1. INTRODUCTION

Antimicrobial resistance is a global health threat, and the effort to explore new antimicrobial compounds has undergone development.1,2) ATP synthase, an F-type ATPase (F-ATPase) family enzyme, has emerged as a target molecule for antimicrobials since the recent approval of bedaquiline as the first new multidrug-resistant tuberculosis agent. This diarylquinoline compound binds to the subunit \( \alpha \) of Mycobacterium tuberculosis ATP synthase.3,4) The mycobacterial ATP synthase differs from its mammalian homologs in detailed structure and the regulation of activity.5) Furthermore, those characteristics are different among other bacterial F-ATPases. Therefore, studying family enzymes in bacteria is valuable for discovering novel antimicrobials against pathogens. This review focuses on the well-studied Escherichia coli ATP synthase and also discusses the \( \text{H}^+ \)-pumping F-ATPase of Streptococcus mutans.

2. STRUCTURE AND FUNCTION OF F-TYPE ATPASE

Members of the F-ATPase family of enzymes are found in the membranes of various organisms6,7) (Fig. 1A). The membranes of the mitochondria and chloroplasts and bacterial plasma membranes have ATP synthases that consist of multiple subunits with the membrane-embedded \( F_0 \) portion and the catalytic sites of membrane-peripheral \( F_1 \) portion. These couple to synthesize ATP through an electrochemical \( \text{H}^+ \) gradient (\( \text{H}^+ \) gradient or \( \text{H}^+ \) motive force) generated by the electron transport chain, which is present in the same membrane. Crystal structures of purified \( F_1 \) reveal a central stalk that penetrates into the head piece, which contains the catalytic sites.8,15) Cryo-electron microscopy (cryo-EM) analysis of the \( F_0F_1 \) complex has revealed a membrane-embedded subunit assembly and a stalk connecting \( F_1 \) and \( F_0 \) at the periphery.16,26)

The \( F_1 \) has three catalytic sites with different nucleotide affinities at the three interfaces of alternately assembled \( \alpha \) and \( \beta \) subunits8) (Fig. 1B). The affinity of these sites changes sequentially, resulting in the successive binding of ADP and Pi and release of ATP (the binding change mechanism).7) During ATP synthesis, conformational changes in the catalytic \( \beta \) subunit are induced by the rotation of the central stalk consisting of amino- and carboxy-terminal helices of the \( \gamma \) subunit at the center of the \( \alpha_3\beta_3 \) hexamer (Figs. 1A, B). This rotational catalysis was revealed by observing the \( F_1 \) motor in the presence of ATP, in which the fluorescent actin probe connected to the \( \gamma \) subunit exhibited continuous counterclockwise rotations viewed from the membrane side against the fixed \( \alpha_3\beta_3 \). Moreover, ATP synthesis was shown to be induced by the forced clockwise rotation of the \( \gamma \) subunit attached to magnetic beads and placed in a magnetic field.31,32)

The \( \text{H}^+ \) pathway of \( F_0 \), which is formed from two half channels that open on each side of the membrane, consists of the \( a \) subunit and the \( c \)-ring—composed of 8–15 \( c \) subunits, varying in number among species.33,36) The \( c \) subunit has an essential glutamic acid (Glu) or aspartic acid (Asp) residue that binds \( \text{H}^+ \) from one half of the channel and releases it into the other one, leading to \( c \)-ring rotation37,39) (Fig. 1). Thus, the electrochemical \( \text{H}^+ \) gradient causes the continuous rotation of the \( c \)-ring, which is coupled to \( \text{H}^+ \)-transport across the membranes. However, the detailed mechanism of how \( \text{H}^+ \) translocate between the \( c \)-subunit residues and water-filled half channels is unclear. Because the \( \gamma \) subunit is bound to the \( c \)-ring, the mechanical rotation causes a conformational change in the \( \beta \) subunits. Therefore, the \( \text{H}^+ \) gradient across the membrane is harnessed to ATP synthesis (Fig. 1B). In fact, the rotation...
3. ROTATION INHIBITION PREVENTING ATP HYDROLYSIS

To estimate the actual rotation rate in solution, a small bead probe without substantial viscous drag was bound to the rotor portion of purified *E. coli* F_{0}F_{1}. The γε-ring rotation was observed with an average rotation rate of approximately 200 rotations per second upon ATP hydrolysis. This rate was consistent with the rotation rate of the γ subunit with the same probe using purified F_{1} with an excess of the ε subunit. Without the ε subunit, the rotation of the γ subunit was 2-fold faster. Because the ε subunit is readily released from purified *E. coli* F_{1}, these results indicate that the ε subunit inhibits the ATP-dependent rotations of F_{1} and F_{0}F_{1}. Indeed, releasing the ε subunit from *E. coli* F_{1} is known to stimulate F_{1}-ATPase activity.

