

Regions of *Rhodobacter sphaeroides* Cytochrome c_2 Required for Export, Heme Attachment, and Function

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Cytochrome c_2 is a periplasmic redox protein involved in both the aerobic and photosynthetic electron transport chains of *Rhodobacter sphaeroides*. The process of cytochrome c_2 maturation has been analyzed in order to understand the protein sequences involved in attachment of the essential heme moiety to the cytochrome c_2 polypeptide and localization of the protein to the periplasm. To accomplish this, five different translational fusions which differ only in the cytochrome c_2 fusion junction were constructed between cytochrome c_2 and the *Escherichia coli* periplasmic alkaline phosphatase. All five of the fusion proteins are exported to the periplasmic space. The four fusion proteins that contain the NH₂-terminal site of covalent heme attachment to cytochrome c_2 are substrates for heme binding, suggesting that the COOH-terminal region of the protein is not required for heme attachment. Three of these hybrids possess heme peroxidase activity, which indicates that they are functional as electron carriers. Biological activity is possessed by one hybrid protein constructed five amino acids before the cytochrome c_2 COOH terminus, since synthesis of this protein restores photosynthetic growth to a photosynthetically incompetent cytochrome c_2 -deficient derivative of *R. sphaeroides*. Biochemical analysis of these hybrids has confirmed CycA polypeptide sequences sufficient for export of the protein (A. R. Varga and S. Kaplan, *J. Bacteriol.* 171:5830–5839, 1989), and it has allowed us to identify regions of the protein sufficient for covalent heme attachment, heme peroxidase activity, docking to membrane-bound redox partners, or the capability to function as an electron carrier.

Cytochrome c_2 (cyt c_2) is a soluble electron carrier located in the periplasm of the purple nonsulfur photosynthetic bacterium *Rhodobacter sphaeroides* (39). This c -type cytochrome is similar to the soluble cyt c of mitochondria (11, 35). *R. sphaeroides* cyt c_2 is an essential component of the cyclic photosynthetic redox chain (37, 40, 50), and it also functions in one of the two branches of the aerobic respiratory electron transfer chain of this bacterium (19).

There are several domains within the cyt c_2 polypeptide that have been implicated in various aspects of the maturation or function of this protein. DNA sequence analysis has identified an NH₂-terminal sequence of 21 amino acid residues which is absent in the mature polypeptide. This sequence contains all of the features of the typical procaryotic signal peptide (16, 25), and it is presumably necessary for localization of the cyt c_2 polypeptide (CycA) to the periplasm. Heme is covalently attached to two cysteines in the cyt c_2 polypeptide via thioether linkages to the sequence 15-Cys-Gln-Thr-Cys-18. His-19 and Met-100 are noncovalent axial ligands to the iron in the porphyrin ring (35) (the amino acid numbers used in this report refer to the amino acid residues of the mature cyt c_2 protein). Finally, Lys-10, Lys-95, Lys-97, Lys-99, and Lys-105 have been implicated in the docking of cyt c_2 to membrane-bound redox complexes during physiological electron transfer reactions (20, 21, 47). These positively charged residues, which are all located near the solvent-exposed heme edge of the protein, interact with negatively charged carboxylate groups on the cyt c_2 redox partners (47).

Although cytochromes such as cyt c_2 play an essential role in cell physiology, very little is known about the regulation of cyt c_2 synthesis, the processing and maturation of the CycA polypeptide, or how CycA is secreted into the peri-

plasmic space. The available information suggests that *cycA*-specific mRNA is translated into an ~15,500- M_r cyt c_2 precursor protein which is subsequently processed (via cleavage of the 21-residue signal sequence) to an ~13,500- M_r polypeptide (13). In order for CycA to function in electron transport, heme must be covalently attached to either the precursor or the processed polypeptide, and it must be exported to the periplasmic space. The order and cellular location of these processes are unknown and must be analyzed in more detail to define the cyt c_2 biosynthetic pathway in molecular terms. A detailed molecular model for cyt c_2 biosynthesis is also necessary to allow a thorough examination of those systems which may regulate cyt c_2 synthesis at the posttranscriptional level (5).

To increase our understanding of cyt c_2 maturation and heme attachment to CycA, we have constructed translational fusions between CycA and the *Escherichia coli* periplasmic alkaline phosphatase (*phoA* gene product; 6). The goal of these experiments was to use these hybrids to identify CycA determinants required for heme attachment, proper subcellular localization, and function as an electron carrier. The construction of the *cycA-phoA* fusions in *E. coli* and an analysis of the CycA-PhoA fusion proteins in both *E. coli* and *R. sphaeroides* are described.

MATERIALS AND METHODS

Bacterial strains and plasmids. *R. sphaeroides* strains were grown at 32°C in Siström's minimal medium A (43) under previously described growth conditions (5, 13, 23, 46). Tetracycline (1 µg/ml), spectinomycin (50 µg/ml), or kanamycin (25 µg/ml) was used when appropriate for *R. sphaeroides*. Strain 2.4.1 is a wild-type *R. sphaeroides* isolate, and CYCA1 is a cyt c_2 -deficient derivative of 2.4.1 with a kanamycin resistance cassette interrupting the *cycA* gene (14). Plasmid pC2P404.1 contains the intact *cycA*

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operon as an ≈ 2.7 -kb *Pst*I restriction endonuclease fragment (13) cloned into the *Pst*I site of the broad-host-range plasmid pRK404 (12) such that the direction of *cycA* transcription is the same as that of the vector *lac* and tetracycline promoters.

