Field-driven ATP synthesis by the chloroplast coupling factor complex reconstituted into liposomes

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1. INTRODUCTION

Since the development of the chemiosmotic theory [1] there has been a continuous effort to experimentally simplify the energy-transducing apparatus in chloroplasts as well as in mitochondria and bacteria. This has been done by structural or functional isolation and characterization of partial reaction sequences. One direction of functional isolation involved replacing the electron transport-induced energization of the thylakoid membrane by an energization with an artificially generated proton gradient, ΔpH [2], and later with an artificially generated membrane potential difference, $\Delta\Psi$ [3–5]. Another direction of research was addressed to structural aspects, i.e., purification and biochemical characterization of the chloroplast ATPase activities, of the CF_1-part (for review see [6]) and of the CF_0–F_1 complex [7,8].

The combination of both structural and functional aspects lead to the study of the energy-transducing apparatus in model systems. Here the first step was to reconstitute purified energy generating membrane components like bacteriorhodopsin [7] or photosystem I [9] together with purified CF_0–F_1 into liposomes. In these cases the membrane was energized by a light-driven proton transport, and coupled ATP synthesis was observed. The last step should be ATP formation catalyzed by the reconstituted ATPase when energized with an artificially generated ΔpH or $\Delta\Psi$. This has been done by energizing CF_0–F_1 proteoliposomes by ΔpH [8] and by energizing macroliposomes reconstituted with TF_0–F_1 from the thermophilic bacterium PS 3 by $\Delta\Psi$ [10].

In this work purified CF_0–F_1 is reconstituted into asolectin liposomes and the membrane is energized artificially by an external electric field. ATP synthesis is observed (luciferin/luciferase and ^{32}P assay) with an ATP yield per pulse comparable to the native membranes. From the results it is concluded that: (1) no proteins other than the coupling factor complex are necessary to observe ATP synthesis, i.e., direct energy transfer from the electron transport chain via protein–protein interaction is excluded; and (2) the possible existence of special proton pathways in the natural thylakoid membrane, which connect electron transport and phosphorylation reactions [11,12] is not essential for ATP synthesis in this model system.

2. MATERIALS AND METHODS

Chloroplast coupling factor complex (CF_0–F_1) was prepared as a protein–lipid (asolectin)–Triton X-100-micelle following the procedure of Pick and Racker [8] as described elsewhere [9]. SDS-gel electrophoresis (12.5–18% acrylamide) [13] revealed that the preparations were free of contaminating proteins, assuming a 4-subunit CF_0-part [8,14,15]. After incorporation into preformed

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; TPT, triphenyltin-chloride; CF_0–F_1, proton translocating chloroplast coupling factor complex; DTT, dithiothreitol.

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asolectin liposomes by immediate dilution [9,16], this material catalyzed uncoupler-sensitive ATP–P_i exchange at rates of 100–300 nmol/mg of protein and min. The preparations contained 3–5 mg protein/ml and were stored under liquid nitrogen.

Protein concentration was determined as described elsewhere [17]. For calculating the number of CF_0–F_1 molecules, a mole mass of M = 430 000 g/mol was used [18]. For the external electric field experiments, reconstitution of CF_0–F_1 into liposomes was carried out by a procedure similar to that described for the H^+-translocating ATPase of the thermophilic bacterium PS 3 [19]. Soybean phospholipids (asolectin, from Associated Concentrates, USA) were partially purified before use [19] and stored at −80°C. 500 mg of this purified asolectin were suspended in 10 ml of a solution containing 2% (w/v) Na–cholate (pH 8), 1% (w/v) Na–deoxycholate (pH 8), 10 mM Tricine–NaOH (pH 8), 0.1 mM EDTA (pH 8) and 0.5 mM DTT. Cholic acid (Sigma) was recrystallized as described earlier [20]. Sodium deoxycholate (Sigma) was used without further purification. The asolectin–detergent solution was subjected to sonic oscillation in an ice bath for 4 × 30 s (Branson sonifier B12, at 20 kHz and 150 Watts) and either used immediately or stored at −30°C for up to 6 days.

