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Photosynthetic Electron Transport Chain of Chlamydomonas reinhardi. IV. Purification and Properties of Plastocyanin[1]

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Summary. The copper protein plastocyanin has been found to be an essential component of the photosynthetic electron transport chain of *Chlamydomonas reinhardi*, and in this paper we describe a method for its isolation and purification from the wild-type strain. In addition, we describe some of its properties and compare them with those reported for spinach plastocyanin.

The plastocyanin was extracted from acetone powders prepared from intact cells, and it was purified by ion exchange chromatography on DEAE cellulose and gel filtration on Sephadex G-75. The yield of the purified protein ranged from plastocyanin equivalent to 2.0 to 2.5 μg atoms copper per 1000 μmoles chlorophyll. In general the absorption spectrum of plastocyanin from *C. reinhardi* resembled that of the plastocyanin from spinach. Some spectral differences were found in the ultraviolet region where, in contrast to spinach plastocyanin, that of *C. reinhardi* had a greater absorbance (relative to peaks in the visible) and less evidence for phenylalanine fine structure. The normal oxidation-reduction potential of *C. reinhardi* plastocyanin was found to be + 0.37 volts, the same as reported for spinach plastocyanin. The molecular weight of *C. reinhardi* plastocyanin has been estimated to be 13,000 ± 2000. In contrast, the value for spinach plastocyanin has been found to be 21,000.

Experiments with mutant strains of *Chlamydomonas reinhardi* have revealed that plastocyanin and cytochrome 553 are essential components in the photosynthetic electron transport chain (6, 8, 12). It became important, therefore, to isolate and identify some of the properties of these proteins as derived from the wild-type strain. Ferredoxin is known to be essential in the photosynthetic electron transport chain for the photoreduction of NADP (16). Though at present there are no mutant strains of *C. reinhardi* that lack ferredoxin, such strains may be found and, therefore, the isolation and characterization of this protein from the wild-type strain is of some interest. In this paper we describe a procedure for isolating and purifying plastocyanin from the wild-type strain of *C. reinhardi*, and we describe some of its properties. A subsequent paper (7) describes the isolation, purification, and some of the properties of cytochrome 553 and ferredoxin from wild-type *C. reinhardi*, and another paper (8) describes photosynthetic electron transport in 2 mutant strains of *C. reinhardi*, one lacking plastocyanin and the other cytochrome 553.

Materials and Methods

Organisms and Conditions of Growth. The wild-type strain (137C) of *C. reinhardi* was grown with vigorous aeration in 20 liter carboys each containing 12 liters of Tris-acetate-phosphate medium (6). The temperature during growth was 25° and the light intensity, from daylight fluorescent lamps, was 10,000 lux at the outside of each carboy. Two, 12-liter cultures were harvested before they had reached maximum cell density. Cells were harvested at room temperature by continuous-flow centrifugation, and they were resuspended in 0.002 M phosphate buffer, pH 7.0, at a density equivalent to about 2.0 mg chlorophyll per ml. The chlorophyll content was determined by a modification (2) of the procedure of Mackinney (13).

The Preparation of DEAE Cellulose and Sephadex G-75 for Column Chromatography. DEAE cellulose of fine mesh size (obtained from Schleicher and Schuell) was washed by a modification of the procedure described by Peterson and Sober (14). About 10 grams dry weight were washed in sequence with 2 liter quantities of 1.0 N KOH, 0.1 N HCl, distilled water, and 0.01 M phosphate buffer, pH

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7.0. The final washing with the buffer was repeated at least 3 times.

The cellulose was allowed to come to room temperature and was washed 3 times with the starting buffer before it was poured into columns. The columns were chilled in the cold room (4°) prior to use. They were not reused because the DEAE cellulose could not be washed free of impurities after use.

