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LIGHT-INDUCED OXIDATION–REDUCTION REACTIONS IN A CELL-FREE PREPARATION FROM THE BLUE-GREEN ALGA *NOSTOC MUSCORUM*:

THE ROLE OF CYTOCHROME *f*, CYTOCHROME b_{558}, C550, AND P700 IN NONCYCLIC ELECTRON TRANSPORT

DAVID B. KNAFF

Department of Cell Physiology, University of California, Berkeley, Calif. 94720 (U.S.A.)

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SUMMARY

1. Cytochrome *f* (λ_{max} = 554 nm, E_m = +0.35 V) and cytochrome b_{558} (λ_{max} = 558 nm, E_m = +0.35 V) were photooxidized by Photosystem I and photoreduced by Photosystem II in a cell-free preparation from the blue-green alga *Nostoc muscorum*. The steady-state oxidation levels of both cytochromes were affected by noncyclic electron acceptors and by inhibitors of noncyclic electron transport. These results are consistent with the hypothesis that the mechanism of NADP reduction by water involves a Photosystem II and a Photosystem I light reaction operating in series and linked by a chain of electron carriers that includes cytochrome *f* and cytochrome b_{558}.

2. Phosphorylation cofactors shifted the steady-state of cytochrome *f* to a more reduced level under conditions of noncyclic electron transport but had no effect on cytochrome b_{558}. These observations suggest that the noncyclic phosphorylation site lies before cytochrome *f* (on the Photosystem II side) and that cytochrome *f* is closer to this site than is cytochrome b_{558}.

3. A Photosystem II photoreduction of C550 at 77 °K was observed, suggesting that in blue-green algae, as in other plants, C550 is closely associated with the primary electron acceptor for Photosystem II. A Photosystem I photooxidation of P700 at 77 °K was observed, consistent with P700 serving as the primary electron donor of Photosystem I.

INTRODUCTION

The photosynthetic electron transport pathways in blue-green algae are of particular interest because blue-green algae appear to be the oldest oxygen-evolving organisms on our planet. Microfossils consistent with the presence of the blue-green

Abbreviations: DCMU, 3-(3′,4′-dichlorophenol)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DPIP, 2,6-dichlorophenolindophenol; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

algae have been found in rocks that are probably $3.2 \cdot 10^9$ years old[1]. In spite of this fact, relatively few studies on electron transport in blue-green algae have been made.

The cytochrome content of several blue-green algae[2–5] has been reported, and there have been reports of light-induced cytochrome photooxidations in these organisms. Amesz and Duysens[6] reported that a cytochrome analogous to the cytochrome *f* of higher plants could be photooxidized by Photosystem I and photoreduced by Photosystem II in *Anacystis nidulans*. Olson and Smillie [7] confirmed that finding and further reported that under certain conditions a Photosystem I photooxidation of a *b*-type cytochrome could be observed. Ogawa and Vernon[5] reported the photooxidation of cytochrome b_{557} in a cell-free preparation from *Anabaena variabilis* but did not determine whether the photooxidation was a Photosystem I reaction or a Photosystem II reaction.

This communication reports light-induced oxidation–reduction reactions of cytochrome *f*, cytochrome b_{558}, C550 (a newly discovered component that has been proposed to be the primary electron acceptor of Photosystem II, refs 8–11), and P700 (the reaction center chlorophyll of Photosystem I, ref. 12) in a cell-free preparation from the blue-green algae *Nostoc muscorum* capable of high rates of noncyclic electron transport from water to NADP. The results of this investigation suggest that NADP photoreduction in this alga proceeds through Photosystem II and Photosystem I light reactions operating in series and that cytochromes *f* and b_{558}, C550, and P700 participate in this electron flow from water to NADP.

METHODS

The material used (Fraction A) was a subcellular preparation from the blue-green alga *N. muscorum* grown as described by Arnon *et al.*[13]. The photochemical activity[13] of Fraction A included photoreduction of NADP with water as the electron donor at rates of the order of 200 μmoles of NADP/mg of chlorophyll *a* per h, coupled with a substantial noncyclic photophosphorylation.

