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A SPECTROSCOPIC ANALYSIS OF LOW-FLUORESCENT MUTANTS OF *CHLAMYDOMONAS REINHARDTI* BLOCKED IN THEIR WATER-SPLITTING OXYGEN-EVOLVING APPARATUS

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SUMMARY

1. A spectral analysis of six mutant strains of the unicellular alga, *Chlamydomonas reinhardti*, having lesions associated with the water-splitting oxygen-evolving apparatus is described.

2. The chloroplast fragments from these mutant strains have a functional Photosystem II reaction center as shown by the normal photoreduction of C-550 and the photooxidation of cytochrome b_{559} at low temperature, and by their ability to carry out a 3-(3,4-dichlorophenyl)-1,1-dimethylurea-sensitive photoreduction of $NADP^+$ at room temperature in the presence of electron donors which donate electrons specifically to Photosystem II.

3. The mutant chloroplast fragments contain equal amounts of the ascorbate-reducible cytochrome b_{559} and C-550 with all of the cytochrome b_{559} present participating in the low-temperature photoreaction. In contrast the wild-type chloroplast fragments contain twice the amount of high-potential cytochrome b_{559} as C-550 with only half of the cytochrome b_{559} present participating in the low-temperature photoreaction.

4. The only component difference found between the mutant and the wild-type chloroplast fragments was that the lfd chloroplast fragments contained only half as much ascorbate-reducible cytochrome b_{559} as the wild type. Equal proportions, respectively, of cytochrome c_{553}, low-potential cytochrome b_{559}, and cytochrome b_{564} are found in both the mutants and wild-type strains.

5. The data indicate that there exist two functionally distinct pools of ascorbate-reducible cytochrome b_{559} both of which function in the water-splitting oxygen-evolving apparatus. The lfd mutant strains are unable to split water and evolve oxygen because they lack one of the two pools of ascorbate-reducible cytochrome b_{559}.

INTRODUCTION

The isolation of mutant strains of algae with lesions in their photosynthetic apparatus has proved to be a valuable experimental approach to elucidate the electron

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transfer reactions of photosynthesis[1,2]. In the present study we have used mutant strains of the algae *Chlamydomonas reinhardti* blocked in their capacity to evolve oxygen to explore the mechanisms of oxygen evolution in photosynthesis.

Two classes of mutant strains of *C. reinhardti* which have normal Photosystem I activity but which cannot evolve oxygen have been isolated and characterized as previously described[3]. One class, designated lfd, is characterized by a low invariant fluorescence yield. These mutants are blocked between water and Photosystem II[3], similar to the block imposed on normal chloroplasts by washing with high concentrations of Tris[4]. Normal electron transport reactions such as a 3-(3,4-dichlorophenyl)-1,1-dimethylurea-sensitive photoreduction of $NADP^+$ and the fluorescence of variable yield which are absent in these strains can be restored to chloroplast fragments of the lfd mutants by adding electron donor compounds which donate electrons specifically to Photosystem II[3]. The other class of mutant strains designated hfd, is characterized by a high invariant fluorescence yield. Photosystem II activity, in contrast to the lfd strains, cannot be restored to chloroplast preparations of the hfd mutant strains.

A spectroscopic analysis of the hfd mutant strains was recently reported[5]. Chloroplast fragments from the hfd mutant strains are not capable of the low-temperature photoreaction, *i.e.* the photoreduction of C-550 and the photooxidation of cytochrome b_{559}, associated with the reaction centers of Photosystem II. A fourth derivative analysis (the 4th derivative analysis of absolute spectra[6,7] permits the resolution and identification of absorption bands that are otherwise observed only in difference spectra) of the absolute spectra of the chloroplast fragments from the wild-type and mutant strains under varying redox conditions showed that while the wild-type fragments contain C-550, cytochrome c_{553}, high- and low-potential forms of cytochrome b_{559} and cytochrome b_{564}, the mutant strains are totally deficient in C-550 and in high-potential cytochrome b_{559}. The hfd mutants, thus, were found to be defective in their Photosystem II reaction centers.

