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Threonine Side Chain Conformational Population Distribution of a Type I Antifreeze Protein in Interacting with Ice Surface Studied via ^{13}C - ^{15}N Dynamic REDOR NMR

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Abstract

Antifreeze proteins (AFPs) provide survival mechanism for species living in subzero environments through lowering the freezing points of their body fluids effectively. The mechanism is attributed to AFP's ability to inhibit the growth of seed ice crystals through adsorption on specific ice surfaces. We have applied dynamic REDOR (Rotational Echo Double Resonance) solid state NMR to study the threonine (Thr) side chain conformational population distribution of a site-specific Thr $^{13}\text{C}_\gamma$ and ^{15}N doubly labeled type I AFP in frozen aqueous solution. It is known that the Thr side chains together with those of the 4th and 8th Alanine (Ala) residues commencing from the Thr (the 1st) in the four 11-residue repeat units form the peptide ice-binding surface. The conformational information can provide structural insight with regard to how the AFP side chains structurally interact with the ice surface. χ -squared statistical analysis of the experimental REDOR data in fitting the theoretically calculated dynamic REDOR fraction curves indicates that when the AFP interacted with the ice surface in the frozen AFP solution, the conformations of the Thr side chains changed from the anti conformations as in the AFP crystal structure to partial population in the anti conformation and partial population in the two gauche conformations. This change together with the structural analysis indicates that the simultaneous interactions of the methyl groups and the hydroxyl groups of the Thr side chains with the ice surface could be the reason for the conformational population change. The analysis of the theoretical dynamic REDOR fraction curves shows that the set of experimental REDOR data may fit a number of theoretical curves with different population distributions. Thus, other structural information is needed to assist in determining the conformational population distribution of the Thr side chains.

Keywords

Antifreeze Protein; dynamic REDOR NMR; Threonine Side Chain Conformations; Interaction with ice surface; χ -squared statistical analysis

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Introduction

Antifreeze proteins (AFPs) were found existing in the body serums of fish, insects and plants living in subzero environments due to their ability to depress the freezing point of water.[1–13] The mechanism of action for the freezing point depression is attributed to the growth inhibition of seed ice crystals arising from AFP's ability to adsorb on specific ice surfaces.[14–16] The same mechanism is also responsible for the inhibition of recrystallization of ice crystals where bigger ice crystals grow with the sacrifice of smaller ones.[17] The HPLC6 isoform of type I AFP has an α -helical secondary structure and contains four 11-residue repeat units commencing with Thr residues.[18] This AFP has the following sequence: Asp-Thr-Ala-Ser-Asp-Ala-Ala-Ala-Ala-Ala-Leu-Thr-Ala-Ala-Asn-Ala-Lys-Ala-Ala-Ala-Glu-Leu-Thr-Ala-Ala-Asn-Ala-Ala-Ala-Ala-Ala-Thr-Ala-Arg. Previous studies of side chain mutations by Baardsnes et al suggested that the AFP ice binding side chains comprise those of the Thr residues and the conserved 4th and 8th Ala residues starting from the Thr (the 1st residues the repeat units).[19] Afterwards, ¹³C spin lattice relaxation NMR of site-specific ¹³C labeled AFPs provided direct structural information to verify the AFP's ice binding side chains.[20, 21] In the relaxation NMR experiments, methyl group ¹³C labeled type I AFP at the Ala₁₇ and Ala₂₁ side chains,[20] that at the Ala₈, Ala₁₉ and Ala₃₀ side chains[20] and that at the Thr₁₃ and Thr₂₄ side chains[21] were individually used. The dynamics of the methyl group's C-C chemical bond rotations and that of the molecular reorientation of water in contacting the methyl groups in the ice surface directly revealed which surface of the α -helical structured peptide interacted with the ice surface. The use of the site-specific ¹³C labeled AFPs are due to two reasons: Firstly, the line shapes and the chemical shift of the methyl ¹³C peaks belonging to different residues did not give any resolution to identify the different methyl groups. This was especially worse for the frozen AFP solution. Secondly, the low natural abundance of ¹³C (1.108 %) was not feasible to give good enough signal to noise ratio within a reasonable NMR experimental time. Thus, site-specific isotope labeled AFP is essential for this study. To gain more detailed structural information with regard to how the Thr side chains interact with the ice surface, we have carried out dynamic ¹³C-¹⁵N REDOR (Rotational Echo Double Resonance) NMR [22, 23] on a site-specific Thr ¹³C _{γ} and amide ¹⁵N doubly labeled type I AFP. Ideally, dynamic REDOR NMR is able to provide the information of conformational population distribution due to the variation of the internuclear distance of weakly coupled spin pair ¹³C-¹⁵N. The population distribution of the Thr side chains can in turn provide certain insight into how the Thr side chains structurally interact with the ice surface. We hope that this study together with our previous relaxation NMR results will provide useful information for theoreticians to model the nature of the interaction of the AFP with the ice surface.