Chlorophyll *a* was determined by the method of Arnon[14]. Cytochrome *f* and cytochrome b_{558} content was calculated from the reduced *minus* oxidized (hydroquinone *minus* ferricyanide) difference spectra and represents the average of six determations. An extinction coefficient (550 nm *minus* 540 nm) of 12.4 $mM^{-1} \cdot cm^{-1}$, based on the spectrum of cyrochtome *f* from spinach chloroplasts (Wada, K., unpublished) was used for cytochrome *f* and it was assumed that cytochrome b_{558} did not contribute to the absorbance at 550 nm. An extinction coefficient (560 nm *minus* 540 nm) of 13.5 $mM^{-1} \cdot cm^{-1}$ based on the spectrum of cytochrome b_{558} from *Euglena gracilis*[15] was used for cytochrome b_{558} and it was assumed that cytochrome *f* did not contribute to the absorbance at 560 nm.

Cytochrome absorbance changes at physiological temperatures were measured with a dual wavelength spectrophotometer as described previously[16] with 540 nm as the reference wavelength. C550 photoreduction at 77 °K was measured as described previously[8] with 538 nm as the reference wavelength. P700 photooxidation at 77 ° K was measured using the spectrophotometer in the split beam mode. After the baseline was recorded, the reference cuvette was masked and the sample cuvette was illuminated for 15 s. The spectrum was recorded and the original baseline was

substracted to yield the light *minus* dark difference spectrum. The half-band width of the measuring beam was 2.0 nm. The half-band widths of the actinic light beams were 10 nm. The intensity of the 715-nm beam used for Photosystem I illumination was $1.0 \cdot 10^4$ ergs/cm^2 per s. Similar results were obtained with 706- or 700-nm light. The intensities of the 664- and 670-nm beams used for Photosystem II illumination were $2.5 \cdot 10^4$ ergs/cm^2 per s and $3.0 \cdot 10^4$ ergs/cm^2 per s, respectively.

Oxidation–reduction potentials were measured aerobically as described previously[17] and the values reported are the average of four determinations.

RESULTS

Content and potentials of cytochromes f and b_{558}

As seen in Fig. 1, there are two peaks in the hydroquinone *minus* ferricyanide difference spectrum of *Nostoc* cell fragments in the cytochrome α-band region. The peak at 554 nm indicates the presence of cytochrome *f* (refs 2–5, 7), and the peak at 558 nm indicates the presence of cytochrome b_{558}, which presumably is similar to the cytochrome b_{557} observed in *A. variabilis* by Ogawa and Vernon[5]. The cytochromes are present in equimolar amounts: There is one cytochrome *f* per 350± 40 chlorophyll *a* molecules and one cytochrome b_{558} per 320±40 chlorophyll *a* molecules.

Fig. 2 shows oxidation–reduction titrations of cytochromes *f* and b_{558}. The average of four determinations gave $E_m = +0.35 \pm 0.01$ V as the midpoint potential for both cytochrome *f* and cytochrome b_{558} at pH 7.8. Similar values have been obtained for cytochrome *f* in a wide variety of plants[15,17–20] and for cytochrome b_{558}

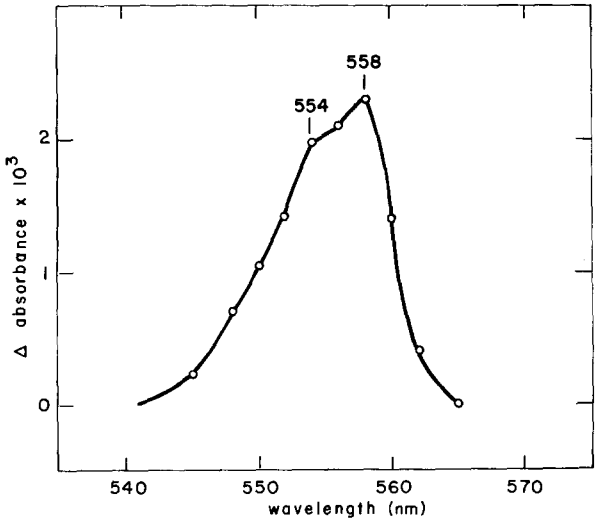


Fig. 1. The hydroquinone-reducible cytochromes of *N. muscorum*. The reaction mixture contained (per 1.0 ml) Fraction A (equivalent to 25 μg chlorophyll *a*) and the following (in μmoles): *N*-Tris (hydroxymethyl)methylglycine buffer (pH 7.8), 50; $MgCl_2$, 10; K_2HPO_4, 5; and $K_3Fe(CN)_6$, 2. To the 5.0-ml reaction mixture ($E_h = +515$ mV) was added 10 μmoles of hydroquinone in a vol. of 20 μl, which lowered the potential to +276 mV. A separate sample was used for each point.

