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ARTICLE *in* BIOCHEMISTRY · APRIL 1988

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Phenotypic and Genetic Characterization of Cytochrome c_2 Deficient Mutants of *Rhodobacter sphaeroides*†

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Received September 23, 1987; Revised Manuscript Received December 1, 1987

ABSTRACT: *Rhodobacter sphaeroides* mutants lacking cytochrome c_2 (cyt c_2) have been constructed by site-specific recombination between the wild-type genomic cyt c_2 structural gene (*cycA*) and a suicide plasmid containing a defective *cyc* operon where deletion of *cycA* sequences was accompanied by insertion of a Kn^R gene. Southern blot analysis confirmed that the wild-type *cyc* operon was exchanged for the inactivated *cycA* gene, presumably by double-reciprocal recombination. Spectroscopic and immunochemical measurements, together with genetic complementation, established that the inability of these mutants to grow under photosynthetic conditions was due to the lack of cyt c_2. The cyt c_2 deficient strains reduced photooxidized reaction center complexes approximately 4 orders of magnitude more slowly than the parent strain. The phenotype and characteristics of these mutants were restored when a wild-type *cyc* operon was introduced on a stable low copy number plasmid. These experiments provide the first genetic evidence for the obligatory role of cyt c_2 in wild-type cyclic photosynthetic electron transport in *R. sphaeroides*. We have also observed that the *R. sphaeroides* cyt c_2 deficient strains spontaneously gave rise to photosynthetically competent pseudorevertants at a frequency which suggests that the cyt c_2 independent photosynthetic electron transport which suppresses the phenotype of the cyt c_2 deficient strains was the result of a single mutation elsewhere in the genome.

The facultative photoheterotrophic bacterium *Rhodobacter sphaeroides* (recently redefined from the genus *Rhodopseudomonas*; Imhoff et al., 1984) provides an excellent model system to study physiological control of membrane biogenesis, photosynthesis, and function of electron-transport chains [see Staehelin and Arntzen (1986) for recent reviews]. Growth of this bacterium under chemoheterotrophic conditions is supported by a branched aerobic respiratory chain (Zannoni et al., 1980) consisting of a cytochrome c_2 (cyt c_2) dependent pathway with components structurally and functionally similar to those of mitochondrion, and a cyt c_2 independent redox chain (Zannoni & Baccarini-Melandri, 1980). Photoheterotrophic growth conditions induce synthesis of the intracytoplasmic membrane (ICM) which contains bacteriochlorophyll (Bchl)–protein complexes and redox components which convert light energy to cellular energy (Kaplan & Arntzen, 1981; Donohue & Kaplan, 1986).

Cyt c_2 is proposed to function as a mobile periplasmic redox carrier (Prince et al., 1975). In cells grown chemoheterotrophically with high oxygen tensions, cyt c_2 transfers electrons from the membrane-bound ubiquinol:cyt c_2 oxidoreductase (cyt b/c_1) complex (Gabellini et al., 1982) to a terminal cyt a/a_3 oxidase (Gennis et al., 1982). Under photoheterotrophic conditions, cyt c_2 completes the cyclic photosynthetic redox chain by transferring electrons from the cyt b/c_1 complex to the photooxidized reaction center (RC) complex of the ICM (Prince et al., 1974; Overfield et al., 1979; Bowyer et al., 1980).

Although the spectroscopic and kinetic parameters of photosynthetic electron flow in *R. sphaeroides* demonstrate the function of cyt c_2 in the proton-pumping two-electron Q-cycle (Crofts et al., 1983), genetic evidence for the obligate role of cyt c_2 is lacking (Daldal et al., 1986). Cyt c_2 independent electron transfer between cyt b/c_1 and RC complexes has recently been demonstrated in both wild-type and cyt c_2 deficient mutants of the closely related bacterium *Rhodobacter capsulatus* (Prince et al., 1986). In this paper, we document that *R. sphaeroides* strains devoid of cyt c_2 are unable to grow under photosynthetic conditions and thus provide the first genetic evidence for the obligate role of cyt c_2 in wild-type *R. sphaeroides* cyclic photosynthetic electron flow. In addition, we report that *R. sphaeroides* cyt c_2 deficient strains generate pseudorevertants which are capable of cyt c_2 independent growth under photosynthetic conditions.

Materials and Methods

Growth of Bacteria. As indicated in the text, we utilized both *R. sphaeroides* wild-type strain 2.4.1 and Ga, a previously described 2.4.1 derivative containing a mutation in carotenoid biosynthesis (Cohen-Bazire et al., 1956; Crofts et al., 1974). Both strains were originally obtained from Dr. W. R. Sistrom, University of Oregon. *R. sphaeroides* strains were grown at 32 °C chemoheterotrophically in Sistrom's minimal medium A (Leuking et al., 1978) either by vigorous shaking on a gyratory shaker or by sparging with a mixture of 30% O_2, 69% N_2, and 1% CO_2. Liquid photoheterotrophic cultures were maintained either by placing in completely filled culture vessels or by sparging with a mixture of 95% N_2 and 5% CO_2 using

† This work was supported by Grant 86-CRCR-1-2217 from the USDA Competitive Research Grants Program to T.J.D., by Grants GM31667 and GM15590 from the National Institutes of Health to S.K., and by Grants GM26305 and GM35438 from the National Institutes of Health to A.R.C. A North Atlantic Treaty Organization postdoctoral fellowship from the Science and Engineering Research Council, United Kingdom, supported A.G.M.

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previously described light intensities (Donohue et al., 1986) and estimates for cell number per Klett unit (no. 66 filter; Tai & Kaplan, 1985). Anaerobic growth on solid media was accomplished with BBL anaerobic gas pack jars and hydrogen–carbon dioxide generators. Gratuitous ICM synthesis was induced either by growth in a low-oxygen atmosphere (0.1% O_2, 99.4% N_2, and 0.5% CO_2) or by dark anaerobic respiration with dimethyl sulfoxide (DMSO) as a terminal electron acceptor. Dark anaerobic growth conditions used Sistrom's minimal medium A lacking succinate but supplemented with 20 mM glucose, 0.2% yeast extract, and 80 mM DMSO. Where appropriate, 1 μg/mL tetracycline or 25 μg/mL kanamycin was used for growth of *R. sphaeroides*. Photoheterotrophic cultures of *R. sphaeroides* grown in the presence of tetracycline were placed behind either a Corning CS 7-69 filter (620–1100 nm) or a Carolina Biological far-red 750 filter to minimize antibiotic photooxidation (Hasan & Khan, 1986) and the generation of growth-inhibitory products.

