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Copper-induced expression, cloning, and regulatory studies of the plastocyanin gene from the cyanobacterium *Synechocystis* sp. PCC 6803

Linda M. Briggs,[1] Vincent L. Pecoraro[1] and Lee McIntosh[2]*
[1] *Department of Chemistry, University of Michigan, Ann Arbor, MI 48109, USA;* [2] *DOE Plant Research Laboratory & Department of Biochemistry, Michigan State University, East Lansing, MI 48824, USA (* author for correspondence)*

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Abstract

Plastocyanin can be detected in *Synechocystis* sp. PCC 6803 when 3 μM copper is added to the growth medium, BG-11. The plastocyanin gene (*petE*) was cloned from a genomic λ EMBL 3 library by screening with the *petE* gene from *Anabaena* sp. PCC 7937. The *Synechocystis* 6803 *petE* gene is present as a single copy and, as deduced from the DNA sequence, encodes a precursor protein of 126 amino acids. The predicted 29 amino acid transit peptide shows substantial homology to the *Anabaena* 7937 transit peptide, thought to direct the plastocyanin precursor to the thylakoid lumen. Putative promoter sites -16 and -38 base pairs from the start of the *petE* gene have been identified. The deduced amino acid sequence has the greatest homology (61%) to the green alga *Scenedemus obliquus* plastocyanin. Despite the lower homology, the copper binding residues and certain aromatic residues remain highly conserved. Northern hybridization analysis indicates that the *Synechocystis* sp. PCC 6803 *petE* gene is not transcriptionally regulated since the accumulation of *petE* mRNA appears to be independent of the copper concentration in the growth media. The possibility of an additional polypeptide needed to facilitate the electron transfer from plastocyanin to $P700^+$ is also discussed.

Introduction

Plastocyanin, a small (M_r 10000) protein composed of a single polypeptide which contains one copper atom per molecule, is one of the mobile electron carriers connecting photosystem I (PS I) and photosystem II (PS II). Cupric (Cu^{2+}) plastocyanin oxidizes the membrane-bound cytochrome b_6/f complex and then diffuses to the reaction center of PS I where the cuprous (Cu^+) plastocyanin reduces $P700^+$ [22, 37].

In higher plants, nuclear-encoded plastocyanin is translated in the cytoplasm as a precursor protein that contains a transit peptide at the amino terminus. The transit peptide, which ranges from 34–80 amino acids depending on the organism, contains the information necessary for translocation to the thylakoid lumen [32]. During translocation the transit peptide is proteolytically removed leaving mature plastocyanin (10000 kDa) in the thylakoid lumen [30].

Plastocyanin, which is constitutively produced

in higher plants, is only found in some species of green algae and cyanobacteria [12, 15, 26]. The functional replacement for plastocyanin is a soluble iron-heme protein, cytochrome c553. Whether plastocyanin or cytochrome c553 functions in the cell is determined by the amount of copper available in the growth medium. Under conditions of copper deficiency the small (M_r 9000) cytochrome c553 replaces plastocyanin as the reductant of $P700^+$ [26]. Sandmann has shown, in some *Nostoc* cultures, that as the copper content is depleted with time the amount of plastocyanin decreases with a concomitant increase in cytochrome c553 [26]. Two different modes of metal-based regulation have been reported for plastocyanin in the literature. In the filamentous cyanobacterium *Anabaena* sp. PCC 7937 the steady-state level of *petE* transcript was shown to increase with an increased level of copper supplied to the growth medium [35]. The situation is very different in the green alga *Chlamydomonas reinhardtii*. *PetE* mRNA levels were independent of copper concentration; instead, plastocyanin is regulated by the degradation of the apoprotein in copper-deficient cultures [19].

Our studies of the *petE* gene and induction of plastocyanin synthesis in cyanobacteria is part of a larger effort to understand the molecular basis of gene regulation brought about by changes in the intracellular and extracellular concentrations of metal ions. Herein we (1) report the cloning of the *petE* gene, which is present as a single gene copy that encodes for a precursor protein of 126 amino acids; (2) demonstrate that the Cu^{2+} concentration regulates plastocyanin synthesis either at the translational or post-translational level and, (3) identify possible *cis*-acting regions at − 16 and − 38 base pairs from the *petE* gene.

Materials and methods

Materials

Restriction endonucleases and other DNA-modifying enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, MD) and New England Biolabs (Beverly, MA) and used according to the suppliers' instructions. Nitrocellulose filters were obtained from Schleicher & Schuell (Keene, NH). The dideoxy 'Sequenase' sequencing kit was obtained from United States Biochemical Corporation (Cleveland, OH). Radiolabeled nucleotide was purchased from Amersham Corporation (Arlington Heights, IL). All sequence analysis was performed using the sequence analysis software package of the genetics computer group, University of Wisconsin [7].

