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Thiol modulation of the chimeric ATP synthase complex of bacterial F_1 containing the regulatory region of the γ subunit of chloroplast ATP synthase

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Introduction

The ATP synthase of chloroplast (CF_oCF_1) is activated by the proton gradient across the thylakoid membrane and modulated by the formation and the reduction of the disulfide bridge between two cysteine residues on the γ subunit (*thiol-modulation*) (Nalin & McCarty 1984, Mills & Mitchell 1984). These two cysteines (^{199}Cys and ^{205}Cys, the numbers are for spinach chloroplast F_1-γ) are located on the additional amino acid stretch region, 'regulatory region' (from ^{193}Ser to ^{241}Leu), of CF_1-γ (Miki, et al. 1988). In the soluble CF_1 reduction of the disulfide bond in γ causes elicitation of the latent ATP hydrolyzing activity. To discover which residues in the region were important for this regulation, we studied the properties of the chimeric complex, which was prepared from the recombinant CF_1-γ subunit and the recombinant TF_1-α and β subunits (Hisabori, et al. 1997, 1998). The ATPase activity of this chimeric complex was regulated by the redox conditions as we predicted. Then we prepared a mutant in the regulatory region of γ subunit and investigated their regulatory function in the complex. From this study, we found key residues, the deletion of which reversed the regulatory function. The ATPase activity of this mutant complex was activated under oxidizing condition and inactivated under reducing conditions (Konno et al. 2000). In addition, we found that the region following the regulatory cysteines is important for the suppression of the enzymatic activity.

In F_1-ATPase, the fact that the rotation of the γ subunit is coupled with ATP hydrolysis was proven by a biochemical method for mitochondrial F_1 (Duncun et al. 1995) and by the direct observation using a giant probe, fluorescence-labeled actin filaments, attached to the γ subunit of bacterial F_1 (Noji, et al. 1997, 1999; Omote et al. 1999) and CF_1 (Hisabori, et al. 1999). The regulation of the enzyme activity of CF_oCF_1, therefore, may be *via* regulation of the rotation itself, although the rotation of γ and the accompanying subunits in the F_oF_1 complex have not yet been proven directly (Sambongi, et al. 1999; Tsunoda, et al. 2000, 2001). Here, to understand the function of this regulatory region in the complex in more detail, we adopted two different strategies; observation of the enzyme regulation at the single molecule level and regulation of the enzyme activity of bacterial F_oF_1, when the regulatory region of CF_1-γ was introduced into the counter-part of the γ subunit.

The newly constructed chimeric $\alpha_3\beta_3\gamma$ complex was redox sensitive (Bald, et al. 2000). Using this complex we successfully observed the regulation of the rotation. In addition we

succeeded in expressing an F_oF_1-ATP synthase complex from the thermophilic bacterium, in which γ includes the CF_1-γ regulatory region, in *Eschericia coli*. ATP hydrolysis activity of the engineered F_oF_1 complex was modulated under the redox conditions.

Materials and methods

Biotin-PEAC maleimide was purchased from Dojin (Kumamoto, Japan). Streptavidin-coated microspheres (mean diameter: 530 nm) were from Bangs Laboratories, Inc. (Fishers, IN, USA). 9-Amino-6-chloro-2-methoxy-acridine (ACMA), dithiothreitol (DTT) and carbonyl cyanide p-(tri-fluoromethoxy)phenyl-hydrazone (FCCP) were from Sigma (St. Louis, MO, U.S.A.). 4-acetamido-4'-maleimidyl-stilbene-2, 2'-disulfonate (AMS) was from Molecular Probes (Eugene, OR, U.S.A.). Other chemicals were of the highest grade commercially available.

For the studies of the regulation of the rotation of γ, we first constructed the partial complex of thermophilic F_1, $\alpha_3\beta_3\gamma_{TCT}$ (Bald, et al. 2000). In this complex, the central 114 amino acid region of the γ subunit was substituted with the counter-part from the γ subunit of CF_1, 148 amino acid residues. This complex showed the specific redox regulation properties. When the complex was purified accoding to the method described in (Bald, et al. 2000), the cysteines in the regulatory region of the complex were completely in the oxidized state. Then we incubated this fully oxidized complex over-night with a 2-fold excess of Biotin-PEAC maleimide to introduce biotin at the position of newly introduced cysteine (^{107}Ser was replaced to Cys). The biotinylated complex was then purified by gel filtration chromatography and was reduced by incubation for 1 h with DTT plus chloroplast thioredoxin-*f* (Stumpp, et al. 1999). For the observation of rotation under the reducing conditions, the biotinylated, reduced $\alpha_3\beta_3\gamma_{TCT}$ complex (1-5 μM) in an assay mixture containing DTT was infused into the flow-cell and immobilized. Then streptavidin-coated microspheres were then attached on the complex (Yasuda, et al. 2001). The rotation was initiated by the infusion of 2 mM Mg-ATP. To oxidize the immobilized complex, 100 μM $CuCl_2$ was introduced. Rotation of the microsphere was monitored with a conventional optical microscope, recorded on a video tape recorder, and analyzed.