CF_0–F_1-micelles (250 μl, 4 mg/ml of protein) and 750 μl of the asolectin–detergent solution were mixed and dialyzed against 1 litre of 10 mM tricine–NaOH (pH 8), 2.5 mM $MgCl_2$, 0.2 mM EDTA and 0.25 mM DTT at 30°C for 20 h with stirring. After 2, 16 and 18 h the dialyzing medium was changed. In order to reduce the ion-conductivity of the solution used in the external field experiments, the dialyzing solution was diluted with water 1:4 and 1:10 after 16 and 18 h dialyzing time, respectively. The resulting proteoliposomes had diameters between 100 and 300 nm, and the suspensions contained about 1 mg/ml protein (CF_0–F_1) and 30–40 mg/ml phospholipids. They could be stored at 4°C for at least three days without any significant loss of ATP synthesis activity. Further characterization of these liposomes will be reported elsewhere. For the control measurement with CF_0–F_1 in the micellar state (i.e., before reconstitution), the excess ATP from the preparation procedure was removed by dialysis against the tenfold diluted dialyzing solution for 3 h as described above. Liposomes without CF_0–F_1 were prepared as described above but without coupling factor complex. The reaction mixture for external field experiments contained 1 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.1 mM ADP–KOH (pH 8), 0.3 mM tricine–NaOH (pH 8) and reconstituted liposomes giving a final protein concentration of 100–200 μg/ml. This suspension (1 ml) was exposed to external electric field pulses. In a control experiment an aliquot was not exposed to field pulses in order to check for the possible influence of the adenylate kinase activity of CF_0–F_1 [20]. The amount of ATP synthesized was measured with the luciferin/luciferase technique. Some preliminary experiments were also carried out using the ^{32}P incorporation into ADP. In these experiments radioactive phosphate (10 μCi/ml) was also present. The reaction mixture exposed to the field pulses and the control mixture were denatured by addition of 10 μl 40% trichloroacetic acid. The inorganic phosphate was precipitated with molybdate [21], and the organic ^{32}P remaining in the supernatant was counted by Cerenkov radiation in a scintillation counter (Packard).

In experiments with the luciferin/luciferase technique, 10 μl of the exposed reaction mixture were added directly to a test solution of 100 μl luciferin/luciferase assay (LKB kit) and 400 μl of a solution containing 0.1 M Tris–acetate (pH 7.75) and 3 mM EDTA. The resulting luminescence was measured in a LKB luminometer 1250. The luciferin/luciferase assay gave a constant light output for more than 30 min. The light output was calibrated by addition of a known amount of ATP. The quench time for both techniques was about 1 min (mainly due to discharging of the system), and no correction was made for ATP hydrolysis which might have occurred after membrane energization. Indeed, ATPase activation during dialysis (20 h at 30°C) of the enzyme in the presence of DTT has been revealed by ANS fluorescence-measurements. However, the rate of hydrolysis can be neglected due to the low ATP concentration in the external field experiments (Rögner, unpublished observation).

The electric field pulses were generated in the following way: A high voltage capacitor (150 nF) was charged up to 20 kV by a power supply (DEL Electronics Corp). The capacitor was discharged after triggering by a thyratron (English Electric

Valve Co., CX 1191D) through the reaction mixture. The decay of the voltage was measured via a high voltage probe (Tektronix, P6019) by a storage oscilloscope (Nicolet 2090-3B). The decay was exponential with the half-life time depending on the resistance of the reaction medium (half-life time between 60–270 μs). In most experiments the half-life time was 200 μs. The cuvette was made from Trovidur and was equipped with two platinum electrodes. In the ^{32}P measurements, the cuvette contained a reaction volume of 1 ml (distance between electrodes 1 cm). Whereas, a cuvette with a reaction volume of 0.3 ml (distance between the electrodes 0.5 cm) was used in the luciferin/luciferase measurements.

