Efficient ATP synthesis by thermophilic *Bacillus* F$_o$F$_1$-ATP synthase

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**Introduction**

F$_o$F$_1$-ATP synthase (F$_o$F$_1$) synthesizes ATP in the F$_1$ portion when protons flow through F$_o$ to rotate the shaft common to F$_1$ and F$_o$. Rotary synthesis in isolated F$_1$ alone has been shown by applying external torque to F$_1$ of thermophilic origin. Proton-driven ATP synthesis by thermophilic *Bacillus* PS3 F$_o$F$_1$ (TF$_o$F$_1$), however, has so far been poor in vitro, of the order of 1 s$^{-1}$ or less, hampering reliable characterization. Here, by using a mutant TF$_o$F$_1$ lacking an inhibitory segment of the $\varepsilon$-subunit, we have developed highly reproducible, simple procedures for the preparation of active proteoliposomes and for kinetic analysis of ATP synthesis, which was driven by acid-base transition and K$^+$-diffusion potential. The synthesis activity reached $\sim$16 s$^{-1}$ at 30 °C with a $Q_{10}$ temperature coefficient of 3–4 between 10 and 30 °C, suggesting a high level of activity at the physiological temperature of $\sim$60 °C. The Michaelis–Menten constants for the substrates ADP and inorganic phosphate were 13 $\mu$m and 0.55 mm, respectively, which are an order of magnitude lower than previous estimates and are suited to efficient ATP synthesis.

**Abbreviations**

$\Delta$W, membrane potential; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; F$_o$F$_1$, F$_o$F$_1$-ATPase; $[K^+]_{\text{out}}$, internal K$^+$ concentration; $[K^+]_{\text{sub}}$, external K$^+$ concentration; OG, n-octyl-$\beta$-D-glucoside; pH$_{\text{in}}$, pH inside the liposomes; pH$_{\text{sub}}$, pH outside the liposomes; PMF, proton motive force; TF$_o$F$_1$, *Bacillus* PS3 F$_o$F$_1$-ATPase; TF$_o$F$_1$$^{\text{AC}}$, mutant *Bacillus* PS3 F$_o$F$_1$-ATPase lacking the C-terminal domain of the $\varepsilon$-subunit; TF$_o$F$_1$$^{\text{WT}}$, *Bacillus* PS3 wild-type F$_o$F$_1$-ATP synthase.
ATP is synthesized. Conversely, when the PMF is lower, \( F_1 \) wins, and protons are pumped back by reverse rotation of \( F_0 \). ATP-driven rotation has been characterized in detail, particularly for isolated \( F_1 \) [5–9].

\( F_1 \) alone, without \( F_0 \), can synthesize ATP when its rotor is forced to rotate in the reverse direction by an artificially applied force. \( F_1 \) is thus a reversible molecular machine that can interconvert chemical and mechanical energies in either direction. This has so far been shown for a subcomplex, \( \alpha_3\beta_3\gamma \), of \( F_1 \) derived from a thermophile, Bacillus PS3 [10,11]. The whole ATP synthase of the thermophile (\( \text{TFoF}_1 \)), however, has performed rather poorly in the past in \textit{in vitro} studies. The maximal turnover rate, \( V_{\text{max}} \), has been reported to be 0.1 s\(^{-1}\) at 36 °C [12], 1–3 s\(^{-1}\) at 40 °C [13–15] and up to 7 s\(^{-1}\) at 40 °C in the presence of cholesterol [16]. In line with the rather low activities, reported Michaelis–Menten constants, \( K_m \), for substrates are high: 0.3 mM for ADP and 10 mM for \( P_i \) [14], or 0.4 mM for ADP and 6.3 mM for \( P_i \) [15]. ATP synthases from other sources generally show an activity more than an order of magnitude higher, and \( K_m \) values are correspondingly lower [17–21].