The thermophilic F_oF_1 complex was over-expressed in *E. coli* cell as the membrane-integrated protein using the newly constructed expression plasmid pTR19-ASDS (Suzuki, T. unpublished). The F_oF_1 complex containing γ_{TCT} was obtained by the substitution of the γ subunit gene in pTR19-ASDS with the appropriate gene. After the induction of the complex in *E. coli*, the plasma membrane was collected and the inside-out vesicles containing the chimeric F_oF_1 complex together with the respiratory chain were prepared by the method described in (Zhang & Fillingame 1994). ATP-driven proton pump activity was measured by fluorescence photometer FP-6300DS (JASCO, Tokyo) using ACMA as a probe (Zhang & Fillingame 1994). For the measurement of ATP hydrolysis activity, an ATP regenerating system (Stiggal, et al. 1979) was used. ATP hydrolysis activity was measured in the presence of 5 mM KCN and 1 μM FCCP.

Results and Discussion

Observation of the regulation at the single molecule level

Immobilization and chemical modification of CF_1 is difficult as over-expression and genetic manipulation of this enzyme has not yet been established. Therefore, we introduced about half of the CF_1-γ subunit including the regulatory region into thermophilic F_1 (TF_1-WT) carrying His-tags in the β subunit together with a single cysteine residue on γ, which was used for the fixation of the probe on the complex. The new chimeric mutant, $\alpha_3\beta_3\gamma_{TCT}$, was expressed in *E.*

coli (Bald, et al. 2000) and purified. The main question regarding the regulation of CF_1-ATPase activity is; when the activity of CF_1 in the oxidized state is only 30% of the activity in the reduced state, are all enzymes active in the oxidized state, but working with only 30% of the full velocity, or are only 30 % active but these working at full velocity? To address this question, we tried to observe the regulation of the complex at the single molecule level. The fluorescence from the dye-labeled actin filament, which was used as a probe for the early single molecule observations of F_1-ATPase (Noji, et al. 1997, 1999; Omote, et al. 1999; Hisabori et al. 1999), is quenched very quickly under the oxidizing conditions and was not suitable for this study. Then we adopted the polystyrene microsphere as a probe for detection of rotation and observed rotation (Fig. 1). By switching from reducing to oxidizing conditions and *vice versa*, we examined the real-time redox-control of rotation of the γ subunit at the single molecule level. We could observe continuous counter-clockwise rotation under the reducing conditions (Fig. 2). This rotation frequently stopped when the enzyme was in the oxidizing conditions. The rotation of the particles typically accelerated again, following a lag

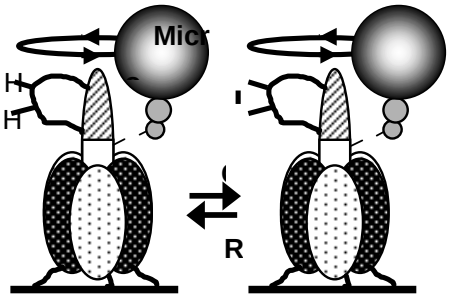

Fig. 1. Single molecule observation of the rotation of γ and the redox regulation. The Chimeric $\alpha_3\beta_3\gamma$ complex containing the regulatory region derived from CF_1-γ was immobilized on the glass surface via His-tags. Biotinylated microspheres (diameter : about 500 nm) were then attached to γ using streptavidin. Reduction of the disulfide bond was performed by the infusion of DTT plus thioredoxin-*f* directly into the micro-chamber.

