Detecting proton flux across chromatophores driven by F_0F_1-ATPase using *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt

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Abstract

N-(Fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (F-DHPE) is a lipid fluorescence dye sensitive to pH changes and is used in this study for detecting proton flux through F_0F_1-ATPase within chromatophores driven by ATP hydrolysis. F-DHPE is easily labeled to the outer surface of chromatophores. In the range of pH 7.0 to 9.0, fluorescence intensity is sensitive to pH changes. The sensitivity is especially great in the range of pH 8.2 to 9.0, so pH 8.6 was chosen as the appropriate experimental condition. It is shown that added ATP not only acts as a fluorescence quencher but also can be hydrolyzed by F_0F_1-ATPase to pump protons into chromatophores, resulting in fluorescence restoration. A stimulator ($NaSO_3$) and various types of inhibitors (NaN_3, 5′-adenylyl imidodiphosphate [AMP–PNP], and *N,N′*-dicyclohexylcarbodiimide [DCCD]) of F_0F_1 confirmed that fluorescence restoration is caused by ATP-driven proton flux. When loaded with one antibody (anti-β antibody) or two antibodies (anti-β antibody and sheep to rabbit second antibody), F_0F_1-ATPase exhibits lower proton pumping activities, as indicated by fluorescence restoration. The possible mechanism of the inhibition of antibodies on proton pumping activity is discussed.
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F_0F_1-ATPase is a key enzyme in the biological world and is one of the most ubiquitous abundant proteins on the earth. F_0F_1-ATPase is a complex of two parts, F_0 and F_1, mechanically coupled by a common central stalk. The membrane-embedded F_0 efficiently converts the proton-motive force into mechanical rotation of the central stalk inside the F_1, and the rotation causes cyclic conformational changes in F_1, driving ATP synthesis. The enzyme can also function in the reverse direction, hydrolyzing ATP and using the released energy to pump protons across the membrane [1–5].

Taking advantage of the single molecule observation, many researchers [6–9] have analyzed the rotary torque and speed, mechanism of force generation, and architecture of the rotor and stator of this new smallest biological rotary motor as Boyer had hypothesized [1]. Recently, the nanotechnology has enabled the design and production of a variety of nanodevices and molecular machines. The study of molecule motors has the potential to power these nanodevices, which have been attracting increasing interest. Biophysical analysis of many enzymes has revealed their roles as nanomotors, such as kinesin dynein, myosin, DNA helicase, and RNA polymerase as linear stepped motors, but the rotary motors are only flagella motor and ATP synthase [10]. The force generation and size scale of these bimolecular

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motors are compatible with nanofabricated structures. Because the splendid biological rotary motor would be powerful in interdisciplinary sciences, many scientists have attempted to develop the rotation of ATP motors as microdevices for applying in nanotechnology. Although the direct observation of rotation of F_1-ATP synthase by attaching actin fluorescence filament to the γ subunit of F_1 observed by fluorescence microscopy has been achieved, the actin filament load was 1 to 2 μM long with a 10-nm diameter of F_1-ATPase. In filaments with frequent lower average speeds, pauses and fluctuation should in part be of a Brownian origin [7,9]. The slower movement is under higher load. By precise analysis of the single molecule observation system, only approximately 1% of the actin fluorescence filament was rotating.

The fluorescence technique is one of the most powerful methods for studying the structure and function of biomacromolecules [11]. F_0F_1-ATPase proton channel was detected by 9-amino-6-chloro-2-methoxyacridine (ACMA)[1] quenching, but this approach detected only the membrane energized for the pH gradient form [12]. Fluorescein-phosphatidylethanolamine (FPE) was used for measuring pH changes in the internal compartment of phospholipid vesicles [13]. *N*-(Fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (F-DHPE) is also a lipid fluorescent probe that is sensitive to pH changes and has been used to measure pH changes adjacent to the bilayer surface [14]. In this article, we report its use for measuring the proton flux through F_0F_1-ATPase within chromatophores driven by ATP hydrolysis, as indicated by fluorescence restoration responding to pH changes in the external chromatophores.

Materials and methods

Chemicals

F-DHPE and 5′-adenylyl imidodiphosphate (AMP–PNP) were purchased from Molecular Probes (USA). *N,N*′-Dicyclohexylcarbodiimide (DCCD) and ATP were obtained from Sigma (USA). Second antibody (sheep to rabbit) was obtained from Dingguo (China). All other analytically purified reagents were purchased domestically.

