J. Membrane Biol. 104, 179–191 (1988)

The Journal of
Membrane Biology

Pump and Displacement Currents of Reconstituted ATP Synthase on Black Lipid Membranes

B. Christensen,† M. Gutweiler,‡ E. Grell,† N. Wagner,‡ R. Pabst,‡ K. Dose,‡ and E. Bamberg†
†Max Planck Institute for Biophysics, D-6000 Frankfurt am Main 71, Federal Republic of Germany, ‡Institute for Biochemistry, Johannes Gutenberg University, D-6500 Mainz, Federal Republic of Germany

Summary. Purified ATP synthase (F_0F_1) from *Rhodospirillum rubrum* was reconstituted into asolectin liposomes which were than adsorbed to a planar lipid bilayer. After the addition of an inactive photolabile ATP derivative (caged ATP), ATP was released after illumination with UV light, which led to a transient current in the system. The transient photocurrent indicates that the vesicles and the planar membrane are capacitatively coupled. Stationary pump currents were obtained after addition of protonophores. These currents are specifically inhibited by oligomycin and stimulated threefold by inorganic phosphate (P_i). In analogy oligomycin-sensitive pump currents in the reverse direction coupled to net ATP synthesis were induced by a light-induced concentration jump of ADP out of caged ADP, demonstrating the reversibility of the pump. For this, a preformed proton motive force and P_i were necessary.

In a second series of experiments, proteoliposomes containing both ATP synthase and bacteriorhodopsin were adsorbed to a planar bilayer. The system was excited by a laser flash. The resulting photocurrents were measured with a time resolution of 2 μsec. In the presence of ADP, the signal was modulated by the electrical activity of ATP synthase. ADP-induced charge displacements in ATP synthase, with time constants of 11 and 160 μsec were obtained. The kinetics of the charge movements were slowed down by F_0 specific inhibitors (DCCD or oligomycin) and were totally absent if ADP binding to F_1 is prevented by the catalytic site-blocking agent NBD-Cl. The charge displacement of ATP synthase is coupled only to the membrane potential induced by the electrical activity of bacteriorhodopsin. The charge movements are interpreted as conformational transitions during early steps of the reaction cycle of ATP synthase.

Key Words ATP synthase · bacteriorhodopsin · caged ATP · black lipid membrane · *Rhodospirillum rubrum*

Introduction

According to the chemiosmotic theory (Mitchell, 1961), ATP synthesis in chloroplasts, bacteria and mitochondria catalyzed by ATP synthase is driven by an electrochemical gradient of protons across the membrane. The ATP synthase (EC. 3.6.1.34) consists of a catalytic part, F_1, and a membrane integrated portion, F_0, functioning as a proton channel. After solubilization and purification (Schneider et al., 1980), the enzyme has been reconstituted into liposomes together with the light-driven proton pump bacteriorhodopsin, in its monomerized form (Wagner et al., 1987). The illumination of the proteoliposomes effects an electrochemical gradient across the liposomal membrane, and ATP synthesis can be recorded (Racker & Stoeckenius, 1974). Electrogenic properties of ion pumps such as ATP synthase or bacteriorhodopsin were demonstrated by electrical measurements on planar lipid membranes as was first shown by Drachev et al. (1974, 1976*a*,*b*). In these studies reconstituted proteoliposomes were adsorbed onto a preformed black lipid membrane, so that the liposomes are capacitatively coupled to the external measuring circuit. After addition of ATP, an oligomycin-sensitive transmembrane electric potential was built up within minutes. Alternatively, ATP synthase may be incorporated directly into the planar membrane (Borisova, Babakov & Kolomytkin, 1984; Hirata et al., 1986). Small pump currents with a poor signal-to-noise ratio have been obtained, leading also to unsatisfactory time resolution. Previous papers have shown that the method of capacitive coupling yields much better results in amplitude and time resolution (Drachev et al., 1976*b*; Herrmann & Rayfield, 1978; Fahr, Läuger & Bamberg, 1981; Bamberg, Hegemann & Oesterhelt, 1984; Fendler et al., 1985). Therefore, in this paper the method of adsorption of proteoliposomes to a planar lipid membrane was chosen. For the ATP synthase proteoliposomes, a light-induced concentration jump of ATP or ADP was used to initiate the electric activity of the ion pump. This was achieved by the use of caged nucleotides (caged ADP or caged ATP) as previously shown for the Na^+K^+-ATPase (Fendler et al., 1985). The time resolution is limited to milliseconds

by the photolysis of the caged compounds (Kaplan, Forbush & Hoffman, 1978; McCray et al., 1980). Using the bacteriorhodopsin/ATP synthase proteoliposomes, kinetics of the electric currents of the ATP synthase could be determined in the microsecond range.

Materials and Methods

Chemicals

P^3-1-(2-nitro)phenylethyladenosine-5'-diphosphate and the analogous triphosphate (caged ADP and caged ATP) were prepared as described previously as triethylammonium salts (Kaplan et al., 1978; Fendler et al., 1985).

The protonophore 1799 (2,6-dihydroxyl-1,1,1,7,7,7-hexafluoro-2,6-bis(trifluoromethyl)heptan-4-one) was kindly provided by Dr. P. Heydtler, Du Pont de Nemours, Wilmington. Monensin was a gift from Dr. G. Szabo, Galveston, TX. Tetraphenylborate ($TPhB^-$) was purchased from Fluka, Buchs, CH. DCCD, NBD-Cl and oligomycin were from Sigma, Munich, FRG. All uncouplers and inhibitors were dissolved in ethanol p.A. and added to both sides of the black lipid membrane to a final ethanol concentration of less than 1% (vol/vol). All other reagents were of analytical grade from Merck, Darmstadt, FRG.

Proteins and Reconstitutions

The ATP synthase was prepared from *Rhodospirillum rubrum* FR 1 cells (Schneider et al., 1980). Bacteriorhodopsin was prepared from *Halobacterium halobium* strain S9 (Oesterhelt & Stoeckenius, 1974). Monomerization and subsequent delipidation was obtained after the procedure applied by Pabst et al. (Pabst, Nawroth & Dose, 1984; Pabst & Dose, 1985).

Preparation of bacteriorhodopsin/ATP synthase proteoliposomes was achieved by a one-step detergent dialysis or a quick centrifugation procedure (Wagner et al., 1987). In short, 20 mg of asolectin were sonicated in 2.0 ml standard buffer (20 mM Tris/acetate, pH 8.0, 50 mM KCl, 0.2 mM EDTA) at 20°C for 10 min under nitrogen with a microtip of a Branson sonifier B 12 (40 W). After addition of ATP synthase and bacteriorhodopsin, a final volume of 3 ml containing 2 mg/ml asolectin, 0.1 mg/ml ATP synthase and 0.1 mg/ml bacteriorhodopsin was dialyzed for 24 hr, against standard buffer or centrifuged over a Sephadex G-50 column preequilibrated with standard buffer. Preparation of bacteriorhodopsin liposomes or ATP synthase liposomes was achieved by the same procedure.

Measurement of light-driven ATP synthesis of the bacteriorhodopsin/ATP synthase liposomes was performed by means of the luciferin/luciferase assay as described by Lemasters and Hackenbrock (1979). Light-driven ATP synthesis rates of 150–280 nmol ATP/min mg ATP synthase, corresponding to about 10–20% of the in vivo activity, are routinely obtained. ATP hydrolysis and its stimulation by the uncoupler FCCP were determined for ATP synthase liposomes by measurement of the liberated orthophosphate according to Arnold et al. (1976).

Planar Lipid Bilayer Setup

For the formation of a positively charged black lipid membrane, a solution of 1.5% wt/vol diphytanoyl-phosphatidylcholine (Avanti Biochemicals, Birmingham, AL) and 0.025% (wt/vol) octadecylamine (Riedel de Haën, Hannover, FRG) in *n*-decane (Dancshazy & Karvaly, 1976) was spread over a 1-mm hole in the septum of a Teflon chamber separating two compartments containing standard buffer, 1 mM $MgCl_2$ and 1 mM KH_2PO_4 (P_i) in the case of bacteriorhodopsin/ATP synthase liposomes and standard buffer with varying amounts of $MgCl_2$ in the case of ATP synthase liposomes. Proteoliposomes (max. 30 μg protein) were added at least 10 min after complete blackening of the lipid membrane to the rear compartment of the cuvette, whereas the light entered the cell through the front compartment and was focused on the black lipid membrane. The membrane cell was connected to the external measuring circuit via platinized platinum electrodes for the fast kinetics of the pump currents. All other experiments were performed with Ag/AgCl electrodes. To avoid artificial photoeffects, the electrodes were separated from the aqueous compartments of the cell by agar-agar salt bridges.

