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MUTANT STRAINS OF *CHLAMYDOMONAS REINHARDI* WITH LESIONS ON THE OXIDIZING SIDE OF PHOTOSYSTEM II

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SUMMARY

1. Two mutant strains of the unicellular green alga, *Chlamydomonas reinhardi*, having lesions on the oxidizing side of photosystem II, have been obtained using a selection and screening technique that facilitates the isolation of photosynthetic mutant strains having lesions either on the oxidizing or reducing side of photosystem II.

2. Chloroplast fragments obtained from the two strains lack Hill activity. However, they exhibit normal rates of $NADP^+$ photoreduction with an artificial electron donor to photosystem I. Fluorescence yield changes are absent in the chloroplast fragments obtained from both strains.

3. In the presence of electron donor systems to photosystem II, the mutant strains exhibit fluorescence yield changes and a 3(3,4-dichlorophenyl)-1,1-dimethylurea-sensitive photoreduction of $NADP^+$ that are characteristic of the wild-type strain.

4. Based on what is generally regarded to be the nature of photosystem II, mutant strains having lesions on the oxidizing side of photosystem II should exhibit an invariant high fluorescence yield. The abnormally low fluorescence yield exhibited by the mutant strains can be explained if it is assumed that there is a back reaction between a component on the reducing side of photosystem II and one on the oxidizing side.

INTRODUCTION

The reactions that occur on the water-splitting, oxidizing side of photochemical system II (photosystem II-ox) are responsible for the extraction of four electrons and protons from water and the production of molecular oxygen. These reactions are among the most poorly characterized of all the events that take place during photosynthesis by algae and higher plants. Information concerning events on photosystem II-ox can be obtained with mutant strains of the unicellular green alga, *Chlamydomonas reinhardi*, that have lost the capacity to carry out reactions on this side of photosystem II. In this paper we describe a method for the selection and

Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol.

screening of the mutant strains, and we characterize some of their photosynthetic properties.

MATERIAL AND METHODS

Organisms and their culture

The wild-type strain of *C. reinhardi* (137c) and two different mutant strains (*lfd-2*, and *lfd-27*) were grown in Tris–acetate phosphate medium[1] under conditions previously described[2]. Chloroplast fragments were prepared from cells in the logarithmic phase of growth according to the method of LEVINE AND GORMAN[3]. The chlorophyll content of the chloroplast fragments or of whole cells was determined by a modification[4] of the method of MACKINNEY[5]. Cell numbers were obtained with the aid of a hemacytometer.

Photosynthetic reactions

Rates of oxygen evolution were measured at 25° with a Clark-type oxygen electrode (Yellow Springs Instrument Co. Model No. 53) contained in a 1-ml capacity thermostated lucite cuvette. Illumination was with white light at 40000 lux obtained with a 500-W lamp (Sylvania C5A).

Rates of $NADP^+$ or 2,6-dichlorophenolindophenol (DCIP) photoreduction were measured under aerobic conditions with an Aminco-Chance double beam spectrophotometer. A 650-nm actinic beam of an intensity of $1.4 \cdot 10^4$ erg·cm^{-2}·sec^{-1} was provided to the samples from the side. The measuring and reference wavelengths for the determination of the rate of $NADP^+$ photoreduction were 340 and 390 nm, respectively. The phototube was protected from the actinic light by a Corning 5874 filter in combination with a Wrattan 18A filter. For determining the rate of photoreduction of DCIP, the measuring and reference wavelengths were 600 and 460 nm, respectively. The phototube was protected from the actinic light with a Corning 4076 filter and a Wrattan 57 filter.

Fluorescence excitation spectra

Fluorescence excitation spectra were measured with a modified Aminco-Bowman spectrofluorimeter fitted with an EMI 9558C phototube. The fluorescence emission was detected at 750 nm in order to avoid scatter artifacts in the excitation spectra that would occur were the emission measuring wavelength below 700 nm. A plastic cutoff filter (Rhom and Haas No. 58015) that only transmits above 700 nm was placed before the entrance slit of the emission monochromator to reduce the stray light reaching the phototube.

Fluorescence yield measurements

Fluorescence yield measurements were made with a specially constructed instrument similar to that described by YAMASHITA AND BUTLER[6]. The fluorescence of chloroplast fragments, excited by a low intensity (70 erg·cm^{-2}·sec^{-1}) modulated (300 Hz) 650-nm measuring beam, was detected with an EMI 9558C phototube. The resulting modulated signal was demodulated by a lock-in amplifier (PAR-HR8) tuned to the frequency of the modulated exciting light. High intensity unmodulated 650-nm actinic illumination ($2.4 \cdot 10^4$ erg·cm^{-2}·sec^{-1}) sufficient to saturate photosynthesis was

provided at right angles to the phototube and the measuring beam. The fluorescence produced by the unmodulated actinic beam was not detected by the lock-in amplifier. The phototube, situated directly behind the cuvette, was blocked with a pair of Corning filters (2030 and 9830) that pass only fluorescence of wavelengths greater than 680 nm.

