Supporting Online Material for
Axle-Less F₁-ATPase Rotates in the Correct Direction


*To whom correspondence should be addressed. E-mail: kazuhiko@waseda.jp

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**Supporting References**

**Online Movie 1**
Rotation of a 40-nm gold bead attached to γ-ΔN22C43 (2 mM ATP).
This movie corresponds to N22C43 in Fig. 3D, -1 s to 15 s. Blue frames indicate irregular motions, and green frames backward steps. Original images (390×390 nm² = 7×7 pixels) have been Gaussian-filtered and 8× magnified to aid perceive rotation. Original movie at 125 frames s⁻¹ is replayed at 25 frames s⁻¹ (5× slow replay).

**Online Movie 2**
Rotation of a 40-nm gold bead attached to γ-ΔN18C40 (2 mM ATP).
This movie corresponds to N18C40 in Fig. 3D, 0 s to 8 s. Blue frames indicate irregular motions, and green frames backward steps. Original images (390×390 nm² = 7×7 pixels) have been Gaussian-filtered and 8× magnified. Original movie at 250 frames s⁻¹ have been re-sampled at 125 frames s⁻¹ and is replayed at 25 frames s⁻¹ (5× slow replay).

**Online Movie 3**
Rotation of a 40-nm gold bead attached to γ-ΔN14C36 (2 mM ATP).
This movie corresponds to lower N14C36 in Fig. 3A, 17 s to 22 s. Blue frames indicate irregular motions. Original images (390×390 nm² = 7×7 pixels) have been Gaussian-filtered and 8× magnified. Original movie at 500 frames s⁻¹ have been re-sampled at 125 frames s⁻¹ and is replayed at 25 frames s⁻¹ (5× slow replay).

**Online Movie 4**
Rotation of a 40-nm gold bead attached to wild-type TF1 (2 µM ATP).
This movie corresponds to wild type in Fig. 3C, 0 s to 1 s. Original images (390×390 nm² = 7×7 pixels) have been Gaussian-filtered and 8× magnified. Original movie at 8,000 frames s⁻¹ have been re-sampled at 1,000 frames s⁻¹ and is replayed at 25 frames s⁻¹ (40× slow replay).

**Online Movie 5**
Rotation of a 0.29-µm polystyrene bead duplex attached to $\gamma$-ΔN7C29 (2 mM ATP).
This movie corresponds to the lowest N7C29 in Fig. 4A, 0 s to 40 s. Original movie at 500 frames s$^{-1}$ have been re-sampled at 25 frames s$^{-1}$ (real time replay).

**Online Movie 6**
Rotation of a 0.29-µm polystyrene bead duplex attached to wild type TF1 (200 nM ATP).
This movie corresponds to wild type in Fig. 4A, 0 s to 40 s. Original movie at 500 frames s$^{-1}$ have been re-sampled at 25 frames s$^{-1}$ (real time replay).
Materials and Methods

Molecular genetics and confirmation of truncation

Mutations were made on plasmid pKABG1/HC95 that carries genes for the \( \alpha \) (C193S), \( \beta \) (His10 at amino terminus), and \( \gamma \) (S107C, I210C) subunits of TF1 (S1, S2). We introduced \( \gamma \) C-terminal truncations in pKABG1/HC95 as previously described (S3). First, we transferred a \( B_{\text{gl}II} \)-\( M_{\text{lu}I} \) fragment of plasmid pKABG1/HC95 containing the region coding \( \gamma \) (thick arrow in the diagram) into a plasmid derivative of pET-42b(+) (Novagen, Tokyo) in which Xho\(_I\)-Eco\(_{RI}\) region had been replaced with a fragment lacking an HindIII site. The resultant plasmid contained a sole HindIII-Nhel region, which encompassed the \( \gamma \) C-terminus. To mutate this region, we prepared an HindIII-Nhel fragment with a desired \( \gamma \) truncation by PCR amplification using pKABG1/HC95 as template and the following primers.

