Photosynthetic Electron Transport Chain of Chlamydomonas reinhardi VI. Electron Transport in Mutant Strains Lacking Either Cytochrome 553 or Plastocyanin[1]

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Summary. A mutant strain of *Chlamydomonas reinhardi, ac-206*, lacks cytochrome 553, at least in an active and detectable form. Chloroplast fragments of this mutant strain are inactive in the photoreduction of NADP when the source of electrons is water, but they are active when the electron source is 2,6-dichlorophenolindophenol and ascorbate. The addition of either cytochrome 553 or plastocyanin, obtained from the wild-type strain, has no effect upon the photosynthetic activities of the mutant strain. Cells of the mutant strain lack both the soluble and insoluble forms of cytochrome 553, but they possess the mitochondrial type cytochrome *c*. Thus, the loss of cytochrome 553 appears to be specific.

Another mutant strain, *ac-208*, lacks plastocyanin, or possesses it in an inactive and undetectable form. Chloroplast fragments of *ac-208* are inactive in the photoreduction of NADP with either water or 2,6-dichlorophenolindophenol and ascorbate as electron donors. However, these reactions are restored upon the addition of plastocyanin. The addition of cytochrome 553 has no effect. The measurement of light-induced absorbance changes with *ac-208* reveal that, in the absence of plastocyanin, light fails to sensitize the oxidation of cytochrome 553, but it will sensitize its reduction. However, the addition of plastocyanin restores the light-induced cytochrome oxidation.

A third mutant strain, *ac-208* (sup.) carries a suppressor mutation that partially restores the wild phenotype. This mutant strain appears to possess a plastocyanin that is less stable than that of the wild-type strain.

The observations with the mutant strains are discussed in terms of the sequence of electron transport System II → cytochrome 553 → plastocyanin → System I.

Whole cells of certain mutant strains of *Chlamydomonas reinhardi* are unable to fix carbon dioxide at the wild-type rate (16), and their chloroplast fragments are inactive in the photoreduction of NADP when the source of electrons is from water (5, 15). In addition, certain of the light-induced absorbance changes characteristic of the wild-type strain are absent in the mutant strains (14).

In a brief report (5) we described 2 mutant strains in which the content of either cytochrome 553 or plastocyanin was affected. The purpose of this paper is to describe the properties of these mutant strains in detail and to provide additional evidence (14) that the 2 metallo-proteins function in the photosynthetic electron transport chain in the sequence System II → cytochrome 553 → plastocyanin → System I.

Materials and Methods

Organisms and Growth Conditions. The wild-type strain (137c) of *C. reinhardi* and 3 mutant strains *ac-206*, *ac-208*, and *ac-208* (sup.) were used in the experiments described here. The third strain, referred to as *ac-208* (sup.) is a double mutant strain that carries, in addition to the primary mutation *ac-208*, a secondary suppressor mutation that partially restores the wild phenotype.

Cells were cultured in Tris-acetate phosphate medium (5) and were harvested while in the exponential or early stationary phase of growth. The strains were cultured at 4000 lux except for *ac-208* which was cultured at 1000 lux until the last 24 hours of growth when the light intensity was increased to 4000 lux. The lower light intensity was found to be necessary in order to prevent the cells from clumping during growth. Light was provided by daylight fluorescent lamps and the temperature was 25°.

Preparation of Chloroplast Fragments. Chloroplast fragments for the measurement of DPIP and

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Table I. *Plastocyanin Extracted from Wild Type and from the Mutant Strains*

The plastocyanin was extracted from acetone-treated cells and purified through step one (6).

Strain	Plastocyanin μg atoms Cu/1000 μmoles chlorophyll
Wild type	2.3
ac-206	3.9
ac-208	<0.05
ac-208 (sup.)	<0.05

NADP photoreduction and for the measurement of light-induced absorbance changes were prepared by ultrasonic disruption of the cells as previously described (14) except they were not washed. For certain experiments, however, chloroplast fragments were prepared from cells disrupted by grinding in sand (5).

Assay Conditions. Chlorophyll was determined by a modification (1) of the procedure of Mackinney (17). The photoreduction of NADP and of DPIP was measured in a Cary model 14 recording spectrophotometer using the method described by Smillie (18). Light-induced absorbance changes were detected by the method previously described (14).

Carbon dioxide fixation by whole cells was measured as the uptake of acid by the use of a Radiometer titrimeter with a Radiometer TTA31 titration assembly.

Quantitative Determination of Cytochrome 553 and Plastocyanin. The plastocyanin content was determined in extracts that had been carried through step 1 of the purification procedure (6), and the cytochrome content was determined in cells that had been either extracted with 80 % aqueous acetone (6, 7) or on preparations that had been carried through step 1 of the purification procedure (6).

