Low Redox Potential Promotes Sulphide- and Light-dependent Hydrogen Evolution in Oscillatoria limnetica

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Anoxygenic photosynthetic electron transport from sulphide, culminating in either H₂ evolution or CO₂ photoassimilation, was shown to include the segment from plastoquinone to ferredoxin in the cyanobacterium Oscillatoria limnetica. Both sulphide-dependent H₂ evolution and CO₂ photoassimilation were inhibited by plastoquinone analogues. In the former reaction, the block was bypassed by reduced N,N',N',N'-tetramethyl-p-phenylenediamine (TMPD). The link between this segment of electron transport and the hydrogenase enzyme was shown to limit the rate of sulphide-dependent H₂ evolution. The rate of flow of electrons through this pathway was lower than would be expected either from the amounts of available enzyme, as measured by the in vitro oxidation of reduced methyl viologen, or from rates of electron transport to CO₂ photoassimilation. When the strong reductant sodium dithionite was added to intact cells, the resulting low redox potential significantly improved photosynthetic sulphide-dependent H₂ evolution. Hydrogenase activity in cell free extracts was similarly affected by dithionite. It is suggested that ambient redox potential controls electron flow through the hydrogenase, so that surplus reducing power is removed via H₂ evolution.

INTRODUCTION

Light-dependent utilization of sulphide electrons by the cyanobacterium Oscillatoria limnetica requires a preliminary induction period in the presence of sulphide (Cohen et al., 1975a; Belkin & Padan, 1978a, b). Low redox potential shortens the length of this adaptation. In the presence of dithionite (10 mM) the lag at pH 6.8 is eliminated (Belkin & Padan, 1983). Once adaptation is complete and sulphide electrons enter the photosynthetic electron transport chain, they can be channelled to three different acceptors: (a) CO₂, when a normal mode of anoxygenic photosynthesis takes place (Cohen et al., 1975a, b; Oren et al., 1977, 1979; Oren & Padan, 1978; Padan, 1979); (b) protons, when no CO₂ is available or when CO₂ fixation is blocked (Belkin & Padan, 1978a, b); (c) N₂, when no combined nitrogen is present (Belkin et al., 1982).

The anoxygenic CO₂ photoassimilation reaction is unaffected by DCMU, the photosystem II inhibitor (Cohen et al., 1975a) and inhibited by the ferredoxin-blocking agent DSPD (Belkin & Padan, 1978b). The sulphide-dependent H₂ evolution reaction is mediated by an hydrogenase (Belkin & Padan, 1978a, b) and like the former reaction it is insensitive to DCMU and inhibited by DSPD. Both systems thus share photosynthetic electron transport driven by photosystem I. This communication deals with this electron transport segment and its linkage to the hydrogenase for H₂ evolution.

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP-INT, 2,4-dinitrophenyl ether of 2-iodo-4-nitrothymol; DSPD, dicarboxyliciminedipropanediamine; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; MES, 2-(N-morpholino)ethanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; TAPS, tris(hydroxymethyl)methylaminopropanesulfonic acid; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine.

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Light-driven H₂ production is basically an energy-wasteful process. However, under certain conditions, it can be utilized to rid the cells of excess reducing power, as suggested by Hillmer & Gest (1977). To investigate the influence of low redox potential on H₂ evolution in *O. limnetica*, we studied the effect of the strong reductant sodium dithionite on both intact cells and cell-free extracts. The rate-limiting step for sulphide-dependent dithionite.

**METHODS**

*Growth of cyanobacterium. Oscillatoria limnetica* (Cohen et al., 1975a) was grown aerobically or anaerobically as previously described (Oren et al., 1977; Oren & Padan, 1978). Exponential growth phase, in both cases, lasted 5–6 d.

**Preparation of cells for experiments.** Cells from exponential phase, aerobically grown cultures were harvested, washed and resuspended (10–20 μg chlorophyll a ml⁻¹) in an anaerobic growth medium lacking Na₂CO₃ and containing 10 μM-DCMU. The medium was buffered with 25 mM-HEPES for the pH range 7.0–8.2, and 25 mM-MES or TAPS were used for lower and higher pH, respectively. Anaerobically grown cultures were similarly treated after precipitation of the sulphur deposited during anaerobic growth by a low-speed centrifugation (1000 g) for 15 s.

