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$[Ln(DPA)_3]^{3-}$ Is a Convenient Paramagnetic Shift Reagent for Protein NMR Studies

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Site-specific labeling of proteins with paramagnetic lanthanide ions (Ln^{3+}) generates pronounced effects in NMR spectra, providing unique long-range structural information.[1] In particular, lanthanides with an anisotropic magnetic susceptibility tensor ($\Delta\chi$ tensor) produce pseudocontact shifts (PCSs) that are readily measured as the difference in chemical shifts observed between samples with paramagnetic and diamagnetic lanthanide tags. Nonvanishing $\Delta\chi$ tensors also lead to weak molecular alignment in the magnetic field with an alignment tensor directly proportional to the $\Delta\chi$ tensor, greatly facilitating the refinement of protein structures by PCSs and residual dipolar couplings (RDCs).[2]

Many lanthanide complexes have been developed for site-specific tagging of proteins via covalent bonds.[3] They rely either on fusions with lanthanide-binding peptides or on chemical reaction with one or two[4] free thiol groups. Consequently, use of these lanthanide tags usually requires protein engineering. The Cu^{2+}−iminodiacetic acid complex has been shown to bind specifically and noncovalently to histidine side chains,[5] but the affinity of lanthanides for histidines is too low for this strategy. Here we present a lanthanide compound, the 3:1 complex between pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA) and lanthanides, $Na_3Ln(DPA)_3$, that binds noncovalently to many proteins in the manner of a lanthanide shift reagent.

The $[Ln(DPA)_3]^{3-}$ complex is very stable and provides 9-fold coordination of the Ln^{3+} ion, leaving no coordination site for water. Crystal structures show a propeller-like arrangement of the DPA molecules around the lanthanide with a C_3 symmetry axis (Figure S1).[6] A cocrystal structure of hen egg-white lysozyme with $[Eu(DPA)_3]^{3-}$ revealed binding at four different sites in the vicinity of arginine side chains.[7]

We first probed the interaction of $[Tb(DPA)_3]^{3-}$ with the N-terminal domain of the *E. coli* arginine repressor (ArgN) for which we have previously obtained PCS data using a covalently binding Ln^{3+}−DPA tag.[8] Addition of increasing amounts of $[Tb(DPA)_3]^{3-}$ resulted in increasing shifts of the protein NMR signals, indicating fast chemical exchange between bound and free protein, and a dissociation constant of ∼14 μM (Figure S2). Backbone amides experienced PCSs of up to 1 ppm in the 1:1 complex of ArgN and $[Tb(DPA)_3]^{3-}$ (Figure 1a). A 50% excess of the lanthanide complex did not result in a 1:2 complex, as no new PCSs were observed. As expected for binding to a single site, experimental and back-calculated PCSs correlated closely (Figure 1b). The principal axes of the $\Delta\chi$ tensor fitted to the PCSs obtained with $[Tb(DPA)_3]^{3-}$, $[Tm(DPA)_3]^{3-}$, and $[Yb(DPA)_3]^{3-}$ were closely aligned (Table 1), resulting in a strong correlation of the PCSs of the Tb and Tm complexes (Figure 1c) which facilitated the resonance assignment of the paramagnetic NMR spectrum.

The binding site of the $[Ln(DPA)_3]^{3-}$ complexes in ArgN positions the lanthanide near the side chains of Arg48 and Lys45 (Figure 2). In addition, binding is aided by proximity to the N-terminus of helix 3 and the overall positive charge of the protein.