Results

The Plastocyanin and Cytochrome 553 of the Mutant Strains. The plastocyanin of the wild-type and the mutant strains as determined after purification through step 1 (6) is shown in table I. The oxidized and reduced spectra of the partially puri-

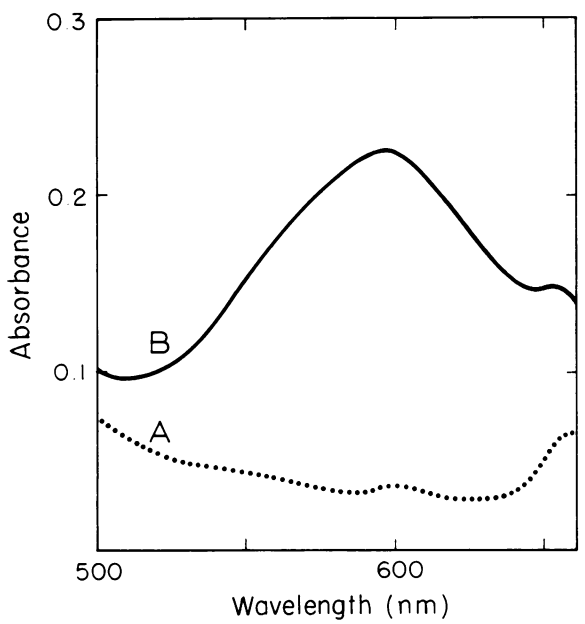

FIG. 1. The reduced spectrum (A) and oxidized spectrum (B) of the plastocyanin of *ac-206* after purification through step 1 (6). The reduction was achieved with sodium ascorbate and the oxidation was achieved with potassium ferricyanide. These spectra and those in subsequent figures were obtained with a Cary model 14 recording spectrophotometer.

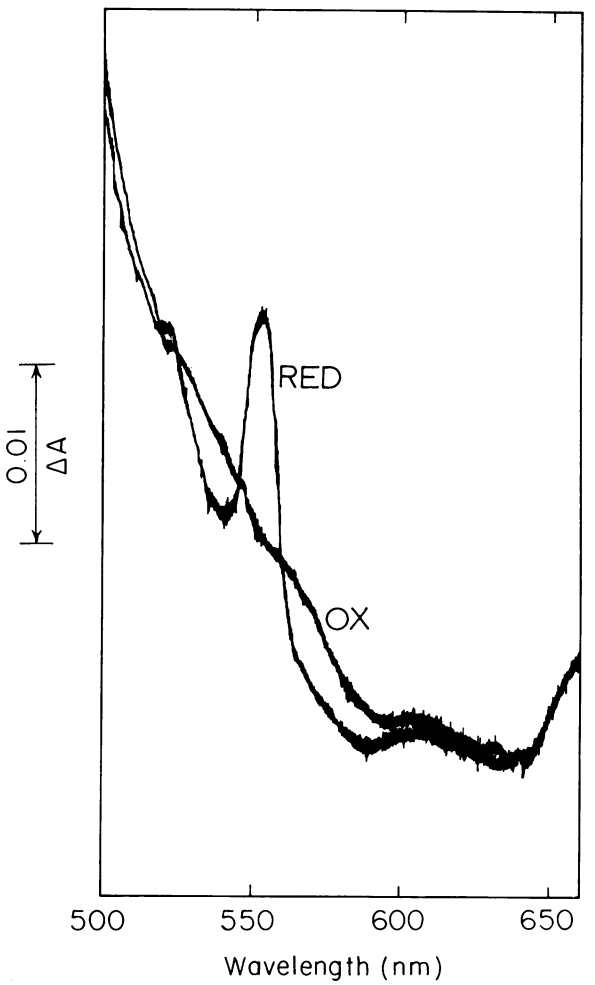

FIG. 2. The reduced (RED) and oxidized (OX) spectra of an extract of *ac-208* purified through step one (6). Reduction was with sodium ascorbate and oxidation was with potassium ferricyanide. The 0 to 0.1 slide wire of the spectrophotometer was used.

fied plastocyanin from *ac-206*, shown in figure 1, were nearly identical with the corresponding spectra for the wild-type strain (6). Both *ac-208* and *ac-208* (sup.) appeared to lack plastocyanin, for none could be detected when the sensitivity of the method was such that a plastocyanin content 2 % of that of the wild-type strain could have been detected. Figure 2 shows the oxidized and reduced spectra, at high sensitivity, of the partially purified fraction of *ac-208* corresponding to the fraction of the wild-type that contained plastocyanin. The spectra clearly show the soluble form of cytochrome 553, but there is no trace of plastocyanin. Essentially identical spectra were obtained for *ac-208* (sup.).