Preparation of chromatophores

Chromatophores were prepared from the cells of *Rhodospirillum rubrum* according to [15,16] in buffer containing 0.1 mM Tricine–NaOH (pH 8.0), 0.25 M sucrose, and 5 mM $MgCl_2$. After centrifugation, the chromatophores were resuspended in buffer containing 0.1 mM Tricine–NaOH (pH 8.0), 0.25 M sucrose, 5 mM $MgCl_2$, and 50% glycerol. The suspension was rapidly frozen in liquid nitrogen and stored at –80 °C. The concentration of bacteriochlorophyll (BChl) in the samples was determined spectrophotometrically (880 nm) according to the method of Clayton [17].

Unidirectional labeling of F-DHPE

F-DHPE (3 μl, 1 mg/ml, dissolved in ethanol) was added to 1 ml chromatophores. After incubating for 30 min at room temperature, free F-DHPE was washed away by centrifuging at 10,000g for 30 min at 4 °C three times. The labeled chromatophores are called fluorescent chromatophores in the following text.

Preparation of anti-thermophilic bacterium Bacillus PS3 β-subunit antibody

The β-subunit of F_0F_1-ATPase from thermophilic bacterium *Bacillus PS3* (TF$_1$β) was expressed in *Escherichia coli* JM103 [18] and purified as in [19]. The antibody was prepared according to [20]. The antibody was purified by precipitation with 33% $(NH_4)_2SO_4$ and stored at –20 °C before use.

Loading of antibodies onto F₀F₁-ATPase within chromatophores

The β-antibody was incubated with fluorescent chromatophores at 37 °C for 60 min. Redundant free β-antibody was washed by centrifuging at 10,000g for 10 min three times, and the second antibody (sheep to rabbit) was incubated with fluorescent chromatophores loaded with β-antibody at 37 °C for 60 min. The free second antibody was also washed away by centrifuging at 10,000g for 10 min three times.

Fluorescence assay

ATP-dependent fluorescence quenching and restoration were measured in 1 ml assay buffer (0.1 mM Tricine–NaOH [pH 8.6], 50 mM KCl, and 5 mM $MgCl_2$) under constant stirring at 37 °C. After the addition of 7 μg fluorescent chromatophores, the reaction was initiated by adding 2 mM ATP in the F-4500 fluorescence instrument (Hitachi). Fluorescence was excited at 496 nm and registered at 519 nm.

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[1] *Abbreviations used:* ACMA, 9-amino-6-chloro-2-methoxyacridine; FPE, fluorescein-phosphatidylethanolamine; F-DHPE, *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt; AMP–PNP, 5′-adenylyl imidodiphosphate; DCCD, *N,N*′-dicyclohexylbarbodiimide; BChl, bacteriochlorophyll; TF$_1$β, thermophilic bacterium *Bacillus PS3* β-subunit.

Results and discussion

F-DHPE labeled to the outer surface of chromatophores

F-DHPE is known as a lipid fluorescent probe and can be easily incorporated into phospholipid vesicles. As shown in Fig. 1A, fluorescence intensity of chromatophores labeled with F-DHPE (curve a) was higher than that of chromatophores as the control (curve c). Cu^{2+} is a well-known fluorescence quencher. After the addition of 1 mg/ml $CuCl_2$, the fluorescence intensity of chromatophores labeled with F-DHPE was even lower than that of the control (curve b vs. curve c), indicating that the F-DHPE was totally labeled to the outer surface of chromatophores because F-DHPE can insert into the membrane of chromatophores. This result shows that the lipid fluorescent probe F-DHPE was labeled to the outer surface of chromatophores unidirectionally in our system. Therefore, the F-DHPE-labeled chromatophores can be used in later studies.

Although we know that F-DHPE is sensitive to pH changes, in our study we wanted to determine its sensitivity to pH changes in detail and find the appropriate pH range for later experiments. Chromatophores labeled with F-DHPE in indicated pH buffers (7.0, 7.4, 7.8, 8.2, 8.6, and 9.0) were added to the fluorescence assay. Fig. 1B shows that fluorescence intensity increased with increasing pH values. A considerable change of fluorescence intensity appeared from pH 8.2 to 9.0. Therefore, a pH value of 8.6 was chosen for the following tests.

