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The antimycin sensitivity of flash-induced ATP synthesis in photosystem I-enriched subchloroplast vesicles

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Photosystem I-enriched vesicles, derived from spinach thylakoids by mild digitonin treatment, reconstituted with native ferredoxin and redox-poised with NADPH and oxygen, display antimycin-sensitive cyclic photophosphorylation. The effects of 1–30 μM antimycin point to electron transfer inhibition: (i) they are cancelled after addition of 10 μM pyocyanin and (ii) they resemble the effects of 1–5 μM DBMIB, but differ from those of FCCP, nigericin or valinomycin. Some vesicle preparations fail to show antimycin sensitivity in the absence of pyocyanin [(1985) FEBS Lett. 192, 271–274] and have apparently lost the antimycin-sensitive component.

Antimycin A; ATP synthesis; Cyclic electron transfer; Single turnover activation; Photosystem I; Subchloroplast vesicle

1. INTRODUCTION

Several studies have demonstrated the antimycin sensitivity of cyclic photophosphorylation and electrical potential formation [1–11], but only one paper [5] provided direct evidence for antimycin sensitivity of the light-induced redox changes of cytochromes *b*-563 and *c*-554 in chloroplasts. On the other hand, there are several instances in which antimycin appeared to have no effect at all in chloroplasts [10–14], or in which it had apparently no direct effect on the chloroplast cytochrome *bf* complex [13,14,16,17], in contrast to the mitochondrial situation [15]. High-affinity binding sites for antimycin (with an apparent dissociation constant of 4 nM) have been observed in chloroplasts [4,16]. However, they were not found on the isolated cytochrome *bf* complex, but in a membrane protein fraction from which the *bf* complex had been removed. Antimycin sensitivity has tentatively been associated with a postulated ferredoxin-plastoquinone oxidoreductase, involved in cyclic electron transfer around PS I [1,14,16,18]. Since antimycin can have uncoupling properties [1,2], it may not always be directly evident from data on photophosphorylation and electrical potential generation, whether antimycin affects electron transfer, indeed.

In phosphorylation experiments, reducing conditions appeared to enhance the effect of antimycin [3,10,11]. In agreement with this, antimycin appeared to be ineffective especially in situations where PS II activity, and the corresponding input of reducing equivalents to the cyclic electron transfer system, were absent [12]. It was proposed that the reduction level of ferredoxin was the most important factor in this respect [10,11], which corroborates the idea that antimycin acts on ferredoxin-dependent quinone reduction.

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Abbreviations: Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DNP-INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether; $\Delta\tilde{\mu}H^+$, transmembrane difference of electrochemical potential; FCCP, carbonyl-cyanide-*p*-trifluoromethoxy-phenylhydrazone; PS I or II, photosystem I or II

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We used PS I vesicles that contain the essential complexes for cyclic electron transfer, such as the cytochrome *bf* complex and plastocyanin, but lack PS II activity [18–20]. Native ferredoxin is reconstituted just before experimentation and the necessary redox poise is imposed by NADPH, in the presence of oxygen [8,21]. At optimal redox poise (i.e. at a molar NADPH/O_2 ratio of 3–5), antimycin A-sensitive cyclic phosphorylation was previously observed in these vesicles under steady-state conditions [8], but not under single-turnover conditions [21]. The present work reveals antimycin sensitivity also under single-turnover conditions. The results point to electron transfer inhibition rather than uncoupling.

2. MATERIALS AND METHODS

The vesicle isolation procedure, the reaction conditions and the chemicals were as described previously [21,22]. Vesicles were stored in liquid nitrogen. The reaction mixture contained 10 mM $KHCO_3$, 2 mM K_2HPO_4, 1.75 mM NADPH, 39 μM ADP, 5 μM diadenosyl pentaphosphate, 5 μM ferredoxin, 40 mM Tes-KOH buffer (pH 8.0) and 350 μM O_2. The temperature was 10°C. The inhibitors DBMIB and DNP-INT were kindly donated by Professor A. Trebst (Ruhr-Universität, Bochum, FRG). Antimycin A was purchased from Sigma (St. Louis, MO, USA). After addition of antimycin or DBMIB (in ethanol), 5 min of incubation were allowed before testing the effect. The ethanol, always below 1% (v/v), was checked to have no effect itself. Single-turnover flashes were fired: (i) in a train, at variable frequency, or (ii) in a train of small flash groups, each consisting of 2–6 (10–30 ms-spaced) flashes, at variable group frequency. Before calculation of the relative inhibition caused by antimycin, the flash-induced ATP synthesis was corrected for the dark ATP hydrolysis just before or just after the flash train.