Theory

To calculate the theoretical REDOR curves in corresponding to the dynamic average of the dipolar couplings due to the conformational population distribution, in the following we will briefly review the dynamic REDOR theory and then derive the dynamic REDOR formulae for our specific case. The dipolar coupling angular frequency (rad s⁻¹) of an unlike spin pair of I and S with half integer magnetic quantum numbers under magic angle spinning (MAS) is given as[22]

$$\omega_D(\alpha, \beta, \tau) = \pm \frac{1}{2} D (\sin^2 \beta \cos 2(\alpha + \omega_r \tau) - \sqrt{2} \sin 2\beta \cos(\alpha + \omega_r \tau)) \quad (1)$$

where α and β are the azimuthal angle and the polar angle, respectively, as defined by the internuclear vector with respect to the MAS axis, ω_r is the angular frequency of MAS, τ is the evolution time, $D = (\gamma_I \gamma_S h / 2\pi r^3)(\mu_0/4\pi)$ being the dipolar coupling constant (rad s^{-1}) in which γ_I and γ_S denote the gyromagnetic ratios of I spin and S spin, respectively, h is the Plank's constant, μ_0 is the permeability constant and r is the internuclear distance. The average dipolar coupling frequency (s^{-1}) under the REDOR pulse sequence and MAS for a full rotor period is given as

$$\bar{\nu}_D(\alpha, \beta) = \pm \frac{\sqrt{2}}{\pi} D \sin 2\beta \sin \alpha \quad (2)$$

Equation (2) is applicable to the REDOR pulse sequence where all the π pulses are placed in the half and full rotor period. The three conformers of a Thr side chain are shown in Figure 1(a) to (c), and (d) to (e) as well. The internuclear distance of the anti conformer is different from those of the gauche conformers. Thus, the dynamically averaged dipolar coupling frequency is the population weighted average of them as below:

$$\bar{\nu}_D(\alpha_1, \beta_1, \alpha_2, \beta_2, \alpha_3, \beta_3) = \pm \frac{\sqrt{2}}{\pi} \sum_{i=1}^3 P_i D_{SI_i} \sin 2\beta_i \sin \alpha_i \quad (3)$$

where the index i labels the three conformations and P_i denotes the corresponding population (where $P_1 + P_2 + P_3 = 1$). This Equation is valid when the correlation time of the C_α - C_β chemical bond rotation is much shorter than the inverse dipolar coupling frequency. The I spin (here ^{13}C) observable I_{xobs} along the x axis in the rotating frame of reference due to the dipolar dephasing by the S spin (here ^{15}N) is given as:

$$\begin{aligned} I_{\text{xobs}} &\propto \cos(\bar{\nu}_D(\alpha_1, \beta_1, \alpha_2, \beta_2, \alpha_3, \beta_3) N_r T_r) \\ &\propto \cos(N_r T_r \frac{\sqrt{2}}{\pi} \sum_{i=1}^3 P_i D_{SI_i} \sin 2\beta_i \sin \alpha_i) \end{aligned} \quad (4)$$