Growth conditions

Synechocystis sp. PCC 6803 was grown in BG-11 with 5 mM N-tris[hydroxymethyl] methyl-2-aminoethane sulfonic acid (TES) [24]. Plastocyanin synthesis was induced by adding $CuSO_4$ to BG-11, to a final concentration in the medium of 3 μM Cu^{2+}.

Immunoblotting

Cells harvested at room temperature were washed first in 10 mM EDTA, 20 mM N-[2-hydroxyethyl]piperazine-N-[2-ethanesulfonic acid]) (HEPES) pH 7.5, then in 1.5 M KCl, 5 mM EDTA. After washing, the cells were brought to 0.1 mg/ml cholorophyll for lysis in 0.4 M sucrose, 10 mM EDTA, 20 mM HEPES pH 7.5 and treated with 2% lysozyme for 30 minutes at 31 °C, followed by sonication with acid-washed glass beads (Sigma no. G2506, St Louis, MO).

Proteins were run on a 17.5 to 10% SDS-polyacrylamide gel, then transferred to mitrocellulose under 0.9 Amp current for 2 hours with cooling [33]. Protein concentrations were determined using the Lowry assay [16]. The nitrocellulose was probed with rabbit polyclonal antibodies raised against purified spinach plastocyanin by multiple subcutaneous injections into rabbits [34]. Antibody cross-reactivity was visualized using protein A alkaline phosphatase [3] (Sigma no. P9650). The portion of nitrocellulose containing Bio-Rad (Richmond, CA) low molecular weight markers was removed and stained with india ink.

Library screening

Duplicate plaque lifts to nitrocellulose filters from a λEMBL3 phage library [6] were baked at 80 °C in *vacuo* for 0.5–1 hour followed by prehybridization at 55 °C in 6 × SSC, 5 × Denhardt's, 0.5% SDS, for 40 minutes. Hybridization with the random-primer-labeled probe [9] was carried out at 55 °C in 3 × SSC, 0.1% SDS, 5 × Denhardt's [17]. The hybridized plaque lifts were washed three times in 3 × SSC, 55 °C totaling 34 minutes. Kodak XAR film was exposed to the washed filters using intensifying screens. Positive plaques were picked and rescreened using the same protocol. Phage DNA isolated from liquid culture [4] was subcloned into pBluescript obtained from Stratagene (La Jolla, CA).

DNA isolation and Southern blotting

Cesium-purified genomic DNA isolated from *Synechocystis* 6803 [38] was used for Southern blot analysis [32]. 10 μg (per digest) of genomic DNA was run on 0.8% agarose gel, transferred to a nitrocelluose filter, baked at 80 °C for 1 hour, and prehybridized using 6 × SSC, 5 × Denhardt's, 0.5% SDS and 0.1 mg/ml denatured salmon sperm DNA. Overnight hybridization was performed at 65 °C by adding the random-primer-labeled probe directly to the prehybridization solution. Filters were washed two times in 6 × SSC at 40–47 °C for 20 minutes, two times in 1 × SSC at 65 °C for 20 minutes and three times in 0.1 × SSC at 67–71 °C for a total of 1 hour. Filters were exposed as described above.

DNA sequencing

CsCl-purified plasmid DNA prepared by the alkaline lysis method [17] was sequenced first using the chemical modification and chain cleavage method [18]. The dideoxy chain termination method with ^{32}P-dATP as radiolabel [27] was used with primers based on sequence obtained from the chemical cleavage method.

RNA isolation and northern blotting

Total RNA was isolated from *Synechocystis* 6803 according to the procedure of Golden [10]. Total RNA was run on a 1.2% agarose/formaldehyde gel, 3 hours at 100 V. The gel was soaked in 10 × SSC for 45 minutes before setting up the overnight transfer to nitrocellulose. Nitrocellulose was prehybridized in 3 × SSC, 1 × Denhardt's, 0.1% SDS, 30 mM Tris, 0.1 mM EDTA, 50% formamide, 40 μg/ml salmon sperm DNA. Randomprimer-labeled probe was added directly to the prehybridization solution for the overnight hybridization at 45 °C. Filters were washed for 3 hours at 55–60 °C in 0.1 × SSC, 1 hour at 70–72 °C in 0.1 × SSC, then exposed as described above.