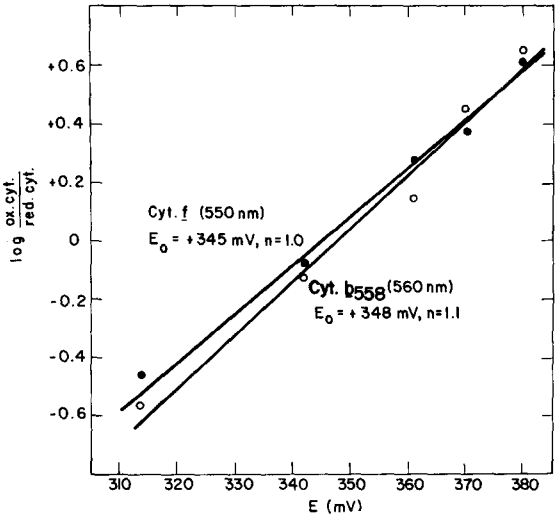


Fig. 2. The oxidation–reduction potential of *Nostoc* cytochromes *f* and b_{558}. Reaction mixture as in Fig. 1 except that 0.5 μmole of $K_3Fe(CN)_6$–$K_4Fe(CN)_6$ mixtures chosen to give the indicated potentials replaced the 2 μmoles of $K_3Fe(CN)_6$. Hydroquinone was added as in Fig. 1 to completely reduce the cytochromes. ●, cytochrome *f* measured at 550 nm; ○, cytochrome b_{558} measured at 560 nm.

from *E. gracilis*[15] and the high-potential form of cytochrome b_{559} in higher plants[10,17,19,21].

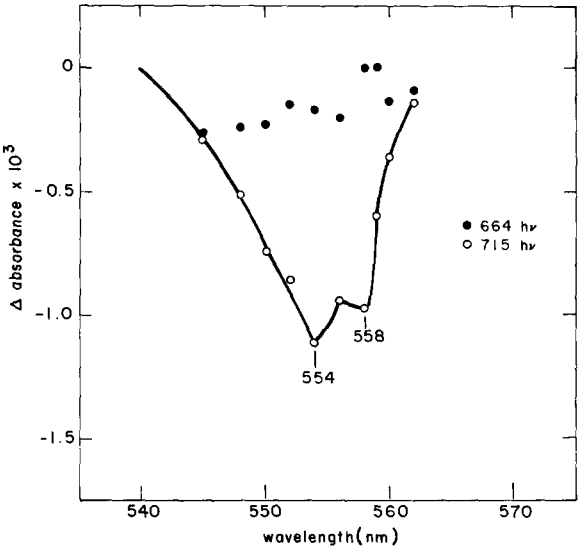


Fig. 3. Spectrum of cytochrome photooxidation by Photosystem I in a subcellular preparation of *Nostoc* in the presence of ascorbate. Reaction conditions as in Fig. 1 except that 2 μmoles of sodium ascorbate replaced the $K_3Fe(CN)_6$.

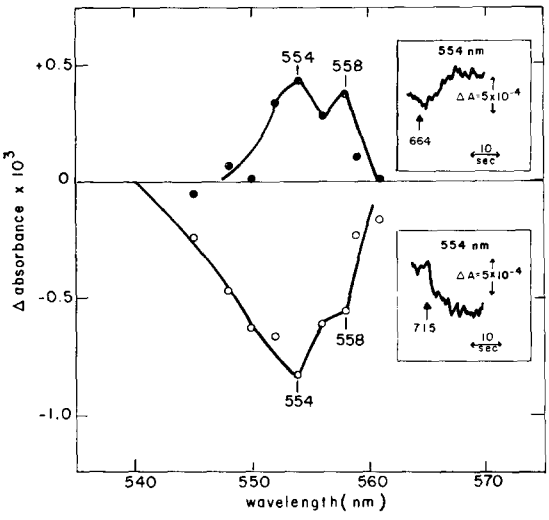


Fig. 4. Photosystem II cytochrome photoreduction and Photosystem I cytochrome photooxidation in a subcellular preparation of *Nostoc*. Reaction mixture as in Fig. 1 except that $K_3Fe(CN)_6$ was omitted. Closed circles show the effect of Photosystem II light (664 nm) and open circles, the effect of Photosystem I light (715 nm).

