ATP HYDROLYSIS AND MEMBRANE POTENTIAL IN SPINACH CHLOROPLASTS

Jean Michel GALMICHE and Guy GIRAULT

Service de Biophysique, Département de Biologie, Centre d'Etudes Nucléaires de Saclay, BP no. 2, 91190 Gif-sur-Yvette, France

Received 8 July 1980

1. Introduction

In chloroplasts the synthesis of ATP [1] as well as its hydrolysis [2] need the activation of the ATPase system. This ATPase activity has been observed in intact chloroplasts [3] but also in chloroplasts which have lost their external membranes and are freely permeated by nucleotides [4]; the latter chloroplasts, when kept in the dark at 0°C, slowly lose their activity in several hours and recover it after a short period of illumination [4,5].

In the chemiosmotic hypothesis, energy transduction in the membranes is a reversible mechanism [6] and it is assumed that the redox reactions as well as the ATP hydrolysis can generate an electrogenic translocation of H^+ across the thylakoids. The resulting 'proton-motive force' is the sum of an electrical ($\Delta\psi$) and of a concentration-dependent (ΔpH) component. In chloroplasts, ATP hydrolysis [2,4] is coupled to an inward transport of H^+ (ΔpH) but a concomitant change of the membrane potential ($\Delta\Psi$) has not yet been evidenced, contrary to what has been observed in other similar systems, mitochondria or bacterial chromotophores.

In chloroplasts the membrane potential ($\Delta\psi$) has been characterized by the ΔA of intrinsic (carotenoïds [1] or extrinsic [7] probes:

(i) Attempts to find a carotenoïd ΔA_{515}, in response to hydrolysis of ATP in chloroplasts have been unsuccessful. But these negative results can well be explained by the difficulty of monitoring slow ΔA in a spectral range where the absorbance of the pigments is high and exhibits sharp changes as a function of the wavelength.

(ii) The extrinsic probe, oxonol VI, has been used in chromatophores [8] and in chloroplasts [9] and has been shown to respond to the light- or K^+ diffusion-induced potential. Oxonol VI ΔA in response to the transmembrane potential were measured at ~600 nm, a wavelength range where the absorbance of the chloroplasts is minimum and hence the light scattering does not interfere too much in the measurements of the transmitted light.

Here we investigated the reliability of the oxonol VI ΔA to monitor the membrane potential as proposed [10,11]. Then we used oxonol VI to demonstrate a change in the membrane potential ($\Delta\psi$) during ATP hydrolysis by chloroplasts in complete darkness.

2. Materials and methods

Chloroplasts, able to hydrolyse ATP in the darkness, were prepared as in [4]. In brief, freshly harvested spinach leaves were illuminated for 30 min in ice-cold water. Chloroplasts were rapidly isolated using a standard technique [12] in 0.4 M sucrose, 0.05 M tricine, 0.01 M NaCl, at pH 7.8, then washed once in 0.4 M sucrose and finally resuspended in 0.4 M sucrose.

The absorption spectra of chloroplasts in the presence or absence of oxonol were recorded on a double-beam spectrophotometer Perkin Elmer 356, used in the split-beam mode with the same reference (cuvette with buffer). The spectra were averaged by adding the signals of 5–10 different expt under identical conditions. Difference absorption spectra were obtained by subtracting two spectra differing in only one experimental parameter (i.e., presence of a chemical). ΔA_{515} or ΔA_{580} were measured using a laboratory-made spectrometer [13]. Two xenon flashlamps were fired simultaneously or alternately at fixed rates.

All reactions were run in the following medium: 40 mM tricine, 5 mM $MgCO_3$, 20 mM $KHCO_3$, at pH 8. ATP synthesis was measured by following the rate of incorporation of ^{32}p from $^{32}P_i$ into ATP. Flash-

Elsevier/North-Holland Biomedical Press

induced ATP synthesis was determined by the bioluminescent firefly luciferin–luciferase reaction.

3. Results and discussion

3.1. *Oxonol and light-induced membrane potential*

Interaction of oxonol VI with chloroplasts produces a red shift of its absorption band which is further increased by illumination (fig. 1). We characterize the red shift by the maximum amplitude of the ΔA_C (fig. 1).

Flash-induced ΔA_{600} were measured. In the absence of oxonol the changes are of small amplitude. With oxonol added, much larger signals are observed and their difference spectrum has the same shape as the difference spectra of fig. 1. Kinetics of ΔA_{580} are shown in fig. 2. It seems the ΔA_{580} are influenced by the different compounds in the same way as ΔA_{515}: methylamine is completely inactive, valinomycin + K^+ or ATP decrease the magnitude and accelerate the dark decay of the signal.

