Active-Site Structure of the Thermophilic F₀c-Subunit Ring in Membranes Elucidated by Solid-State NMR

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ABSTRACT  F_oF₁-ATP synthase uses the electrochemical potential across membranes or ATP hydrolysis to rotate the F_o-c-subunit ring. To elucidate the underlying mechanism, we carried out a structural analysis focused on the active site of the thermophilic c-subunit ring (TF_o,c) in membranes with a solid-state NMR method developed for this purpose. We used stereo-array isotope labeling (SAIL) with a cell-free system to highlight the target. TF_o,c oligomers were purified using a virtual ring His tag. The membrane-reconstituted TF_o,c oligomer was confirmed to be a ring indistinguishable from that expressed in E. coli on the basis of the H⁺-translocation activity and high-speed atomic force microscopic images. For the analysis of the active site, 2D ¹³C-¹³C correlation spectra of TF_o,c rings labeled with SAIL-Glu and -Asn were recorded. Complete signal assignment could be performed with the aid of the C₁₃-C₃ correlation spectrum of specifically ¹³C,¹⁵N-labeled TF_o,c rings. The C₅ chemical shift of Glu-56, which is essential for H⁺ translocation, and related crosspeaks revealed that its carboxyl group is protonated in the membrane, forming the H⁺-locked conformation with Asn-23. The chemical shift of Asp-61 C₁ of the E. coli c ring indicated an involvement of a water molecule in the H⁺ locking, in contrast to the involvement of Asn-23 in the TF_o,c ring, suggesting two different means of proton storage in the c rings.

INTRODUCTION

H⁺-driven F_oF₁-ATP synthase plays a major role in energy production in most organisms. It consists of a water-soluble F₁ part and a membrane-integrated F_o part (Fig. 1A). It converts the electrochemical potential generated by the H⁺ gradient across membranes into the rotation of the c-subunit ring (red in the figure) in F_o and then into that of the γ subunit in F₁, or vice versa (1). The c ring comprises 8–15 c subunits depending on the biological species (2–7). The mechanism underlying the energy conversion at F_o is one of the major unresolved issues regarding F_oF₁-ATP synthase. The proton translocation across F_o is the key process in the energy conversion and consists of five steps, namely, H⁺ transfer through the first H⁺ channel, H⁺ transfer between the a and c subunits, the rotation of the c ring, H⁺ transfer between the c and a subunits, and H⁺ transfer through the second H⁺ channel. The conserved acidic amino acid residue in the c subunit is known to be essential for this process. Therefore, the active-site structure of the c ring, including the conserved acidic residue, plays an important role in the second and fourth steps. The crystal structures of the c rings of H⁺-driven F_oF₁-ATP synthases in detergent micelles have been reported for chloroplasts (8), a cyanobacterium (9), Bacillus pseudofirmus (10), and yeast mitochondria (11). Those of F-type (5) and V-type (12,13) Na⁺-driven ATPases from bacteria have also been reported. In the case of H⁺-driven F_oF₁, the structure of the active site, including the conserved acidic amino acid, was found to be different in the abovementioned crystals. This may be due to the different detergent conditions. Therefore, investigation of a membrane-embedded c ring is required to clarify the structure and function of the active site.

We previously reported a solution structure of the c subunit of thermophilic Bacillus PS3 H⁺-driven F_oF₁-ATP synthase (TF_o,F₁) (14). However, the structure of its c-subunit ring (TF_o,c ring) has not yet been determined. The number of subunits in the ring was determined to be 10 using a combination of gene engineering and a biochemical method (4). The amino acid sequence of TF_o,c in one-letter codes is as follows:

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MHLGV LAAAI¹⁰ AVGLG ALGAG²⁰ IGNGL
IVSRT³⁰ IEGIA RQPGL⁴⁰ RPVLQ TTMPI⁵⁰
GVALV EALP¹⁵ IGVVF SFYL⁷⁰ GR
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Here, E56 is the conserved glutamate. The solution structure comprised two a helices folded in a hairpin style, as found in every crystal c-ring structure. The conserved Glu is thought to accept/release a proton from/to a proton channel upon interaction with the a subunit. The purpose of this work is to elucidate the structure of the active site of the TF_o,c ring in lipid membranes using solid-state NMR (ssNMR) in combination with the stereo-array isotope labeling technique.
specific labeling. The TF₀c ring is suitable for such extensive work because of its stability.

