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Improved Purification of Thermophilic F_oF_1-ATP Synthase *c*-Subunit Rings and Solid-State NMR Characterization of Them in Different Lipid Membranes

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Abstract ATP synthase produces ATP, a major energy source for metabolic processes in organisms, from ADP and inorganic phosphate in cellular membranes. ATP synthase is known as a rotary motor, in which the *c*-subunit ring functions as a rotor. In this work, we have tried to develop a more general preparation procedure of thermophilic F_oc-ring (TF_oc-ring) for NMR measurements. The expression of TF_oF_1 is easily affected by various experimental conditions such as temperature, shape and size of a flask, a volume of medium, and shaking rate of an incubator. Accordingly, we have tried to optimize the expression conditions of TF_oF_1. TF_oc-rings were purified from TF_oF_1 according to a reported method. We modified purification procedures to improve purity and yield of TF_oc. On top of them, we found a new combination of detergents for the purification at anion-exchange column chromatography. To examine the effect of lipid environments on the structure, the TF_oc-rings were reconstituted into two kinds of lipid bilayers, namely, saturated and unsaturated lipid ones. Then, we have compared characteristics of the TF_oc-ring structures in these membranes with solid-state NMR.

Keywords motor protein, membrane protein, ring His-tag, detergent, CP/MAS NMR

Introduction

ATP synthase produces ATP, a major energy source for metabolic processes in organisms, from ADP and inorganic phosphate (P_i) in cellular membranes. ATP synthase consists of two parts, F_o- and F_1-portions. Thus, it is also designated as F_oF_1-ATPase. In bacteria, the cytoplasmic F_1 domain comprises $\alpha_3\beta_3\gamma\delta\varepsilon$ subunits, which has ATP-synthesizing/hydrolyzing activity.[1,2] The isolated F_1 is water-soluble. The membrane-embedded F_o domain comprises $ab_2c_{8\text{-}15}$ subunits, and forms a channel for transportation of proton. ATP synthase is a rotary motor, in which the *c*-subunit ring functions as a rotor. Downhill proton flow through F_o causes

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rotation of the rotor (*c*-ring) and rotation of the F_1-γε (rotor stalk; $F_o c_{8\text{-}15}$-F_1γε). The rotation of γ induces conformational changes in the β-subunits, which results in ATP synthesis at the F_1 catalytic sites.[3-10]

Although the *c*-subunit ring has an important role in ATP synthesis, the high resolution structure of the *c*-subunit ring in lipid membranes is still unknown. In this work, we investigated the effect of lipid species on the structure of the *c*-subunit ring of thermophilic *Bacillus* PS3 ATP-synthase (TF_oc-ring) in lipid membranes, using solid-state NMR. Since the function of a membrane protein is influenced by the interaction with lipids in membranes, it is important to examine the effect of lipids on the structure. The TF_oc-subunit ring comprises ten *c*-subunits, and each subunit is composed of 72 amino acid residues.[7] The structure of TF_oc monomer in organic solvent was determined as a hairpin conformation composed of two α-helices.[11] The TF_oc-ring is embedded in a membrane, and 76% amino acid residues are hydrophobic. The hydrophobic transmembrane region causes following obstacles in the study of membrane proteins. First, a low level of expression of membrane proteins makes it difficult to obtain enough amount of the protein of interest. Second, membrane proteins are easy to aggregate during the purification due to its hydrophobicity. Third, it is not easy to isolate the target protein from similar transmembrane proteins. Finally, the isolated membrane proteins have to be reconstituted into lipid membranes in vitro for high resolution analysis.[12] Although a standard method was reported[13], we need sophisticated techniques to obtain enough amount of the protein. In this work, we have tried to develop a more general procedure of TF_oc-ring preparation for NMR measurements. The expression of TF_oc-subunits is easily affected by various experimental conditions such as temperature, shape and size of a flask, a volume of medium, and shaking rate of an incubator. Accordingly, we have tried to optimize the expression conditions and purification procedures to improve purity and yield of TF_oc. On top of them, we found a new combination of detergents for isolation of TF_oc-ring from the other TF_o components in anion-exchange column chromatography. To examine the effect of lipid environments on the structure, the TF_oc-rings were reconstituted into two kinds of lipid bilayers, namely, saturated and unsaturated lipid ones. Then, we have compared characteristics of the TF_oc-ring structures in these membranes with solid-state NMR.