Sephadex G-75, in bead form, was suspended in 4 liters of 0.01 M phosphate buffer, pH 7.0, containing 0.1 M KCl. The suspension was stirred for 30 minutes to allow the beads to become fully swollen and dispersed. The beads were then washed several times in the KCl-buffer solution. A column was prepared in a 2.6 cm (I.D.) × 55 cm tube fitted with a Teflon metering valve at the bottom. The packing process was carried out as described in the brochure, *Sephadex in Gel Filtration* (distributed by Pharmacia Fine Chemicals, Inc.) using the KCl-phosphate buffer. After the packing process was completed, a single layer of Kimwipe, cut to fit, was placed on top of the gel bed to protect it from minor disturbances. It was found that use of a filter paper disc, as recommended in the brochure, seriously disturbed the flow of liquid into the column. After packing, at room temperature, the column was kept in the cold room at 4°. It was reused in several separate experiments, being washed after each use with about 500 ml of the KCl-phosphate buffer solution.

Gradient Elution from DEAE Cellulose. Gradients of increasing salt concentration were generated with a 2-stage, constant-volume system (14). Elution was carried out at constant pH. The reservoir contained 0.6 M KCl in either 0.02 M phosphate buffer, pH 7.0, or 0.05 M acetate buffer, pH 5.2. Initially the first mixing chamber contained 0.2 M KCl in either the phosphate or acetate buffer, and the second mixing chamber contained either the phosphate or the acetate buffer. The total volume of liquid in each of the 2 mixing chambers was 200 ml. The conditions listed above gave a very nearly linear gradient of KCl concentration from 0 to 0.3 M over a 265 ml volume of flow. Gradient elution was carried out at 4°.

Purification and Properties of Plastocyanin

The procedure described here has been modified after the methods of Katoh (9) and Katoh, Shiratori, and Takimiya (10). The modifications achieved a rapid and economical method for obtaining plastocyanin of high purity from cells of *C. reinhardi*.

The Quantitative Determination of Plastocyanin. The plastocyanin content of protein samples was obtained by measuring their absorbance at 597 nm after oxidation with potassium ferricyanide. This value was corrected for the absorbance by contaminating pigment after reduction with sodium ascorbate. For crude extracts, containing relatively large amounts of contaminating pigments, complete oxidized and reduced spectra were made. In all cases, the amount of plastocyanin was calculated by use of the extinction coefficient of oxidized plastocyanin, 4.9 $cm^2/\mu g$ atom Cu (10).

The U-V absorbance of each sample was measured at 278 nm, and the ratio A_{278}/A_{597} was taken as the absorption index (10). Since the absorbance at 278 nm is an approximate measure of total protein and the absorbance at 597 nm (oxidized-minus-reduced) is a measure of the amount of plastocyanin, the absorption index is inversely related to the purity of the plastocyanin.

Preparation of the Crude Extract. After the cells had been harvested and suspended in 0.002 M phosphate buffer, pH 7.0, all subsequent operations were carried out in a cold room at 4°. The cell suspension was added quickly, with stirring, to 4 volumes of acetone chilled to −20°. The cells were then collected by filtration under suction through a double layer of Whatman No. 1 filter paper. The filter cake was sucked free of excess acetone and immediately thereafter it was resuspended in 0.01 M phosphate buffer, pH 7.0, at a cell concentration equivalent to 1 mg chlorophyll per ml. The suspension was then left at 0° for at least 6 hours. The cell debris was centrifuged out at 25,000 × *g* for 10 minutes and the clear supernatant collected.

Purification Step 1. The crude extract was applied to a column (1.3 × 10 cm) of DEAE cellulose equilibrated with 0.01 M phosphate buffer, pH 7.0, at a flow rate of 50 to 100 ml per hour. The column was then eluted with 20 ml of 0.05 M phosphate buffer, pH 7.0, followed by 30 ml of 0.05 M phosphate buffer containing 0.15 M KCl, and finally by 30 ml of 0.05 M phosphate buffer containing 0.40 M KCl. The effluent was collected in 5.7 ml fractions, and these were analyzed for their plastocyanin content and absorbance at 278 nm. The plastocyanin, in the reduced state, was found in the fractions eluted by the buffer containing 0.15 M KCl. Ferredoxin was found in the fractions eluted by the buffer containing 0.40 M KCl (7).