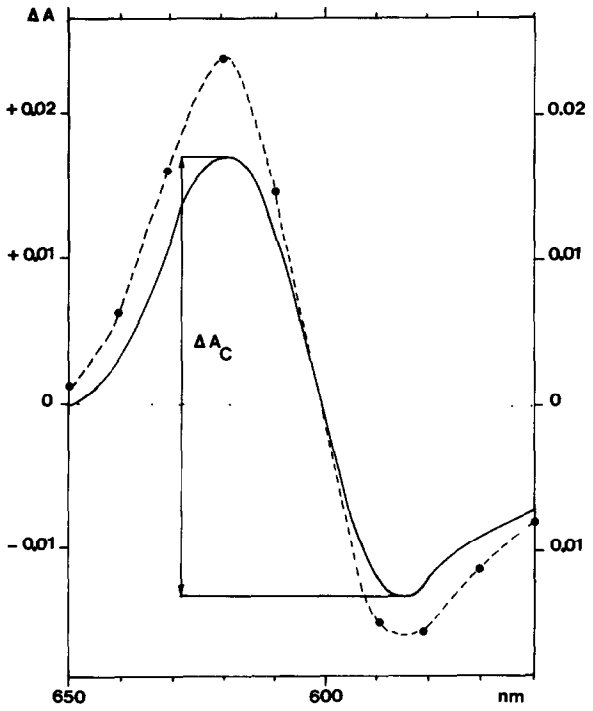

Fig. 1. Absorbance changes of oxonol VI after interaction with chloroplasts. Chloroplasts were suspended in the normal medium at 20 μg equiv. chl/ml. Oxonol VI was 2.45 μM and temp. 10°C. (——) Difference between two difference absorption spectra [(oxonol with chloroplasts – chloroplasts) – (oxonol in buffer – buffer)] in the dark. (– –) Difference as precedently but in the light. Spectrum was deduced from the ΔA (light – dark) observed with the chloroplasts suspensions at the different wavelengths marked by (•).

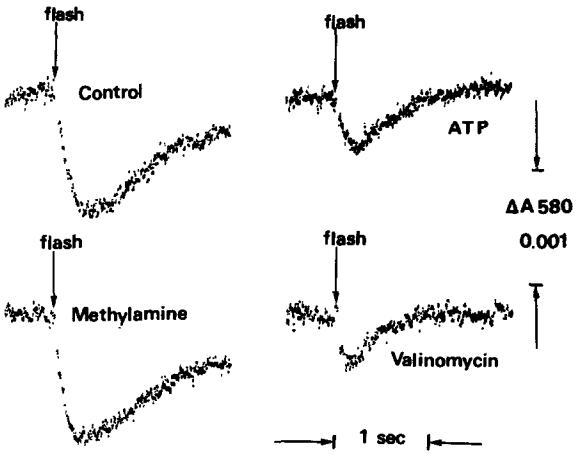

Fig.2. ΔA_{580} induced by a train of flashes in chloroplast suspensions. Conditions as in fig. 1 but with 10 μM PMS (phenazine methosulfate). When present, methylamine was 10 mM, ATP 200 μM and valinomycin 25 nM. The signal is the average effect of 40 trains of 5 flashes (1 flash each 3 ms) given every 8 s.

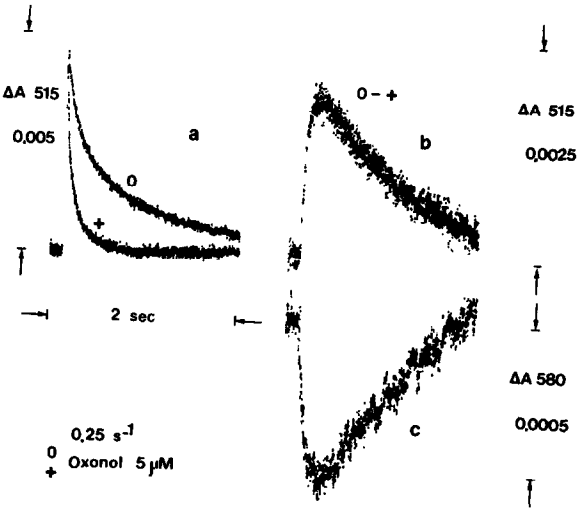

Fig.3. ΔA_{515} and ΔA_{580} induced by a single turnover flash. Conditions were as in fig. 2 but oxonol VI, when present, was 5 μM. ΔA_{515} and ΔA_{580} are the average effects of, respectively, 100 and 200 single flashes given every 4 s. (a) ΔA_{515} measured in the absence (0) or in the presence (+) of oxonol. (b) Difference of ΔA_{515} without (0) minus with (+) oxonol. (c) ΔA_{580}.