Experimental Methods

Expression and Purification- The expression and purification were carried out according to the reported methods.[13] ATP-synthase of thermophilic *Bacillus* PS3 (TF_oF_1) was expressed in *E. coli* cells, strain DK8, transformed with the pTR19-ASDS-cHis2R vector.[14] Here, Ser2 was replaced with His. The 2×YT and CHL-IS media were used for non-labeled and labeled samples, respectively. The 2×YT medium included tryptone (16 g/L), yeast extract (10 g/L), NaCl (5 g/L), NaOH (pH 7.0). The CHL-IS medium was composed of the isotope-labeled chlorella extract medium, CHL-I (0.7 g ^{13}C, ^{15}N-cholrella extract, 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g $^{15}NH_4Cl$, 0.5 g NaCl per liter; CHLORELLA Industry Co., Japan), and ^{13}C-glucose (1 g/L, ISOTECK, Inc., Miamisburg, USA), ^{13}C-glycerol (2.4 g/L, Cambridge Isotope Laboratories, Inc. Andover, USA), ^{13}C, ^{15}N-algal amino acid mixture (1 g/L; CHLORELLA Industry Co.), cysteine (50 mg/L), $MgSO_4{\cdot}7H_2O$ (0.25 g/L), ampicillin (0.1 g/L), thymine (50 mg/L), and thiamine (2 mg/L). Cells were grown on an agar plate with the 2×YT medium with 100 mg/L ampicillin at 37 ℃ for 17 hrs. Ten colonies were inoculated to 66 ml of 2×YT and cells were grown at 37 ℃ and 200 rpm with a shaker SI-300R (Lab companion, Korea). When OD_{600} of the culture reached to 0.5, 5 ml of the culture was inoculated to 250 ml of CHL-IS and incubated at 37 ℃ and 220 rpm for 26 hrs with the same shaker. For non-labeled samples, 1.5 ml of the culture was inoculated to 1.5 L of 2×YT and incubated at 37 ℃ and 130 rpm for 26 hrs with a shaker VS-8480 (Vision scientific Co., Ltd., Korea). The cells were harvested by centrifugation at 7100 rpm and 4 ℃ for 15 min with a centrifuge (Supra

22K, Hanil Science Industrial Co., Ltd., Korea) and washed with 5 mM HEPES-KOH buffer (pH 7.5) and 2 mM EDTA buffer. The cell pellets were suspended in 340 ml of 10 mM HEPES-KOH buffer (pH 7.5) containing 740 μl 2-mercaptethanol. Cells were disrupted with a French Press (J5-598A, SLM Aminco, Urbana, USA) at 18191 psi 3-times and subjected to sonication (2510, Branson, Danbury, USA). The lysates were centrifuged at 10000 rpm and 4 ℃ for 20 min with an ultracentrifuge (Ultra 5.0, Hanil Science Industrial Co., Ltd.). The supernatant was centrifuged at 39000 rpm and 4 ℃ for 3 hrs. The pellet was suspended in 370 ml of 7 M urea, 5 mM EDTA, 10 mM HEPES-KOH buffer (pH 7.5), and stirred at 25 ℃ for 30 min for removal of F_1. This solution was centrifuged at 39000 rpm and 30 ℃ for 3 hrs and washed with milliQ water 3-times at 39000 rpm, and 4 ℃ for 1.5 hrs. The pellet was suspended in 250 ml of 2% sodium deoxycholate (DOC, Waco Pure Chemical Industries, Ltd. Japan), 10 mM Tris-HCl buffer (pH 7.5) with protease inhibitor cocktail (PIC, 1 tablet/L; Roche, Switzerland). The solution was sonicated for promotion of solubilization (102C, Branson) and mildly stirred for 30 min at room temperature. After centrifugation at 39000 rpm and 30 ℃ for 45 min, the supernatant was diluted four-fold with 100 mM KCl, 20 mM imidazole, 20 mM potassium phosphate (KPi) buffer (pH 7.5), including PIC. Then, pH was adjusted to 8.0, and 30 ml of Ni-NTA resin (Qiagen, Germany) was added. After stirred in ice for 1 hr, this was loaded to a column and washed with a 20 mM KPi buffer (pH 7.5) solution including 0.15% n-decyl-β-D-maltoside (DM, Dojindo Laboratories, Japan), 0.5% DOC, 20 mM imidazole, and 100 mM KCl. The column was eluted with the same buffer except for containing 200 mM imidazole. The TF_o*c*-subunit fraction was collected and was diluted ten-fold with QA buffer (0.15% DM, 0.2 mM EDTA, 10 mM Tris-HCl buffer (pH 8.0)) and loaded to an anion exchange Hitrap Q column (HQ; GE Healthcare, Sweden). After washed with QA buffer, the column was eluted with a 0–42.5 mM NaCl gradient in QA buffer. The purity of the TF_o*c*-rings was confirmed with Tricine-SDS-PAGE.

