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ENHANCED SENSITIVITY TO PLASTOQUINONE INHIBITORS OF FERREDOXIN PHOTOREDUCTION BY PHOTOSYSTEM II IN INSIDE-OUT CHLOROPLAST VESICLES

Ban-Dar Hsu, George M.-S. Tang, and Daniel I. Arnon

Division of Molecular Plant Biology
University of California, Berkeley, CA 94720

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SUMMARY: New evidence is presented in support of the concept that reducing power for photosynthesis is generated solely by photosystem II (the oxygenic photosystem) when it transfers electrons from water to ferredoxin without the collaboration of photosystem I, the anoxygenic photosystem responsible for cyclic photophosphorylation. Membrane vesicles of opposite sidedness were prepared from spinach chloroplasts by the two-phase partition method: inside-out-vesicles greatly enriched in photosystem II and right-side-out vesicles containing both photosystems and having the same sidedness orientation as unfractionated chloroplast membranes. In both types of vesicles, plastoquinone analogues were used to inhibit light-induced electron transport from water to ferredoxin and from water to native photosystem I acceptors, the membrane-bound iron-sulfur centers A and B. In right-side-out vesicles the photoreduction of iron-sulfur centers A and B was more sensitive to plastoquinone inhibitors than the photoreduction of ferredoxin, whereas the converse was found in inside-out vesicles in which a greatly enhanced sensitivity of ferredoxin reduction to plastoquinone inhibitors was detected: the photoreduction of ferredoxin was about 80% inhibited at low concentrations of plastoquinone inhibitors that had practically no effect on the photoreduction of iron-sulfur centers A and B. These findings appear to exclude the possibility that these photosystem I contaminants were involved in the photoreduction of ferredoxin by the PSII-enriched inside-out vesicles.

Albertsson et al. (1) have recently reported that spinach chloroplast vesicles, greatly enriched in photosystem II (PSII) and turned inside-out with respect to the original sidedness of the membrane, gave a significant oxygenic reduction of $NADP^+$ when the participation of photosystem I (PSI) was excluded. ("Oxygenic reduction" denotes reduction by electrons that originate from water.) These findings are in conflict with the widely held Z scheme in which a collaboration of PSII and PSI is required for the oxygenic reduction of $NADP^+$: PSII photooxidizes water and transfers electrons to PSI, the only photosystem deemed competent to reduce ferredoxin and

Abbreviations: PSI and PSII, photosystems I and II; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DNP-INT, dinitrophenyl ether of iodonitrothymol.

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hence $NADP^+$ (2,3). While incompatible with the Z scheme, these findings (1) strongly support an alternative scheme of photosynthetic electron transport which envisions that PSII (renamed the oxygenic photosystem) drives the noncyclic electron transport from water to ferredoxin without the collaboration of PSI (renamed the anoxygenic photosystem) whose role is limited to cyclic electron transport and phosphorylation (4,5).

Noninvolvement of PSI in the oxygenic reduction of ferredoxin and $NADP^+$ would profoundly alter present concepts of photosynthesis and needs therefore multifaceted experimental support. In previous experiments with inside-out vesicles the participation of PSI in the reduction of $NADP^+$ was ruled out by evidence that these preparations had no significant PSI components accessible to reduction by plastocyanin (1), a copper protein electron carrier essential in the Z scheme as a donor to PSI (2,3) but found also to be required for $NADP^+$ reduction by the oxygenic photosystem in the inside-out PSII vesicles that functioned independently of PSI (1).

Inside-out vesicles usually include some contamination by right-side-out vesicles (6) which trap native plastocyanin and have PSI components that are inaccessible to externally added plastocyanin (7). Adherents of the Z scheme could argue that the observed oxygenic reduction of $NADP^+$ in inside-out vesicles may have been due to this PSI contamination from right-side-out vesicles.

We report here that, with water as electron donor, chloroplast vesicles of opposite sidedness exhibited strikingly different patterns of sensitivity to plastoquinone inhibitors in the photoreduction of ferredoxin on the one hand, and of PSI components (iron-sulfur centers A and B) on the other. These findings are incompatible with centers A and B being electron donors to ferredoxin and the notion that photoreduction of ferredoxin by PSII in inside-out vesicles is due to PSI contaminants.

