PsaE Is Required for in Vivo Cyclic Electron Flow around Photosystem I in the Cyanobacterium Synechococcus sp. PCC 7002

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Electron transfer rates to P700+ have been determined in wild-type and three interposon mutants (psaE-, ndhf-, and psaE- ndhf-) of Synechococcus sp. PCC 7002. All three mutants grew significantly more slowly than wild type at low light intensities, and each failed to grow photoheterotrophically in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and a metabolizable carbon source. The kinetics of P700+ reduction were similar in the wild-type and mutant whole cells in the absence of DCMU. In the presence of DCMU, the P700+ reduction rate in the psaE mutant was significantly slower than that observed for the wild-type strain. The further addition of methyl viologen caused the rate of P700+ reduction in the wild type to become as slow as that for the psaE mutant in the absence of methyl viologen. Given the ability of methyl viologen to intercept electrons from the acceptor side of photosystem I, this response reveals a lesion in the PSI acceptor side of photosystem I, which is to transform the energy of a photon into stable chemical free energy in the form of reduced Fd and NADPH. In this role, PSI functions as a light-energy-stored plastocyanin-Fd oxidoreductase (for reviews, see Chitnis and Nelson, 1991a; Golbeck and Bryant, 1991; Bryant, 1992; Golbeck, 1992a; Sétif, 1992). PSI also functions in a cyclic process, transferring electrons through the Cyt b6/f complex so that additional protons can be translocated across the membrane for the purpose of supplying additional ATP. The mechanistic details of this process are largely unknown. The cyanobacterial PSI reaction center is a pigment-protein complex comprising at least 12 polypeptides (labeled PsaA through PsaF and PsbA through PsbM). Most of the Chl's and other cofactors associated with light absorption and charge separation are associated with the PsaA and PsbA polypeptides. The PsaC protein contains the F3 and F9 terminal electron acceptors (Oh-oka et al., 1987, 1988; Wynn and Malkin, 1988). The PsAD protein is important for stabilizing and orienting the PsA protein on the PSI core (Li et al., 1991), as well as "docking" soluble Fd for efficient electron transfer from F3 and/or F9 (Zanetti and Merati, 1987; Wynn and Malkin, 1988).

The function of PsaE is not known with certainty. Its addition to PsaE-deficient PSI complexes in vitro leads to higher rates of Fd and/or flavodoxin reduction (Golbeck, 1992b; Strotmann and Weber, 1993). However, this implied function cannot be the major role for this protein, because no obvious phenotype was found to be associated with the psaE mutations in photosynthetically grown Synechocystis sp. PCC 6803 (Chitnis et al., 1989) or Synechococcus sp. PCC 7002 (Bryant et al., 1990). In particular, there is no effect on rates of photoautotrophic growth or oxygen evolution in saturating light, and there is no effect on noncyclic electron transport (Bryant et al., 1990; Zhao et al., 1993). Chitnis and Nelson (1991b) reported that PSI complexes of a psaE mutant of Synechocystis sp. PCC 6803 have increased turnover relative to those of the wild-type strain. We made the observation that a psaE mutant strain of Synechococcus sp. PCC 7002 was less stable to increased growth temperature than the wild type (Golbeck and Bryant, 1991). This phenotype led us to suspect that there might exist a functional lesion in the PSI complex that becomes apparent only at elevated temperatures. This function could be associated with the need for additional ATP to cope with the increased demands of ion...
pumping and metabolism at elevated temperatures. Most recently, we found that the psaE mutant of Synechococcus sp. PCC 7002 grew much more slowly than the wild-type strains at low light intensities (Zhao et al., 1993). Moreover, the Synechococcus sp. PCC 7002 psaE mutant could not grow at all under phototroph conditions (Zhao et al., 1993), and the Synechocystis sp. PCC 6803 psaE mutant grew only extremely slowly (if at all) under these conditions (our unpublished results). This phenotype suggested that mutations in psaE might affect the cyclic electron transport pathway around PSI.

The existence of an electron cycle around PSI in vivo has not been demonstrated directly, but it has been inferred from indirect measurements. The most severe experimental limitation in the study of the cyclic process is the paucity of unique spectroscopic signals. Most workers in the field presuppose the participation of components of linear photosynthetic electron transfer, such as P700 and the entire PSI electron acceptor chain (A0, A1, Fd, Fe, and F0), along with Fd, plastoquinone, the Cyt b6/f complex, and plastocyanin (or Cyt c553). Because the cycle may use soluble electron transport components, such as Fe or NADPH, this largely precludes studies of refined PSI particles. In a series of seminal papers, Biggins and co-workers inferred the existence of cyclic electron flow by measuring the kinetics of Cyt f turnover (Biggins, 1974a, 1974b) and P700+ reduction (Maxwell and Biggins, 1976, 1977) in PSI mutants and in the presence of various PSI and PSI inhibitors. The slow kinetics of P700+ reduction under conditions appropriate for cyclic flow led them to conclude that cyclic electron transport does not contribute appreciably to photosynthesis in oxygen-evolving autotrophs. More recently, Myers (1986, 1987) sought evidence of cyclic flow from analysis of the steady-state fraction of P700 reduced as a function of the intensity of light and found that the "total return electron flow" (cyclic plus respiration in the light) cannot be much larger than dark respiration. He also concluded that cyclic electron flow is not a significant process in cyanobacteria at ordinary light intensities (Myers, 1987).