Table 1
Photophosphorylations in chloroplast suspension under flashing or continuous light in the presence of variable concentrations of oxonol VI

Oxonol VI (μM)	Light flash repetition: 0.07 s^{-1} (ATP . 1000 chl^{-1} . train of flashes^{-1})	Continuous light (μmol ATP . h^{-1} mg chl^{-1})
0	1.2	170
0.6	1	179
1.25	0.43	174
2.5	0.35	167

Flash-induced phosphorylations: Chloroplasts 20μg at equiv. chl were added to 1 ml basic medium with 0.002 mM ADP, 2 mM P_i, 100 μM methylviologen and 0.2 ml luciferin–luciferase mixture (prepared by dissolving 1 vial ATP monitoring reagent LKB in 10 ml distillated water); Temp. 5°C. A train of 5 flashes (1 flash each 3 ms) was given at a repetition rate of 0.07 s^{-1}. Continuous light phosphorylation: conditions as above but ADP was 2 mM and P_i 5 mM; suspensions were illuminated for 1 min in white light of saturating intensity

The rise of the flash-induced ΔA_{580} is much slower than that of ΔA_{515}, in agreement with [9]. We also found that oxonol accelerates the dark decay of the carotenoid shift (fig. 3), a property which has been observed in bacterial chromatophores [8]. The ΔA_{580} exhibits roughly the same shape as the difference in ΔA_{515} observed without and with oxonol, as a function of the time after the flash (fig. 3).

The results are satisfactorily explained by the proposal [8] that the dye, being a lipo-soluble anion, distributes across the membrane according to the membrane potential, which is positive inside, with a concomitant dissipation of the potential. A confirmation of such an electro-impelled transfer of the dye is the inhibition by oxonol of the flash-induced synthesis of ATP, without inhibition of photophosphorylation in continuous light (table 1), as observed in the presence of valinomycin + K^+ [14]. Oxonol is a permeant anion, valinomycin + K^+ a permeant cation complex, and both accelerate the decay of the membrane potential.

We thus consider that, in the presence of oxonol VI, the ΔA_{580} measures the increase in oxonol concentration inside the thylakoids caused by an electro-impelled transfer. As the equilibration of the dye through the membrane in response to the membrane potential is slow, the kinetics of the ΔA_{580} are different to those of the potential changes supposedly probed by the ΔA_{515}. These properties of oxonol VI, although precluding its use as probe of fast changes of the membrane potential, confirm the usefulness of the dye to check slow changes of this potential.

3.2. *Formation of a membrane potential during hydrolysis of ATP*

When added to the chloroplast suspension, ATP substantially increases the red shift of the absorption band of oxonol VI. The addition of sulfocyanide partially reverses the effect of ATP (fig. 4). A part of the change observed after addition of ATP is not reversed by sulfocyanide and is therefore independent of any transmembrane potential or of the charges at the interfaces of the thylakoids. This type of change is equally induced by ATP, ADP or AMP and does not require the presence of Mg^{2+} (expt 1,6,7, table 2).

The part of the ΔA reversed by addition of sulfocyanide is observed only in the presence of Mg^{2+} (expt 1, table 2) and is not observed in the presence of ADP or AMP (expt 7, table 2). The magnitude of this change is decreased or suppressed by treatments which are known to inhibit the ATPase activity of the coupling factor in situ [4]:

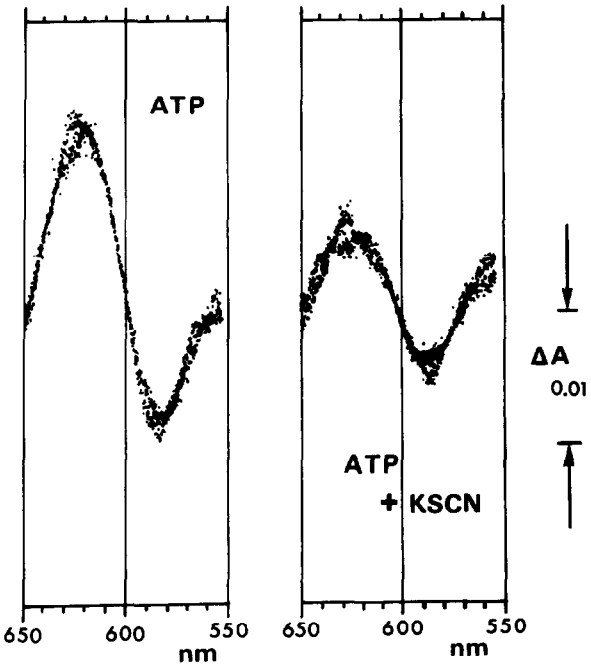

Fig.4. Absorbance changes of oxonol in chloroplasts suspensions after addition of ATP. Conditions were as in fig. 1. When present, ATP was 20 μM and KSCN 10 mM. Difference absorption spectra were the differences between two recorded spectra: left curve, with (ATP) – (control without ATP); right curve, with (ATP +KSCN) – (control).