The structural analysis of membrane proteins by ssNMR has been extensively carried out (15–18). However, it is still challenging. We performed ssNMR analysis of $^{13}\text{C}$, $^{15}\text{N}$ uniformly and specifically labeled $\text{FoF}_1$ subunit rings from *Escherichia coli* (19,20). Although site-specific isotope labeling is a risky and tedious task. In contrast, the fully labeled sample suffered from severe signal overlapping. Therefore, we developed a more efficient, specific labeling method in this work. Cell-free expression can provide a variety of methods for isotope labeling and reconstitution of $\text{FoF}_1$. We used stereo-array isotope-labeled amino acids (SAIL-AA) (21) and a wheat-germ extract (WGE) system for specific labeling of $\text{FoF}_1$ rings. The most critical point in cell-free production is the formation of active rings from the synthesized $c$-subunit monomers. We examined this by referring to the $c$ rings prepared from $\text{FoF}_1$-ATP synthase. Since the active-site structures in crystals in micelles were different from one another, we focused our attention on this difference. We successfully prepared specifically isotope-labeled $\text{FoF}_1$ rings and obtained structural information about the active site in lipid membranes. Our results provide insights into the chemical mechanism underlying the proton locking in $\text{FoF}_1$ rings and H⁺ transfer at the interface of the $c$ and $a$ subunits.

**MATERIALS AND METHODS**

The experimental details are presented in the Supporting Material.

**Synthesis of $\text{FoF}_1$ in a WGE system**

A mutated protein, S2H-$\text{FoF}_1$, was synthesized from messenger RNA (mRNA) in a WGE HPOWG translation mixture at 26°C for ~24 hr with a supply of amino acids from the dialysis buffer according to a previously described method (22). Because only S2H-$\text{FoF}_1$ was used in this work, the term “S2H-$\text{FoF}_1$” will be employed instead of S2H-$\text{FoF}_1$ hereafter. For isotope labeling of Ala, Gly, Val, Glu, and Asn, $^{13}\text{C}$,$^{15}\text{N}$-labeled ones were used in the amino acid mixture. SAIL-Asn and -Glu were used at 0.12 mM with the other amino acids deuterated. After the reaction, the collected product was solubilized and applied to Ni-NTA chromatography (23). The column was eluted with an imidazole gradient (20–200 mM) in 100 mM KCl, 0.5% sodium deoxycholate (DOC), 0.15% decyl maltoside (DM), and 20 mM HEPES-KOH buffer (pH 7.5), and the $\text{FoF}_1$ fraction was collected. This fraction was used for reconstitution into membranes in some cases. Otherwise, this fraction was further applied to an anion exchange HiTrap Q (HQ) column. The HQ column was eluted with a NaCl gradient (0–50 mM) in 0.2 mM EDTA, 10 mM Tris-HCl buffer (pH 7.5). The purity of $\text{FoF}_1$ was confirmed by Tricine-SDS-PAGE and western blotting.

**Reconstitution of $\text{FoF}_1$ oligomers into lipid bilayers**

We solubilized 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DMPC-$d_{14}$, 98% $^2\text{H}$, 95% chemical purity) in a 0.5% DM solution. This solution was slowly titrated into the protein solution (protein concentration: 0.5 mg/mL; lipid/protein (molar ratio) = 24) under mixing at room temperature. The detergent was removed with Bio-Beads SM-2 (20–50 mesh) followed by dialysis against 100 mM KCl, 0.4 mM NaN₃, 20 mM HEPES-KOH/Tris-HCl buffer (pH 7.5), and then against 0.4 mM NaN₃, 10 mM HEPES-KOH/Tris-HCl buffer (pH 7.5). The collected precipitate was subjected to freeze-and-thaw treatment 10 times. In the case of SAIL-AA-labeled samples, the buffer was replaced with 0.4 mM NaN₃, 10 mM deuterated Tris-$^2\text{HCl}$ buffer (in $^2\text{H}_2$O, pH 7.5).

**Activity analysis and high-speed atomic force microscopy imaging**

Active $\text{FoF}_1$ was reconstituted into liposomes. Then, the proton translocation activity was measured according to a previously described method (23,24). A laboratory-built, high-speed (HS) atomic force microscope was used (25). Atomic force microscopy (AFM) images were obtained in the tapping mode. A droplet of $\text{FoF}_1$/DMPC-$d_{14}$ liposome solution was deposited on a freshly cleaved mica surface. After incubation for 5 min, the surface was washed with a solution of 10 mM MgCl₂, 50 mM KCl,
20 mM Tris-HCl (pH 7.5). The liposomes retained on the surface were subjected to HS-AFM in the same buffer.

