Evidence for rotation of V_1-ATPase

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V_oV_1-ATPase is responsible for acidification of eukaryotic intracellular compartments and ATP synthesis of Archaea and some eubacteria. From the similarity to F_oF_1-ATP synthase, V_oV_1-ATPase has been assumed to be a rotary motor, but to date there are no experimental data to support this. Here we visualized the rotation of single molecules of V_1-ATPase, a catalytic subcomplex of V_oV_1-ATPase. V_1-ATPase from *Thermus thermophilus* was immobilized onto a glass surface, and a bead was attached to the D or F subunit through the biotin-streptavidin linkage. In both cases we observed ATP-dependent rotations of beads, the direction of which was always counterclockwise viewed from the membrane side. Given that three ATP molecules are hydrolyzed per one revolution, rates of rotation agree consistently with rates of ATP hydrolysis at saturating ATP concentrations. This study provides experimental evidence that V_oV_1-ATPase is a rotary motor and that both D and F subunits constitute a rotor shaft.

Two subclasses of the ATPase/ATP-synthase superfamily that catalyze the exchange of the energy of proton translocation across membranes and the energy of ATP hydrolysis/synthesis are V_oV_1-ATPase and F_oF_1-ATP synthase (1–3). V_o and F_o are the integral membrane proton channel portion. V_1 and F_1 are the soluble portion that contain catalytic sites for ATP hydrolysis/synthesis. V_oV_1-ATPases exist in membranes of various intracellular acidic compartments of eukaryotic cells (1, 4) and plasma membranes of Archaea and some eubacteria (5–7). F_oF_1-ATP synthases are responsible for ATP production in mitochondria, chloroplasts, and respiring bacteria (2). Several years ago, rotation of the centrally located γ subunit in the surrounding $\alpha_3\beta_3$ hexamer cylinder in the isolated F_1 was video-imaged (8), and the rotary catalysis mechanism of F_oF_1-ATP synthase was established (3, 9). Given the functional and structural similarity between V_oV_1-ATPase and F_oF_1-ATP synthase, it has been assumed that V_oV_1-ATPase would use a similar rotary mechanism to the F_oF_1-ATP synthase. However, without a precise knowledge of the atomic structure or even accurate subunit arrangement in V_oV_1-ATPase, the attempts to prove this assumption have been unsuccessful.

We previously identified V_oV_1-ATPase in an aerobic thermophilic eubacterium, *Thermus thermophilus* (5, 10, 11). The V_1 portion of *T. thermophilus*, which is ATPase-active and hence called V_1-ATPase, is made up of four subunits: A (63.6 kDa), B (53.1 kDa), D (24.7 kDa), and F (11.7 kDa) with a stoichiometry of $A_3B_3D_1F_1$ (5). The A subunit contains a catalytic site, and the A and B subunits are arranged alternately, forming a hexameric cylinder similar to the $\alpha_3\beta_3$ of F_oF_1-ATPase. The D subunit most likely fills the central cavity of the A_3B_3 cylinder (11). Cross-linking studies have suggested that the D subunit forms part of the central stalk, and the F subunit is assumed to be associated with the D subunit (12–14). Here we report the visual demonstration of ATP-dependent rotation of V_1-ATPase. The results also show that both D and F subunits constitute a rotor shaft with respect to the stator A_3B_3 cylinder.

