The cbb_3 Terminal Oxidase of *Rhodobacter sphaeroides* 2.4.1: Structural and Functional Implications for the Regulation of Spectral Complex Formation†

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ABSTRACT: We have previously shown that the flow of reductant through the cbb_3 terminal cytochrome *c* oxidase of *Rhodobacter sphaeroides* is essential to the repression of photosynthesis (PS) gene expression in the presence of oxygen by inhibiting the functional activity of the Prr two-component activation system. To gain further insight into the role of the cbb_3 oxidase and the cognate *ccoNOQP* operon in the oxygen regulation of PS gene expression, we constructed nonpolar, in-frame deletions within the *ccoN* and *ccoQ* genes. Whereas mutations in *ccoN*, *ccoQ*, and *ccoP* resulted in PS gene expression in the presence of oxygen, only the *ccoQ* mutation showed both the normal flow of reductant through the cbb_3 oxidase and the absence of any alteration in the relative levels of spheroidene and spheroidenone, as is observed for those mutations in the *cco* operon that result in the loss of terminal oxidase activity. Consistent with these findings is the observation that extra copies of the *ccoNOQP* operon *in trans* resulted in the decreased formation of both the B800−850 and B875 spectral complexes under anaerobic growth conditions. These results in conjunction with our earlier findings indicate that (1) the flow of reductant through the cbb_3 terminal oxidase is a prerequisite to the regulation of PS gene expression by the Prr two-component regulatory system, (2) the CcoQ protein is involved in conveying the signal derived from reductant flow through the cbb_3 terminal oxidase to the Prr regulatory pathway, (3) there is reductant flow through this terminal oxidase under anaerobic conditions, and as a result, the activity of the Prr system is still subject to cbb_3 regulation, and (4) the acceptor for reductant flow through cbb_3 under anaerobic conditions is in whole or in part involved in the conversion of spheroidene to spheroidenone.

The purple non-sulfur photosynthetic bacterium *Rhodobacter sphaeroides* is able to grow by aerobic and anaerobic respiration and photosynthetically in the light under anaerobic conditions. The photosynthetic apparatus of this bacterium is normally synthesized under conditions of low O_2 (less than 3%) or anaerobiosis. Previous studies revealed that the activation of photosynthesis (PS)[1] gene expression under low O_2 tensions is mediated mainly through the intervention of three key regulatory systems, namely, the FnrL, Prr, and AppA−PpsR systems (*1*) during the transition from aerobic to either anaerobic conditions or conditions of low O_2 growth. *R. sphaeroides* possesses two functional terminal cytochrome *c* oxidases, the aa_3- and cbb_3-type cytochrome *c* oxidases, which both belong to the heme−copper oxidase superfamily (*2*, *3*). The cbb_3 oxidase encoded by the *ccoNOQP* operon is expressed primarily under microaerobic and anaerobic conditions (*2*, *4*, *5*), reflecting its high affinity for O_2. The *ccoN* product is the catalytic subunit and contains the binuclear center consisting of the B-type high-spin heme and Cu_B. CcoO and CcoP are membrane-bound mono- and diheme cytochromes *c*, respectively, and transfer the electrons from cytochrome c_2 to CcoN.

CcoQ of *R. sphaeroides* is predicted to be a small membrane-bound polypeptide consisting of 67 amino acids. It was demonstrated in *Bradyrhizobium japonicum* that the CcoQ analogue, namely, FixQ, is a true subunit of the cbb_3 oxidase (*6*) and that an in-frame deletion mutation in *fixQ* has no apparent effect on assembly or on the activity of the cbb_3 oxidase (*7*).

Previous studies in our laboratory revealed that insertion mutations in *ccoP* as well as *rdxB*, which is the first gene of the *rdxBHIS* operon located 234 bp downstream of the *cco* operon in *R. sphaeroides*, resulted in the induction of the photosynthetic apparatus under aerobic growth conditions accompanied by increased PS gene expression, as well as the accumulation of high levels of the carotenoid spheroidenone relative to spheroidene under photosynthetic conditions (*8*). These results have been interpreted to suggest that a pathway involving the cbb_3 oxidase/Rdx protein through its interaction with O_2 serves as an oxygen sensor to generate an inhibitory signal for PS gene expression. This signal obligatorily communicates with the PrrBA two-component activation system to regulate PS gene expression (*9*).

However, the manner in which the Prr system interacts with the cbb_3 oxidase/Rdx protein remained to be clarified.

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[1] Abbreviations: ALA, 5-aminolevulinic acid; Bchl, bacteriochlorophyll; cbb_3 or cbb_3 oxidase, cbb_3-type cytochrome *c* oxidase; Crt, carotenoid; DMSO, dimethyl sulfoxide; ICM, intracellular membrane; HPLC, high-performance liquid chromatography; *o*-NPG, *o*-nitrophenyl β-D-galactopyranoside; PCR, polymerase chain reaction; PS, photosynthesis; RC, reaction center; SIS, Sistrom's medium A; SE, spheroidene; SO, spheroidenone.

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Table 1: Bacterial Strains and Plasmids Used in This Work

