F$_o$ of ATP synthase is a rotary proton channel: Obligatory coupling of proton translocation with rotation of c-subunit ring*

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Running title: H$^+$-flow/c-ring rotation in ATP synthase

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SUMMARY

Coupling of proton flow and rotation in $F_o$ motor of ATP synthase was investigated using the thermophilic Bacillus PS3 enzyme expressed functionally in Escherichia coli cells. Cysteine residues introduced into the N-terminal regions of subunits $b$ and $c$ of ATP synthase ($bL2C/cS2C$) were readily oxidized by treating the expressing cells with CuCl$_2$ to form predominantly a $b$-$c$ cross-link with $b$-$b$ and $c$-$c$ cross-links being minor products. The oxidized ATP synthases, either in the inverted membrane vesicles or in the reconstituted proteoliposomes, showed drastically decreased proton pumping and ATPase activities compared with the reduced ones. Also, the oxidized $F_o$, either in the $F_1$-stripped inverted vesicles or in the reconstituted $F_o$-proteoliposomes, hardly mediated passive proton translocation through $F_o$. Careful analysis using single mutants ($bL2C$ or $cS2C$) as controls indicated that $b$-$c$ cross-link was responsible for these defects. Thus, rotation of the $c$-oligomer ring relative to subunit $b$ is obligatory for proton translocation; no rotation of the $c$-ring, no proton flow through $F_o$. 
ATP synthases catalyze ATP synthesis/hydrolysis coupled with a transmembrane H⁺(proton)-translocation in bacteria, chloroplasts and mitochondria (1-3). The enzyme is composed of two portions, a water-soluble F₁, which has catalytic sites for ATP synthesis/hydrolysis (4), and a membrane-integrated F₀, which mediates H⁺-translocation. The bacterial enzyme has the simplest subunit structure, α₃β₃γ₁δ₁ε₁ for F₁ and a₁b₂c₁₀-₁₁(7) for F₀. F₁ is easily and reversibly detached from F₀ by removal of Mg²⁺ in a low ionic strength solution. F₁ is by itself a rotary motor driven by ATP hydrolysis (5,6), in which a central stalk made of γ and ε subunits rotates relative to the surrounding α₃β₃ hexamer ring (7,8). Remaining F₀ sector in the membrane acts as a proton channel that mediates passive proton translocation across membrane.

F₀ in ATP synthase is thought to work as a rotary motor driven by the energy of proton translocation down the electrochemical potential. Structural studies on F₀ with electron microscopy (9) and atomic force microscopy (10-12) have suggested that subunits a and b₂ are peripherally located outside of a ring of subunit c oligomers (c-ring). A low-resolution crystal structure of an F₁+c₁₀ subcomplex from yeast mitochondria revealed a tight interaction between γε subunits of F₁ and c-ring of F₀ (13). The cross-links were readily made between introduced cysteines of subunit c and γε subunits of F₁ without losing functional coupling between F₁ and F₀ (14). ATP facilitated movement of subunit c relative to subunit a that was assessed by the a-c cross-link (15). A side stalk, made of b₂ and δ, connects stator of F₀ and that of F₁, and prevents stators being dragged by rotation of the central stalk(16). From these and other observations, it is generally accepted that c-ring rotates relative to stator subunits, ab₂ (17-19). Thus, proton influx into the cytoplasm through F₀ (in the case of mitochondria, into the matrix) would cause rotation of c-ring and hence the central γε stalk, which then enforces each catalytic site in F₁ to synthesize ATP.
As a reverse reaction, ATP hydrolysis in $F_1$ drives reverse rotation of the $\gamma\epsilon$ stalk and $c$-ring, which causes proton efflux through $F_o$.