ssNMR measurements

NMR measurements were performed with Varian Infinity-plus 600 and 700 spectrometers operating at 14.09 and 16.44 T, respectively. Broadband double- and triple-resonance magic-angle-spinning (MAS) probes for 3.2 and 2.5 mm rotors were used. The rotor size and MAS rate used for measurements of two-dimensional (2D) spectra were 3.2 mm and 12.5 kHz unless otherwise specified. The amount of protein used was ~3 mg/3.2 mm rotor. The probe temperature was set at 233 K. The sample temperature was approximately −25°C, because the water signal disappeared in the region of the set temperature from −15 to −20°C due to freezing. 2D dipole-assisted rotational resonance (DARR) (26) and 2D C_{13} correlation (27) spectra under MAS were recorded with a 3 s repetition time. The $^{13}$C chemical shift (CS) was referenced to 2,2-dimethylsilapentane-1-sulfonic acid (DSS) by using the methine carbon signal of adamantane at 40.5 ppm relative to DSS.

RESULTS

Cell-free production of TF$_{6,c}$ with the WGE system and isolation of TF$_{6,c}$ oligomers

In the absence of a detergent and liposomes, a 1.5–2 mg TF$_{6,c}$/mL reaction mixture was obtained as precipitate. However, the precipitate could not be efficiently solubilized by 2% DOC. To improve the recovery, we examined the effect of an additive such as detergent micelles (Tween 20, NP-40, Triton-X, sucrose monolaurate, or dodecyl maltoside (DDM)) or soybean phosphatidylcholine (sPC) liposomes on expression. The best recovery was achieved in the presence of sPC liposomes. Thus, we decided to synthesize TF$_{6,c}$ with WGE in the presence of 0.44% sPC liposomes. We also used 2% DOC to promote ring formation in the solubilization step because it has been used to isolate TF$_{6,c}$ rings from E. coli membranes (23). To isolate the TF$_{6,c}$ rings, we used a virtual His tag. Although TF$_{6,c}$ monomer has only one His at the second position, a decamer ring, for example, should have a noncovalent ring His$_{10}$ tag. The tricine-SDS-PAGE images in Fig. S1 A indicate the feasibility of this His tag in Ni-NTA chromatography. The obtained TF$_{6,c}$ oligomer amounted to a 0.8–1.6 mg/ml reaction mixture. Then, this fraction was applied to an HQ column. The Tricine-SDS-PAGE image at each purification step is presented in Fig. S1 A. Blue native PAGE revealed that the obtained oligomers are homogeneous (Fig. 1 B). To determine whether the isolated oligomers are functional TF$_{6,c}$ decamers, we measured the H$^+$-translocation activity of F$_5$F$_1$ reconstituted from the isolated TF$_{6,c}$ oligomers, the TF$_{6,c}$ab$_2$ complexes, and TF$_1$ in sPC liposomes (23). The results are presented in Fig. 1 C. An active F$_5$F$_1$ will translocate H$^+$ from the outside to the inside of the liposome at the expense of ATP added to the outside. The acidification of the inside quenches the fluorescence of 9-amino-6-chloro-2-methoxyacridine (ACMA). The quench rate of the reconstituted TF$_5$F$_1$ including cell-free c oligomers was 69.5%, whereas that of reconstituted TF$_5$F$_1$ including the intact TF$_{6,c}$ ring isolated from TF$_5$F$_1$ expressed in E. coli membranes was 71%, i.e., the activity of the former was almost the same as that of the latter. The analysis of the activity of mutant TF$_{6,c}$ incorporating genetically fused TF$_{6,c}$ oligomer (from 2-mer to 14-mer) revealed that only TF$_{6,c}$F$_1$ incorporating the decamer ring was active (4). Therefore, we can conclude that the TF$_{6,c}$ oligomers obtained from the cell-free system are decamer rings as in the case of the intact ones.