Reagents

L-Ascorbate was obtained from Sigma Chemical Corp. and *p*-aminophenol hydrochloride, *p*-hydroquinone, and *p*-phenylene diamine hydrochloride were obtained from Eastman Organic Chemicals. All were used in aqueous solutions that were prepared daily.

RESULTS

Selection and screening for photosystem II-ox mutant strains

The selection of the mutant strains is based on the observation that under anaerobic conditions and in the presence of a photodynamic dye such as erythrosin B, cells that can evolve oxygen are killed upon exposure to high light intensities whereas mutant cells that produce little or no oxygen, and thus have a low internal oxygen concentration, are protected from photodynamic killing.

The selection procedure was applied to cells that had been treated with ultraviolet irradiation to induce mutations. 10 ml of wild-type cells at a concentration of $0.5 \cdot 10^6$–$2 \cdot 10^6$ cells/ml in Tris–acetate phosphate medium were placed in an open glass petri dish, agitated with a magnetic stirrer, and irradiated for 2 min with a 15-W G.E. germicidal lamp placed 48 cm from the surface of the medium. After irradiation the cell suspension was diluted 5-fold in Tris–acetate phosphate medium and incubated in the dark for 12–24 h. Erythrosin B was then added to 10 ml of the culture in a stoppered thermostated glass cuvette to give a final concentration of the dye of $1 \cdot 10^{-4}$ M, and the suspension was deoxygenated by bubbling with a CO_2–N_2 (5:95, v/v) mixture for 15 min in the dark. Following deoxygenation, but with continuing gassing with the CO_2–N_2 mixture, the suspension was irradiated for 40 min with light ($2 \cdot 10^5$ lux) from a 1000-W projector lamp (G.E. DRS). This period of exposure was found to be sufficient to kill 90% of the wild-type cells. After being irradiated, the cell suspension was diluted and plated to Tris–acetate phosphate medium to give about 200 colonies per plate. Following incubation in the light for 4–5 days, the plates were screened for mutant colonies by a modification of the procedure of BENNOUN AND LEVINE[7]. Each plate was illuminated with light (27000 lux) from an Osram HBO-200 high pressure mercury arc lamp. The light was passed through a water filter and a Corning 4305 filter that cut off all wavelengths above 640 nm. The fluorescence of the colonies on the plates activated by this light was viewed by eye through goggles fitted with Corning 2030 filters that pass only wavelengths of light above 640 nm.

It has previously been shown[6] that when isolated spinach chloroplasts are washed with Tris buffer, photosystem II-ox activity is lost and a decreased level of fluorescence results. We therefore sought colonies having a lower level of fluorescence than is characteristic of colonies of wild-type cells. Colonies exhibiting low fluorescence were picked and replated to Tris–acetate phosphate medium. Cells from the colonies that formed were tested for growth on minimal medium and on Tris–acetate phos-

phate medium. Colonies of cells that grew on minimal medium were discarded. Those that did not were retained for analysis of their photosynthetic and genetic properties.

The photosynthetic properties of lfd-2 and lfd-27

Two low-fluorescence mutants, *lfd-2* and *lfd-27*, were isolated by the procedure described above. Both mutant strains evolve little or no oxygen, although their chlorophyll content falls within the range generally found for wild-type cells and their ratios of chlorophyll *a* to *b* are similar to wild-type. Table I shows that chloroplast

TABLE I

REACTIONS OF THE PHOTOSYNTHETIC ELECTRON-TRANSPORT CHAIN OF *lfd-2*, *lfd-27*, AND WILD-TYPE *C. reinhardi* CHLOROPLAST FRAGMENTS

All reactions were measured at 25° in the appropriate reaction mixture (1 ml final volume) containing chloroplast fragments (equivalent to 12.5 μg chlorophyll/ml) prepared by the sonication of cells. Each reaction mixture contained in μmoles: Potassium phosphate buffer (pH 7), 10; KCl, 20; $MgCl_2$, 2.5. For the Hill reaction with $NADP^+$ the reaction mixture contained in μmoles: ferredoxin purified from wild-type *C. reinhardi*, 0.005; $NADP^+$, 0.5; and 50 munits Fd-$NADP^+$ reductase purified from wild-type *C. reinhardi*. For the Hill reaction with DCIP the reaction mixture contained 0.05 μmoles of DCIP. For the photoreduction of $NADP^+$ using the DCIP–ascorbate couple, the reaction mixture contained in μmoles: DCIP, 0.05; ascorbate, 5; DCMU, 0.02; ferredoxin purified from wild-type *C. reinhardi*, 0.005; $NADP^+$, 0.5; and Fd-$NADP^+$ reductase purified from wild-type *C. reinhardi*, 50 munits.

