Single-turnover flash-induced ATP synthesis in photosystem I-enriched subchloroplast vesicles

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Flash-induced ATP synthesis coupled to cyclic electron flow in photosystem I-enriched subchloroplast vesicles was studied by continuous registration of luciferin-luciferase luminescence. Cyclic electron flow was elicited in the presence of the artificial mediator phenazine methosulphate or of native ferredoxin, the latter redox-poised by NADPH and oxygen. ATP hydrolysis is observed in dark-adapted vesicles in the presence of ferredoxin without requirement for previous activation of the ATPases. Ferredoxin-catalyzed ATP synthesis proceeds at a constant (maximal) rate between NADPH/O_2 ratios of 1.5 and 11. It was sensitive to FCCP, DCCD, DBMIB, DNP-INT and HQNO, but not to antimycin A.

ATP synthesis *Single turnover* *Photosystem I* *Subchloroplast vesicle* *(Spinach)* *Ferredoxin* *Redox balance*

1. INTRODUCTION

PS I-enriched vesicles derived from spinach chloroplasts by mild digitonin treatment [1] contain all the natural redox components necessary for cyclic electron transfer [2–5] and photophosphorylation [3], except ferredoxin. Light-induced steady-state phosphorylation can be elicited in these vesicles after addition of the natural mediator ferredoxin in the presence of NADPH and O_2 (to provide an appropriate redox balance) [3–5] as well as after addition of the artificial mediator PMS [1]. Under non-phosphorylating conditions, a clear steady-state light-induced proton uptake was observed, in the presence of either PMS [1], ferredoxin and NADPH (cyclic electron transfer), or duroquinol and methyl viologen (linear electron transfer) (Krab, K., Hotting, E.J. and De Wolf, F.A., unpublished). Here, we report on ATP synthesis in these vesicles under single-turnover conditions, and ATP hydrolysis, continuously monitored by luciferin-luciferase luminescence [6–10].

Abbreviations: Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP-INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; PMS, *N*′-methylphenazonium methosulphate; PS, photosystem

2. MATERIALS AND METHODS

PS I vesicles were isolated from market spinach according to [11], stored under liquid nitrogen and diluted to 50 (single-turnover experiments) or 5 (steady-state experiments) (μg Chl)·ml^{-1} in a medium containing 10 mM $KHCO_3$, 2 mM K_2HPO_4, 40 mM Tes-KOH buffer, pH 8.0, 38 μM ADP, 4 μM diadenosyl pentaphosphate and PMS or ferredoxin and NADPH as indicated, at 10°C. Steady-state actinic light was passed through a Calflex KG3, a K6 (Balzers, Liechtenstein) and 2 RG630 (Schott, FRG) filters. Further conditions were as in [7]. DNP-INT was a gift from Professor A. Trebst (Ruhr-Universität, Bochum) and nigericin from the Lilly Laboratories (Indianapolis,

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IN). DBMIB was obtained from the CEN-Saclay (Gif-sur-Yvette).

3. RESULTS

In agreement with earlier results [1,2,4], we observed a higher steady-state phosphorylation rate in PS I vesicles in the presence of PMS (550 nmol ATP · min^{-1} · $(mg\ Chl)^{-1}$) than in the presence of ferredoxin (150–300 nmol · min^{-1} · $(mg\ Chl)^{-1}$) (not shown). Flash-induced PMS-catalyzed ATP synthesis could only be established with groups of 6 flashes (fig.1a,b), but not with single flashes (frequencies up to 1 Hz). There was a lag in the onset of ATP synthesis which was longer at 10 μM PMS (trace a) than at 50 μM PMS (trace b). This lag was absent in subsequent flash trains, even after 7–15 min of darkness. After the lag period, the ATP yield per flash group depends on the PMS concentration: it was 12 pmol · $(mg\ Chl)^{-1}$ at 10 μM PMS and 58 pmol · $(mg\ Chl)^{-1}$ at 50 μM PMS (i.e. 1.0 μmol PMS · $(mg\ Chl)^{-1}$), and was not further enhanced by additional PMS (up to 100 μM). Under steady-state conditions, optimal phosphorylation was observed at 1.0 μmol PMS · $(mg\ Chl)^{-1}$, in agreement with [1] (not shown).

