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

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F$_1$-ATPase (F$_1$) is a multisubunit water-soluble domain of F$_o$F$_1$-ATP synthase and is a rotary enzyme by itself. Earlier genetic studies using yeast suggested that two factors, Atp11p and Atp12p, contribute to F$_1$ assembly. Here, we show that their mammalian counterparts, AF1 and AF2, are essential and sufficient for efficient production of recombinant bovine mitochondrial F$_1$ in *Escherichia coli* cells. Intactness of the function and conformation of the *E. coli*-expressed bovine F$_1$ was verified by rotation analysis and crystallization. This expression system opens a way for the previously unattempted mutation study of mammalian mitochondrial F$_1$.

F$_o$F$_1$-ATP synthase (F$_o$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$_o$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°

Abbreviations

AMPPNP, adenylyl-imidodiphosphate; Au-bead, colloidal gold particles; CBB, Coomassie Brilliant Blue; F$_1$, F$_1$-ATPase; F$_o$F$_1$, F$_o$F$_1$-ATP synthase; fps, frames per second; IF1, inhibitory factor-1; Pi, inorganic phosphate; rps, revolutions per second.

rotation of a central rotor shaft composed of γδε-sub-units relative to a surrounding stator ring of α$_3$β$_3$-sub-units (eukaryotic subunit composition) [4,5]. Extensive studies on rotation of bacterial F$_1$ 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 F$_1$ requires knowledge on how chemical events occurring to F$_1$ induce its structural changes and trigger rotation. In this respect, much structural information has been accumulated for bovine F$_1$, rather than bacterial F$_1$, by X-ray crystallography [10,11]. However, rotation of mitochondrial F$_1$ was not demonstrated until recently because of the absence of *in vitro* expression system of mitochondrial F$_1$ genes that enables genetic modification necessary for single-molecule observation, such as introduction of the His-tag.

We recently succeeded in expressing human mitochondrial F$_1$ 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 F$_1$ in *E. coli*. Early genetic works using *Saccharomyces cerevisiae* identified two mitochondrial proteins of Atp11p and Atp12p as molecular chaperones necessary for assembly of F$_1$ [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 F$_1$ was not tested directly. We thus expressed the five subunits of bovine mitochondrial F$_1$ 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 F$_1$ in *E. coli*. ATP-driven rotation and crystallization confirmed intactness of *E. coli*-expressed bovine F$_1$.

Experimental procedures

Expression of bovine F$_1$ in *E. coli*

The expression plasmid for bovine F$_1$ was constructed in the same manner as performed previously for human F$_1$ [5]; five genes coding subunits of bovine F$_1$ (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 α-γ-β-δ-ε-*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 F$_1$ as performed previously [4]. The resulting plasmid, pBF1, was introduced into F$_o$F$_1$-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 F$_1$ from thermophilic *Bacillus* PS3 in *E. coli* [22], the growth rate of the *E. coli* was not significantly affected by the expression of bovine F$_1$. It is assumed that submillimolar concentration of ADP in cytoplasm is enough to keep F$_1$ in the inactive state of so-called MgADP-inhibition, a general feature of F$_1$ 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 F$_1$ are the same as those for human F$_1$, 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/HCl (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 F$_1$ was about 2–3 mg per 6-L-culture. Authentic bovine F$_1$ was prepared from bovine heart as reported [24] with a modification; gel-filtration was performed with a Superdex200 column in 20 mM Tris/HCl (pH8.0), 200 mM NaCl, 0.1 mM ATP, and 0.5 mM EDTA. To avoid cold dissociation of bovine F$_1$, 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 F$_1$

Rotation of a single molecule of bovine F$_1$ was observed by the procedures described in ref. [5]. Two cysteine residues were introduced into a globular domain of γ-subunit (γAla99Cys and γSer191Cys). Images of a rotating submicron polystyrene bead attached to the γ-subunit of immobilized bovine F$_1$ 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

(MEMRECAM GX-8S; NAC Image Technology Inc., Tokyo, Japan) [5].