Table 1

Influence of antimycin A on the flash-induced ATP yield at the onset of flashing

Group frequency (Hz)	Number of flashes per group	Relative ATP yield (%) at the following antimycin A concentrations (μM)			
		1	10	15	30
0.4	1	–	66	–	–
	6	–	52	–	–
0.7	1	–	57	46	12
1.4	1	71	52	–	–
	3	–	29	–	–
	6	–	18	–	–

The yields in the absence of antimycin (0.1–0.6 ATP per 10^3 Chl per group) were set to 100%. Flash groups were fired in a train at the indicated frequency and comprised the indicated number of (10 ms-spaced) flashes. Each train was preceded by 50 s darkness. Further conditions as in section 2

3. RESULTS

Table 1 shows that, in contrast to some previous PS I vesicle batches [21], the present batch was sensitive to 1–30 μM antimycin A, under single-turnover conditions and at an NADPH/O_2 ratio of 5. In the absence of inhibitor and in the beginning of the experiment, flash-induced ATP synthesis proceeded at a constant rate (figs 1–2a,b), as in previous studies [21,22]. After 30–60 min at 10°C, the ATP synthesis in the present vesicles started to

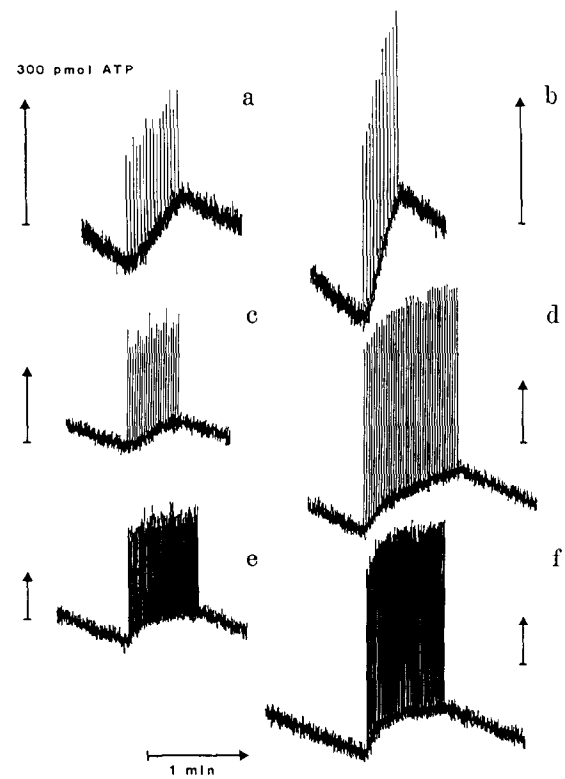

Fig.1. Flash-induced ATP synthesis monitored by luciferin/luciferase luminescence. All vertical arrows correspond to 300 pmol ATP. Each spike reflects a transient artifact due to one single flash or one flash group containing 6 (10 ms-spaced) flashes. a,c,e, single flashes; b,d,f, flash groups; a,b, freshly thawed vesicles; c–f, after 40 min at 10°C. The single flashes or flash groups were fired at: 0.47 Hz (a,b); 0.68 Hz (c,d); 1.56 Hz (e); or 1.24 Hz (f). Further conditions as in section 2.

decrease during the course of flashing (fig.1d–f). In trains of single flashes this was most apparent at flash frequencies above 2 Hz (cf. traces c and e of fig.1), but in trains of small flash groups, it was already observed at lower flash frequency (cf. traces d and f of fig.1). A similar pattern as in fig.1d–f could be induced immediately at the beginning of the experiment by addition of 10 μM antimycin A (cf. traces a–d in fig.2). Lower concentrations of antimycin were less effective. The decrease of the yield (fig.2c–f) appeared to be reversible: after a dark period, ATP synthesis proceeded initially again at a faster rate (fig.2e–f). This initial (flash-induced) rate (figs 1d–f, 2c–f) increased in concurrence with the length of the preceding dark period (fig.2e,f; table 2). Apparently, a reversible saturation or (contrarily) exhaustion occurred somewhere in the system. Once induced, the effect of antimycin (fig.2c–f) could be abolished again by subsequent addition of 10 μM pyocyanin, a mediator of artificial electron transfer between the reducing side of PS I and plastocyanin (fig.2g). Pyocyanin similarly abolished the effect of ageing, even after more than 90 min at 10°C, as shown in fig.2g. If pyocyanin was added before antimycin, the vesicles became completely insensitive to antimycin [this is not shown, but cf. fig.2a (control) with fig.2g (after addition of antimycin plus pyocyanin), which shows essentially the same behaviour]. In the presence of pyocyanin (or else at the beginning of the experiment, under the conditions of figs 1a,b and 2a,b), the ATP synthesis rate was observed to decrease only after about 5 min of continuous flashing, due to accumulation of ATP. In that case, however, subsequent hydrolysis in the dark was also enhanced (fig.2). After correction for this hydrolysis, the ATP synthesis after 7.5 min of

