Photosynthesis of *Prochlorothrix hollandica* under Sulfide-Rich Anoxic Conditions

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The photosynthetic activity and photosystem II fluorescence of *Prochlorothrix hollandica* were studied under anoxic, sulfide-rich conditions. Oxygenic photosynthetic activity with water as the electron donor was highly resistant to inhibition by sulfide. Cells still retained 50% of their oxygenic photosynthetic activity at >1 mM sulfide. In the presence of DCMU [N-(3,4-dichlorophenyl)-N'-dimethylurea], an inhibitor of photosystem II activity, *P. hollandica* cells exhibited a low but significant anoxygenic photosynthetic activity when sulfide was present. This activity increased with higher sulfide concentrations and reached maximal rates at concentrations exceeding 1 mM sulfide. The effects of hydroxylamine on both oxygen evolution and fluorescence induction kinetics were similar to those observed for sulfide. It was concluded that the oxidizing site of photosystem II was the site of sulfide action leading to reduced or even fully inhibited electron donation to photosystem II. These observations bear similarity to the situation in some cyanobacteria in which both hydroxylamine and sulfide inhibit electron donation from H₂O to P₆₈₀. The high resistance of photosystem II to sulfide is related to the hydrophobic nature of the manganese-stabilizing protein in *P. hollandica* (T. S. Mor, A. F. Post, and I. Ohad, *Biochim. Biophys. Acta* 1141:206–212, 1993). The observed sulfide tolerance of *P. hollandica* may confer a competitive advantage in its natural environment, where it forms a dominant fraction of phytoplankton in waters in which sulfide presence is a recurring phenomenon.

Oxygenic photosynthesis is based on the light-mediated electron flow through photosystem II (PS_{II}) and PS_I, thereby generating ATP and reducing power needed for photoassimilation of inorganic carbon. Electron flow starts with the arrival of excitation energy at PS_{II} reaction centers, leading up to the donation of electrons to the primary electron acceptor, Q_A. The PS_{II} reaction centers are repopulated with electrons through a cycle of so-called S states, followed by the evolution of oxygen through water splitting. The latter process is sensitive to sulfide, which acts to inhibit oxygenic photosynthesis at the donor site of PS_{II} (18). Sulfide is in pH-dependent equilibrium with undissociated, water-soluble H₂S gas that can freely cross both the cytoplasmic and chloroplast membranes to reach its target (11). In contrast to the higher plants, many cyanobacteria are capable of coping with sulfide stress. Photosynthesis in some cyanobacteria exhibits resistance to sulfide (8). Yet other species are capable of using sulfide as an electron donor for the photoassimilation of carbon (7, 8), and electron donation occurs after PS_{II}. This type of photosynthesis is called anoxygenic photosynthesis, since it involves PS_I activity only and thus no oxygen is evolved (7, 10, 19). Efficient anoxygenic photosynthesis depends on sulfide-induced de novo protein synthesis (1, 10), and an inducible sulfide-quinone oxidoreductase that facilitates electron transport from sulfide to PS_I has been purified and characterized (2). Both facultative anoxygenic photosynthesis (in which sulfide-resistant oxygenic photosynthesis and sulfide-dependent anoxygenic photosynthesis operate in concert) and complete replacement of oxygenic photosynthesis by anoxygenic photosynthesis have been observed in cyanobacteria alone (8, 19).

Prochlorophytes are a group of photosynthetic prokaryotes

that lack pycobisomes (5, 21). Instead they possess a chlorophyll *a/b* antenna which is distinct from known eukaryotic antenna structures (15, 20). Their thylakoid organization and regulation of photosynthetic activity resemble those found in the chloroplast of eukaryotic organisms (4, 22, 23). However, phylogenetic studies indicate that prochlorophytes form a heterogeneous group among the cyanobacteria (20, 25) without any relationship to the chloroplast lineage. Like cyanobacteria they are capable of oxygenic photosynthesis, and free-living prochlorophyte species are reported to be major contributors to primary production in their natural environments (see reference 21 for a review). The filamentous free-living freshwater species *Prochlorothrix hollandica* is the only prochlorophyte that thrives in environments where the presence of sulfide is a recurring phenomenon (13, 26). Hence photosynthetic activity of *P. hollandica* is subject to sulfide presence. We report here on PS_{II} resistance to sulfide and on the presence of anoxygenic photosynthetic capability in the filamentous prochlorophyte *P. hollandica*.

