The Photosynthetic Electron Transport Chain of *Chlamydomonas reinhardi*

II. COMPONENTS OF THE TRIPHOSPHOPYRIDINE NUCLEOTIDE-REDUCTIVE PATHWAY IN WILD-TYPE AND MUTANT STRAINS*

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Previous studies (1-3) have described the application of mutant strains of *Chlamydomonas reinhardi* to an investigation of the enzymatic pathway of triphosphopyridine nucleotide reduction during photosynthesis. Two of these mutants, *ac*-115 and *ac*-141, appear to be genetically blocked in the light-dependent O₂-evolving step of photosynthesis. The validity of this conclusion rests on the following observations. (a) The mutant cells fail to evolve oxygen in the presence of benzoquinone. (b) Chloroplast fragments isolated from these mutants will not support a Hill reaction with 2,6-dichlorophenolindophenol or ferricyanide, nor with cytochrome *e* or TPN in the presence of added photosynthetic pyridine nucleotide reductase. (c) Chloroplast fragments can reduce TPN upon illumination if ascorbate and 2,6 dichlorophenolindophenol are provided as electron donors. (d) The reactions shown by these mutants and those given by Mn²⁺-deficient cells or cells treated with inhibitors of O₂ evolution are similar. Although *ac*-115 and *ac*-141 are both genetically blocked in the O₂-evolving step of photosynthesis, it is unlikely that the blocks are identical since the two mutants fall into different linkage groups (1). Both the light-dependent O₂-evolving step and a second light-dependent step coupled to TPN reduction are operative in a third mutant, *ac*-21, but there appears to be a genetic block in the sequence of dark exergonic reactions linking the two light-dependent events.

In recent years a number of new components of the photosynthetic electron transport chain have been identified. These include Mn²⁺, quinones, plastocyanin, *b*-type and *f*-type cytochromes, photosynthetic pyridine nucleotide reductase, and pyridine nucleotide transhydrogenase (4). The existence of similar or identical compounds in wild-type *C. reinhardi* is established in this and the preceding paper (3). The concentrations of these substances in wild-type cells are compared with those in the three mutant strains showing impaired photosynthesis. The results obtained are related to the enzymatic studies previously described (3) and to the pathway of TPN photoreduction in photosynthetic cells.

EXPERIMENTAL PROCEDURE

Organisms—*Chlamydomonas reinhardi*, wild-type strain 137c, and three mutant strains, *ac*-21, *ac*-115, and *ac*-141, having impaired photosynthesis were used in the experiments described in this paper. The characteristics of these strains and the growth conditions employed are described in the preceding paper (3).

**Analyses**—Spectrophotometric determinations of chlorophyll *a* and chlorophyll *b* were made on 80% acetone extracts of cells (5). One-dimensional descending chromatography of the 80% acetone extracts was carried out on Whatman No. 1 paper with 1% 1-propanol in Skellysolve B as the developing solvent (6).

Plastocyanin was extracted and partially purified by (NH₄)₂SO₄ precipitation and column chromatography (7). Acetone-dried powders of cells were extracted for 6 hours with 0.02 M potassium phosphate, pH 7.0. The plastocyanin in the extract was precipitated between 50% and 100% saturated (NH₄)₂SO₄. The precipitate was dissolved in, and then dialyzed against, the same phosphate buffer. A few crystals of K₃Fe(CN)₆ were added to oxidize the plastocyanin, which was then absorbed on a diethylaminoethyl cellulose column. The latter was washed with 0.05 M potassium phosphate, pH 7.0, and the blue plastocyanin band was eluted with 0.1 M potassium phosphate, pH 7.0. The plastocyanin content was determined spectrophotometrically with the extinction coefficient given by Katoh *et al.* (7), \( E_{1%}^{1%} = 4.9 \times 10^4 \text{ cm}^2 \text{ g}^{-1} \text{ atom of copper} \).

