External electric field driven ATP synthesis in chloroplasts: a slow, ATP synthase-dependent reaction

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Electric field jump *Cross-membrane potential* *Phosphorylation* *ATP synthesis* *Inhibitors of EFP* *Coupling factor 1*

1. INTRODUCTION

The process of energy transduction in chloroplasts involves several sequential stages. Light induced charge separation triggers a series of redox reactions which lead to the formation of a transmembrane electric potential difference and a proton concentration gradient. In a well-coupled system these gradients drive the generation of ATP from ADP and P_i. This final step of energy transduction and the mode by which it leads to ATP synthesis are not yet understood. One type of experimental approach aimed at the elucidation of the energy transduction process involves reduction of the number of factors which participate in it. Thus, an artificially imposed transmembrane proton concentration gradient has been demonstrated to activate ATP synthesis in systems in which electron transfer was completely blocked [1]. It was also shown that the ATP synthase enzyme complex could catalyze ADP phosphorylation when reconstituted into liposomes in absence of the components of the electron transport chain [2]. Recently a novel method has been presented in which formation of ATP from ADP and phosphate, catalyzed by thylakoid membranes can be driven by an external electric field [3]. In such a system not only electron transfer but also the formation of bulk to bulk ionic gradients is presumably absent during activation. As previously stated [4] this approach has clear kinetic advantages and has been shown to be effective in other energy transducing systems as well [5–7]. A further development of this method involved the use of an enzymatic assay that allowed the continuous monitoring of ATP formation induced by exposure of thylakoid membranes to an external electric field [8]. Information regarding the time course of ATP synthesis has been thus obtained indicating that the external electric field induced ATP synthesis proceeds for relatively long periods (seconds) following the termination of the applied electric field pulse.

Further studies of the unusual kinetics of external electric field driven ADP phosphorylation (EFP reaction) are presented in this report. They indicate the direct involvement of the CF_1 portion of the ATP synthase complex in the EFP reaction. The influence of inhibitors on the EFP reaction was investigated and compared to their effect on photophosphorylation, revealing important differences between the two.

2. EXPERIMENTAL PROCEDURES

2.1. *Materials*

Chloroplast thylakoids were prepared from lettuce as previously described [9]. Chlorophyll was determined by a standard method [10]. Hexokinase type V, gramicidin D and tentoxin were purchased from Sigma Chemicals Co., St. Louis, MO. FCCP was from Du-Pont de Nemours and Co., Wilmington, Delaware.

Photophosphorylation and the kinetics of the external electric field driven ATP synthesis were measured by either one of two methods:

(i) $^{32}P_i$ incorporation into ATP was followed by a rapid quenching technique: 0.5 ml of the assay medium containing 200 mM sucrose, 0.5 mM $MgCl_2$ 2.5 mM phosphate, 0.3 mM ADP (freed of ATP by pretreatment with hexokinase and

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glucose), 10 mM Na–HEPES buffer and thylakoid membranes at pH 8.0, were placed in a 1.0 ml plastic cuvette with two parallel stainless steel electrodes and exposed to an external electric field (usually 1200 V × cm^{-1} of 50 Hz AC, 20–50 ms duration) at 21°C in the dark. At different time intervals following the exposure to the field, 0.3 ml of 2 M perchloric acid were rapidly injected to the cuvette. The quenched sample was then processed and assayed for the amount of [^{32}P]ATP as previously described [11]. The rapid injection set-up was presented elsewhere [12]. The sequence of events was electronically controlled.

(ii) The ATP dependent light emission in the coupled luciferase enzymatic assay was continuously monitored as was previously described [8]. The output of the photomultiplier (EMI 9526 shielded by two Corning 4-96 filters) was fed into a differential amplifier (Tektronics 3A9) connected to a storage oscilloscope (Tektronics 7623) and a chart recorder. Photophosphorylation was measured in the same set up either before or after the measurement of the EFP reaction. Illumination (up to 2 × 10^5 ergs · cm^{-2} · s^{-1}) was provided through an RG 665 cut off filter, for preset time intervals controlled by an electronic shutter.