The experimental procedure for measuring the ATP-driven pump currents after a photo-induced concentration jump of ATP out of caged ATP has been described by Fendler et al. (1985). Under the illumination conditions of this setup (OSRAM HBO 200W2, 125 msec flash duration, 3.7 W/cm² in the plane of the membrane), each UV flash liberates about one third of the caged nucleotide into free nucleotide as described by Nagel et al. (1987). For the reversal of the pump currents a concentration jump of ADP out of caged ADP was applied. The solution contained standard buffer, 0.5 mM $MgCl_2$, either 50 μM ATP and 45 μM caged ADP or no ATP, 1 mM P_i and 450 μM caged ADP. A series of control experiments was performed in order to avoid possible UV-induced electrical artifacts. The maximal sensitivity was 0.02 nA/cm². Without any additions and also with ATP synthase proteoliposomes adsorbed to the black lipid membrane, no light-induced effects were observed. The caged nucleotides (190 μM) show a small UV artifact under the conditions (ca. 0.02 nA/cm², 20 mM Tris, pH 8.0) as already described (Fendler et al., 1985; Nagel et al., 1987). Since this signal depends on the buffer concentration (it increases up to 8 nA/cm² at 0.1 mM Tris, *data not shown*) we assigned it to the irreversible proton release during the photolytic release reaction of ATP out of caged ATP (Kaplan et al., 1978; McCray et al., 1980) and not to the hydrophobic nitrobenzyl group interacting with the planar membrane as originally suggested by Fendler et al. (1985). Therefore, strongly buffered solutions (at least 10 mM) are necessary.

The experimental setup for the time-resolved photoelectric measurements on bacteriorhodopsin/ATP synthase proteoliposomes is similar to that described by Fahr et al. (1981). The adsorption of the vesicles to the black lipid membrane was determined by the increase of photocurrents upon illumination with yellow light ($\lambda \geq 450$ nm, 0.4 W/cm²) in the plane of the planar membrane. Excitation of bacteriorhodopsin with a short actinic laser flash (575 nm wavelength, 10 nsec pulse duration) was performed with an excimer laser pumped dye laser (EMG 100, FI 2002, both from Lambda-Physik, Göttingen, FRG). The transient photocurrents were amplified with a homemade *I-V*-converter (10^7 V/A), recorded and averaged on a fast digital oscilloscope (Biomation 4500, Gould, Seligenstadt, FRG).

Description of the Compound Membrane

A quantitative description of the bacteriorhodopsin proteoliposome/black lipid membrane system was given by Herrmann and Rayfield (1978) in an equivalent circuit diagram. The description is also valid for ATP driven pumps like the Na^+K^+-ATPase (Fendler et al., 1985; Borlinghaus, Apell & Läuger, 1987) and the Ca^{2+}-ATPase from sarcoplasmic reticulum (Hartung et al.,

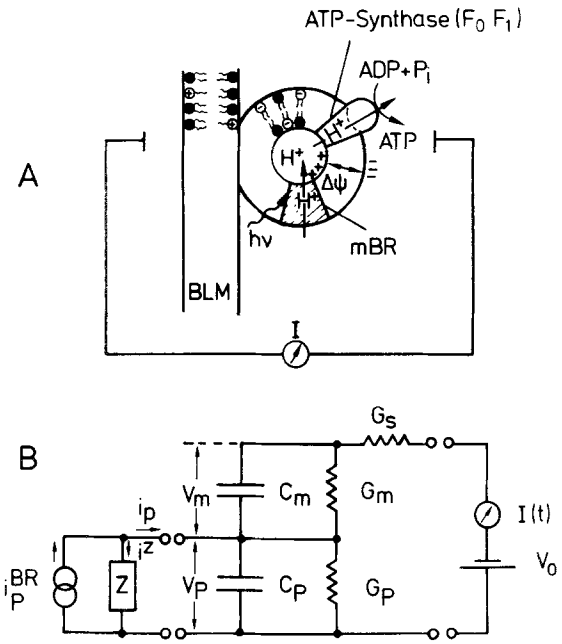

Fig. 1. Schematic representation of the composed membrane system. (*A*) Proposed arrangement of the bacteriorhodopsin/ATP synthase proteoliposomes adsorbed to the underlying planar lipid membrane. (*B*) Equivalent circuit diagram of the two membranes in series G_m, G_p, conductivity of the black lipid membrane and the proteoliposome membrane, respectively. C_m, C_p, capacitance of the black lipid membrane and the proteoliposome membrane, respectively. I_p^{BR} pump current generator, Z time-dependent ATP synthase impedance, i^Z current in Z, i_p current flow into the network: G_s conductance of the external circuit

1987). A similar analysis for photocurrents with a time resolution of 1–2 μsec was obtained for purple membranes (bacteriorhodopsin membrane sheets from *H. halobium* adsorbed to a black lipid membrane system (Fahr et al., 1981). For the bacteriorhodopsin/ATP synthase proteoliposomes, the ATP synthase complex has to be inserted as an additional element in the equivalent circuit. The schematic representation of the compound membrane and the equivalent circuit is given in Fig. 1*A* and *B*.

In the absence of ADP the bacteriorhodopsin-induced pump current $I(t)$ is given by (Fahr et al., 1981)

$$I(t) = \frac{C_m}{C_m + C_p} I_{po}(t) \tag{1}$$

with

$$I_{po}(t) = \sum_{i=1}^{4} a_i e^{-t/\tau_i}$$

where C_m and C_p are the capacitances of the underlying planar film and of the vesicular membrane respectively. I_{po} is the primary pump current. Intrinsic relaxation processes τ_i of bacteriorhodopsin can be measured within the time window (Fahr et al., 1981).

$$\tau_s \ll \tau_i < \tau \tag{2}$$

$$\tau_s = \frac{C_{tot}}{G_s} \tag{3}$$

$$\tau = \frac{C_m + C_p}{G_m + G_p}. \tag{4}$$

C_{tot} is the total capacitance of the compound membrane, G_s the conductance of the external circuit, G_m, G_p represent the conductance of the lipid bilayer and of the vesicular membrane, respectively. The experimentally obtained time window goes from 2 μsec to 0.5 sec. (Fahr et al., 1981). The data were processed on a PDP-11 minicomputer system (DEC, Munich, FRG). The fit is performed by means of the program DISCRETE (Provencher, 1976). The obtained photocurrent was fitted according the equation (Fahr et al., 1981)

$$I(t) = \sum_{i=1}^{4} a_i \exp(-t/\tau_i) \tag{5}$$

where a_i represents the photocurrent amplitudes.

The light-induced displacement current in bacteriorhodopsin yields a time-dependent change of the potential across the vesicular membrane, which presumably induces corresponding charge displacements on the ATP synthase complex represented in the scheme as a time-dependent impedance Z. The current going into the network is the difference of i_p^{BR}, which is the bacteriorhodopsin induced pump current and of i^Z, which is the shunt current in Z.

Control experiments were carried out with bacteriorhodopsin liposomes under the same conditions as the corresponding experiments with bacteriorhodopsin/ATP synthase liposomes.

With the assumption that the ATP synthase displacement currents do not change the intrinsic kinetics of bacteriorhodopsin, the ATP synthase contribution to the observed photocurrents can be obtained by substraction of the bacteriorhodopsin signal from the composed current.

Results

Effects of Caged Nucleotides on Enzymatic Activity of Reconstituted ATP Synthase

The effect of caged nucleotides (caged ATP and caged ADP) was studied under ATP hydrolysis and ATP synthesis conditions, respectively. ATP synthase proteoliposomes showed no ATP hydrolysis in the presence of caged ATP (600 μM) and Mg^{2+} (250 μM). UV illumination (λ ≥ 300 nm, 0.6 mW/cm², 30 min) of a solution of 600 μM caged ATP resulted in a photolytic release of 350 μM ATP. The addition of ATP synthase proteoliposomes (5 μg ATP synthase) and Mg^{2+} (250 μM) to this solution led to a specific ATPase activity of 1.4 μM ATP min^{-1} mg^{-1}. Control experiments with 360 μM ATP showed a specific ATPase activity of 1.6 μM ATP min^{-1} mg^{-1}. The influence of caged ADP on the ATP synthesis activity was tested with bacteriorhodopsin/ATP synthase proteoliposomes. Irradiation of bacteriorhodopsin with visible light λ ≥ 450 nm was used for the creation of the necessary pmf across the vesicular membrane. As shown in Fig. 2 no ATP synthesis occurs in the presence of caged ADP (600 μM) and 250 μM Mg^{2+}, during light-driven H^+ pumping by bacteriorhodopsin. After previous

