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Expression of Anabaena PCC 7937 Plastocyanin in Synechococcus PCC 7942 Enhances Photosynthetic Electron Transfer and Alters the Electron Distribution between Photosystem I and Cytochrome-c Oxidase*

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The petE gene encoding plastocyanin precursor protein from the cyanobacterium Anabaena PCC 7937 was introduced in the cyanobacterial host strain Synechococcus PCC 7942. The host normally only uses cytochrome c553 as Photosystem I (PS I) donor. The heterologous gene was efficiently expressed using the inducible Escherichia coli lac promoter. Accumulation of plastocyanin protein depended on the presence of Cu++. The protein was accurately targeted to the thylakoid lumen, from which it could be isolated in the mature form. Redox difference spectroscopy proved the presence of a Cu++ ion in the holoenzyme. Isolated heterologous plastocyanin was functional in reconstitution of in vitro electron transfer to PS I. The presence of Anabaena plastocyanin in Synechococcus thylakoid membranes increased PS I electron transfer rate 2.5 times. Analysis of P700 redox and PS II fluorescence transients in vitro showed a faster electron transfer through PS I because of enhanced electron supply in the presence of plastocyanin. In addition, the distribution of electrons between photosynthetic and respiratory electron transfer changed. Plastocyanin preferentially donates electrons to PS I rather than to the respiratory cytochrome-c oxidase complex and is not functionally equivalent to cytochrome c553.

Plastocyanin (PC)1 is a small (97–105 amino acids) blue colored protein with a reversibly oxidizable copper atom in its catalytic site (1). It is active in the thylakoid lumen as a soluble mobile electron carrier in oxygenic photosynthesis between two complexes embedded in the thylakoid membrane, the cyt b₆/cyt f complex and (P700 in) the PS I reaction center (1, 2). PC is synthesized in the cytoplasm as a precursor protein with an N-terminal extension that contains the information needed for its routing to the thylakoid lumen. The extension is cleaved off by a specific peptidase during the transport across the thylakoid membrane (3–5). It remains at question whether a part of the PC pool becomes tightly bound to the PS I reaction center or whether the apparent associations are only weak (6).

PC occurs in plants, green algae and cyanobacteria. Algae and cyanobacteria that do not contain PC use an iron heme group containing, soluble c-type cyt (c553 or c555) instead (7). A third class of organisms contains the genetic information for both proteins. In these organisms, the presence of copper in the growth medium is the factor that determines which gene is utilized (8–12). In the green alga Chlamydomonas reinhardtii, petE mRNA encoding PC is constitutively expressed, but copper supply is needed to support the accumulation of PC because of the rapid degradation of the apoprotein (13). In the cyanobacterium Anabaena PCC 7937 and Synechocystis PCC 6803, expression of the petE gene is regulated by copper at the level of transcription initiation (11, 12). In all of these organisms, copper-limitation induces expression of cyt mRNA encoding cyt c553, and the cells then switch to the use of cyt c553 as an alternate electron carrier (9, 11, 12, 14, 15).

c-type cyt electron carriers are present in respiratory chains in all organisms, as well as in the photosynthetic chain in all photosynthetic prokaryotes and some eukaryotic algae (14). Interaction of the photosynthetic and respiratory electron transfer chains in these organisms is clearly established, and in the prokaryotic organisms, cyt c553 has a clear role in this interaction (16–19). Participation of PC in respiratory electron transfer in these organisms has been demonstrated only in vitro (19,20). Plants and most green algae have completely replaced the c-type cyt in the photosynthetic chain by the more recent copper protein PC (7, 14). The reason for this apparent specialization is unknown, but might be related to the structure and the reaction mechanisms of both proteins. Gene expression conditions and protein structures of PC and cyt c553 are different, but their routing to the thylakoid lumen, physical properties (solubility, size, and redox potential), and physiological function are equivalent (21, 22).

In higher plants and green algae, PC and cyt c553 are acidic, but in cyanobacteria, the IEP of both PC and cyt c553 ranges from 3.8 in unicellular strains to 9.5 in the filamentous species (23). This variance is striking, charge is important in the interaction of PC with PS I (24–26), and most PS I proteins are highly conserved (27, 28). When isolated from the same organism, the IEP of PC and cyt c553 is always similar (29). This linked variation could be the result of parallel evolution of these proteins in response to a change in a common reaction partner (9, 23, 26). For the interaction of (acidic) PC with its
redox partners, especially the negative charges present in the characteristic negative patch of this protein are thought to be important (24-26). In the communication between PC (or cyt c<sub>553</sub>) and P700, the 18-kDa psaF gene product has been suggested to be involved (25, 29-32). It likely enhances the interaction of the negatively charged PC with the negative charges present on P700 with its positively charged residues (25). How this interaction operates in the case of basic PC (or cyt c<sub>553</sub>) is unknown (33).

