F_OF_1-ATP synthase (F_OF_1) has been investigated by using several single-molecule analytical techniques as well as by biochemical methods. The studies have advanced our understanding of the rotary catalysis and inhibitory mechanisms. However, because most of the studies were performed with bacterial F_OF_1s, molecular mechanisms of factors unique in eukaryotic F_OF_1, such as IF_1, ε subunit (no counterpart in bacterial F_OF_1) and inhibitory compounds, still remains to be clarified. To overcome the gap, we constructed a heterologous expression system for human F_1-ATPase (HF_1) using *E. coli* as a host cell. Five kinds of genes encoding for human F_1-ATPase (α, β, γ, δ, and ε) were cloned from human cDNA library and then, introduced into an expression vector. HF_1 was successfully expressed in F_OF_1-deficient *E. coli* cells. The recombinant HF_1 was purified from the cytoplasmic fraction of the cells by using Ni-Sepharose (histidine-tag was introduced at N-terminus of β subunit) and gel-filtration column chromatography. Thus obtained HF_1 complex showed significant ATPase-activity, of which 95% was inactivated by 2.5 mM sodium azide. The azide-sensitive ATPase was inhibited almost completely (93%) by 1 μM efrapeptins with 25 nM IC_{50}, while F_OF_1s of *Bacillus* PS3 and *E. coli* showed no sensitivity at all to efrapeptins. Also, 87% of ATPase was inhibited by 2.4 μM IF_1 ($IC_{50} = 0.35$ μM). These results suggest the intact characters of the recombinant HF_1. Then, HF_1 was subjected to single molecule analysis to investigate the rotary catalysis mechanism. Biotinylated HF_1 at the rotor moiety was immobilized on Ni-NTA glass surface, and submicron particle(s) was fixed to the rotor. Under the view of a microscope, counterclockwise rotation (when seen from the F_O-side) was observed. This is the first demonstration of rotation of mammalian F_1-ATPase.

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2P.36 Robustness of torque-transmission between β and γ of F_1-ATPase

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F_1-ATPase is a rotary molecular motor in which the rotor subunit, γ rotates against the $\alpha_3\beta_3$ stator ring upon ATP hydrolysis. The catalytic sites reside at the α–β interfaces, mainly on β subunits. A widely accepted scenario is that ATP-binding leads to the inward swing motion of the C-terminal domain of β toward γ, which is then converted into the γ rotation. Therefore, the directly contacting loop of this domain with γ, which includes a highly conserved sequence, 'DELSEED', is thought to have a critical role in torque-transmission. A recent finding that an axle-less F_1-ATPase still shows unidirectional rotation supports the above scenario although torque of the axle-less F_1 is half of that of the wild-type [1]. While it was suggested that the torque-transmission from β to γ subunit is mediated mainly by specific electrostatic interactions at DELSEED loop [2], mutagenesis works on this loop showed that the torque-transmission mechanism is robust against partial Ala substitution [3] or deletion [4]. With attempt to reveal the robustness of the torque-transmission at this region, we have carried out extensive Gly substitutions around DELSEED loop to completely destroy the specific interaction and also structural rigidity. All of 9 amino acids composing the loop were substituted with Gly, and the ATPase and rotation activities of the mutant were examined. Surprisingly, the mutant F_1 was active in ATPase and rotation. We further substituted, with Gly, 1 or 2 turns of both 2 α-helices connected with the loop. However, still mutant F_1 showed active unidirectional rotation although the average rotary velocity is slower than that of the wild-type. Based on these findings, we concluded that the torque-transmission between β and γ is not mediated by any specific electrostatic or steric interaction and it is designed to be highly robust against perturbation.

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2P.37 Chemomechanical coupling of P_i release on F_1-ATPase

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F_1-ATPase ($\alpha_3\beta_3\gamma$) is a rotary motor protein, which couples ATP hydrolysis to the rotary motion. Extensive studies mostly revealed the chemomechanical coupling mechanism of F_1; however, the timing of P_i release remains to be clarified. Each of three β-subunits, which has the catalytic site, follows the same reaction pathway of ATP hydrolysis, but they are always in a reaction phase differing by ±120° from each other. Then, focusing on one β-subunit, β binds ATP at a particular binding angle. After γ rotates 200°, β cleavages the bound ATP into ADP and P_i. The produced ADP is released from β after further 40° rotation at +240° from the ATP-binding angle. Previous studies also suggested two possibilities of the timing of P_i-release. P_i is released: (i) right after ATP cleavage at +200°, or (ii) at +320° from the ATP-binding angle. In this study, we observed the rotating F_1 and measured the equilibrium of ATP cleavage/synthesis with the magnetic tweezers as previously reported [1]. As F_1 released the produced P_i, the equilibrium was shifted to ATP cleavage; therefore, from the time course of the probability of ATP cleavage, we determined the rate of P_i-release at the angle for ATP cleavage and ADP release as 0.021 $s^{-1}$and 1.5 s^{-1}, respectively. We also determined the rate at the proper angle for P_i-release as 2600 s^{-1} by using the fast-framing camera. From these results, we found that P_i release at the angle for ATP cleavage was the uncoupling side reaction and authentically occurred at +320° from the ATP-binding angle. In addition, the rate of P_i release strongly depended on the rotary angle, and the activation energy change was estimated to be $DE = 5.5k_BT/rad$, which was almost 55% of the net rotary torque exerted by F_1, implying that P_i release is the main torque generating step of F_1.