E. coli strains were grown at 37°C in L broth (28). Ampicillin (50 μ g/ml), tetracycline (10 μ g/ml), and spectinomycin (50 μ g/ml) were used to maintain plasmids, and kanamycin (25 μ g/ml) was used to select for Tn5 insertions. *E. coli* CC118 (Δ *phoA20*; 29) was used in experiments to score PhoA activity on plates. Solid media were supplemented with 5-bromo-4-chloro-3-indolyl phosphate (40 μ g/ml; 29). When necessary, plates were also supplemented with 40 μ M isopropylthio- β -D-galactoside.

Derivatives of *E. coli* S17-1 (42) were used as donors for conjugation into *R. sphaeroides* strains, and filter matings were conducted as previously described (14). *R. sphaeroides* exconjugants were selected on solid media in the presence of the appropriate antibiotics under aerobic conditions. Resulting colonies were picked, replated, and screened for their photosynthetic phenotype (5).

Construction of CycA-PhoA fusions. Plasmid pC2P2.71 (13) contains the *R. sphaeroides cycA* gene as a 2.7-kb *Pst*I fragment downstream of the pUC19 (51) *lac* promoter. *E. coli* JM83 (51) containing pC2P2.71 was infected with λ Tn*phoA*-1 (29), and ampicillin- and kanamycin-resistant colonies were selected. Miniprep DNA from a pool of ampicillin- and kanamycin-resistant cells was prepared and transformed into CC118. CC118 transformants were plated on medium containing ampicillin, kanamycin, isopropylthio- β -D-galactoside, and 5-bromo-4-chloro-3-indolyl phosphate to identify *phoA*⁺ Tn*phoA* insertions in pC2P2.71. To ensure that independent Tn*phoA* transpositions were analyzed, one *pho*⁺ strain from each phage-infected culture was chosen for further analysis.

Tn*phoA* insertions into *cycA* were confirmed by restriction endonuclease analysis, and the position of the *cycA-phoA* fusion was determined by sequencing the DNA across the fusion junction, using the oligonucleotide 5'-AA TATCGCCCTGAGCAGCCCG as a primer for plasmid dideoxy DNA sequencing. This primer is complementary to *phoA* 65 bp downstream of the fusion junction between *cycA* and the truncated *phoA* gene from Tn*phoA* (7).

The majority of the Tn5 sequences were deleted by taking advantage of a *Bst*EII site ~ 130 bp downstream of the *phoA* stop codon. Plasmid DNA was digested with *Bst*EII and *Sma*I, and 4.8- to 5.1-kb restriction fragments containing vector sequences plus the *cycA-phoA* fusion were purified. The size of the fragment varied depending on the location of the fusion junction. The *Bst*EII ends were made blunt and ligated to the *Sma*I site (from the pUC19 multiple cloning sequence). The mixture was transformed into CC118, and the resulting ampicillin-resistant colonies were screened for their *pho* phenotype. Finally, 2.1- to 2.4-kb *Pst*I-*Sst*I restriction fragments containing the *cycA-phoA* fusions were purified, cloned into the *Pst*I-*Sst*I sites of the broad-host-range plasmid pRK415 (27), and placed downstream of an omega cartridge encoding spectinomycin resistance (38) to ensure that transcription in *R. sphaeroides* came from *cycA* promoter elements.

Fractionation of cell lysates. To analyze the location of the fusion proteins, *R. sphaeroides* shock fluid was prepared (46), and cytoplasmic, inner membrane, and outer membrane fractions were separated as described previously (46, 49). Malate dehydrogenase assays (31) and reduced (ascorbate)-minus-oxidized (potassium ferricyanide) difference

spectra (13, 24) were determined to estimate the efficiency of fractionation. Protein was quantitated by the method of Markwell et al. (32).

For malate dehydrogenase, 1 U of activity is the amount of enzyme required to convert 1 μ mol of substrate to product per minute under standard assay conditions (31). For alkaline phosphatase, 1 U of activity is the amount of enzyme required to convert 1 nmol of substrate to product per minute under the standard assay conditions (36).

Immunoblotting. For immunochemical analysis, 30 μ g of protein in each cell fraction described above was separated by 10 to 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (NaDodSO₄-PAGE) (10, 13), followed by electrophoretic transfer of the proteins to 0.2 μ m-pore-size nitrocellulose filter paper (8). Immunoblotting was performed as described previously (2, 8, 13), using rabbit antiserum against purified cyt c_2 (13) or alkaline phosphatase. Western immunoblot analysis was also performed, using soluble fractions of photosynthetically grown cells (5, 13).

Heme attachment assays. (i) **Peroxidase activity.** Mid-exponential-phase photosynthetic cultures (0.5×10^9 to 1×10^9 cells per ml) were harvested by centrifugation, washed in buffer containing 0.1 M NaH₂PO₄ and 0.01 M EDTA (pH 7.6), and suspended in 2.5 ml of this buffer before lysis by sonication (46). Membrane and soluble cell fractions were separated by centrifugation in a 70.1 Ti rotor at 40,000 rpm for 3 h (5, 13). Approximately 300 μ g of soluble protein was solubilized at 37°C prior to gradient NaDodSO₄-PAGE. Heme staining was performed by the method of Francis and Becker (18).

