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Redox Regulation of the Rotation of F_1-ATP Synthase*

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Dirk Bald‡§¶, Hiroyuki Noji‖, Masasuke Yoshida‡§, Yoko Hirono-Hara‡, and Toru Hisabori‡**

From ‖PRESTO, ‡Chemical Resources Laboratory, Tokyo Institute of Technology, Nagatsuta 4259, Midori-ku, Yokohama, Kanagawa 226-8503 and §CREST Genetic Programming Team 13, Teikyo University Biotechnology Research Center 3F, Nogawa 907, Miyamae-ku, Kawasaki, Kanagawa 216-0001, Japan

In F_1-ATPase, the smallest known motor enzyme, unidirectional rotation of the central axis subunit γ is coupled to ATP hydrolysis. In the present study, we report the redox switching of the rotation of this enzyme. For this purpose, the switch region from the γ subunit of the redox-sensitive chloroplast F_1-ATPase was introduced into the bacterial F_1-ATPase. The ATPase activity of the obtained complex was increased up to 3-fold upon reduction (Bald, D., Noji, H., Stumpp, M. T., Yoshida, M. & Hisabori, T. (2000) *J. Biol. Chem.* 275, 12757–12762). Here, we successfully observed the modulation of rotation of γ in this chimeric complex by changes in the redox conditions. In addition we revealed that the suppressed enzymatic activity of the oxidized F_1-ATPase complex was characterized by more frequent long pauses in the rotation of the γ subunit. These findings obtained by the single molecule analysis therefore provide new insights into the mechanisms of enzyme regulation.

F_OF_1-ATP synthase, which is located on the mitochondrial inner membranes, chloroplast thyalkoid membranes, and bacterial plasma membranes, synthesizes ATP, which is known as the universal energy "coin" in the cell, from ADP and phosphate at the expense of the proton gradient across the membranes (1). The water-soluble part of ATP synthase, the F_1 region, consists of five subunits with a composition of $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ (2). The hydrophobic F_O part is composed of $a_1b_2c_{10-14}$ (3–5). The subcomplex of F_1 part, $\alpha_3\beta_3\gamma$, is the minimum complex that is capable of ATP hydrolysis (6). Rotation of the γ subunit in the $\alpha_3\beta_3$ hexagon was first postulated by P. D. Boyer and co-workers (8) from the analysis of the cooperative kinetics of F_1-ATPase. A high resolution x-ray structure of a major part of the bovine heart mitochondrial F_1 (7) revealed an alternating hexagonal arrangement of the $\alpha_3\beta_3$ ring with the γ subunit as a central axis. Then the rotation of γ in the central cavity of $\alpha_3\beta_3$ was suggested by biochemical experiments (10), and finally, direct visualization of the rotation of an actin filament attached to the γ subunit of F_1 showed unequivocally that γ rotates unidirectionally during ATP hydrolysis (11–14).

The chloroplast ATP synthase has basically the same structure as those from other sources. However the enzyme activity of chloroplast ATP synthase (CF_OCF_1) from higher plants is modulated in a unique way, reduction of a disulfide bridge in the γ subunit (15, 16). In the solubilized CF_1 part, the reduction of this disulfide bond, which is located in a switch region on γ unique to chloroplast ATP synthase, strongly enhances the ATP hydrolysis activity (ATPase activity). This switch region consists of 37 amino acid residues (Pro^{194}–Ile^{230} in case of the spinach chloroplast enzyme) containing two cysteine residues, but its structure is unknown, and there are no data available on how reduction of the disulfide bond leads to activation of the enzyme. Such redox regulation is a common property of so-called thiol enzymes in the chloroplasts like glyceraldehyde-3-phosphate dehydrogenase (17), fructose-1,6-bisphosphatase (18), sedoheptulose-1,7-bisphosphatase (19), phosphoribulokinase (20), and NADP-malate dehydrogenase (21). The chloroplast thioredoxin, which is reduced by electron flow from photosystem, plays a main role in this regulation system (21). However, to our knowledge there are no data available, neither for chloroplast enzymes nor for enzymes from other organelles, on whether regulation affects the reaction rate of a single enzyme molecule or influences the number of active enzyme molecules. To address this question, observation of the rotation of F_1-ATPase is the useful system, because it allowed us to observe the real-time behavior at the single molecule enzyme level. Then we recently constructed the chimeric $\alpha_3\beta_3\gamma$ complex, in which central 111 amino acid residues of the F_1-γ from a thermophilic bacterium were replaced by the homologous 148 residues derived from spinach CF_1-γ, which includes a switch region (22). In the report, we designated the obtained chimeric γ subunit as γ_{TCT} (thermophilic-chloroplast-thermophilic). The newly designed $\alpha_3\beta_3\gamma$ complex ($\alpha_3\beta_3\gamma_{TCT}$ complex) was susceptible to redox regulation, and the ATPase activity in the reduced state was up to 3-fold higher than that in the oxidized state. In the present study we investigated the differences of the rotation behavior of this engineered complex under the reduced and oxidized conditions. Then we successfully controlled the rotation of the γ subunit by redox switching and succeeded to observed the differences of the behavior of the active enzyme and that of the suppressed enzyme at the single molecule level.

