Redox signaling: globalization of gene expression

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Here we show that the extent of electron flow through the *cbb₃* oxidase of *Rhodobacter sphaeroides* is inversely related to the expression levels of those photosynthesis genes that are under control of the PrrBA two-component activation system: the greater the electron flow, the stronger the inhibitory signal generated by the *cbb₃* oxidase to repress photosynthesis gene expression. Using site-directed mutagenesis, we show that intramolecular electron transfer within the *cbb₃* oxidase is involved in signal generation and transduction and this signal does not directly involve the intervention of molecular oxygen. In addition to the *cbb₃* oxidase, the redox state of the quinone pool controls the transcription rate of the *puc* operon via the AppA–PpsR antirepressor–repressor system. Together, these interacting regulatory circuits are depicted in a model that permits us to understand the regulation by oxygen and light of photosynthesis gene expression in *R.sphaeroides*.

Keywords: *cbb₃* cytochrome *c* oxidase/electron transport chain/gene regulation/photosynthesis/redox sensing

Introduction

The primary environmental factor that governs the expression of the photosynthesis (PS) genes in *Rhodobacter sphaeroides*, is oxygen. The photosynthetic apparatus is synthesized when oxygen tensions fall below ~2.5% (Kiley and Kaplan, 1988). Under anaerobic conditions in the light the cellular level of the B800–850 complex relative to the B875 complex increases differentially and inversely to the incident light intensity (Kiley and Kaplan, 1988; Zeilstra-Ryalls *et al.*, 1998).

Transcriptional regulation of the PS genes in *R.sphaeroides* is achieved by the coordinate action of at least three major regulatory systems: the PrrBA two-component activation system, the AppA–PpsR antirepressor–repressor system and FnrL. The PrrBA two-component system functions as a global anaerobic activation system (Eraso and Kaplan, 1994, 1996; Joshi and Tabita, 1996; Qian and Tabita, 1996). PpsR is a redox-responding protein that is responsible for the aerobic and high-light repression of the *puc* operon and photopigment biosynthesis genes such as *crt* and *bch* (Gomelsky and Kaplan, 1995a; Oh and Kaplan, 2000). The AppA protein appears to modulate the activity of PpsR in response to

changes in cellular redox state (Gomelsky and Kaplan, 1995b, 1997, 1998). FnrL, the homolog of the global anaerobic regulator Fnr, is required for the enhanced expression of selective PS genes (*puc*, *hemA*, *hemN*, *hemZ* and *bchE*) and the *ccoNOQP* operon encoding the *cbb₃* oxidase, as well as for the expression of dimethyl sulfoxide (DMSO) reductase in *R.sphaeroides* under oxygen-limiting conditions (Zeilstra-Ryalls *et al.*, 1995, 1997; Zeilstra-Ryalls and Kaplan, 1998; Mouncey and Kaplan, 1998a,b; Oh *et al.*, 2000).

When grown aerobically, *R.sphaeroides* contains a branched aerobic electron transport chain (ETC) that is terminated with two functional cytochrome *c* oxidases and at least one functional quinol oxidase (Hosler *et al.*, 1992; Shapleigh *et al.*, 1992; Garcia-Horsman *et al.*, 1994b; N.J.Mouncey, E.Gak, M.Choudhary, J.Oh and S.Kaplan, in preparation). The *aa₃*- and *cbb₃*-type cytochrome *c* oxidases belong to the heme–copper oxidase superfamily (Garcia-Horsman *et al.*, 1994a) and take electrons from the *bc₁* complex through both cytochromes *c₂* and *c_y* (Donohue *et al.*, 1988; Myllykallio *et al.*, 1999). *Rhodobacter capsulatus*, a bacterium phylogenetically very close to *R.sphaeroides*, does not contain the *aa₃* cytochrome *c* oxidase. The *cbb₃* oxidase encoded by the *ccoNOQP* operon consists of four non-identical subunits (Toledo-Cuevas *et al.*, 1998). CcoN is the catalytic subunit where the reduction of O₂ occurs, while CcoO and CcoP are monoheme and diheme cytochromes *c*, respectively. CcoQ is the smallest subunit and is not required for *cbb₃* oxidase activity (Zufferey *et al.*, 1998; Oh and Kaplan, 1999).

