Vol. 84, No. 4, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 30, 1978 Pages 901-906

FLASH SPECTROSCOPIC STUDIES OF CYCLIC ELECTRON FLOW IN INTACT CHLOROPLASTS

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Received August 3,1978

Summary: The kinetic behaviours of cytochrome b-563 and cytochrome f are shown to be consistent with their participation in coupled cyclic electron flow in intact chloroplasts. Electron transfer between cytochromes b-563 and cytochrome f is antimycin sensitive. Fluorescence induction studies indicate that plastoquinone may function in a coupled step between the cytochromes.

In chloroplasts, the cyclic electron transport pathway around photosystem I is thought to share with non-cyclic electron flow some or all of the carriers between plastoquinone and P700 (1,2). There is little spectroscopic or other evidence in support of this view, owing to poor turnover of the cycle in the most common object of study -- naked thylakoid membranes. Loss of stromal or weakly bound electron carriers during isolation of the thylakoid presumably accounts for low cyclic activity (3). In this report, flash spectroscopic results with intact chloroplasts are presented for conditions under which the energetic properties of the chloroplast cyclic system have been well defined (4).

Materials and Methods: Intact CO_2-fixing chloroplasts were prepared from spinach as described previously (5). Measurements at 18-22°C were performed with samples composed of chloroplasts in "Reaction Buffer" consisting of 0.36 M sorbitol, 50 mM tricine and 0.3 mM K_2HPO_4 adjusted to pH 8.1. Samples also contained 1200 units of catalase ml^{-1} to prevent H_2O_2 buildup during illumination (6). The chlorophyll concentrations were 50 μg chl ml^{-1} in 1.5 ml for the absorption changes and 20 μg chl ml^{-1} in 3 ml for chlorophyll fluorescence and ATP measurements. Thylakoid membranes were obtained by osmotic rupture of intact chloroplasts in 1.5 ml of 10 mM tricine and 10 mM $MgCl_2$ at pH 8.1. Osmotic strength was restored by the subsequent addition of 1.5 ml of double strength Reaction Buffer. Chlorophyll fluorescence at 680 nm and light induced ATP production were measured as reported earlier (4). Flash induced absorption changes with peaks at 518, 554, and 564 nm were kinetically resolved by the procedure of Dolan and

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

0006-291X/78/0844-0901$01.00/0

Hind (7) with the following modifications: The elimination of the P518 component from the signals at 554 and 564 nm corresponding to cytochromes f and b-563 respectively was achieved by appropriate digital subtractions of the 518 nm signal rather than the 531 nm signal (see ref. 7 and figure 3 legend); the flash repetition rate was 1.5 Hz and limited to the spectral region above 650 nm by Kodak Wratten filters (No. 70), and the flash intensity was doubled by illuminating the sample cuvette from opposing directions perpendicular to the measuring beam, with two EG&G FX201 xenon lamps. A single flash resulted in 80% saturation of the P518 change.

Results and Discussion: Figure 1 shows chlorophyll fluorescence rise curves as a function of time under weak illumination, for intact chloroplasts without added electron acceptors. The fluorescence rise with 0.1 μM DCMU, attributable to Q reduction (8), is rapid and is insensitive to antimycin. In unpoisoned chloroplasts, the partial reduction of Q, registered by the initial rise from F_o (0-1 sec), is not influenced by antimycin, whereas the slow fluorescence rise (2-20 sec) which monitors plastoquinone (PQ) reduction through its equilibrium with Q, is severely inhibited. Although the PQ pool could be kept oxidized by addition of an uncoupler (0.33 mM NH_4Cl), 1 μM antimycin does not uncouple chloroplasts (9). Antimycin does not inhibit linear electron flow (9) as does DCMU (Fig. 1) hence an electron input to PQ, other than by photosystem II, must be postulated.

Figure 2 shows that similar results are observed in unpoisoned, freshly shocked chloroplasts. More importantly, the rapid fluorescence rise, in shocked chloroplasts incubated with NADPH, indicates that reduction of the Q + PQ pool can be driven by NADPH. The traces further show that the reduction is via an antimycin sensitive electron transport system.