strain or plasmid	relevant phenotype or genotype	source or reference
	R. sphaeroides	
2.4.1	wild type	*10*
CCOP1	2.4.1 derivative, *ccoP*::ΩTp^r	*8*
CCONΔ	2.4.1 derivative, in-frame deletion in *ccoN*	this study
CCOQΔ	2.4.1 derivative, in-frame deletion in *ccoQ*	this study
RDXBΔ	2.4.1 derivative, in-frame deletion in *rdxB*	this study
	E. coli	
DH5α*phe*	(Φ80d*lacZ*Δ*M*15) Δ*lacU169 recA1 endA1 hsdR17 supE44 thi1 gyrA96 relA1 phe::Tn10d*Cm	*11*
S17-1	Pro$^-$Res$^-$Mod$^+$*recA*; integrated plasmid RP4-Tc::Mu-Km::Tn*7*	*12*
	Plasmid	
pUC18	Ap^r; *lacPOZ*′	*13*
pBluescript SK+	Ap^r; *lacPOZ*′	Stratagene
pRK415	Tc^r; Mob$^+$ *lacZ*α IncP	*14*
pLO1	Km^r; *sacB* RP4-*oriT* ColE1-*oriV*	*15*
pUI2803	pRK415::4.7-kb *Bam*HI−*Eco*RI fragment containing *ccoNOQP*	*8*
pUI2805	pRK415::5.6-kb *Bam*HI−*Pst*I frafment containing *rdxBHI*′	*8*
pCCO1	pBluescript SK+::4.7-kb *Bam*HI-*Eco*RI fragment containing *ccoNOQP* from pUI2803	this study
pOHQ-1	pRK415::0.86-kb *Pst*I fragment containing *ccoQ*; *ccoQ* is in divergent orientation to Tc resistance gene	this study
pOHQ-2	pRK415::0.86-kb *Pst*I fragment containing *ccoQ*; *ccoQ* is in collinear orientation to Tc resistance gene	this study
pCCONΔ4	pLO1::1.2-kb *Sac*I−*Xba*I fragment containing Δ(*ccoN*)	this study
pCCOQΔ1	pLO1::1.5-kb *Pst*I−*Sac*I fragment containing Δ(*ccoQ*)	this study
pRDXBΔ3	pLO1::2.4-kb *Pst*I−*Sac*I fragment containing Δ(*rdxB*)	this study
pCF200Km	Sp^{r}St^{r}Km^r; IncQ, *puc*::*lacZYA*′	*16*
pUI1663	Sp^{r}St^{r}Km^r; IncQ, *puf*::*lacZYA*′	*17*

In an effort to approach this and other questions we have assessed the role of the *ccoQ* gene product in *R. sphaeroides*. Studies of the CcoQ mutant demonstrated that CcoQ is involved in the transduction of the O_2-sensing signal generated through the cbb_3 oxidase/Rdx protein to a downstream regulatory element, perhaps PrrB or PrrB through PrrC. Either the redox state or the accumulation of a certain redox intermediate of the electron transport chain must be present to act as the signal to regulate PS gene expression as well as to determine the carotenoid composition and ultimately the ratio of the B800−850 to B875 spectral complex.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Rhodobacter sphaeroides* 2.4.1 strains were grown at 30 °C on Sistrom's medium A (SIS) (*18*) containing succinate as the carbon source and supplemented, as required, with the following antibiotics: tetracycline, 1 μg/mL; kanamycin, 50 μg/mL; trimethoprim, 50 μg/mL; and streptomycin and spectinomycin, 50 μg/mL each. Chemoheterotrophic cultures were grown aerobically on a rotary shaker with vigorous shaking using baffled flasks. Photosynthetic cultures were grown at a medium incident light intensity of 10 W/m^2 in completely filled screw-capped glass tubes. The tubes containing photosynthetic cultures were rotated by using a rotary drum to keep cells suspended and mixed. Alternatively, photosynthetic cultures were grown by being sparged with 95% N_2−5% CO_2 (*8*). Anaerobic growth with dimethyl sulfoxide (DMSO) as a terminal electron acceptor was performed in SIS medium supplemented with 0.1% (w/v) yeast extract in the presence of DMSO [0.5% (w/v)] in screw-capped tubes in the dark or light (10 W/m^2). *Escherichia coli* strains were grown at 37 °C on LB medium supplemented, when required, with the following antibiotics: ampicillin, 50 μg/mL; kanamycin, 50 μg/mL; tetracycline, 20 μg/mL; and streptomycin and spectinomycin, 50 μg/mL each.

DNA Manipulations. Genomic DNA from *R. sphaeroides* was isolated according to Ausubel et al. (*19*). Standard protocols or manufacturer's instructions were followed for recombinant DNA manipulations (*20*).

Conjugation Techniques. Plasmids were mobilized by biparental matings from *E. coli* S17-1 strains into *R. sphaeroides* strains as described elsewhere (*21*).

Construction of In-Frame Deletion Mutations: (A) ccoQ. For the construction of the in-frame deletion mutation, two rounds of the polymerase chain reaction (PCR) were carried out. With pCCO1 as the template, two primary PCR reactions were performed with primers DB+ (5′-GGTAAGGGAGCG-CAGGCATGGTGTCGTCGTCCGAGGAGGC-3′) and XBAI (5′-CGACCTTCTAGATCTGGGTGCTGT-3′), and with primers DB− (5′-GCCTCCTCGGACGACGACACCAT-GCCTGCGCTCCCTTACC-3′) and SACI (5′-CCGCTC-GAGCTCGCCGGGCGCGAC-3′) to generate two 40-bp overlapping DNA fragments (620 and 602 bp, respectively), both bearing the same 171-bp in-frame deletion within *ccoQ* in the region of overlap. The 40-mer primers DB+ and DB− are complementary to each other. The two primary PCR products were used as templates for the secondary PCR. The overlap in sequence allows the DNA fragments to recombine during the secondary PCR reaction, producing a complete duplex fragment that can serve as template for the secondary reamplification with the primers SACI and XBAI. The principle and procedure of the PCR mutagenesis used in the construction of the CcoQ mutant are described elsewhere (*22*). The 1202-bp secondary PCR product containing the in-frame deletion of *ccoQ* was purified with the QIAquick gel extraction kit (Quiagen Inc., Santa Clarita, CA) and restricted with the restriction enzymes *Sac*I and *Xba*I. The digested PCR product was cloned into the vector pLO1, which is a suicide vector in *R. sphaeroides*, restricted with

the same enzymes. The resulting plasmid pCCOQΔ1 was transferred from *E. coli* strain S17-1 to *R. sphaeroides* by conjugation. Heterogenotes of *R. sphaeroides*, generated by a single recombination event, were selected for their kanamycin resistance, and homogenotes were obtained from the heterogenotes after a second recombination for sucrose resistance as detailed by Lenz et al. (*15*). The allelic exchange in the homogenotes that produced isogenic CcoQ in-frame deletion mutants was verified by PCR with isolated genomic DNA.

