

F₀F₁-ATPase as biosensor to detect single virus

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Abstract

F₀F₁-ATPase within chromatophore was constructed as a biosensor (immuno-rotary biosensor) for the purpose of capturing single virus. Capture of virus was based on antibody–antigen reaction. The detection of virus based on proton flux change driven by ATP-synthesis of F₀F₁-ATPase, which was indicated by F1300, was directly observed by a fluorescence microscope. The results demonstrate that the biosensor loading of virus particles has remarkable signal-to-noise ratio (3.8:1) compared to its control at single molecular level, and will be convenient, quick, and even super-sensitive for detecting virus particles.

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F₀F₁-ATP synthase in bacterial plasma membranes, mitochondrial inner membranes, and chloroplast thylakoid membranes catalyzes the endergonic synthesis of ATP from ADP and phosphate using a transmembrane proton-motive force (PMF) generated by oxidative phosphorylation or photosynthesis [1–3]. In *Escherichia coli*, the F₁ and F₀ are composed of $\alpha_3\beta_3\gamma\delta\epsilon$ and ab_2c_n , respectively. These two parts are 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 flux through F₀ drives rotation of the c-ring and hence $\gamma\epsilon$, forcing conformational changes in F₁ that result in ATP synthesis from ADP and Pi. Conversely, ATP hydrolysis in F₁ causes the reverse rotation of the rotor [4], hence drives F₀ to pump protons in the reverse direction.

The F₀F₁-ATP synthase is a nanoscale rotary biological motor. Because of its transduction of energy, nanoscale size, and possible practicability in micro/nanotechnology, many scientists have attempted to develop the motor as nanopower devices [5,6]. But, it is difficult to use the power

generated by mechanochemical coupling of the motor outside the cell. For such purposes, Cui et al. [7] reported using fluorescein-DHPE (F-DHPE) labeled on the surface of chromatophores to detect proton flux through F₀F₁-ATPase driven by ATP hydrolysis. Their results showed that the F₀F₁ motors would be a novel application as biosensor to detect loads of molecular at single molecular level.

Rapid, selective, and sensitive detection of virus is central to implement an effective response to viral infection. The current outbreak of avian influenza A was among poultry in all over the world, improving influenza surveillance would be important and urgent. Many methods can be used to detect the virus, including immunological assays, transmission electron microscopy and PCR-based testing of viral nucleic acids, and even optical microscopes in the laboratory, however, most of them are time consuming and require purifying the samples, which makes them inappropriate for clinic and diagnostics. For such reasons, researchers use micro- and nanosensor technology to improve the detection of virus [8].

Here, further development of the biosensor based on F₀F₁-ATPase within chromatophores to detect virus is described. The design of immuno-rotary biosensor (IRB) based on rotary single F₀F₁-ATPase is shown in Fig. 1A.

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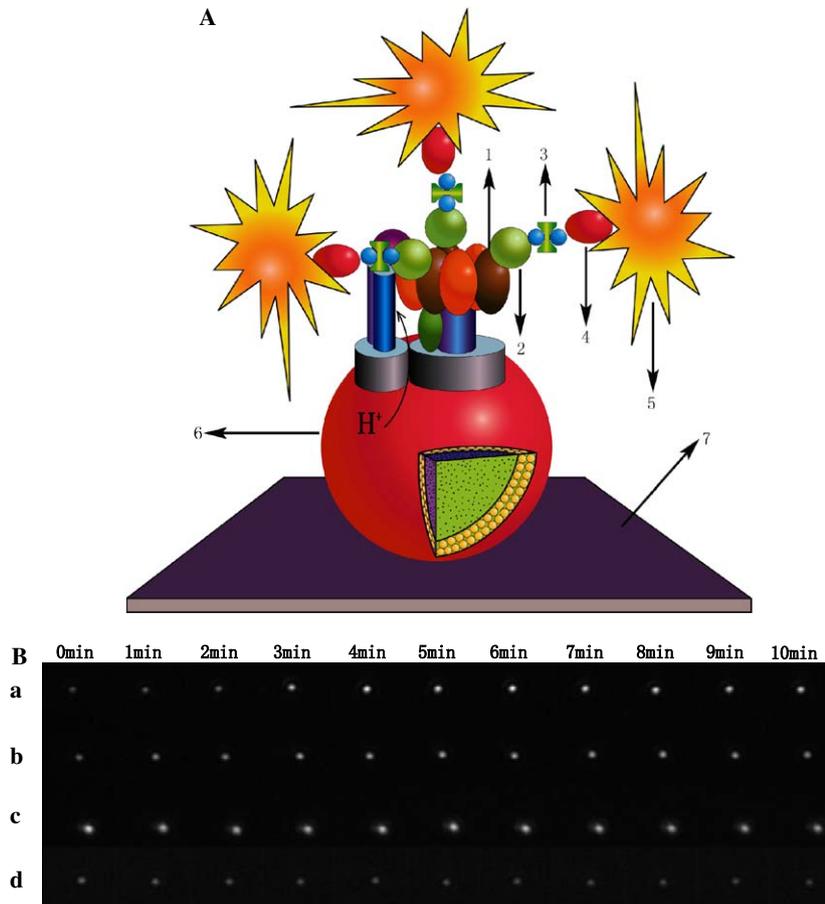