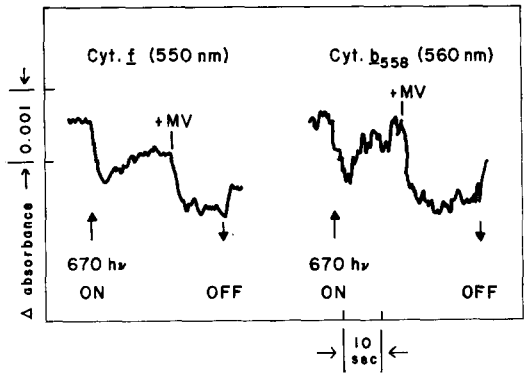
Effect of monochromatic light on cytochromes

Fig. 3 shows that when *Nostoc* cell fragments (preincubated with ascorbate to reduce cytochromes *f* and b_{558}) were illuminated with Photosystem I light (715 nm) both cytochromes were photooxidized. In a typical experiment all of the cytochrome *f* was photooxidized, while approximately 50% of the cytochrome b_{558} was photooxidized. Photosystem II illumination (664 nm) was much less effective in photooxidizing the cytochromes.

Fig. 4 shows that Photosystem II illumination of *Nostoc* cell fragments that were not preincubated with ascorbate resulted in the photoreduction of both cytochromes *f* and b_{558}. (The cytochromes are approximately 50% reduced in freshly prepared Fraction A.) Photosystem I illumination was completely ineffective in photoreducing the cytochromes; instead, it photooxidized the cytochrome *f* and cytochrome b_{558} that were originally reduced in the cell fragments. The Photosystem II photoreduction of cytochromes *f* and b_{558} could also be observed by illuminating the sample with 664-nm light after preillumination with Photosystem I light (715 nm) had photooxidized both cytochromes. The Photosystem II photoreduction of the cytochromes required relatively high levels of $MgCl_2$ (5–10 mM), as did oxygen evolution[13,22], indicating that the source of electrons for the photoreduction of the cytochromes was water.

Effect of noncyclic acceptors

To study the relationship of cytochromes *f* and b_{558} to noncyclic electron transport, the effect of noncyclic acceptors on the steady-state oxidation level of the cytochromes was examined. Fig. 5 shows that addition of methylviologen during Photosystem II illumination shifted the steady-state of both cytochrome *f* and cytochrome b_{558} from predominantly reduced to predominantly oxidized. The



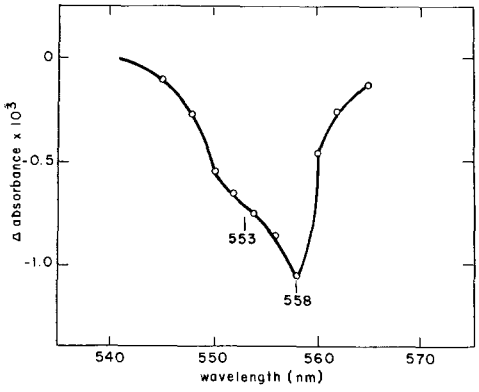


Fig. 5. Effect of a noncyclic acceptor on *Nostoc* cytochromes during Photosystem II illumination. Experimental conditions as in Fig. 3. Where indicated, 50 nmoles of methylviologen (MV) in a vol. of 0.01 ml were added to the 5.0-ml reaction mixture.

Fig. 6. Spectrum of the effect of addition of methylviologen during Photosystem II illumination. Experimental conditions as in Fig. 5.

spectrum of the absorbance change produced by the addition of methylviologen is shown in Fig. 6. The minima at 553 and 558 nm indicate that addition of methylviologen resulted in the oxidation of cytochromes *f* and b_{558}. It is likely that the cytochromes remained in the reduced state during Photosystem II illumination in the absence of acceptor because the oxidation of the cytochromes was rate limiting under these conditions.