Escherichia coli strains harboring pUC19 (Yanisch-Perron et al., 1985), pSup202 (Simon et al., 1983), pRK404 (Ditta et al., 1985), or their derivatives were grown at 37 °C in the presence of ampicillin (50 μg/mL), chloramphenicol (50 μg/mL), kanamycin (50 μg/mL), or tetracycline (20 μg/mL for pSup202 vectors, 10 μg/mL for pRK404 vectors) where appropriate. When necessary, media were additionally supplemented with 40 μM isopropyl β-D-thiogalactoside and 30 μg/mL 5-bromo-4-chloro-3-indolyl β-D-galactoside. Cultures of *E. coli* S17-1 derivatives used as donors for conjugations contained approximately 2×10^6 colony-forming units mL^{-1}(Klett unit)$^{-1}$ (no. 66 filter).

Genetic Techniques. Plasmid DNA was mobilized into *R. sphaeroides* using derivatives of *E. coli* S17-1 (Simon et al., 1983) as donors. Filter matings were conducted on LB plates at 32 °C for 6 h using an input cell ratio of approximately 5×10^8 exponential-phase chemoheterotrophically grown recipients and 5×10^6 exponential-phase donors. The mating mixtures were removed from the filters into 1 mL of Sistrom's medium A, collected by centrifugation in sterile Eppendorf tubes, and suspended in 0.1 mL of the same medium prior to the addition of approximately 10^9 plaque-forming units of *E. coli* bacteriophage T_4D. This mixture was incubated at 37 °C for 15 min to allow infection of the donor cells, and the cells were washed twice in 1 mL of ice-cold Sistrom's medium A prior to plating under chemoheterotrophic conditions for selection of *R. sphaeroides* exconjugants.

Recombinant DNA Techniques. Purified plasmid DNA (Donohue et al., 1986), small-scale plasmid preparations (Maniatis et al., 1982), and bulk *R. sphaeroides* DNA from chemoheterotrophic cells were prepared as previously described (Nano & Kaplan, 1984). Treatment of DNA molecules with restriction endonucleases or other nucleic acid modifying enzymes was performed according to the manufacturer's specifications. The electrophoretic analysis or purification of DNA molecules from gel matrices has been described previously (Donohue et al., 1986). Chromosomal Southern blots (5 μg of DNA per lane) were performed with DNA transferred to nitrocellulose sheets by capillary action using nick-translated DNA probes and previously described stringency conditions (Donohue et al., 1986).

Immunochemical and Spectrophotometric Determination of Cyt c_2. *R. sphaeroides* cells were grown by dark anaerobic respiration, harvested in exponential phase (cell density of approximately 5×10^8 cells/mL), and lysed in a French press (Donohue et al., 1986). Unbroken cells and cell debris were removed by centrifugation (10000*g*, 10 min), the resulting crude cell lysate was fractionated into particulate and soluble fractions by high-speed centrifugation (40000 rpm, 3 h, Beckman 50Ti rotor), soluble and particulate fractions were analyzed by gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Donohue et al., 1986), and separated polypeptides were transferred to 0.2-μm nitrocellulose in preparation for immunoblotting (Chory et al., 1984). Western blotting was performed with rabbit antibody against purified cyt c_2 (Donohue et al., 1986). Goat anti-rabbit antibody conjugated to biotin and streptavidin–β-galactosidase were used to develop the immunoblots. Bluogal (halogenated indolyl β-D-galactoside) was used as a chromogenic substrate (Bethesda Research Laboratories, Gaithersburg, MD), and immunoblots were processed according to the manufacturer's specifications.

Reduced minus oxidized spectra in soluble cell extracts were recorded at room temperature with an SLM Aminco DW2000 spectrophotometer using 1 mM sodium ascorbate as a reductant and 800 μM potassium ferricyanide as an oxidant. The cytochrome content of soluble fractions was estimated by subtracting the absorbance at 540 nm from that at the α-peak maximum and assuming a millimolar extinction coefficient of 20.

Kinetics of Light-Induced Electron Flow. A computer-linked single-beam kinetic spectrophotometer equipped with a Xenon flash lamp (10-μs duration at half-maximal intensity) provided the saturating, single-turnover actinic light flashes (Bowyer et al., 1980). Light-induced redox changes were monitored as follows (Bowyer et al., 1980): Absorbance increases at 542 nm revealed RC P_{870} photooxidation, while the subsequent decrease in absorbance at 542 nm signified P^+_{870} reduction. Redox changes of *c*-type cytochromes (cyt c_1 and cyt c_2, if present) were obtained in the dual-wavelength mode by subtracting absorbance changes at 542 nm from those at 551 nm. A decrease in this parameter indicated oxidation of *c*-type cytochromes while an increase revealed *c*-type cytochrome reduction. Light-induced absorbance increases at 503 nm, known as the electrochromic carotenoid band shift, reflect generation of a membrane potential (Jackson & Crofts, 1971). The initial abrupt rise in absorbance at 503 nm after a saturating flash of actinic light is due to charge separation in the RC; the subsequent slow increase reflects movement of electrons across the membrane via the *b*-type cytochrome in the cyt b/c_1 complex (Jackson & Dutton, 1973).

Light-induced redox changes are reported for *R. sphaeroides* Ga and its derivatives. Exponential-phase cells were harvested by centrifugation, washed, and suspended in a modified Sistrom's minimal medium A containing 50 mM fumarate and 0.5 mM succinate. The cell suspension was allowed to dark-adapt under anaerobic conditions for 30 min before light-induced redox changes were measured and a fresh dark-adapted sample of cells was pumped in from a stirred, anaerobic reservoir for each train of light flashes. Under these conditions, the high-potential components of the redox chain (i.e., cyt c_1, any cyt c_2 present, Rieske FeS center, and P_{870}) become completely reduced, the quinone pool and cyt b_{50} become largely reduced, and the anaerobic environment prevents respiratory oxidation of these redox carriers.