The temperature increase due to Joule heating during the electric pulse was calculated for the field strength used in the experiment. Using an electric field strength of 20 kV · cm^{-1}, a temperature increase of 7.2 K was found for the 1 ml cuvette and 6 K for the 0.3 ml cuvette. However, when 10 kV · cm^{-1} was used the temperature increased 1.8 K and 1.5 K for the 1 ml and the 0.3 ml cuvette, respectively.

The reaction was carried out at room temperature. In order to permit restoration of the original temperature, a delay of 30 s between successive pulses was utilized.

3. RESULTS

Figure 1 shows the ATP-concentration in a suspension of reconstituted liposomes when a series of field pulses was applied.

The level of ATP before the application of pulses was 0.75 μM. This was mainly due to the ATP content of the ADP sample (0.5–1%). During the experiment (about 15 min) the ATP-concentration in the control remained constant. Therefore, the ATP-concentration of the sample was plotted directly in fig.1 (left scale). For the scale on the right, the ATP level was corrected by subtraction of the control value, yielding the amount of ATP generated per CF_0–F_1. The latter was directly proportional to the number of pulses for at least up to 20 pulses. About 0.1 ATP per CF_0–F_1 was generated with each pulse. With the first pulse, an unusually high amount of ATP was observed. If the reconstituted liposomes were preincubated with DCCD (about 30 min), nearly the

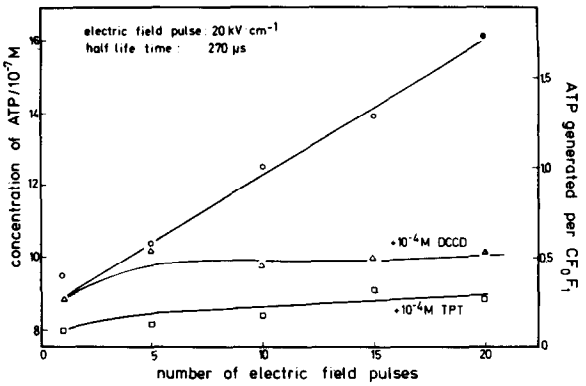

Fig.1. ATP synthesis in a series of electric field pulses. Left: ATP concentration in the reaction medium measured by the luciferin/luciferase assay; ATP concentration before the pulses and in the control during the experiment was 0.75 μM. Right: Amount of ATP generated per CF_0–F_1 (pulsed sample-control). The reconstituted liposomes were incubated at room temperature with the inhibitors for 30 min prior to the application of the pulses. For further details see Materials and Methods.

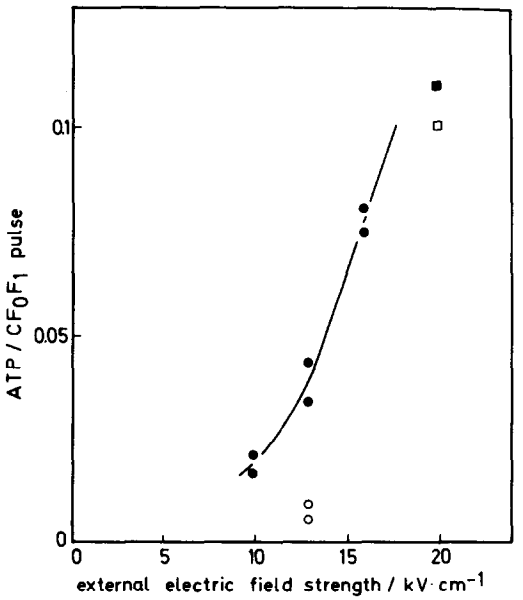

Fig.2. Amount of ATP generated per pulse as a function of the external field strength. Ten pulses were applied (time between the pulses 30 s) and from the resulting amount of ATP the yield per pulse was calculated. The different symbols refer to different CF_0–F_1 preparations. For further details see Materials and Methods.