Similar results were obtained with the physiological acceptor for noncyclic electron transport, NADP (in the presence of ferredoxin and ferredoxin–NADP reductase). No effect of acceptor on the steady-state oxidation level of either cytochrome was observed in Photosystem I light, whether in the presence or absence of the Photosystem I electron donor, reduced 2,6-dichlorophenolindophenol (DPIP).

Effect of inhibitors of noncyclic electron transport

Fig. 7 shows that two inhibitors of noncyclic electron transport, 3-(3′,4′-dichlorophenol)-1,1-dimethylurea (DCMU) and the plastoquinone antagonist

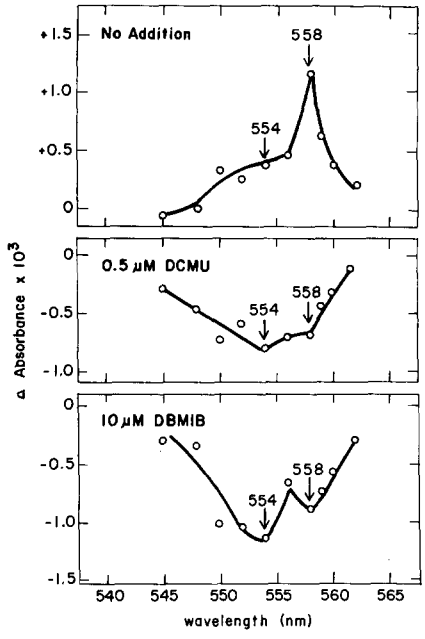


Fig. 7. Effect of inhibitors of noncyclic electron transport on the Photosystem II photoreduction of cytochromes *f* and b_{558}. Reaction mixture as in Fig. 4. DCMU (0.5 μM) or DBMIB (10 μM) were present as indicated.

2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB)[23,24], inhibit the Photosystem II photoreduction of cytochromes *f* and b_{558}. DCMU gave 96% inhibition of noncyclic electron transport from water to NADP at the concentration used (0.5 μM) for these measurements. Although a higher concentration of DBMIB (10 μM) was required to obtain complete inhibition of noncyclic electron transport from water to NADP in the *Nostoc* preparation than was required for spinach chloroplasts[23,24], the inhibitor was specific for the Photosystem II reduction of NADP in the *Nostoc* preparation, as indicated by the lack of inhibition of the Photosystem I reduction of NADP with reduced DPIP as the donor at a DBMIB concentration of 10 μM.

Fig. 8 shows that neither DCMU nor DBMIB had any inhibitory effect on either the rate or extent of the Photosystem I photooxidation of cytochromes *f* or b_{558}. The spectrum of the absorbance change in the cytochrome α-band region produced by Photosystem I illumination was unaffected by either inhibitor (data not shown).

Effect of phosphorylation cofactors

Fig. 9 shows that the addition of ADP to *Nostoc* cell fragments undergoing noncyclic electron transport from water to NADP resulted in a reduction of cytochrome *f* (550 nm was chosen as the measuring wavelength to minimize absorbance changes caused by cytochrome b_{558}) but had little or no effect on cytochrome b_{558} (558 nm). No effect was observed if acceptor was absent or if Photosystem I light (either in the presence or absence of reduced DPIP) was substituted for Photosystem

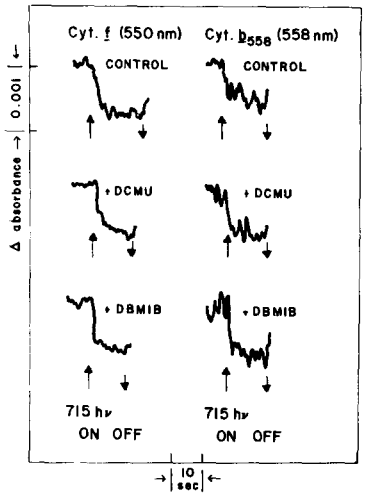


Fig. 8. Effect of inhibitors of noncyclic electron transport on the Photosystem I photooxidation of cytochromes *f* and b_{558}. Reaction mixture as in Fig. 3. DCMU (0.5μM) or DBMIB (10 μM) were present as indicated.

