously reported CLaNP-3 since Yb in CLaNP-3 induced two peaks for certain residues.[13] Apparently, the introduction of the pyridine *N*-oxide arms instead of acetates forces the probes to exist in a single conformation on the protein surface. Some diamagnetic resonances were still visible in the paramagnetic samples, allowing us to estimate the amount of labeled protein to be 80%.

The paramagnetic effect of the Yb-ligated probe on the protein was much larger for **2** than for **1** (Figure 2). From the PCS, the magnetic susceptibility tensors of both probes could be estimated, using eq 1 (Supporting Information) and the crystal structure of Paz E51C/E54C attached to a previously reported two-armed probe, CLaNP-1,[11] PDB entry 1PY0. This yields for the axial and rhombic components 5.3 ± 0.1 and $1.0 \pm 0.4 \times 10^{-32}$ m^3, respectively, for Paz probe **2**, and 2.0 ± 0.1 and $1.6 \pm 0.2 \times 10^{-32}$ m^3 for Paz probe **1** (Supporting Information, Table S1).

A similar effect was observed for the degree of alignment of the proteins in the magnetic field. Residual dipolar couplings (rdc's) were determined,[14] by comparison of the ^{1}H−^{15}N coupling, for the proteins carrying the Yb and Lu probes. Significant rdc's up to nearly 6 Hz could be measured at 14.1 T for Paz labeled with Yb-ligated **2** (Figure 2). This was not the case for the protein with the one-armed probe, which exhibited small rdc's, indicating less alignment. From the rdc's of residues in defined secondary structure elements, tensor values were estimated using eq 2 (Supporting Information) and PDB entry 1PY0 in Module,[15] yielding $\Delta\chi_{ax} = 5.2 \pm 0.4$ and $\Delta\chi_{rh} = 2.1 \pm 0.4 \times 10^{-32}$ m^3, for Paz probe **2** and $\Delta\chi_{ax} = 1.1 \pm 0.4$ and $\Delta\chi_{rh} = 0.4 \pm 0.3 \times 10^{-32}$ m^3, for Paz probe **1** (Supporting Information, Table S1).

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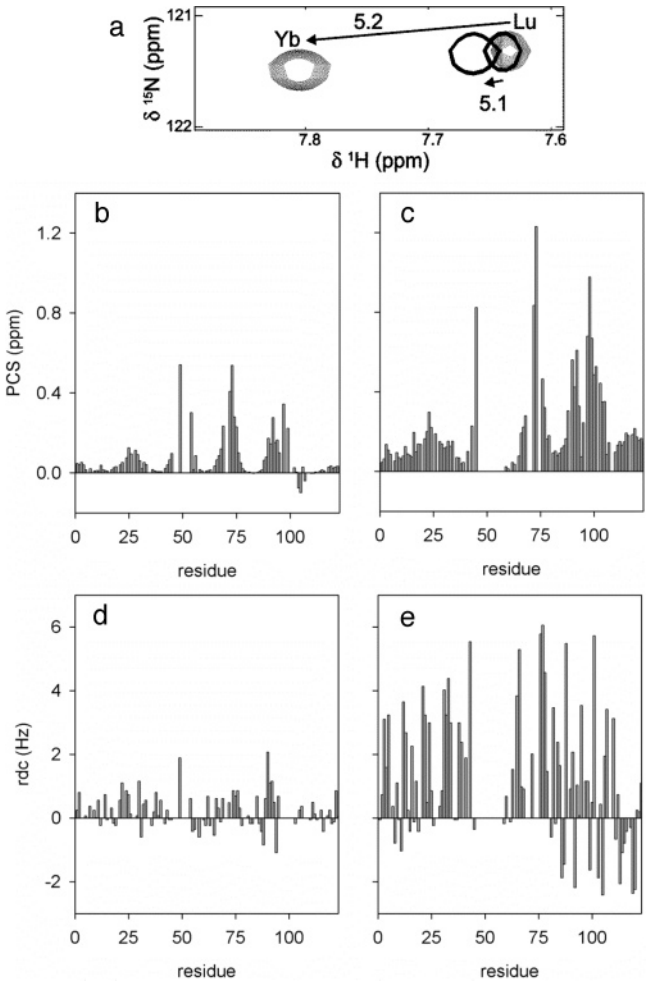

Figure 2. (a) Overlay of [^{15}N−^{1}H] HSQC spectra of Paz with the various probes attached, zoomed in at the resonance of residue A120. Thick circles are used for probe **1**, thin circles for probe **2**, arrows indicate observed shifts from Lu to Yb. PCS (b) and rdc (d) of Paz E51C with Yb ligated **1** attached. PCS (c) and rdc (e) of Paz E51C/E54C with Yb ligated **2** attached. Samples contained 0.1−0.5 mM protein in 20 mM phosphate buffer pH 7. Spectra were acquired at 14.1 T and 293 K.

The largest PCS and rdc values are observed for the two-armed probe. The tensor for Yb ligated in **2** is similar in size to the one of CLaNP-3,[13] which is expected for a probe similar in size and attached to the same position. Apparently, exchanging the acetate arms for pyridine *N*-oxide arms only stabilizes the probe conformation.

The reduced alignment and magnitude of PCS observed when using probe **1** may be explained in two ways. Probe **1** has an axial acetate moiety, whereas in the two-armed probe, this is functionalized into an amide bond. It is known that the axial ligands of DOTA-like rings may cause a difference in tensor magnitude.[16] To check whether this would explain the lower tensor values found for probe **1**, the single E51C mutant of Paz was also labeled with probe **2**. The measured PCS of Yb in probe **2** attached to Paz via only C51 was similar to those obtained when using probe **1** (data not shown), indicating that the difference in the coordinating ligands is not responsible for the difference in tensor magnitude of probes **1** and **2**. The most likely explanation for the smaller tensor magnitude of probe **1** is that it is more mobile relative to the protein backbone due to its single point of attachment.

Because of the reduced mobility of the two-armed probe, the magnitude of the PCS and rdc values measured is also large in comparison with those reported when using other artificial probes. Similar rdc's (up to 8 Hz) have been reported only for much stronger paramagnets, such as Dy, at higher field.[9]

Rdc's are more sensitive to conformational averaging than PCS,[17] which may explain the smaller tensor values from rdc's compared to those from PCS for probe **1**. For probe **2**, these are the same, in line with a low mobility for this probe. Contrary to rdc's, PCS values depend on the third power of the distance between metal and nucleus, and averaging can result in errors in distance determination, as is the case with NOE's. The two-armed attachment strongly reduces possible distance averaging because the metal position is better defined. Thus, it can be predicted more accurately than that of single-armed probes. The quality of the tensor fit for probe **2**, based on the metal position observed for another two-armed probe (CLaNP-1, PDB entry 1PY0), is high, contrary to that for probe **1** (Supporting Information, Figure S2), indicating that the metal positions are similar for CLaNP-1 and probe **2**. This will be important for application of the probe to large protein complexes, in which the probe position cannot be determined independently.

In conclusion, the novel paramagnetic probe CLaNP-5.2 yields large single shifts of the protein nuclei resonances in HSQC spectra and causes significant alignment already at 14.1 T. It is therefore ideally suited to aid in structure determination of protein complexes in which the probe is used to generate long-range distance information.

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Supporting Information Available: Equations 1 and 2 used for tensor calculations, Figures S1 and S2 showing HSQC spectra and tensor fits, and Table S1 presenting the tensor values. This material is available free of charge via the Internet at http://pubs.acs.org.

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