Development of Photosystem II Activity in *Chlamydomonas reinhardi* Mutants

**INSERTION OF PHOTOSYSTEM II UNITS INTO INACTIVE PREEXISTING MEMBRANES VERSUS CONTINUOUS FORMATION OF NEW PHOTOSYNTHETIC MEMBRANES**

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**ABSTRACT**

In a previous work the development of photosystem II activity during the greening process of the y-1 mutant of *Chlamydomonas reinhardi* was studied (Cahen, Malkin, Shochat, Ohad. Plant Physiol 58: 257-267). Measurements of quantum yield, maximal rate of electron transfer, flash yield, and fluorescence induction indicated that photosystem II development consists of two partially overlapping phases: (a) reorganization and integration of preexisting units; and (b) addition of newly formed units to the growing membranes.

In the present work an attempt was made to study these two processes independently. This was achieved using two experimental systems: (a) cells of the y-1 mutant, greened in the presence of chloramphenicol (CAP), whose activity is repaired under conditions which prevent further chlorophyll synthesis and membrane growth (dark or light with addition of cycloheximide [CHI]); (b) green cells of a pale mutant derived from y-1 in which chloroplast membranes are formed gradually during several cell divisions.

In both systems different parameters of 2,6-dichlorophenolindophenol reduction, as well as fluorescence, were measured. The results are interpreted using a kinetic model reported previously. It is concluded that during repair of photosynthetic activity in inactive membranes which were formed previously in the presence of chloramphenicol, photosystem II reaction centers are formed and integrated in preexisting membranes. In the pale green mutant there is complete de novo synthesis of units of constant composition which are added during formation of new chloroplast membranes.

Cells of *Chlamydomonas reinhardi* (y-1) mutant lose their Chl as well as their entire photosynthetic membrane system when grown for several generations in the dark (9). Following renewed illumination, Chl and membrane synthesis is resumed and accompanied by the build-up of photosynthetic ability (greening process) which is accomplished in the absence of cell division (6, 11).

Recently we analyzed the development of PSII activity during the greening process of this mutant using fluorescence induction measurements and determination of the quantum yield, flash yield, and maximal rate (under saturating light conditions) of PSII-mediated electron transport (2). We hoped that by measuring simultaneously partially overlapping reactions of PSII it would be possible to get detailed information on the process of synthesis and assembly of PSII units. From the experimental results and parameters derived therefrom, we concluded that the greening process in this mutant consists of two somewhat superimposed phases: a phase of interconnection and reorganization of preexisting units followed by a phase of addition of complete active units.

The aim of this work was to study these two processes separately. It has been reported previously that dark-grown *C. reinhardi* cells can green in the presence of CAP which prevents the synthesis of chloroplast-made polypeptides (6) required for development of photosynthetic activity (4). Thus, membranes are formed containing near normal amounts of Chl but lacking photosynthetic activity. Such membranes can regain their activity if protein synthesis in the chloroplast is allowed to occur following removal of the drug (repair process). It is possible to achieve this repair without any additional Chl synthesis (4, 11) if the cells are incubated in the dark or, alternatively, in the light but in the presence of CHI. This drug prevents both synthesis of membrane proteins of cytoplasmic origin and of Chl (4, 6). Thus, the repair process could be followed without interference from new Chl synthesis and membrane growth. This system offers the opportunity to study the formation of the photosynthetic units and their integration into a preexisting membrane (4).

A different model for a developing system in which the main phase is that of membrane growth by addition of newly synthesized and completely integrated membrane components, including Chl, is a pale green mutant derived from the y-1 cells (y-1p). In this mutant the greening process is extremely slow as compared to that of the y-1 cells and extends through several generations. The results obtained in this work from the analysis of PSII parameters for the repair process of the y-1 mutant fit nicely with the concept of synthesis of PSII units which are coupled to a preexisting pool of light-harvesting Chl. These results are distinctly different from those obtained for the greening of the y-1p mutant, or the second phase of the ordinary greening of the y-1 mutant.