Results

Plastocyanin induction

When *Synechocystis* 6803 is grown in lab cultures containing 3 μM copper, plastocyanin can be detected. Induction of plastocyanin synthesis was seen by comparison of cell extracts from *Synechocystis* 6803 grown with and without addition of copper to the growth medium BG-11 [24]. Lanes 2 and 3 of the immunoblot in Fig. 1 show whole cell extracts from cells grown in BG-11, with 3 μM Cu^{2+}. Lane 4 is whole cell extract from cells grown in unsupplemented BG-11, that is 0.3 μM Cu^{2+}. The plastocyanin band clearly visible in lanes 2 and 3 (at approximately M_r 10000) is absent in lane 4 (160 μg total protein lanes 2, 3, 4). The nitrocellulose filter shown in Fig. 1 was incubated with rabbit polyclonal antibodies raised against purified spinach plastocyanin. Spinach plastocyanin, M_r 10400 [24], in lane 1 (0.5 μg protein) has a pI of 4.2 [23]. *Synechocystis* 6803 plastocyanin has a calculated [7] pI of 5.6.

Gene cloning

The *petE* gene from *Synechocystis* 6803 was cloned from a λ EMBL3 genomic library [6] by screening with the *Anabaena* 7937 *petE* gene, which was a generous gift from J. Van der Plas [35]. The

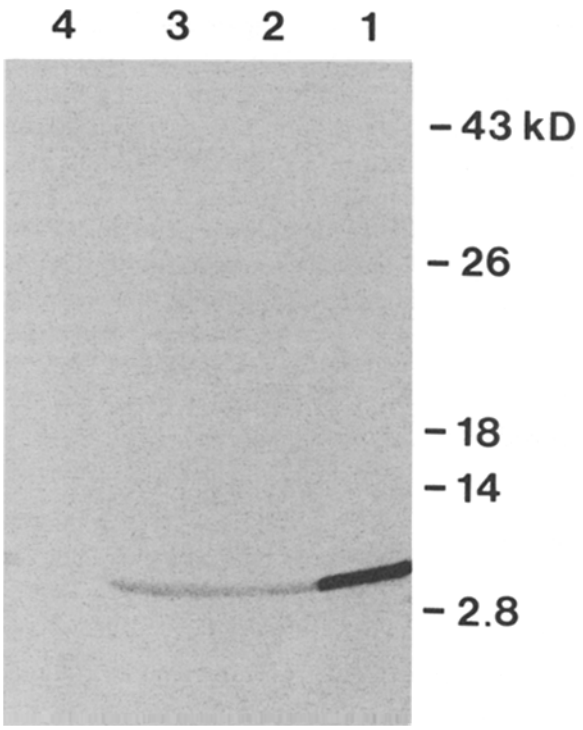

Fig. 1. Western blot of *Synechocystis* sp. PCC 6803 total cellular protein. Proteins run on a 17.5 to 10% polyacrylamide gradient, transferred to nitrocellulose membrane, then incubated with antibodies raised against spinach plastocyanin. Lane 1, 0.5 μg spinach plastocyanin; lanes 2 and 3, total cellular protein from cells grown in BG-11 + 3 μM Cu^{+2} (cells used in RNA prep); lane 4, total cellular protein from *Synechocystis* 6803 grown in BG-11. 160 μg total protein in all *Synechocystis* lanes.

Anabaena probe, a 600 bp *Hin*d III fragment consisting of the 400 bp coding region for plastocyanin and the 200 bp flanking 3′ region, was first used to probe genomic *Synechocystis* DNA digested with *Hin*d III, *Kpn* I, *Bam* HI, *Hin*c II, *Nco* I, *Xba* I, and *Eco* RI. A single hybridization signal for each digest showed early signs that plastocyanin was encoded by a single copy and served to establish hybridization conditions with the heterologous probe (results not shown). The same probe was used to screen plaque blots at 55 °C, 3 × SSC. Southern hybridization analysis of phage DNA isolated from one of the positives, a 17 kb *Sal* I insert in EMBL3, showed strong signals at 4 kb *Nco* I, 9 kb *Eco* RI and 1.8 kb *Hin*d III. The 9 kb *Eco* RI and 1.8 kb *Hin*d III bands were isolated from preparative agarose gels and subcloned into pBluescript.

Further restriction digests and Southern hybridization of the 1.8 kb *Hin*d III subclone narrowed the *petE* region to a 500 bp *Nco* I/*Hin*d III fragment. End labeling with ^{32}P-dATP followed by chemical modification and chain cleavage sequencing [18] revealed the *petE* gene was truncated just upstream of the copper-binding site by the *Hin*d III site. Specific primers for dideoxy chain termination sequencing [27] of the 9 kb *Eco* RI subclone were designed based on the initial sequence obtained. Three sets of primers were made for sequencing the entire region (shown in Fig. 2) several times in both directions.

