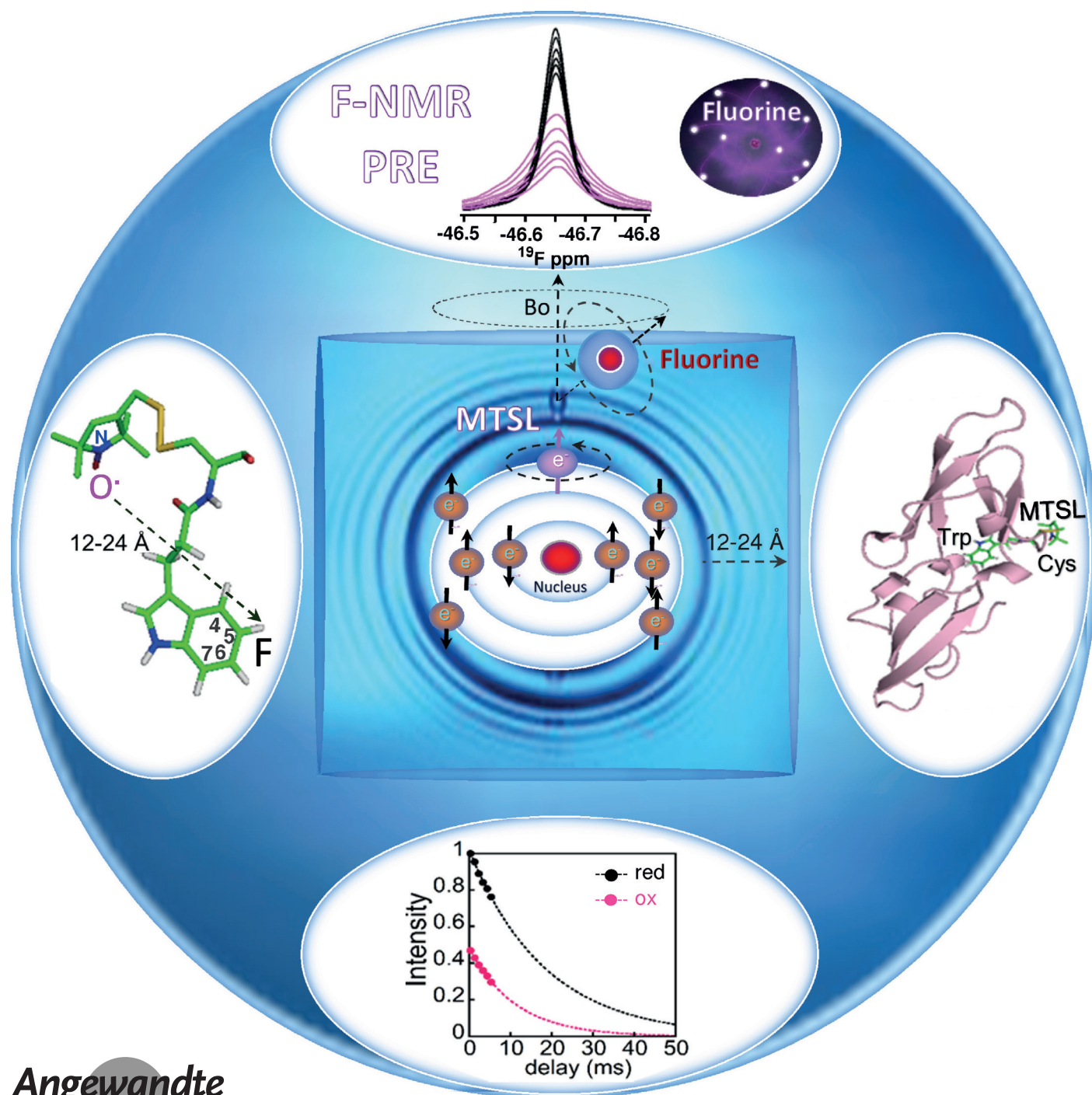# ¹⁹F Paramagnetic Relaxation Enhancement: A Valuable Tool for Distance Measurements in Proteins