Crystallization of *E. coli*-expressed bovine F$_1$

Concentrated recombinant bovine F$_1$ was supplemented with 0.5 mM AMPPNP and 20 mM MgCl$_2$ (the final bovine F$_1$ concentration was 10 mg·mL^{-1}) and used for crystallization. Reservoir solution (70 μL) containing 100 mM Tris/HCl (pH 8.5), 200 mM LiSO$_4$, and 21–23% PEG3350 (Hampton Research, Aliso Viejo, CA, USA) was put into a sitting-drop dish, and the bovine F$_1$ 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 MgCl$_2$, 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 MgCl$_2$, 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 F$_1$, 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 F$_1$ [25] showed that IC$_{50}$ 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 F$_1$ depends on AF1 and AF2

The five genes for bovine F$_1$ were introduced into the *E. coli* expression vector in the same order as in the *E. coli* F$_o$F$_1$ 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 F$_o$F$_1$ 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 F$_1$ 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 F$_1$ while pBF1(-AFs)-harboring cells produced very little, if any, amount of bovine F$_1$ (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 F$_1$ (Fig. 1B). F$_1$ isolated from bovine heart appeared as two split bands in native-PAGE for an unknown reason and bovine F$_1$ produced in *E. coli* also gives two bands. The monomeric β-subunit of bovine F$_1$ produced in *E. coli* migrates in the gel more slowly than that of authentic bovine F$_1$ due to the attached histidine tag (Fig. 1A). Production of bovine F$_1$ 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 F$_1$. We purified bovine F$_1$ from pBF1(+AFs)-harbored *E. coli* cells and confirmed that it has the same subunit composition as authentic bovine F$_1$ by SDS/PAGE analysis (Fig. 1E). The ATPase activity of mitochondrial F$_1$ is known to be inhibited by a specific inhibitor protein of mitochondria, IF1 [25]. Sensitivity of *E. coli*-expressed bovine F$_1$ to IF1 was tested by using bovine IF1 fused to GFP. As shown in Fig. 1F, the ATPase activity of *E. coli*-expressed bovine F$_1$ was inhibited by IF1-GFP in the same manner as observed for authentic bovine F$_1$ (Fig. 1F).

