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Association of the Chloroplastic Respiratory and Photosynthetic Electron Transport Chains of *Chlamydomonas reinhardii* with Photoreduction and the Oxyhydrogen Reaction[1]

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ABSTRACT

The hydrogenase-dependent processes, photoreduction and the dark oxyhydrogen reaction, both of which can support CO_2 assimilation, were compared with aerobic photosynthesis and respiration for their sensitivity to electron transport inhibitors in cells and intact chloroplasts of *Chlamydomonas reinhardii* 11-32/6. Photoreduction but not photosynthesis was inhibited in chloroplasts and the oxyhydrogen reaction detected only in cells was inhibited up to 75 and 90%, respectively, by 150 micromolar rotenone, indicating the involvement of a NAD(P)H-plastoquinone oxidoreductase in the hydrogen utilizing pathways. The oxyhydrogen reaction coupled to CO_2 fixation was inhibited more than 95% by 10 micromolar 2,5 - dibromo - 3 - methyl - 6 - isopropyl - *p* - benzoquinone (DBMIB), a concentration which did not affect respiratory activity. In cells, both photoreduction and the oxyhydrogen reaction exhibited a similar sensitivity to salicylhydroxamic acid (SHAM) showing approximately 90% inhibition by 7 millimolar concentration. Photosynthesis was inhibited only 30% by the same concentration of SHAM. Antimycin A (18 micromolar, 10 micrograms per milliliter) inhibited both photoreduction (80%) and the oxyhydrogen reaction (92%) in cells with the oxyhydrogen reaction being approximately 10 times more sensitive to lower concentrations of the inhibitor. Antimycin A at 18 micromolar concentration did not inhibit photosynthetic CO_2 fixation unless the cells were adapted to an atmosphere of N_2 and the reaction conducted anaerobically. Photosynthesis, photoreduction, and the oxyhydrogen reaction coupled to CO_2 fixation were all inhibited greater than 90% by 10 micromolar carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone. ATP added to chloroplasts adapted to an atmosphere of H_2 could support CO_2 uptake in the dark. These results are interpreted as evidence that photoreduction and the oxyhydrogen reaction involve some common components of thylakoidal electron transport pathways in *Chlamydomonas* including NAD(P)H-plastoquinone oxidoreductase and the plastoquinone pool. An O_2-consuming thylakoidal or mitochondrial reaction is an additional component of the oxyhydrogen reaction.

Under conditions where *Chlamydomonas reinhardii* is adapted to a hydrogen metabolism, this unicellular green alga can catalyze the reduction of CO_2 in the light through the Calvin cycle (16) coupled to the uptake of H_2. Of the two photosynthetic photoactivities, this light-driven process termed photoreduction by Gaffron (7) is dependent solely on the reactions of PSI. To explain involvement of H_2 in the process, Gibbs *et al.* (10) have proposed entry of H_2 catalyzed by hydrogenase either at the level of Fd or of a carrier (a quinone) between the two photosystems.

Adapted *C. reinhardii* can also catalyze the uptake of H_2 coupled to the consumption of O_2 in the dark (6). This oxyhydrogen reaction can be further coupled to CO_2 fixation through the Calvin cycle (16) indicating that this system may be closely associated with the chloroplast. In our studies with intact *C. reinhardii* chloroplasts, which were capable of catalyzing hydrogen-dependent photoreduction (15), we were unable to detect the oxyhydrogen reaction coupled to CO_2 reduction. Studies with *Scenedesmus* cells indicated that the oxyhydrogen reaction had properties in common with both photosynthesis and respiration (5, 16), suggesting that possibly both mitochondrial and chloroplastic reactions were required for the CO_2-coupled oxyhydrogen reaction to occur.

Recent studies on thylakoid fractions of *C. reinhardii* revealed the presence of an NAD(P)H-PQ[3] oxidoreductase similar to that associated with NADH oxidation in mitochondria (11, 12). An uncharacterized NADPH oxidizing system associated with *Chlamydomonas* thylakoids had been previously reported (2) and was interpreted to function in the metabolic pathway for carbohydrate degradation in these algae (8, 9).

