The phototroph-specific β-hairpin structure of the γ subunit of FoF1-ATP synthase is important for efficient ATP synthesis of cyanobacteria

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The FoF1 synthase produces ATP from ADP and inorganic phosphate. The γ subunit of FoF1 ATP synthase in photosynthetic organisms, which is the rotor subunit of this enzyme, contains a characteristic β-hairpin structure. This structure is formed from an insertion sequence that has been conserved only in phototrophs. Using recombinant subcomplexes, we previously demonstrated that this region plays an essential role in the regulation of ATP hydrolysis activity, thereby functioning in controlling intracellular ATP levels in response to changes in the light environment. However, the role of this region in ATP synthesis has long remained an open question because its analysis requires the preparation of the whole FoF1 complex and a transmembrane proton-motive force. In this study, we successfully prepared proteoliposomes containing the entire FoF1 ATP synthase from a cyanobacterium, Synechocystis sp. PCC 6803, and measured ATP synthesis/hydrolysis and proton-translocating activities. The relatively simple genetic manipulation of Synechocystis enabled the biochemical investigation of the role of the β-hairpin structure of FoF1 ATP synthase and its activities. We further performed physiological analyses of Synechocystis mutant strains lacking the β-hairpin structure, which provided novel insights into the regulatory mechanisms of FoF1 ATP synthase in cyanobacteria via the phototroph-specific region of the γ subunit. Our results indicated that this structure critically contributes to ATP synthesis and suppresses ATP hydrolysis.

Photosynthetic organisms utilize FoF1 ATP synthase (FoF1) for a solar-to-chemical energy conversion system to produce ATP, the universal energy currency for cells. Under illumination, photosynthetic electron transport is activated, generating a proton electrochemical gradient (proton motive force, pmf) across the thylakoid membrane, which drives ATP synthesis (1–4). In the dark, or when the pmf is insufficient, FoF1 hydrolyzes ATP as the reverse reaction and transports H+ to the lumen. Therefore, it is reasonable to assume that these organisms have evolved a unique mechanism for regulating FoF1 activity that utilizes pmf for synthesizing ATP.

Although the molecular mechanism underlying its catalytic reactions of ATP synthesis/hydrolysis has been extensively studied over the past decades, the regulation mechanisms of FoF1 exhibit variation among species/organelles and remain unclear. To date, two inhibitory mechanisms, MgADP- and e-inhibition, have been shown to be conserved among bacterial FoF1s and are considered to be important for avoiding the futile ATP hydrolysis reaction and ensuring efficient ATP synthesis (5). In addition, in chloroplasts, it was previously demonstrated that ATP hydrolysis activity is regulated by intramolecular disulfide bond formation/dissociation in the γ subunit (6–8). However, in cyanobacteria, which are the phylogenetic ancestors of chloroplasts (9), this regulation does not occur, as the cyanobacterial γ subunit is devoid of the Cys residues, which are responsible for this redox regulation (10). Furthermore, the cyanobacterial regulatory mechanism for ATP synthase reaction is still obscure.

FoF1 has a dual rotary-motor architecture, which couples the catalytic ATP synthesis/hydrolysis reactions with the electrochemical potential of H+ across the biological membrane (11–14). FoF1 consists of a hydrophilic rotary motor, F1, and an intramembrane rotary motor, Fo (Fig. S1). F1 has a subunit stoichiometry of α3βγδε1, and the rotor shaft moiety composed of the γε subunits rotates inside of the stator subunits, αβεβ, by the energy of ATP hydrolysis. Catalytic sites that are necessary for the ATP synthesis/hydrolysis reactions are present in the β subunit. The membrane-embedded Fo consists of three types of subunits with the stoichiometry of α3βγδε1, where the rotor-ring composed of multimeric c subunits (c-ring) rotates relative to a stator part, ab2. The number of the c subunits in the ring varies among species, from 8 to 15 (15–22). The γ subunit, a part of the rotor shaft of F1 motor, comprises N- and C-terminal helical domains, as well as a protruding globular Rossmann fold domain located between these two helical parts (Fig. 1A). The N- and C-terminal helical domains assemble into an antiparallel coiled-coil stalk, which...
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A

In the central cavity of the catalytic headpiece, $\alpha_3\beta_3$, as a rotor shaft. Moreover, the cyanobacterial and chloroplast $\gamma$ subunits possess an inserted sequence of 30 or 39 amino acid residues within the Rossmann fold (Fig. 1, A and B) (10).

For a long time, the structure of this region remained unknown; however, since 2018, three papers have been published on this subject; two of them pertain to the structures of FoF1 from the chloroplasts of Spinacea oleracea and the other describes the structure of the $\gamma$–$\varepsilon$ subcomplex from a thermophilic cyanobacterium, Thermosynechococcus elongatus BP-1 (T. elongatus) (23–25). It was revealed that this region forms a unique $\beta$-hairpin structure that extends along the central coiled-coil stalk and interacts with the “DELSEED” loop of the $\beta$ subunit (Fig. 1C). This negatively charged loop is highly conserved among FoF1s, and its conformation is important for torque transmission from the catalytic site to the $\gamma$ subunit upon ATP binding, thus affecting ATP hydrolysis activity (26–28).