Materials. All restriction endonucleases and nucleic acid modifying enzymes were obtained from either Bethesda Research Laboratories (Gaithersburg, MD) or New England Biolabs (Beverly, MA) and were used according to the manufacturer's specifications. Nitrocellulose paper used for Southern (0.45-μm pore size) and Western (0.22-μm pore size) blots was from Schleicher & Schuell (Keene, NH). [α-

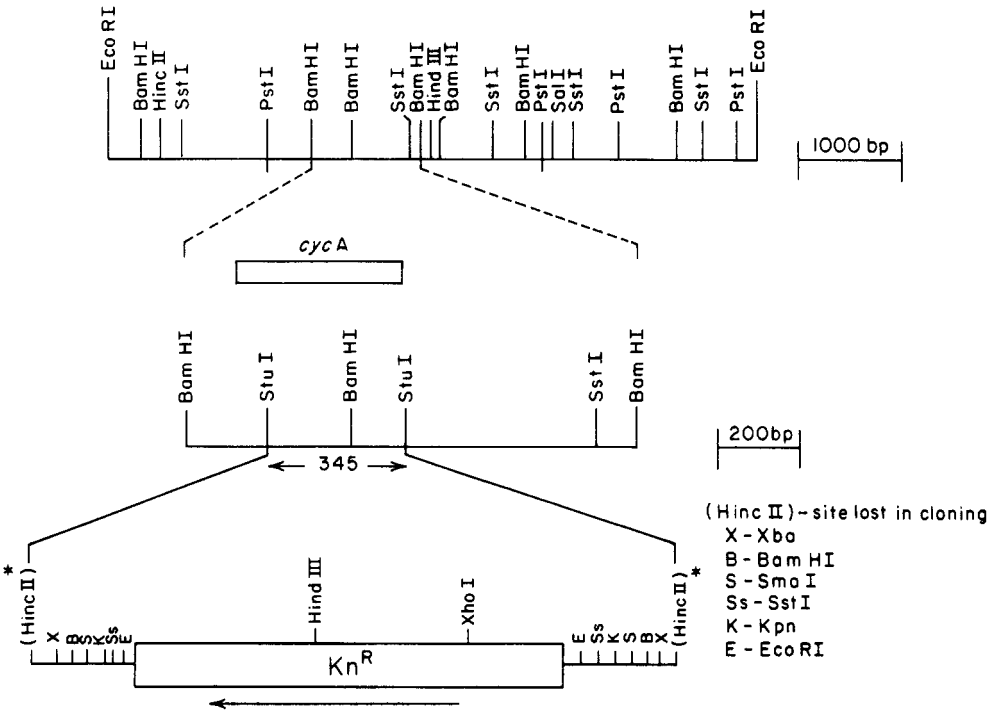

FIGURE 1: Mutagenesis of the *R. sphaeroides cyc* operon. The *R. sphaeroides cycA* gene is contained on a 2700 bp *Pst*I restriction endonuclease fragment within the 6250 bp *Eco*RI restriction endonuclease fragment shown at the top (Donohue et al., 1986). A 345 bp deletion within *cycA* was created by replacing the indicated *Stu*I restriction endonuclease fragment with the approximately 1450 bp Kn^R cartridge as a *Hinc*II restriction endonuclease fragment from plasmid pRME1 (Harayama et al., 1986). These *Stu*I sites were unique within pC2P2.71 which contains the 2700 bp *cycA*-containing *Pst*I restriction endonuclease fragment in pUC19 (Donohue et al., 1986). Restriction endonuclease mapping of the resulting plasmid, pC2P2.71::Kn (Δ*Stu*I), confirmed that transcription of the Kn^R gene was opposite that of the interrupted *cyc* operon (data not shown). The 3750 bp *Pst*I restriction fragment containing the interrupted *cyc* operon (with approximately 600 and 1500 bp of *R. sphaeroides* DNA upstream and downstream of the inactivated *cycA* gene, respectively) was cloned into the pSup202 *Pst*I site to generate pSupC2P2.71::Kn (Δ*Stu*I).

^{32}P]dCTP (800 Ci/mmol) was obtained from New England Nuclear Corp. (Boston MA). All other chemicals were of reagent grade.

RESULTS

Construction of Cyt c_2 Deficient Mutants. The *R. sphaeroides cyc* operon was interrupted by exchanging a 345 base pair (bp) *Stu*I restriction endonuclease fragment containing *cycA* sequences (extending from nucleotide 31 in the coding sequence for the mature cyt c_2 protein to the adenine in the *cycA* opal stop codon; Donohue et al., 1986) with an approximately 1450 bp *Hinc*II Kn^R cartridge (see Figure 1 for details). *R. sphaeroides* cyt c_2 deficient mutants were constructed by conjugation with *E. coli* S17-1 [pSupC2P2.71::Kn (Δ*Stu*I)] donors and selecting for Kn^R *R. sphaeroides* exconjugants under chemoheterotrophic plating conditions. This mutagenesis technique relied on the fact that pSup202 acts as a suicide plasmid in *R. sphaeroides* since its replication origin cannot function in this bacterium. Selection for Kn^R demands stable maintenance of the drug-resistance gene in the *R. sphaeroides* exconjugants, presumably by homologous recombination between the genomic *cyc* operon and the inactivated *cyc* operon on the suicide plasmid. *R. sphaeroides* Kn^R strains arising by double-crossover events should contain an inactive *cyc* operon replacing the wild-type genomic copy and be sensitive to tetracycline (encoded by pSup202). Strains containing the suicide plasmid incorporated into the genome are both Kn^R and Tc^R; this latter class of strains should contain multiple *cyc* operon sequences at least some part of which could be lost by recombination if they are grown in the absence of selection for both antibiotics. The other selectable markers on pSup202 are ineffective for such a screen in *R. sphaeroides* since this bacterium in naturally ampicillin resistant and does not express the pSup202 chloramphenicol resistance gene in vivo.