During several 10-ms observations of the F_{1} and F_{0}F_{1}, their rotations occasionally paused. Detailed observations of F_{1} rotation revealed that the mechanical rotation was stochastic and intrinsically paused for various durations. Although the pauses are partly due to the bound MgADP, which is a universal inhibitor of F-ATPases, addition of the ε subunit decreased average rotation rate to increase total duration of pauses.

The inhibition of rotation coupled with ATP hydrolysis is thought to be important to maintain cellular ATP levels when the H^{+} gradient dissipates. The rotational observation of F_{1} from cyanobacterium and *Bacillus* PS3 revealed the inhibitory effects of the ε subunit; however, their mechanism of inhibition is different from that seen in *E. coli*.

Different inhibition mechanisms exist to prevent the reversal of ATP synthesis in various organisms. In plants, ATP generation progresses through photophosphorylation in the presence of light, whereas the ATP synthase F-ATPase is inactivated in the dark when crosslinking between two specific cysteine (Cys) residues occurs in the γ subunit to inhibit rotation. The disulfide bond is reduced by the ferredoxin/thioredoxin system of energized chloroplasts in the presence of light, and then, ATP synthesis is reactivated. The mitochondria have an endogenous ATPase inhibitor protein called IF1. The dimerized IF1 inhibits ATP hydrolysis in slightly acidic solutions, whereas the oligomerization and inactivation of IF1 occurs at an alkaline pH. Crystal structures of the F_{0}F_{1} complexes reveal that IF1 binds the interface of the α and β subunits and inhibits conformational changes in the β subunit. IF1 was found to be highly expressed in some cancer cells, which suggests its involvement in metabolic reprogramming, resistance to cell death, etc., although its relevance in cancer remains unclear.

In bacteria, mechanisms of ATPase inhibition that do not involve the ε subunit have also been suggested. In the α-proteobacteria *Paracoccus denitrificans*, the ζ subunit, which is similar to IF1, was suggested to inhibit the ATP hydrolysis activity of the F_{0}F_{1}-ATP synthase in the absence of a H^{+} gradient. Mycobacterial α subunit has an extension of approximately 35 amino acids at the carboxy-terminus. The deletion of this region showed substantial ATPase activity of *Mycobacterium smegmatis* ATP synthase, which is barely detectable in the wild-type enzyme. Cryo-EM analysis suggested that the segment interacted with the γ subunit to prevent rotation in the direction of ATP hydrolysis.

The rapid inhibition and reactivation of the ATP synthase caused by the structural changes of these regulatory subunits must be important to adapt in response to environmental changes. In the next section, the mechanism of regulation of the ε subunit of *E. coli* is discussed.
4. REGULATION OF E. COLI ATP SYNTHASE BY THE EPSILON SUBUNIT

Structural analysis of the $\varepsilon$ subunit has revealed $\beta$-sheet and $\alpha$-helix domains in the amino- and carboxy-terminal regions, respectively,$^{64,65}$ (Fig. 1C). Because early analyses of mutant enzymes lacking the carboxy-terminal region of the $\varepsilon$ subunit showed increased ATPase activity, an inhibitory function of the region was indicated.$^{66,67}$ Recent analyses of E. coli $F_1$ have indicated that the region is important for reducing rotation rate.$^{68}$

Based on the tertiary structures of the E. coli $\varepsilon$ subunit in $F_1$ and $F_0 F_1$, the carboxy-terminal helix domain extends along the $\gamma$ subunit and interacts with the $\alpha$ and $\beta$ subunits to prevent $\gamma$-subunit rotation$^{11,18}$ (Fig. 1A). The $\varepsilon$ subunit of thermophilic Bacillus PS3 also showed an extended conformation similar to the E. coli enzyme.$^{12,21,69}$ By contrast, structures of the same subunit in various bacteria, such as M. smegmatis, Caldalkalibacillus thermarum, and Fusobacterium nucleatum, show different helix structures that are folded toward the membrane side in a compact form.$^{13-15,26}$ A similar compact form of the $\varepsilon$ subunit in E. coli was suggested through the structural analysis of isolated $\varepsilon$ subunits and crosslinking experiments between the introduced Cys residues in the $\varepsilon$ subunits.$^{64,65,70}$ The region was suggested to change conformations to regulate ATPase activity in E. coli and Bacillus PS3 enzymes.$^{69-72}$ The highly extended conformation of the $\varepsilon$ subunit visible in crystal and cryo-EM structures was not demonstrated to exist in the membrane $F_0 F_1$ complexes (Fig. 1A), although other results have indicated that the $\varepsilon$ subunit extends into a different form.$^{70}$