Plastoquinone was extracted and determined as follows. A suspension of cells was poured into a large excess of acetone at −20°. The mixture was stirred for 5 minutes and then filtered. The residue was washed with acetone, dried, and extracted twice at room temperature with iso-octane for 2 hours. The combined acetone and iso-octane extracts were evaporated and the residue was taken up in iso-octane. The extracted plastoquinone was partially purified on a Decalco column as described by Crane (8), and its concentration was estimated spectrophotometrically (ΔA₁%ox/ΔA₁%red = 198).

Cytochromes *b*, *c*, and *f* were assayed spectrophotometrically. Since extinction coefficients for cytochromes *b*, *c*, and *f* have not
been determined, an extinction coefficient for cytochrome c (9) was used for all calculations ($ΔE_{450}$ (reduced minus oxidized) = 2.10 x 10$^4$ cm$^{-1}$ per mmole). Cytochrome c was extracted from acetone powders of C. reinhardtii with 0.05 M Tris buffer, pH 7.5. Cytochromes b$_7$ and f$_7$ were examined in cell suspensions which had been extracted previously with 80% acetone at -10°C (10).

Mitochondria were isolated from etiolated pea stems as described by Smillie (11).

**RESULTS**

**Chlorophylls**—The chlorophyll content of wild-type and mutant strains varied depending on growth conditions and the age of the cells. Values for total chlorophyll in wild-type and the mutant strains ranged between 1.4 and 2.1 μg of chlorophyll per 10$^6$ cells. The ratio of chlorophyll a to chlorophyll b varied between 2 and 3, the lower values being obtained toward the end of the logarithmic phase of growth. However, under comparable growth conditions the chlorophyll per cell, and the chlorophyll a to chlorophyll b ratio in wild-type and mutant strains, showed similar changes during their logarithmic growth phases.

**Plastocyanin**—A copper-containing protein, plastocyanin, has been isolated from Chloroplasts (7) and is a naturally occurring oxidant for the Hill reaction (13). As was shown in the preceding paper (3), Chlamydomonas plastocyanin can act as a Hill reaction oxidant for chloroplast fragments isolated from wild-type cells. Since two of the mutant C. reinhardtii strains, ac-116 and ac-141, were deficient in the Hill reaction (3), their plastocyanin content was determined.

The visible absorption spectra of the oxidized and reduced forms of plastocyanin isolated from the wild-type strain are shown in Fig. 1A. The oxidized form showed maximal absorbance at 597 μm. Table I shows the plastocyanin content of the wild-type, ac-21, ac-116, and ac-141 strains. A chlorophyll to plastocyanin ratio of 500 was obtained for wild-type C. reinhardtii. This is somewhat higher than the value of 300 reported for spinach by Katoh et al. (7) and may have resulted from incomplete extraction of the protein. The values obtained for the mutant strains are not sufficiently different from either of these values to indicate that the plastocyanin content of the mutant cells is related to their inability to photoreduce TPN.

**Plastoquinone**—The oxidized and reduced spectra of plastoquinone prepared from wild-type are shown in Fig. 1B. The spectra were similar in preparations of ac-21. These spectra resemble those obtained with purified spinach plastoquinone (8), although the maximum given by the oxidized form appeared at a slightly lower wave length owing to impurities. When identical procedures were applied to ac-116 and ac-141, a yellow fraction was obtained which did not show a definite peak at 254 μm. The addition of sodium borohydride resulted in only a small decrease in absorbance at this wave length. To establish the presence of plastoquinone in ac-116 and ac-141, additional purification was required. The fraction from the Decalso column was evaporated under reduced pressure, dissolved in iso-octane, and absorbed on a second Decalso column (30 × 1.1 cm). Fractions (5 ml each) were obtained by gradient elution with a reservoir of 6% diethyl ether in iso-octane and 300 ml of iso-octane in the mixing container. The ultraviolet spectrum of each fraction was recorded, and the tubes containing plastoquinone were combined. A major contaminant with an absorption maximum at 281 μm was eluted just before plastoquinone. A second contaminating fraction with maxima at 268 and 307 μm was eluted after the plastoquinone. These contaminants accounted for most of the absorption spectrum of the fraction from the first Decalso column.