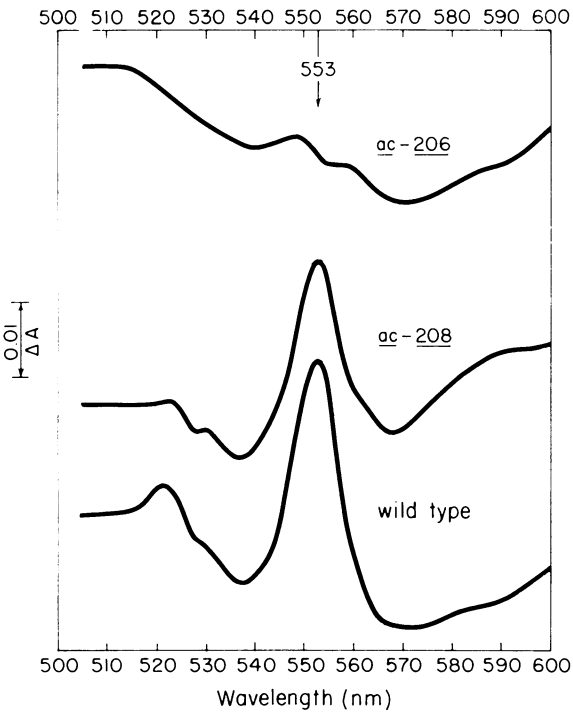

FIG. 3. The reduced-minus-oxidized difference spectra of cells of wild type, *ac-206*, and *ac-208*, after extraction with 80 % acetone at −15°. The extracted cells were collected by centrifugation at 20,000 × *g* for 2 minutes at −15°. They were resuspended in 0.01 M phosphate buffer, pH 7.0 at a chlorophyll concentration of 1 mg per ml as determined prior to acetone extraction. The samples were reduced with sodium ascorbate and oxidized with potassium ferricyanide. The spectra were obtained with model 14 Cary recording spectrophotometer fitted with a scattered light transmission accessory. The 0 to 0.1 slide wire was used, and the slit control and dynode voltage were set at their minimum values. A high intensity light source was used, and the slit widths were less than 0.25 mm throughout the region of the spectrum that was scanned. The optical path length of the cuvettes was 1 cm. Each spectrum has been corrected for the base line obtained with the appropriate untreated samples.

As stated previously (6) 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. This yield may not represent the total plastocyanin content of the cells, for a fraction of the plastocyanin may not be extracted by the methods used, and some of it might be denatured by these methods. It was found for spinach chloroplasts (8) that only one half of their copper content was extracted as plastocyanin. However, the nature of the copper that remained could not be determined. Until a practical method has been found for determining the amount of plastocyanin in situ it will be necessary to assume that in general the extracted plastocyanin represents the minimum value, and that the true content of this protein in the cells is unknown.

Figure 3 shows the reduced-minus-oxidized difference spectra obtained from aqueous suspensions of acetone-extracted cells of wild-type, *ac-206*, and *ac-208*. Whereas the cells of the wild-type and *ac-208* contained nearly the same amount of cytochrome 553, there was no detectable trace in cells of *ac-206*. The aqueous suspension of acetone-extracted cells was also separated into an insoluble fraction (fig 4) and a soluble fraction (fig 5). The complete absence of cytochrome 553 from cells of *ac-206*, at least in the insoluble form, is shown in figure 4. The soluble fractions (fig 5) revealed the α-band of cytochrome *c* (550 nm) in the wild-type and mutant strains, and plastocyanin in wild-type and *ac-206*. However, the soluble form of cytochrome 553 could not be detected in these unpurified preparations (see below). The presence of cytochrome *c* in the soluble fraction from *ac-206* is noteworthy, for it shows that the lack of cytochrome 553 did not extend to other *c*-type cytochromes.

As noted previously (7), the yield of the soluble form of cytochrome 553 from the wild-type strain was variable, but at least a trace of it was always detected in preparations purified through step 1 (6). Figure 2 shows that the soluble form was readily detected in the partially purified extracts of *ac-208* when the spectra were obtained at high sensitivity. However, figure 6 shows that corresponding preparations of *ac-206* recorded at high sensitivity gave no trace of cytochrome 553. Thus, both the soluble and insoluble forms of cytochrome 553 were not detected in *ac-206*. This finding was important because the 2 forms were not spectroscopically identical; the insoluble form had its α-band at 553.5 nm (fig 4) and the soluble form had its α-band at 552.5 nm (7). The results with *ac-206* provided the best evidence that the 2 forms were really the same cytochrome, the insoluble form being presumably modified slightly by its binding to the chloroplast structure.

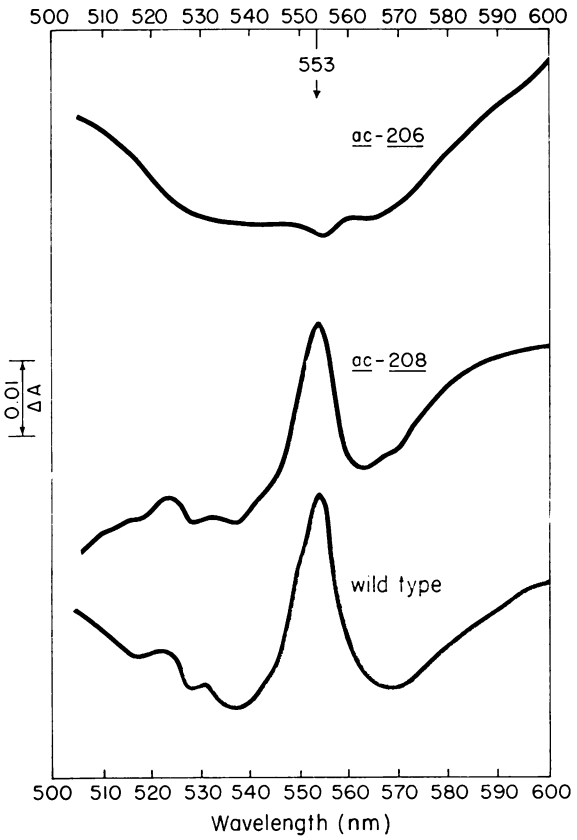

FIG. 4. The reduced-minus-oxidized difference spectra of aliquots of the same, acetone-extracted, preparation shown in figure 3 but after washing with buffer to remove the soluble proteins. The spectra were recorded under the same conditions as described in figure 3.