**MATERIALS AND METHODS**

*C. reinhardi* y-1 and y-1p mutants were grown in semicontinuous cultures on a mineral medium with acetate as the sole

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2 Abbreviations: CAP: chloramphenicol; CHI: cycloheximide; DCIP: 2,6-dichlorophenolindophenol; PSII: photosynthetic unit.
carbon source (9). The y-1p mutant was obtained by UV irradiation of the y-1 cells. The cells were grown in the dark for four to six generations. Under these growth conditions neither mutant synthesizes Chl, and the photosynthetic membranes originally present are diluted among the daughter cells. The dark-grown cells are relatively rich in carotenoids and thus have an orange-yellow color (6).

In some experiments the y-1p cells were grown in alternate light and dark periods (1 day each). This was done in order to reproduce the initial level of Chl for the greening experiments in a relatively consistent manner.

For the repair experiments the y-1 cells were resuspended in fresh growth medium at a final concentration of about $10^8$ cells/ml in order to prevent cell division (9). The cells were first exposed to light for 6 to 9 hr in the presence of CAP (200 $\mu$g/ml), and thus formed membranes devoid of photosynthetic activity. These cells contained only 50 to 60% of the normal Chl level found in cells greened in the absence of CAP. The cells were then washed free of the drug by centrifugation, resuspended in fresh growth medium, and further incubated in the dark or light with addition of CHL (1-1.5 $\mu$g/ml) (4, 6).

The second step (incubation in absence of CAP) of the above procedure, where photosynthetic activity is gradually built up, will be referred to as "repair." The greening of the y-1p cells was carried out by exposing to light dark-grown cells and allowing them to divide for several generations (several days), without any drug addition.

**Measurement of Photosynthetic Activity.** Activity was measured with crude cell-free chloroplast membranes, as previously described (2). For DCIP reduction the reaction mixture consisted of 2 ml TKM buffer (30 mM tris-HCl [pH 8], 10 mM KCl, and 1 mM MgCl$_2$) containing 5 $\mu$m DCIP and chloroplast membranes to give a final concentration of 5 to 10 $\mu$g Chl/ml. The reduction of DCIP was followed spectrophotometrically using an Amino-Chance dual wavelength spectrophotometer provided with side illumination and employed in the double beam mode. The difference in A was measured at 580 to 520 nm ($\Delta$A = $1.05 \times 10^4$ liter·mol$^{-1}$) or 590 to 520 nm ($\Delta$A = 1.2 $\times 10^4$ liter·mol$^{-1}$) depending upon the turbidity of the membrane preparation used. In flash yield experiments the wavelengths used were 625 and 695 nm ($\Delta$A = $1.45 \times 10^4$ liter·mol$^{-1}$). For flash experiments an ILC L-268 lamp was used with a flash duration of $5 \mu$s and at a repetition rate of 5 to 20 flashes/sec. In these experiments the flash light was filtered through a UV cut-off filter only. For quantum yield measurements the exciting beam was filtered through a 685 nm interference filter (5 nm half-width; Baird Atomic Inc.). For maximal rate measurements excitation was passed through a broad band K-6 Balzer's filter (650-690 nm). The photomultiplier was protected with two broad band filters (DT green, Balzer's) and an Andor Corning 4-96 filter transmitting between 500 and 600 nm.

For fluorescence induction measurements the exciting light ($4-6 \times 10^{-8}$ nE cm$^{-2}$·sec$^{-1}$) was filtered through a 520 to 640 nm broad band Balzer's filter and a Schott OG-590 cut-off filter. The photomultiplier received light through a 685 nm Baird Atomic interference filter of a very low intrinsic fluorescence. The signal was displayed on a Tektronics 564 oscilloscope equipped with a memory screen. Far red light was given between measurements on the same sample using a 726 nm Baird Atomic interference filter. When DCMU was used, it was added after the far red illumination. The opening time of the shutter was 2 to 3 msec. For measurements of the fast and slow phases of fluorescence induction a time scale of 10 to 20 msec/division and of 0.1 to 1 sec/division were used, respectively. All measurements were done at room temperature.

Chl was quantitated as described by Vernon (10). Cell concentration was measured with a hemocytometer. For more details, including analysis of data, consult reference 2.

