Expression of mammalian mitochondrial F$_1$-ATPase in 
*Escherichia coli* depends on two chaperone factors, AF1 and AF2

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Keywords
F$_1$-ATPase; F$_0$F$_1$-ATP synthase; molecular chaperone

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(Received 28 September 2016, revised 5 October 2016, accepted 6 October 2016)
doi:10.1002/2211-5463.12143

F$_0$F$_1$-ATP synthase (F$_0$F$_1$) is ubiquitously found in membranes of bacteria, chloroplasts, and mitochondria, and synthesizes ATP from ADP and inorganic phosphate (Pi) driven by downhill proton flow across the membranes [1–3]. F$_1$-ATPase (F$_1$) is a water-soluble catalytic domain of F$_0$F$_1$, which has a subunit composition of $\alpha_3\beta_3\gamma\delta\epsilon$. F$_1$ is a rotary motor, where net hydrolysis of one ATP molecule drives a 120°...
rotation of a central rotor shaft composed of γδε-subunits relative to a surrounding stator ring of αβγδε-subunits (eukaryotic subunit composition) [4,5]. Extensive studies on rotation of bacterial F1 revealed six-step rotation in one revolution, that is, repetition of an 80° rotation by ATP binding to one of the three catalytic β-subunits and a 40° rotation by release of Pi from another β-subunit [4,6–9]. Understanding the rotation mechanism of F1 requires knowledge on how chemical events occurring to F1 induce its structural changes and trigger rotation. In this respect, much structural information has been accumulated for bovine F1, rather than bacterial F1, by X-ray crystallography [10,11]. However, rotation of mitochondrial F1 was not demonstrated until recently because of the absence of in vitro expression system of mitochondrial F1 genes that enables genetic modification necessary for single-molecule observation, such as introduction of the His-tag.

We recently succeeded in expressing human mitochondrial F1 in Escherichia coli cells and reported its nine-step rotation in one revolution [5]. Following up this work, here, we report the expression of bovine mitochondrial F1 in E. coli. Early genetic works using Saccharomyces cerevisiae identified two mitochondrial proteins of Atp11p and Atp12p as molecular chaperones necessary for assembly of F1 [12]. Analyses using a yeast two-hybrid system and immunoprecipitation further showed direct interaction of Atp11p [13] with a β-subunit and of Atp12p with an α-subunit [14]. Mammalian homologs of these chaperones are ATPAF1 and ATPAF2 (AF1 and AF2, hereafter), and their coding genes, ATP11 and ATP12, can respectively complement genetic deficiencies of ATP11 [15] and ATP12 [16] of yeast. AF1 and AF2 have antiaggregation activity toward reduced insulin [17,18] and citrate synthase in vitro, respectively [19]. However, whether AF1 and AF2 are essential for the production of mammalian F1 was not tested directly. We thus expressed the five subunits of bovine mitochondrial F1 in E. coli cells with or without coexpression of AF1 and AF2. The results clearly show that AF1 and AF2 are essential and sufficient for the production of bovine F1 in E. coli. ATP-driven rotation and crystallization confirmed intactness of E. coli-expressed bovine F1.