Reconstitution of TF$_{6,c}$ oligomers into lipid membranes and HS-AFM imaging

Purified TF$_{6,c}$ was reconstituted into DMPC-$d_{54}$ membranes. The detergent was removed by a combination of Bio-Beads treatment and dialysis as described above. The obtained membrane preparation was characterized by sucrose-density gradient (10–44%) ultracentrifugation as in the case of the EF$_{6,c}$ ring (19). The membrane fraction gave a sharp single band that was different from the band for pure DMPC liposomes. To elucidate the macroscopic architecture of the TF$_{6,c}$ oligomers in DMPC-$d_{54}$ membranes, we obtained successive HS-AFM images of the preparation on the mica surface in the presence of buffer at room temperature. As a reference, we observed images of the intact TF$_{6,c}$ rings in DMPC-$d_{54}$ membranes. They were isolated from TF$_5$F$_1$ expressed in E. coli membranes and well characterized in our previous study (23). The images are presented in Fig. 2 A. A torus-shaped substance with a pore at the center could be clearly observed. Since the DMPC-$d_{54}$ membranes are attached to the mica surface, the TF$_{6,c}$ rings should be oriented perpendicular to it. However, it was difficult to discriminate the two different bottom ends (the loop and termini sides) of the TF$_{6,c}$ cylinder. The ring diameter was measured using the top position of well-defined rings (see Supporting Material for Materials and Methods). The diameter of the TF$_{6,c}$ rings expressed in E. coli was 3.8 ± 0.2 nm (average of 35 images). In Fig. 2 B, we can directly confirm the ring structure of the oligomeric TF$_{6,c}$ prepared with the WGE cell-free system. Its diameter was 3.9 ± 0.2 nm (average of 40). Therefore, the oligomeric TF$_{6,c}$ is indistinguishable from the intact TF$_{6,c}$ decamer ring in terms of the macroscopic structure under physiological conditions.

The advantage of HS-AFM is that it allows one to not only obtain images of a c ring under physiological conditions but also follow a change in the same ring as a function of time. As can be seen in Fig. 2 C, a nonring structure was generated from time to time during scanning. Its curvature changed as a function of the scanning time. This could be ascribed to the degradation caused by the cantilever. The change in the curvature length presented in Fig. 2 C can be summarized as 1.2 × N nm (N, integer), suggesting that regular units are removed from the ring by the cantilever. Although there is no direct evidence, the c subunit

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observed circumference is 12.2 nm, \(12.2 = 1.2 \times N_{\text{ring}}\). This leads to the most possible \(N_{\text{ring}} = 10\). The most possible number is consistent with the TF\(_{e,c}\) decamer ring, strongly suggesting that the removed regular unit is a \(c\) subunit.

**ssNMR measurements**

Taking advantage of the WGE cell-free system, we prepared specifically labeled TF\(_{e,c}\) rings reconstituted in DMPC-\(d_{54}\) membranes to analyze its active site. We focused on Glu-56 (E56) and Asn-23 (N23) because we had previously suggested that the side chain of E56 in the C-terminal helix would interact with that of N23 in the N-terminal helix in the absence of the \(a\) subunit in the membrane (14). Because of the low yield and low impurity, the preparation obtained from the Ni-NTA chromatography was used for NMR analysis unless otherwise specified. To obtain information on the TF\(_{e,c}\) structure in general, and site-specific assignment of E56 and N23, Ala, Gly, Val, Glu, and Asn in TF\(_{e,c}\) were specifically \(^{13}\text{C}\)- and \(^{15}\text{N}\)-labeled ([\(^{13}\text{C},^{15}\text{N}\)]AGVEN-TF\(_{e,c}\)). The other amino acid residues were nonlabeled ones. Because Ala (nine residues), Gly (11 residues), and Val (eight residues) are abundant, they can provide information about the general structure. In contrast, there are only one Asn and three Glus that will provide specific information about the active site. A 2D DARR spectrum of [\(^{13}\text{C},^{15}\text{N}\)]AGVEN-TF\(_{e,c}\) under MAS with a mixing time of 15 ms is presented in Fig. 3 A. The spectrum in the aliphatic region is relatively simple and can be readily assigned in terms of amino acids. Because N23 is unique, the assignment is clear on the basis of its spin connectivity (solid lines). The crosspeaks of Ala and Asn can be used to check the intactness of the TF\(_{e,c}\)-ring structure obtained from the cell-free system, because they were also isolated in the DARR spectrum of the uniformly \(^{13}\text{C}\)-labeled TF\(_{e,c}\) ring obtained from TF\(_{e,F1}\) (23). The CSs of N23 \(C^\alpha\), \(C^\beta\), and \(C^\gamma\) were identical for these two spectra. The overlapped crosspeaks of Ala \(C^\beta\)/

\[C^\delta\] of the uniformly labeled TF\(_{e,c}\) ring obtained from TF\(_{e,F1}\) are presented in the inset of the figure. The peculiar pattern is identical to that of this spectrum. Although the crosspeaks of Val \(C^\omega/C^\delta\) of the uniformly labeled TF\(_{e,c}\) ring partially overlap those of Pro, they are also similar to those in this spectra. Since most of Ala, Val, and Asn are distributed in the transmembrane region, it is strongly suggested that the ring structure composed of the TF\(_{e,c}\) monomers produced with the cell-free system is indistinguishable from that of the intact TF\(_{e,c}\) ring also at atomic resolution.

