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Resonance phenomenon of the ATP motor as an ultrasensitive biosensor

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ABSTRACT

We designed a rotary biosensor as a damping effector, with the rotation of the F_0F_1-ATPase driven by Adenosine Triphosphate (ATP) synthesis being indicated by the fluorescence intensity and a damping effect force being induced by the binding of an RNA molecule to its probe on the rotary biosensor. We found that the damping effect could contribute to the resonance phenomenon and energy transfer process of our rotary biosensor in the liquid phase. This result indicates that the ability of the rotary motor to operate in the vibration harmonic mode depends on the environmental conditions and mechanism in that a few molecules of the rotary biosensor could induce all of the sensor molecules to fluoresce together. These findings contribute to the theory study of the ATPase motor and future development of biosensors for ultrasensitive detection.

1. Introduction

The F_0F_1-ATPase, one of the most thoroughly studied enzymes, is an intricate nanomachine that self-assembles from eight different subunits. It is found ubiquitously in the plasma membrane of bacteria, chloroplasts and mitochondria and uses the transmembrane electrochemical potential to synthesize ATP [1]. The protein consists of two rotary motors that act in opposition: the F_1 motor generates a mechanical torque using the hydrolytic energy of ATP, whereas the F_0 motor generates a rotary torque in the opposite direction using a transmembrane proton motive force [2]. The F_0F_1-ATPase is a rotary nanomolecular motor that can generate force torques larger than 100 pN via a mechanism referred to as the "binding change mechanism" [3], and single-molecule technologies have greatly contributed to our understanding of this motor. For example, fluorescence imaging and spectroscopy have demonstrated the physical rotation of isolated F_1 units [4–7] and the F_0F_1 holoenzyme [8–10], and magnetic tweezers have been employed to manipulate ATP synthesis/hydrolysis in the F_1 units [11,12] and proton translation in F_0 units [13]. Regulation of the F_1 rotation speed by adjusting the load on the motor was observed by Noji et al. at the level of a single molecule: the longer the actin filament load was, the slower was the rotation of the F_1 unit [7]. In our previous studies, we also observed that linking longer actin filaments to the F_1 unit could induce a slower rotation of the F_1-γ subunit at the single-molecule level [14]. These results imply a relationship between rotation speed and the load on the ATPase, which gives rise to a damping effect.

Damping theory was first described by Newton and has been verified using such objects as pendulums [16,17]. Damping has an important impact on the dynamic action of nanomachines, affecting their force sensing and other applications, and resonance is the most widely used phenomenon for characterizing force materials. Although the ATPase characteristics have been considered to be a damping effect at the single-molecule level for many years [7], damping of the ATP motor by a resonance energy-transfer processing effect has not yet been reported. This is because, in real physical systems, resonance at the molecular level operates at low temperatures and in a high vacuum. Indeed, direct observation of the resonance effect of ATP motors has previously been difficult using the currently available physical techniques. In this study, a rotary biosensor consisted of the damping effect on an ATP motor, inducing the resonance energy-transfer-processing phenomenon by the receptor (Fig. 1).

Our results indicate that the ATP motor capturing targets can rotate at different speeds and that ATP motors with targets in the same solution also change their rotation speed, thus displaying all of the on or off signals together. The results imply that resonance plays a key role in this phenomenon and that there may be energy transfer between the ATP motors that capture targets and those that do not capture targets. This finding contributes to our understanding of the theory of ATP motor rotation and will also contribute to the design of novel biosensors that are based on this finding for ultrasensitive applications.