(ii) **Radioactive labeling of the heme moiety.** To label cells with ³H-aminolevulinic acid ([³H]ALA), *R. sphaeroides* was grown aerobically in medium supplemented with 0.2% Casamino Acids (Difco) until mid-exponential phase. Cells were then subcultured to medium containing 0.2% Casamino Acids and 10 μ Ci of [³H]ALA per ml and grown aerobically for six to eight doublings to mid-exponential phase. Cells were harvested and fractionated as described above for heme staining. Cold trichloroacetic acid precipitations were performed to quantitate incorporation of [³H]ALA into protein. To remove heme from those proteins in which it is not covalently attached to the polypeptide chain, soluble proteins (70 μ g per lane) were heated at 85°C in solubilization buffer (8) prior to separation by gradient NaDodSO₄-PAGE. The gel was treated with Fluoro-Hance (Research Products International, Mount Prospect, Ill.) before exposure to preflashed X-ray film.

Materials. [³H]ALA (specific activity, 1 to 5 Ci/mmol) was obtained from New England Nuclear, Boston, Mass. The oligonucleotide primer used for sequencing the *cycA-phoA* fusion junction was prepared at the University of Wisconsin-Madison Biotechnology Center.

RESULTS

Construction of the CycA-PhoA fusions in *E. coli*. When *cycA* is placed under control of the *E. coli lac* promoter, the cyt c_2 signal sequence can target either cyt c_2 (33) or a specific CycA-PhoA fusion (48) to the *E. coli* periplasm. Using Tn*phoA*, we identified *pho*⁺ *E. coli* cells containing unique CycA-PhoA fusions at alanine 11 (A11), alanine 30 (A30), leucine 40 (L40), glutamine 81 (Q81), or glutamine 119 (Q119) of the 124-amino-acid mature cyt c_2 protein. These *E. coli* strains contain antigen detectable with antibody to either

TABLE 1. Intracellular localization of enzymatic activities in aerobically grown *R. sphaeroides*^a

| Strain | Fraction | Activity (total U) ^b | |
|------------------|-----------|---------------------------------|-----------------------------------|
| | | Malate dehydrogenase | Alkaline phosphatase ^c |
| 2.4.1 | Periplasm | 0.61 (1) | 1.0 (45) |
| | Cytoplasm | 72.85 (96) | 0.7 (32) |
| | Membrane | 2.14 (3) | 0.5 (23) |
| CYCA1(pC2P404.1) | Periplasm | 0.32 (2) | 0.9 (50) |
| | Cytoplasm | 14.60 (93) | 0.5 (28) |
| | Membrane | 0.75 (5) | 0.4 (22) |
| CYCA1(pC2PA11) | Periplasm | 0.55 (18) | 165 (87) |
| | Cytoplasm | 2.29 (75) | 24 (13) |
| | Membrane | 0.20 (6.6) | ND |
| CYCA1(pC2PA30) | Periplasm | 0.47 (2.5) | 254 (99.6) |
| | Cytoplasm | 17.36 (94) | 0.9 (0.4) |
| | Membrane | 0.69 (4) | ND |
| CYCA1(pC2PL40) | Periplasm | 0.59 (7) | 41.9 (84) |
| | Cytoplasm | 7.60 (90) | 8.2 (16) |
| | Membrane | 0.23 (3) | ND |
| CYCA1(pC2PQ81) | Periplasm | 0.10 (10) | 72.1 (72) |
| | Cytoplasm | 0.85 (83) | 27.8 (28) |
| | Membrane | 0.07 (7) | ND |
| CYCA1(pC2PQ119) | Periplasm | 0.31 (1) | 259 (84) |
| | Cytoplasm | 20.80 (98) | 48.5 (16) |
| | Membrane | 0.20 (1) | ND |

^a Similar trends were observed for photosynthetically grown 2.4.1 and CYCA1(pC2PQ119) cells containing the fusions (i.e., greater than 75% of the cellular alkaline phosphatase activity was detected in the periplasmic fraction).

^b Percent activity is given in parentheses.

^c Values are averages of two repetitions of the assay; errors range from 1 to 13%. ND, not determined.

R. sphaeroides cyt *c*₂ or *E. coli* alkaline phosphatase (data not shown).

Phenotype of CycA-PhoA fusions in *R. sphaeroides*. The five *cycA-phoA* fusions were separated from Tn5 sequences and cloned into a broad-host-range plasmid which can be mobilized into and stably maintained in *R. sphaeroides*. The plasmids have been named pC2A11 to pC2Q119 according to the position of the *cycA-phoA* fusion, and we have designated gene products encoded by these plasmids as A11, A30, etc. While wild-type *R. sphaeroides* is phenotypically *pho* (36, 48), cells containing any of the five CycA-PhoA fusions exhibit a *pho*⁺ (blue) phenotype on 5-bromo-4-chloro-3-indolyl phosphate plates, suggesting that all of the hybrid proteins were exported. In addition, since a transcription-translation stop cartridge was placed upstream of the *cycA* sequences, this result indicates that transcription of the fusions is directed by the *R. sphaeroides cycA* promoter sequences contained on the plasmid.