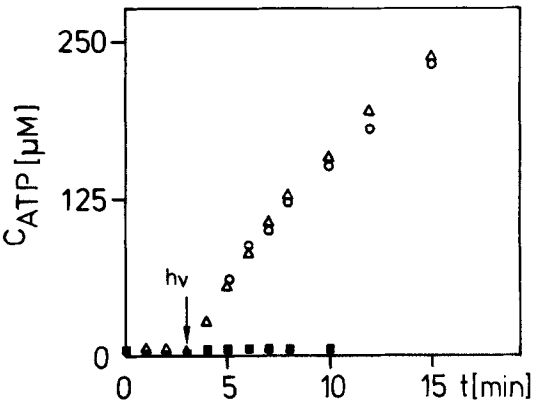

Fig. 2. ATP synthesis in bacteriorhodopsin/ATP synthase proteoliposomes (125 μg ATP synthase/ml) in standard buffer. ■ 600 μM caged ADP, 250 μM Mg^{2+}, 1 mM P_i; △ 600 μM caged ADP (UV illumination 30 min 0.6 mW/cm² yields 350 μM free ADP) 250 μM Mg^{2+}, 1 mM P_i; ○ 360 μM ADP, 250 μM Mg^{2+}, 1 mM P_i. The arrow indicates the start of an irradiation of bacteriorhodopsin (λ ≥ 450 nm, 400 mW/cm²)

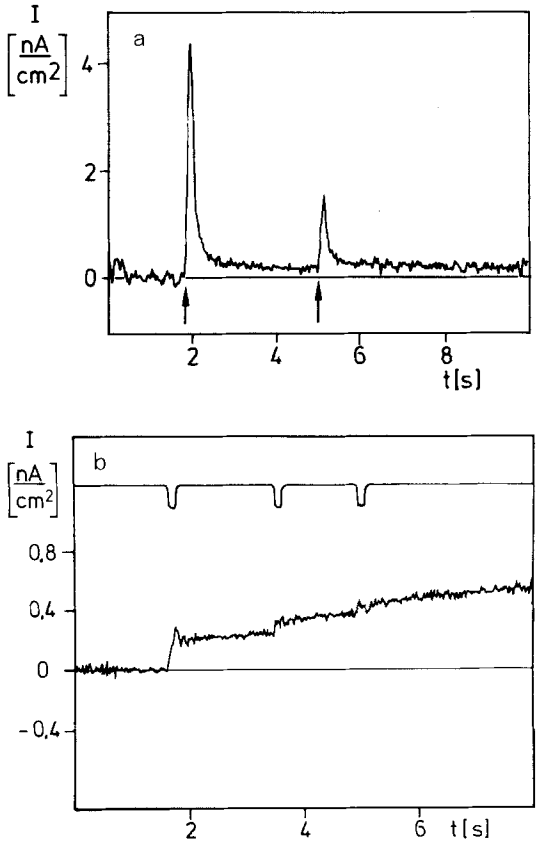

Fig. 3. (*a*) Short-circuit current of ATP synthase proteoliposomes attached to a black lipid membrane after a concentration jump of ATP out of caged ATP. Standard buffer, 0.39 mM P_i, 0.5 mM $MgCl_2$, 0.32 mM caged ATP, addition of 190 μg ATP synthase in liposomes. The arrows indicate a UV flash (3.7 W/cm²) of 125 msec duration. (*b*) Stationary pump currents (lower trace) in presence of the protonophore 1799 (5 μM) addition of 170 μl ATP synthase proteoliposomes (≈17 mg ATP synthase, 0.1 mg protein, 2 mg asolectin/ml). The upper trace indicates the flash duration

illumination (λ ≥ 450 nm) and with UV light similar to that described above for caged ATP, ATP synthesis was observed. Figure 2 also shows that under the same ADP conditions no difference was observed in ATP synthesis activity of the vesicles between the photolytically released ADP and the added ADP.

Pump Currents by ATP Hydrolysis

After addition of reconstituted ATP synthase proteoliposomes and caged ATP to one side of a planar lipid membrane, transient short-circuit currents were elicited upon UV illumination (Fig. 3*a*). The sign of the current is always the same and corresponds to a transport of positive charges to the proteoliposome's free side of the planar film. The ATP-driven current decays in the 100-msec range, indicating capacitive coupling between adsorbed vesicles and the planar film. The decay cannot be accounted for by the consumption of ATP by the enzyme or by the diffusing process of ATP out of the UV light beam under these experimental conditions (*see* Discussion). From that it may be concluded that the vesicles are only attached to the planar film and not integrated to it. Otherwise, stationary pump currents lasting several seconds reflecting the continuous function of the pump should be observed. Further flashing of the membrane system several seconds after the first flash evoked a current with only about 40% of the amplitude of the previous flash (*see* Fig. 3*b*). This is due to the smaller concentration jump in the second flash (67% of the first one) and presumably the build-up of a significant membrane potential across the vesicle membrane, already inhibiting further proton transport by the ATP synthase after a few seconds. Extending the time interval between subsequent flashes to several minutes yielded constant amplitudes of the pump current. The signal was totally abolished by the ATP synthase specific inhibitor oligomycin (30 μM) in less than 10 min. A schematic representation of the experimental situation is given in Fig. 4. The equivalent circuit is same as in Fig. 1b excepted the ATP synthase being the pump element $i_p^{F_0F_1}$, and no shunt impedance Z present.

After the addition of the UV insensitive uncoupler 1799 to the membrane system, stationary pump currents were observed showing the continuous function of the enzyme. The action of the uncoupler is shown schematically in Fig. 4. Fig. 3*b* shows sta-

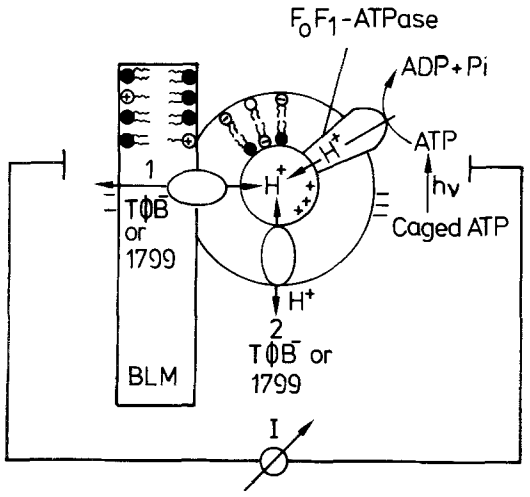

Fig. 4. Schematic representation of the ATP synthase proteoliposomes adsorbed to the underlying planar lipid membrane. The mode of action of the uncoupler 1799 is designated as pathways *1* and *2*, respectively. Only pathway 1 contributes to the measured stationary current. Equivalent circuit is the same as in Fig. 1*b* except for the shunt impedance *Z*

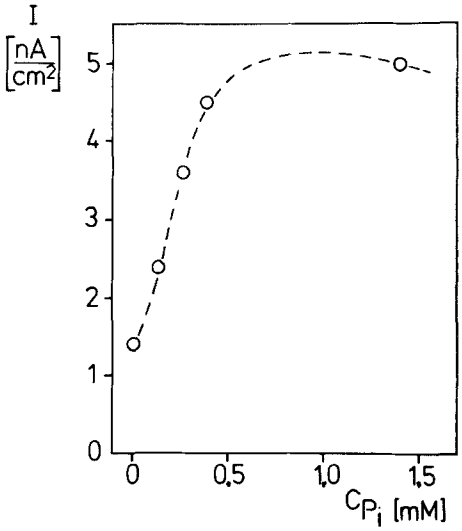

Fig. 5. Stimulation of the peak currents by P_i initiated by a concentration jump of ATP. Conditions were as in Fig. 3*a* except for variable P_i concentration

tionary pump currents of ATP synthase after UV initiated liberation of ATP. The concentration of the nucleotide was constant over the time range of the measurement (8 sec). Again, further flashing evoked smaller current changes because of the decrease of available caged ATP in the light beam, whereas an effect of $\Delta\psi$ is negligible in the presence of the protonophore. The amplitude and direction of these stationary signals were not influenced by an externally applied voltage, i.e., they were not caused by UV light or ATP-induced conductance changes of the uncoupler in the planar lipid membrane. The stationary currents could also be abolished by oligomycin.

The peak currents induced by ATP hydrolysis were stimulated by the reaction product P_i. This is shown in Fig. 5. A half maximal concentration of 0.3 mM P_i and a maximum at about 0.5 mM was observed with a decrease at higher P_i concentrations.