To explore the mechanism of proton flow through $F_o$ and its coupling with rotation, measurement of the proton flow together with $c$-ring rotation is absolutely required. The study has been impeded by unstable nature of ATP synthases; structural and functional integrity of the enzyme is easily damaged during experimental procedures (20,21). For example, the cross-linking between subunit $b$ and $c$ in *Escherichia coli* ATP synthase impairs coupling between proton pumping and ATPase activity (21). To overcome this difficulty, we have established an expression system for a stable ATP synthase of thermophilic *Bacillus* PS3 ($TF_oF_1$) in *E. coli* cells. Cysteine residues introduced into subunits $b$ and $c$ of $TF_oF_1$ were readily cross-linked in the presence of CuCl$_2$. The resultant enzyme lost both proton pumping and ATPase activity, an indication of retaining tight coupling after the cross-linking. The passive proton translocation through $F_o$ was also disabled by this cross-link. Thus, the $c$-ring must rotate for protons to pass through $F_o$.

**EXPERIMENTAL PROCEDURES**

*Construction of an expression vector for $TF_oF_1$* ---- DNA manipulations were carried out by following the methods of the literature (22). A plasmid pTR-k$\varepsilon$2, which is an expression vector for $\alpha_3\beta_3\gamma\epsilon$ subcomplex of *Bacillus* PS3 $F_1$-ATPase (23), was used as a start material. A 4.4-kbp DNA fragment containing *Bacillus* PS3 uncBEFHA' genes (coding for $a$, $c$, $b$, $\delta$, and $\alpha$ subunits of $TF_oF_1$) was amplified by PCR from the plasmid pUC119/TF$_o$F$_1$ (24) using primers, 5'-CCGCGGGAATTCTAAGAAGGAGAT ATACAT-
ATGGAGCATAAAGCGCCGCTTGTCG-3' and 5'-GGCCGATCGGTACCAGCGC-GTCGATCGCTTTAATCC-3' (underlined bases correspond to EcoRI and KpnI sites, respectively). The amplified fragment was digested with EcoRI and KpnI, and ligated into the pTR-ke2 previously digested with the both restriction enzymes. For convenience, a SpeI restriction site was introduced just downstream of uncE gene (c) by the method of Kunkel and Roberts (25) using a synthetic oligonucleotide, 5'-TTTACTTAGGTGATATA-CTAGTCGATATGGAAAGTGA-3'.

Mutated TF₁s were constructed by the Mega-primer method (26), using following mutation primers: 5'-CGGTGCGATACTAGTCGAAAGGAGTGAAACGCGGTGTGGGAAGGCAAACCTATGGGCGCTCG-3' (bLeu2Cys) or 5'-CAATCGCAGCTGCAAGTACACCCAAGCACATGTTGATAGATCCTCCTTCACC-3' (cSer2Cys). In the mutants, the region amplified by PCR was verified by nucleotide sequencing.

Expression and purification of TF₁s.---- E. coli DK8 (23) harboring pTR19-ASDS was aerobically cultivated in 2×YT medium (22) containing ampicillin (100 µg/ml) at 37°C for 20 h. Cells (40 g of wet weight) harvested from the culture were dissolved in 280 ml of buffer-PA3 (10 mM HEPES/KOH, pH 7.5, containing 5 mM MgCl₂ and 10% glycerol), and disrupted by a French press (1200 mbar, twice). After removing the cell debris, membrane fraction was collected by a centrifugation (200,000 × g, 1 h, 4 °C). The membrane fraction obtained was washed, precipitated, and then suspended in 40 ml of buffer-PA3. This suspension was used as the inverted membrane vesicles in this study. After washing the inverted vesicles with a 2-fold volume of buffer-PA3 by centrifugation, the vesicles were dissolved in 120 ml of the buffer-PA3 containing 2 % TritonX-100 and 1 % sodium cholate, and subjected to sonification for 2 min at room temperature.
solution containing solubilized TF$_o$F$_1$ was obtained by a centrifugation (140,000 x g, 15 min, 25 °C). The solution was diluted 3-fold with buffer-K (20 mM potassium phosphate buffer, pH 7.5, containing 100 mM KCl) containing 20 mM imidazole, and applied to Ni-NTA column (Qiagen, Germany). After washing with 10 volumes of buffer-KT (buffer-K supplemented with 0.5 % TritonX-100), TF$_o$F$_1$ was eluted with the buffer-KT containing 200 mM imidazole. Then, the eluted fraction was precipitated by ammonium sulfate in the presence of 1 % sodium cholate (27). The precipitate was dissolved in a small volume of 10 mM HEPES/KOH, pH 7.5, containing 0.1 % Triton X-100. About 10 mg of the purified TF$_o$F$_1$ were routinely obtained from a 1-liter culture. The homogeneity of the purified TF$_o$F$_1$ was judged by a SDS-PAGE analysis (see Fig. 1A).