Purification Step 2. The fractions from step 1 that contained plastocyanin were combined and dialyzed for 12 hours against 2 liters of 0.01 M phosphate buffer, pH 7.0. The plastocyanin, which had been oxidized in the course of spectrophotometric analysis, became fully reduced during the dialysis. This autoreduction has been described by Katoh (9).

After dialysis the plastocyanin was adsorbed onto a column (1.3 × 14 cm) of DEAE cellulose equilibrated with 0.01 M phosphate buffer, pH 7.0. The column was then washed with 20 ml of 0.02 M phosphate buffer, pH 7.0 before elution was started with the gradient of KCl in 0.02 M phosphate buffer, pH 7.0. The effluent was collected in fractions of 5.7 ml, and these were analyzed for their plastocyanin content and absorbance at 278 nm.

The 5 fractions of lowest absorption index, containing about 85 % of the total plastocyanin, were combined and saved for the next purification step.

Purification Step 3. The plastocyanin obtained from the previous step was reduced by adding 1 drop of 0.1 M sodium ascorbate. It was dialyzed for 12 hours against 2 liters of 0.005 M phosphate buffer, pH 6.5 and then for 3 hours against 2 liters of 0.02 M acetate buffer, pH 5.2. There was some precipitation of contaminating proteins during the second dialysis. The precipitate was removed by centrifugation at 25,000 × *g* for 10 minutes. The clear solution was adsorbed onto a column (1.3 × 14 cm) of DEAE cellulose equilibrated with 0.02 M acetate buffer, pH 5.2. The column was then washed with 20 ml of 0.05 M acetate buffer, pH 5.2, and the plastocyanin was eluted with the gradient of KCl in 0.05 M acetate buffer, pH 5.2. The effluent was collected in 5.7 ml fractions and each fraction was analyzed for its plastocyanin and absorbance at 278 nm. The 5 fractions of lowest absorbance index, containing about 95 % of the total plastocyanin were combined and immediately dialyzed for 12 hours against 2 liters of 0.01 M phosphate buffer, pH 7.0.

Purification Step 4. The dialyzed plastocyanin from step 3 was concentrated by adsorbing it on a small column (0.6 × 5 cm) of DEAE cellulose in 0.01 M phosphate buffer, pH 7.0. It was eluted in about 4 ml with 0.20 M KCl in 0.05 M phosphate buffer, pH 7.0, and it was used directly without dialysis for gel filtration.

Gel filtration on Sephadex G-75 was carried out as described by Porath (15). The column, prepared as described earlier, was washed with 300 ml of 0.1 M KCl in 0.01 M phosphate buffer, pH 7.0 before the sample was applied. The flow rate was 2 ml per minute. The effluent was collected in 5.7 ml fractions, and it was analyzed for plastocyanin content and absorbance at 278 nm. The fractions having absorption indices less than 2.1 were combined and saved. They represented about 98 % of the total plastocyanin.

The Storage of Purified Plastocyanin. In order to concentrate the purified plastocyanin it was first dialyzed against 0.01 M phosphate buffer, pH 7.0. It was then adsorbed on a small column (0.6 × 5 cm) of DEAE cellulose and eluted with a minimum volume of 0.20 M KCl in 0.05 M phosphate buffer, pH 7.0. The plastocyanin, in a volume of about 2.5 ml, was dialyzed against 0.005 M phosphate buffer, pH 7.0 and then stored as a frozen solution at −15°.

Results and Discussion

The Yield of Plastocyanin and the Plastocyanin Content of Cells. A culture of wild-type *C. reinhardi* equivalent to 433 mg chlorophyll yielded crude plastocyanin equivalent to 1.12 μg atoms copper as determined after purification through step 1. If it is assumed that all of the plastocyanin content of wild-type cells was extracted as the soluble protein, it can be calculated that the plastocyanin content of the cells was 2.3 μg copper per 1000 μmoles chlorophyll. In other experiments with wild-type cells this value ranged from 2.0 to 2.5 μg atoms copper per 1000 μmoles chlorophyll. By comparison, Katoh, Suga, Shiratori, and Takamiya (11) reported yields of plastocyanin from spinach chloroplasts in the range of 2.5 to 3.3 μg atoms copper per 1000 μmoles chlorophyll.