**Experimental procedures**

**Expression of bovine F1 in E. coli**

The expression plasmid for bovine F1 was constructed in the same manner as performed previously for human F1 [5]; five genes coding subunits of bovine F1 (α, β, γ, δ, and ε) [20] and two genes, ATP11 and ATP12, were amplified by PCR from the cDNA library prepared from the total RNA of bovine heart muscle. The genes were tandemly introduced in the order of e-γ-β-δ-e-ATP11-ATP12 into the expression vector pTR19 [21], which are transcribed from the trc promoter. A histidine tag composed of 10 histidine residues was genetically introduced into the N terminus of the β-subunit of F1 as performed previously [4]. The resulting plasmid, pBF1, was introduced into F1F1-deficient E. coli strain, DK8 [21]. The recombinant E. coli strain was cultivated in 2 × YT medium containing 100 μg·mL⁻¹ ampicillin for 40 h at 29 °C. The culture flasks were shaken for aeration because respiration of cells is necessary for efficient expression even though growth is dependent on glycolysis. As observed in the case of expression of F1 from thermophilic Bacillus PS3 in E. coli [22], the growth rate of the E. coli was not significantly affected by the expression of bovine F1. It is assumed that submillimolar concentration of ADP in cytoplasm is enough to keep F1 in the inactive state of so-called MgADP-inhibition, a general feature of F1 from any sources [23]. The cells were disrupted and the water-soluble fraction was subjected to Ni-affinity column chromatography and gel-filtration column chromatography. Purification procedures for bovine F1 are the same as those for human F1, except the buffers for cell lysis (20 mM potassium phosphate (pH 7.5), 100 mM KCl and 0.1 mM ATP) and for gel-filtration (40 mM Tris/Cl (pH 8.0), 200 mM NaCl, 1 mM EDTA and 0.1 mM ATP). After gel-filtration with Superdex200 10/300GL column (GE Healthcare, Uppsala, Sweden), fractions of a peak having the ATPase activity were collected, concentrated with a centrifugal concentrator (50 kDa, Centricon50; Millipore Corp., Billerica, MA, USA), and used for further analyses. Yield of the purified recombinant bovine F1 was about 2–3 mg per 6-L-culture. Authentic bovine F1 was prepared from bovine heart as reported [24] with a modification: gel-filtration was performed with a Superdex200 column in 20 mM Tris/Cl (pH8.0), 200 mM NaCl, 0.1 mM ATP, and 0.5 mM EDTA. To avoid cold dissociation of bovine F1, all procedures were carried out at a temperature higher than 20 °C. Mutated IF1 (IF1-GFP) used in this study, I60GFPHis, was prepared as reported previously [25].

**Rotation of E. coli-expressed bovine F1**

Rotation of a single molecule of bovine F1 was observed by the procedures described in ref. [5]. Two cysteine residues were introduced into a globular domain of γ-subunit (γ/Ala99Cys and γSer191Cys). Images of a rotating submicron polystyrene bead attached to the γ-subunit of immobilized bovine F1 were captured with a CCD camera (ICL-B0620M; Implex, Minneapolis, MN, USA) at 500 frames per sec (fps) under illumination of a mercury lamp. Rotation of the Au-bead (40 nm diameter) was observed at 25 000 fps with a laser-illuminated center-shielded dark-field microscopic system equipped with a high-speed camera.
Crystallization of *E. coli*-expressed bovine F1

Concentrated recombinant bovine F1 was supplemented with 0.5 mM AMPPNP and 20 mM MgCl2 (the final bovine F1 concentration was 10 mg·mL⁻¹) and used for crystallization. Reservoir solution (70 μL) containing 100 mM Tris/HCl (pH 8.5), 200 mM LiSO4, and 21–23% PEG3350 (Hampton Research, Aliso Viejo, CA, USA) was put into a sitting-drop dish, and the bovine F1 solution and the reservoir solution (each 0.25 μL) were mixed to make one sitting drop. Crystals with the size of 0.05–0.3 mm were grown in approximately 3 weeks at 20 °C. For analysis of the crystals with polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS/PAGE), 10–20 crystals were collected from crystallization drops using a cryoloop, washed four times with 100 μL of wash solution (the reservoir solution supplemented with 0.5 mM AMPPNP, 20 mM MgCl2, and 25 mM NaCl), and dissolved in the SDS/PAGE sample buffer. After electrophoresis, the gel was stained with silver.