Table 2
ΔA_C of oxonol VI caused by nucleotide addition in chloroplast suspension

Expt	Additions	$\Delta A_C \times 10^3$
1	20 μM ATP + Mg^{2+}	9 (4)
	20 μM ATP without Mg^{2+}	5 (0)
2	20 μM ATP	8 (4)
	Valinomycin then 20 μM ATP	4
	20 μM ATP then valinomycin	5
3	20 μM ATP	8 (4)
	Methylamine then 20 μM ATP	4
4	20 μM ATP	9.6
	20 μM ATP then methylamine	11
5	30 μM ATP	9 (4.5)
	30 μM ATP + 100 μM ADP	6.5 (1.9)
6	20 μM ADP	3 (0)
	20 μM ADP without Mg^{2+}	3 (0)
7	20 μM ADP	5 (0)
	20 μM AMP	4 (0)

Conditions were as in fig. 1 but, when indicated, Mg^{2+} was omitted, 10 mM methylamine or 10 mM KSCN were present. ΔA_C was measured as in fig. 5. Numbers in brackets represent the part of ΔA_C which was reversed by addition of KSCN

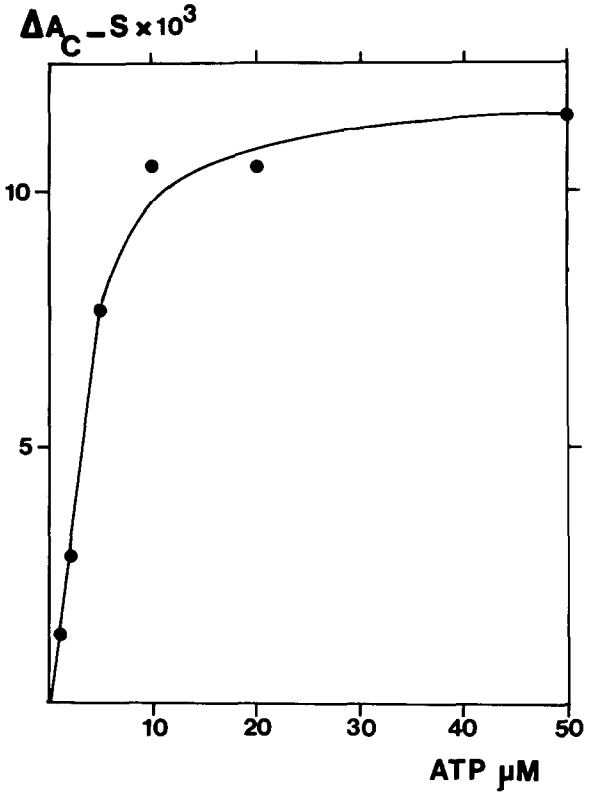

Fig.5. Sulfocyanide-sensitive ΔA_C of oxonol as a function of [ATP]. Conditions were as in fig. 4 but $MgCO_3$ was 10 mM. The maximum amplitude, ΔA_C, of the difference spectra in fig. 4 was determined as shown in fig. 1. The part of ΔA_C sensitive to KSCN, is plotted as a function of [ATP].

(i) By addition of methylamine before supplying ATP to the chloroplasts, in which case the ATPase becomes inactive (expt 2, table 2);
(ii) By omission of Mg^{2+}, on which ATPase activity is dependent (expt 1, table 2);
(iii) By the concomitant addition of ADP which inhibits the hydrolysis of ATP (expt 5, table 2).

If this sulfocyanide-sensitive ΔA depends on the ATPase activity, it is maximum at 10 μM ATP (fig. 5), 10 times lower than the 100 μM required to observe the maximum ΔpH caused by hydrolysis of ATP [4]. This result and the insensitivity to methylamine (expt 4, table 2), when the amine, added after ATP, collapses the ΔpH, show that this ΔA is not directly linked to the extent of the ΔpH formed. The fact that valinomycin + K^+ has the same effect as sulfocyanide (expt 2, table 2) supports the view that this sulfocyanide-sensitive ΔA results from the action of a membrane potential.

4. Conclusion

With chloroplasts in which the ATPase is active, hydrolysis of ATP causes the same type of oxonol VI ΔA as illumination, with the same sensitivity to the chemical tested. In the two cases the changes are not dependent on the extent of the ΔpH and the results are consistent with an inward electro-impelled transfer of the anion through the membrane. We conclude that energization of the thylakoid membrane by hydrolysis of ATP leads to the formation of a membrane potential, inside positive, as that was observed by illumination.

Acknowledgement

We are grateful to Dr C. L. Bashford for his kind gift of oxonol VI.

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