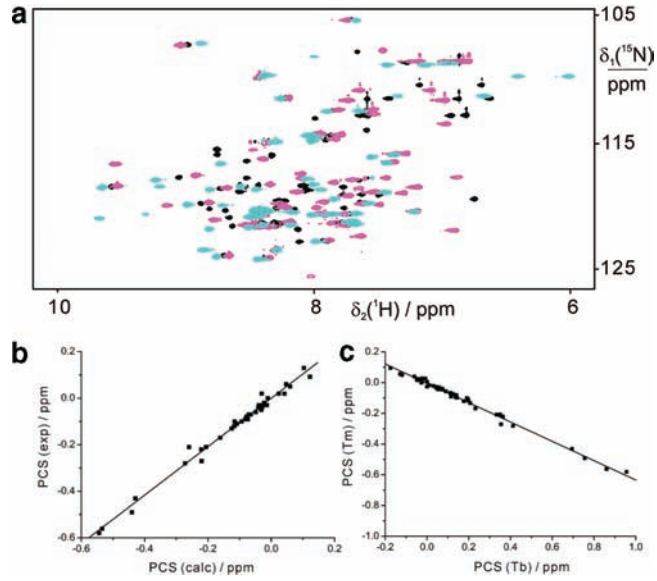

Figure 1. Pseudocontact shifts induced by $[Tb(DPA)_3]^{3-}$ and $[Tm(DPA)_3]^{3-}$ in the protein ArgN. (a) ^{15}N-HSQC spectrum of 0.1 mM ArgN in the presence of 0.15 mM diamagnetic $[Y(DPA)_3]^{3-}$ (black) or paramagnetic $[Tb(DPA)_3]^{3-}$ (cyan) or $[Tm(DPA)_3]^{3-}$ (magenta). (b) Correlation of experimental and back-calculated PCSs induced by $[Tm(DPA)_3]^{3-}$. The $\Delta\chi$ tensor was fitted using the program Numbat (Table 1).[9] (c) Correlation of PCSs induced by $[Tb(DPA)_3]^{3-}$ versus those induced by $[Tm(DPA)_3]^{3-}$. The slope of −1.59 is in agreement with expectations for an axially symmetric $\Delta\chi$ tensor.[10]

Measurements of RDCs of backbone amides at a ^{1}H frequency of 800 MHz showed RDCs ranging from −5 to +4 Hz for the complex with $[Tb(DPA)_3]^{3-}$ and −2 to +3 Hz for the complex with $[Tm(DPA)_3]^{3-}$ (Table S2), indicating that the lanthanide−DPA complexes can be used to align protein samples in a magnetic field for RDC measurements.

Noncovalent binding of $[Ln(DPA)_3]^{3-}$ complexes offers many attractive features for the study of protein structure and interactions. (i) The protein does not need to be modified. (ii) The protein can

Table 1. $\Delta\chi$ Tensor Parameters of $[Ln(DPA)_3]^{3-}$ Complexes Bound to ArgN[a]

Ln^{3+}	$\Delta\chi_{ax}$[b]	$\Delta\chi_{rh}$[b]	α[c]	β[c]	γ[c]
Tb^{3+}	−6.8	−2.8	108	119	110
Tm^{3+}	4.9	1.2	107	116	107
Yb^{3+}	2.0	0.5	103	114	117

[a] The parameters refer to the first conformer of the NMR structure (PDB code 1AOY).[11] The metal coordinates were $x = -11.712$, $y = -4.515$, $z = -9.350$. Fitting was performed using the program Numbat,[9] using the data from all three metal ions simultaneously with a single metal position. The data are reported in the unique $\Delta\chi$-tensor representation.[9] [b] In units of 10^{-32} m^3. [c] Euler angles in degrees in the zyz convention.

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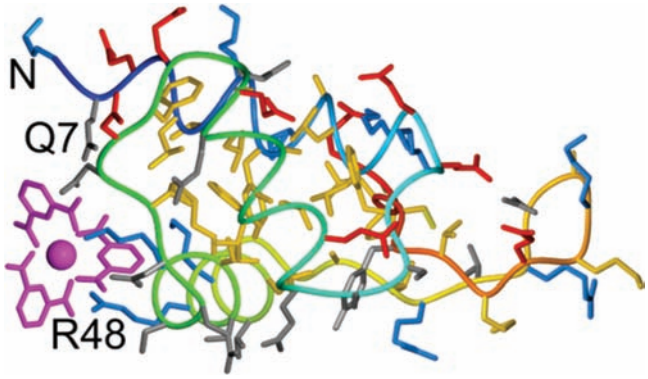