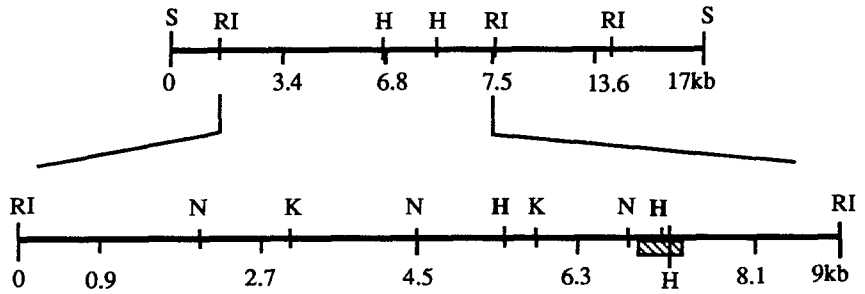

Fig. 2. Top: restriction map of the 17 kb clone isolated from the bacteriophage EMBL3 genomic library. Bottom: restriction map of the 9 kb *Eco* RI subclone. The 1.8 kb *Hin*d III fragment shows the relative position of the plastocyanin gene denoted as (▨). Restriction sites are as follows: H = *Hin*d III; K = *Kpn* I; N = *Nco* I; RI = *Eco* RI; S = *Sal* I.

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          -80                                  -60                                  -40
AAA AAC GTC CCT GAT CGC CTT TTT GGG CAC GGA GTA GGG CGT TAC CCC GGC CCG TTC AAC

          -20                                   -1                   10                   20
CAC AAG TCC CTA TAG ATA CAA TCG CCA AGA AGT ATG TCT AAA AAG TTT TTA ACA ATC CTC
                                                 M   S   K   K   F   L   T   I   L

  30                   40                   50                   60                   70                   80
GCT GGC CTT CTG CTG GTG GTC TCC AGT TTC TTT TTA TCC GTG AGC CCC GCT GCC GCT GCC
 A   G   L   L   L   V   V   S   S   F   F   L   S   V   S   P   A   A   A   A  ▲

  90                  100                  110                  120                  130                  140
AAT GCA ACA GTG AAA ATG GGT TCT GAC AGT GGT GCC CTA GTT TTT GAA CCC AGC ACC GTC
 N   A   T   V   K   M   G   S   D   S   G   A   L   V   F   E   P   S   T   V

 150                  160                  170                  180                  190                  200
ACC ATC AAA GCT GGA GAG GAA GTC AAA TGG GTT AAC AAC AAA CTC TCC CCT CAT AAC ATT
 T   I   K   A   G   E   E   V   K   W   V   N   N   K   L   S   P   H   N   I

 210                  220                  230                  240                  250                  260
GTG TTT GCC GCC GAT GGT GTG GAT GCT GAC ACC GCT GCT AAG CTC TCC CAC AAA GGC TTG
 V   F   A   A   D   G   V   D   A   D   T   A   A   K   L   S   H   K   G   L

 270                  280   HIND III       300                  310                  320
GCC TTT GCC GCT GGT GAA AGC TTC ACC TCC ACC TTC ACT GAG CCC GGC ACC TAC ACC TAC
 A   F   A   A   G   E   S   F   T   S   T   F   T   E   P   G   T   Y   T   Y

 330                  340                  350                  360                  370                  380
TAC TGT GAA CCC CAC CGG GGT GCT GGC ATG GTA GGC AAA GTT GTC GTT GAG TAA TCA GCC
 Y   C   E   P   H   R   G   A   G   M   V   G   K   V   V   V   E   *

 390                  400                  410
AGC TCA ATC TGT GTG TCG TTG ATT TAA GCT T
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Fig. 3. Nucleotide sequence of the *Synechocystis* sp. PCC 6803 *petE* gene and deduced amino acid sequence below the DNA coding region. The arrowhead indicates the assumed processing site for removal of the transit peptide. Putative promoter sequences are underlined. *Hin*d III site is shown near the copper binding site.

PetE gene

The nucleotide sequence coding for the *Synechocystis* 6803 *petE* gene is shown in Fig. 3. The methionine seven amino acid residues beyond the start of the open reading frame is probably the start codon since there are no others in this vicinity. The arrowhead in Fig. 3 shows the putative processing site for removal of the transit peptide. This site was deduced from comparison with the *Anabaena* transit sequence (see Discussion). A portion of the region upstream from the start codon is noteworthy since it bears resemblance to putative promoter sequences reported for ferredoxin genes from *Anabaena* 7120, 7937 and *Synechococcus* 7942 [36]. Comparisons are shown in Fig. 4. The sequence TAGATA, the '– 10' region, is found 16 base pairs upstream from the ATG, and spaced 16 base pairs further upstream the sequence CCCGTT is found, labeled the '– 35' region.