In this paper, we describe an experimental system that can be used to study the cyclic electron transport pathway in whole cells of cyanobacteria. The protocol depends on the application of a spectrometer capable of measuring P700 turnover in whole cells and on inhibitors that block selected portions of the photosynthetic and respiratory electron transport chains. Further dissection of the respiratory and photosynthetic electron transport pathways has been accomplished by the construction and characterization of interposon mutants in which the ndhF (Schlucht et al., 1993), psaE (Zhao et al., 1993), and both genes (this work) have been insertionally inactivated in Synechococcus sp. PCC 7002. The data show that the stromal protein PsAE is necessary for cyclic electron flow around PSI. A preliminary report of these findings was made at the IXth International Congress on Photosynthesis (Yu et al., 1992).

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The unicellular marine cyanobacterium Synechococcus sp. PCC 7002 strain PR6000 (wild type; formerly Agmenellum quadruplicatum strain PR-6; Rippka et al., 1979) and derived mutant strains PR6300 (Schlucht et al., 1993), PR6302 (Zhao et al., 1993), and PR6303 (see below) were maintained in liquid culture at 39°C and on 1.5% agar plates at 34°C in medium containing 1 mg mL-1 of sodium nitrate as described by Stevens et al. (1973). The concentration of glycerol, when added to the culture medium, was 10%. Transformants of Synechococcus sp. PCC 7002 were selected and maintained on media containing 200 µg mL-1 of kanamycin and/or 30 µg mL-1 of streptomycin. Cells grown in liquid were routinely bubbled with air supplemented with 1% CO2. Unless specified otherwise, cells were grown at a light intensity of 230 to 270 µE m-2 s-1 (PAR). Light intensities were measured using a model QSL-100 quantum scalar irradiance meter (Biospherical Instruments, Inc., San Diego, CA). Cell concentrations and growth were monitored turbidometrically at 550 nm or 750 nm with a Bausch and Lomb Spectronic 20 spectrophotometer.

Construction of Mutant Strain PR6303 (ndhF- psaE-)

The cloning and characterization of the ndhF (Schlucht et al., 1993) and psaE (Zhao et al., 1993) genes of Synechococcus sp. PCC 7002 have been described. Routine cloning and DNA manipulations were performed as described by Sambrook et al. (1989) using Escherichia coli strain DH5α. Strain PR6300, which carries an insertionally inactivated ndhF gene (Schlucht et al., 1993), was used as the starting point for the construction of a psaE- ndhF- strain. The psaE gene of Synechococcus sp. PCC 7002 is encoded on a 1621-bp HindIII-BglII restriction fragment (Zhao et al., 1993); a unique BamHI site occurs within the coding sequence near the 5' end of the psaE gene. A 2.0-kb BamHI 'II' fragment, encoding resistance to streptomycin/spectinomycin, was cloned into this BamHI site in a pUC19 derivative harboring the 1621-bp HindIII-BglII fragment. The resulting construction was excised with HindIII and EcoRI and used to transform strain PR6300 to streptomycin resistance as described by Bryant and Tandeau de Marsac (1988). After transformants were repeatedly streaked on selective media to allow segregation of alleles, the DNA configuration surrounding the ndhF- and psaE- genes was confirmed by Southern blot hybridization analyses (Buzby et al., 1983). Small-scale isolations of chromosomal DNAs from 10-ml liquid cultures were performed as described by Murphy et al. (1990). The labeling of DNA probe fragments by the random priming method (Feinberg and Vogelstein, 1983) with [α-32P]dATP (New England Nuclear, Boston, MA) was performed as recommended by Boehringer Mannheim Biochemicals (Indianapolis, IN).

Oxygen Evolution, Oxygen Uptake, and Photosystem Measurements

Chl concentrations were measured by extraction in 80% aqueous acetone (MacKinney, 1941; Arnon, 1949). Oxygen evolution from whole cells was measured with a Clark-type electrode at a Chl concentration of 4 µg mL-1. The electrode chamber temperature was maintained at 37°C with a circulating water bath, and the chamber contents were continu-
ously stirred. Saturating light (1000 µE m⁻² s⁻¹) was provided with a tungsten-halogen source filtered through a 500-nm cut-on filter (Corion, Holliston, MA) and a 2-cm water bath as the heat filter. Oxygen uptake of whole cells was similarly measured in complete darkness.

Thylakoid membranes were isolated from cells in the late-exponential phase of growth. Cells were harvested by centrifugation at 8000 g at room temperature for 10 min, and cell pellets were washed once with buffer H (40 mM Hepes [pH 8.0], 10 mM NaCl, 0.4 M Suc). The pellets were resuspended in buffer H at a Chl concentration of 0.4 mg mL⁻¹, and cells were disrupted by passage through a French pressure cell operated at 20,000 psi at 4°C. Unbroken cells and large debris were removed by centrifugation of the extracts at 50,000 g for 45 min at 4°C, and the pelleted membranes were resuspended in buffer H at a Chl concentration of 1.0 mg mL⁻¹.