II light. The effect of ADP was diminished considerably by the presence of the inhibitors DCMU or DBMIB. Addition of ADP in the absence of phosphate resulted in only a small reduction of cytochrome *f*, presumably because of the low endogenous phosphate level.

The spectrum of cytochrome reduction caused by the addition of ADP is shown in Fig. 10. The single peak at 554 nm indicates that cytochrome *f* was the major cytochrome affected. Although some absorbance changes were observed at wavelengths greater than 558 nm, they had no distinct spectrum and were probably due to scattering changes rather than to the reduction of cytochrome b_{558}.

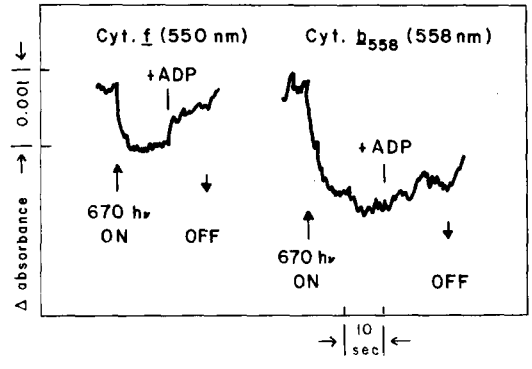


Fig. 9. Effect of addition of ADP on *Nostoc* cytochromes during noncyclic electron transport. Reaction mixture as in Fig. 3 except that spinach ferredoxin, 0.01 μmole per 1.0 ml, NADP, 1 μmole per 1.0 ml, and saturating spinach ferredoxin–NADP reductase were added. Where indicated, 500 nmoles of ADP in a vol. of 0.01 ml were added to the 5.0-ml reaction mixture.

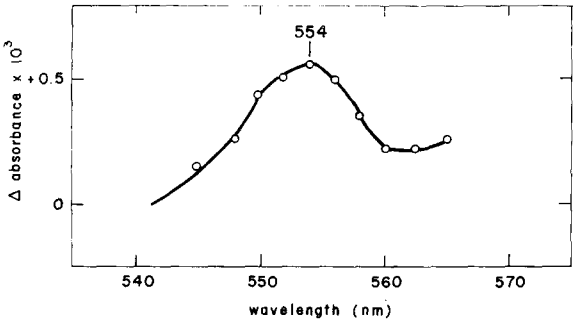


Fig. 10. Cytochrome *f* reduction caused by addition of ADP during noncyclic electron transport. Experimental conditions as in Fig. 9.

C550 photoreduction and P700 photooxidation at 77 °K

Fig. 11 shows the effect of Photosystem II (664 nm) and Photosystem I (715 nm) illumination at 77 °K on *Nostoc* cell fragments preincubated with ferricyanide to chemically oxidize the cytochromes. Photosystem II illumination results in an increase in absorbance at 541 nm and a decrease in absorbance at 547 nm, similar to the absorbance changes produced by the photoreduction at 77 °K of C550 in higher plants[8–11,25]. As was found previously in higher plants, no C550 photoreduction occurred at 77 °K with Photosystem I illumination[8,10,11]. P700 photooxidation at 77 °K (refs 26–29), in contrast to C550 photoreduction, occurred equally in Photosystem I and Photosystem II light. Fig. 12 shows the spectrum of the absorbance change produced by Photosystem I illumination of *Nostoc* cell fragments at 77 °K.

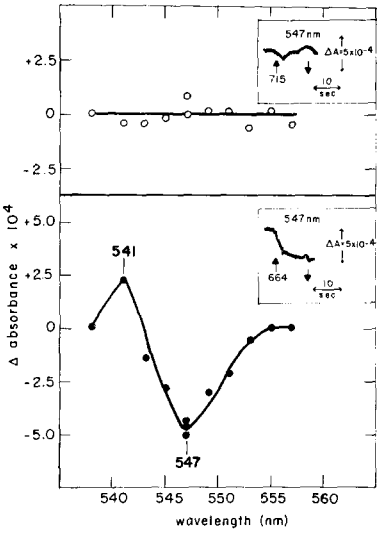


Fig. 11. C550 photoreduction in a subcellular preparation of *Nostoc* at 77 °K. The reaction mixture contained (per 1.0 ml Fraction A (equivalent to 67 μg of chlorophyll *a*), 0.5 ml of glycerol, and the following (in μmoles): *N*-Tris(hydroxymethyl)methylglycine buffer (pH 7.8), 50; $MgCl_2$, 10; and $K_3Fe(CN)_6$, 5.