Materials and Methods

Protein Preparation. *Escherichia coli* strain BL21-CodonPlus-RP (Stratagene) was used for expression of V_1-ATPase from *T. thermophilus* HB8. Plasmid pUCV1, carrying genes for A, B, D, and F subunits under regulation of *lac* promoter, was used for gene expression. The mutant V_1-ATPases (A-His-8-tags/ΔCys/A-S232A/A-T235S/D-E48C/D-Q55C) and (A-His-8-tags/ΔCys/A-S232A/A-T235S/F-S28C/F-S35C) were used for rotation experiments and are referred to as V_1-ATPase in this report unless specified otherwise. The expressed cells were suspended in 20 mM imidazole HCl (pH 8.0) containing 0.3 M NaCl and disrupted by sonication. After removal of heat-labile proteins derived from the host cells by heat treatment at 65°C for 30 min, the solution was applied to a Ni^{2+}-affinity column (Amersham Pharmacia), which then was washed thoroughly and eluted with 0.5 M imidazole HCl (pH 8.0) containing 0.3 M NaCl. The buffer was exchanged to 20 mM Tris·HCl (pH 8.0) containing 1 mM EDTA by ultrafiltration (VIVA-spin, VIVA Science, Gloucestershire, U.K.), and the solution was applied to a RESOURCE Q column (Amersham Pharmacia). The fractions containing V_1-ATPase were concentrated, and contaminating proteins were removed with a Superdex 200 column (Amerhsam Pharmacia). The above purification procedures were carried out at 25°C and completed within 6 h. The purified V_1-ATPase was immediately biotinylated with a 2 M excess of 6-{*N*′-[2-(*N*-maleimido)ethyl]-*N*-piperazinylamido}hexyl D-biotinamide (biotin-$PEAC_5$-maleimide, Dojindo, Kumamoto, Japan) in 20 mM Mops-KOH (pH 7.0) containing 100 mM KCl. After a 15-min incubation at 25°C, proteins were separated from unbound reagent with a PD-10 column (Amersham Pharmacia). The biotinylated V_1-ATPases were kept on ice and used for experiments within 3 days. Specific biotinylation of the D or F subunit was checked by Western blotting using streptavidin-alkaline phosphatase conjugate (Amersham Pharmacia).

Rotation Experiments. A flow cell (5 μl) was made of two coverslips (bottom, 24×36 mm^2; top, 18×18 mm^2) separated by two spacers of 50-nm thickness (8). The glass surface of the bottom coverslip was coated with Ni^{2+}-nitrilotriacetic acid. The biotinylated V_1-ATPase (0.1–1 μM) in buffer A [50 mM Tris·HCl, pH 8.0/100 mM KCl/5 mM $MgCl_2$/0.5% BSA (wt/vol)] was applied to the flow cell and washed with 20 μl of buffer A. The suspension (10 μl) of 0.1% (wt/vol) streptavidin-coated beads (ϕ = 0.56 μm, Bangs Laboratories, Carmel, IN) in buffer A was infused into the flow cell, and unbound beads were washed out with 40 μl of buffer A. Observation of rotation was started after infusion of 10 μl of buffer A supplemented with indicated concentrations of ATP and an ATP-regenerating system (0.2 mg/ml creatine kinase and 2.5 mM creatine phosphate). The rotation of beads was

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Abbreviation: rps, revolutions per second.

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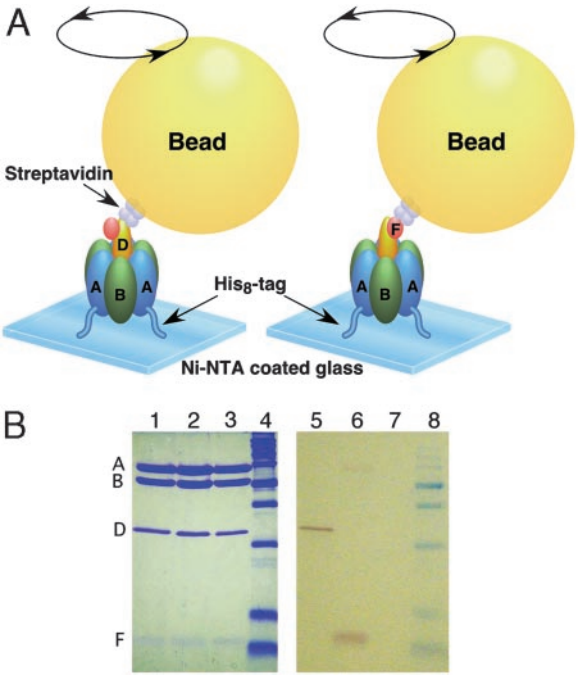

Fig. 1. (*A*) Experimental setup to observe rotation of V_1-ATPase. The V_1-ATPase was fixed on the glass surface with amino-terminal His-8 tags of the A subunits. A bead was attached to the D (*Left*) or F (*Right*) subunit through biotin-streptavidin linkage. Rotation of obliquely attached beads was observed. The arrows indicate the direction of rotation. (*B*) Biotinylation of the D and F subunits. The biotinylated V_1-ATPases were analyzed with 15% acrylamide gel electrophoresis in the presence of SDS. (*Left*) Protein staining with Coomassie brilliant blue. (*Right*) Western blotting stained by alkaline phosphatase-streptavidin conjugate. Lanes 1 and 5, V_1-ATPase that has the biotinylated D subunit; lanes 2 and 6, V_1-ATPase that has the biotinylated F subunit; lanes 3 and 7, nonbiotinylated V_1-ATPase; lanes 4 and 8, molecular mass standards (250, 150, 100, 75, 50, 37, 25, 15, and 10 kDa).

observed with a bright-field microscope (IX70, Olympus, New Hyde Park, NY) at a magnification of ×1,000. Images were video-recorded (30 frames per second) with a charge-coupled device camera. All these procedures and observations were carried out at 25°C.

