Protein Structures

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Using Genetically Encodable Self-Assembling Gd^{III} Spin Labels To Make In-Cell Nanometric Distance Measurements

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Abstract: *Double electron–electron resonance (DEER) can be used to study the structure of a protein in its native cellular environment. Until now, this has required isolation, in vitro labeling, and reintroduction of the protein back into the cells. We describe a completely biosynthetic approach that avoids these steps. It exploits genetically encodable lanthanide-binding tags (LBT) to form self-assembling Gd^{III} metal-based spin labels and enables direct in-cell measurements. This approach is demonstrated using a pair of LBTs encoded one at each end of a 3-helix bundle expressed in E. coli grown on Gd^{III}-supplemented medium. DEER measurements directly on these cells produced readily detectable time traces from which the distance between the Gd^{III} labels could be determined. This work is the first to use biosynthetically produced self-assembling metal-containing spin labels for non-disruptive in-cell structural measurements.*

The behavior of proteins can differ significantly in cells compared to in vitro.[1,2] Double electron–electron resonance (DEER or PELDOR)[3,4] is a proven method for studying the structures of biological systems[5–7] in a wide variety of environments, even inside cells.[8–13] All in-cell DEER measurements to date have been carried out by introducing in vitro labeled proteins or DNA back into the cells by microinjection, electroporation, or hypoosmotic shock. These techniques are invasive and disrupt the integrity of the cell, but they have been necessary because methods for the biosynthetic production of spin-labeled proteins have been lacking. One elegant solution has been suggested by the use of genetic code expansion to biosynthetically produce spin-labeled thioredoxin with an unnatural amino acid containing a nitroxide radical sidechain.[14,15] However, in-cell applications of such an approach have yet to be pursued.

One potential problem with using nitroxide radicals inside cells is their limited stability in the reducing cytosolic environment.[16] A different approach to in vivo biosynthetic spin labeling is to genetically encode a peptide sequence capable of coordinating a paramagnetic metal ion that can subsequently form self-assembled metal spin labels (SAMSL). Two histidine residues that bind a Cu^{II} ion in the presence of iminodiacetate,[17] and Mn^{II} bound to His-tags,[18] are two such examples, as is Gd^{III} bound to high-affinity lanthanide-binding tags (LBT).[19–21] Until now, such SAMSLs have been formed in vitro. Herein, we describe how Gd^{III}:LBT SAMSLs can be produced in vivo and directly used for in-cell distance measurements.

A DNA construct consisting of a pair of LBTs encoded one at each end of a 3-helix-bundle (3Hx)[22] was overexpressed in *E. coli* to produce LBT-3Hx-LBT. Its 4.5 K EPR spectrum after isolation and incubation with Gd^{III} ($S = 7/2$) is shown in Figure 1 A. The particular shape of the spectrum is due to a distributed zero-field interaction ranging from $D =$ 2.2 to 1.5 GHz, with the largest contribution coming from a component with $D = 2.2$ and $E = 0.9$ GHz (see Figure S1 in the Supporting Information). Gd^{III} complexes in frozen solution often do not have unique zero-field interactions. This has been ascribed to variations in the electronic structure of the Gd^{III} ligand spheres.[23] Consistent with the observed zero-field distribution is that water fractionally ligates Gd^{III}:LBT centers in the crystallographic structure of labeled ubiquitin.[21]The dominant zero-field component was responsible for the two distinct central transition ($\Delta m_s = -1/2 \leftrightarrow 1/2$) shoulders at 3.367 and 3.405 T. The EPR spectrum of doubly Gd^{III}:LBT-labeled IL1β-S2R2 show similar central transition shoulders ($D = 1.8$ and $E = 0.6$ GHz).[19]

The same features were present in the spectra of *E. coli* cells overexpressing LBT-3Hx-LBT grown on 500 μM Gd^{III} supplemented medium, thus confirming the presence of Gd^{III}:LBT-3Hx-LBT:Gd^{III} in these cells (Figure 1 B,C). The spectra also exhibited six sharp lines arising from the native Mn^{II}.[24,25] The spectra of cells expressing only the 3Hx protein without the LBTs lacked the shoulders (Figure 1 D). The intensity of the cellular Gd^{III}:LBT-3Hx-LBT:Gd^{III} signal increased with increasing Gd^{III} concentration in the medium (Figure 1 B). Gd^{III} uptake depended on the incubation time, taking up to 4 h to reach equilibrium (Figure 1 C), unlike Mn^{II}, which takes only minutes.[26] Gd^{III} concentrations up to 500 μM in the medium did not affect survival rate, while only slightly diminishing protein overexpression (Figure S2 in the Supporting Information). After 4 h induction on 500 μM Gd^{III}-supplemented medium, the cells had an intracellular Gd^{III} concentration of 3–6 mM and a LBT-3Hx-LBT concentration of 1–2 mM.