where N_r and T_r denote the number of rotor periods and the length of one rotor period, respectively. The dipolar dephasing time τ at the end of N_r rotor cycles is $\tau = T_r N_r$. For a solid powder, the integrated signal intensity over the entire spatial orientations after normalization is:

$$S(\tau)/S_0 = \frac{1}{8\pi^2} \int_{\psi=0}^{2\pi} \int_{\theta=0}^{\pi} \int_{\varphi=0}^{2\pi} \cos(N_r T_r \frac{\sqrt{2}}{\pi} \sum_{i=1}^3 P_i D_{SI_i} \sin 2\beta_i \sin \alpha_i) d\varphi \sin \theta d\theta d\psi \quad (5)$$

where φ , θ , and ψ denote the three Euler angles to specify the orientations of the crystals. In order to calculate the integral, α_i and β_i for $i=1, 2$ and 3 need to be written as the functions of the three Euler angles. Goetz and Schaefer have defined a unit vector to generalize the calculation.[23] The unit vector is defined as from the I spin to the S_i spin as

$$\hat{n}(i) = \vec{r}_{IS}^i / |\vec{r}_{IS}^i| = (x(i)\hat{i} + y(i)\hat{j} + z(i)\hat{k}) \quad (6)$$

Rotation of this unit vector by (φ, θ, ψ) gives a new unit vector $\hat{n}'(i)$ which is related to the original components of the unit vector by

$$\hat{n}'(i) \cdot \hat{y} = [-\cos\psi \sin\varphi - \cos\theta \cos\varphi \sin\psi]x(i) + [\cos\psi \cos\varphi - \cos\theta \sin\varphi \sin\psi]y(i) + [\sin\psi \sin\theta]z(i) \quad (7)$$

$$\hat{n}'(i) \cdot \hat{z} = [\sin\theta \cos\varphi]x(i) + [\sin\theta \sin\varphi]y(i) + [\cos\theta]z(i) \quad (8)$$

After considering

$$\hat{n}'(i) \cdot \hat{y} = \sin\beta_i \sin\alpha_i \quad (9)$$

and

$$\hat{n}'(i) \cdot \hat{z} = \cos\beta_i \quad (10)$$

the dynamically averaged dipolar coupling frequency for a full rotor period can be written as

$$\bar{\nu}_D(\alpha_1, \beta_1, \alpha_2, \beta_2, \alpha_3, \beta_3) = \pm \frac{2\sqrt{2}}{\pi} \sum_{i=1}^3 P_i D_{S_{li}} [\hat{n}'(i) \cdot \hat{z}] [\hat{n}'(i) \cdot \hat{y}] \quad (11)$$

Thus, the normalized REDOR dephasing intensity of a solid powder is obtained as:

$$S(\tau)/S_0 = \frac{1}{8\pi^2} \int_{\psi=0}^{2\pi} \int_{\theta=0}^{\pi} \int_{\varphi=0}^{2\pi} \cos \left(\pm \frac{2\sqrt{2}}{\pi} N_r T \sum_{i=1}^3 P_i D_{S_{li}} [\hat{n}'(i) \cdot \hat{z}] [\hat{n}'(i) \cdot \hat{y}] \right) d\varphi \sin\theta d\theta d\psi \quad (12)$$

The unit vector needs to be found before using Equation 12 to calculate the REDOR intensities. In the following, we will derive the unit vectors of the Thr side chain system