### Figure 1. Comparison of the $\gamma$ subunits from various organisms.

A, the domain architecture of the $\gamma$ subunit from the phototrophs. They possess a unique insertion sequence within the Rossmann fold domain, between the N-terminal and C-terminal $\alpha$ helices. B, a partial alignment of amino acid sequences around the insertion region. The alignment was generated using the Clustal W multiple alignment tool. Cyanobacteria and rhodophytes have the phototroph-specific insertion, which forms a $\beta$-hairpin structure. Green plants and chlorophytes have an additional sequence, which forms the redox-active $\beta$-hairpin structure. T. elongatus, Thermosynechococcus elongatus BP-1; A. 7120, Anabaena sp. PCC 7120; C. reinhardtii, Chlamydomonas reinhardtii; A. thaliana, Arabidopsis thaliana; S. oleracea, Spinacia oleracea; G. stearothermophilus (formerly called Thermophilic bacillus PS3); S. cerevisiae, Saccharomyces cerevisiae; E. coli, Escherichia coli; B. taurus, Bos taurus; I. tartaricus, Ilyobacter tartaricus. C, schematic representations of the $\beta$-hairpin structures from cyanobacteria and chloroplasts. The $\gamma$ subunit from T. elongatus was superimposed on that from S. oleracea; reduced (left) and oxidized (right) forms are shown. The green and orange-red colors indicate the $\gamma$ subunit and the $\beta$-hairpin structure from T. elongatus (Protein Data Bank ID: 5ZWL); the yellow and beige colors indicate the $\gamma$ subunit and the $\beta$ subunit from S. oleracea (Protein Data Bank ID: 6VON; and right, 6VOH); the blue, sky-blue, and gray colors indicate the reduced and oxidized forms of the phototroph-specific $\beta$-hairpin structure, and the redox-active $\beta$-hairpin, from S. oleracea.
Moreover, Yang et al. (25) recently published the structures of both oxidized/reduced F_{o}F_{1} from spinach chloroplasts. In view of the molecular structure, the interactions between the DELSEED-loop of the β subunit and the β-hairpin structure of the γ subunit did not differ significantly between the oxidized and reduced states (25). It is possible that, in cyanobacteria, which are devoid of this small redox-active β-hairpin, the interaction between the DELSEED loop of the β subunit and the β-hairpin structure of the γ subunit differs from that of chloroplasts. As shown in Figure 1C, the tilting angle of the cyanobacterial β-hairpin appears to be slightly different from that of both the oxidized and reduced forms of the spinach γ subunit. As the only structure of the cyanobacterial γ subunit that has been published to date is the γ-e subcomplex (24), its interaction with the DELSEED loop is not fully understood.

In our previous reports, using recombinant α_3β_3γ derived from T. elongatus, we demonstrated that the phototroph-specific insertion region plays an important role in MgADP inhibition (30). The whole-β-hairpin-truncated α_3β_3γ (α_3β_3γ_ΔA198–222) exhibited higher ATP hydrolysis activity than the wildtype form. We also demonstrated that the two-amino-acid truncations at the turn of the β-hairpin structure (α_3β_3γ_ΔA212–213) resulted in a significant increase in ATP hydrolysis activity via the cancelation of MgADP inhibition. Truncation of an even greater number of amino acids did not raise the activity further (α_3β_3γ_ΔA211–214, α_3β_3γ_ΔA210–215, α_3β_3γ_ΔA209–216 and α_3β_3γ_ΔA205–220) (24). These results strongly suggested that the interaction between the DELSEED loop of the β subunit and the two amino acids located at the turn of the β-hairpin structure is sufficient to induce MgADP inhibition upon ATP hydrolysis. This model was supported by our studies using recombinant α_3β_3γ derived from T. elongatus with nick insertion into the proximal region of the β-hairpin structure (between V_222 and T_223) and cross-linking experiments using disulfide bond formation between the central stalk and the β-hairpin structure within the γ subunit (31, 32).

Those findings prompted us to investigate the correlation between the β-hairpin structure and ATP synthesis and H^+-translocation in cyanobacterial F_{o}F_{1}. To date, the role of this structure in ATP synthesis and H^+-translocation has not been elucidated, partly because those measurements require the preparation of the reproducible quality of proteoliposomes (PLs) and quantitative application of ΔpH across the membrane. Here, we set up a one-step mild purification method of cyanobacterial F_{o}F_{1} from Synechocystis sp. PCC 6803 (S. 6803). S. 6803 is a mesophilic cyanobacterium that allows easy genetic engineering, as this strain is capable of natural transformation with high efficiency in double homologous recombination and its genome harbors sufficient homology to T. elongatus: their amino acid sequences of the γ subunit share 73% homology (Fig. 2A). We analyzed the ATP hydrolysis/synthesis and H^+-translocating activities of the wildtype and β-hairpin-truncated F_{o}F_{1} mutants. Based on the detailed biochemical analyses of these phenomena using phototroph-derived F_{o}F_{1}, we here describe the role of the conserved β-hairpin structure in ATP synthesis. Our results demonstrated that the phototroph-specific β-hairpin structure of the γ subunit of F_{o}F_{1} critically contributes to its ATP synthesis activity, in addition to suppressing ATP hydrolysis. We also performed physiological analyses using S. 6803 and found that the intracellular ATP content was significantly decreased in the β-hairpin-truncated mutants. However, under our experimental conditions, there was no noticeable effect other than the decrease in the amount of ATP.