As mentioned earlier, F_0F_1-ATPase is a rotary motor. During ATP hydrolysis, protons are pumped into the chromatophores, resulting in a decrease of concentration of H^+ in the solution. Thus, the pH will increase in a solution of 0.1 mM Tricine with poor buffer capacity, and the fluorescence probe F-DHPE is expected to detect the pH increase. A schematic view of experimental setup is shown in Figs. 2A and B. Fig. 2A is a schematic view of chromatophores labeled with F-DHPE before ATP hydrolysis, whereas Fig. 2B is a schematic view of chromatophores during ATP hydrolysis, resulting in the protons being pumped into the chromatophores.

The ATP hydrolysis activity of newly prepared chromatophores is approximately 108 µmol/h/mg BChl. When 2 mM ATP was added to the reaction buffer, we found that fluorescence was quenched immediately from 900 to 250 U and then increased from 250 to 550 U in 20 min (Fig. 2C). This result indicated that ATP acted as a fluorescence quencher during the fluorescence decrease phase and that the pH value of the vesicles increased during the subsequent fluorescence increase phase because F_0F_1-ATPase hydrolyzed ATP and pump
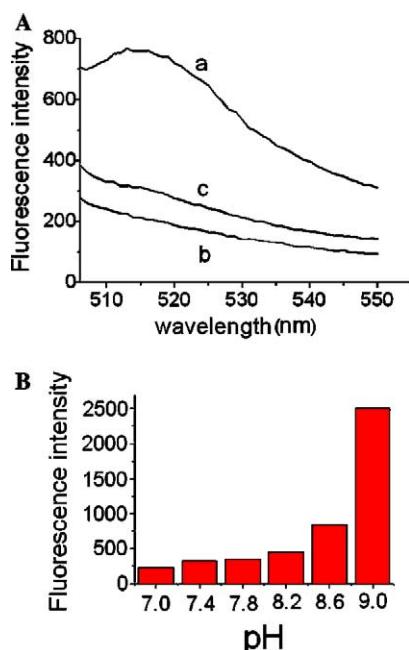

Fig. 1. Characteristics of fluorescence probe F-DHPE labeled to outer surface of chromatophores. (A) Fluorescence intensity after F-DHPE was labeled to the outer surface of chromatophores. (a), fluorescence intensity of chromatophores labeled with F-DHPE; (b), fluorescence intensity of chromatophores labeled with F-DHPE after the addition of 1 mg/ml $CuCl_2$; (c), fluorescence intensity of chromatophores as control. (B) Relationship between fluorescence intensity and pH values.
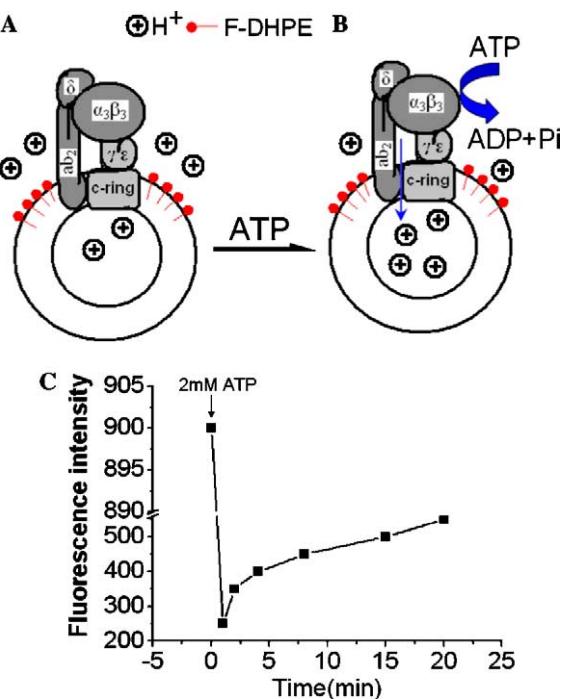

Fig. 2. Schematic view of experimental setup and fluorescence quenching/restoration caused by the addition of ATP. (A) Schematic view of chromatophore before ATP hydrolysis. (B) Schematic view of ATP-driven proton flux across chromatophores. The protons were pumped inside of chromatophores during ATP hydrolysis. (C) Time course of fluorescence quenching and restoration. The assay buffer contained 0.1 mM Tricine–NaOH (pH 8.6), 50 mM KCl, and 5 mM $MgCl_2$. After the addition of 2 mM ATP, the fluorescence intensity immediately decreased greatly to a minimum value and then increased with the ATP hydrolysis process.

protons inside the vesicles. It was concluded that the later fluorescence intensity increase resulted from the simultaneous proton flux through the F_0 channel and decrease of ATP concentration due to the ATP hydrolysis.