Because the thermophilic enzyme is robust and suited to single-molecule studies [3.5–8,10,11], we investigated whether \( \text{TFoF}_1 \) with high synthesis activity can be prepared. The \( \varepsilon \)-subunit, in particular its C-terminal domain, exerts an inhibitory effect both for ATP hydrolysis and ATP synthesis, and deletion of this domain has been shown to increase the synthesis activity [22,23], probably by preventing the formation of the inhibited form. We thus sought for a reconstitution method that leads to a high synthesis activity. We obtained an activity of \( \sim 16 \) s\(^{-1}\) at 30 °C, with a temperature coefficient that suggests a much higher activity at the physiological temperature of the thermophile. \( K_m \) values for the substrates at 30 °C were low and comparable with those of other enzymes, such that, unless \( K_m \) values at physiological temperatures differ significantly, efficient ATP synthesis will be ensured \textit{in vivo}. In addition, the activity at room temperature (25 °C) of \( \sim 10 \) s\(^{-1}\) suggests, on the basis of three ATPs per revolution [24], a rotary rate of \( \sim 3 \) revolutions s\(^{-1}\), which should be readily detected in single-molecule studies under a microscope.

**Results**

**ATP synthesis by mutant \( \text{TFoF}_1 \) lacking the C-terminal domain of the \( \varepsilon \)-subunit (\( \text{TFoF}^{\varepsilon\text{Ac}}_1 \)) reconstituted into liposomes**

A problem in the previous assays was the inhibitory effect of the \( \varepsilon \)-subunit on the ATP synthesis activity. In the absence of a nucleotide in the medium, \( \text{TFoF}_1 \) is resting in a state inhibited by the \( \varepsilon \)-subunit [25], and recent studies suggest the possibility that activation of such \( \text{TFoF}_1 \) to initiate ATP synthesis requires an extra PMF in addition to the thermodynamically required magnitude of PMF [26,27]. \( \text{TFoF}^{\varepsilon\text{Ac}}_1 \), in which the C-terminal region of the \( \varepsilon \)-subunit that is responsible for the inhibitory effect is deleted, has shown a higher rate of ATP synthesis [22], and this was also the case for \textit{Escherichia coli} \( \text{F}_o\text{F}_1 \) [23]. In this work, therefore, we prepared \( \text{TFoF}^{\varepsilon\text{Ac}}_1 \), using as the wild type \( \text{TFoF}_1 \) with a 10-histidine tag at each \( \beta \)-subunit (\( \text{TFoF}^{\text{WT}}_1 \)) [28] (see Experimental procedures). Unless stated otherwise, all results below refer to \( \text{TFoF}^{\varepsilon\text{Ac}}_1 \).

We also improved the assay system to obtain high ATP synthesis activities reproducibly. Previously, \( \text{TFoF}_1 \) was dissolved in solutions containing Triton X-100 during purification and proteoliposome reconstitution procedures [13–15]. However, we found that \( \text{TFoF}_1 \) exposed to Triton X-100 has a strong propensity to form aggregates. In the improved assay, the \( \text{TFoF}_1 \) preparation was dispersed in 6% n-octyl-\( \beta \)-D-glucoside (OG) in the presence of phospholipids, and OG was then removed with Biobeads (see Experimental procedures). The proteoliposomes thus made were very stable, and they retained 90% of ATP synthesis activity after storage for 3 days at 4 °C. This method is simple, does not require preformed liposomes, and is highly reproducible.

The ATP synthesis activity of the proteoliposomes was assayed by acid–base transition. First, the proteoliposomes were equilibrated with an acidic buffer with low K\(^+\) to set the pH inside the liposomes (\( \text{pH}_{\text{in}} \)) to 5.65 and the internal K\(^+\) concentration (\( [\text{K}\text{+}]_{\text{in}} \)) to 0.6 mM. The acidic buffer contained valinomycin to render the membranes permeable to K\(^+\). Then, the proteoliposomes were injected into a basic mixture to change the pH outside the liposomes (\( \text{pH}_{\text{out}} \)) to 8.8 and the external K\(^+\) concentration (\( [\text{K}\text{+}]_{\text{out}} \)) to 105 mM. This would generate a transient PMF of 330 mV, with the calculated membrane potential (\( \Delta \psi \)) of 135 mV (\( [\text{K}\text{+}]_{\text{out}} = 105 \) mM, \( [\text{K}\text{+}]_{\text{in}} = 0.6 \) mM) and \( \Delta \text{pH} \) of 3.2 (\( \text{pH}_{\text{out}} = 8.8, \text{pH}_{\text{in}} = 5.65 \)).

