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# REACTIONS OF *b*-CYTOCHROMES IN THE RED ALGA *PORPHYRIDIUM AERUGINEUM*

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## SUMMARY

1. The kinetics of light-induced absorbance changes due to oxidation and reduction of cytochromes were measured in a suspension of intact cells of the unicellular red alga *Porphyridium aerugineum*. Absorbance changes in the region 540–570 nm upon alternating far-red light and darkness indicated the oxidation of cytochrome *f* and reduction of cytochrome $b_{563}$ upon illumination. The relative efficiencies of far-red and orange light indicated that both reactions were driven by Photosystem I.

2. Experiments with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), with anaerobic cells and in alternating far-red and orange light indicated that cytochrome $b_{563}$ reacts in a cyclic chain around Photosystem I, and that the reduced cytochrome does not react with oxygen or with another oxidized product of Photosystem II. The quantum requirement for the photoreduction was about 6 quanta/equiv at 700 nm. A low concentration of *N*-methylphenazonium methosulphate (PMS) enhanced the rate of reoxidation of cytochrome $b_{563}$ in the dark. In the presence of higher concentrations of PMS a photooxidation, driven by Photosystem I, instead of reduction was observed. These observations suggest that PMS enhances the rate of reactions between reduced cytochrome $b_{563}$ and oxidized products of Photosystem I.

3. In the presence of carbonylcyanide *m*-chlorophenylhydrazone (CCCP) a light-induced decrease of absorption at 560 nm occurred. Spectral evidence suggested the photooxidation of cytochrome $b_{559}$ under these conditions. Inhibition by DCMU and a relatively efficient action of orange light suggested that this photooxidation is driven by Photosystem II.

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## INTRODUCTION

The role of cytochromes in photosynthesis has only been partly elucidated as yet. Experiments with intact cells and cell-free preparations indicate that cytochrome *f* reacts in the photosynthetic chain between the two light reactions (see,

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Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; P700, chlorophyllous pigment absorbing at 700 nm, primary electron donor of Photosystem I; PMS, *N*-methylphenazonium methosulphate.

*e.g.* ref. 1). The function of cytochromes $b_{563}$ and $b_{559}$, however, is less well established.

From measurements with intact algae[2] or algal chloroplasts[3] it was concluded that cytochrome $b_{563}$, like cytochrome *f*, reacts in the noncyclic chain. In contrast to this, experiments with chloroplasts suggested a reaction in a cyclic chain around System I (refs 4, 5) or around both photosystems[6].

For cytochrome $b_{559}$ the evidence indicates an association with System II (refs 7–9). Both photooxidation[10–16] and reduction[4,17–20] by System II have been reported but it is not clear if this compound is located in the main path of electron transport[12,15,21]. This point is further complicated by the observation that two cytochromes $b_{559}$ may exist[22,23] with widely different redox potentials and that the high-potential cytochrome may be converted into one of lower redox potential by various treatments[15,21,23] (see also ref. 24).

In a previous paper[25] we have shown that spectrophotometric measurements with intact cells of the red alga *Porphyridium aerugineum* and of *Chlorella vulgaris* strongly indicate that cytochrome *b* does not react in the chain between the two light reactions. More recent experiments[26] with *Porphyridium* suggested that cytochrome $b_{563}$ may be located in a cyclic chain near System I. In this paper we will report further evidence to support this hypothesis and present some results concerning the reactions of cytochrome $b_{559}$ in this alga.

## MATERIALS AND METHODS

*Porphyridium aerugineum* was grown in liquid culture medium as described elsewhere[27]. The algae were harvested by centrifugation and resuspended in fresh growth medium, gassed with air and 5 % $CO_2$, unless otherwise noted. Before measurement they were transferred to 1-mm cuvettes. The absorbance of the suspension was 0.4/mm at 680 nm, corrected for scattering.

Light-induced absorbance changes were measured by means of a "split-beam" apparatus operating according to the same principle as one described earlier[28] but equipped with two independent measuring beams by use of two monochromators and two photomultipliers.

