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Cytochrome c-553 Is not Required for Photosynthetic Activity in the Cyanobacterium *Synechococcus*

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In cyanobacteria, the water-soluble cytochrome c-553 functions as a mobile carrier of electrons between the membrane-bound cytochrome b_6-*f* complex and P-700 reaction centers of Photosystem I. The structural gene for cytochrome c-553 (designated *cytA*) of the cyanobacterium *Synechococcus* sp. PCC 7942 was cloned, and the deduced amino acid sequence was shown to be similar to known cyanobacterial cytochrome c-553 proteins. A deletion mutant was constructed that had no detectable cytochrome c-553 based on spectral analyses and tetramethylbenzidine-hydrogen peroxide staining of proteins resolved by polyacrylamide gel electrophoresis. The mutant strain was not impaired in overall photosynthetic activity. However, this mutant exhibited a decreased efficiency of cytochrome *f* oxidation. These results indicate that cytochrome c-553 is not an absolute requirement for reducing Photosystem I reaction centers in *Synechococcus* sp. PCC 7942.

INTRODUCTION

Cytochrome *c*-553 functions as a mobile carrier of electrons between the membrane-bound cytochrome b_6-*f* complex and the P-700 reaction center of Photosystem I (PSI) in cyanobacteria and many eukaryotic algae. This iron-heme protein is a distinct subgroup of a family of cytochromes that includes the *c*-type soluble cytochromes in mitochondria, mobile cytochrome *c*-555 in anoxygenic green photosynthetic bacteria, and cytochrome c_2 from purple photosynthetic bacteria (Dickerson, 1980). Cyanobacterial *c*-type cytochromes are water soluble, contain approximately 90 amino acid residues (Aitken, 1976), and have high redox potentials of between 350 mV and 400 mV (Sugimura et al., 1968). In many cyanobacteria, cytochrome *c*-553 appears to be the only species that shuttles electrons from the cytochrome b_6-*f* complex to P-700; however, in some species either plastocyanin or both plastocyanin and cytochrome *c*-553 can serve this function (Sandmann and Boger, 1980).

Cytochrome *c*-553 has a function in photosynthetic electron transport similar to that of plastocyanin in higher plants. Plastocyanin transfers electrons via the reversible oxidation (Cu^{2+}) and reduction (Cu^{+}) of its active copper center (Ho and Krogmann, 1982). Similarly, the bound iron of cytochrome *c*-553 undergoes reversible oxidation-reduction reactions during photosynthetic electron transport.

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In some cyanobacteria, such as *Anabaena variabilis*, the cytochrome b_6-*f* complex is equally active in reducing cytochrome *c*-553 and plastocyanin in vitro (Krinner et al., 1982). Both of these redox proteins have been demonstrated in vivo in some algal and cyanobacterial species; the relative ratio of the two depend upon the availability of copper in the growth medium (Wood, 1978). In the green alga *Chlamydomonas reinhardtii*, plastocyanin accumulation is prevented in copper-deficient cells by specific and rapid degradation of constitutively synthesized apoplastocyanin (Merchant and Bogorad, 1986b). Conversely, cytochrome *c*-552 (analogous to cytochrome c-553 in cyanobacteria) accumulates in cells starved for copper as a consequence of increased levels of mRNA for pre-apocytochrome *c*-552 (Merchant and Bogorad, 1986a, 1987).

Cytochrome *c*-553 can be the physiological electron donor for respiratory as well as photosynthetic electron transport. Both photosynthetic and respiratory electron transport can be inhibited by the addition of antibodies raised to cytochrome c-553 (Alpes et al., 1984). A functional role for this cytochrome in respiratory and photosynthetic electron transport was established by experiments in which cytochrome *c*-553-depleted membranes of *Nostoc muscorum* were made competent in transferring electrons to PSI and cytochrome oxidase by the addition of the cytochrome (Sturzl et al., 1982). In contrast, native cytochrome *c*-553 from *Anacystis nidulans* could only do-

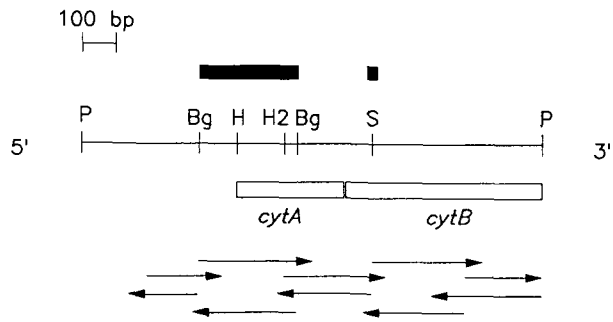

Figure 1. Restriction Map and Sequencing Strategy of the 1.4-kb PstI Clone Containing the Cytochrome *c*-553 Gene from *Synechococcus* sp. PCC 7942.

Arrows below the restriction map indicate the direction and extent of DNA that was sequenced. The cytochrome *c*-553 gene is designated *cytA*, and an unidentified open reading frame initiating immediately after the termination codon of *cytA* is designated *cytB*. The filled boxes above the restriction map indicate the regions of DNA deleted and replaced with a spectinomycin cassette in making deletion mutations in the *cytA* and *cytB*. Restriction enzymes are: P, PstI; Bg, BglII; H, HindIII; H2, HincII; S, SmaI.

nate electrons to PSI and not cytochrome oxidase (Peschek and Schmetterer, 1982).

Extensive amino acid sequence information and a detailed structural analysis of *c*-type cytochromes have helped establish evolutionary relationships between prokaryotic and eukaryotic organisms (Dickerson, 1980; Ludwig et al., 1982; Meyer and Kaman, 1982). The use of the *c*-type cytochromes as a paradigm for protein evolution has resulted in the accumulation of primary structural information for cytochrome *c*-553 from several cyanobacterial species (Ulrich et al., 1982). The complete amino acid sequence of this cytochrome from *A. nidulans* has been reported (Sprinkle et al., 1986). Although the presence of plastocyanin in this cyanobacterium has been suggested on the basis of electron paramagnetic resonance signals (Visser et al., 1974), plastocyanin has not been detected by either immunological (Aitken, 1976) or heterologous gene hybridization techniques (Van der Plas et al., 1989). Therefore, it is assumed that *A. nidulans* belongs to a class of cyanobacteria (which includes *N. muscorum* and *Spirulina platensis*) in which cytochrome *c*-553 is the sole mobile electron carrier capable of reducing P-700 (Sandmann and Boger, 1981).

