Preliminary Estimation of Rotary Torque Produced by Proton-Motive Force in Fully Functional F_0F_1-ATPase

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Abstract: F_0F_1-ATPase is a rotary molecular motor. It is well known that the rotary torque is generated by ATP hydrolysis in F_1 but little is known about how it produces the proton-motive force (PMF) in F_0. Here a cross-linking approach was used to estimate the rotary torque produced by PMF. Three mutant *E. coli* strains were used in this study: SWM92 (δW28L F_0F_1, as control), MM10 (αP280CγA285C F_0F_1) and PP2 (αA334C/γL262C F_0F_1). The oxidized inner membranes from mutant MM10 having a disulfide bridge in the top of γ subunit exhibited good ATP synthesis activity, while the oxidized PP2 inner membranes having a disulfide bridge in the middle of γ subunit synthesized ATP very poorly. We conclude that the rotary torque generated by PMF is sufficient to uncoil the α-helix in the top of γ subunit (MM10) and to overcome the Ramachandran activation barriers (25-30kJ/mol, i.e. about 40-50pN·nm), but cannot cleave the disulfide bond in the middle of the γ subunit (200 kJ/mol, i.e. 330pN·nm) (PP2). Consequently a preliminary estimation is that the rotary torque generated by PMF in the fully functional F_0F_1 motor is greater than 40-50pN·nm but less than 330pN·nm.

Keywords: F_0F_1-ATPase, proton-motive force (PMF), rotary torque, cross-linking.

INTRODUCTION

F_0F_1-ATPase which is found in bacterial plasma membranes, mitochondrial inner membranes, and chloroplast thylakoid membranes, catalyses the endergonic synthesis of ATP from ADP and phosphate using transmembrane proton-motive force (PMF) generated by oxidative phosphorylation or photosynthesis [1-8]. It is composed by two parts: a cytoplasmic F_1 part which includes three catalytic sites for ATP synthesis/ hydrolysis and a membrane-embedded F_0 part which constitutes a proton channel. In *E. coli*, F_1 and F_0 are composed by $\alpha_3\beta_3\gamma\delta\varepsilon$ and ab_2c_n, respectively. These two parts were structurally linked by two stalks, a central stalk of the γ and ε subunits that links to the c subunit ring and an outer stalk of δb_2, linking $\alpha_3\beta_3$ to a subunit. The downhill proton flow through F_0 drives rotation of the c-ring and hence γε, forcing conformational changes in F_1 that result in ATP synthesis from ADP and Pi. Conversely, ATP hydrolysis in F_1 causes the reverse rotation of the rotor [9] that drives F_0 to pump protons in the reverse direction.

It is well known that F_0F_1-ATPase is a rotary molecular motor [10]. By attachment of fluorescent actin filaments, ATP-driven rotation of F_0F_1 was observed directly by microscopy [9, 11-13], and the rotary torque produced by ATP hydrolysis was about 40 pN·nm [11], or even 50-56 pN·nm[14]. But the proton-driven rotation of F_0F_1-ATPase remains more elusive than the ATP-driven rotation. Although the rotation of F_0F_1 driven by proton flux also was observed by FRET [15], there was no information concerning about the rotary torque. The study of molecular motors that have the potential to power nanodevices has attracted increasing interest. Therefore, the study of the molecular mechanisms of rotary torque generated by PMF is important and is receiving considerable attention not only by biologists, but also from scientists in other fields of research.

Cross-linking studies have been widely used for defining the rotor and stator of F_0F_1-ATPase [6]. It has been reported that covalent disulfide-bridging between subunits α and γ at positions αP280C and γA285C did not inhibit the ATPase activity of F_1 and the rotation of γ subunit relative to $\alpha_3\beta_3$ hexagon (Fig. **1**) [16]. Studies on cross-linking between α and γ (αP280C/γA285C) revealed that the torque generated by ATP hydrolysis can overcome the artificial clamping of C-terminal portion of γ probably by unwinding the α-helix to form a swivel joint [16, 17]. But until now it is not known whether covalent disulfide-bridging between α and γ (αP280C/γA285C) inhibits the proton-pumping activity during oxidative phosphorylation. In this paper, by cross-linking study on αP280C/γA285C mutant and another mutant (αA334C/γL262C) where cysteines were introduced in the center position (Fig. **1**), we analyzed the rotary torque generated by PMF during oxidative phosphorylation.