Figure 1 shows the time courses of the luciferase-catalyzed light emission, which directly reflected the increase in the ATP concentration resulting from synthesis by \( \text{TFoF}^{\varepsilon\text{Ac}}_1 \). At the time indicated by the arrow (time zero), the proteoliposome mixture was injected into the basic mixture. ATP synthesis started at the maximum initial rate, which gradually slowed down and leveled off at \( \sim 60 \) s, reflecting dissipation of the
imposed PMF (time constant of the order of 10 s at 30 °C). To determine the activity at the calculated PMF of 330 mV, we estimated the initial velocity of ATP synthesis at time zero by fitting the 0–6-s portion with a single exponential (gray curves in Fig. 1) and converting the velocity to the turnover rate. As can be seen, the initial velocity of synthesis was proportional to the amount of the added proteoliposomes (Fig. 1, traces 1 and 2), giving similar rates of ATP synthesis by TF\textsubscript{oF\textsubscript{1}}\textsuperscript{tAc} of 15 s\(^{-1}\) (trace 1) and 16 s\(^{-1}\) (trace 2). Under the same conditions, TF\textsubscript{oF\textsubscript{1}}\textsuperscript{WT} showed only low activity of 0.7 s\(^{-1}\) (trace 3). The low activity is consistent with previous studies with TF\textsubscript{oF\textsubscript{1}}\textsuperscript{WT}, including one that used acid–base transition to obtain a rate of \(\sim 2\) s\(^{-1}\) at 40 °C [13]. No ATP synthesis was observed when an uncoupler, carbonyl cyanide 4-(trifluoromethoxy)phenylhydradzone (FCCP), was included in the mixture. Nigericin, which acts as an uncoupler in the presence of valinomycin, also abolished ATP synthesis.

Note that the orientation of the enzyme in the reconstituted membrane was not controlled for in this work. We did not apply correction for misoriented TF\textsubscript{oF\textsubscript{1}}, and thus the activity values reported here are probably underestimated. Also note that the catalyzing F\textsubscript{1} was always exposed to the fixed pH\textsubscript{out} of 8.8, and the activity values refer to the catalysis at this pH.

**Dependence on protein/lipid ratio**

To explore optimal conditions for activity assays, we prepared proteoliposomes with a fixed amount of phospholipid (16 mg mL\(^{-1}\)) and varying amounts of TF\textsubscript{oF\textsubscript{1}}\textsuperscript{tAc}, and measured the ATP synthesis activity (Fig. 2). The activity was almost constant, \(\sim 16\) s\(^{-1}\), for the TF\textsubscript{oF\textsubscript{1}}\textsuperscript{tAc}/phospholipid weight ratio of 0.002 to 0.01. These ratios correspond to one to three molecules of TF\textsubscript{oF\textsubscript{1}}\textsuperscript{tAc} per proteoliposome of diameter 170 nm (Fig. 2, inset), a size expected for liposomes prepared in similar ways [29,30]. Beyond this range, the activity started to decrease gradually, although the total amount of ATP synthesized by 50 s increased steadily, at least to the weight ratio of 0.08. Because the measurement accuracy critically depends on the absolute amount of ATP synthesized, in the following experiments we used the proteoliposomes with a weight ratio of 0.02 (final TF\textsubscript{oF\textsubscript{1}}\textsuperscript{tAc} concentration in the reaction mixture of 17 nM).