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Abstract: *Fluorine NMR paramagnetic relaxation enhancement was evaluated as a versatile approach for extracting distance information in selectively F-labeled proteins. Proof of concept and initial applications are presented for the HIV-inactivating lectin cyanovirin-N. Single F atoms were introduced at the 4-, 5-, 6- or 7 positions of Trp49 and the 4-position of Phe4, Phe54, and Phe80. The paramagnetic nitroxide spin label was attached to Cys residues that were placed into the protein at positions 50 or 52. ^{19}F-T_2 NMR spectra with different relaxation delays were recorded and the transverse ^{19}F-PRE rate, ^{19}F-Γ_2, was used to determine the average distance between the F nucleus and the paramagnetic center. Our data show that experimental ^{19}F PRE-based distances correspond to 0.93 of the ^1H_N-PRE distances, in perfect agreement with the gyromagnetic $\gamma^{19}F/\gamma^1H$ ratio, thereby demonstrating that ^{19}F PREs are excellent alternative parameters for quantitative distance measurements in selectively F-labeled proteins.*

*I*n paramagnetic relaxation enhancement (PRE) NMR experiments, the increase in longitudinal or transverse relaxation rates, induced by the presence of a paramagnetic center, is measured. The effect is $\langle r^{-6} \rangle$ distance dependent, and, as a result, information on the distance between the paramagnetic moiety to the measured nuclei is obtained.

In biomolecular NMR, PREs are increasingly used for providing long-range distance information that complements nuclear Overhauser effect (NOE)-derived, short (≤ 5 Å) interproton distance restraints. In cases where NOE data were found limited, distances derived from PREs provided valuable supplemental restraints and permitted either characterization of the global folds for some proteins,[1,2] or delineation of structural properties of disorder proteins.[3,4]

The most widely used spin label, originally developed for EPR studies,[5,6] is the nitroxide radical MTSL (1-oxyl-2,2,5,5-tetramethyl-Δ^3-pyrroline-3-methyl)methanethiosulfonate.[7,8] It enhances the transverse relaxation rate (R_2) of protons and is introduced into proteins by site-specific labeling of cysteine residues.[9–11]

PRE measurements in proteins commonly monitor ^1H line broadening or intensity attenuation of proton resonances, caused by R_2 relaxation rate enhancements, and these are used to derive distances, with an accessible distance range from the MTSL spin label to protons of 13–25 Å. Resonances of residues in close proximity to the spin label are broadened beyond detection, while for long distances, the effect becomes undetectable.

Importantly, for large proteins and proton detection, the application of the PRE method is fraught with difficulties since resonance overlap may preclude individual effects to be measured accurately. In addition, complete or near-complete

assignments have to be available. Therefore, in order to obtain PRE-based structural information for large proteins and protein complexes, other nuclei beyond ^1H may represent viable alternatives.

In addition, current strategies to specifically label side chains, using biosynthetic amino acid precursors, have been proven to offer ideal avenues for solution NMR spectroscopy studies of large proteins.[12–17] However, this method is inherently subject to certain limitations, requiring one to specifically and selectively label many different types of methyl groups in several or in the same sample(s).

Although classical ^1H, ^{13}C, and ^{15}N spectroscopic approaches have been used extensively to study proteins in solution, ^{19}F NMR spectroscopy is gaining increasing popularity. As discussed previously,[18–21] the 100 % naturally abundant ^{19}F atom possesses a spin 1/2 nucleus and a high gyromagnetic ratio that results in excellent sensitivity. In addition, ^{19}F is absent from virtually all naturally occurring biomolecules, thus studies of fluorinated proteins do not suffer from any background signals. Several methods to prepare ^{19}F-modified proteins have been described,[22–27] and recently a simple and inexpensive approach, using the fluoroindole precursor for expressing ^{19}F-Tryptophan-containing proteins, has been reported.[28]

In the present work, we explore ^{19}F PREs for extracting quantitative distance restraints, using the cyanobacterial lectin CV-N as a model system. Two cysteine residues, Cys50 or Cys52, were introduced in solvent accessible positions in the linker region between the two domains for spin-label attachment. PREs were observed on the ^{19}F nucleus, and amide ^1H_N protons were used as controls. To that end, uniformly ^{15}N-labeled protein with site-specific fluorination was generated. Two types of fluorine labels were employed: 1) single fluorine atoms were individually incorporated at the 4-, 5-, 6- or 7-positions of the single tryptophan, Trp49, and 2) all phenylalanines, Phe4, Phe54, and Phe80, were labeled at the 4-positions.