Reversibility of the ATP Synthase

The reversibility of ATP synthase is demonstrated by the following experiments. The energy necessary for ATP synthesis to occur was provided as follows: A low level of ATP (50–100 μM) was added to the membrane system as a continuous background concentration to build up a steady-state level of a proton motive force across the vesicle membrane. Photolytic release of free ADP out of caged ADP was used to start net ATP synthesis. The following behavior was observed (*see* Fig. 6): Without P_i, only the usual UV artifact was observed (trace *A*). The released P_i from the hydrolysed ATP did not interfere with the measurements because of its negligible small concentration. After addition of P_i, a UV flash generated a transient current (trace *B*) in the opposite direction as in the ATP hydrolysis experiment (Fig. 2). The amplitude of the signal is of comparable size as the pump currents associated with ATP hydrolysis. Removal of the free Mg^{2+} by addition of 1 mM EDTA abolished the signal, showing that Mg^{2+} is an essential cofactor for the synthesis of ATP (Fig. 6, trace *C*). In a new experiment, the addition of 30 μM oligomycin totally blocked the signal (Fig. 6, trace *D*). When, however, no ATP but only 450 μM caged ADP and 1 mM P_i were present in the solution, illumination did not induce any pump current. Only after addition of free ATP (100 μM), a synthesis signal as in Fig. 6, trace *B*, was observed. Again, this signal was abolished by oligomycin.

Stationary Properties of Bacteriorhodopsin/ATP Synthase Vesicles Adsorbed to a Planar Lipid Membrane

After addition of bacteriorhodopsin/ATP synthase proteoliposomes to a planar lipid membrane, a capacitive photocurrent developed with time (Fig. 7). The sign of the photocurrent indicates that positive charges are pumped into the vesicles. The extremely small stationary current shows that the vesicles are only attached to the planar lipid membrane

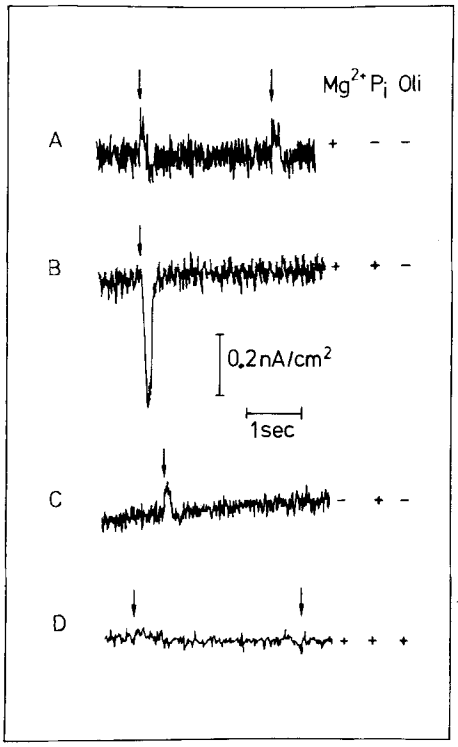

Fig. 6. Short-circuit currents under ATP synthesis conditions. 100 μl ATP synthase proteoliposomes (0.1 mg protein, 2 mg asolectin/ml). ATP was used to energize the proteoliposome membrane to a steady-state pmf. Photolytic release of ADP out of caged ADP was used to start ATP synthesis. The arrows indicate the application of the UV flash (3.7 W/cm², 125 msec duration) for the liberation of ADP out of caged ADP. Solution: standard buffer (0.5 mM $MgCl_2$, 50 μM ATP, 45 μM caged ADP). Trace *A*: Only the artifact is observed; no ATP synthase activity. Trace *B*: An electric current in the reverse direction to Fig. 3*a* is observed. Trace *C*: Removal of Mg^{2+} with 1 mM EDTA depletes the current. Trace *D*: Conditions as trace *B* plus oligomycin (30 μM). The ATP synthase-induced current is blocked

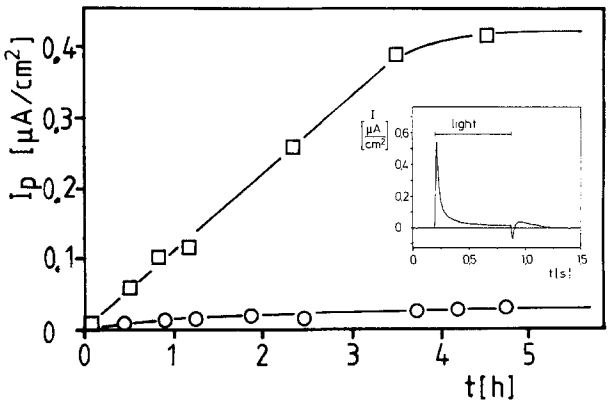

Fig. 7. Development with time of the light-induced pump current of bacteriorhodopsin proteoliposomes (○—○) and bacteriorhodopsin/ATP synthase proteoliposomes, respectively (□—□). The inset shows the capacitive behavior of the proteoliposomes adsorbed to the planar film. Light intensity 0.4 W/cm², $\lambda \geq 450$ nm, 100 μl bacteriorhodopsin proteoliposomes or 100 μl bacteriorhodopsin/ATP synthase proteoliposomes. Other conditions were as in Fig. 3*a* except caged ATP. Standard buffer, 1 mM $MgCl_2$ and 1 mM P_i

and not incorporated into it. Peak currents increased for about 3 hr and were typically 10 times higher than the current obtained by pure bacteriorhodopsin proteoliposomes. After reaching the maximum value, the peak photocurrent remained constant for several hours.

Flash-Induced Charge Displacements in Bacteriorhodopsin/ATP Synthase Proteoliposomes

The experimental situation for bacteriorhodopsin/ATP synthase proteoliposomes adsorbed to the planar lipid membrane is shown in Fig. 1*A*. Figure 1*B* shows the equivalent circuit.

The observed photocurrents under different phosphorylating conditions are shown in Fig. 8*A*. Drastic effects of the substrate ADP and the F_0 directed inhibitor oligomycin were apparent. In control experiments the photocurrents of bacteriorhodopsin proteoliposomes (without ATP synthase) were not influenced by the substrate ADP and the inhibitors (DCCD, oligomycin, *see* Table 1). Therefore the changes of the kinetics of the photocurrents can only be ascribed to the ATP synthase itself.

The numerical analysis revealed further details (*see* Table 1*b*). The kinetics of the photocurrent without any addition (Fig. 8*A*, trace *a*) corresponds to the pure bacteriorhodopsin signal as described by Fahr et al., 1981 (*see* Table 1*a*). A fast unresolved displacement current (1–2 μsec) is followed by an oppositely directed current in the time scale from 10–200 μsec. This latter part of the transient current was assigned to the H^+ movement within bacteriorhodopsin (Keszthelyli & Ormos, 1980; Fahr et al., 1981). In the presence of all substrates, necessary for ATP synthesis (ADP and P_i, Fig. 8*A*, trace *b*), an additional charge displacement process against the bacteriorhodopsin-mediated H^+ movement is clearly resolved. This process is drastically amplified by the addition of oligomycin (Fig. 8*A*, trace *c*) or DCCD (*not shown*).

DCCD and oligomycin alone did not induce any ATP synthase signal (without ADP). However, remarkable differences in the action of the inhibitors were observed if ADP is added (*see* Fig. 9*A* and *B*). Addition of oligomycin prior to ADP prevented the appearance of any ATP synthase contribution in contrast to the marked slow-down effect mentioned

Table 1a. Control of ATP synthase effect on the photocurrent kinetics of bacteriorhodopsin proteoliposomes adsorbed to planar lipid membranes

System	τ_1[a] (μsec)	τ_2 (μsec)	τ_3 (μsec)	τ_4 (μsec)	SNF
Bacteriorhodopsin proteoliposomes	2	9	27 ± 5	110	90
Bacteriorhodopsin proteoliposomes + 1 mM ADP	2	13	37 ± 6	220 ± 140	145
Bacteriorhodopsin proteoliposomes + 30 μM DCCD + 1 mM ADP	2	10	23	100	337
Bacteriorhodopsin proteoliposomes + 1 mM oligomycin + 1 mM ADP	2	9	26 ± 5	103	104
Bacteriorhodopsin proteoliposomes + 30 μM DCCD incubated for 30 hr	1.5	5	24	130	50

Evaluation of control experiment with bacteriorhodopsin proteoliposomes, conditions as in Fig. 7
SNF: Signal noise ratio of the fit (Provencher, 1976).
[a] τ_1 not corrected for limited time resolution of the system. ± standard deviation of the fit, when larger than 10%.