Cohen-Bazire *et al.* (1956) first proposed that the redox state of the ETC might ultimately control the levels of the spectral complexes in *R.sphaeroides* in response to changes in oxygen tension and light intensity. The finding that mutations in the *ccoNOQP* operon led to the formation of spectral complexes even under high oxygen conditions provided the first clue that the *cbb₃* cytochrome *c* oxidase might be an O₂/redox sensor (Zeilstra-Ryalls and Kaplan, 1996; O’Gara and Kaplan, 1997). We proposed that the *cbb₃* oxidase transduces an inhibitory signal to the PrrBA two-component system under aerobic conditions to prevent PS gene expression (O’Gara *et al.*, 1998; Oh and Kaplan, 1999; Oh *et al.*, 2000). However, the nature of the signal and the mechanism by which the *cbb₃* oxidase perceives the signal remained to be determined.

In this report we present evidence suggesting that the extent of electron flux through the *cbb₃* cytochrome *c* oxidase determines the expression levels of the PS genes that are under control of the PrrBA two-component system. The dual function of the *cbb₃* oxidase as both terminal oxidase and O₂ sensor represents a novel mode of redox sensing. Furthermore, we present the first evidence

that the redox state of the quinone pool in the ETC can provide a *cbh₃*-independent signal that is likely to be sensed and transduced by the AppA–PpsR antirepressor–repressor system.

Results

Growth and spectral complex formation in ETC mutant strains of *R.sphaeroides* under aerobic conditions

When grown on SIS agar plates under aerobic conditions, the BC1 and C2Cy mutant strains formed colonies with very deep red pigmentation, compared with the relatively pale pink coloration of the wild type. This phenotype, designated ‘oxygen-insensitive spectral complex formation’, was previously observed for Cco mutant strains as well as the RdxB insertion mutant (RDXB1) (O’Gara and Kaplan, 1997; Oh and Kaplan, 1999). Colonies of the Cy mutant exhibited somewhat greater pigmentation than those of the wild type. In contrast, the C2 or AA3 mutant strains produced colonies with similar coloration to those of the wild-type strain 2.4.1. Under 30% O₂ conditions, growth rates of all ETC mutants with the exception of the BC1 and C2Cy mutant strains were not significantly different. The latter mutants relying exclusively on quinol oxidase function had an ~2-fold greater doubling time than the wild-type strain.

All ETC mutant strains were cultivated under 30% O₂, along with the wild type, and the levels of spectral complexes were quantitatively determined. As shown in Figure 1, the mutants are divided roughly into three groups. The first group comprised the C2 and AA3 mutants which, like the wild-type strain 2.4.1, produced only

Fig. 1. Levels of spectral complexes in *R.sphaeroides* strains grown under aerobic conditions. Strains were grown aerobically by sparging with 30% O₂–69% N₂–1% CO₂ to an OD₆₀₀ of 0.5. The black and gray bars indicate the levels of B800–850 and B875 light harvesting complexes, respectively. All values provided are the average of two independent determinations. Vertical bars represent the standard deviation from the mean.

background levels of spectral complexes. The second group consisted of the BC1, C2Cy, CCONΔ and CCOQΔ mutant strains where spectral complex formation was orders of magnitude higher when compared with the wild type. Approximately the same amounts of B875 complex were produced in the mutants of this group with the exception of the CCOQΔ mutant, whereas the levels of the B800–850 complex showed greater variation

Fig. 2. Analysis of mRNA levels specific for *puc* (A) and *puf* (B) in *R.sphaeroides* strains grown under aerobic conditions. Total RNA was isolated from *R.sphaeroides* strains grown aerobically as described for Figure 1. Approximately 20 µg of total RNA was loaded in each lane. The northern blots were probed with a labeled 0.53 kb *Bam*HI–*Kpn*I fragment from pUI624 and a 0.47 kb *Syl*I fragment from pUI655, which are specific for *puc*- and *puf*-mRNA, respectively. The levels of transcripts were quantitated as described. After background correction, the values were normalized by the levels of processed 23S (23S*) and 16S rRNA. The normalized values are plotted above the northern blots. The transcript levels in the wild type (2.4.1) are set at 100.

(BC1>C2Cy>CCONΔ>CCOQΔ). The Cy mutant belonged to the third group. Although the Cy mutant synthesized spectral complexes in the presence of 30% O₂, the levels were well below those observed for the mutants of group two.