Table II shows the plastoquinone content of wild-type and mutant strains. The ac-21 strain contained slightly less plastoquinone than the wild-type cells. The plastoquinone contents of ac-116 and ac-141 were significantly less, being only one-fifth the amount found in wild-type cells.

**Cytochromes**—A cytochrome was extracted by treating acetone powders of C. reinhardtii with 0.05 M Tris, pH 7.5. This oyo
A comparison of reduced and oxidized samples yields the value obtained for wild-type cells corresponded to 1800 moles of chlorophyll per mole of cytochrome.

Hill and Bonner (10) have shown that cytochrome \( b_6 \) and cytochrome \( f \) can be detected spectroscopically in suspensions of chloroplasts previously extracted with cold 80% acetone. Suspensions of \( C. \) reinhardi, after extraction with 80% acetone, showed similar cytochrome spectra. Cytochrome \( b_6 \) is largely in the oxidized form, and cytochrome \( f \), in the reduced form (10). The difference spectrum of reduced and untreated samples is mainly that of cytochrome \( b_6 \), whereas the difference spectrum of untreated and oxidized samples is mainly that of cytochrome \( f \). A comparison of reduced and oxidized samples yields the sum of the difference spectra of cytochrome \( b_6 \) and cytochrome \( f \). Difference spectra obtained with preparations from wild-type and mutant cells are shown in Fig. 2. Equal numbers of cells were used and the extracted cells were suspended in the same final volumes. The spectra are thus comparable on a per cell basis. Examination of the spectra of reduced minus untreated samples showed that the cytochrome \( b_6 \) content of wild-type and mutant cells was similar except for a somewhat lower value in \( ac-21 \). Significant differences were found for the cytochrome

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cytochrome content</th>
<th>Ratio, ( b_6/ f )</th>
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<tbody>
<tr>
<td></td>
<td>Cytochrome ( b_6 )</td>
<td>Cytochrome ( f )</td>
</tr>
<tr>
<td>Wild-type</td>
<td>90</td>
<td>363</td>
</tr>
<tr>
<td>( ac-21 )</td>
<td>113</td>
<td>489</td>
</tr>
<tr>
<td>( ac-116 )</td>
<td>154</td>
<td>174</td>
</tr>
<tr>
<td>( ac-141 )</td>
<td>100</td>
<td>167</td>
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Discussion

Chloroplast fragments isolated from wild-type \( C. \) reinhardi can, upon illumination, reduce several Hill reaction oxidants (3). TPN can serve as the oxidant provided that photosynthetic pyridine nucleotide reductase is added. In these enzymatic activities \( C. \) reinhardi chloroplasts are quite similar to higher plant chloroplasts. Likewise, some of the components of \( C. \) reinhardi chloroplasts, such as chlorophyll, plastocyanin, and plastoquinone, are identical with or very similar to those found in the chloroplasts of higher plants.

Two cytochromes are prominent in higher plant chloroplasts. Both are bound to the lamellae and are not removed during the usual isolation procedures for chloroplasts. One of these cytochromes (cytochrome \( f \)) has been solubilized and purified by Davenport and Hill (14). Closely related cytochromes have been found in several algae (15–19). A cytochrome identical with or closely related to cytochrome \( f \) has been identified in \( C. \) reinhardi chloroplast fragments by procedures described by Hill and Bonner (10). A \( c \)-type cytochrome has also been identified in extracts of \( C. \) reinhardi. This cytochrome was distinguished from cytochrome \( f \) by its absorption spectrum and its rapid oxidation by mitochondrial cytochrome oxidase. Oxidation of \( f \)-type cytochromes by mitochondria is very slow (19).