We judged that introduction into and subsequent expression of a petE gene in a cyanobacterium, which otherwise only contains the cytA gene encoding cyt c<sub>553</sub> as PS I donor, could be instrumental in answering questions on the efficiency and physiological function of both proteins in photosynthesis and respiration.

Although the presence of PC in the unicellular strain Synechococcus PCC 7942 has been suggested on the basis of electron paramagnetic resonance signals (34), PC has never been detected by optical spectroscopy (35), heterologous gene hybridization (4), or immunological methods (36). The cytA gene (encoding an acidic cyt c<sub>553</sub>) in this strain is constitutively expressed independent of the presence of copper (37). It is therefore assumed that this strain belongs to the group of cyanobacteria in which cyt c<sub>553</sub> is the only electron carrier capable of reducing P700<sup>+</sup> (10, 35). Inactivation of the cytA gene in this strain resulted in decreased cyt f oxidation, pointing to a role of cyt c<sub>553</sub> in the reduction of the respiratory cyt-c oxidase (37). Synechococcus sp. PCC 7942 is well suited as a recipient host, the strain is physiologically and genetically well characterized and is easily transformable. An integration platform for homologous recombination of newly introduced genes has been described (39), and a system for inducible expression of these genes using the Escherichia coli trc promoter and lac<sub>PL</sub> repressor (40) is available. The PC protein we chose to study was the one encoded by the petE gene from the filamentous strain Anabaena sp. PCC 7937 (4). Isolation, purification, and properties of the basic PC protein (IEP = 8.4) has been reported, and its activity in vitro photosynthetic and respiratory electron transfer in Anabaena has been established (20, 23, 41).

This study presents properties of the transgenic, Anabaena PCC 7937 PC containing Synechococcus PCC 7942 strain. The heterologous PC was efficiently expressed, and participated in electron transfer in addition to the endogenous cyt c<sub>553</sub>. The presence of PC caused a larger electron transfer capacity of PS I and altered electron distribution between PS I and cyt-c oxidase.

**EXPERIMENTAL PROCEDURES**

**Materials—**Enzymes for DNA manipulations were purchased from Pharmacia (Uppsala, Sweden); radiolabeled nucleotides and Hybond-N membranes from Amersham Corp. Chemicals used to prepare BG11 medium were from Merck (Darmstadt, Germany). Plant agar was from Boehringer Mannheim. Immobilon<sup>TM</sup> polyvinylidene difluoride membrane was obtained from Millipore Corp. (Bedford, MA, and the Protoblot<sup>®</sup> Western blot system was from Promega (Madison, WI). Equipment and chemicals for SDS-polyacrylamide gel electrophoresis experiments were from Bio-Rad; the densitometer used was the Pharmacia LKB 2400 UltraScan XL with Version 2.0 software, and apparatus and software used in the chlorophyll fluorescence measurements was from Walz (Effeltrich, Germany).

**Strains and Culture Conditions—**E. coli PC2495 (4) was grown as in (42). The cyanobacterial strains Anabaena PCC 7937 (Anabaena variabilis ATCC 28413) and Synechococcus PCC 7942 (Anacystis nidulans R2-SpC) R2-PIM9 (4, 39) were grown in BG11 liquid medium (38) or on BG11 plates containing 0.8% plant agar (39). R2-PIM9 and derivatives were grown in BG11 medium supplemented with 30 µM Na<sub>2</sub>SO<sub>3</sub> and plant agar with 0.8% as above. Cultures for the isolation of PC protein were grown in flasks under vigorous aeration with air at room temperature. Other cultures (60 ml) were grown in 300-ml flasks at 30 °C under continuous fluorescent white light (20–35 µmol m<sup>-2</sup> s<sup>-1</sup>) and shaking at 100 rpm. Copper-free BG11 medium (standard BG11 contains 0.3 µM CuSO<sub>4</sub>) was prepared using Au <sup>+</sup> cobalt trace element solution (38) without CuSO<sub>4</sub>. Glassware was used with rinsed with 6 N HCl to remove traces of copper (9). Copper-free copper was depleted from copper by growing 1.2 × 10<sup>11</sup> cells g<sup>-1</sup> with copper-free BG11. Medium was supplemented with copper by the addition of CuSO<sub>4</sub> to 0.5 µM.

**Cloning Procedures and Plasmids—**DNA restriction fragment purification was according to Ref. 43, and other DNA manipulations were as in Refs. 4 and 42. Construction of petE gene by insertion of an aad gene encoding streptomycin resistance into the E. coli petE gene (4) was performed by homologous recombination at the bla and ori sequences present in both the plasmid and the platform, resulting in restoration of the bla gene (encoding ampicillin resistance), and replacement of the neo gene (encoding kanamycin resistance) in the platform by the DNA insert of the donor plasmid. Plasmids pTrc99S, pMG77B, and pMG95B were transformed into Synechococcus R2-Trc99S, -MG77B, and -MG95B, respectively. For each construct, several independent clones were isolated and used in the experiments.