Fig. 1. (A) Basic design of immuno-rotary biosensor based on single rotary F_0F_1 -ATPase (1) β subunit of F_0F_1 -ATPase; (2) antibody of β subunit; (3) the system of biotin–streptavidin–biotin; (4) the antibody of H9 avian influenza virus; (5) H9 avian influenza virus; (6) F_0F_1 -ATPase within chromatophores; (7) glass surface coated with chitosan. (B) Images of intensity change of fluorescence dots caused by ATP synthesis of single F_0F_1 -ATPase within chromatophore in the time course 10 min. a, IRB loaded with virus particles; b, IRB without loads; c, IRB loaded with two antibodies; d, IRB without adding ADP.

The fluorescence probe F1300 labeled in inner chromatophores was used as a proton flux indicator. The F_1 β subunit site of F_0F_1 -ATPase linked to β antibody–biotin–avidin–biotin–antibody system (especially for Avian virus) was used as a capture reaction receptor. Rotation of single F_0F_1 -ATPase within chromatophores driven by ATP synthesis was observed directly under a fluorescence microscope and recorded with cooled digital CCD camera.

Materials and methods

Chemicals and materials. Fluorescence probe (No. F1300) was purchased from Molecular Probes (Eugene, Oregon, USA). ADP was purchased from Sigma–Aldrich (St. Louis, USA). All other analytically purified reagents were purchased domestically. Tricine–NaOH buffer (0.1 mM tricine, 5 mM $MgCl_2$, and 5 mM KCl, pH 6.5). ATP synthesis buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, 5 mM K_2HPO_4 , and 10% glycerol, pH 8.5). 1× PBS buffer (8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 , and 3.58 g Na_2HPO_4 , pH 7.4).

Chromatophores were prepared from the cells of *Rhodospirillum rubrum* according to [9,10]. The β -subunit of F_0F_1 -ATPase from thermophilic bacterium *Bacillus PS3* ($F_1\beta$) was expressed in *E. coli* JM103 [5] and purified as in [11]; the antibody was prepared according to ref [12], purified by precipitation with 33% $(NH_4)_2SO_4$, and stored at $-20^\circ C$ before use.

The avian H9 influenza A viruses were propagated in the allantoic cavities of 11-day-old embryonated chicken eggs at $37^\circ C$ for 3 days. The allantoic cavities were collected and centrifuged at 4000 rpm for 40 min and then the supernatant was centrifuged again at 100,000g for 2 h. The viruses were resuspended in PBS buffer and used in the following experiments [13]. The H9 influenza A and H9 influenza A antibodies were from Dr. Ni zhiqian (Harbin Veterinary Research Institute).

Methods. The fluorescence probe F1300 was labeled into chromatophore as follows: 3 μ l F1300 (0.0015 mol/L, dissolved in ethanol) was added to 150 μ l chromatophores and then was ultrasonicated in ice for 3 min to make the probe into inner chromatophores. The free fraction was washed by centrifugation at 12,000 rpm for 30 min at $4^\circ C$ three times. The precipitate was resuspended in tricine–NaOH buffer.

The β -subunit antibody was labeled with biotin–streptavidin as follows: 2 μ l of 2 μ M biotin was added in 20 μ l β -subunit antibody at room temperature for 30 min and then added 2 μ l of 2 μ M streptavidin at room temperature for 30 min. The H9 avian influenza virus antibody was labeled with biotin by adding 2 μ l of 2 μ M/ml biotin in 15 μ l H9 avian influenza virus antibody at room temperature for 30 min.