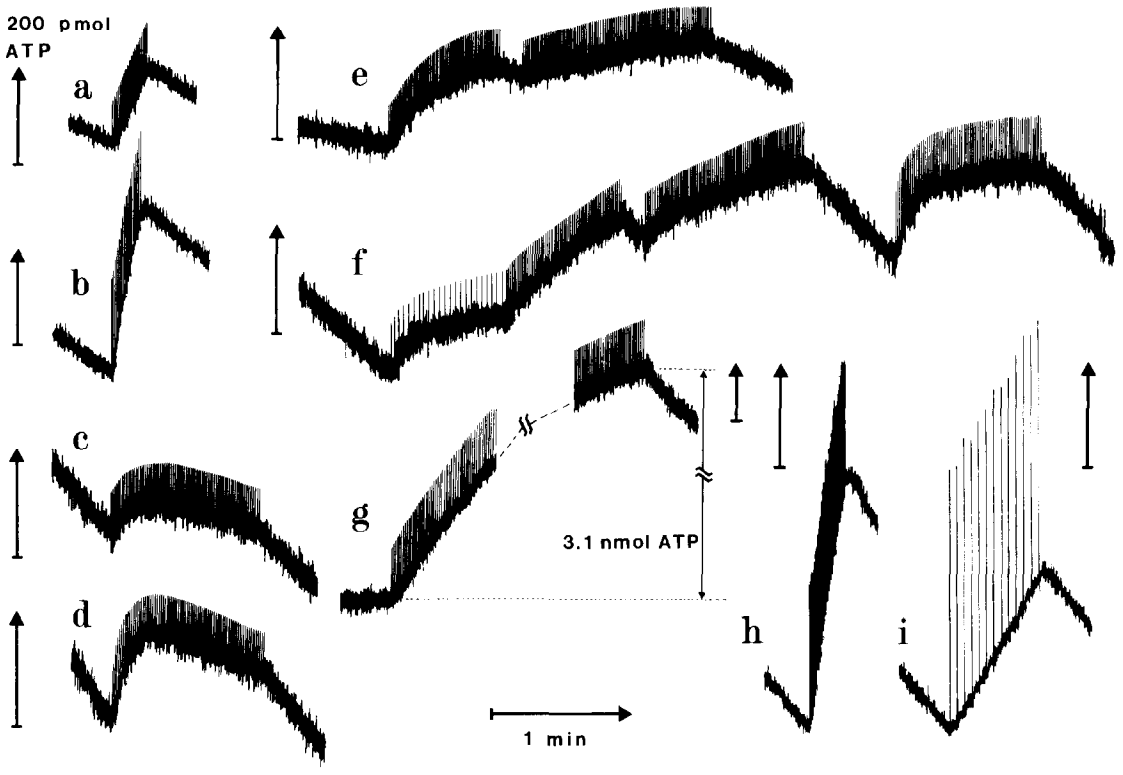

Fig.2. The effects of antimycin A and pyocyanin on flash-induced ATP synthesis. The vertical arrows correspond to 200 pmol ATP. a,c,e,g,h, single flashes; b,d,f,i, groups of 6 (10 ms-spaced) flashes; a,b, control; c–f, same vesicles with 10 μM antimycin; g, after 90 min and subsequent addition of 10 μM pyocyanin (a flash train of 7.5 min duration was applied; only the first and the last part of the original trace is shown); h,i, fresh vesicles without ferredoxin, with 10 μM pyocyanin and 1.75 mM NADPH. In the traces a–g, only part of the flash light artifact is shown (cf. with traces h,i). Further conditions as in section 2.

Table 2

Effect of antimycin A on ATP synthesis in freshly thawed vesicles, at the onset of flashing and after prolonged flashing, in relation to the dark time preceding the flash trains

Experimental conditions	Dark time (s)	Rate ($ATP \cdot Chl^{-1} \cdot s^{-1}$)		
		Initial (I)	After 35 s of flashing (P)	Ratio I/P
A	0	25 to 300	25 to 300	1.0
B	10	22	22	1.0
	57	57	29	2.0
	100	49	20	2.5
	520	57	25	2.3
C	8	31	24	1.3
	14	49	24	2.0
	24	69	29	2.4
	40	80	24	3.3

The experimental conditions were as follows: (A) no inhibitor, flash groups containing 1–6 (10 ms-spaced flashes), group frequency 0.4 to 1.4 Hz (observations of various experiments); (B) single flashes at 1.2 Hz, 10 μM antimycin A; (C) flash groups comprising 6 (10 ms-spaced flashes), 10 μM antimycin A, group frequency 0.4 Hz. Further conditions as in section 2