If the cyclic pathway involves cytochrome b and utilizes the coupled step between PQ and cytochrome f (1,2), both the oxidation and reduction kinetics of cytochrome b-563 and cytochrome f respectively should depend on the coupling state of the chloroplast. These expectations are consistent with the flash kinetics depicted in Figure 3 for intact chloroplasts . With no additions (Fig. 3a), the amplitudes of the cytochrome b-563 and f responses to a flash are approximately equal and their relaxations are

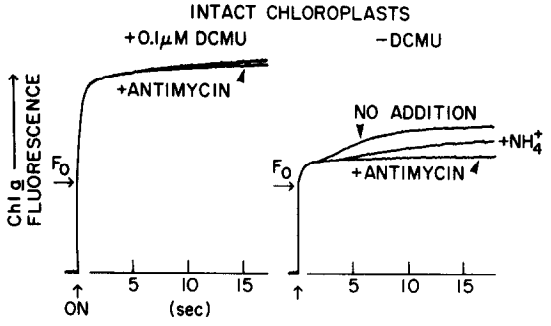

Fig. 1. Fluorescence induction traces for intact chloroplasts without added electron acceptors. The indicated additions were 0.1 μM DCMU, 1.0 μM Antimycin and 0.33 mM NH_4Cl. Measurement conditions under weak (0.5 w/m^2) blue illumination as described in ref. 4.

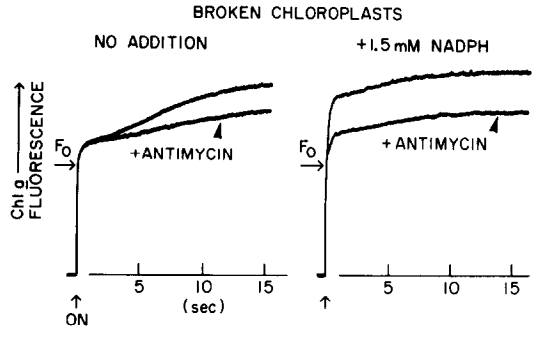

Fig. 2. Fluorescence induction traces for freshly shocked chloroplasts. The indicated additions were 2.0 μM Antimycin and 1.5 mM NADPH. Other measurement conditions as described in Fig. 1 and Methods.

nearly complete within 40 msec. Calculation gives a ratio of 0.93 for the cytochrome b-563 to f heme turnover, using extinction coefficients of 20 and 22 $mM^{-1}cm^{-1}$ respectively (10). This suggests that light driven electron transfer from cytochrome f to b-563 is not in competition with pseudocyclic flow from cytochrome f to oxygen. It is also noteworthy that the amplitude of the cytochrome f signal represents about half of the total chloroplast cytochrome f content of 1.0 heme/500 chlorophylls, as determined from chemical redox titrations.

In the presence of 0.1 μM DCMU, which poises the cyclic pathway, steady state energization is maximal (4) as is the production of ATP (Fig. 3b).