In several independent mutagenesis experiments using *R. sphaeroides* 2.4.1 as a recipient, 21 out of 163 Kn^R exconjugants were Tc sensitive (Tc^S) and unable to grow photosynthetically (representative isolates CYCA1, -10, and -11), 139 were Tc^R and capable of photosynthetic growth (CYCA2 and CYCA3), and 3 were Tc^R and photosynthetically incompetent. When strain Ga was used as a recipient, 10 out of 181 Kn^R exconjugants were Tc^S and photosynthesis incompetent (representative isolate CYCA65), 154 were Tc^R and capable of photosynthetic growth (CYCA19 and -20), and 17 were Tc^R and unable to grow photosynthetically (CYCA14 and -15). Southern blots demonstrated that representative Tc^R, Kn^R exconjugants derived from both 2.4.1 and Ga contained the suicide plasmid integrated in the genome, presumably by an uneven number of crossover events (data not shown). We are currently correlating the site of plasmid integration in the Kn^R, Tc^R derivatives with the existence of cyt c_2 and the photosynthesis phenotype. However, the fact that Tc^R, Kn^R photosynthesis-deficient exconjugants were obtained between 2% and 10% as frequently as those exhibiting a Tc^R, Kn^R photosynthesis-competent phenotype may suggest that the former class arose by plasmid integration events upstream (600 bp of homology) rather than downstream of *cycA* (1500 bp of homology). Uneven- and even-numbered recombination frequencies of 3.9×10^{-4} and 5.5×10^{-5} for strain 2.4.1 and of 4.2×10^{-4} and 1.6×10^{-5} for strain Ga, respectively, were calculated by assuming that the mobilization frequency of the pSup202 replicon was equivalent to that of a stable mobilizable control plasmid, pRK404.

Southern blots demonstrated that the genomic *cyc* operon was exchanged with the inactivated *cyc* operon in strains

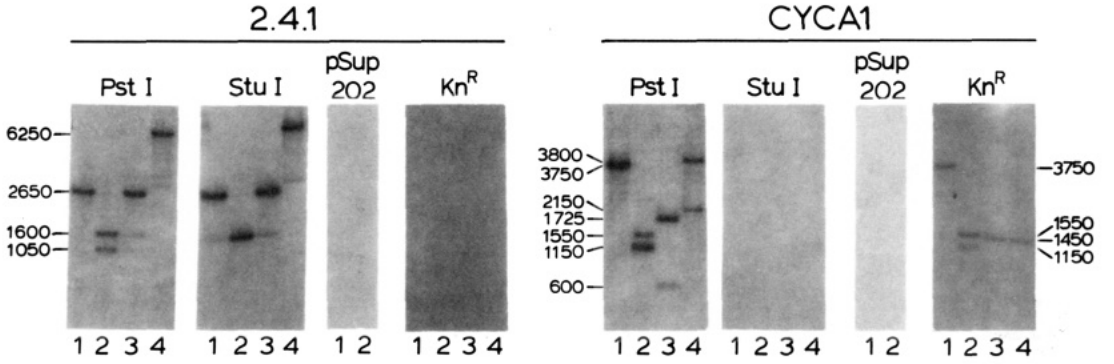

FIGURE 2: Genomic Southern blot analysis confirming the interruption of the *cyc* operon. Lanes 1–4 contain samples of the indicated genomic DNA digested with *Pst*I, *Pst*I–*Hin*dIII, *Pst*I–*Eco*RI, and *Eco*RI, respectively. DNA probes used were as follows: a 2700 bp *cycA*-containing *Pst*I restriction endonuclease fragment described in Figure 1; a 345 bp *cycA*-specific *Stu*I restriction endonuclease fragment which contained sequences replaced by the Kn^R gene in Figure 1; the intact pSup202 suicide plasmid; and a 1450 bp intact Kn^R gene as the *Hin*cII restriction endonuclease fragment shown in Figure 1. The numbers next to the autoradiograms show the sizes of restriction endonuclease fragments predicted to be homologous to the individual DNA probes. When the sizes of these restriction endonuclease fragments were measured relative to restricted bacteriophage λ molecular weight standards, the values obtained were within 10% of those expected.

CYCA1, -10, -11, and -65. The 3750 bp *Pst*I restriction endonuclease fragment in CYCA1 DNA was homologous to the 2700 bp *cycA*-containing *Pst*I restriction endonuclease fragment contained in 2.4.1 DNA (Figure 2, lane 1; *Pst*I and *Stu*I probes). These experiments documented collinearity of the interrupted *cyc* operon and the Kn^R gene on the 3750 bp *Pst*I restriction endonuclease fragment from CYCA1 genomic DNA (lane 1, *Pst*I and Kn^R probes), and they confirmed that the 3750 bp *Pst*I restriction endonucleae fragment from CYCA1 DNA contained the two expected *Eco*RI restriction sites flanking the Kn^R gene (lanes 3 and 4, *Pst*I and Kn^R probes). The predicted restriction endonuclease fragments derived from genomic CYCA1 DNA were homologous to the intact Kn^R gene probe, and, as expected, CYCA1 DNA lacked detectable homology to the 345 bp *cycA*-specific *Stu*I restriction endonuclease fragment which was replaced with the Kn^R gene. In addition, the 1550 bp *Pst*I–*Hin*dIII restriction endonuclease fragment in genomic CYCA1 DNA which hybridized to both the *Pst*I and the intact Kn^R DNA probes (Figure 2) was also homologous to an amino-terminal-specific *Xho*I–*Hin*dIII (Figure 1) Kn^R gene probe (data not shown). This fact confirmed that the direction of transcription of the Kn^R gene in the CYCA1 genome was the same as it was on the suicide plasmid. Finally, the absence of suicide plasmid sequences in CYCA1 genomic DNA was demonstrated by the lack of detectable homology to pSup202. The autoradiogram shown for both CYCA1 and wild-type 2.4.1 DNA with the pSup202 probe was from an exposure which showed strong homology to an equivalent amount of genomic DNA from several Kn^R, Tc^R strains which arose by an uneven number of crossover events (2.4.1 derivatives CYCA2 and CYCA3, and Ga derivatives CYCA14, -15, -19, and -20; data not shown). Results indentical with those shown in Figure 2 were obtained with genomic DNA from other representative Kn^R, Tc^S derivatives of 2.4.1 (CYCA10 and -11) or Ga (CYCA65) which were unable to grow under photosynthetic conditions at 3, 10, or 100 W/m^2 (data not shown).