Hill (10, 20) has identified a \( b \)-type cytochrome (cytochrome \( b_6 \)) in higher plant chloroplasts. Unlike other members of the \( b \) group, this cytochrome is not denatured by cold 80% acetone. \( C. \) reinhardi chloroplasts contain a cytochrome which survives extraction with cold acetone and has an absorption spectrum.
like cytochrome b6. Besides higher plants and C. reinhardii, a b-type cytochrome has been found in chloroplasts of Caulopa prolifera (21) and Euglena gracilis (22). Chance and Sager (22) have demonstrated the oxidation of a b-type cytochrome in a chlorophyll-deficient mutant of C. reinhardii upon illumination under anaerobic conditions.

Analyses of chlorophyll, carotenoid, plastoquinone, plastocyanin, and cytochromes were also conducted on the three mutant strains having impaired photosynthesis. Previous studies (3) indicated that two of these strains, ac-115 and ac-141, were genetically blocked in the light-dependent step of photosynthesis which is linked to oxygen evolution. The third mutant strain, ac-21, appeared to be genetically blocked in a subsequent light-independent step which normally utilizes a reductant produced in the light-dependent oxygen-evolving step.

Wild-type, ac-21, ac-115, and ac-141 contained equivalent amounts of chlorophylls a and b, plastocyanin, and cytochromes c and b6. In addition, all strains contained photosynthetic pyridine nucleotide reductase, transhydrogenase, pyridine nucleotide-linked cytochrome c reductases, and cytochrome c photo-oxidase (3).

Since the details of an investigation of the carotenoids of wild type and the three mutant strains will be presented elsewhere, only a brief summary is presented here. Each of the three mutant strains contain less pigment than the wild-type strain; ac-21, 37.3%; ac-115, 73.8%; and ac-141, 41.6%. In addition, all have a marked decrease in the ratio of b-carotene to a-carotene. Both of these properties are also characteristic of the wild-type strain when it is grown in the dark. Finally, the three strains differ from each other and from wild-type in the kinds and amounts of specific carotenoids. Thus, ac-21 and ac-115 contain traces of cryptoxanthin, which is absent from both wild-type and ac-141; and ac-141 has a lutein content that is more than twice that of wild type.

Though there are differences between the wild-type and mutant strains with respect to the kinds and amounts of carotenoids, these differences do not seem sufficient to account for the inability of the mutant strains to carry out normal photosynthesis.

Of greatest interest is the reduced level of plastoquinone in ac-115 and ac-141. Plastoquinone has been implicated in photosynthesis because it is localized in chloroplasts (24) and because, if isolated chloroplasts are illuminated, the endogenous plastoquinone is reduced (25, 26). In addition, plastoquinone is essential for the Hill reaction. Chloroplasts extracted with petroleum ether are devoid of Hill reaction activity (27, 28) although they can still photoreduce TPN in the presence of ascorbate, 2,6-dichlorophenolindophenol, and photosynthetic pyridine nucleotide reductase (29). The active factor removed by the petroleum ether appears to be plastoquinone, since Hill reaction activity is regained by the addition of purified plastoquinone (28). The question may be raised as to whether the levels of plastoquinone in ac-115 and ac-141 are insufficient to support a Hill reaction. A possible answer to this question can be obtained by referring to Bishop's data (28) in which a relation between Hill reaction activity and the plastoquinone content of sugar beet chloroplasts is given. When the plastoquinone content was reduced by extraction with petroleum ether to between 0.01 to 0.014 mole of plastoquinone per mole of chlorophyll (the range of plastoquinone in ac-115 and ac-141, see Table 11), Hill reaction activity fell to 30 to 50% of the normal value. On this basis the low plastoquinone content of ac-115 and ac-141 could account for reduced Hill reaction activity, but not to a level which would have been undetectable in the assays employed.