Other methods

The ATPase activity was measured in 50 mM HEPES/KOH buffer (pH 7.5) containing 100 mM KCl, 1 mM MgCl2, 1 mM ATP, and the ATP-regenerating system [21] supplemented with 0.2 mM NADH and 0.2 mg·mL⁻¹ lactate dehydrogenase [26]. The reaction was initiated by adding F1, and the change in absorbance at 340 nm was recorded. The ATPase activity was calculated from the slope of absorbance decrease during 400–500 s. For the assay of IF1 inhibition, IF1-GFP was added to the reaction mixture prior to the measurement at the indicated concentration. Previous studies of authentic bovine F1 [25] showed that IC50 of IF1-GFP is 65 nm, while that of wild-type IF1 is approximately 10 nm [5]. Protein concentrations were determined by protein assay kit (Pierce Biotechnology Inc., Rockford, IL, USA), with bovine serum albumin as a standard. All SDS/PAGE and native-PAGE in this study were performed with a gradient polyacrylamide gel (10–20%) and nongradient gel (12%). The proteins were visualized by Coomassie Brilliant Blue (CBB) or by immunoblotting with anti-β and anti-δ antibodies. All data used for this study were measured at least in triplicate.

Results and Discussion

*Escherichia coli* expression of bovine F1 depends on AF1 and AF2

The five genes for bovine F1 were introduced into the *E. coli* expression vector in the same order as in the

E. coli F0F1 operon, α-γ-β-δ-ε, to generate a plasmid pBF1(-AFs). A set of genes, ATP11 and ATP12, were further introduced at the end of the operon as α-γ-β-δ-ε-ATP11-ATP12 to generate a plasmid pBF1(+AFs). These plasmids were individually introduced into the *E. coli* strain that lacks the whole F0F1 operon in the chromosome, and resultant recombinant strains were cultured. The water-soluble fraction of harvested cells was analyzed with polyacrylamide gel electrophoresis in the absence of SDS (native-PAGE) using authentic bovine F1 purified from bovine heart as a control (Fig. 1A–D). Native-PAGE followed by immunoblotting with anti-β antibodies showed that pBF1(+AFs)-harboring cells produced a significant amount of bovine F1 while pBF1(-AFs)-harboring cells produced very little, if any, amount of bovine F1 (Fig. 1A). The band arising from the monomeric β-subunit was seen in all samples. The immunoblotting with anti-δ antibodies confirms pBF1(+AFs)-dependent production of bovine F1 (Fig. 1B). F1 isolated from bovine heart appeared as two split bands in native-PAGE for an unknown reason and bovine F1 produced in *E. coli* also gives two bands. The monomeric β-subunit of bovine F1 produced in *E. coli* migrates in the gel more slowly than that of authentic bovine F1 due to the attached histidine tag (Fig. 1A). Production of bovine F1 in pBF1(+AFs)-harboring cells was confirmed by protein staining as a faint, but distinct band (Fig. 1C, D). These results show that expression of the ATP11 and ATP12 is essential for efficient production of bovine F1. We purified bovine F1 from pBF1(+AFs)-harbored *E. coli* cells and confirmed that it has the same subunit composition as authentic bovine F1 by SDS/PAGE analysis (Fig. 1E). The ATPase activity of mitochondrial F1 is known to be inhibited by a specific inhibitor protein of mitochondria, IF1 [25]. Sensitivity of *E. coli*-expressed bovine F1 to IF1 was tested by using bovine IF1 fused to GFP. As shown in Fig. 1F, the ATPase activity of *E. coli*-expressed bovine F1 was inhibited by IF1-GFP in the same manner as observed for authentic bovine F1 (Fig. 1F).