The chopping frequency of both beams was 250 Hz. The apparatus enabled simultaneous measurement of absorbance changes at two different wavelengths. "Cross talk", even at wavelength pairs which were too close to be separated by means of filters could be avoided by proper choice of the chopping and detecting phases. One of the two vessels could be illuminated by means of projectors equipped with interference and absorption filters. System I light was obtained by means of a filter combination consisting of Schott RG 665, 3 mm, combined with AL 700 to give a band with a maximum at 700 nm. System II light (618 nm) was obtained with AL 618, RG 610, 2 mm and an interference filter with cut-off above 644 nm. A Balzers Calflex C filter was added to each filter combination. The intensities of actinic light were measured by means of a calibrated silicon cell. Automatic timers provided fixed cycles of illumination. Suitable filters placed in front of the photomultipliers served to block the actinic light and to minimize stray light from the monochromators. After amplification and rectification both signals were fed into a Nuclear Chicago Model 7100 signal averager, or recorded directly on a two-channel recorder. Some experiments were done with an apparatus equipped with only one measuring beam[28].

Absorbances of algal suspensions were measured with a spectrophotometer equipped with opal glass. The apparent absorbance at 740 nm was subtracted from the absorbance measured at other wavelengths in order to correct for light scattering.

The experiments were performed at room temperature (21–23 °C).

## RESULTS AND INTERPRETATION

### *Kinetics and difference spectrum in alternating illumination*

When *Porphyridium aerugineum* is illuminated alternately with light mainly absorbed by System I and light mainly absorbed by System II, the oxidation and reduction of Q, the primary electron acceptor of System II, plastoquinone, cytochrome *f* and P700 can be observed spectrophotometrically. Measured under these conditions, the difference spectrum in the $\alpha$-band region showed participation of cytochrome *f* (with $\alpha$ band at 552 nm) but gave no evidence for the reaction of either cytochrome $b_{559}$ or $b_{563}$ in the chain between the two light reactions[25]. Upon alternating light and dark, however, the kinetics in the region 552–565 nm clearly indicated the reaction of more than one component, and difference spectra thus obtained suggested a reaction of both cytochrome $b_{563}$ and cytochrome *f* (ref. 26). Experiments with a better time resolution than applied earlier gave a clearer separation of the spectra of both cytochrome components (Fig. 1). The difference spectra, with bands at 552 and 562–563 nm, and the kinetics at various wavelengths (Fig. 2) indicate that cytochrome *f* is oxidized and $b_{563}$ reduced upon the onset of illumination; on turning off the light, $b_{563}$ oxidation proceeds faster than the reduction of cytochrome *f*.

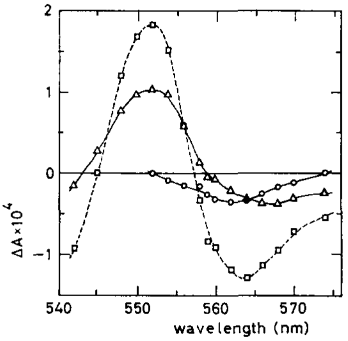


Fig. 1. Absorbance difference spectra of *Porphyridium aerugineum* obtained in alternating 2 s far-red light (700 nm) and 2 s darkness. ○—○, absorbance decrease measured 0.3 s after turning off the light; △—△, absorbance change occurring between 1 and 2 s after cessation of illumination; □- - -□, total absorbance change (dark *minus* light). Intensity of actinic light: 4.7 nEinstein·cm$^{-2}$·s$^{-1}$. The other conditions are given in the text.

The spectrum of the absorbance change occurring between 1 and 2 s after illumination, which probably represents that of cytochrome *f* only, and which is similar to that of Fig. 7 (see below) indicates that the isosbestic point of cytochrome *f* is close to 560 nm. Therefore, this wavelength was used for further studies of the kinetics of cytochrome $b_{563}$. The absorbance changes at 552 nm probably reflect the kinetics of cytochrome *f* only; Fig. 1 indicates that this wavelength is near the isosbestic point of cytochrome $b_{563}$.

