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Enhancing solution structural analysis of large molecular proteins through optimal stereo array isotope labeling of aromatic amino acids

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ABSTRACT

The observation of side-chain peaks of aromatic amino acids is the prerequisite for a high-resolution three-dimensional structure determination of proteins by NMR. However, it becomes difficult with increasing molecular size due to an increased transverse relaxation and the control of the relaxation pathway is needed to achieve the observation. We demonstrated that even for the large molecular size of 82 kDa Malate synthase G (MSG), the aromatic ^{13}C-^{1}H (CH) peaks of Tryptophan (Trp) and Phenylalanine (Phe) residues can be observed with high quality using a systematic stable isotope labeling scheme, Stereo-Array Isotope Labeling (SAIL) method. However, the sequence specific assignments of these peaks relied on the use of amino acid substitutions, employing an inefficient method that required many isotopes labeled samples. In this study, we developed novel SAIL amino acids that allow for the observation of the aromatic ring δ,ζ and the aliphatic β position peak of Phe residues. The application of TROSY-based experiment to the isolated CH moieties resulted in the successful observation of discernible and resolved CH peaks in Phe residues in MSG. In MSG, the sequence-specific assignments of the backbone and C_β positions have already been confirmed. Therefore, using this labeling method, the δ and β position peaks of Phe residues can be clearly assigned in a sequence-specific and stereospecific manner through experiments based on intra-residue NOE. Furthermore, the NOESY experiment also allows for the acquisition of information pertaining to the conformation of Phe residues, such as the $\chi1$ dihedral angle, providing valuable insights for the determination of accurate protein structures and in dynamic analysis. This new SAIL amino acids open an avenue to achieve a variety of NMR analysis of large molecular proteins, including a high-resolution structure determination and dynamics and interaction analysis.

1. Introduction

Solution NMR is an exceptional technique capable of analyzing the structural dynamics intimately associated with the biological function of proteins, at atomic resolution. However, the application of NMR has been limited due to the fact that many functionally important proteins and protein-protein complexes exceed the molecular weight manageable by conventional NMR techniques. To overcome this "molecular weight barrier", Transverse relaxation optimized spectroscopy (TROSY), combined with protein perdeuteration and methyl protonation strategies, has been developed [1–3]. This advancement has enabled the observation of NMR signals of amide and methyl groups in high molecular weight proteins exceeding 100 kDa. However, observing NMR signals of aromatic residues remains a challenge, impeding the collection of crucial NOEs necessary for high-resolution protein structure determination.

A case in point is the NMR studies of malate synthase G (MSG), a single globular protein comprised of 723 amino acids with a molecular

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weight of 82 kDa [4,5]. MSG catalyzes the Claisen condensation of glyoxylate with an acetyl group of acetyl-CoA and thereby produce malate. Despite its large molecular weight, the methyl-protonation strategy proved effective in collecting NOE restraints involving methyl and amide atoms in MSG [6]. The global backbone fold of MSG, determined using these NOE restraints (PDB code: 1Y8B), closely resembled its crystal structure (PDB code: 1D8C). However, there remains difference in the detailed conformation between the NMR and crystal structures [5,6], likely due to the absence of side-chain restraints involving aromatic ring protons. Because the aromatic residues are main constitutes of the hydrophobic core structure, aromatic ring related NOEs are thought to be crucial for efficient and precise structural studies on large molecular proteins [7–10]. Additionally, aromatic amino acid residues, such as Phe and Tyr in proteins exhibit rotational motion around the Cα-Cβ bond axis and flipping around the Cβ-Cγ bond axis. Compared to other residues, aromatic amino acid residues have bulky side chain and often contribute to the formation of a hydrophobic core within proteins, as mentioned earlier. These motions are thought to be caused by Large Amplitude Slow Breathing Motions (LASBM) present in the protein [11–16].

To observe NMR signals of aromatic amino acid residues in large molecular weight systems, understanding the interactions causing the difficult situation. In conventional uniformly ^{13}C and ^{15}N labeled proteins, aromatic and aliphatic CH peaks become broadened due to rapid transverse relaxation and scalar couplings. Additionally, the narrow chemical shift dispersion of these groups complicates analysis due to spectral crowding. One well-recognized relaxation mechanisms for the aromatic CH moiety are the dipolar interaction between the linked proton and carbon atoms and chemical shift anisotropy (CSA) on the carbon. As to the aliphatic CH_2 groups, the geminal ^1H-^1H coupling will also cause line broadening. To mitigate these C-H and H-H interactions, CH TROSY method has been proposed [17,18]. In this method, the dipolar interaction and CSA are destructively interfered, such the aromatic/aliphatic carbon give rises to sharpened peak. Furthermore, the steady-state magnetization of carbon atom also contributes to the sensitivity enhancement.

Despite these advancements, observing aromatic CH and aliphatic CH_2 peaks in large molecular weight systems remains challenging due to complex spin system, strong ^{13}C-^{13}C coupling, dipolar and scalar interaction with adjacent CH moiety and dipolar interactions with remote protons. Systematic optimization of stable isotope labeling holds unparalleled potential in addressing these issues. For instance, interactions with adjacent-CH moieties can be eliminated by substituting adjacent carbon and proton atoms with ^{12}C and ^2H, respectively [10,19–23]. The stereo-array isotope labeling (SAIL) method is particularly effective in optimizing adjacent CH interactions [9,10,23–27]. In aliphatic CH_2 groups, replacing one ^1H with ^2H stereospecifically eliminates geminal ^1H-^1H scalar and dipolar coupling [24]. Regarding the dipolar interaction with remote protons, it can readily be mitigated by the deuteration of the background atoms.