Table II. *Photosynthetic Carbon Dioxide Fixation by Whole Cells of Wild Type,* ac-208, *and* ac-208 (*sup.*)

The cells were suspended in 2 ml of 0.0025 M $KHCO_3$ in the titration vessel of a Radiometer titrimeter. The chlorophyll concentration was 50 μg/ml. The temperature was controlled at 25° and saturating light intensity (30,000 lux) was provided by a projection lamp having a filter to cut out light of wavelengths less than 560 nm. The filter was necessary to protect the glass electrode which was sensitive to blue light. The titrimeter was set to maintain a constant pH of 7.5 in the reaction vessel and to record the amount of 0.01 M HCl added in order to maintain a constant pH. A slow loss of CO_2 was observed in the dark, and the rate of photosynthetic carbon dioxide fixation was calculated as the light-minus-dark difference in the rate of CO_2 disappearance.

Strain	μmoles CO_2 fixed/hr/mg Chl
wild type	140
ac-208	<5
ac-208 (sup.)	63

Photosynthetic Carbon Dioxide Fixation by Whole Cells. Table II shows a comparison of the rates of photosynthetic carbon dioxide fixation by whole cells of wild-type, *ac-208* and *ac-208* (sup.). Whereas the rate exhibited by cells of *ac-208* was 28-fold less than that of wild-type and typical of mutant strains having impaired photosynthesis, the suppressed mutant strain had a rate that was only slightly less than that of the wild-type rate.

Photoreduction of NADP and DPIP by Chloroplast Fragments of the Mutant Strains. As shown in table III, chloroplast fragments of *ac-206* and *ac-208* were incapable of photoreducing NADP at a detectable rate when water was the electron donor (NADP-a). When reduced DPIP was provided as the electron donor (NADP-b) the chloroplast fragments of *ac-206* were able to photoreduce NADP at rates comparable to that of wild-type, but the chloroplast fragments of *ac-208* showed only a negligible rate. On the other hand, both *ac-206* and *ac-208* gave Hill activity that was about 10% of that of wild-type with DPIP as the electron acceptor.

The activity of chloroplast fragments of *ac-208* (sup.) was dependent upon the method of preparation of the chloroplast fragments. When the frag-

Table III. *Photoreduction of NADP and DPIP by Chloroplast Fragments of Wild Type and Mutant Strains*

NADP-a is the photoreduction of NADP with water as the electron donor, and NADP-b is the photoreduction of NADP with reduced DPIP as the electron donor.

For NADP-a the reaction mixture (2.0 ml) contained chloroplast fragments (20–25 μg chlorophyll) and the following in μmoles: potassium phosphate buffer, pH 7.0, 20; KCl, 40; $MgCl_2$, 5; ferredoxin purified from wild-type *C. reinhardi*, 0.005; and NADP, 0.5. For NADP-b the reaction mixture contained, in addition to the above, the following in μmoles: DPIP, 0.1; sodium ascorbate, 10; and DCMU, 0.02. The reaction was run in a cuvette placed in the sample compartment of the spectrophotometer. The cuvette in the reference compartment contained a control reaction mixture from which ferredoxin and NADP were omitted.

For the photoreduction of DPIP, the reaction mixture (2.0 ml) contained chloroplast fragments (20 μg chlorophyll) and the following in μmoles: potassium phosphate buffer, pH 7.0, 20; KCl, 40; $MgCl_2$, 5; and DPIP, 0.1. The DPIP was omitted from the control reaction mixture.

The reactions were run at 25°.

Strain	μmoles of oxidant photoreduced/hr/mg chlorophyll		
	NADP-a	NADP-b	DPIP
Wild type	169	81	269
ac-206	<2	43	18
ac-208	<2	4	29
ac-208 (sup.)	5	9	44

ments were prepared by ultrasonic disintegration (table III), the rate of NADP-a was quite low. However, when the chloroplast fragments were prepared by grinding the cells in sand the rate of NADP-a by the suppressed strain was 35 μmoles NADPH/hr/mg chlorophyll, whereas that of the unsuppressed strain remained at less than 2. It appears, therefore, that grinding with sand is in some respects a more gentle method for disrupting cells than is ultrasonic disintegration. In addition, the former method gave chloroplast fragments active for photosynthetic phosphorylation whereas the latter did not (5).