The diagram in Figure 2 schematically describes the Thr side chain's rotation around the C_α - C_β chemical bond for the convenient derivation of the unit vectors. The nitrogen atom (N) is positioned at the origin, the N- C_α chemical bond is along the z axis and the N, C_α and C_β are placed in the y-z plane. θ' is the angle between the z axis and the C_α - C_γ vector. β' is the angle between the z axis and the C_α - C_β vector. χ is the angle between the C_α - C_β vector and the C_α - C_γ vector. $\vec{S}$ axis is the extension of the C_α - C_β vector. Trajectory of the rotation of the C_γ nuclear around the C_α - C_β chemical bond make a plane A which is perpendicular to the y-z plane and also perpendicular to the $\vec{S}$ axis which is across with plane A at point o. The vector $\vec{B}$ is in the A plane and the y-z plane simultaneously and thus perpendicular to the $\vec{S}$ axis. φ' is the angle that the C_γ has rotated around the $\vec{S}$ axis clockwise (looking into the direction of $\vec{S}$) starting from its anti conformation ($\varphi'=0^\circ$). To find the unit vectors, we will first find the projection of the N- C_γ vector to the x, y and z axes. The box and the dashed lines inside the box are drawn for assisting the derivation of the unit vectors.

For the z projection of the N-C_γ vector:

The length of C_{α-q} can be found by the length of C_{α-C_β} and the angle θ' as:

$$L_{C_{\alpha-q}} = L_{C_{\alpha-C_{\gamma}}} \cos \theta' \quad (13)$$

The angle θ' can be related to χ, β' and φ' by [24]

$$\cos \theta' = \cos \chi \cos \beta' + \sin \chi \sin \beta' \cos \varphi' \quad (14)$$

Thus, the z projection of the N-C_γ vector is:

$$z_{N-C_{\gamma}} = L_{N-C_{\alpha}} + L_{C_{\alpha-q}} = L_{N-C_{\alpha}} + L_{C_{\alpha-C_{\gamma}}} \cos \theta' \quad (15)$$

For the x projection of the N-C_γ vector:

The length of C_{γ-o} is calculated by

$$L_{C_{\gamma-o}} = L_{C_{\alpha-C_{\gamma}}} \sin \chi \quad (16)$$

Thus, the length of C_{γ-p} which is also the x projection of the N-C_γ vector is:

$$x_{N-C_{\gamma}} = L_{C_{\gamma-p}} = L_{C_{\gamma-o}} \sin(\pi - \varphi') = L_{C_{\alpha-C_{\gamma}}} \sin \chi \sin(\pi - \varphi') = L_{C_{\alpha-C_{\gamma}}} \sin \chi \sin \varphi' \quad (17)$$

For the y projection of the N-C_γ vector:

The length of C_{γ-q} can be calculated as

$$L_{C_{\gamma-q}} = L_{C_{\alpha-C_{\gamma}}} \sin \theta' \quad (18)$$

Thus, the length q-p which is also the y projection of the N-C_γ vector is:

$$y_{N-C_{\gamma}} = L_{q-p} = (L_{C_{\gamma-q}}^2 - L_{C_{\gamma-p}}^2)^{1/2} = L_{C_{\alpha-C_{\gamma}}} (\sin^2 \theta' - \sin^2 \chi \sin^2 \varphi')^{1/2} \quad (19)$$

where the following relation will be applied

$$\sin^2 \theta' = (1 - \cos^2 \theta') \quad (20)$$

The direction of the unit vector $\vec{n}(i)$ is defined as from C_γ to N. Thus,

$$\widehat{n}_{C_{\gamma}-N}(i) = (-x_{N-C_{\gamma}}/L_{N-C_{\gamma}})\widehat{i}, -(y_{N-C_{\gamma}}/L_{N-C_{\gamma}})\widehat{j}, -(x_{N-C_{\gamma}}/L_{N-C_{\gamma}})\widehat{k} \quad (21)$$

All the lengths and angles in the above equations can be obtained from the structural data. For ideal cases, $\phi'=0$ is for the anti conformation, $\phi'=120$ for Gauche 2 and $\phi'=240$ (or -120) for Gauche 1 as shown in Figure 1. For the convenience but not introducing significant errors in the theoretical REDOR intensity curves, we have used the theoretical parameters of Thr amino acid structure maximized using Gaussian 03 program. The obtained parameters are: $\chi=35.265^\circ$, $\beta=68.9^\circ$, $L_{C\alpha-C\gamma}=2.490\text{\AA}$, $L_{N-C\alpha}=1.449\text{\AA}$, $L_{N-C\gamma}=3.783\text{\AA}$ for anti conformation ($\phi'=0$) and $L_{NC\gamma}=2.912\text{\AA}$ for the two gauche conformations ($\phi'=\pm 120^\circ$).

