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F_oF_1-ATPase activity regulated by external links on β subunits

Jie Cheng [a,c], Xiao-ai Zhang [a,c], Yao-Gen Shu [b,*], Jia-Chang Yue [a,*]

[a] National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China
[b] Key Laboratory of Frontiers in Theoretical Physics, Institute of Theoretical Physics, Chinese Academy of Sciences, Beijing 100190, China
[c] Graduate School of the Chinese Academy of Sciences, Beijing 100049, China

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ABSTRACT

F_oF_1-ATPase activity is regulated by external links on β subunits with different molecular weight. It is inhibited when anti-β subunit antibody, streptavidin and H9 antibody link on the β subunits successively, but is activated when virus was binded. Western blotting indicated that the employed anti-β antibody target was on the non-catalytic site of the β subunit. Furthermore, an ESR study of spin-labeled ATP (SL-ATP) showed that the affinity of ATP to the holoenzyme increases with increasing external links on the β subunits. This simple regulation method may have great potential in the design of rapid, free labeled, sensitive and selective biosensors.

Introduction

F_oF_1-ATPase is the ubiquitous rotary motor that uses the transmembrane electrochemical potential to synthesize ATP in bacterial cell membranes, chloroplasts, mitochondria and on the plasma membrane of endothelial and tumor cells. The holoenzyme is a complex of two rotary motors, F_o and F_1, which are mechanically coupled by a common central stalk ("rotor"), $c_n\varepsilon\gamma$. The membrane-embedded F_o unit converts the proton motive force (p.m.f) into mechanical rotation of the "rotor", thereby causing the cyclic conformational change of the $\alpha_3\beta_3$ crown ("stator") in F_1, and driving ATP synthesis. A striking characteristic of this motor is its reversibility. It may rotate in the reverse direction for ATP hydrolysis and utilize the excess energy to pump protons across the membrane [1–6]. Furthermore, single molecule experiments have revealed that the enzyme activity can be regulated by manipulating its physical rotation because of tight mechanochemical coupling [7–9].

The regulation of the holoenzyme activity is still an interesting topic [10]. Traditional methods of describing enzyme regulation are chemical in nature, and utilize either the concentration of the substrate or the transmembrane electrochemical potential [11–13]. In fact, the F_oF_1 motor actively converts a chemical reaction (ATP hydrolysis in F_1 or proton transfer in F_o) into the unidirectional physical rotation of the "rotor" that is accompanied with the cyclic conformational change of the "stator". Single molecule assays have indicated that the activity of F_1 or F_o [7–9] can be regulated by varying the load on the "rotor," and the eccentric rotation of the γ subunit is mechanically coupled with the cyclic conformational change of the $\alpha_3\beta_3$ crown at a high efficiency. However, the physical regulation of the activity of the holoenzyme (including F_o and F_1), instead of chemical regulation, is still difficult to understand. One of the reasons is that the "rotor" of F_oF_1 motor is mostly enwrapped by the "stator" $\alpha_3\beta_3$ crown. The other reason is that the exposed fraction of the "rotor" is also partly shaded by the b_2 subunit. Thus, it becomes very difficult to directly regulate the holoenzyme activity by a load on the "rotor".

In view of the fact that the unidirectional rotation of the "rotor" is tightly coupled with the cyclic conformational change of the "stator", what will happen to the activity of the rotary motor if external complexes bind to the "stator"? Our previous investigations [14–18] have demonstrated that the holoenzyme activity can be regulated by external links on β subunits. In this article, we studied the holoenzyme activity regulation in detail with different external links on β subunits, including Western blotting to check the epitope of anti-β antibody, and ESR to analyze the affinity of ATP to the modified holoenzyme.

Materials and methods

Materials. 3-Carboxy-1-oxyl-2,2,5,5-tetramethyl pyrrolidine was purchased from Fisher Scientific Co. (USA). HAT (hypoxanthine aminopterin thymidine), HT (hypoxanthine thymidine), PEG (MW 1500), luciferin, luciferase, adenosine 5′-triphosphate (ATP) (disodium salt) and adenosine-5′-diphosphate (ADP) (disodium salt), were purchased from Sigma–Aldrich Co. (USA). DMEM high glucose medium was purchased from Gibco (USA). Fetal bovine serum was purchased from PAA Co. (Location?). Ni–NTA His Bind Resins was purchased from Qiagen (USA). pET-30a was purchased from Nova-

* Corresponding authors. Fax: +8610 64871293.
E-mail addresses: shuyg@itp.ac.cn (Y.-G. Shu), yuejc@sun5.ibp.ac.cn (J.-C. Yue).