**RESULTS**

**Repair of PSII Activity in CAP-treated Cells.** Several parameters were measured during the repair of photosynthetic activity of y-1 cells following incubation in the light or dark of CAP-treated cells. The time course of the restoration of DCIP reduction as measured by the maximal rate and the quantum yield for this reaction is shown in Figure 1. Both parameters measured show a fast increase during the first 2 hr and then a tendency to level off during the next 1 to 2 hr. The final levels of activity obtained in cells incubated in the light or dark were practically the same.

The changes in the photosynthetic unit size, as derived from measurements of the flash yield of DCIP reduction in typical experiments, are shown in Figure 2. It was found that, while the apparent photosynthetic unit size (flash yield × Chl$^{-1}$) decreases considerably, the effective photosynthetic unit size (apparent size × quantum yield) decreases only slightly in the cells repaired in the light and remains constant for cells repaired in the dark. The effective photosynthetic unit size which was reached was about two times larger than that found in normal light-grown y-1 cells (2).

Typical results of measurements of changes in fluorescence induction parameters are shown in Figure 3. The initial level of the fluorescence experiment ($F_{\text{max}}-F_0$) decreased by about 60%. The variable fluorescence ($F_{\text{max}}-F_V$), which was very low initially, increased during the first 2 hr about 4-fold and leveled off afterward in a manner similar to that observed for the quantum yield (cf. Fig. 1).

Fig. 1. Changes in quantum yield (O, left) and maximal rate (O, right) of DCIP reduction during repair of PSII activity in CAP-treated C. reinhardtii y-1 cells. Dark-grown y-1 cells (initial Chl content, 1.2 $\mu$g Chl/10$^8$ cells) were suspended in fresh growth medium, 10$^8$ cells/ml, with addition of CAP. The cells were illuminated for 6 hr (final Chl content, 5.6 $\mu$g Chl/10$^8$ cells). For repair the cells were washed free of the drug, resuspended in fresh growth medium, and further incubated in the dark (O) or in the light with addition of CHL (O). In the dark-incubated cells the Chl content did not change, while in the light-incubated cells it rose to 6.6 $\mu$g Chl/10$^8$ cells.

Fig. 2. Changes in apparent (O) and effective (O) PSII photosynthetic unit size during repair of activity in CAP-treated y-1 cells. Same experiment as in Figure 1: O: dark repair; O: light repair in presence of CHL.
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FIG. 3. Changes in variable (○) and intrinsic (■) fluorescence during repair of activity in CAP-treated y-1 cells. Experimental conditions as for Figure 1. Initial Chl content at the start of the greening process was 0.6 μg Chl/10⁷ cells, and at the end of the incubation, 6.8 μg Chl/10⁷ cells; after repair in light in the presence of CHI, Chl content was 7.3 μg Chl/10⁷ cells. No change occurred in Chl content during dark repair; light repair, □; dark repair, ■.

FIG. 4. Relative (over-all) changes in the measured (left) and derived (right) parameters during repair of photosynthetic activity in CAP-treated y-1 cells. For explanation of symbols see Table I and text. □: Cells repaired in the light in the presence of CHI; ■: cells repaired in the dark. Data are the average of three experiments. Changes are expressed as the nearest whole number, and calculated as the ratio of the final dark or light values to the initial values of the repair process.

Figure 1 to 3 give an example of the variations of several parameters during the repair process. Figure 4 gives a summary of the variation of all of the measurable parameters (left) available to us and relevant to PSII. This histogram also summarizes the changes during development of parameter values which are computed from the measurables and which are related to the intrinsic properties of the photosynthetic unit (right). The definition of all of these parameters follows our previous definition (2) and is summarized for convenience in Table I.

Table I: Explanation of photosystem II parameters utilized in this work

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
<th>Units</th>
</tr>
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<tbody>
<tr>
<td>MR</td>
<td>Maximal rate of DCIP reduction in saturating illumination</td>
<td>μeq e⁻/mg chl, hr</td>
</tr>
</tbody>
</table>
| QY     | Quantum yield at limiting light conditions | μeq e⁻/mg chl, flash |}
| FY     | Flash yield of DCIP reduction in saturating flashes | μeq e⁻/mg chl, flash |}
| F0     | Fluorescence level of dark adapted membranes at time zero of fluorescence induction | μeq e⁻/mg chl, flash |}
| Fm     | Maximal extent of the fluorescence at the end of the induction period | μeq e⁻/mg chl, flash |}
| A/Fv   | Extent of variable fluorescence (Fv/Fm) | μeq e⁻/mg chl, flash |}
| A'/Fv  | Areas (normalized) above the fluorescence induction curve | sec |
| Fv/f   | Extent of the initial rapid phase of the variable fluorescence | μeq e⁻/mg chl, flash |}