Figure 2. Structure of ArgN with bound $[Yb(DPA)_3]^{3-}$, showing the position of the metal ion (magenta sphere) determined by the PCSs observed in the protein. The first conformer of the NMR structure of ArgN is displayed (PDB code 1AOY).[11] The structure of the DPA complex was taken from the PDB file 2PC2[7] (magenta sticks). The 3-fold symmetry axis of the DPA complex was arbitrarily aligned with the z-axis of the $\Delta\chi$ tensor. The model shows that the fit of the PCSs place the metal position at a reasonable position for multiple interactions with the protein. For example, the side chain amide of Gln7 showed the largest chemical shift changes of all side chain amides in the presence of diamagnetic $[Y(DPA)_3]^{3-}$ (0.07 ppm). Side chains are shown in blue (Arg, Lys), red (Asp, Glu), gray (Asn, Gln, Ser, Thr, Tyr), and yellow (Ala, Cys, Ile, Leu, Met, Phe, Pro, Val). Figure prepared with Molmol.[12]

easily be recovered by dialysis. (iii) Fast chemical exchange allows facile assignment of the paramagnetic NMR spectrum by following the cross-peaks in a titration experiment.[13] (iv) Resonance assignment is further aided by an excellent correlation between PCSs observed for lanthanides and $\Delta\chi$ tensors of opposite sign (Figures 1c and S9).

$[Ln(DPA)_3]^{3-}$ complexes display a greater affinity to proteins than other lanthanide complexes. For example, neither free DPA nor $TmCl_3$ or Tm(DPA)Cl generated any significant chemical shift changes in ^{15}N-HSQC spectra of ArgN. High concentrations of $[Dy(DTPA)]^{2-}$ have previously been recommended to change chemical shifts in a protein.[14] In a 0.1 mM solution of ArgN, however, even 4 mM $[Tb(DTPA)]^{2-}$ generated chemical shift changes of less than 0.06 ppm. For a large excess of reagent over protein, the chances for interactions with more than a single site increase, prohibiting fitting of the PCS data with a single $\Delta\chi$ tensor.

We subsequently tested other proteins for binding of $[Ln(DPA)_3]^{3-}$. Hen egg-white lysozyme (HEWL), the C-terminal domain of *E. coli* DnaG (DnaG-C),[15] and the C-terminal domain of ERp29[16] (ERp29-C) preferentially bound $[Ln(DPA)_3]^{3-}$ complexes at a single site with affinities ranging from 0.3 (ERp29-C) to 2 mM (DnaG-C). In the case of weak binding, the PCSs were reduced but still readily observable (Figures S3−S6). Only T4 lysozyme showed evidence for simultaneous binding at two different sites, while the Tm complex bound only very weakly to the death domain of $p75^{NGFR}$ [17] or to the τ_{C14} domain.[18] Even weak binding led to measurable RDCs. For example, RDCs of ±3.5 Hz were measured for 0.1 mM DnaG-C in the presence of a 4-fold excess of $[Tb(DPA)_3]^{3-}$ at a ^{1}H NMR frequency of 600 MHz.

The cocrystal structure of HEWL with the $[Eu(DPA)_3]^{3-}$ complex displays four different interaction sites, all of which contain an arginine residue, although the interaction with the arginine side chain is not structurally conserved at the atomic level. For HEWL, we detected only a single binding site by NMR (near Arg128). Our NMR measurements confirmed that most proteins capable of binding the $[Ln(DPA)_3]^{3-}$ complex had an arginine residue in the binding site. Arginine residues are known to interact favorably with aromatic groups through cation−π interactions.[19]

In conclusion, $[Ln(DPA)_3]^{3-}$ complexes present widely applicable paramagnetic reagents. Although the binding sites are difficult to predict, binding is, in effect, selective for a single site if the complex is used in a near-equimolar ratio, allowing determination of the $\Delta\chi$ tensor and interpretation of the measured PCSs for structure analysis. Binding to two or more sites would still generate molecular alignment in the magnetic field, yielding RDCs that can readily be interpreted by an average alignment tensor.

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Supporting Information Available: Structure of the $[Lu(DPA)_3]^{3-}$ complex; Titration curves and NMR spectra of proteins with $[Ln(DPA)_3]^{3-}$; Tables of PCSs and ^{15}N−^{1}H RDCs of ArgN induced by $[Ln(DPA)_3]^{3-}$; Sanson−Flamsteed plot of the $\Delta\chi$-tensor fit to ArgN; Table comparing $\Delta\chi$ and alignment tensors of ArgN; Stereoview of Figure 2; Correlation plots of PCSs induced in T4 lysozyme and DnaG-C mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

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