Protonophores induce plastoquinol oxidation and quench chloroplast fluorescence: Evidence for a cyclic, proton-conducting pathway in oxygenic photosynthesis

(photosystem II/cyclic proton transport/uncouplers)

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ABSTRACT The photosynthetic apparatus converts light into chemical energy by a series of reactions that give rise to a coupled flow of electrons and protons that generate reducing power and ATP, respectively. A key intermediate in these reactions is plastoquinone (PQ), the most abundant electron and proton (hydrogen) carrier in photosynthetic membranes (thylakoids). PQ ultimately transfers electrons to a terminal electron acceptor by way of the Rieske Fe-S center of the cytochrome *bf* complex. In the absence of a terminal acceptor, electrons accumulate in the PQ pool, which is reduced to plastoquinol (PQH₂), and also on a specialized PQ, Q_A, which is reduced to an unprotonated semiquinone anion (Q_A^{•-}). The accumulation of Q_A^{•-} is measured by a rise in fluorescence yield and the accumulation of PQH₂ is measured by absorption difference spectrometry. We have found that in the absence of a terminal electron acceptor, two chemically diverse proton-conducting ionophores (protonophores), 2,6-di-*t*-butyl-4-(2',2'-dicyanovinyl)phenol (SF 6847) and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), induced oxidation of PQH₂ and quenching of chloroplast fluorescence, signifying oxidation of Q_A^{•-}. The two protonophores produced the same effects even when the only recognized pathway of PQH₂ oxidation by way of the cytochrome *bf* complex was inhibited by dibromothymoquinone. Two other uncouplers, gramicidin and nigericin, which are not protonophores but facilitate proton movement across membranes by other mechanisms, were ineffective. These findings are consistent with the operation in the oxygen-generating photosystem (photosystem II) of a cyclic, proton-conducting pathway.

The recently described perspective on photosynthesis envisions the operation in the oxygen-generating photosystem (photosystem II; PSII) of a light-induced cyclic pathway for conductance of protons (1). We now report effects of uncouplers on chloroplast fluorescence and the redox state of plastoquinone (PQ) that are consistent with the operation of such a pathway.

The photosynthetic apparatus converts light into chemical energy by a series of reactions that give rise to a coupled flow of electrons and protons that generate reducing power and ATP, respectively (2). A key intermediate in these reactions is PQ, the most abundant redox component in photosynthetic membranes (thylakoids) (3, 4). Because PQ-plastoquinol (PQH₂) oxidoreductions ($\text{PQ} + 2\text{e}^- + 2\text{H}^+ \rightleftharpoons \text{PQH}_2$) involve transfers of hydrogen atoms (electrons plus protons), PQ is both the main electron and the main proton carrier in thylakoids.

In functioning chloroplasts, PQH₂ is ultimately oxidized by a terminal electron acceptor, by way of the Rieske Fe-S center of the cytochrome *bf* complex (5). In the absence of a

terminal acceptor, PQH₂ accumulates; electrons then "back up" and accumulate on a specialized PQ (Q_A). The accumulation of Q_A^{•-} is monitored by a rise in variable fluorescence (*F_v*) (6).

We have found that in the absence of a terminal acceptor, two chemically diverse proton-conducting ionophores (uncouplers), 2,6-di-*t*-butyl-4-(2',2'-dicyanovinyl)phenol (SF 6847) and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), induced oxidation of PQH₂ and dramatically lowered chloroplast fluorescence (signifying oxidation of Q_A^{•-}). The two protonophores produced the same effects when the only recognized pathway of PQH₂ oxidation by way of the cytochrome *bf* complex was inhibited by 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone [DBMIB (dibromothymoquinone)] (4). Two other uncouplers, gramicidin and nigericin, which are not protonophores but facilitate by other mechanisms proton movement across membranes (7), were ineffective.

These findings are consistent with the operation in PSII of a cyclic, proton-conducting pathway that involves oxidation of PQH₂ by way of cytochrome *b*₅₅₉ (1). We discuss here the PQ components of the cycle; the evidence pertaining to cytochrome *b*₅₅₉ is being reported separately (8).

METHODS

Chloroplasts were isolated from spinach leaves (*Spinacia oleracea* var. Marathon) grown in a greenhouse in nutrient solution culture (9) and freshly harvested before each experiment. Previously described procedures were used for chlorophyll estimation (9) and the preparation of thylakoids (10). These consisted of osmotically disrupted chloroplasts that retained the capacity for complete noncyclic electron transport from water to NADP⁺ and photosynthetic phosphorylation (10). Where maintained, anaerobic conditions were established by gassing the samples with N₂ and including in the reaction mixture a glucose/glucose oxidase/catalase oxygen trap (11).