These cells yielded a detectable DEER response (Figures 2 A and Figure S3 in the Supporting Information). A Gd^{III}–Gd^{III} distance of $r_0 = 3.8$ nm with a distribution of 1.5 nm (full width at half height) was obtained from Tikhonov analysis using a regularization parameter of 1000 (Figure 2 B and Figure S4 in the Supporting Information). The same distance

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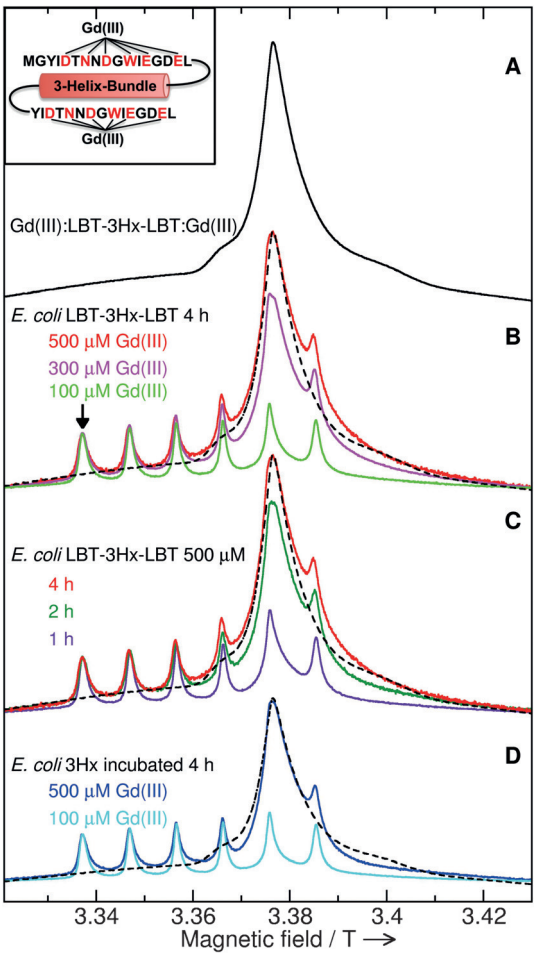

Figure 1. 94 GHz 4.5 K field-swept echo-detected EPR spectra of Gd^{III}:LBT3HxLBT:Gd^{III} A) purified (black and black-dashed in other panels); B) in *E. coli* cells after 4 h induction on medium supplemented with the indicated Gd^{III} concentrations; and C) after various induction times on media supplemented with 500 μM Gd^{III}. D) The spectrum for 3Hx in *E. coli* cells after 4 h induction on medium supplemented with the indicated Gd^{III} concentrations is shown for comparison. All cell spectra were normalized to the intensity of the Mn^{II} resonances. The inset shows the LBT sequences with the Gd^{III}-coordinating residues in red.

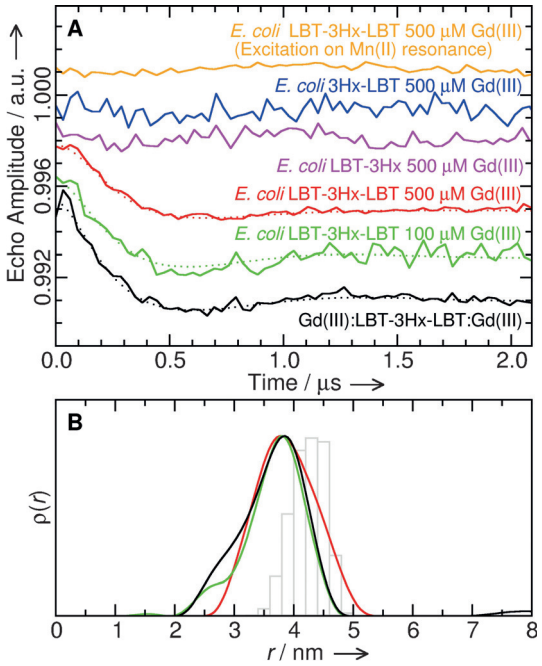

Figure 2. 94 GHz DEER measurements of *E. coli* cells after 4 h of induction of LBT-3Hx-LBT and 3Hx in growth medium supplemented with Gd^{III}. A) Background-corrected DEER time traces (solid line) and fits (dashed lines) based on the Gd^{III}–Gd^{III} distance distributions from Tikhonov analysis shown in (B), together with the distance probability histograms (gray) obtained from molecular dynamics. The time trace of purified 200 μM Gd^{III}:LBT3HxLBT:Gd^{III} is shown in black.