Rotation of *E. coli*-expressed bovine F1

To verify the function of *E. coli*-expressed bovine F1, ATPase-driven rotation was observed by microscopic single-molecule analysis. For this purpose, a submicron polystyrene bead was attached to two introduced cysteine residues of the γ-subunit as a rotation probe. At a low ATP concentration (1 μM), bovine F1 rotates at a speed 2.5 ± 0.3 rps and rotation takes three dwells per revolution, approximately at every 120° rotation.
The dwells become shorter as the ATP concentration increased, indicating that bovine F₁ waits for ATP binding during the dwell to drive the next cycle of rotation. The rate constant of ATP binding ($k_{\text{on}}$) calculated from the lifetime of the dwell ($\tau = 56 \pm 3$ ms) is $1.8 \pm 0.3 \times 10^7$ M$^{-1}$s$^{-1}$ (Fig. 2B). Rotation at a saturating ATP concentration (4 mM) was observed with a rapid camera (a frame per 40 μs) (Fig. 2C). By using colloidal gold particles (diameter, 40 nm) as a rotation probe, viscous friction of the rotating particle did not slow down rotation under the experimental conditions and the rotation speed, 655 ± 38 rps ($N = 6$ molecules), directly reflects the maximum turnover rate of ATP hydrolysis by a single molecule of bovine F₁, that is, ~ 2000 per second. The presence of dwells is suggested from the angle histogram of rotation that awaits extensive analysis. As expected from high sequence conservation between bovine F₁ and human F₁, these motor characteristics of bovine F₁ are similar to those of human F₁.
Crystallization of E. coli-expressed bovine F₁

Bovine F₁ (without a Cys mutation) purified from E. coli cells was subjected to crystallization. A crystal was not made under the reported conditions for crystallization of authentic bovine F₁ [27] probably because of the histidine-tag of the β-subunit of E. coli-expressed bovine F₁. After screening crystallization conditions, we found that crystals were reproducibly formed in the solution containing 0.5 mM AMPPNP and 20 mM MgCl₂ with PEG3350 as precipitant (Fig. 3A). Crystals were collected from the drops, washed, and analyzed by SDS/PAGE (Fig. 3B). All the five F₁ subunits were detected in the gel, confirming that the crystals were of bovine F₁. The result shows that the purified bovine F₁ has a quality high enough to grow crystals.

Conclusions

Bovine F₁, for the first time, was successfully expressed in E. coli cells. As expected, purified bovine F₁ exhibits motor characteristics similar to those of human F₁. It forms good crystals rather easily. We previously spent time and efforts for crystallization of bacterial F₁ [28], but now realize that bovine F₁ is superior to bacterial F₁ in crystallization for the detailed structural study of F₁.

Availability of mutants adds a further advantage to E. coli-expressed bovine F₁ over the native protein.

Without AF1 and AF2, only very little, if any, bovine F₁ was produced by E. coli, indicating that AF1 and AF2 are required for efficient production of bovine F₁. This expression is the first demonstration of the chaperone function of these two factors for assembly of mammalian F₁. As speculated in a previous yeast study [29], mammalian AF2 would bind to α-subunit by mimicking the coiled-coil region of the γ-subunit and then, α- and β-subunit eject their cognate chaperone factors by switching their partner on the way of the assembly. In relation to this, a metabolic disease with a decreased amount of F₀F₁ in mitochondria is attributed to a mutation in the ATP12 gene, suggesting a critical physiological role of these assembly factors in production of functional F₀F₁ [30]. Although we did not test human ATP11 and ATP12 for expressing bovine F₁, sequence similarities of AF1 and AF2 are 93% and 88% such that we would expect them to be interchangeable. We expect that the development and improvement of the present bovine F₁ expression system would open a way to the study of detailed mechanisms of the assembly and a structure-mechanism relationship of mitochondrial F₁.

Acknowledgements

We thank our colleagues in Waseda University, Drs. K. Kinosita, K. Adachi, M. Bertz, R. Chiwata, T. Ogawa in Kyoto-Sangyo University, Drs. Y. Araiso and S. Matsumoto, and those in The University of Tokyo, Drs. H. Noji, R. Watanabe and N. Soga, for valuable discussions. This work was partly supported by JSPS KAKENHI Grant Number 24570149 (TS) and 90049073 (MY) and by the Platform Project for Supporting in Drug Discovery and Life Science Research from Japan Agency for Medical Research and Development (AMED) (TS).

