F_1-ATPase from *Saccharomyces cerevisiae* was inhibited under conditions of ATP hydrolysis with a fragment of yeast IF_1 consisting of residues 1–53 (known as yI1–53). The complex was crystallised in the presence of 0.5 mM ADP and 1.5 mM ATP. In the inhibited structure, residues 17–40 of yI1–53 form an α-helix, of which residues 17–35 are bound in a cleft between the C-terminal domains of the α_{DP}- and β_{DP}-subunits, and residues 36–40 of this α-helix extend beyond the external surface of the F_1-domain. The α-helix interacts mainly with the β_{DP}-subunit, but also with subunits α_{DP} and β_{TP}. Residues 1–16 of yI1–53 form a loop from residues 6 to 16 held together by a salt bridge (residues R9 and D15) and a hydrogen-bonding network involving residues S4, R9 and D15 and residue R9 of the γ-subunit. The N-terminal region from residues 1 to 5 extends into the central aqueous cavity of the enzyme around the central stalk and makes contacts with the α_E-, β_{DP}- and γ-subunits. Many aspects of this structure are similar to those of the structure of bovine F_1-I1–60 [1]. However, the structures differ in several significant respects. First, the α-helix of yI1–53 is tilted more steeply (relative to the central stalk) than its counterpart in the bovine structure, and so the detailed interactions that contribute to binding differ in some respects. Second, the loop structure in residues 6–16 of yI1–53 replaces a second short α-helix from residues 13 to 17 of bovine I1–60. Third, the structures differ in the nucleotide occupancies of catalytic subunits; bovine F_1-I1–60 contains inorganic phosphate bound to the P-loop in the β_E-subunit whereas the β_E-subunit in the yeast complex contains ADP, but no magnesium. In both structures, the β_{DP}- and β_{TP}-subunits are occupied by magnesium ADP. The bovine structure was interpreted as representing a post-hydrolysis "dead-end" state [1], whereas the yeast structure appears to represent a post-hydrolysis, pre-product release intermediate that precedes the "ground state" structure of the enzyme in the hydrolytic cycle.

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2P.33 Kinetic equivalence of membrane potential and pH difference across membrane in ATP synthesis by *Bacillus* PS3 F_OF_1-ATP synthase

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F_OF_1-ATP synthase synthesizes ATP by using proton motive force (*pmf*) that consists of transmembrane gradients of electrical potential ($\Delta\psi$) and proton concentration (ΔpH). The two terms are thermodynamically equivalent according to the chemiosmotic theory of P. Mitchell [1]. But their kinetic equivalence to drive F_O has been reported to be varied by the source of F_OF_1s and preparations. Here, using *Bacillus* PS3 F_OF_1 (TF_OF_1) with a mutation lacking inhibitory effect of ε, we have developed simple and highly reproducible procedures to prepare active proteoliposomes and to analyze kinetics of ATP synthesis which was driven by acid–base transition and K^+/valinomycin diffusion potential. TF_OF_1 showed maximum rates of ATP synthesis of 18 s^{-1} at 30° with K_ms for ADP and Pi, 19 and 500 μM, respectively. Then, the rates of ATP synthesis were determined under several combinations of $\Delta\psi$ and ΔpH. The rates were highly correlated to the *pmf* value calculated from $\Delta\psi$ and ΔpH, although small deviation still remains. Osmotic imbalance between inside and outside of proteoliposomes has only little effect on the kinetics. Importantly, when K^+ concentration inside proteoliposomes is decreased below about 3 mM, it appears that Nernst equation tends to overestimate the valinomycin-induced $\Delta\psi$, which is one of the reasons of the deviation. Taking these results into consideration, we propose that rates of ATP synthesis are solely dependent on the magnitude of *pmf* but not on each of $\Delta\psi$ and ΔpH.

Reference

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2P.34 Isolation of the H^+-ATP synthase from *E. coli* and the stability of its subcomplexes

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The H^+-ATP synthase from *E. coli* ($EFOF_1$) and the hydrophilic part (EF_1) are isolated. The enzyme detergent micelles are purified either by sucrose density centrifugation or by ion exchange chromatography followed by gel permeation chromatography. For activity measurements $EFOF_1$ is reconstituted into phosphatidylcholine/phosphatidic acid liposomes and a transmembrane pH-difference is generated by an acid base transition. The initial rate of ATP synthesis is measured with luciferin/luciferase. The activity of EF_1 is measured by ATP hydrolysis with an ATP regenerating system. Analysis of the different fractions obtained after gel permeation chromatography reveals that the highest ATP synthesis activities and the highest ATP hydrolysis activities are found in different fractions, although the SDS-PAGE does not reveal significant differences in subunit composition of these fractions. Since the e-subunit is known to act as inhibitor of ATP hydrolysis without a significant effect on ATP synthesis, the dissociation of this subunit from the different subcomplexes was investigated. The dissociation constant of e was determined as described in [1]. The following dissociation constants are found: $K_D = 5.2$ nM for EF_1, $K_D = 0.7$ nM for $EFOF_1$ micelles, and $K_D = 0.1$ nM for $EFOF_1$ liposomes.

Reference

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2P.35 Biochemical and single-molecule analyses of human F_1-ATPase

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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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