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2P.38 Assembly and oligomerization of human ATP synthase lacking mitochondrial subunits a and A6L

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ATP synthase from human p^0 (rho zero) cells was almost fully assembled in spite of the absence of subunits a and A6L using clear native electrophoresis (CNE or CN-PAGE). From this we conclude that subunits a and A6L are the last subunits to complete the ATP synthase assembly. Under the CNE conditions small amounts of dimeric and even tetrameric forms of the large assembly intermediate were preserved, suggesting that it associated further into higher order structures in the mitochondrial membrane. This result was comparable to the reduced amounts of dimeric and tetrameric ATP synthases from yeast subunit e and g null mutants detected by CNE. The dimer/oligomer-stabilizing effects of subunits e/g and a/A6L seem additive in human and yeast cells. The mature IF_1 inhibitor was specifically bound to the dimeric/oligomeric forms of ATP synthase and not to the monomer whereas nonprocessed pre-IF_1 still containing the mitochondrial targeting sequence was selectively bound to the monomeric assembly intermediate in p^0 cells and not to the dimeric form. This supports previous suggestions that IF_1 plays an important role in the dimerization/oligomerization of mammalian ATP synthase and in the regulation of mitochondrial structure and function.

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2P.39 Resolving stepping rotation of V-ATPase with an essentially drag-free probe

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Vacuole-type ATPases (V- or VoV1-ATPases), together with F_OF_1 ATP synthases, constitute a superfamily of rotary molecular machines that couple ATP hydrolysis/synthesis in the soluble V_1/F_1 portion with proton (or Na^+) flow in the membrane-embedded V_O/F_O portion through rotation of a common central shaft. Here we have observed at submillisecond resolutions the ATP-driven rotation of isolated V_1 and of the whole V_OV_1 from *Thermus thermophilus*, by attaching a 40-nm gold bead for which viscous drag is almost negligible. At saturating ATP of 4 mM, V_1 rotated at about 60 revolutions/s, with about 5 ms dwells every 120°. Dwell time analyses indicated that at least two events other than ATP binding, one likely ATP hydrolysis, occur in each dwell, as in F_1. Unlike F_1, however, the dwells were at ATP-waiting positions that were resolved at μM ATP. V_OV_1 rotated an order of magnitude slower, and exhibited dwells separated by about 30°. The twelve positions, though not always fully populated, match the twelve-fold symmetry of the V_O rotor in *T. thermophilus*, indicating that the ATP-driven rotation must go through stator–rotor interactions in V_O.

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2P.40 Heterologous expression of the peripheral stalk *Aquifex aeolicus* F_1F_O ATP synthase in *Escherichia coli*

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The hyperthermophilic bacterium *Aquifex aeolicus* possesses a nine-subunit F_1F_O ATP synthase [1]. A part of the complex, called the peripheral stalk, provides the connection between the membrane embedded F_O part and the soluble F_1 part, acting as a stator to counteract the rotation of the catalytic F_1 part during ATP synthesis. Structural information is available to date for the peripheral stalk subunits of the bovine mitochondrial F_1F_O ATP synthase and the *Thermus thermophilus* A_1A_O ATP synthase, respectively [2–5]. However, further structural characterization is necessary because the peripheral stalk is the least conserved component of the complex, differing substantially in composition and stoichiometry among ATP synthase subtypes [5]. In particular, in *A. aeolicus*, the peripheral stalk is exceptional because it is hetero- and not homodimeric and so it differs from that of all other currently known F_1F_O ATP synthases of non-photosynthetic organisms [1]. It mainly contains subunits b_1 and b_2, encoded by genes *aq_1586* and *aq_1587*, which overlap by 1 bp in the genome. We have cloned the two genes and expressed the b_1/b_2 subunits heterologously in *Escherichia coli*. They localize both in *E. coli* membranes and inclusion bodies. Two-dimensional Blue native (2-D BN)/SDS-PAGE, together with peptide mass fingerprint mass spectrometry (PMF-MS) shows that they form a complex in *E. coli* membranes. The b_1/b_2 complex can be isolated from the membranes to a high level of purity in a single chromatographic step. Further studies are in progress to optimize the expression level and to characterize the folding and stability of the b_1/b_2 complex by size exclusion chromatography, circular dichroism and differential scanning calorimetry. The final aim of the project is the determination of the structure of the b_1/b_2 complex by 3-D crystallography.

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