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SULFIDE INHIBITION OF PHOTOSYSTEM II IN CYANOBACTERIA (BLUE-GREEN ALGAE) AND TOBACCO CHLOROPLASTS

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Summary

The present study shows that in the presence of 600 nm light, sulfide acts as a specific inhibitor of photosynthetic electron transport between water and Photosystem II in the cyanobacteria *Aphanothece halophytica* and *Synechococcus* 6311 as well as in tobacco chloroplasts. In the presence of 600 nm light, sulfide affects the fast fluorescence transients as does a low concentration (10 mM) of hydroxylamine; the fluorescence yield decreases in the presence of either chemical and can be restored by the addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea. In chloroplasts, however, NH_2OH, an electron donor at high concentrations (40 mM), relieves the sulfide effect.

In the dark, sulfide affects the cyanobacterial fluorescence transients through decrease of oxygen tension. The fluorescence yield increases in a similar pattern to that observed under nitrogen flushing.

Upon omission of sulfide in *A. halophytica*, the characteristic aerobic fluorescence transients return, consistent with the ease of alternation between oxygenic and sulfide-dependent anoxygenic photosynthesis in many cyanobacteria.

Introduction

It has been demonstrated that many cyanobacteria ('blue-green algae') are able to utilize sulfide as an electron donor in a bacterial-type photosynthesis

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone.

[1,2]. In this process, driven solely by Photosystem I, and uninhibited by DCMU, sulfide is anoxygenically oxidized by these cyanobacteria to elemental sulfur [3,4]. This reaction occurs in *Oscillatoria limnetica* and *Aphanothece halophytica* only after synthesis of new proteins induced upon light incubation of the cells in the presence of high concentrations of sulfide (2 h and 3 mM or 1.5 h and 1.5 mM, respectively) [2,5]. However, oxygenic photoassimulation of CO_2 driven by both Photosystems I and II is inhibited already at low sulfide concentrations (>0.1 mM). Such inhibition is observed in cyanobacterial strains whether capable or incapable of anoxygenic photosynthesis with sulfide as electron donor. These data suggest that Photosystem II is highly sensitive to sulfide.

Photosystem II function can be investigated by analysis of the fluorescence of its associated chlorophyll *a*. Upon illumination with wavelengths exciting Photosystem II, characteristic patterns of fast changes in chlorophyll *a* fluorescence ('fast fluorescence transients') are observed which continue until the steady state of fluorescence yield is reached [6], followed by much slower changes in some photosynthetic systems [6,7]. The rapid transients reflect presumably changes in the reduction state of the electron acceptor of Photosystem II, Q, which when oxidized quenches fluorescence emission. Changes in the fluorescence yield at the early stages of the induction express changes in the ratio between input and output of electrons in Photosystem II which are adjusted by changing the steady-state level of Q reduction [6,8—10]. The subsequent slower changes in fluorescence yield have been correlated with the high energy state of the photosynthetic membranes as well as with changes in quanta distribution between the two photosystems [6].

By studying the effect of sulfide on the fluorescence yield changes of Photosystem II, the present work shows that sulfide in the presence of 600 nm light acts as a reversible photosynthetic electron transport inhibitor between water and Photosystem II in *A. halophytica*, a facultative anoxygenic photosynthesizing cyanobacterium in the presence of sulfide [2]. Similar sulfide inhibition was observed in *Synechococcus* 6311 and tobacco chloroplasts which are incapable of anoxygenic photosynthesis with sulfide as electron donor. Some of the findings described in this paper were published as an abstract [11].

Methods

Organisms and conditions of culture. Aphanothece halophytica (strain No. 7418, R.Y. Stanier culture collection) [2] was grown in 1-l Erlenmeyer flasks containing 500 ml aerobic mineral growth medium [5]. The cultures were incubated for 4 days at 35°C, continuous illumination provided by incandescent flood lamps (Irisol, France, 300 W) at an incident intensity of 50 W/m^2 as measured by a Yellow Springs Instruments photometer, model 65.

