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Voltage-generated torque drives the motor of the ATP synthase

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The mechanism by which ion-flux through the membrane-bound motor module (F_0) induces rotational torque, driving the rotation of the γ subunit, was probed with a Na^+-translocating hybrid ATP synthase. The ATP-dependent occlusion of 1 $^{22}Na^+$ per ATP synthase persisted after modification of the c subunit ring with dicyclohexylcarbodiimide (DCCD), when $^{22}Na^+$ was added first and ATP second, but not if the order of addition was reversed. These results support the model of ATP-driven rotation of the c subunit oligomer (rotor) versus subunit a (stator) that stops when either a $^{22}Na^+$-loaded or a DCCD-modified rotor subunit reaches the Na^+-impermeable stator. The ATP synthase with a Na^+-permeable stator catalyzed $^{22}Na^+_{out}/Na^+_{in}$-exchange after reconstitution into proteoliposomes, which was not significantly affected by DCCD modification of the c subunit oligomer, but was abolished by the additional presence of ATP or by a membrane potential (ΔΨ) of 90 mV. We propose that in the idling mode of the motor, Na^+ ions are shuttled across the membrane by limited back and forth movements of the rotor against the stator. This motional flexibility is arrested if either ATP or ΔΨ induces the switch from idling into a directed rotation. The *Propionigenium modestum* ATP synthase catalyzed ATP formation with ΔΨ of 60–125 mV but not with ΔpNa^+ of 195 mV. These results demonstrate that electric forces are essential for ATP synthesis and lead to a new concept of rotary-torque generation in the ATP synthase motor.

Keywords: electrostatic motor/F_1F_0 ATP synthase/sodium ion exchange/rotary-torque generation

Introduction

The enzyme ATP synthase or F_1F_0 ATPase catalyzes ATP synthesis in mitochondria, chloroplasts and bacteria, utilizing the energy of a transmembrane electrochemical gradient of protons, or in some cases, Na^+ ions (Futai *et al.*, 1989; Fillingame, 1990; Deckers-Hebestreit and Altendorf, 1996; Dimroth, 1997; Weber and Senior, 1997). The enzyme is a multisubunit complex of bimodular architecture and function. The extramembraneous F_1 module has the subunit composition $\alpha_3\beta_3\gamma\delta\varepsilon$ and carries out ATP synthesis coupled to ion movement through the transmembrane F_0 module, which in bacteria has the probable subunit composition ab_2c_{12} (Jones *et al.*, 1998). Based on a high resolution structure of a substantial part of bovine F_1 in combination with biochemical and biophysical data, a mechanism for ATP synthesis has emerged that involves binding changes at the catalytic β subunits elicited by rotation of the asymmetrically bent γ-shaft within an annulus formed by the $\alpha_3\beta_3$ hexagon (Boyer, 1993; Abrahams *et al.*, 1994). Rotation of the γ-subunit by ATP hydrolysis has been shown with an $\alpha_3\beta_3\gamma$ subcomplex of F_1 (Duncan *et al.*, 1995; Sabbert *et al.*, 1996; Noyi *et al.*, 1997) and there is evidence that subunit ε rotates with subunit γ as a unit (Capaldi *et al.*, 1996; Kato-Yamada *et al.*, 1998). In the ATP synthesis mode, rotation is believed to be driven in the opposite direction by the downhill movement of the coupling ions through the F_0 module.

The most intriguing question on the operation of the ATP synthase therefore relates to the mechanism by which this ion flux is utilized to induce rotary torque. The ways in which this may be accomplished are now beginning to emerge. Electron and atomic force microscopic studies of *Escherichia coli* F_0 suggest that the c subunits are assembled into a ring that is flanked at the periphery by the a and the two b subunits (Birkenhäger *et al.*, 1995; Singh *et al.*, 1996; Takeyasu *et al.*, 1996). The b subunits are proposed to be part of the stator that fixes the a subunit to the $\alpha_3\beta_3$ headpiece via association with the δ subunit (Lill *et al.*, 1996; Ogilvie *et al.*, 1997). Subunit a is an integral membrane protein that consists of five or six membrane-spanning α-helices (Yamada *et al.*, 1996; Jäger *et al.*, 1998; Long *et al.*, 1998; Valiyaveetil and Fillingame, 1998) and the c subunits may be folded like a hairpin forming two transmembrane α-helices connected by a hydrophilic loop, as suggested by nuclear magnetic resonance (NMR) analysis of *E.coli* subunit c in chloroform/methanol/H_2O (4:4:1) (Girvin *et al.*, 1998). Evidence from mutational studies indicated that subunits a and c operate together in the coupling ion movement across the membrane (Futai *et al.*, 1989; Fillingame, 1990; Deckers-Hebestreit and Altendorf, 1996; Weber and Senior, 1997; Steffens *et al.*, 1988) which was recently reinforced by studies with the Na^+, Li^+ or H^+-translocating ATP synthase of *Propionigenium modestum* (Dimroth, 1997): each c subunit carries a coupling ion-specific site including the conserved acidic residue (cE65) (Kluge and Dimroth, 1993) that has a unique reactivity towards modification by dicyclohexylcarbodiimide (DCCD). Additional Na^+-binding ligands are cS66 and cQ32 (Kaim *et al.*, 1997). These sites are readily accessible from the electronegative (N) side of the membrane for all c subunits except that at the a subunit interface (Kaim *et al.*, 1998). In this position, the binding site contacts the electropositive (P) side of the membrane through a cation selective channel of subunit a. This channel became Na^+-impermeable by an a subunit triple-mutation (K220R, V264E, I278N) leading to the

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Table I. Occlusion of $^{22}Na^+$ by ATP synthase with a Na^+-impermeable stator mutation (strain MPA762)

Sequence of addition	1 mM ^{22}NaCl	1 mM ^{22}NaCl 2.5 mM ADP	32 µM DCCD 1 mM ^{22}NaCl **2.5 mM ATP**	32 µM DCCD **2.5 mM ATP** 1 mM ^{22}NaCl
[mol Na^+/mol ATP synthase]	<0.01	<0.01	0.98 ± 0.03	<0.01

Where indicated, enzyme was incubated with 32 µM dicyclohexylcarbodiimide (DCCD) for 15 min. The addition sequence was from top to bottom. After 30 s the occluded $^{22}Na^+$ was separated from free $^{22}Na^+$ by ion exchange chromatography on Dowex 50 loaded with K^+ and measured by γ-counting. The mean from three different experiments is depicted.