**Antisera—**Polyclonal antiserum 8806 against Anabaena PCC 7937 PC protein was prepared by immunizing rabbits (New Zealand White) with 300 µg of synthetic peptide 87–109 (representing the first 20 amino acids of mature Anabaena PC protein) in Freund’s complete adjuvant followed by four booster injections in Freund’s incomplete adjuvant on days 14, 29, 42, and 56. Rabbit sera were obtained from day 89.0. Antiserum 8905 against Microcystis aeruginosa PCC 7005 cyt <sup>+</sup>C<sub>553</sub> protein was prepared as above but with purified cyt <sup>+</sup>C<sub>553</sub> using one immunizing and two booster injections of 200 µg of protein each.

**RNA Isolation and Northern Blot Analysis—**Total RNA was isolated from Synechococcus or Anabaena cells as described (11). Aliquots (10 µg) of total RNA were denatured with formaldehyde-formamide and separated on 1.2% formaldehyde-agarose gels. RNA was visualized with an acidine orange and blotted onto Hybond-N nylon filter as described (30). The 400-base pair HindIII-NheI DNA fragment from pPCV6 covering most of the Anabaena petE gene coding region (4) was radiolabeled with the random primer method (45) and used as a probe for petE mRNA as described (11).

**Protein Isolation, SDS-Polyacrylamide Gel Electrophoresis, and Western Blot Analysis—**Anabaena and Synechococcus cultures were grown to approximately 15 µg of chlorophyll a (60 µg of protein, 1 × 10<sup>11</sup> cells/ml) of culture and when necessary induced with IPTG (0.5 mM during the last 16 h of growth). Chlorophyll a concentration was estimated as described previously (46). Cells were harvested by centrifugation for 10 min at 4,000 × g (room temperature) and washed in 10 mM Tris-HCl (pH 8.0), and resuspended in a final volume of 0.6 ml. Protein concentration was determined according to (47) after the addition of SDS to 1% and boiling for 5 min using bovine serum albumin (also in the presence of SDS) as a standard. Aliquots were boiled in sample buffer and separated by electrophoresis in a 15% SDS-polyacrylamide gel electrophoresis system (48, 49). For Western blot analysis, gels were blotted by electrophoresis at 80 V for 90 min onto polyvinylidene difluoride membrane in 25 mM Tris base, 200 mM glycine, 0.02% (w/v) SDS, and 20% methanol. Blots were probed for immunoreactive PC or cyt <sup>+</sup>C<sub>553</sub>, protein with specific antisera in Tris-buffered saline containing 0.1% Tween 20, 2% bovine serum albumin (w/v), and 5% goat serum. Washes were 15 min in Tris-buffered saline containing 0.1% Tween 20. Antiserum and antibodies were visualized using the Promega Protoblot<sup>®</sup> system as suggested by the manufacturers. PC and cyt <sup>+</sup>C<sub>553</sub> contents of protein fractions were determined by comparing the intensity of bands on Western blot with that of serial dilutions of extracts of wild-type Anabaena cells for PC and of Synechococcus cells for cyt <sup>+</sup>C<sub>553</sub> using a densitometer, and checked with redox difference spectroscopy (see below). Normal or cyt <sup>+</sup>C<sub>553</sub> content was set at 3 nmol·µmol chlorophyll a<sup>-1</sup> (10).
PC Isolation—Synechococcus cultures were grown to 15 μg of chlorophyll a·ml⁻¹. R2-MG77B was induced with IPTG as above. Cells were harvested with a tangential filter device (Filtron), washed in buffer A (300 mM d-(−)-mannitol, 20 mM sodium-potassium phosphate, 10 mM MgCl₂, and 50 mM Tricine-NaOH (final pH, 7.8)), and pelleted by centrifugation as above. The rest of the purification procedure was performed at 4°C. Cells were resuspended at a concentration of 1 mg of chlorophyll a·ml⁻¹ in buffer A (10 mM L-ascorbic acid and subse-

quent reoxidized with 2 mM potassium ferricyanide. Spectra were recorded at room temperature on an Aminco DW 2000 spectrophotometer (SLM-Aminco, Urbana, IL). The spectra represented are an average of six repetitive scans.

In vitro PS I Electron Transfer Rate—Anabaena and Synechococcus cultures were grown, induced with IPTG, harvested by centrifugation as above, and resuspended in buffer A (10 mM L-ascorbic acid and subsequently reoxidized with 2 mM potassium ferricyanide. Spectra were recorded at room temperature on an Aminco DW 2000 spectrophotometer (SLM-Aminco, Urbana, IL). The spectra represented are an average of six repetitive scans.

