Copper-mediated Regulation of Cytochrome c553 and Plastocyanin in the Cyanobacterium Synechocystis 6803*

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In certain cyanobacteria and algae, cytochrome c553 or plastocyanin can serve to carry electrons from the cytochrome bf complex to photosystem I. The availability of copper in the growth medium regulates which protein is present. To investigate copper induced control of gene expression we isolated these proteins from the cyanobacterium Synechocystis 6803. Using immunodetection and optical spectroscopy, the steady state levels of cytochrome c553 and plastocyanin were measured in cells grown at different copper concentrations. The results show that in cells grown in 20–30 nm copper, cytochrome c553 was present, whereas plastocyanin was not detected. The opposite behavior was observed in cells grown in the presence of 1 μM copper; plastocyanin was present, whereas cytochrome c553 could not be detected. Both proteins were present in cells grown in 0.3 μM copper. Northern analysis of total RNA, probed with a gene fragment for cytochrome c553, showed that the transcript of the petE gene encoding plastocyanin is detectable. Moreover, immunoprecipitation of [35S]methionine pulse labeled cells shows that apoplastocyanin is synthesized, but degraded rapidly in the absence of copper, indicating post-translational control of plastocyanin synthesis. A different regulatory mechanism for plastocyanin expression appears to work in the green alga Pediastrum boryanum. Based on an in vitro translation system, Nakamura et al. (11) conclude that the synthesis of plastocyanin is regulated at the pre-translational level. The cyanobacterium Anabaena variabilis ATCC 29413 appears to be similar to Pediastrum in that the expression of plastocyanin is regulated before translation (12). Recently the petE gene that encodes plastocyanin in the cyanobacterium Synechocystis 6803 was cloned and sequenced by Briggs et al. (13). Synechocystis 6803 also contains the gene for cytochrome c553. Analysis of total RNA in cells grown on medium containing 0.3 or 1 μM copper showed that the transcript of the petE gene was present at similar levels, leading them to propose that plastocyanin gene expression is regulated at the translational or post-translational level (13). In a cyanobacterium, Synechococcus 7942, that lacks the plastocyanin gene, cytochrome c553 is expressed in the presence or absence of copper (14).

Our work is designed to investigate the regulation of plastocyanin and cytochrome c553 synthesis by copper in the transformable cyanobacterium, Synechocystis 6803 (15–17). Using immunodetection and optical spectroscopy, we demonstrate that the synthesis of plastocyanin and cytochrome c553 responds reciprocally to copper in the growth medium. In contrast to the results of Briggs et al. (13), our analysis of total RNA shows that the transcript of the gene encoding plastocyanin synthesis cytochrome c553 (4, 6, 7). Why plastocyanin is selected over cytochrome c553 when adequate levels of copper and iron are present is unknown. It is noteworthy that through evolution higher plants have selected plastocyanin for photosynthetic electron transport in chloroplasts and a c-type cytochrome, similar to cytochrome c553, for respiratory electron transport in mitochondria.

The molecular processes underlying copper-induced control of the synthesis of plastocyanin and cytochrome c553 are poorly understood, although it is clear that regulation occurs at different levels of gene expression in different organisms. In the eukaryotic green alga Chlamydomonas reinhardtii, Merchant and Bogorad (8–10) have shown that transcription of the gene encoding cytochrome c553 is inhibited in cells grown in the presence of copper, indicating transcriptional regulation. In contrast, they showed that plastocyanin is not present during steady state protein synthesis in cells grown without added copper, but that functional mRNA from the gene encoding plastocyanin is detectable. Moreover, immunoprecipitation of [35S]methionine pulse labeled cells shows that apoplastocyanin is synthesized, but degraded rapidly in the absence of copper, indicating post-translational control of plastocyanin synthesis. A different regulatory mechanism for plastocyanin expression appears to work in the green alga Pediastrum boryanum. Based on an in vitro translation system, Nakamura et al. (11) conclude that the synthesis of plastocyanin is regulated at the pre-translational level. The cyanobacterium Anabaena variabilis ATCC 29413 appears to be similar to Pediastrum in that the expression of plastocyanin is regulated before translation (12). Recently the petE gene that encodes plastocyanin in the cyanobacterium Synechocystis 6803 was cloned and sequenced by Briggs et al. (13). Synechocystis 6803 also contains the gene for cytochrome c553. Analysis of total RNA in cells grown on medium containing 0.3 or 1 μM copper showed that the transcript of the petE gene was present at similar levels, leading them to propose that plastocyanin gene expression is regulated at the translational or post-translational level (13). In a cyanobacterium, Synechococcus 7942, that lacks the plastocyanin gene, cytochrome c553 is expressed in the presence or absence of copper (14).