Strain	*μmoles oxidant reduced/h per mg chlorophyll*		
	$NADP^+$ Hill reaction	*DCIP Hill reaction*	*$NADP^+$ photoreduction with DCIP/ascorbate*
Wild type	148	62	103
lfd-2	0	0	72
lfd-27	4	0	107

fragments of the mutant strains have only insignificant Hill reaction rates with either $NADP^+$ or DCIP as oxidants in comparison to chloroplast fragments obtained from wild-type cells. However, the data in Table I indicate that the activity of photochemical system I is normal in the chloroplast fragments of both mutant strains, for the rate of $NADP^+$ photoreduction using the DCIP–ascorbate couple as the electron donor is essentially the same as in chloroplast fragments obtained from wild-type cells. Thus the mutant strains have lesions that are associated with photosystem II rather than with photosystem I.

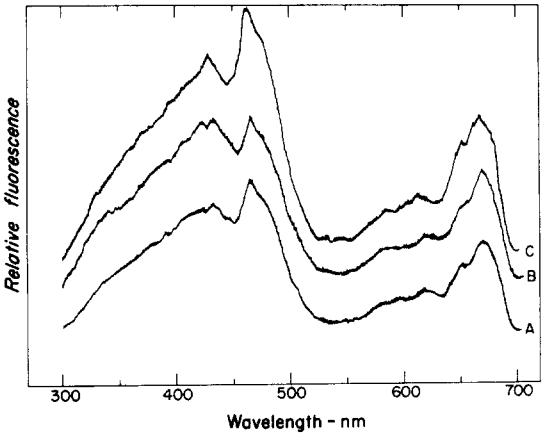
The fluorescence excitation spectra of the mutant strains

The fluorescence excitation spectra of *lfd-2* and *lfd-27* cells and of wild-type cells are shown in Fig. 1. The light-harvesting pigment complex of photosystem II appears to be fully functional in both mutant strains, for their excitation spectra are identical to the spectrum obtained for wild-type.

Restoration of $NADP^+$ photoreduction by photosystem II-ox donors

YAMASHITA AND BUTLER[6,8,9] showed that the oxidizing side of photosystem II can be inactivated in isolated spinach chloroplasts by washing the chloroplasts in Tris buffer or by treating them with ultraviolet light or heat. They showed further

that various electron donor systems could feed into the oxidizing side of photosystem II so as to partially restore the Hill reaction with $NADP^+$. We have found the *p*-hydroquinone–ascorbate donor system to be effective in restoring 60% of the $NADP^+$ Hill reaction in *lfd-27* (Fig. 2). The 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) sensitivity of this reaction indicates that the *p*-hydroquinone–ascorbate couple donates its electrons to PS II before the site of DCMU action. Other electron donor systems such as *p*-aminophenol–ascorbate and *p*-phenylene diamine–ascorbate were also found to be effective in restoring a photosystem II-dependent photosynthetic electron transport to both *lfd-2* and *lfd-27*.



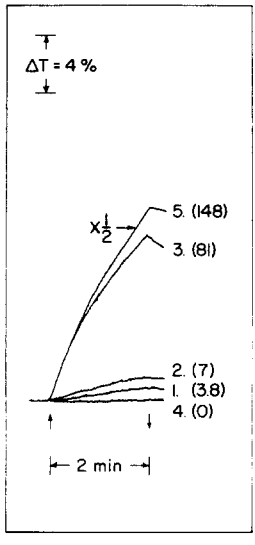


Fig. 1. The fluorescence excitation spectra of (A) *lfd-2*, (B) *lfd-27*, and (C) wild-type cells (equivalent to 10 μg chlorophyll/ml). The cells were suspended in 50% glycerol–Tris–acetate phosphate medium.

Fig. 2. The photoreduction of $NADP^+$ by *lfd-27* and wild-type chloroplast fragments (equivalent to 12.5 μg chlorophyll/ml). The reaction mixture is given in Table I. Curve 1, *lfd-27* with no additions; Curve 2, *lfd-27* with 2 mM ascorbate; Curve 3, *lfd-27* with 0.4 mM *p*-hydroquinone and 2 mM ascorbate; Curve 4, *lfd-27* with 0.4 mM *p*-hydroquinone, 2 mM ascorbate, and 0.01 mM DCMU; Curve 5, wild-type with no additions.