Oxidation of cysteine residues in F$_o$ was performed as follows: Recombinant cells expressing TF$_o$F$_1$ (10 g) were washed and suspended in 40 ml of buffer-PA3 containing 200 µg/ml CuCl$_2$, and incubated at 25 °C for 30 min. The recombinant TF$_o$F$_1$ has one cysteine residue at position 27 of subunit $\alpha$ but this residue has no reactivity under these oxidizing conditions. After adding 10 mM EDTA to the solution, the cells were washed with 40 ml of buffer-PA3 twice, and inverted membrane vesicles were prepared by the procedures described above.

Reconstitution of TF$_o$F$_1$ into liposomes ---- Reconstitution of proteoliposome was performed by the method of Kaim et al (28) with some modifications. Phospholipid (Soybean L-a-phosphatidylcholine, type II-S, Sigma) was suspended in buffer-PA3 at the concentration of 44 mg/ml, and sonicated for 3 min on ice-water to prepare liposomes. Either TF$_o$F$_1$ (0.5 mg) or TF$_o$ (0.2 mg) was added to 200 µl of the liposome solutions. The solution was frozen with liquid nitrogen and thawed at room temperature. After adding an
equal volume of distilled water, the liposomes were briefly sonicated for 15 sec, collected by a centrifugation (200k × g, 20 min, 4 °C), and resuspended in 200 µl of buffer-PA3.

**Analyzes of proton flow through F₀----F₁** was removed from the inverted vesicles by washing with 0.2 mM EDTA (29), and the resultant F₁-stripped inverted vesicles were used for analysis of proton efflux through F₀. Isolation of pure F₀ complexes and the analysis of proton influx through F₀ were performed as described previously (30).

**Other methods** ---- ATPase activity was measured at 37 °C using an ATP-regenerating system (23). The activity that hydrolyses 1 µmole of ATP per min is defined as one unit. DCCD sensitivity was measured as follows: Either the inverted vesicles (10 mg-protein/ml) or proteoliposomes (prepared as above) were added to the equal volume of 100 mM Tris/HCl (pH 8.8) containing 200 µM DCCD. These conditions were chosen to avoid undesired DCCD-labeling of catalytic residue (Glu190) of β subunits of F₁. After an incubation at 25 °C for 30 min, the vesicles were diluted 100-fold and subjected to the measurement of ATPase activity. ATP-driven H⁺-pumping activity of the vesicles was measured by the method of Aggeler et al (31) using a JASCO model-720 spectrofluorometer at 42 °C at 480 nm using an excitation wavelength of 410 nm. Authentic *Bacillus PS3 F₀F₁* was purified from *Bacillus PS3* cells as described previously (27). Two-dimensional SDS-PAGE was carried out as performed previously (23). Protein concentrations were determined using the BCA Protein Assay Kit from Pierce, with bovine serum albumin as a standard.

