Accumulation of Pre-apocytochrome f in a Synechocystis sp. PCC 6803 Mutant Impaired in Cytochrome c Maturation*

(Received for publication, May 24, 1999, and in revised form, August 16, 1999)

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Cytochrome c maturation involves heme transport and covalent attachment of heme to the apoprotein. The 5′ end of the ccsB gene, which is involved in the maturation process and resembles the ccs1 gene from Chlamydomonas reinhardtii, was replaced by a chloramphenicol resistance cartridge in the cyanobacterium Synechocystis sp. PCC 6803. The resulting Δ(M1-A24) mutant lacking the first 24 ccsB codons grew only under anaerobic conditions. The mutant retained about 20% of the wild-type amount of processed cytochrome f with heme attached, apparently assembled in a functional cytochrome b_{6f} complex. Moreover, the mutant accumulated unprocessed apocytochrome f in its membrane fraction. A pseudorevertant was isolated that regained the ability to grow under aerobic conditions. The locus of the second-site mutation was mapped to the remaining part of ccsB. In this pseudorevertant the amount of holocyt f increased, whereas that of unprocessed apocytochrome f decreased. We suggest that the original deletion mutant Δ(M1-A24) expresses an N-terminally truncated version of the protein. The stable accumulation of unprocessed apocytochrome f in membranes of the Δ(M1-A24) mutant may be explained by its association with truncated and only partially functional CcsB protein resulting in protection from degradation. Our attempt to delete the first 244 codons of ccsB in Synechocystis sp. PCC 6803 was not successful, suggesting that this would lead to a lack of functional cytochrome b_{6f} complex. The results suggest that the CcsB protein is an apocytochrome chaperone, which together with CcsA may constitute part of cytochrome c lyase.

In c-type cytochromes, the heme group is covalently bound to the conserved CXXCH motif of the apoprotein in a process called cytochrome (cyt) c maturation (1, 2). This posttranslational process involves heme and apocytochrome transport from the cytoplasm into the periplasm in bacteria, into the intermembrane space in mitochondria, or into the thylakoid lumen in chloroplasts and cyanobacteria, followed by heme attachment to apocytochrome. Remarkably, three separate pathways of maturation of c-type cytochromes evolved among various organisms (1–5).

For Saccharomyces cerevisiae and animal mitochondria only one type of protein, the cyt c lyase, has been shown to be essential for the heme attachment step (6, 7). The lyase was shown to interact directly with both heme (8) and apocytochrome (9), and therefore it is assumed to function in the catalysis of thioether bond formation between heme and cysteine residues of apocytochrome.

In contrast to yeast mitochondria, mutational analysis of Gram-negative bacteria implicated about ten genes in assembly of both soluble and membrane-associated c-type cytochromes. In Escherichia coli, eight of the genes required for cyt c maturation are organized in an operon (10). The gene products involved can be divided into several subprocesses: (a) heme delivery, (b) transfer of reducing power from the cytoplasm to the periplasm, and (c) proper heme attachment. Bacterial c-type apocytochromes are normally synthesized with an N-terminal signal sequence that is recognized by the Sec system for protein translocation across the membrane and by the signal peptidase for processing (11).

The cytochrome c biosynthesis pathway in chloroplasts, cyanobacteria and Gram-positive bacteria is more obscure (5, 12, 13). Only three genes have been identified thus far to be required for cyt c maturation. Functionally, the pathways in chloroplasts and Gram-negative systems are very similar and cyanobacterial cytochromes can be functionally assembled in E. coli (14). However, obvious sequence similarities between the components of the two systems are limited to one putative cytochrome binding site (15) and a protein involved in transfer of reducing power across the membrane (16). In Chlamydomonas reinhardtii, the plastoplast-encoded ccsA gene (15) and a nuclear-encoded ccsI gene (17) were identified as indispensable for heme attachment. The CcsA and CcsI proteins were suggested to function together in a complex, as inactivation of either of the two genes leads to deficiency in both proteins (1, 5). In some systems heme attachment can proceed spontaneously (18–20). This implies that components of the cyt c maturation pathways may not be directly involved in the formation of thioether bonds but rather in bringing together both substrates in the right conformation and in the correct redox state so that heme attachment can occur.