Reconstitution- DM (final concentration 0.5 %), 1-Palmitoyl-2-Oleolyl-*sn*-3-Glycero-phosphatidyl-choline (POPC-d_{31}; 98% ^{2}H lipid/protein weight ratio = 1/4; Icon Services Inc., Summit, USA), and $MgCl_2$ (final concentration 5 mM) were added to the purified *c*-subunit fraction. Also 1,2-diperdeuteriomyristoyl-*sn*-glycero-3-phosphocho line (DMPC-d_{54}, 98% ^{2}H, chemical purity 95%; Cambridge Isotope Laboratories, Inc.) was used in the same way. Bio-Beads SM-2 (Bio-Rad Laboratories, Hercule, USA) were added to the mixture (37.5 mg/mL) to remove detergents and gently mixed overnight at 4 ℃. After filtering Bio-Beads, the solution was dialyzed against dialysis buffer A (5 mM $MgCl_2$, 0.4 mM NaN_3, 10 mM HEPES-KOH buffer (pH 7.5)) for 3 days and then against dialysis buffer B (0.4 mM NaN_3, 10 mM HEPES-KOH buffer (pH 7.5)) for 3 days at room temperature. The precipitate was collected by centrifugation at 39000 rpm and 4 ℃ for 2 hrs. The precipitate was suspended in dialysis buffer B and performed 10 cycles of freeze and thaw process (-20 and 25 ℃ for 25 min, respectively for POPC sample; -20, 4, and 30 ℃ for 15min, respectively for DMPC sample). After centrifugation at 70000 rpm and 4 ℃ for 2 hrs a few times, the pellets were used for solid-state NMR measurements. In the case of POPC, the care was taken to shut down the light to avoid the oxidation of the unsaturated fatty acids.

Solid-state Nuclear Magnetic Resonance- NMR spectra were recorded with a Bruker DRX700 NMR spectrometer at 16.44 T. Broadband triple resonance MAS probes for 3.2 and 2.5 mmΦ rotors were used at 213 K (set temperature). Two-dimensional (2D) ^{13}C homonuclear correlation spectra with the dipolar assisted rotational resonance (DARR) pulse sequence[15] were obtained under magic angle sample spinning (MAS). The MAS rate was 12.5 kHz unless otherwise specified. The mixing times were 15 ms and 200 ms. Data matrices for the TF_o*c*-rings in POPC bilayers were 1024 (t1)×210 (t2) and 1024×250 for at 15 and 200 ms mixing times, respectively. Those for in DMPC were 1024×240 and