Results

Expression of the Anabaena petE Gene in Synechococcus—

The petE gene encoding PC precursor protein from Anabaena PCC 7937 (4) was cloned into the E. coli pTrc99S expression vector, resulting in plasmids pMG77B and pMG95B. The pTrc99S vector (as a negative control) and the petE expression plasmids pMG77B and pMG95B were transferred to the cyanobacterial Synechococcus PCC 7942 R2-PIM9 recipient strain (39). The recombinant clones obtained were called Synechococcus R2-Trc99S, -MG77B, and -MG95B. Fig. 1 shows the genetic content of the inserts in the platform of the transgenic Synechococcus strains. Expression of the Anabaena petE gene in R2-MG77B and -MG95B resulted in the accumulation of PC protein. In R2-MG77B petE expression depended on the presence of IPTG, in R2-MG95B expression was constitutive. The protein was of the correct (10 kDa), mature size when compared with PC protein isolated from Anabaena cells (not shown).

To investigate the role of Cu²⁺ ions in the formation of PC protein in Synechococcus, R2-Trc99S, -MG77B, and -MG95B cells were grown under copper-deficient conditions, diluted 1:100, and grown to 15 μg of chlorophyll a·ml⁻¹ in the absence or presence of Cu²⁺ and IPTG. Cells were harvested, total RNA and protein were isolated, and Northern and Western blot analysis were performed (Fig. 2, A and B). In control experiments with Anabaena, the presence of Cu²⁺ in the medium results in a substantial increase in mRNA level (Fig. 2A, lane 2 versus lane 1), showing the copper-regulated transcriptional activation of the petE promoter (11). In Synechococcus, the amount of petE mRNA was hardly influenced by the presence of Cu²⁺ due to the copper-independent activity of the trc promoter. In R2-MG77B cells, petE mRNA is only present after IPTG induction (lanes 6 and 8 versus lanes 5 and 7); in R2-MG95B cells, petE mRNA is made constitutively (lanes 9 and 10). No petE mRNA was detected when the petE gene was not present in the platform (lanes 3 and 4). Fig. 2B shows the corresponding Western blot. In Anabaena, no PC protein could be detected in the absence of Cu²⁺ (lane 1 versus lane 2). In Synechococcus, even with ample amounts of petE mRNA present in the cell (lanes 6, and 8–10), accumulation of PC protein to a level similar to that in Anabaena cells (lane 2) only occurred when Cu²⁺ was present (lanes 8 and 10). This suggests that the PC protein accumulated in the cell is in the holo-form and contains a Cu²⁺ cofactor. Next, the PC protein was isolated from cells of the transgenic
Synechococcus clones to demonstrate the presence of a Cu\textsuperscript{2+}-center.

Purification and Redox Difference Spectroscopy of PC Protein—PC protein was isolated from cultures of Synechococcus R2-Trc99S (as a negative control), -MG77B (induced with IPTG), and -MG95B by extraction of isolated thylakoids with the nonionic detergent CHAPS, which releases PC from the thylakoid lumen (49, 50). The PC protein was partially purified by ammonium sulfate precipitation and found in the 70–100% fraction in agreement with published data on the purification of the PC protein from Anabaena cells (41). Purification of PC protein from the thylakoids was about 90-fold (data not shown). Western blots of the 70–100% fractions of the three strains showed the absence of PC protein in extracts of R2-Trc99S and its presence in -M77B and -MG95B (Fig. 3A). Otherwise, the 70–100% fractions of the three strains showed similar banding patterns on gels stained with Coomassie Brilliant Blue (not shown). Notably, cyt c\textsubscript{553} was present in equal amounts in the 70–100% fractions of all three strains (Fig. 3B). It was purified about 60-fold. The 70–100% fractions were used for redox difference spectroscopy. Characteristic spectra were obtained for both PC and cyt c\textsubscript{553}, i.e. with the typical broad absorbance peak of holo-PC at 597 nm (Fig. 3C) (6, 12, 41) and the peaks for holo-cyt c\textsubscript{553} at 524 and 555 nm (Fig. 3D) (12, 22). These data prove that the transgenic PC protein is present in the holoform, with a Cu\textsuperscript{2+} ion mounted in its catalytic site. To reveal possible physiological activity of the heterologous PC protein, the 70–100% fractions were used for \textit{in vitro} studies of electron transfer via PS I.