ATP-dependent occlusion of one Na^+ per mutant ATP synthase (Kaim and Dimroth, 1998a; Kaim *et al.*, 1998). It was therefore proposed that ATP hydrolysis elicits rotation of the γ and the ε subunits in concert with that of the c subunit ring versus the a subunit (Dimroth *et al.*, 1998; Kaim *et al.*, 1998). According to our model, with the enzyme operating in ATP hydrolysis, the ion translocation proceeds in three steps: first, the coupling ions bind to the c subunit sites from the cytoplasm; secondly, ATP-driven rotation moves a Na^+, Li^+ or H^+-boarded c subunit into the interface to the a subunit; and thirdly, the coupling ion is released through the a subunit channel to the periplasm. In ATP synthesis, coupling ion movement in the opposite direction (downhill the electrochemical gradient) will also be linked to the rotation of the c subunits versus the a subunit with the provision to induce rotary torque for rotation of the γ subunit that is instrumental in ATP synthesis.

The experiments described in this communication add important information as to the mechanism of ion translocation and torque generation. We show here that Na^+ ions are shuttled freely across the membrane in the idling mode of the motor. The exchange is not affected by partial modification of c subunit sites with DCCD but blocked in the additional presence of ATP, indicating a switch from idling into a directed rotation that stops when a DCCD-modified c subunit strikes against the a subunit. The exchange is also abolished by imposing a membrane potential of ~90 mV but not with Na^+ concentration gradients of up to 195 mV. Accordingly, $\Delta\Psi$ and ΔpNa^+ are kinetically unequivalent driving forces for ATP synthesis and only the former is able to overcome the activation energy barrier under our experimental conditions. ATP synthesis experiments with proteoliposomes harboring the *P.modestum* ATP synthase reinforce this conclusion.

Results

Evidence for intersubunit rotation in the F_0 module

We investigated Na^+ occlusion by an *E.coli*/*P.modestum* ATP synthase hybrid with a triple mutation in its a subunit (aK220R, aV264E, aI278N) (Kaim and Dimroth, 1998a) after destruction of Na^+ binding sites on rotor subunits by covalent modification with DCCD. The idea was to create a motor whose rotation could be blocked either through the mutated stator or through a modified rotor subunit. Running the engine with ATP as the energy source would thereby result in its blockade, but by adding $^{22}Na^+$ prior or after ATP-induced rotation, we could trace the Na^+ ions within the motor's core. The results given in Table I show the occlusion of 1 mol $^{22}Na^+$ per mol DCCD-modified mutant ATP synthase if $^{22}Na^+$ was added first, and ATP second. However, if this order was reversed, occlusion of $^{22}Na^+$ was not observed. These results are consistent with the rotational model: in the first case, the ATP-driven rotation stops and $^{22}Na^+$ becomes occluded when a Na^+-loaded rotor subunit has entered the boundary to the Na^+-impermeable stator channel. In the second case, the rotation stops without $^{22}Na^+$ occlusion when the DCCD-modified rotor subunit strikes against the stator. The $^{22}Na^+$ ions were released from their occluded position with a chase of LiCl but not with a chase of NaCl. This is in accord with previous observations (Kaim *et al.*, 1998), indicating that Li^+ ions passing through the mutated (Na^+-impermeable) stator channel perturb the immobilized position of the rotor so that the bound $^{22}Na^+$ ions gain access to the aqueous environment and are therefore removed from the enzyme during passage through the ion-exchange column.

Idling ATP synthase motor reflected by $^{22}Na^+_{out}$/Na^+_{in} exchange experiments

We reasoned that the idling motor of the ATP synthase of *P.modestum* might be sufficiently mobile to catalyze an exchange of internal and external Na^+ ions. The purified enzyme was therefore reconstituted into proteoliposomes that contained 100 mM NaCl inside and 2 mM ^{22}NaCl outside, and the uptake of radioactivity was determined. The results shown in Figure 1 indicate that the ATP synthase catalyzed the exchange of external $^{22}Na^+$ for internal unlabelled Na^+ ions. The radiolabelled $^{22}Na^+$ was, however, not transported into proteoliposomes that were devoid of internal Na^+ ions. Neither could the influx of $^{22}Na^+$ be induced by a membrane potential of –180 mV (not shown). However, the same proteoliposomes performed active Na^+ pumping to the inside, driven by ATP hydrolysis (not shown). This occurred at an initial rate of 8 s^{-1}, while $^{22}Na^+_{out}$/Na^+_{in} exchange was catalyzed with an initial rate of 7 s^{-1}. As expected, the ATP-driven active pumping of $^{22}Na^+$ into the proteoliposomes was completely abolished by modifying part of the rotor subunits with DCCD (not shown). In contrast, $^{22}Na^+_{out}$/Na^+_{in} exchange was not significantly affected by this treatment (Figure 1). These results are in accord with the notion that ATP-driven $^{22}Na^+$-uptake requires complete revolutions of the c subunit ring which are abolished if individual c subunits are modified with DCCD. For $^{22}Na^+_{out}$/Na^+_{in} exchange activity, however, limited back and forth rotation of the rotor is apparently sufficient which, in agreement with our results, should not be significantly perturbed by modifying individual rotor subunits with DCCD. However, in the presence of ATP, the rate of $^{22}Na^+_{out}$/Na^+_{in} exchange by the DCCD-modified

