Synthesis of ATP induced in pea chloroplasts by single turnover flashes

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1. INTRODUCTION

The onset of the ATP synthesis in chloroplasts is thought to depend on the activation of the enzyme involved, namely the coupling factor CF_1–CF_0 or ATPase system [1].

This ATPase system has been proposed to be reversible [2–4]. Actually, the mechanism for ATP:H_2O oxygen exchange [5] and ATP:P_i exchange [6] both indicate a reversibility of the ATP synthesis on the ATPase system. So the rates of ATP synthesis and hydrolysis depend on the activation of the ATPase system [7,8] which hence can be estimated from the ability of chloroplasts to hydrolyse ATP in the dark [9].

There are conditions in which chloroplasts exhibit a stable ATPase activity [10–13]. Chloroplasts prepared from pre-illuminated leaves [14] have an active ATPase. A stable pH difference of ~ 1.2 is measured across the thylakoid membranes in the dark from the distribution of the [^{14}C]-methylamine inside and outside the thylakoid. ATP hydrolysis in the dark is coupled to an inward transport of H^+ and a concomitant change of the transmembrane electric potential difference [15].

We propose to follow the synthesis of ATP induced by single turnover flashes in such chloroplasts where the ATPase system is fully active. In these conditions a critical difference of pH across the thylakoid membrane is observed. Above this critical value the yield of ATP synthesized/flash is maximum and independent of the number of flashes already fired. By comparing the yield of ATP synthesis/single flash or group of 2 flashes we determine kinetic factors which govern the yield of ATP synthesis. These factors seem not directly related either to the ΔpH or to the transmembrane electric potential difference.

2. MATERIALS AND METHODS

Type B chloroplasts [16] were prepared as in [14] from freshly harvested, 2-weeks-old pea leaves 'pois nains d'Annonay très hatifs, G. Delbard' which were illuminated for 30 min in ice-cold water before grinding.

ATP was directly measured in the stirred chloroplast suspension by recording the luciferin–luciferase luminescence [17]. Luminescence was detected with a cooled EMI 9558 QH photomultiplier screened from actinic illumination by a Specivex DH 485 b optical filter. A home-made device switched off the photomultiplier response while the flashes were fired. The photomultiplier signal was amplified and recorded.

ΔpH was measured by recording the quenching of the atebrin fluorescence [18] in the same device as for ATP measurements but 2.5 μM atebrin was added instead of the luciferin–luciferase system. The photomultiplier was screened with supplementary Specivex H 513 b optical filter and the fluorescence excited by 430 nm monochromatic light.

Absorbance change at 515 nm, ΔA_{515}, was measured in unstirred samples as described [19].

Excitation light flashes were obtained from 2 xenon flashes (general Radio Stroboslav) fired simultaneously and were filtered through a Calflex X 2 (Balzers) and a RG 630 (Schott) optical filter. The flashes were given one-by-one or as groups of

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2 separated by variable time space, one from another. Single flashes or group of flashes were repeated at the 0.1 Hz frequency. The flashes (2 μs at half-intensity, 4.5 μs at half-energy) gave a constant energy sufficient to saturate the ΔA_{515} and the flashes were short enough to induce one turnover only of the electron-transport chain.

All the measurements were performed at 10°C in the following medium: 40 mM Tricine pH 8 (40 mM Tricine–maleate or 40 mM morpholinopropane–sulfonic acid for more acidic pH), 10 mM $MgCO_3$, 20 mM $KHCO_3$, 2 mM K_2HPO_4, occasionally 20 μM ADP and 5 μM diadenosylpentaphosphate and chloroplasts equivalent to 20 μg chl./ml final conc. The luciferin–luciferase system was purchased from LKB. Nigericin was a gift of the Ely Lilly Lab.

3. RESULTS AND DISCUSSION

3.1. *Onset of photophosphorylation*

With chloroplasts where the ATPase system was fully activated we expected that the synthesis of ATP/flash would be independent of the number of flashes already fired at least when the flashing rate was low enough (0.1 Hz) to avoid any substantial accumulation of protons inside the thylakoids.

