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# Characterization of external electric field-driven ATP synthesis in chloroplasts

(electric field jump/cross-membrane potential/phosphorylation/luciferase/inhibitors)

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**ABSTRACT** **External electric field-induced ADP phosphorylation (EFP) in lettuce chloroplasts was monitored with a coupled luciferin-luciferase enzymatic assay. This assay made it possible to follow ATP synthesis in a kinetically competent manner. The EFP reaction was found to be a much slower process than the light-driven reaction in the same system. The amount of ATP synthesized after a single electric field pulse corresponds to many turnovers of the ATP synthase.**

It was first demonstrated in 1976 (1) that exposure of thylakoid membranes to an external electric field in the presence of ADP and $^{32}P_i$ results in a net formation of [$^{32}$P]ATP. Such ATP synthesis was demonstrated also with reconstituted $H^+$-ATPase liposomes (2) as well as with mitochondria and submitochondrial particles (3). External electric field-driven ATP synthesis offers both high time resolution and an easy control over the membrane potential in the vesicles studied. Thus, with the use of fast response measurement techniques, kinetic analysis of the events occurring during and after the exposure to an external electric field should be easily obtained.

The luciferase enzymatic assay method for the determination of ATP allows the detection of very low ATP concentrations (4). The use of the luciferase-coupled enzymatic assay has been shown to permit kinetic analysis of the onset of ATP synthesis by thylakoid membranes in the subsecond time scale (5–7). This method was used to study external electric field-driven ATP synthesis and to compare it with ATP synthesis driven by light. It is demonstrated that most of the electric field-driven ATP synthesis occurs after the exposure to the electric field and for many turnovers of the ATP synthase enzyme complex. In addition, the response of the external electric field-driven ATP synthesis reaction to various uncouplers and energy transfer inhibitors is described.

## MATERIALS AND METHODS

Lettuce chloroplasts were prepared as described (8).

**Coupled Luciferin-Luciferase Assay.** Luciferase was purified from firefly lanterns as described (9) with some modifications. Sephadex G-25 (coarse) (Pharmacia, Sweden) was used to separate the luciferin and dehydroluciferin from the proteins in the homogenized firefly lanterns (Sigma, no. FFT). The column (1.5 cm in diameter and 25 cm high) was washed with 60 ml of a buffer containing 35 mM Tris sulfate and 1 mM EDTA at pH 7.7. The fractions that contained the protein were pooled and added to a DEAE-Sephacel column (Pharmacia) (1 cm in diameter and 18 cm high) that had been preequilibrated with the same Tris sulfate/EDTA buffer. The column was washed with 300 ml of the same buffer (at 20 ml/hr) and luciferase was eluted by a 0.25 mM $MgSO_4$ gradient. Fractions (1 ml) containing luciferase activity were collected and stored at −60°C. The luciferase was essentially free of myokinase activity. It gave a stable light signal for >1 min with an ATP concentration of <0.01 μM, provided that 50 mM KCl was present. The rise time of this signal was ≈200 msec. Activity was essentially maintained at the same level for more than 5 months.

Fifty microliters of the purified luciferase (2 mg of protein per ml) and 0.5 mM D-luciferin (Sigma) were added to the assay system. Light emission due to the presence of ATP was filtered through a Corning 4-96 filter and detected by a photomultiplier (EMI 9524B). At the end of each experiment a known amount of ATP was added for calibration. Calibration signals did not change much throughout this study when similar experimental conditions were employed. Commercial ADP (Sigma) was purified of residual ATP by pretreatment with hexokinase (EC 2.7.1.1) and glucose.

Light filtered through a Schott RG 5 filter at right angle to the analyzing photomultiplier was used to illuminate the chloroplasts in photophosphorylation experiments.

**Fluorometric ATP Measurements.** Fluorometric ATP measurements were done as described (10). Excitation light was at 365 nm with light from a mercury lamp passing through Corning 5840, Schott WG 353, and Schott U2 filters. Fluorometric emission of NADPH was measured by using a Corning 3387 filter.

**Electric Field Jump Setup.** Rectangular electric field pulses were applied to a suspension of chloroplasts in an electric field jump setup (E-jump) apparatus. The sample was placed between two electrodes in a cell (similar to the E-jump cell described in ref. 11) equipped with four quartz windows for optical detection. The two electrodes were connected to a high power pulse generator (Cober 606) capable of delivering direct current up to 2,500 V, with very fast rise and fall times (<1 μsec) and a variable duration of 1 μsec–3 msec. A short electrical pickup by the multiplier was observed when applying the voltage pulses.