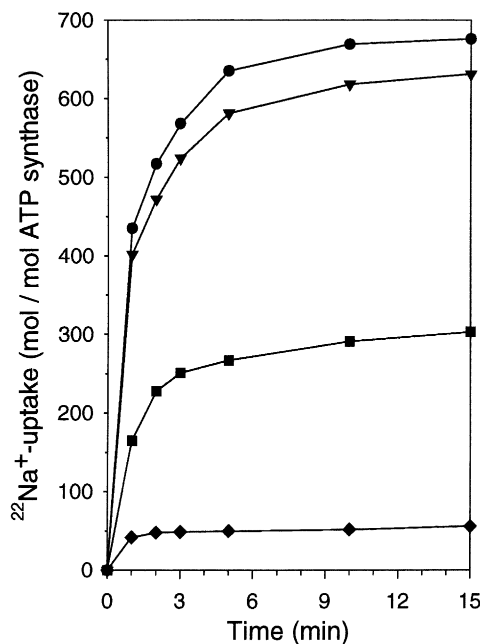

Fig. 1. $^{22}Na^+_{out}/Na^+_{in}$ exchange by reconstituted proteoliposomes containing the ATP synthase of *P.modestum*. Proteoliposomes (14 μl) containing 100 mM NaCl were diluted into 0.7 ml buffer containing carrier-free $^{22}NaCl$ (1 μCi). After separating external $^{22}Na^+$ by cation exchange chromatography, internal $^{22}Na^+$ was analysed by γ counting (●). $^{22}Na^+$ uptake after treatment of proteoliposomes with 32 μM DCCD for 15 min (▼); $^{22}Na^+$ uptake by the DCCD-treated proteoliposomes in the presence of 2.5 mM ATP (■); $^{22}Na^+$ uptake into proteoliposomes without NaCl on the inside (◆).

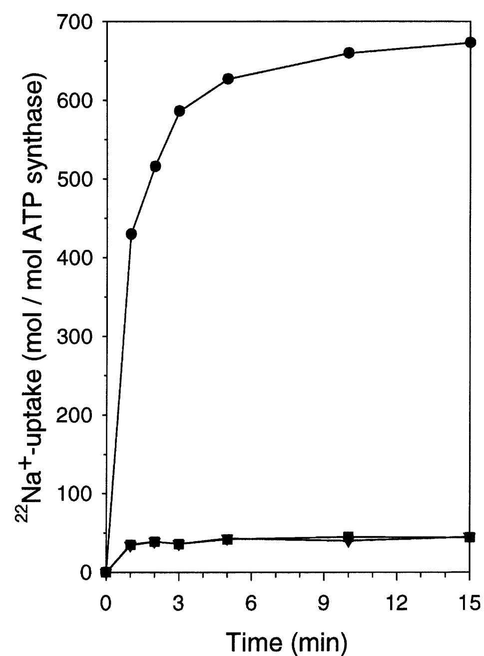

Fig. 2. Effect of the membrane potential (ΔΨ) on $^{22}Na^+_{out}/Na^+_{in}$ exchange by proteoliposomes containing the ATP synthase of *P.modestum*. Proteoliposomes containing 100 mM NaCl were diluted 1:50 into 0.7 ml buffer containing carrier-free $^{22}NaCl$ (1 μCi). $^{22}Na^+$ uptake was determined (●) as described in Figure 1. In other experiments an electric potential (ΔΨ) of ~90 mV (inside positive; ■) or (inside negative; ▼) was applied by valinomycin-induced potassium ion diffusion (for details see Materials and methods).

enzyme was reduced to ~40% (Figure 1). This is the proportion expected if ATP synthase oriented with its ATP-binding site to the exterior became completely inactive and the remaining activity was entirely catalyzed by enzyme oriented in the opposite direction. Please note that the curves represent the average over many proteoliposomes and that 50% of the ATP synthase molecules are oriented to the inside and 50% are oriented to the outside (Kluge and Dimroth, 1992). Under our conditions, the number of ATP synthase molecules per proteoliposome was ~0.5. Therefore, only ~50% of the proteoliposomes participated in $^{22}Na^+_{out}/Na^+_{in}$ exchange in the presence of ATP and the amount of $^{22}Na^+$ taken up under these conditions converged to ~50% of the level reached in its absence. Neither ADP nor the non-hydrolyzable ATP analogue AMP-PNP had any effect on $^{22}Na^+_{out}/Na^+_{in}$ exchange activity (not shown), indicating that the binding of these nucleotides to appropriate sites in F_1 is unable to lock the c subunit assembly in an immobilized state.

Evidence for torque generation in the F_0 module by ΔΨ but not by ΔpNa^+

It is indisputable that the electric potential (ΔΨ) and the concentration gradient of the appropriate coupling ion (ΔpH or ΔpNa^+) are themodynamically equivalent driving forces for ATP synthesis, as predicted by Mitchell's chemiosmotic hypothesis. Whether these forces are also kinetically equivalent was investigated further. We expected that with an appropriate driving force the motor should switch from idling into a torque-generating directed rotation which should terminate $^{22}Na^+_{out}/Na^+_{in}$ exchange. In the experiments described above (Figure 1), $^{22}Na^+$ uptake was observed against a 50-fold concentration gradient of unlabelled Na^+ ions (ΔpNa^+ of ~100 mV). With this gradient, therefore, the motor is retained in an idling mode and apparently unable to generate torque. Torque generation requires unidirectional motion of the rotor relative to the stator which is not compatible with the $^{22}Na^+_{out}/Na^+_{in}$ exchange activity. This significant observation implies that ΔpNa^+ alone is kinetically not suitable as a driving force for ATP synthesis. We therefore analysed the effect of a diffusion potential (ΔΨ) applied by valinomycin-induced K^+ movements on $^{22}Na^+_{out}/Na^+_{in}$ exchange activity. Interestingly enough, the results shown in Figure 2 indicate that the $^{22}Na^+$ exchange activities were essentially abolished by a ΔΨ of approximately ±90 mV. The electric force is therefore apparently transduced by the motor to enforce a rotation against the load torque until the rotor reaches an immobile position.

ΔΨ is an obligatory driving force for ATP synthesis

To substantiate the findings discussed above, the effect of ΔpNa^+ and ΔΨ on ATP synthesis was measured directly with proteoliposomes containing the purified ATP synthase. With proteoliposomes loaded with 200 mM NaCl, ATP synthesis was negligible even at a ΔpNa^+ of 195 mV (Figure 3A). It was indistinguishable from controls when the ΔpNa^+ was abolished by the Na^+ ionophore monensin or when counteracting ΔΨ generation by Na^+-coupled ATP synthesis was eliminated by adding valinomycin in the presence of equal K^+ concentrations on both sides of the membrane. Therefore, this background ATP synthesis probably results from traces of contaminating adenylate kinase. However, after applying a valinomycin-