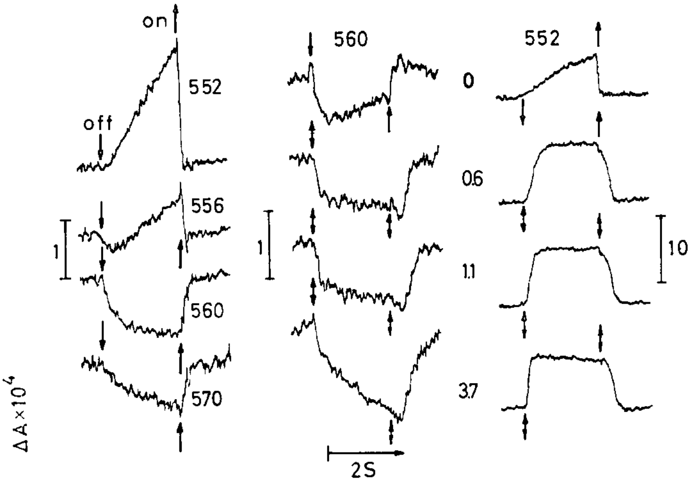


Fig. 2. Kinetics of absorbance changes. An upward moving trace indicates an increase of absorbance. The vertical bars indicate the absorbance scales in $10^{-4}$ absorbance units. Left hand column: kinetics at various wavelengths under the same conditions and with the same sample as used for Fig. 1. An upward pointing arrow indicates the beginning; a downward pointing one the end of illumination. Second and third columns: kinetics at 560 and 552 nm in alternating far-red light (700 nm, 3.4 nEinstein·cm$^{-2}$·s$^{-1}$) and darkness (upper tracings) or light of 618 nm. At the first double arrow of each tracing, far-red light was replaced by orange light; at the second double arrow orange light was replaced by far-red. The numbers give the intensity of orange light in nEinstein·cm$^{-2}$·s$^{-1}$. The tracings are the average of 20–40 experiments.

### *Efficiencies in far-red and orange light*

The light-induced reduction of cytochrome $b_{563}$ was not inhibited by DCMU which suggests that the reduction is driven by System I. This was confirmed by comparison of the relative efficiencies of far-red and orange light in effecting $b_{563}$ reduction. The experiment was done in the presence of DCMU, in order to block the electron

TABLE I

RELATIVE EFFICIENCIES FOR CYTOCHROME PHOTOREACTIONS

The third column gives the efficiency for absorbed quanta at 700 nm, relative to those at 618 nm in bringing about the reaction indicated in the first column. The numbers were calculated from the light curves for the steady-state deflections at 560, 552 and 559 nm, respectively, by the method given by Duysens[32]. The data given in the first two rows were obtained with a different sample as used for the experiments of the last three rows of the table.

| *Type of reaction* | *Additions* | *Relative efficiency 700/618 nm* |
|---|---|---|
| Cytochrome $b_{563}$ reduction | 50 μM DCMU | 2.5 |
| Cytochrome *f* oxidation | 50 μM DCMU | 2.8 |
| Cytochrome $b_{563}$ oxidation | 10 μM CCCP | 0.66 |
| Cytochrome *f* oxidation | 10 μM CCCP | 1.38 |
| Cytochrome *f* oxidation | CCCP and DCMU | 1.41 |

transport chain between the two systems and also to keep the algae in the so-called pigment state 1 (refs 29, 30, 26) in both kinds of illumination[31].

As the first two rows of Table I show, the relative efficiency for far-red light was high, and agreed well with that for cytochrome *f* oxidation.

The quantum requirement for cytochrome $b_{563}$ photoreduction in light of 700 nm was found to vary between 5.3 and 7.0 *hν*/equiv for different samples. The quantum yield was independent of the intensity, up to 4 nEinstein·cm$^{-2}$·s$^{-1}$. Under identical conditions the quantum requirement for cytochrome *f* photooxidation varied between 1.17 and 1.40 *hν*/equiv.

The values for cytochrome *f* were calculated on the basis of a difference in molar extinction coefficient of the reduced and oxidized forms of 15.7 mM$^{-1}$·cm$^{-1}$ at 552 nm (ref. 33). The extinction coefficient of cytochrome $b_{563}$ is not known; we assumed that its differential extinction coefficient at 563 nm was the same as that of cytochrome *f* at 552 nm, and a corresponding value of 13.5 at 560 nm was used in the calculation.

The maximum deflection at 560–563 nm observed in strong (saturating) light corresponded to an amount of cytochrome $b_{563}$ which was only one-sixth of the cytochrome *f* pool. As will be discussed later the total pool of $b_{563}$ may be larger.