Concentrations of P700 and Cyt b₅₉₃ of thylakoids were used to estimate the amounts of PSI and PSII in whole cells. The light-induced P700 absorption change was measured with a dual-wavelength spectrophotometer (DW2a; SLM-Aminco, Urbana, IL) as described by Myers (1986). Actinic light was provided with a tungsten-halogen light, filtered with a 440-nm interference filter (10-nm bandwidth; Corion; Holliston, MA). The difference absorption coefficient of 70 mM cm⁻¹ (Hiyama and Ke, 1972) was used to estimate P700 concentration. Cyt b₅₉₃ concentration was measured according to the method of Murakami and Fujita (Murakami and Fujita, 1991).

P700 Turnover in Vivo

The reduction kinetics of P700⁺ in whole cells was measured using modulated light and phase-sensitive detection with an apparatus similar in principle to that described by Maxwell and Biggins (1976). Modulated light was generated by providing a square-wave pulse train at a frequency of 16 kHz to an electronically switched 833.5-nm laser diode (DLD 300; Melles Griot, Irvine, CA). The 50-mW beam was expanded to a height of 10 mm and a width of 5 mm and passed through a 5-mm-wide sample cuvette with a 1-cm pathlength. A colored glass bandpass filter (λcuton > 800 nm) was inserted between the cuvette and detector to minimize scattered actinic illumination and fluorescence artifacts. The modulated near-IR measuring beam was detected with a photodiode (RCA 30810) operated in the photocathode mode, and the signal was demodulated with a lock-in amplifier (model 5101; EG&G, Sunnyvale, CA). The instrument operated with a time constant of 1 ms. The sample was excited with white light generated from a 150-W quartz-tungsten lamp that was focused on the cuvette after passing through a colored glass filter (λcutoff < 750 nm) to eliminate the near-IR wavelengths. An illumination period of 1.5 s was followed by a dark period of 6 s permitted full activation and recovery of the electron transport components as judged by repeated light-dark cycles. The signal-to-noise ratio was improved by averaging 64 light-dark cycles with a Nicolet 4095A digital oscilloscope (12-bit resolution, 500 µs point⁻¹). The first light-dark cycle was recorded separately and compared with the average; no changes in the kinetics were found during the course of any measurement.

The data were transferred via an RS-232 line to a Macintosh Ici computer for curve fitting using IGOR, a commercial software package (WaveMetrics, Lake Oswego, OR). Single- and double-exponential curve fits were attempted, and the closest fit was chosen; in nearly all cases, a single-exponential curve provided an excellent fit to the P700⁺ reduction kinetics to more than three half-lives. Fresh cyanobacterial cells were harvested by centrifugation at room temperature and resuspended in fresh growth medium (pH 8.2) at cell densities corresponding to 5 to 13 µg of Chl mL⁻¹.

Spectrophotometric Assay of Cyclic Electron Flow

The assay of cyclic electron flow relies on measuring the reduction kinetics of P700⁺ following exposure to light and on the use of mutants and inhibitors to separate cyclic from linear electron flow. The premise of the measurement is that in cyanobacteria the Cyt b₅₉₃ complex is shared by the photosynthetic and respiratory electron transport chains (Scherer, 1990) and that electron flow through this complex is the rate-limiting step in the reduction of P700⁺. The protocol relies on white light to excite both PSI and PSII such that the plastoquinone pool will fill, and the proximal electron donor pool before P700 will deplete, during a 1.5-s illumination period. In the 6-s period of subsequent darkness, electrons will flow through the Cyt b₅₉₃ complex from both the preloaded plastoquinone pool and the respiratory NADH dehydrogenase, and electrons will flow out of the Cyt b₅₉₃ complex to both the Cyt oxidase and P700⁺. Under these conditions, P700⁺ reduction is limited by the electron flow through the rate-limiting Cyt b₅₉₃ complex (and any additional bottlenecks in the pathway to the Cyt complex from the NADH dehydrogenase and the cyclic pathway around PSI).

Inhibitors of PSII and Respiratory Electron Flow

Stock solutions of DCMU, DBMIB, and methyl viologen were prepared to 1 mM in 95% ethanol. The concentration of ethanol added to the sample cuvette never exceeded 0.5%; this amount of ethanol had no effect on the kinetics or extent of P700⁺ reduction in wild-type or mutant cells. Chemical reagents were added to the sample cuvette in the following concentrations: 10 mM glycerol, 10 µM DCMU, 1 mM KCN, 100 µM methyl viologen, 5 µM DBMIB. The P700⁺ reduction kinetics were measured under a variety of conditions: with and without 10 µM DCMU to inhibit electron donation from PSII; with and without 10 µM DCMU and 1 mM KCN to inhibit PSII and electron exit to the respiratory Cyt oxidase; with and without 10 µM DCMU, 1 mM KCN, and 100 µM methyl viologen to inhibit PSII, the Cyt oxidase, and electron entry from the cyclic pathway around PSI; and with and without 5 µM DBMIB to inhibit electron entry from all sources to the Cyt b₅₉₃ complex. These inhibitors, when used in conjunction with strains harboring mutations in the ndhf gene (inactive NADH dehydrogenase; Schluchter et al., 1993), the psaE gene (inactive cyclic pathway around PSI; see below), or both genes, could be used to separate cleanly...
the entry points and exit points of electrons derived from the photosynthetic and respiratory components.