In this study, we demonstrate, using the MSG protein as a model, that discernible and resolved aromatic/aliphatic CH peaks can be observed with high quality using the SAIL CH TROSY method. We focused on the aromatic ζ, δ and aliphatic β position peaks of Phenylalanine (Phe) and employed several types of SAIL Phe. The isotope labeling pattern of SAIL Phe ensures high quality observation of each CH peaks by maintaining adjacent carbon and proton as ^{12}C and ^2H, respectively. Applying CH TROSY experiments to MSG protein, selectively labeled with SAIL Phe on a deuterated background, resulted in well-resolved aromatic and aliphatic CH peaks of Phe. Moreover, this approach using SAIL Phe provides a novel method for amino acid sequence specific signal assignment of these CH peaks. Typically, sequence specific assignment of NMR signals in high molecular weight proteins involves mutational analysis, where individual amino acid residues are substituted with other amino acids. While this method is straightforward, it requires the preparation of numerous stable isotope-labeled samples. Furthermore, substitutions involving aromatic amino acid residues, which form the hydrophobic core structure of proteins, often lead to significant conformational changes or denaturation, making it challenging to unambiguously attribute the target NMR signals. Using the SAIL Phe approach in this study, we were able to clearly capture the intra-residue NOE between the amide, β2/β3, δ, and ζ protons through a simple NOESY experiment. Based on previous researches, for high molecular weight proteins (approximately 100 kDa), sequence-specific signal assignments of the protein backbone and C_β positions can be confirmed by utilizing TROSY based triple resonance NMR experiments [28]. Therefore, by utilizing these intra-residue NOEs, we were able to assign the NMR signals derived from the β2, β3, δ, and ζ positions of Phe residues in a sequence-specific and stereospecific manner. Simultaneously, since we were able to clearly assign the $H_{\beta2}$ and $H_{\beta3}$ signals, it was possible to determine the dihedral angle (i.e., χ1 angle) of the Phe residue side chains. This information plays a crucial role in the determination of solution structures of high molecular weight proteins. These results demonstrate the potential of stereo-specific isotope labeling techniques in NMR analysis of large molecular proteins, significantly contributing to the advancement of next-generation integrative structural biology research.

2. Materials and methods

2.1. Synthesis of new relaxation optimized sail phenylalanines

The L-[β2,ε1,ε2,ζ-$^2H_4$; C′,α,β,δ1,δ2-$^{13}C_5$;^{15}N]-Phe (RO-SAIL_$^{13}CH_{\delta,\beta3}$ Phe), L-[α, β3,ε1,ε2,ζ-$^2H_5$; C′,α,β,δ1,δ2-$^{13}C_5$;^{15}N]-Phe (RO-SAIL_$^{13}CH_{\delta,\beta2}$ Phe), L-[β2,δ1,δ2,ε1,ε2,ζ-$^2H_6$; C′,β-$^{13}C_2$;^{15}N]-Phe (RO-SAIL_$^{13}CH_{\beta3}$ Phe), L-[β3,δ1,δ2,ε1,ε2,ζ-$^2H_6$; C′,β-$^{13}C_2$;^{15}N]-Phe (RO-SAIL_$^{13}CH_{\beta2}$ Phe), L-[α,β3,ε1,ε2-$^2H_4$; β,δ1,δ2,ζ-$^{13}C_4$;^{15}N]-Phe (RO-SAIL_$^{13}CH_{\zeta,\delta,\beta2}$ Phe) and L-

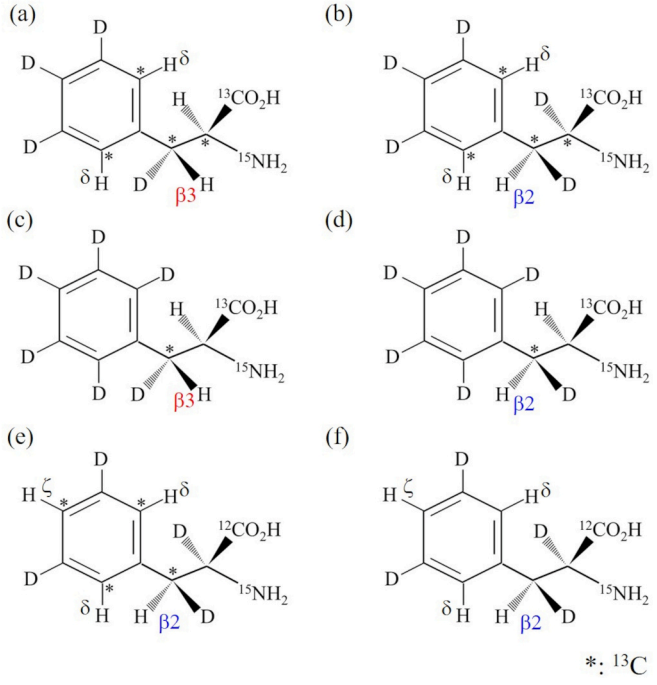

Fig. 1. Relaxation optimized SAIL phenylalanine. The structure of relaxation optimized SAIL phenylalanine. (a) The L-[β2,ε1,ε2,ζ-$^2H_4$; 0,α,β,δ1,δ2-$^{13}C_5$;^{15}N]-Phe (RO-SAIL_$^{13}CH_{\delta,\beta3}$ Phe), (b) L-[α, β3,ε1,ε2,ζ-$^2H_5$; 0,α,β,δ1,δ2-$^{13}C_5$;^{15}N]-Phe (RO-SAIL_$^{13}CH_{\delta,\beta2}$ Phe), (c) L-[β2,δ1,δ2,ε1,ε2,ζ-$^2H_6$; 0,β-$^{13}C_2$;^{15}N]-Phe (RO-SAIL_$^{13}CH_{\beta3}$ Phe), (d) L-[β3,δ1,δ2,ε1,ε2,ζ-$^2H_6$; 0,β-$^{13}C_2$;^{15}N]-Phe (RO-SAIL_$^{13}CH_{\beta2}$ Phe), (e) L-[α,β3,ε1,ε2-$^2H_4$; β,δ1,δ2,ζ-$^{13}C_4$;^{15}N]-Phe (RO-SAIL_$^{13}CH_{\zeta,\delta,\beta2}$ Phe), (f) L-[α,β3,ε1,ε2-$^2H_4$; ^{15}N]-Phe (RO-SAIL_$H_{\zeta,\delta,\beta2}$ Phe). The ^{13}C atoms are shown as asterisk (*). All RO-SAIL Phe were synthesized by SAIL Technologies. Inc.

[α,β3,ε1,ε2-$^{2}H_4$; ^{15}N]-Phe (RO-SAIL_$H_{\zeta,\delta,\beta2}$ Phe) (Fig. 1) were synthesized by SAIL Technologies. Inc., according to previously reported procedure using appropriate isotope sources [9,23,29]. For RO-SAIL_$^{13}CH_{\delta,\beta3}$ Phe (Fig. 1a) and RO-SAIL_$^{13}CH_{\delta,\beta2}$ Phe (Fig. 1b), glycine-^{13}C,^{15}N, diethyl malonate-1,3-$^{13}C_2$, and diethyl oxalate-$^{13}C_2$ were used as the ^{13}C and ^{15}N sources corresponding to the N, C′, α, β, and δ positions. For RO-SAIL_$^{13}CH_{\zeta,\delta,\beta2}$ Phe (Fig. 1e), glycine-^{15}N, diethyl malonate-1,3-$^{13}C_2$, diethyl oxalate-$^{13}C_2$, and acetone-2-^{13}C were used as the ^{13}C and ^{15}N sources corresponding to the β, δ, and ζ positions. For RO-SAIL_$H_{\zeta,\delta,\beta2}$ Phe (Fig. 1f), hydroxybenzoic acid was used, and after inducing H/D exchange at the ε position by heating in deuterium chloride, the hydroxyl group was deoxygenated to give benzaldehyde, which was then converted to compound Fig. 1f according to the method described in previous report [23].