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gen (USA). pMD19-T Simple vector was purchased from TaKaRa Co (Japan). All other analytical purified reagents were purchased domestically.

Preparation of the anti-β subunit monoclonal antibody. The β subunit of F_oF_1-ATPase from the thermophilic bacterium *Bacillus. PS3* ($F_1\beta$) was expressed in *Escherichia coli* JM103 and purified according to Ref. [9]. Six-to-eight-week-old female mice were first injected with Freund's complete adjuvant, then by incomplete adjuvant, and finally by splenocyte intravenous booster. The antigen without adjuvant was injected to splenocytes. After three days, splenocytes were isolated and fused with Sp2/0 mouse myeloma cells using polyethylene glycol. Hybridomas were obtained in 96-well culture plates in RPMI-1640 supplemented with 20% FBS and HAT medium. Cell supernatants were screened for antibodies binding to the β subunit of F_1-ATPase. The positive clones were screened by ELISA, using the recombinant ATPase β subunit as the target antigen [19]. Subcloned cells in 96-well plates were recovered, and injected intraperitoneally into BALB/c mice (10^6 per mouse). Antibodies in the ascites were purified by gel filtration on Sephadex G-200 (Pharmacia?, USA). The monoclonal anti-β subunit antibody (2D5) was prepared according to Ref. [20] and stored at −20 °C.

Cloning of the fragments. The fragments of the β subunit, including domains 1–2, domain 2, and domains 2–3, were PCR-amplified using specific primers. Primers were designed to introduce an NdeI restriction site at the 5′ end, and an HindIII site at the 3′COOH-terminal end (Table 1). Domain boundaries were determined based on structural motifs (Fig. 2A–C). PCR products were purified from 1.5% Tris–acetate/EDTA agarose gels using a gel band purification kit (Amersham), and subcloned into pMD™ 19-T Simple Vector (TAKARA). Competent *E. coli* DH5α (Life Technologies) were transformed with 1 μl ligation mixture/100 μl cells, plated on LB-ampicillin (50 μg/ml) and X-Gal agarose plates, and grown overnight at 37 °C. Colonies were screened for inserts with NdeI and HindIII (TAKARA) digestion, followed by gel purification. The subcloned inserts were ligated into the NdeI- and HindIII-digested pET30a vector (Novagen), using the Clonables kit (Novagen). Competent *E. coli* Novablue cells (Novagen) were transformed with the ligation mixture and grown on LB-kanamycin agarose plates. Colonies were screened for insertion by restriction enzyme digestion and DNA sequencing.

Purification of the fragments and β subunit. BL21(DE3) cells (Solarbio, China) were transformed with pET30a vector containing β subunit or its domains, plated on LB-kanamycin agarose plates, and grown overnight at 37 °C. Then, 2.5 ml LB, containing 30 μg/ml kanamycin was inoculated with one colony and grown at 37 °C, with shaking at 200 rpm, to an $A_{600\ nm}$ of 0.6, and stored overnight at 4 °C. A 50 ml culture (LB, 30 μg/ml kanamycin) was inoculated with 2 ml of the noninduced overnight culture and grown at 37 °C, 250 rpm, to an $A_{600\ nm}$ of 0.60. Isopropyl thio-β-D-galactosidase was added to a final concentration of 1 mM/l and cultures were grown an additional 3 h at 37 °C, 250 rpm. Cells were harvested by centrifugation at 5000*g* for 5 min and stored at −20 °C. Lysates were prepared by denaturing with 8 M/l urea for all proteins. Lysates were purified using Ni–NTA His Bind Resin columns (Novagen) and the resulting proteins were dialyzed against PBS (pH 7.0). The β subunit was isolated and purified from *Rhodopseudomonas palustris* according to Ref. [21].

Table 1
Primer sequences used for construction of fragments. Sequences are based on the DNA of *Rhodopseudomonas palustris* ATP synthase (gene accession RPA0176 KEGG GENES Database). Restriction enzyme sites, in bold, represent for 5′ end primers, an NdeI cleavage site, and for 3′ primers, an HindIII site. Primers read from 5′ to 3′ according with gene sequence.