The immuno-rotary biosensor (IRB) was constructed as follows: 100 μ l of 0.5% chitosan was added into a flow cell at $4^\circ C$ overnight and then free chitosan was washed three times with PBS buffer; the F1300 labeled chromatophores were added into the flow cell at $4^\circ C$ overnight, non-specific binding sites were blocked with 3% BSA for 2 h at $37^\circ C$ and then washed three times with Tricine buffer; 100 μ l biotin–streptavidin labeled β -subunit antibody was added at $37^\circ C$ for 1 h when it was diluted 200 times and then washed three times with PBS buffer; 100 μ l biotin labeled

H9 avian influenza virus antibody was added at room temperature for 10 min when it was diluted 200 times and then washed three times with PBS buffer; at the last, 30 μ l H9 avian influenza virus was added at 37 °C for 1 h and then washed three times with PBS buffer. The prepared IRBs were stored at 4 °C before use.

Detection of the fluorescence change of IRBs at single molecular level was as follows: ADP was added into the ATP synthesis buffer with the final concentration of 2 mM in the flow cell. The fluorescence change of single fluorescence dot was recorded by Olympus IX71 fluorescence microscope with cooled digital CCD cameras (Princeton scientific) for 1 min interval at 37 °C with excitation at 485 nm and emission at 538 nm. The flow cell was kept at 37 °C during the whole experimental process.

The recorded pictures were dealt with the software of Winview/32 (Princeton Scientific).

Results

Fig. 1B shows that F1300 is a reliable proton flux indicator for direct observation of single F_0F_1 -ATPase with a fluorescence microscope. Fig. 1B(a,b,c) show the images of fluorescence intensity changes of IRB loaded of H9 avian influenza virus particles (a), without loads (b), loaded of β antibody and H9 avian influenza virus antibody (c) when 2 mM ADP was added to initialize reaction at 37 °C, respectively. Fig. 1B(d) shows the images of fluorescence intensity changes of IRB without adding ADP. In images a, the result showed that the intensity of fluorescence dot increased from 1568 to 4991 unit in the time course of 0 to 4 min (the highest) and decreased from 4991 to 4049 unit in the time course of 4–10 min; images b show that the intensity of fluorescence dot increased from 2260 to 3400 unit in the time course of 0 min to 5 min (the highest) and decreased from 3400 to 2726 unit in the time course of 5–10 min; therefore, the increasing of fluorescence intensity was coupled with ATP synthesis activity and the decreasing of fluorescence intensity may be caused by quenching. In images c, there is almost no obvious change of fluorescence intensity because of the effects of inhibition of ATP synthesis. In images d, there is obvious decreasing of fluorescence intensity because of quenching only.

Furthermore, the relationship between the changes of fluorescence intensities and various molecule loads of F_0F_1 -ATPase during the ATP synthesis was shown in Fig. 2A. The experiment was described in the methods. The curves (a,b, and c) show the fluorescence intensity changes of IRB loaded with virus particles (a), without loads (b), and only loaded with 2 antibodies (c) when ADP was added to initialize reaction at 37 °C, respectively. The curves d show the fluorescence intensity changes of IRB without adding ADP. In curves a, the fluorescence intensities of IRBs increased more than three times at 5 min compared to the beginning when the IRB was loaded of virus particles (17 samples). In curves b, the fluorescence intensities of IRBs increased not more than 1.5 times at 5 min compared to the beginning when the IRB was without loads (5 samples). In curves c, the fluorescence intensities of IRBs increased less than 1.1 times, and most of the fluorescence intensities even decreased more than 10% at 5 min compared to the beginning when the IRB was loaded