2. MATERIALS AND METHODS

2.1. Chemicals

ACMA was purchased from Molecular Probes. ATP, ADP, NADH, DCCD and CCCP were purchased from Sigma. ATP yields were determined by luciferin/luciferase kit purchased from LKB. All of other analytically purified reagents were purchased domestically.

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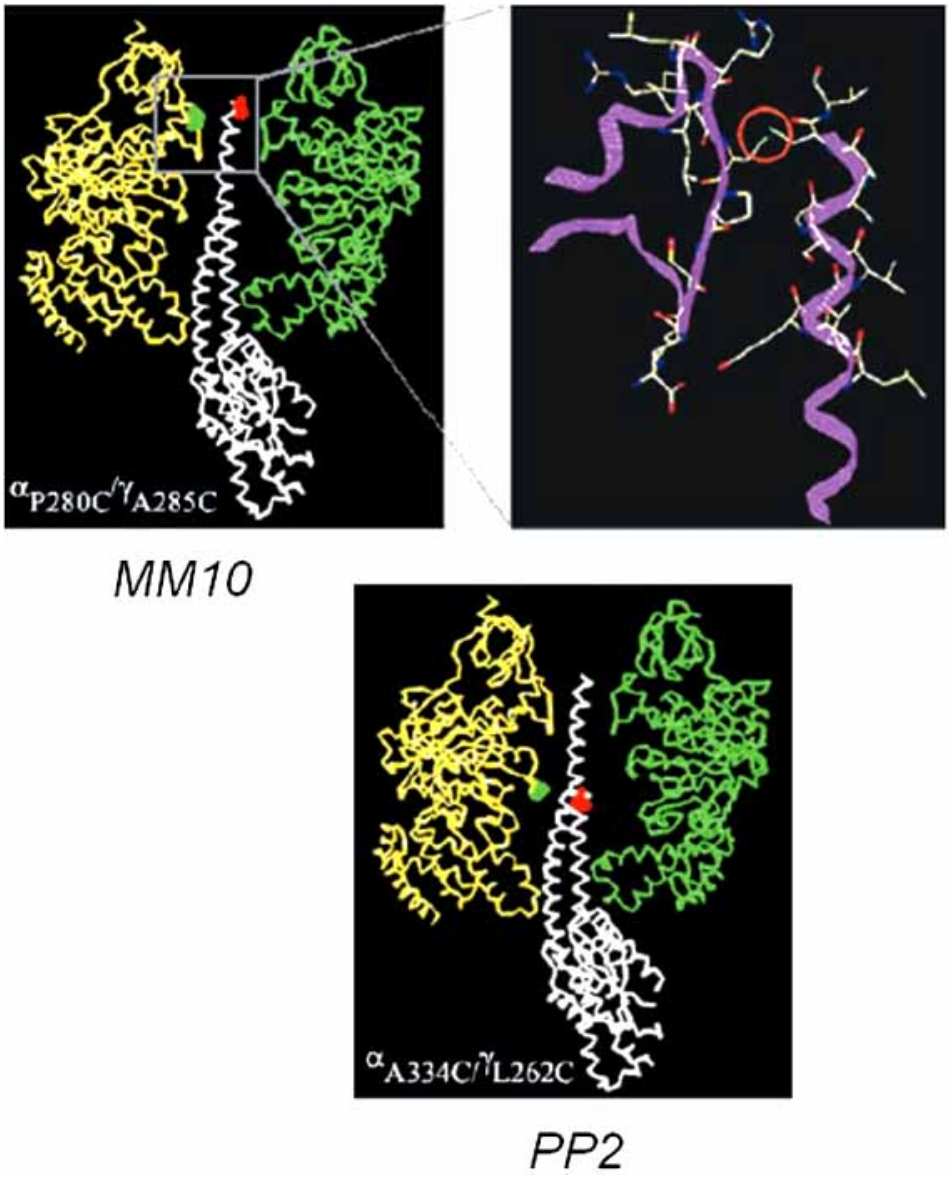

Figure 1. The localization of engineered cysteines in *E. coli* F_1-ATPase mutants MM10 (αP280C/γA285C) and PP2 (αA334C/γL262C) [16].The introduced cysteines (red and green dots) in MM10 and PP2 are at the center and top positions, respectively. (This picture is from [16], with the permission of Dr. S. Engelbrecht).