PS gene expression in ETC mutants of *R.sphaeroides* under aerobic conditions

The expression of the *puc* and *puf* operons, which encode the apoproteins of the B800–850 and B875 complexes, respectively, was investigated at the transcriptional level by means of northern hybridization analyses using total RNA isolated from the wild-type and mutant strains grown under 30% O₂. As shown in Figure 2A, the levels of *puc* transcripts were increased in the BC1, C2Cy, CCONΔ and CCOQΔ mutants by a factor of 8–16, compared with the wild type. The Cy mutant showed an ~5-fold increase in *puc* operon expression when compared with the wild type, whereas only marginal derepression of *puc* operon expression was observed in the C2 and AA3 mutants.

The trend in *puf* operon derepression in the mutants grown under 30% O₂ was similar to that observed for the *puc* operon, although the more extreme variations in mRNA levels observed for the latter were dampened in the former (Figure 2B). What is striking is the relatively consistent levels of derepression of the *puf* operon in the BC1, C2Cy, CCONΔ and CCOQΔ mutant strains (~6% variation), which is not true for *puc* operon expression (~24% variation).

Contributions of the *aa₃* and *cbb₃* terminal oxidases to the electron flux of the ETC

As shown in Table I, the CCONΔ and AA3 mutant strains exhibited ~70.5 and 35.7% of the cytochrome *c* oxidase activity present in the wild type 2.4.1, respectively, when grown under 30% O₂ conditions. This indicates that in *R.sphaeroides* 2.4.1 grown under 30% O₂ conditions the bulk of the reducing equivalents moving to O₂ are proceeding through the *aa₃* terminal oxidase. Yet, inactivation of the *aa₃* oxidase did not lead to the oxygen-insensitive formation of spectral complexes. This observation is crucial to the conclusion that derepression of PS genes observed in the CCONΔ mutant is not a consequence of changes in the 'redox state' of electron

carriers upstream of the *cbb₃* oxidase, e.g. the *bc₁* complex and quinone pool, etc. This fortifies our previous proposal that the *cbb₃* oxidase *per se* functions as an O₂/redox sensor and generates the inhibitory signal that represses PS gene expression in the presence of O₂.

It is the functional state of the *cbb₃* oxidase that determines PS gene expression

If we assume that the *cbb₃* oxidase is the actual O₂/redox sensor, we can then ask how derepression of the *puc* and *puf* operons occurs in the BC1 and C2Cy mutant strains under aerobic conditions. A first possibility is that inactivation of these electron carriers might affect either the transcription of the *ccoNOQP* operon or the assembly and/or maturation of the *cbb₃* oxidase post-transcriptionally. We therefore determined the activity of the *cbb₃* oxidase in the mutant and wild type grown under anaerobic dark–DMSO conditions where the *cbb₃* oxidase is the exclusive cytochrome *c* oxidase synthesized in *R.sphaeroides* (Table I). As expected, the CCONΔ mutant exhibited virtually no cytochrome *c* oxidase activity. In contrast, high *cbb₃* oxidase activities were detected in the wild type and other mutant strains. Therefore, preventing the flow of reducing equivalents to the *cbb₃* oxidase affects neither transcription of the *ccoNOQP* operon nor assembly/activity of the *cbb₃* oxidase.

A second possibility is that interruption of electron flow to the *cbb₃* oxidase resulting from inactivation of the upstream electron carriers might render the *cbb₃* oxidase incapable of generating an inhibitory signal in the presence of O₂. Using a *puf::lacZ* transcriptional fusion, we examined the effect of the *bc₁* complex-specific inhibitor myxothiazol upon *puf* operon expression in both the wild type and AA3 mutant grown under 30% O₂. Figure 3 shows that the promoter activity of the *puf* operon increased with increasing myxothiazol concentration. Importantly, higher promoter activity of *puf* was observed in the wild type at a given concentration of myxothiazol than in the AA3 mutant. Furthermore, promoter activity reached maximal levels at a lower concentration of myxothiazol in the wild type (2 μM) than in the AA3 mutant (3 μM), although the ultimate level of promoter activity was the same for both strains. The concentration of myxothiazol that gave half-maximal *puf* promoter activity

Table I. Cytochrome *c* oxidase activities from *R.sphaeroides* strains

Strain	Relative cytochrome <i>c</i> oxidase activity (%) ^a	Specific activity of <i>cbb₃</i> (μmol/min/mg protein) ^b
2.4.1	100	1.32 ± 0.01
BC1	n.d.	1.15 ± 0.01
C2	n.d.	1.37 ± 0.03
Cy	n.d.	1.33 ± 0.10
C2Cy	n.d.	0.97 ± 0.02
CCONΔ	70.5	0.06 ± 0.00
AA3	35.7	1.23 ± 0.02
CCOQΔ	n.d.	1.16 ± 0.05