This was true with most of the chloroplast preparations but sometimes, even with freshly prepared chloroplasts, the yield of ATP synthesis/flash was low on the first flash and increased until a limit value after the 5–10th flash (fig.1). This progressive increase of the yield was also observed when the chloroplasts were kept, prior to use, 1–2 h in ice-cold 0.4 M sucrose in the dark.

Nevertheless the protons accumulated inside the thylakoids even at a flash rate of 0.1 Hz. The pH difference across the thylakoid membranes, measured by quenching of the atebrin fluorescence, increased progressively with the number of flashes (fig.2). Above a critical value of this ΔpH (observed either since the first flash or after several flashes depending on the state of the chloroplasts) the yield of ATP synthesized/flash became constant and independent of the number of flashes fired (table 1).

Within the precision of the measurements the critical value of this ΔpH (1.32–1.44) was the same at external pH of 7 or 8, after a single flash or after a group of 2 flashes given at 0.1 Hz.

We interpret this critical value of the ΔpH as evidence for a critical energy threshold which is expected for ATP synthesis following the chemiosmotic theory [20,21].

3.2. *External pH dependency of ATP synthesis*

The profile of the response of ATP synthesis to external pH (fig.3) was different when photophosphorylation was induced by a single flash or a group of 6 flashes (3 ms apart). With a single flash the yield of ATP synthesized was at pH 7 only 30% of that synthesized at pH 8 while with a group of 6 flashes it was at pH 7 almost 70% of that at pH 8. In all cases, especially with the group of 6 flashes, flash-induced phosphorylations appeared less sensitive than phosphorylation in continuous light to the external pH [22]; in continuous light phosphorylation was at pH 7 ~ 10% only of that at pH 8.2 (the optimal pH).

The pH dependency of ATP synthesis was sharper than that in [23] when these authors mea-

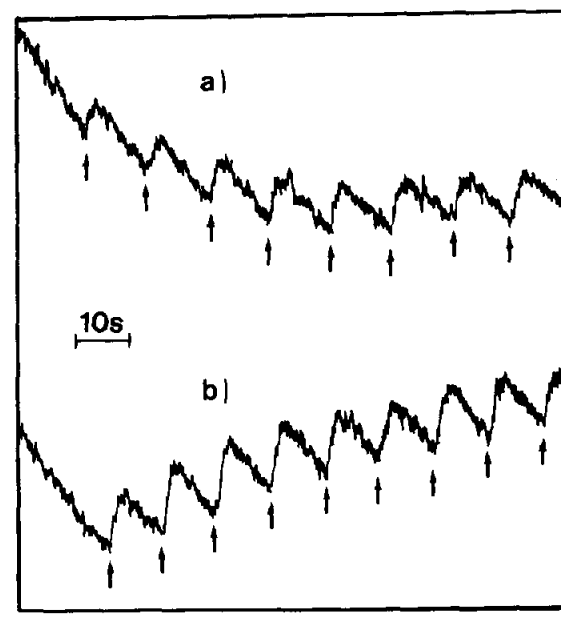

Fig.1. Photophosphorylation induced by single flashes. Conditions were as in section 2. Single flashes were fired at 0.1 Hz. Chloroplasts (equiv.: (a) 80 μg Chl; (b) 60 μg) were kept in complete darkness in 2 ml reaction medium before the first flash was fired. The yield of ATP synthesis . flash $^{-1}$. 1000 Chl $^{-1}$ was: (a) 0.31 on flash 1, 0.43 after flash 5; (b) 0.47.