MATERIALS AND METHODS

Cultivation. *P. hollandica* CCAP 1490/1 was maintained in unialgal cultures at 22°C in mineral BG 11 medium (4, 24). Light-limited batch cultures of *P. hollandica* were grown on an orbital shaker with continuous illumination provided by "warm white" fluorescent tubes at a photon flux density of 20 μmol of photons · m⁻² · s⁻¹.

Photosynthetic activity. Cells from mid-log-phase cultures were collected by centrifugation at 3,000 × g for 10 min at 20°C, washed, and then resuspended in fresh BG 11 medium. The cells were returned to culture conditions for 15 min before photosynthesis measurements were carried out. Photosynthetic rates were measured as the light-driven incorporation of acid-stable radioactive carbon, supplied as 0.1 μCi of NaH¹⁴CO₃ (specific activity, 62 mCi/mmol) (Amersham) in NaHCO₃ (final concentration, 2.5 mM) as described previously (1). Cell suspensions were bubbled with N₂ gas prior to photosynthesis measurements to remove dissolved oxygen and avoid immediate oxidation of added sulfide. The time course of ¹⁴C incorporation due to oxygenic photosynthetic activity was determined after addition of a known concentration of sulfide (pH 8.0), starting immediately upon the addition of the radiolabelled bicarbonate mix. Anoxygenic photosynthetic activities were determined likewise but after addition of 10⁻⁶ M

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FIG. 1. Time course of photosynthetic carbon fixation by *P. hollandica* cells in the presence of 3.5 mM sulfide compared to control cells incubated in the light, cells incubated in the light in the presence of 10^{-6} M DCMU, and cells incubated in the dark.

DCMU [*N*-(3,4-dichlorophenyl)-*N'*-dimethylurea] in order to inhibit photosystem II activity. Light intensity during measurements was $100 \mu\text{mol of photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, allowing maximum photosynthetic activity in *P. hollandica* (4). Sulfide concentrations were monitored over the course of the photosynthesis measurements according to the method of Cline (6). Sulfide remained essentially constant during the course of all experiments addressing the effects of sulfide concentration on photosynthetic carbon fixation. The effects of hydroxylamine (pH 8.0), an inhibitor of photosynthetic oxygen evolution, were determined from oxygen measurements by using a YSI 5330 electrode connected to a homemade multigain amplifier (9). Chlorophyll *a* was determined spectrophotometrically on 90% acetone extracts of cells collected by centrifugation of 10-ml samples at $3,000 \times g$ for 10 min at 4°C. Chlorophyll *a* concentrations were calculated according to the method of Jeffrey and Humphrey (12).

Fluorescence induction. Chlorophyll *a* fluorescence induction was determined with a custom-built fluorometer with digitized data acquisition as described in a previous publication (23). Light from a xenon light source was passed through a broad-wavelength-band blue filter (400 to 550 nm) to provide appropriate excitation light. Emission was detected by a photodiode fitted with a Schott long-pass filter to transmit wavelengths of >680 nm only. Fluorescence induction kinetics were determined for 3 s upon the opening of a Uniblitz 100-2B (Vincent Associates) electronic shutter. The fluorescence signal was converted by an 8-bit A/D converter and subsequently recorded. Fluorescence kinetics were recorded following dark adaptation of the sample for 1 min unless otherwise specified. Maximal fluorescence was determined after addition of 10^{-6} M DCMU. Hydroxylamine and Na-sulfide were added 1 min before the sample was subjected to excitation light.