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1 Sometimes referred to as cytochrome c652 or cytochrome c6.

2 Also known as Anabaena sp. PCC 7937.
plastocyanin is regulated by the availability of copper in the growth media. In addition, we show that the transcript for the gene encoding cytochrome c_{553} is also controlled by copper availability. These results indicate that copper-mediated regulation of the expression of the genes encoding plastocyanin and cytochrome c_{553} is prior to translation in Synechocystis 6803.

MATERIALS AND METHODS

Cell Cultures—Synechocystis sp. PCC 6803 was grown at 30 °C on agar plates as described elsewhere (18) or in Erlenmeyer flasks in BG11 liquid medium prepared without copper (19). In media prepared without added copper, the residual copper concentration determined by atomic absorption spectroscopy was 20-30 nM. When required, cells were grown in the absence of copper by a modification of the procedure described by Ikeuchi and Inoue (25). Cells were collected by centrifugation, washed in 0.1 M sodium phosphate (pH 7.0), and stored at 4° C.

Purification of Plastocyanin—Plastocyanin was isolated from cells grown in the absence of copper by a modification of the procedure described by Ikeuchi and Inoue (25). Cells were disrupted by sonication for 1 min at full power using a Gallenkamp Soniprep 150 macro-tip sonicator in a stainless steel beaker in an ice/salt bath. This procedure was repeated 10 times with 5-10-min intervals between sonication. The cell debris was removed by centrifugation at 10,000 x g for 20 min. Ammonium sulfate was added to the supernatant to 35% of saturation and the precipitate was removed by centrifugation at 12,000 x g for 20 min. Cytochrome c_{553} remained in the supernatant and was precipitated overnight in 100% ammonium sulfate, and then centrifuged at 12,000 x g for 20 min. The pellet was resuspended in 10 ml of medium containing 10 mM Tris-Cl (pH 7.2), 10 mM NaCl and dialyzed (M, cat off 6,000-8,000, Spectrum Medical Industries, Los Angeles) against 1 liter of the Tris-resuspension buffer for 16 h at 4° C, during which time the buffer was changed 4-5 times. The sample containing cytochrome c_{553} (approximately 100 ml after dialysis) was loaded on a 50-ml DEAE-cellulose column equilibrated with the resuspension buffer. The column was eluted with a 10-400 mM NaCl gradient in 10 mM Tris-Cl (pH 7.2). Cytochrome c_{553}, which eluted from the column at approximately 100 mM NaCl, was detected by its a-band absorbance peak at 552.5 nm. The cytochrome c_{553} fraction was concentrated to 3-5 ml in a dialysis bag buried in polyethylene glycol. This procedure yielded approximately 10 mg of cytochrome c_{553}. For further purification, the cytochrome c_{553} fraction was loaded on a Sephacryl S200 (97 x 2.6 cm, Pharmacia) column equilibrated with a buffer containing 20 mM Tricine-NaOH (pH 8.0), 100 mM NaCl. The fraction containing cytochrome c_{553} from this column was loaded on a 50-ml CM-cellulose column equilibrated with 10 mM sodium acetate (pH 5.0), 10 mM NaCl. The column was eluted with 10-200 mM NaCl in the acetate buffer and the pink band containing cytochrome c_{553} was collected.

Purification of Plastocyanin—Plastocyanin was isolated from cells grown in the presence of 1 μM CuSO4 by modification of the procedure described by Lightbody and Krogmann (29). Approximately 20 g of cells were broken and the debris was removed as described above. Ammonium sulfate was added to the supernatant to 35% of saturation and the precipitate was removed by centrifugation at 12,000 x g for 20 min. Plastocyanin remained in the supernatant and was precipitated overnight in 100% ammonium sulfate, and then centrifuged at 12,000 x g for 20 min. The pellet was resuspended in 10 ml of medium containing 10 mM Tris-Cl (pH 7.5), 100 mM NaCl and 1% SDS. The fraction containing plastocyanin from this column was loaded on a 50-ml DEAE-cellulose column equilibrated with a buffer containing 50 mM Tris-Cl (pH 7.5) and eluted with a 1-25 mM NaCl gradient. Plastocyanin eluted from the column at about 10 mM NaCl. This procedure yielded approximately 200 μg of plastocyanin.