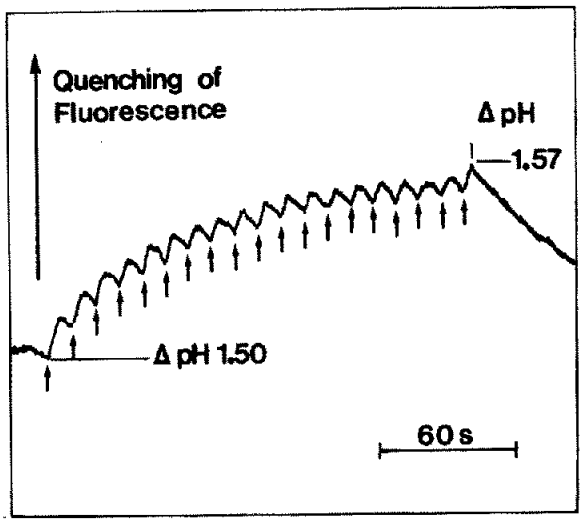

Fig.2. Flash-induced increase of the ΔpH across the thylakoid membranes. Conditions were as in section 2. Single flashes were fired at 0.1 Hz. Chloroplasts (equiv. 40 μg Chl in 2 ml reaction medium) were kept in complete darkness before the first flash was fired. Quenching of the fluorescence of atebrin is reported. ΔpH-value is given at the beginning and at the end of the illumination period.

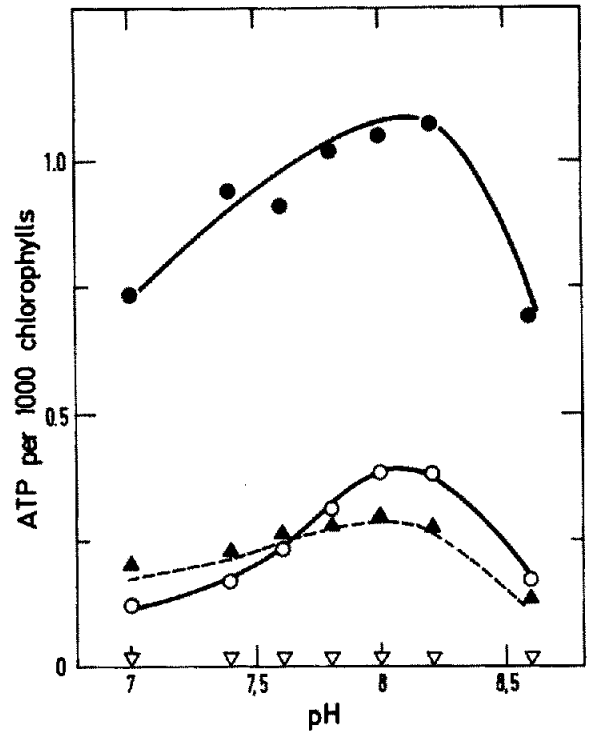

Fig.3. ATP synthesis/flash or group of 6 flashes (3 ms apart) as a function of the external pH. Conditions were as in section 2. Chloroplasts in 2 ml reaction medium were equivalent to 40 μg Chl (○,▽) single flashes, (●,▼) groups of 6 flashes (3 ms apart) were fired at 0.1 Hz: (▽,▼) 10 nM nigericin was added.

Table 1

Critical ΔpH above which the yield of ATP synthesized/flash or group of 2 flashes is independent of the number of flashes fired

Experimental conditions	Critical ΔpH	ATP/ flash	No. exp.
pH 7, Single flash	1.36 ±0.078	0.17	5
pH 7, Group of 2 flashes	1.405±0.016	0.39	7
pH 8, Single flash	1.44 ±0.04	0.45	6
pH 7, Group of 2 flashes	1.416±0.03	0.55	12

Conditions were as in section 2. Chloroplasts in 2 ml reaction medium were equivalent to 40 μg Chl; ATP synthesis and ΔpH were measured simultaneously in 2 separate samples. External pH was 7 or 8 and illumination was done with single flashes or groups of 2 flashes (variable times apart) at 0.1 Hz.

sured the ATP generated by 20 ms light pulses in broken spinach chloroplasts in the presence of 10^{-4} M benzylviologen. Two main differences must be emphasized; we did not use any cofactor; and we measured the true yield of ATP synthesis by taking into account the rate of ATP hydrolysis during the intermediary dark phases. On the contrary, in [23] ATP synthesis was determined from the incorporation of ^{32}P in ATP and the hydrolysis of the newly formed [^{32}P]ATP during the intermediary dark phase of 31 s between 2 successive flashes was neglected.