The C_γ -N unit vectors are given as the following:

$$\begin{array}{lll} \text{Anti,} & \phi'=0, & \hat{n}_{C_\gamma N}(1)=\{0.0000, -0.3645, -0.9310\} \\ \text{Gauche 1,} & \phi'=-120^\circ, & \hat{n}_{C_\gamma N}(2)=\{0.4275, -0.7402, -0.5186\} \\ \text{Gauche 2,} & \phi'=120^\circ, & \hat{n}_{C_\gamma N}(3)=\{-0.4275, -0.7402, -0.5186\} \end{array} \quad (22)$$

The unit vectors might be different for different orientations in the coordinate system. However, the final results of calculation using Equation 12 will be the same. We did numerical calculation using a Mathematica program in order to have the job done in an affordable calculation time. The sum of every 5° was evaluated in the integral calculation. The normalized REDOR intensities only have errors of $\sim \pm 0.001$ compared with the truly integrated REDOR intensities.

Experimental

The sequence of the HPLC6 isoform of type I AFP has been given in the introduction section. The Thr methyl $^{13}\text{C}_\gamma$ and amide ^{15}N double labels were made at the Thr 13 and Thr 24 residues in the second and the third repeat units. The AFP was custom synthesized by Auspep Pty Ltd using a 98 atom % $^{13}\text{C}_\gamma$ and ^{15}N doubly labeled L-Thr amino acid provided by Isotec Inc. The Mass spectra show that the purity of the peptide is $\geq 95\%$. To prepare the NMR sample, 2 mg of the AFP was dissolved in 0.25 ml water in an insert cell of a 7 mm NMR rotor. To freeze the AFP solution, the AFP solution within the insert cell together with the NMR rotor was immersed in liquid nitrogen. Then, the sample was placed in a freezer at -20°C overnight. The purpose of using liquid nitrogen to freeze the sample instead of freezing the sample at a higher temperature was to create a more homogeneous solid solution. The relaxation procedure at -20°C would eliminate glassy ice structures and allow the AFP to find the best structural match with the ice surface which would generate the lowest local Gibbs energy states for the interaction of the AFP with the ice surfaces. After the temperature of the rotor housing of the NMR probe was cooled below -20°C , the NMR rotor containing the frozen AFP was quickly transferred into the probe to avoid any melting of the sample. The temperature was kept at -20°C during the REDOR NMR experiments. Standard procedures were used to calibrate the sample temperatures.[25, 26] For comparison, the REDOR NMR experiment was also done for the dehydrated Thr $^{13}\text{C}_\gamma$ and ^{15}N doubly labeled AFP powder. As control experiments, the REDOR NMR was also carried out for two more solid powders including $^{13}\text{C}_\beta$ - ^{15}N doubly labeled L-Ala amino acid (with 99 atom % ^{13}C and 99 atom % ^{15}N) and $^{13}\text{C}_\gamma$ - ^{15}N doubly labeled L-Thr amino acid (with 98 atom % of both ^{13}C and ^{15}N). Although the L-Ala REDOR NMR has been done by Mehta et al, [27] here we just used it as a control experiment. These amino acids were supplied by Isotec Inc.. The samples were prepared by mixing 5 molar% of the ^{13}C and ^{15}N doubly labeled L-Ala and L-Thr in 95 molar% natural abundant L-Ala and L-Thr (from Aldrich) in water, respectively, and then dried in a 70°C oven. All the NMR experiments for the dehydrated samples were carried out at room temperature.