To obtain sequential information, we recorded a 2D \(C^\omega_{i+1}-C(C^\alpha_i)\) correlation spectrum under MAS of the same sample (Fig. 3 B). Here, the magnetization was transferred from \(^{13}\text{C}\)^\[i\] to \(^{13}\text{C}\)^\[i+1\] through \(^{15}\text{N}\)^\[i\] and \(^{13}\text{C}\) = \(\text{O}_{1}\). Now, well-resolved crosspeaks could be obtained because of the uniqueness of successive pairs (for example, A11V12) in the sequence. Sequential walking could be
performed, for example, from A11V12 to V12G13 through broken lines. The horizontal line crossed the diagonal line at the CS of V12 at the C\text{a} axis. A vertical line starting from this point found the crosspeak of V12G13. There were two crosspeaks, which might be ascribed to V55E56. However, we could identify the correct one because of the sequential connectivity of V55E56 and E56A57 (indicated by solid lines). The other crosspeak was assigned to V52A53, which was the only possible pair in the region. Thus, the C\text{a} CSs of A11, V12, G13, E32, G33, G51, V52, A53, V55, E56, A57, V63, and V64 were obtained unequivocally. From this result, the crosspeak of Glu observed in both ω1/ω2 and ω2/ω1 (aliphatic areas in the DARR spectrum) turned out to be due to E56 C\text{a}/C\text{b}. The obtained CSs are summarized in Table S1. These CSs and the CS confinement based on the C\text{a}/C\text{b} crosspeaks in the DARR spectrum suggest that the backbone structure represented by these residues is composed of α-helical conformations as in the cases of the intact TF\text{c} ring (23) and the solution structure (28).

Then, we focused our attention on E56 and N23. To obtain simple and well-resolved spectra with analyzable intensities, we labeled TF\text{c} with SAIL-Glu and -Asn using the cell-free system (SAIL-EN TF\text{c}). The stereochemical labeling sites are available in Kohno and Endo (22). We used deuterated amino acid residues, except for Glu and Asn. DARR spectra of the SAIL-EN TF\text{c} rings in DMPC-d\text{54} membranes, with mixing times of 100 and 50 ms, are presented in Fig. 4, A and B, respectively. Those with mixing times of 15, 200, and 400 ms are presented in Fig. S2, A–C, respectively. In Fig. 4 A, the C\text{a}/C\text{b} crosspeaks of N23 and E56 can be identified on the basis of the assignment made for the [13C,15N]AGVEN-TF\text{c}-ring signals. The resolution in the carbonyl carbon region was better than that in the aliphatic region. Typical spin connectivity for C\text{a}, C\text{b}, C\text{g}, C\text{d}, and C\text{0} of E56 is represented by solid lines. The CSs of E56 were determined from those crosspeaks. For the assignment of the other two Glu residues, an expansion of the upper half of the carbonyl region is presented in the inset.

**FIGURE 3** 2D 13C–13C correlation DARR (A) and 2D C\text{a,i+1}/C\text{a,i} correlation (B) spectra of [13C,15N]AGVEN TF\text{c}-rings in DMPC-d\text{54} membranes at 14.09 T. (A) Mixing time = 15 ms. The amino-acid-specific assignment is presented with a one-letter code. The data size was 1024(d1) × 408(d2) for 60 × 60 kHz spectral widths, and n = 232 scans. The inset is the Ala C\text{b}/C\text{a} region of the DARR spectrum in Yumen et al. (23). (B) Sequential walking (solid and broken lines) and unambiguous assignment of the crosspeaks are presented; assignment of overlapping peaks is in parentheses. The data size was 1024(d1) × 100(d2) for 60 × 50 kHz spectral widths, and n = 840 scans. A squared sine bell with –80° shift and an exponential with 50 Hz broadening factor and linear prediction were used as window functions in Fourier transformation for the d1 and d2 axes, respectively, in A, and the same window functions with 150 Hz broadening factor were used in B.