Determination of Isoelectric Point (pI)—The pH values of cytochrome c_{553} and plastocyanin were estimated by using an EITF gel electrophoresis system from Bio-Rad. The gel solution contained 0.5% agarose, 5% acrylamide, N,N'-diallyltartardiamide, and 5% sodium deoxycholate. The gel was poured as a 1 ml of H_{2}O and 1 ml of 7% acrylamide, 3.5% N,N'-diallyltartadiamide, 0.1% sodium deoxycholate. The lower tank contained 50 mM phosphoric acid and the upper tank contained degassed 50 mM NaOH. The gel was run at 600 V overnight and then at 1,000 V for an additional 1-2 h. To determine the pH gradient, blank gels were run with every 0.5 cm, 0.1 ml of H_{2}O, 0.1 ml of 50 mM NaOH, and 0.1 ml of 50 mM phosphoric acid. Cytochrome c_{553} was identified as a pink band in the gel and plastocyanin was identified as a blue band, created by soaking the gel in a 5 mM potassium ferricyanide solution. The pH values were determined by comparing the migration distances with the pH gradient of the blank gels.

Antibodies—Cytochrome c_{553} antibodies were raised in mice (BALB/c) treated with 0.5 ml of pristane (pristane: 2,6,10,15-tetramethylpentadecane, Sigma) 2 weeks before the first infection (24). The primary immunization used approximately 20 μg of protein/mouse in 100 μl mixed with an equal volume of Freund's complete adjuvant. Four weeks later, 100 μg was injected with Freund's incomplete adjuvant for the secondary immunization. The ascitic fluid was collected 10 days later. Antibodies against plastocyanin were raised in a rabbit (New Zealand White). About 100 μg of the protein was used for the primary immunization and 50 μg was used for the secondary immunization, given subcutaneously 3 weeks later. Antiserum was collected 3 weeks after the second injection.

Electrophoresis and Immunodetection of Proteins—Protein samples were fractionated on 10-22% gradient SDS-polyacrylamide gels according to Igeuchi and Inoue (25). Cells grown at different copper concentrations were harvested during log phase growth, washed in buffer consisting of 20 mM Tris-Cl (pH 7.5), 100 mM NaCl, and resuspended in 0.1 M sodium phosphate (pH 7.0), and stored at 4° C.

DNA Isolation and Northern Blot—Total RNA was extracted from early-log phase cultures according to the procedure of Reddy et al. (27), except that LiCl-precipitated RNA was not further purified by ethanol precipitation. About 100 μg of total RNA was loaded on a 1.5% agarose gel containing formaldehyde and blotted to GeneScreen Plus membrane (Du Pont-New England Nuclear) according to the manufacturer's instructions. The DNA probe was labeled with 32P using a random oligo-labeling kit from Pharmacia. Hybridization was performed at 63 °C overnight in a solution containing 50 mM Tris-Cl (pH 7.5), 1 M NaCl, 1% SDS. After hybridization, the blot was washed at room temperature for 10 min in 0.1 x SSC and 1% SDS followed by a high stringency wash in 0.1 x SSC and 1% SDS at 65 °C for 45 min.

Optical Spectroscopy—Absorption spectra of plastocyanin were determined using a Cary 219 spectrophotometer at a half-bandwidth of 2 nm. Cytochrome c_{553} spectra were determined using a DW2c spectrophotometer. The monochromators were used with a half-bandwidth of 2 nm and were calibrated using a mercury penlamp. Time resolved light-induced absorbance changes were measured using a built-in photon-counting system as described by Cornog and O'Neil (28). Actinic light was provided by two 300-W nitrogen-halogen lamps, each of which was filtered by a heat filter and a red CS-2-68 blocking filter (Corning Glass Works, Corning, NY). The actinic flash duration was 50-100 ns. To improve the signal to noise ratio 8-32 traces were averaged. Typically at a photostationary state the width of the measuring beam was 1 cm and the half-bandwidth was 3 nm. The amount of cytochrome undergoing redox changes in the light was calculated assuming a reduced minus oxidized extinction coefficients
for cytochrome \( f \) and cytochrome \( c_{553} \) of 20 mM\(^{-1}\) cm\(^{-1}\) (29) and 17.3 mM\(^{-1}\) cm\(^{-1}\) (30), respectively.