The Results of Purification of the Plastocyanin. The course of purification of the plastocyanin is shown in table I. The amount of plastocyanin in the crude extract could not be determined accurately because the high concentration of light-scattering impurities caused erratic fluctuations of the apparent absorbance at 597 nm. However, once the plastocyanin had been purified through step 1 it was readily visible and could be determined easily and accurately. Also, its absorption index decreased sharply during the purification reaching a limiting value at about 2.0 (table I). This limiting value is considered to correspond to very nearly pure plastocyanin from *C. reinhardi.* An absorption index of 1.9 was attainable, but when the index fell below 2.0, the plastocyanin preparations were found to be unstable, and they returned to a higher index upon storage or upon further attempts to achieve higher purification. A similar observation (10) has been made for spinach plastocyanin.

Figure 1 shows the elution diagram of the chromatography carried out at step 2. Chromatography at steps 3 and 4 gave an increasing homogeneity of the plastocyanin as seen by an increasingly close correlation between the curves for total protein and for plastocyanin.

Table I. *The Course of Purification of Plastocyanin from Wild-Type* C. reinhardi

Stage of purification	Plastocyanin (μg atoms Cu)	Absorption index (A_{278}/A_{597})
Crude extract	ca 1.0	1400
Step 1 (stepwise elution, pH 7.0)	1.12	47
Step 2 (gradient elution, pH 7.0)	0.91	5.6
Step 3 (gradient elution, pH 5.2)	0.70	2.3
Step 4 (gel filtration, pH 7.0)	0.60	2.0

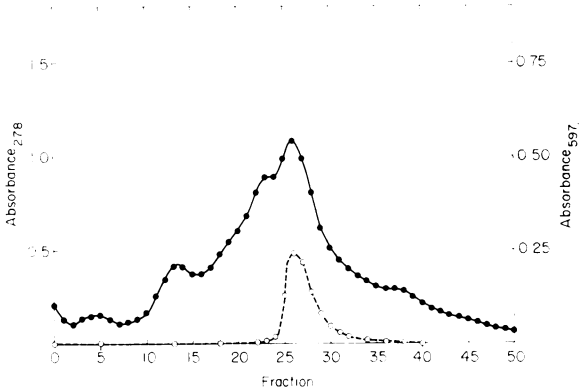

FIG. 1. The elution pattern of total protein (solid line) and plastocyanin (broken line) from DEAE cellulose in step 2 of the purification procedure described in the text. The total protein was measured as the absorbance at 278 nm, and the plastocyanin was measured as the oxidized-minus-reduced absorbance difference at 597 nm. The oxidation was by potassium ferricyanide and the reduction by sodium ascorbate. The ordinant scales were chosen so that the ordinant of A_{278} and A_{597} would coincide with the ratio A_{278}/A_{597} equal to 2.0, the absorbance ratio characteristic of pure plastocyanin of *C. reinhardi* (see text). The spectra shown here and in succeeding figures were obtained with a Cary model 14 recording spectrophotometer.

Other Purification Techniques. Several techniques, in addition to that described above were tried in preliminary attempts to purify plastocyanin from *C. reinhardi.* Ammonium sulfate precipitation (10) was found to be unsatisfactory, for the plastocyanin was not precipitated at either pH 7.0 or 5.2 until at least 80% saturation was reached and only about one-half of the plastocyanin was recovered in the precipitation at 100% saturation with ammonium sulfate. Though much of the plastocyanin could be detected in the supernatant it was not readily recovered.

Chromatography on hydroxyapatite gel by the technique of Tiselius, Hjerten, and Levin (17) was found to be effective, but the technique did not achieve any further purification when applied to plastocyanin having an absorption index of 2.0. However, this technique should be considered as a potential fifth step under circumstances where the 4-step procedure is inadequate.