Dependence of restoration of fluorescence intensity on proton flux during ATP hydrolysis

It has been reported that sulfite can stimulate the ATPase activity of chromatophores [21]. The effect of
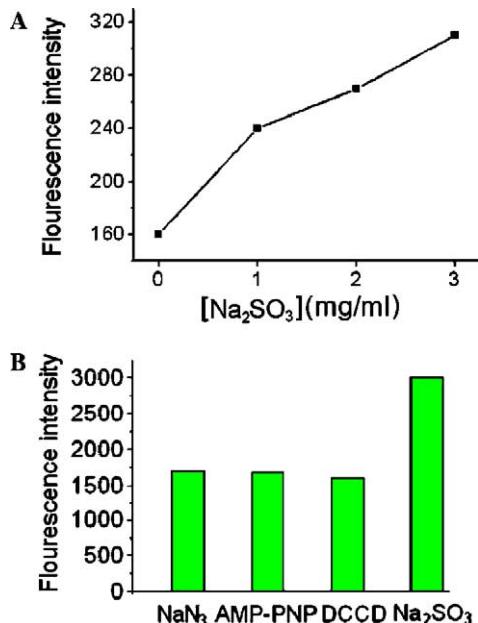

Fig. 3. Fluorescence intensity regulated by proton flux. (A) Linear relationship between the fluorescence intensity and the Na_2SO_3 concentration. According to the procedure in Fig. 2C, 2 mM ATP was added to the fluorescence assay buffer containing chromatophores in the presence of Na_2SO_3, and the restored fluorescence was assayed for 1 min. (B) Effect of inhibitors on the restored fluorescence intensity after the addition of 2 mM ATP. Here, 5 mM NaN_3, 3 mg/ml Na_2SO_3, and 20 μM AMP–PNP were added directly into the assay buffer, and 20 μM DCCD was incubated with chromatophores for 1 h at room temperature prior to the fluorescence assay according to (A).

sulfite as a stimulator on the proton flux is shown in Fig. 3A (fluorescence restoration was assayed according to Fig. 2C and the following tests of fluorescence restoration). The result shows clearly the linear relationship between the fluorescence intensity and the Na_2SO_3 concentration. When 3 mg/ml Na_2SO_3 was added to the assay buffer, the fluorescence intensity was twice as high as that of the control (~320 vs. 160 U), indicating that the increase of fluorescence intensity was consistent with ATP hydrolysis by F_0F_1-ATPase with the proton pumping. In addition, the F_1 inhibitors NaN_3 and AMP–PNP and the F_0 channel inhibitor DCCD were added to assay buffer containing 3 mg/ml Na_2SO_3 to confirm further that the fluorescence intensity was regulated by proton flux, as shown in Fig. 3B. Roughly half of the fluorescence intensity was retained compared with the sample treated only with 3 mg/ml Na_2SO_3 (Fig. 3B). This result implies that NaN_3 (5 mM), AMP–PNP (20 μM), and DCCD (20 μM) inhibited the ATPase activity of chromatophores and blocked the ATP hydrolysis-driven proton pump, slowing the fluorescence intensity restoration.