Synechococcus 6311 (*Anacystis nidulans*), (R.Y. Stanier culture collection) was grown in BG 11 medium [12] under identical conditions.

Cell and chloroplast preparations. Aphanothece cells were harvested and resuspended in fresh growth medium as previously described [5]. *Synechococcus* cells were similarly prepared in the latter medium which, however, contained distilled water instead of the Turks Island Salt solution. Cell suspensions

(0.4 — 0.6 μg chlorophyll *a* per ml) were incubated for 2 h in completely filled 100-ml Erlenmeyer flasks under growth conditions as above. For induction of anoxygenic CO_2 photoassimilation in *Aphanothece*, sulfide (1.5 mM) was added at the onset of incubation.

Chloroplasts were isolated from *Nicotiana tabacum* var. xanthi according to Avron [13] and stored at −180°C with 30% (v/v) ethyleneglycol [14]. When used, they were resuspended in buffer containing 200 mM sucrose, 100 mM NaCl and 20 mM Tris (pH 7.8) at a final chlorophyll concentration of 20 μg/ml.

Fluorescence measurements. For the fluorescence measurements, cell or chloroplast preparations were preincubated in the dark at room temperature for 10 min, followed, when indicated, (see legends) by Na_2S addition, N_2 flushing, and/or preillumination.

The measurements were performed at room temperature in a locally built spectrofluorimeter. Excitation light was provided by a slide projector equipped with a 500 W incandescent lamp. The excitation light was passed through a 600 nm interference filter (Schott, half band width 14 nm). The incident light intensity was 35.9 W/m^2 as measured by a calibrated silicone-cell light meter. The emitted fluorescence, in perpendicular direction to the excitation beam, was filtered through a 685 nm interference filter (half band width 3 nm) and measured as described previously [15]. The cuvette used was a standard cell of 1 cm optical path. provided with a magnetic bar enabling the sample to be stirred. For each measurement, a new sample (2.5 ml) was used. When indicated, Na_2S, DCMU (DuPont), and/or neutralized $NH_2OH \cdot HCl$ were added to a final concentration of 1 mM, 10 μM, and 10 or 40 mM, respectively.

Other measurements. Chlorophyll determinations in cyanobacterial cultures were carried out as previously described [5]. The chlorophyll content of the chloroplast suspensions was determined according to Arnon [16].

The absorbance of *Aphanothece* cells at different wavelengths was determined with the aid of a locally built integrating sphere photometer and the appropriate interference filters.

Results and Discussion

Aerobically grown *A. halophytica* showed fast transients in chlorophyll *a* fluorescence at 685 nm upon illumination with 600 nm light (Fig. 1a). From a basic level (O), the fluorescence yield rose to an intermediary value (I), then declined somewhat to a dip (D), and finally rose to a peak (P). From this level, the yield dropped at a somewhat slow rate to a quasi-steady-state level (S). It was followed by a much slower increase to a level, greater than P and not complete within a 20 min period (not shown). These transients are also characteristic of other cyanobacteria [17] as well as eukaryotic algae [10,18]. Isolated chloroplast preparations of higher plants, however, show only rapid transients similar to the O → I → D → P part only [9].

Whereas, the O-level of the fluorescence yield is quite constant under particular experimental conditions, the O → I → D → P → S rapid transients are variable, as they are affected by changes in the ratio between electron transport rate into and from Photosystem II (see Introduction). Accordingly, the fast