In this paper we report on a spectral analysis of the lfd mutant strains which have lesions on the oxidizing side of Photosystem II. Difference spectra and a fourth derivative analysis of absolute spectra show that all these mutants have normal Photosystem II reactions centers (as indicated by the low-temperature photoreactions) but lack approximately 50 % of the high-potential cytochrome b_{559}.

MATERIAL AND METHODS

A spectral analysis similar to that reported for the hfd strains[5] was performed with chloroplast fragments from the lfd strains. Absorption spectra of the chloroplast fragments were examined in the 525–575-nm spectral region with a single beam spectrophotometer on line with a PDP 8/I computer. Chloroplast fragments were prepared as previously described[5]. Strains examined in this study include, lfd 2, lfd 27, lfd 13, lfd 17, lfd 15 and lfd 29.

RESULTS

Light-induced absorbance changes of chloroplast fragments from wild-type cells of *C. reinhardti* and from the low-fluorescent mutant, lfd 13, at −196 °C are compared in Fig. 1. The light *minus* dark difference spectra of both samples show

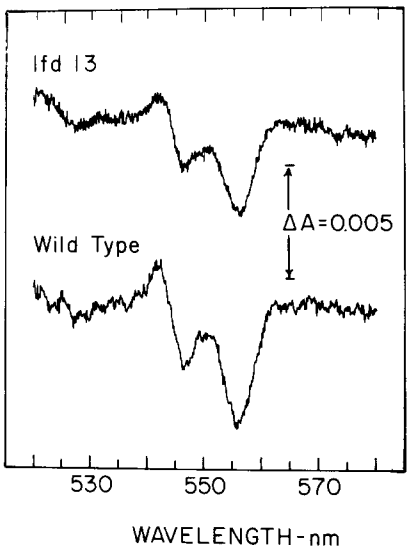

Fig. 1. Light *minus* dark low-temperature (−196 °C) difference spectra (sample irradiation with 645 nm light for 30 s at −196 °C *vs* same sample before irradiation) of ascorbate-reduced (20 μmoles) wild-type and of lfd 13 chloroplast fragments (equivalent to 100 μg chlorophyll in 0.5 ml).

the normal photoreduction of C-550 and photooxidation of cytochrome b_{559}. Similar results were obtained with the other five lfd mutant strains that have been isolated. Thus, the lfd mutants appear to have normal Photosystem II reaction centers which is consistent with the original work indicating that these mutants were blocked between water and Photosystem II[3].

Fourth derivatives of the absolute absorption spectra of chloroplast fragments from the wild-type and the lfd 13 mutant cells are presented in Figs 2A and 2B, respectively. The spectra were measured at −196 °C at different stages of reduction (obtained with ferricyanide before (D) and after (L) illumination at −196 °C, with ascorbate before (D) and after (L) illumination at −196 °C, and with dithionite). The spectra of the unirradiated ferricyanide-oxidized chloroplast fragments from both the wild type and the mutant (Strain lfd 13) show only the 4th derivative band at 545 nm due to the oxidized band of C-550. After irradiation the 4th derivative spectra show that the absorption band of C-550 shifts to 543 nm. (The absorption band shift of C-550 was shown previously with spinach chloroplasts[8].) Addition of ascorbate to either the wild-type or lfd mutant chloroplast fragments reduces cytochrome c_{553}, giving a split band with maxima at 547 and 552 nm at −196 °C, and cytochrome b_{559} with a maximum at 557 nm. C-550 is oxidized in the unirradiated ascorbate-reduced samples and its absorption band is not resolvable from the 547-nm band of cytochrome c_{553}. A comparison of the 4th derivative spectra (Asc D) of the two samples indicates less ascorbate-reducible cytochrome b_{559} in the mutant. On irradiation of the ascorbate-reduced samples at −196 °C (Asc L) the C-550 in both samples is reduced, the cytochrome b_{559} of the mutant is totally oxidized but only a part of the cytochrome b_{559} of the wild type is oxidized. The amount of cytochrome b_{559} which can be photooxidized at −196 °C is limited to the amount of C-550 photoreduced because the photoreaction requires that C-550 serve as the primary electron