**Cell-free extracts.** Cells were washed, resuspended (50 μg chl. a ml⁻¹) in 10 mM-HEPES buffer, pH 8.0, and preincubated for 5 min in the dark at 35 °C under N₂; lysosome was then added to a final concentration of 1 mg ml⁻¹. Cell disruption was followed microscopically and by release of phycocyanin into the medium, as measured at A₅₃₅. Complete cell burst was evident after 30–45 min incubation.

**H₂ evolution.** Serum bottles (15 ml) containing 2 ml of intact or disrupted cell suspension were sealed with serum stoppers, flushed with N₂ for 20 min to obtain anaerobic conditions and incubated at 35 °C with shaking, with or without illumination (2.5 mJ cm⁻² s⁻¹). For induction, 3-5 mM-Na₂S was added and the cells were preincubated for 3 h in the light. They were then washed and resuspended in the N₂-flushed medium lacking Na₂S. All additions and withdrawals were made anaerobically with syringes after the N₂ flushing. H₂ was determined in gas samples as previously described (Belkin & Padan, 1978a). H₂ evolution from reduced methyl viologen was assayed in cell-free preparations in the dark, containing, in 2 ml, 25 μg chlorophyll a ml⁻¹, with 2.5 mM-methyl viologen reduced by 10 mM-sodium dithionite, in 20 mM-HEPES, pH 8.0 (35°C).

**Redox titrations.** Determination of redox potential was carried out in an anaerobic water-jacketed (35 °C) chamber, using a platinum electrode (Ingold, type 10-805-3000) with a calomel reference electrode (Radiometer, type K401). Both electrodes were connected to a pH meter (Radiometer model 26). For simultaneous pH monitoring a combined pH electrode (El-Hama, type 1160-400) was also inserted in the assay chamber and connected to an El-Hama PBS730 pH meter; samples of sodium dithionite (200 mM, in anaerobic 250 mM-MES or HEPES buffer) were added stepwise to 20 ml of the standard reaction medium which included 3-5 mM-Na₂S. Redox potential was recorded after each addition. H₂ evolution was determined in gas samples with or without illumination (2.5 mJ cm⁻² s⁻¹).

**CO₂ photoassimilation.** This was determined in intact *O. limnetica* cells under identical conditions to the H₂ evolution assay, but with NaH¹⁴CO₃ injected after the N₂ flushing to a final concentration of 14 mM and a specific activity of 0.06 Ci mol⁻¹ (2.2 GBq mol⁻¹). The radioactivity incorporated into the cells was determined as previously described (Oren et al., 1977).

**Chlorophyll and sulphide.** There were determined according to previously described methods (Mackinney, 1941; Trüper & Schlegel, 1964).

**Chemicals.** Methyl viologen, HEPES, MES, TAPS, and TMPD were purchased from Sigma and NaH¹⁴CO₃ from Amesham. DCMU and FCCP were obtained from Dupont, Wilmington, Delaware, U.S.A. DBMIB and DNP-INT were a gift from Professor M. Avron and Dr Y. Shachak of the Weizmann Institute of Science, Rehovot. Na₂S.9H₂O was from BDH and sodium dithionite from Riedel, Seelee-Hannover, F.R.G.

**RESULTS**

The sulphide-dependent electron transport shared by H₂ evolution and CO₂ photoassimilation

The effect of the plastoquinone antagonist DBMIB on sulphide-dependent H₂ evolution was examined. Activity was inhibited by 75–80% by 2 x 10⁻⁵ M-DBMIB (Table 1), localizing the quinone in the electron transport sequence from sulphide. The electron carrier TMPD, which, when reduced, donates electrons to plastocyanin (Trebst & Reimer, 1977) and therefore bypasses the DBMIB-blocked site, restored activity to the original rate. Similar results were
Low redox- and sulphide-dependent H₂ evolution

Table 1. Effect of quinone analogues on sulphide-dependent hydrogen evolution

Two-d-old cells from an anaerobically grown O. limnetica culture were washed and resuspended (14 µg chl. a ml⁻¹) in 2 ml of the anaerobic growth medium in the absence of CO₂, and in the presence of 10 µM-DCMU and 25 µM-HEPES, pH 7.5. Following a 20 min flushing with N₂ and an anaerobic addition of 3 mM-Na₂S, H₂ evolution was assayed in the light.