(B) ccoN. For the construction of the *ccoN* deletion mutation, a 546-bp *Nar*I fragment within *cco*N was deleted. The 2.1-kb *Pst*I−*Xho*I fragment from pCCO1 was cloned into pBluescript SK+ digested with the same enzymes, yielding the plasmid pCCONΔ1. Following the digestion of pCCONΔ1 with *Nar*I, the plasmid was self-ligated to remove the 546-bp internal *Nar*I fragment (pCCONΔ2). The 1.5-kb *Kpn*I−*Xba*I fragment from pCCONΔ2 was cloned into pUC18 digested with the same enzymes to add the restriction sites for *Pst*I and *Sac*I to both flanking sides of the fragment for the next cloning step (pCCONΔ3). The 1.5-kb *Pst*I−*Sac*I fragment from pCCONΔ3 was finally cloned into pLO1 to give the plasmid pCCONΔ4. The allelic exchange using pCCONΔ4 was performed in the same way as described above for construction of the CcoQ mutant.

(C) rdxB. For the introduction of an in-frame deletion into *rdxB*, a 234-bp *Nco*I fragment was removed from the gene. To this end, the 2.7-kb *Pst*I−*Sac*I fragment from pUI2805 was cloned into pBluscript SK+ digested with the same enzymes. The resulting plasmid pRDXBΔ1 was subject to *Nco*I partial restriction. The plasmids, from which 234-bp *Nco*I fragment is deleted, were isolated from a 0.8% (w/v) agarose gel following electrophoresis and religated, yielding the plasmid pRDXBΔ2. Finally, the 2.4-kb *Pst*I−*Sac*I fragment from pRDXBΔ2 was cloned into pLO1 (pRDXBΔ3). Mutant selection was carried out as described above.

Quantitative Analysis of Spectral Complexes. The harvested cells were resuspended in ICM buffer (10 mM KH_2PO_4/K_2HPO_4 and 1 mM EDTA, pH 7.2) and disrupted by passage through a French pressure cell (ca. 0.9 cm diameter piston, 90 MPa). Cell-free crude extracts were obtained following two rounds of centrifugation in a benchtop microcentrifuge at 13 000 rpm for 15 min to remove cell debris. Spectra were recorded with a UV 1601PC spectrophotometer (Shimadzu Corp., Columbia, MD). The B800−850 and B875 complex levels were determined by the method of Meinhardt et al. (*23*) from the spectral data collected.

Carotenoid and Bacteriochlorophyll Analyses. For extraction of photopigments, 0.5 mL of crude cell extract was mixed with 1.5 mL of acetone/methanol (7:2 v/v) followed by vigorous vortexing for 1 min. After centrifugation in a benchtop microcentrifuge at 13 000 rpm for 15 min, the supernatant was used for quantitation of carotenoid and bacteriochlorophyll as described previously (*18*). The ratio of spheroidenone to spheroidene was determined by high-performance liquid chromatography (HPLC) with a Shimadzu system equipped with an SPD-M10AV diode array detector as described by Yeliseev et al. (*24*).

Enzyme Assays and Protein Determination. Cultures of *R. sphaeroides* were grown to mid-exponential phase. Cells were harvested, resuspended in the appropriate buffer, and disrupted by passage through a French pressure cell. Cell-free crude extracts were obtained by centrifugation at 13 000 rpm for 15 min.

β-Galactosidase activity was assayed spectrophotometrically by determining the initial conversion rate of the substrate analogue *o*-nitrophenyl β-D-galactopyranoside (*o*-NPG) to *o*-nitrophenol at 420 nm and 30 °C for 1 min with a UV 1601PC spectrophotometer (*25*). The standard assay mixture (1 mL) is composed of 200 μL of *o*-NPG [0.2% (w/v)], appropriate volume (10−50 μL depending on activity) of crude cell extract, and β-galactosidase buffer (50 mM potassium phosphate buffer, pH 7.0, containing 10 mM KCl, 1 mM $MgSO_4$, and 10 mM β-mercaptoethanol).

Cytochrome *c* oxidase activity was measured spectrophotometrically by monitoring the oxidation of reduced horse heart cytochrome *c* (Sigma, St. Louis, MO) at 550 nm and 30 °C (*26*). The standard assay mixture (1 mL) is composed of 20 μL of 1.25 mM reduced cytochrome *c*, appropriate volume (10−50 μL) of crude cell extract, and 20 mM phosphate buffer (pH 7.5) containing 5 μM myxothiazol (Sigma). Alternatively, the presence of cytochrome *c* oxidase was tested by the NADI reaction as described elsewhere (*27*). In this assay, colonies that contain an active cytochrome *c* oxidase turn blue within 1 min.

Protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL) with bovine serum albumin as the standard protein.

RESULTS

Construction of In-Frame Deletion Mutation of ccoQ. In an attempt to determine the function of the *ccoQ* gene, an in-frame deletion mutation of *ccoQ* (strain CCOQΔ) was constructed to avoid any polar effect of the mutation on the *ccoP* gene located immediately downstream of *ccoQ*. A 171-bp DNA segment of the *ccoQ* gene consisting of 204-bp nucleotides was deleted by the recombination PCR approach outlined in Materials and Methods. In addition, the in-frame deletion mutation of *ccoN* (strain CCONΔ), whose product contains the active site of the cbb_3 oxidase, was also constructed for the purpose of control studies with the CcoQ mutant. When grown on SIS agar plates in the presence of O_2, deletion mutations in the *ccoQ* and *ccoN* genes gave rise to colonies with deep red pigmentation, compared with the light red coloration of the wild type. This phenotype was previously observed for the *ccoP* or *rdxB* insertion mutations of *R. sphaeroides* 2.4.1, which produce the photosynthetic apparatus under aerobic conditions (*8*). The CcoQ and CcoN mutants are stable under aerobic conditions, as judged from the uniform red colony pigmentation, as opposed to PpsR and PRRB78 mutants, which show a very dark red pigmentation and a high degree of genetic instability under these same conditions (*28*, *29*).