The oxidation of reduced PQ by O_2 has also been shown in *Chlamydomonas* thylakoids (3) indicating that the components necessary for a minimal respiratory pathway may be present in the algal chloroplast. Hiyama *et al.* (13) have proposed from spectrophotometric measurements with a pale green mutant of *C. reinhardii* a chloroplastic cyclic pathway involving Cyt-b_{563} and its interaction with O_2. Under conditions where mitochondrial metabolism is suppressed it is possible to speculate that these chloroplast systems may play an important role in cellular energy transduction or in the regulation of chloroplast reactions.

In the present study we evaluate the possible role of the thylakoidal electron transport pathways of *C. reinhardii* in photoreduction and the oxyhydrogen reaction. This was accomplished by comparing the sensitivity of photoreduction and the oxyhydrogen reaction with aerobic photosynthesis and respiration to electron transport inhibitors in cells and in isolated chloroplast preparations.

EXPERIMENTAL METHODS

Cell Culture and Synchronization. Cultures of *Chlamydomonas reinhardii* 11-32/b were grown synchronously and photoautotrophically with CO_2 as the only carbon source as previ-

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[3] Abbreviations: PQ, plastoquinone; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; FCCP, carbonyl cyanide-*p*-trifluoromethyoxyphenylhydrazone; SHAM, salicylhydroxamic acid.

ously described (14). Cultures were routinely harvested midway through the dark cycle by centrifugation at 1300*g* for 2 to 3 min and resuspended at approximately 1 mg Chl/ml in 20 mM Tricine-NaOH (pH 7.8), 2 mM EDTA, and 1 mM $MgCl_2$. Chl was quantitated spectrophotometrically in 80% acetone (1).

Measurement of Activities. The oxyhydrogen reaction, photoreduction, and photosynthesis were followed by the incorporation of $^{14}CO_2$ into nonvolatile material at 25°C. We have not been able to detect the oxyhydrogen reaction in our isolated chloroplast preparation; thus, this reaction has been measured only in the intact cell. Cells equivalent to 20 μg Chl in the buffer described above containing 10 μM DCMU were adapted in septated vials under an atmosphere of H_2 followed by flushing with H_2 for 30 min. For the measurement of photosynthesis, DCMU was omitted from the buffer and the samples were not adapted to H_2 and assayed under air unless otherwise specified. When used, chloroplasts were isolated from lysed protoplasts (14) and assayed in the presence of 50 mM mannitol as osmoticum.

After adaptation (if required), 5 μmol $NaHCO_3$ containing 2 to 10 μCi ^{14}C/μmol were added to a final volume of 1 ml and the reaction started by the onset of illumination (100 W/m^2) for photoreduction and photosynthesis or the addition of O_2 to a final concentration of 1% (v/v) in the atmosphere above the reaction mixture for the dark oxyhydrogen reaction. At various time intervals, 100 μl aliquots were withdrawn with a syringe and injected onto planchets containing 0.2 ml of 1 N HCl. After drying over low heat the samples were counted for incorporation of ^{14}C with a Nuclear Chicago gas flow counter.

Methyl viologen reduction was measured by the method of Erbes and Burris (4) under an atmosphere of H_2. An O_2 electrode was used to evaluate the effect of inhibitors on respiration. Cells equivalent to 50 μg Chl in the same buffer as used for the photosynthetic measurements were assayed for O_2 consumption in the dark at 25°C. Inhibitors were injected after a linear rate was obtained.