RESULTS AND DISCUSSION

Photosynthetic ^{14}C fixation by *P. hollandica* cells at $100 \mu\text{mol of photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ occurred in a linear fashion for more than 4 h in control cells (Fig. 1). A constant but low photosynthetic activity was apparent in the presence of 3.5 mM sulfide. This activity was significantly higher than those observed for cells incubated either in the dark or in the light in the presence of DCMU, a herbicide that specifically inactivates PS_{II}. Photosynthetic carbon fixation was progressively inhibited with increasing sulfide concentrations (Fig. 2), and 50% inhibition of photosynthetic activity was observed at approximately 1.2 mM sulfide. The ability to maintain oxygenic photosynthesis at such concentrations is indicative of high resistance to sulfide in *P. hollandica*. Green chloroplasts and many cyanobacterial species are fully inhibited by sulfide at concentrations as low as 200 μM (7, 8, 10, 11, 18). The inhibitory effect

FIG. 2. Effects of ambient sulfide concentration on sulfide-tolerant oxygenic photosynthesis rates (●) and sulfide-dependent anoxygenic photosynthesis rates (▲) determined in the presence of 10^{-6} M DCMU for cultures of *P. hollandica*. Photosynthesis measurements started immediately upon the addition of sulfide and continued for 4 h. Error bars indicate standard deviations of mean values. Chl, chlorophyll.

of sulfide was fully reversible, and upon its removal cells immediately resumed photosynthetic activity at the rates observed in control cells. In the presence of DCMU we observed light-driven carbon fixation with rates increasing with sulfide concentration and saturating at about 2 mM (Fig. 2). This finding suggests that sulfide may function as an electron donor in linear electron flow through PS_I in *P. hollandica*, as has been reported for a number of cyanobacteria (7, 8, 10). This anoxygenic photosynthetic activity, present immediately on exposure of aerobically grown cells to sulfide, occurred at 1 to 4% of the rate of oxygenic photosynthesis. Rates of anoxygenic photosynthesis in some cyanobacteria increase during a 2- to 10-h induction period following transfer to sulfide-rich conditions (7, 10), the time required for de novo synthesis of protein involved in electron transport between sulfide and PS_I (1, 2, 10). Rates exceeding the observed rates of anoxygenic photosynthesis could not be induced by prolonged incubation (4 to 24 h) of *P. hollandica* in medium supplied with sulfide.

Hydroxylamine is a well-known inhibitor of PS_{II} activity which, like sulfide, affects the water-splitting process at the donor site of PS_{II} (18). Oxygen evolution, at a photon flux of $100 \mu\text{mol of photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, was progressively inhibited as the concentration of hydroxylamine increased above 10^{-4} M (data not shown). At a hydroxylamine concentration of 1.25 mM, photosynthetic oxygen evolution was reduced to 50% of the activity of control cells. Both hydroxylamine and sulfide inhibited photosynthetic carbon fixation at concentrations ranging from 0.5 to 5 mM, thus indicating that the donor site of PS_{II} in *P. hollandica* was indeed well protected against the action of both compounds. In this respect it is interesting to note that the manganese-stabilizing protein of *P. hollandica* is much more hydrophobic than its counterpart in common green algae and cyanobacteria (16, 17). The hydrophobic nature of this protein could result in an oxygen-evolving complex which is more deeply embedded in the thylakoid membrane (17). Such a configuration may provide an effective shield against the action of both sulfide and hydroxylamine, since it would lead to less exposure of the protein to inhibiting compounds present at the luminal side of the thylakoid membrane.

FIG. 3. Digitized representation of fluorescence induction curves of *P. hollandica* cells which were light adapted (thin solid curve), dark adapted with and without DCMU (dotted curves), and dark adapted with and without DCMU in the presence of 1 mM H_2S (thick solid curves) prior to measurement. F_{max} was determined in the presence of 10^{-6} M DCMU for all treatments. Note that the interrupted x-axis indicates the change in time scale between the determination of F_0 and the other fluorescence parameters. A.U., arbitrary units.