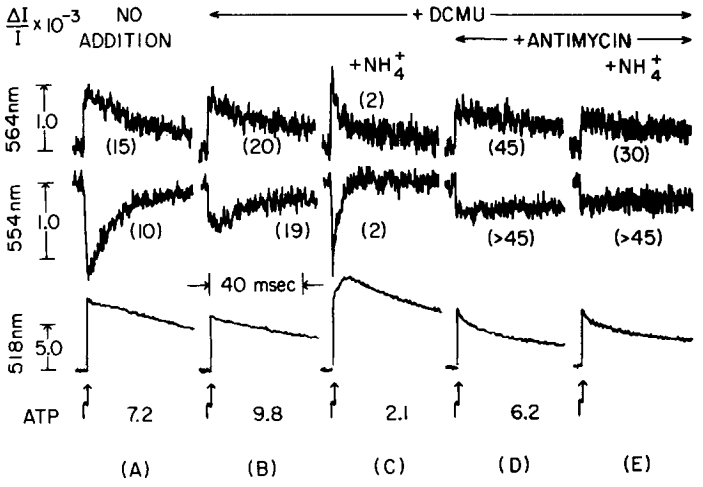

Fig. 3. Kinetic traces for the flash induced absorption changes at 564 nm, 554 nm and 518 nm in intact chloroplasts. The P518 traces represent the accumulated average of 128 sweeps at 518 nm. The cytochrome b-563 response was obtained by digital subtraction of 42 sweeps at 518 nm from 512 sweeps at 564 nm. The cytochrome f response was derived by digitally subtracting 56 sweeps at 518 nm from 512 sweeps at 554 nm. Otherwise the absorption changes were recorded with a 50 μ sec signal averager address time and as described in Methods and ref. 7. Numbers in parentheses give the estimated half times (in msec) for relaxation based on a simple first order decay. ATP values indicate the amount of endogenous stromal ADP (nmoles/mg chl) phosphorylated during 15 sec of continuous red (Corning 2-58, 100 w/m^2) light. Additions were 0.1 μM DCMU in traces B-E, 3.3 mM NH_4Cl in traces C and E, 1.0 μM Antimycin in trace D and 2.0 μM Antimycin in trace E.

However, the large electrochemical gradient from cyclic turnover retards electron flow between cytochromes b-563 and f, and in repetitive flashes the signal amplitudes also decline. Conversely, an uncoupling concentration of NH_4Cl (Fig. 3c) accelerates the oxidation of cytochrome b-563 and reduction of cytochrome f to a common $t_{0.5}$ of 2.0 msec, and decreases by 79% the chloroplast ATP level induced by light.

The response of tightly coupled chloroplasts to antimycin is very different from that provoked by uncoupler, although the ATP level is also depressed (cf. Fig. 3c, 3d). Antimycin inhibits the oxidation of cytochrome b-563 reduced in a flash (Fig. 3d) to > 40 msec, from a control $t_{0.5}$ of 20 msec, and there is consequent loss of signal amplitude owing to incomplete relaxation between flashes. In the presence of both antimycin and uncou-

pler, traces identical to Fig. 3d are obtained (Fig. 3e) thus antimycin decreases uncoupled electron transfer between the cytochromes (Fig. 3c) by a factor of > 10. Antimycin is clearly a selective inhibitor of photosynthesis and not an uncoupler (9,11).

In Fig. 3c the secondary rise in absorbance at 518 nm is kinetically comparable to the electron flow from cytochrome b-563 to cytochrome f. This rise is not present in samples containing antimycin, even if NH_4Cl is added (Figs. 3d, 3e). Nigericin or monensin plus Na^+ could be substituted for NH_4Cl with similar results. Occurrence of such a phenomenon milliseconds after the 12 μsec flash, and well beyond residual flash "tails", is evidence for an electrochromic effect generated by postillumination transfer of electrons through the cyclic route and a coupling site. This contradicts a recent suggestion that electrochromic effects in chloroplasts result only from the membrane potential generated by primary photoevents (12).

We conclude: (i) The lower level of PQ reduction in the presence of antimycin, implied by fluorescence studies (Figs. 1,2) is evidence for inhibition of cyclic electron flow by antimycin between cytochrome b-563 and the PQ pool. (ii) Cyclic electron flow is tightly coupled to ATP formation in intact chloroplasts. This is exemplified by the ten-fold acceleration in the rate of cytochrome b-563 oxidation and cytochrome f reduction under uncoupled conditions. The cyclic coupling site lies, presumably, between PQ and cytochrome f and is shared with the linear system as concluded elsewhere for broken (1,2) and intact chloroplasts(13). (iii) The antimycin sensitive component of the P518 response, in intact chloroplasts, may be related to the dark electron flow responsible for the slow electrochromic shift present in photosynthetic bacterial chromatophores (14)and algae (15). The absence of this component in isolated thylakoids (12) could be the result of poor endogenous cyclic activity following the loss of soluble intermediates (3) during preparation.

Acknowledgements: This research was carried out at Brookhaven National Laboratory under the auspices of the United States Department of Energy. The authors would like to thank Drs. David Crowther and John Mills for their helpful discussions in preparing this report.

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