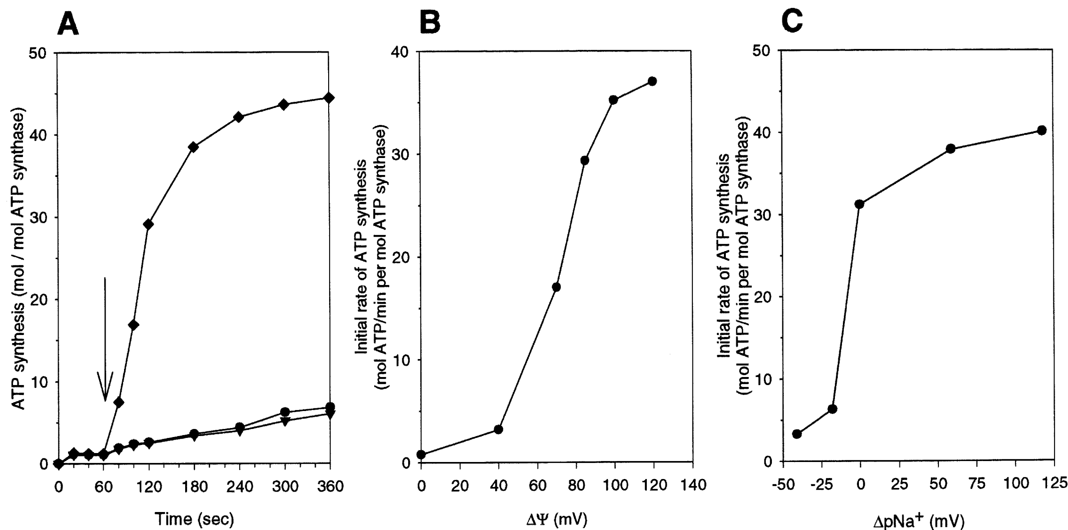

Fig. 3. (**A**) Effect of the electric potential (ΔΨ) and the Na^+ concentration gradient (ΔpNa) on ATP synthesis by the *P.modestum* ATP synthase. Proteoliposomes were reconstituted in 2 mM Tricine–LiOH buffer pH 7.2 containing 10 mM NaCl (K^+ concentration ~0.1 mM) as described in Materials and methods, and diluted into reaction buffer I. After addition of 20 μM valinomycin (↓) a calculated K^+ diffusion potential of 195 mV was induced and ATP synthesis was determined (◆). The effect of a calculated ΔpNa^+ of 195 mV on ATP synthesis was determined as described in Materials and methods by dilution of proteoliposomes to yield interior and exterior Na^+ concentrations of 200 and 0.1 mM, respectively (●). The same results were obtained with proteoliposomes containing 10 mM KCl on either side of the membrane and 20 μM valinomycin or no valinomycin. ATP synthesis with a ΔpNa^+ of 195 mV and 20 μM monensin (a Na^+ ionophore) was determined as control (▼). (**B**) Dependence of the rate of ATP synthesis on ΔΨ. Proteoliposomes were reconstituted in 5 mM sodium phosphate buffer pH 7.2 containing 5 mM NaCl and 1 mM KCl, and diluted 1:10 into the same buffer containing 5 mM $MgCl_2$, 0.1 mM ADP and the following KCl concentrations: 1, 5, 15, 28, 50 and 108 mM. The reactions were started by adding 20 μM valinomycin. (**C**) Effect of ΔpNa^+ on the rate of ATP synthesis driven by a calculated K^+/valinomycin diffusion potential of 195 mV. Proteoliposomes were reconstituted in 5 mM sodium phosphate buffer pH 7.2 containing 5 mM NaCl and 0.1 mM KCl and diluted 1:10 into 5 mM potassium phosphate buffer pH 7.2 containing 195 mM KCl 5 mM $MgCl_2$, 0.1 mM ADP and the following NaCl concentrations: 50, 20, 10, 1 and 0.1 mM. The reactions were started by adding 20 μM valinomycin.

induced K^+-diffusion potential of 195 mV and no ΔpNa^+ (10 mM NaCl present on both sides of the membrane), ATP synthesis immediately commenced, approaching a steady state at ~3 min, when 45 mol ATP were synthesized per mole of reconstituted enzyme. Hence, the electric potential but not a Na^+ concentration gradient of the same size can be used as the exclusive driving force for ATP production. The results shown in Figure 3B indicate that very little ATP is formed at a ΔΨ of ⩽40 mV. The initial rate of ATP synthesis increased exponentially with a midpoint potential of ~70 mV and approached a maximum at a ΔΨ of 120 mV, when 37 mol ATP were formed per mole ATP synthase per minute. The exponential dependence of the reaction rate on the electric potential is indicative of a rate-limiting voltage-dependent reaction step (Läuger, 1991). The effect of ΔpNa^+ on the velocity of ATP synthesis at a constant ΔΨ of 195 mV is shown in Figure 3C. The initial rates of ATP synthesis increased slightly when ΔpNa^+ was raised from 0 to 59 or 118 mV by varying external Na^+ concentrations at an internal Na^+ concentration of 10 mM. However, when the external Na^+ concentration was increased to 20 or 50 mM, corresponding to ΔpNa^+ values of –18 or –41 mV, respectively, the rate of ATP synthesis was severely diminished to almost background levels. These results are congruent with the Na^+ flux data described above, indicating that the electric potential is kinetically essential to drive ATP synthesis. ΔpNa^+ is not used as the exclusive energy source, but the rate of ATP synthesis is dependent on the Na^+ concentrations present on either side of the membrane. This kinetic effect is expected because the efficiency of Na^+ binding to the stator and release from the rotor depends upon these Na^+ concentrations. Our results agree with previous reports on $\Delta\mu Na^+$-driven ATP synthesis by reconstituted proteoliposomes containing the ATP synthase of *P.modestum* (Dmitriev *et al.*, 1993; Kaim and Dimroth, 1994). We show here for the first time that ATP synthesis can be energized by ΔΨ in the absence of ΔpNa^+. The slow rate of ΔpNa^+-induced ATP synthesis in the absence of ΔΨ by this system (Dmitriev *et al.*, 1993) could not be confirmed.

