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Mechanically driven proton conduction in single δ -free F₀F₁-ATPase

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Abstract

In order to observe mechanically driven proton flux in F_0F_1 -ATPase coupled with artificial driven rotation on F_1 simultaneously, a double channel observation system was established. An artificial δ -free F_0F_1 -ATPase was constructed with α_3 , β_3 , ϵ , γ , and c_n subunits as rotator and a, b_2 as stator. The chromatophore was immobilized on the glass surface through biotin–streptavidin–biotin system, and the magnetic bead was attached to the β subunit of δ -free F_0F_1 -ATPase. The mechanically driven proton flux was indicated by the fluorescence intensity change of fluorescein reference standard (F1300) and recorded by a cooled digital CCD camera. The mechanochemical coupling stoichiometry between F_0 and F_1 is about $4.15 \pm 0.2H^+$ /rev when the magnetic field rotated at 0.33 Hz (rps). © 2006 Elsevier Inc. All rights reserved.

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F₀F₁-ATPase is a nanoscale rotary motor and actually a combination of two motors' function together. The catalytic synthesis of ATP from ADP and phosphate uses transmembrane proton-motive force (PMF) generated by oxidative phosphorylation or photosynthesis. The downhill proton flux through F_0 drives rotation of the c-ring and hence γ , ε , forcing conformation change in F₁ that results in ATP synthesis from ADP and Pi. Conversely, ATP hydrolysis in F_1 causes the reverse rotation of the rotor that drives F₀ to pump protons in the reverse direction [1,2]. The exact mechanism of reversible cycle of F_0F_1 -ATPase has been well known [3], but to prove this concept in single molecule is still unsolved. It has been reported that the mechanically driven ATP synthesis/hydrolysis in F₁-ATPase was completely reversible by artificial force [4]. But direct observation of the proton conduction is difficult since the transport may be very

small for the single molecule patch-clamp approach. The greater challenge is to directly observe mechanochemical coupling between F_1 and F_0 in single molecule. The study on molecular mechanisms of proton transport in F_0F_1 -ATPase has received attentions not only from biologists, but also from multi-scientists, because the mechanism of proton transport across membrane as energy sources is so important in many fields.

In F₀F₁-ATPase, there are two "stalks" (ϵ , γ , and b₂ δ) connecting F₀ to F₁. If the δ subunit is removed, only one center "stalk" (ϵ , γ) exists in the F₀F₁-ATPase, as illustrated in Fig. 1. When the rotation between F₁/ γ and F₁/ $\alpha_3\beta_3$ is prevented by ADP and NaN₃ [5], α , β , γ , ϵ , and c-ring in δ -free F₀F₁-ATPase were fixed together as new rotor and ab₂ acted as new stator. It has been reported that such model of hybrid δ -free F₀F₁-ATPase was used to image the rotation of ATP motor driven by proton-motive force [6,7]. And this system is also suitable for studying mechanically driven proton conduction coupled with artificially driven rotation of the new rotor simultaneously at single molecule level.

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Fig. 1. Schematic view of the δ -free F_0F_1 -ATPase within chromatophore. A, α_3 , β_3 , δ , a and b_2 subunits as stator, c-ring and $\gamma\epsilon$ as rotator in the natural F_0F_1 -ATPase; B, α_3 , β_3 , γ , ϵ subunits and c-ring in δ -free F_0F_1 -ATPase were reconstructed as new rotor by ADP and NaN₃ [5], and the new stator formed by ab_2 .

In this study, an artificial δ -free F_0F_1 -ATPase within chromatophore was reconstructed to research mechanochemical coupling between F_0 and F_1 parts. The chromatophore was immobilized on the glass surface of sample dish through biotin–streptavidin–biotin system; the magnetic bead was attached to the β subunit of δ -free F_0F_1 -ATPase and driven by a pair of magnets. The experimental schematic view of δ -free F_0F_1 -ATPase within chromatophore is shown in Fig. 1.