2.2. Preparation of Inner Membranes

The *E. coli* strain pSWM92/DK8 expressing δW28L F_1F_0 as a control strain in this study was kindly presented by Dr. Alan. E. Senior [18]. The *E. coli* strain pPP2/DK8 (αA334C/γL262C) and plasmid pMM10(αP280C/γA285C) were kindly presented by Prof. Siegfried Engelbrecht [16]. The plasmid pMM10 was transformed into *E. coli* strain DK8[19]. The three *E. coli* strains were cultured in LB medium supplemented with ampicillin at 50 μg/ml for 12 hours at 37°C, and harvested by centrifugation (7700 g, 30 min, 4°C). The cell pellets were stored at -80°C.

Before preparation of the inner membranes, the cells were thawed at ambient temperature and resuspended in French press buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 5 mM $MgCl_2$). The suspension was subjected to the French press procedure at 16,000 psi. Unbroken cells were collected by centrifugation (25,000 g, 20 min, 4°C) and discarded. Then the supernatant fraction containing inner membranes was collected by centrifugation (200,000 g, 90 min, 4°C). The pellets (inner membrances) were resuspended in French press buffer, and stored in liquid N_2. Proteins concentration of membranes was determined by BCA protein assay kit with bovine serum albumin as a standard.

2.3. Oxidation and Reduction of Inner Membranes

Oxidized and reduced inner membranes were prepared by treating with 250 μM $CuCl_2$ and 50 mM DTT for 24 hours at 22°C, respectively. 1 mM EDTA was added to the oxidized membrane vesicles to chelate the free Cu^{2+}, and to the DTT treated vesicle solution to adjust the conditions before assaying of ATP synthesis [20]. Cross-linked products were analyzed by SDS-PAGE (15%), followed by immunoblotting for identification with polyclonal antibody against α

subunit used at dilutions of 1:100,000 (Dr. Joerg Greie, AG Mikrobiologie, Prof. Dr. K. Altendorf, personal communication).

2.4. Other Methods

ATP hydrolysis activity of inner membranes was measured at 37°C by determination of Pi yields [21]. ATP-driven proton translocation was determined by measuring the quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) on inner membranes using a Hitachi F-4500 spectrofluorometer, (37°C, excitation at 410 nm and emission at 480 nm): 5 μl-sample of membrane vesicles(180 μg) was diluted into 1.6 ml fluorescence assay buffer (10 mM Hepes-KOH, 5 mM $MgCl_2$, 300 mM KCl, pH 7.5) containing 0.3 μg/ml ACMA; the fluorescence quenching was initiated by the addition of 1 mM ATP and terminated by addition of 10 μM CCCP. ATP synthesis was determined as follows [20,22]: 70μg of inner membranes were resuspended in 2 ml of buffer containing 25 mM Tris-HCl, 5 mM $MgCl_2$, 10% glycerol, 0.4 mM ADP, 5mM K_2PO4, and the reaction was initiated by addition of 1 mM NADH. After incubation at 37°C for 5 min, the synthesis was stopped by 4% trichloroacetic acid. The amount of ATP was determined with the luciferin/luciferase system (LKB kits) by measuring the emitted light using an ATP chemiluminometer.

3. RESULTS

3.1. Formation of α-γ Cross-Links

Three different mutant inner membranes were used: SWM92 (δW28L), MM10 (αP280C/γA285C) and PP2 (αA334C/γL262C). There is just one cysteine at γ87 in SWM92, so it was used as control in experiments. As for MM10 and PP2, cysteine residues were introduced at positions 280 and 334 in α, and 285 and 262 in γ (Fig. **1**). It has been reported that disulfide bridges can easily form between α and γ when it was treated with $CuCl_2$ in these two mutants F_1 [16]. In the preparation of inner membranes, no DTT or $CuCl_2$ was added, so in the following experiment, we treated the vesicles with 200 μM $CuCl_2$ or 50 mM DTT to ensure of the complete oxidation or reduction. As shown in (Fig. **2**), oxidation with $CuCl_2$ generated the quantitative formation of the α-γ in the mutants of MM10 and PP2, which was confirmed by immunoblotting with the α-subunit antibody.