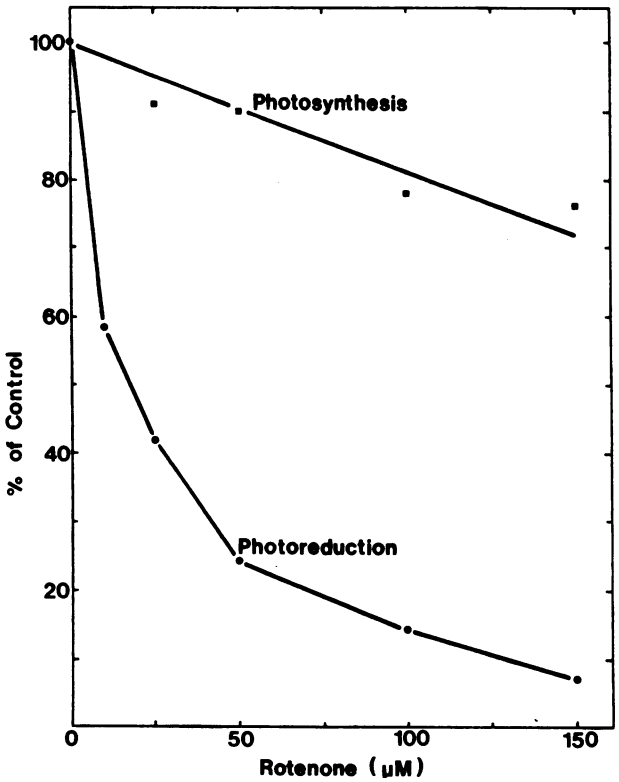

FIG. 1. Effect of rotenone on light-dependent reactions in chloroplasts. Chloroplasts were assayed at 20 μg Chl/ml in 20 mM Tricine-NaOH, pH 7.8, 1 mM $MgCl_2$, 2 mM EDTA containing 50 mM mannitol and 10 μM DCMU for photoreduction (●) or 120 mM mannitol for photosynthesis (■). Control rates were 0.95 μmol CO_2 fixed/mg Chl·h for photoreduction and 14.6 μmol CO_2/mg Chl·h for photosynthesis. Each concentration of mannitol is the optimum for the respective system.

RESULTS

Rotenone. Godde and Trebst (12) found that the NAD(P)H-PQ oxidoreductase of the *Chlamydomonas* thylakoid was inhibited by rotenone, a well known respiratory inhibitor. In *C. reinhardii* cells 150 μM rotenone inhibited respiration 70 to 90% as measured with the O_2 electrode (data not shown). Under anaerobic conditions 150 μM rotenone also inhibited H_2-dependent photoreduction of CO_2 up to 75% in chloroplasts (Fig. 1), indicating the involvement of the NAD(P)H-PQ oxidoreductase in hydrogen uptake coupled to CO_2 fixation. The same concentration of rotenone had little effect on light-driven CO_2-dependent O_2 evolution in these cells (data not shown). The oxyhydrogen reaction coupled to CO_2 reduction in whole cells was also inhibited by approximately 90% by 150 μM rotenone, but because of the presence of 1% O_2 in the reaction mixture this inhibition could be attributed to an effect on either the thylakoid PQ oxidoreductase or mitochondrial ubiquinone oxidoreductase.

DBMIB. Photoreduction, which occurs in the presence of DCMU, was previously shown to be sensitive to DBMIB (15), indicating the importance of electron flow through PQ, possibly as a cyclic pathway, for the coupling of H_2 and CO_2 uptake. The oxyhydrogen reaction coupled to CO_2 fixation was also found to be sensitive to DBMIB (Fig. 2) at concentrations inhibitory of photosynthetic electron transport but having no effect on respiration. The electron transport components involved in both photoreduction and the oxyhydrogen reaction must interact with the PQ pool of the algal thylakoid.

SHAM. Similar to DBMIB, SHAM inhibits photoreduction and the oxyhydrogen reaction coupled to CO_2 reduction in *C. reinhardii* cells by approximately 90% (Fig. 3) but at 1000-fold

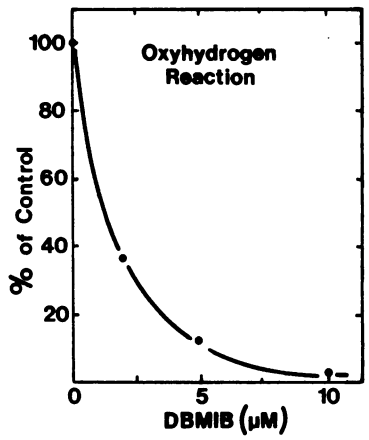

FIG. 2. DBMIB inhibition of the oxyhydrogen reaction coupled to CO_2 fixation in *C. reinhardii* cells. The reaction was started by the addition of 1% (v/v) O_2 to the atmosphere above the solution and the incorporation of $^{14}CO_2$ followed in the dark. In the absence of DBMIB, a rate of 1.8 μmol CO_2 fixed/mg Chl·h was measured.