Further analysis of the mode of action of sulfide on PS_{II} activity in *P. hollandica* was obtained from fluorescence induction kinetics in dark-adapted cells upon exposure to excitation light. The fluorescence induction signal consists of two main components: (i) a fast fluorescence rise associated with fluorescence emission by excited chlorophylls of PS_{II} and its antenna and (ii) a slow fluorescence rise associated with enhanced chlorophyll *a* fluorescence caused by lower rates of charge separation in PS_{II} reaction centers containing reduced Q_A , the primary electron acceptor (for a review see reference 14). Cells immediately respond to the excitation light with initial fluorescence (F_0), which reaches an intermediary plateau after 3 to 4 ms (Fig. 3). The F_0 values for *P. hollandica* were similar to those seen in dark-adapted cells of green algae like *Chlamydomonas reinhardtii* which have oxidized Q_A and PQ pools (22). Preillumination with 710-nm light was not instrumental in further lowering F_0 in control cells of *Prochlorothrix*, indicating that its quinone pool is largely oxidized in the dark (22). From F_0 , fluorescence continued to rise in intensity following a typical transient before reaching steady-state fluo-

rescence (F_s) after approximately 1.5 s (Fig. 4). This transient, the so-called Kautsky transient, is related to dynamic changes in the redox state of the Q_A pool during establishment of steady-state electron flow between PS_{II} and PS_{I} . In the presence of DCMU, the efficient inhibition of electron transfer from Q_A to PQ caused all reaction centers of PS_{II} to eventually contain reduced Q_A , and the maximal fluorescence intensity (F_{max}) was reached after 100 ms (Fig. 3). The range between F_0 and F_{max} is the range of variable fluorescence (F_v). Since F_0 is indicative of PS_{II} with fully oxidized Q_A and F_{max} is indicative of PS_{II} with fully reduced Q_A , F_v thus reflects the reduced state of Q_A , the result of electron flow from the oxygen-evolving complex to the acceptor site of PS_{II} . F_v values obtained for *P. hollandica* are reminiscent of those found in green chloroplasts rather than those found in cyanobacteria (22). F_0 values for light-adapted cells were 50% higher than those seen in dark-adapted *Prochlorothrix*. A Kautsky transient was absent from light-adapted cells. F_s in light-adapted *Prochlorothrix* was reached after less than 1.5 s and was maintained until the end of the measurement (Fig. 3). Addition of 1 mM sulfide to dark-adapted *P. hollandica* cells yielded a fluorescence transient in which F_0 was nearly that of dark-adapted *P. hollandica* cells (Fig. 3). The inhibitory effect of sulfide on the potential of PS_{II} to generate reduced Q_A was immediately apparent from the observation that F_v only initially reached the level of F_s in light-adapted cells, after which it declined. Steady-state fluorescence was reached after 2 s and was only slightly higher than F_0 . This implies that in the presence of sulfide, PS_{II} of *P. hollandica* retains a low but significant potential to reduce Q_A which could support the lower rates of oxygenic photosynthesis shown in Fig. 1 and 2. Consistent with the observations discussed above on sulfide and hydroxylamine inhibition of photosynthesis, these findings show that sulfide lowers the rate at which Q_A is reduced by PS_{II} . This notion is further supported by the observation that maximal fluorescence levels were attained at a lower rate in sulfide-treated cells (Fig. 3). These findings are very similar to those reported for *Aphanethece halophytica* and *Synechococcus* strain PCC 6311 (18) indicating that (i) sulfide acts at the PS_{II} reaction centers in *P. hollandica* and (ii) residual oxygenic photosynthesis can be maintained in *P. hollandica* in the presence of sulfide.