RESULTS

Construction and Properties of the psae<sup>−</sup> ndhf<sup>−</sup>

Strain PR6303

A psae ndhf double mutant was constructed by interposon mutagenesis of the ndhf<sup>−</sup> strain PR6300 (Schluchter et al., 1993). A construction in which the psae gene was interrupted by a 2.0-kb DNA Ω fragment, which encodes resistance to spectinomycin/streptomycin, was used to transform strain PR6300 to streptomycin resistance. After single colonies were repeatedly streaked to allow segregation of alleles, several transformants were selected for Southern blot hybridization analyses to confirm the construction of the mutations. Figure 1 shows the results obtained with one such isolate, designated PR6303, and the chromosomal configuration at the psae locus was investigated (Fig. 1A). As expected, the psae-specific probe hybridizes to a 6.8-kb EcoRI fragment in the EcoRI digest of the PR6000 DNA (Fig. 1A, lane 1) and to an 8.8-kb EcoRI fragment in the EcoRI digest of the PR6003 DNA (Fig. 1A, lane 3). The larger fragment in the mutant strain results from the 2.0-kb Ω fragment, which was introduced in the BamHI site within the psae gene. The configuration at the ndhf locus was studied (Fig. 1B). The ndhf probe fragment hybridized to an EcoRI fragment of 6.8 kb for the wild-type strain PR6000 (Fig. 1B, lane 1) and to an EcoRI fragment of 8.1 kb for the mutant strain PR6303 (Fig. 1B, lane 3). The increase of 1.3 kb is that expected due to the insertion of a 1.3-kb PstI fragment encoding the aphH gene into the PstI site within the ndhf<sup>−</sup> coding sequence (Schluchter et al., 1993). The results shown in Figure 1 also demonstrate that the mutant strain PR6303 is homozygous for both mutant loci, because fragments with sizes observed for the wild-type strain were not observed in digests of PR6030 DNA.

The physiological characteristics of strain PR6303 (Table I) were similar to those of single psae<sup>−</sup> and ndhf<sup>−</sup> strains of Synechococcus sp. PCC 7002 (Schluchter et al., 1993; Zhao et al., 1993). As expected, strain PR6303 could not grow under phototrophic conditions. Under autotrophic growth conditions at high light intensity, the growth rate of mutant strain PR6303 was equal to that of the wild-type strain PR6000; however, at low light intensities, the mutant PR6303 exhibited doubling times that were significantly longer than those of the wild type, as previously observed for strains harboring mutations in either psae or ndhf alone (Schluchter et al., 1993; Zhao et al., 1993).

Figure 1. Fluorogram of Southern blot hybridization experiment to confirm construction of mutant strain PR6303. A, Total chromosomal DNAs were isolated from strain PR6000 (lanes 1 and 2) and strain PR6303 (lanes 3 and 4). DNAs were digested with EcoRI (lanes 1 and 3) and HindIII (lanes 2 and 4). The blot was probed with a 0.85-kb BamHI-BglII fragment encoding the psae and 3′ flanking sequences (Zhao et al., 1993). B, Total chromosomal DNAs were isolated from strain PR6000 (lanes 1 and 2) and strain PR6303 (lanes 3 and 4). DNAs were digested with EcoRI (lanes 1 and 3) and HindIII and EcoRI (lanes 2 and 4). The blot was probed with a 1.65-kb HindIII-HinclI fragment encoding most of the ndhf gene (Schluchter et al., 1993). The numbers to the left indicate the positions of HindIII fragments of bacteriophage λ used as size markers (in kb).

Table I. Physiological and biochemical parameters for Synechococcus sp. PCC 7002 strains PR6000 (wild type) and PR6303 (psae<sup>−</sup> ndhf<sup>−</sup>)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PR6000</th>
<th>PR6303</th>
</tr>
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<tr>
<td>Doubling times (h)</td>
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<td></td>
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<tr>
<td>1% CO₂, 280 μE m⁻² s⁻¹</td>
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<td>3.8</td>
</tr>
<tr>
<td>1% CO₂, 34 μE m⁻² s⁻¹</td>
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<td>26.4</td>
</tr>
<tr>
<td>+DCMU, +glycerol, 230 μE m⁻² s⁻¹</td>
<td>20.4</td>
<td>No growth</td>
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Biochemical parameters<sup>a</sup> |
| O₂ evolution (μmol of O₂ Chl⁻¹ h⁻¹) | 380 | 420 |
| O₂ uptake (μmol of O₂ Chl⁻¹ h⁻¹) | 12 | 5 |
| CHI/P700 | 126 ± 7<sup>b</sup> | 123 ± 8 |
| CHI/Cyt <sub>DD</sub> <sub>59</sub> | 232 ± 8 | 210 ± 10 |
| PSI/PSII | 1.80 | 1.71 |

<sup>a</sup>Values for cells grown under 1% CO₂ and 230 μE m⁻² s⁻¹.  
<sup>b</sup>Mean ± se.