Rotation of *E. coli*-expressed bovine F$_1$

To verify the function of *E. coli*-expressed bovine F$_1$, 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 F$_1$ rotates at a speed 2.5 ± 0.3 rps and rotation takes three dwells per revolution, approximately at every 120° rotation
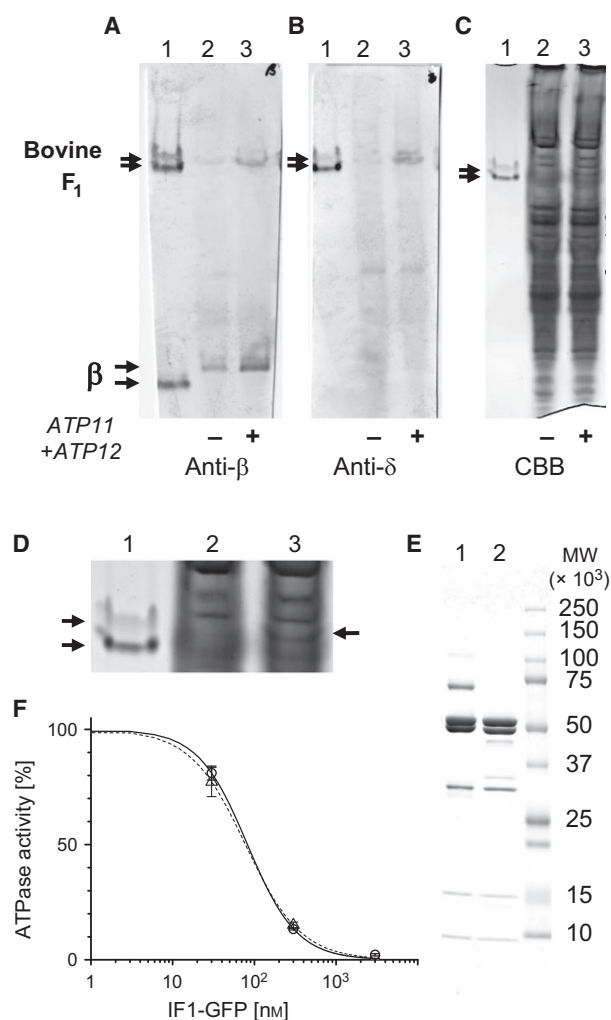
Fig. 1. Expression of bovine F$_1$ in *Escherichia coli*. (A–D) Native-PAGE analysis of water-soluble fraction of *E. coli* cells expressing bovine F$_1$ without (plasmid pBF1(-AFs), lane 2) or with (pBF1 (+AFs), lane 3) simultaneous expression of the two genes, *ATP11* and *ATP12*. Lane 1 represents authentic bovine F$_1$ purified from bovine heart muscle. The gels were analyzed by immunoblotting using anti-β-subunit (A) or anti-δ-subunit antibodies (B), or by protein staining with CBB (C, D). (D) The region containing a band of bovine F$_1$ in C is enlarged. Arrows indicate the band of bovine F$_1$. (E) SDS/PAGE analysis of authentic bovine F$_1$ (lane 1) and purified *E. coli*-expressed bovine F$_1$ (lane 2). (F) Inhibition of ATPase activity of bovine F$_1$ by IF1. GFP-fused IF1 was used. The ATPase activity in the absence of IF1-GFP is set to 100%. Solid line, *E. coli*-expressed bovine F$_1$; dotted line, authentic bovine F$_1$.
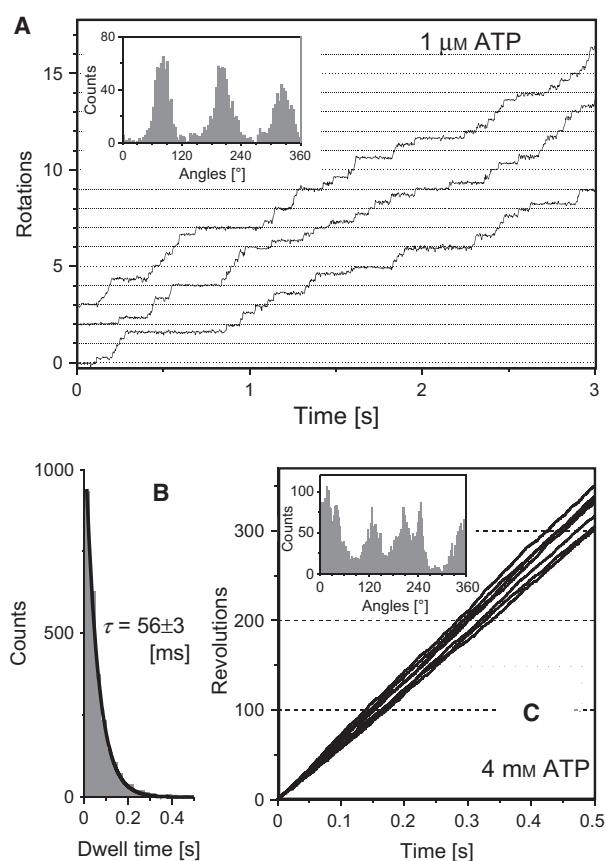

Fig. 2. Single-molecule analysis of rotation of *Escherichia coli*-expressed bovine F$_1$. (A, B) Rotation of bovine F$_1$ at 1 μM ATP observed with a camera 500 fps. (A) Time-courses of the rotation. The inset is an angle histogram of the rotation. (B) A histogram of duration of dwells ($N = 3672$ dwells, three molecules) observed in the rotation. The bin width was 0.25 s. The histogram was best simulated with a single-exponential decay function with a lifetime of 56 ± 3 ms. (C) Time-courses of rotation at a saturating ATP concentration, 4 mM. Rotation was analyzed at 25k fps using Au particles (diameter was 40 nm) as a rotation probe. The inset is an angle histogram of the rotation. The averaged rotation speed over 0.5 s was 655 ± 38 rps ($N = 6$ molecules).