3. RESULTS

3.1. *Time course of ATP synthesis as followed by rapid quenching*

ATP synthesis generated by exposure of thylakoid membranes to an external electric field, was previously detected by following the change in the extent of ATP dependent light emission of the coupled luciferase assay [8]. Slow synthesis lasting several seconds was observed in response to a voltage pulse of a few milliseconds. To ensure that this slow rate of ATP formation does not reflect a change in the response of the coupled luciferase assay due to the application of the external electric field, the kinetics of external electric field-driven ATP synthesis were measured using radioactive labeling coupled with a rapid mixing–quenching technique.

Fig.1. illustrates the time course of [^{32}P]ATP formation in response to an external electric field applied to the chloroplast suspension containing ADP and $^{32}P_i$. The amount of [^{32}P]ATP formed reached a maximum around 0.8 s after the termination of the field pulse; this was followed by a decrease in the amount of [^{32}P]ATP to a level around 10% of the maximal amount, after a few seconds. The initial slow rate of [^{32}P]ATP formation following the exposure of the thylakoids to the external electric field is in agreement with observations obtained by measurements using the luciferase assay (fig.1-insert) [8].

3.2. *Involvement of the ATP synthase coupling factor (CF_1) in the EFP reaction*

In order to demonstrate the dependence of the EFP reaction on the CF_1 component of the ATP synthase complex, the chloroplasts were depleted of their CF_1 by means of NaBr treatment. This treatment has been shown to selectively remove practically all the CF_1 complexes from the thylakoid membranes [13]. Figure 2 illustrates the response of both NaBr treated and non-treated particles to an external electric field and to a short light pulse. ATP synthesis was monitored by following the ATP dependent light emission of the coupled luciferase assay. The capacity to carry out both the light driven ATP synthesis and the EFP reaction were practically absent in the NaBr treated particles.

Tentoxin, a cyclic tetrapeptide produced by the fungus *Alternaria alternata*, was shown to be a potent and highly specific inhibitor interacting directly with the CF_1 portion (probably with the $\alpha\beta$ part) of the ATP synthase complex [15–17] in lettuce chloroplasts. Its effect could therefore yield direct information regarding the role of CF_1 in the EFP reaction. Figure 3 depicts the effect of tentoxin on the EFP reaction and on photophosphorylation. Both reactions were completely inhibited by addition of 2.2 μM tentoxin to the reaction medium. EFP inhibition by tentoxin has been observed by both the radioactive labeling and the coupled enzymatic assay (fig.1 and insert). Two minutes preincubation with tentoxin were allowed and relatively high concentrations were used due to the slow forward rate of binding [18].

3.3. *Comparison of the EFP reaction with photophosphorylation*

Table 1 summarizes the effect of some inhibitors on the EFP reaction as measured by [^{32}P]ATP for-