### *Interaction with System II*

Fig. 2 compares the kinetics at 560 and 552 nm in alternating System I light of fixed intensity and System II light of varying intensity. The kinetics at 552 nm showed the same pattern as described earlier[25]. The results show that System II light had little effect upon the rapid reoxidation of cytochrome $b_{563}$ that occurred upon turning off the System I illumination. At high intensities of System II light a second, slow decrease of absorbance was observed at 560 nm. The difference spectrum (not shown) suggested that this decrease was caused by a further oxidation of cytochrome $b_{563}$. This oxidation may be due to a reaction with an oxidized product of System II, which is probably not oxygen, in view of the results obtained with anaerobic algae to be described below.

The oxidation might also be an indirect effect, *e.g.* due to a change in the ADP/ATP ratio. After System II light, a delay occurred in the reduction of cytochrome $b_{563}$ that might reflect a temporarily enhanced rate of electron transport towards the Calvin cycle.

### *Effects of various reactants and inhibitors*

Anaerobiosis did not bring about a drastic change in the kinetics of either cytochrome *f* or $b_{563}$. For these experiments, the algae were gassed for about 1 h with $N_2$ and 5 % $CO_2$ instead of air and 5 % $CO_2$. DCMU was added to prevent $O_2$ evolution. After transfer to the measuring cuvettes, the suspension was kept in the dark for 30 min in order to further remove oxygen by respiration. As Fig. 3 shows, the kinetics at 560 nm were very similar to those of a control in the presence of air, indicating that the reoxidation of cytochrome $b_{563}$ upon darkening is not caused by oxygen. The difference spectrum was also similar to that obtained under aerobic conditions. The dark reduction of cytochrome *f* was faster in the absence than in the presence of oxygen.

PMS, at 1 μM, accelerated the dark oxidation of cytochrome $b_{563}$ and the dark reduction of cytochrome *f* (Fig. 3). As already reported[26], a photooxidation of cytochrome $b_{563}$ was observed in the presence of higher concentrations of PMS. As Fig. 4 shows, the difference spectrum in far-red light under these conditions showed the absence of net cytochrome *f* oxidation, presumably because rapid re-reduction of cytochrome *f* or P700 occurred in a cyclic reaction. The absence of a delay in the increase of the yield of chlorophyll *a* fluorescence upon switching to System II after System I light also indicated that cytochrome *f* stayed reduced during the System I illumination. The negative bands at 563 and 434 nm were apparently due to cytochrome $b_{563}$. The photooxidation was presumably driven by System I: it was insensitive to DCMU and absorbed quanta of 680 nm were more active than of 618 nm in bringing about the reaction.

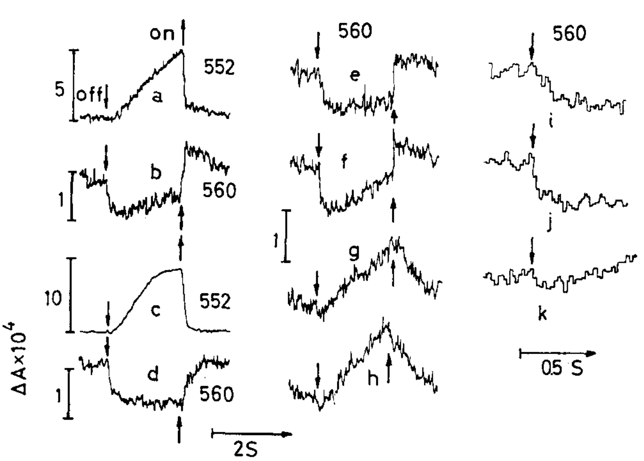


Fig. 3. Absorbance changes at the wavelengths indicated in alternating far-red light and darkness. Tracings a and b, controls in the presence of air; Tracings c and d, in the absence of oxygen and with 50 μM DCMU; Tracings e–h show the effect of PMS; e, control; f, 1 μM; g, 2 μM; h, 5 μM PMS; Tracings i–k correspond to Tracings e–g and show part of the kinetics upon darkening at an expanded time scale. The intensity of actinic light was 4.8 nEinstein·cm$^{-2}$·s$^{-1}$. See Fig. 2 for further explanation and conditions.