Kinetic equivalence of ΔΨ and the pH gradient (ΔpH) has been reported for a number of proton-translocating ATP synthases (Bokranz *et al.*, 1985; Slooten and Vandenbranden, 1989; Junesch and Gräber, 1991; Turina *et al.*, 1991). Initial rate measurements seemed to indicate that ΔpH could act as the sole driving force for ATP synthesis and/or added force to the ΔΨ applied. The experimental set-up for ΔpH generation was the 'acid bath procedure' (Jagendorf and Uribe, 1966); the proteoliposomes or organelles carrying the ATP synthase were first incubated for a few minutes with succinate, maleinate or malonate at pH ~5.0 and the mixture was subsequently diluted into an equal volume of buffer pH ~8.5 containing ADP, phosphate and Mg^{2+}. ATP synthesis commenced immediately and stopped after a few seconds. Unfortunately, it has never been considered that this procedure may create a substantial membrane potential by diffusion of the single protonated species of these acids. For instance, the pK_a values of maleinate are 1.8 and 6.0. Therefore, at pH 5.0, the undissociated maleinate species hardly exists and that with one protonated carboxylate predominates. If this species traverses the membrane, the proton dissociates in the external medium of high pH, resulting in a

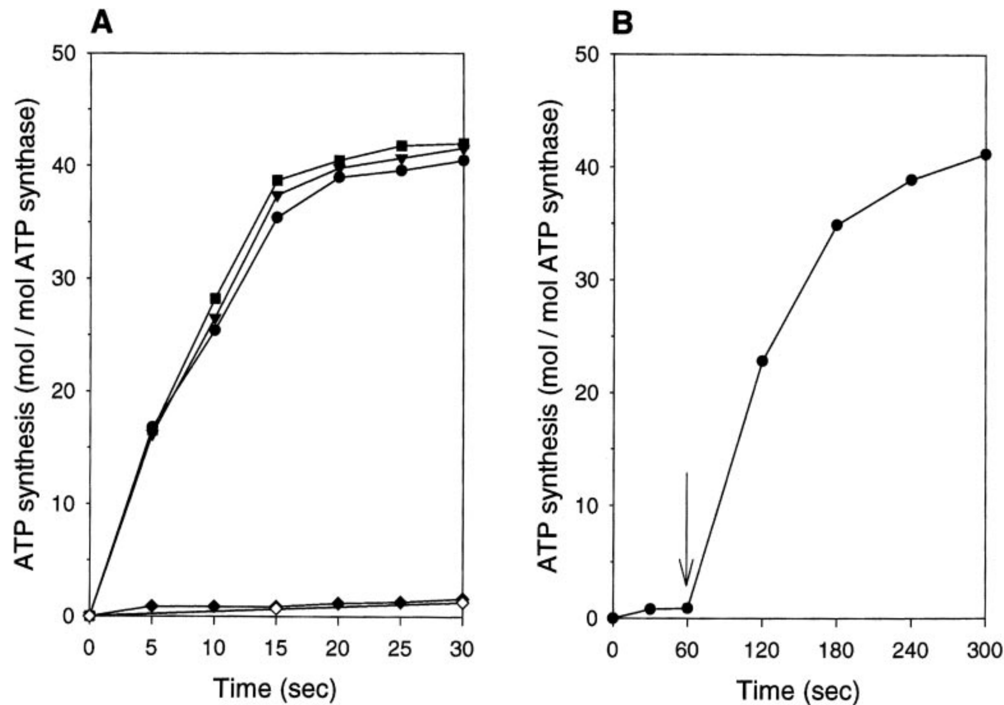

Fig. 4. (**A**) ATP synthesis by proteoliposomes after an acid–base transition. Proteoliposomes were reconstituted with the *P.modestum* ATP synthase in 5 mM potassium phosphate buffer pH 7.2 containing 10 mM NaCl and subjected to an acid–base transition with succinate (●), malonate (▼), maleinate (■), fumarate (◆) or acetate (◆) as the acidic buffer, as described in Materials and methods. Similar results as with fumarate or acetate were obtained if proteoliposomes were reconstituted in the absence of NaCl and subjected to the acid–base transition with succinate as acidic buffer (no NaCl added to reaction buffer III) (◆). In control experiments, proteoliposomes were reconstituted in the presence of 100 mM MES–NaOH buffer pH 5.0 and 10 mM NaCl. They were collected by centrifugation (200 000 *g*; 45 min) and resuspended in reaction buffer III to yield an external pH of 8.4. ATP synthesis was subsequently determined with samples (◇). In the experiment shown in (**B**) the reaction buffer III contained in addition 200 mM KCl. After 60 s, a potassium ion diffusion potential was induced by adding 20 μM valinomycin (↓). ATP formation was measured with samples as described under Materials and methods (●).

large gradient of the singly protonated species. As the transport is electrogenic, a $\Delta\Psi$ of ~150 mV can be generated.

If this were a valid explanation, proteoliposomes containing the ATP synthase of *P.modestum* should synthesize ATP in response to the acid-bath procedure. We observed that this was indeed the case (Figure 4A). ATP was rapidly synthesized after an acid–base transition with succinate, malonate or maleinate and with 10 mM NaCl on either side of the membrane. The initial rate of ATP synthesis with the *P.modestum* enzyme was about five times higher than by energizing the liposomes with a potassium diffusion potential, but the total amount of ATP synthesized was approximately equal. ATP formation was highly dependent on the nature of the acid used to bring the pH of the liposome interior down to pH 5.0, and with acetate or fumarate no ATP synthesis was observed. The rationale for this finding is described in the Discussion. In controls where the dicarboxylate buffer inside the proteoliposomes was replaced by methylethane sulfonate (MES) buffer pH 5.0, which is certainly unable to generate a diffusion potential, no ATP was produced under otherwise identical conditions. However, by applying a K^+/valinomycin diffusion potential to these proteoliposomes, ATP synthesis commenced immediately (Figure 4B). Hence, ATP synthesis was not impaired by the preparation of the proteoliposomes in MES buffer pH 5.0. Importantly, ATP was also not synthesized in the absence of Na^+ ions (Figure 4A), thus indicating that its formation was not due to the proton-translocating side activity of this enzyme (Laubinger and Dimroth, 1989).

We describe elsewhere that with succinate as the acidic buffer a diffusion potential of 125–130 mV was created following the acid–base transition by the procedure used in ATP synthesis experiments (Kaim and Dimroth, 1998b). We measured the potential by the distribution of ^{14}C-thiocyanate between internal and external volumes of (proteo)liposomes. However, no potential was found with MES instead of succinate as the acidic buffer. Furthermore, the reconstituted ATP synthase from *E.coli* catalyzed ATP formation after an acid–base transition with succinate, maleinate or malonate but not with MES as the acidic buffer. ATP was also synthesized at $pH_{in} = pH_{out} = 7.2$ with a K^+/valinomycin diffusion potential. The ATP synthesis rate corresponded exponentially to the potential approaching a turnover of 1 s^{-1} at $\Delta\Psi > 100$ mV. Hence, we conclude that the electric potential is essential in overcoming the activation energy of a voltage-dependent reaction step in the ATP synthesis mechanism.