A forward primer containing an HindIII-site:

\[
5'\text{-GGAAGCTTCTGCCGCTCACTGAC-3'}
\]

Reverse primers containing an Nhel-site:

- for \( \gamma\Delta C25 \),
  \[
  5'\text{-GGGCTAGCTTATTAAGGCGTCAATGCTGAATGAGCTCG-3'}
  \]
- for \( \gamma\Delta C29 \),
  \[
  5'\text{-GGGCTAGCTTATTAAGGCGTCAATGCTGAATGAGCTCG-3'}
  \]
- for \( \gamma\Delta C32 \),
  \[
  5'\text{-GGGCTAGCTTATTAAGGCGTCAATGCTGAATGAGCTCG-3'}
  \]
- for \( \gamma\Delta C36 \),
  \[
  5'\text{-GGGCTAGCTTATTAAGGCGTCAATGCTGAATGAGCTCG-3'}
  \]
- for \( \gamma\Delta C40 \),
  \[
  5'\text{-GGGCTAGCTTATTAAGGCGTCAATGCTGAATGAGCTCG-3'}
  \]
- for \( \gamma\Delta C43 \),
  \[
  5'\text{-GGGCTAGCTTATTAAGGCGTCAATGCTGAATGAGCTCG-3'}
  \]

The HindIII-Nhel portion of the amplified fragment was transferred back to the modified pET-42b(+), and its \( B_{\text{gl}II}\)-\( M_{\text{lu}I} \) fragment was introduced into pKABG1/HC95 to produce the expression plasmid pKABG1/HC95(\( \gamma\Delta C \)).

For N-terminal truncations, we first introduced into pKABG1/HC95 an \( N_{\text{sp}V} \) site immediately upstream the \( \gamma \)-coding region and an \( S_{\text{pel}I} \) site ~300 bases down stream by silent mutations using QuickChange XL Site-Directed Mutagenesis kit (Stratagene, CA). We refer to this plasmid as pKABG1-gN. Primers used for the \( N_{\text{sp}V} \) site were

\[
5'\text{-CGCCTGTtGATGAAACCCGCGTTCGAAAAAGGAGGTGAACCAACCATGCG-3'}
\]

and

\[
5'\text{-GCGATGGGTTTCACTCCCTTCTTCTGAAACACGGGTCTTCTACAAAGCG-3'}
\]

and those for the \( S_{\text{pel}I} \) site were

\[
5'\text{-GCAACGTTGTGCGACCTAGTGACACGAACGTTTGTACACAGGC-3'}
\]

and

\[
5'\text{-CGTTTTTGGACGTTTGGTACACTAGTCGCAACACGGTTGC-3'}
\]
where underscores show mutations.

We then introduced the \(\gamma-\Delta C\) mutations into pKABG1-gN by replacing its SpeI-MluI portion with a truncated sequence. The latter was obtained by PCR using pKABG/HC95(\(\gamma-\Delta C\)) above as template and a forward primer containing an SpeI site

\[5'-GCAACGTGTTGCGACTAGTGTAAC-3'\]

and a reverse primer containing an MluI site

\[5'-CTTGGATAAAGCTGCCTTTGTC-3'.\]

Finally, we introduced N-terminal truncations by replacing the \(NspV\)-SpeI portion that encompasses the \(\gamma\) N-terminus with a truncated sequence. The truncated sequences were obtained by PCR using pKABG1-gN as template and following primers.

A reverse primer containing an SpeI-site:

\[5'-GCAACGTGTTGCGACTAGTGTAAC-3'\]

Forward primers containing an NspV site:

for \(\gamma-\Delta N4C25\), 5'-GAACCCGTGTTGCGACTAGTGTAAC-3'

for \(\gamma-\Delta N7C29\), 5'-GAACCCGTGTTGCGACTAGTGTAAC-3'

for \(\gamma-\Delta N11C32\), 5'-GAACCCGTGTTGCGACTAGTGTAAC-3'

for \(\gamma-\Delta N14C36\), 5'-GAACCCGTGTTGCGACTAGTGTAAC-3'

for \(\gamma-\Delta N18C40\), 5'-GAACCCGTGTTGCGACTAGTGTAAC-3'

for \(\gamma-\Delta N22C43\), 5'-GAACCCGTGTTGCGACTAGTGTAAC-3'

where underscores show mutations.

The mutant lacking \(\gamma\) entirely, the \(\alpha_3\beta_3\) complex, was produced by introducing into pKABG1-gN two \(XhoI\) sites, one between \(NspV\) site and the beginning of the \(\gamma\) coding region and the other between the end and \(NheI\) site, and then removing the \(XhoI\)-\(XhoI\) portion. The replacement sequence was obtained by PCR using pKABG1-gN as template and a forward primer containing an \(NheI\) site and an \(XhoI\) site

\[5'-AAATTTGCTAGCGCTCGACTCGTTGCTGTAAC-3'\]

and a forward primer containing an \(NspV\) site and an \(XhoI\) site

\[5'-GAACCCGTGTTGCGACTAGTGTAAC-3'.\]

where underscores show mutations.