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<th>Additions (m)</th>
<th>H₂ evolution rate</th>
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<tr>
<td></td>
<td>µmol (mg chl. a)⁻¹ h⁻¹</td>
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<tr>
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<td>DBMIB (2 x 10⁻⁵)</td>
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<td>DBMIB (2 x 10⁻⁵) and TMPD (10⁻⁴)</td>
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<td>DNP-INT (2.5 x 10⁻⁵)</td>
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<td>DNP-INT (2.5 x 10⁻⁵) and TMPD (10⁻⁴)</td>
<td>10.8</td>
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obtained with another plastoquinone inhibitor, DNP-INT, (Trebst et al., 1978) (Table 1). Although the results described in Table 1 refer to experiments conducted with anaerobically grown cells, similar results were obtained with cells induced with sulphide for only 3 h. Similar to its effect on H₂ evolution, DBMIB inhibited sulphide-dependent CO₂ photoassimilation by 80% (not shown). Both reactions were previously shown to be inhibited by the ferredoxin blocking agent DSPD (Belkin & Padan, 1978b). Hence it is suggested that the segment of the photosynthetic electron transport chain shared by both sulphide-dependent H₂ evolution and CO₂ photoassimilation includes the quinone, and proceeds through photosystem I up to ferredoxin.

The hydrogenase activity limits the rate of sulphide-dependent hydrogen evolution

The electrons destined for H₂ evolution exhibited much slower transport rates than those channelled to CO₂ fixation. After a 3 h induction, only 2.4 µequiv. e⁻ (mg chl. a)⁻¹ h⁻¹ were expelled as molecular H₂, whereas the rate of electron transfer to CO₂ was 280 µequiv. e⁻ (mg chl. a)⁻¹ h⁻¹. Similar results were obtained with anaerobically grown cells, although in the latter system both activities markedly increased. After 3 d anaerobic growth, anoxygenic CO₂ photoassimilation rates increased twofold to 530 µequiv. e⁻ (mg chl. a)⁻¹ h⁻¹, whereas rates of electron transfer to H₂ evolution increased more than 12-fold to 30 µequiv. e⁻ (mg chl. a)⁻¹ h⁻¹. Even after this dramatic increase, however, nearly 95% of the electron transfer potential exhibited by the CO₂ photoassimilation reaction was not utilized for H₂ evolution. It was concluded that a rate-limiting step exists in the electron pathway from sulphide to H₂ and that this must be due to factors located beyond the common segment shared by both reactions, namely the specific electron pathway diverged to hydrogenase, and/or hydrogenase itself.

Hydrogenase potential activity can be estimated in cell-free preparations by measuring dark H₂ evolution from dithionite-reduced methyl viologen. After a 3 h induction period, the amount of enzyme present in O. limnetica cells maintained a rate of H₂ evolution equivalent to 18.6 µequiv. e⁻ (mg chl. a)⁻¹ h⁻¹, which is nearly eight times higher than the light- and sulphide-dependent reaction via the electron transport chain. This pattern did not change significantly during anaerobic growth, since both the enzyme level and the rate of photosynthetic H₂ evolution increased similarly. The total hydrogenase activity was then 140 µmol H₂ evolved (mg chl. a)⁻¹ h⁻¹, which was five times higher than the photosynthetic reaction (Fig. 1). Thus the actual light-dependent expression of the enzyme comprised, at best, only 20% of the total potential activity of the enzyme. It is thus suggested that the rate-limiting factor in sulphide-dependent H₂ evolution is not the total hydrogenase level, but rather its in vivo activity.

Low redox potential modulates hydrogenase activity

Dithionite cannot serve as an electron donor for photosynthetic reactions in aerobically or anaerobically grown O. limnetica (Oren, 1978 and Fig. 2). However, when it was added to cells, induced for 3 h in the presence of sulphide, a fivefold increase from 1 to 5 µmol H₂ (mg chl.
H₂ evolution during anaerobic growth in the absence (○) or presence (●) of 10 mM-sodium dithionite in the light at pH 8-0, and dark H₂ evolution by disrupted cells (▲) in the presence of 2.5 mM-methyl viologen reduced by 10 mM-sodium dithionite.

Fig. 2. Acceleration by sodium dithionite of sulphide-dependent H₂ evolution in 3 h induced O. limnetica cells. The pH was 7.3 and the following additions were made at zero time: □, none; ■, sodium dithionite (10 mM); ▲, Na₂S (3.5 mM); ●, Na₂S and sodium dithionite.

a)-1 h-1 occurred in light- and sulphide-dependent H₂ evolution. Although to a lesser extent, even the already enhanced rates of H₂ evolution of anaerobically grown cells were significantly increased by the addition of sodium dithionite to the reaction mixture (Fig. 1). In all cases, the light saturation behaviour of H₂ evolution did not change (not shown), and the reactions were carried out under light-saturating conditions.