3.2. Effects of Oxidation on the ATPase Activity of Inner Membranes

The effect of disulfide bridge formation on purified MM10 and PP2 F_1 has been demonstrated [16], but we have no information about it in intact F_0F_1-ATPase embedded in inner membranes. So, in this paper, the ATP hydrolysis activities of inner membranes were assayed. The results showed that the ATP hydrolysis activity of PP2 was inhibited greatly by the oxidation, up to 88% (Fig. **3A**, 0.045 units/mg membrane protein compared with 0.385units/mg membrane protein). The ATP hydrolysis activity of SWM92 was less inhibited by the oxidation (Fig. **3A**, 0.331 units/mg membrane protein compared with 0.423 units/mg membrane protein). The inner membranes of MM10 were also not affected by the oxidation, same to the purified MM10 F_1 (Fig. **3A**, 0.282 units/mg membrane protein compared with 0.291 units/mg membrane protein).

It is well known that DCCD is a well-characterized F_0F_1 inhibitor, reacting covalently with D61 of c subunits to irreversibly block both ATP synthesis and hydrolysis [23]. As shown in (Fig. **3A**), the ATP hydrolysis activities of the inner membranes were inhibited by DCCD, indicating that the ATP hydrolysis activities were presented by the F_0F_1-ATPase embedded in the inner membranes, and the coupling between F_1 and F_0 was not disrupted in all the preparation procedures.

Fig. **3B** shows the ATP-driven proton pumping activities of inner membranes, corresponding with the ATP hydrolysis activities as shown in Fig. **3A**. The reduced and oxidized SWM92 inner membranes behaved significant fluorescence quenching of ACMA (about 85%, Fig. **3B** SWM92), indicating that the ATP-driven proton pumping activities of in-

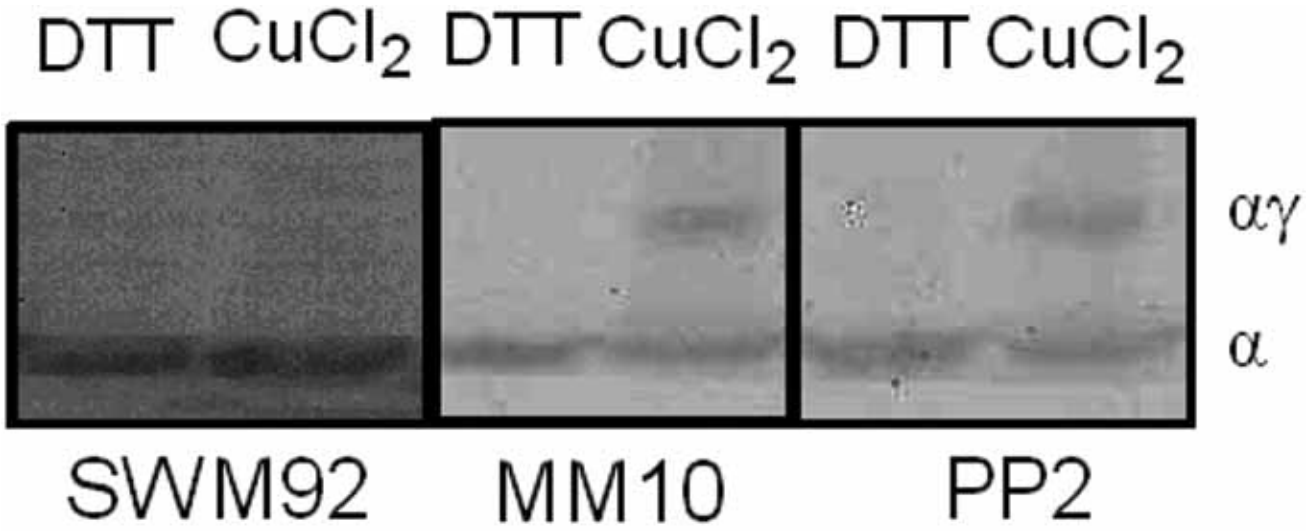

Figure 2. Formation of the α-γ cross-links *via* disulfide bonds in the inner membranes. The cross-linked products were identified with anti-α subunit immunoblotting.

ner membranes was not effected by the oxidation. Oxidation MM10 inner membranes show about 60-70% quenching (Fig. **3B** MM10), so the effect of oxidation was also minor. However, when the oxidized inner membranes of PP2 were subjected to the fluorescence assay, small (about 20%, Fig. **3B** PP2) fluorescence quenching was induced in response to the addition of ATP; while the reduced inner membranes showed remarkable quenching (over 80%, Fig. **3B** PP2), indicating that the formation of disulfide bridge inhibited the proton pumping activity of inner membranes, i.e., fixed the stator and rotor of F_0F_1-ATPase.