For compounds RO-SAIL_$^{13}CH_{\beta3}$ Phe (Fig. 1c) and RO-SAIL_$^{13}CH_{\beta2}$ Phe (Fig. 1d), glycine-1-^{13}C, ^{15}N and benzaldehyde (ring-d5, carbonyl-^{13}C) were used as the ^{13}C and ^{15}N sources corresponding to the ^{13}C and ^{15}N atoms at the C′ and β positions according to the procedure [29], and by combining racemization and optical resolution at the α position, two isomers were synthesized.

2.2. Sample preparation

The MSG proteins were prepared following previously described procedures [28] with slight modification. Briefly, the *E.coli* strain BL21 (DE3) pLys S cells was transformed with pMSG-B, encoding the MSG protein fused with His-tag at its C-terminus [5]. The *E. coli* cells were cultured at 37 °C in 200 mL of D_2O/M9 medium (7.0 g/L Na_2HPO_4 (anhydrous), 3.0 g/L KH_2PO_4 (anhydrous), 0.50 g/L NaCl, 1.0 g/L [U-^{2}H] D-glucose, 1.0 g/L $^{15}NH_4Cl$, 20 mg/L thiamin hydrochloride, 20 mg/L (+)-Biotin, 1.6 mg/L $FeCl_3$ (anhydrous), 0.24 g/L $MgSO_4$ (anhydrous), 6.3 mg/L $MnCl_2$ (anhydrous), 11 mg/L $CaCl_2$ (anhydrous), 0.10 mg/L folic acid, 0.10 mg/L choline chloride, 0.1 mg/L nicotinamide, 0.1 mg/L D-pantothenic acid, 10 μg/L riboflavin, 0.1 mg/L pyridoxal hydrochloride, 0.2 mg/L *myo*-inositol). At that point, 0.6 mg isotope labeled Phe was added to this medium. When the OD_{600} reached around 0.3, 2.4 mg of isotope labeled Phe was added to the 200 mL D_2O/M9 growth medium and resume the cultivation. Then the OD_{600} reached around 0.4 (about 1–1.5 h later), isopropyl -β-D- thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the growth was continued for 8 h at 37 °C with shaking, and then the cells were collected by centrifugation.

The purification of the MSG protein was performed as described previously [28] with slight modification. Briefly, we additionally performed anion-exchange chromatography after Ni-NTA column purification. Finally, the protein was dialyzed against an NMR buffer containing 20 mM sodium phosphate (pH 7.1), 20 mM magnesium chloride ($MgCl_2$), 5 mM DL-1,4-DTT (d_{10}-DTT) and 1 mM sodium azide (NaN_3). Unless stated, the water in the NMR buffer was 99 % H_2O/1 % D_2O.

2.3. NMR spectroscopy

The NMR measurements were performed using Avance III 950, 900 and Avance III HD 800 spectrometer equipped with cryogenic probe (Bruker Biospin) at 37 °C, unless stated otherwise.

The concentrations of the MSG samples were 0.1–0.3 mM. We used the slot tube, which provides higher signal-to-noise ratio and efficient use of sample mass compared to conventional NMR sample tubes [30]. In 2D ^{1}H-^{13}C TROSY HSQC experiments on MSG proteins selectively labeled by RO-SAIL Phe, the spin-state-selective coherence transfer (S^3CT) was employed in the pulse sequence [31,32].

In 2D ^{1}H-^{13}C TROSY HSQC experiments for observing aromatic CH signals on (RO-SAIL_$^{13}CH_{\delta,\beta3}$ or_$^{13}CH_{\delta,\beta2}$ Phe; [U-^{2}H; ^{15}N])-labeled MSG, the data size and spectral width were 256 (t_1) × 2048 (t_2) and 1360 Hz (ω_1, ^{13}C) × 14,400 Hz (ω_2, ^{1}H), respectively. The carrier frequencies of ^{1}H and ^{13}C were 4.7 and 128 ppm, respectively. The number of scans/FID was 64. The repetition time was set to 4 s due to use of highly deuterated labeled sample. The experiment duration was 9.3 h. For observing aliphatic β CH signals of Phe residues on (RO-SAIL_$^{13}CH_{\beta3}$, or_$^{13}CH_{\beta2}$ Phe; [U-^{2}H; ^{15}N])-labeled MSG, the spectral width and the carrier frequencies were 2700 Hz (ω_1, ^{13}C) × 14,400 Hz (ω_2, ^{1}H), 4.7 ppm (^{1}H) and 37 ppm (^{13}C), respectively.

The 3D ^{13}C-edited NOESY experiment was performed for aromatic / aliphatic signal assignment of (RO-SAIL_$^{13}CH_{\delta,\beta3}$, or_$^{13}CH_{\delta,\beta2}$ Phe; [U-^{2}H, ^{15}N])-labeled MSG with a NOE mixing time of 100 ms. The data size and the spectral width were 128 (t_1) × 20 (t_2) × 2048 (t_3) points and 14,400 Hz (ω_1, ^{1}H) × 2700 Hz (ω_2, ^{13}C) × 14,400 Hz (ω_3, ^{1}H), respectively. The number of scans/FID was 32. The carrier frequencies of ^{1}H and ^{13}C were 4.7 and 37 ppm, respectively. The repetition time was set to 4 s due to use of highly deuterated labeled sample. The experiment duration was 91 h.

The 2D ^{1}H-^{1}H NOESY experiment was performed for aromatic / aliphatic signal assignment and inter residue NOE analysis of (RO-SAIL_$H_{\zeta,\delta,\beta2}$ Phe; [U-^{2}H, ^{15}N])-labeled MSG with a NOE mixing time of 200 ms. The data size and the spectral width were 512 (t_1) × 2048 (t_2) points and 9500 Hz (ω_1, ^{1}H) × 9500 Hz (ω_2, ^{1}H), respectively. The number of scans/FID was 16. The carrier frequency of ^{1}H was 4.7 ppm. The repetition time was 5 s and the experiment duration was 12 h.

All NMR spectra were processed by using the TopSpin software (Bruker Biospin). The pulse sequence of ^{1}H-^{13}C TROSY HSQC and acquisition parameters for each experiment were deposited in Zenodo (https://zenodo.org/doi/10.5281/zenodo.13283807).