## Results

### Purification of F_{o}F_{1}, ATP synthase and construction of the β-hairpin-truncated mutants of the γ subunit

To investigate the role of the β-hairpin on ATP synthesis function, we purified F_{o}F_{1} preparations from S. 6803, as follows. First, the His_{10}-tag sequence was genetically fused to the N terminus of the β subunit in S. 6803 (Fig. S2A). The resulting S. 6803 was termed WT strain in the present study. Then, thylakoid membranes were prepared from the WT strain and solubilized with lauryl maltose neopentyl glycol (LMNG). The F_{o}F_{1} preparation was purified from the solubilized fraction by Ni-affinity chromatography. The resulting F_{o}F_{1} preparation was subjected to SDS-PAGE (Fig. 3A, lane WT). Nine types of bands were observed on the gel, which were assumed to correspond to the nine subunits of WT-F_{o}F_{1}. The band intensity of the eight bands other than that corresponding to the c subunit agreed with the expected subunit stoichiometry of S. 6803 F_{o}F_{1}, i.e., α_3β_3γ_εabbδcc (24). The validity of the bands was confirmed by peptide mass fingerprinting after trypsin digestion, N-terminal sequencing, or immunoblot analyses (Table 1 and Fig. 3B). The SDS-PAGE analysis additionally identified a band corresponding to the c subunit above that of the α subunit on the gel (Fig. 3, A and B). As observed previously in the c subunit of Propionigenium modestum (33), the c subunits of S. 6803 formed a stable c-ring architecture that was resistant to the electrophoretic analysis, although the number of c subunits in the ring remained obscure (which was previously reported as 14 in the literature) (21). Furthermore, N-terminal sequencing of the a subunit pinpointed the location of the proteolytic cleavage position between Ala^{42} and Ala^{43}. Based on the genomic sequence of the a subunit in the KEGG database (https://www.genome.jp/kegg; accession number, sl11322), an N-terminal 42-residue segment is suggested to be processed. However, a multiple sequence alignment showed that an N-terminal region composed of 28 residues in S. 6803 was not present in those of other cyanobacteria. This result suggests that the translation of the a subunit starts at a second methionine, Met^{29}, of the open reading frame provided in the database (Fig. S3). If so, the 14 N-terminal residues are the correct segment, which is processed by proteolytic digestion in S. 6803. Because the N terminus of the a subunit is located on the luminal side of thylakoid membranes (23), the N-terminal 14-residue segment is expected to act as a signal peptide for transport across the membrane. However, further analyses are necessary to confirm this function.

Next, we prepared two mutated strains of S. 6803, ΔA212–213 and ΔA205–220, via the genome manipulation of
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WT-$S$. 6803 (Fig. 2, B and C). The complete segregation and replacement of endogenous $sl\ell 1327$, which encodes the $\gamma$ subunit, with the mutated gene were validated by PCR analysis (Fig. 2D) and DNA sequence analysis. The mutants carried the genetic deletion of $\Delta 212$–213 or $\Delta 205$–220 of the $\gamma$ subunit, respectively. Therefore, the two amino acids located at the tip of the turn region or the entire $\beta$-hairpin structure were not present in the two mutants (Fig. 2B). We then purified the mutant $F_oF_1$ using the same procedure as that used for the WT strain. No significant differences were observed in subunit stoichiometry (Fig. 3, A and B) or expression level, which was assessed based on immunoblotting using $\beta$ subunit–specific antibodies (Fig. 2, E and F), between the WT and the mutant strains.

The extent of MgADP inhibition was assessed by the ratio of activation of ATP hydrolysis by lauryl dimethylamine oxide (LDAO). LDAO is a nonionic detergent and considered to be effective in the release of $F_1$ from the MgADP-inhibited state.
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ATP hydrolysis activities of reconstituted proteoliposomes. The complex was purified by membrane solubilization with lauryl maltose neopentyl glycol, a nonionic detergent, and subsequent nickel affinity chromatography. Then, 10 μg of proteins was loaded per lane. Western blot detection of the αβγ complex was performed by immunoblotting with specific anti-c-subunit antibodies; chemiluminescence was measured.

Figure 3. Purification of FoF1, ATP synthase and measurements of the ATP hydrolysis activity of reconstituted proteoliposomes. A, purity of the FoF1 complexes. The complex was purified by membrane solubilization with lauryl maltose neopentyl glycol, a nonionic detergent, and subsequent nickel affinity chromatography. Then, 10 μg of proteins was loaded per lane. Western blot detection of the c-ring. The proteins electrophoresed on 16% polyacrylamide gels were transferred onto a PVDF membrane, followed by immunoblotting with specific anti-c-subunit antibodies; chemiluminescence was measured. C, ATP hydrolysis activity of proteoliposomes was measured using an ATP-regenerating system. The assay was conducted at 30 °C. The activities were determined from the slope obtained in the steady state. The results of four independent experiments were averaged (mean ± SD). The asterisks indicate statistical significance (p < 0.005, Welch’s t test).