This paper describes characterization of a Synechocystis sp. PCC 6803 mutant carrying a 5′ deletion of the open reading frame slr2087 (nomenclature according to Cyanobase), which is similar to ccs1 and which we have named ccsB. Moreover, an attempt is reported to inactivate a much larger portion of slr2087 as well as slr1513, coding for CcsA.
**Cytochrome c Maturation Mutant**

**TABLE I**

| Organism          | ccsA | ccsB | ccsC |
|-------------------|------|------|------|
| *Synechocystis*   | (sll1513) | (slr2087) | (sll0621, sll0686) |
| *Chlamydomonas*   | ccsA |      |      |
| *Porphyra*        | yc5  | ycf4 | ORF240 |
| *Myco bacterium*  | Rt0528 | ccsA | ccsB |
| *Bacillus*        | resC | resB | ccdA |

* Not yet identified.

**EXPERIMENTAL PROCEDURES**

**Growth Conditions**—Wild type and mutants of *Synechocystis* sp. PCC 6803 were grown in liquid BG-11 medium (21) at 30 °C at 40 μE/m²s on a rotary shaker. Strains were grown with 5 mM glucose unless phototrophic conditions are indicated. For anaerobic growth, the strains were grown in a 1% CO₂, 99% N₂ atmosphere.

**Oxygen Evolution**—Oxygen evolution was measured as described earlier (22) using a Clark-type electrode in the presence of 1 mM K₂Fe(CN)₆ and 0.1 mM dimethyl-p-benzoquinone (DMBQ) for PS II activity, or 10 mM NaHCO₃ for whole chain electron transport.

**Fluorescence Measurements**—Chlorophyll a fluorescence induction and decay were detected with a Walz pulse-amplitude-modulation fluorometer and recorded using the program FIP (QA Data, Turku, Finland). Actinic illumination came from a pulse-amplitude-modulation 102 L LED lamp. For fluorescence induction 1 μs, 2.5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) was added, with an equal volume of glass beads (90-μm diameter), and kept on ice. The cells were broken in a MiniBeadBeater. The homogenate was centrifuged in a microcentrifuge at 14,000 rpm for 10 min at 4 °C. The supernatant represents the soluble cell fraction; the pelleted thylakoids were resuspended in the original volume of thylakoid buffer.

Proteins were separated by SDS-polyacrylamide gel electrophoresis without urea. A 15% polyacrylamide gel (a 15–20% gradient gel for cytochromes) was used for detection of cyt c(ox) was used for separation; the stacking gel was 5.5% polyacrylamide. Samples were solubilized in sample buffer at room temperature for 15 min before loading on the gel. A total of 50 μg of protein was loaded per lane.

**Western Blotting and Heme Staining**—After electrophoresis, proteins were transferred to polyvinylidene fluoride membrane (Millipore, Westboro, MA) using the Bio-Rad Trans-Blot system. Blotted membranes were subjected to immunoblot analysis using antibodies against cyt f, cyt b₆, Rieske, and subunit IV (1:1,000 dilution). Bound primary antibody was detected with an alkaline phosphatase-conjugated secondary antibody.

**RESULTS**

**Gene Nomenclature**—In this paper a unified nomenclature of *ccs* (c-type cytochrome synthesis) genes will be used that is appropriate for organisms using the chloroplast/cyanobacterial pathway of cyt c maturation. A cyt c (cyt c maturation) nomenclature has been proposed for organisms using the pathway of Gram-negative bacteria (10). As shown in Table I the current nomenclature of genes involved in cyt c maturation in chloroplast, cyanobacteria, and Gram-positive bacteria is very different for different organisms. In this paper, *ccsA* will refer to the cyanobacterial homologue of the *ccsA* gene of *C. reinhardtii*; *ccsB* will denote the *Synechocystis* sp. PCC 6803 homologue of *C. reinhardtii* Ccs1, whose product is thought to form a complex with CcsA; last, *ccsC* will denote the two putative *Synechocystis* sp. PCC 6803 genes homologous to *Bacillus subtilis* ccdA, which codes for the protein suggested to be involved in the transfer of reducing power across the membrane.