Here we report the isolation and characterization of the gene encoding pre-apocytochrome *c*-553 from the unicellular, naturally transformable cyanobacterium *Synechococcus* sp. PCC 7942. The gene encodes a polypeptide of 111 amino acids with a probable 24-residue NH_2-terminal signal peptide. Transcriptional studies indicate that the expression of the gene is not regulated by Cu^{2+} concentration. As a first approach to the analysis of the functional properties of cytochrome *c*-553, constructs were designed that enabled us to inactivate the *cytA* gene in vivo. Surprisingly, a mutant strain lacking this cytochrome exhibits little difference from wild-type cells in its overall photosynthetic activity when grown in saturating light. However, electron flow from the cytochrome b_6-*f* complex to P-700 is less efficient in the mutant strain, especially under non-saturating light conditions.

RESULTS

Cloning and Sequence Analysis of the *Synechococcus* sp. PCC 7942

The amino acid sequences for cyanobacterial cytochromes *c*-553 are very similar; approximately 35% of the residues are invariant (Sprinkle et al., 1986). From the amino acid sequence for the *A. nidulans* cytochrome *c*-553, we designed three sets of mixed oligonucleotides for use as hybridization probes. The oligonucleotides were complementary to the coding strand of the structural gene between amino acids 37 to 42, 47 to 57, and 53 to 59. These regions were selected for their limited codon redundancy or their high degree of conservation. All possible coding sequences were not included in the population of probes to limit the complexities of the mixtures.

In DNA gel blot hybridization, all three synthetic oligonucleotides hybridized to a 1.4-kb PstI fragment of *Synechococcus* sp. PCC 7942 genomic DNA. Three hybridizing clones were isolated from a PstI genomic library and one of these, pCCP145, contained a single insert of 1.4 kb.

The 1.4-kb PstI insert was cloned into M13mp19 and sequenced. A restriction map of the 1.4-kb PstI fragment and a strategy for sequencing the *cytA* gene are shown in Figure 1. The nucleotide sequence of the *cytA* gene from *Synechococcus* sp. PCC 7942 is shown in Figure 2. The predicted amino acid sequence is highly homologous to that of the *A. nidulans* cytochrome *c*-553 except for the presence of a 24-amino acid extension on the amino terminus of the *Synechococcus* sp. PCC 7942 protein. Based on amino-terminal sequences of other cyanobacterial cytochrome *c*-553 polypeptides, the mature protein probably begins at Ala-25 and comprises 87 amino acids; the calculated molecular weight of the mature protein is 8935 with an estimated pI of 5.7. The sequence AAG, complementary to the 3′ end of the 16S rRNA (Tomioka and Sugiura, 1983), is 12 bases upstream from the initiator methionine codon. Immediately downstream of the *cytA* gene termination codon, an unidentified open reading frame (designated *cytB*) is initiated and continues through the remainder of the 1.4-kb PstI clone and into an adjacent PstI restriction fragment. This potential protein contains at least 188 amino acid residues and shows no strong sequence homology to any known proteins.

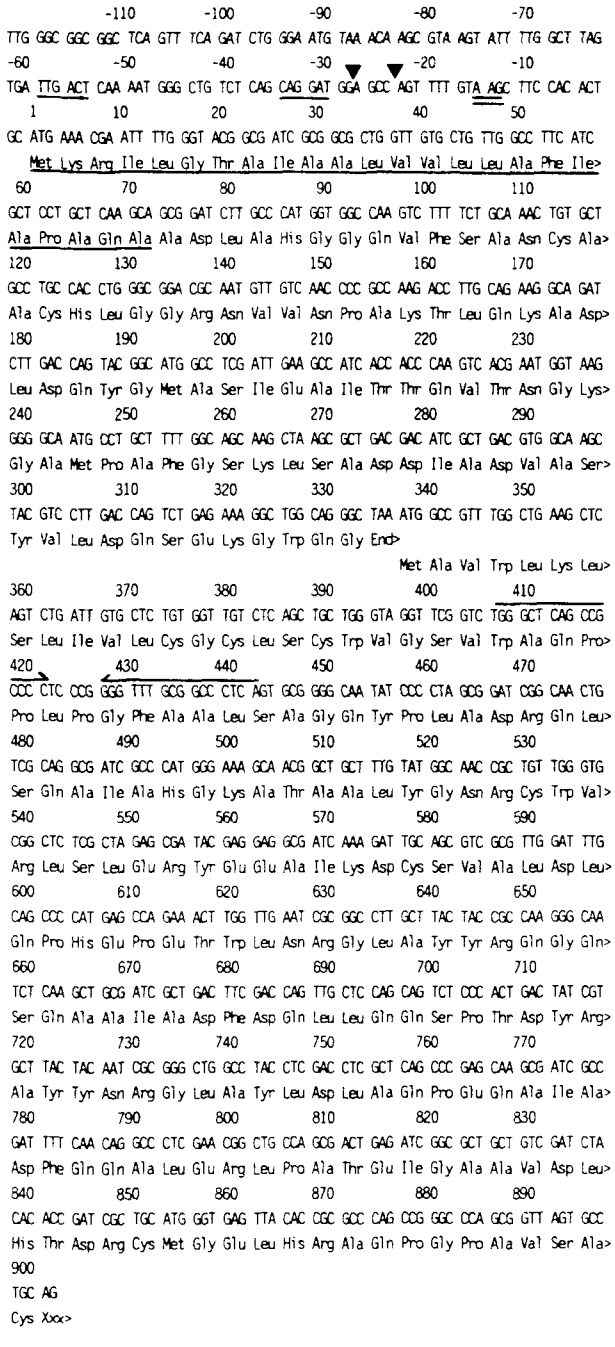

Figure 2. Nucleotide Sequence of the *cytA* and *cytB* of *Synechococcus* sp. PCC 7942 and Deduced Amino Acid Sequences of the Proteins.

A putative ribosome binding site is doubly underlined. A stem and loop structure that may function as a *cytA* transcription terminator/stabilizer is underlined by arrows. Sequences located in the putative −10 and −35 regions of the promoter are underlined. The vertical arrowheads indicate the potential sites of transcription initiation. The amino acids composing the cytochrome *c*-553 signal sequence are underlined. The presumed initiation codon of *cytB* is at residue 337.