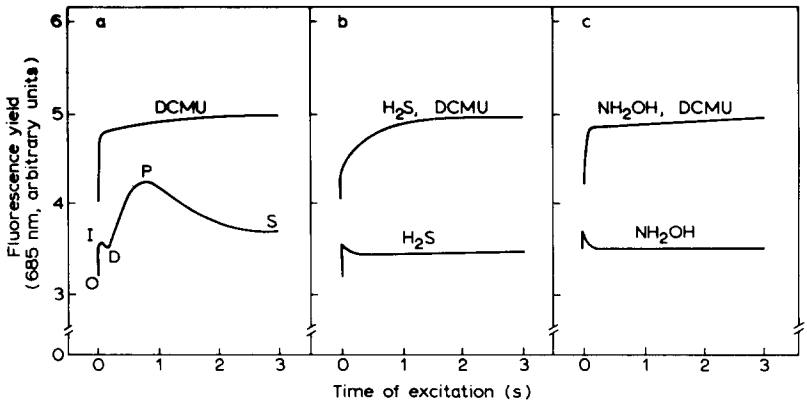

Fig. 1. Chlorophyll *a* fluorescence yield at 685 nm in *A. halophytica* as a function of duration of excitation. (a and c) Aerobic cells. (b) Cells induced to anoxygenic photosynthesis, 2 h incubation in the light in the presence of 1 mM Na_2S. Where indicated on the traces DCMU (10 μM) or NH_2OH (10 mM) was added immediately before excitation. Cell density, 0.39 μg chlorophyll *a* per ml; excitation wavelength, 600 nm; intensity, 35.9 W/m^2.

transients were interpreted as follows [6]: the O → I transient, an increase in electron transport into Photosystem II upon activation of the water-splitting system; the I → D transient, an increase of electron transport from Photosystem II to Photosystem I; and finally, the D → P transient, an increase in the reduction state of Q upon reaching the steady state of photosynthetic electron transport [18—20]. Other parameters of the photosynthetic system are possibly involved in the somewhat slower P → S transient and the very slow subsequent ones (see Introduction).

The effect of an increase in reduction state of Q on the fast fluorescence transients is demonstrated by the addition of DCMU which blocks electron transport at a site after Q in the photosynthetic transport chain [21]: a peak higher than the P-level was obtained with faster kinetics and there were no intermediary transients (Fig. 1a).

In the presence of sulfide (⩾0.1 mM), oxygenic CO_2 photoassimilation was blocked in aerobically grown *A. halophytica* cells as in *O. limnetica* cells [5]. This inhibition of oxygenic photoassimilation is also revealed after sulfide induction of Photosystem I-driven anoxygenic photosynthesis [4]. In cells induced to anoxygenic photosynthesis, after 2 h light incubation in the presence of sulfide, the fluorescence transients obtained were similar to the O → I → D transients revealed in the absence of sulfide but the D → P rise was missing and the final fluorescence yield was very nearly that of the constant, base level (Fig. 1b).

The drop in fluorescence yield evoked by sulfide could be interpreted as damage of Photosystem II. This possibility, however, may be excluded: addition of DCMU to cells of low fluorescence yield, increased the yield to the high level observed in the untreated control in the presence of DCMU (compare Fig. 1b with 1a). Note, however, the slower kinetics of the DCMU effect in the sulfide-treated cells. An alternative working hypothesis is suggested: the insertion of sulfide produces a severe rate-limiting step in electron transport at the

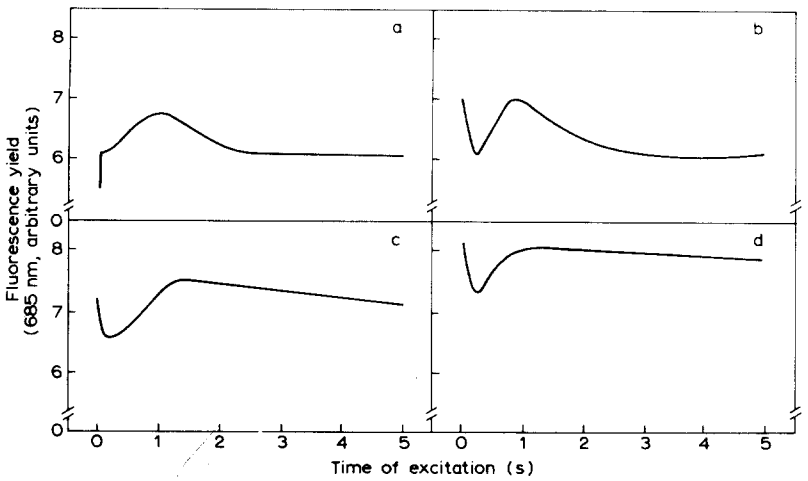

Fig. 2. Chlorophyll *a* fluorescence transients in aerobic *A. halophytica* cells after periods of dark incubation in the presence of 1 mM sulfide. (a) No sulfide. (b, c and d) 0.25, 5 and 10 min after the addition of Na_2S (1 mM), respectively. Cell density, 0.44 μg chlorophyll *a* per ml; excitation wavelength, 600 nm; intensity, 35.9 W/m^2. Emission measured at 685 nm.