**Fig. 1.** ATP synthesis by TF\textsubscript{oF\textsubscript{1}}\textsuperscript{tAc} or TF\textsubscript{oF\textsubscript{1}}\textsuperscript{WT} reconstituted in liposomes. The ATP synthesis reaction was initiated by injection of 100 μL of the acidified proteoliposome mixture into 900 μL of the basic mixture at the point indicated by the arrow (time zero), and luciferin emission was monitored at 30 °C. The final concentrations of TF\textsubscript{oF\textsubscript{1}}, ADP and P\textsubscript{i} were 17 nM (8.5 nM in trace 2), 0.5 and 10 mM, respectively. At 60 s, 100 pmol of ATP was added three times for calibration. The imposed PMF calculated from the Nernst equation is 330 mV (pH\textsubscript{out} = 8.8, pH\textsubscript{in} = 5.65, [K\textsuperscript{+}]\textsubscript{out} = 105 mM, [K\textsuperscript{+}]\textsubscript{in} = 0.6 mM). The scale at the top is based on the average proteoliposome diameter of 170 nm. The inset shows the size distribution estimated by dynamic light scattering.

**Fig. 2.** Effect of TF\textsubscript{oF\textsubscript{1}}\textsuperscript{tAc}/lipid ratio on ATP synthesis activity. Proteoliposomes were made by mixing 20–600 μg of TF\textsubscript{oF\textsubscript{1}}\textsuperscript{tAc} and 8 mg of lipid in 500 μL and adding BioBeads. The initial rate of synthesis and the amount of ATP synthesized by 50 s are shown. The imposed PMF was 330 mV (pH\textsubscript{out} = 8.8, pH\textsubscript{in} = 5.65, [K\textsuperscript{+}]\textsubscript{out} = 105 mM, [K\textsuperscript{+}]\textsubscript{in} = 0.6 mM). The scale at the top is based on the average proteoliposome diameter of 170 nm. The inset shows the size distribution estimated by dynamic light scattering.
Dependence on temperature

The results in Figs 1 and 2 were obtained at 30 °C. To determine the activity at the physiological growth temperature of Bacillus PS3 (≈ 60 °C or above) and to investigate the possibility of single-molecule experiments at room temperature, we examined the temperature dependence of the ATP synthesis activity. Unfortunately, the luciferase system was not perfectly stable above 30 °C, so we analyzed the activity between 10 and 30 °C (Fig. 3). Lowering the temperature greatly decreased the initial rate of synthesis, but the rate after 60 s did not differ much (Fig. 3). At 10 °C, synthesis of ATP started after a short lag. The reaction of luciferin/luciferase was sufficiently fast (~ 0.1 s) at 10 °C, and the reason for the lag is unknown. There may also be a slight lag at 15 °C. We ignored these lag phases, and estimated the maximal rates of ATP synthesis (Fig. 3B). The activity increased three-fold to four-fold per 10 °C, or the Q_{10} temperature coefficient was 3–4 in this range. The Arrhenius plot (Fig. 3B, right) indicates an activation energy of 110 kJ mol\(^{-1}\) in this range, and simple extrapolation would suggest an activity at the physiological temperature (≈ 60 °C) of ~ 1000 s\(^{-1}\). Although such an extrapolation is not warranted, the physiological activity is probably above 100 s\(^{-1}\).

Dependence on substrate concentrations

At 30 °C, we examined how substrate concentrations affect the rate of ATP synthesis. The ADP concentration was changed from 1 μM to 1 mM at a saturating concentration (10 mM) of Pi (Fig. 4). The data are fitted well with the Michaelis–Menten equation with a K_{ADP} of 13 μM and a V_{max} of 17 s\(^{-1}\). We also changed the Pi concentration from 0.1 to 30 mM at a saturating concentration (0.5 mM) of ADP. The results also conformed to the Michaelis–Menten equation, with a K_{Pi} of 0.55 mM and a V_{max} of 16 s\(^{-1}\) (Fig. 5).

Discussion

We have developed simple and reproducible procedures for the preparation of active TFoF1 proteoliposomes and conditions for real-time monitoring of ATP synthesis. The synthesis activity reported here is an order of magnitude higher than that in previous reports on TFoF1 [12–16]. Note that most of the previous work was performed at 40 °C, whereas our measurements here were made at 30 °C. The primary reason for the increase in activity is the removal of the inhibitory C-terminal segment of the \(\varepsilon\)- subunit, as seen in Fig. 1. In addition, we noticed that complete solubilization of TFoF1 with proper detergents and a low protein/lipid ratio are keys to high activity. Also, Bio-beads need to be selected from among several lots to obtain maximal activity under the protocol described here, or else the amount of added Bio-beads and incubation time should be optimized for each lot.