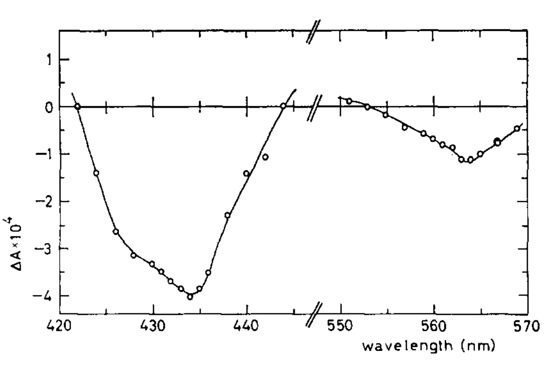


Fig. 4. Absorbance difference spectrum (far-red light *minus* dark) obtained with 25 μM PMS. The light intensity was 2.3 nEinstein·cm$^{-2}$·s$^{-1}$. Other conditions as for Fig. 3.

Antimycin A, at concentrations up to 0.1 mM, had little effect upon the kinetics of cytochrome $b_{563}$ in alternating far-red light and darkness. The same was true for 2-heptyl-4-hydroxy-quinoline-*N*-oxide. At a concentration of 50 $\mu$M it enhanced the absorbance change due to $b_{563}$ by about 50 %. The same effect was caused by 5 $\mu$M DBMIB. The difference spectrum in far-red light was similar to that without the inhibitor, and clearly showed the photoreduction of cytochrome $b_{563}$. The reduction of cytochrome *f* by System II was nearly completely inhibited at this concentration (*cf.* ref. 34).

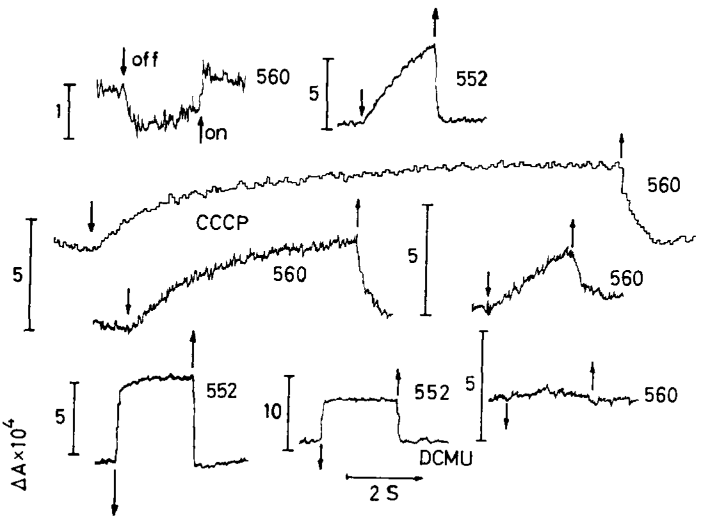


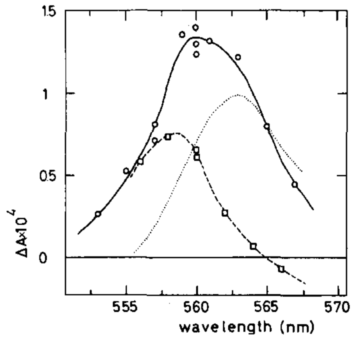
Fig. 5. Kinetics at 560 and 552 nm in alternating far-red light (700 nm) and darkness. Upper tracings: without additions. The other tracings were obtained in the presence of 10 $\mu$M CCCP. For the two tracings at the lower right the suspension contained 50 $\mu$M DCMU in addition. Light intensity: 4.8 nEinstein·cm$^{-2}$·s$^{-1}$. See Fig. 2 for further explanation.