RESULTS

Purification of Cytochrome \( c_{553} \) and Plastocyanin from \textit{Synechocystis} 6803—Cytochrome \( c_{553} \) migrated as a single 9-kDa band on an SDS-polyacrylamide gel after purification on the DEAE-cellulose, Sephacryl S200, and CM-cellulose columns (Fig. 1, lane 1). Based on the results of sequencing the N-terminal end of the protein (data not shown) we estimate that the protein is more than 99% pure. The visible reduced minus oxidized absorption spectrum of the cytochrome is shown in Fig. 2A. The peak of the cytochrome \( \alpha \)-band is 552.5 nm (Fig. 2B).

Purified plastocyanin migrated as a single 12-kDa band on an SDS polyacrylamide gel (Fig. 1, lane 2). The visible absorption spectrum of oxidized plastocyanin shows a peak at 601 nm (Fig. 2C).

In cyanobacteria the isoelectric points of cytochrome \( c_{553} \) and plastocyanin have been found to be similar within the same species (5). Our data are in keeping with this observation; the isoelectric points of cytochrome \( c_{553} \) and plastocyanin are 5.6 and 6.2, respectively, as measured on isoelectric focusing gels. It is noteworthy that the isoelectric point of plastocyanin observed by isoelectric focusing (6.2) is similar to the value (5.6) predicted by Briggs et al. (13) based on the gene sequence of plastocyanin from \textit{Synechocystis} 6803.

Effect of Copper on Cell Growth—Copper is known to prevent the growth of cyanobacteria (31) and to inhibit the activity of photosystem II (32). Fig. 3 shows that the growth rate of \textit{Synechocystis} 6803 is not affected by copper concentrations of 20–30 nm to 1 \( \mu \)M, whereas at a copper concentration of 2 \( \mu \)M the rate of growth is significantly lower. We found that copper concentrations above 5 \( \mu \)M prevented growth.

Copper Regulation of the Expression of Cytochrome \( c_{553} \) and Plastocyanin—Western blot analysis show that cells grown in medium containing 20–30 nm copper have no detectable plastocyanin (Fig. 4A, lane 1), whereas cytochrome \( c_{553} \) is present (Fig. 4B, lane 1). Cytochrome \( c_{553} \) responds in the opposite manner; cells grown in medium containing 1 \( \mu \)M copper have no detectable cytochrome \( c_{553} \) (Fig. 4B, lane 3), whereas plastocyanin is present (Fig. 4A, lane 3). Cells grown in medium containing 0.3 \( \mu \)M copper (normal BG11; Ref. 19) show the presence of intermediate levels of both plastocyanin and cytochrome \( c_{553} \) (lane 2 of Fig. 4, A and B).

The reciprocal relationship between the steady state levels of plastocyanin and cytochrome \( c_{553} \) is demonstrated clearly in Fig. 5. Cells were transferred from a copper-deficient medium to a medium containing 0.3 \( \mu \)M copper and the levels of both proteins were determined by immunodetection. As described above, cells grown in 20–30 nm copper contain cytochrome \( c_{553} \) but no detectable plastocyanin (Fig. 5, lanes 0). Within 30 h after the addition of copper to the medium, the steady state level of cytochrome \( c_{553} \) is drastically lowered, a decrease that is compensated for by a significant synthesis of plastocyanin (Fig. 5, lanes 1). The steady state level of plastocyanin was maximum in lane 2, after 98 h, at which time cytochrome \( c_{553} \) was not detectable. After 98 h the level of plastocyanin began to decrease, while the level of cytochrome \( c_{553} \) increased. This reversal in the steady state levels of the two proteins is likely due to depletion of the copper supply in

![Fig. 1. SDS-polyacrylamide gel of purified cytochrome \( c_{553} \) and plastocyanin isolated from \textit{Synechocystis} 6803. The proteins were fractionated on a gradient gel of 16–22% polyacrylamide containing 7.5 M urea and subsequently stained with Coomassie Blue. Lane 1, cytochrome \( c_{553} \); lane 2, plastocyanin.](image-url)
the medium during cell growth, which blocks the synthesis of plastocyanin and induces the synthesis of cytochrome $c_{553}$. Over the same period, cells grown in 1 $\mu$M copper did not show any change in the level of plastocyanin while no cytochrome was detected at any stage of growth (data not shown).

Merchant et al. (10) recently determined that the number of copper ions per cell required to suppress the synthesis of cytochrome $c$ in *Chlamydomonas* is approximately 9-10. We find a similar result for *Synechocystis* 6803. Under conditions where no cytochrome $c_{553}$ was detected (0.3 $\mu$M copper, Fig. 4B, lane 3), the number of copper ions per cell was approximately 4-10.

