LIGHT-ACTIVATED ATPase AND ATP-DRIVEN REVERSE ELECTRON TRANSPORT IN INTACT CHLOROPLASTS

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1. Introduction

Following light-activation the latent ATPase of broken chloroplasts can hydrolyse ATP at high rates [1–3], and drive energy-dependent processes like proton uptake [4–6], reverse electron transport [6–8], and stimulation of post-illumination chlorophyll luminescence [9,10].

To observe ATP hydrolysis and reverse coupling reactions in broken chloroplasts, it is necessary to preilluminate chloroplasts with intense light in the presence of a thiol compound such as dithiothreitol (DTT) [1–10].

Here, I show that light-activated ATPase activity occurs also in intact chloroplasts without any addition of DTT. ATP hydrolysis and reverse coupling reactions are initiated by the addition of dihydroxyacetonephosphate (DHAP) which passes the chloroplast envelope via the phosphate translocator [11–13] and thus induces a rapid increase of the internal [ATP] [14,15].

2. Materials and methods

Intact chloroplasts were isolated from freshly harvested spinach according to [16] as modified [17]. No cysteine or ascorbate were added to the isolation medium. The yield of intact chloroplasts was estimated by the ferricyanide method [18] and was routinely 85–95%. Storage times at 0°C were ⩽3 h, at which time ATP- or DHAP-induced responses were decreased by ~25%. The standard reaction mixture contained: 0.33 M sorbitol, 40 mM Hepes/KOH buffer (pH 7.8), 10 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM KH_2PO_4 and chloroplasts at ~25 μg chl/ml. Envelope-free chloroplasts were obtained from intact chloroplasts by a brief (5 s) osmotic shock of 10 μl chloroplasts (at ~2 mg chl/ml in resuspension medium) in 350 μl water followed by addition of 360 μl double strength medium. To activate the ATPase the chloroplast suspension was illuminated for 3 min with heat-filtered white light from a tungsten–halogen lamp (~300 W/m²). The reaction mixture was continuously stirred and maintained at a constant temperature of 15°C. The measuring system and the methodology employed were as in [6,8–10] with variants as indicated in the figure legends. DHAP was obtained by hydrolysis of the dimethylketal, purchased from Sigma. Ketal (25 mg) dissolved in 2 ml water, was treated for 1 min with 1 g Dowex ion-exchange resin, and the filtrate was hydrolysed for 3 h at 38°C. ATP was purchased in form of the Mg-salt from Sigma. The ATP and the DHAP solutions were adjusted to pH 7.8 with KOH.

Abbreviations: 9-AA, 9-aminoacridine; PSII, photosystem II; Q, primary electron acceptor of PSII and quencher of chlorophyll fluorescence; DTT, dithiothreitol; PMS, phenazonium-methosulfate; DHAP, dihydroxyacetonephosphate; DCCD, dicyclohexylcarbodiimide; chl, chlorophyll

3. Results and discussion

In fig.1 intact chloroplasts were illuminated for 3 min with intense white light before being shocked in water and resuspended in isotonic medium. No cofactors for ATPase activation as DTT or PMS [1–10] were added. Following 3 min dark adaptation, ATP was injected and induction of the reverse reactions was recorded. Upon ATP addition the following changes were observed:

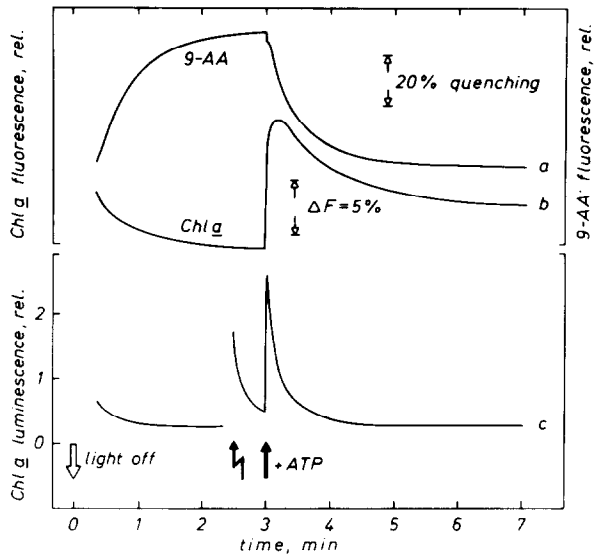

Fig.1. ATP-induced reverse coupling reactions in envelope-free chloroplasts. (a) Quenching of 9-AA (3×10^{-6} M) fluorescence. (b) Increase of chl *a* fluorescence. Fluorescence is monitored by a weak (1 mW/m²) 480 nm measuring beam, which by itself does not cause significant Q-reduction. (c) Stimulation of post-illumination chl *a* luminescence. Luminescence is triggered by two saturating, single turn-over Xe flashes given at 1 s interval 30 s before ATP-addition. Chloroplasts were light-activated while still intact, without any additions, then rapidly shocked in water and resuspended in isotonic medium. The time scale refers to the dark-time following a 3 min pre-illumination period. Monitoring was initiated within ~20 s after the activating light was turned off. The initial traces represent the relaxation of the chloroplasts into the dark-adapted state, i.e., decay of the proton gradient, reoxidation of Q and decay of post-illumination luminescence.