Whereas increasing sulfide concentrations did not affect the F_{max} , we observed a steep decline in the F_s of the cells (determined after 2.5 s) between concentrations of 0 and 1 mM sulfide, followed by a shallow decline until at 5 mM F_s reached

FIG. 4. (A) F_s (●) and F_{max} (■) in the presence of 10^{-6} M DCMU (■) of *P. hollandica* cells as a function of increasing sulfide concentrations. The dotted line indicates the average level of F_0 . (B) F_0 (●) and F_{max} (■) in the presence of 10^{-6} M DCMU (■) of *P. hollandica* cells as a function of increasing sulfide concentrations. The dotted line indicates the average level of F_{max} . A.U., arbitrary units.

the level of F_0 (Fig. 4A). This pattern of sulfide-controlled decrease in the reduced state of PS_{II} paralleled the pattern of inhibition of carbon fixation at increasing sulfide concentrations. The low but continued photosynthetic carbon fixation in the presence of sulfide is accompanied by distinct PS_{II} resistance to this compound, as shown from the fluorescence patterns. Sulfide concentrations at <5 mM could not fully inhibit a flux of electrons through P_{680} , resulting in a partial reduction of Q_A . Hydroxylamine addition yielded fluorescence induction curves with characteristics identical to those obtained for sulfide-treated *P. hollandica* cells (data not shown), as is observed in cyanobacteria (18). F_0 and F_{max} were not affected by the addition of hydroxylamine, but the Kautsky gradient was strongly reduced and the cells attained a lower F_s than did control cells. These findings confirm the above observation that the oxidizing site of PS_{II} is indeed the site of action of both sulfide and hydroxylamine.

The addition of DCMU caused a pronounced rise in F_0 in dark-adapted control cells (Fig. 3). The rise in F_0 became more pronounced with increasing sulfide concentrations, and F_0 approached 80% of F_{max} values at a concentration of 5 mM (Fig. 4B). If electron flow from the donor site of PS_{II} is efficiently inhibited by sulfide, one would expect F_0 levels in the presence of DCMU to be similar to those of control cells. However, the increase in fluorescence with elevated sulfide concentrations indicates an increase in the reduced state of PS_{II} and thus an increase of Q_A^- -containing PS_{II} s in the dark. Hence, sulfide was somehow involved in reducing Q_A to Q_A^- in the dark, and Q_A^- accumulated due to the DCMU-impaired flow of electrons to PQ. If sulfide is capable of reducing Q_A , one would expect it to be capable of reducing PQ also, possibly mediated by a quinone reductase as reported for *Oscillatoria limnetica* (1, 2). Such a scenario would be consistent with the anoxygenic photosynthetic capacity observed in *P. hollandica*, since it would enable electron transfer from sulfide to PS_I via the PQ pool.

Both the presence of an anoxygenic photosynthetic capability and the resistance of PS_{II} to sulfide have interesting ecological implications. Upon inhibition of PS_{II} activity, sulfide may act as an electron donor to PS_I in *Prochlorothrix* cells. The physiological importance of this phenomenon remains unclear due to the low rates of anoxygenic photosynthesis. However, such rates may suffice to meet the low maintenance requirements of cyanobacteria (27), including those of *Prochlorothrix*. Furthermore, the very presence of such an activity opens the perspective that not only sulfide but other compounds as well may function as alternative electron donors in photosynthetic carbon fixation. It was reported that dimercaptopropanol is an efficient electron donor in anoxygenic photosynthesis by *Oscillatoria limnetica* (3). The employment of alternative electron donors in anoxygenic photosynthesis could assist the cells in surviving extended periods of anoxic stress.

P. hollandica is found in shallow turbid lakes throughout northwestern Europe (26). The presence of organic matter, the shallow photic layer, and the relatively large impact of sediment on water chemistry in these lakes all contribute to frequently recurring anoxic conditions and hence sulfide accumulation (14). Since many algae suffer irreversible damage by even minute concentrations of sulfide, an ability to photosynthesize in the presence of sulfide provides cells with important means for survival (8, 10, 11, 19). The unusually high resistance of PS_{II} to sulfide stress may thus be of competitive advantage to *Prochlorothrix*, dominant among phytoplankton in shallow Dutch lakes (26).

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