Southern hybridization analysis of *Synechocystis* genomic DNA with the random primer labeled 1.8 kb *Hin*d III fragment shown in Fig. 5 indicates that the *petE* gene is present in a single copy. The 1.8 kb *Hin*d III fragment consists of the 400 bp coding region at its 3′ end and the remaining 1.4 kb being the upstream 5′ region. For the *Hin*d III, *Eco* RI, and *Kpn* I digests a single hybridization signal appears at 1.8 kb, 8.9 kb and 2.2 kb, respectively. There are two bands present in the *Nco* I digest because the first and more

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                "-35"                                   "-10"
(1)  TTT  CAAGTT  ATATACTTGGATTTTTCTCG  TAGTAT  CAGAATTG - 98bp ATG - Ferredoxin
(2)  TTT  CAAGTT  ATATACTTTGATTTTTCCCG  TAGTAT  CAGAATTG - 98bp ATG - Ferredoxin
(3)  GGC  CGAGTT  TGAGCCGT-GATTAC-CCC-  TACGAA  CTTTCCGG - 169bp ATG - Ferredoxin
(4)  GGC  GCAGTT  CGCCCTTTGGCAA--CCCA-  TAGTAT  CAATGGGA - 63bp ATG - Ferredoxin
(5)  GG-  CCCGTT  CAACCAC----AAGTCCCTA  TAGATA  CAATCGCCA - 6bp ATG - Plastocyanin

(6)  AGA  TTGTCA  TATTTGGTGTCGATTT----  TATTTA  AAATGAAA - 6bp ATG - Plastocyanin
(7)  ----  TTGACA  ------- 17bp ---------  TATAAT  ---------- Consensus E. coli promoter
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Fig. 4. Comparison of putative promoter sequences for ferredoxin and plastocyanin from (1) *Anabaena* sp. PCC 7120; (2) *Anabaena* sp. PCC 7937; (3) *Synechococcus* sp. PCC 7942 P1; (4) *Synechococcus* sp. PCC 7942 P2 [35]; (5) *Synechocystis* sp. PCC 6803; (6) *Anabaena* sp. PCC 7937 [34]; (7) Procaryotic promoter sequence. The '– 10' and '– 35' regions are based on consensus procaryote sequences. The sequences shown in lines 3 and 4 are two putative promoters for the same gene in *Synechoccus* 7942. Gaps are inserted to maximize homology.

intense signal at 2.2 kb has a larger overlapping area with probe than the second less intense signal at 4.5 kb which is where the plastocyanin-coding region actually lies (see Fig. 2).

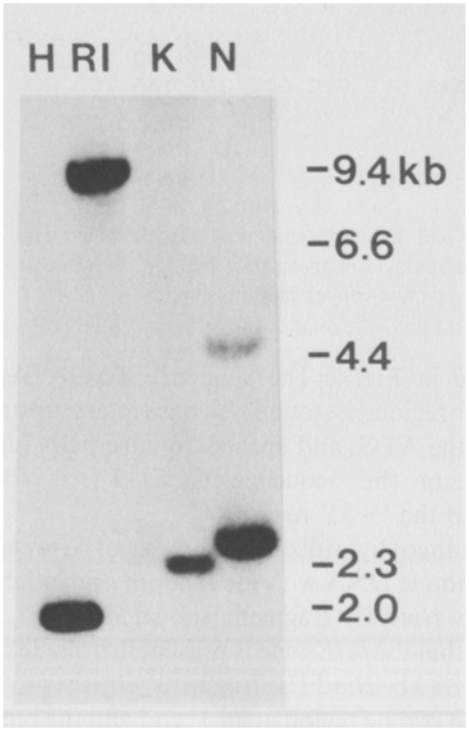

Fig. 5. Southern hybridization of 1.8 kb *Hind* III fragment to total *Synechocystis* sp. PCC 6803 genomic DNA run on 0.8% agarose gel blotted to nitrocellulose, hybridization at 65 °C, 6× SSC. Genomic DNA was digested with H = *Hind* III; RI = *Eco* RI; K = *Kpn* I; and N = *Nco* I. Molecular size markers in kb are lambda DNA digested with *Hind* III.