That the inability of these Kn^R, Tc^S exconjugants to grow photosynthetically was due to disruption of the genomic *cyc* operon was further supported by the ability of the wild-type *cyc* operon to complement this phenotype in trans when it was supplied on a stable plasmid. Plasmids pC2P404.1 and pC2P404.2 contain the intact *cyc* operon from strain 2.4.1 cloned in the broad host range, low copy plasmid pRK404 (Ditta et al., 1985), such that the direction of *cyc* operon transcription is the same and opposite that of the pRK404 *lac* promoter, respectively (data not shown). When either plasmid pC2P404.1 or plasmid pC2P404.2 was mobilized into CYCA1, -10, -11, or -65, 100% of the Tc^R exconjugants were capable of photosynthetic growth at all light intensities tested with generation times similar to those of the respective *cycA*$^+$ parent containing the plasmid vector (data not shown). A detailed analysis of growth and regulation of the plasmid-encoded *cyc* operon at the RNA and protein level will be the subject of a separate paper.

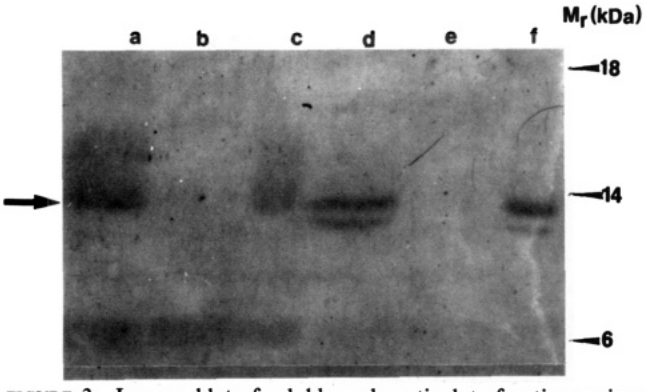

FIGURE 3: Immunoblot of soluble and particulate fractions using antibody against cyt c_2. Cell extracts were electrophoresed on 11.5–18% linear gradient SDS–PAGE gels (Donohue et al., 1986). Lanes a–c contain membrane fractions: (a) 40 μg of protein from 2.4.1 (pRK404); (b) 40 μg of protein from CYCA1 (pRK404); (c) 6 μg of protein from CYCA1 (pC2P404.1). Lanes d–f contain soluble fractions: (d) 40 μg of protein from 2.4.1 (pRK404); (e) 40 μg of protein from CYCA1 (pRK404); (f) 6 μg of protein from CYCA1 (pC2P404.1).

Immunological and Spectrophotometric Analysis of Cytochrome c_2. Cyt c_2 was measured immunochemically in soluble (lanes a–c) and particulate (lanes d–f) fractions of cells grown via dark anaerobic respiration (Figure 3). Anti-cyt c_2 serum detected two electrophoretic forms of cyt c_2, with apparent molecular weights of 13 500 and 12 500, in soluble fractions of 2.4.1 (pRK404) grown via dark anaerobic respiration (Figure 3, lane d). In contrast, the cyt c_2 in the particulate fraction consisted of only the large molecular weight form of cyt c_2 (Figure 3, lane a, see arrow). CYCA1 (pRK404) lacked immunochemically detectable cyt c_2 in either cell fraction (Figure 3, lanes b and e), while extracts from CYCA1 (pC2P404.1) contained the wild-type pattern of cyt c_2 species in both the soluble and particulate fractions (Figure 3, lanes c and f). These data correlate the phenotype of strain CYCA1 with the lack of cyt c_2 and demonstrate that the two

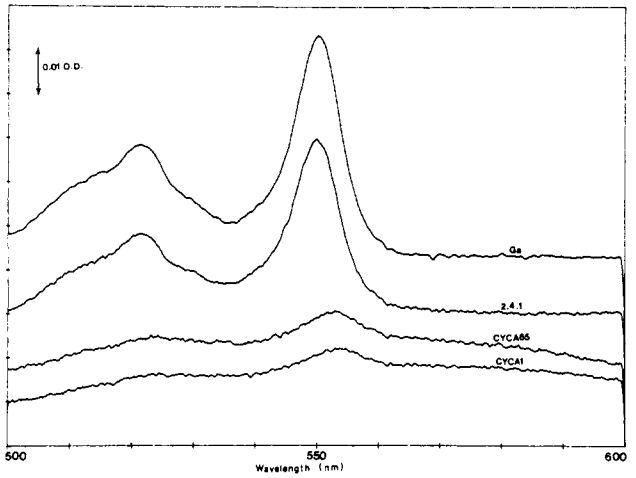

FIGURE 4: Reduced minus oxidized spectra of soluble extracts. Cell extracts were prepared and analyzed as described under Materials and Methods. The protein concentrations for soluble extracts analyzed were as follows: Ga, 4.8 mg/mL; 2.4.1, 3.4 mg/mL; CYCA65, 4.1 mg/mL; CYCA1, 3.2 mg/mL. The α-peak maxima obtained for the redox carrier(s) present in both Ga and 2.4.1 were approximately 550 nm, while those measured for CYCA65 and CYCA1 were approximately 554 nm.

electrophoretic cyt c_2 species in the soluble fraction are the product of the *cycA* gene.

Spectrophotometric analysis of soluble extracts confirmed the absence of an ascorbate-reducible *c*-type cytochrome with an α-band maximum at 550 nm (i.e., cyt c_2) in strains CYCA1 and CYCA65 (Figure 4). Both strains contain detectable amounts of an ascorbate-reducible *c*-type cytochrome in soluble extracts with an α-band maximum of approximately 554 nm when grown anaerobically in the dark with DMSO as an external electron acceptor. This 554-nm-absorbing species may be the soluble cyt c_{554} (Meyer & Cusanovich, 1985) which is reported to be induced when cells are grown via DMSO respiration (Ward et al., 1983). Assuming a millimolar extinction coefficient of 20 for those soluble redox carriers at the α-band maximum observed in the soluble extracts, we calculate total *c*-type cytochrome specific activities of 455 and 458 pmol/mg of soluble protein for 2.4.1 and Ga, respectively. These values are in excellent agreement with our previous estimates of total ascorbate-reducible *c*-type cytochrome (which we assumed to be all cyt c_2) in $cycA^+$ strains (Donohue et al., 1986). The *c*-type cytochrome level in CYCA1 and CYCA65 calculated from the data in Figure 4 is 73 and 78 pmol/mg of soluble protein, respectively. Assuming that the $cycA^-$ mutation does not affect synthesis of the 554 nm-absorbing species and that the 554-nm-absorbing species is due to a single redox carrier, this suggests that cyt c_2 is present in approximately 8-fold excess over cyt c_{554} when grown via DMSO respiration.