Chloroplast fluorescence and absorbance difference measurements for quinones were performed as described (6, 12). Differential extinction coefficients (mM⁻¹·cm⁻¹) of 13 at 263 nm for PQ and 19.6 at 257 nm for 2,5-dimethylbenzoquinone (DMQ) were used. DBMIB was kindly supplied by A. Trebst (Ruhr-Universität, Bochum, F.R.G.), nigericin was provided

Abbreviations: PS, photosystem; PQ, plastoquinone; PQH₂, plastoquinol; Q_A and Q_B, specialized membrane-bound forms of PQ; DMQ, 2,5-dimethylbenzoquinone; diuron (DCMU), 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB (dibromothymoquinone), 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; SF 6847, 2,6-di-*t*-butyl-4-(2',2'-dicyanovinyl)phenol; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; *F_v*, variable fluorescence.

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by Hoffman-LaRoche, and SF 6847 was provided by Sumitomo Chemical (Osaka, Japan). FCCP and gramicidin were purchased from Sigma.

RESULTS

The carbonylcyanide phenylhydrazones [FCCP and carbonylcyanide *m*-chlorophenylhydrazone (CCCP)] are widely used proton-conducting ionophores that uncouple electron transport from ATP formation in oxidative and photosynthetic phosphorylation (7). In other studies these uncouplers were observed to influence the oxidizing (water-splitting) side of PSII and were characterized as ADRY reagents (accelerators of deactivation reactions of the water-splitting system Y) (13).

A puzzling effect of FCCP and CCCP on PSII was that, at low concentrations, they quench chloroplast fluorescence emanating from PSII. This fluorescence reflects the redox state of Q_A . Q_A is the first stable electron acceptor of PSII; it is a specialized, tightly bound PQ that is reduced only to the unprotonated semiquinone form Q_A^- (14). Fluorescence yield is high when Q_A is predominantly in the reduced state (Q_A^-) and decreases when it is in the oxidized state (15). Thus, the quenching of fluorescence is tantamount to reoxidation of Q_A^- .

Different explanations were put forward to explain fluorescence quenching by FCCP or CCCP (16–18), including “direct quenching actions of oxygenated reaction products or a cyclic electron flow between reduced electron carriers and such intermediates” (19) but excluding the protonophoric properties of these uncouplers. Since proton conductance in PSII is of considerable conceptual interest (1), we reinvestigated the possibility that fluorescence quenching by FCCP may be related to its proton-conducting properties. We were especially interested to determine whether another protonophore, chemically different from FCCP, would also quench chloroplast fluorescence. For this purpose we used SF 6847, a ditertiary phenol derivative, known as the most potent protonophoric uncoupler of electron transport and ATP formation (20). The effect of the two protonophores, FCCP and SF 6847, was compared to that of two other uncouplers, which facilitate proton movement across membranes by other mechanisms: gramicidin, a channel-forming ionophore, and nigericin, an ionophore that catalyzes the exchange of K^+ for H^+ (7).

Fig. 1 shows that, in the absence of an electron acceptor, SF 6847 and FCCP dramatically quenched the yield of F_V , signifying that accumulation of Q_A^- was abolished. Gramicidin and nigericin were ineffective (see Fig. 3). Low fluorescence persisted for the duration of the illumination period (several minutes). Thus, light-induced electron flow in the absence of an electron acceptor continued for an extended period without an accumulation of Q_A^- . The concentration of the uncouplers was only $5 \mu M$ —too low for them to act as electron acceptors, even if this were chemically possible.

In seeking an explanation of these results, we first sought to establish that fluorescence quenching was not due to damage of the photochemical apparatus by SF 6847 and FCCP. To this end we measured fluorescence in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea [diuron (DCMU)], an inhibitor that prevents electron transfer from Q_A to PQ. As shown in Fig. 2, in the presence of diuron, neither FCCP nor SF 6847 quenched fluorescence, an indication that the antenna and reaction center of PSII were not damaged and Q_A was being fully photochemically reduced to Q_A^- .

Next to be considered was the role of oxygen as a terminal electron acceptor. We sought to exclude the possibility that uncouplers quenched fluorescence because they increased the rate of electron flow from Q_A^- by way of PSI to molecular oxygen. We measured fluorescence under strictly anaerobic

FIG. 1. Quenching of F_V by protonophores SF 6847 and FCCP. The reaction mixture contained osmotically disrupted chloroplasts (equivalent to $50 \mu g$ of chlorophyll per ml), 5 mM $MgCl_2$, 50 mM Tricine (pH 7.5), and, where added, $5 \mu M$ SF 6847 or $5 \mu M$ FCCP. The reaction mixtures were incubated for 2 min in the dark and then illuminated by light filtered by Corning CS 4-96 and CS 3-69 filters. Intensity of illumination, $45 \text{ microeinsteins} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (1 einstein = 1 mol of photons). F_0 , initial fluorescence.

conditions, including an ambient oxygen trap, whose effectiveness in excluding traces of oxygen was previously tested by the oxygen-sensitive redox state of bound iron sulfur centers of PSI (11). Fig. 3 shows that the protonophores SF 6847 and FCCP markedly quenched fluorescence in the

FIG. 2. No quenching of F_V in the presence of diuron (DCMU). Experimental conditions were as in Fig. 1 except that $10 \mu M$ diuron was present throughout.