Table 1b. Evaluation of the photocurrent kinetics of bacteriorhodopsin proteoliposomes, bacteriorhodopsin/ATP synthase proteoliposomes

System	Pos. amplit.	Neg. amplitudes			Pos. amplit.	SNF
	τ_1 (μsec)	τ_2 (μsec)	τ_3 (μsec)	τ_4 (μsec)	τ_{ADP} (μsec)	
Bacteriorhodopsin proteoliposomes	2	9	27 ± 5	110 ± 25	—	90
Bacteriorhodopsin/ATP synthase proteoliposomes	1.6	9	40	120	—	120
Bacteriorhodopsin/ATP synthase proteoliposomes + 1 μM ADP	1.8	—	44	93	11	110
Bacteriorhodopsin/ATP synthase proteoliposomes + 1 mM ADP + 30 μM oligomycin	—	—	45	210 ± 70	18	35
Bacteriorhodopsin/ATP synthase proteoliposomes + 1 mM ADP + 0.5 μM TPB^-	2	9	27	110	—	525
Bacteriorhodopsin/ATP synthase proteoliposomes + 1 mM ADP + 30 μM oligomycin + 0.5 μm TPB^-	2	8	24	110	—	540

SNF: Signal noise ratio of the fit (Provencher, 1976). τ_1: Not resolved in the fit. τ_{ADP}: Relaxation time, measured in the presence of ADP, occurs with negative amplitude.

Table 1c. Determination of the displacement currents by the ATP synthase (displaced charge and relaxation times)[a]

System	τ_{1ADP} (μsec)	τ_{2ADP} (μsec)	ΔQ_1 (pc)	ΔQ_2 (pc)
Bacteriorhodopsin/ATP synthase proteoliposomes + 1 mM ADP − background illumination	12	160	0.18	−0.4
Bacteriorhodopsin/ATP synthase proteoliposomes + 1 mM ADP + 30 μM oligomycin − background illumination	15	480	0.27	0.05
Bacteriorhodopsin/ATP synthase proteoliposomes + 1 mM ADP + background illumination (12 mW/cm²)	21	—	0.18	−0.16
Bacteriorhodopsin/ATP synthase proteoliposomes + 1 mM ADP + 30 μM oligomycin + background illumination (12 mW/cm²)	35	Nonmeasurable	0.27	Nonmeasurable

Determination of the ATP synthase displaced charges and of the relaxation times.
[a] Data obtained by the difference of the bacteriorhodopsin/ATP synthase signal minus the bacteriorhodopsin signal.

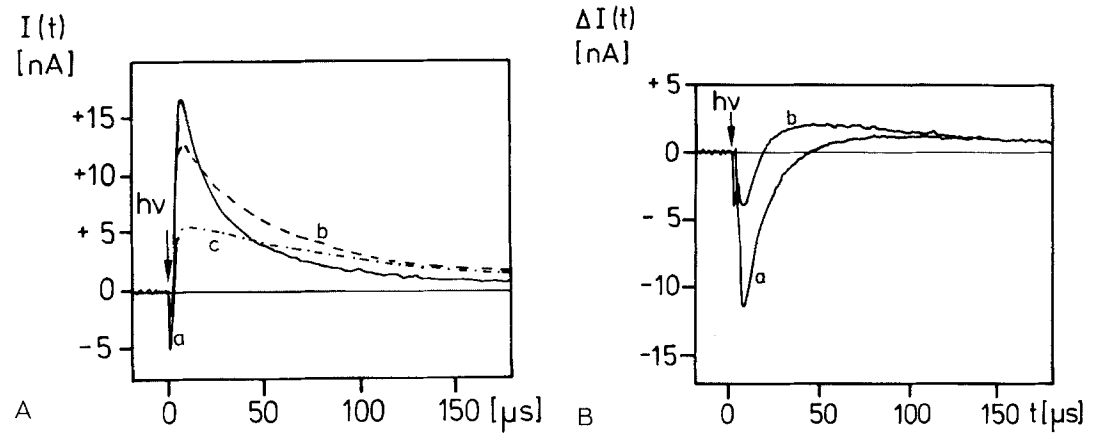

Fig. 8. (*A*) Photocurrents of bacteriorhodopsin/ATP synthase proteoliposomes adsorbed to a black lipid membrane after a laser flash (570 nm, 10 nsec, 3.5 μJ). Conditions were as in Fig. 7. Trace *a*: In the absence of ADP. Trace *b*: After addition of 1 mM ADP. Trace *c*: 1 mM ADP, 30 μM oligomycin. The same behavior is observed with 10–30 μM DCCD instead of oligomycin (*not shown*). (*B*) Difference of the photocurrents (traces *b* or *c* minus trace *a* from *A*) in order to eliminate the contribution of the bacteriorhodopsin induced signal. (*a*) 1 mM ADP + 30 μM oligomycin. (*b*) 1 mM ADP

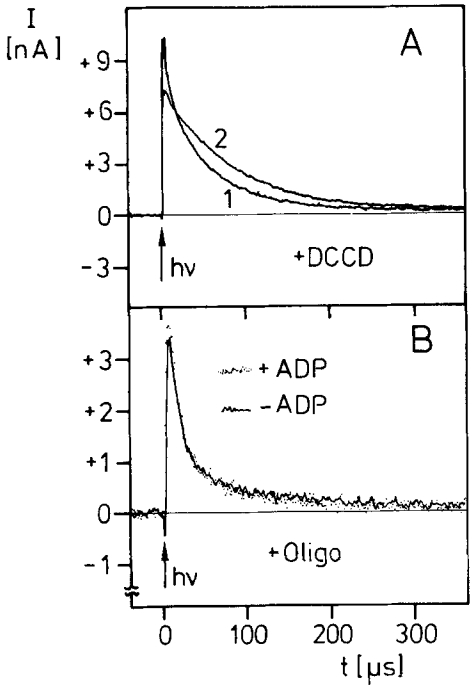

Fig. 9. Influence of the sequence of addition of the inhibitors DCCD and oligomycin on current kinetics of bacteriorhodopsin/ATP synthase proteoliposomes. (*A*) Bacteriorhodopsin/ATP synthase proteoliposomes preincubated with 30 μM DCCD (18 hr). Trace *1*: Photocurrent kinetics without ADP. Trace *2*: Same conditions as in trace *1*, but with 1 mM ADP. Standard buffer, 1 mM $MgCl_2$, 1 mM P_i, 100 μl bacteriorhodopsin/ATP synthase proteoliposomes. (*B*) Analogue experiment with 30 μM oligomycin starting in the presence of 1 mM ADP. Standard buffer 1 mM $MgCl_2$, 1 mM P_i, + 1 mM ADP, 100 μl bacteriorhodopsin/ATP synthase proteoliposomes. Light intensity of the laser in both sets of experiments, 15 μJ. The fast component in the experiments was not resolved

above in the reverse sequence. However, in the case of DCCD, the sequence of additions had no effect on the signal.

In any case tested so far, the presence of ADP (and P_i) was necessary for the ATP synthase signal to appear. The influence of the catalytic site directed inhibitor NBD-Cl was investigated also. The addition of NBD-Cl (1 mM) (Linnett & Beechey, 1979) prevented the ADP-induced charge displacement.

In order to eliminate the underlying charge displacement by bacteriorhodopsin, the following evaluation procedure was applied. Difference signals obtained by substraction of the signals without any additions (e.g. trace *a* in Fig. 8*A*) from the signal containing both ATP synthase and bacteriorhodopsin contribution (e.g. traces *b* or *c*, Fig. 8*A*) are shown in Fig. 8*B*. The resulting difference signals of the displacement currents represent the ATP synthase response on a fast generation of an electrochemical gradient across the vesicular membrane by bacteriorhodopsin. After 10 μsec, the signal can be fitted to a sum of 2 exponentials where a fast (τ_{1ADP}) component in the 10-μsec range, and a slower component (τ_{2ADP}) of about 160 μsec. For the process (τ_{1ADP}) the translocated charge was measured depending on the added ADP concentration. A high affinity of 9 μM was found (Fig. 10).