## RESULTS

**Time Course of the External Electric Field-Driven ATP Synthesis.** Exposure of a chloroplast suspension to an external electric field for 0.3–3.0 msec results in net synthesis of ATP. Fig. 1 demonstrates a typical kinetic pattern of such ATP synthesis as monitored by the coupled luciferase-luciferin enzymatic assay. The chloroplasts were exposed to an ≈0.5-sec light pulse, followed by a 1-msec exposure to an external electric field. Observation that the light-driven reaction is much faster than the external electric field-driven reaction was facilitated by the rapid response of the luciferase enzymatic assay. Thus,

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Abbreviations: EFP, electric field-induced ADP phosphorylation; DCCD, *N,N'*-dicyclohexylcarbodiimide.

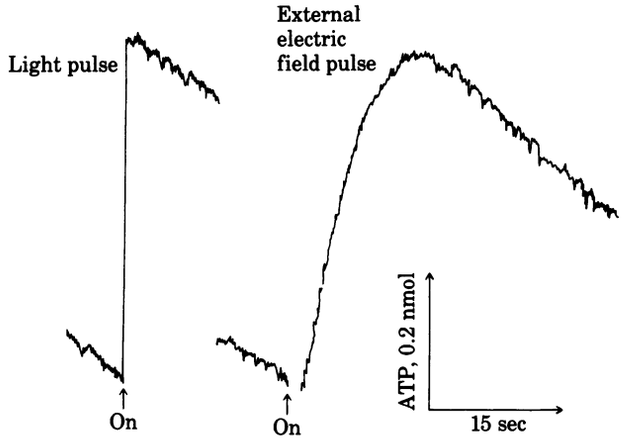


FIG. 1. Light- and external electric field-driven ADP phosphorylation in chloroplasts followed by a coupled luciferase assay. Chloroplasts (62 μg of chlorophyll) were added at room temperature (23°C) to a 1-ml reaction mixture containing 200 mM sucrose, 10 mM Na-Hepes, 0.5 mM $MgCl_2$, 5 mM $P_i$, 50 mM KCl, 0.06 mM $P^1,P^5$-diadenosine pentaphosphate, 0.225 mM ADP, 0.5 mM luciferin, 40 μl of luciferase (0.5 mg/ml), and 0.05 mM phenazine methosulfate (pH 8.0). For the light-driven reaction, light was passed through a Schott RG 5 filter for 540 msec, followed by calibration with 1 nmol of ATP. Exposure to an external electric field of 1,700 V/cm was for 1.3 msec, followed by calibration with 1 nmol of ATP.

the electric field-induced ADP phosphorylation (EFP) occurs after the external electric field has been turned off, with a velocity much slower than that of the light-driven reaction.

The amount of ATP synthesized in the EFP experiment described in Fig. 1 is 7.8 nmol/mg of chlorophyll. This is equivalent to 6.0 mol of ATP per mol of $CF_1$ ATP synthase [assuming that all the $CF_1$ molecules activate and that $CF_1$ concentration is 1.3 nmol/mg of chlorophyll (12)]. The shortest turnover time reported for light-driven ATP synthesis in chloroplasts is 2.2 msec (13). However, for the EFP reaction a turnover time of 0.2 msec would be necessary if the reaction time was limited to the short exposure of the external electric field.

To assess the validity of the assay as a quantitative method, an independent assay was used to determine the amount of ATP formed after an external electric field pulse. Hexokinase, glucose, glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and NADP were added to the reaction medium instead of luciferase and D-luciferin (Fig. 2). Changes in ATP concentration were calculated from the change in NADPH fluorescence. In either assay, between 7 and 11 nmol of ATP per mg of chlorophyll per pulse were obtained with similar pulse width and voltage amplitude. Because the concentration of the substrates is far below the $K_m$ values for the enzymes in the NADPH fluorescence assay, the slow rate observed in the fluorescence assay detects the rate-limiting step after the formation of ATP (glucose-6-phosphate dehydrogenase reaction) and not the rate of ATP synthesis itself. Thus, this method was used to determine the extent of ATP formation rather than its rate. It should be noted that the luciferin-luciferase assay was done in the presence of 50 mM KCl to obtain a more stable emission response. A temperature increase of 2–4°C during the exposure to the electric field occurs at this ionic strength. However, when KCl was omitted from the reaction medium and measurements were done at low ionic strength employing either the luciferin-luciferase assay (not shown) or NADPH fluorescence (Fig. 2), similar amounts of ATP were produced. Thus, it may be concluded that the contribution of a temperature increase to the EFP reaction could not be significant.