Cyanovirin-N variants were prepared as described previously,[29a,b] using 4, 5, 6, or 7-fluoroindole,[28] and 4-F-Phe in the growth medium for fluorine labeling. Proteins were expressed in sufficient quantities for paramagnetic/diamagnetic labeling, and structural models were created using the mtsslWizard in Pymol,[30] based on the wild-type CV-N solution structure [PDB code 2EZM] as the template.

In order to explore a wide range of ^{19}F PRE effects, three different MTSL-tagged systems were created: the Cys50-MTSL-modified 5F-Trp49 CV-N variant (Figure 1 A, left), four Cys52-MTSL-modified 4F-, 5F-, 6F-, or 7F-Trp49 CV-N variants (Figure 1 A, right), and the Cys52-MTSL-modified 4-F Phe4, Phe54 and Phe80 CV-N variants (Figure 1 A, center).

^1H-^{15}N HSQC spectra of ^{S52C}CV-N, MTSL-modified ^{S52C}CV-N, and wild-type CV-N were compared to ascertain that neither the introduction of the Cys, nor the spin label perturbed the protein structure in any significant manner (see Figure S1 in the Supporting Information). The chemical shifts of all proteins are essentially identical and only moderate changes were observed for residues in close proximity to the spin label (Figure S1).

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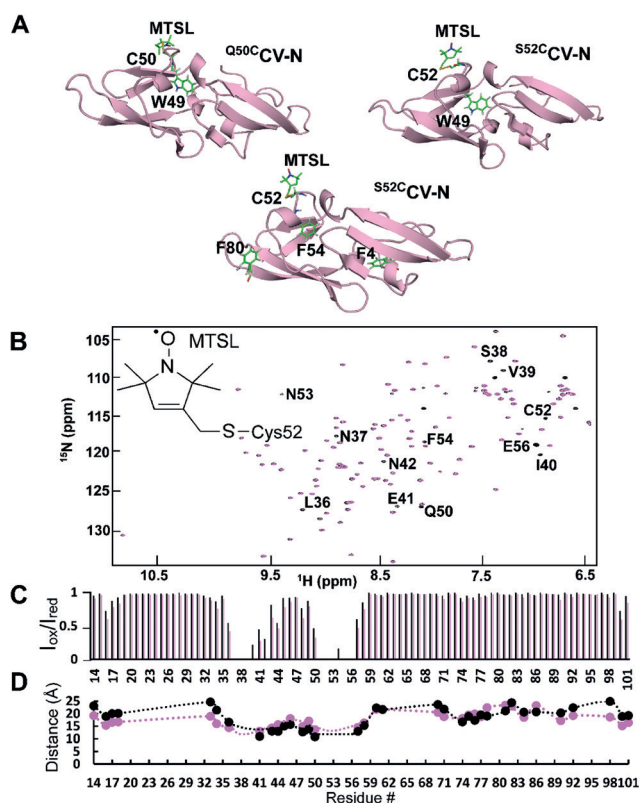
Figure 1. ^1H_N-PREs measured on MTSL-modified ^{S52C}CV-N. A) Ribbon representation of Cyanovirin-N variants used for ^{19}F PRE measurements. The Cys50 and Cys52 with the attached MTSL as well as Trp49, Phe4, Phe54, and Phe80, which are fluorine labeled in the various proteins, are shown in stick representation. B) Superposition of ^1H-^{15}N HSQC spectra of reduced (black) and oxidized (magenta) uniformly ^{15}N- and spin-labeled ^{S52C}CV-N. Resonances, the linewidths of which are most severely affected by paramagnetic broadening, are labeled by residue name and number. C) ^1H_N-PRE signal intensity ratios for oxidized and reduced MTSL-labeled ^{S52C}CV-N, using 1 ms (black) and 10 ms (magenta) relaxation delays. D) Distances extracted from ^1H-PREs (magenta) and those measured from the molecular model of the MTSL-modified ^{S52C}CV-N (black).