Table 1

| Subunit | ID    | Theoretical mass (kDa) | MALDI-TOF MS Scorea | Coverage (%)b | N-terminal sequencingc |
|---------|-------|------------------------|---------------------|--------------|------------------------|
| α       | sll1326 | 54,000                 | 97                  | 24           | VIXIPDEISS             |
| β       | sll1329 | 53,100                 | 188                 | 44           | Not detected           |
| γ       | sll1327 | 34,600                 | 124                 | 39           | PNLXAIKD               |
| δ       | sll1322 | 30,700                 | Not detected        | ALEVGQ       |
| ε       | sll1325 | 20,000                 | 98                  | 70           | MXGLY                  |
| β'      | sll1324 | 19,800                 | 104                 | 29           | Not detected           |
| b'      | sll1323 | 16,200                 | 100                 | 30           | Not detected           |
| e       | slr1330 | 14,600                 | 56                  | 39           | TLTVRVT                |
| c       | sl2615  | 8000                   | Not detected        | Not detected |

a Undetermined amino acids are indicated with an "X."

b Score obtained by Mascot (http://www.matrixscience.com).

c Theoretical mass, including the His-tag.

Preparation of PLs and measurement of ATP hydrolysis/synthesis activities

The obtained FoF1 preparations were, respectively, reconstituted into soybean liposomes to obtain FoF1 PLs. As observed in the analyses of αβγ derived from T. elongatus (24), ATP hydrolysis activities of the mutant PLs were higher than those of the WT PLs (Fig. 3C). The activity was roughly 20- and 23-fold in Δ212–213 (1.0 ± 0.2 μmol Pi min⁻¹ mg⁻¹) and Δ205–220 (1.2 ± 0.2 μmol Pi min⁻¹ mg⁻¹) compared with the WT PLs (0.051 ± 0.005 μmol Pi min⁻¹ mg⁻¹) (Fig. 3C). The degree of the enhancement was similar in the two mutants. Subsequently, H⁺-translocating activity of the PLs was analyzed using an ΔpH indicator, ACMA (Fig. 4). The addition of ATP drove its hydrolysis, followed by coupled proton translocation into PLs, which was observed as the fluorescence quenching of ACMA. The Δ212–213 and Δ205–220 PLs showed a higher proton-translocating activity than WT PLs, the activity of which was diminished in the presence of the H⁺-ionophore FCCP. However, the increases of the activity were relatively moderate when compared with those of the ATP hydrolysis analysis: slopes of the quenching were 0.0066 ± 0.0015, 0.025 ± 0.0094, and 0.049 ± 0.017 IU s⁻¹ for WT, Δ212–213, and Δ205–220, respectively (average ± SD, n = 3).

ATP synthesis activities were then analyzed using the acid-base transition method with valinomycin-induced diffusion potential of K⁺ (Fig. 5A) (35, 36) as follows: first, PLs were incubated with an acidic buffer (pH 5.6) to acidify the inside of the PLs. The acidified PLs were rapidly injected into the assay mixture, which included a basic buffer (pH 8.8), K⁺, ADP, Pi, and luciferin/luciferase. The increase in luminescence intensity was monitored in real time at 30 °C using a luminometer (see the Experimental procedures section for details). The injection of PLs into the assay mixture resulted in the formation of ΔpH between the inside and outside of PLs. Simultaneously, the injection induced the diffusion of K⁺ from the outside to inside of PLs with the assistance of valinomycin, resulting in the formation of an inside-positive ΔΨ across the lipid bilayer of PLs. These two energies, termed ΔpH and ΔΨ, are driving forces of ATP synthesis by FoF1 (37). The amounts of ATP synthesized in the reaction mixture were calibrated by the

(34). For this purpose, the F1-enriched (“crude F1”) fractions were obtained by chloroform extraction (Fig. S4A). The results indicated that the Δ212–213 and Δ205–220 mutations led to a significant increase of ATP hydrolysis activity (Fig. S4B), and LDAO activation ratios were significantly lower than those from WT (Fig. S4C), which is consistent with the results from T. elongates (24). These results indicate that ATP hydrolysis was inhibited by MgADP in WT.
addition of 50 pmol ATP three times at the end of the measurements. The activity was given as the initial rate of synthesis, which was obtained by fitting to the following equation:

\[ y = y_0 + a \times \left[ 1 - \exp\left(-b \times (x-x_0)\right) \right] \]

over 0 to 45 s after the addition of PLs. As a result, the ATP synthesis activity was 5.0 ± 0.3, 1.3 ± 0.2, and 1.9 ± 0.3 s⁻¹ for WT, Δ212–213, and Δ205–220, respectively (average ± SD, Fig. 5B). We further confirmed that those activities were abolished by the addition of the uncoupler nigericin (Fig. S5).

Based on the results reported above, we demonstrated that, in the two β-hairpin-truncated mutants, i.e., Δ212–213 and Δ205–220, the ATP hydrolysis activity was increased, and the ATP synthesis activity was decreased, compared with the WT strain. This raised the possibility that the β-hairpin truncation led to the functional uncoupling between Fo and F₁ motors, i.e., the energy (torque) obtained from pmf at Fo cannot be sufficiently transferred to F₁'s catalytic site to synthesize ATP, because of the structural instability resulting from the truncation. However, as contradicted to this interpretation, those mutants showed significant increases in ATPase-driven H⁺-translocating activity by the mutation (3.8-fold in Δ212–213 and 7.5-fold in Δ205–220) (Fig. 4). This suggests that the regulative effect, rather than the simple uncoupling effect, confers the above-mentioned feature to those mutants. Therefore, we evaluated the rate of uncoupling by comparing the ATP hydrolysis/synthesis and proton-translocation activities shown in Figures 3C, 4 and 5B. Figure 6A indicates the relative ratio of ATP hydrolysis to H⁺-translocating activities. The relative ratios to the WT obtained for Δ212–213 and Δ205–220 were 5.2 ± 2.2 and 3.0 ± 1.1. This suggests that, in those mutants, F₉F₁ was partially uncoupled to different extents and that the uncoupling effect was stronger in Δ212–213 than it was in Δ205–220. Figure 6, B and C indicates the relative ratios of ATP synthesis to H⁺-translocating activities and the relative ratios of ATP synthesis to ATP hydrolysis activities, respectively, both of which were remarkably decreased in Δ212–213 and Δ205–220 compared with the WT; this shows that the preference of F₉F₁ between ATP synthesis and ATP hydrolysis significantly shifted from ATP synthesis to ATP hydrolysis.
synthesis to ATP hydrolysis in the two mutants. These calculations led us to conclude that the β-hairpin structure is a regulatory module that facilitates the achievement of efficient ATP synthesis by F_{o}F_{1}.