Domains	5′ Primer sequence	3′ Primer sequence
1 and 2	TT**CATATG**GCTACAC CCGCCAATCAGACCG	AT**AAGCTT**GACGATC GAGGCGGACAGCATG
2	TT**CATATG**CAGTCGA CCGAGGCTGAAATTC	AT**AAGCTT**GACGATC GAGGCGGACAGCATG
2 and 3	TT**CATATG**CAGTCGA CCGAGGCTGAAATTC	TAT**AAGCTT**GGCGGC GAGCTTCTTGCCCTTC
Full length	TT**CATATG**GCTACAC CCGCCAATCAGACCG	TAT**AAGCTT**GGCCGCC TCGGCGGCGAGCTTCT

Western blotting. Purified, recombinant β domain proteins under reducing conditions were separated by SDS–PAGE and transferred to polyvinylidene difluoride. Membranes were blocked by 5% BSA overnight at 4 °C, then incubated with the anti-β subunit monoclonal antibody at 2.5 mg/ml for 2 h at room temperature. After washing three times, the polyvinylidene difluoride membrane was incubated with goat anti-mouse, HRP-conjugated secondary antibody. The blot was developed by using a super-enhanced chemiluminescence detection kit (Applygen Technologies Inc., Beijing, China).

Preparation of chromatophores. Chromatophores were prepared from the cells of *R. palustris*, according to Ref. [22] in TSM buffer (0.1 mM Tricine–NaOH, 0.25 M sucrose, 5 mM $MgCl_2$, pH 8.0). *R. palustris* cells were grown in the medium for 72 h at 33 °C and illuminated with white light. Harvested cells were washed twice in TSM buffer and then disrupted by ultrasonication. The suspension was centrifuged for 30 min at 25,000*g*. The chromatophore in the supernatant was collected by centrifugation at 25,000*g* for 90 min, and then resuspended in TSM buffer. Bacteriochlorophll (BChl) was determined by using the spectrophotometric extinction coefficient (860 nm) according to Clayton [23]. Under the electron microscope, the diameter of the obtained chromatophore was estimated to be about 100 nm.

Preparation of samples with different external links on the β subunit. To compare the holoenzyme activity with different external links, we have prepared 5 samples: (1) sample 1 is the native F_oF_1-ATPase within the chromatophore; (2) sample 2: 100 μl (50 mg/ml) chromatophore (sample 1) was incubated with 1 μl (10 mg/ml) anti-β antibody at 37 °C for 60 min, and then the free anti-β antibody was washed away by centrifugation at 4 °C for 30 min. (The anti-β antibody was labeled with biotin in the 1:10 and free biotin was dialyzed); (3) sample 3: sample 2 was incubated with equivalent streptavidin in 37 °C for 40 min; (4) sample 4: sample 3 was incubated with biotinylated influenza virus anti-H9 antibody at 37 °C for 30 min, and the free antibody was washed; (5) sample 5: sample 4 was incubated with 10 μl influenza H9 virus for 60 min at 37 °C and then the sample was washed three times. All the samples with different links were treated as Fig. 1.

Measurement of F_oF_1-ATPase activity. The ATP synthesis activity was measured by the luciferin–luciferase method with a computerized ultra-weak luminescence analyzer (type BPCL manufactured at the Institute of Biophysics, Academia Sinica, Beijing, China). Three microliters (1 μg) of chromatophores were reacted in 50 μl ATP synthesis buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, 5 mM K_2HPO_4, 2 mM ADP, 10% glycerol, pH 8.0) at 37 °C for 5 min. The ATP synthesis reaction was stopped by adding 1/10 volume of 4% TCA, and then 3 μl liquor was diluted with 100 μl assay mix dilution buffer. The luminescence was measured for 10 s after the injection of luciferin–luciferase reagent. The ATP hydrolysis activity was measured with the same method. Similarly, 1 μg chromatophores were incubated at 37 °C for 5 min in 50 μl ATP hydrolysis buffer (50 mM Tris–HCl, 2 mM ATP, 2 mM $MgCl_2$, 10 mM KCl, pH 8.0). The ATP synthesis/hydrolysis reaction was stopped by TCA, and then 3 μl liquor was tested in the same way.

Preparation of spin-labeled ATP (SL-ATP). The spin-labeled ATP, 3′-*O*-(1-oxyl-2,2,5,5-tetramethyl-3-carbonyl pyrrolidine) adenosine 5′-triphosphate, was synthesized by the method described in Refs. [24,25]. ATP was esterified by 3-carboxy-1-oxyl-2,2,5,5-tetramethyl