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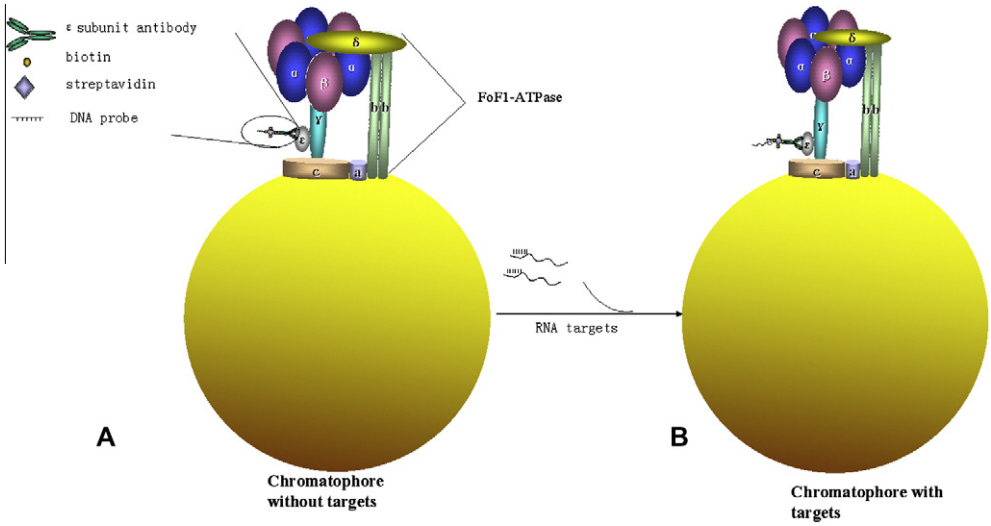

Fig. 1. Schematic illustration of the rotary biosensor. (A) Rotary biosensor without targets. (B) Rotary biosensor with targets.

2. Materials and methods

2.1. Cell lines and reagents

Thermomicrobium roseum wa0073 (ATCC27502) was purchased from ATCC, USA. The luciferase/luciferin ATP detection kits were purchased from Promega Corporation (USA). ADP, (+)-biotin N-hydroxysuccinimide ester and NeutrAvidin were purchased from Sigma–Aldrich (St. Louis, USA). The RNA extraction kits were purchased from Tiangen, and the RNA probes were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The HIV-1 positive serum was obtained from the Chinese Center for Disease Control and Prevention.

2.2. Preparation of chromatophores containing the F_0F_1-ATPase

Thermomicrobium roseum was cultured at 60°C for 24 h, and the cells were collected by centrifugation at 5,000×*g* for 20 min. The pellets were resuspended in 20 ml buffer A, containing 20 mM Tris–HCl (pH 8.0), 2 mM $MgCl_2$, 100 mM NaCl, and 10% glycerin (v/v), and sonicated for 3 min. The lysate was centrifuged for 30 min at 5000×*g* at 4 °C, and the supernatants were collected and centrifuged at 180,000×*g* for 90 min at 4 °C. The precipitate containing the chromatophores was resuspended in buffer A and stored at −80 °C for further use.

The concentration of F_1 was determined as follows: 0.8 ml chromatophores and 0.12 ml 64 mM ATP (final concentration, 8 mM) were stirred gently at 4 °C for 60 min. LiCl at 1 M (final concentration, 4 M) was then added dropwise with vigorous stirring at 4 °C for 30 min. After centrifugation at 145,000×*g* for 90 min, the concentration of F_1 in the supernatant was measured using the Bradford method. The final concentration was 30 mg/ml; the molecular weight of the F_1 subunit is 380 kDa, thus the concentration of F_1 was approximately 80 μM.

2.3. Preparation of monoclonal antibodies against the ε subunit

The ε subunit was expressed and purified according to a previously published method [17]. The ε subunit monoclonal antibodies were prepared according to the method of Hanly et al. [18] and were precipitated by 33% $(NH_4)_2SO_4$. Then the IgGs were purified using a HiTrap Protein G HP (GE Healthcare) according to the manuscript and stored at −20 °C before use.

