Coupling of proton flow and rotation in the F₀ 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 β and c of ATP synthase (βL2CεS2C) were readily oxidized by treating the expressing cells with CuCl₂ 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 F₀, either in the F₁-stripped inverted vesicles or in the reconstituted F₀-proteoliposomes, hardly mediated passive proton translocation through F₀. Careful analysis using single mutants (βL2C or εS2C) as controls indicated that the 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; if there is no rotation of the c-ring there is no proton flow through F₀.

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, α₂βγεδ₂F₁F₀, which is 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 α₂β₃ hexamer ring (7, 8). Remaining F₀ sector in the membrane acts as a proton channel that mediates passive proton translocation across the 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 α and b₂ are peripherally located outside of a ring of subunit c oligomers (c-ring). A low resolution crystal structure of an F₁+ε₁₀ subcomplex from yeast mitochondria revealed a tight interaction between γε subunits of F₁ and the 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 the 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 the stator of F₀ and that of F₁ and prevents the stators from being dragged by rotation of the central stalk (16). From these and other observations, it is generally accepted that the 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 the 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 F₁ drives reverse rotation of the γε stalk and c-ring, which causes proton eﬄux through F₀. To explore the mechanism of proton flow through F₀ and its coupling with rotation, measurement of the proton flow together with the c-ring rotation is absolutely required. The study has been impeded by the unstable nature of ATP synthases; the structural and functional integrity of the enzyme is easily damaged during experimental procedures (20, 21). For example, the cross-linking between subunits 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 (TF₀F₁)₃ in E. coli cells. Cysteine residues introduced into subunits b and c of TF₀F₁ were readily cross-linked in the presence of CuCl₂. 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 F₀ was also disabled by this cross-link. Thus, the c-ring must rotate for protons to pass through F₀.

EXPERIMENTAL PROCEDURES

Construction of an Expression Vector for TF₀F₁—DNA manipulations were carried out by following the methods of the literature (22). A plasmid pTR-ke, which is an expression vector for α₂βγε subcomplex of Bacillus PS3 F₁-ATPase (23), was used as a start material. A 4.4-kilobase pair DNA fragment containing Bacillus PS3 uncBEFHA genes (coding for a, b, c, d, δ, и, and ε subunits of TF₀F₁) was amplified by PCR from the plasmid pUC119/TF₀F₁ (24) using primers 5′-CCCGCCGGAAATTCTAAGAAGGGAGATATACATATGGAGCATAAAGCGCCGCTTGTCCG-3′ and 5′-GGCCGATCGGTTACCCGGGCTCGATCGTCTTTATTAAAGCCGCGGAGCGCGCTTGTCCG-3′.
Expression and Purification of TF-F1 in E. coli Cells—An operon structure containing unc genes coding for TF-F1 was introduced into a downstream of a strong promoter, trc, and then expressed in a F-F1-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 TF-F1 is functional as ATP synthase in the E. coli cell. TF-F1, consisting of eight subunits was constitutively expressed in the plasma membranes, which amounted to ~20% of the whole membrane proteins. N-terminal sequencing of the recombinant TF-F1 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.

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located in the periplasmic surface of plasma membrane (21) and as expected, the introduced cysteine residues in \( \text{bL2C/cS2C} \) formed a disulfide cross-link by treating \( \text{E. coli} \) cells with an oxidant, 200 \( \mu \text{M} \) \( \text{CuCl}_2 \). 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; \( \text{bL2C}, 5.8 ± 0.1 \) units/mg; \( \text{cS2C}, 5.3 ± 0.1 \) units/mg; \( \text{bL2C/cS2C}, 6.8 ± 0.2 \) units/mg of membrane protein).

Mutant \( \text{TF}_o^b \alpha \) were purified from the vesicles and analyzed by SDS-PAGE after incubation with or without 50 \( \mu \text{M} \) DTT at 25 °C for 1 h (Fig. 2A). The mutant \( \text{TF}_o^b \alpha \) incubated with DTT showed eight bands, the same as that of wild type (lanes 1–4). However, those not exposed to DTT had an additional one (\( \text{bL2C} \) and \( \text{cS2C} \), lanes 6 and 7) or three bands (\( \text{bL2C/cS2C} \), 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 \( \text{b-b, b-c, and c-c} \) as indicated by arrows in Fig. 2A. Cross-link yields in \( \text{bL2C/cS2C} \) were analyzed by two-dimensional SDS-PAGE, first in non-reducing and second in reducing conditions (Fig. 2B). The three bands were separated in the second 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 this into account, the yields of \( \text{b-b} \) and \( \text{b-c} \) cross-links in \( \text{bL2C/cS2C} \) were estimated from the densities of spots to be 16 ± 5 and 68 ± 7%, respectively. A further increase of the cross-links of \( \text{b-b} \) or \( \text{b-c} \) was not observed in \( \text{bL2C/cS2C} \) even though either 500 \( \mu \text{M} \) \( \text{CuCl}_2 \) or 1 \( \text{mM} \) copper/phenanthroline was used as an oxidant. It is worth mentioning that a significant amount of \( \text{b-c} \) cross-link was formed (yield ~40%) even in the membranes that were not treated with CuCl2 (data not shown).\(^3\) It appears evident that there is close proximity between the N-terminal ends of subunit b(s) and (or more) of subunit c(s).