Author contributions

TS and MY conceived and designed the experiments and wrote the paper. TS developed the E. coli expression system of bovine F₁ with NI and JS and the X-ray crystallographic system with YW and TE, and single-molecule analytical system. TH gave critical suggestions for experimental systems and interpretations throughout this study.

References

1 Yoshida M, Muneyuki E and Hisabori T (2001) ATP synthase – a marvellous rotary engine of the cell. Nat Rev Mol Cell Biol 2, 669–677.
2 Boyer PD (2002) A research journey with ATP synthase. *J Biol Chem* **277**, 39045–39061.

3 Senior AE, Nadanaciva S and Weber J (2002) The molecular mechanism of ATP synthesis by F_1F_0-ATP synthase. *Biochim Biophys Acta* **1553**, 188–211.

4 Noji H, Yasuda R, Yoshida M and Kinoshita K Jr (1997) Direct observation of the rotation of F_1-ATPase. *Nature* **386**, 299–302.

5 Suzuki T, Tanaka K, Wakabayashi C, Saita E and Yoshida M (2014) Chemomechanical coupling of human mitochondrial F_1-ATPase motor. *Nat Chem Biol* **10**, 930–936.

6 Yasuda R, Noji H, Kinoshita K Jr and Yoshida M (1998) F_1-ATPase is a highly efficient molecular motor that rotates with discrete 120 degree steps. *Cell* **93**, 1117–1124.

7 Yasuda R, Noji H, Yoshida M, Kinoshita K Jr and Itoh H (2001) Resolution of distinct rotational substeps by submillisecond kinetic analysis of F_1-ATPase. *Nature* **410**, 894–904.

8 Adachi K, Owak K, Nishizaka T, Furuike S, Noji H, Itoh H, Yoshida M and Kinoshita K Jr (2007) Coupling of rotation and catalysis in F_1-ATPase revealed by single-molecule imaging and manipulation. *Cell* **130**, 309–321.

9 Furuike S, Hassain MD, Maki Y, Adachi K, Suzuki T, Kohori A, Itoh H, Yoshida M and Kinoshita K Jr (2008) Axle-less F_1-ATPase rotates in the correct direction. *Science* **319**, 955–958.

10 Walker JE and Dickson VK (2006) The peripheral stalk of the mitochondrial ATP synthase. *Biochim Biophys Acta* **1757**, 286–296.

11 Bason JV, Montgomery MG, Leslie AG and Walker JE (2015) How release of phosphate from mammalian F_1-ATPase generates a rotary substep. *Proc Natl Acad Sci USA* **112**, 6009–6014.

12 Ackerman SH (2002) Atp11p and Atp12p are chaperones for F_1-ATPase biogenesis in mitochondria. *Biochim Biophys Acta* **1555**, 101–105.

13 Wang ZG and Ackerman SH (2000) The assembly factor Atp11p binds to the beta-subunit of the mitochondrial F_1-ATPase. *J Biol Chem* **275**, 5767–5772.

14 Wang ZG, Sheluho D, Gatti DL and Ackerman SH (2000) The alpha-subunit of the mitochondrial F_1-ATPase interacts directly with the assembly factor Atp12p. *EMBO J* **19**, 1486–1493.

15 Wang ZG, Schmid KJ and Ackerman SH (1999) The *Drosophila* gene 2A5 complements the defect in mitochondrial F_1-ATPase assembly in yeast lacking the molecular chaperone Atp11p. *FEBS Lett* **452**, 305–308.

16 Wang ZG, White PS and Ackerman SH (2001) Atp11p and Atp12p are assembly factors for the F_1-ATPase in human mitochondria. *J Biol Chem* **276**, 30773–30778.