Other Assays. Protein concentrations of V_1-ATPase were determined from UV absorbance calibrated by quantitative amino acid analysis; 1 mg/ml gives a 0.59 OD at 280 nm. ATP hydrolysis activity was measured by the rate of NADH oxidation coupled with pyruvate kinase and lactate dehydrogenase (Roche, Mannheim, Germany) as described (15).

Results and Discussion

Enzymes Suitable for Rotation Observation. The observation system of V_1-ATPase rotation is similar to that used for F_1-ATPase (Fig. 1*A*; ref. 16). Rotation was visualized by a bead obliquely attached to the D or F subunit, which was illuminated as a bright-field image under the optical microscopic field. To immobilize the enzyme to the Ni^{2+}-nitrilotriacetic acid-coated glass surface, His-8 tags were added to the amino termini of the A subunits. Differing from most eukaryotic counterparts, V_1-ATPase from *T. thermophilus* is ATPase-active. However, this enzyme has strong propensity to lapse into the so-called MgADP-inhibited form (see ref. 17) during catalytic turnovers; half the population of the wild-type V_1-ATPase molecules are subject to this inhibition within ≈5 min after the addition of substrate ATP, and most molecules stop ATP

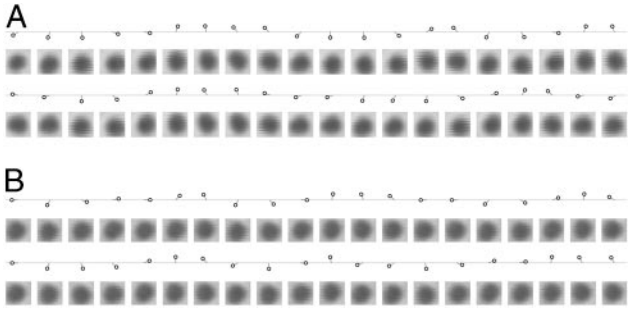

Fig. 2. Sequential images of a rotating bead at 4 mM ATP. A bead attached to the D subunit (*A*)and that attached to the F subunit (*B*) are shown. Centroid positions are shown above the images. The interval between images is 33 msec.

hydrolysis in 10 min (15). This time interval is too short to find and analyze the rotating molecules. Several mutant forms of the enzyme were generated to overcome this disadvantage. It was found that the S232A/T235S double substitution in subunit A suppressed the problem of MgADP inhibition, and the ATPase activity of the mutant enzyme remained for 1 h after the addition of substrate.

The rotation probe, a bead, was attached to the presumed rotor-shaft subunits, D and F, through the biotin-streptavidin linkage. *T. thermophilus* V_1-ATPase contains nine cysteine residues in the three copies of the A subunit (Cys-28, Cys-255, and Cys-507) and three in each of the three B subunits (Cys-264). We substituted all of these with serine residues to ensure the specificity of the reaction of the newly introduced cysteine with the biotin-maleimide reagent. It is difficult to predict which residue should be replaced by cysteine, because the atomic structure of V_1-ATPase is not currently known. Therefore, we tested 22 mutant V_1-ATPases containing single- or double-cysteine residues introduced at various positions in the D subunit. Of these, the nine mutants allowed biotinylation of the introduced cysteine(s) and two showed measurable rotation. Similarly, five mutants of the F subunits were tested, and the three that biotinylated all showed rotation. For this study we used the mutant V_1-ATPases (A-His-8-tags/ΔCys/A-S232A/A-T235S/D-E48C/D-Q55C) and (A-His-8-tags/ΔCys/A-S232A/A-T235S/F-S28C/F-S35C) to observe rotation of the D and F subunits, respectively, and all the data in this article are those obtained with these mutants.