higher concentrations. In contrast to DBMIB inhibition, SHAM reduced photosynthetic CO_2 fixation by only 25 to 30% at these concentrations indicating that the site of inhibition by SHAM was not a component of the linear electron transport pathway. Under similar conditions, using a mutant (F-60) of *C. reinhardii*, it was reported that respiratory carbohydrate breakdown was inhibited by SHAM only if KCN was also present (8), indicating that the effect of SHAM was on the alternate mitochondrial

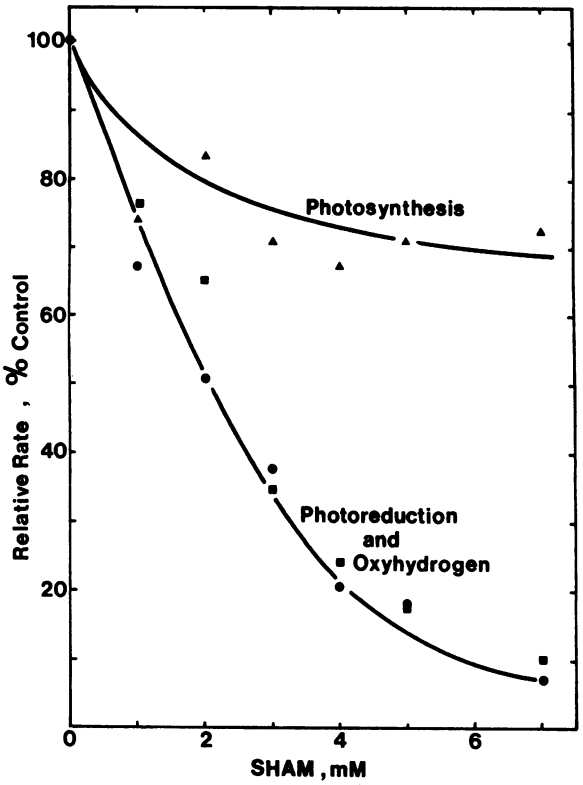

FIG. 3. Effect of SHAM on photosynthesis, photoreduction and the oxyhydrogen reaction coupled to CO_2 fixation in *C. reinhardii* cells. The control rates for photosynthesis (▲), photoreduction (●), and the oxyhydrogen reaction (■) were 144, 16, and 2.5 μmol CO_2 fixed/mg Chl·h, respectively.

respiratory pathway as expected. In contrast to this finding with the mutant, we found SHAM inhibition of both the oxyhydrogen reaction and photoreduction in the absence of KCN (Fig. 3), and using thylakoids isolated from *C. reinhardii* wild type we observed that 6 mM SHAM (and 10 μM DBMIB to a lesser extent) catalyzed a rapid uptake of O_2 when isolated thylakoids were supplied with 1 mM NADH or NADPH (data not shown). We have interpreted this as a competitive relation between PQ and SHAM for electrons from the NAD(P)H-PQ oxidoreductase followed by a rapid autooxidation of reduced SHAM. The parallel inhibition of photoreduction and the oxyhydrogen reaction by SHAM suggests that the reduction of SHAM may also occur under microaerobic and anaerobic conditions.

Antimycin A. Antimycin A was also found to be a potent inhibitor of both the oxyhydrogen reaction and photoreduction (Fig. 4) with the oxyhydrogen reaction exhibiting approximately 10 times greater sensitivity than photoreduction. Under similar conditions aerobic photosynthesis (Fig. 4) and respiration (data not shown) were not significantly inhibited. Anaerobically, photosynthetic CO_2 fixation was sensitive to antimycin A (Fig. 5) depending on the level of O_2 present in solution. In the presence of glucose and glucose oxidase to trap photosynthetically produced O_2, the sensitivity of anaerobic photosynthesis to antimycin A was enhanced (Fig. 5).