**Light-induced Cytochrome Absorption Changes in Whole Cells**—The kinetics of the light-induced absorption change at 553-540 nm in *Synechocystis* 6803 cells grown in the absence of added copper are shown in Fig. 6. The wavelength dependence of the absorbance change for cells grown without and with added copper is shown in Fig. 7A. For cells grown without added copper, the absorbance change is dominated by cytochrome $c_{553}$, whereas for cells grown in the presence of copper the spectrum is due to cytochrome $f$, with an $\alpha$-band peak at 556.8 nm and an unidentified component with a peak near 550 nm. Subtraction of the spectrum for cells grown without added copper from that of cells grown in the presence copper reveals the light-induced spectrum of cytochrome $c_{553}$ (Fig. 7B). This assignment is confirmed by comparing the light-induced spectrum with the chemical difference spectrum of the purified cytochrome (Fig. 7B). Based on Fig. 7, we calculate that the amount of cytochrome $c_{553}$ turning over in the light in cells grown without added copper is 1 cytochrome $c_{553}$/175 chlorophyll. The amount of cytochrome $f$ turning over in the light in cells grown in the presence of 1 $\mu$M copper is 1 cytochrome $f$/650 ± 70 chlorophyll (calculated using three different cell cultures and ignoring the contribution of the unknown component with a peak around 550 nm). The light-induced redox behavior of cytochrome $c_{553}$ (Fig. 6) and of cytochrome $f$ (data not shown) is similar to that of cytochrome $f$ in plants (discussed in Ref. 33). At the onset of illumination there is a rapid absorbance decrease that corresponds to the oxidation of the cytochromes by photosystem I. After the actinic light is turned off, the absorbance increase corresponds to the dark reduction of the cytochromes by electrons from the plastoquinol pool.

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**Fig. 3.** Growth at different copper concentrations of *Synechocystis* 6803 cells. At time 0, cells growing exponentially in a copper-deficient medium were suspended in BG11 medium supplemented with 0 $\mu$M copper (solid circles), 0.3 $\mu$M copper (open circles), 1 $\mu$M copper (crosses), 2 $\mu$M copper (solid triangles). The starting density of the cultures was adjusted to 0.1 $\mathrm{A}$ at 730 nm.

**Fig. 4.** Western blot of the soluble protein extract of *Synechocystis* 6803 cells grown at different copper concentrations. Proteins were fractionated and Western blotting was performed as described under "Materials and Methods." Panel A, Western blot probed with anti-plastocyanin antibodies. Lane 1, proteins extracted from cells grown in 20-30 nM copper; lane 2, proteins extracted from cells grown in 0.3 $\mu$M copper; lane 3, proteins extracted from cells grown in 1 $\mu$M copper. Panel B, Western blot probed with anti-cytochrome $c_{553}$ antibodies. Lane assignments as in panel A.

**Fig. 5.** Western blot analysis showing the time course of the steady state concentration of plastocyanin and cytochrome $c_{553}$ in *Synechocystis* 6803 cells induced by the addition of copper. At time 0, cells were transferred from a medium without added copper, to a medium containing 0.3 $\mu$M copper. Under these conditions the doubling time of the cells was about 12 h. Proteins were extracted and Western blot analysis was performed as described under "Materials and Methods." Panel A, Western blot of soluble cell extract probed with anti-plastocyanin antibodies. Cellular growth conditions were: lane 0, cells grown in 20-30 nM copper (time 0); lane 1, cells grown for 30 h in medium containing 0.3 $\mu$M copper; lane 2, cells grown for 98 h after addition of copper; lane 3, cells grown for 124 h after addition of copper; lane 4, cells grown for 146 h after addition of copper. Panel B, Western blots of soluble cell extract probed with anti-cytochrome $c_{553}$ antibodies. Lane assignments as in panel A.

**Fig. 6.** Kinetics of the light-induced absorbance change at 553-540 nm observed in cells of *Synechocystis* 6803 grown in the absence of added copper. Cells were suspended at a chlorophyll concentration of 10 $\mu$M in a medium containing 25 mM HEPES/NaOH (pH 7.5).
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**DISCUSSION**

The roles of a number of metals, e.g. mercury, cadmium, and copper, as activators or repressors of expression of various genes have been well documented in recent years (34–37). Regulation by copper of the steady state levels of plastocyanin and cytochrome $c_{553}$ in a number of cyanobacteria and algae offers an attractive system to study the effect of copper ions on the expression of the corresponding genes. In *Synechocystis* 6803 we have shown that in the presence of 1 μM copper, expression of the cytA gene encoding cytochrome $c_{553}$ is repressed, while expression of the petE gene encoding plastocyanin is turned on (Fig. 4). Cells grown at low copper concentrations (20–30 nM) exhibit the inverse behavior, the expression of the petE gene encoding plastocyanin is repressed, while the expression of the cytA gene encoding cytochrome $c_{553}$ is turned on. Moreover, both plastocyanin and cytochrome $c_{553}$ could be detected in cells grown in the presence of 0.3 μM copper, indicating that the inhibition of the expression of cytochrome $c_{553}$ is incomplete at intermediate levels of copper.