(a) ~50% quenching of 9-AA fluorescence indicating the build-up of a proton concentration gradient across the thylakoid membrane;
(b) A rapid increase of chl *a* fluorescence, reflecting partial reduction of Q by reverse electron flow;
(c) Rapid stimulation of post-illumination chl *a* luminescence, indicating an increased rate of charge recombination at PSII reaction centres.

These ATP-induced responses are practically identical to the corresponding responses in thylakoid preparations activated in presence of DTT [6,8–10]. Hence, light activation of the ATPase takes place in the intact chloroplast, and does not require addition of DTT.

In order to induce ATP hydrolysis and coupled reverse reactions in the unbroken intact chloroplast, the [ATP] within the stroma has to be increased. In this case external addition of ATP is ineffective, as the envelope of intact chloroplasts permits passage of ATP at only rather low rates [19,20]. A rapid increase of internal [ATP] is, however, possible upon external addition of dihydroxyacetonephosphate (DHAP). Passage of DHAP across the envelope occurs via the phosphate translocator [13], and within the stroma conversion of DHAP to 3-phosphoglycerate is coupled with the phosphorylation of ADP to ATP [15,21]. As shown in fig.2, addition of DHAP to light-activated intact chloroplasts does indeed result in induction of reverse reactions:

(a) Following a lag period there is ~10% quenching of 9-AA fluorescence;
(b) There is a biphasic reduction of the PSH acceptor Q;
(c) Post-illumination luminescence is rapidly stimulated.

These DHAP-induced responses are qualitatively similar to the ATP-induced responses in fig.1. The DHAP-induced reduction of Q is more pronounced than the ATP-induced response in broken chloroplasts (compare traces (b) in fig.1,2). This difference may be due to the lack of reducing equivalents in the broken chloroplasts as compared to the intact system, since upon rupture of the envelope the endogenous reductants are diluted in the medium. For further comparison, table 1 summarizes the

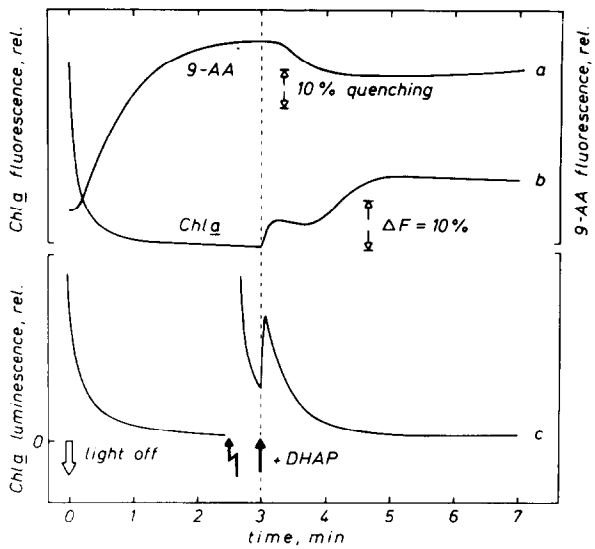

Fig.2. DHAP-induced reverse coupling reactions in intact chloroplasts. DHAP was added to 2×10^{-4} M final conc. Other conditions as in fig.1, except that shocking was omitted.

Table 1
DHAP- and ATP-stimulation of post-illumination chl *a* luminescence in relation to conditions of intactness, light activation and presence of DTT

Intact during activation	Intact during reaction	Light-activated	DTT present	Type of addition	Relative response
+	+	+	+	DHAP	100
+	+	+	–	DHAP	54
/	+	–	–	DHAP	0
+	–	+	+	DHAP	0
+	+	+	+	ATP	33
+	–	+	–	ATP	425
–	–	+	+	ATP	240
–	–	+	–	ATP	12
+	+	+	–	ATP	6
/	–	–	+	ATP	8

Intact chloroplasts displayed, according to the ferricyanide method [18], an intactness of 89%. DTT, when added, was present at 5 mM. DHAP as well as ATP were applied at 10^{-4} M

extent of DHAP- and ATP-induced reactions, as measured by the relative stimulation of post-illumination chl *a* luminescence, for a variety of conditions. The following points may be noted:

(i) Although presence of DTT is not obligatory for activation of the ATPase in intact chloroplasts, it does enhance the reaction;
(ii) The DHAP- and the ATP-induced responses are dependent on preceding light activation;
(iii) For the DHAP response an intact outer membrane is essential;
(iv) Light activation of broken chloroplasts does require addition of DTT;
(v) Light activation of intact chloroplasts in the absence of DTT appears superior to light activation of envelope-free chloroplasts in the presence of DTT;
(vi) ATP does not pass the envelope in appreciable amounts.