**Dependence of the Amount of ATP Synthesized on the Number of Pulses Applied, on the Pulse Width, and on the Voltage of the External Electric Field.** The amount of ATP produced in the EFP reaction was linearly dependent on the number of pulses to which the chloroplast membranes were exposed with 1-sec intervals between pulses. This was verified with either the luciferase (Fig. 3) or the fluorometric assay (not shown). When the time of exposure to an external electric field was varied by increasing the pulse width, a nonlinear dependence was observed (Fig. 4). This may suggest that exposure of the same enzyme population to an external electric field reaches a saturation, indicating an accumulation up to a saturation level of some intermediate that is being used to drive the ATP synthesis reaction.

The dependence of the amount of ATP synthesized on the voltage of the applied external electric field is shown in Fig. 5. In general, an increase in the amount of the ATP produced per pulse is observed when the voltage is increased. However, the dependence is nonlinear. The available sensitivity of the ATP-monitoring assay did not allow experiments at lower voltages to establish whether a voltage threshold exists below which ATP synthesis cannot occur. Analysis at much higher voltages is complicated by heating problems as well as by dielectric breakdown (14).

**Effect of Selenate on ATP Synthesis Driven by an External Electric Field.** An adenylate kinase activity has been described to be bound to the chloroplast membranes (15). It was shown to copurify with the chloroplasts' ATP synthase complex $CF_1$

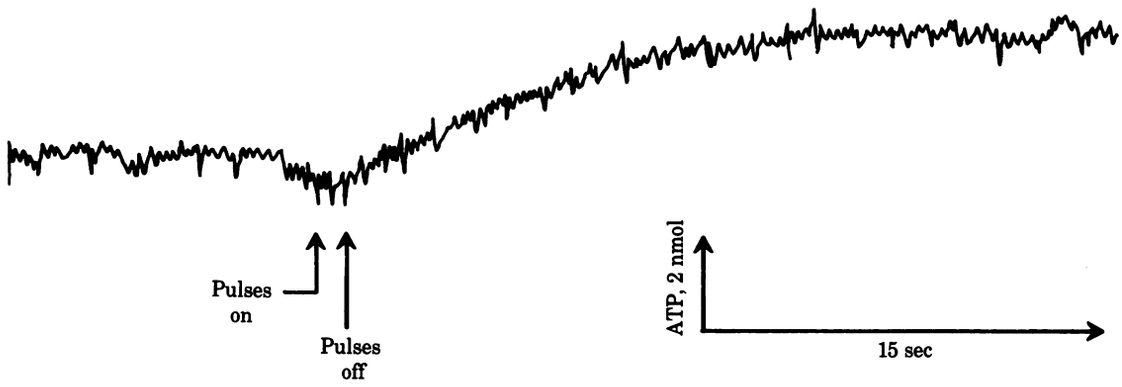


FIG. 2. Fluorometric assay of external electric field-driven ATP synthesis. Chloroplasts (62 μg of chlorophyll) were added to a 1-ml reaction mixture containing 200 mM sucrose, 10 mM NaHepes, 0.5 mM $MgCl_2$, 5 mM $P_i$, 0.225 mM ADP, 20 mM glucose, 9 mM NADP, 5 units of glucose-6-phosphate dehydrogenase, and 25 units of hexokinase. Excitation light was at 365 nm. Emission was measured with a 460-nm cut-off filter (Corning 3387); 3 pulses of 1.3-msec duration and of 1,800 V/cm were applied at 1-sec intervals.