3. Results and discussions

3.1. The observation of CH_δ and $CH_{\beta2/\beta3}$ peaks in Phe residues of large molecular proteins

First, we prepared novel RO-SAIL_$^{13}CH_{\delta,\beta3}$ Phe (Fig. 1a) labeled MSG on a deuterated background and measured 2D ^{13}C aromatic TROSY HSQC spectra. In our previous study, we had designed SAIL amino acids with relaxation optimization for the ζ-position of Phe residues [27]. Despite the presence of 19 Phe residues in MSG, we successfully observed all$^{13}C^{1}H_\zeta$ signals with high sensitivity and sharpness. In this current sample, we applied a similar relaxation optimization strategy to the aromatic ring CH_δ position of Phe residues. As a result, we achieved highly sensitive observation of $^{13}C^{1}H_\delta$ signals as anticipated (Fig. 2a). While we were able to observe$^{13}C^{1}H_\delta$ signals for 19 Phe residues, some signals exhibited reduced sensitivity when compared to others. Such variations in sensitivity were less pronounced for $^{13}C^{1}H_\zeta$ signals of Phe in MSG. In general, it is well-known that the aromatic ring of Phe residues undergoes flipping motion around the Cβ-Cγ bond axis. The sensitivity and linewidth of signals at the δ position may vary depending on this rotational motion, potentially explaining the observed variation in the $^{13}C^{1}H_\delta$ signals of each Phe residue in MSG. The ring flipping motion of aromatic amino acid residues in proteins is coupled with the dynamics of surrounding amino acid residues, and is thought to reflect the large amplitude breathing motions throughout the protein [11–16]. In the context of elucidating the function of MSG, the dynamics of Phe residues are of particular interest. To obtain more detailed information, our focus was on the observation of the aliphatic β-methylene signals of Phe residues.

In the conventional uniformly isotope labeling method, even in the case of small proteins, aliphatic ^{13}C-$^{1}H_2$ resonances exhibit low sensitivity and resolution due to the intricate relaxation processes involving inter ^{13}C-^{1}H and ^{1}H-^{1}H dipolar interactions, ^{13}C-^{13}C strong coupling and chemical shift anisotropies in ^{13}C and ^{1}H. To mitigate these nuclear relaxation effects, the "CH_2-TROSY" experiments [18] were proposed. However, sensitivity enhancement of CH_2 signals proved insufficient for large proteins. Furthermore, the problem of chemical shift degeneracy of methylene protons, which hampers precise stereo specific and sequence

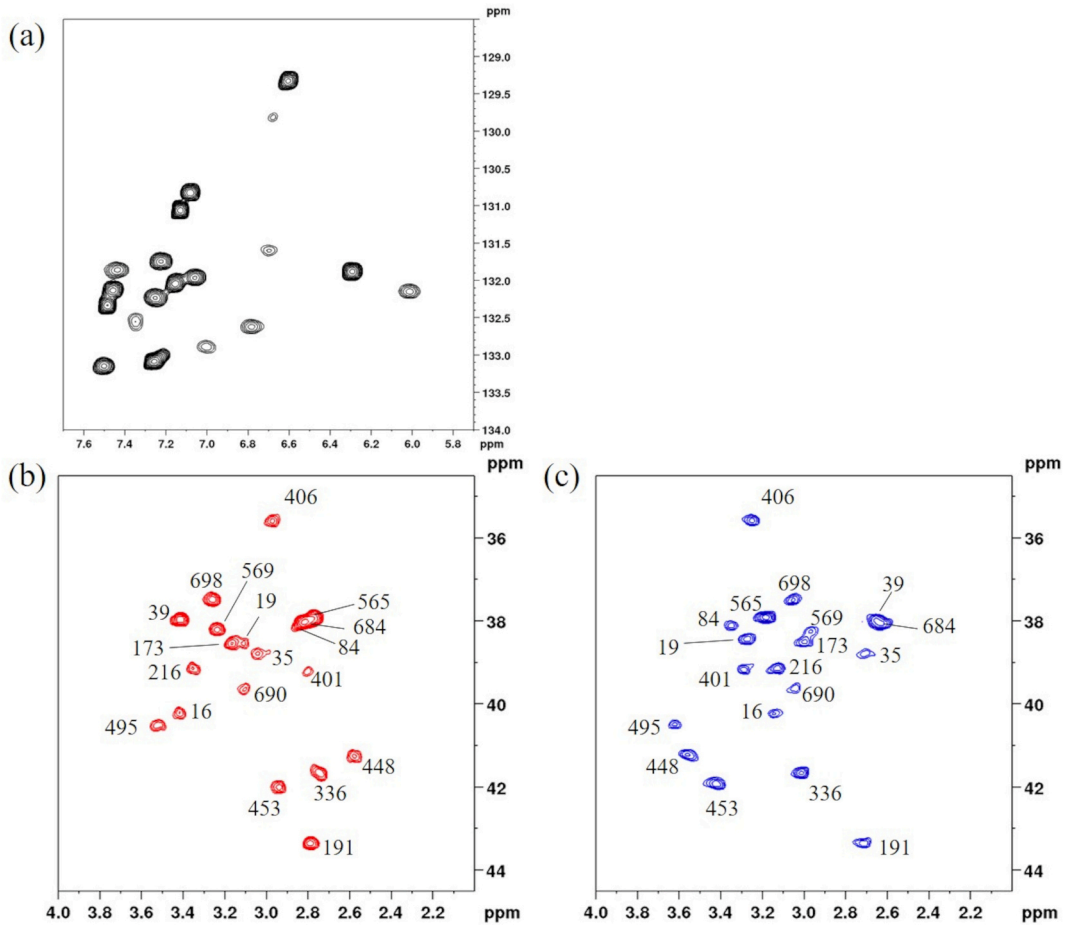

Fig. 2. 2D ^{13}C-^{1}H TROSY spectra of Phe residues in RO-SAIL Phe labeled MSG. The 900 MHz 2D ^{13}C-^{1}H TROSY spectrum (aromatic region) of the RO-SAIL_$^{13}CH_{\delta,\beta3}$ Phe; [U-^{2}H, ^{15}N]-labeled MSG (a) and 2D ^{13}C-^{1}H TROSY spectrum (aliphatic region) of the RO-SAIL_$^{13}CH_{\beta3}$, or_$^{13}CH_{\beta2}$ Phe; [U-^{2}H; ^{15}N])-labeled MSG (b,c). These sample are solved in 20 mM sodium phosphate, 20 mM $MgCl_2$, 5 mM d_{10}-DTT and 5 % D_2O at pH 7.1.

specific signal assignments, remains unsolved with this approach.