The two mutants exhibited similar Michaelis–Menten kinetics with K_m values from 0.3 to 0.5 mM and a k_{cat} value of ≈10 sec^{-1}. These values are in the same range of those of the wild-type enzyme (15). Biotin was introduced specifically into either the D or F subunit in the mutant V_1-ATPases (Fig. 1*B*). It was confirmed that beads observed under microscope were attached to the V_1-ATPases through biotin-streptavidin linkage, because very few beads were found when nonbiotinylated mutant V_1-ATPases were used. Specific immobilization of the V_1-ATPase molecules on the glass surface coated with Ni^{2+}-nitrilotriacetic acid through a His-8 tag was confirmed from the nearly complete loss of the beads from the glass surface by washing the flow cell with 2 M imidazole buffer.

Rotation of the D Subunit. We found rotating beads attached to the D subunit in V_1-ATPase when the flow cell was infused by a buffer containing ATP (Figs. 2*A* and 3). Five to 10 rotating beads were usually found in the 0.2-mm^2 area of a single flow cell. However, the number of rotating beads declined with time, and very few rotating beads were found 30 min after the addition of ATP. Rotations were unidirectional and, similar to F_1-ATPase, directions were always counterclockwise when

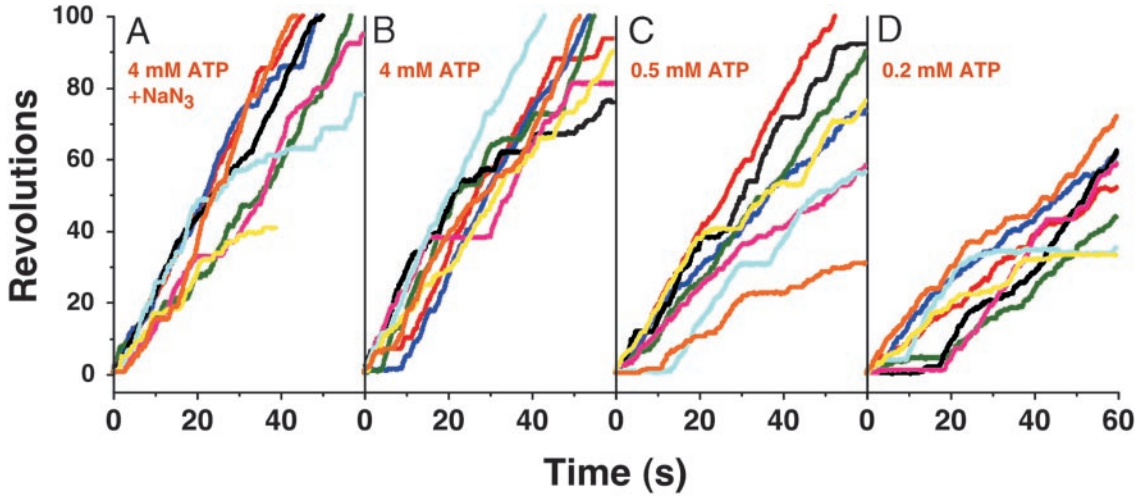

Fig. 3. Time courses of rotation of beads attached to the D subunit. (*A*) Bead rotation at 4 mM ATP in the presence of sodium azide (0.5 mM). (*B–D*) Bead rotation in the absence of sodium azide at 4 (*B*), 0.5 (*C*), and 0.2 (*D*) mM ATP.

viewed from the membrane (V_o) side (8). When the infusing buffer did not contain ATP, obvious unidirectional rotation apart from Brownian fluctuation was not found. It is known that azide inhibits ATPase activity and hence rotation of F_1-ATPase (8), but it does not inhibit ATPase activity of V_1-ATPase (5). Consistently, azide did not affect the observed rotation of V_1-ATPase at 4 mM ATP (Fig. 3 *A* and *B*) and 0.1 mM ATP (data not shown).