*Analysis of the Spectral Complexes in the CCOQ*Δ. To confirm that the deep red coloration of the CcoQ and CcoN mutants under aerobic conditions was due to the presence of photosynthetic spectral complexes, crude extracts from cultures of these mutant strains grown aerobically were prepared and the levels of the light-harvesting complexes (B800−850 and B875) as well as the photopigments, bacteriochlorophyll (Bchl) and carotenoid (Crt), were determined spectrophotometrically (Table 2). For comparison,

Table 2: Levels of Spectral Complexes and Photopigments in *R. sphaeroides* Strains Grown under Aerobic Conditions

strain[a]	B800−850[b]	B875[b]	Bchl[c]	Crt[c]
2.4.1	0.1 ± 0.1	0.1 ± 0.0	0.4 ± 0.1	0.4 ± 0.1
CCONΔ	1.0 ± 0.2	6.4 ± 0.6	2.3 ± 0.4	1.2 ± 0.1
CCOQΔ	0.7 ± 0.1	5.7 ± 0.9	1.9 ± 0.5	1.0 ± 0.1
CCOP1	0.8 ± 0.1	5.4 ± 0.7	1.9 ± 0.5	1.0 ± 0.1

[a] Strains were grown aerobically on a rotary shaker with vigorous shaking to an OD_{600} of 0.4−0.5. [b] The levels of B800−850 and B875 spectral complexes are expressed as nanomoles per milligram of protein. [c] Photopigment values are expressed as micrograms per milligram of protein. All values provided are the average of two independent determinations.

Table 3: Spectral Complex Formation and Carotenoid Composition in *R. sphaeroides* Strains Grown under Photosynthetic and Dark Anaerobic (DMSO) Conditions[a]

	photosynthetic condition			anaerobic dark condition	
strain	B800−850	B875	SE/SO[b]	B800−850	B875
2.4.1	20.5 ± 2.2	9.2 ± 0.7	3.1	12.7 ± 0.7	7.4 ± 0.1
CCONΔ	18.0 ± 0.9	9.1 ± 0.1	0.1	14.5 ± 0.8	5.6 ± 0.4
CCOQΔ	21.0 ± 0.5	9.9 ± 0.1	2.9	16.9 ± 0.3	6.5 ± 0.1
CCOP1	22.2 ± 0.4	8.5 ± 0.5	0.2	15.4 ± 1.3	5.6 ± 0.9

[a] Strains were grown photosynthetically in screw-capped tubes at a light intensity of 10 W/m^2 or anaerobically in the dark with DMSO to an OD_{600} of 0.5−0.7. The levels of B800−850 and B875 spectral complexes are expressed as nanomoles per milligram of protein. All values provided are the average of two independent determinations. [b] For the determination of carotenoid composition, photosynthetic cultures were grown by continuous sparging with a mixture of 95% N_2−5% CO_2 at an incident light intensity of 10 W/m^2. The carotenoid composition is expressed as the ratio of spheroidene (SE) to spheroidenone (SO). The total levels of Crt synthesized in the mutant strains was similar to that formed in 2.4.1.

crude extracts were also prepared from the wild-type and the CcoP mutant strain grown under the same conditions. As anticipated, wild-type cells produced only background levels of both light-harvesting complexes under aerobic conditions. In contrast, the CcoQ, CcoN, and CcoP mutant strains exhibited 7−10-fold and 54−64-fold increased levels of the B800−850 and B875 complexes, respectively. Consistent with these results, the cellular levels of the photopigments, Bchl and Crt, also increased in the three Cco mutants, compared with those levels in the wild type. These data indicated that the in-frame deletion mutations in *ccoN* and *ccoQ* led to the same phenotype as previously observed for the *ccoP* mutation, namely, the oxygen-insensitive formation of the photosynthetic complexes, which is normally repressed in the wild type in the presence of oxygen. The substantial increase in the B875 complex, compared with the B800−850 complex, can be explained by the hierarchy of spectral complex formation, in the order B875, B800−850, under limiting Bchl conditions such as for these aerobic conditions observed here (*30*, *31*).

The levels of the spectral complexes were also determined with the wild-type and the mutant strains grown anaerobically, either in the presence of light (10 W/m^2) or in the dark with DMSO as a terminal electron acceptor. As shown in Table 3, the levels of spectral complexes were substantially increased, as anticipated, over cells grown aerobically. The wild type and three Cco mutants produced more spectral complexes under photosynthetic conditions than under anaerobic dark DMSO conditions. No major difference in

Table 4: β-Galactosidase Activities from Cell Extracts of *R. sphaeroides* Strains Bearing *puc*::*lacZ* (pCF200Km) or *puf*::*lacZ* (pUI1663) Transcriptional Fusion Plasmids[a]

strain	pCF200Km (*puc*::*lacZ*)	pUI1663 (*puf*::*lacZ*)
2.4.1	51 ± 10	64 ± 3
CCOQΔ	469 ± 11	320 ± 8

[a] Strains were grown aerobically on a rotary shaker with vigorous shaking to an OD_{600} of 0.4−0.5. The β-galactosidase activity is expressed as nanomoles per minute per milligram of protein. All values provided are the average of two independent determinations.

levels of the spectral complexes was observed between the wild type and the Cco mutants under either growth condition. Under anaerobic dark DMSO conditions, levels of the B800−850 were slightly elevated (∼14−33%) in the Cco mutants when compared to the wild type, which is often observed. Taken together, these data obtained from spectral analyses using the in-frame deletion mutants confirmed earlier results (*8*), which demonstrated that the *cbb$_3$* cytochrome *c* oxidase generates a signal that inhibits PS gene expression in response to oxygen.

Analyses of puc and puf Expression in the CcoQ Mutant Using puc::lacZ and puf::lacZ Reporters. The oxygen-insensitive formation of the photosynthetic spectral complexes in the CcoQ mutant suggested that the genes encoding the B800−850 and B875 structural polypeptides (encoded by the *puc* and *puf* operons, respectively) were likely to be derepressed, as previously observed in the CcoP mutant (*8*, *9*). By use of *lacZ* transcriptional fusions, the promoter activities of the *puc* and *puf* operons under aerobic conditions were determined in the wild type and CcoQ-minus background (Table 4). Mutation in *ccoQ* was similar in its effect on *puc* and *puf* operon expression, as was that in *ccoP* under aerobic conditions. The *puc* and *puf* operons were derepressed under aerobic conditions in the CcoQ mutant by factors of 9.2 and 5, respectively, when compared to the wild type. These data provide further evidence for the importance of CcoQ in the transcriptional regulation of photosynthesis gene expression in response to oxygen.