In the aim of achieving highly sensitive observation or the NMR signals of β-methylene group of Phe in large molecular proteins, we designed RO-SAIL_$^{13}CH_{\beta3}$ Phe and RO-SAIL_$^{13}CH_{\beta2}$ Phe (Fig. 1c,d). In these RO-SAIL Phe, ^{13}C-^{13}C coupling effect for β position was completely eliminated. Furthermore, one of the two methylene protons (i.e. $H_{\beta2}$ or $H_{\beta3}$) is selectively deuterated. To suppress the effect of ^{1}H-^{1}H dipole interactions, all protons in the aromatic ring are also deuterated. As to the α proton of phenylalanine, it is substituted with deuterium through *E.coli* cultivation in a deuterated M9 medium, enabling substantial reduction of ^{1}H-^{1}H dipole interactions for the β protons of Phe when incorporated into the target proteins. In practice, we successfully observed high-resolution and high-sensitive $^{13}CH_{\beta2/3}D_{\beta3/2}$ signals of Phe from perdeuterated MSG specifically labeled with RO-SAIL_$^{13}CH_{\beta3}$ Phe or _$^{13}CH_{\beta2}$ Phe (Fig. 2b,c). The amino acid sequence-specific assignment of Phe $^{13}CH_{\beta2/3}D_{\beta3/2}$ signals can be achieved by utilizing the $^{13}C_{\beta}$ chemical shift data obtained from TROSY-based triple resonance experiments using uniformly ^{2}H, ^{13}C, ^{15}N-labeled MSG (BMRB Entry 5471) [28]. While some signals in each spectrum were overlapping, we successfully assigned the NMR signal of the β-methylene for all Phe residues in MSG.

3.2. The assignment of aromatic $^{13}CH_{\delta}$ signals of SAIL Phe residues in MSG

Following the assignment of the NMR signals at the β methylene of Phe in MSG, we proceeded with the assignment of aromatic ring CH_{δ} signals. In the NMR analysis of large molecular proteins, the assignment of signals originating from side chains of amino acid residues, such as methyl groups and aromatic rings, predominantly employs techniques that utilize amino acid substitution [33]. While this method facilitates signal assignment, it necessitates the preparation of numerous amino acid variants and takes a considerable amount of time for NMR measurements. Additionally, introducing mutations into aromatic amino acid residues often leads to the collapse of the tertiary structure of proteins. In such cases, optimizing amino acid mutations would be necessary, requiring even more experiments. Therefore, we propose the use of NOESY experiments employing new SAIL aromatic amino acids as an alternative method. We performed ^{13}C edited 3D NOESY-HSQC experiment on deuterated MSG labeled with RO-SAIL_$^{13}CH_{\delta,\beta3}$ Phe or RO-SAIL_$^{13}CH_{\delta,\beta2}$ Phe. In this experiment, we could clearly observe intra-residue NOE signals among amide proton (H_N), $H_{\beta2/3}$ and H_{δ} within approximately 12 h of measurement time (Fig. 3). Leveraging previous studies [28] and our SAIL method referenced in the prior section, we have confirmed the assignment of the H_N and $H_{\beta2/3}$ signals of Phe within MSG. By analyzing the intra-residue NOE signals, we can straightforwardly determine the sequence-specific assignment of the aromatic H_{δ} signals of Phe within MSG. In practice, for all 19 Phe residues within MSG, it was possible to observe intra-residue NOEs from $H_{\beta2}$ and $H_{\beta3}$ to H_{δ}, allowing for the unambiguous determination of sequence-specific assignments (Table S1; BMRB Entry 26,349). Although this method involves the use of expensive SAIL amino acids, it offers an advantage over the signal assignment method using amino acid variants, which also requires costly deuterated reagents and stable isotope-labeled amino acids. Hence, we propose this method as an alternative signal assignment technique that can save experimental time. Notably,