The effect of dithionite on the rate of H₂ evolution was pH independent. In the presence of 10 mM of this compound, a constant maximal rate of 6 µmol H₂ (mg chl. a)-1 h-1 was observed throughout the pH range 6.5-9.0. When the reaction was measured in the presence of sulphide alone, however, the same high rates were attained only at pH values greater than 8.2; activity decreased to 4 µmol H₂ (mg chl. a)-1 h-1 at pH 8.0 and was virtually zero at pH 7.1.

Dithionite has a very low redox potential of about -450 mV (Van Dijk & Veeger, 1981), which may be the factor responsible for its effect on H₂ evolution. Sulphite, the oxidized decomposition product of dithionite, had no significant effect on the reaction. To further test the effect of low redox potential, cells were exposed to different dithionite concentrations at two pH values, and both ambient redox potentials and H₂ evolution were measured. At the two pH values tested, 6.6 (Fig. 3a) and 7.5 (Fig. 3b) the redox potentials maintained by sulphide, prior to the introduction of dithionite, were around -120 mV and -180 mV respectively, and H₂ evolution rates were minimal. These rates dramatically increased with the addition of dithionite and with the ensuing decrease in redox potential. Even though different concentrations of dithionite were needed to create identical redox potentials at the two pH values, maximal rates were observed in both cases when the redox was lowered to -420 mV. It is concluded that at least up to pH 7.5, the low ambient redox potential imposed by dithionite is indeed the factor responsible for the acceleration of H₂ evolution rates, and that a certain redox value, around -420 mV, allows maximal in vivo activity.

The effect of redox potential on hydrogenase activity in cell-free extracts was measured at pH 7.5, the optimum pH for the enzyme and also the cytoplasmic pH in many prokaryotes including cyanobacteria (Falkner et al., 1976; Padan et al., 1981). The hydrogenase-mediated H₂ evolution was found to be dependent on redox potential in a pattern very similar to that of intact cells (Fig. 4).
Low redox- and sulphide-dependent $H_2$ evolution

Fig. 3. Ambient redox potential (○) and sulphide-dependent $H_2$ evolution (●) at pH 6.6 (a) or pH 7.5 (b). After the N$_2$-flushing, cells were incubated in the presence of 3.5 mM Na$_2$S and various concentrations of sodium dithionite. $H_2$ evolution was measured after linear rates had been established following the lag period.

Fig. 4. Redox potential (○) and in vitro hydrogenase activity (●) with different concentrations of Na$_2$S$_2$O$_4$ at pH 7.5. Maximal activity (100%) was $1.2 \mu$mol $H_2$ evolved (mg chl. a)$^{-1}$ h$^{-1}$.

DISCUSSION

Both anoxygenic sulphide-dependent $H_2$ evolution and CO$_2$ photoassimilation were shown to be dependent on photosystem I and to share the photosynthetic electron pathway up to ferredoxin. This common chain includes the plastoquinone site, since the quinone inhibitors DBMIB and DNP-INT inhibited both reactions. Furthermore, $H_2$ evolution was restored in the DBMIB-inhibited system when reduced TMPD, which bypasses the block (Trebst & Reimer, 1977), was added to the reaction mixture. Phosphorylation coupled to $H_2$ evolution has been implicated, since the uncoupler FCCP accelerates $H_2$ evolution (Belkin & Padan, 1978 a). This coupling site is most probably located at the quinone level, since FCCP has no enhancing effect on the reduced TMPD bypass to plastocyanin (Belkin et al., 1982). These results are in accordance with recent data implying a quinone in photophosphorylation or oxidative phosphorylation (Mitchell, 1979).