FIG. 3. Quenching of F_v by uncouplers under anaerobic conditions. The reaction mixtures were as in Fig. 1 except that they were gassed with N_2 and contained, in addition, an oxygen trap consisting of 40 mM glucose, 1200 units of catalase, and 13 units of glucose oxidase per ml. The concentration of each uncoupler was 5 μ M.

absence of ambient oxygen. Gramicidin and nigericin were, as under aerobic conditions, virtually ineffective.

It appeared therefore that fluorescence quenching by SF 6847 and FCCP could not be attributed to their induction of an accelerated electron transport by way of PSI to molecular oxygen. There remained the possibility that protonophores affected fluorescence indirectly by inducing a reoxidation of PQH₂. Electrons pass from Q_A^- to a second bound PQ, Q_B , which is also reduced to the unprotonated semiquinone form (Q_B^-) but, which, unlike Q_A^- , exchanges electrons with the PQ pool. As two electrons are transferred sequentially to Q_B , there is an uptake of protons from the outer aqueous phase (stroma) resulting in the formation of PQH₂ (4). Thus, in the absence of a terminal acceptor, PQH₂ accumulates and its accumulation is accompanied by an accumulation of Q_A^- , as evidenced by the rise in fluorescence. Conversely, quenching of fluorescence by SF 6847 and FCCP, signifying reoxidation of Q_A^- , should be accompanied by reoxidation of PQH₂.

Fig. 4 shows that these expectations were verified. The effect of SF 6847 and FCCP on the redox state of PQ paralleled the effect of these protonophores on fluorescence (Fig. 1). In the absence of protonophores, electrons accumulated in the PQ pool, reducing it to PQH₂, but in the presence of either SF 6847 or FCCP there was no accumulation of electrons in the PQ pool (Fig. 4).

Fig. 4, jointly with Fig. 1, indicated that in the presence of SF 6847 or FCCP and in the absence of an added acceptor there was a light-induced electron flow from the reaction center of PSII to Q_A and then to the PQ pool and beyond. The known pathway for electron flow beyond PQ requires oxidation of PQH₂ by the Rieske Fe-S center of the cytochrome *bf* complex, a step that is strongly inhibited by DBMIB (4). Thus, it would be expected that in the presence of DBMIB, neither SF 6847 nor FCCP could quench fluorescence and electrons would accumulate in the PQ pool and in Q_A^- .

Fig. 5 shows that this proved not to be the case. In the presence of DBMIB, FCCP and SF 6847 lowered fluorescence as drastically as in the absence of DBMIB. The slight lowering of fluorescence by DBMIB in the control sample (Fig. 5) did not occur in most experiments. It appeared, therefore, that the protonophore-induced electron flow from Q_A^- to PQ and beyond may have occurred not through oxidation of PQH₂ by the cytochrome *bf* complex but by another pathway. In that pathway, the most likely oxidant of PQH₂ would be cytochrome *b*₅₅₉, whose reduction is not inhibited by DBMIB (21).

FIG. 4. Effect of protonophores on PQ-PQH₂ transition as monitored by absorbance change $\Delta A_{263} - \Delta A_{285}$. Other experimental conditions were as in Fig. 1.

Striking as the effects of SF 6847 and FCCP were in inducing oxidation of Q_A^- and PQH₂ it seemed unlikely that these protonophores could prevent the accumulation of electrons on Q_A and in the PQ pool at all incident light intensities. Since the protonophores do not damage the reaction center of PSII (Fig. 2), it seemed probable that, under high light intensity, the inflow of electrons into Q_A and the PQ pool would be more rapid than the outflow, with the result that Q_A^- and PQH₂ would accumulate, even in the presence of the protonophores.

Figs. 6 and 7 show that this was indeed the case. As light intensity increased, there was an increase in F_v , indicative of an accumulation of Q_A^- , even in the presence of SF 6847 and FCCP (Fig. 6). Likewise, at high light intensities there was an accumulation of PQH₂ in the presence of SF 6847 and FCCP (Fig. 7).

The magnitude of electron "outflow" from PQH₂ in the presence of FCCP and SF 6847 can be estimated from Fig. 8, in which the rate of electron flow to the artificial electron acceptor DMQ is plotted as a function of incident light intensity, in the absence of protonophores (control) and in the presence of FCCP and SF 6847. The differences in rates

FIG. 5. Quenching of F_v by FCCP (5 μ M) and SF 6847 (5 μ M) in the presence of DBMIB (1 μ M). Other experimental conditions were as in Fig. 1.