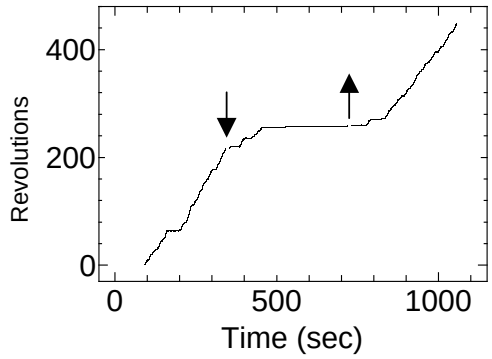

Fig. 2. Typical redox-controlled rotation of the microsphere attached to $\alpha_3\beta_3\gamma_{TCT}$. Rotation is induced by 2 mM Mg-ATP and observation was started under the reducing conditions. Then the solution in the micro-chamber was exchanged to the oxidizing conditions at the downward arrow. At the position of the upward arrow, the buffer was again switched back to the reducing conditions. The line represents the anti-clockwise rotation of the microsphere.

when the buffer conditions were switched back to the oxidizing conditions. This result suggests that the behavior of the chimeric γ subunit in the $\alpha_3\beta_3$ hexagon is similar to that in the wild-type complex when it was in the reducing conditions and the oxidation of the regulatory region disturbs the smooth rotation.

F_oF_1 complex and redox regulation

In chloroplast, CF_1-γ is the target for thioredoxin and the reduction of the disulfide bond in γ is directly coupled with the photosynthetic electron flow *via* ferredoxin, ferredoxin-thioredoxin reductase and the chloroplast thioredoxin. The significance of the activation of CF_oCF_1 by the reduction of the γ subunit however is ambiguous because it is difficult to reduce only the γ subunit of CF_1 without affecting the other components in the chloroplast. In fact many enzymes involved in photosynthesis activity are obviously regulated by the redox conditions in the chloroplast.

Here we constructed the new expression plasmid named pTR19-ASDS for expression of thermophilic bacterial F_oF_1 complex in *E. coli*, and then substituted the gene for the γ subunit

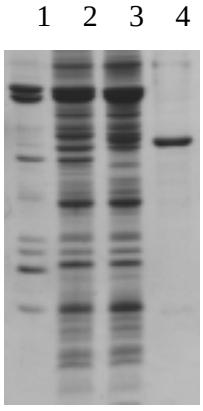

Fig. 3. The chimeric F_oF_1 complex expressed in *E. coli*. The crude membrane fractions were obtained from pTR19-ASDS transformed *E. coli* cell. *Lane* 1, wild type TF_oTF_1 complex; *lane* 2, inside-out visicle with TF_oTF_1; *lane* 3, inside-out visicle with TF_oTF_1 containing γ_{TCT}; *lane* 4, the recombinant γ subunit of CF_1.

with that for the intra-molecular chimeric γ (γ_{TCT}) (Bald et al. 2000) (Fig. 3). The introduction of γ_{TCT} did not affect the stoichiometry of the subunits of the membrane integrated complex F_oF_1, suggesting the complex was assembled in the correct manner.

The reduction of the disulfide bond on this γ subunit in the presence of DTT plus thioredoxin was then assessed using the thiol modifier AMS. In the case of the complex, $\alpha_3\beta_3\gamma_{TCT}$, the reduction of the disulfide bond was easily accomplished (Fig. 4, *lanes* 1 and 2). However γ_{TCT} was hardly reduced in the F_oF_1 complex. It is well known that the energization of the thylakoid membrane by illumination is required for the reduction of γ in membrane-bound CF_oCF_1. Therefore, we tried to reduce γ in the presence of NADH to promote the electron flow *via* bacterial electron transport pathway. Although the membrane potential was formed when 200 μM NADH was added to the inside-out membrane vesicle, the disulfide bond on γ was not fully reduced even when DTT and thioredoxin were supplemented into the reaction medium. Nevertheless, we were finally able to reduce the disulfide bond by using the combination of 2-

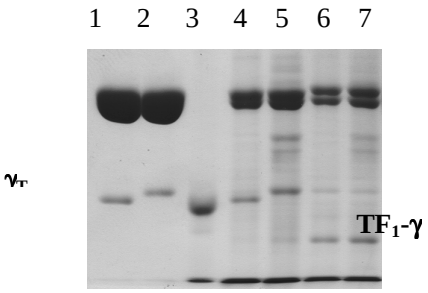

Fig. 4. Reduction and oxidation of the γ subunit visualized by AMS labeling. *Lane* 1, the oxidized form $\alpha_3\beta_3\gamma_{TCT}$; *lane* 2, the reduced form $\alpha_3\beta_3\gamma_{TCT}$; *lane* 3, the recombinant CF_1-γ; *lane* 4, the oxidized form TF_oTF_1 with γ_{TCT}; *lane* 5, the reduced form TF_oTF_1 with γ_{TCT}; *lane* 6, TF_oTF_1 under the oxidizing conditions; *lane* 7, TF_oTF_1 under the reducing conditions. For lanes 4-7, membrane fraction was directly labeled with AMS and loaded.

mercaptoethanol and dithiothreitol (Fig. 4, *lanes* 4 and 5). Thus the structure around the regulatory region in the F_oF_1 complex is very different from that of the isolated F_1-ATPase.