17 Hinton A, Zuiderweg ER and Ackerman SH (2003) A purified subfragment of yeast Atp11p retains full molecular chaperone activity. *J Biol Chem* **278**, 34110–34113.

18 Sheluho D and Ackerman SH (2001) An accessible hydrophobic surface is a key element of the molecular chaperone action of Atp11p. *J Biol Chem* **276**, 39945–39949.

19 Hinton A, Gatti DL and Ackerman SH (2004) The molecular chaperone, Atp12p, from Homo sapiens. *In vitro* studies with purified wild type and mutant (E240K) proteins. *J Biol Chem* **279**, 9016–9022.

20 Knowles AF and Penefsky HS (1972) The subunit structure of beef heart mitochondrial adenosine triphosphatase. Isolation procedures. *J Biol Chem* **247**, 6617–6623.

21 Suzuki T, Ueno H, Mitome N, Suzuki J and Yoshida M (2002) F_0 of ATP synthase is a rotary proton channel. Obligatory coupling of proton translocation with rotation of c-subunit ring. *J Biol Chem* **277**, 13281–13285.

22 Matsui T and Yoshida M (1995) Expression of the wild-type and the Cys-/Trp-less alpha beta gamma complex of thermophilic F_1-ATPase in *Escherichia coli*. *Biochim Biophys Acta* **1231**, 139–146.

23 Jault JM, Dou C, Grodsky NB, Matsui T, Yoshida M and Allison WS (1996) The alpha beta gamma subcomplex of the F_1-ATPase from the thermophilic *Bacillus* PS3 with the beta T165S substitution does not entrap inhibitory MgADP in a catalytic site during turnover. *J Biol Chem* **271**, 28818–28824.

24 Walker JE, Fearnley IM, Gay NJ, Gibbon BW, Northrop FD, Powell SJ, Runswick MJ, Saraste M and Tybulewicz VL (1985) Primary structure and subunit stoichiometry of F_1-ATPase from bovine mitochondria. *J Mol Biol* **184**, 677–701.

25 Bason JV, Runswick MJ, Fearnley IM and Walker JE (2011) Binding of the inhibitor protein IF1 to bovine F_1-ATPase. *J Mol Biol* **406**, 443–453.

26 Suzuki T, Suzuki J, Mitome N, Ueno H and Yoshida M (2000) Second stalk of ATP synthase. Cross-linking of gamma subunit in F_1 to truncated F_0 subunit prevents ATP hydrolysis. *J Biol Chem* **275**, 37902–37906.

27 Lutter R, Abrahams JP, van Raaij MJ, Todd RJ, de Meirleir L, Seneca S, Lissens W, de Clercq I, Eyskens R, Noji H, Yoshida M, Mitome N, Ueno H and Yoshida M (1995) Expression of the human mitochondrial F_1-ATPase assembly gene 2A5 complements the defect in Drosophila. *FEBS Lett* **379**, 790–794.

28 Shirakihara Y, Shiratori A, Tanikawa H, Nakasako M, Knowles AF and Penefsky HS (1972) The subunit structure of beef heart mitochondrial adenosine triphosphatase. Isolation procedures. *J Biol Chem* **247**, 6617–6623.

29 Shirakihara Y, Shiratori A, Tanikawa H, Nakasako M, Yoshida M and Suzuki T (2015) Structure of a thermophilic F_1-ATPase inhibited by an e-subunit: deeper insight into the e-inhibition mechanism. *FEBS J* **282**, 2895–2913.

30 Ludlam A, Brunzelle J, Pribyl T, Xu X, Gatti DL and Ackerman SH (2009) Chaperones of F_1-ATPase. *J Biol Chem* **284**, 17138–17146.

31 de Meirleir L, Seneca S, Lissens W, de Clercq I, Eyskens R, Gerlo E, Smet J and Van Coster R (2004) Respiratory chain complex V deficiency due to a mutation in the assembly gene ATP12. *J Med Genet* **41**, 120–124.