Table 1

ATP yield obtained in 10 field pulses (field strength 20 kV . cm $^{-1}$, half-life time of the field 200 μs, time between successive pulses 30 s)

Conditions	ATP yield	ATP / CF_0–F_1 · 10 pulses
Reconstituted liposomes		1.0
Liposomes without CF_0–F_1		< 0.02
CF_0–F_1 without liposomes		< 0.02
Reconstituted liposomes + 10^{-4} M DCCD		0.5
Reconstituted liposomes + 10^{-4} M TPT		0.3

For further details see Materials and Methods

same amount of ATP was found after the first pulses as without DCCD. Whereas, after about 5 pulses, the yield was drastically reduced so that between the fifth and the 20th pulse almost no ATP was generated. If the proteoliposomes were preincubated with the same concentration (100 μM) of TPT for about 30 min, the inhibition was more complete than that induced by DCCD.

The amount of ATP generated per CF_0–F_1 as a function of the external electric field strength (fig.2) showed a strong increase up to the highest field strength used in our experiments (20 kV . cm $^{-1}$). ATP yields varied with the different preparations, e.g., experiments carried out with 13 kV . cm $^{-1}$ showed ATP yields which differed by a factor of 4–5. It should be noted that in these preparations the rate of ATP–P_i exchange also differed by about the same factor [14]. Control experiments comparing the micellar and liposomal state of CF_0–F_1 are listed in table 1. The ATP yield in 10 field pulses was 1 ATP/CF_0–F_1 (see fig.1).

Practically no ATP was found when liposomes without CF_0–F_1 were pulsed. Similarly, when the CF_0–F_1-micelle was pulsed without reconstitution, no ATP was formed. The formation of ATP in reconstituted liposomes was reduced to 50% of the control value by DCCD and to 30% by TPT.

Preliminary experiments with the ^{32}P assay gave a value of 0.4 ATP/CF_0–F_1 in 10 pulses. The yield decreased to about 50%, when the enzyme was inhibited by TPT. The difference in yield between both methods are in the range of the scattering of data for different preparations (see fig.2).

4. DISCUSSION

The experiments presented above show that the CF_0–F_1 complex, reconstituted into asolectin liposomes, is able to synthesize ATP if the system is energized by an external electric field. The method used for energization of the liposomes is somewhat different from the one used earlier for isolated chloroplasts [3,4]. Its advantage is that stronger electric fields (up to 30 kV . cm $^{-1}$) can be applied to sufficiently energize small vesicular compartments like proteoliposomes or submitochondrial vesicles (see [23]). However, its disadvantage is that the field strength does not remain constant during the pulse but decays exponentially after an initially high value. Pulse duration can only be varied, if the resistance of the reaction medium is changed. Therefore, we are unable to calculate the rate of ATP synthesis by this method.

ATP synthesis was measured by the luciferin/luciferase assay which is specific for ATP. There is a possibility that the increase in ATP concentration is not due to net ATP synthesis but to a release of tightly-bound ATP or to a release of ATP trapped inside the liposomes through a di-electric breakdown of the membrane. This can be excluded by two different arguments: Firstly, ATP synthesis is also observed with incorporation of ^{32}P into ADP. (The ^{32}P assay measures unspecifically the synthesis of phosphate derivatives so that other reactions, e.g., ATP–^{32}P exchange, might interfere. However, the results of both assays taken together prove that ATP was newly synthesized.) The second argument is derived from the studies with the energy-transfer inhibitors DCCD and TPT. The latter are known to inhibit ATPase catalyzed reactions [24,25]. In the presence of either one of these inhibitors no ATP synthesis could be observed after the first five pulses in a series of field pulses (fig.1). This also demonstrates in an independent way that new ATP is generated in the reconstituted system catalyzed by the membrane-bound ATPase.