^aCytochrome *c* oxidase activities from cells grown aerobically by sparging with 30% O₂–69% N₂–1% CO₂ to an OD₆₀₀ of 0.5–0.6. Oxygen consumption was measured polarographically with an oxygen electrode. The rate of oxygen consumption of the wild-type membrane fraction is set at 100 and the relative activity is expressed for the other strains. n.d., not determined.

^bStrains were grown anaerobically in the dark with DMSO as a terminal electron acceptor. Cytochrome *c* oxidase activity was measured spectrophotometrically by monitoring the oxidation of pre-reduced horse heart cytochrome *c*. All values provided are the average of two independent determinations.

Fig. 3. Effect of myxothiazol on the expression of *puf* in the wild-type and AA3 mutant strains of *R.sphaeroides* grown under aerobic conditions. The wild-type (2.4.1) and AA3 mutant strains harboring the *puf::lacZ* transcriptional fusion plasmid pUI1663 were grown aerobically by sparging with 30% O₂–69% N₂–1% CO₂ to an OD₆₀₀ of ~0.25–0.3. After addition of a given concentration of myxothiazol dissolved in methanol, the cultures were grown under the same growth conditions for an additional 3 h. Cell-free crude extracts were used to determine β-galactosidase activity. All values provided are the average of three independent determinations. Error bars represent the standard deviation from the mean.

was ~1 and 1.3 μM for the wild-type and AA3 mutant strains, respectively.

What caused the differences in the kinetic behavior of *puf* operon expression between the wild type and the AA3 mutant as a function of myxothiazol concentration under high oxygen conditions? The most plausible explanation is that there are more electrons available to the *cbb₃* oxidase in the AA3 mutant in the presence of myxothiazol. Therefore, our hypothesis that the extent of electron flow through the *cbb₃* oxidase determines the expression levels of the PS genes that are under control of the PrrBA two-component system, is supported.

Another possibility is that it is a reduction in the proton motive force or cellular energy charge (ATP/ADP) that brings about derepression of the *puc* and *puf* operons in the BC1 and C2Cy mutants grown under 30% O₂ conditions. To address this point, we investigated the effect of an uncoupler of oxidative phosphorylation upon *puc* and *puf* operon expression in the wild type 2.4.1 grown under 30% O₂. As shown in Figure 4A, the growth rate of 2.4.1 was inversely related to the concentration of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) in the growth medium and growth ceased in the presence of 5 μM CCCP. As shown in Figure 4B, the expression of both *puc* and *puf* remained at basal levels regardless of an increase in CCCP concentration. However, the addition of 3 μM myxothiazol induced *puf* expression in the presence of 1–4 μM CCCP. These data and those above clearly suggest that it is not a decrease in the proton motive force and/or cellular levels of ATP but the blocking of electron flow through the *bc₁* complex to the *cbb₃* oxidase that causes derepression

Fig. 4. Effect of CCCP on the growth of *R.sphaeroides* 2.4.1 (A) and the expression of *puc* and *puf* in *R.sphaeroides* 2.4.1 grown under aerobic conditions (B). Growth curves were determined by cultivating the wild type 2.4.1 under aerobic conditions in SIS medium containing a given concentration of CCCP. The wild type 2.4.1 harboring either *puf::lacZ* (pUI1663) or *puc::lacZ* transcriptional plasmid (pCF200Km) was grown aerobically as described for Figure 3. After addition of a given concentration of CCCP dissolved in ethanol, cultivation continued under the same growth conditions for an additional 3 h. Myxothiazol was added to the control group (2.4.1 containing pUI1663) to a final concentration of 3 μM in addition to CCCP. Cell-free crude extracts were used to determine β-galactosidase activity.

of *puc* and *puf* operon expression in the BC1 and C2Cy mutant strains.