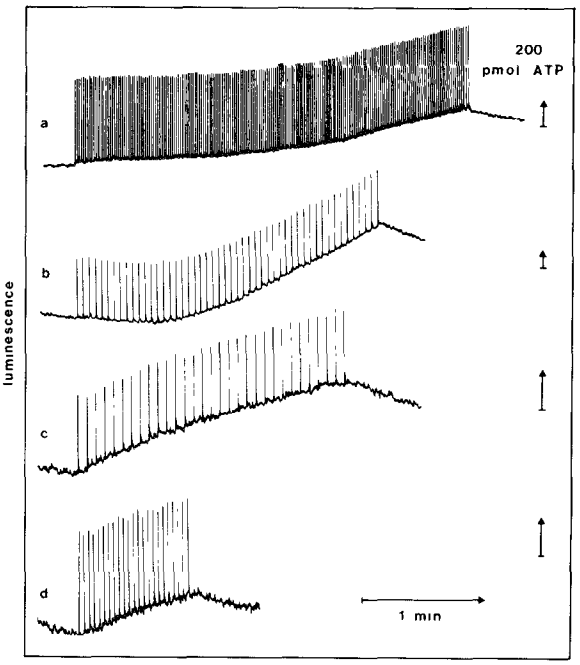

Fig.1. Flash-induced ATP synthesis in PS I vesicles monitored by luciferin-luciferase luminescence. The vertical arrows correspond to 200 pmol ATP. The spikes represent transient artefacts. (a–c) Groups of 6 flashes (within the groups the flashes were separated by 3-ms intervals), (d) single flashes. Additions: (a) 10 μM PMS, (b) 50 μM PMS, (c,d) 5 μM ferredoxin and 2 mM NADPH. Oxygen concentration was 350 μM. Further conditions as in section 2.

In the presence of the natural mediator ferredoxin, a prominent flash-induced ATP synthesis was also observed (fig.1c,d). In this case, single flashes at low flash frequency were sufficient; for instance, 66 pmol ATP · $flash^{-1}$ · $(mg\ Chl)^{-1}$ at 0.1 Hz or 86 pmol ATP · $flash^{-1}$ · $(mg\ Chl)^{-1}$ at 0.15 Hz. In different experiments at flash frequencies around 0.4 Hz, the ATP yields varied from about 20 to 100 pmol · $flash^{-1}$ · $(mg\ Chl)^{-1}$. In freshly prepared (not frozen) vesicles, a yield of 75 pmol ATP · $flash^{-1}$ · $(mg\ Chl)^{-1}$ was obtained with single flashes at 0.04 Hz. Significantly, in contrast to what has been observed in chloroplasts [6–8], there was no lag phase in flash-induced ATP synthesis, regardless of preceding dark periods. Apparently, membrane energization and/or ATPase activation were not limiting factors for initial ATP synthesis under these conditions.

Even in vesicles that had been kept in the dark during storage as well as during the preparation of the experiment, ATP hydrolysis occurred before flashing, in the presence of ferredoxin, showing that the ATPases were already in an activated state. Modulation by thiol agents or light activation [12] was not necessary. Some dark ATP hydrolysis occurred in the absence of ferredoxin above 1 μM ATP, at 50 (μg Chl) · ml^{-1}. This hydrolysis was increased 3–4-times by addition of 5 μM ferredoxin in the dark; NADPH had no influence. After several minutes of darkness, flashing caused a (variable) increase of ATP hydrolysis (typically from about 50–100 to 1000–2000 pmol · min^{-1} · $(mg\ Chl)^{-1}$), which relaxed again in the course of 5–7 min after the flashes. Also an increase of flash frequency enhanced this rate.

Net single-turnover ATP synthesis was maximal at 2.5–10 μM ferredoxin (at 50 (μg Chl) · ml^{-1}), roughly in agreement with steady-state experiments [3]. However, a distinct phosphorylation optimum at $NADPH/O_2$ ratio of 5, as found under steady-state conditions [3], was not observed here (fig.2): between $NADPH/O_2$ ratios of 1.5 and

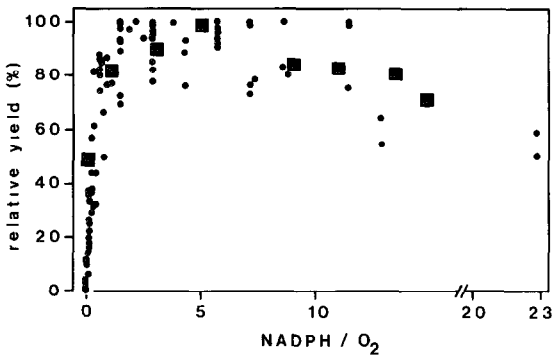

Fig.2. Relative ATP yield per flash as a function of NADPH concentration. Ferredoxin concentration was 5 μM; oxygen concentration was 350 μM (●) or 50 μM (■). Single flashes or groups of 3 flashes (within the groups the flashes were separated by 50-ms intervals). Further conditions as in section 2. The 100% yields varied between 10 and 150 pmol ATP · (flash)$^{-1}$ · (mg Chl)$^{-1}$.