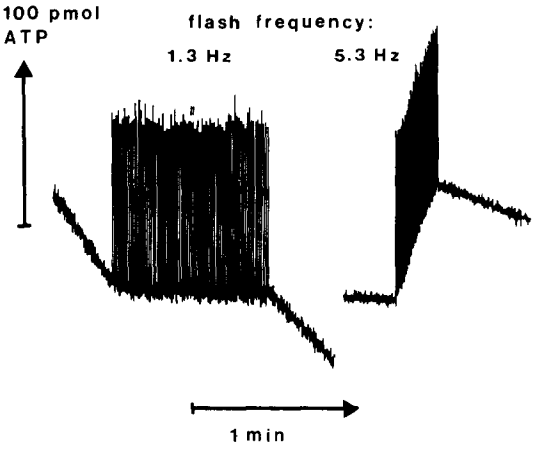

Fig.3. The behaviour of flash-induced, ferredoxin-mediated ATP synthesis in the presence of FCCP. Single flashes were fired at the indicated frequencies; no electron transfer inhibitors were present; the FCCP concentration was 250 nM. Further conditions as in section 2.

flashing at 1.2 Hz appeared equal to the initial rate ($0.14\ ATP \cdot (10^3\ Chl)^{-1} \cdot s^{-1}$, see fig.2), in contrast to what was observed in the presence of antimycin or in aged vesicles. These results suggest that antimycin (or ageing) partially inhibited electron transfer from ferredoxin to plastocyanin (which is by-passed in the presence of pyocyanin) and/or the associated translocation of protons.

The ATP synthesis rates in the pyocyanin-mediated system were not higher than in the native, ferredoxin-mediated system (cf. figs 1a,b, 2a,b and 2h,i), indicating that presumably the pyocyanin-mediated system did not turn over much faster than the native system. Thus, it seems unlikely that a higher proton-motive force in the pyocyanin-mediated system masked an uncoupling effect of antimycin. This is corroborated by the observation that the ageing-, or antimycin-induced effect (figs 1d–f and 2c–f) could not be induced by ionophoric agents like FCCP (fig.3, see also [22]), nigericin or valinomycin (not shown, see [22]). In a previous paper [22], we showed that protonophoric agents like FCCP or nigericin affect ATP synthesis more with a train of single flashes than with a train of flash groups, whereas the reverse was true for electron transfer inhibitors like DNP-INT or DBMIB. Also in the present vesicle batches, the effect of DBMIB was smaller with single flashes than with flash groups (38% inhibition with single flashes; 45–60% inhibition with groups consisting of 6 (10 ms-spaced) flashes). Table 1 shows that in this respect, antimycin, at 10 μM, behaved like a true electron transfer inhibitor rather than an uncoupler.

4. DISCUSSION

The present results demonstrate antimycin A inhibition of cyclic photophosphorylation in PS I vesicles under single-turnover conditions. Comparison of the results obtained with antimycin, DBMIB, uncouplers and pyocyanin strongly indicates that the effect of antimycin reflects partial inhibition of electron transfer from ferredoxin to plastocyanin, rather than uncoupling.

Hosler and Yocum [10,11] have suggested that antimycin sensitivity is only observed at a sufficient reduction level of ferredoxin. In our PS I vesicles, PS II activity is absent and the only source of reducing equivalents is NADPH. The redox poise of the system is set by manipulating the $NADPH/O_2$ ratio in the reaction mixture [8,21]. It is possible, that this is not a proper way to obtain a reproducible and sufficient reduction level of ferredoxin. However, several attempts to elicit an-

timycin sensitivity at a molar NADPH/O_2 ratio of 5 failed in some previous vesicle batches [21], whereas antimycin inhibition was obtained without failure in the present batch at the same NADPH/O_2 ratio and under the same conditions. More likely, the antimycin-sensitive component [14,16] was absent in the PS I vesicle batches used in [21]. Possibly, it is sometimes lost during the preparation of the vesicles (which would indicate that it is not an integral membrane component, but is rather weakly attached to the external thylakoid membrane surface). Alternatively, it could be sometimes absent in the spinach used to prepare the vesicles. Preliminary data, obtained in our laboratory with recently prepared vesicle batches, indeed failed to show high-affinity binding sites for antimycin, even in the presence of bovine serum albumin (cf. [4]) (Krab, K. and Scholts, M.J.C., unpublished). These batches were prepared according to exactly the same procedure as the presently or previously used [21] batches. The average ATP yield per single flash in the presently discussed antimycin-sensitive vesicle batches (figs 1–3) was a factor 1.5 to 2 higher than in the antimycin-insensitive batches used in [21,22] (0.08–0.10 ATP·$(10^3$ Chl$)^{-1}$ versus 0.05–0.06 ATP·$(10^3$ Chl$)^{-1}$), at a flash frequency of 0.5–1 Hz and at the onset of flashing. Possibly, the presence of the proposed antimycin-sensitive component enhances the rate of cyclic electron transfer and/or the efficiency of the electron transfer-associated proton translocation.

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