Gene Copy Number

Electrophoretically distinct species of cytochrome *c*-553 have been identified in a number of naturally isolated cyanobacteria, suggesting the existence of multiple *cytA* genes (Ho and Krogmann, 1984). To examine this possibility, the 1.4-kb PstI fragment containing *cytA* was used to probe digests of *Synechococcus* sp. PCC 7942 genomic DNA at low stringency (hybridization in 6×SSC at 54°C, washing in 5×SSC, 0.1% sodium dodecyl sulfate at room temperature). The hybridization results showed one signal for each restriction enzyme digest, suggesting that only a single copy of *cytA* is present in the *Synechococcus* sp. PCC 7942 genome (results not shown). These results do not rule out the existence of a divergent gene copy encoding an isozyme of cytochrome *c*-553 that was not detected under the conditions of hybridization described above.

Transcript and Promoter Analysis

The cytochrome *c*-553 content of some cyanobacteria and eukaryotic algae is tightly regulated by the availability of copper to the cell (Sandmann, 1986; Merchant and Bogorad, 1987). In the green alga *C. reinhardtii*, cytochrome *c*-552 mRNA is only detectable in copper-deficient cells. Hence, total RNA from *Synechococcus* sp. PCC 7942 cells grown in media with and without copper was examined for the presence of the *cytA* transcript. As shown in Figure 3, the *cytA* transcript is not very abundant in *Synechococcus* sp. PCC 7942 and was detected only after lowering the stringency of the hybridization conditions. A 280-bp BglII fragment with part of the *cytA* gene hybridized to a 480-base transcript of RNA extracted from both cells grown in the presence or absence of copper. Thus, the amount of copper present during growth does not appear to affect the relative accumulation of the *cytA* transcript.

A gene-specific *cytB* fragment appeared to hybridize to a 1200-base transcript, although the signal was very weak and required twice the exposure time (Figure 3). This signal was also detected with the *cytA* gene-specific probe. Because the signal was both weak and diffuse (it extends from 500 bases to 1200 bases), the 1200-base transcript is probably being rapidly degraded. This degradation seemed specific because a DNA fragment encoding ferredoxin (Reith et al., 1986) hybridized to a transcript of approximately 450 bases that was not degraded. RNA gel blot hybridization with DNA fragments beginning 100 bp upstream of the *cytA* gene failed to hybridize to the mRNA (results not shown).

The 5′ end of the *cytA* transcript was located by primer extension and mung bean nuclease protection experiments. The primer extension experiments, shown in Figure 4, localized the 5′ end to position −23, whereas the mung bean nuclease protection experiments resulted in the generation of protected fragments that began at both positions −23 and −27 (results not shown). Hence, transcription initiation may occur at position −27 or at both −23 and

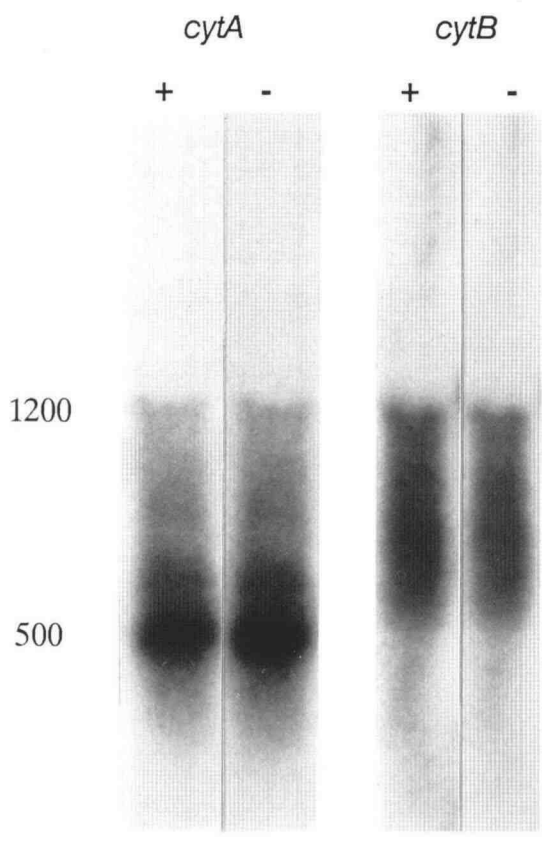

Figure 3. RNA Gel Blot Hybridization Analysis of *Synechococcus* sp. PCC 7942 RNA from Cells Grown with (+) and without (−) Cu^{2+}.

Total RNA was denatured, electrophoresed, transferred to nitrocellulose, and hybridized to gene-specific probes for *cytA* and *cytB*. The size estimate of the transcripts is in nucleotides and is derived from RNA size markers.

−27. Consistent with this conclusion was the presence of the sequence TTGACt in the −35 region that is very similar to the *Escherichia coli* consensus sequence (TTGACA). There is no sequence in the −10 region similar to the TATAAT sequence of *E. coli* (Rosenberg and Court, 1979). Approximately 80 bp downstream from the *cytA* gene termination codon is a palindromic sequence (Figure 2; nucleotide 406 to 442) that may serve to terminate transcription or stabilize the transcript. If the *cytA* transcript terminates at the end of the stem-loop structure (position 442 in Figure 2), the length of the mRNA would be approximately 470 nucleotides, which is close to the size estimated from RNA gel blot hybridizations.

Insertional Inactivation

To determine whether *cytA* and *cytB* are essential for photosynthetic activity, we used cartridge mutagenesis to construct in vitro deletion or insertion derivatives of the clone pCCP145 in which *cytA* and *cytB* genes were interrupted by a gene that confers resistance to spectinomycin (Figure 1). These constructs were used to transform wild-type cells of *Synechococcus* sp. PCC 7942 to spectinomycin resistance. Because the constructs were cloned in pUC9, a plasmid that cannot replicate in *Synechococcus* sp. PCC 7942, spectinomycin resistance must result from integration of the plasmid DNA into the cyanobacterial genome. The resulting Sp^R transformants were screened for sensitivity to ampicillin, a phenotype indicative of a double, homologous recombination that would replace the wild-type *cyt* genes with the interrupted sequences (Golden et al., 1986). Ampicillin-resistant transformants, probably the result of a single recombinational event, were not analyzed further.