Kinetics of P700 Oxidation in Wild-Type Synechococcus sp. PCC 7002

The light-induced oxidation of P700 in whole cells of wild-type Synechococcus sp. PCC 7002 is shown in Figure 2 (top). The upward-pointing arrow indicates the onset of actinic illumination; the downward-pointing arrow indicates the onset of darkness. Upon illumination following a 6-s dark period, the oxidation of P700 undergoes an oscillation before attaining a final, steady-state level. This oscillation is abolished by addition of either 10 μM DCMU (Fig. 2, bottom), 100 μM methyl viologen, or 5 μM DBMIB (data not shown). The effect of DCMU can be explained by its ability to prevent the PSI-generated electrons from reaching the Cyt b₆/f pool, thereby masking
Role of PsaE in Cyclic Electron Flow around PSI

Wild-type

OFF

No Addition

half-time: 41.6 ms

ON

OFF

10 μM DCMU

half-time: 485 ms

ON

1 s

Figure 2. Kinetics of P700 oxidation and reduction in whole cells of Synechococcus sp. PCC 7002. Top, Untreated wild-type cells; bottom, after addition of 10 μM DCMU. Fresh cyanobacterial cells were harvested by centrifugation at room temperature and resuspended in fresh growth medium (pH 8.2) at cell densities corresponding to 10 μg of Chl mL⁻¹. The shutter was opened for 1.5 s following a dark period of 6 s to permit full activation and recovery of the electron transport components. The signal-to-noise was improved by averaging 64 light-dark cycles. The P700⁺ reduction kinetics were fit to a single exponential (smooth line in +DCMU trace).

any rate limitation on the reducing side of PSI. The tacit assumption is that the NADH dehydrogenase is not able to supply electrons at a rate that would allow the accumulation of reduced plastoquinone (see below). Methyl viologen functions as a very efficient electron acceptor, preventing the oscillation by intercepting electrons from the reduced PSI acceptors. The plastoquinone antagonist DBMIB blocks all electron flow from reduced plastoquinone generated by PSII or the NADH dehydrogenase and, additionally, blocks cyclic electron flow around PSI.

Postillumination Kinetics of P700 in Wild-Type and Mutant Strains: No Inhibitors

The postillumination kinetics of P700⁺ reduction are more readily interpreted because the kinetics after 1.5 s depend only on the flow of electrons to P700⁺ through the Cyt b₆/f bottleneck and not on the outflow of electrons from the acceptor side of PSI. The P700⁺ reduction kinetics were found to be first order in the wild-type strain (Fig. 2) and all mutant strains of Synechococcus sp. PCC 7002. A single monophasic decay is consistent with (but not necessarily exclusively attributable to) the existence of one rate-limiting step in P700⁺ reduction. After illumination with white light, the postillumination reduction kinetics of P700⁺ in the wild-type and mutant strains PR6300, PR6302, and PR6303 of Synechococcus sp. PCC 7002 show similar half-times of 38 to 45 ms (Table II). The reduction kinetics of P700⁺ were remarkably constant and did not deviate more than 10% when determined at different times in the exponential growth phase of the culture. These kinetics corroborate the metabolic behavior of the phenotypes in vivo and show that PsaE is not required for PSI linear electron flow during high light photosynthesis. The relative insensitivity of P700⁺ reduction rates to the absence of NdhF and PsaE is only possible if the fluxes through the NADH dehydrogenase and through the PSI cyclic pathway are a small fraction of the PSII flux (see below).

P700⁺ Reduction in Wild-Type and Mutant Strains

Effect of DCMU

In the presence of DCMU, the P700⁺ reduction in wild-type Synechococcus sp. PCC 7002 shows a half-time of 485 ms, a rate nearly 12-fold slower than that of the control (Table II). The effect of DCMU was reversed when the cells were resuspended in fresh growth medium buffered with 10 mM bicarbonate/carbonate (pH 9.0) (data not shown). In the presence of DCMU, electrons available for P700⁺ reduction are derived predominantly from the NADH dehydrogenase and from cyclic electron flow around PSI. In the psaE mutant, the half-time of P700⁺ reduction increases to 589 ms in

<table>
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<tr>
<td>DBMIB, DCMU</td>
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* MV, Methyl viologen; e⁺, electrons.
**Effect of DBMIB**

DBMIB is a potent inhibitor of the Cyt b$_{6}$/f complex (Cramer et al., 1991) and would be expected to inhibit electron entry from PSI, from the respiratory NADH dehydrogenase complex, and (probably) from the cycle around PSI. As shown in Table I, the half-time for P700$^{+}$ reduction increases 27-fold from 41.6 to approximately 1110 ms in the wild-type strain PR6000; the psaE$^{-}$/PR6302, and ndhF$^{-}$/PR6303 strains of *Synechococcus* sp. PCC 7002. The further addition of glycerol to stimulate respiration (data not shown) or DCMU (Table II) to additionally block PSI in the *Synechococcus* sp. PCC 7002 strains has no effect. The very slow reduction rate of P700$^{+}$ in the presence of DBMIB may represent a physiologically insignificant "leak" due to the relatively high midpoint potential of P700$^{+}$ (E$_{m}$ = +430 mV) and the presence of an oxidizable donor in the lumen of the thylakoid. In summary, the data suggest that electrons from the cyclic electron transport pathway as well as from the NADH dehydrogenase enter the photosynthetic electron transport pathway between the inhibition points defined by DCMU (second electron accepting plastoquinone of PSI) and DBMIB (the Cyt b$_{6}$/f complex).