donor side of Photosystem II. The residual flow of electrons does not allow oxygenic CO_2 photoassimilation nor a substantial net reduction of Q. Therefore, the fluorescence yield is very low. Addition of DCMU enables Q reduction by the residual slow electron flow and a high fluorescence yield is restored, albeit at a slower rate as compared to the DCMU control lacking sulfide. Indeed, the specific sulfide effects are very similar to those of hydroxylamine which at low concentrations ($\leqslant$10 mM) is a specific electron transport inhibitor at the donor side of Photosystem II in many photosynthetic systems [10,22].

In the presence of hydroxylamine ($\geqslant 10^{-4}$ M), oxygenic CO_2 photoassimilation in *A. halophytica* (not shown) and in other cyanobacteria [23] and eukaryotic algae [10] is inhibited. Its effect on fluorescence yield both in eukaryotic algae [10] and in the cyanobacterium *A. halophytica* is very similar to that induced in the latter by sulfide: the variable fluorescence yield decreased but could be restored by DCMU (compare Fig. 1b with 1c).

When the aerobic cells were preincubated in the presence of Na_2S (1 mM) in the dark, the pattern of fluorescence transients changed, attaining the final pattern after 10 min (Figs. 2a–2d). As compared with the control cells, incubated in the absence of sulfide, the dark sulfide-treated cells showed a higher initial level of fluorescence yield which decreased to a more pronounced dip, then rose again to a higher level (compare Fig. 2a with Figs. 2b–2d). Thereafter, there was almost no further decrease in fluorescence. Similar phenomena have been previously observed in *Chlorella* cells [18–20,24,25] and in the present work with *A. halophytica* upon flushing the experimental systems in the dark with nitrogen for 70 min (Fig. 3b). Longer periods of flushing (125 min) of *A. halophytica* cells caused disappearance of the transients, the fluorescence yield remaining at its high initial level (Fig. 3c). Hence, in the dark, the sulfide effect on fluorescence yield must be due to a decrease in oxygen tension caused by chemical oxidation of the sulfide. When the oxygen

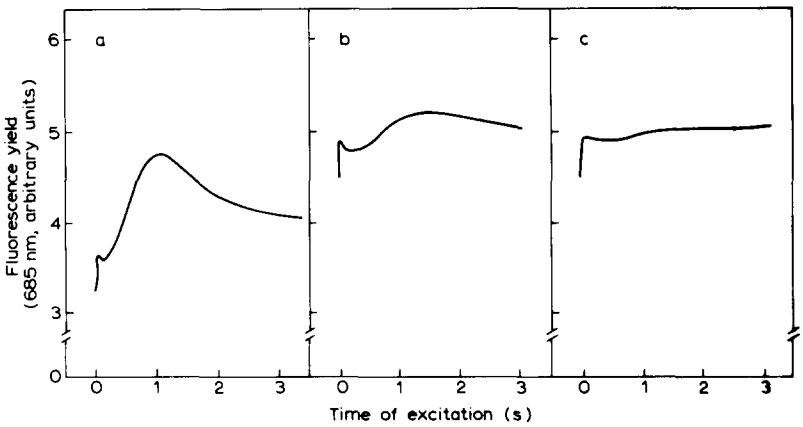

Fig. 3. Chlorophyll *a* fluorescence transients in *A. halophytica* cells after different periods of nitrogen flushing in the dark. a, 0 min; b, 70 min; c, 125 min flushing of nitrogen in the dark into aerobically grown cell suspensions. Cell density, 0.39 μg chlorophyll *a* per ml; excitation wavelength, 600 nm; intensity, 35.9 W/m^2. Emission measured at 685 nm.