**FIGURE 4** (A and B) 2D 13C–13C correlation DARR spectra of SAIL-EN TF\text{c} rings in DMPC-d\text{54} membranes at 16.44 T with mixing times of 100 (A) and 50 (B) ms. Connectivities for N23 and E56 are presented as broken and solid lines, respectively, and those for E32 and E39 are indicated by solid and broken arrows, respectively. (A) The top half of the carbonyl carbon region is expanded in the inset. The data size was 1024(d1) × 210(d2) for 70 × 70 kHz spectral widths, and n = 80 scans. A squared sine bell with –80° shift and an exponential with 50 Hz broadening factor and linear prediction (d1) and an exponential with 50 Hz broadening factor and linear prediction (d2).
of Fig. 4 A. The two Glus are designated as Eₐ and Eₜ for convenience. They are indicated by solid and broken arrows, respectively. Because the two C\(^{13}C\)/C crosspeaks indicated by the two arrows in the inset are separated from each other, the assignment (except for Eₜ C\(^{13}C\)) could be performed using the connectivity with them (see Supporting Material for Results). The Eₐ C\(^{13}C\) CS was determined from the isolated C\(^{13}C\)/C crosspeak in Fig. 4 B. The CS differences of (Eₐ-Eₕ) and (Eₜ-Eₕ) for C\(^{13}C\) were 1.1 and 0.3 ppm, respectively. Because the CS difference between E₃₂ and E₅₆ in Fig. 3 B was larger than 1.0 ppm, Eₐ and Eₜ should be assigned to E₃₂ and E₃₉, respectively. The assignment was also confirmed with the connectivities in the spectra at 15, 200, and 400 ms mixing times (Fig. S2). The assigned CSs are summarized in Table S2. CSs obtained from SAIL signals were corrected using a calibration table kindly provided by Prof. Masatsune Kainosho.

The CS of E₅₆ C\(^{13}C\) is significantly different from those of E₃₂ and E₃₉. It is 174.5 ppm for E₅₆, but 183.1 and 182.6 ppm for E₃₂ and E₃₉, respectively. According to a report by Gu et al. (29), isotropic CSs of E₃₂ and E₃₉ C\(^{13}C\) are in the range for deprotonated carboxyl groups. In contrast, that of E₅₆ C\(^{13}C\) is basically in the range for protonated carboxyl groups. However, the isotropic CS of the deprotonated carboxyl group was reported to go down to 172 ppm in crystals (29). To make the protonation state of E₅₆ convincing, we performed CS tensor analysis of E₅₆ C\(^{13}C\).

There is a strong correlation between the principal CS tensor elements (\(\delta_{11}, \delta_{22}, \text{ and } \delta_{33}\)) and chemical structures in solid state (29,30), namely, \(\delta_{11}\) is larger than 250 ppm for the protonated carboxyl groups. Also, \(\delta_{22}\) provides information about the strength of a hydrogen bond involving the carboxyl group. To determine the principal CS tensor elements of E₅₆ C\(^{13}C\), we measured 1D spectra of the SAIL-EN TFₜₙ₋ₜₜₙ C ring (purified with NTA-Ni and HQ columns) in membranes at spinning rates of 4.0, 4.3, 4.5, and 5.0 kHz at 16.44 T. The spectra at spinning rates of 5.0 and 4.5 kHz are presented in Fig. 5 A. In spite of the low signal/noise ratio, the intensity change could be followed, as indicated by arrows in the figure. The side-band intensity data for the four spinning rates were analyzed with Herzfeld-Berger analysis software (HBA 1.6.12) (31,32). Details of the analysis are described in the Supporting Material for Results. The obtained principal elements of the CS tensor were (264 ± 11, 139 ± 7, 121 ± 10) ppm. Because \(\delta_{11}\) is larger than 250 ppm, we can safely conclude that this carboxyl group is protonated. Furthermore, the value of \(\delta_{22}\) suggests that the E₅₆ carboxyl group is involved in weak hydrogen bonding (29).

To obtain structural information around E₅₆, we carried out a \(^1\)H-driven spin-diffusion experiment on the SAIL-EN TFₜₙ₋ₜₜₙ C rings with a mixing time of 700 ms. The relevant parts of the spectrum are presented in Fig. 5 B and Fig. S2 D, in which the crosspeaks of N2₃ C\(^{13}C\) with E₅₆ C\(^{13}C\), C\(^{15}C\), and C\(^{15}C\) can be seen, revealing that they are close to each other.

### DISCUSSION

#### Strategy for analyzing the active site of the TFₜₙ₋ₜₜₙ C ring in membranes

To analyze the active site of the TFₜₙ₋ₜₜₙ C ring in membranes, we used specific isotope labeling and CP/MAS NMR. Because this is a membrane protein, we had to develop a method for expression with the WGE cell-free system, formation and purification of the rings, and their reconstitution into membranes. To obtain well-defined structural information about the active site by ssNMR, we used SAIL-Glu and -Asn. This work revealed that the combination of expression with the WGE cell-free system, formation and purification of the rings, and their reconstitution into membranes. This work revealed that the combination of expression with the WGE cell-free system, formation and purification of the rings, and their reconstitution into membranes.

Cotranslational insertion of membrane proteins into liposomes in the WGE cell-free system was previously reported (33). Although TFₜₙ₋ₜₜₙ C rings were artificially produced in the cell-free system, they assembled into decamer rings as in the case of TF₉ₕ₋₉ₕ₋₉ₚᶜ formation in vivo. This was verified by the proton-translocating activity, and warranted by the
macroscopic and microscopic structures based on HS-AFM images and NMR spectra, respectively. This fact strongly suggests that the number of $c$ subunits that form a ring in lipid membranes is determined by their primary sequence.