**Effect of CN$^{-}$**

To investigate further the relative activity of the respiratory electron transport chain, we measured the rate of P700$^{+}$ reduction after inhibiting the Cyt oxidase with KCN. Several important enzymes that affect the consumption of reducing power (Rubisco, nitrate reductase, nitrite reductase, sulfate reductase, etc.) may also be inhibited under these conditions. In the absence of added DCMU, the addition of CN$^{-}$ to the wild-type and mutant strains of *Synechococcus* sp. PCC 7002 did not have a noticeable effect on the rate of P700$^{+}$ reduction (data not shown). When Cyt oxidase is inhibited with CN$^{-}$ in the presence of DCMU, the half-time for P700$^{+}$ reduction decreased 3.2-fold to 153 ms. An acceleration in P700$^{+}$ reduction would be expected if there were an additional exit point for electrons between the Cyt b$_{6}$/f complex and P700$^{+}$. The addition of CN$^{-}$ to the DCMU-inhibited ndhF mutant resulted in a decrease in the half-time for P700$^{+}$ reduction to 485 ms. Under these conditions, electron entry from PSI and the NADH dehydrogenase complex are blocked, and electron exit to the Cyt oxidase is blocked, and the remaining source of electrons for P700$^{+}$ reduction will be a putative cycle around PSI. The ndhF psaE mutant PR6303 shows a half-time larger than either single mutant alone, 1138 ms, a result that is due to the DCMU-induced block in PSI and to lesions in both the PsaE-dependent cyclic pathway and the NADH dehydrogenase. Because the three major sources of electrons to P700$^{+}$ are blocked, the reduction kinetics are identical with those observed for the wild-type cells in the presence of DBMIB (Table II).

**Effect of Methyl Viologen**

A final test of the hypothesis that cyclic electron flow occurs when cyanobacteria are inhibited with DCMU and CN$^{-}$ would be to intercept the electrons from the acceptor side of PSI before their reentering the intersystem electron transport chain. The herbicide methyl viologen (paralquat) has the ability to accept electrons from this region of the electron acceptor chain. As shown in Table II, the addition of methyl viologen to wild-type *Synechococcus* sp. PCC 7002 cells in the presence of DCMU and CN$^{-}$ leads to a 1.5-fold increase in the half-time for P700$^{+}$ reduction from 153 to 249 ms. Methyl viologen has no effect on the paseE mutant, a result that can be explained by the absence of cyclic electron flow around PSI. This implies that the 250 ms half-time seen in the paseE mutant in the absence of methyl viologen is due only to the respiratory component, with no contribution by the cyclic pathway. In the presence of DCMU and CN$^{-}$, the addition of methyl viologen to strains PR6300 (ndhF$^{-}$) and PR6303 (psaE$^{-}$/ndhF$^{-}$) slows the rate of P700$^{+}$ reduction to DBMIB-like levels (see above). Under these conditions, electron flow through PSI (due to DCMU), the NADH dehydrogenase (due to the ndhF mutation), Cyt oxidase (due to CN$^{-}$), and the PSI cyclic system (due to methyl viologen and/or the paseE mutation) is completely blocked.

**DISCUSSION**

We investigated the function of the PsaE protein by measuring the growth rates and reduction kinetics of P700$^{+}$ in whole cells of the wild-type and three interposon mutants (psaE$^{-}$, ndhF$^{-}$, and psaE$^{-}$/ndhF$^{-}$) of *Synechococcus* sp. PCC 7002. All mutant strains failed to grow phototrophically in the presence of DCMU and a carbon source (glycerol), conditions under which the wild-type strain grows well (Lambert and Stevens, 1986). Under photautotrophic conditions at high light intensity, the mutants and wild type had nearly equal growth rates, but the doubling times of the mutants were significantly longer than that of the wild-type strain at lower light intensities (Schluchter et al., 1993; Zhao et al., 1993; Table I). Oxygen-uptake experiments indicated an inactive respiratory electron transport chain in the ndhF mutant
The physiological behavior of the psaE mutants in *Synechococcus* sp. PCC 7002 suggested that the defect might reside in the cyclic electron transport pathway around PSI (Zhao et al., 1993). The conditions under which cyclic electron flow around PSI could be detected included: (a) in comparison of the wild type and psaE mutant in the presence of DCMU, in which only the respiratory complex and the cyclic pathway are active, and (b) in comparison of the wild type and psaE mutant in the presence of DCMU and CN−, in which only the cyclic pathway is active. The best diagnostic assay appears to be analysis of P700+ reduction rates when the cyanobacteria are inhibited with DCMU and CN−. Under these conditions the further deceleration in the rate of P700+ reduction by methyl viologen indicates that the cycle around PSI is active. Addition of methyl viologen to the wild-type strain under these conditions results in a P700+ reduction rate that is identical with that for the psaE mutant in the absence of methyl viologen (Table II). In contrast, addition of methyl viologen to the ndhf mutant under these conditions (DCMU + KCN) results in a P700+ reduction rate that is similar to that for the wild-type strain in the presence of DBMIB (Table II). The difference in behavior of these two mutants under these conditions clearly demonstrates the occurrence of the cyclic electron transport pathway that depends on the presence of PsaE in the cells.