Absorption Spectra. Figure 2 shows the absorption spectra of the oxidized and reduced forms

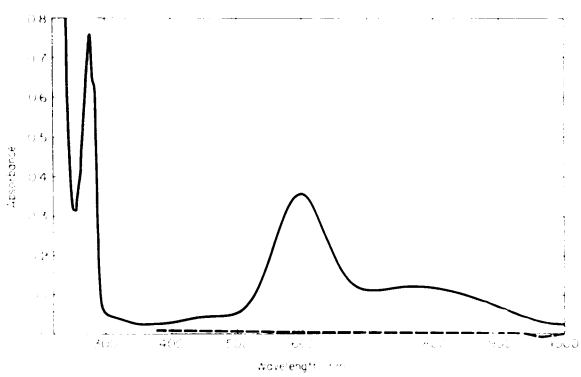

FIG. 2. The oxidized spectrum (solid line) and the reduced spectrum (broken line) of the plastocyanin purified from wild-type *C. reinhardi.* The protein was oxidized by potassium ferricyanide and then dialyzed to remove the ferri- and ferro-cyanides. The reduced spectrum was obtained by addition of sodium ascorbate. The slight dip in the spectrum at 970 nm is believed to be an artifact due to the strong water absorption band at that wavelength.

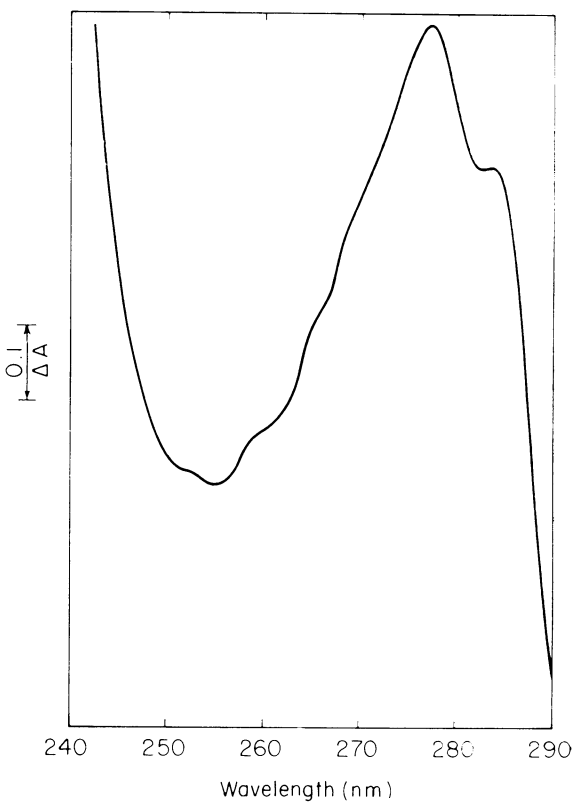

FIG. 3. Detail of the ultraviolet absorption band of the plastocyanin purified from wild-type *C. reinhardi.* The plastocyanin concentration in this figure was not the same as in the previous figure.

of purified plastocyanin from wild type *C. reinhardi.* The oxidized plastocyanin was found to have 3, broad, overlapping peaks in the visible region of the spectrum. The major peak was located at 597 nm and 2 minor peaks were located at 760 and 460 nm. The minor peak at 760 nm was extremely broad and extended beyond 1000 nm into the near infrared. The 3 visible peaks disappeared when the plastocyanin was reduced, and the fully reduced protein was colorless. The U-V

peak of plastocyanin is shown in more detail in figure 3. The absorbance maximum was at about 278 nm and indications of a fine structure attributable to phenylalanine residues can be seen.

From its absorption spectrum, the plastocyanin of *C. reinhardi* resembles the plastocyanin of spinach (10). The 3 visible peaks of the 2 proteins have maxima at very nearly the same wavelengths and have the same relative heights and widths. The chief spectral differences lie in the ultraviolet region where in contrast to spinach plastocyanin, that of *C. reinhardi* has a greater absorbance relative to the visible peaks and a less pronounced phenylalanine fine structure.

Oxidation-Reduction Properties. The plastocyanin of *C. reinhardi* was readily oxidized by potassium ferricyanide and reduced by sodium ascorbate, cysteine, or hydroquinone. The reduced plastocyanin showed no detectable tendency to autoxidize even after thorough dialysis to remove any excess reductant. However, the oxidized plastocyanin in partially purified preparations showed a marked tendency to autoreduce during the course of dialysis. This tendency decreased with further purification and when fully purified the plastocyanin remained at least 90 % oxidized. However, even the purest preparation would not remain completely oxidized in the absence of excess oxidant. These preparations underwent partial autoreduction to the 96 to 97 % oxidized level when dialyzed to remove excess oxidant.