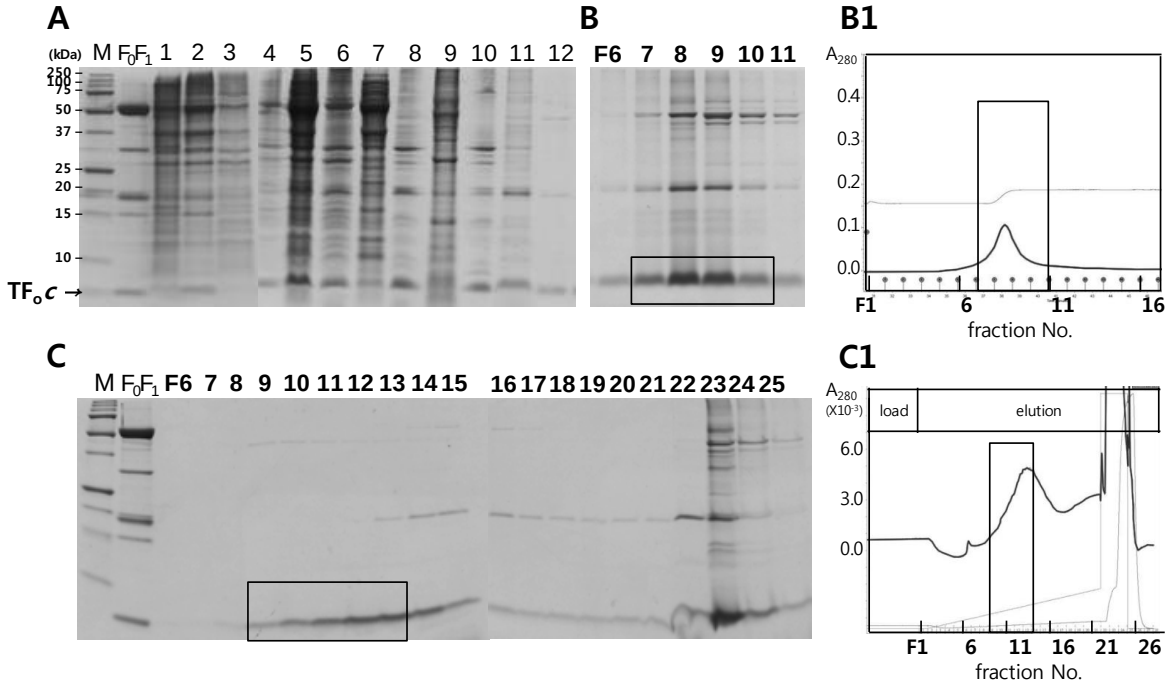

Figure 1. Expression and purification of labeled TF_oc-subunit rings. (A) The Tricine-SDS-PAGE image of the preparation at each expression or purification step. Lane 1, cells in a 200 ml main culture with two-step preculture (OD_{600}=1.441); Lane 2, cells in a 250 ml main culture with single-step preculture (OD_{600}=1.213); Lane 3, cells in a 1.5 L main culture with single-step preculture (OD_{600}=0.722); Lanes 4 and 5, pellet and supernatant (sup) after cell disruption, respectively; Lane 6 and 7, membrane fraction and its sup, respectively; Lane 8 and 9, urea-treated membrane fraction and its sup, respectively; Lane 10, pellets after solubilization with DOC; Lane 11, solubilized membrane fraction; Lane 12, TF_oc fraction at Ni-NTA; M, molecular weight markers. (B) Fractions No. **6–11** in B1. (B1) The elution pattern in Ni-NTA chromatography. (C) TF_oc fractions No. **7–25** in C1. (C1) The elution pattern in HQ chromatography. Load, loading pass.

1024×280 for at 15 and 200 ms, respectively. They were zero-filled to 2048×2048. The spectral widths were 100×100 kHz for POPC samples and 70×70 kHz for DMPC samples.

The obtained FID signals were processed with Felix (Felix NMR Inc., San Diego, CA). Fourier transformations were performed with exponential, sine-bell, and sine-squared window functions with baseline correction. The spectra were analyzed with SPARKY, which was developed by T. D. Goddard and D. G. Kneller in UCSF.[16]

Results and Discussion

Expression and Purification of TF_oc-rings- The TF_oc-subunit rings were expressed in the *E. coli* strain DK8 transformed with the pTR19-ASDS-cHis2R vector. In this vector, Ser2 was replaced with His to introduce a virtual ring His-tag. The His layer in a ring structure will act as a non-bonded ring His-tag in Ni-NTA chromatography. The expression of the F_oF_1 gene in 1.5 L of a rich medium, 2×YT, provided an enough amount of non-labeled TF_oc-rings. For producing isotope-labeled one, however, we had to use a semi-chemical medium, CHL-IS. The expression in this medium was not good enough, when we took the two-step inoculation according to the reported method.[13] When the cells precultured with 2×YT were directly added to the final CHL-IS culture at 50-fold dilution (The medium was removed by centrifugation.), the expression

efficiency was enhanced as can be seen in lane 2 in comparison with lane 1 in Figure 1A. OD_{600} of the former was lower than that of the latter, since better expression suppressed the cell-growth. Furthermore, the aeration was optimized, using a 250 ml of culture in a 1 L round flask as sown in lanes 2 and 3 of Figure 1A. In this case, OD_{600} of the latter was lower than that of the former, because of less aeration.

