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# Application of Site-Specific $^{19}F$ Paramagnetic Relaxation Enhancement to Distinguish two Different Conformations of a Multidomain Protein

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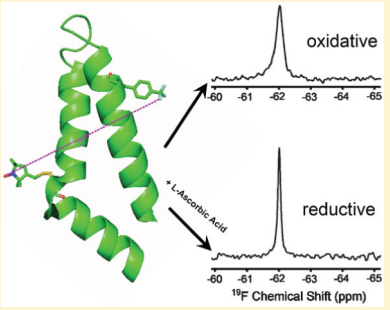
Supporting Information

**ABSTRACT:** Paramagnetic relaxation enhancement (PRE) provides long-distance restraints in solution NMR protein structural studies. It has been shown previously that L27tan, a protein with tandem L27-domains, has two possible conformations. Here, $^{19}F$ was site-specifically introduced to L27tan via the incorporation of an unnatural amino acid, trifluoromethyl-phenylalanine (tfmF). Different $^{19}F$ signal intensity attenuations were observed at different L27tan sites, due to different distances between the site-specifically incorporated tfmF and site-directed spin radical labeling. Analysis of the $^{19}F$ detection PRE showed that the L27tan protein had a closed conformation in solution. This $^{19}F$ detection PRE method could be further applied in distance measurements for proteins of large size, including multidomain proteins or membrane proteins.




**SECTION:** Biophysical Chemistry

Due to the high sensitivity of $^{19}F$ to its surrounding environment, the fact that it produces no background signals in a protein sample, and its high NMR detection sensitivity, $^{19}F$ NMR spectroscopy has been widely applied to investigate protein structure changes, dynamics, folding/unfolding, and aggregation states.[1] Recently, an unnatural amino acid, trifluoromethyl-phenylalanine (p-tfmF), was site specifically introduced to a protein.[2,3] Detection of the $^{19}F$ chemical shift and a side-chain internal mobility analysis of large size proteins and membrane proteins was achieved and was shown to raise a new molecular weight level than proton detection NMR.[4,5] Here, we are presenting that this unnatural amino acid-based $^{19}F$-NMR method can also be applied to perform distance measurements in large proteins, including multidomain proteins.

Paramagnetic relaxation enhancement (PRE) is widely applied in protein structural studies using solution NMR, to obtain quantitative long distance data.[6] An unpaired electron in nitroxide spin radical MTSL (*S*-(2,2,5,5- tetramethyl-dihydro-1H-pyrrol-3-yl) methyl methane sulfonothioate), introduced via cystein-based site-directed spin labeling (SDSL), will enhance the longitudinal or transverse relaxation of protein amide protons.[7] In amide proton detection PRE experiments, amide proton line width broadening or intensity attenuation are typically used to derive a quantitative distance in the range of 14−23 Å [6]. Besides nitroxide radicals, different paramagnetic probes, such as $Cu^{2+}$, $Mn^{2+}$, and other metal ions, can also enhance the longitudinal and transverse relaxation of amide protons in close proximity.[8] Detection nuclei other than protons might be more effective in PRE measurements, due to their ability to avoid water suppression and low background signals from solvents. Recently, $^{13}C$ detection-based PRE measurements were applied to derive distances for structure determinations or protein interface analysis.[9] As described in this study, tfmF-based $^{19}F$ spin incorporation might provide a new method to achieve inter-residue distance measurements between site-specific incorporated detection nuclei ($^{19}F$) and site-directed labeled spin radicals.

Normally, the enhanced transverse relaxation of detection nuclei, arising from the dipole−dipole interaction between the nucleus and the unpaired electrons with an isotropic g-tensor, can be calculated according to the Solomon equation.[10] Using this equation, different quantitative distance ranges between amide proton and unpaired electrons in the paramagnetic center were calculated, in the range of 9−18 Å for $Cu^{2+}$, 16−25 Å for $Mn^{2+}$, and 14−23 Å for MTSL;[8] while the quantitative distance range between $^{19}F$ spin and MTSL was calculated to be 13−21 Å, without considering side chain motions of tfmF and Cys-MTSL (see Supporting Information). The results of these calculations showed that $^{19}F$ detection PRE can be applied for qualitative protein distance measurements in a reasonable range. That is, if the distance is beyond 21 Å, no $^{19}F$ peak attenuation or line broadening should be observed, while the $^{19}F$ NMR resonance should broaden out with zero intensity, if the distance is less than 13 Å.