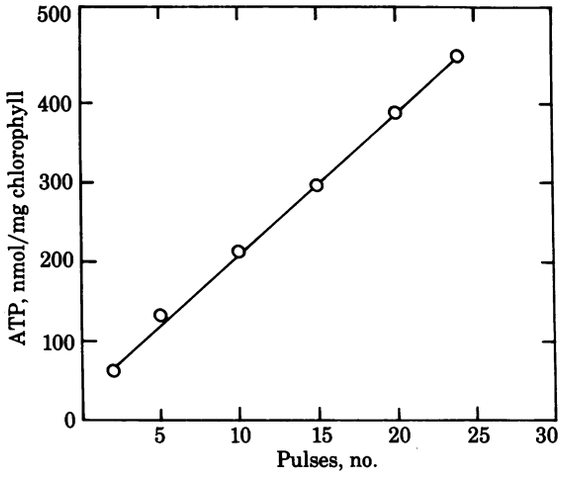


FIG. 3. EFP reaction as a function of the number of external electric field pulses. Chloroplasts (48 μg of chlorophyll) were added to a cuvette containing 1 ml of the same reaction mixture as in Fig. 1. Pulses of 1.25-msec duration and of 1,880 V/cm were applied at 1-sec intervals.

(16). To determine whether the ATP synthesis observed may be due to activation of such bound adenylate kinase by the external electric field, selenate was added. Selenate was shown to inhibit photophosphorylation in chloroplasts in a manner competitive with phosphate (17). Table 1 describes the results of adding 7.5 mM selenate to the reaction mixture that contained 2.5 mM phosphate. The reaction was inhibited by >70%. The inhibition was reversed by an excess of phosphate, with a return to >80% of the original activity. Arsenate also inhibits photophosphorylation in chloroplasts by competition with phosphate (18). However, because arsenate also inhibits the luciferase reaction (19), it could not easily be used with this assay. At the concentration used, selenate barely affected the luciferase reaction; thus, it is concluded that ATP synthesis induced by an external electric field involves the phosphorylation of ADP by inorganic phosphate.

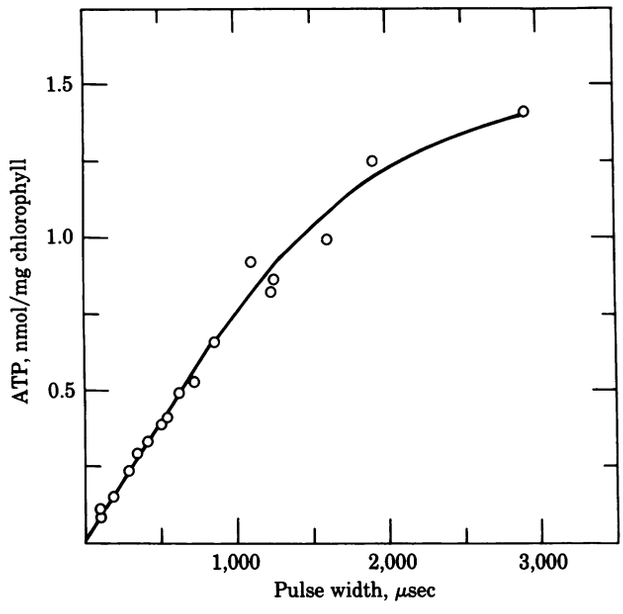


FIG. 4. The amount of ATP synthesized in an EFP reaction as a function of the duration of the external electric field. Chloroplasts (174 μg of chlorophyll) were added to a reaction mixture as in Fig. 1. Single pulses of varying duration were applied at 1,500 V/cm.

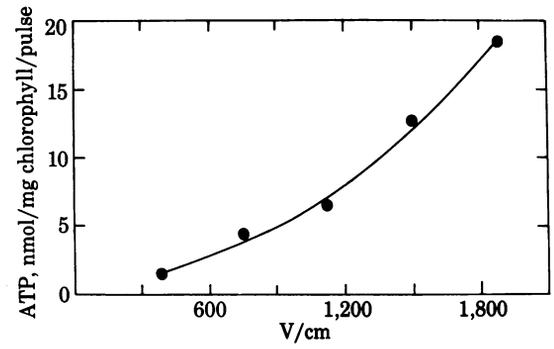


FIG. 5. The amount of ATP synthesized in an EFP reaction as a function of the voltage applied. Chloroplasts (55 μg of chlorophyll) were added to a cuvette containing 1 ml of the same reaction mixture as in Fig. 1. Ten pulses of 1.25-msec duration at varying voltage values were applied at 1-sec intervals.

**Effect of Ionophores and Energy Transfer Inhibitors on EFP.** Proton transporting ionophores such as gramicidin or nigericin are known uncouplers of light-driven ATP synthesis (20, 21). Pore-type ionophores—such as gramicidin—induce ion conductance that is much faster (few nanoseconds) than that (few microseconds) of the carrier-type ionophores—such as valinomycin or nigericin (22).