\textit{In Vitro PS I Electron Transfer—}Aliquots of the various 70–100% fractions (50 \(\mu\)g of protein, reduced with ascorbate) were added as electron donors to osmotically shocked, washed \textit{Synechococcus} R2-Trc99S thylakoid membranes. The relative initial rates of \textit{in vitro} photosynthetic electron transfer were measured. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea was present to inhibit electron flow between PS II and PS I, and KCN was present to inhibit electron drain via cytochrome-c oxidase. This way, differences in electron donation to PS I could be assigned to the PC content of the various 70–100% fractions. Protein fractions from \textit{Synechococcus} strains R2-MG77B and -MG95B clearly reconstitute light-dependent \textit{in vitro} PS I activity in wild-type thylakoid membranes more efficiently than the protein fraction from R2-Trc99S, which contains no PC (Table I). Differences in the enhancement of the PS I rates cannot be due to the involvement of variable contents of \textit{Synechococcus} cyt c\textsubscript{553} as donor to PS I, as this protein is present in equal amounts in all extracts used (see Table I). The results indicate that the heterologous \textit{Anabaena} PC can participate in photosynthetic electron transfer in \textit{Synechococcus}. We then investigated whether the PC protein as it is present in transgenic \textit{Synechococcus} thylakoid membranes could lead to a higher PS I rate using reduced DCCIP as artificial electron donor (Table II). Thylakoid membranes containing PC protein (induced R2-MG77B and R2-MG95B) showed up to 2.5-fold higher initial PS I rates than membranes in which no PC protein was present (R2-Trc99S and noninduced R2-MG77B). The rates measured depended on the amount of PC present in the membranes. R2-MG95B membranes even reached initial PS I rates also -95B membranes ever reached initial PS I rates also obtained with \textit{Anabaena} membranes. The PC present in the isolated osmotically shocked thylakoids may constitute a membrane-associated pool, reflecting the occurrence of PC in both bound and soluble forms (6). Western blots of the thylakoid membrane preparations used in this experiment (Fig. 4) clearly show that the membranes differ in PC content (Fig. 4A) but that all have a normal wild-type level cyt c\textsubscript{553} (Fig. 4B). There are at least 4 immunoresponsive cyt c bands. The lowest band represents cyt c\textsubscript{553} given its apparent molecular mass of about 12 kDa and its disappearance upon inactivation of the cyt c\textsubscript{553} gene (37). Furthermore, cyt c\textsubscript{553} was retained in the 70–100%...
The PC and cyt c553 contents represent final concentration. The PC concentration was as indicated, the cyt c553 concentration was 1.3 ± 0.3 μmol mg chlorophyll a⁻¹.

<table>
<thead>
<tr>
<th>Protein extract (50 μg)</th>
<th>[PC] (μmol)</th>
<th>PS I rate (nmol O₂ min⁻¹ mg chlorophyll a⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>40 ± 2 (100)</td>
</tr>
<tr>
<td>R2-Trc99S</td>
<td>&lt;0.01</td>
<td>52 ± 3 (130)</td>
</tr>
<tr>
<td>R2-MG77B + IPTG</td>
<td>0.16 ± 0.02</td>
<td>69 ± 4 (173)</td>
</tr>
<tr>
<td>R2-MG95B</td>
<td>0.69 ± 0.05</td>
<td>128 ± 7 (320)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain</th>
<th>nm</th>
<th>PS I rate (nmol O₂ min⁻¹ mg chlorophyll a⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R2-Trc99S</td>
<td>&lt;1</td>
<td>198 ± 19 (100)</td>
</tr>
<tr>
<td>R2-MG77B</td>
<td>&lt;1</td>
<td>234 ± 34 (138)</td>
</tr>
<tr>
<td>R2-MG77B + IPTG</td>
<td>7 ± 1</td>
<td>420 ± 43 (212)</td>
</tr>
<tr>
<td>R2-MG95B</td>
<td>60 ± 17</td>
<td>491 ± 19 (248)</td>
</tr>
<tr>
<td>Anabaena PCC7937</td>
<td>63 ± 16</td>
<td>481 ± 41 (243)</td>
</tr>
</tbody>
</table>

**FIG. 4.** Western blot of the transgenic Synechococcus strains used for *in vitro* and *in vivo* PS I measurements. Western blots were prepared with 10-μg aliquots of the thylakoid membrane preparations used in Table II. Two independent clones of each recombinant Synechococcus strain were tested. A, blot probed with an anti-Anabaena PC antibody; lanes 1 and 2, R2-Trc99S; lanes 3 and 4, R2-MG77B (not induced); lanes 5 and 6, R2-MG77B (induced); lanes 7 and 8, R2-MG95B. B, as in A, probed with an anti-cyt c553 antibody.

Fraction separated from the other immunoreactive bands (Fig. 3B) and displaying the typical cyt c553 redox difference spectrum (Fig. 3D). The main conclusion from the PS I rate measurements in Tables I and II is that in the transgenic Synechococcus strains, the Anabaena PC protein can clearly speed up electron donation into the P700⁺ reaction center of PS I in the presence of normal amounts of cyt c553, the authentic PS I donor.