Under the illumination regimen used and in the presence of DCMU alone, only a small contribution from cyclic electron transport is observed in the ndhf mutant (1039 ms half-time in the presence of DCMU for the ndhf mutant compared to 1100 ms half-time for the psaE ndhf double mutant; Table II). There are probably two explanations for this result. First, in the presence of DCMU alone, there are many possible sinks for electrons on the acceptor side of PSI. In addition to its effects in inhibiting Cyt oxidase, CN− is an inhibitor of Rubisco, nitrite reductase, sulfite reductase, and possibly other metallo- or hemeproteins (Wishnick and Lane, 1969; Knaff and Hirasawa, 1991). Thus, in the presence of CN−, the pool of acceptors for electrons on the acceptor side of PSI becomes significantly smaller. Complete reduction of the pool would be expected to increase the flow of electrons through the cyclic pathway.

Second, the redox status of the plastoquinone pool at the end of the dark period is significantly different in the strains with a functional NADH dehydrogenase (wild type and the psaE mutant) as compared to those strains carrying the ndhf mutation. In the wild-type and psaE mutant strains, the plastoquinone pool would be expected to be largely reduced at the end of the dark period because of respiratory electron transport, whereas in the ndhf and the psaE ndhf mutant strains, the plastoquinone pool would be largely oxidized because of efflux of electrons from the pool to Cyt oxidase. If only a small number of electrons are available in the plastoquinone pool at the end of the dark period, there can only be a small number of electrons available for reduction of P700+ after the illumination period if cyclic electron transport is the only source of those electrons. Room temperature fluorescence induction measurements for the mutant and wild-type strains are consistent with this interpretation (J. Zhao and D.A. Bryant, unpublished data).

The reduction rates of P700+ in control, DCMU-poisoned, and methyl viologen-treated wild-type cells of *Synechococcus* sp. PCC 7002 were qualitatively similar to those observed in a variety of species by Maxwell and Biggins (1976, 1977). Although the absolute rates of P700+ reduction vary substantially from species to species, the pattern of behavior to inhibitors and electron acceptors was similar. As shown in the model (Fig. 3), the plastoquinone pool is reduced by the electrons from PSII, and this pathway can be inhibited selectively by the addition of DCMU. Electrons may also flow through the NADH dehydrogenase complex to plastoquinone, and this pathway can be eliminated by insertional inactivation of the ndhf gene. The third route by which electrons may return to P700+ involves a cycle around PSI and requires the participation of the psaE gene product in an as-yet unspecified manner. The very slow reduction of P700+ in the presence of DBMIB is considered a leak rate that is not physiologically significant.

In the wild-type and mutant strains of *Synechococcus* sp. PCC 7002, the P700+ reduction kinetics are first order under the conditions of inhibition shown in Table II. Given the appropriate mutants and inhibitors described above, it is possible to isolate each entry and exit point into the shared electron transport pathway. If the entry and exit points for electrons are independent of each other, then the sum of the individual rate constants should equal the rate constant for P700+ reduction in wild-type cells under control conditions. The simple differential equation

\[
\frac{d[P700^+]}{dt} = k - k_{\text{leak}}
\]

Figure 3. Model of electron flux leading to the reduction of P700+ in *Synechococcus* sp. PCC 7002. Plastoquinone may be reduced by electrons from PSII, and this pathway can be inhibited by addition of DCMU. Electrons may also flow through the NADH dehydrogenase to the level of plastoquinone, and this pathway is eliminated by a mutation in the ndhf gene. The third route by which electrons may return to P700+ requires the participation of the psaE gene product in an as-yet unspecified manner. The numbers refer to the relative fluxes through the various pathways expressed in percentage of total electron flow to P700+.Copyright © 1993 American Society of Plant Biologists. All rights reserved.
\[ \frac{dP700^+}{dt} = -\mu_T P700^+ \]

\[ \mu_T = (\mu_{PSII} + \mu_N + \mu_C + \mu_L - \mu_{on}) \]

describes the rate of \( P700^+ \) reduction as a function of time. The lumped rate constant \( \mu_T \) is made up of the following terms: \( \mu_{PSII} \) is the relative contribution from PSII, \( \mu_N \) is the relative contribution from the NADH dehydrogenase, \( \mu_C \) is the relative contribution from the cyclic electron pathway around PSI, \( \mu_L \) is the very small and probably nonphysiological contribution that is DBMIB insensitive, and \( \mu_{on} \) is the relative consumption rate constant for the Cyt oxidase. The terms \( \mu_{PSII} \) and \( \mu_{on} \) can be experimentally adjusted to zero by the addition of DCMU and KCN, respectively. Evaluation of the remaining terms can then be determined by considering the \( P700^+ \) reduction rate data for the appropriate mutant strains, because \( \mu_N \) is zero for the \( ndhF \) mutant, and \( \mu_C \) is zero for the \( psaE \) mutant.