Recently, Merchant et al. (10) proposed a scheme to explain the copper-induced regulation of the expression of the genes encoding plastocyanin and cytochrome $c_{553}$ in *C. reinhardtii*. In this organism, they demonstrated that the petE gene is transcribed and translated to form the plastocyanin apoprotein, even when copper is absent from the growth medium. However, in the absence of copper, the apoprotein is degraded rapidly (half-life of 16–18 min) (8). Transcription of the gene encoding cytochrome $c_{553}$, on the other hand, is completely suppressed in the presence of copper (8–10). Based on these data, Merchant et al. (10) proposed that copper ions play a dual role in regulation of gene expression. First, copper binding stabilizes the apoprotein. Second, excess copper ions that are not needed to form holoplastocyanin form a complex with a regulatory protein that represses the synthesis of the message for cytochrome $c_{553}$. Although the underlying mechanism of this dual process has yet to be revealed, the scheme offers an explanation for the opposing role of copper in regulating the steady state levels of plastocyanin and cytochrome $c_{553}$ in *Chlamydomonas*.

Our data show that copper regulation in *Synechocystis* 6803 is similar to *Chlamydomonas* in at least one aspect: the expression of the cytA gene, which encodes cytochrome $c_{553}$, is repressed by copper in the growth medium (Fig. 8, panel A). It appears, however, that the regulatory activity of copper operates only in organisms that are capable of synthesizing both cytochrome $c_{553}$ and plastocyanin. In *Synechococcus* 7942, a cyanobacterium that does not appear to have the petE gene, cytochrome $c_{553}$ is synthesized in the presence or absence of copper (14). The absence of the cytA transcript in *Synechocystis* 6803 cells grown in low copper indicates repression of cytA transcription or destabilization of the transcript.

Copper regulation of the plastocyanin petE gene in *Synechocystis* 6803 differs from that of *Chlamydomonas*. In *Synechocystis* 6803 the petE transcript is not detected in cells grown in low copper medium, leading us to conclude that plastocyanin...

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anin gene expression is controlled by copper primarily at the level of gene transcription or message stabilization. A similar observation has been reported for the cyanobacterium *Anabaena* 29413 by van der Plas *et al.* (12). They showed that expression of petE gene is repressed at the transcriptional level when copper is absent from the growth medium.

Our conclusion differs from that of Briggs *et al.* (13), who suggested that the petE gene in *Synechocystis* 6803 is not regulated at the transcriptional level, but at the translational or post-translational level. Their conclusion is based on the observation that the level of the petE mRNA is the same in cells grown in 0.3 or 3 μM copper, whereas plastocyanin is detectable by spinach antiplastocyanin antibody only in cells grown in 3 μM copper. In contrast, we found using anti-Synechocystis plastocyanin antibody that plastocyanin was detectable in cells grown in 0.3 μM copper medium, but that the level decreased as the cell density increased (Fig. 5A), presumably because the concentration of free copper in the medium decreased as copper ions were incorporated into apoplastocyanin. In the presence of 0.3 μM copper, we observed a high level accumulation of petE mRNA in early-log phase cells, which is consistent with the result of Briggs *et al.* (13). However, the petE mRNA steady state level decreased during later stages of growth (data not shown). In our view, the critical observation is that in cells grown at a 20–30 nM copper, there is a significant decrease of petE transcript level (Fig. 8B), indicating the gene expression is inhibited at a pre-translational level. Since copper is required in at least two steps of plastocyanin synthesis, mediating activation of gene expression and assembly of the functional protein, it is possible that the two processes are affected differentially by copper, which may explain the result by Briggs *et al.* that the same cells grown in the presence of 0.3 μM copper show similar petE transcript level but different protein level. It is noteworthy that two copper-dependent steps have been suggested to control plastocyanin synthesis in the green algae *Scenedesmus obliquus* (38).

**REFERENCES**