**FIG. 5.** P700 redox transient measurements. Culture samples of the transgenic Synechococcus strains were pre-treated with 20 s of strong far-red light (19 W m⁻²), after which a pulse of white light was given in addition to the ongoing excitation with far-red light. P700 redox changes were monitored as absorbance changes at 820 nm. The duration of the white light pulse was 5 s. For reasons of presentation, only 1.5 s of the maximal oxidized state plateau in white light is shown. After switching off of the white light, a fast decline of the P700⁺ signal by re-reduction was recorded at a time base of 1 ms, the subsequent slow reoxidation was recorded at 3 ms. The break in the time axis has been indicated in the traces (1). Time axis is for *trace A* only. *trace A* shows after white light on; *trace B* and *trace C* show the fast decline of the P700⁺ signal by re-reduction and the subsequent slow reoxidation. The P700⁺ signal is relatively low. Next, an additional strong pulse of actinic white light was given for 5 s. This results in an increase of the P700⁺ signal due to the use of the full PS I-antenna complement. After switch off of the white light, P700⁺ absorbance decreases to the lower far-red level. The kinetics of these transients report on the electron supply rates to PS I in which PC and cyt c553 are involved. Electrons resulting from the water-splitting activity of PS I that are temporarily stored in the chain of electron carriers between PS I and PQ pool, re-reduce P700⁺. This is reflected in the fast drop of the P700⁺ signal after the white light pulse. After depletion of the electrons stored between PS I and P700⁺ reoxidation proceeds (the far-red background light remains on). The balance between influx from and influx of electrons into P700⁺ determines the rate of reoxidation. The influx depends on the content of the soluble PS I electron donors cyt c553 and (when present) PC. Table III summarizes the rates of fast P700⁺ reduction and slow P700⁺ reoxidation. The transient P700⁺ decline is faster when PC protein is present, in Synechococcus PCC 7942. In the test system used here (Table II), saturating amounts of artificial electron donor and acceptor, warrant the highest possible PS I rate. Addition of the proton gradient uncoupler NH₄Cl to 10 mM did not result in a further increase of the electron transfer rate (not shown). The *in vitro* data on the stimulating role of PC in electron transfer capacity in Synechococcus do not necessarily reflect the *in vivo* regulation of electron transfer. To extend our observations to intact cells of the Synechococcus strains, we studied light flash-induced redox transients of P700⁺ as a function of the presence of PC as electron donor.
Heterologous PC Changes Electron Transfer in Synechococcus

The methods used were according to Fig. 5. Rates are expressed in mV s⁻¹. Measurements were in triplicate, except for R2-MG77B. Numbers in brackets indicate the relative differences in percent.

<table>
<thead>
<tr>
<th>Strain</th>
<th>P700⁺ reduction rate</th>
<th>Effect</th>
<th>P700 reoxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-KCN</td>
<td>+KCN</td>
<td>-KCN</td>
</tr>
<tr>
<td>R2-Tcr99S</td>
<td>39.9 ± 1.3 (100)</td>
<td>42.2 ± 0.7</td>
<td>5.8 ± 2.5</td>
</tr>
<tr>
<td>R2-MG77B</td>
<td>51.3 (129)</td>
<td>54.3</td>
<td>5.8</td>
</tr>
<tr>
<td>R2-MG95B</td>
<td>66.2 ± 2.8 (166)</td>
<td>68.9 ± 1.2</td>
<td>4.1 ± 1.2</td>
</tr>
</tbody>
</table>

![Table III](image)

Relative P700 absorbance change upon exposure of dark adapted cells to far-red illumination, effects of KCN

Culture samples of recombinant Synechococcus strains were preincubated in darkness during 5 min. R2-MG77B was induced as described. After exposure to far-red light (19 watts⁻¹) P700 was partially oxidized, and the P700⁺ signal peak height was monitored during 20 s. The absorbance decrease between the original dark and the far-red induced one was determined. Peak heights are expressed in mV. Measurements were in triplicate, except for R2-MG77B. Numbers in brackets indicate the relative differences in percent.

<table>
<thead>
<tr>
<th>Strain</th>
<th>P700 absorbance change</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-KCN</td>
<td>+KCN</td>
</tr>
<tr>
<td>R2-Tcr99S</td>
<td>0.15 ± 0.04 (100)</td>
<td>0.21 ± 0.04 (100)</td>
</tr>
<tr>
<td>R2-MG77B</td>
<td>0.14 (93)</td>
<td>0.16 (100)</td>
</tr>
<tr>
<td>R2-MG95B</td>
<td>0.08 ± 0.015 (53)</td>
<td>0.08 ± 0.012 (38)</td>
</tr>
</tbody>
</table>

![Table IV](image)