The physiological effect of the β-hairpin truncation

Next, we investigated the physiological effect of β-hairpin truncation in cyanobacterial cells. The absorption spectra revealed that pigmentation was not affected in cyanobacterial mutant cells (Fig. 7A), as there was no difference in chlorophyll concentration between WT and the mutant cells (Fig. 7B). We also confirmed that the size of those cells was almost identical (1.89 ± 0.15, 1.96 ± 0.13, and 1.89 ± 0.12 μm/cell for WT, Δ212–213, and Δ205–220, respectively). These observations permitted us to estimate intracellular compounds via normalization either to chlorophyll content or cell density, which was reflected in the optical density at 750 nm (OD750).

As shown in Figure 7D, there was no significant difference in the growth rates between the WT and the mutant cells under continuous light conditions. Sunamura et al. previously reported that the γ_{Y}198–222 mutant of S. 6803, in which the whole γ-insertion region was deleted, exhibited a reduction of the intracellular ATP content and that the growth of γ_{Y}198–222 was not significantly different from that of the wildtype cells under continuous light conditions and light/dark (8/16 h) conditions (30). The present results shown in Figure 7D were analogous to the previous results as expected. Here, we investigated the intracellular ATP content of the mutants, i.e., Δ212–213 and Δ205–220, and further explored the physiological effect of the decrease in ATP level. Figure 8A indicates the steady-state level of intracellular ATP content. Under continuous light conditions, the ATP levels were decreased to 72% in Δ212–213 and 65% in Δ205–220 compared with the WT (Fig. 8A, white bars). The differences in ATP level relative to the WT increased after dark treatment for 24 h, to 50% and 53%, respectively (Fig. 8A, gray bars). We then investigated the short-term kinetics of both ATP and ADP content during a light-to-dark transition (Fig. 8B). As reported in the literature, in the WT cells, the ATP content was decreased within a few minutes after the transfer from the light to the dark. During the dark period, in cyanobacterial cells, the ATP level was gradually increased by oxidative phosphorylation (38). Similar to previous reports (30), the ATP content was decreased in Δ212–213 and Δ205–220 compared with the WT. The two analyses depicted in Figure 8, A and B showed that the ATP level was not significantly different between the two mutants under light or dark conditions. The light-to-dark transition analysis yielded the following remarkable finding: the degree of the relative decrease in ATP level in the mutants was more significant than that observed in the WT. Although we analyzed the photosynthetic activity under the conditions where the ATP levels were significantly affected in the mutants (Fig. S6), there were no remarkable differences between WT and the mutants. Furthermore, we found that the total amount of ATP + ADP was comparatively stable over the experimental period (Fig. 8B), indicating that the dynamics of ATP level reported above mainly reflect the conversion of ADP/ATP. Under continuous light conditions, the ratio of ATP to ATP + ADP was calculated as 73%, 58%, and 57% in the WT, Δ212–213, and Δ205–220 strains, respectively. After a 24-h incubation in the dark, this ratio was decreased to 40%, 23%, and 30%, respectively. These results prompted us to investigate the effects of this phenomenon on the bioenergetic metabolism. Figure 8C depicts the intracellular glycogen content under continuous light and dark conditions. Glycogen levels were gradually reduced after transfer from the light to the dark, which resulted from respiration and autofermentation (39). Contrary to expectations, despite the significant decrease in ATP level, the results obtained for the mutants were not significantly different from those recorded for WT cells.

Discussion

The phototroph-specific β-hairpin structure of S. 6803 facilitates the synthesis and suppresses the hydrolysis of ATP

Here, we established a single-step method for the preparation of S. 6803 F_{o}F_{1}. There were no significant differences in the subunit composition and stoichiometry of the F_{o}F_{1} preparation compared with those of the enzyme purified using the conventional method (40). The obtained F_{o}F_{1} was reconstituted into soybean liposomes to analyze its functions in detail. The resulting F_{o}F_{1} PLs exhibited sufficient activity for the comparative analyses of ATP synthesis/hydrolysis and proton translocation between the WT and the mutant strains. Targeted genome manipulation is relatively easy in S. 6803, whereas it is possible but not easy in some other cyanobacteria, green algae, and higher plants. In the present study, we investigated for the first time the role of the phototroph-specific β-hairpin region of F_{o}F_{1} in the ATP synthesis/hydrolysis activity of the enzyme. Although we could not exclude the possibility that a certain amount of mutant F_{o}F_{1} was