Cells were disrupted by French press and the membrane fraction was obtained by ultracentrifugation. The F_1-portion was removed from the membrane fraction by 7 M urea treatment and then it was solubilized with 2% DOC solution. In this step, we examined different sonication conditions. If sonication is too weak, rings will not be well solubilized, and if sonication is too strong, isolated rings will be broken down. We found that the sonication with 80% amplitude for 40 sec was the best in our purification set-up. Then, Ni-NTA affinity column chromatography and Hitrap Q anion exchange column chromatography were used to isolate TF_oc-rings.

The SDS-PAGE images of the over-all purification steps were presented in Figure 1A. Figure 1B1 and 1B provide the elution pattern in the Ni-NTA affinity chromatography and the SDS-PAGE images of TF_oc fractions in it, respectively. This result clearly shows that the non-covalent ring His-tag worked efficiently for isolation of the ring structures.

In the anion-exchange chromatography, the residual imidazole concentration originated from the Ni-NTA elution influences the binding interaction between TF_oc-rings and HQ resins. Using more Ni-NTA fractions means higher imidazole concentration in the HQ column. At a low concentration of imidazole (3 TF_oc fractions at Ni-NTA), TF_oc-rings eluted out in a late stage (Figure 2A). At a high concentration of imidazole (5 TF_oc fractions at Ni-NTA), most of TF_oc passed out in the loading pass (Figure 2C). Thus, imidazole acted as salt and interrupted the interaction between TF_oc-rings and HQ resins. When we used 4 TF_oc fractions obtained in the Ni-NTA affinity chromatography, the yield of pure TF_oc-rings was 6 mg. Since the yield was enough to perform solid-state NMR measurements, we used 4 fractions at Ni-NTA for the purification in the HQ anion-exchange chromatography. Presented in Figure 1C1 and 1C are the elution patterns in the HQ anion-exchange chromatography and the SDS-PAGE images of TF_oc fractions in it, respectively, in the purification of the ^{13}C,^{15}N-labeled TF_oc-rings. The purified TF_oc-rings were reconstituted into lipid membranes by removing detergents with Bio-Beads SM-2 and dialysis. If only Bio-Beads were used, the yield was low because of adsorption of the proteins to the beads. If only the dialysis was used, the

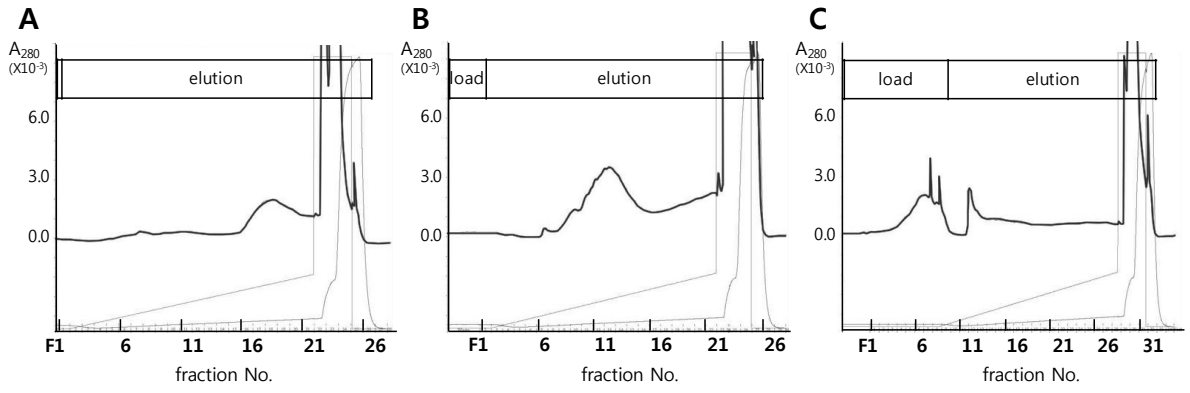

Figure 2. Elution patterns for the application of 3 (A), 4 (B), and 5 (C) TF_oc fractions at Ni-NTA to a HQ column. More Ni-NTA fractions being applied to a HQ column means higher residual imidazole concentration in the HQ column. Load, loading pass.