The Michaelis–Menten constants for the substrates, 13 μM for ADP and 0.55 mM for Pi, obtained here are low enough to ensure efficient ATP synthesis under cellular conditions where the ADP concentration is expected to be submillimolar and the Pi concentration several millimolar. There is no guarantee that the K_{m} values at the physiological temperature of the thermo-
phile are close to our experimental values at 30 °C, but the lower $K_m$ values are more advantageous than the previous values of 0.3–0.4 mM for ADP and 6–10 mM for $P_i$ [14,15]. These previous values may, in part, reflect the properties of the $e$-subunit-inhibited fraction. It is also possible that ADP and/or $P_i$ help to convert the inhibited form to an active form, and the measured $K_m$ might be influenced by these activation processes.

As noted above, the reported ATP synthesis activity of TFoF1 has so far been much lower and the $K_m$ values for ADP and $P_i$ higher than those of F1,F1 from other sources. Bovine enzyme in submitochondrial particles gave, in its high-activity mode, a $V_{\text{max}}$ of $\sim 420$ s$^{-1}$ at 30 °C [17], a $K_{m}^{\text{ADP}}$ of 50–100 μM, and a $K_{m}^{P_i}$ of $\sim 2$ mM (PMF unknown) [18]. Yeast mitochondrial ATP synthase reconstituted in liposomes showed a $V_{\text{max}}$ of 120 s$^{-1}$ at 25 °C and an apparent $K_{m}^{P_i}$ lower than 1.5 mM at a pH on the $F_1$ side below 8 (PMF of 250–300 mV) [19]. The reconstituted chloroplast enzyme gave a $V_{\text{max}}$ up to $\sim 400$ s$^{-1}$ and a $K_{m}^{P_i}$ of 0.35 or 0.97 mM, depending on the reconstitution protocol (PMF of $\sim 300$ mV) [20]. *E. coli* ATP synthase in liposomes showed a $V_{\text{max}}$ of $\sim 30$ s$^{-1}$ at room temperature, a $K_{m}^{\text{ADP}}$ of 27 μM, and a $K_{m}^{P_i}$ of 0.7 mM (PMF of $\sim 330$ mV) [21]. Another report on the *E. coli* enzyme [23] gave a $V_{\text{max}}$ of 16–20 s$^{-1}$ at 24–25 °C, a $K_{m}^{\text{ADP}}$ of 100 μM and a $K_{m}^{P_i}$ of 4 mM for the wild type, and a $V_{\text{max}}$ of $\sim 60$ s$^{-1}$, a $K_{m}^{\text{ADP}}$ of 25 μM and a $K_{m}^{P_i}$ of 3 mM for an $e$ΔC mutant (PMF of $\sim 260$ mV). This last
result obtained with the bacterial enzyme is qualitatively similar to that obtained with TFoF1, in that C-terminal truncation of the ε-subunit increases \( V_{\text{max}} \) while decreasing \( K_m \) values for ADP and Pi. The present results on TFoF1 place this thermophilic enzyme among those with regular synthesis activities, and, with regard to \( K_m \) values, at the low end. Note that the \( V_{\text{max}} \) of TFoF1 at its physiological temperature of \( \sim 60^\circ\text{C} \) or above is expected to be much higher than 16 s\(^{-1}\) (Fig. 3).

The demonstration of substantial ATP synthesis by TFoF1 around room temperature should be a large step towards single-molecule observation of rotation-catalyzed ATP synthesis under an optical microscope. The thermophilic enzyme is quite stable, remaining active for days at room temperature. This stability greatly facilitates micromanipulation work, which is tedious both in preparation and observation (both take hours). Indeed, much of the mechanical characterization of F1 has been achieved with F1 derived from the thermophile, Bacillus PS3. We hope to answer, by using TFoF1, the fundamental questions of how protons rotate F1 and how rotation leads to ATP synthesis. So far, even the demonstration of proton-driven rotation has been difficult [31], but a major obstacle, the low activity, has now been removed.