**RESULTS**
Expression of TF₉F₁ in E. coli cells ----

An operon structure containing unc genes coding for TF₉F₁ was introduced into a downstream of a strong promoter, trc, and then, expressed in a F₉F₁-deficient E. coli strain, DK8. The resultant recombinant strain (DK8/pTR19-ASDS) acquired an ability to form colonies on succinate minimum medium plates within 2 days (the diameter is about 2 mm), which is almost the same growth rate of a wild-type E. coli strain (JM109), indicating that the recombinant TF₉F₁ is functional as ATP synthase in E. coli cell. TF₉F₁ consisting of eight subunits was constitutively expressed in the plasma membranes, which amounted to ~20 % of the whole membrane proteins (Fig. 1A, lane 1). The purified TF₉F₁ was comprised of eight kinds of subunits, the same as authentic TF₉F₁ purified from the original Bacillus PS3 cells² (Fig. 1A, lane 2 and 3). The membranes of expressing E. coli exhibited 1.0 unit/mg of proteins (at 37 °C) of ATPase activity. More than 80 % of the activity were inactivated by a 50-min incubation with 50 µM DCCD or by a 20-min incubation with 100 µM DCCD (Fig. 1B). This inhibition is comparable or slightly more efficient than that (75 %) observed for the authentic TF₉F₁ purified from Bacillus PS3 cells (27). This inactivation is due to labeling subunit c but not labeling catalytic glutamic acid (Glu190) in β subunit of F₁ since ATPase activity was unaffected by DCCD treatment when measured in the presence of lauryldimethylamine oxide (LDAO), which unleashes ATPase activity of F₁ from Fₒ. The membrane ATPase was inhibited almost completely by 5 mM azide, an inhibitor of ATPase activity of F₁ and ATP synthase.

Cross-linking b and c subunits at the N-terminal regions ----

Two cysteine residues were introduced into TF₉F₁ by substituting Leu2 of subunit b and Ser2 of subunit c to obtain a mutant TF₉F₁, bL2C/cS2C. To use as controls, we also made two single mutants,
bL2C or cS2C, that had one substituted cysteine in subunit \( b \) or \( c \). These mutants were expressed in \( E. coli \) DK8 cells. N-terminal regions of subunits \( b \) and \( c \) are located in the periplasmic surface of plasma membrane (21) and as expected, the introduced cysteine residues in \( bL2C/cS2C \) formed a disulfide cross-link by treating expressing \( E. coli \) cells with an oxidant, 200 \( \mu \)M CuCl\(_2\). After careful removal of the oxidant, inverted membrane vesicles were prepared from the cells. In the presence of LDAO, the vesicles from the mutants showed ATPase activities similar to that of the wild-type, indicating that the wild-type and mutants were expressed at comparable levels (wild-type, 6.6±0.1 units/mg; \( bL2C \), 5.8±0.1 units/mg; \( cS2C \), 5.3±0.1 units/mg; \( bL2C/cS2C \), 6.8±0.2 units/mg-membrane protein).

Mutant TF\(_{\text{oF1}}\)s were purified from the vesicles, and analyzed by SDS-PAGE after incubation with or without 50 mM DTT at 25 °C for 1h (Fig. 2A). The mutant TF\(_{\text{oF1}}\)s incubated with DTT showed eight bands, the same as that of wild-type (lanes 1-4). However, those not exposed to DTT had additional one (\( bL2C \) and \( cS2C \), lanes 6 and 7) or three band(s) (\( bL2C/cS2C \), lane 8). Based on the N-terminal peptide sequences and estimated molecular sizes of the bands, these new bands were identified as disulfide cross-linked products of \( b-b \), \( b-c \), and \( c-c \) as indicated by arrows in Fig. 2A. Cross-link yields in \( bL2C/cS2C \) were analyzed by a two-dimentional SDS-PAGE, 1st in non-reducing and 2nd in reducing conditions (Fig. 2B). The three bands were separated in the 2nd electrophoresis into spot(s) corresponding to monomeric subunit \( b \) and/or \( c \). In bacterial ATP synthase, subunit \( b \) has been known to exist as a homodimer (32) and only one of two copies of subunit \( b \) is assumed to lie adjacent to the \( c \)-ring that is able to form a cross-link (21). Taking into account of this, the yields of \( b-b \) and \( b-c \) cross-links in \( bL2C/cS2C \) were estimated from the densities of spots to be 16±5% and 68±7%, respectively. A further
increase of the cross-links of $b$-$b$ or $b$-$c$ was not observed in $bL2C/cS2C$ even though either 500 µM CuCl$_2$ or 1 mM Cu/phenantroline was used as an oxidant. It is worth mentioning that a significant amount of $b$-$c$ cross-link was formed (yield ~40%) even in the membranes that was not treated with CuCl$_2$ (data not shown)$^3$. It appears evident that the close proximity between N-terminal ends of one of subunit $b$(s) and one (or more) of subunit $c$(s).