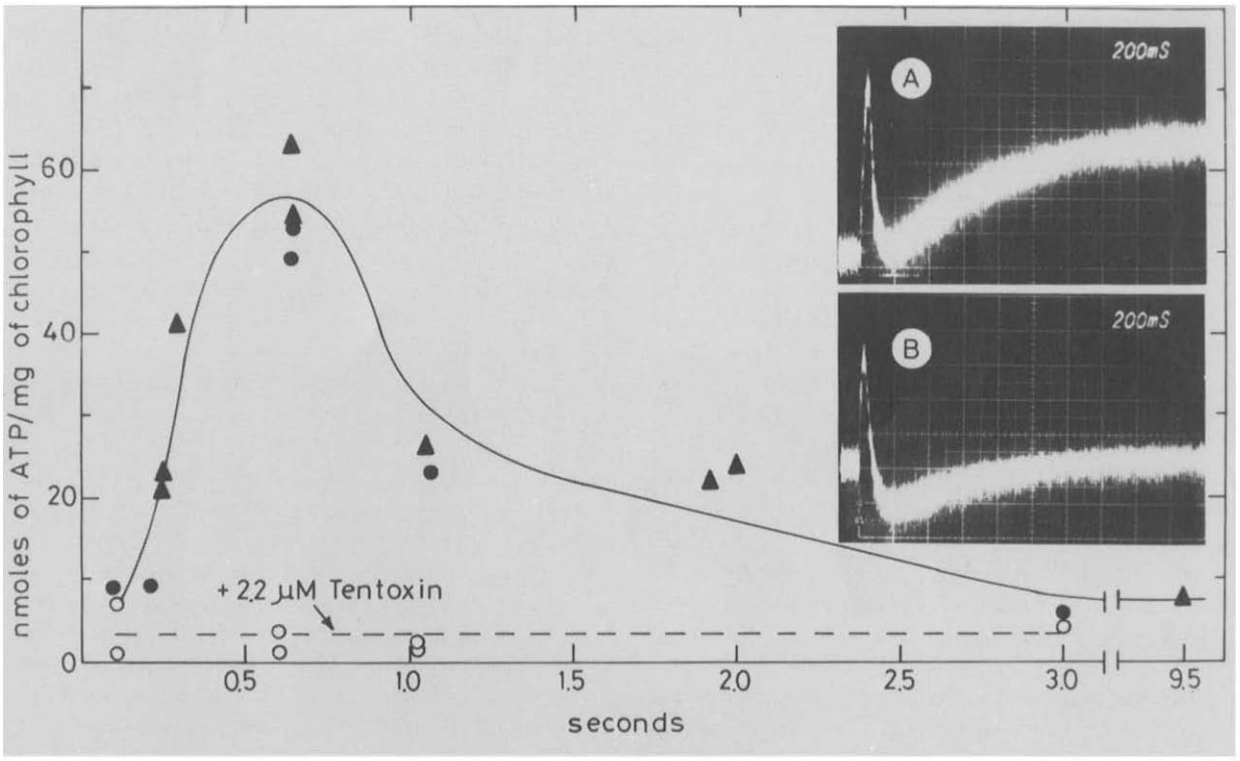

Fig.1. Time course of ATP formation in the EFP reaction in presence and absence of tentoxin. Abscissa: time after application of the electric field. Ordinate: amount of ATP formed. 0.5 ml of a reaction mixture containing 200 mM sucrose, 0.5 mM $MgCl_2$, 2.5 mM $^{32}P_i$, 0.5 mM ADP and 10 mM Na–HEPES and chloroplasts (75 μg (▲) or 166 μg (●) at pH 8.0 and 21°C were placed in a cuvette between two stainless steel electrodes 0.3 cm apart. The mixture was exposed to 1200 V/cm AC field for 50 ms and automatically quenched by rapid injection of 0.5 ml of 2 M perchloric acid. When indicated 2.2 μM tentoxin (○) was present in the assay medium and 2 min incubation time allowed before the exposure to the electric pulse. 1 μmol of cold ATP was then added to the quenched reaction mixture and the sample analyzed as described in [11]. Every point represents the mean value of three independent measurements on the same chloroplasts. Insert: 0.8 m of a reaction mixture containing 200 mM sucrose, 0.5 mM $MgCl_2$, 2.5 mM P_i, 0.5 mM ADP (purified of residual ATP by pretreatment with glucose and hexokinase), 0.42 mM D-luciferin, 50 λ (0.5 mg protein/ml) luciferase and 150 μg of chlorophyll at pH 8.0 and 21°C were exposed to a 30 ms of 1200 V/cm AC pulse. (A) control, (B) in presence of 7.7 μM tentoxin. The initial spike is an electric field-induced artefact unrelated to ATP synthesis being much faster than the assay's response time.

mation at 0.8 s following the external electric field pulse and at steady state illumination. The use of radioactive labeling eliminates the problems which result from modulation of the sensitivity of the assay method by the inhibitors that are tested as in the case of, e.g., arsenate and luciferase [19]. Full inhibition of both the EFP reaction and of photophosphorylation was obtained with 2.2 μM tentoxin.

FCCP (1 μM) (a weak acid type uncoupler), caused 70% inhibition of the light driven reaction, but did not have any effect on the EFP reaction. At 10 μM FCCP full inhibition of both reactions was obtained; however, at such high concentrations of this reagent, inhibition may not be due to uncoupling (since the system is fully uncoupled at 2–3 μM FCCP), but rather to a more direct effect on the ATP synthase.