Chl/Chl  | Ratio of active chlorophyll to total chlorophyll |
β        | Fraction of total reaction centers that can transfer electrons to DCIP |
Cv/Chl  | Concentration of reaction centers per chlorophyll |
PQ/Chl  | Concentration of total PS II acceptors pool (presumably mostly plastoquinone) |
PSU eff | Effective photosynthetic unit size (Total chlorophyll per reaction center) |

For more details and derivation of these parameters see reference 2.

FIG. 5. Changes in the quantum yield (○) and maximal rate (■) of DCIP reduction as a function of Chl content during greening of y-1p cells. After growing in the dark for three to four generations, the cells were allowed to continue and divide in light/dark cycles for four generations. The cell concentration was kept constant (2 × 10⁷ cells/ml) using a semicontinuous culture apparatus (2).

FIG. 6. Relative over-all changes in measured and derived parameters during the greening process of y-1p mutant cells (□). For comparison, average data for greening y-1 mutant cells (taken from ref. 1) are shown (■). The y-1 cells were grown in the dark for five generations and greened under continuous illumination, without cell division taking place, for 5 to 9 hr (1). Changes are calculated as the ratio of the final and initial values in the greening process for both y-1 and y-1p mutants.
nal Chl content reached is lower (7–10 μg/10^7 cells) as compared with that of the Chl content in the y-1 and wild type cells (25–50 μg Chl/10^7 cells) (2, 9).

Development of PSII Activity during Greening of y-1 Mutant Cells. The maximal rate of DCIP reduction per Chl increases as a function of Chl concentration in the y-1p cells when these are kept in continuous cultures for three to four generations. The increase in quantum yield is rather similar to that of the maximal rate (Fig. 5; cf. also Fig. 6). In Figure 6 typical results are shown of the changes in additional measured parameters, including those obtained from fluorescence induction experiments. The changes are again shown in the form of a histogram and compared to those occurring during greening of the y-1 mutant (2). Figures 4 and 6 can be compared using Table II, in which the final values for the parameter obtained for the various experimental systems are summarized. All of the observable and derived parameters from the fluorescence induction measurements fluctuate slightly around a constant value. The effective photosynthetic unit size increases (3X) as the Chl content per cell rises.

DISCUSSION

Previously it has been shown that chloroplast membranes of C. reinhardtii y-1 formed in the presence of CAP are photosynthetically inactive (4, 7). Since such membranes contain many of the electron transport carriers, such as Cyt f, plastocyanin, and ferredoxin (6) in almost normal amounts, as well as Chl a and b and most of the major membrane polypeptides (4, 5), it was suggested that their lack of activity is due to the absence of the PSII and PSI reaction centers (7). The activity of the PSII complex was shown to be associated with the presence of several membrane polypeptides whose synthesis is inhibited by CAP and which are thought to be synthesized in the plastid (1, 8). Thus, the repair of PSII activity in CAP-treated cells was explained as being due to synthesis and insertion of polypeptides connected with the formation of the PSII reaction center. The results of the present study demonstrate that this is indeed the case. This conclusion is based on the fact that during the repair process, the number of PSII reaction centers increases. At the same time, the dark-adapted fluorescence level (F60/Chl) decreases, indicating that the Chl which is already present in the membrane is being integrated into active units as soon as they are formed.

Our analysis gives us the possibility of distinguishing between reaction centers active only in primary charge separation (which is not connected to the main electron transfer chain of PSII, i.e., electron transfer from H₂O to DCIP), and reaction centers which are active in electron transport to the plastoquinone pool. This distinction is indicated by the ratio of the fast component of the variable fluorescence rise (similar to the rise in the presence of DCMU) to the total variable fluorescence extent (F60/Chl). In the repair process in the light there is a decrease in this fraction of nonconnected reaction centers from about 0.4 to 0.2, which agrees with the over-all picture of the repair process.