The CycA-PhoA fusions are localized to the *R. sphaeroides* periplasm. To determine the localization of the fusion proteins, cytoplasmic, membrane, and periplasmic fractions were also assayed for alkaline phosphatase activity and malate dehydrogenase as a cytoplasmic marker (Table 1). In all of the fusion-containing strains examined, greater than 72% of the alkaline phosphatase activity was found in the periplasmic space. In addition, the preferential distribution of malate dehydrogenase activity in the cytoplasmic frac-

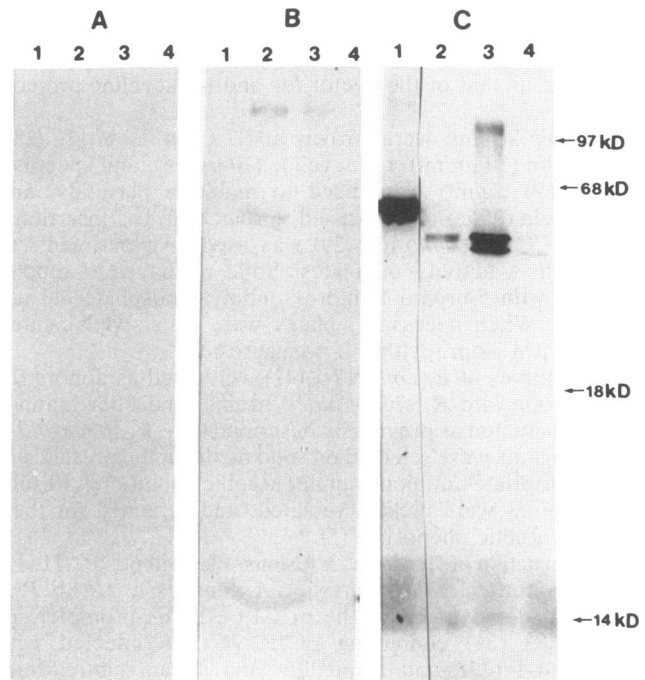


FIG. 1. Immunoblot of cellular fractions prepared from photosynthetically grown cells by the shock fluid method described in Materials and Methods. Periplasmic fractions (C) were prepared by osmotic shock, followed by separation of the cytoplasmic fraction (A) and cytoplasmic membranes (B); 30 μ g of protein was loaded in each lane. Lanes: 1, 2.4.1(pC2Q119); 2, 2.4.1(pC2L40); 3, 2.4.1(pC2A30); 4, 2.4.1(pC2PA11).

tions confirms that significant amounts of cell lysis did not occur during shock fluid preparation. The low levels of alkaline phosphatase present in 2.4.1 and CYCA1(pC2P404.1) are due to the endogenous *R. sphaeroides* enzyme(s), which previous experiments have shown to be present in both periplasmic and cytoplasmic fractions (36, 48).

Since alkaline phosphatase is enzymatically active in *E. coli* only when it is exported (3), the cellular location of the fusion proteins was also determined by immunoblot analysis of cell fractions prepared from cells grown aerobically (data not shown) or photosynthetically (Fig. 1), using antiserum against either cyt *c*₂ or *E. coli* alkaline phosphatase. As expected from the data in Table 1, the majority of the antigen detected by either antiserum was found in the periplasmic fraction (Fig. 1C shows the results obtained with cyt *c*₂ antiserum). Immunoblot analysis of equivalent amounts of cytoplasmic and membrane protein from cells grown either aerobically (data not shown) or photosynthetically detected only minor amounts of antigen in the cytoplasm or either membrane fraction (Fig. 1A and B). Identical results were obtained when antiserum against alkaline phosphatase was used to localize antigen in these fractions (data not shown). This finding indicates that all of the fusion proteins are being efficiently exported to the periplasm. The efficient localization of all five fusions to the periplasm confirms a previous observation that the signal sequence and the amino-terminal 10 amino acids of cyt *c*₂ are sufficient to direct export of a specific CycA-PhoA hybrid to the periplasm (48).

When soluble fractions of photosynthetically grown cells were probed with either antiserum, the largest-molecular-