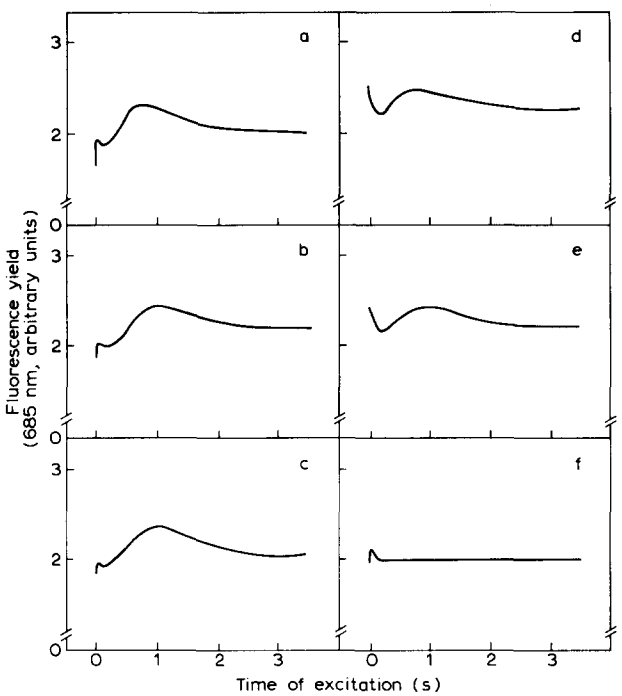

Fig. 4. The effect of sulfide in the presence of 702 nm (mainly system I) or 600 nm (preferentially system II) light on the chlorophyll *a* fluorescence transients in *A. halophytica*. Aerobically grown cells were preincubated in the absence (a—c) or in the presence of Na_2S (1 mM) (d—f) in the dark (a, d), 1 min at 702 nm light, followed by 4 min in the dark (b, e) or at 600 nm light for 1 min followed by 4 min in the dark (c,f). Preillumination light was obtained with the aid of a 702 nm interference filter (Baird Atomic, half band width 12 nm) or a 600 nm interference filter (Schott, half band width 14 nm); incident light intensities were 46 or 6.8 W/m^2, respectively, yielding equal absorbance at both wavelengths. Thereupon, excitation (600 nm; intensity, 35.9 W/m^2) was initiated. Cell density, 0.39 μg chlorophyll *a* per ml. Emission measured at 685 nm.

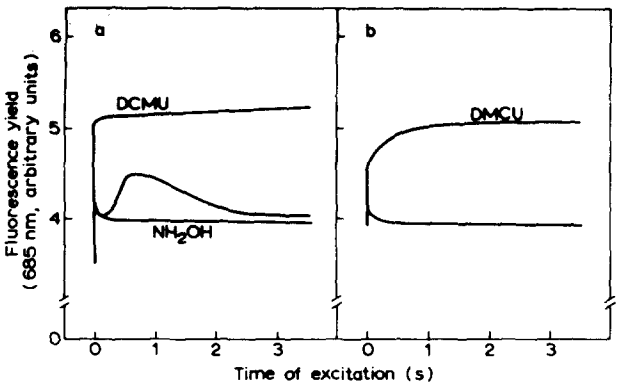

Fig. 5. Chlorophyll *a* fluorescence transients in *Synechococcus* 6311 (*Anacystis nidulans*) (a) Aerobic cells. (b) Incubation with 1 mM Na_2S for 5 min in the light, followed by 10 min dark incubation. Where indicated on the traces, DCMU (10 μM) or NH_2OH (10 mM) was added immediately before excitation. Cell density, 0.8 μg chlorophyll *a* per ml; excitation wavelength, 600 nm; intensity, 35.9 W/m^2. Emission measured at 685 nm.

concentration is lowered, overreduction of the electron transport system is responsible for the effects on fluorescence yield [6,24].