Interestingly, Jagendorf and Uribe (1966) observed in their classical study that ATP synthesis by the chloroplast ATP synthase was accelerated 15-fold over basal level by preincubation with 10 mM succinate, although the same ΔpH was generated by the acid–base transition (with glutamate pH 4 as acidic and Tris pH 8 as basic buffer). This effect can now be explained by generation of a succinate diffusion potential as a mandatory driving force also for the chloroplast ATP synthase. Hence, the depend-

ence of ATP synthesis on voltage is probably a general principle of F_1F_0 ATP synthases.

Discussion

Two different models of intersubunit rotation in F_0 have been proposed with different implications for the torque-generating mechanism. Arguments disfavouring the model with two staggered stator (a subunit) half channels communicating with rotor (c subunit) sites from the two sides of the membrane (Vik and Antonio, 1994; Duncan *et al.*, 1995; Junge *et al.*, 1997; Elston *et al.*, 1998) have been described elsewhere (Dimroth *et al.*, 1998; Kaim and Dimroth, 1998a; Kaim *et al.*, 1998). In these models, the sites become occluded while the rotor revolves through the lipid phase from one stator half channel to the next. Here, we would like to concentrate and elaborate on the alternative model that we have proposed recently (Dimroth *et al.*, 1998; Kaim *et al.*, 1998) with special emphasis on the results reported in this study. We envision the path of Na^+ or H^+ ions through the F_0 motor from the periplasm of a bacterial cell via an a subunit stator channel to the binding site of that c subunit of the rotor at the stator–rotor interface. In turning the rotor, the bound ion becomes accessible to the cytoplasm and dissociates. There are two important restraints for rotation based on observations from the *P.modestum* ATP synthase. First, rotor sites entering the stator boundary during ATP hydrolysis-driven rotation of the ring must be filled, and secondly, these sites must be empty in order to leave the stator boundary. If during ATP synthesis rotation is reversed, the incoming sites must be empty and the outgoing sites must be filled. These features derive from the observation that ATP hydrolysis by the *P.modestum* ATP synthase is Na^+-dependent (Laubinger and Dimroth, 1987; Kluge and Dimroth, 1993), i.e. rotor sites must be filled to enter the stator boundary. They further derive from the observation that ATP hydrolysis by the a subunit triple mutation with the Na^+-impermeable stator channel was blocked after the occlusion of one Na^+ per ATP synthase (Kaim *et al.*, 1998). Hence, the torque generated by ATP hydrolysis is apparently insufficient to turn a Na^+-boarded rotor subunit out of the rotor/stator interface.

An interesting facet of the motor is its idling mode in the absence of external driving forces as discovered by the free exchange of external $^{22}Na^+$ for internal unlabeled Na^+ ions. Importantly, the rate of this exchange reaction was about the same as that of ATP-driven Na^+ pumping. It is therefore unlikely to involve slow steps that are unrelated to the common catalytic mechanism. It is also important that the Na^+ exchange rate was not significantly affected by modification of part of the rotor sites with DCCD which completely blocked ATP-driven Na^+ translocation. Hence, the idling motor apparently shuttles Na^+ ions across the membrane by only limited back and forth rotation of the rotor versus the stator in accordance with the one channel model (Dimroth *et al.*, 1998; Kaim *et al.*, 1998). To catalyze the Na^+ exchange reaction by the two channel mechanism would, on the other hand, require almost complete revolutions of the rotor to make alternative contacts to the two stator half channels and this should be prevented if a single rotor site of the ring connecting these channels is modified by DCCD.

From these exchange experiments we conclude that the motor persists in an idling mode where it is not able to perform work at a ΔpNa^+ of ~100 mV, but switches into a torque-generating directed rotation by applying a $\Delta\Psi$ of $\pm$90 mV. These data are consistent with our previous results on Na^+ ion fluxes across the isolated motor (F_0) reconstituted into proteoliposomes. The system catalyzed efficient $^{22}Na^+_{out}/Na^+_{in}$ exchange, but was unable to perform $^{22}Na^+$ uptake if a ΔpNa^+ of –180 mV was applied. Na^+ uptake was strictly dependent on $\Delta\Psi$ (inside negative) and significant activities required electric potentials well above –40 mV. Upon applying $\Delta\Psi$ (inside positive) of sufficient magnitude the flux of Na^+ ions was reversed (from the inside to the outside) (Kluge and Dimroth, 1992). The following general mechanism therefore seems to apply: the idling motor either isolated or combined with the catalytic part of the machine shuttles Na^+ ions across the membrane because the rotor has limited mobility. This mobility persists in the presence of Na^+ concentration gradients. These gradients are therefore unable to switch the motor from idling to directed rotation of the rotor against the stator. In contrast, voltage induces the switch; $^{22}Na^+_{out}/Na^+_{in}$ exchange is therefore replaced by the electrically-driven directed Na^+ movement through the motor. With the isolated F_0 motor, the electric field apparently drives continued revolutions of the rotor against the stator leading to an accumulation of Na^+ ions on the negative side of the membrane. In the F_1F_0 ATP synthase, however, Na^+ movement through the motor is arrested after a fraction of one revolution when the load torque elicited by the catalytic F_1 moiety prevents further rotation of the rotor. This system is therefore unable to catalyze $\Delta\Psi$-driven Na^+ accumulation without coupling to ATP synthesis.