**Effects of $b$-$c$ cross-link on the activities of $TF_oF_1$** ---- The inverted vesicles prepared from the cells oxidized by CuCl$_2$-treatment were incubated with or without 50 mM DTT for 1 h and H$^+$-pumping activity was analyzed. DTT-treated vesicles of all mutants showed substantial ATP-driven H$^+$-pumping activities, comparable with that of the wild-type (Fig. 3A, left panel). Therefore, the introduced cysteine residue(s) at position 2 of subunit $b$ and that of subunit $c$, alone or together, does not significantly affect the $F_o$ function. This was also the case for the vesicles of mutants $bL2C$ and $cS2C$ that were not reduced by DTT. However, the vesicles of a mutant $bL2C/cS2C$, without DTT treatment, had drastically decreased H$^+$-pumping activity (Fig. 3A, right panel). Membranes of the oxidized vesicles from $bL2C/cS2C$ are capable of holding electrochemical potential of protons generated by NADH oxidation, as described later, and proton-leak cannot be a reason of the apparent loss of H$^+$-pumping. The inactivation of $bL2C/cS2C$ by oxidation was also observed for ATPase activity. Oxidized vesicles from $bL2C/cS2C$ without DTT treatment retained only 37% of the ATPase activity of that of the DTT-treated ones whereas activities of the vesicles from the single mutants (and wild-type) were hardly affected by oxidation-reduction treatment (Fig. 3B). The inhibition of ATPase was completely recovered by adding 0.1% LDAO in the ATPase assay mixture (data not shown),
confirming that the failure was not in F₁ but in F₀. The same experiments were repeated for the proteoliposomes reconstituted from purified TF₁,F₁ and soybean phospholipids (Figs. 3C and 3D). As observed for the inverted vesicles, inactivation of H⁺-pumping and ATPase activities was evident only for the oxidized bL2C/cS2C. To summarize the results, only oxidized TF₁,F₁ containing double mutations bL2C/cS2C has a defect in ATP hydrolysis and in H⁺-pumping. This defect is caused by a b-c cross-link and cannot be ascribed to the b-b and c-c cross-links. This is because, as shown in Fig. 2A, the amount of b-b and c-c cross-links produced in the oxidized bL2C/cS2C was too little to account for the observed inactivation. Furthermore, the oxidized single mutants, bL2C and cS2C, contained more b-b and c-c cross-links, respectively, than those in the oxidized bL2C/cS2C as shown in Fig. 2A but still their activities were not inactivated significantly. On the contrary, the amount of b-c cross-link in oxidized bL2C/cS2C (~68 %) agrees fairly well with the degree of inactivation (~60 % for ATPase activity). Thus, prevention of movement of c-ring relative to subunit b is fatal for the catalytic function of ATP synthase with proper coupling.