Fluorescence yield properties of chloroplast fragments

The fluorescence yield properties of the chloroplast fragments of the mutant strains *lfd-2* and *lfd-27* in the absence of an electron donor system are unlike those of wild-type. Fig. 3 compares the fluorescence properties of chloroplast fragments from wild-type and *lfd-27* cells in the presence and absence of an electron donor system; the properties of *lfd-2* are identical to *lfd-27*. In contrast to chloroplast fragments from wild-type cells, the *lfd-27* fragments show no fluorescence yield changes upon actinic illumination in the absence of electron donor, indicating that the rate of electron transfer to Q is insufficient to bring about any appreciable reduction of Q. However, the addition of the *p*-hydroquinone–ascorbate electron donor system partially restores the fluorescence yield changes that are characteristic for wild-type chloroplast fragments. This observation demonstrates that the electron donor system

provides its electrons on the oxidizing side of photosystem II and indicates that the lesions in *lfd-2* and *lfd-27* are on the oxidizing side of photosystem II. A depressed fluorescence yield for wild type is seen in the presence of DCMU. This effect of DCMU is not understood.

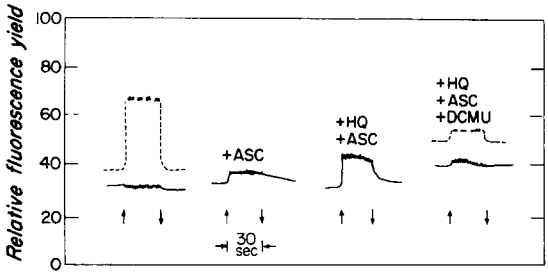


Fig. 3. Fluorescence yield of *lfd-2* and wild-type chloroplast fragments (equivalent to 3 μg chlorophyll/ml). The reaction mixture contained in μmoles: potassium phosphate (pH 7), 10; KCl, 20; $MgCl_2$, 2.5; and where indicated *p*-hydroquinone (HQ), 0.4; ascorbate (ASC), 2; and DCMU, 0.01. Red actinic light (650 nm; $2.4 \cdot 10^4$ erg·cm^{-2}·sec^{-1}) turned on at upward arrow and off at downward arrow. Traces drawn with dotted line are for wild-type chloroplast fragments and traces drawn with solid line are for *lfd-2* chloroplast fragments.

DISCUSSION

Abnormally high fluorescence yields from algal and higher plant chloroplasts are produced by genetic lesions on the reducing side of photosystem II (ref. 7) or the presence of an inhibitor of electron transport such as DCMU[10]. The abnormally low fluorescence yields that occur in Tris-washed, heat-treated, and ultraviolet-irradiated spinach chloroplasts[6,8,9] and in the *lfd* mutant strains of *C. reinhardi*, have been shown to be correlated with a lesion on the oxidizing side of photosystem II.

Considering what is generally regarded to be the nature of photosystem II (refs. 10–12), high fluorescence yields could arise for several reasons: (1) the electron transport chain might be unable to reoxidize Q; (2) there might be a loss or inactivation of Q; and (3) there could be an irreversible bleaching or loss of the reaction-center pigment of photosystem II. An irreversible bleaching of the reaction-center pigment would occur if there were no electron transfer to the photosystem II reaction center because of a loss or inactivation of either Z, the electron donor to the photosystem II reaction-center[12], or some component between Z and water.

Among mutant organisms in which a high fluorescence yield has been reported, there are those that are unable to perform a Hill reaction with oxidants such as DCIP or ferricyanide and that have lesions clearly closely associated with photosystem II (refs. 13–16). It has been suggested[13–16] that the lesion in these mutant strains is on the reducing side of photosystem II and that they lack Q. However, as pointed out above, any lesion on the oxidizing side of photosystem II that results in a loss or an irreversible bleaching of the reaction-center pigment of photosystem II would also produce all of the characteristics reported for these mutant strains. Accordingly a re-evaluation of the properties of these mutant strains would be of some interest.

Since lesions on the oxidizing side of photosystem II should be expected to result in an abnormally high fluorescence yield, the observation of the low fluorescence

yield in Tris-washed, heat-treated, or ultraviolet-irradiated spinach chloroplasts and in the *lfd* mutant strains of *C. reinhardi* is unexpected. This apparent contradiction between experimental findings and theoretical expectations can be resolved, however, if it is assumed that a back reaction exists between the primary reductant of photosystem II and either the oxidized photosystem II reaction-center pigment or some intermediate on the oxidizing side of photosystem II. Such a back reaction would result in the restoration of an active reaction center that could once again serve as a trap for the excitation energy of the light-harvesting chlorophyll of photosystem II. If a back reaction does indeed occur between the primary electron donor of photosystem II and the reaction-center pigment, the experimental observation of the low fluorescence yield predicts that the reaction-center of photosystem II is either a low or non-fluorescing species of chlorophyll or, alternatively, that it is some pigment other than chlorophyll. Experiments are underway to test the back-reaction hypothesis and its predictions.

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