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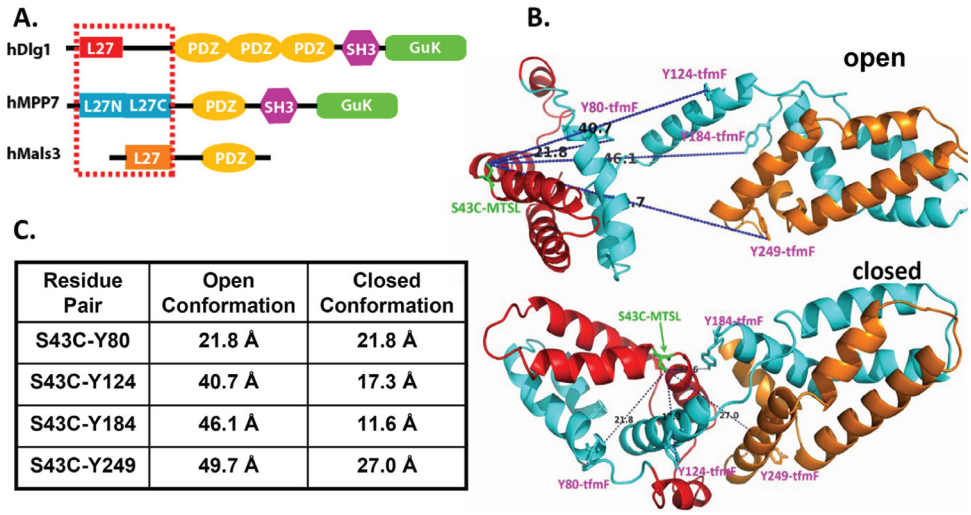


C.

| Residue Pair | Open Conformation | Closed Conformation |
|---|---|---|
| S43C-Y80 | 21.8 Å | 21.8 Å |
| S43C-Y124 | 40.7 Å | 17.3 Å |
| S43C-Y184 | 46.1 Å | 11.6 Å |
| S43C-Y249 | 49.7 Å | 27.0 Å |

**Figure 1.** (A) L27tan consists of four tandem L27 domains from human Dlg1, MPP7 and Mals3 proteins. (B) Cartoon presentations of two alternative conformations of the four tandem L27 domains in L27tan: open (upper panel) or closed (lower panel). (C) Distances between Ser43 hydroxyl proton and Tyrosine phenol protons (at different sites) from L27tan, in open and closed conformations.

In this study, a qualitative distance analysis between site-specific $^{19}$F nucleus and MTSL was applied to distinguish open and closed conformation of a multiple-domain protein: L27tan. L27tan is a protein containing four tandem L27 domains from human Dlg1, human Mpp7 (L27N and L27C), and human Mals3[11] (Figure 1A). Previously, crystals of L27tan were obtained and single anomalous dispersion (SAD) method was applied to determine its structure, and the Fourier map calculated from the initial SAD phases showed two alternative conformations (closed vs open) of this molecule (Figure 1B).[11] In both the closed and open conformations of L27tan, pronounced differences were found in the distances measured between Tyr80 and Ser43, Tyr124 and Ser43, Tyr184 and Ser43, and Tyr249 and Ser43 (Figure 1B,C). In our previous studies, some biochemical and functional results have verified that there is only one conformation that exists for L27tan in aqueous buffer.[11] An amide proton detection PRE method has been applied to distinguish between the closed and open conformations of L27tan.[11] The observed Tyr184 amide proton intensity attenuation with respect to a spin radical labeling at Ser43 site was applied to ascertain a closed conformation of L27tan. To achieve Tyr184 amide proton assignment, resonance disappearance occurring with the Tyr184Phe mutation of L27tan has been designed for the protein [11]. However, this amide proton detection PRE method can not easily be applied to other Tyr sites, because it is difficult for resonance observation or assignments for this protein with more than 250 residues. Especially, this L27tan protein is not stable at ambient temperature, and it can not tolerate NMR acquisition time of 60 min or longer. Therefore, some fast NMR methods should be developed.