**Generation of the ccsB Mutants**—The *Synechocystis* sp. PCC 6803 *ccsB* (slr2087) gene was identified by its similarity to Ccs1 from *C. reinhardtii* (38% identity at the protein sequence level). Two deletion mutants were generated. In the first mutant, a chloramphenicol resistance marker (1.5 kilobases) from pACYC184 was introduced around the translation start site of *ccsB*, replacing the region between the *Nae* I site 53-bp upstream from the translation start site and the BglII site 72-bp downstream of the translation start site of the *ccsB* gene. This mutant will be referred as Δ(M1-A24), the name reflecting the amino acids that have been deleted. In the second mutant, Δ(M1-I244), the deleted region was between the BclI sites 82-bp upstream and 732-bp downstream of the translation start site of the *ccsB* gene. Interestingly, the phenotypes of both mutants were different. The Δ(M1-A24) transformant did not segregate its wild type and mutant genome copies when grown under ambient aerobic conditions (data not shown), but segregation was obtained easily under anaerobic conditions. *Synechocystis* sp. PCC 6803 contains multiple genome copies per cell, and lack of segregation of wild-type and mutant gene copies in the presence of high concentrations of a selectable marker usually indicates that the presence of the particular gene that is attempted to be deleted is indispensable for cell survival. Segregation of the Δ(M1-A24) mutant was checked by PCR (Fig. 1). However, we were unable to segregate the Δ(M1-I244) mutant in both aerobic and anaerobic conditions (data not shown). However, the partially segregated Δ(M1-I244) mutant also appeared to be oxygen sensitive. As the Δ(M1-I244) mutant did not segregate, the corresponding transformant was not further characterized.

**Δ(M1-A24) Mutant Characterization**—Consistent with the conditions necessary for segregation, the Δ(M1-A24) mutant depended on anaerobic conditions for growth. As shown in Table II, under anaerobic conditions the Δ(M1-A24) mutant grew photoautotrophically at a rate about two-thirds that of wild type. Interestingly, the Δ(M1-A24) mutant grew in air if PS II was deleted or inhibited by atrazine (Table II). When exposed to oxygen, the Δ(M1-A24) mutant cultures
TABLE II
Aerobic and anaerobic growth of wild type and the Δ(M1-A24) mutant in the presence or absence of PS II

| Strain                  | Growth conditions | PA      | PM/PH | aerobic | anaerobic |
|-------------------------|-------------------|---------|--------|---------|-----------|
| Wild type               |                   | 12.7 ± 1.8 | 9.2 ± 1.5 | 13.3 ± 1.5 | 9.6 ± 0.8 |
| Δ(M1-A24)              |                   | 18.6 ± 3.0 | 10.5 ± 2.0 | ∞        | ∞         |
| Δ(M1-A24)/PS II-less   |                   | ∞        | 18.2 ± 2.6 | 21.5 ± 2.9 |
| PS II-less              |                   | ∞        | 17.5 ± 2.3 | 16.8 ± 2.1 |
| Δ(M1-A24) + atrazine    |                   | ∞        | ND*     | 19.7 ± 2.5 |

* ND, not determined.

TABLE III
Photosynthetic electron transport in wild type and the Δ(M1-A24) mutant

| Strain                  | Oxygen evolution (μmol O₂/mg chl h⁻¹) |
|-------------------------|--------------------------------------|
|                         | (H₂O to DMBQ) | Whole chain (H₂O to CO₂) |
| Wild type               | 400        | 380 |
| Δ(M1-A24)              | 300        | 65  |

continued to grow for about 20 h, followed by an inhibition of further growth (data not shown). The growth rate of wild-type cells was similar in both aerobic and anaerobic conditions (Table II). However, after several days of exposure to oxygen, the mutant culture started to grow again when it was returned to anaerobic conditions. Therefore, oxygen did not kill the Δ(M1-A24) mutant strain, but it prevented its growth.