Kinetics of Light-Induced Electron Transport. The cyt c_2 deficient strains were incapable of photosynthetic growth, so the experiments in Figure 5 utilized cells grown by dark anaerobic respiration to induce ICM synthesis. Virtually identical results were obtained using cells grown in a low oxygen atmosphere (data not shown). The data in Figure 5 were generated with Ga derivatives to minimize interference from light-induced carotenoid absorbance changes in strains constructed from wild-type 2.4.1.

Reduction of photooxidized P^+_{870} was very rapid in the Ga cells and CYCA65 containing the *cyc* operon in trans on plasmid pC2P404.2 (Figure 5). The kinetics of P^+_{870} reduction observed in these two strains were consistent with previously reported half-times of 3 and 200 μs for the fast and slow phase of P^+_{870} reduction, respectively (Overfield et al., 1979; Bowyer et al., 1979). The redox conditions employed provided a highly reduced quinone pool since the absorbance changes observed upon a second saturating flash of light were greatly reduced. P^+_{870} reduction was significantly slower in CYCA65; consequently, RC oxidation was more easily visualized in this strain on this time scale (Figure 5). The 35-ms half-time of P^+_{870} reduction in CYCA65 (Figure 6) was approximately 4 orders of magnitude slower than the fast phase reported for the parent Ga strain (Overfield et al., 1979; Bowyer et al., 1979). Although P^+_{870} reduction was accompanied by *c*-type cytochrome oxidation in Ga and CYCA65 (pC2P404.2), the slowed P^+_{870} reduction in CYCA65 was not accompanied by detectable *c*-type cytochrome oxidation (Figure 5). Since the decay of the carotenoid change in CYCA65 showed a half-time in excess of 100 ms (Figure 6), a major fraction of the slow P^+_{870} reduction in CYCA65 was not due to direct P^+_{870} reduction by reduced quinone in the Q_A site (Wraight, 1979; Norris et al., 1986). In addition, phase III of the carotenoid band shift (which reflects turnover of the cyt b/c_1 complex) was present in Ga and CYCA65 (pC2P404.2) but undetectable in CYCA65, suggesting that the P^+_{870} reduction observed was independent of electron flow through the cyt b/c_1 complex.

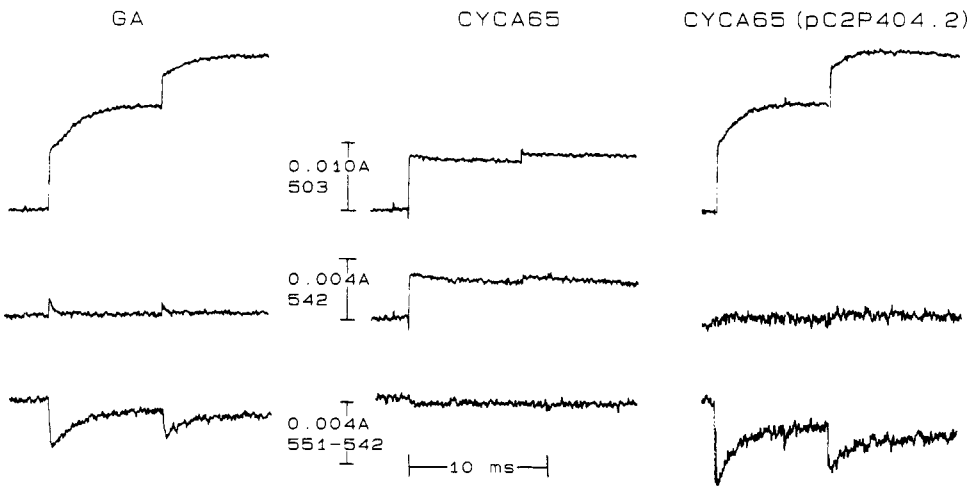

FIGURE 5: Kinetics of light-induced changes in the electrochromic carotenoid band shift (503 nm), P_{870} (542 nm), and *c*-type cytochromes (551–542 nm). Whole cells of *R. sphaeroides* strains Ga, a cyt c_2 deficient mutant derived from Ga (CYCA65), and CYCA65 harboring an intact *cyc* operon on plasmid pC2P404.2 were poised as described under Materials and Methods and analyzed at RC concentrations (Bowyer et al., 1981) of 0.3, 0.26, and 0.5 μM, respectively. The train of two saturating light flashes was given 8.2 ms apart, the traces shown are an average of 16 scans, and the instrument response time was 40 μs.

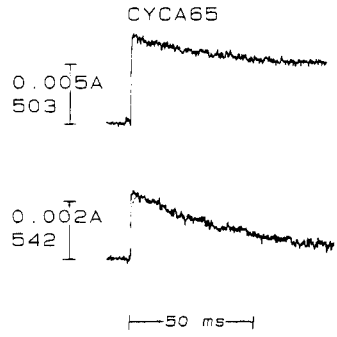

FIGURE 6: Light-induced electron transport in strain CYCA65. The experimental design was identical with that shown in Figure 5 except that a single flash was employed and the kinetics were monitored over a longer time scale with a response time of 200 μs to estimate half-times of membrane potential decay (503 nm) and P^+_{870} reduction (542 nm).

Finally, the data in Figure 5 demonstrate that the kinetics of light-induced electron flow returned to those of the Ga parent when strain CYCA65 contained the wild-type *cycA* gene in trans on plasmid pC2P404.2.