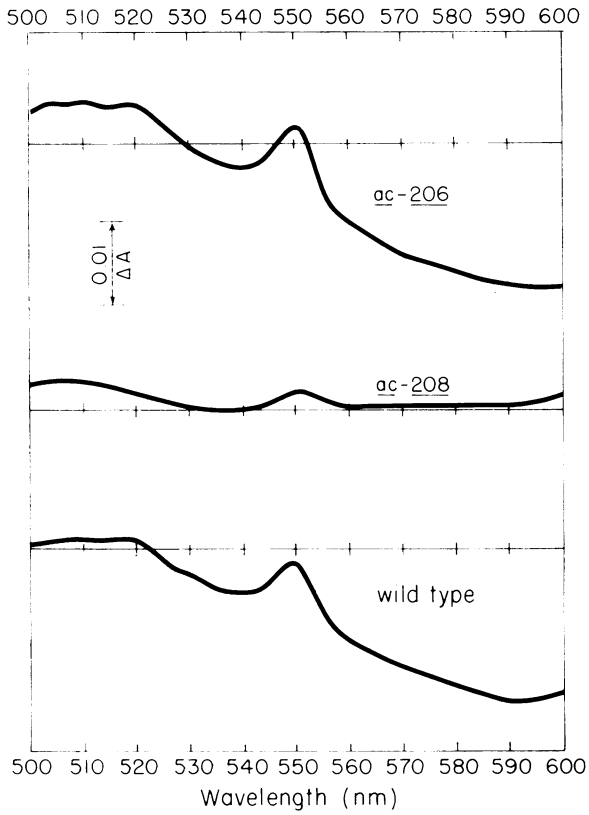

FIG. 5. The reduced-minus-oxidized difference spectra of the water soluble components of the acetone-extracted cell preparation shown in figure 3. The cell preparations were centrifuged for 10 minutes at 20,000 × *g* and the supernates were collected. They were reduced with sodium ascorbate and oxidized with potassium ferricyanide. The 0 to 0.1 slide wire was used on the spectrophotometer.

Readdition Experiments. When plastocyanin from wild-type *C. reinhardi* was added to chloroplast fragments of *ac-208* their photosynthetic activity was at least partially restored. Table IV gives the results of readdition experiments in which NADP and DPIP photoreduction were measured. The restoration effect was more pronounced with chloroplast fragments of the suppressed strain of *ac-208* than with the unsuppressed strain. In a control experiment with *ac-206*, the addition of plastocyanin failed to restore the lost photosynthetic activity, and with the wild-type strain the added plastocyanin had only a slight stimulating effect.

Figure 7 shows the dependence of the restored activity of chloroplast fragments of suppressed *ac-208* upon the plastocyanin in the reaction mixture. Full restoration required a plastocyanin concentration equivalent to 0.007 μg atoms copper per ml. Table V shows that the restoration was completely reversed simply by washing the chloroplast fragments in buffer.

When the chloroplast fragments of *ac-208* and *ac-208* (sup.) were prepared by grinding the cells in sand (table VI) they did not show as great an effect of added plastocyanin as when chloroplast fragments were prepared by disrupting the cells by ultrasonic disintegration (table IV). This result suggested that the restoration of NADP photoreduction may have depended upon a certain degree of structural degradation or disruption of the chloroplasts possibly at a molecular level. Indeed, as shown in table VI, when chloroplast fragments from *ac-208* (sup.) were mistreated either by ultrasonic treatment or by aging at 25°, the NADP-a activity with added plastocyanin actually increased whereas the activity without added plastocyanin de-

Table IV. *Effect of Added Plastocyanin on the NADP and DPIP Photoreduction*

The reaction mixtures and conditions of assay were the same as described in table III. Where indicated, plastocyanin equivalent to 0.02 μg atoms Cu, purified from the wild type strain, was added to the reaction mixtures.

Strain	Addition	μmoles oxidant photoreduced/hr/mg chlorophyll		
		NADP-a	NADP-b	DPIP
ac-208	none	<2	4	16
	plastocyanin	22	22	36
ac-208 (sup.)	none	5	9	44
	plastocyanin	105	69	180
ac-206	none	<2	...	...
	plastocyanin	<2	...	...
Wild type	none	94	40	126
	plastocyanin	102	60	147

Table V. *Reversal by Washing of the Restoration of NADP Photoreduction in Suppressed* ac-208

The reaction mixtures and conditions of assay were the same as given in table III. However, where indicated, plastocyanin was added to give a concentration of 0.01 μg atoms Cu per ml. The chloroplast fragments were washed with 0.01 M phosphate buffer, pH 7.0, containing 0.02 M KCl, and 2.5 mM $MgCl_2$ by centrifuging at 10,000 × *g* for 10 minutes. They were then resuspended in fresh medium.

Treatment	NADP-a μmoles NADPH/hr/mg chlorophyll
Plastocyanin added and fragments washed	7
Plastocyanin added, fragments washed, and plastocyanin added again	73

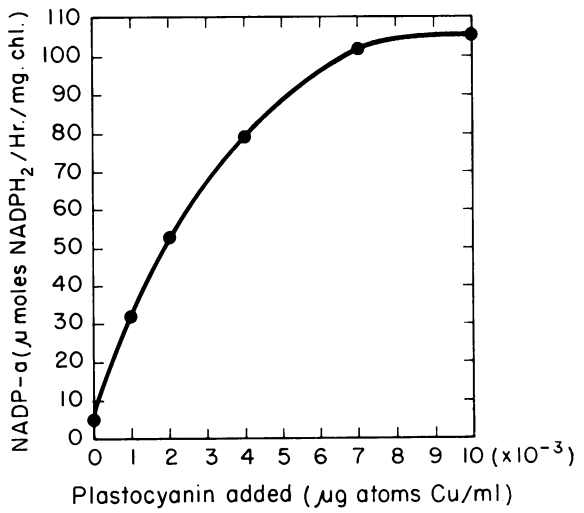

FIG. 7. The NADP-a activity of chloroplast fragments of *ac-208* (sup.) as a function of the concentration of purified plastocyanin added to the reaction mixture. The methods for assay were the same as described in table III. Plastocyanin, purified from the wild-type strain, was added to give the indicated final concentrations.