Inactivation of the ccsB homologue in C. reinhardtii led to a nonphotosynthetic phenotype and to a cyt b₆f deficiency (17). Therefore, it was important to determine whether the Δ(M1-A24) mutant of Synechocystis sp. PCC 6803 is also impaired in photosynthetic electron transport through the cyt b₆f complex. For this reason, oxygen evolution rates of the Δ(M1-A24) mutant and wild type were measured, and the rate of electron transport through PS II to the artificial electron acceptor DMBQ (which can oxidize the plastoquinone pool) was compared with that of whole-chain electron transport to CO₂ (Table III). The two rates were similar for wild type, but the rate of whole-chain electron transport in the Δ(M1-A24) mutant was about 5-fold lower than electron transfer involving only PS II. This indicates that photosynthetic electron transport was blocked beyond PS II and is consistent with an inhibition at the level of the cyt b₆f complex.

A similar conclusion was drawn from chlorophyll fluorescence induction and decay measurements in the Δ(M1-A24) mutant. Chlorophyll fluorescence increases with reduction of Qₐ in PS II, and reduced Qₐ is oxidized by the plastoquinone pool. If photosynthetic electron transport is blocked at or beyond the cyt b₆f complex, oxidation of the plastoquinone pool would be expected to be slower and consequently Qₐ oxidation after illumination should be slower as well. Indeed, after illumination variable fluorescence in the Δ(M1-A24) mutant decayed with a half-time about 4-fold slower than in wild type, suggestive of slower electron flow out of the plastoquinone pool (Fig. 2A). Fluorescence induction was faster in the mutant than in wild type, indicative of a more rapid net reduction of the plastoquinone pool in the mutant upon illumination (Fig. 2, B and C). In the presence of 1 μM DBMIB (a cyt b₆f complex inhibitor), the induction curve of the wild type was very similar to that of the mutant with or without DBMIB, whereas in the mutant, DBMIB barely affected the fluorescence induction curve (Fig. 2, B and C). These data are fully consistent with inhibition at the level of the cyt b₆f complex in the Δ(M1-A24) mutant.

Processing of cyt f in the Δ(M1-A24) Mutant—To determine the accumulation of c-type cytochromes and the cyt b₆f complex in the Δ(M1-A24) mutant more directly, the amount of cyt b₆f complex subunits in thylakoid membranes was monitored using Western blotting and heme staining. In cyanobacteria, several c-type cytochromes are localized on the luminal side of the thylakoid membrane, including cyt f, cyt c₅₅₃, which is a soluble electron carrier between cyt b₆f complex and PS I, and cyt c₅₅₃, a PS II luminal protein. Unlike in C. reinhardtii, in cyanobac-
The cyt $b_{5f}$ complex appears to be indispensable, possibly because of its role in respiration, making a cyanobacterial mutant completely deficient in this complex unlikely to survive. The $\Delta$(M1-A24) mutant was found to have significantly decreased amounts of cyt $f$, cyt $b_6$, and cyt $c_{553}$ in its thylakoids (Fig. 3).

Surprisingly, in the mutant, a band with decreased mobility was recognized by the cyt $f$ antibody; we assign this band to unprocessed pre-apocyt $f$. The observed 2–3-kDa difference is consistent with the upper band being the unprocessed protein. This difference in electrophoretic mobility is similar to that between C. reinhardtii pre-apocyt $f$ and apocyt $f$ (24). Even though the upper band accumulated to high levels in thylakoid membranes of the $\Delta$(M1-A24) mutant (Fig. 3), this band was not visible upon heme staining. Therefore, this band does not covalently bind heme. However, the lower band in the mutant corresponding to processed cyt $f$ (about 20% of the wild-type amount) stained with heme at an intensity corresponding to the amount of processed cyt $f$ in the membrane (Fig. 3). This is consistent with the 5-fold reduction in linear electron flow (Table III) and suggests that most or all of the processed cyt $f$ in the mutant carries covalently attached heme.

The other cyt $b_{5f}$ complex subunits, including cyt $b_6$, accumulated to about 20% of the amount in wild type (Fig. 4; note that for wild type, 5-fold less thylakoids were loaded than for the $\Delta$(M1-A24) mutant). In the range of protein concentrations used the immunoreaction observed is approximately proportional to the amount of antigen (not shown). Therefore, the amount of cyt $b_6$, Rieske, and subunit IV polypeptides is proportional to the amount of processed holocyt $f$ and the amount of functional cyt $b_{5f}$ complex. This implies that the unprocessed pre-apocyt $f$ is not part of the cyt $b_{5f}$ complex (Fig. 3).

