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Distance Determination in Proteins inside *Xenopus laevis* Oocytes by Double Electron−Electron Resonance Experiments

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Pulsed ESR techniques, such as DEER (double electron−electron resonance) and electron DQC (double quantum coherence), combined with site-directed spin labeling (SDSL), are receiving increasing attention in structural biology because they provide a means for accurate determination of relatively long distances between electron spin centers introduced into proteins of interest.[1,2] With these methods, distributions of interspin distances of SDSL proteins in a range from 1.5 to 8 nm have been reported. This broad range of measurable distances is comparable to that of the FRET (Förster Resonance Energy Transfer) technique, but because the spin-label probes are smaller than the fluorophores, perturbations on the target molecules can be reduced. Because of this long measurable distance, together with a wider applicability to different sample states (e.g., crystallization is not required), pulsed ESR techniques complement conventional techniques of structural biology such as solution/solid state NMR and X-ray crystallography.

In-cell NMR spectroscopy, an MR-based in situ observation of proteins within living cells, has been successfully employed for the study of the conformations and interactions of proteins in *Escherichia coli* cells[3,4] and oocytes of *Xenopus laevis*.[5,6] Recently, we reported in-cell NMR experiments using mammalian somatic cells.[7] On the other hand, to the best of our knowledge, measurement of interspin distances of SDSL proteins inside cells by pulsed ESR methods has not been previously reported. In this study, we examined the feasibility of DEER experiments for distance measurements of SDSL proteins inside oocytes of the African clawed frog, *Xenopus laevis*.

One intrinsic difficulty of ESR measurement of SDSL proteins inside cells comes from the short persistence of radical spins in cells. A nitroxide radical, the most commonly used radical species for SDSL, can be chemically reduced in the cytoplasmic environment. It was reported that the ESR signals of nitroxide radicals attached to the extracellular regions of membrane proteins disappeared after the protein had been internalized from the plasma membrane into the cytosol.[8] Therefore, the persistence of such radical species in cells is a major concern in performing the DEER experiments. Thus, we first examined the lifetime of nitroxide spin labels in *Xenopus* oocytes using continuous wave (CW)-ESR. A human ubiquitin derivative bearing cysteine substitutions at Ser20 and Gly35 (designated S20C-G35C) was labeled using 3-maleimido-PROXYL, which attaches nitroxide spin labels to the thiol groups of those cysteines[9] (Figure 1A and B). It is important to note that the maleimide conjugation, unlike disulfide linkage, is resistant to reductive cleavage. The SDSL protein was injected into oocytes, which were then incubated for various periods and washed, and then frozen for CW-ESR measurement. The spectra of the oocytes displayed a pattern similar to that of an *in vitro* reference spectrum (Figure S1A), suggesting the observed signals can be attributed to the nitroxide radicals. The ESR signal intensities decreased with increasing incubation time, presumably due to reductive conversion of the nitroxide radicals to ESR-silent hydroxyl amines. The estimated half-life of the intracellular "active" spin labels was approximately 50 min (Figure S1B). Approximately, 30 μM of the nitroxide radicals remained active in the cells even after 2 h of incubation (Figure S1B).

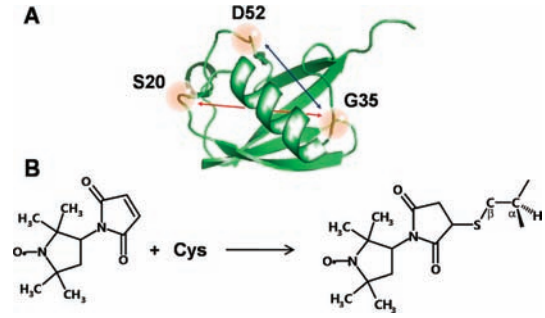

Figure 1. Site-directed spin labeling. (A) Ribbon diagram of the structure of ubiquitin. Three spin-labeled sites, Ser20, Gly35, and Asp52, were substituted with cysteine and (B) reacted with 3-maleimido-PROXYL. Distances measured are indicated with red (S20-G35) and blue (G35-D52) arrows.

We then acquired DEER data on oocytes injected with SDSL ubiquitin derivatives.[10] In addition to S20C-G35C, we labeled a ubiquitin derivative with cysteine substitutions at Gly35 and Asp52 (designated G35C-D52C) for the experiments.

Figure 2A and 2B show dipolar modulation DEER echo curves for spin labeled ubiquitin derivatives both *in vivo* and *in vitro*. The interspin distances (r) and their distribution widths ($\sigma_{\langle r \rangle}$, the standard deviation of the distance) were estimated using the single-Gaussian fit method from time domain data after subtraction of the effects of intermolecular dipolar interactions[11,12] (Figure 2C, 2D, and Supplementary Table 1). The obtained r values were confirmed to be consistent with those extracted by the Tikhonov regularization method[13] implemented in DeerAnalysis2009[12] (Supplementary Figure S2).