Effects of \( \text{b-c} \) Cross-link on the Activities of \( \text{TF}^b \alpha \) —The inverted vesicles prepared from the cells oxidized by \( \text{CuCl}_2 \) treatment were incubated with or without 50 \( \mu \text{M} \) DTT for 1 h, and \( \text{H}^+ \)-pumping activity was analyzed. DTT-treated vesicles of all mutants showed substantial ATP-driven \( \text{H}^+ \)-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, do not significantly affect the \( \text{F}_0 \) function. This was also the case for the vesicles of mutants \( \text{bL2C} \) and \( \text{cS2C} \) that were not reduced by DTT. However, the vesicles of a mutant \( \text{bL2C/cS2C} \) (without DTT treatment) had drastically decreased \( \text{H}^+ \)-pumping activity (Fig. 3A, right panel). Membranes of the oxidized vesicles from \( \text{bL2C/cS2C} \) are capable of holding the electrochemical potential of protons generated by NADH oxidation, as described later, and proton leak cannot be a reason for the apparent loss of \( \text{H}^+ \) pumping. The inactivation of \( \text{bL2C/cS2C} \) by oxidation was also observed for ATPase activity. Oxidized vesicles from \( \text{bL2C/cS2C} \) 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 recov-

\(^3\) The membrane vesicles without \( \text{CuCl}_2 \) treatment showed levels of ATPase activity and proton pumping intermediate between the \(+\text{DTT}\) and \(-\text{DTT}\) level.
ered by adding 0.1% LDAO in the ATPase assay mixture (data not shown), confirming that the failure was not in F0 but in F1. The same experiments were repeated for the proteoliposomes reconstituted from purified TF0F1 and soybean phospholipids (Fig. 3, C and D). 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 TF0F1 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 the 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. The electrochemical potential of protons was generated across the 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 F0 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 F0 vesicles of bL2C/cS2C (Fig. 4A, bL2C/cS2C). However, when the same inverted F0 vesicles of bL2C/cS2C were subjected to the test without prior DTT treatment, remarkable fluorescence quenching was induced in response to the addition of NADH. The magnitude of quenching by NADH matched well the one observed for the inverted vesicles, without F1-stripping treatment, prepared from cells expressing wild-type F0/F1 (data not shown). This result clearly indicates that b-c cross-link blocks proton efflux through F0. It also implies that b-c cross-link does not make F0 leak protons.

To confirm further the above contention, F0 was isolated from purified TF0F1 and reconstituted into proteoliposomes. F0 proteoliposomes were incubated with or without DTT and then loaded with 0.5 M KCl. Membrane potential (inside negative) was generated by the addition of valinomycin, and downhill proton influx through F0 was assessed by monitoring fluorescence quenching of ACMA (Fig. 4B). In the case of F0 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 F0. F0 proteoliposomes of bL2C/cS2C with prior DTT treatment displayed a similar extent of fluorescence quenching. On the contrary, the quenching was greatly suppressed when F0 proteoliposomes of bL2C/cS2C without prior DTT treatment were examined. In any case, preincubation of F0 proteoliposomes with DCCD resulted in complete abolishment of valinomycin-induced quenching. These results, together with those of the inverted F0 vesicles, led to the conclusion that proton efflux and influx through F0 are blocked by b-c cross-link.

DISCUSSION

The major message of this report concerns the relation between the c-ring rotation and the proton flow through F0. As illustrated in Fig. 5, the F0 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 F0 and F1 in the cross-link containing TF0F1 (Fig. 3). Regardless of the directions of proton translocation, either the periplasmic side to the cytoplasmic side or the cytoplasmic side to the periplasmic side, translocations were equally blocked by the cross-linking. The inactivation is reversible; reduction of the disulfide restored the proton translocation by F0 and ATP-driven proton pumping by TF0F1. These results strongly indicate that protons cannot pass through F0 without rotation of the c-ring, or conversely, rotation of the c-ring must accompany proton translocation. Cross-linking of b and c subunits caused neither a proton leak nor the unleashing of activation of ATPase of F1. Thus the possibility that the cross-link itself disrupts the function of F0 is minimal, if not null. In our experimental setups, proton translocations across membranes down the Δψ, 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 Δψ. Dimroth’s group has detected 22Na+/Na+ exchange across proteoliposome membranes in the absence of Δψ through F0 isolated from Na+-transporting ATP synthase of Propionigenium modestum (33). They did not examine the movement of the c-ring but assumed that the back-and-forth thermal rotary motion of the c-ring in F0 was responsible for the exchange.
Fillingame and his colleagues (21) demonstrated by using the 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. 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 F₀ is a rotary proton channel in which the F₁-ring can rotate in either direction corresponding to either efflux or influx of protons. B. F₀ is in the oxidized form. The b-c cross-link physically prevents rotation of the c-ring, and protons can no longer pass through F₀. F₀ is derived from TF₁ (bL2c/s2c) that has cysteines at N-terminal regions of subunits b and c.

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