The proposed voltage-dependent switch of the motor from idling into a torque-generating directed rotation that is coupled to ATP synthesis is nicely complemented by the observation that ATP formation obligatorily requires an electric potential as the driving force. This statement is in contrast to the general opinion that ΔpH and $\Delta\Psi$ are not only thermodynamically but also kinetically equivalent driving forces for ATP synthesis. The most detailed studies on this subject were performed by Gräber and colleagues with reconstituted chloroplast ATP synthase (Junesch and Gräber, 1991; Turina *et al.*, 1991). In these studies and in all others with reconstituted proteoliposomes that we are aware of, the pH gradient was produced by the acid bath procedure with succinate as the acidic buffer (Jagendorf and Uribe, 1966). As this procedure in addition generates a diffusion potential of $>$100 mV (Kaim and Dimroth, 1998b) it is no longer tenable to conclude that ATP synthesis is energized by ΔpH only. The observed increase in the rate of ATP synthesis by increasing the size of the K^+/valinomycin diffusion potential is, however, in accord with the results presented here. The diffusion of dicarboxylic acids in the monoanionic form across the membrane seems to be a specific property for succinate, malonate and maleinate, respectively. Acetate, on the other hand, is known to diffuse through the membranes only as the protonated species, providing the basis for ΔpH determination by the distribution of acetate between the two compartments of the membrane (Rottenberg, 1979). Accordingly, ATP synthesis was not supported if the acid-bath procedure

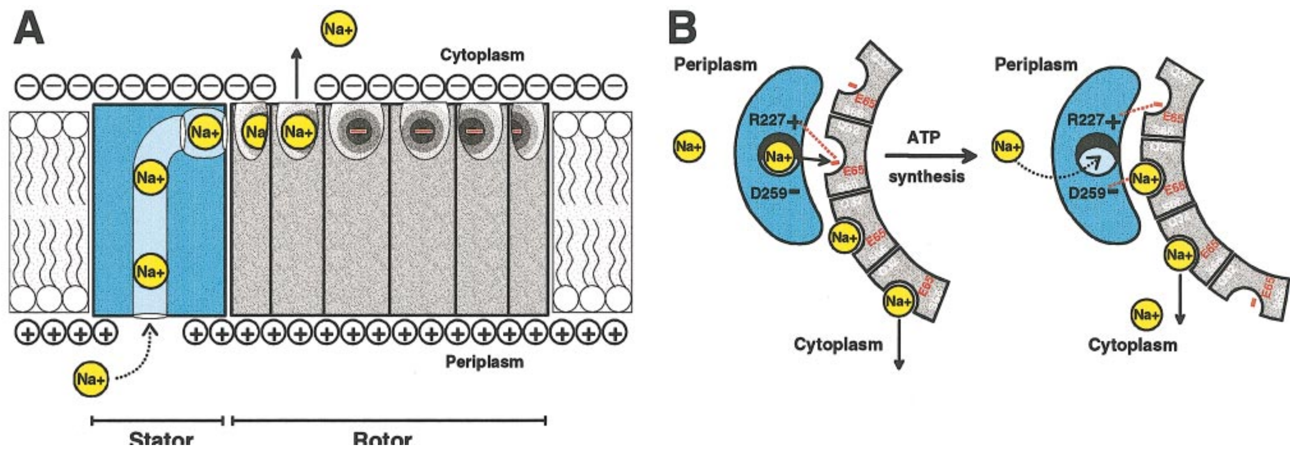

Fig. 5. Model of the ATP synthase motor. (**A**) Longitudinal section through the motor. During ATP synthesis, Na^+ ions are driven by the electric potential (indicated by the circles with positive and negative signs on the two membrane sides) from the periplasm through the stator channel (subunit a) into a site, tentatively placed near the negatively charged cytoplasmic surface. Subsequent Na^+ ion transfer to an empty rotor site (on subunit c) induces rotation. This makes the bound Na^+ accessible to the cytoplasm where it dissociates. (**B**) Cross section through the motor, showing the mechanism for torque generation (view from the cytoplasm). As indicated by the red dotted line, the positive stator charge (R227) electrostatically attracts an empty rotor site with the negatively charged E65 residue. After transfer of Na^+ from the stator to this rotor site, a new empty rotor site becomes attracted by R227 and unidirectional rotation continues.

was performed with acetate instead of one of the dicarboxylic acids mentioned above. We therefore reasoned that these dicarboxylates may diffuse through the membrane as monoanions by forming a ring structure in which the two carboxylates are bridged with the proton. In this way, the negative charge would become highly delocalized, facilitating diffusion of these compounds across the membrane. To check this hypothesis we performed the acid bath procedure with fumarate which is unable to form a ring. No ATP formation was seen under these conditions. A key element to translocate a charged compound across the membrane appears to be delocalization of its charge. Examples are thiocyanate, tetraphenylborate, dinitrophenolate and also, as shown here, certain dicarboxylates.

ATP synthesis has also been measured with submitochondrial particles (Thayer and Hinkle, 1975). The highest rates were observed by applying a K^+/valinomycin diffusion potential and an acid–base transition with malonate pH 5.0 as the acidic buffer. The rates were reduced to 25 and 15%, respectively, if the synthesis was driven either by the acid–base transition or a K^+/valinomycin diffusion potential only. The conclusion of a ΔpH-driven ATP synthesis again is equivocal because a diffusion potential should have been generated by the acid–base transition with malonate.

A hypothetical mechanism linking $\Delta\Psi$-driven Na^+ ion movement through the motor to the generation of rotary torque for ATP synthesis is shown in Figure 5. In this model we place the Na^+-binding rotor sites near the membrane boundary. This position of the rotor sites is not crucial for our model, however, which would also work with binding sites placed more to the centre of the membrane and connected to the cytoplasmic surface by appropriate access channels within the c subunits. As discussed elsewhere (Kaim *et al.*, 1998) rotor sites are freely accessible from the cytoplasm except for those at the stator interface. In the NMR structure of the *E.coli* subunit c monomer in chloroform/methanol/water (4:4:1) the putative H^+ binding Asp61 residue is located in the centre of an extended hydrophobic domain comprising two antiparallel α-helices and was therefore modeled to be positioned in the middle of the membrane (Girvin *et al.*, 1998). However, there is no guarantee that this structure reflects that of the c subunit oligomer in the natural environment of the membrane. In dodecylsulfate micelles, *P.modestum* subunit c folds differently, and according to this structure the Na^+ binding site is likely to be localized at the membrane boundary (Dimroth *et al.*, 1998). We anticipate voltage-dependent binding of a coupling ion at the stator site which we intuitively place near the negatively charged surface. The subsequent transfer of Na^+ (or H^+) to the adjacent rotor site may be facilitated by electrostatic attraction through the negatively charged cE65 residue, augmented perhaps by a low dielectric environment when in contact with the stator. As the occupied rotor site can leave the stator interface only in one direction (counterclockwise movement if viewed from the cytoplasm), the sense of rotation is determined.