The DEER data in the oocytes gave interspin distances, r, similar to those obtained from the *in vitro* reference (Figure 2C and 2D; Supplementary Table 1). The r values of the S20C-G35C and G35C-D52C proteins obtained from the DEER measurements in

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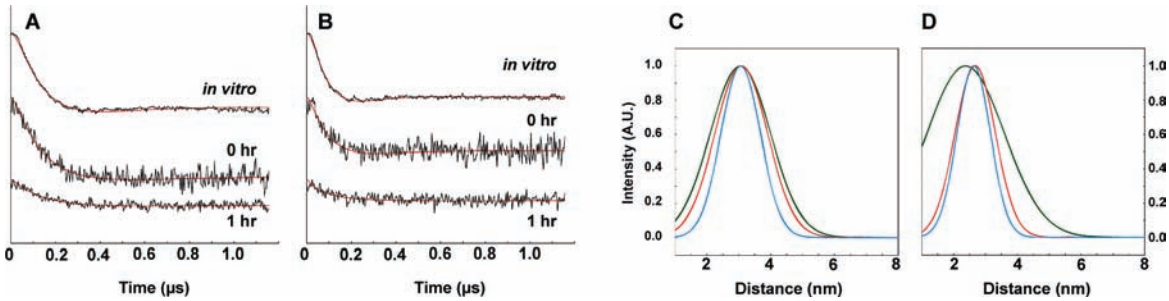

Figure 2. (A and B) Constant-time DEER spectra of S20C-G35C (A) and G35C-D52C (B) ubiquitin *in vitro* and in cell (at 0 and 1 h after injection). An exponential decay component due to intermolecular interactions has been subtracted from the spectra. The fits to the data are plotted by the red lines. (C and D) Distance profiles of S20C-G35C (C) and G35C-D52C (D) measured *in vitro* (blue) and in cells either 0 h (red) or 1 h (green) after injection into cells. The profiles were obtained using the single-Gaussian fit method from time domain data.

oocytes were 3.14 and 2.60 nm, respectively, and the *in vitro* values were 3.11 and 2.65 nm, respectively. This observation suggests that the *r* values inside the oocytes were similar to those determined *in vitro* and that the ubiquitin derivatives display no large structural differences between an intracellular and an *in vitro* environment, which is consistent with our previous observation.[9] The distance between Cα atoms at the positions of the labeled residues ($R_{\alpha\alpha}$) in S20C-G35C and G35C-D52C was estimated to be 2.3 and 1.8 nm, respectively, from the crystal structure of wild-type ubiquitin (PDB ID: 1UBQ). The differences between their DEER-derived *r* values *in vitro* and $R_{\alpha\alpha}$, 0.81 and 0.85 nm, respectively, can be attributed to side chain tethering of the nitroxide moiety and the backbone. For methanethiosulfonate (MTSSL)-labeled proteins, the difference between *r* and $R_{\alpha\alpha}$ was estimated to range from 0 to ~1 nm, depending on the conformation of the tethering chain.[1,2] Because the chain length of the 3-maleimido-PROXYL-labeled proteins is longer than that of MTSSL-labeled proteins, the difference between *r* and $R_{\alpha\alpha}$ could be larger than that of MTSSL-labeled proteins. Thus, we concluded that the deviations of DEER-derived *r* values *in vitro* and in cells from $R_{\alpha\alpha}$ are due to the side chain.

The $\sigma_{\langle r\rangle}$ values in oocytes incubated for 1 h are substantially larger than those without incubation and those from the *in vitro* reference spectrum. The large $\sigma_{\langle r\rangle}$ after the 1-h incubation may be due to uncertainty originating from the relatively poor signal-to-noise ratio of the DEER data. The incubation also led to an increase in unpaired spin labels, which may have underscored ESEEM and slightly shortened the obtained *r* values.

Although $\sigma_{\langle r\rangle}$ may have a relatively large uncertainty, DEER signals from SDSL proteins incubated in oocytes for 1 h still seem adequate for the estimation of most populated interspin distances. The results suggest that the in-cell pulsed ESR measurements can be applied to studying protein conformational changes that occur within 1 h. With this time window, a variety of biochemical reactions and cellular events are in the scope. For example, stimulation of *Xenopus* oocytes with progesterone, a steroid hormone, causes a decrease in cAMP levels within minutes, leading to maturation of the oocytes in a few hours. Fertilized eggs of *Xenopus laevis* develop to Stages 3−4 (4- to 8-cell embryo) in 2 h.[14] Therefore, the pulsed ESR spectroscopy of SDSL proteins in these cells may provide a means to analyze the conformational changes of proteins involved in those cellular and developmental events.

Although $^{1}H-^{15}N$ correlation NMR experiments of proteins *Xenopus* oocytes have been reported,[5,6] *de novo* structural information such as internuclear distances has not been derived from such experiments. In contrast, pulsed ESR is capable of providing long-range distance information on proteins in the intracellular environment, as shown in this study, and thus can complement in-cell NMR.

Microinjection can be implemented on many other cells. However, for in-cell ESR, its applicability is limited to relatively large cells such as the oocytes of zebrafishes, because it requires a large number of spin-labeled proteins. For mammalian cultured cells, one may utilize Cell Penetrating Peptide[7] or other vector systems to deliver SDSL-proteins into the cells, although the lifetime of spin labels may be a critical issue.

In summary, we presented here for the first time SDSL-DEER measurements of proteins in *Xenopus* oocyte cells. Our data suggest that the method can potentially be used to detect conformational changes of proteins associated with cellular events that occur within 1 h. With this time window, a variety of biochemical reactions and cellular events are within scope.

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Supporting Information Available: Detailed experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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