3.3. *Sensitivity of ATP synthesis to uncouplers*

As reported in [24] flash-induced photophosphorylation was inhibited by valinomycin in the presence of K^+. This shows that flash-induced transmembrane electric potential is a determinant

factor in the fixation of the energy needed for ATP synthesis.

Nigericin in the presence of K^+ is a potent uncoupler of the steady-state photophosphorylation in chloroplasts. It has been thought that in flashing light or in the initial period of illumination photophosphorylation only depends on the transmembrane electric potential [25,26]. In such conditions nigericin in the presence of K^+ should not inhibit ATP synthesis. The results in [25,26] supported this prediction at least at external pH 7. On the contrary, at external pH 7 or 8, very low concentrations of nigericin inhibited flash-induced phosphorylation in our chloroplasts. In this range of nigericin concentration the slow rise of the absorbance change at 515 nm (ΔA_{515}, measuring the transmembrane electric potential difference) became prominent [27]. Indeed, with 10 nM nigericin, phosphorylation induced by a single flash was completely abolished (fig.3). At the same [nigericin] 75% of the phosphophorylation induced by a group of 6 flashes (3 ms apart) was inhibited (fig.3).

Nigericin at nM levels did not inhibit ATP hydrolysis in the dark nor the concomitant inward flux of protons (table 2). However the ΔpH across the thylakoid membranes dropped below the critical value. Single flashes given at 0.1 Hz frequency did not accumulate enough protons inside the thylakoids to raise the ΔpH above the limit value and consequently ATP was not synthesized. With groups of 6 flashes given at the 0.1 Hz frequency the influx of protons was higher and after several cycles the ΔpH increased above the critical value and thus phosphorylation started again, so much the more as the transmembrane electric potential difference (the other factor of energization of the membrane) was twice this observed with a single flash.

Table 2

ΔpH induced in the dark by hydrolysis of ATP

Exp. no.	Conditions in the dark	ΔpH
1	Control	1.660
	+ 50 μM ATP	1.893
	+ 5 nM nigericin	1.645
	+ 10 nM nigericin	1.572
	+ 20 nM nigericin	1.394
2	Control	1.550
	+ 5 nM nigericin	1.310
	+ 50 μM ATP	1.653
3	Control	1.430
	+ 10 nM nigericin	1.180
	+ 50 μM ATP	1.515

Conditions were as in section 2. Chloroplasts in 2 ml reaction medium were equivalent to 40 μg Chl

Contrary to an often expressed statement [25,26] the onset of phosphorylation does not only depend on the transmembrane electric potential difference. It must be recalled that chloroplasts, even in the dark, can present a significant ΔpH across the membrane. Ignoring that presence can lead to misinterpret the role of this ΔpH on the onset of photophosphorylation and to mis-estimate the value of the light-induced ΔpH. Nigericin acts by decreasing the value of the ΔpH and the results of its action, reported above, confirm the necessity of a critical value of the ΔpH to observe a flash-induced ATP synthesis.

3.4. *ATP synthesis by a group of two flashes*

Fig.4 gives the mean value of the amount of ATP synthesized by each flash of a group of 2 flashes fired every 10 s, as a function of the dark period, t_D, between 2 successive flashes of the group.

This mean value increased with t_D until an optimum, reached for ~1000 ms at external pH 8 and ~500 ms at pH 7. Assuming that the first flash of the group was as effective as a single flash given at the 0.1 Hz same frequency, the yield of ATP synthesized by the second flash of the group was, at the optimum t_D, 1.6-times and 3-times the yield synthesized on the first flash, at pH 8 and at pH 7, respectively.