FCCP. FCCP at a concentration of 10 μM inhibits greater than 90% the CO_2 fixation associated with photosynthesis, photoreduction, and the oxyhydrogen reaction (data not shown), indicating the requirement for coupled ATP synthesis in these processes. To demonstrate the role of light (photoreduction) or O_2 (oxyhydrogen) in the production of ATP in these processes *C. reinhardii* chloroplasts were adapted under an atmosphere of 100% H_2 and provided with ATP in the dark. Added ATP

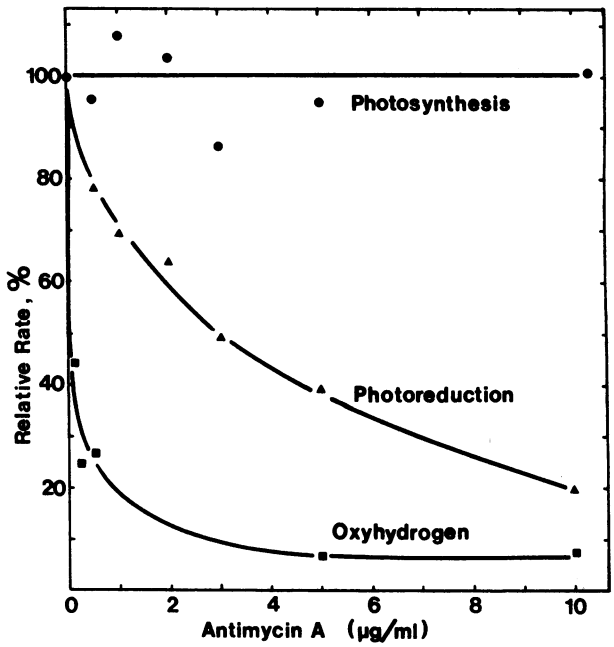

FIG. 4. Antimycin A inhibition of the oxyhydrogen reaction coupled to CO_2 fixation and photoreduction in *C. reinhardii* cells. In the absence of antimycin A the rates of photosynthesis (●), photoreduction (▲) and the oxyhydrogen reaction (■) were 72, 29.7, and 0.98, μmol CO_2 fixed/mg Chl·h, respectively.

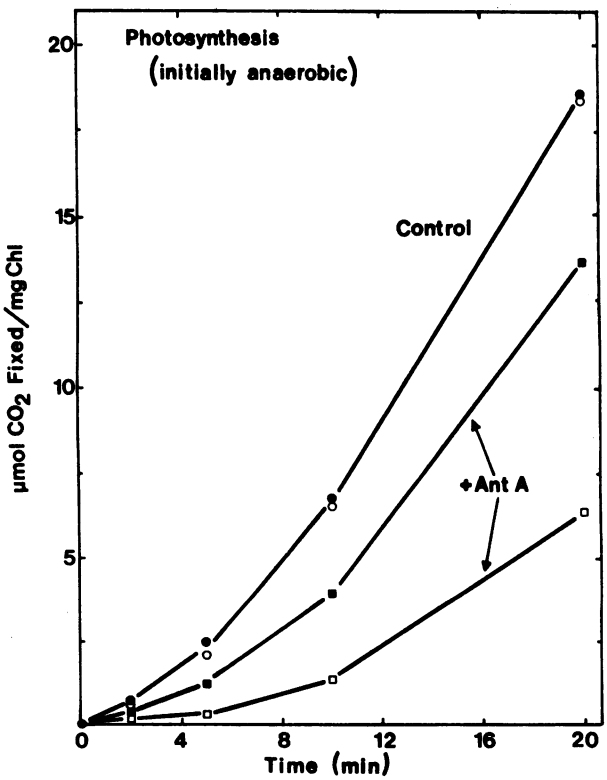

FIG. 5. Effect of antimycin A on anaerobic photosynthetic CO_2 fixation. *C. reinhardii* cells were resuspended in the reaction buffer described for photosynthesis and adapted to an atmosphere of N_2. As for aerobic photosynthesis the reaction was started by the addition of NaH $^{14}CO_3$ and the onset of illumination. All samples contained 25 mM glucose and: no further additions (●), 1 unit glucose oxidase (○), 10 μg/ml antimycin A (■), and 1 unit glucose oxidase + 18 μM (10 μg/ml) antimycin A.

increased the amount of CO_2 fixed by these plastids approximately three-fold over a control containing no ATP (Fig. 6). Since only ATP addition was required, the stromal NADPH was maintained at a level high enough to drive CO_2-fixation anaerobically. We conclude that the need for light or 1% O_2 in CO_2 fixation under anaerobic conditions is to provide coupled electron transport through PQ for the synthesis of ATP. The low level of CO_2 fixation detected in the control (Fig. 6) may have been due to endogenous levels of ATP produced by substrate level phosphorylation within the chloroplast.