As the $\Delta$(M1-A24) mutant ceased to grow in ambient air conditions, we investigated whether the accumulation of the cyt $b_{5f}$ complex changed upon exposure of the mutant strain to air. The amounts of the cyt $b_{5f}$ subunits remained stable for the first 40 h of the experiment (Fig. 4), long after the cells had ceased to grow. Protein degradation was obvious only after 70 h in aerobic conditions (Fig. 4).

Plastocyanin Is Indispensable in the $\Delta$(M1-A24) Mutant—As the $\Delta$(M1-A24) mutant accumulated some processed cyt $f$ and cyt $c_{553}$ with heme bound, it was interesting to determine whether cyt $c_{553}$ is properly synthesized as well. Cyt $c_{553}$, together with plastocyanin, shuttles electrons from the cyt $b_{5f}$ complex to PS I in Synechocystis sp. PCC 6803. Fig. 5 indicates that in our growth conditions the level of holocyt $c_{553}$ in wild type is very low. However, upon deletion of the plastocyanin gene ($petE$) the cyt $c_{553}$ level increased dramatically. Interestingly, after transformation of the $\Delta$(M1-A24) mutant with a $petE$ deletion construct (25) the resulting $petE$ deletion transformants did not segregate and no holocyt $c_{553}$ was apparent in the partially segregated mutant (Fig. 5). This suggests that, unlike holocyt $f$ (Fig. 3) and cyt $c_{553}$, the $\Delta$(M1-A24) mutant is unable to make holocyt $c_{553}$.

Supplementation of the $\Delta$(M1-A24) Mutant with Heme and/or Reductant—Accumulation of the unprocessed pre-apocyt $f$ that lacked covalently bound heme raised the possibility that the $\Delta$(M1-A24) mutant may be deficient in heme transport and reduction. This would be similar to the situation in yeast mitochondria, where heme-deficient mutants accumulate pre-apocyt $c_1$ in vivo (26) and where attachment of the reduced heme was necessary for processing of the cyt $c_1$ in vitro (27, 28). To determine whether the $\Delta$(M1-A24) phenotype could be overcome in part by supplementation of extra heme and/or reductant, $\Delta$(M1-A24) cells were grown on plates with 100 $\mu$g of protein/lane was loaded on a polyacrylamide gel (10 $\mu$g for wild type). Proteins were identified immunologically.

**Pseudorevertants**—Second-site mutants (pseudorevertants) were selected in which the $\Delta$(M1-A24) mutant phenotype was suppressed at least partially. Several pseudorevertants that were able to grow in air were selected. During the initial screening, most of the pseudorevertants were found to be deficient in PS II activity, which is consistent with the data that were presented in Table II. However, one pseudorevertant was phototrophic with about 50% of the wild type growth rate under aerobic conditions and with 70% of the whole chain electron transport rate of wild type (data not shown). This pseudorevertant showed an increased accumulation of holocyt $f$ (Fig. 6A) and a decreased but still detectable level of pre-apocyt $f$ (Fig. 6B). Chromosomal DNA from this pseudorevertant complemented the original $\Delta$(M1-A24) strain, using growth at ambient oxygen levels as a selection criterion. Functional complementation was used to localize the second-site mutation. The complementing PCR fragment contained a single point mutation in the noncoding region of the chloramphenicol resistance cassette in between the resistance marker and the remainder of the $ccsB$ gene (Fig. 7A). The mutation (T to G)
transformants with a deletion at the petE locus did not segregate. Identified by heme staining using enhanced chemiluminescence, the soluble fraction was loaded and cytochromes were identified by heme staining using enhanced chemiluminescence.

Unlike for the ccsA mutant, the ccsB gene coding for the first 24 amino acid residues of the corresponding protein. The Δ(M1-A24) mutant grew photoautotrophically in anaerobic conditions, indicating the presence of a functional cyt b₅f complex. It exhibited about 20% of the wild-type rate of whole chain electron transport, demonstrating that in Synechocystis sp. PCC 6803 the introduced ccsB partial deletion does not lead to a total lack of the cyt b₅f complex. The oxygen evolution and fluorescence induction and decay measurements (Table III, Fig. 2) were consistent with impairment of photosynthetic electron transport beyond the plastoquinone pool in the Δ(M1-A24) mutant. Consistent with a decreased rate of whole chain electron transport, the Δ(M1-A24) mutant accumulated about 20% of holocytf. In addition, the amount of cyt e₅₅₀ was very much reduced in the Δ(M1-A24) mutant whereas cyt e₅₅₃ was undetectable (Fig. 5), indicating that the mutant is impaired in a pathway common to c-type cytochromes.