After the purification of homozygous mutants (Williams, 1988), genomic DNA was isolated, digested with various restriction enzymes, and analyzed by DNA gel blot hybridizations to pCCP145. The restriction fragments of genomic DNA from the two mutant strains were the sizes expected from a double, homologous recombination event. As shown in Figure 5, the $cytA^-$ and $cytB^-$ mutant strains hybridized to 3.1-kb and 3.4-kb PstI restriction fragments, respectively. Wild-type copies of the interrupted DNA were not detected, indicating that both of the mutants had segregated to pure, stable cultures. At low cell densities under nonsaturating light conditions, the $cytA^-$ mutant grew only slightly slower than the wild-type strain (results not shown).

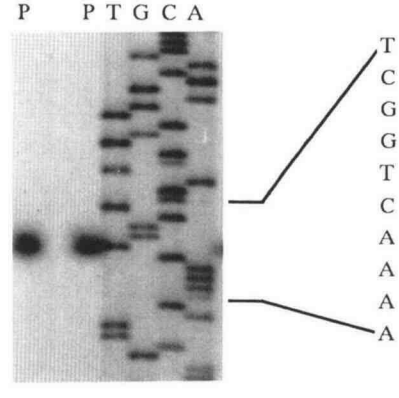

Figure 4. Identification of the Start Site for the *cytA* Transcript.

A synthetic oligonucleotide complementary to the 5′ end of the *cytA* coding region was used in a primer extension assay with total RNA. The primer-extended products (P) were electrophoresed in parallel with a sequencing ladder (A,C,G,T) generated by using the same primer on an appropriate M13 recombinant clone.

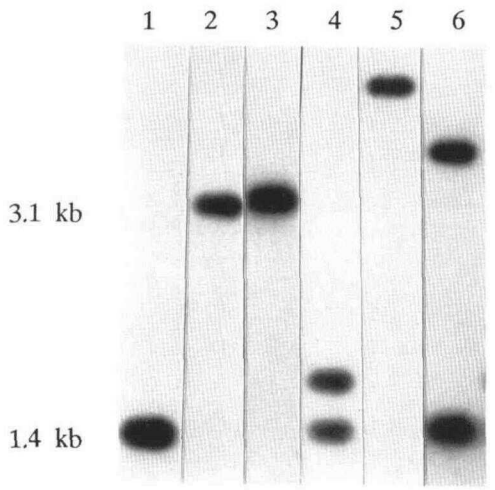

Figure 5. DNA Gel Blot Hybridizations Confirming that the Recombinant Clones Containing the Inactivated *cytA* and *cytB* Genes Had Integrated into *Synechococcus* sp. PCC 7942 by a Double Recombination Event.

Lanes 1 and 4 contain wild-type genomic DNA, lanes 2 and 5 contain *cytA*$^-$ mutant DNA, and lanes 3 and 6 contain *cytB*$^-$ mutant DNA. Lanes 1 through 3 were digested with PstI, whereas lanes 4 through 6 were digested with HincII. The probe was the 1.4-kb PstI fragment from pCCP145. Size markers are in kilobases.

Analysis of Mutant Phenotype

Each of the mutants was tested for the presence or absence of the cytochrome c-553 polypeptide. Cytochrome c-553 can be identified as a band on polyacrylamide gels by 3,3′,5,5′-tetramethylbenzidine (TMBZ) and hydrogen peroxide to localize heme-dependent peroxidase activity. Analysis of soluble protein extracts enriched for cytochrome c-553 showed that both wild-type and the *cytB*$^-$ mutant contained both cytochromes c-553 and c-550. In contrast, as shown in Figure 6A, the *cytA*$^-$ mutant lacked cytochrome c-553 but still contained cytochrome c-550.

Recently, Serrano et al. (1989) have used immunocytochemical techniques to localize cytochrome c-553 to the periphery of the cells and to thylakoid membranes in the cyanobacterium *Anabaena variabilis*. Because the *cytA* gene appeared to encode a signal sequence, it seemed likely that cytochrome c-553 was either located exclusively in the lumen of the thylakoid membranes or in both the thylakoid lumen and periplasmic space (where it might have some function in respiratory electron transport). To determine whether it was present in the latter compartment, both wild-type and mutant strains were subjected to conditions of osmotic shock, and the periplasmic proteins released were analyzed by polyacrylamide gel electrophoresis. As shown in Figure 6A, osmotic shock treatment released cytochrome c-553 from wild-type cells; it was not detected in the population of osmotic shock proteins of the *cytA*$^-$ mutant.

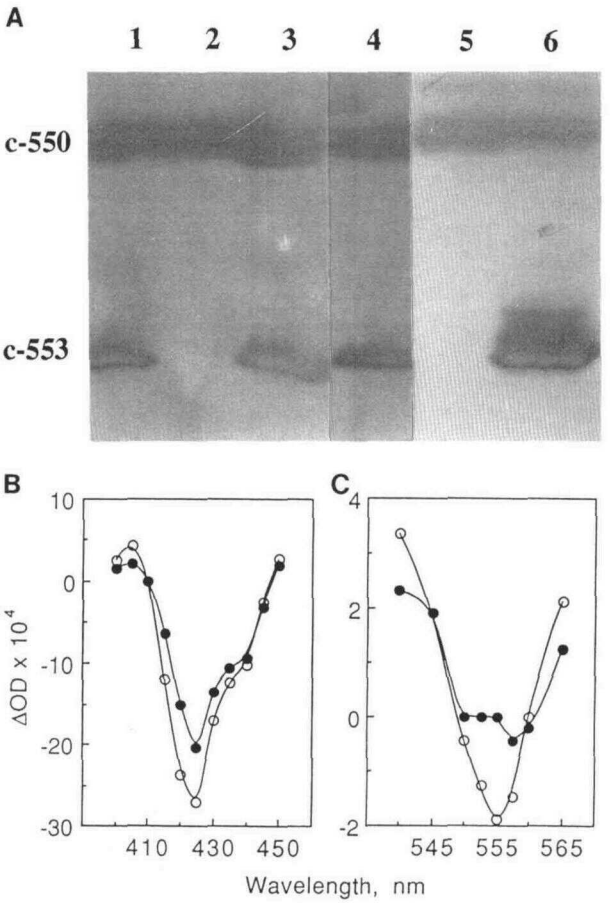

Figure 6. Absence of Cytochrome c-553 in the *cytA*$^-$ Mutant.