TF_oc-rings were partially denatured before getting into the membranes.[13] The obtained membrane sample was used for solid-state NMR measurements.

Effect of a new detergent combination- It was difficult to get a completely separated peak of the *c*-subunit rings in the HQ anion-exchange chromatography so far under our experimental conditions. We found a new combination of detergents, namely, 0.075% DM and 0.075% n-Dodecyl-β-D-maltoside (DDM, Dojindo Laboratories, Japan) that can provide a completely isolated peak of TF_oc-rings at the HQ step. We used the mixture of 0.075% DM and 0.075% DDM in the washing buffer and elution buffer of the Ni-NTA column as well. Then, obtained TF_oc-ring fractions were applied to the HQ column and eluted in the presence of 0.075% DM and 0.075% DDM. As can be seen in Figure 3A, the TF_oc-ring peak was completely isolated from that of *a*/*b*-subunits. However, a part of the TF_oc-rings would be lost in the loading pass in this case. Since the fractions at Ni-NTA were also concentrated, 3 fractions were applied to the HQ column. Both of the *c*- and *a*/*b*-subunit peaks were eluted out later and the separation became greater as can be seen in Figure 3B. The Tricine SDS-PAGE images in Figure 3B1 confirmed the complete separation of the TF_oc-ring peak from that of the *a*/*b*-subunits.

Because DDM is more hydrophobic than DM, it has more affinity to TF_oc than to TF_oa/b. More binding of DDM would reduce the number of DOC in TF_oc-ring