Effect of Inhibitors on Hydrogenase. Using H_2-dependent methyl viologen reduction as a probe for soluble hydrogenase activity (4), we determined that with the exception of SHAM none of the inhibitors used in this study affected hydrogenase activity. At 5 mM, SHAM reduced hydrogenase activity by approximately 20% but had no effect at 2.5 mM.

DISCUSSION

The results of the inhibitor studies presented herein with *C. reinhardii* cells and chloroplasts and those using subchloroplast preparations (11, 12) indicate that the photosynthetic electron transport chain of *Chlamydomonas* has many properties in common with the mitochondrial electron transport system. The presence of a NAD(P)H-PQ oxidoreductase in *C. reinhardii* thylakoids indicates that like the cyanobacteria (17) the PQ pool functions as the crossover point between oxidative and reductive reactions in the chloroplast. Our results with DBMIB (Fig. 2), an inhibitor of PQ oxidation (11), and rotenone (Fig. 1), an inhibitor of PQ reduction (12), indicate that electron flow through PQ via the NAD(P)H-PQ oxidoreductase is an important aspect of the oxyhydrogen reaction and photoreduction. The inhibition of these reactions by antimycin A, which is known to affect a *b*-type Cyt associated with cyclic electron transport, may indicate a function for other components of the electron transport chain in association with the PQ-oxidoreductase. The extreme sensitivity of the oxyhydrogen reaction to antimycin A (Fig. 4) may indicate the presence of an additional antimycin A-sensitive component in the pathway coupling H_2 and O_2 consumption. We speculate that the PQ-oxidation process observed by Bennoun (3) functions as the oxidase for the oxyhydrogen reaction and may be antimycin A sensitive. The inhibitions of hydrogenase-dependent processes by SHAM (Fig. 3) may indicate similarities between the chloroplast oxidative pathway and the alternate

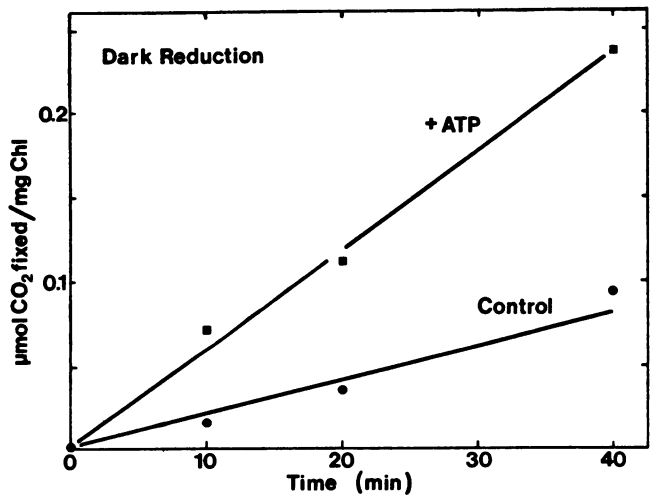

FIG. 6. Dark reduction of CO_2 by anaerobically adapted *C. reinhardii* chloroplasts. Chloroplasts were anaerobically adapted as for photoreduction. The reaction was started by the addition of NaH $^{14}CO_3$ and 10 mM ATP, and followed in the dark.