Then we examined the difference in the ATP hydrolysis activity of chimeric F_oF_1 in the membrane under the reducing and oxidizing conditions. The ATP hydrolysis activity of the cimiric F_oF_1 was accelerated about 1.4 to 1.8-fold when the complex was reduced. In contrast, these remarkable changes were not observed in the case of the wild type F_oF_1 complex.

Next, the redox regulation of the proton pump activity was measured. Even with the freshly prepared thylakoid membrane, the ATP-driven proton pump activity was not observed irrespective of the reduction or the oxidation of CF_1 when it was measured in the absence of membrane potential (Fig. 5A). In contrast, the inside-out vesicle containing TF_oTF_1 showed strong activity (Fig. 5B). When the γ subunit of CF_1 was introduced into the TF_oTF_1 complex, again the ATP-driven proton pumping activity was not observed (data not shown). In contrast, the proton pumping activity of the F_oF_1 containing γ_{TCT} was somehow much stronger than that of TF_oTF_1 complex (Fig. 5C). We failed to observe the redox regulation of this proton pumping activity because the membrane became leaky when $CuCl_2$, which assists the oxidation of γ in the presence of oxygen, was added. Thus, it is difficult for us to describe on the role of redox regulation of CF_oCF_1 for the physiological role of this enzyme yet.

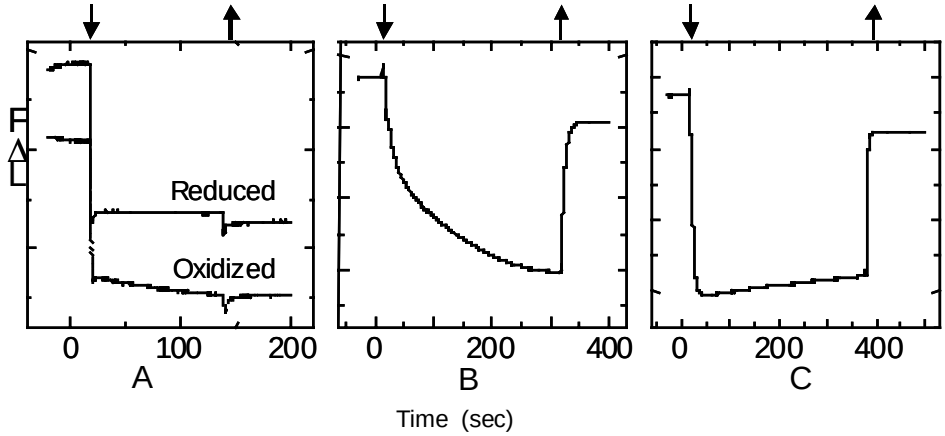

Fig. 5. The proton pumping activity promoted by ATP hydrolysis was monitored by ACMA fluorescence. At the position of the downward arrows, ATP was added and at the upward arrows, FCCP was added. **A**, thylakoid membrane. **B**, the inside-out membrane with TF_oTF_1. **C**, the inside-out membrane with TF_oTF_1 containing γ_{TCT}. The activity was measured at 25°C (**A**) or 45°C (**B** and **C**).

Conclusions

Taken together, we successfully introduced the regulatory region of CF_1-γ into the γ subunit of the bacterial F_1 subcomplex or F_oF_1 in a correct functional form. The unique characteristics of CF_oCF_1, specifically the redox regulation, was readily conferred to the bacterial F_oF_1 by this modification. We could then observe the redox regulation of the rotation of γ at the single molecule level, as well as the ATP hydrolysis activity of the membrane bound F_oF_1. This implies that the regulatory region of CF_1-γ is the independent functional unit.

The single molecule observation of the rotation of this redox sensitive enzyme will provide new insights into the understanding of the redox regulation at the molecular level. The introduction of the regulatory region of CF_1-γ into the bacterial F_oF_1 provided us a helpful model system for the study on the thiol modulation of ATP synthase.

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