The rotation of many beads continued for hundreds of revolutions, but trajectories of rotations were not smooth but rather ragged; rotations sometimes were interrupted by pauses. The cause of these interruptions may be investigated after the angular and time resolutions of the observation system are improved sufficiently. The average rotation rate at 4 mM ATP was calculated to be ≈2.6 revolutions per sec (rps) for the beads showing apparently uninhibited rotation that continued >20 sec without interruption longer than 2 sec. Rotations at 1 mM ATP (data not shown) appeared very similar to those observed at 4 mM ATP, and the average rotation rate of ≈2.4 rps was obtained. Given that the bulk phase kinetics indicates a k_{cat} of ≈10 sec^{-1}, this average rate of rotation shows that three ATPs are hydrolyzed per revolution. At these ATP concentrations, ATP binding to the enzyme occurs fast and is not rate-limiting to the turnover of the ATPase reaction (V_{max} conditions). Agreement of the rate of rotation and rate of ATP hydrolysis under V_{max} conditions indicates that some catalytic events occurring within the enzyme, rather than ATP binding to the enzyme and the viscous frictional load of the rotating beads,‖ determines the rate of continuous rotation. At 0.5 mM ATP, the average rotation rate is 2.2 rps, slightly lower than that of the V_{max} conditions (Fig. 3*C*). At 0.2 mM ATP, the substrate ATP binding to the enzyme becomes the rate-limiting step in the whole catalytic cycle, and the rotation rate slowed significantly (Fig. 3*D*). These results, along with the prediction that the D subunit is a highly helical polypeptide, suggest that the D subunit is a functional homologue of the γ subunit, a major rotor-shaft subunit of F_1-ATPase.

‖The viscous frictional load of the rotating bead at 2.6 rps is calculated to be ≈15 pN·nm (18). The rotation is not impeded by the load of this magnitude, because the ATP hydrolysis rate of immobilized, bead-attached V_1-ATPase estimated from the rate of bead rotation is nearly the same as the ATP hydrolysis rate of free V_1-ATPase in the bulk medium, where the frictional load is negligible.

Rotation of the F Subunit. Beads attached to the F subunit also rotated. Usually, one to three rotating beads were found in the 0.2-mm^2 area of a flow cell at 4 mM ATP (Figs. 2*B* and 4). During observation of rotation of the F subunit, rotating bead was sometimes detached from glass surface and floated into solution. It is most likely that the dissociation of F subunit-attached bead from A_3B_3D might decrease the number of rotating beads per flow cell. The direction of rotation was always counterclockwise. The average rate of rotation is ≈2.5 rps, the same as observed for beads attached to the D subunit. The close proximity of the F subunit to the D subunit has been suggested by cross-linking studies (12) and the complex formation of these subunits in some yeast mutant V_oV_1-ATPase strains (13). It now is clear that the F subunit associates with the D subunit, forming a rotor apparatus in V_oV_1-ATPase.

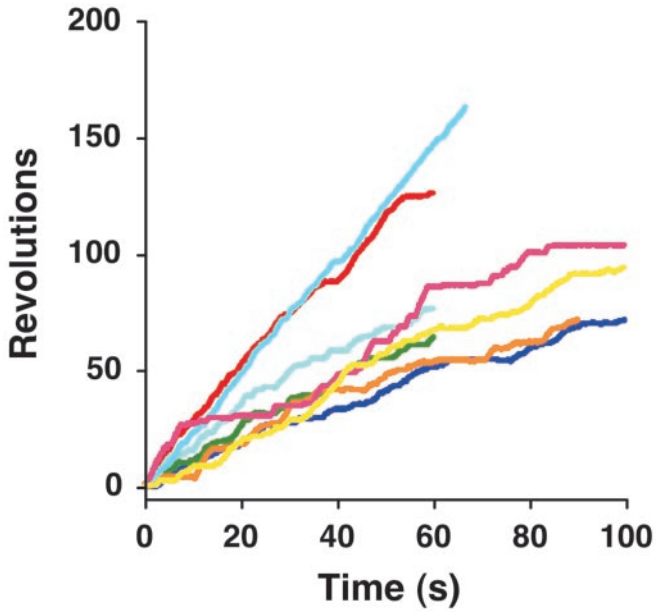

Fig. 4. Time courses of rotation of beads attached to the F subunit. A bead was attached to the F subunit, and the rotation was observed at 4 mM ATP. The details of experimental conditions are as described in *Materials and Methods*.

Conclusion. We visualized the ATP-dependent rotation of the V_1-ATPase. The direction of the rotation is the same as that of F_1-ATPase. The D and F subunits rotate relative to the A_3B_3 ring. Thus, strong evidence is now given to the contention that not only F_oF_1-ATP synthase but also V_oV_1-ATPase work through a rotary catalysis mechanism.

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