

The divalent metal binding sites of beef heart mitochondria F1ATPase were studied by FT-ESEEM spectroscopy, using Mn(II) as a paramagnetic probe, which replaces the naturally occurring Mg(II) and maintains the enzyme catalytic activity. Purified F1ATPase still containing three endogenous tightly bound nucleotides, named MF1(1,2), was obtained under mild conditions, whereas a harsher treatment gave a fully nucleotide depleted enzyme, named MF1(0,0). When MF1(1,2) was loaded with Mn(II) in 1:0.8 ratio, the spectrum showed evidence of a nitrogen interacting with the metal, while this interaction was not present in the spectrum of MF1(0,0) loaded with Mn(II) in the same ratio. However, when MF1(0,0) was loaded with 2.4 Mn(II), the spectrum showed metal-nitrogen interaction resembling that of MF1(1,2) loaded with Mn(II) in 1:0.8 ratio. When MF1(1,2) was loaded with 2.4 Mn(II) the metal-nitrogen interaction signal remained and a phosphorous coordination to the metal was also evident, indicating a binding of Mn^{2+} to a site containing a tightly bound nucleotide but metal free. These results strongly support the role of the metal alone in structuring the catalytic sites of the enzyme while ESEEM technique appears to be a sensitive and suitable spectroscopic method for conformational studies of MF1 with the advantage of using proteins in frozen solution.

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S1.21 Thermodynamic constraints in the reversal of adenine nucleotide translocase during the reversal of F₀-F₁ ATP synthase caused by respiratory chain inhibition: Critical role of substrate-level phosphorylation

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Mitochondria are the main ATP producers in the cell. However, during respiratory chain inhibition mitochondria become ATP consumers due to reversal of the F₀-F₁ ATP synthase. The current belief is that under these conditions, extramitochondrial ATP is carried into the matrix chiefly through the reversal of the ANT. For this, the mitochondrial membrane potential must reach values more positive than both the reversal potential of the ATP synthase (Erev-ATPase) and that of the ANT (Erev-ANT). Here we show that in mitochondria capable of substrate-level phosphorylation, inhibition of the respiratory chain shifts the membrane potential to a range bracketed by the Erev-ATPase and the Erev-ANT, the latter being more negative than the former. As a consequence of this, reversal of the ATP synthase generates a sufficient membrane potential to oppose the ANT from operating in reverse mode, for as long as substrate-level phosphorylation is maintained. During respiratory chain inhibition the ANT can only be reversed by a concomitant uncoupling when the membrane potential attains values more positive than the Erev-ANT, or by incapacitating substrate-level phosphorylation. The latter maneuver shifts the Erev-ANT towards more negative values than the prevailing mitochondrial membrane potential.

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S1.22 Determination of unidirectional H⁺ transport in F-type ATPases

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F-type ATP synthases are ubiquitous enzymes, which can either synthesize ATP using an electrochemical gradient or hydrolyze ATP to generate a membrane potential. We have investigated the properties of these two working modes in the Na⁺ dependent enzyme from *Propionigenium modestum* and the H⁺-dependent enzyme from *E. coli*. We found that ΔpNa and $\Delta\psi$ are required for efficient ATP synthesis in the Na⁺-dependent enzyme. In accordance with this, we were able to show that both driving forces are capable of energizing Na⁺-transport through F₀, when the enzyme was driven in synthesis direction. Interestingly, ΔpNa alone could not stimulate ion transport in the reverse direction as found during hydrolysis, whereas a $\Delta\psi$ served efficiently as driving force. The two directions are therefore kinetically not equivalent. Furthermore, we observed different K_D values for Na⁺ for either transport direction. Whereas the K_D for Na⁺ in hydrolysis direction is in the range of 1 mM, it was determined to be 15 mM during ATP synthesis. To test whether this asymmetry is present in H⁺ dependent ATP synthases as well, we developed a fluorescent assay for monitoring transport in either synthesis direction or in hydrolysis direction through the F₀ part of *E. coli* and chloroplast ATP synthase. The assay allowed quantitative determination of initial transport rates for H⁺ dependent ATP synthases from different organisms in a well defined *in vitro* system.

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S1.23 The H⁺/ATP ratio of H⁺-ATP synthases from chloroplasts, *E. coli* and mitochondria

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The H⁺/ATP ratio is an important parameter for energy balance in cells and for the mechanism of coupling between proton transport and ATP synthesis. Rotational catalysis predicts that the H⁺/ATP coincides with the ratio of the c-subunits to the β -subunits, implying that a value of 4.7 is expected in the chloroplast ATP synthase and a value of 3.3 is expected in the mitochondria and *Escherichia coli* enzyme. This ratio can be determined based on the energetics given by the chemiosmotic theory. The isolated enzymes were reconstituted into liposomes. The internal phase of the liposomes was equilibrated with the acidic medium during reconstitution, so that the internal pH could be measured with a glass electrode. An acid-base transition was carried out and the initial rates of ATP synthesis or ATP hydrolysis were measured with luciferin/luciferase as a function of ΔpH at constant $Q = [ATP]/([ADP][P_i])$. From the shift of the equilibrium ΔpH as a function of Q , the standard Gibbs free energy for phosphorylation and the H⁺/ATP ratios were determined.