Transcriptional analysis

Northern hybridization analysis was performed to compare steady-state *petE* mRNA levels from cells grown with and without additional copper in the growth medium. Total RNA isolated from cells grown in BG-11 containing 0.3 μM and 3 μM Cu^{+2} was separated and denatured on a 1.2% agarose/formaldehyde gel, then transferred to a nitrocellulose filter. The nitrocellulose filter

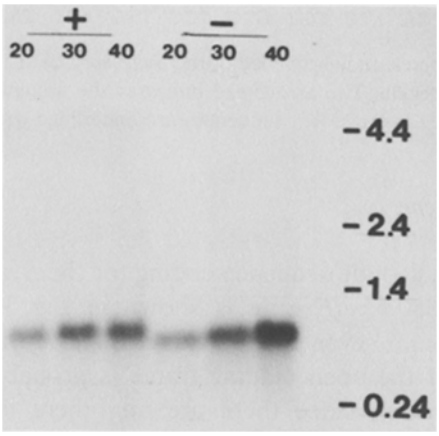

Fig. 6. Northern hybridization analysis of total RNA from *Synechocystis* sp. PCC 6803 grown with and without Cu^{2+} added to the growth medium BG-11. Total RNA was separated on a 1.2% agarose/formaldehye gel transferred to nitrocellulose and hybridized with a 380 bp fragment derived from the *Synechocystis* 6803 *petE* gene. The first three lanes labeled + are RNA from cells grown with 3 μM Cu^{2+} in BG-11 20, 30, 40 μg respectively. The next three lanes labeled − are RNA from cells grown in BG-11 (0.3 μM Cu^{2+}) also 20, 30, 40 μg respectively.

was prehybridized (42 °C) followed by overnight hybridization (45 °C, 50% formamide). Results of the northern hybridization, using a 380 bp section of the *petE* coding region as a probe, indicate a single specific transcript between 655–755 bases as shown in Fig. 6. Three total RNA lanes (20, 30, 40 μg) were run for each growth condition, with the increasing band intensities paralleling the increasing amount of total RNA per lane. There is no change in the steady-state *petE* mRNA level as a function of copper available to the cell.

Discussion

In a survey of 15 species of cyanobacteria for formation of plastocyanin and cytochrome c553 Sandmann observed plastocyanin in *Synechocystis* ATCC 27178 [26]. We report conclusive evidence for the presence of plastocyanin in another strain, *Synechocystis* 6803, namely plastocyanin synthesis due to an increased level of copper in the growth media. The *petE* gene was cloned from a genomic phage library using the *Anabaena petE* gene as a probe. At the nucleotide level the 600 bp region of the *Anabaena* probe shows 62% identity to the *Synechocystis petE* gene [7]. In *Synechocystis* 6803 plastocyanin is encoded as a 126 amino acid precursor protein with a probable 29 amino acid transit sequence at the amino terminus. The transit sequence shows substantial homology to the same region of the 139 amino acid precursor plastocyanin reported for *Anabaena* 7937 [35]. In higher plants, where plastocyanin is nuclear-encoded, the transit peptide serves to direct the precursor protein across the chloroplast and thylakoid membranes. The chloroplast and thylakoid import domains of the transit peptide have been been identified using the pea chloroplast system [29]. Even though the chloroplast envelope is missing in cyanobacteria the thylakoid membrane must be traversed for plastocyanin to function as the reductant of

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                1          10         20         30         40
Synechocystis    nAtVKMGsD sGaLvFEPSt vTIKAGEeVk WvNNklsPHN IVFaaD...g
Anabaena       etytVKLGsD kGlLvFEPak lTIKpGDtVe FlNNkvpPHN VVFDatlnPA
Scenedesmus      AnVKLGaD sGaLvFEPat vTIKAGDsVT WtNNAGFPHN IVFDEDaVPA.
Chlorella       dvtVKLGaD sGaLvFEPSs vTIKAGEtVT WvNNAGFPHN IVFDEDeVP..
Entermorpha     aAiVKLGgD DGsLaFvPnn iTVgAGEsIe FiNNAGFPHN IVFDEDaVPA
Populus          vdVlLGaD DGsLaFvPSE fsVpAGEkIv FkNNAGFPHN VlFDEDaVPs
Spinacia         veVlLGgg DGsLaFlPgD fsVasGEeIv FkNNAGFPHN VVFDEDeIPs
Silene           AeVlLGss DGgLaFvPSD lsIasGEkIT FkNNAGFPHN dlFDEDeVPA
Consensus      --A-VKLG-D DG-L-FEPSD -TIKAGE--T F-NNAGFPHN IVFDED-VPA