Materials and methods

Chemical and materials. Fluorescein reference standard (FRS, Catalog Number: F1300) was purchased from Molecular Probes (Eugene, Oregon, USA). Ni–NTA magnetic beads were purchased from Micromod Partikeltechnologie GmbH (Germany). Streptavidin and N,N'-dicyclohexyl-carbodiimide (DCCD) were purchased from Sigma. Biotin-AC₅-sulfo-OSu was purchased from Dojindo (Japan). Lipid–biotin was purchased from Avanti. All other analytically purified reagents were purchased domestically. Tricine–NaOH buffer (0.1 mM Tricine, 5 mM MgCl₂, and 5 mM KCl, pH 8). PBS buffer (8 g NaCl, 0.2 g KCl, 0.2 g KH₂PO₄, and 3.58 g Na₂HPO₄, pH 7.4).

Chromatophores were prepared from the cells of *Rhodospirillum rubrum* according to Ref. [8,9].

Methods. F₁-deleted chromatophores were prepared by EDTA-treatment according to Ref. [10]. The EDTA-treated chromatophores were washed twice (50,000 rpm, 30 min, 4 °C) with TSM buffer (50 mM Tricine–NaOH, pH = 8.0, 0.25 M sucrose, and 4 mM MgCl₂) and then dissolved in TSM buffer. The concentration of bacteriochlorophyll and the ATP hydrolysis activity were measured.

The ε subunit was expressed and purified as follows: the gene encoding of ϵ subunit of F₀F₁-ATPase was amplified by PCR from the plasmid pSWM92 which is a generous gift from Prof. Senior AE. An NdeI restriction site and an N-terminal 6×His tag was introduced by the forward primer 5'-CACC-ATATGCATCACCATCACCATCACGCAAT GACTTAC, and a Hind III restriction site was introduced by the reverse primer 5'-AAGCTTGCAAAAAAAAGCCAG-CCTGTTTCCAG. PCR mega primer mutagenesis was carried out to introduce the Gly57Cys mutation. The sequence of the primer used for mutation is 5'-CGTGACA GTGCTGTTTCACGA. The amplified PCR product was ligated to pGEM®-T Easy vector, then the fragment was subcloned to expression vector pET22b(+) by double-digesting by NdeI and HindIII and ligating to NdeI/Hind III-cut plasmid pET22b(+). The constructed pET22b(+) was transformed into E. coli BL21 (DE3) used to express and purify the ɛ subunit. Mutation was confirmed by sequence analyses. The ϵ subunit purification was confirmed by SDS polyacrylamide gel-electrophoresis. Reconstituted δ -free F₀F₁-ATPase within chromatophores: the PS3 F₁ was donated by Prof. C. Montemagno (UCLA, USA). The F1 protein $(\alpha_3\beta_3\gamma)$ was purified according to Ref. [11]. Then 5 µg (Bchl) F₁-deleted chromatophores, 10.4 µg ε subunit and 50 µg purified F₁ ($\alpha_3\beta_3\gamma$) were incubated at 25 °C for 12 h in the reconstitution buffer (50 mM Tricine-NaOH, pH 8.0, 4 mM ATP, 25 mM MgCl₂, 1 mM dithiothreitol, and 10% glycerol). After reconstitution, they were centrifuged at 180,000g for 30 min. The pellets were resuspended in buffer (50 mM Tricine-NaOH, pH 8.0, 10% glycerol, and 25 mM MgCl₂) and washed three times. The reconstituted chromatophores were stored at -20 °C. The ATP hydrolysis activity of the reconstituted chromatophores was measured by quantifying the amount of produced phosphate.

Experiment process. The fluorescence probe, F1300 was labeled into reconstituted δ -deleted chromatophores through sonication for 5 min in ice, and the free fraction was washed three times by centrifugation (15,000 rpm, 30 min, 4 °C). The pellets were resuspended in Tricine–



Fig. 2. The experimental system which was established to study mechanochemical coupling between proton conduction in F_0 and rotation of F_1 -ATPase. (1), an electrically driven device with a pair of permanent magnets N–S; (2), the magnetic field N–S was suspended on top of the sample cell; (3), sample dish with chromatophores; (4), fluorescence microscopy with double channel observation system; (5), the channel for fluorescence observation and recording by CCD; (6), the switch for control anticlockwise or clockwise rotation of magnetic driven device; (7), another channel for visible light observation of magnetic beads rotation and recording by video tape recorder.