Dithionite markedly accelerated the rate of sulphide- and photosystem I-dependent $H_2$ evolution in _O. limnetica_. It was however, incapable of replacing sulphide as an electron donor for anoxygenic photosynthetic $H_2$ evolution and may thus only alleviate a rate-limiting step. This could occur at three levels in the pathway of the electrons from sulphide to $H_2$: the hydrogenase, the photosynthetic electron transport and the linkage between these two components. The level of the hydrogenase enzyme in these cells as measured in vitro was very
high, and is therefore unlikely to limit the rate of sulphide- and light-dependent electron flow to H₂ evolution in vivo. Whereas the enzyme could maintain a rate of H₂ evolution equivalent to 18.6 and 140 μequiv. e⁻ (mg chl. a)⁻¹ h⁻¹ in 3 h induced or anaerobically grown cells respectively, the corresponding light-dependent activity was only 13–21%. The electron transport rate through the pathway from plastoquinone to ferredoxin, which is common to both CO₂ photoassimilation and H₂ evolution, cannot include the rate-limiting step of the latter, since the electron flow culminating in CO₂ fixation proceeds at least 18 times faster. Therefore it is concluded that the specific electron pathway to the hydrogenase, branching from the main transport chain, includes the rate-limiting step to H₂ evolution. This step is most probably the one affected by dithionite.

Simultaneous measurements of the ambient redox potential imposed by dithionite on intact cells and the in vivo sulphide-dependent H₂ evolution, as well as similar experiments with cell-free enzyme preparations, revealed that the lower the redox value, the higher the activity. The redox potential below which maximal H₂ evolution rates were attained was around -420 mV, similar to the midpoint potential of both the H₂/H⁺ couple and ferredoxin. Assuming that dithionite reduces both the external and the cytoplasmic redox potentials, it is suggested that the in vivo activity of the hydrogenase is modulated by the environmental redox potential. Dithionite had no effect on the rate of H₂ evolution above pH 8.0, when even in its absence similar rates of sulphide-dependent H₂ evolution were measured. At this point the ambient redox potential maintained by sulphide was measured to be -200 mV. It is suggested that, for an unknown reason, at this pH such a potential suffices to allow optimal flow of photosynthetically driven electrons to hydrogen.

The influence of redox potential on hydrogenase activity has been described for the purified enzymes of several bacteria (Chen, 1978; Van Dijk & Veeger, 1981; Fernandez et al., 1982). In most of these cases, similarly to our results, maximal activity was reached below a certain potential, around -400 to -450 mV. The importance of the redox state in the activity of ferredoxin has also been described (Carter et al., 1972; Cammack & Christou, 1978) and control of photosynthetic enzymes by redox potential via the ferredoxin/thioredoxin system has been suggested (Buchanan, 1980).

Enhanced H₂ evolution under highly reducing conditions was caused, in our case, by externally supplied dithionite. We suggest, however, that this effect mimics a physiological control mechanism, with H₂ evolution acting as a valve regulating the intracellular redox environment. Such a role has been attributed to the nitrogenase-mediated H₂ production of photosynthetic bacteria (Hillmer & Gest, 1977) and to the hydrogenase-involving reactions of several heterotrophic bacteria (Adams et al., 1981) and eukaryotic algae (Kessler, 1974; Hallenbeck & Benemann, 1979). The existence of such a redox-controlled 'switch' has recently been suggested in Proteus mirabilis, where electrons can be selectively channeled towards the reduction of either protons or other oxidants (Krab et al., 1982).

Since H₂ evolution is very wasteful in terms of energy consumption, it is essential that it operates only when the system is over-reduced. Indeed, in O. limnetica, in the presence of ascorbate-reduced TMPD under anaerobic conditions, the rate of H₂ evolution is very low (not shown). In the presence of sulphide, under similar conditions, the rate increases concomitantly with the lowering of the redox potential to -200 mV. Addition of dithionite, yielding an ambient redox potential of -420 mV, enhances the rate to the maximum. However, the rate of H₂ evolution is always much lower than that of CO₂ photoassimilation, although both reactions share a common segment of the electron transport chain. Furthermore, in the presence of CO₂, H₂ evolution is drastically inhibited, even in the presence of dithionite, implying that when CO₂ photoassimilation takes place electron transport is safeguarded against leakage of electrons to H₂. Similarly, in intact algal cells, light-dependent H₂ production was observed only in the absence of CO₂ photoassimilation (Kessler, 1974).

The ancient origin of the cyanobacteria, in the early Precambrian period (Schopf, 1974), implies that in the past they had to cope with an extremely reducing environment. Oscillatoria limnetica is one of the cyanobacterial species that retains a large part of the metabolic
mechanism of its ancestors, namely the anoxygenic photosynthetic apparatus. The redox-controlled H₂ valve may very well be another metabolic relic of this organism’s evolutionary history.

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