The SAIL-EN $TF_{o,c}$ rings in the DMPC membranes provided high-resolution ssNMR spectra and structural information in combination with the $^{13}$C-$^{15}$N]AGVEN-$TF_{o,c}$ rings. The sensitivity was better for the SAIL-EN $TF_{o,c}$ ring than for the AGVEN-labeled one in spite of similar intrinsic line widths (see Supporting Material for Discussion). The better sensitivity likely is due to the higher efficiency in CP and DARR for the SAIL-EN $TF_{o,c}$ rings. The longer $T_1$ of protons and carbons would improve CP efficiency, and the longer $T_2$ and $T_1$ of carbons may suppress the decay in the evolution and mixing periods of DARR, respectively (26,34,35). The efficient DARR in the presence of only a limited number of protons revealed that the combination of a single $^{13}$C-$^1$H group for heterogeneous broadening and a single $^1$H in the direct neighbor for homogeneous broadening is the core spin system for generating an effective $^{13}$C polarization transfer through DARR. The method developed in this work also can be applied to focused analysis of other membrane proteins in lipid membranes.

Structure and function of the active sites of $F_{o,c}$ rings in membranes

The active-site structure of the $c$ subunit involving the conserved acidic amino acid residue is a key factor in elucidating the proton-transfer mechanism at the interface between the $a$ and $c$ subunits in $F_o$. This is also important for understanding the mechanism of the proton translocation across $F_o$. Our results provide structural information about the active site of the $TF_{o,c}$ ring embedded in membranes. The CS of $E56$ C$^d$ showed that its carboxyl group was protonated in membranes. The presence of the crosspeaks between $N23$ C$^f$ and $E56$ C$^d$, C$^r$, and C$^g$ in the spin diffusion spectrum revealed that the side chain of $E56$ points to the inside of the ring and interacts with $N23$, forming a proton-locked conformation. Furthermore, $\delta_{22}$ of $E56$ C$^d$ suggested that the carboxyl group is involved in weak hydrogen bonding. This is also supported by the unique isotropic CS of $N23$ C$^r$ (170.6 ppm), which is smaller than the average value found in the BMRB database (176.5 ppm). Actually, the isotropic CS of an amide carbon was reported to be proportional to the length of a hydrogen bond in peptide bonds because of a change in $\delta_{22}$ (36), i.e., the smaller the CS, the longer the hydrogen bond.

Our results can be compared with the active-site structures of the $F_{o,c}$ rings reported thus far. Actually, they were all different from one another. The cyanobacterium $c_{14}$ assumed a proton-locked conformation with the conserved carboxyl group on the C-terminal (outer) helix, forming hydrogen bonds with a glutamate side chain on the N-terminal (inner) helix and others (9). Here, the protonated carboxyl group is locked by these hydrogen bonds. The chloroplast $c_{14}$ assumed a modified proton-locked conformation, missing the hydrogen bond with the glutamine in spite of its presence in the N-terminal helix (8), and Bacillus pseudofirmus $c_{13}$ assumed a water-involved ion-locked conformation, with the carboxyl group hydrogen bonding only with a water molecule (10). In this case, there is no polar amino acid residue in the active-site region of the N-terminal helix. The presence of the water molecule is similar to the Na$^+$-coordination structure in $F_{o,c}$ from Ilyobacter tartaricus (5). Since the acidic side chains pointed into the rings in all three cases, they were specified as closed conformations in general. In contrast to them, the conserved carboxyl group (E59) of the yeast mitochondrial $c_{10}$ (Y$F_{o,c}$) was located outside of the ring in both the protonated and deprotonated states (11). They are called open conformations. The reason for the open-form formation was attributed to the property of detergent by molecular-dynamics simulation. Actually, the Y$F_{o,c}$E59 exhibited a closed form in a crystal structure of the Y$F_{o,c}$E59 complex (37). Our work reveals that the closed form is the correct conformation in lipid membranes. The open form was assumed to be a conformation at the interface between the $a$ and $c$ subunits (11).

Furthermore, classification of the $c$ subunits as an E/D-only type or mixed type on the basis of their primary sequence was proposed to be useful for understanding the active-site structure (10). In the former, the conserved Glu or Asp is the only residue that can be predicted to be involved in ion coordination, whereas the latter has more polar residues. To make the difference clearer, we use the term “E/D-plus type” instead of mixed type in this discussion. It turned out so far that the closed conformation in the E/D-only-type ring ($c_{13}$) carries a water molecule coordinated to the carboxyl group, whereas those in the E/D-plus-type rings ($c_{14}$ and $c_{15}$) do not in the high-resolution crystal structures.