A key element in this mechanism is the voltage-driven immediate reoccupation of the empty stator site which prevents Na^+ ions moving backwards from the rotor to the stator and therefore assures unidirectional rotation. We further envisage electrostatic interactions between charged amino acid residues of the stator and rotor as essential for the generation of rotary torque. In this respect it is important that the stator carries the conserved and positively charged residues D259 and R227 (*P.modestum* numbering) of which at least the latter is functionally essential (Valiyaveetil and Fillingame, 1997). Thus, an empty rotor site with the negatively charged cE65 residue might be electrostatically attracted by aR227. Subsequently, the negative rotor charge is balanced by boarding Na^+ from the stator site. This allows further rotation of the rotor through the electrostatic attraction of the next empty rotor subunit by aR227 and so forth. We intuitively place aR227 and aD259 on opposite sides of the stator site. aD259 may be dispensable for torque generation in the ATP synthesis mode of the enzyme. It may be important, however, to assure ATP-driven coupled Na^+ pumping when the engine operates in the reverse direction. In the

presence of the negative stator charge, ATP-driven rotation of unloaded rotor sites beyond the stator boundary which would inevitably lead to uncoupling, could be prevented by repulsion of cE65 and aD259. Hence, our concept for the ATP synthase's motor function involves electrostatic interactions between stator and rotor sites that are periodically modulated by charge balancing as the coupling cations move across. The bacterial flagellar motor may operate by a similar electrostatic mechanism (Elston and Oster, 1997).

Materials and methods

Bacterial strains and media

The bacterial stains *E.coli* MPA762 (Kaim and Dimroth, 1998a) and *P.modestum* were used in this work. *Escherichia coli* MPA762 was cultivated routinely in M13 minimal media supplemented with thiamine (0.1 mg/l) and 35 mM succinate or 10 mM glucose (Kaim and Dimroth, 1993). The phenotype of *E.coli* MPA762 was verified by screening on succinate minimal agar plates containing Na^+ concentrations of 10 mM or below 50 μM, respectively (Kaim and Dimroth, 1995). *Propionigenium modestum* cells were grown on succinate under strictly anaerobic conditions as described (Laubinger and Dimroth, 1988).

Biochemical procedures

The ATP synthase was purified and determined by its ATP hydrolysis activity as described (Kaim and Dimroth, 1993, 1994). The $^{22}Na^+$ occlusion experiments by the ATP synthase were performed as described (Kaim *et al.*, 1998).

$^{22}Na^+_{out}/Na^+_{in}$-exchange by the P.modestum ATP synthase

The ATP synthase of *P.modestum* was reconstituted into liposomes according to the freeze–thaw sonication method (Kluge and Dimroth, 1992). Proteoliposomes in KP-buffer (5 mM potassium phosphate pH 7.2) were incubated overnight with 100 mM NaCl. Fourteen microliters of the Na^+-loaded F_1F_0-liposomes (1.0 mg of lipid) were diluted into 0.7 ml assay buffer at 25°C [2 mM Tricine–KOH buffer pH 7.2, containing 5 mM $MgCl_2$, 200 mM choline chloride and 5 μl $^{22}NaCl$ (1 μCi, carrier free)]. The exchange was terminated after appropriate incubation periods by adsorbing external $^{22}Na^+$ by cation exchange chromatography on Dowex 50 loaded with K^+ as described (Laubinger and Dimroth, 1988). The columns were washed with 2× 0.3 ml 2 mM Tricine–KOH buffer pH 7.2, containing 5 mM $MgCl_2$ and 200 mM sucrose and the radioactivity of the eluate corresponding to internal $^{22}Na^+$ was measured by γ counting. To induce a positive diffusion potential 14 μl of the Na^+-loaded proteoliposomes were incubated for 1 min with 20 μM valinomycin and diluted into 0.7 ml assay buffer containing 200 mM KCl instead of 200 mM choline chloride. To induce a negative diffusion potential the proteoliposomes in KP buffer were incubated overnight with 200 mM KCl and 100 mM NaCl followed by incubation for 1 min with 20 μM valinomycin and dilution of 14 μl into 0.7 ml assay buffer.

Measurement of ATP synthesis

ATP synthesis was determined by the luciferin–luciferase assay as described (Kaim and Dimroth, 1994). The ATP synthase of *P.modestum* was reconstituted into proteoliposomes by the freeze–thaw sonication procedure (Kluge and Dimroth, 1992). Energization was performed by two different methods.

(i) Valinomycin-induced K^+ diffusion: proteoliposomes were diluted 1:10 into reaction buffer I (2 mM Tricine–LiOH pH 7.2, 200 mM KCl, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 mM ADP, 5 mM potassium phosphate) and the electric potential was induced by adding 20 μM valinomycin to the reaction mixture. Samples of 50 μl were taken after different incubation periods and the reaction was terminated with 5 μl 20% trichloroacetic acid. The ATP content was determined with 11 μl of the centrifuged samples. To determine whether ΔpNa^+ can drive ATP synthesis, the reconstituted proteoliposomes were loaded by overnight incubation with 200 mM NaCl. External Na^+ was exchanged by K^+ by passing the suspension over a column of Dowex 50 loaded with K^+ and the proteoliposomes were collected by centrifugation (200 000 *g*; 45 min). After resuspension in 0.5 ml 2 mM Tricine–LiOH buffer pH 7.2 containing 200 mM choline chloride the suspension was diluted 1:10 at 25°C into reaction buffer II [2 mM Tricine–LiOH pH 7.2, 200 mM choline chloride, 5 mM $MgCl_2$, 0.1 mM ADP, 5 mM potassium phosphate (intrinsic Na^+ concentration 0.1 mM)], and the ATP formed was determined with samples treated as described above.

(ii) Acid–base transition: proteoliposomes were incubated for 1 min with 100 mM of succinate, maleinate, malonate, fumarate or acetate, pH 5.0. To each suspension 3 ml 5 M NaOH was added followed by rapid mixing with an equal volume of reaction buffer III (100 mM glycylglycine pH 8.5, 10 mM NaCl, 5 mM potassium phosphate, 5 mM $MgCl_2$, 0.1 mM ADP) to yield a final pH of 8.1–8.3. The ATP formed was analyzed with samples treated as described above.

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