Mutations Which Suppress the Phenotype of Cyt c_2 Deficient Strains. The previously presented data demonstrated that the inability of the cyt c_2 deficient strains to grow photosynthetically could be attributed to a defect in RC reduction. During a physiological analysis of these strains, we noted that photosynthetically competent, Kn^R cells could be obtained in liquid cultures if chemoheterotrophically grown cells were incubated under photoheterotrophic conditions (100 W/m^2) for periods of time (i.e., 5–7 days, or longer) well past that required for chemoheterotrophic cultures of either the parent or a complemented strain to adapt to photosynthetic conditions. We subsequently isolated spontaneous photosynthetically competent derivatives of the cyt c_2 deficient strains by seeding petri plates with steady-state chemoheterotrophically grown cells and incubating these under photoheterotrophic conditions (10 W/m^2). Spontaneous Kn^R pseudorevertants which were capable of photosynthetic growth were obtained at a frequency of between 0.8 and 5 in 10^{-7} for the cyt c_2 deficient 2.4.1 derivatives CYCA1, -10, and -11 and approximately 9 in 10^{-7} for the cyt c_2 deficient Ga derivative CYCA65. The majority of the *cycA* coding sequence was deleted in the cyt c_2 deficient strains, and our preliminary analysis confirms that these pseudorevertants lack soluble, ascorbate-reducible cyt c_2. Thus, the frequency at which the photosynthetically competent pseudorevertants were obtained suggests that they resulted from a single mutation at another locus.

DISCUSSION

We have constructed *R. sphaeroides* cyt c_2 deficient mutants via homologous recombination between the chromosomal *cyc* operon and a defective *cycA* gene on a suicide plasmid. Our experiments have confirmed that the inability of these mutants to grow under photosynthetic conditions was due to alteration of the genomic *cyc* operon and the resulting lack of cyt c_2. Although the technology used has been described previously (Simon et al., 1983), to our knowledge this report represents the first successful application of this technique in *R. sphaeroides*. The general utility of this method for constructing *R. sphaeroides* mutants is documented by our recent isolation of strains lacking Bchl-binding proteins (Davis et al., 1988).

The presence of cyt c_2, the capacity for photosynthetic growth, and wild-type kinetics for RC reduction were all restored when strains harboring an altered genomic *cyc* operon contained the wild-type *cycA* gene with approximately 500 bp of upstream DNA on a stable low copy plasmid. This is consistent with our previous conclusion that at least one potential promoter for *cycA* expression was contained in this region (Donohue et al., 1986). We are currently determining how expression of the extrachromosomal *cyc* operon in the complemented strains is regulated at the RNA and protein levels relative to the genomic operon in wild-type strains.

Our experiments confirm the lack of spectroscopically and immunologically detectable cyt c_2 in CYCA1 and CYCA65 and present the first biochemical evidence for multiple electrophoretic forms of soluble cyt c_2 in cells grown by dark anaerobic respiration. We have previously shown that cyt c_2 and ICM Bchl-binding protein levels are similar in cells grown photosynthetically or by dark anaerobic respiration (Donohue et al., 1986), and we have noted an identical distribution of cyt c_2 species in particulate and soluble fractions of photosynthetically and DMSO grown cells (unpublished results). The two soluble electrophoretic forms of cyt c_2 represent mature species of this redox protein since they are both present in purified cyt c_2 preparations from photosynthetic cells, they both stain positive for heme after SDS–PAGE (unpublished results), and they both have an apparent molecular weight less than the cyt c_2 precursor polypeptide (Donohue et al., 1986).

Our working hypothesis is that the two soluble forms of cyt c_2 result from specific posttranslational modification(s) of the *cycA* protein which serve to direct the individual species to different environments and that the partitioning observed reflects the fact that the M_r 13 500 cyt c_2 form is tightly associated with the periplasmic leaflet of the ICM in vivo. If periplasmic cyt c_2 had simply been encapsulated within chromatophores during cell breakage, then one would also expect the small electrophoretic form to reside in the particulate fraction. The existence of two kinetically distinct populations of cyt c_2 molecules in chromatophore preparations has been proposed previously (Prince et al., 1974; Dutton et al., 1975, 1978). It seems unlikely that the kinetic behavior observed in earlier studies could be solely attributed to the two cyt c_2 species reported here since both kinetic phases were of similar amplitude and could also be explained in terms of individual spectroscopic contributions of cyt c_2 and the cyt c_1 of the cyt b/c_1 complex. However, if the small cyt c_2 form were loosely membrane-associated, it still might exhibit different kinetics of electron transfer to the RC than the large, membrane-associated cyt c_2 species.

We have demonstrated that *R. sphaeroides* cyt c_2 deficient strains are unable to grow under photosynthetic conditions due to their inability to rapidly reduce photooxidized RC complexes. That these mutants were also deficient in light-induced turnover of the cyt b/c_1 complex supports the function of cyt c_2 in the modified Q-cycle model for wild-type *R. sphaeroides* cyclic photosynthetic electron transport (Crofts et al., 1983). The ability of *R. sphaeroides* cyt c_2 deficient mutants to grow either under chemoheterotrophic conditions or via dark anaerobic respiration is also consistent with the existence of cyt c_2 independent routes for aerobic (Zannoni & Baccarini-Melandri, 1980; Zannoni et al., 1980) and dark anaerobic respiration (Richardson et al., 1986; Ward et al., 1983; Ferguson et al., 1987).

The inability of the *R. sphaeroides* cyt c_2 deficient mutants to grow under photosynthetic conditions is in marked contrast to the phenotype of *R. capsulatus* cyt c_2 deficient strains (Daldal et al., 1986). Light-induced electron transfer in *R. capsulatus* strains lacking cyt c_2 results in both cyt c_1 oxidation and turnover of the cyt b/c_1 complex, while the *R. sphaeroides* cyt c_2 deficient mutants showed no detectable light-induced

c-type cytochrome oxidation or turnover of the cyt b/c_1 complex (as measured by phase III of the carotenoid band shift). The *R. capsulatus* strains lacking cyt c_2 reduce photooxidized RC with two kinetic phases with the major component having a half-time on the order of tens of milliseconds, very similar to that found for the analogous *R. sphaeroides* mutants. Analysis of light-induced redox changes in both wild-type and cyt c_2 deficient *R. capsulatus* strains inferred that approximately 20% of the RC were reduced at a much more rapid rate (half-time <100 μs) by a previously undescribed cyt c_2 independent route, demonstrated that this process was not mediated by a soluble redox carrier, and suggested that cyt c_1 of the cyt b/c_1 complex was the direct cyt c_2 independent electron donor to the RC (Prince et al., 1986).