NaOH buffer. The fluorescence chromatophores labeled with biotin as follows: the lipid–biotin in ethanol was added into the F1300 labeled chromatophores (100/5 V:V), after incubated in room temperature for 30 min, the free fraction was washed by centrifugation.

Glass-bottomed dishes coated with poly-lysine were free samples from Matteck Company (USA). The surface of the dish was modified as follows: first, 100-200 µl Biotin-AC5-sulfo-OSu was added into the dish and incubated for 1 h at 37 °C; after washing for three times with PBS buffer, 100-200 µl of 10 nM streptavidin was added into the dish and incubated for 10 min at 37 °C, then free streptavidin was washed with PBS three times; at last, 100-200 µl lipid biotin labeled fluorescence chromatophores were added into the dish; after incubated for 10 min at 37 °C, the free chromatophores were washed with buffer A (0.1 mM Tricine-NaOH, pH 8.0, 50 mM KCl). At this time, all of the chromatophores were linked to the glass surface through biotin-streptavidin-biotin system. Then 100-200 µl 250 nm Ni–NTA coated magnetic beads were added into the sample dish; after 15 min incubation at room temperature, the free beads were washed with buffer A for three times. Then buffer B (0.1 mM Tricine-NaOH, pH 8.0, 50 mM KCl, 5 mM NaN₃, and 2 mM ATP) was added into the dish for the rotation observation.

To observe mechanochemical coupling between driven rotation of magnetic beads on F_1 and proton flux from F_0 , a double channel observation system was established. The schematic view of our experimental system is shown in Fig. 2. The rotation of magnetic bead was driven by a magnetic tweezer, which was composed by an electrically driven device and a pair of magnets together. The mechanically driven proton flux was indicated by the fluorescence intensity change of F1300, which was sensitive to pH change [12]. The fluorescence intensity of single chromatophore was observed by Olympus IX71 fluorescence microscope and recorded by digital CCD cameras (Princeton Scientific) with excitation at 485 nm and emission at 538 nm. The recorded pictures were dealt with by the software of Winview/32 (Princeton Scientific).

Results

It has been reported that the fluorescence probe (F1300) which is sensitive to pH-changes was labeled in inner chromatophore as a proton flux indicator [12]. Here, we further found that the fluorescence intensity of F1300 in single chromatophore was nearly linear to pH value at the region 6-9, which is shown in Fig. 3. Therefore, F1300 is an appropriate pH indicator, and serves as a reliable quantitative indicator for the mechanically driven proton conduction in single δ -free F₀F₁-ATPase.



Fig. 3. Fluorescent probe (F1300) sensitive to pH-changes in inner chromatophore was used as a proton flux indicator. It clearly shows that there was linear relationship between fluorescence intensities and pH values from 6 to 9. Each of the data was averaged from 10 chromatophores.

The δ -free F_0F_1 -ATPase within chromatophore was immobilized on the glass surface, and the magnetic bead was attached to the β subunit of δ -free F₀F₁-ATPase (Fig. 1). When the magnetic field was rotating in counterclockwise, the magnetic bead attached to β subunit of F₁ portion also rotated in counterclockwise viewed from F₁ side to F_0 side which can be observed by a channel of visible light. Meanwhile, the increase of fluorescence intensity of chromatophore was recorded by CCD in another channel. Reversely, when the magnetic field was rotating in clockwise, the magnetic bead attached to β subunit of F₁ portion also rotated in clockwise viewed from F₁ side to F₀ side, and the fluorescence intensity of chromatophore was decreased. The relationship between rotation of magnetic field and change of fluorescence intensity in time course is shown in Fig. 4A. First, when the magnetic field was rotating in counterclockwise, the fluorescence intensity increased from 23 to 215 U, within 18 min, each with 2 min interval, respectively. In reverse, when rotary magnetic field was changed to clockwise, fluorescence intensity decreased from 215 to 12 U, within time course 18-30 min, each with 2 min interval, respectively. Fig. 4B shows that the real proton transfer in single F₀F₁-ATPase was observed directly by fluorescence microscopy which was corresponding to the curve in Fig. 4A. The rotation direction of magnetic field is indicated in figure (R is counterclockwise and S is clockwise, respectively), and the rotation velocity is 0.33 Hz.