A Bruker Avance™ 600 WB NMR spectrometer attached with a Doty 7 mm triple resonance MAS probe was used. REDOR pulse sequence with a single ^{13}C π pulse in the middle of the pulse sequence and a series of ^{15}N π pulses at the half and full rotor positions (except the position of the ^{13}C pulse) was used.[22] Proton high power decoupling (HPDec) was employed during the ^{13}C and ^{15}N pulse sequence and the acquisition period. CP (Cross Polarization) contact time of 700–2000 μs and MAS (Magic Angle Spinning) rate at 2.0 kHz were used. REDOR fraction defined as $(S_0 - S)/S_0$ where S_0 denotes the integrated rotational echo signal intensity of the ^{13}C transverse magnetization (without the ^{15}N π pulses) and S refers to the integrated dipolar dephased signal due to the ^{15}N local field (with the ^{15}N π pulses), is used to present the REDOR experimental data and the theoretically calculated curves.

Results and Discussion

Figure 3 shows the experimental REDOR fractions of the Ala amino acid solid powder (solid squares) and the Thr amino acid solid powder (open circles) with the increase of dipolar dephasing time. The errors bars were added according to the minimum experimental signal to noise ratios. The corresponding theoretical REDOR fraction curves (solid curve for Ala and dotted curve for Thr) were calculated using the ^{13}C - ^{15}N internuclear distances of 2.466 Å for Ala[28] and 3.81 Å for Thr[29] in their crystal structures. The internuclear distance of Thr amino acid is corresponding to the anti-conformation of the side chain with the dihedral angle of 179.8°. The experimental data fit the theoretical curves within the given errors. The REDOR fraction curve of Ala grows much faster than that of Thr as the dipolar coupling constant is inversely proportional to the cube of the internuclear distance. [22] For comparison, the theoretical REDOR fraction curve (dash-dotted curve) for the internuclear distance of 2.91 Å corresponding to the theoretical gauche conformation of the Thr side chain is also given in Figure 3. This curve grows faster than that of the anti conformation because the internuclear distance of the gauche conformation is shorter than that of the anti conformation. The dependence of the REDOR fraction curve on the internuclear distance, thus on the conformation of the Thr side chain shows that REDOR analysis may provide conformational information for the Thr side chains of the AFP when interacting with ice surface.

Figure 4 shows the ^{13}C REDOR NMR spectrum (a) and the ^{13}C rotational echo spectrum (b) of the dehydrated Thr $^{13}\text{C}_\gamma$ - ^{15}N doubly labeled AFP solid powder with the dipolar dephasing time of 6 ms. The peaks at 19.9 ppm show the chemical shift of methyl groups. The ratio of the two peaks is 0.90 showing the ^{13}C dipolar dephasing due to the ^{15}N spin. Figure 4 also shows the ^{13}C REDOR NMR spectrum (c) and the ^{13}C rotational echo spectrum (d) of the frozen Thr $^{13}\text{C}_\gamma$ - ^{15}N doubly labeled AFP solution with the dipolar dephasing 6 ms. The ratio of the two peaks is 0.79. The ^{13}C peaks in (c) and (d) look broader than those of the dehydrated sample as shown in (a) and (b). This is because the AFP molecules were more amorphous and the Thr side chains were more mobile in the frozen solution which does not allow the MAS to effectively average the linewidth. (See the following discussion of the REDOR results for the mobility.)

The experimental REDOR fractions of the dehydrated Thr $^{13}\text{C}_\gamma$ - ^{15}N doubly labeled AFP solid powder are shown in Figure 3 (solid down triangles). The experimental data fit the theoretical anti Thr amino acid curve within the given experimental errors showing the anti conformations for the Thr-13 and Thr-24 side chains in the dehydrated AFP solid powder. The crystal structures of the winter flounder type I AFP acquired at 0 °C and −180 °C show that the Thr side chains at residues 13 and 24 possess anti conformations with the dihedral angles of 176.68° for Thr-13 and 174.30° for Thr-24 in the slightly bowed chain, and 173.13° for Thr-13 and −174.52° for Thr-24 in the straight chain.[30] Our REDOR result of

the dehydrated AFP sample agrees with the Thr side chain conformations in the crystal structure. Note that the ^{13}C - ^{15}N internuclear distance changes due to dihedral angle's differences within $\pm 6^\circ$ can not be detected due to the experimental errors. The experimental REDOR fractions of the frozen Thr $^{13}\text{C}_\gamma$ - ^{15}N doubly labeled type I AFP solution as a function of dipolar dephasing time is also shown in Figure 3 as the solid up triangles. These experimental data falls between those of the ideal gauche-conformation and the ideal anti-conformation of the Thr side chain. To find out the population of the anti, gauche 1 and gauche 2 conformations (shown in Figure 1), we have performed a number of calculations for different population distributions using Equation 12 and the unit vectors in Equation 22.