2.4. Preparation of an HIV-1 RNA biosensor using F_0F_1-ATPase within chromatophores

Single-chain ε subunit antibodies fused to a 6×His tag were obtained from Beijing ABT Genetic Engineering Technology Co., Ltd. NTA and NHS-biotin were mixed in PBS (pH 8.0) for 4 h at room temperature, and $NiSO_4$ was then added to the NTA-biotin mixture and incubated for 10 min. Avidin (50 μg/ml) was mixed with 1 μM of a biotinylated HIV-1 RNA probe and incubated for 10 min at room temperature. The NTA-biotin was then mixed with the NeutrAvidin–biotin-HIV-1 RNA probe (5′-GATGAATAGAATAAAAGTT GCAATA-3′) and incubated for 10 min at room temperature before adding the 6×His tag-fused single-chain ε subunit antibody. Lastly, the chromatophore was mixed with this mixture and incubated for one hour. After centrifugation at 100,000×*g* for 10 min at 4 °C, the pellet containing the HIV-1 RNA biosensor was washed three times and then resuspended in 200 μl buffer containing 0.05 mM Tricine–NaOH (pH 8.5), 5 mM $MgCL_2$, and 20% glycerol.

2.5. Extraction of HIV-1 RNA from HIV-1-positive serum

The RNA was extracted from HIV-1-positive serum using a QIAGEN RNeasy Mini Kit according to the manufacturer's instructions. A 50 μl aliquot of RNase-free water was used to elute the HIV-1 RNA from 200 μl of HIV-1-positive serum, RNA extracted from HIV-1-negative serum was used as the negative control. The concentration of total RNA from HIV-1-positive serum and HIV-1-negtive serum was adjusted into the same concentration. Specific HIV-1 primers were used to determine the concentration of HIV-1 RNA among the total RNA in the serum sample using qRT-PCR and a standard curve generated with serially diluted known copies of HIV-1 RNA.

2.6. Detection of HIV-1 RNA using the HIV-1 RNA biosensor

To optimize the HIV-1 RNA biosensor concentration for low concentrations of HIV-1 RNA, the HIV-1 RNA biosensor was diluted tenfold with PBS. The HIV-1 RNA was then adjusted to 1000 fg/ml and 100 mg/ml for the assay; 100 mg/ml RNA extracted from HIV-1-negative serum was used as the negative control. The ATP synthesis reactions were carried out at 37 °C for 40 min. The reaction system contained the following: 10 μl diluted HIV-1 RNA biosensor, 28 μl HIV-1 RNA sample, 1 μl DMSO, 1 μl 1 U/ml RNase inhibitor, 50 mM Tricine-NaOH (pH 8.0), 5 mM $MgCl_2$, 5 mM KH_2

PO_4, and 0.35 mM ADP. A luminescence instrument was used to measure the ATP synthesis activity of the HIV-1 RNA biosensor.

3. Results and discussion

3.1. Rotational type of biosensor comprising an F_0F_1-ATPase within the chromatophore

We constructed a rotational biosensor with an F_0F_1-ATPase within the chromatophore. The ε subunit of the F_1 subunit acts as the capture reaction receptor and was linked to an antibody–biotin–NeutrAvidin–biotin–antibody system. The rotation of a single F_0F_1-ATPase molecule driven by ATP synthesis could be detected by the fluorescent intensity, which alters the ATP concentration after ATP synthesis, and was measured using a luciferin/luciferase ATP detection kit.

The damping effect, which induces a resonance phenomenon, is illustrated in Fig. 2. Fig. 2A shows that, when the concentration of ATPase is 1.2×10^8 molecules/well, the biosensor can be used for the ultrasensitive detection of HIV-1 RNA in the liquid phase; in contrast, the same concentration of HIV-1 RNA cannot be distinguished when the concentration of ATPase is 1.05×10^8 molecules/well (Fig. 1B). The reason for this phenomenon may be the effect of the environment on the coupling mechanisms in the resonance biosensor. Indeed, the concentration of the ATPase biosensor was so important that there would be no induced resonance phenomenon if the concentration was not suitable.