**Experimental procedures**

**Preparation of TFoF1**

In this work, we used TFoF1 with a 10-histidine tag at the N-terminus of each β-subunit [25] as the wild type (TFoF1\(^{WT}\)). The mutant lacking the C-terminal domain of the ε-subunit (TFoF1\(^{ε\text{-Asp87}}\)) was produced by inserting a stop codon after ε-Asp87. TFoF1\(^{ε\text{-Asp87}}\) and TFoF1\(^{ε\text{-mal}}\) were expressed in an F6,F1-deficient E. coli strain (DK8) with the expression plasmids pTR19-ASDS and pTR19-ASDS-εAc, respectively, and purified as previously described [25], with the following modifications. The membrane fraction containing TFoF1 was solubilized at 30 °C in a solution containing 10 mM Hepes, 5 mM MgCl\(_2\), 10% (v/v) glycerol, 0.5% (w/v) cholic acid and 2% (v/v) Triton X-100, with the pH adjusted to 7.5 with KOH. The suspension was centrifuged at 235,000 g for 60 min. The supernatant was diluted sixfold with M-buffer (20 mM KP, and 100 mM KCl, pH 7.5). To this solution, Ni\(^{2+}\)–Sepharose resin (GE Healthcare, Uppsala, Sweden) that had been pre-equilibrated with W-buffer [M-buffer containing 20 mM imidazole and 0.15% (w/v) n-decyl-β-D-maltoside (Dojindo, Kumamoto, Japan), with the pH adjusted to 7.5 with HCl] was added, and the suspension was gently stirred on ice for 30 min. The resin suspension was then poured into an open column and washed with 10 volumes of W-buffer. Protein was eluted with M-buffer containing 200 mM imidazole and 0.15% n-decyl-β-D-maltoside, with the pH adjusted to 7.5 with HCl, and diluted three-fold with 20 mM Hepes, 0.2 mM EDTA and 0.15% n-decyl-β-D-maltoside, with the pH adjusted to 7.5 with NaOH. The suspension was applied to a RosourceQ column (6 mL; GE Healthcare) equilibrated with the same buffer. Elution with a linear gradient of 0–500 mM Na\(_2\)SO\(_4\) produced two closely located protein peaks. The second peak contained TFoF1, which was concentrated by a centrifugal concentrator with a cutoff molecular mass of 50 kDa (Amicon Ultra; Millipore, Country Cork, Ireland) to a final volume of \( \sim 1 \) mL. The purified TFoF1 preparation was divided into aliquots of 25–50 μL, frozen with liquid N\(_2\), and stored at –80 °C until use. The molar concentration of TFoF1 was determined by absorbance with a molar extinction coefficient at 280 nm of 253 000 M\(^{-1}\) cm\(^{-1}\). Protein mass was calculated by taking the molecular mass of TFoF1 as 530 kDa.

**Reconstitution of TFoF1 into liposomes**

Crude soybean l-α-phosphatidylecholine (Type II-S; Sigma, St. Louis, MO, USA) was washed with acetone [32] and suspended to a final concentration of 32 mg mL\(^{-1}\) in R-buffer (20 mM Tricine, 20 mM succinic acid, 80 mM NaCl and 0.6 mM KOH, with the pH adjusted to 8.0 with NaOH). The suspension was incubated for 30 min with gentle stirring, to allow the lipid to swell. The lipid was further dispersed by brief sonication with a tip-type sonicator (UR-20P; Tomy Seiko, Tokyo, Japan) for 30 s. This suspension was divided into aliquots, frozen with liquid N\(_2\), and stored at –80 °C until use. Reconstitution of TFoF1 into liposomes was performed as follows. The lipid suspension (250 μL) was mixed with 250 μL of TFoF1 in R-buffer containing 10 mM MgCl\(_2\) and 12% (w/v) OG, and the mixture (total volume, 500 μL; concentration of TFoF1, 40–1200 μg mL\(^{-1}\)) was stirred gently at 25 °C for 1 h. To this solution, 200 μL of Biobeads (SM-2; BioRad, Hercules, CA, USA), which had been pre-equilibrated with R-buffer, was added. The mixture was stirred gently for 30 min at 25 °C, and 300 μL of Biobeads was added to the mixture. After another 1.5 h of incubation, the liposome suspension was transferred to a new tube, leaving the Biobeads behind. The concentration of TFoF1 in the final mixture was 75–2300 nm. The average diameter of the proteoliposomes was estimated by dynamic light scattering (HB-550; Horiba, Kyoto, Japan) to be 170 nm (Fig. 2).