The ATP response of 'intact chloroplasts' in presence of DTT reflects the amount of envelope-free chloroplasts in the preparation. Comparison with the corresponding response of 100% broken chloroplasts could be used to estimate the percentage of intact chloroplasts in the preparation (86% in the above experiment).

The DHAP-induced as well as the ATP-induced reverse reactions were measured with variable amounts of added substrate. Half-maximal responses were induced by 4×10^{-5} M DHAP and 10^{-5} M ATP, as determined from the relative stimulation of chl *a* luminescence.

A preliminary survey of inhibitor sensitivity of the DHAP reaction (stimulation of post-illumination luminescence by 10^{-4} M DHAP) yielded the following results:

(1) There is inhibition by energy transfer inhibitors of the ATPase. 50% inhibition is found with 10^{-4} M DCCD or with 10^{-3} M phlorizin.
(2) The uncoupler desaspidin inhibits by 70% at 10^{-8} M. NH_4Cl inhibits by 50% at 2×10^{-4} M.
(3) H_2O_2 which oxidizes sulfhydryl groups, inhibits by 50% at 3×10^{-5} M.
(4) Phosphate inhibits by 50% at 1.5 mM, presumably by affecting the transport of DHAP across the envelope [14,21].
(5) Glycerate and 3-phosphoglycerate inhibit by 50% at 3×10^{-4} M and 5×10^{-5} M, respectively.

These substances are known to enter the intact chloroplast rapidly and to consume ATP [15,21]. Furthermore 3-phosphoglycerate also competes with DHAP for transport via the phosphate-translocator [13] and also interferes with DHAP oxidation [21].

From these findings and from the data presented in fig.2 and in table 1 one may conclude that also in intact chloroplasts, under conditions resembling those in vivo, light-activated ATPase catalyses ATP hydrolysis and coupled reverse reactions in the dark.

In [15] ATP-hydrolysis in illuminated chloroplasts was observed to rapidly decline the dark ($t_{1/2} \sim 2$ s). In the above experiments clear ATPase responses were found 3 min after cessation of light activation. Plots of DHAP- and ATP-induced responses (relative stimulation of post-illumination luminescence) versus dark-times >3 min are shown in fig.3. With intact chloroplasts and DHAP addition a half-maximal response is found after 7 min dark-time. With broken chloroplasts and ATP-addition half-maximal response is found after 5.5 min dark-time.

The chloroplasts in [15], which showed rapid decay of ATP-hydrolysis in the dark, were qualitatively very similar to the chloroplasts used here. The apparent discrepancy in the results, therefore, may be due to difference in the reaction conditions. In the DHAP-induced reaction there is sustained production and hydrolysis of ATP. Possibly only an increase in the ATP/ADP ratio beyond some threshold level will result in ATP hydrolysis.

These results suggest that intact chloroplasts con-

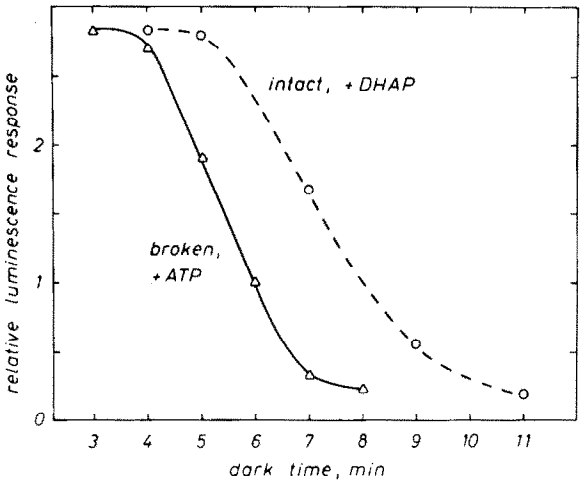

Fig.3. Dependence of ATP- and DHAP-stimulated chl *a* luminescence on dark-time following light activation. The amplitude of the stimulated luminescence peak is taken as measure of ATPase activity. Conditions for ATP- and DHAP-induced reactions as in fig.1 and 2, respectively.

tain a substance exhibiting similar properties to DTT. The ferredoxin–thioredoxin system or glutathione [22] may be proper candidates. No ATP hydrolysis was observed without preceding light activation. This finding is in contrast to the report [23] claiming that class I chloroplasts show considerable ATPase activity without pre-illumination. Rapid passage of ATP through the envelope was also found [23], while here, in general agreement with [18–20], no substantial ATP influx was detected.

A meaningful role for chloroplast ATPase activity in vivo might be at the level of regulation of the overall photosynthetic process. The ATP/ADP ratio in intact chloroplasts [24] as well as in intact cells [25] is surprisingly constant. This suggests some regulatory mechanism, which could include both the adenylate kinase reaction [26] and the ATPase reaction.

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