To confirm the reproducibility of mechanochemical coupling between F_0 and F_1 , we obtained 35 traces of repetitious-cycles of fluorescence intensity change coupled with magnetic field rotating in counterclockwise/clockwise, which strongly indicated that there was mechanochemical coupling between the rotation of the new rotor and proton conduction in F_0 portion. Fig. 4C shows 7 traces selected from 35 traces. Although the repetitious-cycles in Fig. 4C last less than 60 min, most of other repetitious-cycles like Fig. 4A can last more than 90 min (data not shown).



Fig. 4. (A) Relationship between change of fluorescence intensity and rotation of magnetic bead attached to the F_1 . The rotation direction of magnetic field is indicated in the figure, R is counterclockwise and S is clockwise. (B) Fluorescence microscope images of chromatophores (dot) corresponding to the curve in (A). (C) The repetitious-cycles of fluorescence intensity change coupled with magnetic field rotation in counterclockwise/clockwise. The rotation direction of magnetic field changed in the counterclockwise/clockwise every 4 min.

To further confirm the mechanism of mechanochemical coupling between proton conduction through F₀ and rotation of F₁, inhibitory effect of DCCD was assessed by observing the rotation of F₀F₁-ATP motor. Fig. 5A shows that there was no obvious change of fluorescence signal after the addition of DCCD, whether magnetic field was rotated clockwise or counterclockwise. This means that the proton conduction in F_0 motor was inhibited by adding DCCD. Fig. 5B shows the images of chromatophores (dot) corresponding to the curve in Fig. 5A. The reproducibility of DCCD inhibitory effect was confirmed by five different chromatophores, the results shown in Fig. 5C. These results demonstrated that γ subunit and c-ring rotated together coupling with the proton transport through F_0 , only in the absence of DCCD. It has been well known that insert DCCD to F_0 chemically caused uncoupling between the F_1 and F_0 , but the effect of uncoupling between F_1 and F_0 in force conformation has been poorly understood. This finding indicates that DCCD not only affects chemical uncoupling of proton conduction in F₀ c ring, but also affects mechanochemical uncoupling between the F₀ c-ring and $F_1\gamma$ subunit directly.

Discussion

In our experiment system, native photosynthetic membrane was chosen; it has a crowded environment with close packing of protein complexes, and remarkably low diffusion in comparison with eukaryotic membranes [13] to reduce the lateral diffusion of enzyme molecular and prevent the disturbance of membrane. When the F_0F_1 -ATPase were crowded in the high protein content lipid bilayer membrane, it would be difficult to move freely in the chromatophore; the relative rotation of the whole enzyme molecular which is no contribution to proton conduction also could be limited by such crowed environments, but could not be absolutely prevented. This is the primary reason for our low probability to success in experiments; but if the enzyme molecular once became coupled, it can repeat several cycles (counterclockwise/clockwise) and most of the coupling can last more than 90 min (data not shown).

The chromatophores used in our experiments had 0.79 F_0F_1 -ATPase in average, and the average diameter was 76 nm [12]. B.A. Feniouk et al. [14] had reported that the proton buffer capacity in inner chroamtophores was about 2.1 (± 0.7) × 10⁻⁸ mol m⁻². From the linear relationship between fluorescence intensity and pH from 6 to 9 in chromatophores which is shown in Fig. 3, we could calculate the coupling stoichiometry. We took the inner chromatophore membrane as the main effect factor for pH buffering, and considered that there was only one F_0F_1 -ATPase for one chromatophore in the successful experiments; from formula (1) and the fluorescence intensity data, we could get that the stoichiometry of mechanochemical coupling was about 4.15 ± 0.2 H⁺/rev (averaged from 35 chromatophores) when magnetic field was rotated at 0.33 Hz (rps).