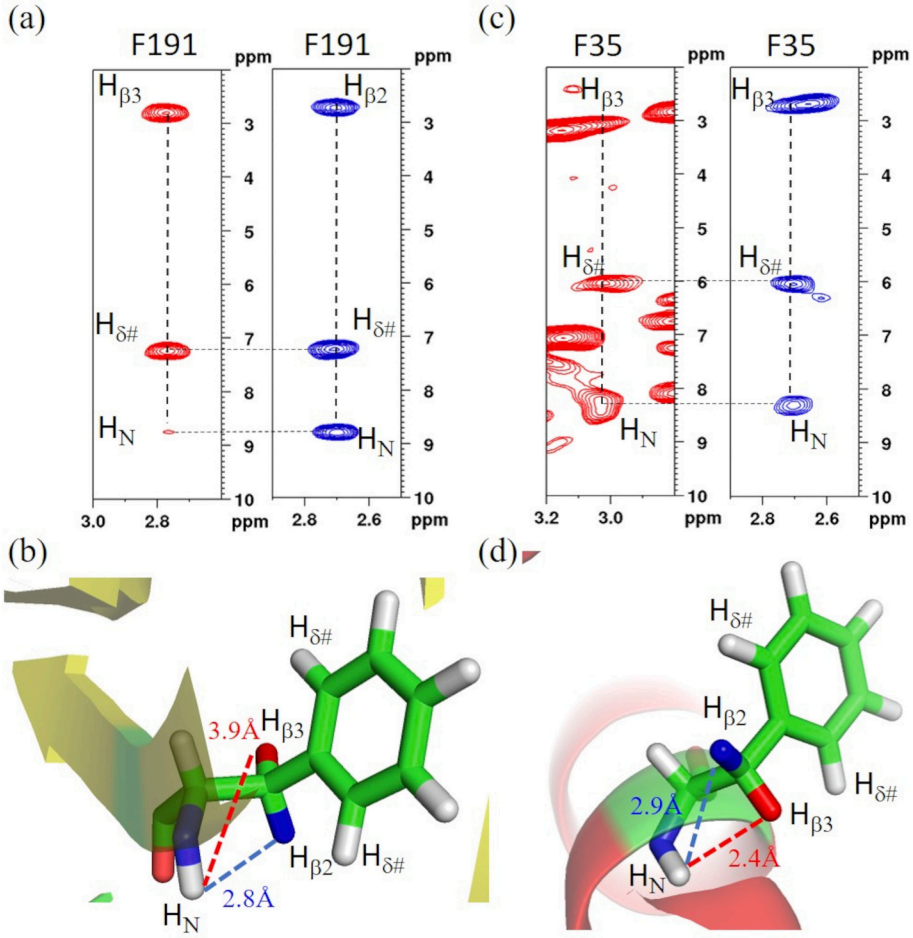

Fig. 3. Sequence specific signal assignment for RO-SAIL Phe residues in MSG. Unambiguous signal assignment for Phe residues in MSG using inter-residue NOEs among NH, β CH and δ CH group. Additionally, by comparing the NOE intensities between $H_{\beta2/\beta3}$ and H_N, the aromatic ring side chain dihedral angle ($\chi1$) can be determined. (a) NOESY spectrum showing $H_{\beta3}$ (red) and $H_{\beta2}$ (blue) of F191. The NOE signal between $H_{\beta2}$ and H_N is observed with high sensitivity, whereas the NOE signal between $H_{\beta3}$ and H_N is weak. This indicates that the H_N of F191 is closer to $H_{\beta2}$ than to $H_{\beta3}$, suggesting that the dihedral angle $\chi1$ is around −60°. (b) Examination of the crystal structure of MSG (PDB code: 1D8C) shows that the distance between H_N and $H_{\beta2}$ of F191 is 2.8 Å, while the distance between H_N and $H_{\beta3}$ is 3.9 Å. Additionally, $\chi1$ is −64°, which is consistent with the results obtained from the NOESY spectrum. (c) NOESY spectrum showing $H_{\beta3}$ (red) and $H_{\beta2}$ (blue) of F35. The intensities of the NOE signals between $H_{\beta3}$-H_N and $H_{\beta2}$-H_N are almost the same, suggesting that $\chi1$ is around 180°. (d) In the crystal structure, the distance between $H_{\beta3}$ and H_N of F35 is 2.9 Å, which is almost the same as the distance between $H_{\beta2}$ and H_N (2.4 Å). In this case, $\chi1$ is 166°, which is consistent with the NMR spectrum results. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in this experiment, clear intra-residue NOE between $H_{\beta2/3}$ and H_N was also detected (Fig. 3). This observation allows us to capture the side chain dihedral angles (i.e. $\chi1$) of Phe.

In general, it is known that the dihedral angle $\chi1$ (NH – Cα – Cβ - Cγ) of each amino acid residues are distributed at either 60°, 180°, or −60°. Since the value of $\chi1$ can define the conformation of the amino acid side chain, determining $\chi1$ for bulky aromatic amino acid residues can provide useful information for the determination of protein tertiary structure. Normally, the determination of $\chi1$ requires several triple resonance NMR experiments (i.e., HNHB and HN(CO)HB experiments), but it is difficult to obtain sufficient signal sensitivity for low-concentration proteins or large molecular proteins. However, using our new RO-SAIL method, $\chi1$ can be determined by performing a simple and highly sensitive NOESY-TROSY experiment. For example, examining the NOESY spectrum from $H_{\beta2}$ and $H_{\beta3}$ of the Phe191 (F191) in MSG, a strong NOE signal is observed between $H_{\beta2}$ and H_N, while the NOE signal between $H_{\beta3}$ and H_N is very weak (Fig. 3a). This indicates that the H_N of F191 is spatially closer to $H_{\beta2}$ than to $H_{\beta3}$, corresponding to a conformation with $\chi1$ at −60°. On the other hand, for the Phe35 (F35), the NOE between $H_{\beta2}$ and H_N and the NOE between $H_{\beta3}$ and H_N showed similar signal intensities. In other words, the H_N of F35 is at approximately the same distance from its own $H_{\beta2}$ and $H_{\beta3}$, corresponding to a conformation with $\chi1$ at 180°. Calculating $\chi1$ for F191 and F35 in the crystal structure of MSG (PDB code: 1D8C) yields −64° and 166°, respectively [5], which is in good agreement with this NOESY experiment (Table S2). These results demonstrate that the NOESY experiment using SAIL-Phe not only allows for the sequence-specific assignment of NMR signals at the aromatic δ position and aliphatic β position but also provides structural information for Phe in MSG.

3.3. Further improvement of the SAIL Phe

Our previous studies have demonstrated that by applying stable isotope labeling considering the nuclear relaxation processes for each amino acid residue, it is possible to observe ^{13}CH signals originating from aromatic and aliphatic groups, such as Phe and Trp, with high sensitivity even in 82 kDa MSG. Additionally, we proposed new method for the sequence specific assignment of these signals. Building upon our achievements, we have newly designed a stable isotope labeling pattern that allows for more efficient observation of NMR signals from Phe in large molecular proteins (Fig. 1e,f).

In the RO-SAIL_$^{13}CH_{\zeta,\delta,\beta2}$ Phe shown in Fig. 1e, we have designed a

stable isotope labelling pattern optimized for nuclear relaxation processes to simultaneously observe the ^{13}CH signals at the β,δ and ζ positions. Furthermore, this amino acid enables the observation of NOE between H_δ and H_ζ, allowing for the signal assignment of ζ position without relying on amino acid substitutions. Indeed, using this RO-SAIL_$^{13}CH_{\zeta,\delta,\beta2}$ Phe, we were able to observe each^{13}CH signal with high sensitivity as expected, and the signal assignment using $H_{\beta2/\beta3}$-H_δ NOE was also confirmed (Fig. 4, Fig. S1). On the other hand, while NOE signals between H_δ and H_ζ were observed, the chemical shifts of these protons tend to degenerate, often resulting in serious overlap between the H_δ-H_ζ NOE peak and diagonal peak in the NOESY-TROSY experiment (Fig. S1). Therefore, it was challenging to obtain sufficient resolution for unambiguous signal assignment. This problem is expected to be more serious during NMR measurement for much larger molecular proteins or liquid-liquid phase separation system which are directly related to biological phenomena. In light of these considerations, we have devised SAIL amino acids that ultimately eliminate nuclear relaxation mechanisms. Until now, we have focused on observing highly sensitive and sharpened ^{13}C-^{1}H correlation signals by eliminating surrounding relaxation sources and utilizing the TROSY effect for specific CH groups of each amino acid residue. In this case, in terms of ^{1}H observation, the signal is broadened due the influence of nuclear relaxation from the directly bonded ^{13}C (i.e. the dipolar coupling between ^{1}H and ^{13}C). By eliminating nuclear relaxation from ^{13}C and limiting the observed nucleus to a specific proton, it is expected to ultimately observe a sharpened ^{1}H signal [34,36]. We have already synthesized a phenylalanine with ^{1}H labeled only at ζ position and introduced it into perdeuterated MSG. As a result, we have successfully observed the $^1H_\zeta$ signal originating from Phe in MSG as a very sharp signal with a linewidth of about 10 Hz [34]. Consequently, we synthesized a new RO-SAIL amino acid, RO-SAIL_$H_{\zeta,\delta,\beta2}$ Phe, in which only the ζ,δ and β positions of Phe are labeled with ^{1}H, while the other atoms are labeled with ^{2}H and ^{12}C (Fig. 1f). Upon measuring the ^{1}H 1D spectrum of perdeuterated MSG labeled with RO-SAIL_$H_{\zeta,\delta,\beta2}$ Phe under deuterated buffer, highly sharpened $^1H_\delta$ and $^1H_\zeta$ signals of Phe in MSG were observed (Fig. 4b). The linewidth of each ^{1}H signal was approximately 10 Hz, therefore, part of signals that were overlapped in 2D CH TROSY spectrum obtained with RO-SAIL_$^{13}CH_{\zeta,\delta,\beta2}$ Phe were clearly separated in this ^{1}H 1D.