Effects of loaded antibodies on the proton flux driven by ATP hydrolysis

According to the rotational catalysis mechanism [1], the rotation of the central $\gamma\varepsilon c_n$ rotor relative to the $\alpha_3\beta_3$ hexagon is critical for operation of the catalytic sites, and the rotation of $\gamma\varepsilon c_n$ relative to the a-subunit is critical for proton transport during ATP hydrolysis. The polyclonal antibodies of the β-subunit can bind at various sites of F_0F_1-ATPase and are assumed to affect the ATP-driven proton-pumping activity of F_0F_1-ATPase. The experimental setup shown in Fig. 4A demonstrates the above assumption. Fig. 4A (a) shows no antibody on F_0F_1-ATPase and the chromatophores as the control. Fig. 4A (b) shows polyclonal antibody of the β-subunit (first antibody) loaded onto
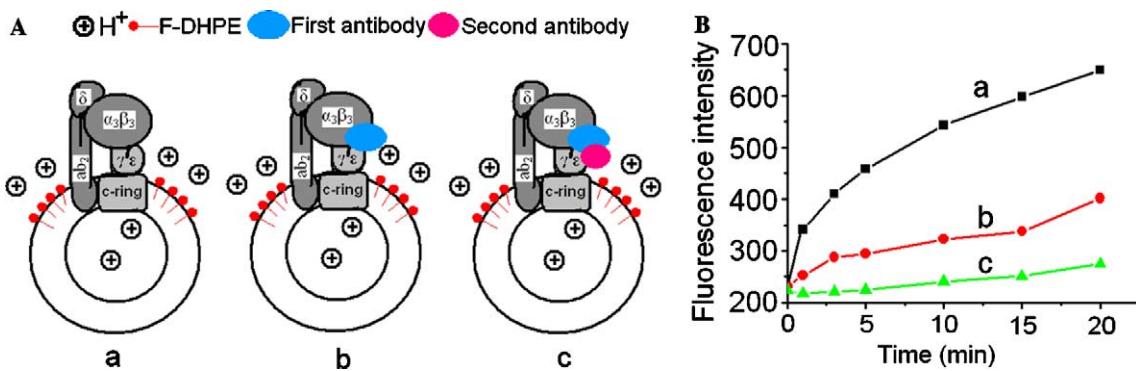

Fig. 4. Effects of antibodies (first antibody of β-subunit and second antibody) on proton flux driven by ATP hydrolysis. (A) Experimental setup: (a) without antibody loaded; (b) first antibody of β-subunit; (c) first antibody of β-subunit and second antibody (sheep to rabbit). The antibodies were loaded onto the F_1F_0-ATPase of chromatophores as described in Materials and methods. (B) Measurement of proton flux by F_1F_0-ATPase with antibodies. a, without loading; b, in the presence of the first antibody of the β-subunit; c, in the presence of the first antibody of the β-subunit and the second antibody (sheep to rabbit). Fluorescence restoration was assayed according to Fig. 2C.

F_0F_1-ATPase. Fig. 4A (c) shows polyclonal antibody of the β-subunit (first antibody) loaded onto F_0F_1-ATPase and the second antibody (sheep to rabbit) loaded onto the anti-β-antibody. Fig. 4B shows the restoration of fluorescent chromatophore samples with various antibodies resulting from ATP hydrolysis. In Fig. 4B, curve (a) shows that the fluorescence intensity was restored greatly (from 220 to 650 U) in 20 min at 37 °C. Curve (b) shows that after the addition of 2 mM ATP, the fluorescence intensity of chromatophores with the first antibody was restored from 220 to 400 U in 20 min at 37 °C. Curve (c) shows the fluorescence restoration of chromatophores loaded with two kinds of antibodies; after adding 2 mM ATP, the fluorescence activity was restored from 220 to 270 U in 20 min at 37 °C. It was calculated from Fig. 4B that the rate constants of fluorescence restoration in 1 min were approximately 2.1/s, 0.5/s, and 0.0/s for no antibodies, one antibody, and two antibodies loading, respectively. Therefore, it was concluded that the loaded antibodies inhibit the ATP-driven proton-pumping activity of F_0F_1-ATPase. We propose that the loss of ATPase activity of chromatophores was not likely caused by the binding of antibodies at active sites of F_0F_1-ATPase given that the joining of second antibodies to first antibodies aggravated the inhibition. Therefore, the inhibition may have been indirect. Considering the "stator" role of the β-subunit and the "rotor" role of $\gamma\varepsilon c_n$, it is possible that the antibodies loaded onto the β-subunits spatially is close to $\gamma\varepsilon c_n$, blocking the rotation of $\gamma\varepsilon c_n$. Further studies are needed to better understand the mechanism of indirect inhibition of antibodies to proton-pumping activity of F_0F_1-ATPase.

Summary

A new method for the detection of proton flux driven by F_0F_1-ATPase using fluorescent probe F-DHPE attached to the lipid surface of chromatophores has been established. It is well known that ACMA and acridine orange have been widely used for detecting ATP-driven proton-pumping activity of F_0F_1-ATPase and proton translocation across F_0 driven by proton-motive force. This study showed that F-DHPE also has such a potential application. Therefore, further investigation and optimization of this simple method are necessary for its application in the study of the proton transduction of F_0F_1-ATPase.

Acknowledgments

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