Figure 6. Comparative analyses of ATP hydrolysis/synthesis and proton-translocating activities. A, the relative ratio of ATP hydrolysis to H^{+}-translocating activities, compared with WT. The ATP hydrolysis activities shown in Figure 3C were converted to s^{-1} (0.48 ± 0.05, 9.4 ± 1.7, and 11 ± 1.7 for WT, Δ212–213, and Δ205–220, respectively). The relative H^{+}-translocating activities were calculated from the slope obtained in the steady state (Fig. 4, 0.0066 ± 0.0015, 0.025 ± 0.009, and 0.049 ± 0.017 IU s^{-1} for WT, Δ212–213, and Δ205–220, respectively). B and C, the relative ratio of ATP synthesis to ATP hydrolysis activities, compared with WT. The ATP synthesis activities shown in Figure 5B were used for calculation (5.0 ± 0.3, 1.3 ± 0.2, and 1.9 ± 0.3 s^{-1} for WT, Δ212–213, and Δ205–220, respectively).
uncoupled, especially in the Δ212–213 mutant, the accelerated H⁺-translocating activities of the mutants and data analyses allowed us to conclude that ATP synthesis was suppressed and ATP hydrolysis was accelerated in the β-hairpin-truncated mutants. Overall, the results presented here indicate that the β-hairpin region of the γ subunit of FoF1 from S. 6803 critically contributes to its ATP synthesis activity and suppresses ATP hydrolysis.

Because the synthesis and hydrolysis of ATP by FoF1 are thermodynamically reversible, it is not readily explainable why the β-hairpin structure oppositely affected those two reactions. In contrast, MgADP inhibition is a regulatory mechanism that achieves such contradictory functions. As mentioned above, this inhibitory mechanism results from the persistent occupation of the catalytic site by MgADP derived from ATP hydrolysis and is spontaneously canceled during ATP synthesis. This is consistent with our previous findings that MgADP inhibition was partly canceled in the β-hairpin-truncated mutants (24). Those findings led us to conclude that the phototroph-specific β-hairpin structure suppresses ATP hydrolysis by facilitating MgADP inhibition. This was possibly explained by the interaction between the DELSEED region of the β subunit and a part of the γ subunit, although structural studies are necessary to prove this hypothesis. It was recently reported that the ε subunit of cyanobacterial FoF1 has a different inhibitory mechanism compared with other organisms (41) and that the binding of the ε subunit caused a relative conformational change in the γ subunit (32). Consequently, the β-hairpin structure of the S. 6803 γ subunit, assisted by the ε subunit, might confer stiffness to the γ subunit and facilitate torque transmission during ATP synthesis.

**Regulation of the intracellular ATP level in cyanobacteria**

As shown in Figure 8A, the intracellular ATP level was significantly decreased in both Δ212–213 and Δ205–220 compared with the WT cells. After a 24-h dark adaptation, the
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ATP levels in those mutants were decreased to 19% to 20% of the normal level (i.e., WT, continuous illumination conditions). These results support our hypothesis that the \( \beta \)-hairpin structure plays a role in the inhibition of ATP hydrolysis and the acceleration of ATP synthesis. We further demonstrated that their cell shape/size, pigmentation, growth rate, and glycogen content were not significantly different from those of the WT cells (Figs. 7, A–D and 8C). This was very similar to the case of IF1, which is an inhibitory protein of mitochondrial ATP synthase and considered to play a role in preventing futile ATP hydrolysis (42). Contrary to previous expectations, it was reported that IF1-knockdown or knockout cells did not show any apparent phenotype, except after the exposure of cells to extreme conditions (e.g., high concentrations of reactive oxygen species) (43, 44). In cyanobacteria, Forchhammer et al. reported that the intracellular ATP level was strictly regulated during the awaking phase from dormancy caused by nitrogen chlorosis (45, 46). Those authors argued that the dormant cells keep the minimum intracellular ATP concentration to ensure survival and that this level is increased in two steps during the recovery phase. Future research using starving cells in similar extreme conditions should further elucidate the physiological function of the \( \beta \)-hairpin structure and its evolutionary significance for phototrophic organisms.

**Experimental procedures**

**Materials**

ATP, ADP, 9-amino-6-chloro-2-methoxy-acridine (ACMA), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazine (FCCP), and valinomycin were obtained from ORIENTAL YEAST, Millipore, Invitrogen Life Technologies, Wako, and Sigma Aldrich, respectively. N-octyl-\( \beta \)-D-glucopyranoside (OG) and lauryl maltose neopentyl glycol (LMNG) were obtained from Anatrace. Pyruvate kinase, lactate dehydrogenase, and NADH were obtained from Roche Diagnostics.

**Bacterial strain and culture conditions**

Cells of the glucose-tolerant strain \( S. \) 6803 (47) were grown at 30 \( ^\circ \)C in liquid BG11 medium (48) supplemented with 20 mM Heps-NaOH (pH 7.5) and bubbled with 1% (v/v) CO\(_2\)-enriched (or ambient) air under continuous illumination with white fluorescent lamps (40 \( \mu \)mol photons m\(^{-2}\)s\(^{-1}\)). For antibiotic selection and maintenance of mutant strains, cells were grown on 1.5% (w/v) BG11 agar plates (Bacto Agar, Difco) supplemented with 0.3% (w/v) sodium thiosulfate.

