proposed as a regulator of the enzyme to avoid wasteful ATP hydrolysis. The regulatory function has been studied biochemically, but not physiologically. Here, physiological importance of the regulation with the ε subunit was investigated by using *E. coli* and *B. subtilis* mutants which is defective in the regulatory domain of the subunit.

In *E. coli*, a strain without the ε regulation showed lower growth rate without any other changes in cellular parameters (ex. Cellular ATP pool, membrane potential) in high- or low-salt conditions compared to wild type. When the wild type and the mutant strains were co-cultured and kept in stationary-phase in the low-salt condition, the mutant showed 1-log decrease in cell survival compared to the wild type after 28 days, suggesting an inability to compete with the wild type in the condition.

In the *B. subtilis* mutant of the ε–regulation, although no defect was observed in its growth in various conditions including the high- and low-salt conditions, the mutant were less efficient to form colonies after heating in stationary-phase in normal minimal medium, suggesting that the mutant is deficient to form heat-resistant spores. While germination and outgrowth from purified spores seemed to be normal, spore yield decreased in the mutant. These results show that the mutant has impaired spore formation ability.

This study in general suggests that the ε–regulation benefits the survivability of the cells in some unfavorable environments.

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1P47

A Liposome System For Light-Driven ATP-Synthesis

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In nature the regeneration of ATP is accomplished by the highly complex, proton-driven membrane protein ATP-Synthase[1]. For the production of ATP the ATP-Synthase requires a membrane energized via a proton gradient. Therefore, the membrane-integrated protein Bacteriorhodopsin can be used, which is able to transform light energy into a proton gradient[2,3].

The aim of this work is to establish a lipsome-based system with integrated *E. coli* ATP-Synthase and Bacteriorhodopsin from *Halobacterium salinarum*. After illumination Bacteriorhodopsin should acidify the liposome interior and thus provide the engery for the ATP-Synthase. As a consequence, the ATP-Synthase should produce ATP in the presence of adenosindiphosphate and phosphate.

All required components for the liposome system were produced in a high purity:

- E. coli ATP-Synthase was overexpressed and purified
- Bacteriorhodopsin was isolated from Halobacterium salinarum
- Phosphatidylcholine liposomes with defined size (diameter: 100 nm to approx. 10 μm) were produced via extrusion or electroswelling

Bacteriorhodopsin was reconstituted into the liposome membrane in the correct direction to pump protons into the liposome interior.

To track intravesicular pH changes generated by Bacteriorhodopsin, the fluorescence-based measurement of pH in the liposome was established using the pH-dependent fluorescent dye pyranine.

Finally, illumination of the Bacteriorhodopsin liposome system resulted in a time-dependent acidification of the liposome interior. This membrane energization is the precondition for the ATP-synthesis to regenerate ATP.

ATP-regenerating liposomes can provide energy for a broad range of biological processes, e.g cell-free protein synthesis.

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1P48

Single Molecule Analysis of Inhibitory Pausing States of V_1-ATPase

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V_1-ATPase, the hydrophilic domain of V-ATPase, is a rotary motor fueled by ATP hydrolysis. Having 3 catalytic sites, 3 step – rotation is observed at low [ATP] and in each 120º step, ATP binding and hydrolysis occur at same angle. Previously, some biochemical studies indicated the presence of a strongly-inhibited state of V_1-ATPase, which was analyzed at single molecule level, in this study. Here we showed that *Thermus thermophilus* V_1-ATPase had two types of inhibitory pauses interrupting continuous rotation: a short pause (*SP*, 4.2 s) that occurred frequently during rotation and a long inhibitory pause (*LP*, >30 min) that terminated all active rotation. Both pauses occurred at the same angle with ATP binding and hydrolysis. Kinetic analysis revealed that the time constants of inactivation into and activation from *SP* were too short to correspond to biochemically predicted ADP inhibition, suggesting that *SP* is a newly identified inhibitory state of V_1. The time constant of inactivation into *LP* was 17 min, consistent with one of the two time constants governing the inactivation process observed in bulk ATPase assay. When forcibly rotated in the direction of rotation by using magnetic field, V_1 in *LP* resumed active rotation. Including ADP in chamber buffer suppressed the probability of mechanical activation, suggesting that mechanical rotation enhanced inhibitory ADP release. These features were highly consistent with mechanical activation of ADP-inhibited F_1, suggesting that *LP* represents the ADP-inhibited state of V_1. Mechanical activation largely depended on the direction and angular displacement of forced rotation, implying that V_1-ATPase rotation modulates the off rate of ADP.