*Effects of b-c cross-link on proton flow through F₀ ----* Inverted vesicles that were prepared from CuCl₂-oxidized cells were washed with 0.2 mM EDTA to obtain F₁-stripped inverted vesicles. Electrochemical potential of protons was generated across membrane of inverted F₀ vesicles by the respiratory chain on the vesicles, using NADH as a substrate, and the downhill proton efflux through F₀ was assessed by monitoring fluorescence quenching of ACMA. Without F₁, protons taken up in vesicles by respiration easily diffused out through F₀ and only a small fluorescence quenching was maintained at steady state as a balance between activities of respiration and proton flow through F₀ (Fig. 4A,
wild-type). Prior reducing treatment of the vesicles by DTT did not change the result significantly. Inverted Fo vesicles prepared from single mutants, bL2C and cS2C, behaved similarly; the quenching was small and the effect of DTT treatment was minor (Fig. 4A, bL2C and cS2C). Also the extent of fluorescence quenching was small for the DTT-treated inverted Fo vesicles of bL2C/cS2C (Fig. 4A, bL2C/cS2C). However, when the same inverted Fo vesicles of bL2C/cS2C were subjected to the test without prior DTT-treatment, remarkable fluorescence quenching was induced in response to addition of NADH. The magnitude of quenching by NADH well matched the one observed for the inverted vesicles, without Fo-stripping treatment, prepared from cells expressing wild-type FoF1 (data not shown). This result clearly indicates that b-c cross-link blocks proton efflux through Fo. It also implies that b-c cross-link does not make Fo proton-leaky.

To confirm further the above contention, Fo was isolated from purified TFoF1 and reconstituted into proteoliposomes. Fo proteoliposomes were incubated with or without DTT and then loaded with 0.5 M KCl. Membrane potential (inside negative) was generated by addition of valinomycin and downhill proton influx through Fo was assessed by monitoring fluorescence quenching of ACMA (Fig. 4B). In the case of Fo proteoliposomes of the wild-type, irrespective of whether they were treated with DTT or not, valinomycin induced significant fluorescence quenching that reflected proton flow through Fo. Fo proteoliposomes of bL2C/cS2C with prior DTT treatment displayed similar extent of fluorescence quenching. The quenching was greatly suppressed, on the contrary, when Fo proteoliposomes of bL2C/cS2C without prior DTT treatment were examined. In any cases, preincubation of Fo proteoliposomes with DCCD resulted in complete abolishment of valinomycin-induced quenching. These results, together with those of the inverted Fo vesicles, lead to the conclusion that proton efflux and influx through Fo are
DISCUSSION

The major message of this report concerns the relation between the c-ring rotation and the proton flow through F_o. As illustrated in Fig. 5, the F_o with a disulfide cross-link between subunits b and c was unable to mediate proton translocation (Fig. 4). With prevented proton translocation, ATP hydrolysis was also prevented, suggesting the retention of tight coupling between F_o and F_1 in the cross-link containing TF_oF_1 (Fig. 3). Regardless of the directions of proton translocation, either periplasmic side to cytoplasmic side or cytoplasmic side to periplasmic side, translocations were equally blocked by the cross-linking. The inactivation is reversible; reduction of the disulfide restored the proton translocation by F_o and ATP-driven proton pumping by TF_oF_1. These results strongly indicate that protons cannot pass through F_o without rotation of c-ring, or conversely, rotation of c-ring must accompany proton translocation. Cross-linking of b and c subunits caused neither proton leak nor unleashing activation of ATPase of F_1. Thus a possibility that the cross-link itself disrupts the function of F_o is minimum, if not null. In our experimental setups, proton translocations across membranes down the \( \Delta \mu H^+ \) were measured. Previous data from other laboratories indicate that the coupling of proton translocation and c-ring rotation is maintained even in the absence of \( \Delta \mu H^+ \). Dimroth’s group has detected \(^{22}\text{Na}^+/{\text{Na}}^+\) exchange across proteoliposome membranes in the absence of \( \Delta \mu H^+ \) through F_o isolated from Na\(^+\) transporting ATP synthase of Propionigenium modestum (33). They did not examine the movement of c-ring but assumed that the back-and-forth thermal rotary motion of c-ring in F_o was responsible for the exchange.
Fillingame and his colleagues demonstrated by using b-c cross-linking of uncoupled E. coli ATP synthase that the subunit c adjacent to subunit b is mobile and exchanges with subunits c that initially occupied other positions (17). This exchange occurs independently of ATP, suggesting thermal motion of the c-ring. Although not tested by their experiments, it is natural to assume that this thermal motion accompanies proton translocation. Taken together, it is safe to conclude that isolated F_o is a rotary proton channel, in which the ‘friction’ between rotor and stator is small enough to allow free fluctuating rotary motion even by environmental thermal energy. Nevertheless, this friction may differentiate the F_o channel from other open ion channels; the ion conductance of the former is several orders lower than that of the latter (F_o, ~7 s^{-1} (33); potassium channel, >10^6 s^{-1} (34); aquapolin-1, >10^9 s^{-1} (35)). Once ΔµH⁺ is applied, the F_o rotary channel starts to rotate unidirectionally and protons are transported also unidirectionally across membranes.