Table 1

The influence of uncoupler and inhibitors of energy transfer and cyclic electron flow on flash-induced ATP synthesis in PS I vesicles

Addition	Concentration (μM)	Relative ATP yield (%) when applying	
		Groups of 3 flashes	Single flashes
None		100	100
FCCP	0.10	83	49
	0.25	24	0
	1.00	0	0
DCCD	40	62	55
DBMIB	1.0	49	52
DNP-INT	10.0	n.d.	38
HQNO	10.0 (2 experiments)	63, 87	50, 75
Antimycin A	1.0–10.0	100	100

Ferredoxin and NADPH were present at 5 μM and 1–2 mM, respectively. Oxygen concentration was 350 μM. Flash (flash group) frequency was 0.4 Hz. Within the flash groups the flashes were separated by 50-ms intervals. Further conditions were as in section 2. The 100% yields varied between 25 and 100 pmol ATP · (flash)$^{-1}$ · (mg Chl)$^{-1}$. n.d., not determined

11 (at 350 μM O_2) the observed yield per flash was more or less constant. At 50 μM O_2, 60% activity was still found at an NADPH/O_2 ratio of 40 and activity was only abolished at a ratio of 90 (not shown, same experiment as in fig.2). Previous studies [3,5] showed that single-turnover cytochrome redox changes and membrane potential formation were still normal at an NADPH/O_2 ratio of 1.3, while steady-state phosphorylation was already abolished. Apparently, single-turnover ATP synthesis does not require such an accurate redox poise as steady-state ATP synthesis.

In agreement with earlier observations on ATPase inhibition [13], 40 μM DCCD inhibited 40–50% (table 1). ATP synthesis was completely abolished at 500 μM DCCD, presumably due to inhibition of the cytochrome b_6/f complex [14]. Table 1 also shows that FCCP, DBMIB, DNP-INT and HQNO inhibit in accordance with their effect on steady-state phosphorylation and flash-induced electron transfer [3,5]. None of these inhibitors induced a lag in the onset of phosphorylation. In contrast to what is usually observed in chloroplasts [15,16], antimycin A (up to 10 μM) had no effect at all on cyclic photophosphorylation in these vesicles.

4. DISCUSSION

The present results confirm that these vesicles are well coupled [3]. Both the occurrence of PMS-catalyzed phosphorylation and the uncoupler-sensitive phosphorylation in the presence of ferredoxin may be explained by a proton-mediated mechanism of energy transduction in these vesicles. In the case of ferredoxin-catalyzed phosphorylation, proton translocation may occur coupled to electron transfer through the cytochrome b_6/f complex [4,5]. However, it has not been possible to demonstrate bulk proton uptake by PS I vesicles under single-turnover conditions (detection limit: 1–4 protons per vesicle) [11].

One possible explanation for this discrepancy is that in these vesicles, energy transduction does not involve the bulk phases. This could also explain the immediate onset of ferredoxin-mediated phosphorylation, since the (relatively slow) formation of a bulk proton gradient would not be essential.

Alternatively, the absence of flash-induced proton uptake [11] and the immediate onset of ferred-

oxin-catalyzed photophosphorylation could be explained by the small size of these vesicles (diameter 50–100 nm [1,2]). The observed ATP yields correspond to about 0.02–0.87 ATP molecules per vesicle (0.005–0.023 per reaction centre). A single free proton per vesicle (about 0.03–0.15 per reaction centre) corresponds to a pH_{in} of about 4.2–5.4. Depending on internal buffer capacity, a proton gradient sufficient to drive the observed ATP synthesis could be generated with only a few protons and in a very short time. The artificial mediator PMS presumably drives phosphorylation by bulk phase proton translocation [17]. The lag observed for PMS-mediated phosphorylation (only in the first flash train) could then either reflect the time needed (i) to reach a threshold proton gradient (cf. [9,10]), (ii) to activate the ATPases [7,12], or (iii) to establish the appropriate redox poise of the PMS system for electron transfer. The absence of a lag in the ferredoxin system (poised by NADPH and oxygen) and in subsequent flash trains in the PMS system points to the latter 2 possibilities.

The ineffectiveness of antimycin A in these vesicles agrees with observations on NADPH-dependent CO_2 fixation in C_3 chloroplasts in the presence of DCMU, and in C4 chloroplasts, which lack PS II activity [18]; in pea chloroplasts in the presence of DCMU [16], residual phosphorylation ($950\ nmol \cdot min^{-1} \cdot (mg\ Chl)^{-1} = 20\%$) was observed in the presence of antimycin. In these systems, cyclic electron transfer possibily proceeds via a special, antimycin-insensitive pathway [19]. Such a (ferredoxin-$NADP^+$ reductase-dependent) pathway has recently been demonstrated in spinach chloroplasts; it was only observed when ferredoxin was in a relatively oxidized state (in the presence of $NADP^+$) [20]. Thus, the absence of reducing power exerted by PS II is possibly responsible for the observed antimycin insensitivity (cf. [19] and discussion in [20]).

Thus, the present results show that these vesicles are interesting tools for mechanistic studies of photosynthetic energy transduction associated with cyclic electron flow around PS I.

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