By measuring a simple ^{1}H-^{1}H 2D NOESY spectrum of this sample, we also successfully obtained the NOE signals among H_ζ, H_δ, and $H_{\beta2}$ with high sensitivity. Consequently, it allows us to unambiguous signal assignment for each aromatic/aliphatic signal of Phe even in 82 kDa MSG (Fig. 5, Table S1; BMRB Entry 26,349).

3.4. *The study of dynamic property of aromatic amino acid residues in large molecular proteins by RO-SAIL NMR methods*

As we mentioned above, RO-SAIL method allows for the specific observation of highly sensitive aromatic and aliphatic CH resonances and their related NOE signals, even in 82 kDa MSG. Such a specific signal observation can also be partially achieved using isotope labeling techniques with amino acid precursors [35,36]. All SAIL amino acids introduced here can be obtained from SAIL Technologies (https://www.sail-technologies.co.jp/en/index.html) at approximately €1–6/mg. Although SAIL amino acids are more expensive compared to amino acid precursors, the labeling rates of each site are highly controlled in SAIL amino acids. In addition to the SAIL amino acids introduced in this paper, a variety of amino acids with different labeling patterns are available, making them more practical for structural and dynamics analysis of proteins.

Our new method allows for highly sensitive observation of NMR signals from aromatic amino acid residues in large molecular proteins, and it is expected to be useful for analyzing their dynamics. In the 2D CH TROSY HSQC spectrum, CH_δ signal of F19 and F401 are specifically broadened, even for CH_ζ signal of it shows highly sensitive signal in same spectrum (Fig. 4). Additionally, intra residue NOEs between $H_{\beta2}$ and H_δ was observed to be split into two peaks, and the linewidth was relatively broadened than that of other Phe residues (Fig. 5). From these

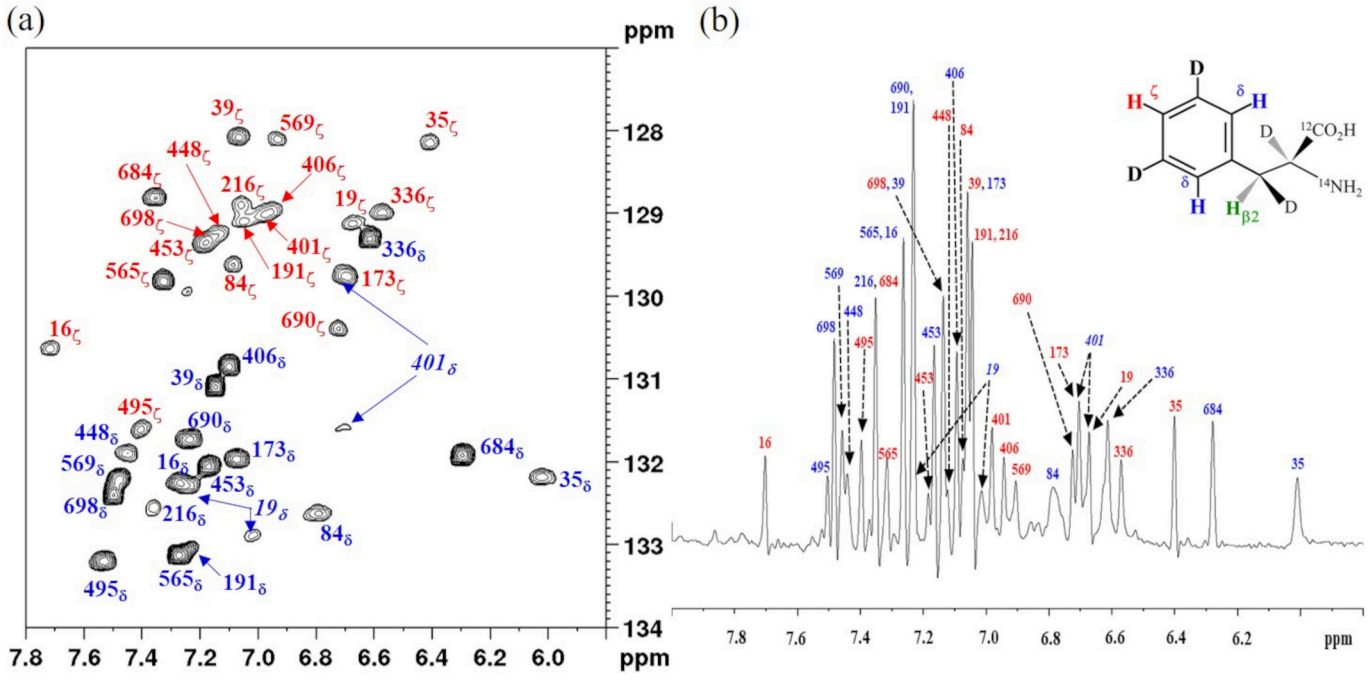

Fig. 4. A highly efficient and sensitive NMR analysis using novel RO-SAIL Phe. (a) The 900 MHz 2D ^{13}C-^{1}H TROSY spectrum (aromatic region) of the RO-SAIL_$^{13}CH_{\zeta,\delta,\beta2}$ Phe; [U-^{2}H, ^{15}N]-labeled MSG. By utilizing RO-SAIL_$^{13}CH_{\zeta,\delta,\beta2}$ Phe, it is possible to simultaneously observe the CH signals at the δ and ζ positions of the aromatic ring. Additionally, NOE analysis from the δ position allows for the sequence-specific assignment of signals at the ζ position (Fig. S1). However, there are cases where the signals at the δ and ζ positions overlap, making unambiguous signal assignment difficult. (b) The 950 MHz 1D ^{1}H spectrum (aromatic region) of the RO-SAIL_$H_{\zeta,\delta,\beta2}$ Phe; [U-^{2}H, ^{15}N]-labeled MSG. In this case, the ^{1}H signals derived from the aromatic ring are completely free from nuclear relaxation processes from surrounding ^{13}C and ^{1}H. As a result, it is possible to observe highly sensitive and sharpened ^{1}H signals even in high molecular weight proteins.