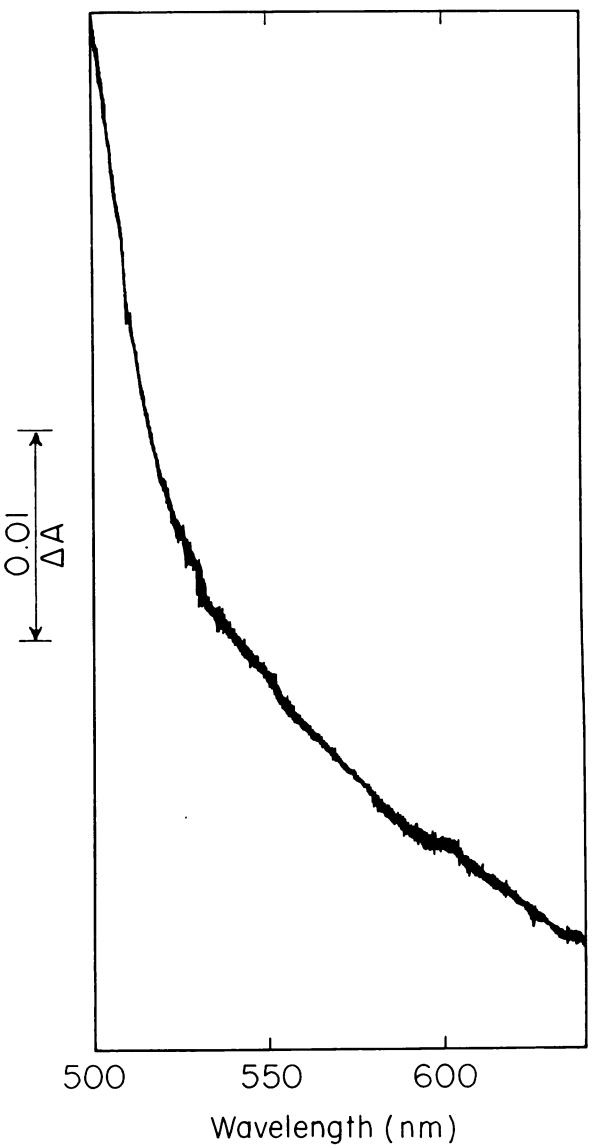

FIG. 6. The reduced spectrum of an extract of *ac-206* purified through step 1 (6). Reduction was achieved with sodium ascorbate. The 0 to 0.1 slide wire was used to record the spectrum.

creased. The effort to demonstrate a similar effect with chloroplast fragments of unsuppressed *ac-208* failed.

The light-induced absorbance changes exhibited by chloroplast fragments of *ac-208* have already been described (14). It was found that cytochrome 559 and cytochrome 553 were reduced by red light but that they were not oxidized by far-red light. Figure 8 shows that upon the addition of plastocyanin to the chloroplast fragments of *ac-208*, far-red light was capable of sensitizing the oxidation of the 2 cytochromes.

The attempt to restore the photosynthetic activity of chloroplast fragments of *ac-206* by the addition of cytochrome 553 obtained from the wild-type strain failed (table VII). Addition of the cytochrome also failed to duplicate the effect that plastocyanin had in restoring the activity of chloroplast fragments of the suppressed *ac-208*. The small restorative effect was probably due to a slight contamination of the cytochrome with plastocyanin. It was estimated that the cytochrome used in this experiment contained plastocyanin equivalent to about 0.3 μg atoms Cu per μmoles of cytochrome 553. This estimate was based upon the oxidized-minus-reduced absorption difference at 597 nm of

the cytochrome preparation assuming that the pure cytochrome would have no absorption difference at this wavelength.

Discussion

The results presented above strongly suggest that *ac-208* is unable to synthesize plastocyanin, at least in an active and detectable form, and that the lack of plastocyanin is the cause of the mutant strain's inability to carry out normal photosynthesis. Furthermore, the addition of plastocyanin from wild type to the chloroplast fragments of *ac-208* restored the mutant strain's missing photosynthetic activities. The restoration of these activities had a high degree of specificity with respect to both the chloroplast fragments used and the protein added.

Plastocyanin failed to restore any photosynthetic activity to chloroplast fragments of *ac-206* (table IV) and cytochrome 553 apparently could not substitute for plastocyanin in restoring activity to chloroplast fragments of *ac-208* though limitation in the quantity and purity of the cytochrome 553 available prevented an adequate test of this point. The specificity for plastocyanin versus cytochrome 553 is of particular significance because these 2 proteins are identical in their normal oxidation-reduction potential and similar in their molecular size, as judged by chromatography on Sephadex G-75 and electrostatic charge as judged by chromatography on DEAE cellulose (6, 7).