EXPERIMENTAL PROCEDURES

Materials—Biotin-$PEAC_5$ maleimide was purchased from Dojin (Kumamoto, Japan). Streptavidin-coated microspheres (mean diameter, 530 nm) was from Bangs Laboratories, Inc. (Fishers, IN). DTT[1] was from Sigma. Other chemicals were of the highest grade commercially available.

Preparation of the Complex—The original chimeric $\alpha_3\beta_3\gamma$ complex was prepared as described (22). As a result of the substitution of the central region of γ from thermophilic F_1 (11) with the counterpart from the chloroplast F_1-γ, a cysteine residue used for the labeling with biotin-maleimide (12) was deleted. Therefore an additional cysteine

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¶ Present address: Dept. of Structural Biology, Free University of Amsterdam, De Boelelaan 1087, 1081 Amsterdam, Netherlands.

** To whom correspondence should be addressed. E-mail: thisabor@res.titech.ac.jp.

[1] The abbreviations used are: DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; BSA, bovine serum albumin.

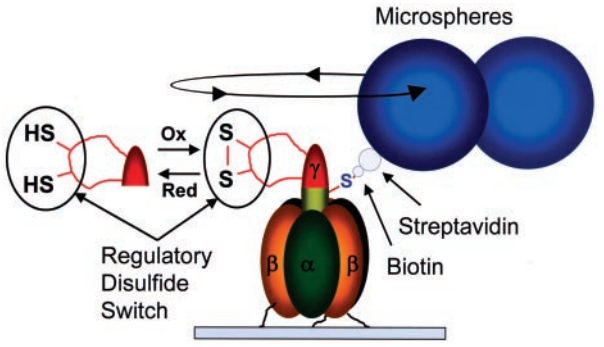

FIG. 1. **The experimental system for single molecule observation of redox control.** The $\alpha_3\beta_3\gamma_{TCT}$ complex was immobilized onto a slide glass via histidine tags, and microspheres (*blue*) were attached to a cysteine residue (*light blue*) on the γ subunit with a biotin/streptavidin linkage (*gray*). The switch region containing two Cys residues is shown as a *red line*. The disulfide bond in this region is reduced by DTT plus thioredoxin (*Red*) and reformed by oxidation with $CuCl_2$ (*Ox*), thus modulating the activity. *HS*, sulfhydryl group.

residue was introduced in place of Ser^{107} (the numbering is for the engineered γ subunit (22)) on the imported sequence by site-directed mutagenesis. This introduced cysteine did not affect the regulatory function nor the enzyme activity of the chimeric complex. This new $\alpha_3\beta_3\gamma_{TCT}$ complex was obtained as described (6). Briefly, the complex was expressed in *Escherichia coli* strain JM 103 and purified directly from the cell extract using a nickel-nitrilotriacetic acid super-flow column (Qiagen). We found that the cysteines in the regulatory region of the complex were in the oxidized state after completing the purification process. We therefore used this complex as the original oxidized complex. We then incubated the fully oxidized complex overnight with a 2-fold excess of Biotin-$PEAC_5$ maleimide. The complex was purified by gel filtration chromatography on a $G3000SW_{XL}$ column (7 × 300 mm; Tosho, Tokyo, Japan). Reduction of the complex was carried out by incubation for 1 h with 200 μM DTT + 4 μM spinach chloroplast thioredoxin-*f* as described (23).

Single Molecule Observation of the Rotation and the Redox Control—In this study, we examined the real-time control of the chimeric complex under the redox conditions using chloroplast thioredoxin plus DTT for reduction or $CuCl_2$ for oxidation. The low concentration of $CuCl_2$ is known to catalyze the formation of the disulfide bond by facilitating the oxidation in the presence of oxygen without having any other inhibitory effect on the ATPase activity of F_1 (24).