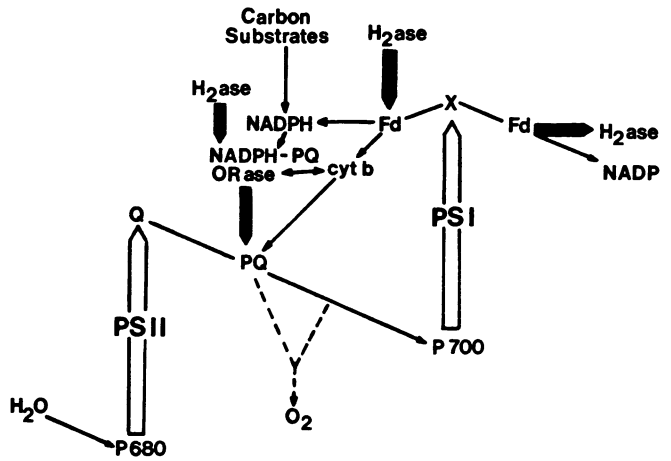

FIG. 7. Proposed scheme for the association of hydrogenase and NAD(P)H-PQ oxidoreductase with the photosynthetic and respiratory electron transport systems of the chloroplast. Abbreviations: P680, reaction center of PSII; P700, reaction center of PSI; Q, primary electron acceptor PSII; X, primary electron acceptor of PSI; H_2ase, hydrogenase; NAD(P)H-PQ ORase, NAD(P)H-PQ oxidoreductase. Inhibitor sites: antimycin A, Cyt-*b* reactions; rotenone, NAD(P)H-PQ ORase; DBMIB, PQ reactions.

cyanide-insensitive respiratory pathway of mitochondria but it is also possible that under anaerobic (or microaerobic) conditions SHAM acts as an autooxidizable PQ competitor.

In Figure 7, we present a scheme depicting the proposed relationship of hydrogenase, NAD(P)H-PQ oxidoreductase, and respiratory and photosynthetic electron transport pathways. Under anaerobic conditions in the light, overreduction of Fd is relieved by the evolution of H_2 with either H_2O or an endogenous carbon substrate acting as electron donor. Under an H_2 atmosphere, H_2 can also serve as an electron source but due to the reduced nature of Fd in the presence of light and inhibition of photoreduction by rotenone, we propose that hydrogenase donates electrons to the NAD(P)H-PQ oxidoreductase complex. In the oxyhydrogen reaction which was also sensitive to rotenone, we propose hydrogenase could again lead to the reduction of Fd or NAD(P)H-PQ reduction in the sequence of "dark" reactions.

It is worth noting that the presence of a NAD(P)H-PQ oxidoreductase in these thylakoids would allow the functioning of a cyclic electron transport pathway through NADPH in addition to the Fd-Cyt-*b* sequence established in higher plants. An interaction between Cyt-*b* and the oxidoreductase suggested by antimycin A inhibition could constitute a third cyclic pathway in the *Chlamydomonas* cell. Clearly the purpose and function of parallel cyclic pathways require further study.

In contrast to photoreduction, the oxyhydrogen reduction coupled to CO_2 fixation has not been detected in the isolated *Chlamydomonas* chloroplast following anaerobic adaptation (15). The hydrogenase of *C. reinhardii*, which when solubilized loses activity rapidly in an atmosphere of 1% O_2 (5) may have a similar sensitivity in the isolated plastid and may be measureable *in vivo* only under strictly anaerobic conditions. Confirmation of the tentative pathways proposed in Figure 7 may require constitution of algal thylakoids with more O_2-stable hydrogenases. An alternative explanation is that the oxyhydrogen sequence ($H_2 + O_2 \rightarrow H_2O$ + ATP) in isolated plastids is either poorly or not coupled to ATP synthesis. The FCCP inhibition of the cellular oxyhydrogen reaction and the stimulation of dark CO_2 uptake in the isolated chloroplast by exogenous ATP (Fig. 6) could also be interpreted as evidence for participation of mitochondrially produced ATP in this reaction as proposed by Gaffron (7).

Finally, it seems likely that *C. reinhardii* may have retained the chloroplastic respiratory pathway because of the selective advantage provided to the alga under the wide range of environmental conditions that the cells experience in nature. The ability to cycle electrons and poise the reduction level of the photosynthetic apparatus under anaerobic or microaerobic conditions could allow more efficient CO_2 fixation and enhanced growth under unfavorable conditions or survival under more severe conditions.

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