                50         60         70         80         90         100
Synechocystis    vdadtAakLS HkgLafAaGE SFtsTFTE.. .pGTYtYYCE PHrGAGMVGK VvVe.
Anabaena         ksadlAkSLS HkqLLmsPGq StStTFpada pAGdYsFYCE PHrGAGMVGK ITVas
Scenedesmus        gVnAdaLS HDDyLNAPGE SYtakF...D TAGeYGYFCE PHQGAGMVGK ViVq
Chlorella         sganAeaLS HEDyLNAPGE SYSakF...D TAGTYGYFCE PHQGAGMkGt ITVq
Entermorpha     GVD..AdaiS aEDyLNskGq tvvrkL...t TpGTYGvYCD PHsGAGMkmt ITVq.
Populus         GVDVskiSMS eEDLLNAkGE tFeVaLsDK. ..GeYtFYCs PHQGAGMVGK ViVn.
Spinacia        GVDaakiSMS eEDLLNAPGE tYkVTLTEK. ..GTYkFYCs PHQGAGMVGK VTVn.
Silene          GVDVtkiSMp eEDLLNAPGE eYSVTLTEK. ..GTYkFYCa PHaGAGMVGK VTVn.
Consensus       GVDV-A-SLS HEDLLNAPGE SYSVT-TEKD TAGTYGFYCE PHQGAGMVGK VTV--
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Fig. 7. Alignment of amino acid sequences of plastocyanin from cyanobacteria, green algae, and chloroplasts. (1) *Synechocystis* sp. PCC 6803; (2) *Anabaena* sp. PCC 7937 [34]; (3) *Scenedesmus obliquus* [20]; (4) *Enteromorpha prolifera* [27]; (5) *Chlorella fusca* [13]; (6) *Populus nigra italica* [10]; (7) *Silene pratensis* [29]; (8) *Spinacia oleracea* [24]; (9) Consensus = consensus amino acid sequence. Consensus residues in capital letters, nonconsensus residues in lower-case letters, alignment has a gap weight = 3.0 and length weight = 0.1 [7]. Active site residues are in bold type, numbering is not based on a particular sequence.

$P700^+$. Therefore, a transit peptide or N-terminal extension is thought to direct transport of plastocyanin across the thylakoid membrane in cyanobacteria. There are striking similarities in comparing the N-terminal extensions of *Synechocystis* and *Anabaena* plastocyanin (Fig. 7), namely the pair of positively charged residues, lysines or arginines spaced eight amino acids upstream from the conserved LLVVSSF sequence which is spaced 4–5 amino acids from the putative [P-X-A-A-A] processing site. Furthermore, cleavage at this site would result in a mature protein of 97 amino acids, nearly all higher-plant plastocyanins are 99 residues, *Chlorella fusca* [14] and *Enteromorpha prolifera* [28] are 98 and *Scenedesmus obliquus* [21] is 97 residues. For purposes of comparison we are assuming glutamine is the first amino acid. Comparison of the cyanobacterial transit sequences to those available for higher plants show only a general conservation of hydrophobic and neutral amino acids with basic residues at the beginning of the thylakoid domain. Otherwise, the cyanobacterial transit sequences show little homology to the much larger higher-plant sequences which are highly conserved.

Figure 6 displays 8 mature plastocyanin sequences and a calculated consensus sequence, the protein sequences being aligned with gaps inserted to maximize similarity [7]. *Synechocystis* plastocyanin has the greatest homology to *Scenedesmus* plastocyanin [21] (61% identical amino acids), followed by *Anabaena* where 56% of the amino acids are identical. Homology to higher-plant sequences is reduced to an even greater extent with a range of 38–50% identical residues for all of the higher-plant sequences. The sequence divergence among the lower organisms is in contrast to the higher-plant sequences which are highly conserved (usually >75% similarity). Both the *Anabaena* and *Synechocystis* plastocyanin lack the so called 'acidic patch' (residues 42–45) so highly conserved among the higher-plant sequences, and thought to be important for protein recognition [2]. However, unlike the basic *Anabaena* 7937 protein, with a pI of 7.5–8.0 [12], *Synechocystis* 6803 plastocyanin has a calculated pI of 5.6 [7], which is consistent with other unicellular cyanobacteria [12]. The unusual variation in isoelectric point for plastocyanin, and cytochrome c553 which varies in a parallel manner [12] is certainly interesting from a taxonomical view point. But more to the heart of the matter is how this relates to the functioning of plastocyanin as a catalyst for electron transfer to $P700^+$. The large diversity seen in plastocyanin amino acid sequences among procaryotes and eucaryotes is in contrast to the very highly conserved amino acid sequences for the apoprotein (*psa*A and *psa*B) of the PS I reaction center [5]. This seems a bit unusual, if they are physiological partners, unless an additional polypeptide facilitates electron transfer to $P700^+$. This idea has been proposed in the past, when purified PS I particles, lacking the 20 kDa polypeptide (subunit III), were inactive in plastocyanin or cytochrome c553 mediated NADP photoreduction [1]. More recent evidence by Malkin supports this as well; his results show that plastocyanin can be covalently crosslinked to PS I particles by a single specific PS I subunit, the 19 kDa polypeptide, a protein located on the lumenal side of the membrane [39].