**Construction and transformation of \( S. \) 6803 mutants**

Genetic engineering of \( S. \) 6803 was performed using the homologous recombination technique. Construction for the addition of a His\(_{10}\)-tag into the N terminus of the \( \beta \) subunit is described in the supplemental information (Fig. S2). The
mutant was selected using the chloramphenicol acetyltransferase gene. Transformants of S. 6803 were selected on BG11 plates for chloramphenicol (20 μg ml\(^{-1}\)) resistance. These mutants ("WT" in the text) were then applied as a parental cell line for further mutagenesis. The deletion of the insertion region of the γ subunit was performed using a plasmid in which sll1327 was cloned adjacent to the kanamycin-resistance gene (30). A plasmid for the deletion of amino acids 205–220 of the γ subunit (Δ205–220/E204G/K221G, “Δ205–220” in the text) was constructed using the megaprimer PCR method (49). For the first PCR, two primers were used: sll1327_Fw as a forward primer and sll1327_del205_220_Rv as a reverse primer. For the second PCR, slr1411_Rv and the DNA fragment obtained from the first PCR were used as primers. The resultant DNA fragment was cloned into the pGEM-T Easy vector (Promega) according to the manufacturer’s instructions. A plasmid for the deletion of amino acids 212–213 of the γ subunit (Δ212–213/R211G/K214G, “Δ212–213” in the text) was constructed using the quick-change method with PrimeSTAR Max (Takara) according to the manufacturers’ instructions, using primers sll1327_del212_213_Fw and sll1327_del212_213_Rv. Plasmids selected by DNA sequencing were mixed with S. 6803 cells, and the transformants were selected and subcultured in the presence of 10 μg ml\(^{-1}\) chloramphenicol and 10 μg ml\(^{-1}\) kanamycin. The primers used for the preparation of mutants are listed in Table S1.

Purification of F\(_{0}\)F\(_{1}\) complexes from S. 6803

One liter of cells grown under normal conditions (30 °C, 1% CO\(_{2}\), continuous light illumination, 40 μmol photons m\(^{-2}\)s\(^{-1}\)) was harvested at the late log phase by centrifugation, followed by flash-freezing in liquid nitrogen and storage at −80 °C until use. The cells resuspended in a buffer containing 20 mM Hepes-KOH (pH 8.0), 10 mM NaCl, 0.1 mM MgCl\(_{2}\), and 0.1 mM ATP were broken by vortexing with zircon beads and the homogenate was centrifuged for 10 min at 3000g at 4 °C to remove cell debris. The supernatant was then centrifuged at 125,000g for 30 min at 4 °C to precipitate thylakoid membranes. The membranes were washed with a buffer containing 20 mM Hepes-KOH (pH 8.0), 0.1 mM MgCl\(_{2}\), 10% glycerol, and 0.1 mM ATP, followed by solubilization in 1% LMNG for 60 min at 4 °C. Solubilized membrane proteins were obtained after centrifugation at 201,000g for 30 min at 4 °C and subjected to Ni-affinity chromatography in a buffer containing 10 mM potassium phosphate, pH 8.0, 100 mM K\(_{2}\)SO\(_{4}\), 0.1 mM MgCl\(_{2}\), 0.1 mM ADP, 0.005% LMNG, and 60 to 200 mM imidazole. The obtained F\(_{0}\)F\(_{1}\) preparations were concentrated using Amicon Ultra (Millipore) with a buffer containing 10 mM potassium phosphate, pH 8.0, 100 mM K\(_{2}\)SO\(_{4}\), 0.1 mM MgCl\(_{2}\), 0.1 mM ADP, and 0.005% LMNG and then flash-frozen in liquid nitrogen and stored at −80 °C after the addition of glycerol at a final concentration of 10%. The concentration of the purified F\(_{0}\)F\(_{1}\) was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) with BSA as the protein standard.

SDS-PAGE, immunoblotting, and N-terminal Edman sequencing

Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Quick CBB (Wako). For immunoblotting, separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Immun-Blot, Bio-Rad). Antibodies against the c subunit were obtained from Agrisera, and those against the β subunit were as described in the literature (31). Chemiluminescence was detected using horseradish peroxidase–conjugated secondary antibodies and ECL Prime (Life Technologies) and visualized on a LAS 3000 mini instrument (GE Healthcare). Images were digitized using the ImageJ software. Otherwise, the separated proteins were transferred onto a membrane (Sequi-Blot PVDF Membrane, Bio-Rad). The N-terminal sequences were determined by Edman degradation on a peptide sequencer (PPSQ21, Shimadzu), based on a previous study (50).

In-gel digestion and peptide-mapping analysis

Proteins stained with Coomassie Brilliant Blue R-250 were excised from the SDS-PAGE gel and in-gel digested using trypsin. The resulting peptides were analyzed by mass spectrometry as described (50). The parameters used for database searches were as follows: database, Cyanobase_S6803GTI; enzyme, trypsin; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M); mass values, monoisotopic; peptide mass tolerance, ± 100 ppm; max missed cleavages, 2.