Fig. 5. (A) The mechanochemical coupling between F_0 c-ring and $F_1\gamma$ subunit could be interrupted by DCCD. (B) Fluorescence microscope images of chromatophores (dot) corresponding to the curve in (A). (C) The reproducibility of DCCD inhibitory effect.

 $\beta = d[H^+]/dpH \tag{1}$

This low value of coupling ratio compared to the expected value (about 10 H⁺/rev) was mainly due to the rotation of whole enzyme claimed above. Perhaps for each rotation of the beads, the whole enzyme made about 0.6 turns rotation relative to the surface of chromatophore, and with only about 0.4 turns rotation between F_0/a and F_0/c . Therefore it reduced proton pumping. In this study, we directly observed the mechanically driven proton influx or efflux in F_0 coupled with rotation of F_1 at single molecular level, and the exactly mechanism will be studied later.

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References

- P.D. Boyer, The ATP synthase—A splendid molecular machine, Annu. Rev. Biochem. 66 (1997) 717–749.
- [2] J. Weber, A.E. Senior, ATP synthesis driven by proton transport in F_1F_0 -ATP synthase, FEBS Lett. 545 (2003) 61–70.
- [3] M. Yoshida, E. Muneyuki, T. Hisabori, ATP synthase—A marvellous rotary engine of the cell, Nat. Rev. Mol. Cell. Bio 2 (2001) 669–677.
- [4] H. Itoh, A. Takahashi, K. Adachi, H. Noji, R. Yasuda, M. Yoshida, K. Kinosita Jr., Mechanically driven ATP synthesis by F₁-ATPase, Nature 427 (2004) 465–468.
- [5] E. Muneyuki, M. Makion, H. Kamata, Y. Kagawa, M. Yoshida, H. Hirata, Inhibitory effect of NaN3 on the F0F1 ATPase of submitochondrial particles as related to nucleotide binding, Biochim. Biophys. Acta 16 (1993) 62–68.

- [6] Y.H. Zhang, J. Wang, Y.B. Cui, J.C. Yue, X.H. Fang, Rotary torque produced by proton motive force in F₀F₁ motor, Biochem. Biophys. Res. Commun. 331 (2005) 370–374.
- [7] Y.B. Cui, Y.H. Zhang, J.C. Yue, P.D. Jiang, Direct observation of the clockwise light-driven rotation of F_0F_1 -ATP synthase complex, Chinese Sci. Bull. 49 (2004) 1342–1347.
- [8] G.E. Zippora, K. Daniel, Selective extraction and reconstitution of F_1 subunits from Rhodospirillum rubrum chromatophores, Methods Enzymol. 126 (1986) 528–538.
- [9] S. Philosoph, A. Binder, G.E. Zippora, Coupling factor ATPase complex of Rhodospirillum rubrum, J. Biol. Chem. 252 (1977) 8747– 8752.
- [10] B.A. Melandri, A. Baccarini-Melandri, A.S. Pietro, H. Gest, Role of phosphorylation coupling factor in light-dependent proton translocation by *Rhodopseudomonas* capsulate membrane

preparations, Proc. Natl. Acad. Sci. USA 67 (1970) 477-484.

- [11] C. Montemagno, G. Bachand, Constructing biological motor powered nanomechanical devices, Nanotechnology 10 (1999) 225–231.
- [12] X.L. Liu, Y. zhang, Y.C. Yue, P.D. Jiang, Z.X. Zhang, F₀F₁-ATPase as biosensor to detect single virus, Biochem. Biophys. Res. Commun. 342 (2006) 1319–1322.
- [13] S. Bahatyrova, R.N. Frese, C.A. Siebert, J.D. Olsen, K.O. van der Werf, R. van Grondelle, R.A. Niederman, P.A. Bullough, C. Otto, C.N. Hunter, The native architecture of a photosynthetic membrane, Nature 430 (2004) 1058–1062.
- [14] B.A. Feniouk, M.A. Kozlova, D.A. Knorre, D.A. Cherepanov, A.Y. Mulkidjanian, W. Junge, The proton driven rotor of ATP synthase: ohmic conductance (10 ms), and absence of voltage gating, Biophys. J. 86 (2004) 4094–4109.