Here, one-dimensional $^{19}$F detection NMR experiments and further PRE measurements provide an alternative and efficient way to distinguish the two different conformations of L27tan. First, the unnatural amino acid $^{19}$F-tfmF was site-specifically introduced at four different sites in L27tan. To minimize protein conformation perturbations, four Tyr sites (80, 124, 184 and 249) were selected for $^{19}$F-tfmF incorporation. Codons for the four Tyr residues were mutated respectively to the amber stop codon (TAG) in expression plasmids of L27tan. $^{19}$F-tfmF was incorporated at four different sites through overexpressing the four L27tan proteins in rich medium, containing 1. 0 mM tfmF (detailed procedures in the Supporting Information). All Cys residues in L27tan were mutated to serine, and, consequently, a residue Ser43 was mutated to Cys for further spin radical labeling. In previous studies, both Cys-less L27tan and S43C-L27tan proteins have been proved to adopt conformation similar to that of wild-type L27tan.[11] The overexpressed S43C-L27tan proteins with tfmF incorporation at each of four Tyr sites were purified using $Ni^{2+}$-NTA affinity chromatography, then labeled with spin radical MTSL as described in the Supporting Information.

One-dimensional $^{19}$F NMR spectra were collected for the four protein samples (tfmF labeling at sites 80, 124, 184, 249) respectively. Due to site-specific $^{19}$F-tfmF incorporation, $^{19}$F resonance assignments to the four different sites were very straightforward. For each instance of $^{19}$F-tfmF incorporation, a pair of one-dimensional $^{19}$F spectra was collected, in the absence or presence of a reductive reagent, L-ascorbic acid. Comparing to the $^{19}$F signals of samples with reductive reagent, $^{19}$F signal intensity attenuations of L27tan in oxidative condition were observed, which can be used to qualitatively derive distances between S43C-MTSL and $^{19}$F-tfmF at four different Tyr sites (Figure 2).

Distances between the hydroxyl proton of Ser43 and Tyr sites were calculated for the open and closed conformations of L27tan, and are shown in Figure 1C. Although spin radical MTSL attached to the Cys site brought distance variations due to side chain motions, only short distance differences (<5 Å) will be introduced, which is minor in those measured distance differences between closed and open conformations of L27tan (Figure 1C).

The distances between Ser43 and Tyr80 are similar in the two conformations, and both of them were slightly longer than 21 Å. Shown in Figure 2A,C, the $^{19}$F peak intensities of Y80-tfmF were similar in both reductive and oxidative conditions, which is consistent with the measured distances in the two conformations. For the $^{19}$F spectra of Y184-tfmF (Figure 2B,C), a single $^{19}$F peak was observed in the reductive condition, while strong peak intensity attenuation was observed in the oxidative condition. This strongly indicated that the distance between Y184-tfmF and S43C-MTSL was less than 13


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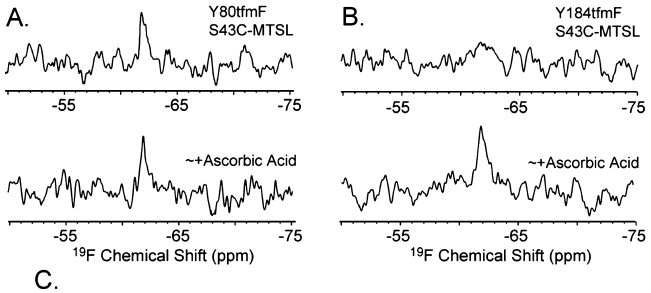