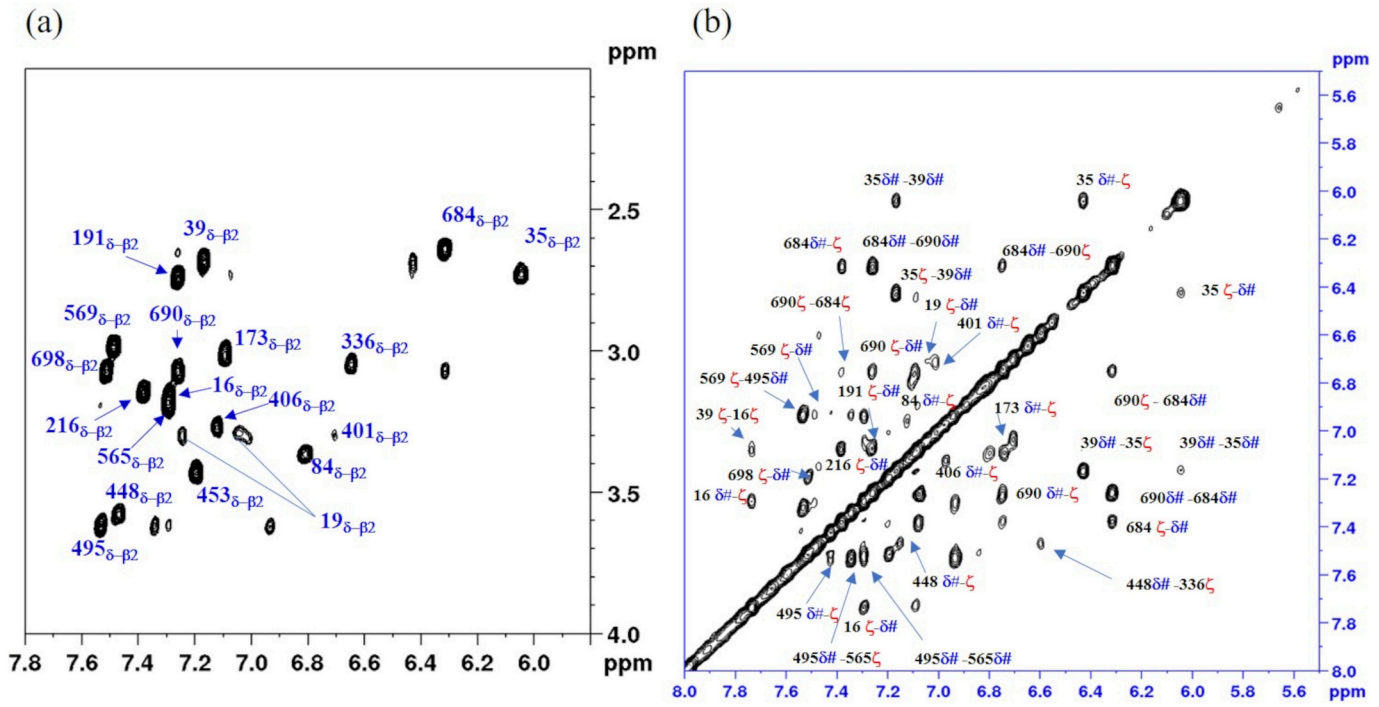

Fig. 5. Assignment of NMR signals of Phe residues in MSG using the novel RO-SAIL method. The 950 MHz 2D ^{1}H-^{1}H NOESY spectrum of the RO-SAIL_$H_{\zeta,\delta,\beta2}$ Phe; [U-^{2}H, ^{15}N]-labeled MSG. (a) aromatic (H_δ, H_ζ) – aliphatic ($H_{\beta2}$) and (b) aromatic-aromatic region of 2D NOESY spectrum. Each NMR signal is observed with high sensitivity and sharpness, allowing for efficient assignment and structural information determination using NOE signals among H_δ, H_ζ and $H_{\beta2}$.

results, it is thought that a reduction in the aromatic ring flip motion has occurred in MSG. Interestingly, these dynamic property of aromatic amino acid residues in large molecular proteins cannot be observed clearly using conventional NMR analysis and crystal structure analysis. We already reported the quantitative analysis of aromatic ring flip motion in FKBP12 by original SAIL NMR method [12]. To achieve a detailed understanding of the correlation between protein dynamics and their biological functions, we are advancing the application and optimization of the RO-SAIL method. Specifically, we are investigating the analysis of changes in protein activation volumes through the combination of high-pressure NMR systems [16], as well as real-time monitoring of protein dynamics within living cells using in-cell NMR techniques. Recently, the analysis of aromatic ring flip motions in large proteins and protein crystals has been reported by combining labeling techniques using SAIL and amino acid precursors for aromatic amino acids with fast MAS solid-state NMR measurements [13,37]. In the near future, these methods are expected to be applied in conjunction with DNP techniques for the dynamic analysis of membrane proteins and proteins in phase-separated states [38].

4. Conclusions

In this study, we experimentally demonstrated for 82 kDa of MSG that its aromatic and aliphatic CH signals become observable by systematic site-specific isotope labeling scheme. The RO-SAIL method opens up applicability of NMR for a high-resolution structure determination and characterizations of aromatic-relevant dynamics, such as the aromatic ring flipping motion of phenylalanine and tyrosine residues, in large proteins.

The successful observation of the aromatic and aliphatic CH peaks in this study raises the possibility that other type of side-chain atoms become observable by controlling the relaxation pathways with site-specific isotope labeling and, if needed, the TROSY principle. Capturing the structural dynamics of proteins at atomic resolution, and quantitatively, is challenging with techniques such as X-ray crystallography and cryo-electron microscopy analysis, and is even beyond the reach of three-dimensional structure predictions, including those for Alphafold. In the next generation of structural biology, the development of versatile structural dynamics analysis methods utilizing NMR is highly anticipated.

CRediT authorship contribution statement

Yohei Miyanoiri: Writing – original draft, Visualization, Project administration, Investigation, Funding acquisition, Conceptualization. **Mitsuhiro Takeda:** Writing – review & editing, Resources, Investigation, Funding acquisition. **Kosuke Okuma:** Visualization, Resources. **Tsutomu Terauchi:** Writing – review & editing, Visualization, Resources. **Masatsune Kainosho:** Writing – review & editing, Supervision, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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