CYTOCHROME F AND PLASTOCYANIN: THEIR SEQUENCE IN THE PHOTOSYNTHETIC ELECTRON TRANSPORT CHAIN OF CHLAMYDOMONAS REINHARDI*

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The elucidation of the mechanism of photosynthetic electron transport and photosynthetic phosphorylation depends, in part, upon a knowledge of the intermediates in the photosynthetic electron transport chain and the sequence in which they act. Mutant strains of the unicellular green alga *Chlamydomonas reinhardi* that cannot carry out normal photosynthesis[1] can aid in obtaining this knowledge. In this paper we describe results obtained with *ac-206*, a mutant strain that lacks cytochrome *f*, and *ac-208*, a mutant strain that lacks the copper protein, plastocyanin. Since its discovery by Katoh,[2] plastocyanin has been presumed to play a role in photosynthetic electron transport. In this paper we present evidence for its participation and site of action with respect to cytochrome *f* in the photosynthetic electron transport chain of *C. reinhardi*.

Organisms and Methods.—The organisms used in the experiments described below were the wild-type strain of *C. reinhardi* (137c), and the mutant strains *ac-206* and *ac-208* derived from the wild type by ultraviolet irradiation.[3]

Cells, in the logarithmic phase of growth, were harvested either from 300-ml shake cultures or from vigorously aerated 12-liter cultures grown in the light at 25°C. The growth medium differed from that previously described.[4] Tris-acetate buffer, 0.02 *M*, pH 7.2, was used in place of both phosphate buffer and sodium acetate. Potassium phosphate buffer, 0.001 *M*, pH 7.0, was also added. Light was provided by daylight fluorescent lamps at an intensity of 4000 lux for cultures of wild type and *ac-206*, and at 2000 lux for cultures of *ac-208*.

Chloroplast fragments for the measurement of the photoreduction of NADP and DPIP were prepared by the sonic disruption of 5-ml suspensions of cells for 30 sec according to the method described by Levine and Volkmann.[5] Chloroplast fragments for the measurement of photosynthetic phosphorylation were prepared by grinding a paste of cells in purified sand. After grinding, the disrupted cells were suspended and washed in the following medium: 0.01 *M* potassium phosphate buffer, pH 7.5; 0.02 *M* KCl; 0.0025 *M* $MgCl_2$; 0.001 *M* $MgNa_2$ EDTA; and 0.001 *M* reduced glutathione. The separation of chloroplast fragments from whole cells was then carried out as described by Levine and Volkmann.[5] The photoreduction of NADP and DPIP was measured as previously described.[6] Cyclic and noncyclic photosynthetic phosphorylation were measured by the techniques described by Avron[7] and Avron and Shavitt.[8] Chlorophyll was determined by a modification[9] of the procedure of MacKinney.[10] Purified PPNR was prepared from wild-type *C. reinhardi* according to the procedure described by Tagawa and Arnon.[11]

Plastocyanin was measured, after partial purification, by a modification of the method of Katoh, Shiratori, and Takamiya.[12] Twenty-four liters of cell culture were harvested and cells were resuspended in 0.002 *M* phosphate buffer, pH 7.0, to a chlorophyll concentration of about 2 mg per ml. All subsequent procedures were carried out in a cold room at 4°C. The cell suspension was added quickly, under rapid stirring, to 4 vol of acetone at about −25°C (the temperature after mixing was about −10°C). The cells were then collected as quickly as possible by filtration and resuspended in 0.01 *M* phosphate buffer, pH 7.0. The suspension was allowed to stand at 4°C for at least 6 hr, after which the cell debris was centrifuged out at 20,000 × *g* for 10 min, and the clear supernate was collected. This was then allowed to drain through a column (about 1.3 × 13 cm) of fine-meshed DEAE cellulose (Selectacel DEAE 40) equilibrated with 0.01 *M* phosphate buffer, pH 7.0. 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 with 0.15 *M* KCl. The column effluent was collected in 5-ml fractions. The plastocyanin was nearly always confined to the first 20 ml of

column effluent after the addition of the phosphate buffer containing the KCl. The plastocyanin so obtained was found to be sufficiently pure to permit an accurate measurement of its amount from the difference between the spectra of the oxidized and reduced pigment.