At pH 7 the mean value of the ATP yield/flash was always higher with the group of 2 flashes than with the single flash.

At pH 8 the mean value of the ATP yield/flash was lower with the group of 2 flashes than with the single flash when t_D was < 10 ms (fig.4). For $t_D > 10$ ms we obtained the same qualitative results as at pH 7.

Those results cannot be explained either by changes of the ΔpH or of the transmembrane electric potential difference depending on the t_D value.

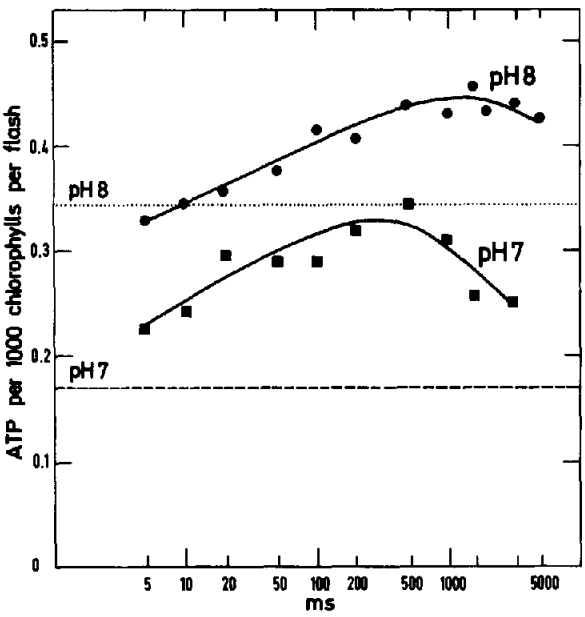

Fig.4. Mean value of ATP synthesized/flash observed with a group of 2 flashes (t_D ms apart). Conditions were as in section 2. Chloroplasts in 2 ml of the reaction medium were equivalent to 40 μg Chl. External pH was (•) 8 or (▪) 7. Groups of 2 flashes (variable t_D ms apart) were given at the frequency 0.1 Hz. The mean value of the maximum amount of ATP synthesized/flash in the group of 2 was calculated from 4–5 different experiments. When the group of 2 flashes was replaced by a single flash the yield of ATP synthesis is indicated (• • •) at pH 8; and (---) at pH 7.

Actually, at any value of t_D, the ΔpH extents observed with a group of 2 flashes were more or less identical. On the other hand, the initial level of the ΔA_{515}, reporting the transmembrane electric potential difference, was maximum for t_D = 5–20 ms then decreased for t_D > 200 ms, then the signal at 515 nm observed for each of the 2 flashes of the group became of the same order of magnitude as the signal obtained with the single flash. So there is no simple correlation between the changes in the yield of ATP synthesis as a function of t_D and the changes of the ΔpH and of the ΔA_{515}. Similar experiments in bacterial chromatophores have led the authors [28,29] to propose that the coupling between electron flow and ATP synthesis was more direct than that expected on the base of the original chemiosmotic model.

These results are satisfactorily interpreted by assuming the formation of a specific state of the membrane after the first flash. This state should have a life-time of several hundred ms, shorter at pH 7 than at pH 8, and should act on the yield of the ATP synthesized by the second flash. A certain amount of this specific state should be present in the dark at pH 8, prior to illumination, but not at pH 7 where it should decompose almost completely.

This state can hardly be identified with some active state of the ATPase system as far as the rate of ATP hydrolysis in the dark is constant and independent of previous flash illumination. We favor the proposal that this specific state is a pH-sensitive redox state of one or of some membrane component(s). This state takes a long time (500–1000 ms) to develop after the flash. After such a long period the electrons displaced by the flash should have moved throughout the electron transport chain. So the specific state may be the result of the redox poise of a pool of components where the electrons are fed from this chain. But we cannot decide whether this redox potential, close to the membrane, conditions the effects of the flash on the charges transfer across the membrane or on the activity of the ATPase system.

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