All mutations above were confirmed by DNA sequencing (FASMAC Co., Ltd., DNA sequencing services, Atsugi, Japan). In \(E.\ coli\) strain JM103 \(\Delta\text{(uncB-uncD)}\) which has lost the ability to express authentic \(F_oF_1\)-ATPase (\(S4\)), all mutants were expressed to a level similar to wild type, as seen in fig. S1. The N-terminal five residues of expressed \(\gamma\) were confirmed by Edman degradation (APRO Life Science Institute, Inc., Naruto, Japan) as shown in table S1. The initiation residue Met-1 was absent in the wild type, \(\gamma-\Delta N7C29\) (~30% had Met-1), and \(\gamma-\Delta N11C32\). The mutants \(\gamma-\Delta N4C25\), \(\gamma-\Delta N7C29\), \(\gamma-\Delta N11C32\), and \(\gamma-\Delta N14C36\) were also checked by MALDI-TOF mass spectrometry (APRO Life Science
Institute) as shown in table S1. Peaks corresponding to $\alpha$, $\beta$, and $\gamma$ subunits were obvious except for $\gamma$-$\Delta$N14C36, for which the $\gamma$ peak appeared on a side of the $\beta$ ($z=2$) peak.

[The amino-acid sequence here and in Fig. 1B in the main text is from ref. S5, except that our numbering in this work and in ref. S3 starts from Met-1, which is absent in the expressed wild type. Recently we have found that the actual sequence of TF$_1$ that we used here (and in ref. S3) is slightly different from the original one in ref. S5 and that the C-terminus of the wild type in this work is Gln-285 and not Gln-283, counting from Met-1 (T. Suzuki and M. Yoshida, unpublished). Because the differences are not in the N- and C-termini, we adopt the published sequence for description of truncation. The expected masses in the table below, however, have been calculated on the basis of the actual sequence.]

![Fig. S1](image)

**Fig. S1.** Expression of $\alpha$$\beta$$\gamma$ subunits of TF$_1$ in *E. coli*. *E. coli* pellets were dissolved in 2% SDS and, after centrifugation to remove insoluble materials, applied to 12.5% polyacrylamide gel containing 0.1% SDS. Stained with Coomassie Brilliant Blue R-250.
**Table S1.** Confirmation of γ truncations

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Amino acid sequence at N-terminus of γ (right to left)*</th>
<th>Experimental mass m/z (z = 1)</th>
<th>Expected mass of γ m/z</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>-DRLSA</td>
<td>54714.8 (expected)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-ΔN4C25</td>
<td>-TKIDM</td>
<td>54720</td>
<td>53360 28720 28728.2  Met5-Leu258</td>
<td></td>
</tr>
<tr>
<td>γ-ΔN7C29</td>
<td>-ANIRT (~70%) -NIRT (~30%)</td>
<td>54720</td>
<td>53360 27800 27812.0  Thr9-Arg254</td>
<td></td>
</tr>
<tr>
<td>γ-ΔN11C32</td>
<td>-TKKTA</td>
<td>54720</td>
<td>53360 26940 26945.0  Ala13-Glu251</td>
<td></td>
</tr>
<tr>
<td>γ-ΔN14C36</td>
<td>-QSTKM</td>
<td>54720</td>
<td>53360 26350 26347.4  Met15-Arg247</td>
<td></td>
</tr>
<tr>
<td>γ-ΔN18C40</td>
<td>-AK(T)iM (T: unclear)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-ΔN22C43</td>
<td>-VMEMM</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Italic M indicates undigested Met-1.

**Purification of F₁ and the measurement of ATP hydrolysis activity**

Mutant TF₁’s were expressed in *E. coli* strain JM103 Δ(uncB-uncD), purified and biotinylated as described (S6), except that heat treatment and passage through a butyl-Toyopearl column (for removal of bound nucleotide) were omitted. (Samples of wild type, γ-ΔC17, and γ-ΔC21 for hydrolysis assay were passed through a butyl column.) The passages through a Ni²⁺-NTA superflow column (Qiagen, Hilden, Germany) and a size-exclusion column (Superdex 200, 10/300 GL, GE Healthcare UK Ltd., UK) and biotinylation were done and purified TF₁ was kept at room temperature. Mutant proteins were used immediately, within at most three days.