The restoration of photosynthetic activity in *ac-208* by plastocyanin was found to be dependent upon the concentration of plastocyanin added to the reaction mixture. Full restoration of the NADP-a reaction to chloroplast fragments of *ac-208* (sup.) was achieved with plastocyanin equivalent to 0.007 μg atoms Cu per ml (fig 8). Since the chlorophyll concentration in the reaction mixture was 0.11 μmoles per ml, and assuming a photosynthetic unit of 200 chlorophyll molecules (10), the full restoration of activity evidently required plastocyanin equivalent to more than 100 copper atoms per photosynthetic unit. It seemed probable, therefore, that the added plastocyanin was not being bound to the active centers of the chloroplast fragments but was interacting with them in a loose, reversible fashion. This idea was confirmed by the results of the experiment described in table V in which it was found that the restoration effect was completely

Table VI. *Photoreduction of NADP, with and without the Addition of Plastocyanin, by Chloroplast Fragments Prepared from* ac-208 *and Suppressed* ac-208 *by Sand Grinding*

The methods and conditions of assay were the same as described in table III except that the cells were disrupted by grinding in sand. The aging was done by allowing the chloroplast fragments to stand at 25° for the indicated period of time. Where indicated, plastocyanin equivalent to 0.02 μg atoms Cu was added to the reaction mixture.

Strain	Treatment of chloroplast fragments	Addition	NADP-a μmoles NADPH/hr/mg chlorophyll
ac-208	None	None	1
		Plastocyanin	6
	Aged, 15 minutes	None	0
		Plastocyanin	4
ac-208 (sup.)	None	None	35
		Plastocyanin	59
	Sonicated 30 sec.	None	16
		Plastocyanin	66
	Aged, 60 minutes	None	4
		Plastocyanin	76

Table VII. *Photoreduction of NADP by Chloroplast Fragments of* ac-206 *and* ac-208 *(sup.) with and without the Addition of Cytochrome 553 or Plastocyanin*

The methods and conditions of assay were the same as described in table III except that the reaction mixtures had a final volume of 0 8 ml rather than 2.0 ml. All components of the reaction mixture were scaled down proportionately. Where indicated, 0.002 μmole of cytochrome 553 or plastocyanin equivalent to 0.008 μg atoms Cu purified from wild type *C. reinhardi* was added.

Strain	Addition	μmoles NADPH/hr/mg chlorophyll NADP-a	NADP-b
ac-206	None	4	44
	Cytochrome 553	4	44
ac-208 (sup.)	None	0	
	Cytochrome 553	9	
	Plastocyanin	63	

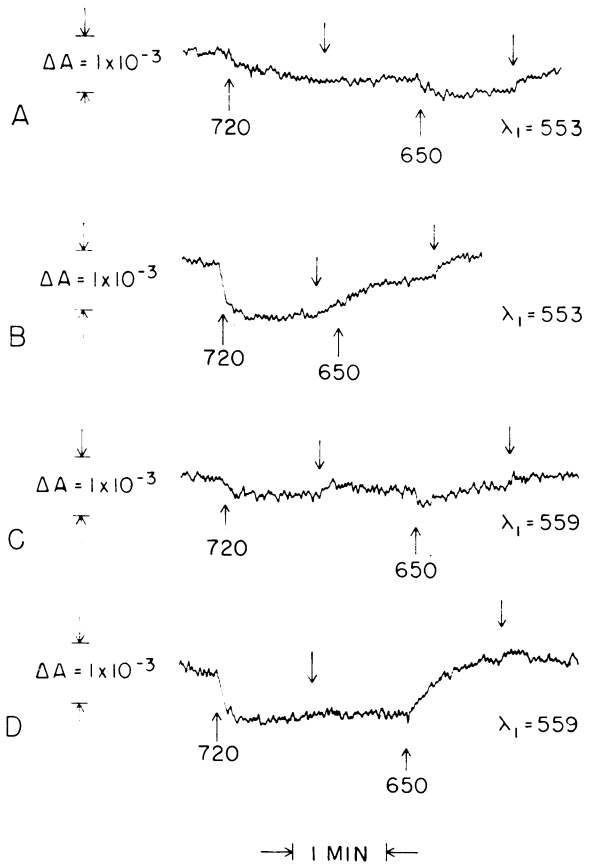

FIG. 8. Light-induced absorbance changes at 553 nm (A and B) and 559 nm (C and D) in chloroplast fragments of *ac-208*. The wavelength of the reference beam was 542 nm The actinic illumination was either 650 or 720 nm. Arrows pointing upward indicate actinic illumination on, and arrows pointing downward indicate actinic illumination off. The preparation of chloroplast fragments in B and D contained plastocyanin equivalent to 0.0028 μg atoms Cu.

reversed when the preparation of chloroplast fragments containing added plastocyanin was washed with buffer.

Katoh and Takamiya (9) reported a restoration effect by plastocyanin similar to that described here. They observed that prolonged ultrasonic treatment of *Brassica* chloroplasts caused a loss of the NADP-a and NADP-b activities and that these activities were restored upon the addition of plastocyanin.