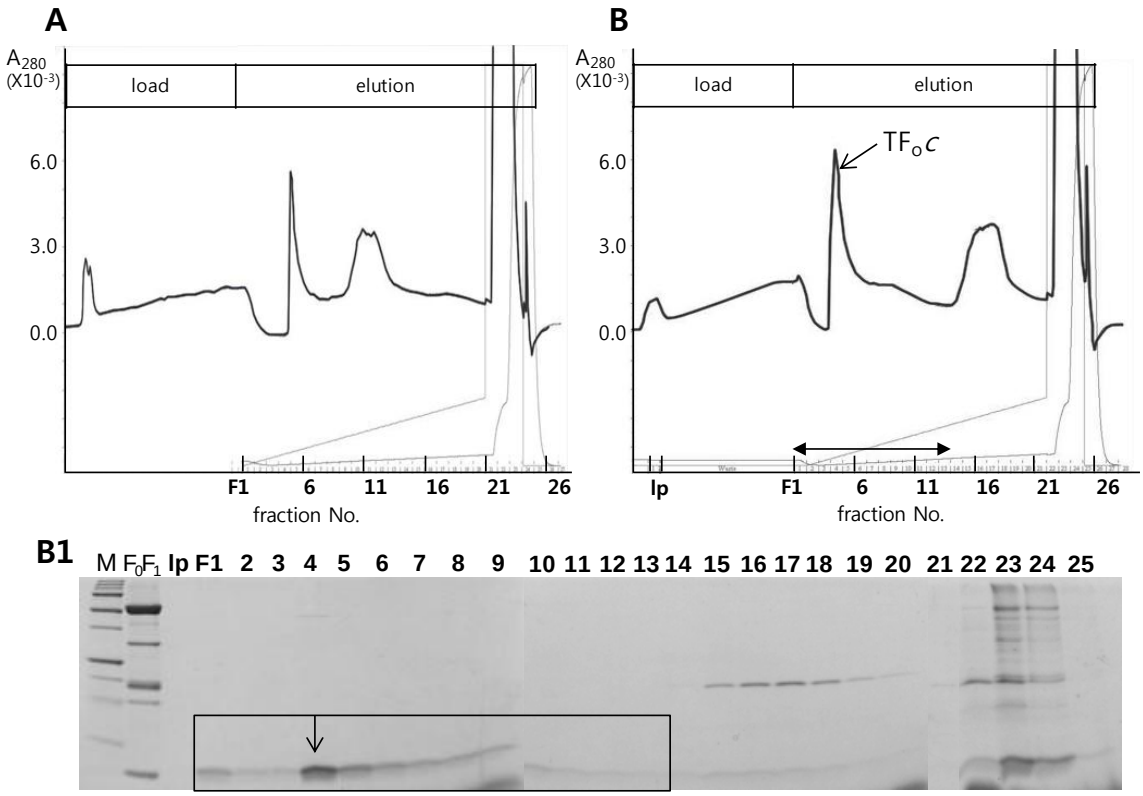

Figure 3. HQ elution patterns in the presence of the mixture of 0.75% DDM and DM. The peaks of *c*-subunit and *a*/*b*-subunits were completely separated. Four (A) and three (B) fractions at Ni-NTA were applied to the HQ column (load, loading pass). (B1) The Tricine-SDS-PAGE images for fractions No. **1–25** in B. There were no proteins in the fraction **lp**.

micelles, leading to a weaker interaction between TF_oc-ring micelles and HQ resins. Thus, TF_oc was eluted out earlier. Here, imidazole concentration influences the *a*/*b*-subunit more than the *c*-subunit. Taking advantage of the new combination of detergents, we could achieve a high-quality as well as high-yield. The ring structure of the isolated TF_oc was confirmed by the previous report.[13]