(A) Analysis of wild-type and *cyt* mutants for the presence of cytochrome c-553. Total soluble proteins enriched for cytochrome c-553 (lanes 1 to 3) and osmotic shock extracts (lanes 4 to 6) were analyzed for cytochrome content by electrophoresis on a polyacrylamide gel followed by TMBZ-H_2O_2 staining. Proteins isolated from the wild-type strain are in lanes 1 and 4, the *cytA*$^-$ mutant in lanes 2 and 5, and the *cytB*$^-$ mutant in lanes 3 and 6. Cytochrome c-553 and c-550 are designated c-553 and c-550, respectively.

(B) Difference spectra of the light-induced absorption changes in the γ band region of the wild-type strain (○) and the *cytA*$^-$ mutant (●).

(C) Difference spectra of the light-induced absorption changes in the α band region of the wild-type strain (○) and the *cytA*$^-$ mutant (●). Actinic flash intensity was 410 W m^{-2}. The extent of the absorption change was measured at approximately 10 msec after the onset of the actinic flash.

Spectra of light-induced absorption changes in whole cells of wild-type and the *cytA*$^-$ strain are shown in Figures 6B and 6C. The *cytA*$^-$ mutant has an altered profile in the 420-nm and 553-nm range. Both of these changes are consistent with the absence of cytochrome *c*-553, which has a sharp γ band at 416 nm and an extensive asymmetrical α band absorption in the 552-nm to 554-nm region (Bohme et al., 1980). At 434 nm, the absorbance change was very similar in the mutant and wild-type strains, indicating similar quantities of oxidizable P-700 (Nanba and Katoh, 1983). Because the samples were diluted to equal chlorophyll concentrations for these experiments, and in cyanobacteria, chlorophyll is associated primarily with PSI (Gantt, 1986), both mutant and wild-type strains appear to have similar sizes and numbers of PSI.

To determine the effect of inactivating the *cytA* gene on photosynthetic electron transport, light-dependent oxygen evolution was measured with a Clark-type electrode. The rate of oxygen evolution for the wild-type and the *cytA*$^-$ mutant was essentially the same under a variety of light conditions. In addition, photoacoustic measurements (Bults et al., 1982) showed nearly identical efficiencies of photosynthetic energy storage in the two strains (results not shown). Because cytochrome *c*-553 mediates electron transfer from cytochrome *f* to PSI, the efficiency of this step in the photosynthetic electron transport chain was determined by measuring the initial rate of the light-induced absorbance change at 420 nm, which is indicative of oxidation of both cytochromes *f* and *c*-553. The initial oxidation rate is shown in Figure 7 and was measured over a range of actinic flash intensities in the *cytA*$^-$ mutant and wild-type strains. Simple regressions drawn through the light-limited region of the curves in Figure 7 (0 to 200 W m^{-2}) gave a slope for the *cytA*$^-$ data that was 54% of that for the wild-type data. This result was repeatable (mean value of *cytA*$^-$ slope/wild-type slope was 58% with a standard deviation of 17.1%, n=5) and persisted in the presence of 50 μM DCMU (data not shown), indicating that, with equal input of light to PSI, the *cytA*$^-$ mutant was considerably less efficient at oxidizing cytochromes upstream of PSI. In the experiment of Figure 7, light-saturated rates of cytochrome oxidation also differed between the *cytA*$^-$ and wild-type strains.

DISCUSSION

Analysis of Protein Sequences

The gene encoding cytochrome *c*-553 from the unicellular cyanobacterium *Synechococcus* sp. PCC 7942 was isolated by hybridization with three sets of oligonucleotides. Based on the gene sequence, the protein appears to be synthesized as a precursor polypeptide of 111 amino acids. The mature protein probably begins at residue 25 (alanine), based on a comparison with cytochrome *c*-553 of *A. nidulans* (Ludwig et al., 1982). The amino acid sequence of cytochrome *c*-553 of *Synechococcus* sp. PCC 7942 differs from the *A. nidulans* protein at nine positions and contains an additional 2 residues at the C terminus (Figure 8A). Three glutamate residues reported in the *A. nidulans* polypeptide are not in the *Synechococcus* sp. PCC 7942 polypeptide, which accounts for the difference in pI values of 3.85 and 5.7 for cytochrome *c*-553 of *A. nidulans* and *Synechococcus* sp. PCC 7942, respectively. The interaction of cytochrome *c*-553 with P-700 is believed to be critically dependent on the electrostatic charge of the cytochrome (Davis et al., 1980). As shown in Figure 8A, a comparison of the amino acid sequence of the mature protein with corresponding sequences from other cyanobacteria and eukaryotic algae demonstrates identity of 55% to 90%. It is interesting to note that cytochrome *c*-553 of *Synechococcus* sp. PCC 7942 is more homologous to some eukaryotic algal cytochromes than to cyanobacterial cytochromes. Additionally, it is more similar to cytochrome *c*-553 polypeptides of other unicellular cyanobacteria than it is to those of filamentous cyanobacteria. In general, the unicellular cyanobacteria have acidic cytochrome *c*-553 polypeptides, whereas basic proteins predominate in filamentous cyanobacteria (Ho and Krogmann, 1984).

Signal Sequence Analysis

The amino-terminal extension on cytochrome *c*-553 is

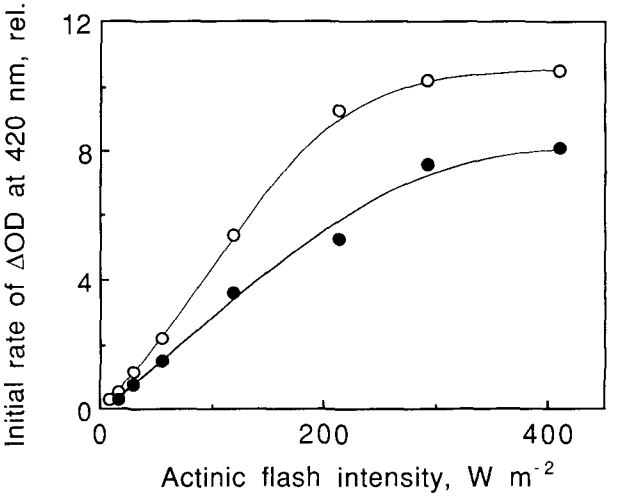

Figure 7. Relative Rates of the Light-Induced Absorbance Change at 420 nm Versus Intensity of the Actinic Flash in Wild-Type (○) and *cytA*$^-$ (●) Strains of *Synechococcus* sp. PCC 7942.