FIG. 6. Effect of light intensity on rise of F_v in the presence of protonophores. The actinic light was filtered by Corning CS 4-96 and CS 3-72 filters and varied over the range shown. Other experimental conditions were as in Fig. 1. μE , microeinstein.

(control – FCCP and control – SF 6847) suggest a minimum outflow capacity of 10 and 30 μmol of e^- (mmol of chlorophyll) $^{-1} \cdot \text{s}^{-1}$ in the presence of FCCP and SF 6847, respectively.

DISCUSSION

It is now well established that PSII reduces Q_A , the specialized, membrane-bound PQ that undergoes reduction to the unprotonated semiquinone anion (Q_A^-), which, in turn, reduces the PQ pool. Electron transfer from Q_A^- (by way of Q_B) to PQ is accompanied by uptake of protons from stroma and yields PQH_2 (4, 14).

When fully reduced, the PQ pool remains in the reduced state if no terminal electron acceptor is present. Electrons then back up and accumulate on Q_A , whose redox state can be monitored by the yield of F_v : a low yield of F_v indicates a predominantly oxidized state of Q_A and a high yield indicates a reduced state (Q_A^-) (6, 14, 15).

FIG. 7. Effect of light intensity on PQ- PQH_2 transition in the presence of protonophores. Experimental conditions were as in Fig. 4. μE , microeinstein.

FIG. 8. Effect of light intensity on the reduction of DMQ in the presence of protonophores. Experimental conditions were as in Fig. 1 with the addition of 50 μM DMQ; light path, 0.178 cm. Flattening corrections were applied as described by McCauley *et al.* (6). μE , microeinstein; Chl, chlorophyll.

In the presence of a terminal electron acceptor, which exercises a "pull" on the electron transport chain, PQH_2 is oxidized by the Rieske Fe-S center of the cytochrome *bf* complex. The oxidation separates electrons and protons, electrons being subsequently transferred to cytochrome *f* and plastocyanin, whereas protons previously taken up from stroma are liberated into the thylakoid lumen (4).

The main import of our findings is that, in the absence of an apparent terminal electron acceptor, protonophores prevented the accumulation of electrons in Q_A^- and in the PQ pool. The possibility that protonophores produced these effects by damaging the photochemical apparatus or accelerating its oxidation by ambient oxygen was experimentally ruled out. The outflow of electrons—i.e., oxidation of Q_A^- and PQH_2 —was induced by SF 6847 and FCCP even in the presence of DBMIB, an inhibitor that effectively blocks electron transfer by the only recognized pathway—i.e., from PQH_2 to the Rieske Fe-S center (4, 5). Thus, in the presence of protonophores and DBMIB, PQH_2 could not have been oxidized by the Rieske Fe-S center but must have been oxidized by an alternate pathway that is resistant to DBMIB inhibition. The oxidant of PQH_2 by this alternate pathway would most likely be cytochrome b_{559} (1), which undergoes reduction in the presence of DBMIB (21).

Next to consider is the nature of the alternate electron transport pathway. Fluorescence quenching by protonophores persisted during an extended illumination period, indicating a continuing flow of electrons through Q_A and PQ (Fig. 1). If this electron flow actuated by PSII were noncyclic (linear) in character, it would result in the production of appreciable oxygen from oxidation of water—events that could occur only in the presence of a terminal acceptor. No significant oxygen production was detected in the presence of the protonophores, and, as stated, no acceptor was added and the possibility that ambient oxygen served as an electron acceptor was also experimentally ruled out (Fig. 3). Thus, the possible induction by the protonophores of a linear electron flow is not supported by the evidence.

We concluded therefore that, in the presence of protonophores, Q_A and PQ were undergoing repeated reduction and oxidation by a light-induced, cyclic electron transport pathway peculiar to PSII, in which cytochrome b_{559} oxidized PQH_2 and was itself oxidized by P680, the photoactive chlorophyll in the PSII reaction center. Under the influence of light P680 served both as electron donor and acceptor and PQ served as a proton shuttle. A schematic representation of the cyclic pathway is given below.

As discussed more extensively elsewhere (8), the oxidation of cytochrome b_{559} by the reaction center of PSII that occurs even at cryogenic temperatures (22) is one of the best established but least understood characteristics of that redox component, whose intimate association with PSII is well known (23). It is noteworthy that an alternate pathway for the oxidation of Q_A and PQH_2 by way of cytochrome b_{559} was revealed only when special provision was made for the conductance of protons, as was done here with protonophores. The possible role of this cycle as a proton conduit under physiological conditions will be discussed separately.

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