The isolation of *R. sphaeroides* cyt c_2 deficient pseudorevertants which were capable of photosynthetic growth demonstrated that cyt c_2 independent pathways for RC reduction can also exist in this bacterium. Preliminary analysis of one such strain which grew photosynthetically with wild-type generation times (CYCA65R7) indicates that light-induced oxidation of a *c*-type cytochrome in whole cells is approximately 35-fold faster (1-ms half-time) than the half-time of P^+_{870} reduction in CYCA65 (unpublished results). CYCA65R7 also exhibited a phase III of the electrochromic carotenoid band shift with a half-time of approximately 7 ms and an amplitude of about 50% of the fast phase. The slow half-time of phase III of the carotenoid band shift compared to the Ga parent might indicate a reduced coupling efficiency between cyt b/c_1 and RC complexes in CYCA65R7. Finally, the kinetics of light-induced electron flow in spheroplast preparations from both Ga and CYCA65R7 are similar to those of whole cells of CYCA65 (i.e., slow P^+_{870} reduction and lack of detectable *c*-type cytochrome oxidation), suggesting that the intervening redox carrier in CYCA65R7 is a periplasmic protein. We are currently examining the cytochrome profile and the kinetics of light-induced electron flow in this and other cyt c_2 deficient pseudorevertants in more detail.

At present, we do not know the nature of the mutation which suppresses the inability of *R. sphaeroides* cyt c_2 deficient cells to grow under photosynthetic conditions or whether these apparently extragenic suppressors can arise from mutations in one or more genes. Experiments are in progress to determine whether these pseudorevertants arise from mutations causing (i) increased synthesis of one or more of the functionally cryptic *R. sphaeroides* periplasmic redox carriers (Meyer & Cusanovich, 1985), (ii) structural gene changes in previously characterized redox proteins which allow them to function in light-induced electron flow, or (iii) synthesis of a previously unreported redox carrier which can replace cyt c_2 in photosynthetic electron flow (i.e., a plastocyanin-type redox carrier). Such an analysis will be very informative with regard to the function and characteristics of redox proteins in photosynthetic electron transport.

ACKNOWLEDGMENTS

We thank M. Rott, J. Brandner, and B. MacGregor for technical assistance with some aspects of these experiments, P. J. Kiley and R. B. Gennis for many useful discussions during the course of these experiments, and S. Harayama for plasmid pRME1.

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Ethanol-, Fasting-, and Acetone-Inducible Cytochromes P-450 in Rat Liver: Regulation and Characteristics of Enzymes Belonging to the IIB and IIE Gene Subfamilies†

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Received May 29, 1987; Revised Manuscript Received October 1, 1987

ABSTRACT: Two major forms of hepatic microsomal cytochrome P-450 were purified from starved and acetone-treated rats. On the basis of amino acid sequence analysis, they were identified as P-450j and P-450b. Ethanol or acetone treatment of rats caused a 9-fold increase in the amount of P-450j in liver microsomes accompanied by similar increases in the rate of NADPH-dependent metabolism of carbon tetrachloride, acetone, and benzene. Immunological experiments indicated that P-450j constitutes the major catalyst of the microsomal metabolism of the latter agents and contributes by about 50% to microsomal P-450-dependent ethanol oxidation under the conditions used. The P-450j-dependent catalytic activities had a high rate of turnover. In contrast, this was not the case for the immunodetectable P-450j, indicating the occurrence of inactive forms of this protein in microsomes. Starvation or ethanol or acetone treatment caused 10–30-fold increases in the amount of both mRNA and apoprotein of P-450b,e compared to control. Run-on experiments and the concomitant increases of the P-450b,e gene products at the mRNA and protein levels indicated the appearance of mainly a transcriptional activation by acetone, ethanol, or starvation. Fasting exerted, in addition, a pronounced synergistic effect on acetone-dependent induction of P-450b,e mRNA (3-fold), apo-P-450b,e (4.3-fold), P-450j mRNA (2-fold), and apo-P-450j (2-fold). No increase of mRNA coding for P-450j, compared to control, was seen after acetone or ethanol treatment alone. The results indicate that effects of ethanol, acetone, and/or starvation on drug and xenobiotic metabolism are caused by the induction of P-450 forms belonging to at least two gene subfamilies.

Ethanol is known to affect drug metabolism in two principally different ways. The acute effect of the alcohol is mainly exhibited by inhibition of drug oxidation, probably caused by competitive interactions with cytochromes P-450 (Rubin & Lieber, 1968; Rubin et al., 1970, 1971). The chronic effect of ethanol on drug metabolism is the opposite, i.e., an increase of the metabolism rate [cf. Linnoila et al. (1979) and Khanna et al. (1976)], and includes a proliferation of the smooth endoplasmic reticulum (Iseri et al., 1966), as well as an increase in the amount of hepatic cytochrome P-450 (Rubin et al., 1968; Villeneuve et al., 1976; Ekström et al., 1986). The rate of drug clearance from the blood is enhanced after ethanol consumption [cf. Kater et al. (1969)], as examplified by the elimination of, e.g., meprobamate and pentobarbital from the blood of man or rats (Misra et al., 1971). The alcohol effect on clearance appears mainly due to an enhanced rate of hepatic metabolism of the compounds, as evidenced by studies with meprobamate (Misra et al., 1971), aminopyrine (Vesell et al., 1971), propanolol (Prichard & Schneck, 1977), and rifamycin (Grassi & Grassi, 1975).

Ethanol treatment of rats results in an enhanced rate of microsomal metabolism of halogenated hydrocarbons, e.g., carbon tetrachloride (CCl_4)[1] (Maling et al., 1975), chloroform

† Supported by grants from Magn. Bergvalls Stiftelse and the Swedish Medical Research Council.
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[1] Abbreviations: P-450, cytochrome(s) P-450; PEG, poly(ethylene glycol); HFBA, heptafluorobutyric acid; TBA, thiobarbituric acid; A, rats treated with acetone (5 mL/kg) for 1 day; SA, rats starved for 24 h and subsequently treated with acetone (5 mL/kg) for 1 day; SA^2, rats starved for 24 h and subsequently treated with acetone (5 mL/kg) for 2 days; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; CCl_4, carbon tetrachloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid. The gene nomenclature is given in Nebert et al. (1987).

0006-2960/88/0427-1925$01.50/0