In order to study the influence of the electrical field ($\Delta\psi$) or the pH gradient (ΔpH) on the ATP synthase related charge displacements, several uncoupling agents were applied to the membrane system. Whereas the electroneutral H^+/cation-exchanger monensin (which may be used to abolish a

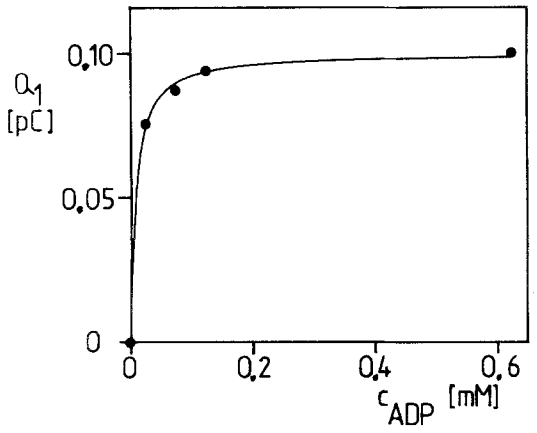

Fig. 10. ATP dependence of the displaced charge $\Delta Q_1 = a_1 \times \tau_{1ADP}$. No background light, flash energy 2 μJ, standard buffer, 1 mM $MgCl_2$, 1 mM P_i, least squares fit to a saturation curve

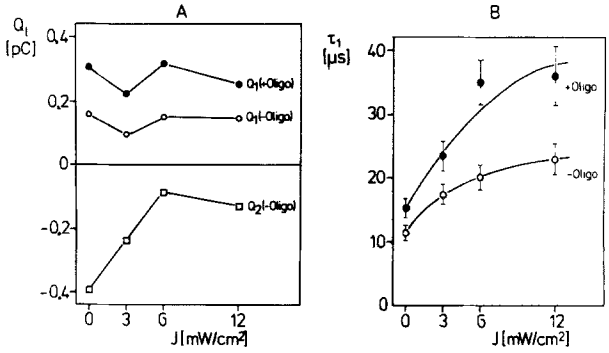

Fig. 11. (*A*) Displaced charge $\Delta Q_i = a_i \cdot \tau_i$ ($i = 1, 2$) depending on the background light ($\lambda \geq 450$ nm; $J = 0$–12 mW/cm²) in the presence and absence of oligomycin (30 μM) Q_1, Q_2 indicate process τ_{1ADP} and τ_{2ADP}, respectively. (*B*) Dependency of τ_{1ADP} on the background light intensity in the presence and absence of oligomycin (30 μM). Standard buffer, 1 mM ADP, 1 mM $MgCl_2$, 1 mM P_i, 100 μl bacteriorhodopsin/ATP synthase proteoliposomes added

pH gradient across the vesicle membrane) had no effect on the behavior of the system, the lipophilic anion $TPhB^-$ (which increases the electrical conductivity of the vesicle membrane and the planar film) totally abolished the electrical response induced by ATP synthase. Only the bacteriorhodopsin signal remained (Table 1*b*).

The influence of a steady-state proton motive force on the relaxation kinetics of ATP synthase and the translocated charges of both processes are shown in Fig. 11*A* and *B*. Simultaneous illumination with a low level (0–12 mW/cm²) of steady-state yellow light ($\lambda \geq 450$ nm) during the laser experiments causes a small fraction of bacteriorhodopsin (0–15%) to work and thereby to establish a steady-state proton motive force. Whereas the translocated charge in the first process is independent of background illumination, the charge displacement of the second process is reduced with increasing light intensity to $\frac{1}{4}$ of the initial value (note process 2 has a negative amplitude, Fig. 11*A*). The corresponding relaxation times of both processes (τ_{1ADP}, τ_{2ADP}) are slowed down by a factor of 2. Oligomycin or DCCD effected the transient kinetics. τ_{1ADP} is increased by a factor of 4 compared to the situation without background light and inhibitors (Fig. 11*B*). The amplitude of process 2 (τ_{2ADP}) became unmeasurably small in the presence of background light and the inhibitors. Therefore only the displaced charge for process 1 could be determined in this situation. Figure 11*A* shows that the translocated charge of this process is increased by a factor of 2 and is background light independent. Further evaluation is given in Table 1a–c.

Discussion

Pump Currents Generated by a Light-Induced Concentration Jump of ATP

The results reported above show that the previously introduced method of electrical measurements of ATPase-induced pump currents (Fendler et al., 1985; Borlinghaus et al., 1987; Nagel et al., 1987; Hartung et al., 1987) is applicable to a fully reconstituted ATP synthase vesicle system. The method was extended to observe pump currents associated with the reverse reaction, net ATP synthesis at the expense of a proton motive force. After photolytic liberation of ADP or ATP out of caged ADP or caged ATP, respectively, full enzymatic activity was obtained. This demonstrated that the photolytic decay products, especially the nitrosoacetophenone, does not interfere functionally with the reconstituted enzyme. So, the photolytic concentration jump may also be a valuable tool for time resolved enzymatic measurements of the reconstituted ATP synthase. The transient currents observed after light-triggered liberation of ATP (Fig. 3*a*) with decay times of 40–120 msec indicate that there is no substantial incorporation of ATP synthase into the black lipid membrane. The smaller peak currents obtained with ATP synthase (0.5–1 nA/cm²) as compared to the routinely observed 10 nA/cm² in the Na^+K^+-ATPase system (Nagel et al., 1987) may be explained by the fact that the ATP synthase has a specific hydrolytic activity for ATP about 10 times lower (1.6 μmol P_i/mg ATP synthase min, (Wagner et al., 1987) than the Na^+K^+-ATPase

system used in the above-mentioned study (15–20 μmol P_i/mg min).

In previous studies (Drachev et al., 1976*a,b*; Borisova et al., 1984; Hirata et al., 1986), pump currents or open-circuit potentials of reconstituted ATP synthase systems on planar black lipid membranes were evoked by addition of ATP. Currents or potentials with rise times from seconds up to several minutes due to the slow diffusion and mixing of the added ATP in the setup were observed. The use of a light flash to start the reaction has the advantage of an exact synchronization of the pump molecules, which is essential for the detection of the observed small signals. The time resolution in our experiments is determined by the reaction rate for photolysis of caged ATP which is about 30 msec at pH 8.0 (McCray et al., 1980) and not by diffusion times inherent in the system. As demonstrated by the stationary pump currents in the presence of uncoupler, the concentration of liberated ATP remains constant for over 10 sec. So, the light-induced concentration jump of ATP approaches the ideal rectangular form in time with a rise time determined by the flash duration (125 msec) and a considerably lower decay time (over 10 sec) determined by diffusion of the ATP out of the irradiated volume. Consumption by the enzyme in solution may be estimated to be less than 0.4 μmol ATP/sec, which is considerably less than 1%/sec of the released ATP (normally 63 μM).

The stimulating effect of the ATP hydrolysis product P_i (Fig. 5) on the peak currents is in good agreement with earlier observations by Baltscheffsky and Lundin (1979) on intact *Rhodospirillum rubrum* chromatophores at low levels of ATP. They observed a half maximal P_i stimulation of the ATP hydrolysis rate at 0.3 mM P_i with a maximum at 0.5 mM for the light-activated ATP synthase and attributed this to a considerable decrease of the K_m for ATP. This regulatory effect for the enzyme is reflected by the dependence of the pump currents on P_i with a very similar concentration dependence as in the native system.

The extension of the method to elicite reversed pump currents coupled to ATP synthesis by photolytic release of ADP out of caged ADP at the expense of a steady-state proton motive force across the vesicle membrane (Fig. 6) shows the reversibility of the ATP synthase, depending on the phosphorylation conditions. The observed currents are not due to a temporary inhibition of the ongoing hydrolysis of ATP, which is necessary for the generation of the proton motive force, because the obtained currents opposite in sign to the ATP-induced currents are only observed in the presence of ADP, P_i and Mg^{2+}. The amplitudes are of the same magnitude as those coupled to ATP hydrolysis. Therefore, the ATP synthase is reversibly working with comparable effectiveness in both directions. Bacteriorhodopsin was not suitable for the energization of the system in the concentration jump experiments, because UV light excites bacteriorhodopsin, inducing photocurrents in the membrane system. These photocurrents cover the expected electrical signal of the ATP synthase.

Flash-Induced Charge Displacements in Bacteriorhodopsin/ATP Synthase Proteoliposomes

The kinetic experiments on bacteriorhodopsin/ATP synthase liposomes attached to a planar bilayer have shown that the photocurrent kinetics of bacteriorhodopsin is a tool to study fast electrical events in the μsec range in ATP synthase. The coupling of ATP synthase to bacteriorhodopsin directly gives access to the time resolution achieved in single turnover photoelectric experiments, which is about 2 μsec in photocurrent measurements (Fahr et al., 1981) and may be improved to several nanoseconds in photovoltage experiments (Benz & Läuger, 1976; Varnadore et al., 1982). Measurements on ATP synthase kinetics by means of stopped-flow techniques are limited in time resolution to several milliseconds and detect only the final step in the reaction cycle, the release of synthesized ATP (Smith, Stokes & Boyer, 1976; Horner & Moudrianakis, 1983, 1985, Gräber, Junesch & Schatz, 1984).

The main advantage of using bacteriorhodopsin for exciting single turnovers in the ATP synthase is its insensitivity towards all effectors of ATP synthase tested so far. Renthal et al. (1981. 1985) reported an interaction of high concentrations (1 mM) of DCCD with bacteriorhodopsin incubated under illumination over night. Obviously, these are very drastic conditions. With the much lower concentrations used in our reconstituted bacteriorhodopsin systems we were not able to detect any influence of DCCD on the photocurrents of bacteriorhodopsin alone in accordance with experiments of Nelson et al. (1977), who could detect neither any incorporation of radioactive DCCD nor any influence on light-driven pH changes in reconstituted bacteriorhodopsin proteoliposomes.