with a distribution of 1.2 nm was obtained for purified Gd^{III}:LBT-3Hx-LBT:Gd^{III}. The distribution profile in this case exhibited a shoulder at 2.5 nm (Figure 2B). Similar features have been attributed to non-central transitions;[19,27,28] however, it is not clear that this attribution applies in our case since the amplitude of this feature varied significantly even for spectra obtained under similar conditions (Figure 2B). Cells overexpressing singly labeled Gd^{III}:LBT-3Hx and 3Hx-LBT:Gd^{III} did not exhibit any modulation (Figure 2), although their EPR spectra were similar to those of cells expressing Gd^{III}:LBT-3Hx-LBT:Gd^{III}. Likewise, no modulations were detected at the magnetic field and pump frequency corresponding to the lowest-field Mn^{II} line (Figure 1B, black arrow, and Figure 2A). These control measurements confirmed that the DEER response detected from cells overexpressing LBT-3Hx-LBT indeed arose from intramolecular dipolar interactions between the two biosynthetically produced Gd^{III}:LBT SAMSLs.

The modulation depth for isolated Gd^{III}:LBT-3Hx-LBT:Gd^{III} was 0.6% and 0.3% in cells. The lower in-cell value was likely due to dipolar interactions between the Gd^{III}:LBT and other paramagnetic species, in particular cellular Mn^{II}[11] and any free Gd^{III}. 35 GHz DEER measurement on isolated labeled IL1β-S2R2 had a modulation depth of 1.5%.[19] This was likely due to the more than twice as high microwave powers used at the lower frequency. Higher modulation depths (2–5%) have been observed for isolated and in-cell proteins labeled with other Gd^{III} spin labels. In these, the zero-field interactions were smaller and consequently the Gd^{III} EPR spectra were considerably narrower, thus allowing a greater number of Gd^{III} labels to be pumped and detected.

The in-cell distance distribution for the Gd^{III}:LBTs was comparable to other in-cell Gd^{III} measurements. Gd^{III}:DOTA-labeled ubiquitin introduced into HeLa cells by hypoosmotic shock showed a distribution of 2 nm (r_0 = 3.5 nm).[12] A similar distribution was obtained for Gd^{III}:PyMTA-labeled polyproline (r_0 = 3.7 nm) in vitro.[11] After microinjection into *Xenopus laevis* oocytes, two components were detected, each with 1 nm distributions centered at r_0 = 2.6 and 3.8. They were attributed to two different helical conformations of the polyproline.[11] The same label rigidly attached to a stiff synthetic polymer showed a distribution of 1.3 nm, both in vitro and in cells (r_0 = 3.0 nm).[11] Although the LBT sequence[21] is bulkier and less structurally defined than the simple organic chelators, the comparable distance distribution suggests that once the SAMSL is formed, it becomes as compact and structured as the simple metal spin labels. Interestingly, the in vitro distance distribution of labeled IL1β-S2R2 was considerably lower

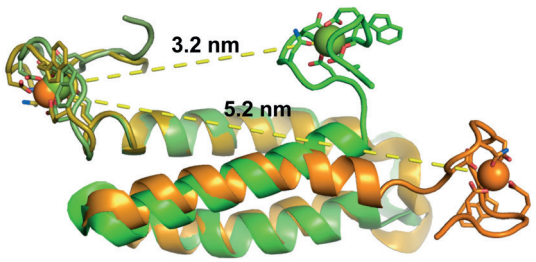

Figure 3. The structure of LBT-3Hx-LBT complexed with metal ions (spheres). The specific structures shown are those of Ca^{II}:LBT-3Hx-LBT:Ca^{II} with the longest (brown) and shortest (green) metal–metal distances obtained from MD calculations.

(0.5 nm).[19] This is likely due to the more rigid location of the LBTs, which were integrated into the loops of the protein[19] rather than at the ends as in our case.

Molecular dynamics (MD) calculations were carried out using Ca^{II} ions as surrogates for Gd^{III} since the latter lack force field parameters (see the Supporting Information). Although based on crude approximations, they did lead to a reasonable picture in which the metal-to-metal distances spanned 3–5 nm (Figure 3), with the shortest distances corresponding to a conformation where the SAMSLs were bent towards each other and the longest to a fully extended structure. As seen in Figure 2B, r_0 was 4.3 nm with a distribution of about 1 nm (see the Supporting Information). Interestingly, the N-terminal SAMSL appeared to be integrated into the helical structure, thus making it less flexible.

The DEER responses were also obtained from cells grown with five-times less Gd^{III} (100 μM Gd^{III}, Figure 1B), a concentration at which the Gd^{III} resonances were difficult to differentiate from those of the Mn^{II}. In these cells, the Gd^{III}:LBT-3Hx-LBT:Gd^{III} concentration was 300 μM at most, which is comparable to some of the most abundant native proteins in *E coli*.[29] In conclusion, our measurements show that it is in principle possible to carry out in-cell structural measurements on proteins bearing SAMSLs in native protein environments without recourse to more invasive approaches. With further optimization, this method undoubtedly has the potential to become an important tool for in-cell structural investigations.

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