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S1.24 Effect of Pi and ADP on the intrinsic uncoupling in the isolated and reconstituted ATP synthase of *E. coli*

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We have recently shown that the H^+/ATP ratio can significantly decrease during ATP hydrolysis by the ATPsynthase of *Rb. capsulatus*, when the concentration of either ADP or Pi is maintained at a low level. This same phenomenon has then been observed in isolated membranes of *E. coli*. We have now purified the ATPsynthase of *E. coli* and reconstituted it into liposomes, in order to verify whether the same behavior could be observed in the isolated enzyme. The ATP hydrolysis and proton pumping activity were measured under the same experimental conditions. The hydrolysis was measured either with the colorimetric pH indicator Phenol Red or with an ATP regenerating enzymatic assay, and the proton pumping was evaluated by a calibrated ACMA assay. The hydrolysis activity was inhibited by Pi with an apparent K_d of 400 μM , while the steady state ΔpH was stimulated up to 200 μM Pi and was only slightly inhibited up to 1000 μM Pi. Both the inhibition of ATP hydrolysis and the stimulation of proton pumping as a function of Pi were lost upon ADP removal by an ADP trap. We conclude that the isolated and reconstituted ATPsynthase of *E. coli* can vary its degree of coupling as a function of Pi and ADP.

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S1.25 Immobilization of the H^+ -ATPsynthase on glass surface and single molecule fluorescence spectroscopy

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During ATP synthesis the rotor subunits of the H^+ -ATPsynthase $\gamma\epsilon c_{10}$ rotate versus stator subunits $\alpha_3\beta_3 \delta ab_2$. This rotation has been shown by single pair Fluorescence Resonance Energy Transfer (spFRET). The double labeled enzyme was integrated into liposomes and the diffusion time through the confocal volume of the microscope limited the observation time. In order to extend the observation time the proteoliposomes were immobilized on a glass surface. To immobilize the proteoliposomes two techniques have been used. First, the enzyme was reconstituted into liposomes containing biotinylated lipids. They are immobilized with streptavidin on a biotinylated surface. Second, the H^+ -ATPsynthase carried a his-tag on the β -subunits. After modification of the surface with BSA or Silan proteoliposomes were immobilized via the his-tagged enzyme. With this method the observation time of a single enzyme was significantly increased, however also photobleaching of the organic fluorophores was increased. Therefore, quantum dots (QD₅₈₀ and QD₆₀₀) with hydrophilic shells have been used as fluorescence donors. They were bound covalently to the b-subunits of the H^+ -ATPsynthase and Atto 647N was used as acceptor. This allows the observation of a single enzyme in the second to minute time range.

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S1.26 Structure of the C-terminal domain of the ϵ subunit of chloroplast-type F_1

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The chloroplast F_1 -ATPase ϵ subunit inhibits ATP hydrolysis with ATP-independent manner, whereas the bacterial ϵ is ATP-dependent. To understand the structure–function relationship of ϵ , we prepared the chimera ϵ subunit combining the N-terminal domain from *Thermosynechococcus elongatus* and the C-terminal α -helical domain from spinach CF_1 (ϵ_{NB_CC}) or EF_1 (ϵ_{NB_CE}). The ϵ_{WT} from *T. elongatus* and ϵ_{NB_CC} inhibited the ATP hydrolysis activity of cyanobacteria F_1 to a similar extent, whereas ϵ_{NB_CE} was less potent. The solution structures of ϵ_{WT} and ϵ_{NB_CC} solved by NMR were so-called “retracted-state”, which was similar to that of EF_1 - ϵ or TF_1 - ϵ . However, the length of α -helices in the C-terminal domain of these CF_1 -type ϵ was longer than that of the bacterial ϵ . Interestingly, the loop between two helices of the ϵ_{NB_CC} was formed by only four residues and the retracted position of the helices was quite different from the previous reports. Significance of the unique conformation of the C-terminal α -helices is discussed.

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S1.27 Step size of proton-driven c ring rotation in single F_0F_1 -ATP synthase by FRET

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A mean ratio of 4.0 protons transported per synthesized ATP has been determined for the *E. coli* F_0F_1 -ATP synthase recently. However, the F_0 part likely contains 10 c subunits corresponding to 3.3 H^+/ATP . Synthesis of ATP is performed by a stepwise internal rotation of subunits in F_0F_1 . Sequential conformational changes of single enzymes are monitored in real time by fluorescence resonance energy transfer, FRET. Therefore two different fluorophores have to be attached to those protein domains, which move during function. We investigated the step size of proton-driven c subunit rotation in F_0F_1 by single-molecule FRET between a and c. ‘Duty cycle optimized alternating laser excitation’ minimized FRET artefacts. Rotary movements with stochastic single step sizes between 36° and 144° were determined by Hidden Markov Models. As the two coupled motors of F_0F_1 showed apparently different step sizes, contributions of rotor and stator subunits for transient energy storage can be located using FRET. Monitoring c rotation we identified the action mode of the allosteric inhibitor aurovertin B, which modulates single F_0F_1 activity by slowing down rotation upon ATP hydrolysis, but acts differently during ATP synthesis.

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S1.28 Mechanochemical coupling of F_1 -ATPase and intracellular ATP imaging

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