The development of capacitive photocurrents with time after addition of the vesicles is a direct and easy measure for the adsorption process to the black lipid membrane. Since bacteriorhodopsin/ATP synthase proteoliposomes adsorb much better to a black lipid membrane than pure bacteriorhodopsin proteoliposomes under the same conditions,

one may conclude that the population of vesicles adsorbed to the black lipid membrane is composed mainly of vesicles containing ATP synthase or ATP synthase and bacteriorhodopsin, whereas vesicles containing only bacteriorhodopsin are less concentrated on the interface of the black lipid membrane. This effect favors contributions of ATP synthase to the displacement currents because unwanted contributions of pure bacteriorhodopsin vesicles are minimized by this selective adsorption effect.

The evaluation of Fig. 8*A*, trace *a*, shows four exponentials comparable to the normal bacteriorhodopsin signal (Table 1*b*, first two lines). After activation of the ATP synthase by ADP and its blockade by the specific inhibitors oligomycin or DCCD the kinetics are changed drastically. Two new processes with a time constant of 10 and 160 μsec, respectively, in the absence of oligomycin were observed in the difference signals. Since the time constant τ_{1ADP} is very similar to τ_2 of the pure bacteriorhodopsin signal (10 μsec), it could be assumed that the bacteriorhodopsin current is simply shunted out by a very fast process ($\tau \ll 2$ μsec) of the ATP synthase. Therefore also the fast process τ_1 (2 μsec) should be affected by the addition of ADP. This apparently is not found. Alternatively, although τ_{1ADP} is in the range of τ_2, it may be concluded, that τ_{1ADP} reflects a charge displacement in the ATP synthase oppositely directed to the bacteriorhodopsin displacement current. This interpretation is strengthened because τ_{1ADP} significantly changes in the presence of background light with and without oligomycin (Fig. 11*B*, Table 1*b* and *c*), which cannot be detected at all for the pure bacteriorhodopsin photocurrent. τ_{2ADP} (160 μsec) is not comparable with a relaxation process in bacteriorhodopsin (Fahr et al., 1981, and Table 1*a* and *b*). τ_{2ADP} increases after the addition of oligomycin to 480 μsec similarly as it was observed for τ_{1ADP} (Table 1*b*) indicating the strong dependency of the kinetics on the ATP synthase specific blocking agent oligomycin. Integration of the time-dependent currents yields the displaced charge per process ($\Delta Q_i = a_i\tau_i$) as shown in Fig. 11. For relaxation process τ_{1ADP} the corresponding charge ΔQ was determined at different ADP concentrations yielding a high affinity (9 μM) which is comparable with the ADP binding obtained by enzymatic measurements (Wagner et al., 1987).

The different effects of tetraphenylborate and the electroneutral H^+/cation exchanger monensin strongly suggest that the coupling between bacteriorhodopsin and ATP synthase is mediated on the μsec time scale by the electrical potential difference ($\Delta\psi$) and not by a pH difference. Control experiments on the action of tetraphenylborate with bacteriorhodopsin proteoliposomes showed no influence on the photocurrent in the microsecond time range, indicating similar increase of the conductances both of the liposomal and the underlying planar lipid membrane, so that the ratio between the liposomal conductance (G_p) and the conductance of the underlying liposomal membrane (G_m) remains constant. The disappearance of the ATP synthase signal after the addition of tetraphenylborate is explained by the high concentration of the lipophilic anion in the liposomal membrane compared to the enzyme, so that the ATP synthase contribution is shunted out.

An interpretation of the effect of background light-induced membrane potential (*see* Fig. 11) on the ATP synthase kinetics can be given only unsatisfactorily. The ratio of ATP synthase in its ground state and its activated state will be reduced by the proton motive force. Therefore the amplitudes of the displacement currents should be decreased, as it was obtained in the experiment.

The absolute necessity of both ADP and P_i for eliciting an ATP synthase displacement current signal and its prevention by previous application of the F_1 specific inhibitor NBD-Cl clearly shows that the observed charge displacements are coupled to the functional state of F_1. The sudden change of the membrane potential by the light excitation of bacteriorhodopsin effects rather the membrane spanning F_0 part of the enzyme than F_1, which is situated outside of the membrane dielectric. Since displacement currents only appear after binding of free ADP to F_1, an interaction of both parts of the enzyme complex must occur.

In this context, the slow-down effect caused by both F_0 specific inhibitors DCCD and oligomycin may be interpreted as a substantial change in the coupling between F_0 and F_1. Recently, Penefsky (1985) showed that these inhibitors cause a release of tightly bound ATP from ATP synthase. This was interpreted (Penefsky, 1985; Ferguson, 1986) as a conformational change relayed from F_0 to the catalytic sites on F_1 similar to the conformational changes necessary for ATP formation and activation of the enzyme by a proton motive force. This further supports the interpretation that the observed signals are related to the catalytic events during ATP synthesis. The effects of the inhibitors are primarily the conformational changes discussed above and not the blocking of passive proton conduction through isolated F_0 part (Sebald & Hoppe, 1981). This view is further strengthened by recent findings of oligomycin-stimulated release of tightly bound ATP from submitochondrial particles (Lundin, da Silva & Baltscheffsky, 1987). Penefsky (1985) postulated a change in the ionization state of

essential amino acid residues in F_0 to be the cause for the conformational changes transmitted to F_1 during the catalytic cycle and also during inhibitor binding to F_0. The electrical measurements reported here show a doubling in the amount of charge moving in the fast process resolved here upon inhibitor binding to F_0, strongly supporting this hypothesis.

The different effects of oligomycin and DCCD on the signals, when applying them to the system before ADP is added, may be interpreted in a more drastic conformational change of the binding site of ADP caused by oligomycin in the absence of ADP, preventing the ADP binding necessary for the displacement current to occur. This agrees with the observation of Strid and Baltscheffsky (1986) who showed a reduced sensitivity of ATP synthase to the inhibition by oligomycin in the presence of ADP and different modes of action of DCCD and oligomycin on the ATP synthase from *R. rubrum* (Strid & Baltscheffsky, 1986). In a different kind of investigation, Penefsky (1985) showed that oligomycin or DCCD impair the binding of substrate at the catalytic site, probably by conformational changes in F_0 relayed to the sites at F_1. Our observations suggest that these changes are more drastic in the case of oligomycin, however, in the absence of ADP. Although the overall mode of action of DCCD and oligomycin on the energy transfer reactions on ATP synthase seem to be similar, some characteristic differences between them have emerged. It was shown that oligomycin and DCCD may have different binding sites (Glaser et al., 1982; Glaser & Norling, 1983; Hoppe & Sebald, 1984), the binding site for oligomycin being sensitive to different conformations of F_1 (Glaser et al., 1983). Whereas the binding site of DCCD on F_0 of many different sources has been established with great precision by radioactive cross-linking, only indirect information on the oligomycin binding site exists and so differences in their respective mode of action are not surprising.

The authors wish to thank K. Fendler for critical and helpful discussions. The excellent technical assistance of A. Hüby, G. Schimmack and E. Lewitzki is gratefully acknowledged. Work was supported partially by the Deutsche Forschungsgemeinschaft SFB 169 to E. B. and grant Nr. Do 192/5-2 to K. D.