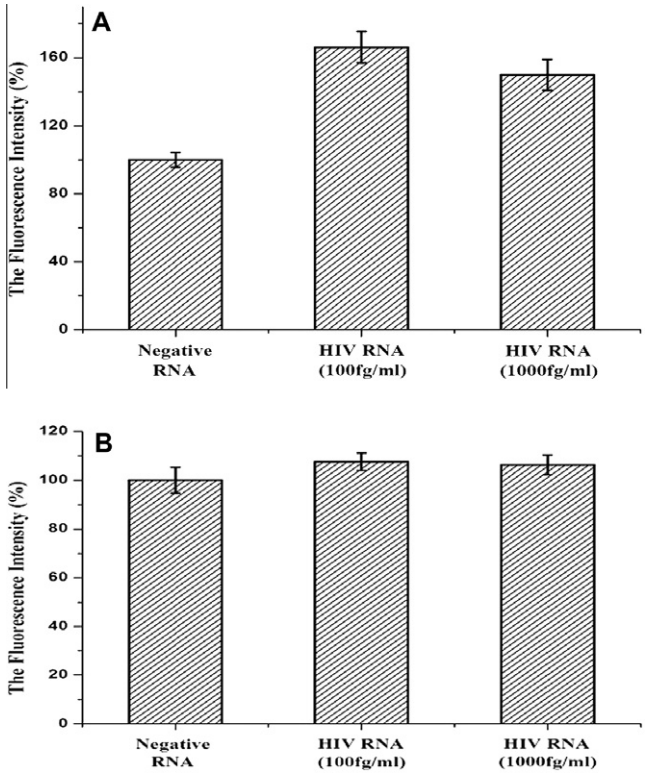

Fig. 2. Change in the fluorescence intensity with different concentrations of the biosensor. (A) Change in the fluorescence intensity with the loading of HIV-1-RNA onto the HIV-1-RNA rotary biosensor at 1 μM in the liquid phase and the damping effect-induced resonance phenomenon. Samples of 100 and 1000 fg/ml HIV-1 RNA were detected using the HIV-1-RNA rotary biosensor; RNA from an HIV-1-negative serum sample was used as the control. (B) Change in the fluorescence intensity with the loading of HIV-1-RNA onto the HIV-1-RNA rotary biosensor at a 0.84 μM in the liquid phase, and the damping effect without the resonance phenomenon was observed. Samples of 100 and 1000 fg/ml HIV-1 RNA were detected using the HIV-1-RNA rotary biosensor; RNA from an HIV-1-negative serum sample was used as the control.

Thus, different concentrations of the ATPase biosensor gave different results when the targets were captured, as the resonance effect plays an important role in our detection system. We investigated an additional environmental effect on the coupling mechanisms of the resonance biosensor (Fig. 3). The rate of the change in the florescence intensity increases from 1.2 to 1.3 and 1.4 with increasing concentrations of HIV-1 RNA from 6 to 60 and 600 fg, respectively. This result implies that it is possible to detect HIV-1 RNA using this resonance biosensor in a linear manner and under resonance-suitable conditions without the PCR amplification steps. According to this resonance principle, our resonance biosensor can detect HIV-1 RNA with a sensitivity of 35 molecules/well (Fig. 3, column 0.01 pg/ml) when the number of ATPase molecules was approximately 2.0×10^6 per well. When there is no resonance effect, a few RNA molecules could not induce sufficient changes in the signal from the ATPase, as shown in Fig. 1B. Because the environmental effects on the coupling mechanisms in the resonance biosensor are important for detection, it is necessary to optimize the conditions for the detection of different targets.