**DISCUSSION**

The petE gene from Anabaena PCC 7937 was efficiently expressed in Synechococcus PCC 7942 R2-PiMS using the E. coli trc promoter. Uncoupling the petE gene from its own promoter, which has a response to copper on the level of transcription initiation (11), gave the opportunity to study the involvement of copper in the biosynthesis of PC in a cyanobacterium at the protein level. Whereas transcription initiation no longer depended on the presence of Cu²⁺-ions, the stability of halo-PC protein did, as earlier documented for the green alga Chlamydomonas reinhardtii (13) and Scenedesmus obliquus (22). The heterologously expressed PC precursor protein was properly processed and targeted; it could be isolated from the thylakoid lumen as a mature sized protein with the Cu²⁺ cofactor inserted. Its redox difference spectrum was identical to spectra of PC proteins isolated from their original hosts, Anabaena PCC 7937 (41) and Synechocystis PCC 6803 (12).

Introduction of PC enhanced the PS I electron transfer rate, both in isolated thylakoid membranes and in whole cells of Synechococcus. Reconstitution of in vitro PS I transfer using Anabaena PC had earlier been shown with Anabaena thylakoid membranes (20). Our results indicate that electron donation by PC to PS I is functionally similar in Anabaena and (transgenic) Synechococcus, suggesting an analogous docking of the basic Anabaena PC on the PS I complex in both strains. This is striking, because the IEP of the endogenous PS I electron demonstrating that heterologous PC participates in electron donation to P700 in vivo. The decline rate measured correlates with the amount of PC in the cell (compare Tables II and III).

If PC presence would only result in a larger electron transfer capacity, one would expect that reoxidation of P700⁺ has a similar rate in all three strains since during this stage the PQ pool is only partially filled (see below). In this case, the resulting diminished electron flux would engage only few electron carriers. Table III shows that the rates are not equal; in strains containing PC, reoxidation is slower. The more PC is present, the slower the reoxidation becomes. Apparently conditions of low electron flux favor electron transfer by PC to PS I. To test whether this could be related to the fact that photosynthetic and respiratory electron transfer pathways interact in wild-type Synechococcus PCC 7942 cells by common use of cyt c,₅₅₃ in both routes (16, 19, 37), the effects of inhibition of the respiratory cyt c oxidase complex on the P700⁺ reoxidation and on its reoxidation were studied. The rate of reduction did not change substantially in the presence of the respiratory inhibitor KCN in all strains studied (Table III). The small differences (4–5%) represent the flux of electrons normally passing into the respiratory cyt c oxidase complex. The equal effects of KCN treatment correlate most probably with the identical amounts of cyt c,₅₅₃ present in all strains (Fig. 4B). It appears that PC is confined to electron donation into PS I and does not contribute electrons to cyt-c oxidase. From this it is concluded, that these two electron donors are nonequivalent. The effects of KCN on P700 reduction are rather low, which shows that competing electron passage via cyt-c oxidase is limited under conditions of low electron flux favoring electron transfer by PC to PS I (Fig. 6). Further changes are effected by state transitions. Table V illustrates that the wild-type (R2-Tcr99S) shows a higher fluorescence peak height with KCN present. The fluorescence yield in the PC-overproducing strain R2-MG95B is independent of the presence of KCN. The results indicate that appreciable control on PS II to PS I photosynthetic electron transfer is exerted by the cyt c oxidase complex. The presence of PC diverts this cyt c,₅₅₃-catalyzed electron drain.

![Table III](image)

![Table IV](image)
Heterologous PC Changes Electron Transfer in Synechococcus

Fig. 6. PS II fluorescence transient measurements. Culture samples of recombinant Synechococcus strains R2-Trc98S and -MG95B were adapted to actinic background light, after which far-red light was added to overexcite PS I until a stable signal was obtained. PS II fluorescence peak-height resulting from switch-off of the far-red light was determined using a PAM chlorophyll fluorescence measuring system. Trace A, R2-Trc98S without KCN; trace B, R2-Trc98S with KCN. The break in the time axis has been indicated in the traces (I). Arrow indicates switch off of the far-red light.

Table V
PS II fluorescence transient measurements

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fluorescence change μV</th>
</tr>
</thead>
<tbody>
<tr>
<td>-KCN</td>
<td>+KCN</td>
</tr>
<tr>
<td>R2-Trc98S</td>
<td>46.7 ± 5.0</td>
</tr>
<tr>
<td>R2-MG95B</td>
<td>54.8 ± 3.2</td>
</tr>
</tbody>
</table>

donors in Synechococcus (c553, IEP = 3.8) (23, 37) and Anabaena (PC, IEP = 8.4 and c553, IEP = 8.9) (4, 23, 24, 54) are very dissimilar. The amino acid sequence of the negative patch of PC, thought to be involved in the binding to PS I, differs substantially between acidic PC proteins and Anabaena PC; instead of seven negative charges in this region, it only contains two, plus one positive charge (4, 30). It is possible that the basic Anabaena PC, in contrast to the endogenous acidic cyt c553, bypasses the positively charged psaF gene product in Synechococcus (33) during its interaction with PS I. This would explain its fast kinetics in electron donation to P700. The high efficiency of Anabaena PC in electron donation to PS I had earlier been demonstrated in vitro (24).