Rates were determined as the slopes of the absorbance change versus time in the initial, approximately linear region of the response.

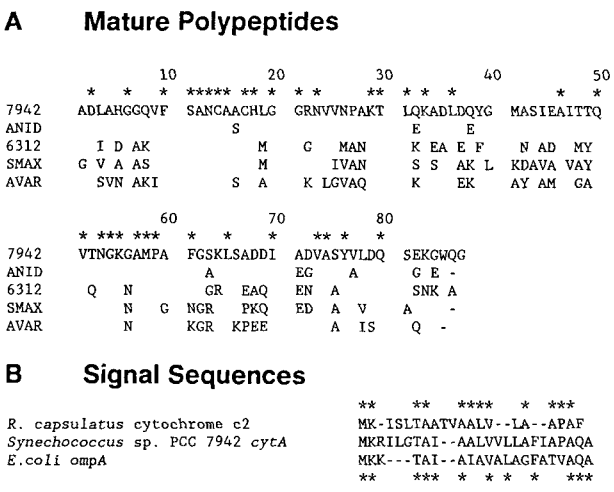

Figure 8. Amino Acid Similarity of Cyanobacterial Cytochrome *c*-553 Polypeptides.

(A) Comparison of the mature *Synechococcus* sp. PCC 7942 cytochrome *c*-553 polypeptide with selected cyanobacterial cytochrome *c*-553 amino acid sequences. Strains are designated as follows: 7942, *Synechococcus* sp. PCC 7942; ANID, *A. nidulans*; 6312, *Synechococcus* sp. PCC 6312; SMAX, *S. maxima*; AVAR, *Anabaena variabilis*. Only differences in the amino acid sequences between *Synechococcus* sp. PCC 7942 and the other cyanobacteria are indicated by an amino acid designation. Asterisks (*) indicate highly conserved residues.

(B) Comparison of the *Synechococcus* sp. PCC 7942 *cytA* signal sequence with *E. coli ompA* signal sequence and the cytochrome c_2 signal sequence of *R. capsulatus*.

probably 24 amino acids and its structural characteristics resemble those of other prokaryotic signal peptides (von Heijne, 1986). The amino-terminal domain of the signal peptide has two basic amino acids following the initiator methionine, and the hydrophobic central region is rich in alanine, leucine, and isoleucine residues (10 of 13). The carboxy-terminal domain of the presequence has the sequence Ala-Gln-Ala, which strongly resembles a prokaryotic signal-protease processing site (Ala-X-Ala). In prokaryotes, the "−1, −3 rule" predicts that the residue at position −1 relative to the cleavage site is small and hydrophobic (such as alanine), whereas the amino acid at −3 is neither charged, large, nor polar. In addition, a proline residue that breaks ordered secondary structure is often found at position −4. Thus, the amino-terminal extension on cytochrome *c*-553 displays all of the major features of a prokaryotic signal sequence (von Heijne, 1986; Gierasch, 1989). This sequence also shows similarities to the *Rhodobacter capsulatus* cytochrome c_2 and *E. coli ompA* signal sequences (Daldal et al., 1986; Gierasch, 1989) (Figure 8B) and is analogous to the signal-like sequences that comprise the second domain (the first domain is a transit peptide) of leader peptides associated with proteins targeted to the thylakoid lumen in higher plants (Smeekens et al., 1985; Tyagi et al., 1987).

In cyanobacteria, proteins can be routed to either the thylakoid or cytoplasmic membranes. The relationship between these membranes has not been resolved. Although it has been reported that the membrane systems are topologically distinct (Stanier and Cohen-Bazire, 1977), it has also been suggested that thylakoids arise from invaginations of the cytoplasmic membrane (Nierzwicki-Bauer et al., 1983). The finding that cytochrome *c*-553 is released by osmotic shock suggests that this protein resides in both the thylakoid lumen (Serrano et al., 1989) and the periplasmic space. This is corroborated by the fact that immunocytochemical techniques have localized the *Anabaena variabilis* cytochrome *c*-553 to the periphery of the cells (Serrano et al., 1989). The functional equivalent of cytochrome *c*-553 in *Rhodobacter sphaeroides* (Kiley and Kaplan, 1988) is also a periplasmic protein. Thus, the cytochrome *c*-553 signal sequence functions in transporting protein across both membranes or, alternatively, the thylakoid lumen is directly connected to the periplasm of the cells.

Gene Regulation

The *cytA* gene is transcribed as a 480-base mRNA and its abundance is not influenced by the copper levels in the growth medium. This is in contrast to the *Anabaena variabilis petEI* gene encoding the mobile electron carrier plastocyanin, which appears to be regulated by copper concentrations. *cytA* may be cotranscribed with a contiguous unidentified reading frame designated *cytB*. The 5′ end of the *cytA* transcript has been mapped and the −35 region has a strong resemblance to its *E. coli* counterpart. However, there have not been any studies done on cyanobacterial promoters that demonstrate that these sequences are functionally important in gene regulation.

Directed Mutations of the Cytochrome c-553 Gene

Deletion of the *cytA* gene of *Synechococcus* sp. PCC 7942 resulted in a mutant strain capable of photoautotrophic growth. Because the *cytA* mutant does not exhibit a readily detectable phenotype under normal light conditions, cytochrome *c*-553 is not required for photosynthetic electron transport in *Synechococcus* sp. PCC 7942. This result is somewhat surprising because a plastocyanin mutant of *C. reinhardtii* is incapable of growing photoautotrophically in the absence of cytochrome *c*-552 (Wood, 1978; Merchant and Bogorad, 1987).

The role of cytochrome *c*-553 in photosynthetic and respiratory electron transport in *Synechococcus* sp. PCC 7942 remains unclear. Our results can be explained by several hypotheses. First, cytochrome *c*-553 function may be replaced by plastocyanin or an alternate electron carrier.