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REFERENCES

FOOTNOTES

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Abbreviation used are: TFₐF₁, ATP synthase from a thermophilic *Bacillus* strain PS3 (recombinant TFₐF₁ investigated in the present study has a histidine-tag of 10 residues at N-terminus of the β subunit); DCCD, *N*,*N*'-dicyclohexylcarbodiimide; LDAO, lauryldimethylamine oxide; DTT, dithiothreitol; ACMA, 9-amino-6-chloro-2-methoxyacridine; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate with prior reducing procedures (termed as reducing SDS-PAGE when necessary for clarification); non-reducing SDS-PAGE, SDS-PAGE without prior reducing treatment.

On the gel of Fig. 1A, a band of recombinant *b* subunit (*lanes 1 and 2*), moved slightly slower than the counterpart of the TFₐF₁ purified from *Bacillus* PS3 cells (*lane 3*). N-terminal sequencing of the recombinant subunit revealed that a N-terminal segment comprising 11 residues is not processed in the *E. coli* cells, different from the authentic enzyme (23). Low mobility of recombinant β subunit is ascribed to 10 residues of a histidine-tag introduced at N-terminus.

The membrane vesicles without CuCl₂-treatment showed levels of ATPase activity and proton pumping intermediate between +DTT and –DTT level.
FIGURE LEGENDS

FIG. 1. Expression of TF₆F₁ in E. coli cells.  A. SDS-PAGE analysis.  lane 1, membrane fraction of E. coli cells expressing TF₆F₁ (E. coli DK8/pTR19-ASDS); lane 2, purified recombinant TF₆F₁; lane 3, authentic TF₆F₁ purified from Bacillus PS3 cells.  B. Inactivation of ATPase activity with DCCD.  The inverted vesicles prepared from E. coli cells expressing the recombinant TF₆F₁ were incubated with 50 (closed squares) or 100 µM DCCD (closed circles) at 25 °C for the indicated periods and ATPase activities were measured at 37 °C.  The ATPase activities after a treatment with 100 µM DCCD for indicated periods were measured in an ATPase assay solution containing 0.1 % LDAO (open circles).

FIG. 2. Cross-link formation through disulfide bonds in the mutant TF₆F₁s.  A. SDS-PAGE analysis of cross-link formation in bL2C, cS2C, and bL2C/cS2C mutants. The purified TF₆F₁s from the oxidized cells were incubated with or without 50 mM DTT, and applied to the gel.  B. Two-dimensional SDS-PAGE analysis of purified bL2C/cS2C from oxidized cells: 1st dimension, non-reducing SDS-PAGE; 2nd dimension, reducing SDS-PAGE.