Although the whole structure of $TF_{o,c}10$ is not yet known, our result clearly reveals that its conserved carboxyl group is protonated and assumes a proton-locked conformation in lipid membranes. Our result is not consistent with the modified proton-locked conformation of $c_{14}$, because of the N23/E56 interaction. $TF_{o,c}10$ is the simplest example of the E/D-plus type, since it has only N23 and E56 as the polar residues in the active-site pocket. The protonated state of E56 in $TF_{o,c}10$ and the weak nature of its hydrogen bonding to N23 strongly suggest the absence of a water molecule in this pocket, as in the cases of $c_{14}$ and $c_{15}$ (Fig. 6 A). In contrast, the E. coli $F_{o,c}$-subunit ring (EF$_{o,c}$10) is classified to the E/D-only type, the structure of which is also not yet known. It carries no polar residue other than the conserved D61 in the active-site pocket. We performed an ssNMR analysis of EF$_{o,c}$10 in lipid membranes (20). The CS of D61 C$^F$ was 179.6 ppm. Because this was determined using a chemically synthesized EF$_{o,c}$ with specific $^{13}$C labeling at A24 C$^d$ and E56 C$^d$, this value is reliable. This CS is
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significantly different from the 174.5 ppm of E56 C$^\beta$ determined in this work. This fact reveals that the chemical nature of the conserved carboxyl group of EF$_{c_{10}}$ is significantly different from that of TF$_{c_{10}}$. The CS of D61 C$^\gamma$ suggests that its carboxyl group is either protonated and involved in a strong hydrogen bonding, or is deprotonated and involved in hydrogen bonding (29). Since there is no polar residue to form hydrogen bonds or to coordinate a cation in the active-site pocket, the only possible hydrogen-bonding partner should be a water molecule, as in the case of c$_{13}$ (Fig. 6B). The involvement of water is also consistent with the CSs of propionic acid and butyric acid diluted in water, namely, 180.02 and 179.22 ppm for COOH, respectively, and 184.95 and 184.10 ppm for COO$^-$, respectively (38). The shorter side chain of D61 may provide a space for the water in the pocket. The presence of water in the pocket may also explain the surviving activity of EF$_{c_{10}}$ on the double mutations A24D/D61G and A24D/D61N (39). Although the essential carboxyl group is located on the inner helix in this case, the hydrogen-bonded water molecule would mediate the proton transfer between D24 and the proton channels, responding to the electrostatic interaction with the conserved Arg in the $a$ subunit. Now, we can conclude that the correlation between the water involvement in proton locking and the E/D-only type also holds for EF$_{c_{10}}$. This strongly suggests that there are two means of proton storage in the c rings in lipid membranes, namely, polar-group-involved proton locking and water-involved proton locking, as presented in Fig. 6. The weak hydrogen bond in TF$_{c_{10}}$ and the presence of the open form in the protonated state in YF$_{c_{10}}$ strongly suggest that the barrier between the closed and open forms in the protonated state is low enough to facilitate the closed/open conversion through thermal fluctuations. The major form will be determined by the hydrophilicity of the environment induced by the interaction with the $a$ subunit or lipids. The deprotonation and protonation probably take place in the open form. In the water-involved type, however, there is a possibility that the carboxyl group leaves the proton in the pocket upon conversion from the closed to the open form under the effect of the positive charge in the $a$ subunit, because the water molecule can stabilize it. The deprotonation in the active site generates an ion pair similar to the Na$^+$-locking type. If this is the case, they may have similar mechanisms for cation transfer. There may be also an effect of F$_1$ on the structure of the active site, which we cannot evaluate at this point.

In summary, we have elucidated the chemical nature of the conserved acidic amino acid residue of the TF$_{c_{10}}$ and EF$_{c_{10}}$ rotors in lipid membranes using ssNMR in combination with the stereo-array isotope specific labeling. It was revealed that the conserved E56 of the former assumed a proton-locked conformation through an interaction with N23. However, D61 of the latter was involved in the interaction with a water molecule. In the case of TF$_{c_{10}}$, the fluctuation of the E56 side chain between the closed and open forms could easily take place because of weak hydrogen bonding with N23.

SUPPORTING MATERIAL

Three figures, three tables, References (40–44), detail of Materials and Methods, PAGE images of the preparations in expression and purification, and relevant NMR data and analysis are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(13)05762-7.

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REFERENCES