Cytochrome c Oxidase Activities. Zufferey et al. (*6*, *7*) reported that an in-frame deletion mutation in *fixQ*, the homologue of the *ccoQ* gene, had no effect on either the activity or the assembly of the *cbb$_3$* oxidase of *B. japonicum*, although its product is a subunit of the terminal oxidase. To investigate the effect of the *ccoQ* mutation on the *cbb$_3$* oxidase, cytochrome *c* oxidase activity was determined in the CcoQ mutant by using reduced cytochrome *c*. The controls consisted of the wild-type as well as the CcoN and CcoP mutant strains. Crude extracts from cells grown under photosynthetic conditions (10 W/m^2) or under anaerobic dark DMSO conditions were used in order to minimize the interference caused by the *aa$_3$*-type cytochrome *c* terminal oxidase. Under these conditions the *cbb$_3$* oxidase is the predominant or exclusive terminal oxidase synthesized in *R. sphaeroides* (*2*, *32*). As shown in Figure 1, the CcoQ mutant had essentially identical cytochrome *c* oxidase activities as the wild type under both growth conditions. In contrast, the CcoN and CcoP mutant strains showed about ∼10% of the activity detected in the wild type under photosynthetic conditions and no activity under anaerobic dark DMSO conditions. These results confirm that the cytochrome *c* oxidase expressed under anaerobic conditions is exclusively

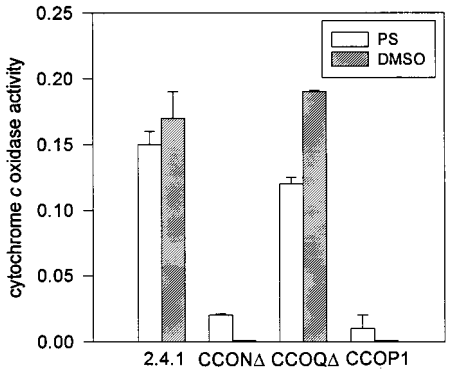

FIGURE 1: Cytochrome *c* oxidase activities from cell extracts of *R. sphaeroides* strains grown photosynthetically (open bars) or anaerobically in the dark with DMSO as a terminal electron acceptor (hatched bars). Cells were cultured photosynthetically in screw-capped glass tubes at an incident light intensity of 10 W/m^2. The cytochrome *c* oxidase activity was expressed as micromoles per minute per milligram protein. All values provided are the average of two independent determinations. Vertical bars represent the standard deviation from the mean.

the *cbb*$_3$-type oxidase. Cytochrome *c* oxidase activity was also tested in the Cco mutants by the NADI reaction. Consistent with the result obtained with reduced cytochrome *c*, the wild-type and the CcoQ mutant colonies were observed to turn blue in the first 1−2 min after the aerobically grown plates were flooded with the NADI reagent (NADI-positive phenotype). In contrast, the CcoN and CcoP mutants were NADI-negative during the first 15 min of the reaction. These data clearly show that the *ccoQ* gene product is not essential for either the assembly or the activity of the *cbb*$_3$ oxidase, as was observed for *B. japonicum*. Given these results as well as those obtained previously, we suggest that the *ccoQ* gene product is directly involved in the transduction of an inhibitory signal emanating from the flow of reductant through the *cbb*$_3$ oxidase in the presence of oxygen. Mutations in *ccoN* and *ccoP*, on the other hand, led to the loss of the *cbb*$_3$ oxidase activity, as expected, but as in the case of the *ccoQ* mutation they also led to spectral complex formation under aerobic growth conditions. Thus, both reductant flow and transduction of the signal through CcoQ are essential to the O_2 control of spectral complex formation. It was recently reported in *Rhodobacter capsulatus* that most of the mutations in *ccoN*, *ccoO*, or *ccoP* resulted in a failure of oxidase assembly (*27*). By analogy, the *fixN* and *fixO* gene products proved to be necessary for *cbb*$_3$ oxidase assembly in *B. japonicum* (*7*). Analogously, this may also be true in *R. sphaeroides*.

Complementation of the CcoQ Mutant. To ensure that the CcoQ mutant strain possesses no additional mutations other than in *ccoQ*, complementation analyses were performed. Plasmids bearing the entire *cco* operon or *ccoQ* alone were introduced *in trans* to complement the *ccoQ* mutation. Complementation was judged as a return of B800−850 and B875 levels to approximately those found in the wild type under aerobic conditions. Plasmid pUI2803 is based on the vector pRK415 and contains the entire *ccoNOQP* operon as well as its own regulatory region, so that the *cco* operon is transcribed from its own promoter. The pRK415 derivatives, pOHQ-1 and pOHQ-2, contain only the *ccoQ* gene cloned in both orientations. In the case of pOHQ-2 the *ccoQ* gene can be expressed from the tetracycline resistance gene promoter on the plasmid. As shown in Table 5, introduction of pUI2803 into the CcoQ mutant led to complete complementation; i.e., the CcoQ mutant with pUI2803 produced wild-type levels of the B800−850 and B875 complexes; i.e., complete repression of the spectral complex formation in the presence of O_2. In the CcoQ mutant bearing pOHQ-1 the same levels of the photosynthetic spectral complexes were detected as in the mutant containing pRK415. In contrast, introduction of pOHQ-2 into the mutant resulted in an approximately 70% reduction, as judged from the levels of residual B875 complexes and a total reduction in B800−850 levels. These complementation results can be readily explained either by the difference in promoter strengths between the promoter of the *cco* operon and that of the tetracycline resistance gene on pRK415, or by the fact that the coordinated transcription/expression of *ccoNOQP* might be important for the efficient assembly of their products in the membrane. These complementation data confirmed that the CcoQ mutant strain contains a mutation only in the *ccoQ* gene.

Table 5: Complementation of *R. sphaeroides* CCOQΔ with Plasmids Containing the *ccoQ* Gene

strain[a] (plasmid)	relevant genotype of plasmid	B800−850	B875
2.4.1 (pRK415)[b]		0.2 ± 0	0.1 ± 0
CCOQΔ (pRK415)		0.5 ± 0	2.9 ± 0.2
CCOQΔ (pUI2803)	*ccoNOQP*$^+$	0.2 ± 0	0.2 ± 0.05
CCOQΔ (pOHQ-1)[c]	*ccoQ*$^+$	0.5 ± 0	3.0 ± 0.1
CCOQΔ (pOHQ-2)[c]	*ccoQ*$^+$	0.2 ± 0	0.9 ± 0.1

[a] Strains were grown aerobically on a rotary shaker with vigorous shaking to an OD_{600} of 0.4−0.5. [b] pRK415 is the parental vector of pUI2803, pOHQ-1, and pOHQ-2 and was used as the negative control. [c] The *ccoQ* gene is oriented divergently and collinearly to the tetracycline resistance gene of pRK415 in pOHQ-1 and pOHQ-2, respectively. The levels of B800−850 and B875 spectral complexes are expressed as nanomoles per milligram of protein. All values provided are the average of two independent determinations.