For MTSL-modified ^{S52C}CV-N, ^1H_N PREs were measured. The superposition of the ^1H-^{15}N HSQC spectra of reduced (black) and oxidized (magenta) proteins (Figure 1B) clearly shows severe intensity attenuation of several amide resonances. The equivalent resonances are present with normal intensities in the diamagnetic state (black contours).

The PRE relaxation profile (I_{ox}/I_{red} vs. residue number) delineates intensity loss for amide resonances of residues in the vicinity of the spin-labeled Cys52 (Figure 1C). Relaxation rates ^1H_N-Γ_2 were extracted from cross-peak intensity ratios in the oxidized and reduced spectra, and distance restraints were derived according to the Solomon–Bloembergen Equation (1),

$$\Gamma_2 = \frac{1}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \gamma^2 g^2 \mu_B^2 S(S+1) r^{-6} \left\{ 4\tau_c + \frac{3\tau_c}{1+(\omega\tau_c)^2} \right\} \quad (1)$$

using a rotational correlation time (τ_c) of 4.5 ns, based on data available for CV-N in solution.[31]

Distances around the paramagnetic center extracted from ^1H_N-PREs (magenta) and calculated from the ^{S52C}CV-N molecular model (black) are provided in Figure 1D, illustrating that the ^1H_N-Γ_2 derived distances are in good agreement with those back-calculated from the MTSL-modified ^{S52C}CV-N model structure.

1D ^{19}F NMR spectra of 4F-, 5F-, 6F-, and 7F-Trp49-labeled ^{S52C}CV-N proteins as well as for the free 4F-, 5F-, 6F, and 7F-Trp amino acids are superimposed in Figure 2. The
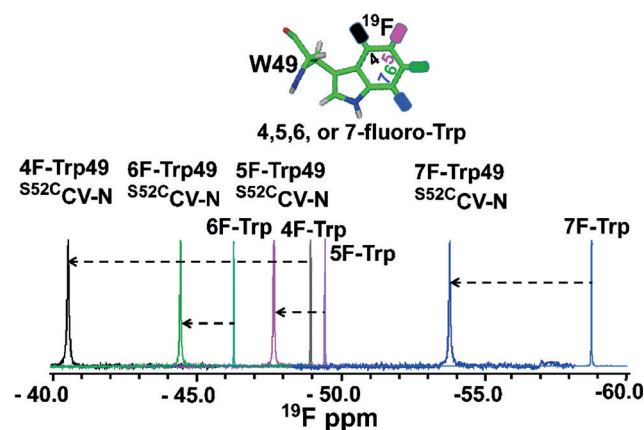

Figure 2. Chemical structure and 1D ^{19}F NMR spectra of 4F-, 5F-, 6F-, 7F-Trp as well as 4F-, 5F-, 6F-, 7F-Trp49-modified ^{S52C}CV-N. Chemical shift differences between the free amino acid and the amino acid when incorporated into the ^{S52C}CV-N protein are indicated by the dashed lines.

free amino acid ^{19}F NMR signals are located at -49, -49.6, -46.1, and -58.8 ppm, respectively, while the equivalent Trp resonances in the ^{S52C}CV-N protein are found at -40.2, -47.5, -44.1, and -53.8 ppm, respectively. All protein resonances are downfield shifted, by varying degrees, compared to the free amino acids, and the largest differences are observed for 4F- ($\Delta\delta = 9$ ppm) and 7F-Trp ($\Delta\delta = 5$ ppm). The overall downfield shifts may arise from the fluorine being embedded into the proteinaceous environment. The most remarkable feature is the wide spread of chemical shifts, which alleviates overlap problems and can be exploited in protein studies.