The results described here for the suppressed strain of *ac-208* are informative though they present a somewhat more complicated situation than the unsuppressed *ac-208*. Cells of *ac-208* (sup.) carried out normal photosynthesis at a rate 50 % of that of wild type when measured as whole cell CO_2 fixation (table II). Since no plastocyanin was detected in the extracts of *ac-208* (sup.), an obvious conclusion would be that the presence of plastocyanin is not obligatory for photosynthesis. However, there are reasons for believing that there is an alternative explanation for these results. Chloroplast fragments of *ac-208* (sup.) prepared by the relatively gentle method of grinding with sand showed a substantial NADP-a reaction (table VI) but little or no reaction when prepared by the ultrasonic treatment of the cells (table IV). However, the activity lost by virtue of the ultrasonic treatment was fully restored by the addition of plastocyanin (table IV). These results suggest that the cells of *ac-208* (sup.) may have contained a structurally modified plastocyanin which was at least partially active in photosynthesis but which was less stable than the plastocyanin of wild type. This hypothesis readily explains the failure to detect plastocyanin in the extracts of *ac-208* (sup.) because the extraction procedure involved treatment with 80 % aqueous acetone which might be expected to denature the unstable plastocyanin. An important, but no doubt difficult, project for future investigation would be to isolate and characterize the unstable plastocyanin from *ac-208* (sup.), if it exists.

Cells of *ac-206* contained no detectable cytochrome 553 in either the soluble or insoluble form. Since the cytochrome in both forms was readily detected in wild type and in the other mutant strains used here, it can be concluded that the inability of cells of *ac-206* to carry out normal photosynthesis was the result of either the lack of cytochrome 553 or the synthesis of the cytochrome in some inactive form. The simultaneous absence of both the soluble and insoluble forms of cytochrome 553 from cells of *ac-206* provided evidence that these 2 forms represent the same cytochrome. Since the cells of *ac-206* contained the mitochondrial-type cytochrome *c*, it was evident that the loss of cytochrome 553 was specific and did not extend to other *c*-type cytochromes.

The attempt to restore the photosynthetic activity of chloroplast fragments of *ac-206* by addition of cytochrome 553 from wild type failed, but the failure probably means that the site of action of the cytochrome in the chloroplast fragments was not accessible to the soluble cytochrome from the external medium.

The sequence of function of cytochrome *f* (or cytochrome 553) and plastocyanin in the photosynthetic electron transport chain has been the subject of much interest recently. Both cytochrome *f* and plastocyanin have been observed to be oxidized by system I and reduced by system II, indicating that they act in the chain between the 2 photochemical systems (3, 13). However, these observations did not permit a definitive conclusion regarding the position of these 2 components in the photosynthetic electron transport chain. It has been proposed (11, 12) that they act in parallel to transfer electrons from system II to system I, but this proposal is incompatible with the results presented here. When either plastocyanin or cytochrome 553 are absent

there is no photoreduction of NADP when the electrons come from water. Therefore, it appears that cytochrome 553 and plastocyanin lie in series rather than parallel in the photosynthetic electron transport chain of *C. reinhardi*.

Fork and Urbach (4) and Avron (2) concluded from the effects of the copper chelating agent, salicylaldoxime, on light-induced absorbance changes of cytochrome *f* that the cytochrome lies between plastocyanin and system I. This sequence is inconsistent with the results obtained with *ac-206* and *ac-208*. First, in the absence of plastocyanin chloroplast fragments of *ac-208* exhibited only the light-induced reduction of cytochromes 553 and 559 (14). Second, the light-induced oxidation of cytochromes 553 and 559 returned when plastocyanin was added to the chloroplast fragments (fig 8). Third, since *ac-206*, which lacks cytochrome 553, could photoreduce NADP when the source of electrons was from reduced DPIP (table III) the site of the entry of these electrons must be on the system I side of cytochrome 553. However, with *ac-208*, in which the cytochrome is present, there was only negligible photoreduction of NADP when reduced DPIP was the electron donor (table III). Therefore, the site of entry of these electrons must be either at plastocyanin or on the system II side of plastocyanin. Fourth, upon addition of plastocyanin to the chloroplast fragments of *ac-208* they were able to carry out the photoreduction of NADP with reduced DPIP as the electron donor (table IV). Accordingly, the only sequence compatible with these results is system II → cytochrome 553 → plastocyanin → system I.

There is no direct way to rationalize the differences between the results presented here and those obtained in the investigations using salicylaldoxime except to point out that different organisms were used by Fork and Urbach (4) and by Avron (2). In addition, San Pietro and Katoh (7a) have shown for spinach chloroplasts that salicylaldoxime does not act as a specific inhibitor of plastocyanin, for the photoreduction of NADP from DPIP and ascorbate, a reaction that depends upon the presence of plastocyanin, is insensitive to salicylaldoxime. Furthermore, they have shown that even though salicylaldoxime can cause the almost instantaneous inhibition of certain photosynthetic reactions, its effect upon the reduction of the copper of plastocyanin takes several hours. Finally, preliminary experiments in this laboratory have revealed that salicylaldoxime at a concentration of 10^{-2} M inhibits the light-induced reduction of both the cytochrome 553 and cytochrome 559 in the wild-type strain and in *ac-208* which is, of course, devoid of plastocyanin.

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