Cytochrome *f* was measured spectrophotometrically in cells that had been extracted with 80% aqueous acetone at −15°C. After extraction the cells were collected by centrifugation at −15°C and resuspended in 0.01 *M* phosphate buffer, pH 7.0, for measurements of the reduced-minus-oxidized difference spectrum. Spectra were obtained using a model 14 Cary recording spectrophotometer having a light-scattering attachment. The 0–0.1 slide wire was used, and the slit control and dynode voltage were set to their minimum values. A special, high-intensity light source was used, and the slit widths were less than 0.25 mm wide throughout the region scanned.

Results.—The plastocyanin content of wild type, *ac-206*, and *ac-208* is given in Table 1. Several attempts were made to demonstrate the presence of plastocyanin in *ac-208*, without success. It was estimated that plastocyanin equivalent to as little as 1 Cu atom per 10,000 chlorophyll molecules would have been detected by the method employed.

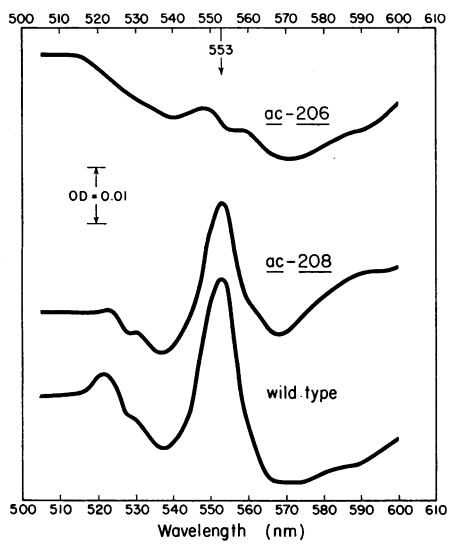

FIG. 1.—The reduced minus oxidized difference spectra of cells of wild type, *ac-206*, and *ac-208* after extraction with 80% aqueous acetone. The cells of each strain were suspended at a concentration equivalent to 1 mg chlorophyll per ml as determined before acetone extraction. The cuvettes, having a vertical light path, were filled to a depth of 1 cm. The samples were reduced with sodium ascorbate or oxidized with potassium ferricyanide. Each spectrum has been corrected for the base line seen with the appropriate untreated samples.

Wild type and *ac-208* both contain a cytochrome having an α band at 553 nm (Fig. 1) indicative of cytochrome *f*, which is found generally in green plants including *C. reinhardi*.[13] All efforts to detect cytochrome *f* in preparations of *ac-206* failed (Fig. 1).

Chloroplast fragments of wild type, *ac-206*, and *ac-208* were assayed for their ability to photoreduce NADP and DPIP. The photoreduction of NADP was measured in two ways: either as a Hill reaction with water as the electron donor, or in the presence of DCMU with DPIP and ascorbate as the electron donors. The data in Table 2 show that chloroplast fragments of both mutant strains were incapable of photoreducing NADP in a Hill reaction, and there was a Hill reaction with DPIP but at a rate lower than wild type. The rate of NADP photoreduction with DPIP and ascorbate as electron donors was comparable in wild type and *ac-206* but was negligible in *ac-208*.

TABLE 1

PLASTOCYANIN CONTENT OF WILD TYPE, *ac-206*, AND *ac-208*

Strain	Plastocyanin content (μg atoms Cu/1000 μmoles chlorophyll)
Wild type	2.3
ac-206	3.9
ac-208	<0.1

The plastocyanin content was determined by measuring the oxidized-reduced absorbance difference at 597 nm using the extinction coefficient of Katoh *et al.*[12] Plastocyanin was oxidized with potassium ferricyanide and reduced with sodium ascorbate.

TABLE 2

PHOTOREDUCTION OF NADP AND DPIP BY CHLOROPLAST FRAGMENTS OF WILD TYPE, *ac-206*, AND *ac-208*

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

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

For NADP photoreduction when the source of electrons was from water, the reaction mixtures (2.0 ml) in the test and control cuvettes contained chloroplast fragments (20–25 μg chlorophyll) and the following in μmoles: $MgCl_2$, 5.0; KCl, 40; and phosphate buffer, pH 7.0, 20. For NADP photoreduction when the source of electrons was from DPIP and ascorbate, the test and control cuvettes contained, in addition to the above, the following in μmoles: DPIP, 0.1; ascorbate, 10; and DCMU, 0.02. An excess of purified PPNR from wild-type *C. reinhardi* and 0.5 μmoles of NADP were included in the test cuvettes only.