Stability of Pre-apocytf—The observation that unprocessed pre-apocytf accumulated to high levels in the thylakoid membrane of the Δ(M1-A24) mutant is unparalleled in other cyt maturation pathways studied. Apocytf usually is much less stable than its holo-form because of the presence of a periplasmic/luminal degradation system that removes nonfunctional proteins (24, 30). The situation observed in the Synechocystis sp. PCC 6803 Δ(M1-A24) mutant is quite different from that in ccs2aA null mutants of C. reinhardtii. Although in this alga only the ccsA mutant has been characterized thoroughly, the CcsA and CcsB homologues require each other for accumulation in vitro (1, 5), and the ccsB mutant is expected to have the same phenotype as the ccsA mutant. In the ccsA mutant of C. reinhardtii both apocytf and apocytf e₅₅₃ were processed and very unstable with half-lives of about 10 min, suggesting that processing does not depend on heme attachment in this system (24, 31). The fact that the pure Δ(M1-I244) ccsB mutant as well as the ccsA mutant could not be obtained in Synechocystis sp. PCC 6803 suggests a lethal phenotype of these mutations. This
corroborates the view that $\Delta$(M1-A24) is not a null mutant for ccsB. The accumulation of unprocessed pre-apocyt $f$ without heme bound in the $\Delta$(M1-A24) mutant of Synechocystis sp. PCC 6803 suggests that the mutant is impaired in both processing and heme attachment. The fact that in the $\Delta$(M1-A24) mutant (a) about 20% of the protein was processed, contained heme, and was assembled into a functional cyt $b_{6}f$, complex, and (b) no heme binding to the unprocessed pre-apocyt $f$ was detected, indicate that processing and heme attachment are closely coordinated in Synechocystis sp. PCC 6803.

The Role of CcsB—Important insight regarding the function of the CcsB protein and the phenotype of the $\Delta$(M1-A24) mutant was obtained from characterization of the photoautotrophic pseudorevertant, functionally complementing the noncoding region of the chloramphenicol resistance marker upstream of the truncated ccsB gene, and led to the formation of a new putative start codon. This stretch of nucleotides contains several possible start codons and several possible ribosome binding sites in the original $\Delta$(M1-A24) mutant (Fig. 7A) raising the possibility that a shorter and modified form of CcsB and was assembled into a functional cyt $b_{6}f$, complex, indicating that processing and heme attachment are closely coordinated in Synechocystis sp. PCC 6803.

We hypothesize that the reason for accumulation of pre-apocyt $f$ in the $\Delta$(M1-A24) mutant is protection of the apocytochrome and/or heme against oxidation. Interestingly, a potential functional analog of CcsB from Rhodobacter capsulatus, Ccl2, accumulates to 20-fold-higher levels when grown aerobically than when under anaerobic conditions (31). Ccl2 is viewed as a protein reducing apocyt $c$ and protecting it against oxidation, which may explain its accumulation when oxygen is present.

A CcsA-CcsB complex is necessary for cyt $c$ maturation in chloroplasts and cyanobacteria. CcsA, which has a putative heme binding site on the luminal side of the thylakoid membrane (15, 32), may function as a heme transporter/coordinator. We suggest that CcsB serves as an apocytochrome chaperone, which binds pre-apocyt $f$ and facilitates its processing by signal peptidase and heme attachment by CcsA. Both processing and heme attachment may take place in the CcsA-CcsB complex; both processes must be tightly coordinated as the N-terminal amino acid of the processed cyt $f$ serves as a heme ligand. We propose that the CcsA-CcsB complex is the site of heme attachment, with CcsB binding the apoprotein and with CcsA binding a heme and facilitating its linkage to apocytochrome.

Acknowledgments—We thank Dr. Alice Barkan, University of Oregon, and Dr. Richard Malkin, University of California, Berkeley for providing us with the antibodies used in this study.

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