However, by utilizing TMBZ staining and polyacrylamide gel electrophoresis, we did not detect any new types of cytochromes in total soluble or membrane protein preparations from the *cytA*$^-$ mutant. Plastocyanin is probably not present in *Synechococcus* sp. PCC 7942 based on both attempts to detect the protein (Aitken, 1976) and the gene using heterologous probes (Van der Plas et al., 1989). However, plastocyanin has been reported in some unicellular cyanobacteria (Aitken, 1976) and perhaps it is only synthesized in *Synechococcus* sp. PCC 7942 in the absence of cytochrome *c*-553 (e.g., in the *cytA*$^-$ strain or under iron-deficient conditions).

An alternate hypothesis is that the cytochrome b_6-*f* complex can donate electrons directly to the P-700 reaction center. An analogous direct electron transfer may occur in some of the photosynthetic bacteria (Prince et al., 1986). Fitch et al. (1989) have reported that cytochrome c_2 is absolutely required for photosynthetic growth of *R. sphaeroides* and, therefore, it is probably the sole component involved in transferring electrons between the cytochrome bc_1 complex and the reaction center in wild-type cells. In photosynthetic bacteria, cytochrome c_2 is structurally and functionally analogous to cyanobacterial cytochrome *c*-553. In contrast, the cytochrome bc_1 complex from the photosynthetic bacterium *R. capsulatus* is probably capable of donating electrons directly to the reaction center (Prince et al., 1986). In *R. capsulatus*, cytochrome c_1 (analogous to cytochrome *f* in cyanobacteria and plants) can rapidly transfer electrons directly to approximately 20% of the reaction centers. In wild-type strains, a further 20% of the reaction centers are rapidly reduced by cytochrome c_2 and the remaining reaction centers are reduced more slowly via an unidentified route (Prince et al., 1986). Under nonsaturating light conditions, *R. capsulatus* strains lacking cytochrome c_2 grow more slowly than wild type (Daldal et al., 1986).

The organization of the two photosystems within the thylakoid membrane in cyanobacteria has some differences from that of the thylakoids of green algae and higher plants. Cyanobacterial thylakoids have no appressed grana regions and the PSI and PSII complexes may be closely associated (Gantt, 1986). Such a lack of separation between the reaction center complexes may reduce the need for a highly diffusible electron carrier such as cytochrome *c*-553 and allow direct electron transfer between the cytochrome b_6-*f* and PSI complexes. In the case of the *cytA*$^-$ mutant, such direct electron transfer may be less efficient than electron transfer in wild type, which is facilitated by the presence of cytochrome *c*-553. This reduced efficiency may have little effect on the overall efficiency of whole chain electron transfer, which is believed to be limited at the step of plastoquinol oxidation. Thus, similar efficiencies of photosynthesis in the *cytA*$^-$ and wild-type strains are consistent with the reduced efficiency of electron transfer from cytochrome *f* to PSI observed in the mutant.

In most cyanobacteria, cytochrome *c*-553 also plays a role in respiration via electron donation to the membrane-bound cytochrome oxidase complex (reviewed in Peschek, 1987). Thus, a lesion in cytochrome *c*-553 may also affect respiratory electron transport in *Synechococcus* sp. PCC 7942. However, cytochrome *c*-553 of *A. nidulans* was not able to perform this function (Peschek and Schmetterer, 1982). Based on the phenotype of the *cytA*$^-$ mutant, cytochrome *c*-553 probably does not play a role in respiratory electron transport of *Synechococcus* sp. PCC 7942. Therefore, the ability to inactivate the cytochrome *c*-553 gene without causing lethality may be restricted to specific cyanobacteria such as *Synechococcus* sp. PCC 7942.

METHODS

Strains and Culture Conditions

Synechococcus sp. PCC 7942 (*Anacystis nidulans* R2) were axenically grown in liquid and solid (1.0% agar) BG-11 (Allen, 1968) medium buffered to pH 8.0 with 20 mM Hepes. Liquid cultures were grown in 60-mL glass tubes, bubbled with air enriched to 3% CO_2, and maintained at 29°C. Illumination was from incandescent bulbs at 60 μmol m^{-2} sec^{-1} unless otherwise stated. Copper-free medium was prepared by eliminating copper sulfate from the trace element solution. Water used to prepare copper-free medium was passed through a Chelex 100 (200 mesh to 400 mesh) ion-exchange column (Bio-Rad) and glassware was washed with 0.5 M HCl. Where appropriate, BG-11 was supplemented with spectinomycin (25 μg/mL) and ampicillin (1 μg/mL). *Escherichia coli* strains DH5α (Bethesda Research Laboratories) and JM109 were used as the hosts for plasmid libraries and constructions.

DNA Analysis

Total genomic DNA was extracted from *Synechococcus* sp. PCC 7942 essentially by the method of Tandeau de Marsac et al. (1982). The methods used to clone and sequence the cytochrome *c*-553 gene (*cytA*) have been described previously (Laudenbach et al., 1988). Briefly, a *Synechococcus* sp. PCC 7942 PstI clone bank was constructed in the *E. coli* vector pUC9 and transformed into *E. coli* JM109. After ensuring that the DNA fragment of interest was present in the clone bank by DNA gel blot hybridization with the oligonucleotide probes, the plasmid clone bank was electrophoresed in 0.7% low-melting-temperature agarose (Maniatis et al., 1982), fractionated into six size classes, and purified from the agarose gel. Half of each fraction was screened with the oligonucleotide for the presence of the desired insert. The remaining DNA from the positively hybridizing fraction of the library was used to transform JM109 directly, and 100 small-scale plasmid preparations from individual transformants were prepared by lysozyme-Triton X-100 treatment of the cultures (Maniatis et al., 1982). Clones with the fragment of interest were identified by a second round of hybridization. The 1.4-kb PstI insert from the isolated recombinant was cloned into M13mp18 and M13mp19 for DNA sequencing.

Hybridization with ^{32}P-Labeled Probes

DNA restriction fragments were electrophoresed in 0.7% agarose gels in a Tris-borate-EDTA buffer system (Maniatis et al., 1982). DNA gel blot transfer of DNA to nitrocellulose filters was performed as described in Maniatis et al. (1982). Radiolabeled oligonucleotides were used at 1×10^6 cpm/mL final in hybridization solution [6 × SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.2% BSA, 0.2% PVP, 0.2% Ficoll, 0.1% SDS, and 0.05 mg/mL denatured, sonicated carrier DNA]. Hybridizations were performed at 30°C for two probes and at 37°C for the third probe. The filters were washed three times in 2 × SSC, 0.1% SDS at room temperature and then air dried and exposed to x-ray film (Kodak XAR5). Oligonucleotides were end labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase (Maniatis et al., 1982). Restriction fragments were radiolabeled by the random oligonucleotide priming method (Feinberg and Vogelstein, 1984). All probes were separated from unincorporated nucleotides on a Sephadex G-50-150 column.