Reconstitution into PLs

PLs were reconstituted as described (36), with some modifications. Crude soybean 1-α-phosphatidylcholine (type II-S; Sigma) was suspended at a final concentration of 32 mg ml\(^{-1}\) in Rec-buffer (15 mM MES-Tricine, 2 mM KOH, 5 mM NaCl, 2.5 mM MgCl\(_{2}\), and 50 mM sucrose, with the pH adjusted to 8.0 with NaOH). The suspension was incubated for 5 min, followed by brief sonication with a water bath sonicator and centrifugation at 125,000g for 30 min at 20 °C. After two or three repetitions of these procedures, the suspension was divided into aliquots, frozen in liquid nitrogen, and stored at −80 °C until use. The reconstitution of F\(_{0}\)F\(_{1}\) into liposomes was performed as follows. The lipid suspension was mixed with an equal volume of Rec-buffer and 2% (w/v) N-dodecyl-β-D-glucoside (OG), followed by incubation for 10 min at room temperature. To this solution, 200 mg of BioBeads (SM-2, Bio-Rad) was added until the mixture became unpure. F\(_{0}\)F\(_{1}\) (90 μg) was then added to the solution (final concentration, 0.15 mg ml\(^{-1}\)). The mixture was incubated at 4 °C overnight, followed by flash-freezing in liquid nitrogen and storage at −80 °C until use.

ATP hydrolysis/synthesis and proton-translocating activities

ATP hydrolysis activity was measured using an ATP-regenerating system as described (31), with some modifications. The assay was conducted at 30 °C. For measurements of ATP synthesis activity, we applied the acid–base transition
method with valinomycin-induced diffusion potential of K⁺, as described (36). The PL suspension (30 μl) was acidified by mixing with 70 μl of an acidic buffer (50 mM MES-Tricine, 2 mM KOH, 50 mM NaCl, 50 mM sucrose, 10 mM Na₂HPO₄, and 2.5 mM MgCl₂, adjusted to pH 5.6 by adding NaOH) supplemented with 0.2 nM valinomycin, 0.5 mM ADP, and 0.01 mM P₁,P₅-di(adenosine-5’)-pentaphosphate (Ap5A), followed by a 10-min incubation at 30 °C. The mixture was then injected into 900 μl of a basic buffer (300 mM Tricine, 200 mM KOH, 50 mM NaCl, 50 mM sucrose, 10 mM Na₂HPO₄, and 2.5 mM MgCl₂, adjusted to pH 8.8 with NaOH, 0.5 mM ADP, and 0.01 mM Ap5A) supplemented with 100 μl of a luciferin/luciferase-containing solution (ATP Bioluminescence Assay Kit CLS II, Roche Diagnostics). The luminescence was detected by a luminometer (Luminescence, AB2200, ATTO). After 50 s, 5 μl of 10 μM ATP was added three times, for calibration. The initial rate of ATP synthesis was calculated from the exponential fit of the initial 0 to 45 s after the injection of the acidified PLs. ATP-driven proton-translocating activity was measured using a fluorometer (FP8500, Jasco) and fluorescence quenching of ACMA (excitation at 410 nm, emission at 480 nm) at 30 °C, as described (51). PLs (50 μl; final concentration, 6.25 μg ml⁻¹) were injected into a buffer containing 15 mM MES-Tricine, 2 mM KOH, 5 mM NaCl, 2.5 mM MgCl₂, and 50 mM sucrose, adjusted to pH 8.0 with NaOH and 0.3 μg ml⁻¹ ACMA. The reaction was initiated by adding 2 mM ATP. When indicated, 1 μg ml⁻¹ FCCP was added.

Measures of Intracellular Chlorophyll Content, Cell Density, and Absorption Spectra

For the extraction of chlorophylls, cells were suspended in 100% methanol, followed by sonication and centrifugation at 20,000g for 10 min, to precipitate cell debris. Chlorophyll content (μg ml⁻¹) was calculated from the following equation: chlorophyll content = 13.4 × A₆₆₅. Cell density was monitored as absorbance at 750 nm (A₇₅₀) on a spectrophotometer (UV-1800, Shimadzu). For measurements of cell absorption spectra, cells were collected by centrifugation and resuspended in BG11 medium (A₇₅₀ = 1.0), followed by measurements using a spectrophotometer equipped with an integrating sphere (V-650, Jasco).

Microscopic Analysis

Bright-field microscopic analysis of cells grown under continuous light conditions (40 μmol photons m⁻² s⁻¹) was performed using a PlanApo N 60×/1.45 oil objective fitted on an Olympus IX73 microscope.

Intracellular ATP/ADP Level Determination

Intracellular ATP/ADP level was determined according to the literature (30), with some modifications. Cell culture (100 μl) was withdrawn and added to 20 μl of 12% perchloric acid. After incubation on ice for at least 30 min, the solution was centrifuged at 20,000g for 10 min at 4 °C to precipitate cell debris. Subsequently, 100 μl of the supernatant was neutralized with 200 μl of 2 M Tris-acetate, pH 7.7. The levels of ATP or ATP + ADP were quantified after incubation for 3 h at 25 °C in the absence or presence of pyruvate kinase in a buffer (10 mM Tris-acetate, pH 7.7, 10 mM KCl, 1 mM MgCl₂, ±10 mM phosphoenolpyruvate, and ±57 μg ml⁻¹ pyruvate kinase). The luminescence was quantified using CLSII (Sigma-Aldrich) and a luminometer, Tristar (Berthold Technologies).

Quantification of Intracellular Glycogen Content

Glycogen was quantified based on a previous study (52). Cells were suspended in 500 μl of 3.5% (v/v) sulfuric acid and boiled at 100 °C for 120 min, followed by centrifugation at 20,000g for 10 min. Then, 6.7 μl of supernatant was mixed with 1 ml of the reaction mixture of LabAssay Glucose (Wako), followed by measurements according to the manufacturer’s instructions.

Data Availability

All data are contained within the article and can be shared upon request (thisabor@res.titech.ac.jp).

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