From above results, the oxidized inner membranes from mutant MM10 exhibited good ATP hydrolysis activity, while the oxidized PP2 inner membranes lost most of the ATP hydrolysis activity, due to the rotary torque produced by ATP hydrolysis in intact F_0F_1-ATPase can uncoil the α-helix in the top of γ subunit, but can not cleave the disulfide bond in the middle of the γ subunit.

3.3. Effects of Oxidation on the ATP Synthesis Activity of Inner Membranes

The effect of oxidation on the ATP synthesis activities of inner membranes was shown in Fig. **4A**. Inner membranes from SWM92 and MM10 treated with 200μM $CuCl_2$ retained 80.2% (23.55 nmolATP/min/mg protein vs.29.37 nmolATP/min/mg protein) and 79.3% (19.37 nmolATP /min/mg protein vs.24.33 nmolATP/min/mg protein) ATP synthesis activities relative to the reduced inner membranes,

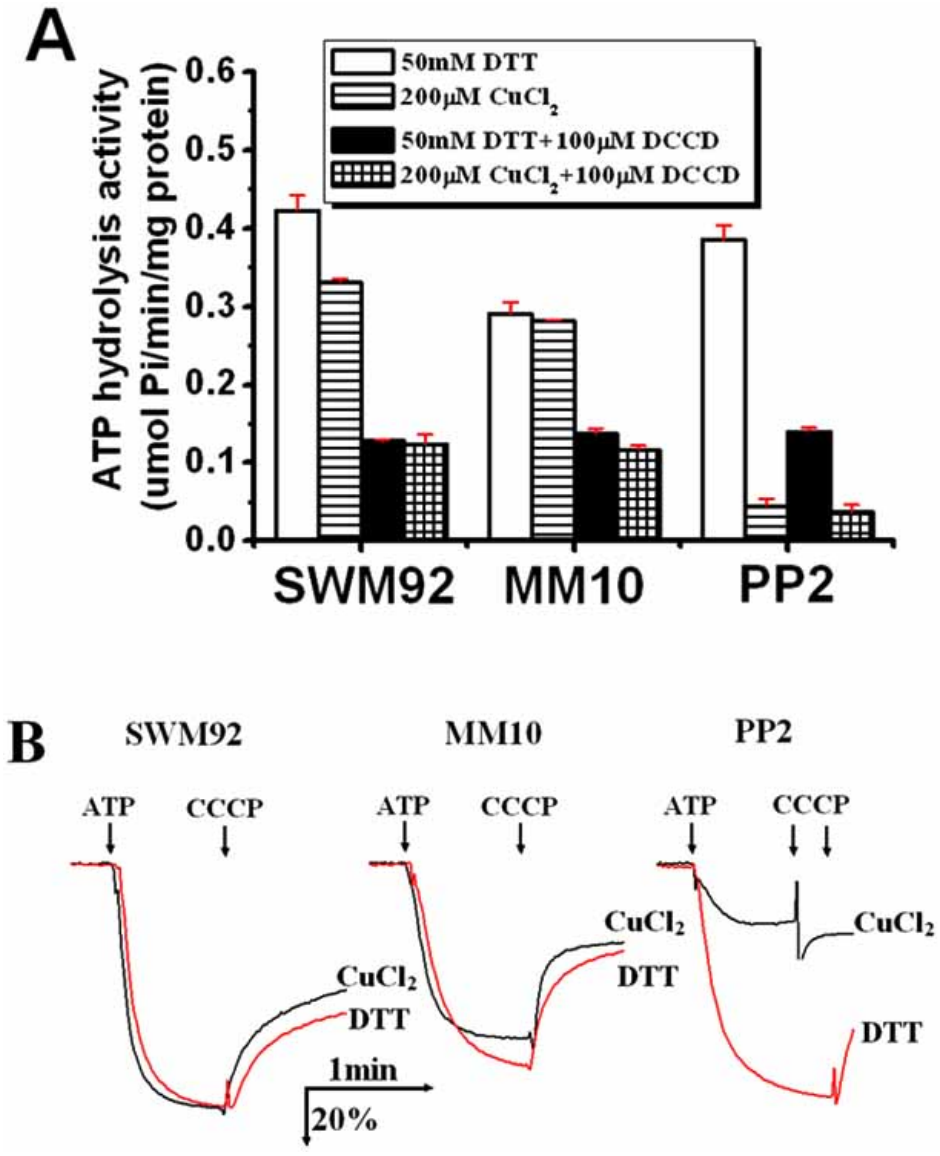

Figure 3. ATP hydrolysis activity and proton pump activity of inner membranes. A. Hydrolysis activity of inner membranes. The hydrolysis activities were assayed by determination of Pi yields at 37°C. Inner membranes were incubated with or without 100 μM DCCD prior to the analysis at 22 °C for 1 hour. B. ATP-driven proton pump activities of inner membranes. ATP-driven proton pump activities were monitored by ACMA quenching in buffer (10 mM Hepes-KOH, 5mM $MgCl_2$, 300 mM KCl, pH 7.5) supplemented with 0.3 μg/ml ACMA. The inner membranes were added to the solution to a final concentration of about 110 μg/ml. The reaction was initiated by adding 1 mM ATP and terminated by addition of 10 μM CCCP.

respectively. So we can conclude that the ATP synthesis of MM10 inner membranes was not affected by the oxidation, implying that the rotary torque produced by PMF could uncoil the disulfide bridges of α-helix in the top of γ subunit. However, the $CuCl_2$ treated PP2 inner membranes lost most of the ATP synthesis activity (94.0%, 1.40 nmolATP/min/mg protein vs. 23.51 nmolATP/min/mg protein), indicating that the cross-linking fully blocked the rotation of rotor relative to stator which due to the rotary torque produced by PMF can not cleave the disulfide bond in the middle of the γ subunit.

All of the inner membranes in this study show great DCCD sensitivities which were shown in Fig. **4B**. In the DCCD sensitivity test, no ATP produced in the DCCD incubated PP2 inner membranes checked by luciferin/ luciferase system, so we showed 100% sensitivity in Fig. **4B**.

4. DISCUSSION

This paper studied the ATP hydrolysis and synthesis activities of MM10 and PP2 inner membranes in oxidation and reduction conditions. As shown in (Fig. **3**), the F_0F_1-ATPase with a disulfide cross-linking between the C-terminal of γ