Carotenoid Composition in the CcoQ Mutant Grown under Photosynthetic Conditions. The Crt content of *R. sphaeroides* is primarily composed of spheroidene (SE) and spheroidenone (SO). SO, the end product of the Crt biosynthetic pathway, is synthesized via the oxidation of SE in a reaction catalyzed by the *crtA* gene product. In the presence of oxygen SO is the primary Crt synthesized in cells, whereas under photosynthetic conditions the relative accumulation of SE and SO is determined by both the incident light intensity and external electron sources and acceptors, both of which affect the cellular redox state (*24*). An insertion mutation in *ccoP* was previously reported to lead to the accumulation of SO relative to SE under photosynthetic conditions (*8*). To investigate the effect of the *ccoN* and *ccoQ* mutations on SO accumulation, photopigments were extracted from the CcoN and CcoQ mutants grown photosynthetically (10 W/m^2) and subjected to HPLC analysis. As shown in Table 3, the carotenoid compositions found in the CcoP and CcoN mutants were almost exclusively SO relative to SE, whereas SE is the major carotenoid in the CcoQ mutant as well as in the wild type. From these data we conclude that alteration in the cellular redox state, caused by inactivation of the *cbb*$_3$ oxidase, is responsible for the accumulation of SO relative to SE in the CcoN and CcoP mutants. This assumption is supported by the fact that the CcoQ mutant, which is

Table 6: Spectral Complex Formation in *R. sphaeroides* 2.4.1 after Overexpression of the *ccoNOQP* Operon[a]

	aerobic condition		photosynthetic condition		
strain (plasmid)	B800−850	B875	B800−850	B875	B800−850/B875
2.4.1 (pRK415)	0.1 ± 0	0.1 ± 0	18.8 ± 1.7	7.8 ± 0.5	2.4
2.4.1 (pUI2803)	0.1 ± 0	0.1 ± 0	8.5 ± 0.3	5.9 ± 0.1	1.4
RDXBΔ (pRK415)	nd[b]	nd	17.1 ± 1.5	6.9 ± 0.0	2.5
RDXBΔ (pUI2803)	nd	nd	13.5 ± 0.3	5.2 ± 0.2	2.6

[a] Strains were grown aerobically on a rotary shaker with vigorous shaking to an OD_{600} of 0.4−0.5. Strains were grown photosynthetically at a light intensity of 10 W/m^2 to an OD_{600} of 0.5−0.6. pUI2803 contains the *ccoNOQP* operon and is based on pRK415. The levels of B800−850 and B875 spectral complexes are expressed as nanomoles per milligram of protein. All values provided are the average of two independent determinations. [b] nd, not determined.

unaffected in its cbb_3 oxidase activity, showed the same carotenoid composition as the wild type. This finding takes on greater significance when we consider what might serve as a terminal electron acceptor for the flow of reductant through the cbb_3 oxidase under anaerobic conditions.

Effect of ccoNOQP Present in Multiple Copies in trans. Thus far, we have demonstrated that either when the flow of reductant through the cbb_3 oxidase is interupted or when the oxidase is unable to communicate with the Prr system through the *ccoQ* gene product, the inhibitory signal acting upon the Prr activation system is removed and spectral complex formation occurs. The corollary to these findings might suggest that enhanced cbb_3 oxidase activity should diminish spectral complex formation relative to wild type. To investigate the effect of the overexpression of the cbb_3 oxidase on spectral complex formation, pUI2803, containing the entire *cco* operon, was introduced into the wild type, and the levels of the spectral complexes were measured for cells grown both aerobically and photosynthetically (10 W/m^2). As a control, the wild type containing the parental vector of pUI2803, pRK415, was included in the experiment. The wild type bearing pUI2803 showed a significant reduction in spectral complex levels under photosynthetic conditions, as compared with the wild type containing pRK415 (Table 6). Formation of the B800−850 complex was more severely affected than that of the B875, leading to a decreased ratio of B800−850 to B875 complexes.

Similarly, the overexpression of the *ccoNOQP* operon in the *rdxB* in-frame deletion mutant (RDXBΔ) diminished the levels of both spectral complexes under photosynthetic conditions, as anticipated (Table 6). However, the effect on the RdxB mutant was not as great as that in the wild type, as also anticipated, since mutation of *rdxB* is known to result in increased spectral complex formation. Furthermore, no shift of spectral complex formation in favor of the B875 complex was observed in the RdxB mutant.

Effect of DMSO Reductase Activity on Spectral Complex Formation. As shown in Table 7, the addition of DMSO to photosynthetic cultures of the wild type and the CcoP mutant led to similarly decreased photosynthetic spectral complex levels, especially the B800−850 complex in both strains. DMSO reductase takes electrons from the Q-pool of the electron transport chain to reduce DMSO to dimethylsulfide (*33*). Therefore, the induction of DMSO reductase, by providing DMSO to photosynthetic cultures (*34*), brings about an alteration in the redox state (probably a more oxidized state) of the electron transport chain, which results in the inhibition of spectral complex formation, especially the B800−850 complex. This result implies that the multiple copy effect of the *cco* operon might be due to an alteration in the redox state of the electron transport chain generated by increased reductant flow through the cbb_3 oxidase. The fact that the DMSO effect resulting in decreased spectral complex formation was also observed in the CcoP mutant supports our conclusion that the change in redox poise generated by the expression of DMSO reductase is sensed and transmitted in a cbb_3- (also CcoQ-) independent manner, because the CcoP mutant of *R. sphaeroides* lacks cbb_3 activity and likely fails to assemble the cbb_3 oxidase in the same manner as for *R. capsulatus* (*27*). The data presented here not only confirm our earlier inferences but in addition extend our conclusions that there is reductant flow through the cbb_3 oxidase that takes place anaerobically (*8*, *9*). This conclusion is now inescapable and raises the question as to which molecular species serves as an electron acceptor from the cbb_3 oxidase in the absence of oxygen.