DNA Sequence Determination

Sequencing in both M13mp19 and pUC9 was carried out by the dideoxy chain-termination method (Sanger et al., 1977) using the modified T7 DNA polymerase (Tabor and Richardson, 1987) with either M13 universal and reverse primers or synthetic deoxyoligonucleotides. Ambiquities in G–C-rich regions were resolved by substituting inosine for guanine in the sequencing reactions. Subclones for sequencing were generated by restricting and religating the original clones with appropriate restriction enzymes or by creating sets of ordered deletions using the procedure of Dale et al. (1985).

RNA Analysis

Total RNA from *Synechococcus* sp. PCC 7942 was isolated by the method described by Laudenbach et al. (1988). For RNA gel blot analysis, 10 μg to 20 μg of RNA was electrophoresed in 1.1% agarose gels after denaturation with glyoxal and dimethyl sulfoxide. RNA gel blotting and hybridization were done by the method of Thomas (1983), except that hybridizations and washes were performed at 37°C. Commercially prepared RNA size markers (Bethesda Research Laboratories) were used to estimate the size of the hybridization signals.

To identify the 5′ end of the *cytA* transcript, both primer extension assays and mung bean nuclease mapping experiments were performed. For primer extension assays, a 29-mer (5′-GCAGAAAAGACTTGGCCACCATGGGCAAG-3′) complementary to the *cytA* sequence from positions 107 to 79 (see Figure 2) was annealed to 20 μg of total RNA from *Synechococcus* sp. PCC 7942 and extended using avian myeloblastosis virus reverse transcriptase (Pharmacia). The endpoints of the primer extension products were precisely located by sequencing with the same primer (on an appropriate M13 clone) and electrophoresing the sequencing reactions next to the primer extension products. Mung bean nuclease protection experiments (Murray, 1986) were used to corroborate the location of the 5′ end of the transcript. The 1.4-kb PstI fragment cloned into M13mp19 was radiolabeled by complementary strand synthesis utilizing 1 μg of template, 1.5 units of the Klenow fragment of DNA polymerase I, and the oligonucleotide used for primer extension. The probe was digested with PstI to generate a labeled fragment of specific length, and the ^{32}P-labeled, single-stranded DNA was separated from the M13 template by electrophoresis on an alkaline denaturing agarose gel. One-fifth of the labeled DNA was precipitated together with 40 μg of RNA. RNA-DNA hybridization, mung bean nuclease digestions, and gel electrophoresis were performed as described previously (Murray, 1986; Reith et al., 1986).

Insertional Inactivation

A 1.4-kb PstI fragment, encoding the *cytA* gene, was cloned into the PstI site of pUC9 (designated pCCP145) and used for subsequent constructions. pHP45Ω (Prentki and Krisch, 1984) was digested with either BamHI or SmaI, and the 2-kb DNA fragment with the spectinomycin (Sp) resistance cassette was recovered by electroelution from a low-melting-temperature agarose gel. To inactivate *cytA*, the BamHI resistance marker was ligated into pCCP145 digested with BglII and transformed into DH5α. Colonies were selected for growth on Amp and Sp, and their plasmid content was analyzed for the presence of the proper construction. The resulting recombinant, designated pCCP158, deleted the first half of *cytA* and replaced it with the Sp cassette. To inactive *cytB*, the SmaI resistance marker was ligated into the SmaI site of a modified version of pCCP145. The resulting clone was designated pCCP260.

Plasmid DNA from pCCP158 and 260 was used to transform *Synechococcus* sp. PCC 7942 to Sp^R by the method of Laudenbach et al. (1988). The resulting colonies, obtained at a frequency expected for a simple double cross-over event, were visible within 7 days and analyzed for DNA content by DNA gel blot hybridization.

Protein Analysis

Total soluble proteins were isolated as described previously (Green and Grossman, 1988). Periplasmic protein fractions were prepared according to Block and Grossman (1988). To isolate soluble proteins enriched for cytochrome *c*-553, total soluble proteins were brought to 50% saturation by the addition of ammonium sulfate and stirred overnight at 4°C. After centrifugation at 8000*g* for 10 min, the supernatant was brought to 95% ammonium sulfate and left at 4°C for at least 1 hr. The precipitated proteins were pelleted by centrifugation at 10,000*g* for 10 min. Proteins were resuspended in a minimum volume of breakage buffer and dialyzed overnight against a large excess of breakage buffer. The proteins were precipitated by the addition of trichloroacetic acid to 10% and analyzed by SDS-PAGE (Green and Grossman, 1988). The polypeptides were resolved by electrophoresis on SDS-PAGE on a linear 5% to 15% gradient gel using the Laemmli buffer system (Laemmli, 1970) and were visualized by staining with Coomassie Brilliant Blue G-250. Peroxidase activity, associated with the heme cofactors of proteins such as cytochromes, was localized using the 3,3′,5,5′-tetramethylbenzidine-hydrogen peroxide method developed by Thomas et al. (1976).

Measurement of Light-Induced Absorbance Changes

Light-induced absorbance changes in suspensions of intact cells diluted to equal chlorophyll *a* were measured in the millisecond time range using a laboratory-built, single-beam spectrophotometer combined with a signal averager (Nicolet 1010, Nicolet Instruments, Madison, WI). Red actinic light was from a tungsten filament lamp fitted with a 3-cm water filter and red cut-off filters (Schott RG2 or RG5, Schott Glass Technologies, Duryea, PA). The photomultiplier tube of the spectrophotometer (EMI 9558-B, EMI, Thorn EMI, Fairfield, NJ) was fitted with three blue-green glass filters (Corning 4-96, Corning Glass, Corning, NY) to absorb the actinic light. Actinic flashes were of 250-msec duration and given at 6-sec intervals. Signals from four to eight flashes were averaged to improve the signal-to-noise ratio, and the traces were plotted for analysis. Relative rates of cytochromes *f* and *c*-533 oxidation were measured as the initial rates of the light-induced absorbance change at 420 nm in samples that had been diluted to equal chlorophyll *a* absorption, measured at 680 nm.

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