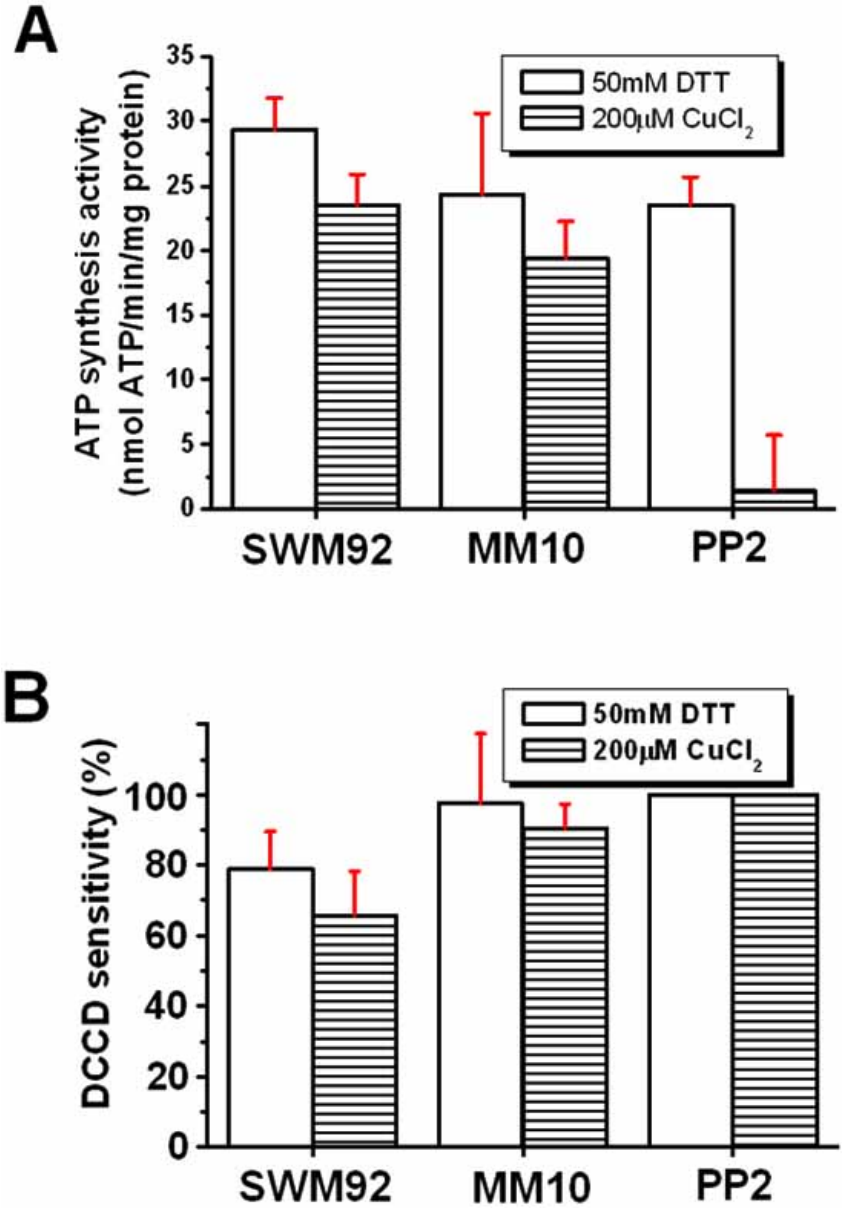

Figure 4. Effects of oxidation on the ATP synthesis activities and DCCD sensitivities of inner membranes. A. ATP synthesis activities of inner membranes treated with 50 mM DTT and 200μM $CuCl_2$. The inner membranes were treated with DTT and $CuCl_2$ as in Fig. **2**. The ATP synthesis activities were measured as described in "Materials and methods". B. DCCD sensitivity of inner membranes. Inner membranes were incubated for 60 min at 22°C with 40 μM DCCD before the ATP synthesis activities were assayed. The ATP synthesis activity of PP2 inner membranes was inhibited completely by DCCD (no ATP was produced by checking with luciferin/luciferase system). Therefore, we show 100% sensitivity of PP2 inner membranes to DCCD.

subunit and α subunit (MM10) was able to hydrolyze ATP to pump the proton into the vesicles, but the oxidized PP2 inner membranes lost most of the ATP hydrolysis activity. These results were in accordance with the results from the study of ATP hydrolysis activity and rotation activity of F_1 of such two mutants [16]. As mentioned above, the torque produced by ATP-driven rotation (about 51 pN·nm [16]) can uncoil the α-helix in the top of γ subunit and overcome Ramachandran activation barriers [16], but cannot cleave the disulfide bond (the energy is about 200 kJ/mol [16]). Significantly, PP2 inner membranes lost most of ATP synthesis activity after oxidation, indicating that the disulfide bond was not cleaved during the ATP synthesis. But the MM10 inner membranes were not affected by the oxidation with $CuCl_2$ (Fig. **4A**).