3.2. Possible mechanisms of the resonance biosensor

Although it has been difficult to observe resonance effects in ATP motors directly using physical techniques, changes in the rotary speed of ATP motors would be a gold standard for measuring this resonance effect because the ATP rotary motor can induce changes in the ATP concentration, which could indicate the resonance effect. As shown in Fig. 4, the resonance phenomenon occurs at a multi-molecular level rather than at the level of a single molecule [15]. Fig. 4A-a1 and Fig. 4A-a2 demonstrate the same rotary speed, and Fig. 4A-b1 and Fig. 4A-b2 show different rotary speeds due to the capture of the RNA. Fig. 4A-c1 and Fig. 4A-c2 show the same rotary speed, which may be due to the resonance effect mechanism with energy transfer from c1 to c2. Fig. 4B illustrates that, with many molecules together in the liquid phase, the resonance phenomenon should play a key role in the coupling of individual molecules at different states during the reaction at a low concentration of targets. This resonance phenomenon may be very specific because the ATP motor motion state exists in two states: with and without targets binding to the ATP motor. Furthermore, there are resonance phenomena with energy-transfer processing when a few targets bind to the ATP motor, which could induce the molecules to fluoresce together, indicating that the rotary motor can work in the vibration harmonic mode depending on the environmental condition. This simple method was used in the present study to focus on this new phenomenon and the

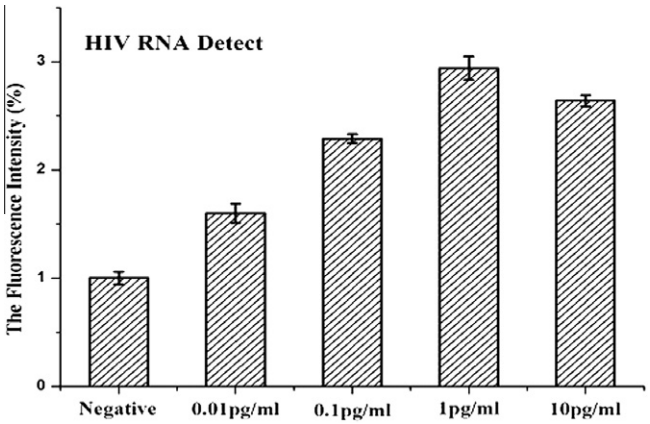

Fig. 3. Relative ATP synthesis activities of the F_0F_1-ATPase at different concentrations of HIV-1 RNA induced the resonance phenomenon. Samples of 0.01, 0.1, 1 and 10 pg/ml HIV-1 RNA were detected using the HIV-1 RNA biosensor; RNA from an HIV-1-negative serum sample was used as the control.