Along with the characteristic copper-binding residues His-38, Cys-82, His-85, Met-90, the aromatic residues at positions 30, 81, as well as Tyr 78 (3 amino acids upstream from cys),

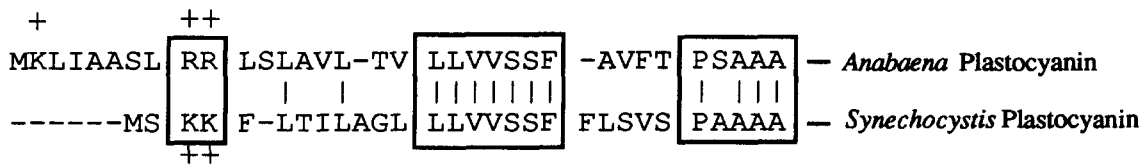

Fig. 8. Alignment of transit peptide sequences from *Anabaena* sp. PCC 7937 and *Synechocystis* sp. PCC 6803. The + indicates positively charged residues at neutral pH. Areas are boxed to show similarities. Gaps are inserted to maximize homology.

Phe 15, and Phe 42 continue to be invariant in the growing collection of plastocyanin sequences, (numbering here is based on *Synechocystis* 6803 plastocyanin). Tyrosine, phenylalanine, and tryptophan have been pinpointed because an interaction of the π-system of these residues may provide an electron transfer pathway [8].

The similarities between the plastocyanin 5′ non-coding region and putative promoter sequences for ferredoxin (Fig. 4) are particularly interesting since ferredoxin like plastocyanin is also metal cation regulated. Under iron-limiting conditions ferredoxin is replaced by flavodoxin in green algae and cyanobacteria [13]. Ferredoxin, a small (M_r 12000) iron-sulfur protein opposite plastocyanin on the stromal side of the thylakoid membrane, shuttles electrons from PS I to $NADP^+$ through the catalyst ferredoxin $NADP^+$ reductase [13]. As seen in the sequence comparison shown in Fig. 4 the TAGATA sequence 16 base pairs directly in front of the start codon is similar to the TATAAT '– 10' consensus sequence for procaryotes. This, along with the CCCGTT sequence that is positioned 16 base pairs further upstream, is similar to the putative promoter sequence reported for ferredoxin [36] differing by 2 nucleotides in each case. On the other hand, the 5′ non-coding region upstream from the *Anabaena* 7937 *petE* gene shows greater similarity to the consensus *E. coli* promoters than to the putative ferredoxin promoters. Also, in the *Synechocystis* 6803 sequence shifting the '– 10' region two base pairs upstream affords the sequence TATAGA, which closely resembles the *E. coli* '– 10' region.

It is clear from the immunoblotting results that plastocyanin accumulation in *Synechocystis* 6803 is regulated by the copper available to the cells. Total RNA isolated from the same cells used in the protein analysis shows no difference in steady-state *petE* transcript levels. It seems then that copper is a factor in the regulatory process post-transcriptionally, as it is in the green alga *Chlamydomonas reinhardtii*, where there is no change in *petE* mRNA levels as the copper concentration decreases. Plastocyanin does not accumulate under copper deficiency because the apoprotein is rapidly degraded [19]. On the other hand, cytochrome c553 is transcriptionally regulated in *Chlamydomonas* [20]. Even though the growth conditions for *Anabaena* and *Synechocystis* were not identical, the evidence suggests that the plastocyanin regulatory process is quite different in the two cyanobacteria. In *Anabaena* 7937 there is a difference in mid log phase *petE* mRNA level when grown in 1 μM versus no Cu^{+2} [35]. Yet in *Synechocystis* 6803 there is not a difference in mid log phase *petE* mRNA level in 3 μM versus 0.3 μM Cu^{+2} as present in 'normal' BG-11. Furthermore, in *Synechocystis* 6803 the effect of changing copper concentration is seen at the protein level. The data suggest an interesting paradox, the *Synechocystis* 6803 plastocyanin regulatory process and, to a lesser extent, the amino acid sequence show a greater similarity to the eucaryotic green alga than to the closer phylogenetic relative, the filamentous cyanobacterium *Anabaena*.

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