By addition of gramicidin D, a pore type ionophore, the light driven phosphorylation was completely inhibited, but no effect on the EFP reaction was observed. It seems as though interference with the ionic permeability of the membrane does not affect the EFP reaction to the same

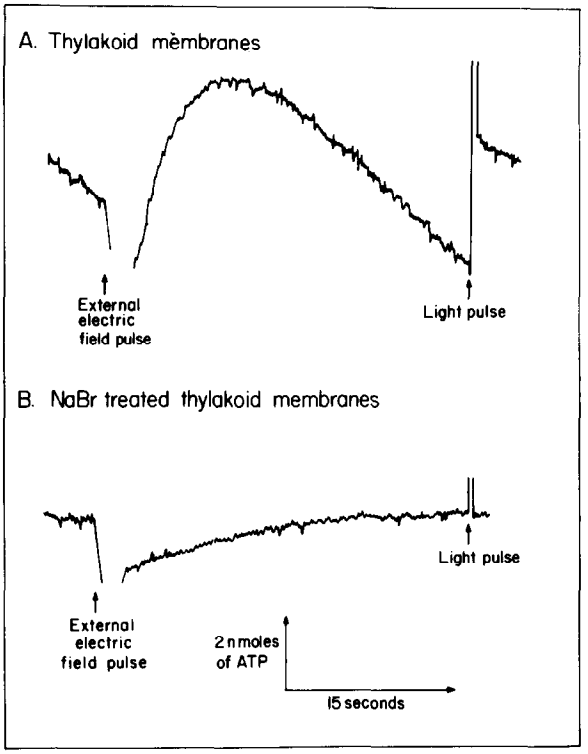

Fig.2. The EFP reaction in NaBr treated particles. Chloroplasts (52 μg of chlorophyll/ml of either NaBr treated or non-treated particles) were added to a reaction mixture containing 200 mM sucrose, 0.5 mM $MgCl_2$, 5 mM P_i, 50 mM KCl, 10 mM Na–HEPES, 0.225 mM ADP (purified of ATP by pretreatment with glucose and hexokinase), 0.05 mM phenazine methosulfate and 0.06 mM P^1,P^5 diadenosine pentaphosphate, 0.5 mM luciferin and 40 λ luciferase (0.5 mg protein/ml) at pH 8.0 and 21°C. The mixture was placed in a cuvette between two gold electrodes as described in [8] and exposed to a 1800 V/cm DC field for 1.3 ms. An exposure to light for 1 s followed. NaBr treated particles were prepared as described in [13].

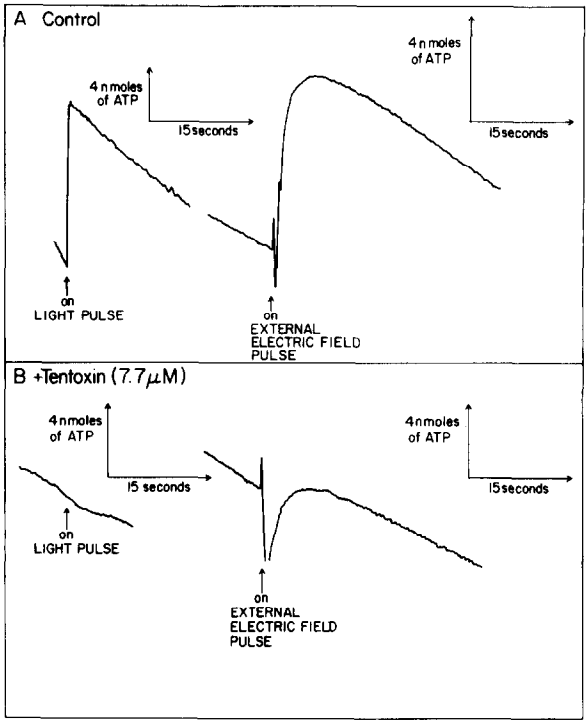

Fig.3. The effect of tentoxin on the EFP reaction and on photophosphorylation. 0.8 ml of reaction mixture containing the same as in the insert to fig.1 but also with 0.06 mM phenazine methosulfate were exposed to a 2-s light pulse and then to a 1200 V/cm AC field for 30 ms. Two minutes preincubation time with 7.7 μM tentoxin were allowed in experiment B.

extent as it does the membrane's photophosphorylation capacity.

Arsenate inhibits photophosphorylation due to its being competitive with phosphate [20]. In our experiments the inhibitory effect of arsenate was significant in both cases but greater upon photophosphorylation than on the EFP reaction.

Cold treatment of chloroplasts (at −79°C) was shown to decrease – under certain conditions – their photophosphorylating capacity due to both decrease in the degree of coupling and the release of the CF_1 component from the membrane [21]. Under the same conditions the EFP reaction is also severely decreased. There is a similar, very significant decay of the extent of EFP reaction in chloroplasts stored at 0°C for several hours following their preparation (not shown).