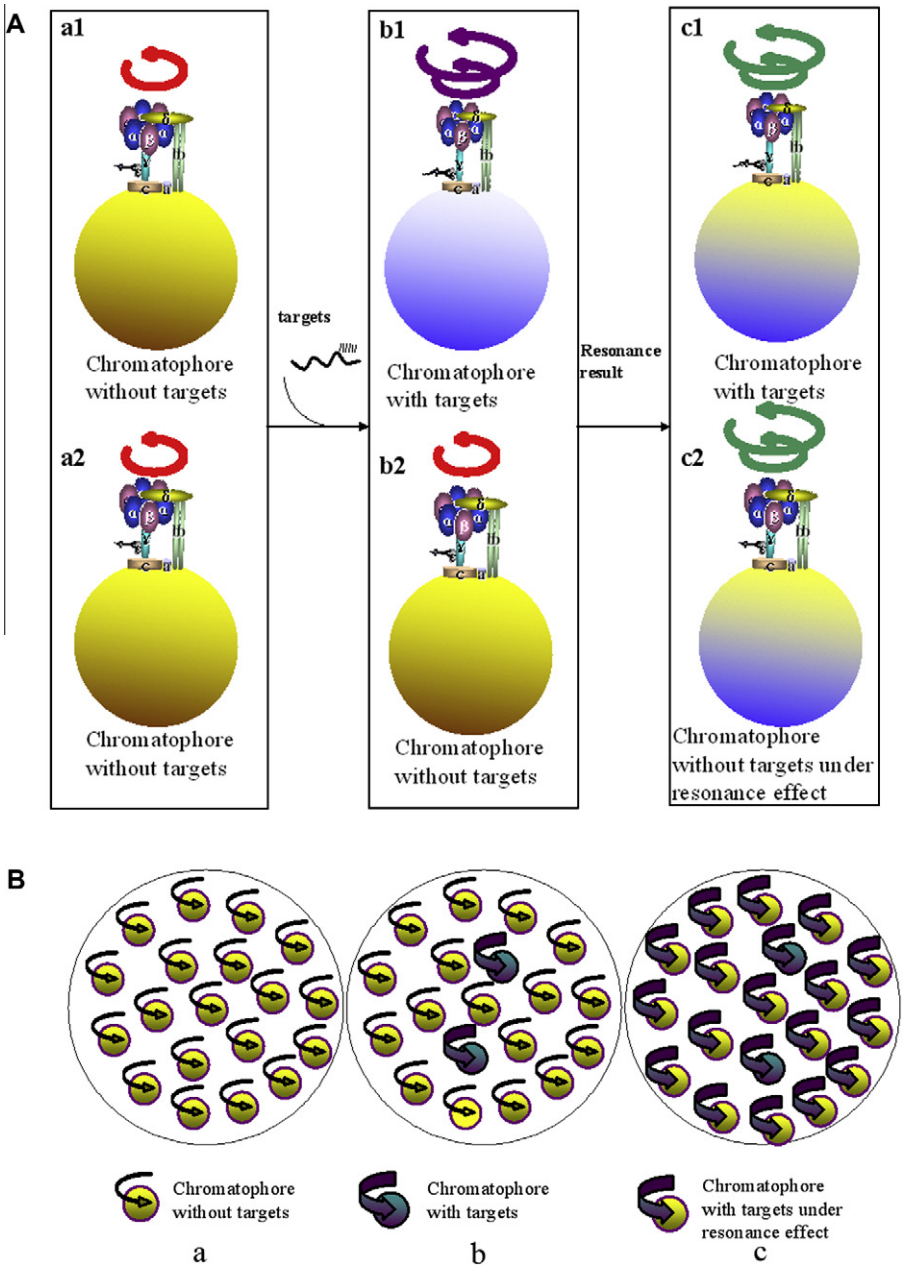

Fig. 4. Schematic diagram of the resonance biosensor. (A) a1 and a2 were all without HIV-1 RNA targets at the same rotary speed; b1 was the status of a1 with the RNA target at a higher rotary speed, and B2 was the same as a2 without the resonance effect. c1 was at the same status as b1 after the resonance, and c2 was the status without the RNA target and at a higher rotary speed after the resonance effect. The higher rotary speed of c1 with RNA and c2 without RNA may be due to the resonance effect mechanism with energy transfer from c1 to c2. (B) Schematic diagram of the resonance biosensor. The group effect of resonance is indicated. (a) All of the chromatophores are at a relative lower rotary speed without RNA target binding. (b) Only a few chromatophores bound to RNA and gained a higher rotary speed, whereas the others were at the original speed without the resonance effect. (c) After the energy transfer from the chromatophore with RNA to the chromatophore without RNA, the resonance effect induced the increase of the rotary speed of all of the chromatophores.

application of the ATP biomotor. However, the resonance effect mechanism is rather complicated and requires further study.

In general, the physical size of a mechanical biosensor is often reduced to the minimum that is detectable, with added mass being proportional to the total mass of the device: nanofabrication technique systems achieve mass resolution while being operable on a nanogram resolution scale. Although the signals are dependent on a single point of one signal, the signal assay requires more complexes devices and is difficult in practical term.

Our finding provides the basis for study of a new theory on ATP motor rotation movement and contributes to the development of biosensors for ultrasensitive applications. With our system, damping of the resonance biosensor would induce a collective signal, measuring a few molecules without depending on nanofabrication techniques because, physically, resonance is the tendency of a system to oscillate at maximum amplitude at certain frequencies. Furthermore, the mechanical biosensor demonstrating that the resonance phenomenon could be utilized as an ultrasensitive biosensor, providing several advantages over previous biosensors.

Acknowledgments

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