Characterization of TF_oc-rings in different lipid

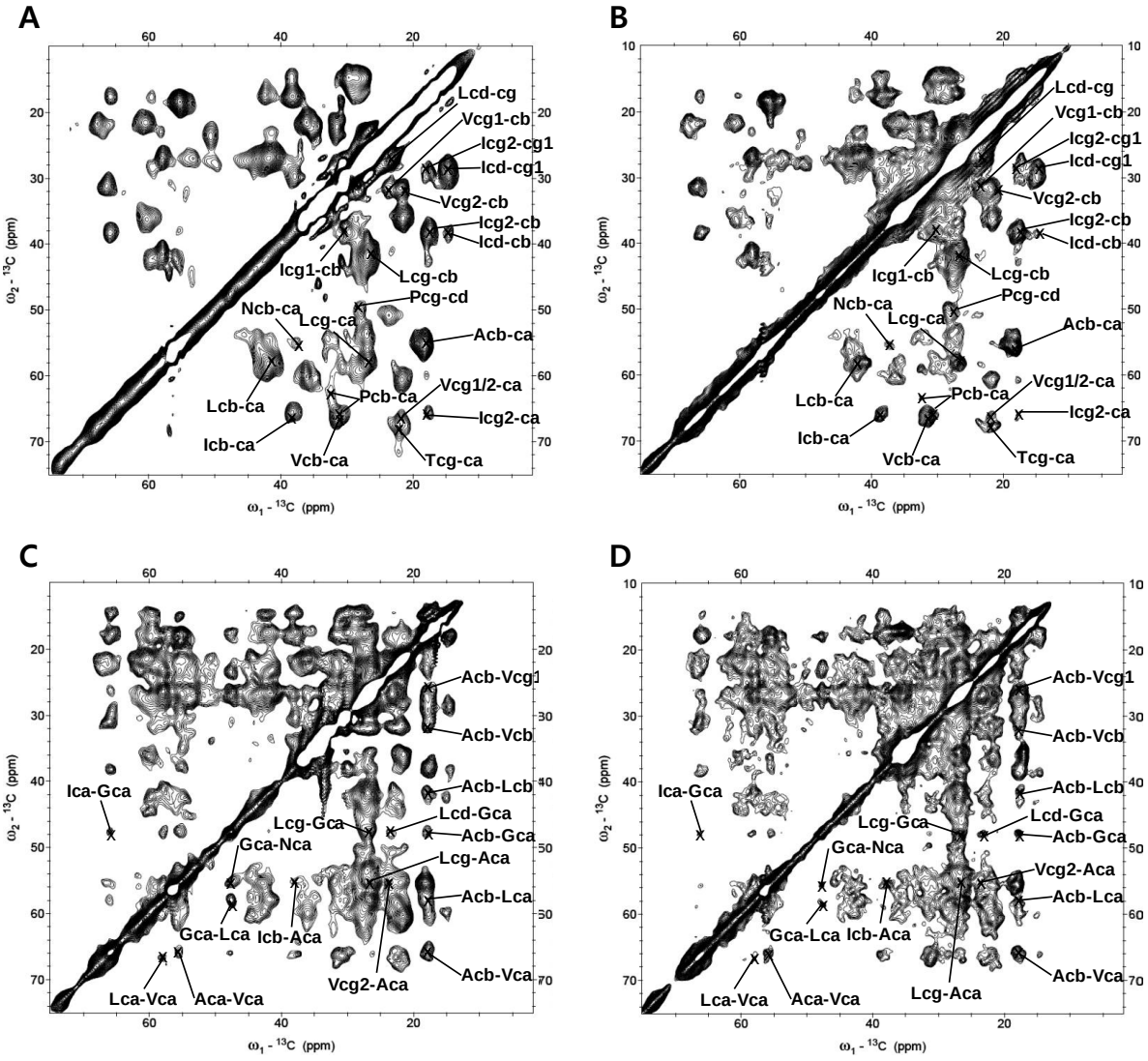

Figure 4. Two dimensional ^{13}C homonuclear correlation DARR spectra of TF_oc-rings in membranes. (A) and (C), in POPC membranes with 15 and 200 ms mixing times, respectively; (B) and (D), in DMPC membranes with 15 and 200 ms mixing times, respectively. Used window functions were sine-bell (70°) for d1 and d2 of A, squared sine-bell (80°) and exponential (50 Hz) for d1 and d2 of B, respectively, squared sine-bell (80°) and exponential (50 Hz) for d1 and d2 of C, respectively, and squared sine-bell (70°) and exponential (50 Hz) for d1 and d2 of D, respectively. For POPC and DMPC samples, 2.5 and 3.2 mmϕ rotors were used, respectively. Partial assignment on amino acid residue base is presented using single letter codes in the spectra. The attached character, ca, cb, cg, and cd stand for C^{α}, C^{β}, C^{γ}, and C^{δ}, respectively.

membranes- Using the obtained samples, we could measure ^{13}C-^{13}C correlation two-dimensional DARR spectra of the uniformly ^{13}C-labeled TF_oc-rings in lipid membranes at 213 K under magic angle sample spinning. To examine the effect of lipids on the protein structure, saturated and unsaturated lipids, DMPC-d_{54} and POPC-d_{31}, were used. The spectra with 15 ms mixing time in Figure 4A and 4B mainly provide intra-residue correlations of TF_oc-ring carbons. These chemical shifts are related to the secondary structure of TF_oc-rings. The spectra with 200 ms mixing time in Figure 4C and 4D include inter-residue correlations of TF_oc-ring carbons. These cross peaks can provide information on a tertiary structure of TF_oc-rings based on through-space distances. Signals of the sample in POPC were generally strong, while the spectrum of the sample in DMPC showed better resolution. Since DMPC carries fully saturated hydrocarbon chains and POPC has one unsaturated one, the phase transition temperature of the former membranes (~18 ^oC)[17] is higher than that (~-3 ^oC)[18] of the latter. Therefore, POPC should be more mobile than DMPC at the same temperature. More mobility in the gel state may have resulted in broader signals in TF_oc-ring/POPC. POPC carries more protons than DMPC. This would increase the cross-polarization efficiency, leading to higher intensity of the signals in TF_oc-ring/POPC. The assignment in terms of amino acid residues are made and indicated in the spectra. Although there is a difference in resolution, we could compare these peaks in detail. It revealed that these spectra are essentially similar to each other, suggesting that TF_oc-rings both in DMPC and in POPC membranes have similar structures. This means that although the lipid-protein interactions affect the dynamics of the rings, they do not change the structure itself. Since POPC membranes are similar to the physiological lipids, it would be appropriate for analysis of functions of the TF_oc-rings. In contrast, DMPC membranes would be suitable for detailed structural analysis because of the better resolution of the TF_oc-ring spectra.

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