FIG. 3. Effect of cross-linking on H⁺-pumping and ATPase activities.  A. H⁺-pumping activity of the inverted vesicles.  H⁺-translocation was monitored by fluorescence quenching of ACMA in buffer-PA4 (10 mM HEPES/KOH, pH 7.5, containing 100 mM KCl and 5 mM MgCl₂) supplemented with 0.3 µg/ml ACMA.  The inverted vesicles prepared from the oxidized cells were added to the solution to be a final concentration of 0.5 mg-membrane protein/ml.  The reaction was initiated by adding 1 mM ATP and
terminated by 1 µg/ml of FCCP.  **B.** ATPase activities of the inverted vesicles.  ATPase activities were measured by the ATP-regenerating system in buffer-PA5 (50 mM HEPES/KOH, pH 7.5, containing 100 mM KCl, 5 mM MgCl₂) supplemented with 1 mM ATP, 5 mM KCN, and 1 µg/ml FCCP at 37 °C.  Bars indicate ATPase activities of samples treated with (black) or without DTT (white).  **C.** H⁺-pumping activity of reconstituted TF₇₅F₁-proteoliposomes.  The proteoliposomes were added to be a final concentration of 21 µg-TF₇₅F₁/ml.  **D.** ATPase activity of reconstituted TF₇₅F₁-proteoliposomes.  Analytical conditions of C and D are the same as those of A and B.  In these analyses, the inverted vesicles and reconstituted TF₇₅F₁-proteoliposomes were incubated with or without 50 mM DTT at 25 °C for 1h, prior to the measurement.

**FIG. 4.** Effect of cross-linking on proton translocation through Fₒ. Vesicles and proteoliposomes were incubated with or without 50 mM DTT prior to the analysis.  **A.** Proton efflux through Fₒ of the F₁-stripped inverted vesicles (final conc. 40 µg-membrane protein/ml) was monitored by fluorescence quenching of ACMA.  Electrochemical potential of protons (inside positive) was generated by adding 0.2 mM NADH to the assay mixture.  **B.** Proton influx through Fₒ of the reconstituted F₁-proteoliposomes (final conc. 5 µg-TF₇₅/ml) was analyzed in 10 mM HEPES/NaOH (pH 7.5) containing 5 mM KCl and 0.5 M sucrose as performed previously (30).  Membrane potential (inside negative) was generated by supplementing 2.5 ng/ml valinomycin to proteoliposomes loaded with 0.5 M KCl inside.  Effect of DCCD on the proton translocation was measured for the proteoliposomes that were treated with 50 mM DTT and then treated with 100 µM DCCD before the analysis.

**FIG. 5.** Proton flow through Fₒ coupled with c-ring rotation and its prevention by the
**b-c cross-linking.**  
A. $F_o$ in the reduced form. The $c$-ring can rotate in either direction corresponding to either efflux or influx of protons.  

B. $F_o$ in the oxidized form. The $b$-$c$ cross-link physically prevents rotation of $c$-ring and protons can no longer pass through $F_o$. $F_o$ is derived from $TF_3F_1 (bL2C/cS2C)$ that has cysteines at N-terminal regions of subunits $b$ and $c$. 
**A**

ATP $\rightarrow$ FCCP $\rightarrow$ ATP $\rightarrow$ FCCP

1 min 10%

bL2C/cS2C bL2C cS2C bL2C/cS2C Wild-type

**B**

![Graph showing ATPase activity with DTT and -DTT](image)

**C**

ATP $\rightarrow$ FCCP $\rightarrow$ ATP $\rightarrow$ FCCP

1 min 10%

bL2C/cS2C bL2C cS2C bL2C/cS2C Wild-type

**D**

![Graph showing ATPase activity with DTT and -DTT](image)
TOP, Fig. 4. Suzuki et al.

A

Wild-type  bL2C  cS2C  bL2C/cS2C

NADH  FCCP

+DTT  -DTT  +DTT  -DTT  +DTT  -DTT

2 min  10%

B

Wild-type  bL2C/cS2C

Val  FCCP

+DCCD  -DTT  +DTT  -DTT  +DTT  -DTT

2 min  20%
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