We introduced Cys substitutions into $\gamma$Leu260 and $\varepsilon$Thr134, which are close to each other in the crystal structure with the highly extended $\varepsilon$ subunit$^{11}$ (Fig. 2A). The membrane fraction actively hydrolyzed ATP when dithiothreitol was added to prevent Cys–Cys crosslinking. When the oxidizing agent was added, the Cys residues were crosslinked, and ATPase activity decreased with increased crosslinking$^{77}$ (Fig. 2B). These results suggest that the highly extended conformation of the $\varepsilon$ subunit in the crystal and cryo-EM structures was feasible in $F_0 F_1$ and inhibited rotation. We observed the ATP-driven rotation of $F_0 F_1$, which contained the $\varepsilon$ subunit (14kDa) fused with an unrelated protein, either cytochrome $b_{562}$ (12kDa) or flavodoxin reductase (28kDa) at the carboxy-terminus.$^{73}$ Thus, it is likely that specific interactions, and not steric hindrance, between the $\varepsilon$ subunit and other subunits inhibit rotation. Taken together, conformational changes in the $\varepsilon$ subunit regulate rotation rate to inhibit ATP hydrolysis. The interactions between the loop regions of the $\beta$ and $\varepsilon$ subunits, containing $\beta$Ser104 and $\varepsilon$Glu381, respectively, were suggested as at least one of the important interactions for preventing wasteful rotation.$^{68}$

However, mutant strains of E. coli with a truncated carboxy-terminal region of the $\varepsilon$ subunit can grow by utilizing oxidative phosphorylation, suggesting that the inhibitory function is not critical under certain experimental conditions.$^{56,74-76}$ In vivo analysis of the $\varepsilon$ subunit structure under different culture conditions suggests that in energetically unfavorable states, some ‘inserted’ forms (into $\alpha_2 \beta_2$) are more abundant, whereas with sufficient nutrition, the forms switch to the compact ‘uninserted’ form.$^{77}$ It is conceivable that E. coli cells survive when the cellular environment is altered, by lowering H$^+$ gradient across the plasma membrane caused by insufficient nutrition or due to transient O$_2$ depletion. When ATP synthesis cannot be maintained through oxidative phosphorylation, the $\varepsilon$ subunit may change the conformation to prevent wasteful ATP hydrolysis. Although it is unknown how environmental factors affect this conformational change, interactions with adjacent subunits may be involved.

The $\varepsilon$ subunit largely interacts with the $\gamma$ subunit (Figs. 1, 2). When the $\gamma$Leu218 in the $\varepsilon$ subunit was substituted with Pro, the ATPase activity of the mutant enzyme increased 2-fold compared with the wild type.$^{73}$ Structural alterations in the $\gamma$ subunit were detected as differences in electrophoretic mobility. The highly extended structure of the $\varepsilon$ subunit was reduced compared with the wild type in the presence of ATP, which was indicated by a reduction in the crosslinking between Cys residues introduced into $\gamma$ and $\varepsilon$ subunits (Fig. 2B). Structural changes in the $\varepsilon$ subunit caused by the $\gamma$Leu218Pro mutation partially impaired the $\varepsilon$ subunit from extending or holding its conformation, causing an increase in ATPase activity.$^{73}$

![Fig. 2. Positions of Cys Substitutions in the $\gamma$ and $\varepsilon$ Subunits and Effects of Crosslinking on ATPase Activities](image_url)