In contrast to the above, when Na_2S ($\geq$0.1 mM) was added to aerobic *A. halophytica* cells in the presence of 600 nm light, absorbed preferentially in Photosystem II (at an intensity of $\geq$6.8 W/m^2) for at least 10 s (followed by a 4 min dark period), a completely different pattern of fast fluorescence transients was observed (Fig. 4f). Nitrogen flushing of the aerobic cells in the presence of 600 or 702 nm light for 1 min, followed by a 4 min dark period, did not affect the fluorescence pattern of the aerobic control cells (not shown). The replacement of 600 nm light by an equally absorbed amount of quanta of 702 nm light in the presence of sulfide (Fig. 4e) yielded the fluorescence pattern observed after 5 min dark incubation in the presence of sulfide (Fig. 4d). It would seem that in the presence of 600 nm light, sulfide has a specific effect on Photosystem II fluorescence. This effect may underly the prompt inhibition of oxygenic CO_2 photoassimilation in *A. halophytica* and possibly also in other cyanobacteria [5] upon the addition of sulfide. Indeed, *Synechococcus* 6311 (*Anacystis nidulans*) which is incapable of utilizing sulfide showed fluorescence patterns similar to those of *A. halophytica* both in presence and in absence of sulfide (compare Fig. 5 with Fig. 1). It is noteworthy that Photosystem II light enhances hydroxylamine inhibition of photosynthetic electron transport as well as recovery when the inhibitor is removed [26,27]. It is thus suggested that sulfide specifically inhibits electron transport at a site very close to (or identical with) that of the hydroxylamine inhibitory site. Illumination with Photosystem II light serves to expose these sites.

If this interpretation of the sulfide effects is correct, introduction of electron donors between the site of inhibition and light reaction II should alleviate the inhibitory effect and restore the variable fluorescence pattern observed in non-inhibited systems. At high concentrations (50 mM) hydroxylamine has been shown to act as such an electron donor, able to release electron flow through Photosystem II from its inhibitory effects at low concentrations [22]. Simi-

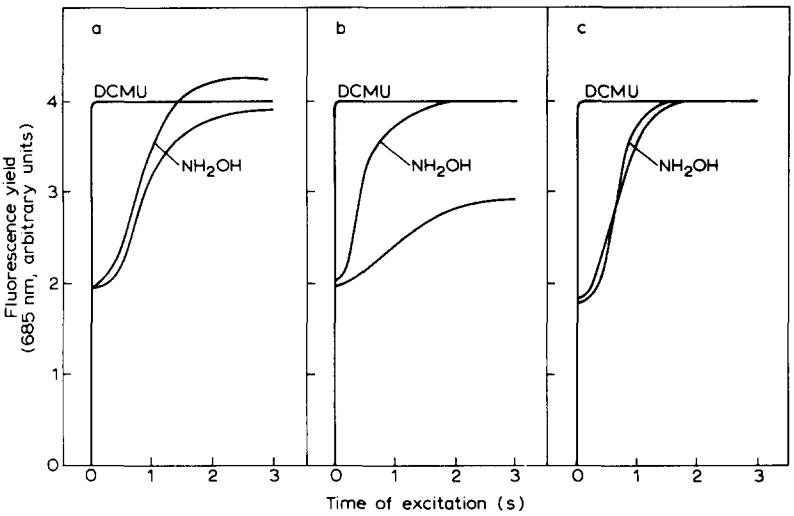

Fig. 6. Chlorophyll *a* fluorescence transients in tobacco chloroplasts. (a) In the absence of sulfide. (b) Incubation in the presence of 1 mM sulfide for 3 min in the light (600 nm; intensity, 35.9 W/m^2) followed by a 2 min dark period. (c) Incubation in the presence of 1 mM Na_2S in the dark for 10 min. Where indicated on the traces, NH_2OH (40 mM) or DCMU (10 μM) was added immediately before excitation. Chlorophyll concentration, 20 μg per ml; excitation wavelength, 600 nm; intensity, 35.9 W/m^2. Emission measured at 685 nm.