Valinomycin at 1 μM, nigericin at 10 μM, or gramicidin at 10 μM with 10 mM KCl (Table 2) did not abolish the EFP reaction under the conditions studied. Nigericin at 1 μM and gramicidin at 10 μM caused the complete inhibition of the light-driven ATP synthesis.

The reason for the noninhibitory response of the EFP reaction to these ionophores is still obscure. It may indicate that the formation of an ion concentration difference across the chloroplast membranes—which seems to be a prerequisite for the light-driven phosphorylation reaction—is not necessary in the EFP reaction.

The effect of the energy transfer inhibitor Dio-9 is demonstrated in Table 2. Dio-9, as well as *N,N'*-dicyclohexylcarbodiimide (DCCD) (not shown), did not have an inhibitory effect on the EFP reaction at concentrations at which the photophosphorylation reaction (measured concomitantly, as described in Fig. 1) was completely inhibited (9 μg/ml and 100 μM, respectively). Thus, the site of binding of these two inhibitors does not seem to participate in the EFP reaction. It should be emphasized that measurements of proton transport during the EFP reaction must be performed before one could suggest the elimination of protons as participants in this reaction.

## DISCUSSION

When a suspension of thylakoid membranes is subjected to an external electric field, a local electric field is established across

Table 1. Effect of selenate on electric field-driven ATP synthesis

| Addition | ATP synthesized, nmol/mg of chlorophyll | % of control |
|---|---|---|
| Phosphate (2.5 mM) | 98.7 | 100 |
| Phosphate (2.5 mM) + selenate (7.5 mM) | 28.0 | 28 |
| Selenate (7.5 mM) + phosphate (15.0 mM) | 81.5 | 83 |

Chloroplasts (54 μg of chlorophyll) were added to a 1-ml reaction mixture as in Fig. 1. Chloroplasts were exposed to single pulses of 4,000 V/cm and of 300-μsec duration. Calibration with externally added ATP (1 nmol) was done after each addition.

Table 2. Effect of gramicidin and Dio-9 on electric field-driven and on light-driven ATP synthesis

| | ATP synthesized | |
|---|---|---|
| | Electric pulse* | Light pulse† |
| Control | 147 | 265 |
| Dio-9 (9 μg/ml) | 110 | 0 |
| Gramicidin (10 μM) + Dio-9 (9 μg/ml) | 108 | 0 |

Chloroplasts (34 μg of chlorophyll) were added to a cuvette containing a 1-ml reaction mixture as in Fig. 1. Exposure to a train of 10 pulses of 1,800 V/cm and of 900-μsec duration was followed each time by a light pulse (250 msec or 1 sec, as indicated) and calibration with 1 nmol of ATP.
* nmol of ATP/mg of chlorophyll.
† μmol of ATP/mg of chlorophyll per hr.

the membrane that is much higher than the externally applied one. This involves polarization of the membrane bilayer itself as well as ion displacement along the membrane's surface (cross-membrane potential gradient formation). When a vesicular membrane with a radius $R$, a thickness $d$, and a much lower conductivity than either the inner or the outer medium is placed in an external electric field of strength $E$, the intensity of the radial electric field at each point of the membrane ($Em$) is given by (23):

$$Em = \frac{3R}{2d} \cos \theta, \qquad [1]$$

in which $\theta$ is the angle between the direction of the external electric field and that normal to the surface at that point. Such angular dependence of the local electric field predicts an electroselection of membrane events in the vesicles. This indeed was clearly demonstrated in studies of electric stimulation of delayed luminescence in swollen thylaloids (24). A similar electroselection would be expected to result in an induced activation of only a limited fraction of the total ATP synthase population in the exposed chloroplasts. Such electroselection is based on the following considerations: (*i*) The sign of polarization is opposite in the two hemispheres of the vesicles, so that only one hemisphere has the right polarity for activation. (*ii*) The angular dependence of the local field would be reflected by activation of more of the ATP synthase molecules that are situated near the pole ($\theta = 0°$) than those situated near the equatorial region ($\theta = 90°$) where the local field vanishes. (*iii*) Assuming the existence of some threshold for a cross-membrane potential difference required for the EFP reaction, enzyme molecules exposed to a local electric field of a value below this threshold would not be activated.