As far as we know, there was little information about the rotary torque produced by F_0F_1-ATPase during the ATP synthesis. Although several models of rotation driven by proton flux through the F_0 of this synthase have been proposed [5], there was no data about the rotary torque produced by PMF. Junge *et al* assumed that the torque generated by PMF in F_0 is 40 pN·nm [24], matching the torque of F_1 in holoenzyme. Recently, in our studies of direct observation the light-driven rotation of F_0F_1-ATPase, about 28pN·nm of the torque was calculated [25]. However, in that work, the F_0F_1-ATPase was not intact. Here, our results showed that the rotary torque produced by PMF, as well as the torque generated by ATP hydrolysis, was sufficient to uncoil the α-helix in the top of γ subunit. It was calculated that the heights of the activation barriers along the Ramachandran coordinates yielded values of 25-30 kJ/mol [16], so the energy of PMF should be greater than 25-30 kJ/mol. This is insufficient to cleave the disulfide bond in the middle of the γ subunit which needs 200 kJ/mol energy. In such indirect way, it is possible to make a preliminary estimate that the torque generated by PMF is greater than 25-30 kJ/mol (40-50pN·nm) but and less than 200 kJ/mol (330 pN·nm). Further studies are clearly necessary for fully understanding the exact rotary torque during oxidative phosphorylation.

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ABBREVIATIONS

DCCD = N,N'-Dicyclohexylcarbodiimide

ACMA = 9-Amino-6-chlro-2-methoxyacridine

FCCP = Carbonyl cyanide p-triflouromethoxyphenyl-hydrazone

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