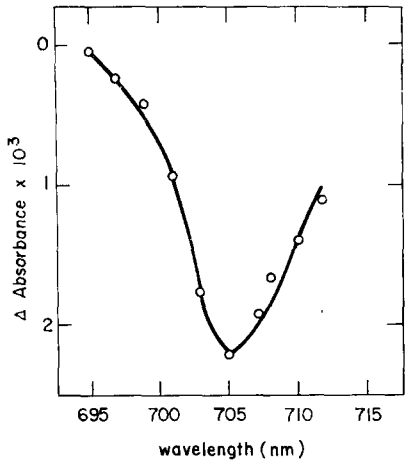


Fig. 12. Photosystem I photooxidation of P700 in a subcellular preparation of *Nostoc* at 77 °K. Reaction mixture as in Fig. 11 except that sodium ascorbate replaced $K_3Fe(CN)_6$. The sample cuvette was illuminated for 15 s with 715-nm light.

DISCUSSION

There is widespread agreement that photosynthetic electron transport from water to NADP involves Photosystem II and Photosystem I light reactions operating in series and connected by a chain of electron carriers that includes cytochrome *f* (see review, ref. 30, for a more detailed discussion of this scheme). Much of the evidence for this formulation comes from the "antagonistic" effect of Photosystem I and Photosystem II illumination on cytochrome *f*, the cytochrome becoming oxidized during Photosystem I illumination and reduced during Photosystem II illumination[6,31–36].

There is less agreement on the behavior of *b*-type cytochromes in plant material. Photooxidation by Photosystem I of cytochrome b_{559} has been observed by Levine and Gorman[34] in fragments from the green alga *Chlamydomonas reinhardti* and of cytochrome b_{558} by Ben-Hayyim and Avron[36] in lettuce chloroplasts. Although Cramer and Butler[35] reported a slow Photosystem I photooxidation of cytochrome b_{559} in spinach chloroplasts, other workers report that no cytochrome b_{559} photooxidation is not observed at physiological temperatures in untreated spinach chloroplasts[16,37], intact pea leaves[37], or intact cells of the red alga *Porphyridium aerugineum*[38]. In these cases, cytochrome b_{559} photooxidation could be observed only after one of a number of treatments: At 77 °K (refs 9–11, 16, 21, 28, 29), or after inhibition of oxygen evolution by treatment with concentrated Tris buffer[16,39,40], or after treatment with the uncouplers carbonylcyanide *m*-chlorophenylhydrazone (CCCP) or carbonylcyanide *p*-trifluoromethyloxyphenylhydrazone (FCCP)[35,37,38,41,42] (a treatment that changes the midpoint potential of cytochrome b_{559}, ref. 42). A further complication was the observation that the photooxidation of cytochrome b_{559} could be either a Photosystem II reaction[10,11,16,37–40] or a Photosystem I reaction[37,41,42], depending on the treatment of the chloroplasts.

All of the reactions of cytochrome b_{558} in *Nostoc* described above were mea-

sured at physiological temperatures with a preparation capable of high rates of NADP reduction and capable of coupled phosphorylation. Inhibitory treatments, such as treatment with Tris buffer or addition of CCCP, that might introduce cytochrome reaction pathways not present under physiological conditions were avoided.

The fact that cytochrome b_{558} in *Nostoc* showed the same response to monochromatic light, Mg^{2+} concentration, noncyclic acceptors, and noncyclic inhibitors as did cytochrome *f* implies that the two cytochromes are members of the same electron transport pathway from water to NADP. Furthermore, the fact that Photosystem II reduced the cytochromes while Photosystem I oxidized the cytochromes suggests that both cytochromes function between a Photosystem II and a Photosystem I light reaction.