(Fig. 2A). The dwells become shorter as the ATP concentration increased, indicating that bovine F$_1$ waits for ATP binding during the dwell to drive the next cycle of 120° rotation. The rate constant of ATP binding (k_{on}) calculated from the lifetime of the dwell ($\tau = 56 \pm 3$ ms) is $1.8 \pm 0.3 \times 10^7$ M$^{-1} \cdot$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 ($N = 7$ molecules), directly reflects the maximum turnover rate of ATP hydrolysis by a single molecule of bovine F$_1$, 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 F$_1$ and human F$_1$, these motor characteristics of bovine F$_1$ are similar to those
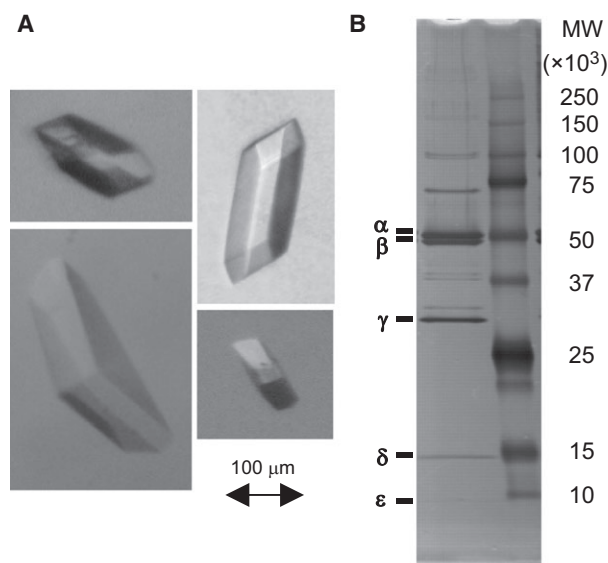
Fig. 3. Crystals of *Escherichia coli*-expressed bovine F$_1$. (A) Crystals of bovine F$_1$ were routinely obtained by sitting-drop vapor diffusion method as stated in *Experimental Procedures*. (B) SDS/PAGE analysis of the crystals. The gel was stained with silver.

of human F$_1$ (k_{on}, $2.7 \pm 0.3 \times 10^7$ M^{-1}·s^{-1}; rotation speed, 705 ± 75 rps) [5].

Crystallization of *E. coli*-expressed bovine F$_1$

Bovine F$_1$ (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 F$_1$ [27] probably because of the histidine-tag of the β-subunit of *E. coli*-expressed bovine F$_1$. After screening crystallization conditions, we found that crystals were reproducibly formed in the solution containing 0.5 mM AMPPNP and 20 mM MgCl$_2$ with PEG3350 as precipitant (Fig. 3A). Crystals were collected from the drops, washed, and analyzed by SDS/PAGE (Fig. 3B). All the five F$_1$ subunits were detected in the gel, confirming that the crystals were of bovine F$_1$. The result shows that the purified bovine F$_1$ has a quality high enough to grow crystals.

Conclusions

Bovine F$_1$, for the first time, was successfully expressed in *E. coli* cells. As expected, purified bovine F$_1$ exhibits motor characteristics similar to those of human F$_1$. It forms good crystals rather easily. We previously spent time and efforts for crystallization of bacterial F$_1$ [28], but now realize that bovine F$_1$ is superior to bacterial F$_1$ in crystallization for the detailed structural study of F$_1$.

Availability of mutants adds a further advantage to *E. coli*-expressed bovine F$_1$ over the native protein.

Without AF1 and AF2, only very little, if any, bovine F$_1$ was produced by *E. coli*, indicating that AF1 and AF2 are required for efficient production of bovine F$_1$. This expression is the first demonstration of the chaperone function of these two factors for assembly of mammalian F$_1$. 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 F$_o$F$_1$ in mitochondria is attributed to a mutation in the *ATP12* gene, suggesting a critical physiological role of these assembly factors in production of functional F$_o$F$_1$ [30]. Although we did not test human *ATP11* and *ATP 12* for expressing bovine F$_1$, 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 F$_1$ expression system would open a way to the study of detailed mechanisms of the assembly and a structure-mechanism relationship of mitochondrial F$_1$.

Acknowledgements

Author contributions

TS and MY conceived and designed the experiments and wrote the paper. TS developed the *E. coli* expression system of bovine F$_1$ 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.

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