Figure 5 shows the general trends of the theoretical dynamic REDOR fraction curves with different conformational population distributions. All the colored curves are labeled with P_A , P_{G1} and P_{G2} in the insets corresponding to anti, gauche 1 and gauche 2 conformational populations, respectively. The growths of the curves become slower with the increase of the anti population reflecting the decreased average dipolar interactions. For the same anti population, the growths of the curves become slower when the two gauche populations become closer. It was also observed that the two curves with the populations ($P_A=a$, $P_{G1}=b$, $P_{G2}=c$) and ($P_A=a$, $P_{G1}=c$, $P_{G2}=b$) are identical. Thus, only one of the two identical curves is given in the Figure. Because of this, an experimental REDOR curve can not be distinguished between the two sets of population distributions if no other structural information is available for assisting in the structural determination.

To find the population distribution of the AFP Thr side chain's conformations, we have compared the experimental REDOR data with the theoretical REDOR curves as shown in Figure 6 from (a) to (f) where the experimental data are shown as the up triangles and the populations (P_A , P_{G1} and P_{G2}) corresponding to the theoretical curves are given in the insets. To quantitatively analyze the data, we have performed χ^2 -squared statistical analysis [31–33] where the χ^2 is defined as

$$\chi^2 = \frac{1}{\sigma^2} \sum_{i=1}^N (E_i - S_i)^2 \quad (23)$$

where E_i and S_i represent the experimental and theoretically simulated REDOR fractions, respectively, σ^2 is the average mean-squared percentage uncertainty of the experimental REDOR fractions and N denotes the total number of experimental REDOR points.

The χ^2 values for the corresponding theoretical REDOR curves are also given in the insets. Smaller χ^2 is corresponding to better fitting of the experimental data to the theoretical curve. Figure 7 shows the contour plot of χ^2 values versus P_{G1} and P_{G2} where $P_A = 1 - P_{G1} - P_{G2}$. Analysis of the plot shows that the lowest χ^2 trajectory follows the function $P_{G1}^2 + P_{G2}^2 = r^2$ where $r = 0.5 \pm 0.03$. As examples, the experimental data may belong to the theoretical curves with the population distributions of ($P_A=0.30$, $P_{G1}=0.40$, $P_{G2}=0.30$) or ($P_A=0.30$, $P_{G1}=0.30$, $P_{G2}=0.40$); ($P_A=0.40$, $P_{G1}=0.11$, $P_{G2}=0.49$) or ($P_A=0.40$, $P_{G1}=0.49$, $P_{G2}=0.11$); and ($P_A=0.50$, $P_{G1}=0.00$, $P_{G2}=0.50$) or ($P_A=0.50$, $P_{G1}=0.50$, $P_{G2}=0.00$). Figure 6 and Figure 7 show that the all anti Thr side chain conformations in the AFP crystal structure have changed to partial anti conformation and partial gauche conformations in the frozen AFP solution. We believe that the interaction of the Thr side chains with the ice surface is the reason for the population changes. In the pairs of population sets (jointed together with or), only one of the two should represent the experimental case. It has been known that the side chains of the Thr residues and those of the 4th and 8th Ala residues commencing from the Thr_s (the 1st) in the four 11-residue repeat units form the AFP ice binding surface.[19–21]

As shown in Figure 1, zooming into the local area around the Thr 13 residue as an example, we found that gauche 1 conformation (e) allows both the methyl group and the hydroxyl group to simultaneously touch the ice binding surface. However, the anti conformation (d) and the gauche 2 conformation (f) lift the hydroxyl group and the methyl group, respectively, away from the surface. Thus, we believe that the van der Waals interaction of the methyl group together with the hydrogen bonding interaction of the hydroxyl group with the ice surface could have played the simultaneous roles to stabilize the gauche 1 conformation which in turn could have enhanced the adsorption of the AFP to the ice surface. Therefore, it is likely that gauche 1 conformer has a larger population than gauche 2 conformer.