^{19}F-Γ_2 relaxation experiments on MTSL-modified, uniformly ^{15}N-labeled ^{Q50C}CV-N, containing 5F-Trp49, were used to probe the lower PRE-based distance limit for fluorine. Resonance intensities, extracted from the oxidized and reduced spectra, were converted into paramagnetic relaxation rate enhancements (^{19}F-Γ_2). 1D ^{19}F NMR spectra of oxidized (magenta) and reduced (black) protein, recorded with different relaxation delays (Figure 3, and Figure S2), yielded a fluorine PRE-derived distance between the paramagnetic center attached to Cys50 in ^{Q50C}CV-N and the fluorine atom at the 5-position of the Trp49 indole ring of 11.9 Å, in good agreement with the predicted distance (13.1 Å) based on the modeled structure (Table S1).

Additional ^{19}F-Γ_2 relaxation experiments were carried out on MTSL-modified, uniformly ^{15}N-labeled ^{S52C}CV-N, containing 4F-, 5F-, 6F-, or 7F-Trp49 as the fluorine probe to evaluate more distances.
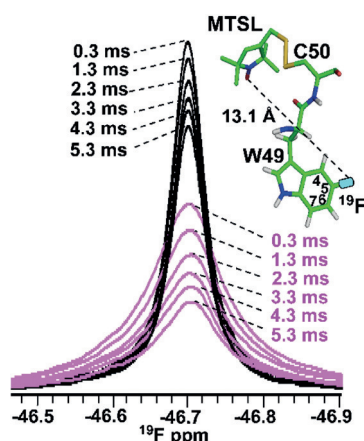
Figure 3. ¹⁹F PREs on 5F-Trp49 in MTSL-modified ^{Q50C}CV-N. The ¹⁹F NMR resonance of oxidized (magenta) versus reduced (black) 5F-Trp49 ^{Q50C}CV-N is superimposed for 0.3, 1.3, 2.3, 3.3, 4.3, and 5.3 ms relaxation delays. The PRE-derived distance is 11.9 ± 0.5 Å and the equivalent distance from the structural model of MTSL-modified ^{Q50C}CV-N is 13.1 Å and is shown on the model by a dashed line.

¹⁹F-Γ_2 relaxation rates were extracted from 1D ¹⁹F resonance signal intensity ratios (I_{ox}/I_{red}) recorded for oxidized (magenta) and reduced (black) spin-label-modified ^{S52C}CV-N proteins, containing 4F-, 5F-, 6F-, or 7F-Trp49 (Figure 4, and Figure S3). The extracted distances from the PRE data between the paramagnetic center and the 4F-, 5F-, 6F-, and 7F positions of Trp49 in ^{S52C}CV-N are 15.2 ± 0.9, 16.4 ± 1.2, 19.3 ± 0.9, and 18.6 ± 1.4 Å, respectively, in excellent agreement with those predicted from the structural model of MTSL-modified ^{S52C}CV-N which are 16.4, 18.2, 19.6, and 19.3 Å (Table S1).