No significant difference is observed between cells repaired in the dark or those repaired in the light in presence of CHI which inhibits the synthesis of both Chl and membrane polypeptides of cytoplasmic origin (4–6). This result agrees with other data showing that repair of photosynthetic activity and synthesis of the missing polypeptides can occur in the dark in these cells without further Chl synthesis (7).

Compared to the repair process described above, where the major feature is the integration of PSII units into a preexisting membrane, the slow greening process of the y-1p mutant seems to consist of continuous addition of complete and active units, including Chl, to a developing membrane. This conclusion is based on the fact that the extent of the variable fluorescence (F60/Chl) remains constant throughout the greening and only small changes occur in all of the measured and derived parameters, while at the same time the Chl content of the membranes increases as much as 7-fold. The parallel increase in the maximal rate of electron transfer and the quantum yield agrees with the concept that new complete units are added throughout the entire greening process. The increase in these parameters can be explained mainly by the increase in the parameter β, the degree of connectivity.

It is interesting to note that while in the greening of y-1 cells the effective PSU size was practically constant for most of the greening process, except for the initial phase when it decreased severalfold (2), here in the slow greening of the y-1p cells the effective PSU size increased, as in the slow greening of a mutant of Chlorella studied by Dubertret and Joliot (3). Earlier we considered that the development of PSII activity in the C. reinhardtii y-1 mutant consists of two processes, namely reorganization and integration of PSII units as well as addition of complete units, formed de novo, to the developing membrane. These two processes partially overlap in time, the former prevailing during the initial phase of the greening, and the latter dominating the linear phase of Chl and membrane accumulation (2). The two experimental systems described here demonstrate the possible existence of these phases separately, each in its own system. In the CAP-treated y-1 cells, reorganization and integration occur during the repair process, while complete units are added to the developing thylakoid of the y-1p mutant.

The results of this work give further confidence to the reliability of the analytical method developed previously (2), for the interpretation of the data. Thus, one can see that different derived parameters behave independently and in a logically predictable way in different experimental systems. Comparing Figures 4 and 6, one can see the substantial differences between the development of several derived parameters in the various experimental systems. For example, in the repair of CAP-treated cells, the ratio of active light-harvesting Chl to total Chl (Chl/Chl) increases 3- to 7-fold. This is in complete contrast with the development of this ratio in y-1 and y-1p cells where the Chl synthesized has a constant activity. The degree of connectivity of active reaction centers to the plastoquinone pool (β) changes only slightly (1-2X) during the repair process or during greening of the y-1p cells, but very significantly during the fast greening of the y-1 cells (20X). This behavior is more likely to occur in a rapidly developing system where many components are synthesized slightly different times, rather than in a slowly developing system where the rate of organization is much faster than the synthesis of any component.

Acknowledgments—We wish to thank S. Shohat for her help during the performance of some of the experiments with the y-1 mutant; the y-1p mutant was obtained from the zoology laboratory at the Oslo University, Norway, in cooperation with A. Løvlie during a sabatical period spent by I. O. supported by the Norwegian Research Council.

Table II: final values of some measured and derived parameters during light and dark repair of CAP-treated y-1 cells, and greening of the y-1 and y-1p mutant cells.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>y-1</th>
<th>CAP (Light)</th>
<th>CAP (Dark)</th>
<th>y-1p</th>
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<tbody>
<tr>
<td>Chl</td>
<td>μg/10^7 cells</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
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<tr>
<td>M4</td>
<td>mg Chl x hr</td>
<td>500</td>
<td>300</td>
<td>200</td>
<td>400</td>
</tr>
<tr>
<td>QY</td>
<td>Chl</td>
<td>18</td>
<td>8</td>
<td>17</td>
<td></td>
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<tr>
<td>Fv/F0</td>
<td>μg/mg Chl</td>
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<td>5.5</td>
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<td>5.5x10^-4</td>
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<tr>
<td>Fv/Chl</td>
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<td>1.8</td>
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<tr>
<td>Fv/Chl</td>
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<td>A/yo</td>
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<tr>
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<tr>
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<td>Chl/μs</td>
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<td>400</td>
<td>500</td>
<td>350</td>
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</table>

Data normalized to equal experimental conditions for the various experiments. Data for the y-1 cells were taken from reference 2.
LITERATURE CITED