For the photoreduction of DPIP, the reaction mixtures (2.0 ml) in the test and control cuvettes contained chloroplast fragments (20–25 μg chlorophyll) and the following in μmoles: $MgCl_2$, 5; KCl, 40; and phosphate buffer, pH 7.0, 20. DPIP, 0.1 μmole, was included in the test cuvette only.

The reactions were run at 25°C.

Cyclic photosynthetic phosphorylation with PMS as the electron carrier occurred at comparable rates in chloroplast fragments of wild type and *ac-206*, and at a greatly reduced rate in *ac-208* (Table 3). The relative insensitivity of this reaction to DCMU indicated that it was a true, rather than an oxygen-dependent, cyclic photosynthetic phosphorylation. Neither mutant strain exhibited noncyclic photosynthetic phosphorylation coupled to the photoreduction of potassium ferricyanide (Table 3) even though detectable rates of ferricyanide reduction were obtained. The coupling ratio of about 0.3 obtained with wild type is characteristic of chloroplast fragments prepared from cells that are disrupted by grinding in sand as described above.

Discussion.—Photosynthesis in green plants involves two different light reactions.[14] One reaction, termed system II, is driven by red light and mediates the formation of a strong oxidant and a weak reductant resulting in oxygen evolution and the reduction of cytochrome *f*. The other reaction, termed system I, is driven by far-red light; it mediates the formation of a weak oxidant and a strong reductant resulting in cytochrome *f* oxidation and the reduction of NADP. The search for the components of the photosynthetic electron transport chain and the assignments of

TABLE 3

PHOTOSYNTHETIC PHOSPHORYLATION BY CHLOROPLAST FRAGMENTS OF WILD TYPE, *ac-206*, AND *ac-208*

Strain	Cyclic Photosynthetic Phosphorylation with PMS as Electron Carrier μmoles Pi esterified/Hr/mg Chlorophyll		Noncyclic Photosynthetic Phosphorylation Coupled to Ferricyanide Photoreduction		
	Without DCMU	With DCMU	μmoles Ferricyanide reduced/hr/mg chlorophyll	μmoles Pi esterified/hr/mg chlorophyll	Coupling ratio
Wild type	476	292	726	105	0.29
ac-206	150	158	17	<0.5	<0.06
ac-208	14	10	64	<0.5	<0.02

The reactions were run at 25°C in 25-ml Erlenmeyer flasks. The reaction mixtures (2.0 ml) contained chloroplast fragments (60–120 μg chlorophyll) and the following in μmoles: glycylglycine-NaOH buffer, pH 8.0, 40; KCl, 40; $MgCl_2$, 5; ADP, 5; AMP, 5; and potassium phosphate, pH 8.0, 10, containing 5×10^5 cpm of P^{32}. For cyclic photosynthetic phosphorylation the reaction mixtures contained 0.067 μmole of PMS. The reaction mixtures were continuously flushed with nitrogen. Where indicated, 0.02 μmole of DCMU was added. For noncyclic photosynthetic phosphorylation, 2 μmoles of potassium ferricyanide were added to the reaction mixtures.

After a 10-min equilibration in the dark, the reactions were initiated by turning on the lights (40,000 lux). The reactions were terminated by turning off the lights and by adding 0.2 ml of 20% TCA to each flask. The esterification of P^{32} was determined according to the procedure described by Avron.[7] The photoreduction of potassium ferricyanide was determined by the method of Avron and Shavitt.[8]

their roles and positions with respect to the two photochemical systems is a major preoccupation of research in photosynthesis. Duysens and Amesz[15] were the first to show that cytochrome *f* lies between photochemical systems I and II. Recently, de Kouchkovsky and Fork[16] have shown that plastocyanin also lies between the two systems. However, the nature of these observations did not permit a definitive conclusion regarding the positions of these two components in the photosynthetic electron transport chain. Kok, Rurainski, and Harmon,[17] and Kok and Rurainski[18] have proposed that plastocyanin and cytochrome *f* function in parallel to transfer electrons from system II to system I. Their proposal is incompatible with the data presented here because in *ac-208*, which lacks plastocyanin but has cytochrome *f*, there is no photoreduction of NADP from water. Similarly, there is no photoreduction of NADP from water in *ac-206* which lacks cytochrome *f* but has plastocyanin. Therefore, both cytochrome *f* and plastocyanin must lie in series rather than in parallel in the photosynthetic electron transport chain of *C. reinhardi*.