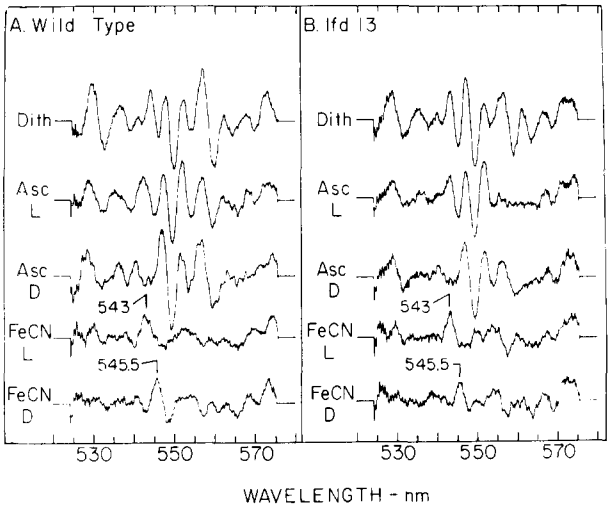

Fig. 2. Fourth derivative spectra of chloroplast fragments (equivalent to 100 μg chlorophyll in 0.5 ml) from (A) wild-type (acl$^+$) and (B) mutant lfd 13 cells at −196 °C in the presence of, as indicated, either 20 μmoles ferricyanide (FeCN), 20 μmoles ascorbate (Asc) or a few grains of dithionite (Dith), measured before (D) and after (L) irradiation with 645 nm light at −196 °C.

acceptor[9]. Addition of dithionite to the chloroplast fragments reduced C-550, cytochrome c_{553}, some additional cytochrome b_{559} that was not reduced by ascorbate, and cytochrome b_{564}. The only significant difference between the 4th derivative spectra of the dithionite-reduced samples is that less cytochrome b_{559} appears to be present in the mutant chloroplast fragments.

Differences in the cytochromes of the wild-type and mutant chloroplast fragments are also revealed in room-temperature absorption spectra taken at different states of reduction (Fig. 3). A comparison of the difference spectra between no addition and ferricyanide shows that while cytochrome c_{553} and cytochrome b_{559} are reduced in freshly prepared untreated chloroplast fragments from wild-type cells, these cytochromes are either oxidized or absent in the chloroplast fragments from the mutant strain. Addition of ascorbate to the mutant chloroplast fragments establishes that some of the cytochromes are present but in contrast to fragments from the wild-type strain are oxidized in the untreated sample. The ascorbate–ferricyanide difference spectrum of the mutant chloroplast particles shows a broad band due to the sum of the α bands of cytochromes c_{553} and b_{559}. It is apparent, however, by comparing the ascorbate−ferricyanide difference spectra of the mutant and the wild-type chloroplasts that the mutant lacks some of the cytochrome b_{559} present in the wild type. This is confirmed in the difference spectrum, wild type *minus* lfd 13, between the two ascorbate−ferricyanide difference spectra which shows that an appreciable part (approximately 50 %) of the ascorbate-reducible cytochrome b_{559} in the wild-type chloroplast fragments is missing from the mutant fragments. Addition of dithionite to the ascorbate-reduced samples reduces additional cytochrome b_{559} (by definition, the low-potential cytochrome b_{559}) and cytochrome b_{564}. The dithionite−ascorbate difference spectra and the corresponding difference spectra between the wild type and the mutant establish that wild-type and mutant samples contain equal amounts of

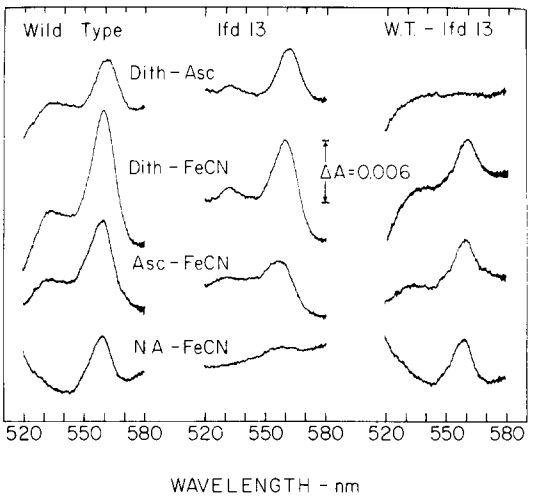

Fig. 3. Room-temperature chemical difference spectra of chloroplast fragments (equivalent to 200 μg chlorophyll in 1 ml) from cells of wild type and of the lfd 13 mutant strain, and the computer generated wild-type *vs* lfd difference spectra (W.T.—lfd 13). NA—FeCN, no addition *vs* ferricyanide (2 μmoles); Asc—FeCN, ascorbate (20 μmoles) *vs* ferricyanide (2 μmoles); Dith—FeCN, dithionite (a few grains) *vs* ferricyanide (2 μmoles); Dith—Asc, dithionite (a few grains) *vs* ascorbate (20 μmoles).