The normal oxidation-reduction potential of the plastocyanin was determined according to the method of Davenport and Hill (4). The plot of log (oxidized plastocyanin/reduced plastocyanin) versus log (ferricyanide/ferrocyanide) is shown in figure 4. The slope of the line in figure 4 indicates that the oxidation and reduction of the plastocyanin is by a single electron transfer. From the slope of the line and using + 0.43 volts as the normal potential of the ferricyanide-ferrocyanide couple (3) at neutral pH, the normal oxidation-reduction potential for the plastocyanin was calculated to be + 0.37 volts at pH 7.0. This value is the same as that obtained for spinach plastocyanin (10).

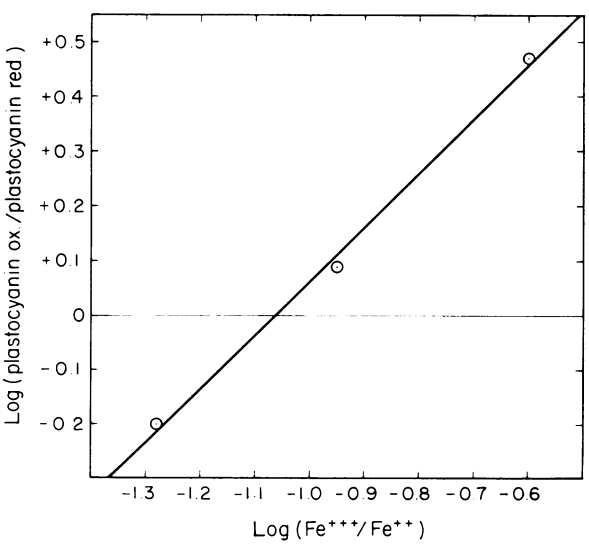

FIG. 4. The plot of log (oxidized plastocyanin/reduced plastocyanin) versus log (ferricyanide/ferrocyanide). The plastocyanin was equilibrated with mixtures of potassium ferri- and ferrocyanide, and its percentage oxidation determined spectrophotometrically at 597 nm.

Molecular Size. Since gel filtration of Sephadex can separate solutes on the basis of their molecular size (15), it was used to estimate the molecular size of plastocyanin. The calculations for the estimate were according to the equation (5):

$$V_e = V_o + K_D V_i$$

where the distribution coefficient, K_D, is characteristic for the solute, and the void volume, V_o, and inner volume, V_i, are characteristic of the column. It has been shown (1) that for a series of globular proteins the distribution coefficient on Sephadex G-75 is approximately related to molecular weight.

The distribution coefficient, K_D, of the plastocyanin was calculated to be 0.48, as determined after elution of a partially purified preparation of the protein from Sephadex G-75. Comparison with the results of Andrews (1) indicated that plastocyanin had a molecular size comparable to that of a globular protein having a molecular weight of 13,000 ± 2000. In contrast, the molecular weight of spinach plastocyanin was found to be 21,000 (10). The value for the molecular weight of *C. reinhardi* plastocyanin will be somewhat in error, however, if the plastocyanin has an unusually high degree of asymmetry or hydration.

Plastocyanin and Photosynthetic Electron Transport. Plastocyanin has been shown to be an essential component of the photosynthetic electron transport chain of *C. reinhardi* (6, 8, 12). The isolation and purification from the wild-type strain was done in conjunction with a mutant strain in which photosynthetic electron transport is blocked (6, 12) and as a subsequent paper will show (8), plastocyanin is either missing or inactive in the mutant strain. Accordingly, the procedures described here were essential for demonstration that plastocyanin was affected in the mutant strain. In addition, the purified plastocyanin from the wild-type strain was used in reconstitution experiments with the mutant strain in which it was shown that the addition of plastocyanin restored photosynthetic electron transport (8).

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