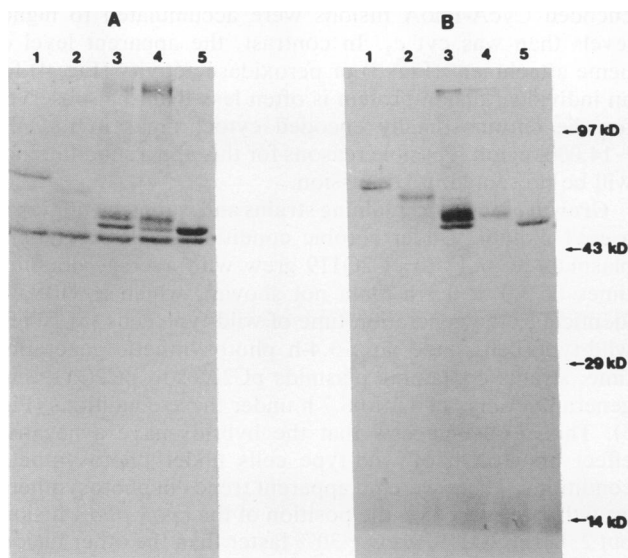


FIG. 2. Immunoblot of soluble cell extracts prepared from photosynthetically grown cells, using antiserum against *E. coli* alkaline phosphatase (A) or *R. sphaeroides* cyt c_2 (B); 30 μ g of protein was loaded in each lane. The M_r of wild-type cyt c_2 (c_2) is \sim 13,500, and that of wild-type alkaline phosphatase (PHOA) is \sim 47,000. Positions of migration of purified alkaline phosphatase and cyt c_2 on this gel system are indicated by arrowheads. Lanes: 1, 2.4.1(pC2Q119); 2, 2.4.1(pC2Q81); 3, 2.4.1(pC2L40); 4, 2.4.1(pC2A30); 5, 2.4.1(pC2A11).

weight species detectable in each strain is within 17% of the estimated molecular weight of the fusion proteins determined from the known DNA sequences (Fig. 2A). Although a hybrid protein of approximately the expected size for a full-length gene product can be detected with use of the alkaline phosphatase antiserum, it is evident that several of the CycA-PhoA fusions are being degraded (Fig. 2A). The immunoblot results suggest that cyt c_2 determinants are cleaved from the fusion proteins, since a protein of molecular weight similar to that of wild-type *E. coli* alkaline phosphatase (\sim 47,000 M_r ; 4) which cross-reacts with anti-alkaline phosphatase but not anti-cyt c_2 serum (see below) is found in each of the fusion-containing strains. No PhoA antigen is detected in strains lacking the PhoA-encoding plasmids (data not shown).

Fusion proteins of approximately full length are also clearly detected in soluble fractions probed with antiserum to cyt c_2 (Fig. 2B). The ability of the cyt c_2 antiserum to react with the A11 hybrid (Fig. 1 and 2B) indicates that this serum is able to recognize epitopes in the first 10 amino acids of mature cyt c_2 . The degradation described above is also apparent in these samples (Fig. 2B), but the \sim 47,000- M_r species is not detected by the cyt c_2 antiserum. No antigen less than or equal to 47,000- M_r is detected with the anti-cyt c_2 serum in any of the extracts from aerobically grown CYCA1 cells containing the fusions or photosynthetically grown CYCA1(pC2Q119) (data not shown). This finding suggests that there is no detectable accumulation of CycA peptides cleaved from the amino terminus of the apparently full length gene products.

The copy number of RK2-derived plasmids has been estimated to be \sim 5 to 10 per *R. sphaeroides* genome (5, 9). From the immunoblot analysis with cyt c_2 antiserum, it is evident that the fusion proteins are being accumulated to

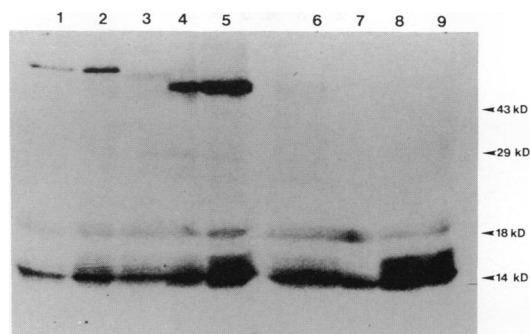


FIG. 3. [3 H]ALA labeling of aerobically grown cells. Soluble extracts of the cells were separated by NaDodSO $_4$ -PAGE, and the gel was treated for fluorography, dried, and exposed to preflashed X-ray film; 60 μ g of soluble protein was loaded per lane. Lanes: 1, CYCA1(pC2Q119); 2, 2.4.1(pC2Q119); 3, 2.4.1(pC2Q81); 4, 2.4.1(pC2L40); 5, 2.4.1(pC2A30); 6, 2.4.1(pC2A11); 7, CYCA1; 8, 2.4.1(pC2P404.1); 9, 2.4.1.

higher levels than is the wild-type cyt c_2 encoded by the 2.4.1 chromosome.

Covalent heme attachment to the fusion proteins. To determine whether heme is covalently bound to any of the hybrid proteins, cells were grown aerobically in the presence of [3 H]ALA. ALA is the first committed precursor in the pathway for heme synthesis (26), so labeling the cells with [3 H]ALA and high-temperature solubilization prior to NaDodSO $_4$ -PAGE allowed us to destroy noncovalent heme-protein interactions and directly monitor covalent attachment of heme to proteins in soluble extracts (Fig. 3). We specifically used aerobically grown cells for this experiment to minimize ALA incorporation into bacteriochlorophyll, which is made only under photosynthetic conditions (8). It is evident from these data that 3 H-heme is attached to a protein of molecular weight similar to that predicted for the full-length fusion protein in cells containing any of the fusions except A11 (Fig. 3, lanes 1 to 6). Therefore, all of the hybrids with a fusion junction downstream of the heme attachment site are substrates for covalent heme binding in vivo. The [3 H]ALA-labeled proteins in the \sim 14-kDa region are the multiple wild-type *R. sphaeroides* c -type cytochromes present in aerobically grown cells (5, 35).