15 μl of the biotinylated, reduced $\alpha_3\beta_3\gamma_{TCT}$ complex (1–5 nM) in an assay mixture containing 10 mM MOPS-KOH (pH 7.0), 50 mM KCl, and 200 μM DTT was infused into the flow cell (11) and incubated for 15 min at room temperature for immobilization. The flow cell was washed with 30 μl of assay mixture supplemented with 10 mg/ml BSA. Next 15-μl streptavidin-coated microspheres (100 nM to 2 μM) in the assay mixture plus BSA were infused. After 15 min of incubation the assay was started by addition of 70 μl of assay mixture/BSA supplemented with 2 mM Mg-ATP, and the rotation was observed under the reduced conditions (R-phase) for about 5 min and then the mixture was exchanged with 150 μl of assay mixture/BSA/Mg-ATP plus 100 μM $CuCl_2$ as oxidant, and the rotation was observed (O-phase). Switching back to R-phase was carried out by exchanging the O-phase mixture with assay mixture/BSA/Mg-ATP plus 200 μM DTT and 4 μM thioredoxin-*f* (R2-phase). Rotation of the microsphere was monitored with a conventional optical microscope type IX70 (Olympus, Tokyo, Japan) and recorded with a video tape recorder. Recorded images were analyzed with the image analysis software provided by R. Yasuda (25).

ATP Hydrolysis Activity—ATP hydrolysis activity was measured by quantifying the amount of produced phosphate (26). The reaction mixture contained 50 mM MOPS-KOH (pH 7.0), 100 mM KCl, 2 mM $MgCl_2$, and 2 mM ATP supplemented with 200 μM DTT + 4 μM thioredoxin-*f* (for R- and R2-phase) or 100 μM $CuCl_2$ (for O-phase), and the reaction was initiated by the addition of the enzyme solution. After incubation for 5 min at room temperature, the reaction was terminated by the addition of 2.4% (w/v) trichloroacetic acid, and the amount of produced phosphate was determined. To mimic the solution exchange that we used in the rotation assay, we reduced or oxidized the enzyme, removed the reductant/oxidant by desalting through a column, and injected this enzyme solution in the reaction mixture containing the oxidant/reductant.

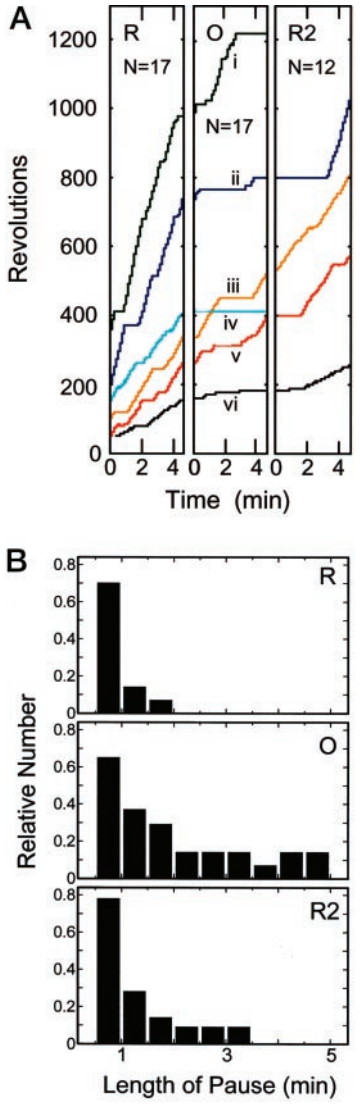

FIG. 2. **Rotation of the γ subunit under the reducing and oxidizing conditions.** *A*, rotation of the γ subunit was recorded in three phases (R-, O-, and R2-phases). Rotation is induced by 2 mM Mg-ATP. Observation was started under the reducing conditions (*R*), then switched to the oxidizing conditions (*O*), and finally switched back to the reducing conditions (*R2*). In each phase particles were observed for about 5 min, and solution exchange from one phase to the next took about 40 s. Each *line* represents the anti-clockwise rotation of the microsphere, and the origin of the each line at time 0 in each phase is arbitrary on the *vertical axis*. *Trace i* and *ii*, 2 microspheres attached (diameter 300 nm); *trace iii*, *iv*, and *v*, 2 microspheres (500 nm); *trace vi*, 3 microspheres (500 nm). *B*, the number of pauses per observed particle in each phase for 5 min was counted as a function of length of the pause and averaged over the total number of particles and then normalized for the number of particles in each phase (R-, O-, and R2-phases).

RESULTS AND DISCUSSION

Redox Control of Rotation of γ—In the present study, the redox control of the activity of the redox-sensitive $\alpha_3\beta_3\gamma_{TCT}$ complex was observed by monitoring the rotation of the γ subunit. Because the fluorescence from the dye-labeled actin filament, which was used for the former studies (11–14), is quenched very quickly under the oxidizing conditions, we could not use the filament as a probe for our study. Instead of that, we adopted the polystyrene microsphere method of detection (27) (Fig. 1). This microsphere method allowed us to use both redox conditions for the observation of the rotation behavior of the single molecule complex with a conventional microscope. In addition we could exchange the buffer in the microflow cell directly without seriously damaging the rotating enzymes.