Table 7: Effect of DMSO on the Production of Spectral Complex Formation in *R. sphaeroides* Strains under Photosynthetic Conditions[a]

	-DMSO		+DMSO	
strain	B800−850	B875	B800−850	B875
2.4.1	25.9 ± 3.1	9.2 ± 0.2	15.2 ± 0.7	7.6 ± 0.2
CCOP1	26.3 ± 2.2	10.5 ± 0.5	13.5 ± 2.6	7.0 ± 0.3

[a] Strains were grown photosynthetically with (+DMSO) or without (-DMSO) supplementation of DMSO to a final concentration of 0.5% (v/v) at a light intensity of 10 W/m^2 to an OD_{600} of 0.5−0.6. The levels of B800−850 and B875 spectral complexes are expressed as nanomoles per milligram of protein. All values provided are the average of two independent determinations.

DISCUSSION

R. sphaeroides possesses multiple respiratory oxidases, which are composed of two cytochrome *c* oxidases, the aa_3- and cbb_3-type oxidases, as well as at least two quinol oxidases whose genes have been recently identified (*2*, *35*). It was previously reported in our laboratory that mutations in the *ccoNOQP* operon as well as in the first gene of the *rdxBHIS* operon, *rdxB*, could lead to induction of the photosynthetic apparatus under aerobic growth conditions (*8*, *9*, *36*). Derepression of the photosynthesis genes in the absence of a functional cbb_3 oxidase was shown to be mediated through the two-component PrrBA regulatory system, which serves as a global activator for PS gene expression (*9*). The cbb_3 oxidase consists of four nonidentical subunits and is encoded by the *ccoNOQP* operon (*4*, *6*, *37*). The *ccoN*, *ccoO*, and *ccoP* gene products are essential for both the activity and the assembly of the functional cbb_3 oxidase. In contrast, FixQ (CcoQ homologue) was shown not to be required either for the assembly or for the activity

of the oxidase in the legume root symbiotic bacterium *B. japonicum* (*7*). Amino acid sequence alignments revealed that the membrane-spanning regions located at the N-terminal half of the CcoQ/FixQ from various organisms (*Azorhyzobium*, *Bradyrhizobium*, *Rhizobium*, *Paracoccus*, and *Rhodobacter*) are highly conserved (*4*). Interestingly, the CcoQ proteins from the photosynthetic bacteria, *R. capsulatus* and *R. sphaeroides*, contain two conserved histidines in the C-terminal domain, which are absent in the other bacteria.

To examine the function of CcoQ in *R. sphaeroides*, an in-frame deletion mutation of *ccoQ* was constructed, in which 57 amino acids extending from the second codon were removed from CcoQ, leaving the remaining 10 amino acids. Because the CcoQ mutant had cbb_3 oxidase activity similar to the wild type, this indicates that CcoQ is not necessary for either the activity or the assembly of the cbb_3 oxidase in *R. sphaeroides* as in *B. japonicum*. However, as is the case for the loss of cbb_3 oxidase activity, photosynthetic spectral complexes were synthesized under aerobic conditions in the CcoQ mutant. In this respect, the phenotype of the CcoQ mutant is similar to that of the RdxB insertion mutant, since alteration of RdxB also results in aerobic spectral complex formation without affecting the activity of the cbb_3 oxidase (*8*). We also demonstrated very substantially increased *puc* and *puf* operon transcriptional activity under aerobic conditions when *ccoQ* was disrupted similar to levels that are observed under anaerobic growth conditions. Therefore, this suggests that CcoQ is involved in the transduction of the inhibitory signal generated by the cbb_3 oxidase in the presence of oxygen and the signal mediated by CcoQ is not the result of an alteration in cellular redox state resulting from the inactivation of the cbb_3 oxidase. Thus, CcoQ behaves as a transponder, detecting the state of cbb_3 and transmitting that information. CcoQ does not appear to be involved in reductant flow. CcoQ also lacks any known motifs associated with redox centers such as an iron−sulfur center and flavin binding region. However, very preliminary data suggest that His^{39} of CcoQ is involved in transmitting the critical signal (unpublished results).

Previous studies revealed that mutations in the *cco* operon resulted in the activation of not only those PS genes regulated by the PrrBA two-component activation system, e.g., *puc* and *puf* (*8*), but also *hemA*, which encodes the 5-aminolevulinic acid (ALA) synthase and which is also regulated by FnrL, a global anaerobic activator (*36*, *38*) as well as others. Additionally, it was observed that a *puc*::*lacZ* transcriptional fusion containing the FnrL binding site (pCF200Km) showed 12-fold induction under aerobic conditions in the *ccoP*-minus background as compared to the wild type, whereas only 3-fold induction was observed with the same transcriptional fusion containing no FnrL binding site (pCF250Km) under the same conditions (*8*). Therefore, FnrL, as well as the PrrBA two-component system, appears to respond to those signal changes caused by the inactivation of the cbb_3 oxidase. This is also true for the DMSO reductase (DOR) system (*39*), which can be turned on aerobically in a cbb_3 oxidase mutant strain.