Several fusion proteins contain heme peroxidase activity. We also wished to determine whether the heme attached to the hybrid proteins was functional in a heme peroxidase assay. To test this, soluble extracts from photosynthetically grown strains were solubilized at 37°C, separated by NaDodSO $_4$ -PAGE, and stained for heme peroxidase activity (Fig. 4). We used photosynthetically grown cells for this analysis to increase *cycA* expression and thus increase our ability to detect low levels of peroxidase activity that might be associated with the hybrids. Extracts from 2.4.1(pC2Q119), 2.4.1(pC2L40), and 2.4.1(pC2A30) contain a heme-staining protein whose M_r corresponds well to the predicted molecular weight of the full-length fusion protein (Fig. 4, lanes 4, 5, and 7). The peroxidase activity of these hybrids is apparently very low since it was necessary to use \sim 300 μ g of cell extract protein per lane to demonstrate activity of the hybrids. Even after prolonged incubation, strains containing the A11 or the Q81 fusion protein contained no detectable heme-staining species in the region of the gel corresponding to the molecular weight of the fusion proteins (Fig. 4, lanes 3 and 6). Also note that all of the

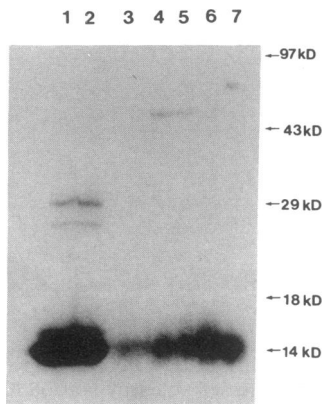


FIG. 4. Heme peroxidase staining of soluble proteins from photosynthetically grown cells. Each sample contains 300 μ g of protein separated by NaDodSO₄-PAGE. Lanes: 1, 2.4.1; 2, CYCA1(pC2P404.1); 3, 2.4.1(pC2A11); 4, 2.4.1(pC2A30); 5, 2.4.1(pC2L40); 6, 2.4.1(pC2Q81); 7, 2.4.1(pC2Q119). The 300 μ g of protein loaded per lane is \sim 6-fold greater than that required to monitor peroxidase activity from wild-type *cyt c₂* in these samples. Thus, the 29-kDa heme-staining species visible in lanes 1 and 2 could be due to incomplete solubilization of the large amount of low- M_r cytochromes in these samples.

strains contain heme-staining proteins in the 14,000- M_r region from the low-molecular-weight soluble *c*-type cytochromes present in photosynthetic cultures (35).

Although the proteins in the 14,000- M_r region of the gel correspond to several *c*-type cytochromes (35), *cyt c₂* is the predominant *c*-type cytochrome in photosynthetically grown cells (34). Thus, the ratio of the 47,000- to 70,000- M_r to 14,000- M_r heme-staining proteins should reflect the relative abundance of these polypeptides. Western blot analysis with *cyt c₂* antiserum in Fig. 1 and 2 showed that the plasmid-

encoded *CycA-PhoA* fusions were accumulated to higher levels than was *cyt c₂*. In contrast, the apparent level of heme attachment (Fig. 3) or peroxidase activity (Fig. 4) for an individual fusion protein is often less than that observed for the chromosomally encoded cytochromes in the M_r - \sim 14,000 region. Possible reasons for this apparent difference will be presented in Discussion.

Growth of fusion-containing strains and complementation of a *cyt c₂* mutant. Under aerobic conditions, cells containing plasmids pC2A11 to pC2Q119 grew with average doubling times of 3.0 ± 0.9 h (data not shown), which is virtually identical to the generation time of wild-type cells (5). While wild-type cells have an \sim 3.4-h photosynthetic generation time, strains containing plasmids pC2A11 to pC2Q119 had generation times of 4.1 to 6.7 h under these conditions (Fig. 5). This result suggests that the hybrids have a negative effect on growth of wild-type cells under photosynthetic conditions. There were no apparent trends in photosynthetic growth rates based on the position of the *cyt c₂*-*phoA* fusion, but 2.4.1(pC2Q119) grew \sim 30% faster than the other fusion-containing strains.

Since several of the fusion proteins were substrates for heme attachment or had heme peroxidase activity, we sought to determine whether any of these proteins might function as an electron carrier *in vivo*. CYCA1 is a *cyt c₂*-deficient derivative of 2.4.1 that is incapable of photosynthetic growth (14). When the fusion-encoding plasmids were mobilized into CYCA1, cells containing pC2Q119 were able to grow photoheterotrophically. Under aerobic conditions, CYCA1(pC2Q119) grows with wild-type generation times. However, CYCA1(pC2Q119) grows with a photoheterotrophic doubling time approximately twice that of 2.4.1 and \sim 30% slower than that of 2.4.1(pC2Q119). The photosynthetic growth of CYCA1(pC2Q119) suggests that the Q119 *CycA-PhoA* hybrid functions in electron transport to restore the photosynthetic defect of CYCA1.