C.

| Residue Pair (S43C-MTSL) | $^{19}$F Intensity Reduced (x $10^4$) | $^{19}$F Intensity Oxidized(x $10^4$) | $^{19}$F Intensity Ratio (oxi./red.) |
|---|---|---|---|
| Y80-tfmF | 6.23 ± 1.78 | 5.78 ± 1.80 | 0.93 ± 0.19 |
| Y124-tfmF | 6.54 ± 1.22 | 4.86 ± 2.02 | 0.74 ± 0.17 |
| Y184-tfmF | 7.22 ± 1.82 | 2.15 ± 1.81 | 0.30 ± 0.13 |
| Y249-tfmF | 5.04 ± 1.54 | 4.91 ± 1.95 | 0.98 ± 0.25 |

**Figure 2.** One-dimensional $^{19}$F spectra of L27tan with $^{19}$F-tfmF substituting for Tyrosine at different sites, and spin radical MTSL attached at Ser43Cys, in the absence (upper spectrum) and presence (lower spectrum) of L-ascorbic acid: (A) Tyr80tfmF; (B) Tyr184tfmF;. (C) $^{19}$F signal intensity ratios between oxidized and reduced states of Ser43Cys-MTSL, at four different Tyr-tfmF sites (80, 124, 184, and 249).

Å. Therefore, L27tan is in a closed conformation (closed: 11.6 Å, open: 46.1 Å in Figure 1C). To verify this, a 74% intensity attenuation of $^{19}$F peak of Y124-tfmF was measured under oxidative conditions (Figure 2C and Supporting Information Figure S2.C). These results indicated that the distance between Y124-tfmF and S43C-MTSL was between 13 and 21 Å, which reconfirmed that the L27tan was in a closed conformation (closed: 17.3 Å, open: 40.7 Å in Figure 1C). Since distances between Tyr249 and Ser43 are larger than 21 Å in either the open or closed conformation (49.7 Å and 27 Å, Figure 1C), there should be no $^{19}$F signal intensity attenuation (0.98 in Figure 2C, and Supporting Information Figure S2.D) measured for Y249-tfmF in the absence or presence of L-ascorbic acid.

In this study, since the L27tan protein is not stable at high concentration and ambient temperature (25 °C) (data not shown), only 0.2 mM proteins were applied for $^{19}$F NMR spectra collection. About 512 free induction decays were accumulated with 2 s acquisition delay. Therefore, around 18 min were required to collect a one-dimensional $^{19}$F NMR spectrum with a reasonable sensitivity. This quick one-dimensional single peak $^{19}$F spectrum collection is superior to conventional $^{1}$H−$^{15}$N two-dimensional correlation spectra, which require longer acquisition time (at least 1.5 h, or 90 min) for L27tan protein (more than 250 residues) in 0.2 mM concentration. At the same time, the one-peak $^{19}$F spectrum provides an instant resonance assignment (Figure S1), which can not easily achieved for a protein of this large size. Therefore, the one-dimensional, single-peak $^{19}$F NMR method is more efficient than conventional proton-detected NMR in PRE-based distance analysis.

In summary, the site-specific $^{19}$F detection PRE method was successfully applied to detect the closed conformation of L27tan, distinguishing between its two possible conformations in solution. The results of this study clearly demonstrated that site-specific $^{19}$F incorporation and spin radical labeling provide a new method to qualitatively measure distance between two residues in a protein. This method has significant advantages over conventional amide proton detection PRE methods: higher sensitivity, straightforward resonance assignments, and specific distance analysis. Especially, our previous studies have demonstrated that a tfmF-based $^{19}$F NMR spectrum can be acquired for very large proteins [5]. Therefore, considering all of the advantages, the site-specific $^{19}$F detection PRE method has good potential in distance measurements and conformational change analysis of supersize proteins, including protein complexes and membrane proteins.

## ■ ASSOCIATED CONTENT

### Supporting Information

Descriptions of $^{19}$F detection PRE distance calculations, sample preparations, and NMR experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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