A. The $\gamma$Leu260 and $\varepsilon$Thr134 residues of the $\gamma$ (cyan) and $\varepsilon$ (pink) subunits, respectively, were replaced with Cys. Two residues are located nearby in the reported $F_1$ structure analyzed through X-ray.$^{51}$ The site of $\gamma$Leu218 of the $\varepsilon$ subunit is also indicated.$^{58}$ B. The crosslinking of membrane $F_0 F_1$ was performed by adding various concentrations of CuSO$_4$ and analyzing through Western blotting using the anti-$\gamma$ subunit antibodies (open squares). ATPase activities of the treated membrane samples are measured (closed diamonds). Results for the membranes carrying F-ATPases with $\gamma$Cys260/$\varepsilon$Cys134 (purple) and $\gamma$Pro218/$\varepsilon$Cys260/$\varepsilon$Cys134 (orange) substitutions are shown.$^{77}$ Reprint from BBA-Bioenergetics, vol. 1860, Yamakita et al., “The carboxyl-terminal helical domain of the ATP synthase $\gamma$ subunit is involved in $\varepsilon$ subunit conformation and energy coupling,” 361–368 (2019), with permission from Elsevier.
5. THE F-ATPASE OF S. MUTANS, AN H\textsuperscript{+}-PUMP-ING ENZYME

S. mutans is indigenous to the human oral cavity where it forms biofilms and is an etiological agent of dental caries.\textsuperscript{78–80} The bacteria catabolize sugars through glycolysis and secrete organic acids, resulting in an acidic environment (Fig. 3A). F-ATPase subunit genes are expressed under acidic conditions, suggesting a role for the H\textsuperscript{+} pump in maintaining a neutral cytoplasmic pH.\textsuperscript{81,82} In fact, inhibitors to \textit{E. coli} ATP synthase prevented growth and survival at pH 5.3 and 4.3, respectively.\textsuperscript{83} Inhibitors of F-ATPase are promising antimicrobial agents for preventing caries, while the catalytic subunits are highly homologous across species, from bacteria to mammals. Thus, it is important to elucidate the unique properties of \textit{S. mutans} F-ATPase.

We cloned the 8 subunit genes that form a cluster in the genome and constructed an expression system in an \textit{E. coli} strain that eliminated its own F-ATPase (ATP synthase).\textsuperscript{84} The ATP-dependent active H\textsuperscript{+} transport by the \textit{S. mutans} enzyme was maximal around pH 5.5 but decreased at pH 7\textsuperscript{84} (Fig. 3B). This result was consistent with the role of this enzyme in acidic adaptation. When a chimeric enzyme consisting of \textit{S. mutans} F\textsubscript{0} (SF\textsubscript{0}) and \textit{E. coli} F\textsubscript{1} (EF\textsubscript{1}) was constructed, its H\textsuperscript{+}-transport exhibited similar pH dependency with the \textit{S. mutans} enzymes\textsuperscript{84} (Fig. 3B).

To identify the amino acid residues involved in H\textsuperscript{+} transport at an acidic pH, we focused on the \textit{c} subunit, which is predicted to contain two transmembrane helices (TM1 and TM2).\textsuperscript{37} The conserved Glu53 residue, which is located at the center of TM2, was found to be essential for H\textsuperscript{+} transport\textsuperscript{84} (Fig. 3C). Mutant membranes that carried substitutions of Ser17Ala and Glu20Ile in TM1, which are located adjacent to Glu53 in the hairpin structure of the \textit{c} subunit, exhibited H\textsuperscript{+}-transport at pH 7.\textsuperscript{84} Because H\textsuperscript{+} transport through the two half channels was mediated by the H\textsuperscript{+}-binding and H\textsuperscript{+}-release of Glu53, the optimal pH should depend on the p\textsubscript{K\textsubscript{a}} of the carboxyl moiety. In the \textit{E. coli} c subunit, Ile28 positionally corresponds with Glu20 of \textit{S. mutans}. A previously reported Ile28Glu mutant of \textit{E. coli} changed the pH sensitivity of H\textsuperscript{+} transport of the membrane.\textsuperscript{85} Thus, the side chain of Glu20 is considered to affect the p\textsubscript{K\textsubscript{a}} of essential Glu53 to release H\textsuperscript{+} at pH 6. We also exhibited that the \textit{E. coli} strain carrying the \textit{S. mutans} c subunit grew better at pH 5.5 than at pH 7.5.\textsuperscript{86}

6. CONCLUSION

Among bacterial F-ATPases, ATP synthases or active H\textsuperscript{+}-transporters may differ in their regulatory mechanisms. The elucidation of this mechanism and characterization of the H\textsuperscript{+} pathway will contribute to the development of antimicrobial inhibitors. Because bedaquiline, an antituberculosis drug, is a
specific *M. tuberculosis* ATP synthase inhibitor, discovery of F-ATPase inhibitors for other pathogenic bacteria may lead to the discovery of novel antibiotic drugs.

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**Conflict of Interest** The author declares no conflict of interest.

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