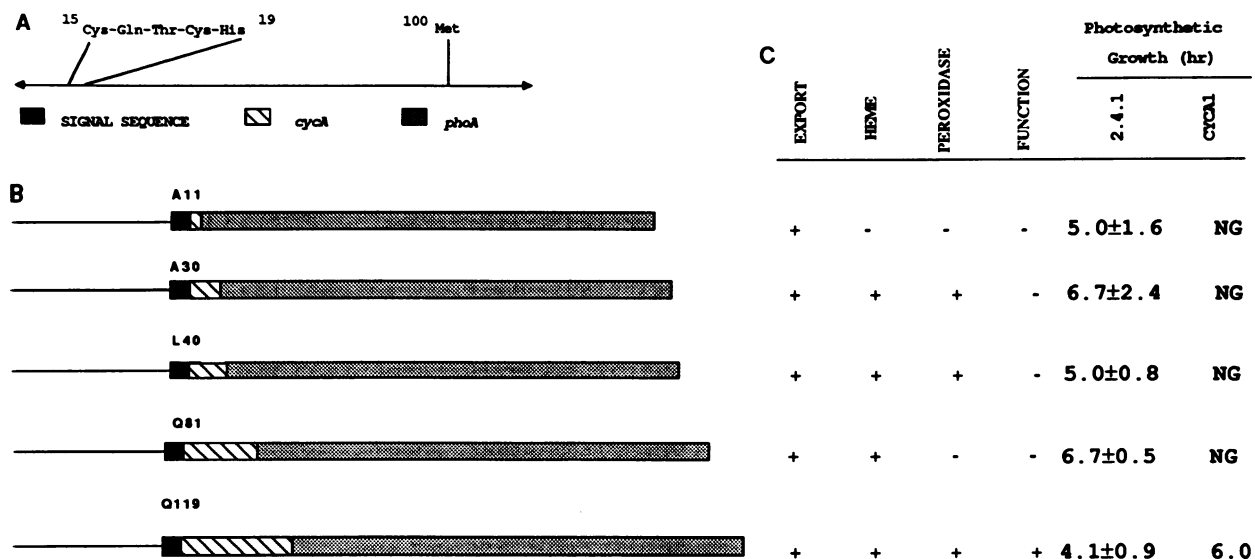


FIG. 5. Depiction of the *cyt c₂* amino acid sequence (A) and the five *CycA-PhoA* fusions (B) and summary of the phenotypes of the fusion-containing strains (C). The mature *cyt c₂* is composed of 124 amino acids, and precyt *c₂* has an additional 21-amino-acid signal sequence located at the amino terminus. The positions of the amino acids that interact with heme are also indicated. A summary of the pertinent activities of each *CycA-PhoA* fusion, including the photosynthetic generation times of the wild type (2.4.1) and the *cyt c₂* mutant (CYCA1) harboring the fusions is provided. NG, no growth.

DISCUSSION

Five *cycA-phoA* translational fusions were constructed in *E. coli*. Cells containing each of these hybrid proteins demonstrate alkaline phosphatase activity in vivo on indicator plates. A similar phenotype was seen when the fusion-encoding plasmids were placed in *trans* to the *R. sphaeroides* chromosome. Since a transcriptional-translational stop cartridge has been inserted immediately upstream of the *R. sphaeroides* DNA sequences on the plasmid, expression of these *cycA-phoA* constructions indicates that transcription of these fusions is directed by the *R. sphaeroides cycA* upstream sequences contained on the plasmids.

The *phoA*⁺ phenotype in *R. sphaeroides* suggests that each fusion protein is localized to the periplasm. This result was not unexpected, since we analyzed only those hybrids which were *pho*⁺ in *E. coli* and since Varga and Kaplan had previously demonstrated that a slightly different CycA-PhoA hybrid which contained only 10 amino acids of mature cyt c_2 was exported to the periplasm of *R. sphaeroides* (48). The periplasmic location was confirmed by alkaline phosphatase activity assays and immunological analysis of cell fractions prepared from the fusion-containing strains.

If we assume that the PhoA sequences do not contribute to export of the hybrids, our results would suggest that cyt c_2 export does not require heme attachment. If this were true, cyt c_2 maturation would differ from that of eucaryotic cyt c since import of apocyt c into mitochondria (44, 52) is coupled to heme attachment (15) and since only those apocyt c proteins which are substrates for heme attachment are imported into mitochondria (15). Thus, it is possible that the cellular constraints for *R. sphaeroides* cyt c_2 export are different than those for import of cyt c into mitochondria.

Although antiserum to either alkaline phosphatase or cyt c_2 reacts with a protein of approximately the predicted M_r for a full-size CycA-PhoA hybrid, it is apparent that some degradation of individual CycA-PhoA fusion proteins is occurring. The precise cause of this degradation is unknown, but cleavage of other PhoA protein fusions has been observed in both *R. sphaeroides* (36, 48) and *E. coli* (1, 3, 30, 45). In fact, we see similar degradation of the CycA-PhoA hybrids in *E. coli* CC118 (data not shown), so it is apparently not related to the physiological function of cyt c_2 in electron transport. Our results suggest that the CycA portion of the fusion proteins is being cleaved from the hybrids at specific sites. This conclusion is supported by the fact that the smallest protein detected with PhoA antiserum migrates to the same position on the NaDodSO₄-PAGE gel as wild-type *E. coli* alkaline phosphatase. In addition, this ~47,000- M_r species was not detectable with use of antiserum to cyt c_2 or by any of the heme detection methods that we used. Finally, we were unable to detect a pool of accumulated low-molecular-weight proteolysis products which either cross-react with cyt c_2 serum or contain heme.