The yield from the first pulse was unusually high (this was not observed in all preparations tested!). One might conclude that, in addition to the newly synthesized ATP, there is some release of preformed ATP with the first pulse (e.g., release of

tightly-bound ATP or dielectric breakdown of some big vesicles containing trapped ATP from the preparation medium of CF_0–F_1). A difference was found in the degree of inhibition by TPT and DCCD during the first pulses. No reason can be given for this behavior. However, it might reflect different sites of attack by TPT and DCCD in the CF_0–F_1 complex.

The magnitude of the transmembrane potential difference, $\Delta\Psi$, across a spherical vesicle, induced by an external electric field, E_{ext} may be estimated from:

$$\Delta\Psi = 1.5 E_{ext} \cdot r_a \cdot \cos\alpha$$

where r_a is the radius of the vesicle and α is the angle between the normal of the membrane and the direction of the external field [26].

With $\vec{E}_{ext} = 2 \cdot 10^4$ V · cm^{-1} (the highest field strength used in our experiments) and $r_a = 0.15$ μm, the maximal $\Delta\Psi$ at the poles (i.e., at $\alpha = 0$) is 450 mV. Since the $\Delta\Psi$ declines to zero at the equator of the vesicle, the average $\Delta\Psi$ over one half of the vesicle is lower ($\overline{\Delta\Psi} = \frac{1}{2}\ \Delta\Psi_{max}$). At the other half of the vesicle the polarity of $\Delta\Psi$ is opposite. This means that at this half ATP synthesis cannot take place. Since the ATP yields listed in figs.1 and 2 and table 1 always refer to all the CF_0–F_1 in the reaction medium, the actual yield is higher by a factor of at least 2, taking into account only that half of the vesicle which is correctly polarized.

The yield reported here is rather high compared to the ATP yield obtained in electric field pulses with chloroplasts [3,4] (about 0.3 ATP/CF_0–F_1 per pulse, pulse duration 30 ms, $E_{ext} = 1100$ V · cm^{-1}). However, a comparison is difficult since in the experiments reported here the external field strength, which is decisive for ATP-formation (fig.2), was not constant but decayed with a half-life time of about 200 μs. Furthermore, because of the very different size distributions of the reconstituted vesicles and the chloroplast membrane compartments the average transmembrane $\Delta\Psi$ might be different and can only be estimated roughly.

The experiments described above show that ATP synthesis can be driven independently from photosynthetic electron transport. This conclusion can also be drawn from experiments where either a bulk–bulk ΔpH [2] or a bulk–bulk $\Delta\Psi$ [3–5] is generated artificially across the thylakoid membrane. However, it could be considered that the generation of a ΔpH or $\Delta\Psi$ changes the redox states [27] or the conformation [28] of a component of the electron transport chain which could be the actual intermediate between electron transport and ATP synthesis. If so, this component would also drive H^+-translocation. This would require the coupling factor complex to have intimate contact with components of the electron transport chain.

In the present work the purified CF_0–F_1 is reconstituted into liposomes without any other protein component. Energization of these liposomes with external electric field pulses led to ATP synthesis. Neither a special redox component of the electron transport chain nor a special structural arrangement in the thylakoid membrane (i.e., direct contact between ATPase and proteins of the electron transport chain or other membrane proteins) should be prerequisites for ATP synthesis. This conclusion is in accordance with the results reported for the reconstituted ATPase of the thermophilic bacterium PS 3 [10], which showed ATP synthesis by external electric field pulses independent of the bacterial electron transport chain. The same holds true for proteoliposomes of CF_0–F_1 energized by ΔpH which also showed ATP synthesis [8].

Furthermore, the results of this type of experiment with a highly simplified thylakoid membrane model and artificial membrane energization again question the existence of a special pathway for protons within the thylakoid membrane. This pathway would connect the site of H^+-generation and the coupling factor complex, necessary for ATP synthesis [11,12]. However, this problem can only be solved by examining the coupling device of the thylakoid membranes in their native, undisturbed state, with these results from the model systems in mind.

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