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1P49

Direct observation of the rotation of F_oF_1-ATP synthase driven by the proton motive force

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F_oF_1-ATP synthase (F_oF_1) is a rotary motor protein which carries out ATP synthesis by coupling to the rotary motion driven by the proton motive force (*pmf*) across the membrane. F_oF_1 consists of two molecular motors; ATP-driven motor (F_1) and proton-driven motor (F_o). In 1997, we for the first time observed the ATP-driven rotation of F_1 [1], and have thoroughly elucidated its operating principle as a rotary motor protein in this decade. On the other hand, the proton-driven rotation of F_1 or whole enzyme; F_oF_1, was recently observed by the single-molecule FRET [2]. However, much remained to be understood about the energy conversion mechanism from the proton transport to the rotary motion of F_oF_1, because the previous studies mainly focused on the rotary behaviors, such as the direction and the step size of the rotation. In this study, to reveal this energy conversion mechanism, we developed the novel experimental system which enabled to monitor the amplitude of *pmf* and the rotary motion of F_oF_1, simultaneously. In this system, we used the pH-sensitive fluorophore; pHrodo, for the measurement of proton gradient across the membrane, which is the main component of *pmf*. In addition, to directly observe the rotary motion of F_oF_1, we attached the gold nanoparticle ($f = 80$ nm) to the rotor part and its rotation was visualized by the total internal reflection dark-field illumination system (TIRFDF). In the presence of *pmf*, F_oF_1 showed the clockwise rotation when viewed from F_o to F_1, and accelerated its rotational rate depending on the amplitude of *pmf*, which was essentially consistent with the previous biochemical study [3]. In addition to the rotational rate, we obtained various information related to the "dynamics", such as the stepwise rotary motion coupled with the proton transport and ATP synthesis, which provide a clue for further understanding of energy conversion mechanism of F_oF_1 in the physiological condition. We will show the details at this meeting.

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1P50

Expression, purification and crystallization of central stalk and peripheral stalk of F_1F_o ATP synthase from *Aquifex aeolicus* in *Escherichia coli*

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Aquifex aeolicus is a hyperthermophilic eubacterium, its genome has been sequenced [1]. The F_1F_o ATP synthase has been purified from this organism, which contains nine subunits α, β, γ, δ and ε of F_1 part and subunits a, b_1, b_2 and c of F_o part [2]. Two stalks connecting F_1 and F_o could also be shown clearly by electron microscopic single particle analysis [2]. This project is mainly focused on these two stalks: central stalk and peripheral stalk of F_1F_o ATP synthase from *A. aeolicus*. The central stalk includes subunits γ and ε. Vectors were already constructed to express $F_1\gamma$ and $F_1\varepsilon$ separately and co-express both of them in *Escherichia coli*. Target proteins $F_1\gamma$ and $F_1\varepsilon$ were verified by western blot with anti- γ or ε antibodies, respectively. High level expression of $F_1\gamma$ and $F_1\varepsilon$ was achieved. The proteins were identified as subunits γ and ε of F_1F_o ATP synthase from *A. aeolicus* by mass spectrometry. $F_1\varepsilon$ was successfully purified after two step purifications. The molecular weight of $F_1\varepsilon$ was determined as 23.51 kDa by size exclusion chromatography. The crystallizations of $F_1\varepsilon$ have been set up. After initial purification of $F_1\gamma$ by Ni-NTA affinity chromatography, however, it was found that the protein was unstable, ease to form aggregations. The preparation of this protein is under optimizations. Co-expression of a subcomplex $F_1\gamma\varepsilon$ was also successful, but the yields need to be improved. The expression vectors of peripheral stalk have been constructed. Further experiments are ongoing.

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1P51

The subunit of the α-proteobacterial F_1F_O-ATP synthase in *Paracoccus denitrificans*: a novel control mechanism of the central rotor

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The novel inhibitory subunit of the F_1-ATPase in *P. denitrificans* (PdF_1) and related α-proteobacteria, namely the subunit [1] works kinetically as a total inhibitor of the sulfite-activated PdF_1-ATPase with an IC_{50} of 220 nM according to a non-competitive mechanism after being reconstituted into the PdF_1 lacking the endogenous and ε subunits. In contrast, the ε subunit of the *P. den* ATP synthase was unable to inhibit the sulfite-activated PdF_1-ATPase, thus confirming that rather than ε plays a central inhibitory role in the α-proteobacterial F_1-ATPase. The secondary and tertiary structures of the isolated subunit were resolved in solution by circular dichroism and 2D-NMR spectroscopy, respectively. The structural data show that the subunit is a globular 100% α-helix protein folded in a 4-helix bundle forming a central globular domain and a protruding part where the N- and C-termini interact closely with each other. The tertiary N-C domain of the subunit contains the inhibitory region of the molecule, as shown by limited proteolysis and functional inhibitory reconstitution experiments. Further limited proteolysis analyses show that during PdF_1-ATPase activation by sulfite the subunit exposes its N- and C-termini while the ε subunit buries its C-terminal domain in the F_1 structure, thus giving clues on the orientation of binding to the PdF_1-ATPase. Moreover, the subunit can be reversibly cross-linked with rotor (γ,ε) and stator (α,β) subunits of the PdF_1-ATPase, as found before for the IF_1 inhibitor of mitochondrial F_1-ATPase [2] together, the data strongly suggests that this novel mechanism of regulation of the subunit involves the control of the intrinsic gyration of the central stalk of the α-proteobacterial F_1F_O-ATP synthase, similar to the inhibitory mechanisms of the ε and IF_1 subunits in eubacterial and mitochondrial ATP synthases, respectively, but with a totally different tertiary structure. Taken together the data opens a new line on the regulation of the intrinsic gyration of the bacterial F_1F_O nanomotor.