4. DISCUSSION

Transmembrane electrical potential difference has been considered to be involved in most of the energy transduction processes in biological membranes. The mode by which it is involved at the molecular level in the activation of processes cata-

Table 1

Effect of inhibitors on the EFP reaction

Chloroplasts	Addition	EFP[a] nmol [^{32}P]ATP formed / mg of chlorophyll	% of control	Photophosphorylation[b] nmol [^{32}P]ATP formed / mg of chlorophyll x h	% of control
Fresh		4,25 ± 0.25	100	982 ± 26	100
	2.2 μM Tentoxin	0.29 ± 0.23	7	11 ± 1	1
	10.0 μM Gramicidin	4.47 ± 0.36	103	39 ± 1	4
	1.0 μM FCCP	4.80 ± 0.03	110	303 ± 24	31
	10.0 μM FCCP	0.10 ± 0.10	2	2 ± 0	0
	10.0 mM Arsenate	3.24 ± 0.03	75	466 ± 14	47
Cold-treated[c]		1.13 ± 0.37	26	300 ± 45	30

[a] Chloroplasts were exposed to an AC electric field of 1200 v/cm for 50 ms at 50 Hz in a reaction medium as described in the legend to fig.1. The reaction was rapidly quenched at 800 ms following the electric field pulse. Chlorophyll concentration in the reaction medium was 144 μg/ml. When tentoxin was added, 2 min preincubation time was allowed

[b] Photophosphorylation was carried out in the same reaction mixture as described for EFP reaction except for the addition of 5 mM glucose 10 U/ml hexokinase and 0.05 mM phenazine methosulfate. The supernatant from PCA quenched and deproteinized samples was extracted with IBB as described in [25] and steady state phosphorylation rates were determined from the amounts of glucose-6-^{32}P formed

[c] Cold treatment conditions (at −79°C) for the chloroplasts, suspended in 30% (v/v) ethylene glycol, were as described in [21]

lysed by membrane bound enzymes is still obscure. We have studied a system in which external electric fields applied to a suspension of thylakoid membranes from lettuce served both to activate and provide a driving force for their ATP synthesizing machinery. As has been previously stressed [4], the magnitude and time course of the induced transmembrane electrical potential can be easily controlled by this method. The kinetic behaviour of this reaction (EFP) studied previously [8] indicated that ATP synthesis driven by an external electric field occurs slowly, mostly following the termination of the exposure to the field, and apparently for several turnovers of the ATP synthase (assuming the ATP synthase to chlorophyll ratio is 1.3×10^{-6} [22]).

In the present work we have used an independent technique to follow ATP synthesis during the first seconds following the exposure to an external electric field. We have shown that de novo ATP synthesis from ADP and phosphate occurs in response to an external electric field. This synthesis peaks at 0.8 s following the field (and the onset of the reaction) and involves the ATP synthesizing complex CF_1. Removal of CF_1 from the thylakoid membranes as well as introduction of the CF_1 specific inhibitor tentoxin, results in the total inhibition of both the external electric field driven reaction and the light driven ATP synthesis. From fig.1 one can calculate an initial rate of ATP synthesis of 200–400 μmol ATP · (mg of chlorophyll × h)$^{-1}$. The decrease in the total amount of ATP observed following the field induced ATP synthesis might be due to a concomitant activation of the latent ATPase activity in the same system. In the absence of reducing agents (e.g., dithioerythrol), a wide range of ATPase activities can be obtained depending on the assay conditions (see, e.g. [23] and [24]). This apparent ATP hydrolysis might explain the reason for the low yields of ATP reported previously [3] when the interval between the termination of the external electric field and the quenching time was large enough to allow significant hydrolysis of the newly synthesized ATP. It may also explain the relatively low ATP formation reported in table 1.

The slow appearance of the ATP synthesis activity at a rather high initial velocity indicates that a major portion of the ATP synthase complexes is active in the process, a fraction which is larger than

that which is effectively activated by the external electric field, according to [3]. The relation of the EFP reaction to that of the light driven phosphorylation, remains obscure. Although both reactions are carried out by the same ATP synthase enzyme complex, one needs more information about the nature of the intermediates in the EFP reaction before one can assert whether they are common to the photophosphorylation reaction.

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