### *Cytochrome $b_{559}$ photooxidation in the presence of CCCP*

Addition of CCCP to a suspension of *Porphyridium aerugineum* caused a drastic change in the absorbance kinetics. As Fig. 5 shows, the dark re-reduction of cytochrome *f* became very fast, and at 560 nm a slow absorbance increase instead of decrease was observed upon darkening. At 0.1, 1 and 10 $\mu$M CCCP, the spectrum of this slow absorbance change had a maximum at 559–560 nm (Fig. 6), and indicated the photooxidation of cytochrome $b_{559}$ upon illumination. At 50 $\mu$M CCCP, the difference spectrum appeared to be distorted, and had a maximum at somewhat shorter wavelength. The difference between the spectra at 1 and 50 $\mu$M CCCP (dotted line of Fig. 6) had a maximum at 562–563 nm. This might suggest that at 50 $\mu$M, a photoreduction of cytochrome $b_{563}$ occurred in addition. At 10 $\mu$M CCCP the dark reduction of cytochrome $b_{559}$ took about 6–10 s for completion. The maximum decrease in absorbance in the light observed after such a long dark time was $3.5 \cdot 10^{-4}$ absorbance unit, as compared to $8 \cdot 10^{-4}$ to $9 \cdot 10^{-4}$ for cytochrome *f* at 552 nm, suggesting that the pool of cytochrome $b_{559}$ was 2–3 times smaller than that of cytochrome *f*.

Microscopic inspection of the cells showed that treatment of about an hour with 10 μM or higher concentrations of CCCP resulted in breakage of the cells. At 25 μM fragmentation of the chloroplast occurred. Some of these fragments only sedimented after centrifugation at 25 000 × *g* during 1.5 h. At this concentration, 15 and 30 % of the phycocyanin was released and solubilized after 45 and 90 min treatment (at room temperature), respectively. Release of phycocyanin did not occur at 10 μM CCCP.

DCMU inhibited the absorbance changes at 559 nm in the presence of CCCP. Only absorbance changes attributable to cytochrome *f*, with α and β bands at 552 and 521 nm were observed in the presence of this inhibitor (Figs 5 and 7).



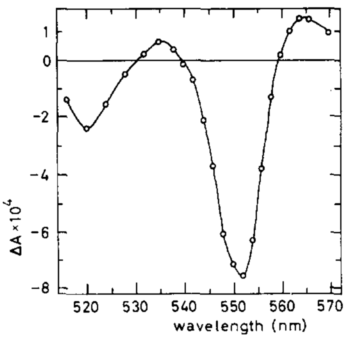


Fig. 6. Absorbance difference spectra of the slow absorbance change in the dark, measured between 0.2 and 2 s after darkening in the presence of 1 μM (○—○) and 50 μM CCCP (□- - -□). The intensities of far-red actinic light were 4.7 and 4.3 nEinstein·cm$^{-2}$·s$^{-1}$, respectively. The dotted line gives the difference between the two curves.

Fig. 7. Absorbance difference spectrum (orange light *minus* dark) obtained in the presence of 10 μM CCCP and 25 μM DCMU. The intensity of actinic light was 7.1 nEinstein·cm$^{-2}$·s$^{-1}$ (618 nm).

As shown in Table I, orange light was about 50 % more effective in bringing about cytochrome $b_{559}$ oxidation than far-red light (700 nm). Cytochrome *f* photooxidation was driven more effectively by far-red light, with or without DCMU. This suggests that the photooxidation of cytochrome $b_{559}$ is driven by Photosystem II. The absence of an effect of DCMU on the relative efficiencies for far-red and orange light in bringing about cytochrome *f* oxidation indicates that electron transport between Systems I and II or between System II and water[35] was disrupted by the CCCP treatment. The lower number for the relative efficiency of far-red as compared to orange light for cytochrome *f* photooxidation with CCCP (rows 4 and 5 of Table I) than without this substance (row 2) may be due to a conversion to something resembling pigment state 2 (refs 29–31) under these conditions.

## DISCUSSION

The data given in this article are most plausible explained by the assumption that cytochrome $b_{563}$ functions in a cyclic chain around Photosystem I, being reduced *via* the primary electron acceptor of System I, and, perhaps indirectly, oxidized by P700. This agrees with conclusions drawn from experiments with spinach chloroplasts[4,5]. It contrasts with the hypothesis of Nishimura[2] and of Ikegami and co-workers[3] who, working with intact red algae and with *Euglena* chloroplasts proposed a reaction site for cytochrome $b_{563}$ in the chain between the two light reactions.