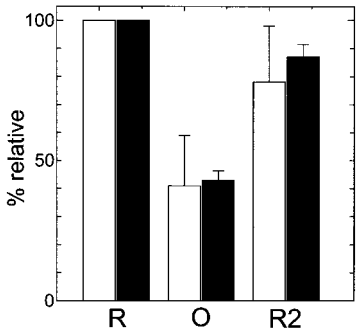

FIG. 3. **The comparison of the rotation rate and the ATPase activity.** The ratio of rotation rates (*open bars*) in the R-, O-, and R2-phases were calculated for each particle and averaged (R-phase normalized to 100%). The ATPase activity (*shaded bars*) under the same conditions was measured by quantitation of the produced phosphate, and the activity at R-phase was set to 100%. *Bars* show the standard deviation of the indicated number of observed rotating particles (shown in Fig. 2*A*) and of three independent experiments for ATPase activity.

We first observed the rotation of the complex under the reducing conditions and labeled this phase as R-phase. Here we could observe several well rotating particles under the microscope, and we typically observed continuous counter-clockwise rotation with several brief pauses (Fig. 2*A*, R-phase). These brief pauses may be attributable to the special state of F_1-ATPase known as ADP inhibition (9). The enzyme trapped in this inhibition state can easily recover and re-start the rotation because of the high ATP concentration in the reaction mixture. After switching to the O-phase, characteristic, long pauses appeared (Fig. 2*A*, O-phase). This phenomenon was observed irrespective of the size and the number of attached microspheres that act as the load on the rotating γ subunit. Despite these long pauses in the O-phase, the rotation in most cases did not stop completely over the whole phase. For the periods between the long pauses, *i.e.* when the enzyme in the O-phase did rotate, the rotation rate was not distinguishable from that observed in the R-phase. When the complex was switched back to the reducing conditions (Fig. 2*A*, R2-phase), the average rotational rate typically increased again compared with that in O-phase although it was in most cases lower than in R-phase because of a lag.

Analysis of Pause—To characterize the rotation under the different conditions, pause lengths and the frequency of the pauses of each rotating particle were analyzed. All pauses with >30-s length were counted for each phase, and their frequencies were calculated based on the length of the pauses (Fig. 2*B*). This analysis revealed a nearly constant number of short pauses (<1 min) in all three phases; however the pauses longer than 1 min were significantly more frequent in the O-phase. Although we observed a higher frequency of the long pauses during the R2-phase than that during the R-phase, they were obviously residual effects of the prior oxidizing conditions (O-phase). For 10 of 12 particles observed, the averaged rotation rate in the O-phase was lower (<85%) than in both R- and R2-phase. In contrast, zero of ten of the wild type particles lacking the switch region showed such a tendency (data not shown).

Redox Control of the Enzyme Activity—We compared the average rotation rates with the ATP hydrolysis activity to confirm that the observed particles for which rotation was analyzed in this study were representative of the whole population (Fig. 3). The average ratio of rotation rates was 100:41:78 for the R-, O-, and R2-phases. This ratio was consistent with the ratio of the ATP hydrolysis activity (100:43:87) under similar conditions, implying the observed regulation of the rotation in the present study is the actual regulated enzyme behavior. Thus our single molecule observation study can reliably provide new insights into the molecular mechanism of F_1-ATPase, especially in the suppressed state. Under the oxidizing conditions, the rotating particle shows two different patterns, the counter-clockwise rotation whose rate is indistinguishable from that of the enzyme under the reducing conditions, and the very long pause. Particularly, we could observe rotation in the O-phase after long pauses (Fig. 2*A*, *traces ii*, *iii*, *v*, and *vi*), although this rotation in some cases only lasted for several seconds (Fig. 2*A*, *traces ii* and *vi*). We regard it as highly unlikely that a single molecule is oxidized and then reduced again under the same oxidizing conditions. Therefore, the oxidized complex must assume two independent states, in one state F_1 works in the usual manner, as under the reducing conditions, in the other it assumes a non-rotating form. The oscillation between these two states lowers the number of revolutions of the γ subunit, resulting in lower enzyme activity under oxidizing conditions than under reducing conditions in contrast to the rate of rotation of all molecules being diminished uniformly.

Conclusions—-In summary, we have successfully attached a redox-sensitive switch to a molecular motor enzyme, F_1, and could control the rotation *via* this switch, thus creating the first nanomotor of this kind. Mechanistically, we could prove the existence of two distinct states of the oxidized enzyme. To the best of our knowledge our present study is the first to elucidate this kind of precise details of the regulation mechanism of an enzyme, and it describes one of the most significant physiological phenomena, regulation of ATP synthase, occurred on the enzyme molecule itself, at the single molecule level.

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