References

Arnold, A., Wolf, H.V., Ackerman, B., Bader, H. 1976. An automated continuous assay of membrane-bound and soluble ATPases and related enzymes. *Anal. Biochem.* **71**:209–213

Baltscheffsky, M., Lundin, A. 1979. Flash induced increase of ATPase activity in *Rhodospirillum rubrum* chromatophores. *In:* Cation Flux across Biomembrane. Y. Mukohata and L. Packer, editors. pp. 209–218. Academic, New York

Bamberg, E., Hegemann, P., Oesterhelt, D. 1984. Reconstitution of the light-driven electrogenic ion pump halorhodopsin in black lipid membranes. *Biochim. Biophys. Acta* **773**:53–60

Benz, R., Läuger, P. 1976. Kinetic analysis of carrier-mediated ion transport by the charge-pulse technique. *J. Membrane Biol.* **27**:171–191

Borisowa, M.P., Babakov, A.V., Kolomytkin, O.V. 1984. H^+-ATPase in a planar lipid bilayer. *Biol. Membr.* **1**:187–190

Borlinghaus, R., Apell, H.-J., Läuger, P. 1987. Fast charge translocations associated with partial reactions of the Na,K-pump: I. Current and voltage transients after photochemical release of ATP. *J. Membrane Biol.* **97**:161–178

Dancshazy, Z., Karvaly, B. 1976. Incorporation of bacteriorhodopsin into a bilayer lipid membrane: A photoelectric-spectroscopid study. *FEBS Lett.* **72**:136–138

Drachev, L.A., Frolov, V.N., Kaulen, A.D., Liberman, E.A., Ostroumov, S.A., Plakunova, V.G., Semenov, A.Y., Skulachev, V.P. 1976*a*. Reconstitution of biological molecular generators of electric current. Bacteriorhodopsin. *J. Biol. Chem.* **251**:7059–7065

Drachev, L.A., Jasaitis, A.A., Kaulen, A.D., Kondraskin, A.A, Liberman, E.A., Nemecek, I.B., Ostroumov, S.A., Semenov, A.Y., Skulachev, V.P. 1974. Direct measurement of electric current generation by cytochrome oxidase. H^+-ATPase and bacteriorhodopsin. *Nature (London)* **249**:321–324

Drachev, L.A., Jasaitis, A.A., Mikelsaar, H., Nemecek, I.B., Semenov, A.Y., Semenova, E.G., Severina, I.I., Skulachev, V.P. 1976*b*. Reconstitution of biological molecular generators of electric current. *J. Biol. Chem.* **251**:7077–7082

Fahr, A., Läuger, P., Bamberg, E. 1981. Photocurrent kinetics of purple-membrane sheets bound to planar bilayer membranes. *J. Membrane Biol.* **60**:51–62

Fendler, K., Grell, E., Haubs, M., Bamberg, E. 1985. Pump currents generated by the purified Na^+K^+-ATPase from kidney on black lipid membranes. *EMBO J.* **4**:3079–3085

Ferguson, S. 1986. Towards a mechanism for the ATP synthase of oxidative phosphorylation. *Trends Biol. Sci.* **11**:100–101

Glaser, E., Norling, B. 1983: Kinetics of interaction between the H^+-translocating component of the mitochondrial ATPase complex and oligomycin or dicyclohexylcarbodiimide. *Biochem. Biophys. Res. Commun.* **111**:333–339

Glaser, E., Norling, B., Kopecky, J., Ernster, L. 1982. Comparison of the effects of oligomycin and dicyclohexylcarbodiimide on mitochondrial ATPase and related reactions. *Eur. J. Biochem.* **121**:525–531

Gräber, P., Junesch, U., Schatz, G.H. 1984. Kinetics of proton-transport-coupled ATP synthesis is chloroplasts. Activation of the ATPase by an artificially generated ΔpH and $\Delta\psi$. *Ber. Bunsenges. Phys. Chem.* **88**:599–608

Hartung, K., Grell, E., Hasselbach, W., Bamberg, E. 1987. Electrical pump currents generated by the Ca^{2+} ATPase of sarcoplasmic reticulum vesicles adsorbed on black lipid membranes. *Biochim. Biophys. Acta* **900**:209–220

Herrmann, T.R., Rayfield, G.W. 1978. The electrical response to light of bacteriorhodopsin in planar membranes. *Biophys. J.* **21**:111–125

Hirata, H., Ohno, K., Sone, N., Kagawa, Y., Hamamoto, T. 1986. Direct measurement of the electrogenicity of the H^+-ATPase from thermophilic bacterium PS3 reconstituted in planar phospholipid bilayers. *J. Biol. Chem.* **261**:9839–9843

Hoppe, J., Sebald, W. 1984. The proton conducting F_0-part of bacterial ATP synthases. *Biochim. Biophys. Acta* **768:**1–27

Horner, R.D., Moudrianakis, E.N. 1983. The effect of permeant buffers on initial ATP synthesis by chloroplasts using rapid mix-quench techniques. *J. Biol. Chem.* **258:**11643–11647

Horner, R.D., Moudrianakis, E.N. 1985. Millisecond kinetics of ATP synthesis driven by externally imposed electrochemical potentials in chloroplasts. *J. Biol. Chem.* **260:**6153–6159

Kaplan, J.H., Forbush, B., III, Hoffman, J.F. 1978. Rapid photolytic release of adenosine-5′-triphosphatase from a protected analogue: Utilization by the Na : K pump of human red blood cell ghosts. *Biochemistry* **17:**1929–1935

Keszthelyi, L., Ormos, P. 1980. Electrical signals associated with the photocycle of bacteriorhodopsin. *FEBS Lett.* **109:**189–193

Lemasters, I.J., Hackenbrock, C.R. 1979. Continuous measurement of adenosine triphosphate with firefly luciferase luminescence. *Methods Enzymol.* **56:**530–544

Linnett, P.E., Beechey, R.B. 1979. Inhibitors of the ATP synthetase system. *Methods Enzymol.* **55:**472–518

Lundin, M., Silva, L.P. da, Baltscheffsky, H. 1987. Energy-dependent formation of free ATP in yeast submitochondrial particles, and its stimulation by oligomcyin. *Biochim. Biophys. Acta* **890:**279–285

McCray, J.A., Herbette, L., Kihara, T., Trentham, D.R. 1980. A new approach to time-resolved studies of ATP-requiring biological systems: Laserflash photolysis of caged ATP. *Proc. Natl. Acad. Sci. USA* **77:**7237–7241

Mitchell, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. *Nature (London)* **191:**144–148

Nagel, G., Fendler, K., Grell, E., Bamberg, E. 1987. Na^+ currents generated by the purified $(Na^+ + K^+)$-ATPase on planar lipid membranes. *Biochim. Biophys. Acta* **901:**239–249

Nelson, N., Eytan, E., Notsani, B.-E., Sigrist, H., Sigrist-Nelson, K., Gitler, C. 1977. Isolation of a chloroplast N,N′ dicyclohexyl-carbodiimide-binding proteolipid, active in proton translocation. *Proc. Natl. Acad. Sci. USA* **74:**2375–2378

Oesterhelt, D., Stoeckenius, W. 1974. Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *Methods Enzymol.* **31:**667–678

Pabst, R., Dose, K. 1985. Rapid delipidation of the membrane protein bacteriorhodopsin by high performance liquid chromatography. *J. Chromatogr.* **350:**427–434

Pabst, R., Nawroth, T., Dose, K. 1984. Time dependent monomerization of bacteriorhodopsin in triton X-100 solutions analyzed by high-performance liquid chromatography. *J. Chromatogr.* **285:**333–341

Penefsky, H.S. 1985. Mechanism of inhibition of mitochondrial adenosine triphosphatase by dicyclohexylcarbodiimide and oligomycin: Relationship to ATP synthesis. *Proc. Natl. Acad. Sci. USA* **82:**1589–1593

Provencher, S.W. 1976. An eigenfunction expansion method for the analysis of exponential decay curves. *J. Chem. Phys.* **64:**2772–2777

Racker, E., Stoeckenius, W. 1974. Reconstitution of purple membrane vesicles catalyzing light-driven proton uptake and adenosine triphosphate formation. *J. Biol. Chem.* **249:**662–663

Renthal, R., Cothran, M., Espinoza, B., Wall, K.A., Bernard, M. 1985. Light activates the reaction of bacteriorhodopsin aspartic acid-115 with dicyclohexylcarbodiimide. *Biochemistry* **24:**4275–4279

Renthal, R., Dawson, N., Villarreal, L. 1981. Light-induced reaction of dicyclohexylcarbodiimide with bacteriorhodopsin. *Biochem. Biophys. Res. Commun.* **101:**653–657

Schneider, E., Friedl, R., Schwulera, V., Dose, K. 1980. Energy-linked reactions catalyzed by the purified ATPase complex (F_0F_1) from *Rhodospirillum rubrum* chromatophores. *Eur. J. Biochem.* **108:**331–336

Sebald, W., Hoppe, J. 1981. On the structure and genetics of the proteolipid subunit of the ATP synthase complex. *Curr. Topics Bioenerg.* **12:**1–6

Smith, D.J., Stokes, B.O., Boyer, P.D. 1976. Probes of initial phosphorylation events in ATP synthesis by chloroplasts. *J. Biol. Chem.* **251:**4165–4171

Strid, A., Baltscheffsky, M. 1986. Different actions of energy-transfer inhibitors. *In:* Proc. 4. EBEC Conference, Prague. Vol. 4, p. 275

Varnadore, W.E., Jr., Arrieta, R.T., Duchek, J.R., Huebner, J.S. 1982. Erythrosin and pH gradient induced photo-voltages in bilayer membranes. *J. Membrane Biol.* **65:**147–153

Wagner, N., Gutweiler, M., Pabst, R., Dose, K. 1987. Coreconstitution of bacterial ATP synthase with monomeric bacteriorhodopsin into liposomes. *Eur. J. Biochem.* **165:**177–183

Received 28 February 1988