METHODS

Chloroplasts were isolated from freshly-harvested spinach leaves (Spinacia oleracea, var. Marathon) grown in a greenhouse in nutrient solution (8). Previously described procedures were used for the estimation of

chlorophyll (8) and isolation and purification of ferredoxin (9). Glucose oxidase (type VII) and bovine catalase were purchased from Sigma Chemical Co. DBMIB and DNP-INT were added as methanol solutions. (DNP-INT was first dissolved in a drop of dimethyl formamide.) Equal concentrations of methanol were added to the control treatments.

Inside-out vesicles enriched in PSII and right-side-out vesicles containing both PSII and PSI were prepared by mechanical disruption of spinach thylakoid membranes in a Yeda press followed by fractionation by an aqueous polymer two-phase (dextran-polyethylene glycol) partition method (6,10,11), with some modifications (1). For the particular batches of dextran (Dextran 500, Pharmacia Fine Chemicals) and polyethylene glycol (Mol. wt. 3350, Sigma Chemical Co.) used, the concentration of each polymer during the partition treatment was 6.0% (w/w). The polyethylene-glycol-rich top phase repetitively partitioned two times (T2) was enriched in right side-out vesicles and the dextran-rich bottom phase repetitively partitioned three times (B3) was enriched in inside-out vesicles. After phase partition the vesicles were collected by centrifugation (1), resuspended in a solution of 100 mM sucrose, 20 mM NaCl and 50 mM Na phosphate pH 7.4, and stored at 77K, in the presence of 5% dimethyl sulfoxide.

The effluent from the first ("high salt") Yeda pressings was separated by centrifugation into a pellet used for the preparation of vesicles (1) and a supernatant that was centrifuged at 180,000 x g for 45 min. and dialyzed overnight against 10 mM Na phosphate, pH 7.4, to remove excess salt. The dialyzed solution, designated Y-1, was used as a source of plastocyanin to stimulate oxygenic $NADP^+$ reduction by the inside-out vesicles depleted of plastocyanin (1).

The oxygenic photoreduction of ferredoxin and of the membrane-bound iron-sulfur centers A and B by the inside-out (B3) and the right-side-out (T2) vesicles was measured by EPR spectroscopy as previously described (5). Instrument settings are given in figure legends. The effect of plastoquinone inhibitors on electron transport from water to PSI was measured (in the absence of added ferredoxin) by the amplitude of the EPR signals characteristic of reduced membrane bound iron-sulfur centers A and B which form part of the acceptor complex of PSI (see review, 12). Reduced centers A and B give signals at g = 1.86, 1.94 and 2.05 (center A) and at 1.89, 1.92 and 2.05 (center B); when fully reduced the g = 1.86 signal of center A undergoes a shift to 1.89 (12). The effect of plastoquinone inhibitors on photoreduction of ferredoxin by water was measured by the amplitude of the characteristic signals of reduced ferredoxin at g = 1.89, 1.96 (main signal) and 2.05.

Because of the considerable overlap between the signals of the reduced ferredoxin and the iron-sulfur centers, the EPR tubes were scanned at two temperatures, 15K for the iron-sulfur centers and 56K for ferredoxin; at the higher temperature the signals of the iron-sulfur centers broaden and cease to be detectable (13). To obtain large signals of the iron-sulfur centers relatively high concentrations of chloroplasts had to be used. These resulted also in high concentrations of other membrane-bound components including plastoquinone, and necessitated the use of relatively high concentrations of plastoquinone inhibitors (14).

RESULTS

Chlorophyll a/b ratios. As stated, two types of chloroplast vesicles of opposite sidedness were isolated from the same preparation of spinach thylakoids: right-side-out vesicles (T2) containing both PSII and PSI and having the same sidedness orientation as unfractionated thylakoids, and inside-out vesicles (B3) greatly enriched in PSII but also usually contain-

ing some PSI derived mainly from a contamination (6) by right-side-out vesicles (see Fig. 1 in ref. 1). The chlorophyll a/b ratio of the right-side-out vesicles was 2.7, similar to 3.0 for the unfractionated thylakoids, and distinctly higher than the ratio of 2.1 for inside-out vesicles. Low chlorophyll a/b ratios denote enrichment in PSII (15).