The rate of ATP hydrolysis by the mutants was measured at 10-20 nM F₁, without biotinylation, at 23°C by coupling the reaction with consumption of NADH (S3); the rate was estimated in the initial ~20 s period (fig. S2).
**fig. S2.** Time courses of ATP hydrolysis at 2 mM ATP by 10 nM mutant F₁. A decrease in absorbance by 0.622 corresponds to consumption of 100 μM ATP. Hydrolysis rates were estimated in the initial 20-s portion (thick black), as shown by the thin black lines. Temperature, 23°C.

**Observation of rotation with gold beads**

We modified the surface of 40-nm gold beads (EM.GC40, a suspension at 9×10¹⁰ particles ml⁻¹, BBInternational, Cardiff, UK) with polyethylene glycol (PEG) and PEG-biotin by adding 0.2 mM 2-aminoethanthiol (Tokyo Chemical Industry, Tokyo), 0.1 mM NHS-m-dPEG (molecular weight: 1214, Quanta BioDesign, Powell) and 0.1 mM NHS-dPEG4-Biotin (Quanta BioDesign) to the bead suspension and letting the reaction proceed at room temperature for more than one day. Before use, we washed the gold beads extensively with buffer A (10 mM Mops-KOH, pH 7.0, 50 mM KCl) to remove free biotin.

Biotinylated F₁ (10-100 nM) in 10 mM Mops-KOH, 20 mM imidazole-HCl, pH7.0, 400 mM KCl, or in buffer A for wild type, γ-ΔC17, and γ-ΔC21, was infused into an observation chamber made of Ni-NTA coated bottom and uncoated top coverslips (S3). After washing out unbound F₁ with buffer A, we infused 10 mg ml⁻¹ BSA in buffer A to
prevent nonspecific binding, and then 40 µg ml\(^{-1}\) streptavidin (Pierce, Rockford) in buffer A. After extensive washing with buffer A, we infused the modified gold beads and washed away excess beads with buffer A. Finally, we infused buffer A containing 2 mM MgATP, 2 mM MgCl\(_2\), and an ATP regenerating system (0.2 mg ml\(^{-1}\) creatine kinase and 2.5 mM creatine phosphate).

Bead rotation was observed at 23°C by laser dark-field microscopy (S2) with some modifications (S. Furuike, unpublished), on an inverted microscope (Olympus IX70). Images were captured with a high-speed CMOS camera (FASTCAM-DJV, Photron, Tokyo) at 125 to 8,000 frames s\(^{-1}\) as an 8-bit AVI file. Centroid of bead images was calculated as described (S2).

**Observation of rotation with polystyrene beads**

Biotinylated TF\(_1\) (10-100 nM) was infused into an observation chamber made of Ni-NTA coated bottom and uncoated top coverslips (S3). Streptavidin-coated polystyrene beads of diameter 0.29 µm (Seradyn) were infused, followed by buffer A containing 2 mM MgATP, 2 mM MgCl\(_2\) and the ATP regenerating system. Rotation was observed at 23°C on an inverted microscope (Olympus IX71) with a stable mechanical stage (KS-O, Chuukousha-Seisakujo, Japan). Ordinary transmission images of the beads were captured with a CCD camera (IPX-210M, Lynx) at 500 frames s\(^{-1}\) as an 8-bit AVI file. The apparent torque was estimated from the instantaneous speed during stepping rotation as described (S3).
**fig. S3.** Estimation of torque from rotation of 0.29-µm bead duplexes. (A) Torque estimation from the instantaneous rotary speed during 120° steps. Thin colored lines show 30 consecutive steps, thick cyan lines being the average. Individual step records have been shifted vertically by a multiple of 120° to obtain the overlap. Time zero for each step record was assigned by eye to the data point closest to 60°. Straight red lines fit the cyan line between 30° and 90°. Torque was calculated as the slope of the red line (in radian s⁻¹) times the frictional drag coefficient of the bead duplex (0.28-0.34 pN·nm·s). Wild type gave torque values of 44±4 pN·nm (mean±SD, n = 3); γ-ΔN4C25, 23±3 pN·nm (n = 3); γ-ΔN7C29, 22±2 pN·nm (n = 3); γ-ΔN11C32, 18 pN·nm (n = 1). (B) Torque estimation from triplets of 120°-steps. Colored thin lines show 10 consecutive triplets in Fig. 4A, γ-ΔN4C25, arrow. Thick cyan line, average; red line, fit between 150° and 210°; yellow dotted line, fit between 60° and 300°. The yellow line gives a torque of 8.3 pN·nm, which should be an underestimate because some of the curves are stuck in the middle.
Supporting References