PC and cyt c553 are not equivalent in the transgenic Synechococcus strains; the heterologous PC was an effective donor for PS I but could not substitute cyt c553 as donor for cyt-c oxidase. Preference for PS I can be a general property of PC, or the result of poor interaction with the Synechococcus cyt-c oxidase complex. Anabaena PC was effective in the reconstitution of both respiratory and photosynthetic electron transfer in Anabaena thylakoid membranes (20), but preferences for electron carriers with different IEP appear to be different among cyanobacterial strains, at least in vitro (18, 20). Whereas basic PC and cyt c553 were active in reconstitution of respiratory electron transfer in all strains tested, they could only reconstitute photosynthetic electron transfer in filamentous strains. On the other hand, acidic PC or cyt c553 could not reconstitute respiratory electron transfer in any of the strains tested and were only effective in photosynthetic electron transfer in unicellular strains (18). In marked contrast, our results obtained in the unicellular strain Synechococcus (which contains an acidic cyt c553) demonstrate that both in vitro and in vivo the basic Anabaena PC is highly active in photosynthetic electron transfer. Preliminary work on expression of the acidic PC from the plant Arabidopsis thaliana (55) in Synechococcus suggested that this protein was not active in photosynthetic electron transfer. It will be interesting to determine whether the acidic cyanoabacterial PC from Synechocystis PCC 6803 (56) or the basic cyt c553 from Anabaena PCC 7937 (54) are active in photosynthetic electron transfer in Synechococcus (work in progress).

The relative inhibitory effect of KCN on electron transfer differed between the various experiments (Tables III-V). We expect that the competition between cyt-c oxidase and PS I depends on the filling status of the PQ pool, which reflects the physiological status of the cells. Competition between PS I and cyt-c oxidase could be nearly abolished by the introduction of PC. Cyt-c oxidase may represent a regulatory switch in cyanobacteria such as Synechococcus for the removal of excess electrons. Release of the regulatory switch by abolishing the competition between PS I and cyt-c oxidase in Synechococcus through the introduction of PC does not seem to be harmful for the cell under the growth conditions tested.

In the development of cyanobacteria, species containing only cyt c553 or both PC and cyt c553 have evolved. The difference in IEP of these electron carriers in this group of organisms is striking. If PC and cyt c553 are not equivalent, and if IEP of these proteins is a determinant of their relative efficiency as electron donor, cyanobacterial species may have different properties with regard to their possibilities to adapt to given growth conditions (for instance the capacity for respiratory electron flow (16, 58)).

The data presented have another interesting link to existing evolutionary hypotheses. All photosynthetic organisms originating from a very early stage of evolution-like photosynthetic bacteria and brown and red algae, contain cyt c553 only (7, 10). PC in cyanobacteria is relatively rare, the gene is expressed only in the presence of copper, and is always accompanied by the gene encoding cyt c553. In green algae, PC is ubiquitous, and the gene is constitutively expressed. Cyt c553 is absent from the chloroplasts of most species, and when present, its physiological function is strictly limited to the replacement of PC in the absence of copper (15, 59). In higher plant chloroplasts, PC is the exclusive PS I electron donor. Dominant presence of PC as the primary soluble electron carrier in the photosynthetic electron transfer pathway is linked with the occurrence of organelles specialized in photosynthesis. The emergence of the mitochondrion as a cyt-c oxidase containing organelle specialized in respiration coincides with the disappearance of cyt c553 from the chloroplast (7). Studies on the interchangeability of these electron carriers could be instrumental in understanding these evolutionary patterns.

The rate of photosynthetic electron transfer in Synechococcus was higher in the presence of PC. This was surprising, because oxidation of cyt c553 by PS I already represented the fastest step in the electron transfer chain of Synechococcus (half-time <20 μs), and the rate-limiting step is the oxidation of PQ (half-time 2,000 μs) (57). Electron transfer to PS I in wild-type Synechococcus cells is not hampered by a mere shortage of electron donor; cyt c553 in Synechococcus is present in excess over P700 centra (35).

Given the change in the distribution of electrons between PS I and cyt-c oxidase in the strains containing heterologous PC, we expect an impact on overall photosynthesis in the cell. Studies on the energy storage by photosacoustic spectroscopy in the recombinant Synechococcus strains are in progress. Preliminary measurements indicated that Synechococcus clones expressing PC had higher photosynthetic oxygen evolution rates under nonsaturating light conditions than wild-type Synechococcus.
REFERENCES


Heterologous PC Changes Electron Transfer in Synechococcus