For an estimate of the upper distance limit for fluorine PREs we used the uniformly ¹⁵N- and 4-fluoro-Phe-labeled ^{S52C}CV-N protein. Three phenylalanines are present in the protein, Phe4, Phe54 and Phe80. A superposition of oxidized
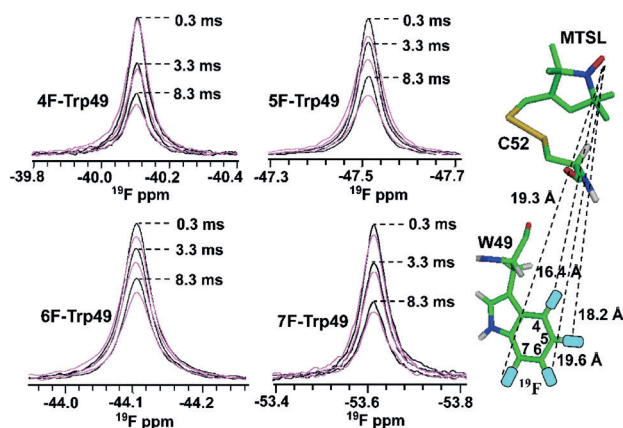
Figure 4. ¹⁹F PREs of 4F-, 5F-, 6F-, 7F-Trp49 containing MTSL-modified ^{S52C}CV-N. The ¹⁹F NMR resonances of oxidized (magenta) versus reduced (black) 4F-, 5F-, 6F-, and 7F-Trp49 ^{S52C}CV-N are superimposed for 0.3, 3.3, and 8.3 ms relaxation delays. PRE-derived distances are: 15.2 ± 0.9, 16.4 ± 1.2, 19.3 ± 0.9, and 18.6 ± 1.4 Å, with the equivalent distances from the structural model of MTSL-modified ^{S52C}CV-N 16.4, 18.2, 19.6, and 19.3 Å (shown by dashed lines on the model).
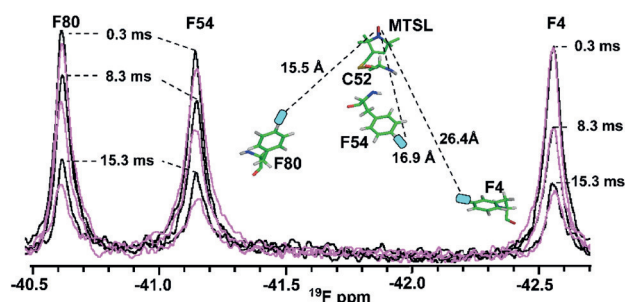
Figure 5. ¹⁹F PREs on 4-F Phe4, 4-F Phe54, and 4-F Phe80 for MTSL-modified ^{S52C}CV-N. The ¹⁹F NMR resonances of reduced (black) versus oxidized (magenta) 4-F Phe-containing MTSL-modified ^{S52C}CV-N are superimposed for 0.3, 8.3, and 15.3 ms relaxation delays. Resonance assignments for Phe4, Phe54, and Phe80 are indicated. The PRE-derived distances are: 23.9 ± 3.1, 16.1 ± 1.4, and 15.7 ± 0.8 Å with the equivalent distances from the structural model of MTSL-modified ^{S52C}CV-N 26.4, 16.9, and 15.5 Å (shown by dashed lines between the MTSL and the three Phe side chains in the model).

(magenta) and reduced (black) 1D ¹⁹F NMR spectra, recorded with different relaxation delays, is provided in Figure 5.

1D-¹⁹F Phe4, Phe54, and Phe80 assignments were obtained using ^{S52C}CV-N F80A and F54A individual mutants (Figure S4).

The three distances extracted from the experimentally measured ¹⁹F PREs between the paramagnetic center and the fluorine atoms at the 4-positions of Phe4, Phe54, and Phe80 are 23.9 ± 3.1, 16.1 ± 1.4, and 15.1 ± 0.9 Å, respectively, in excellent agreement with those measured from the MTSL ^{S52C}CV-N model, which are 26.4, 16.9, and 15.5 Å. Given that all three distances were measured on the same protein, the good agreement reflects the fact that fluorine PRE-based distances are of sufficient accuracy to be used as restraints in protein structure determinations.

In summary, we demonstrated that ¹⁹F-PRE-based distance measurements constitute an excellent alternative to traditional ^1H_N-PRE-based distance extraction. Using CV-N as a model system, we tested the methodology with MTSL-tagging at two positions in the protein and placement of fluorine at several positions, around the indole ring of Trp49, as well as in three Phe residues. In agreement with expectation, based on the gyromagnetic $\gamma^{19}F/\gamma^1H$ ratio, ¹⁹F-PRE-based distances can be measured over the 12–24 Å range, which corresponds to 0.93 of the ^1H_N-PRE distance range (13–25 Å). We suggest that the largest advantage of using ¹⁹F PRE-based distance measurements over proton PREs is the fact that spectral overlap does not impede their extraction and that any specific distances can be targeted by judicious placement of the MTSL label as well as the fluorine atom(s). This will be particularly beneficial for large proteins, protein complexes or membrane proteins.

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