Right-side-out vesicles: effect of plastoquinone inhibitors. One of the main tenets of the Z scheme is that plastoquinone, the most abundant redox component of chloroplasts (16,17), serves as an essential link in the transfer of electrons from PSII to PSI (3,18). Strong support for this conclusion was derived from the inhibition of oxygenic reduction of $NADP^+$ by plastoquinone inhibitors, DBMIB (19,18) and DNP-INT (14,18).

Concurrently with intersystem electron transport, plastoquinone serves, according to the Z scheme, as a transmembrane shuttle of protons from the outside (stroma side) to the thylakoid's inside aqueous space

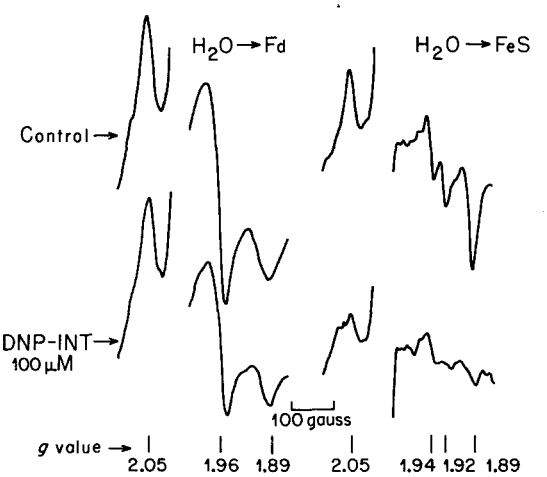

Fig. 1. Differential inhibitory effect of DNP-INT on the photoreduction by water of ferredoxin and bound iron-sulfur centers A and B in right-side-out chloroplast vesicles (T2). The reaction mixture, equilibrated with N_2, contained T2 vesicles (0.4 mg chlorophyll/ml), 50 mM MOPS (morpholinopropanesulfonic acid, pH 6.7), 5 mM $MgCl_2$, 10 mM glucose, 12% methanol, and saturating amounts of glucose oxidase and catalase. 10 μM spinach ferredoxin and DNP-INT were added as indicated. The EPR tubes were illuminated for 30 sec at room temperature and immediately frozen in liquid N_2 under continuing illumination (650 nm, 5 x 10^5 ergs $cm^{-1}sec^{-1}$). EPR spectrometer field setting, 3430 ± 200G; microwave power, 10 mW; modulation amplitude, 10G; gain, 1 x 10^5. EPR spectra were recorded at 56K for $H_2O \longrightarrow Fd$ and at 15K for $H_2O \longrightarrow FeS$. (The free radical signals in the g = 2.0 region are omitted.)

(lumen) (2). Note that Z scheme assigns no role to plastoquinone in the transport of protons released in the photooxidation of water by PSII.

By contrast, in the alternative scheme, plastoquinone is given the important additional function of transporting protons liberated by PSII during the photooxidation of water, a reaction we envision as occurring in the lipophilc domain of the thylakoid (4,5). Thus, both schemes predict that plastoquinone inhibitors should inhibit intersystem electron transfer from water to PSI, but only the alternative scheme predicts that they would also inhibit photooxidation of water involving solely PSII (4,5). It will be noted that the recently observed inhibition by DBMIB and DNP-INT of the oxygenic reduction of $NADP^+$ in inside-out vesicles under experimental conditions that appeared to exclude the participation of PSI (1), was in accord with the alternative scheme.

Fig. 1 shows that in the right-side-out vesicles the reduction of PSI components represented by the iron-sulfur centers A and B was more sensitive to DNP-INT inhibition (ca. 70% at g = 1.89) than the reduction of ferredoxin (ca. 30% at g = 1.96). These results are similar to those obtained earlier (20) with unfractionated thylakoids that have the same surface orientation.

Similar results were obtained with DBMIB (data not shown).

Inside-out vesicles: effect of plastoquinone inhibitors. Completely inverse sensitivity to plastoquinone inhibitors was obtained in the inside-out vesicles (Fig. 2). Photoreduction of ferredoxin was unexpectedly found to be very sensitive to plastoquinone inhibitors: it was about 80% inhibited (g = 1.96) at 10 μM DNP-INT, a low (relative to the high chlorophyll) concentration of DNP-INT which had practically no effect on the photoreduction of iron-sulfur centers A and B (Fig. 2).

Similar results were obtained with DBMIB (data not shown).