Given these as well as results obtained previously (*8*, *9*, *38*), we believe the most reasonable interpretation of these findings is as follows. The flux of reductant through the cbb_3 oxidase, although we cannot eliminate the state of a critical intermediate, is monitored by the CcoQ protein, where

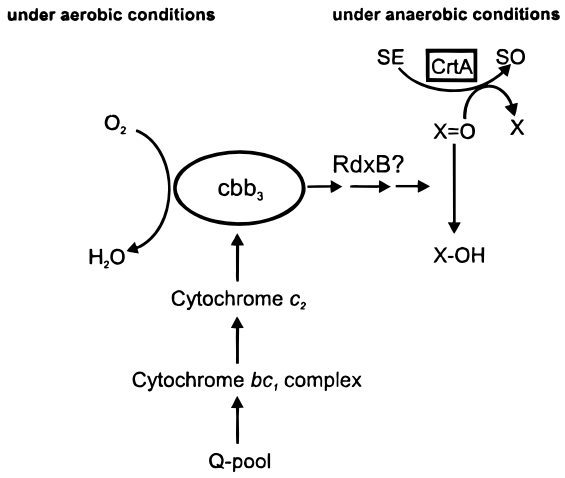

FIGURE 2: Hypothetical pathway for the flow of reducing power through the cbb_3 oxidase under anaerobic conditions. Hypothetical electron sink X═O is the e^- acceptor for the reductant moving through the cbb_3 oxidase under photosynthetic conditions, giving rise to X−OH. In the absence of this flow the oxidized form of X−OH is maintained, namely, X═O, which is used as the O donor in the conversion of SE to SO, catalyzed by CrtA, resulting in the formation of X. See text for additional details.

structural interactions between it and the active oxidase complex permit it to transduce this information to either the PrrB membrane-bound histidine kinase directly or to another element, e.g., PrrC (*28*), that ultimately communicates with PrrB. On the other hand, the activation of DMSO reductase, which siphons the Q-pool of reductant in photosynthetic cells, as well as increasing the gene dosage of *ccoNOQP* in such cells alters the redox state of the photosynthetic electron transport chain. This redox signal might be transmitted, at least in part, by FnrL or AppA−PpsR to control the levels and ratio of the B800−850 and B875 complexes in a cbb_3-independent manner.

The data presented here further support our earlier conclusion that reductant must flow through the cbb_3 oxidase even under anaerobic photosynthetic conditions and this flow is similarly involved in the regulation of PS gene expression (*9*). The difficulty had been, until the present, as to what might serve as the ultimate acceptor for this flow of reductant under these conditions? We believe we can now suggest an answer to this question. In the CcoQ mutant we have dissociated the signal transduction pathway controlling PS gene expression from the differential accumulation of SO relative to SE as observed when we eliminate the flow of reductant through the cbb_3 oxidase. In Figure 2, we present a model whereby the flow of reductant through cbb_3 under photosynthetic conditions is ultimately used at the last step in Crt biosynthesis by keeping the hypothetical oxidant X═O in the reduced form, namely, X−OH. The nature of X−OH or X═O is unknown at the present time. However, we do know that the O which is incorporated into SO under anaerobic conditions does not come from either O_2 or H_2O (*40*). Nontheless, this balance between SE and SO, in whole or in part, is dependent upon electron flow through the cbb_3 oxidase under photosynthetic conditions will, in turn, contribute to the relative cellular abundance of the B800−850 and B875 spectral complexes (*24*). We have shown that SO is the nonpreferred Crt for the assembly of the B800−850 complex, whereas the reaction center (RC) and B875 complexes show no Crt preference for their assembly (*24*).

All of the preceding discussion is derived directly from the results presented here.

However, there remains one additional observation that must be accommodated within this overall scheme. We have previously shown (*8*) that RdxB mutants (a membrane-bound iron−sulfur protein) are cbb_3-positive, show increased PS gene expression aerobically, and show the altered Crt composition typical of the cbb_3 mutants. Because the double mutant of cbb_3 and RdxB was quantatatively similar to either cbb_3 or RdxB alone, we reasoned that RdxB must lie in the same pathway as cbb_3 (*8*, *9*). Considering what we presently know regarding the relationships of the various electron transport components to one another, it is difficult to imagine RdxB coming after the Q-pool but before cbb_3. Thus, we are left with placing RdxB after the cbb_3 in the anaerobic shunt (that flow of reductant through cbb_3 other than to O_2). This can explain how RdxB could be involved in determining the relative levels of SE and SO (see Figure 2). However, it is not immediately clear how this placement of RdxB can result in the lack of transmission of the signal through cbb_3 that serves to inhibit PS gene expression in an RdxB mutant. If we assume that the presence of RdxB following the cbb_3 oxidase normally creates a backpressure to the flow of reductant through the cbb_3 oxidase, perhaps by directly interacting with the cbb_3, and in the absence of RdxB this backpressure is removed, then either the flux or the accumulation of a particular intermediate can be altered, thus altering the signal to CcoQ. This scheme is supported by those data in which the *ccoNOQP* operon is present in multiple copies in an *rdxB*-minus background where *rdxB* is mutated by an in-frame deletion (see Table 6). In the absence of RdxB, reductant flow through cbb_3 oxidase under anaerobic conditions is greatly diminished. On the other hand, the overexpression of the cbb_3 oxidase could provide for some increase of flow resulting in an alteration in redox state of the electron transport chain by siphoning electrons from cytochrome c_2. This explains why the overexpression effect of cbb_3, although present, is not as great in the RdxB mutant as that in the wild type. There are other plausible schemes that can be formulated to explain these findings, although they are not as straightforward as that proposed here.

However, the simplicity of this presumptive model enables the CcoQ protein to monitor cbb_3 activity either aerobically or anaerobically, which in turn will be influenced by the flow of reductant into or out of the Q-pool upstream of cbb_3 or through downstream flow to O_2 or RdxB or to other acceptors. Although we have no evidence that the cbb_3 pathway can influence the PpsR−repressor/AppA−antirepressor function, the fact that the repressor system is subject to redox controls (*29*) under both aerobic and photosynthetic growth conditions strongly suggests that these two apparently separate pathways must, at some point, intersect. This is certainly true for the anaerobic regulator FnrL, which helps to regulate *ccoNOQP*, *dor* gene expression, *hemA*, *puc*, and perhaps *rdxB* as well as other critical genes involved in PS gene expression (*41*). In turn, active FnrL is probably dependent upon cellular redox (*36*, *38*).

These results raise one additional point. As for the cbb_3 oxidase, many prokaryotic and eukaryotic electron transport chains possess extra polypeptides that do not appear to function within the actual electron transport chain, e.g., the role of the *dorB* gene (*33*), but yet their role remains unknown. Like the CcoQ protein, could these extra polypeptides be employed to transmit the state of electron flow through the redox chain to other cellular compartments or receptors? A regulatory mechanism such as this by connecting a cellular redox chain to the regulation of gene expression represents a new paradigm in the regulation of gene expression. We believe that we are now in a position to determine both the pathway and mechanism of signal transduction from O_2 to active PrrA.

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