Conclusion

Through χ -squared statistical analysis to fit the $^{13}\text{C}_\gamma$ - ^{15}N REDOR NMR data to the theoretical dynamic REDOR curves, we found that anti conformation of the Thr side chains in the AFP crystal structure changed to the possible population distributions from ($P_A=0.30$, $P_{G1}=0.40$, $P_{G2}=0.30$) to ($P_A=0.50$, $P_{G1}=0.50$, $P_{G2}=0.00$) along the trajectory defined by the function $P_{G1}^2 + P_{G2}^2 = 0.5^2$. This change indicates that the simultaneous interactions of the methyl groups and the hydroxyl groups of the Thr side chains with the ice surface could release a good amount of energy to compensate the higher energy state of the gauche 1 conformation compared with the anti conformation. We think that the Thr side chains dynamically populated among the three conformers (Figure 1) when interacting with the ice surface. Our previous ^{13}C relaxation NMR result indicates that the average distance of the Thr methyl groups to the ice surface is larger than that of the ice-binding Ala methyl groups. [21] This larger distance is now correlated to the current REDOR result in which the Thr methyl groups could have jumped away from the ice surface when populated in the gauche 2 conformation. Because of this, $P_{G2}=0$ is less likely.

The results of the theoretical calculations of the dynamic REDOR NMR show that the set of experimental REDOR data may fit a number of theoretical curves. Thus, other structural information is needed to assist in determining the conformational population distribution of the Thr side chains.

Acknowledgments

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Figure 1.

(a), (b) and (c) show the Thr side chain conformations where the β carbon atom is over the α carbon atom in each of the diagrams. The Thr13 side chains inside the given squares in (d), (e) and (f) show the relative orientations with respect to the local structure in a section of the Type I AFP. (Nitrogen is shown in blue color, oxygen red, carbon black and hydrogen grey.)

Figure 2. Diagram describing the C $_{\alpha}$ -C $_{\beta}$ chemical bond rotation of the Thr side chain.

Figure 3.

Experimental REDOR fraction data of the ^{13}C and ^{13}N doubly labeled L-Ala (solid squares), Thr (open circles), Thr of the frozen type I AFP solution (up triangles) and Thr of the dehydrated (dry) type I AFP solid powder (down triangles). The corresponding theoretical curves were calculated using the C-N internuclear distances as given in the inset.

Figure 4. ^{13}C REDOR NMR spectrum (a) and the ^{13}C rotational echo spectrum (b) of the dehydrated Thr $^{13}\text{C}_\gamma$ - ^{15}N doubly labeled AFP solid powder and ^{13}C REDOR NMR spectrum (c) and the ^{13}C rotational echo spectrum (d) of the frozen Thr $^{13}\text{C}_\gamma$ - ^{15}N doubly labeled AFP solution with the dipolar dephasing time of 6ms. The dominant peaks at 19.9 ppm show the chemical shifts of methyl groups.

Figure 5. Theoretical C_{γ} -N REDOR fraction curves as a function of the conformational populations P_A , P_{G1} and P_{G2} corresponding to anti, gauche 1 and gauche 2 conformers, respectively.

Figure 6.

Theoretical dynamic REDOR fraction curves as shown from (a) to (f) where the conformational populations are given in the insets, and the experimental REDOR fraction data of the frozen Thr ^{13}C and ^{13}N doubly labeled type I AFP solution (up triangles).

Figure 7.
Contour plot of χ -squared deviations versus the two gauche populations P_{G1} and P_{G2} .