Fork and Urbach[19] concluded from the effects of a copper chelating agent on light-dependent absorbance changes of cytochrome *f* that the cytochrome lies between plastocyanin and system I. This sequence for cytochrome *f* and plastocyanin is inconsistent with the results presented here. Since *ac-206*, which lacks cytochrome *f*, can photoreduce NADP when DPIP and ascorbate are the electron donors, the site of entry of these electrons must be *after* cytochrome *f*. However, with *ac-208*, in which cytochrome *f* is present but plastocyanin is absent, there is negligible photoreduction of NADP when DPIP and ascorbate are the electron donors. Therefore, the site of entry of these electrons must be *at* or *before* plastocyanin. It follows that the sequence in the electron transport chain of *C. reinhardi* is system II, cytochrome *f*, plastocyanin, system I.

The experiments with *ac-206* and *ac-208* also provide evidence regarding the site of photosynthetic phosphorylation. The absence of noncyclic photosynthetic phosphorylation coupled to ferricyanide photoreduction, and the presence of cyclic photosynthetic phosphorylation catalyzed by PMS in *ac-206* support the view that photosynthetic phosphorylation is coupled with electron flow at a site on the system I side of cytochrome *f*. The absence of noncyclic photosynthetic phosphorylation and the possible existence of cyclic photosynthetic phosphorylation in *ac-208* suggest that the coupling site may even be on the system I side of plastocyanin.

The greatly reduced rate of cyclic photosynthetic phosphorylation in *ac-208* further suggests that the main site of entry of electrons carried by PMS lies at or before plastocyanin. On the other hand, the relatively high rate of cyclic photosynthetic phosphorylation in *ac-206* shows that the site of entry is *after* cytochrome *f*. These observations, therefore, provide further support for our conclusion that plastocyanin lies between cytochrome *f* and system I.

Summary.—Evidence is presented from experiments with two different mutant strains of *C. reinhardi*, one of which lacks plastocyanin and the other, cytochrome *f*, that: (1) plastocyanin and cytochrome *f* lie in series rather than in parallel in the photosynthetic electron transport chain, (2) the sequence of plastocyanin and cytochrome *f* in the electron transport chain is system II—cytochrome *f*—plastocyanin—system I, and (3) a site of photosynthetic phosphorylation is on the system I side of cytochrome *f* and possibly on the system I side of plastocyanin.

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Abbreviations: AMP and ADP, adenosine monophosphate and adenosine diphosphate, respectively; DCMU, 3-(3,4-dichlorophenyl)-1, 1 dimethyl urea; DPIP, 2,6-dichlorophenolindophenol; NADP, nicotinamide-adenine dinucleotide phosphate; PMS, phenazine methosulfate; PPNR, photosynthetic pyridine nucleotide reductase; Tris, tris (hydroxymethyl) aminomethane.

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DIRECTION OF SYNTHESIS OF MESSENGER RNA IN CELLS OF ESCHERICHIA COLI

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One strand of the DNA duplex is transcribed into a strand of messenger RNA (mRNA) by the action of the DNA-dependent RNA polymerase.[1, 2] The substrates are 5′-nucleoside triphosphates, but it has not been known whether the new nucleotide residues are added at the 5′ end of the nascent mRNA, or at the 3′ end (Fig. 1).

If a labeled nucleoside could be incorporated into the newest portion of a growing mRNA chain, then subsequent degradative procedures could reveal whether the 3′ or 5′ end was preferentially labeled. Bacteria growing under physiologic conditions synthesize macromolecules too fast to permit such nonuniform labeling. We reported the use of temperature reduction to 0° as a means of achieving nonuniform labeling of nascent protein and we thereby demonstrated the polarity of its syn-