When [³H]ALA labeling was used to detect proteins containing covalently bound heme, we found that all of the fusion proteins except A11 have heme attached. The absence of heme attachment to A11 is expected, since the fusion junction is upstream of the covalent heme attachment site. Our results provide the first in vivo demonstration that heme attachment can occur to a CycA protein which lacks the Met-100 noncovalent heme ligand. Therefore, heme attachment to the CycA gene product does not require the presence of all the heme ligands. This lack of a requirement for the COOH-terminal Met axial ligand is similar to that for eucaryotic cyt c (22), since heme is attached to yeast

iso-1-cyt c proteins in which the C-terminal axial ligand (Met-85) is replaced with an Arg or an Ile. Analyses of mutant iso-1-cyt c proteins with single amino acid substitutions have failed to identify any single amino acid outside the covalent heme attachment site that is essential for ligand association (22). The use of the CycA-PhoA fusions is consistent with this notion, since they have allowed us to determine that heme can be attached to a protein which lacks as many as 94 COOH-terminal amino acids of wild-type CycA. The results with our hybrid proteins may suggest that the lyase which attaches heme to CycA does not have an absolute requirement for COOH-terminal domains of the protein for substrate recognition or enzymatic activity.

We also demonstrated that in some cases, the heme attached to a CycA-PhoA fusion had low but detectable heme peroxidase activity. The only heme-containing protein which lacked peroxidase activity was the Q81 fusion protein. The inability to detect peroxidase activity associated with the Q81 protein may be due to the low level of the apparently full length protein found in Western blots (Fig. 1 and 2), or it may also reflect relatively inefficient heme attachment to the Q81 hybrid (Fig. 3).

The precise cytochrome polypeptide sequences necessary for peroxidase activity are unknown. Structural analysis of *b*-type cytochromes suggests that the axial ligands help keep the heme iron in the appropriate plane to allow heme peroxidase activity (17), so both axial heme ligands should be required for heme peroxidase activity. The Q119 fusion protein contains all of the CycA heme ligands (including Met-100), so perhaps it is not surprising that Q119 has peroxidase activity. In contrast, the heme peroxidase activity detected in the A30 and L40 fusion proteins might suggest that the Met-100 axial ligand is unnecessary for cyt c_2 heme peroxidase activity.

There is an apparent disparity between the CycA-PhoA protein levels (estimated by immunological analysis) and the levels of heme-containing protein (estimated by either [³H]ALA labeling or heme peroxidase staining). Western blots suggest that the fusion proteins are present at higher levels than wild-type cyt c_2 , which is consistent with the multicopy state of the RK2 plasmid encoding these fusions (5). However, the level of heme bound to the fusion proteins or their peroxidase activity is not consistent with the protein levels detected by immunoblot analysis. This disparity may be due to the degradation of the fusion proteins evident in the immunoblot analysis. Alternatively, the conformation of individual fusion proteins may reduce the ability of a given fusion to serve as a substrate for heme attachment or reduce the activity of that fusion in a heme peroxidase assay once heme is attached. However, it appears that this disparity is not due to titration of a cellular heme pool by plasmid-encoded proteins, because copy number levels of cyt c_2 are produced in cells that contain the intact *cycA* gene in *trans* (5).

Photosynthetic growth is restored to the cyt c_2 -deficient mutant, CYCA1, by introduction of a plasmid which encodes the Q119 fusion protein. This finding suggests that the Q119 CycA-PhoA polypeptide is in a conformation which allows it to bind to membrane-bound redox partners and function as an electron carrier. CYCA1(pC2Q119) grows with a photosynthetic doubling time approximately twice that of the wild type. Although this phenotype suggests that Q119 can function in electron transport, the reduced growth rate may reflect the fact that this protein is less efficient in either binding to redox partners or function in electron transport than is wild-type cyt c_2 . In addition, the [³H]ALA-

labeling and heme peroxidase activities associated with Q119 also suggest that this protein may be a relatively poor substrate for heme attachment. Further experiments are in progress to discriminate among these possibilities.

Synthesis of individual fusion proteins does not drastically affect growth of aerobically grown cells. However, photosynthetically grown cultures synthesizing a Cya-PhoA protein grow 1.2- to 2.7-fold more slowly than wild-type cells. The precise cause of this slowed photosynthetic growth is not known; it does not appear to be related to the size of the fusion protein. Results with other PhoA fusions in *R. sphaeroides* would also indicate that this growth phenotype is probably not due to a negative effect of this foreign protein on photosynthetic electron transport (36). Several of the fusion proteins are substrates for heme attachment, and one of them (Q119) is apparently able to function in electron transport. Therefore, one possible explanation is that individual fusion proteins interfere with or compete for wild-type cytochrome c_2 binding to components of the cyclic photosynthetic electron transport chain. Since cytochrome c_2 function is required for photosynthetic growth (14), such competition of the fusion protein with cytochrome c_2 for interaction with membrane-bound redox complexes could preferentially affect the growth of photosynthetic cells. Alternatively, high-level synthesis of these fusions could be indirectly affecting synthesis, export, or assembly of other complexes required for photosynthetic growth if they titrate the cellular pool of some export chaperone (for a review, see reference 41). Therefore, in addition to providing future insights into the processes of export and heme attachment to *c*-type cytochromes, these Cya-PhoA fusions may help map domains within cytochrome c_2 which interact with membrane-bound redox partners.

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