these two dithionite-reducible, non-ascorbate-reducible cytochromes. The same results were obtained with the other five lfd mutant strains.

In summary, the mutant chloroplast fragments contain equal amounts of the ascorbate-reducible cytochrome b_{559} and C-550 and all of the cytochrome b_{559} present participates in the low-temperature photoreaction. The wild-type fragments contain twice as much high-potential cytochrome b_{559} as C-550. (It was also noted previously that spinach chloroplasts contain twice as much ascorbate-reducible cytochrome b_{559} as C-550[9]). The only component difference found between the mutants and wild-type chloroplast fragments was that the lfd mutants contained half as much ascorbate-reducible cytochrome b_{559}. Both contained equal proportions, respectively, of cytochrome c_{553}, the low-potential cytochrome b_{559} and cytochrome b_{564}.

DISCUSSION

The spectroscopic analysis of the lfd mutants indicates that these strains which are unable to evolve oxygen are lacking in half of the normal complement of the high-potential cytochrome b_{559}. These results give experimental support to recent speculations[10,11] that "this cytochrome" functions on the water-splitting side of Photosystem II.

Our results suggest that the high-potential cytochrome b_{559} of normal chloroplasts exists in two functionally distinct pools. Half that pool still present in the lfd strains is intimately connected to the reaction centers of Photosystem II so that it can be photooxidized at −196 °C. The lfd mutant data establishes that the other half of the ascorbate-reducible pool of cytochrome b_{559} is also involved in the water-splitting oxygen-evolving apparatus.

The interpretation of two equal pools of the high-potential cytochrome b_{559} is also supported by observations on the effects of lipase digestion on spinach and *Chlamydomonas* chloroplasts (ref. 12 and B. L. Epel, I. Okayama and W. L. Butter, unpublished results). Relatively mild disruptive treatments of chloroplasts such as washing with high concentrations of Tris[11] or mild heat treatment[13] modify the high-potential (hydroquinone-reducible) cytochrome b_{559} to a lower potential form[13] or forms[11] which are reducible by ascorbate but not by hydroquinone. More drastic disruptive treatments such as acetone extraction[14] or more drastic heat treatments[13] result in the cytochrome b_{559} being modified to even lower-potential forms which are reducible by dithionite but not by ascorbate. Lipase digestion of both spinach and *Chlamydomonas* chloroplasts modifies two equal parts of the high-potential cytochrome b_{559} differently: with spinach chloroplasts after lipase treatment, half of the high-potential cytochrome b_{559} requires ascorbate for reduction and half requires dithionite; with *Chlamydomonas* chloroplasts, half remains ascorbate reducible but the other half is destroyed or denaturated further so that it is no longer reducible even by dithionite[12]. These results suggest that the two halves of the high-potential cytochrome b_{559} are differentially sensitive or accessible to the lipase treatment.

In this work it was noted (see Fig. 3) that both cytochrome c_{553} and the residual cytochrome b_{559} are in the oxidized state in untreated chloroplast fragments from the mutant strain lfd 13. In the wild type, in contrast, both are found in the reduced state. Two interpretation of this discrepancy can be offered. It is conceivable that the mutation in the lfd strain has resulted in a membrane modification, the consequence of which has resulted in an apparent alteration of the redox potential of the cytochromes, leading to their becoming autooxidizable. A simpler but as yet untested alternate interpretation is that since the water-splitting apparatus is inactive in this mutant, the two photosystems, which are in themselves fully active, have depleted the endogenous reductant pools of the chloroplast, resulting in the cytochromes being situated in an oxidizing redox environment. Thus in this later explanation the oxidized state of the cytochrome is only a reflection of the redox state of the endogenous redox buffer pool of the chloroplast.

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