**ATP synthesis assay and data analysis**

ATP synthesis by TFoF1 was monitored with a luciferase assay, as previously described [33], in a luminometer (Luminescence AB2200; ATT0, Tokyo, Japan) equipped with a sample injection apparatus. The synthesis reaction was driven by acid–base transition and valinomycin-medi-
ated K⁺-diffusion potential as follows. A basic mixture was prepared by mixing 21 µL of the luciferin/luciferase mixture (2 × concentration, ATP bioluminescence assay kit CLSII; Roche, Mannheim, Germany), 870 µL of B-buffer (200 mM Tricine, 10 mM NaH₂PO₄, 2.5 mM MgCl₂ and 120 mM KOH, with the pH adjusted to 8.8 with NaOH) and 9 µL of 50 mM ADP (A-2754; Sigma), and was incubated for 5 min at 30 °C. In experiments for the determination of $K_m$, the concentration of NaH₂PO₄ above was varied between 0.1 and 30 mM ($K_m^{o}$), and the concentration of ADP between 1 µM and 1 mM ($K_m^{ADP}$). In a separate tube, the proteoliposome suspension (30 µL) was mixed with 68 µL of an acidic buffer (A-buffer: 20 mM succinic acid, 14.7 mM NaH₂PO₄, 2.5 mM MgCl₂ and 0.6 mM KOH, with the pH adjusted to 5.1 with NaOH), 1 µL of 50 mM ADP and 1 µL of 20 µM valinomycin in ethanol. In assays for $K_m$, the NaH₂PO₄ concentration above was varied between 0.147 and 44.1 mM, and the ADP concentration between 1 µM and 1 mM. The resultant proteoliposome mixture was incubated for 5 min at 30 °C to allow equilibration across the membrane. Inclusion of ADP in the proteoliposome mixture improved ATP synthesis activity about two-fold. The ATP synthesis reaction was initiated by injecting 100 µL of the proteoliposome mixture into 900 µL of the basic mixture in the luminometer with a syringe (LC-100; Kusano, Tokyo, Japan), and the change in luciferin emission was monitored continuously. At the end of the reaction (60 s), 10 µL of 10 µM ATP was added three times for calibration. The ADP solution that we used contained ATP amounting to 0.05% or 0.2% ADP, depending on the lot, as determined by the luciferase assay. The amount of contaminating Pi in the reaction mixture was 25 µM as assessed with the EnzChek Phosphate Assay Kit (Invitrogen, Eugene, OR, USA). Unless otherwise indicated, the final concentrations of TF₀F₁, ADP and Pi in the reaction mixture were 17 nM, 0.5 mM and 10 mM, respectively. The activity values reported are the average over three to five measurements, each with a different preparation in most cases, and the error bars in the figures show the range. The pH values of the reaction mixture and the acidified proteoliposome mixture, termed $pH_{out}$ and $pH_{in}$, respectively, were measured with a glass electrode, and $\Delta pH$ is defined as $(pH_{out} - pH_{in})$. The membrane potential was calculated from the Nernst equation, $\Delta \psi = (RT/F)\log([K^+]_{out}/[K^+]_{in})$ or $60 \cdot \log([K^+]_{out}/[K^+]_{in})$ in millivolts for our experiments at 30 °C. The magnitude of the PMF is given (in mV) as $60 \cdot \Delta pH + \Delta \psi$.

**Calculation of $K_m$**

$K_m$ values were estimated by nonlinear fit with ORIGIN (OriginLab). The synthesis activity, $V$, was fitted with the equation $V = (V_{max} \cdot [S])/(K_m + [S])$, where S is ADP or Pi.

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