larly, in eukaryotic phototrophs, high concentrations of hydroxylamine are able to restore the variable fluorescence reduced by low concentration of this agent [10,28]. However, attempts to restore Photosystem II dependent CO_2 photoassimilation and variable fluorescence in sulfide-inhibited *A. halophytica* by addition of 50 mM hydroxylamine (pH 6—8) or other Photosystem II electron donors (benzidine, iodide, diphenylcarbazide) were unsuccessful. These negative results can be ascribed, nevertheless, to the inability of the intact cells to establish appropriate intracellular donor concentration levels. An attempt to overcome this difficulty has been the use of cell-free preparations. However, cell-free systems of *A. halophytica* as well as of other cyanobacteria lose the variable fluorescence patterns characteristic of whole cells [15].

The effect of sulfide was therefore further pursued in another oxygenic photosynthetic system, isolated tobacco chloroplasts, in which hydroxylamine at high concentrations (50 mM) serves as electron donor for Photosystem II [28]. Sulfide, when added to chloroplasts in the dark had no effect on the variable fluorescence yield (compare Fig. 6c with 6a). A light-dependent effect of sulfide on the variable fluorescence yield of chloroplasts was observed, very similar to that in *A. halophytica* (compare Figs. 6a and 6b with Figs. 1a and 1b). After light incubation in the presence of sulfide, the variable fluorescence yield decreased but could be restored by DCMU. Addition of NH_2OH at concentrations sufficiently high to supply electrons to Photosystem II also alleviated the sulfide effect (Fig. 6b). It may thus be concluded that both in eukaryotic and prokaryotic oxygenic photosynthetic systems, sulfide acts as an inhibitor of electron transport at a site preceding light reaction II, the site being exposed upon excitation of Photosystem II.

The light-dependent sulfide effect both in chloroplasts and cyanobacteria was insensitive to the uncoupler FCCP (not shown), but FCCP (10^{-5} M) completely inhibited CO_2 photoassimilation in *A. halophytica* in the presence as as well as in the absence of sulfide. Thus the involvement of $\Delta\tilde{\mu}H$ across the thylakoid membrane in the inhibition is excluded. This is in contrast with the $\Delta\tilde{\mu}H$-dependent inhibition of oxygen evolution observed in whole chloroplasts under anaerobic conditions [29]. Sites at the donor side of Photosystem II, exposed by light II to the external medium have already been demonstrated in other photosynthetic systems [21,27,30]. For example, *p*-(diazonium)-benzenesulfonate inhibition of electron transport between water and Photosystem II is light dependent and not relieved by phosphorylation uncouplers [21,30]. It is possible that upon illumination, these sites or others in the immediate vicinity become susceptible to inhibition by hydroxylamine and sulfide. Binding of the water splitting system's manganese and release of the manganese has been implicated in the mechanism of hydroxylamine's inhibitory effect [31]. This might also be true for sulfide inhibition; sulfide is known to readily bind to many metal-containing enzymes [32].

The specific effect of sulfide is readily reversible; when sulfide was removed, *A. halophytica* shifted to oxygenic CO_2 photoassimilation after a short lag (10 min). The pattern of variable fluorescence characteristic of cells incubated in the absence of sulfide also reappeared within this time (not shown). The reversibility of inhibition in *A. halophytica* and similar cyanobacteria [5] underlies their capability to readily alternate between anoxygenic and oxygenic photosynthesis.

It should be emphasized that Photosystem I is more resistant to sulfide intoxication and/or reducing conditions than Photosystem II; whereas, $\geqslant$0.1 mM concentrations of sulfide completely block oxygenic photosynthesis, anoxygenic photosynthesis can proceed at a 1.5 mM concentration in *A. halophytica* and at 3.5 mM in *O. limnetica* (in both cases at pH 6.8). The refractivity of Photosystem I to sulfide inhibition as well as the option of switching from anoxygenic photosynthesis confer a selective advantage upon these cyanobacteria in ecosystems alternating between anaerobic and aerobic conditions [33]. The difference in resistance of Photosystems I and II to sulfide may possibly reflect the longer history of the former photosystem.

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