The absence of an effect of oxygen indicates that cytochrome $b_{563}$ is not involved in a pseudocyclic or photorespiratory pathway to oxygen. Likewise, the absence of an effect of DCMU indicates that cytochrome $b_{563}$ is not oxidized by System II. This is in agreement with the results of the experiments in alternating System I and II light: apart from a slow additional oxidation of cytochrome $b_{563}$ in System II light which may have been due to indirect effects, the kinetics were the same as in cycles of System I light and darkness. It may be noted that Weikard[6], working with spinach chloroplasts, observed an oxidation of cytochrome $b_{563}$ by System II light. A reoxidation in the dark after far-red light was only observed with PMS, suggesting that without PMS no cycling around System I occurred. Our results indicate that in intact cells, at least of red algae, the reverse situation exists. Here, the first pathway is at most of minor importance, and cytochrome $b_{563}$ is probably oxidized by System I.

The quantum efficiencies, which were about 4 times lower than for cytochrome *f* oxidation, show that the photoreduction of cytochrome $b_{563}$ is a fairly efficient process, and suggest that under suitable conditions a sizable part of the electrons may be directed along the cyclic pathway, presumably to produce ATP. The rather fast dark oxidation, the half-time of which varied between 100 ms (as in Fig. 3) and about 20 ms indicates a fairly high turnover rate. Under all conditions tested, the absorbance changes of cytochrome $b_{563}$ were several times smaller than those of cytochrome *f*. This also applied for the photooxidation in the presence of PMS. This might suggest that in red algae the pool of cytochrome $b_{563}$ is smaller than in spinach chloroplasts[7]. However, it should be kept in mind that the pool of cytochrome $b_{563}$ in *Porphyridium* may be larger than shown by these data, because we had no way to ascertain the absolute oxidation–reduction levels in the light or in the dark in these experiments. Nevertheless, it may be possible that only some reaction centers of System I are equipped to perform a cyclic reaction, or that a small pool of cytochrome $b_{563}$ is shared by several reaction centers.

Little can be said about possible other components of the cyclic chain. The absence of a correlation between the kinetics of cytochrome *f* reduction and of $b_{563}$ oxidation (see Fig. 2, which shows a much faster dark oxidation of cytochrome $b_{563}$ than dark reduction of cytochrome *f*) does not necessarily indicate that the two cytochromes react with each other *via* an intermediate. If one assumes that cytochrome *f* is in redox equilibrium with P700, then the oxidation rate of cytochrome $b_{563}$ should be comparable to the dark reduction rate of P700, which is much faster than of cytochrome *f*. A direct reaction of cytochrome $b_{563}$ with P700 cannot be excluded either. PMS apparently accelerates the reaction of cytochrome $b_{563}$ with cytochrome *f* or P700 (Fig. 3). The experiments with DBMIB, which probably inhibits plastoquinone reactions[34,36,37] suggest that plastoquinone is not part of the cyclic chain.

The difference spectrum and other data obtained in the presence of CCCP suggest that under these conditions cytochrome $b_{559}$ is oxidized by System II. As far as we know the occurrence of this cytochrome had not been observed in red algae so far. Our data bear resemblance to those obtained by Hiller *et al.*[38] with intact leaves of pea and spinach. Like with *Porphyridium*, cytochrome $b_{559}$ photooxidation was only observed after the leaves had been treated with CCCP. The results, however, suggested that both Systems I and II brought about the photooxidation, and unlike in our system, DCMU gave only a partial inhibition. It should be remarked that the concentration of CCCP used for the leaf experiments was 10 times higher than that applied for most of our measurements. A photooxidation of cytochrome $b_{559}$ by System II has been observed at liquid-nitrogen temperature by several authors[10–15]. Photooxidation observed at room temperature with spinach and algal chloroplasts has usually been attributed to Photosystem I (refs 3, 4, 17–20), but with Tris-washed chloroplasts[10] and with System II particles prepared mechanically[16] or with digitonin[39] the oxidation was ascribed to System II. Our results are different from those of Nishimura[2], who only observed photooxidation of a cytochrome with $\alpha$ band at 562 nm in *Porphyridium cruentum* with 12.5 $\mu$M CCCP. Without CCCP, we found no evidence for cytochrome $b_{559}$ photooxidation. It remains to be established if the effect of CCCP is related to other known effects of this compound (or of FCCP), *viz.* its uncoupling action, the enhanced decay rate of intermediates of the oxygen-evolving pathway[40,41] and the conversion of high-potential to low-potential cytochrome $b_{559}$ (ref. 21).

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