The time constant ($\tau$) for the rise of the cross-membrane potential of a spherical vesicular membrane is determined by membrane capacitance ($Cm$), the specific conductivities of the inner and outer media ($\Lambda_i$ and $\Lambda_o$, respectively), and the radius ($R$) of the sphere. This relation is expressed by (25):

$$\tau = Cm\, R \frac{\Lambda_i + 2\Lambda_o}{2\Lambda_i\Lambda_o}. \qquad [2]$$

For the formation as well as for the decay of the induced local field $\tau$ was experimentally determined to be 4 μsec and 10 μsec, respectively, by the kinetic response of the carotenoid absorption change (25–27) and the electric-induced stimulation of delayed luminescence in swollen thylakoids (28). When a medium of higher conductance and thylakoid membranes of smaller diameter are used—as in the case in the experiments reported here—the charging and discharging of the membranes would occur at rates higher than those obtained for swollen thylakoids.

Therefore, bulk-to-bulk ion fluxes (transmembrane potential gradient formation) in a nonleaky vesicle is a process kinetically inferior to the polarization of the membrane, which involves lateral displacement of charges along the surface of the membrane. With the latter process occurring within the time domain of microseconds, an exposure (a few milliseconds in our case) to an external electric field will reflect itself essentially in a rapid onset and decay of the polarized state—the secondary transmembrane ion movement being too slow to significantly contribute to the activated state of the membrane.

Considering these physical characteristics of the interaction of an electric field with a vesicular membrane, the results presented in this study suggest the following.

(*i*) ATP synthesis induced by an external electric field occurs after the decay of the electric field and goes on for many turnovers of the ATP synthase complex. The nonlinear mode of response to an increasing pulse length—as was also indicated in studies with other systems (3)—might imply the possible accumulation of an activated intermediate. Such an intermediate will allow the many enzyme turnovers to occur after the membrane's millisecond polarization time. The slow kinetics of ATP synthesis would reflect the slow decay of this postulated intermediate. These results are not in agreement with previous interpretations based on the assumption that EFP occurs only during the exposure of chloroplasts to the external electric field (1, 29, 30).

(*ii*) External electric field-driven ATP synthesis is not inhibited by uncouplers that abolish transmembrane ion gradients by competition with the imposed cross-membrane potential difference (gramicidin and valinomycin + $K^+$ in the presence of nigericin). Also, it is not significantly inhibited by energy transfer inhibitors (DCCD and Dio-9) at concentrations that completely inhibit the light-driven phosphorylation reaction. The lack of inhibitory effect by ionophores on the observed slow ATP synthesis that follows the exposure to an electric pulse most likely indicates that ionic gradients may not be involved in this process. However, it seems more difficult to understand why ionophores do not inhibit the processes that occur during the exposure to an external electric field. A possible explanation could be that formation of an intermediate occurs still faster than is the turnover rate of the ionophores.

The efficiency of an uncoupler in decreasing the buildup of the polarized state of the membrane depends on the relative kinetic parameters of both processes. The effectiveness of an uncoupler is determined by its local concentration in the membrane, its turnover time, and the concentration of the ion being transported. In addition, the interfacial potential as well as the cross-membrane potential difference would affect some of the rates involved in the ion transport by the uncouplers (31). Therefore, uncouplers of the carrier-type ionophores—such as valinomycin or nigericin in the presence of $K^+$—would not be expected to prevent the submicrosecond charging of the membrane under our conditions (e.g., see ref. 28). However, gramicidin also did not inhibit the EFP reaction, although one would expect it to be effective, having a turnover number larger by a factor of $10^3$ than the turnover number of a translational carrier such as valinomycin (31).

DCCD is not considered to interact with the catalytic site itself (32) and the site of action of Dio-9 is yet unknown (33). Thus, in promoting the EFP reaction the steps of energy transfer that are inhibited by these inhibitors may be bypassed.

Though the synthesis process after the exposure to the electric field is very slow, it is not affected by ionophores. Thus, ionic gradients do not seem to be involved in this process. Whether the EFP reaction involves the same ATP synthesis mechanism as the light-driven ADP phosphorylation reaction

or a completely different mechanism remains to be elucidated. Nevertheless, we believe the data presented indicate that EFP offers a unique opportunity to obtain further insight into the ATP synthase mode of action, activation, kinetics, and energetic requirements.

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