To determine the relative contribution of cyclic electron flow to total flow under the conditions described, we first calculated the relative ratio of electron flux to \( P700^+ \) through the NADH and the cyclic pathways under conditions in which PSII was inhibited with DCMU and the Cyt oxidase was inhibited with KCN. Solving the above expression led to the conclusion that 57% of the electron flux is derived from the dehydrogenase and 30% is derived from the cyclic pathway, leaving 14% that is due to the nonspecific leak. If we assume that the ratios of these fluxes do not change in proportion when PSII is operating (a 1:12 rate change), then, under the experimental conditions provided, the flux from PSII represents 92% of the electrons that lead to the reduction of \( P700^+ \). The cyclic electron transport system around PSI contributes approximately 2.6% of the electron flux, and the NADH dehydrogenase contributes about 5%. The latter contribution is somewhat variable and is a function of the duration of time that the cells are incubated in the dark and also on the amount of an added metabolizable carbon source (glycerol; data not shown).

Given no suppression by normal photosynthetic function, the presumably nonphysiological leak represents approximately 1% of the total flux to \( P700^+ \). These calculations show that the capacity of cyclic electron transport around PSI is small and would be barely noticeable under normal physiological conditions in which PSII supplies the great majority of the electrons. Myers (1987) and Maxwell and Biggins (1976) reached the similar conclusion that cyclic electron transport is quantitatively small relative to noncyclic electron transport at saturating light intensity. However, the growth rate data for the mutants (Schuchter et al., 1983; Zhao et al., 1993; Table I) indicate that both cyclic electron transport and respiratory electron transport via the NADH dehydrogenase are extremely important at low light intensity. The quantitative calculations indicate that there are only three major paths by which electrons are transferred to the plastoquinone pool and/or the Cyt \( b_6/f \) complex in \( Synechococcus \) sp. PCC 7002 (Fig. 3). The major uncertainty is the precise route by which electrons are cycled back into the electron transport chain and the identities of the components involved.

Mi et al. (1992a, 1992b) have used an approach similar to that reported here to study \( Synechococcus \) sp. PCC 7002, \( Synechocystis \) sp. PCC 6803, and interposon mutants of the latter organism that are defective for several \( ndh \) genes. These workers proposed that the NAD(P)H dehydrogenase is the reentry point for electrons in PSI-mediated cyclic electron transport. This proposal has some attractive features, but it also has some serious limitations. The major products of photosynthetic electron transport are reduced \( Fd \) and NADPH, not NADH. Although the substrate specificity of the cyanobacterial NADH dehydrogenase has not yet been carefully studied (Berger et al., 1991), bacterial NADH dehydrogenases of both the type 1 and type 2 classes have little or no activity with NADPH as electron donor (Yagi, 1991). If this were also true for cyanobacteria, a transhydrogenase activity would be required for this complex to function in cyclic electron transport. Little is known about this enzyme in cyanobacteria, and in the only report that we could identify, only marginal transhydrogenase activity was detected (Leach and Carr, 1970). Transhydrogenase activity could not be detected in \( Synechococcus \) sp. PCC 7002 using the acetylpyridine adenine dinucleotide assay (Leaver et al., 1991; J. Zhao and D.A. Bryant, unpublished results).

It is possible that the cyclic pathway in cyanobacteria involves the participation of \( Fd \), because the pathway is inhibited by methyl viologen. Whether electrons return to the Cyt \( b_6/f \) complex directly or to the plastoquinone pool is not known. Many bacteria are known to possess two different types of NADH:ubiquinone oxidoreductases (Wynn et al., 1989). Evidence for the existence of a type 1 dehydrogenase in cyanobacteria has been obtained through the cloning and sequencing of genes with strong sequence similarity to subunits of the mitochondrial and bacterial NADH dehydrogenase (Ogawa, 1991; Ellersiek and Steinmüller, 1992; Ogawa, 1992; Walker, 1992; Schluchter et al., 1993). Recently, this complex was partially purified from \( Synechocystis \) sp. PCC 6803 (Berger et al., 1991). An NADH dehydrogenase with properties similar to those of type 2 enzymes in bacteria was purified and characterized for \( Anabaena variabilis \) (Alpes et al., 1989). However, such an enzyme seems unlikely to provide a major source of electrons for \( P700^+ \) reduction in \( Synechococcus \) sp. PCC 7002, because strain PR6303 in the presence of DCMU exhibits \( P700^+ \) reduction kinetics similar to those of the wild-type strain in the presence of DBMIB. An alternative possibility is that such a type II enzyme is somehow involved in the PsaE-dependent cyclic electron transport pathway. In spite of the uncertainties of the components involved, the experimental system described here clearly establishes the existence of cyclic electron transport in \( Synechococcus \) sp. PCC 7002 and identifies a novel component, PsaE, required for electron transport via this pathway.

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