These findings seem to exclude the possibility that the oxygenic photoreduction of ferredoxin by the PSII-enriched inside-out vesicles was due to the contamination by right-side-out vesicles containing PSI. If this were the case, the inhibition by plastoquinone inhibitors would have shown a

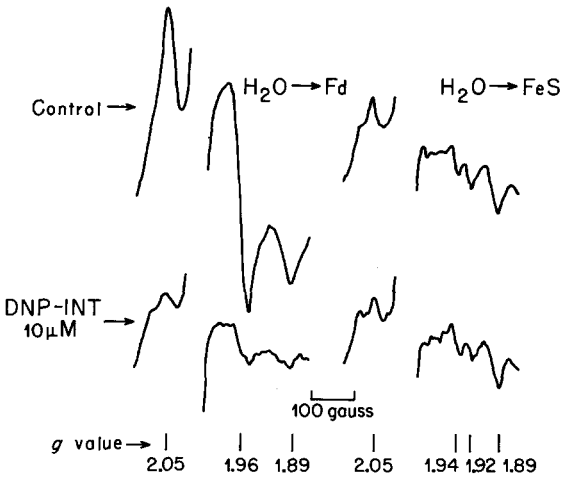

Fig. 2. Differential inhibitory effect of DNP-INT on the photoreduction by water of ferredoxin and bound iron-sulfur centers A and B in inside-out chloroplast vesicles (B3). Experimental conditions were as in Fig. 1 except that the B3 vesicles contained 0.6 mg chlorophyll/ml and plastocyanin (as 0.5 ml of solution Y-1) was added to each sample. Gain, 1 x 10^5.

pattern similar to that in Fig. 1, i.e., ferredoxin reduction would have been less inhibited than the reduction of iron-sulfur centers A and B. That just the opposite was observed suggests that the inside-out vesicles expose to plastoquinone inhibitors a site of plastoquinone function that is more sheltered in right-side-out vesicles. Furthermore, the great difference of sensitivity to plastoquinone inhibitors demonstrates again that, contrary to the Z scheme, centers A and B are not electron carriers in the oxygenic photoreduction of ferredoxin.

DISCUSSION

Prior to the use of the phase partition method, experimental support for the Z scheme was seen in reconstitution experiments of the photosynthetic electron transport chain in which $NADP^+$ reduction was always dependent on the inclusion of both PSII and PSI components (21-23). But as discussed by Albertsson et al. (1), these reconstitution experiments involved detergent-made preparations, and the possibility of detergent-induced artifacts is not excluded. Detergents are not used in the phase partition method; the polymers that are used are likely to protect membrane integrity (24).

The previous paper (1) described how, contrary to the Z scheme, PSII-enriched inside-out vesicles gave a plastocyanin-dependent oxygenic reduction of $NADP^+$ without any apparent involvement of PSI. Adding present findings, the evidence for non-involvement of PSI in the oxygenic reduction of ferredoxin by PSII in inside-out vesicles may be summarized as follows: (i) Externally added plastocyanin is an effective electron donor to P700, the reaction center chlorophyll of PSI, only when P700 is accessible in inside-out vesicles (7); (ii) PSII-enriched, inside-out vesicles which gave a plastocyanin-dependent oxygenic reduction of $NADP^+$ contained no accessible P700 as determined by the addition of chemically reduced plastocyanin (1); (iii) the remaining possibility in defense of the Z scheme, i.e., that the oxygenic reduction of ferredoxin by the PSII-enriched inside-out vesicles was in reality due to a PSI contamination from right-side-out vesicles has now been examined and found unsustainable. We conclude, therefore, that inside-out chloroplast vesicles were oxygenically photoreducing ferredoxin without the collaboration of PSI.

The increased sensitivity of inside-out vesicles to plastoquinone inhibitors in the oxygenic reduction of ferredoxin emphasizes the role of plastoquinone even when no PSI is involved (4,5). Further study is needed to determine which of the four forms of plastoquinone in chloroplasts was rendered more exposed and sensitive. These forms include Q, the one-electron carrier in the PSII reaction center, the two-electron carrier plastoquinone known as B (or R), a third form of plastoquinone functioning in the cytochrome $\underline{b}_6$-$\underline{f}$ complex near the Rieske iron-sulfur center, and finally, the free excess plastoquinone pool (see review, 25).

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