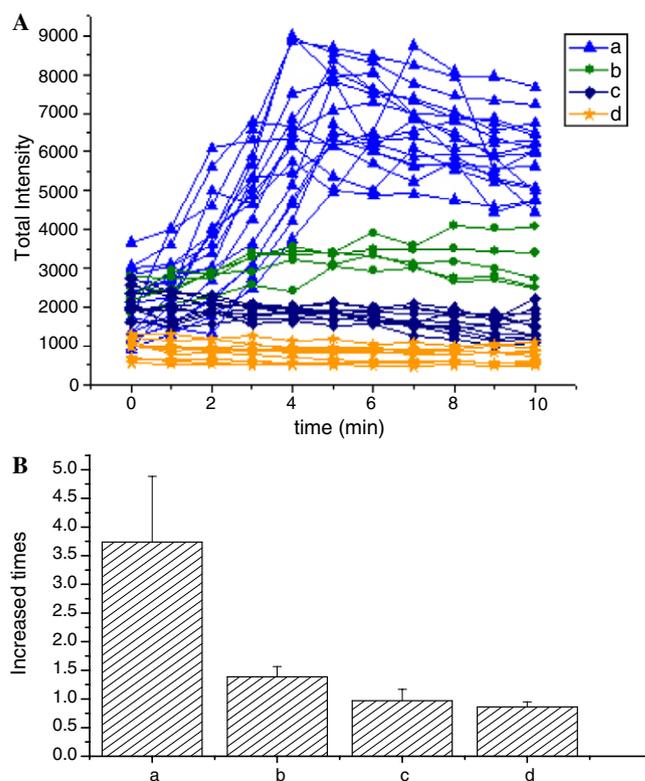


Fig. 2. (A) The fluorescence intensity changes during ATP synthesis of single F_0F_1 -ATPase with various molecule loads. (B) Average increased times of fluorescence intensity after 5 min of ATP synthesis compared to the beginning. a, IRB loaded of virus particles; b, IRB without loads; c, IRB loaded of two antibodies; d, IRB without adding ADP. The statistical results are from 54, 22, 58, and 35 samples for columns (a–d) respectively.

of antibodies only (11 samples). It is noticed that in all of curves a, curves b, and some of the curves c, the increased fluorescence intensity curves showed inflexion points at 4 or 5 min, after this time, the curves decreased and some of them even decreased very quickly. The decrease of fluorescence intensity probably was caused by fluorescence quenching or ATP hydrolysis (for quickly decreased curves). Curves d are control without adding ADP, and the fluorescence intensities decreased more than 15% at 5 min compared to the beginning, which was only caused by quenching (10 samples). From curves a–c, it clearly shows that the rate of fluorescence intensity change is directly proportional to the ATP synthesis activity and can reflect the different load weight on F_0F_1 -ATPase. Therefore, various molecules can be detected directly by the change of fluorescence signal depending on its load weight.

The statistical results are shown in Fig. 2B. The columns (a–d) show that the average increased times of fluorescence intensity after 5 min of ATP synthesis compared to the beginning of the IRB loaded with virus particles, without loads, loaded of two antibodies, and without adding ADP, the increased times were 3.74 (± 1.14), 1.39 (± 0.18), 0.97 (± 0.20), and 0.86 (± 0.09), respectively. For columns c and d, the average increased times were less than 1 because of fluorescence quenching. It is clearly that the

IRB loaded with virus particles (column a) has remarkable signal-to-noise ratio (3.86:1) compared to its control (column c) at single molecular level.

Discussion

F_0F_1 -ATPase is a reversible motor for ATP synthesis/hydrolysis, the protons are pumped out of chromatophores when the F_1 -ATPase rotates clockwise and the ATP is synthesized; reversely, hydrolysis of ATP results in counter-clockwise rotation of F_1 -ATPase and drives protons pump into the chromatophores. And F1300 is pH dependent, the fluorescence intensity of F1300 increases with the increasing of pH. So, the rate of fluorescence intensity change can reflect the rotation rate of F_0F_1 -ATPase and further reflect the weight of loads. Based on this principle, the F_0F_1 -ATPase within chromatophores can be used as biosensor.

It is noticed that the diameter of chromatophore is about 50–90 nm and most of chromatophores are about 0.79 F_0F_1 -ATPase (data not shown). In our experiments, about 62.86% results were positive in detection of virus particles. This means some of chromatophores in absence of F_0F_1 -ATPase or the F_0F_1 -ATPase without loads, but they can be distinguished easily through the increasing of fluorescence intensity. For the purpose of further developing such method, further separating and purifying the chromatophores to improve the performance of IRBs is now undergoing.

To confirm that the IRBs were only in response to H9 virus specifically, the ATP synthesis activity of F_0F_1 -ATPase was measured with the luciferin–luciferase method at different conditions. The ATP synthesis activity of chromatophores (F_0F_1 -ATPase) incubated with H9 virus showed no obvious change. To further validate the above results, the fluorescence intensity changes of IRBs at the ATP synthesis condition (IRBs were fixed on the surface of 96-well plate) was recorded on a fluorescent microplate reader (Fluoroskan Ascent, Labsystems, Finland), where we did not find obvious changes of fluorescence (data not shown). It clearly indicates that only the H9 virus can increase the ATP synthesis activity specifically when it is linked to the F_0F_1 -ATPase by the recognition system.

The activity of F_0F_1 -ATPase did not change when it is incubated with virus particles together, which indicated virus particles cannot directly affect the activity of F_0F_1 -ATPase. F_1 part of ATP synthase is a multi-subunit protein complex and the β subunit is a catalytically active site which demonstrates that the flexibility will affect its performance, so the possible mechanism is that different load weight will affect the conformation of β -subunit. Although it is unclear how it occurs exactly, the application of biosensor is vital to routine inspection and may be used in the ultra-sensitive detection of virus particles in future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbrc.2006.02.103](https://doi.org/10.1016/j.bbrc.2006.02.103).

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