Since the diminished plastoquinone content in ac-115 and ac-141 probably cannot completely account for the loss of Hill reaction activity, two other possibilities ought to be considered. First, chloroplasts may contain more than one type of plastoquinone, and second, there may be more than one site of action of plastoquinone.

Krogmann, Henninger, and Crane (30) have recently identified and separated three plastoquinones from chloroplasts. The original plastoquinone was designated plastoquinone A, and the two new plastoquinones, as plastoquinone B and plastoquinone C. In addition to the three plastoquinones, Henninger, Dilley, and Crane (31) have identified four different tocopherol quinones in chloroplasts isolated from spinach. They have also shown that β- and γ-tocopherol quinone as well as plastoquinones A and B are active in restoring the Hill reaction to spinach chloroplasts that have been extracted previously with organic solvents. The procedures used in the present studies provided only a single plastoquinone fraction, and the possibility exists that one of the plastoquinones or one of the tocopherol quinones is missing in ac-115 and ac-141. This possibility is presently under investigation.

Krogmann (32) and Arnon, Whatley, and Horton (29) have shown that plastoquinone is required by cyclic photophosphorylation catalyzed by N-methylphenazonium methosulfate. This finding indicates that plastoquinone may function in a phosphorylation step as well as in the O2-evolving step; i.e. there may be at least two sites of action of plastoquinone in the pathway of TPN photoreduction and coupled phosphorylation. Krogmann and Duane (33) have expanded these studies and shown that whereas the length of the polyisoprenoid side chain of plastoquinone is critical for photophosphorylation, homologues with shorter side chains are just as active in supporting a Hill reaction. In view of these findings, it is possible that ac-115 and ac-141 are deficient in plastoquinone which is normally active in the Hill reaction and that the residual plastoquinone found in these mutants represents plastoquinone which is active at a different site.

A second point of interest is the increased amount of cytochrome f in ac-115 and ac-141. It was shown previously that TPN photoreduction from ascorbate and 2,6-dichlorophenolindophenol, as well as cytochrome photo-oxidase activity, are at least doubled in these mutants compared with wild-type or ac-21 (3). Photoreduction with ascorbate as the electron donor has been associated with the terminal portion of the electron transport chain, resulting in TPN reduction. This is also true of cytochrome photo-oxidase activity (34). The light-dependent oxidation of cytochrome f has been demonstrated in a number of plant and algae cells (23, 35-37). This oxidation appears to be associated with a light-dependent step in photosynthesis which is not directly coupled with O2 evolution. Light-dependent oxidation of an f-type cytochrome has now been demonstrated in a cell-free system. This oxidation is not inhibited by inhibitors of the Hill reaction. The increased levels of cytochrome f, TPN photoreduction from ascorbate, and cytochrome c

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photo-oxidase activities in ac-115 and ac-141 are consistent with the proposal of Hill and Bendall (38) that cytochrome f is involved in the second light reaction of photosynthesis.

SUMMARY

Wild-type Chlamydomonas reinhardtii cells have been examined for several components of the photosynthetic electron transport chain. Plastoquinone, a copper-containing protein plastoxygenase, and cytochromes b6 and f were identified. The concentrations and absorption spectra of these substances are similar to those found in higher plant chloroplasts. Three mutant strains with impaired photosynthesis were also examined. Similar results were obtained for wild-type cells and ac-116, a mutant which appears to possess a genetic block in a light-independent step subsequent to the light-dependent step of photosynthesis which is coupled to O2 evolution. The other two mutants, ac-115 and ac-141, contained approximately one-fifth the plastoquinone content and double the cytochrome f content of wild-type cells. Both mutants have genetic blocks in the light-dependent O2-evolving step of photosynthesis. The significance of these results in relation to the pathway of triphosphopyridine nucleotide photoreduction in C. reinhardtii is discussed.

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