The effects of the noncyclic inhibitor DCMU reported above are similar to those observed in a wide variety of plant material[31–36]. The effect of the plastoquinone antagonist DBMIB on the photoreactions of cytochrome *f* in *Nostoc* cell fragments reported above is also similar to those reported previously[41,43]. However, the DBMIB inhibition of the Photosystem II photoreduction of cytochrome b_{558} and the lack of inhibition of the cytochrome b_{558} photooxidation observed with *Nostoc* (see Figs 7 and 8) differs from the pattern observed in spinach chloroplasts by Böhme and Cramer[41]. These workers reported that DBMIB inhibited the Photosystem I photooxidation but not the Photosystem II photoreduction of cytochrome b_{559}. These authors[41] interpreted the effect of DBMIB on cytochromes *f* and b_{559} and the reversal of DBMIB inhibition by plastoquinone as indicating that plastoquinone functions between cytochromes *f* and b_{559}. The results obtained with *Nostoc* cell fragments suggest that, if DBMIB functions as a plastoquinone antagonist in blue-green algae as it does in spinach chloroplasts[23,24], plastoquinone is on the reducing (Photosystem II) side of both cytochromes *f* and b_{558} in *Nostoc*.

The effect of ADP on cytochrome *f* in *Nostoc* cell fragments under conditions of noncyclic electron flow from water and the lack of an effect of ADP on cytochrome b_{558} suggests that cytochrome *f* lies closer to the site of noncyclic phosphorylation than does cytochrome b_{559}. These results are similar to those reported for spinach chloroplasts[32,33,44] but differ from those of Ben-Hayyim and Avron[36] who reported similar effects of ADP on both cytochrome *f* and cytochrome b_{558} in experiments with lettuce chloroplasts. The fact that the addition of ADP resulted in a reduction of cytochrome *f* suggests that the phosphorylation site is before (on the Photosystem II side) cytochrome *f*. In terms of the "crossover" theory of Chance and Williams[45], in the absence of ADP the site of electron flow at which energy is conserved is rate limiting. Addition of ADP increases the rate of this step[45] (addition of ADP increased the rate of electron transport from water to NADP in *Nostoc* Fraction A by 30%.) In the case of *Nostoc*, this resulted in a reduction of cytochrome *f* as electron flow into the cytochrome speeded up on addition of ADP.

Although Bendall and Sofrova[11] could not detect C550 in a cell-free preparation from the blue-green alga *Plectonema boryanum*, Fig. 11 shows that C550 is present in the *Nostoc* cell fragments. The fact that C550 is photoreduced at 77 °K in a Photosystem II reaction is consistent with the close association of C550 with the primary electron acceptor for Photosystem II in *Nostoc*. The observation that the Photosystem I photooxidation of P700 occurs at 77 °K in *Nostoc* is consistent with P700 acting as the primary electron donor of Photosystem I (refs

12, 26–29, 46) in *Nostoc*. C550 photoreduction and P700 photooxidation were measured at 77 °K so that only those reactions related to the primary charge separation reactions could occur and secondary electron transfer reactions would be impeded. However, these reactions also occur at physiological temperatures[8,12,16,33,39,40,46] and therefore the low-temperature reactions appear not to be artifacts created by the unique environment present at cryogenic temperatures.

In conclusion, it appears that noncyclic electron transfer from water to NADP in *Nostoc* involves two light reactions operating in series. Photosystem II, which operates efficiently only in short-wavelength light ($\lambda < 680$ nm) produces an oxidant sufficiently electropositive to oxidize water to oxygen and also reduces C550. Reduced C550 feeds electrons into an electron carrier chain that includes cytochromes *f* and b_{558} for transfer to the second light reaction, Photosystem I, which operates most efficiently in long-wavelength light ($\lambda > 680$ nm). Excitation of Photosystem I results in the oxidation of a specialized chlorophyll, P700, and the reduction of an electron carrier sufficiently electronegative to reduce NADP. Electron flow through the chain connecting the two light reactions is coupled to the formation of ATP at a site before cytochrome *f*. Cytochrome *f* and cytochrome b_{558} would appear to function close to each other in the chain, on the basis of their identical oxidation–reduction potentials, but further investigations are needed to determine the sequence of the two cytochromes.

ACKNOWLEDGEMENTS

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