Structural and functional integrity of the *cbb₃* oxidase is required for its O₂-sensing and signal transduction capacity

The catalytic subunit (CcoN) of the *cbb₃* oxidase has a low spin heme *b* and a binuclear center composed of a high spin heme *b₃* and Cu_B, which are involved in intramolecular electron transfer and substrate binding (Garcia-Horsman *et al.*, 1994a; Toledo-Cuevas *et al.*, 1998). Those histidine residues proposed to ligate the low and high spin hemes as well as the Cu_B (Iwata *et al.*, 1995; Zufferey *et al.*, 1998) were changed to alanine by site-

Table II. Relevant phenotypes of a set of histidine replacement mutants of catalytic subunit (CcoN) of *R.sphaeroides cbb₃* oxidase

Strain	Plasmid	30% O ₂ ^a		Dark–DMSO ^b
		B800–850	B875	<i>cbb₃</i> activity
2.4.1	pRK415	0.05 ± 0.01	0.07 ± 0.01	1.20 ± 0.06
CCONΔ	pRK415	0.36 ± 0.03	2.68 ± 0.17	0.01 ± 0.00
CCONΔ	pUI2803	0.05 ± 0.02	0.11 ± 0.01	3.10 ± 0.05
CCONΔ	pUI2803-H405A	0.41 ± 0.01	2.71 ± 0.01	0.09 ± 0.01
CCONΔ	pUI2803-H407A	0.41 ± 0.01	2.38 ± 0.19	1.61 ± 0.02
CCONΔ	pUI2803-H267A	0.41 ± 0.03	2.65 ± 0.10	0.01 ± 0.00

^aFor determination of spectral complex levels, strains carrying the corresponding plasmids were grown aerobically as in Table I. The levels of light harvesting complexes, B800–850 and B875, are expressed as nmol/mg protein.

^bStrains were grown anaerobically in the dark with DMSO as a terminal electron acceptor. Cytochrome *c* oxidase activity was measured spectrophotometrically. *cbb₃* oxidase activity is expressed as μmol/min/mg protein. All values provided are the average of two independent determinations.

directed mutagenesis (His405, His407 and His267 provide the ligands to the high and low spin hemes, and Cu_B, respectively).

We first examined the effect of the histidine to alanine replacements upon spectral complex formation in the mutants grown under 30% O₂ conditions (Table II). The positive control strain CCONΔ (pUI2803) produced only basal levels of the light harvesting complexes under 30% O₂ conditions, like the wild type 2.4.1 (pRK415), indicating that the in-frame deletion of *ccoN* in the CCONΔ mutant is fully complemented by pUI2803. In contrast, the H405A, H407A and H267A mutants as well as the negative control strain CCONΔ (pRK415) exhibited 7- to 8-fold and 22- to 25-fold increased levels of the B800–850 and B875 complexes, respectively, when compared with the positive control strain 2.4.1 (pRK415).

Cytochrome *c* oxidase activity was determined in the mutant strains as well as the control strains grown under anaerobic, dark–DMSO conditions where the *cbb₃* oxidase is the sole cytochrome *c* oxidase. As shown in Table II, the negative control strain CCONΔ (pRK415) showed no *cbb₃* oxidase activity. On the other hand, CCONΔ (pUI2803) exhibited a 2.6-fold increase in *cbb₃* oxidase activity as compared with the wild type 2.4.1 (pRK415), which can be explained by the copy number of the pRK415-based plasmid (four or five copies per cell). While virtually no cytochrome *c* oxidase activity was detected in the H405A and H267A mutants, the H407A mutant showed approximately half the *cbb₃* activity present in the control strain, CCONΔ (pUI2803), but which is still greater than that observed for 2.4.1 (pRK415).

To determine whether the assembly of the *cbb₃* oxidase in the membrane was affected by the histidine replacements, we performed western blot analyses with antibodies against CcoO and CcoP of *R.sphaeroides*. As shown in Figure 5A, the H405A and H267A mutants contained 53–58% and 58–66% of the CcoO and CcoP subunits in their membranes, respectively, relative to the positive control strain, CCONΔ (pUI2803). Therefore, the replacement of the histidine residues involved in the coordination of the binuclear center of CcoN leads to either some assembly defect or instability in the *cbb₃* oxidase in the membrane. In contrast, near control levels of the CcoO and CcoP subunits were detected in the H407 mutant. The nature of the cross-reacting bands in the

Fig. 5. Immunological and spectroscopic analyses of the *cbb₃* oxidase from a set of histidine replacement mutants of CcoN. Membrane fractions were isolated from the strains harboring the corresponding plasmid (in parentheses) grown semi-aerobically by sparging with 2% O₂–97% N₂–1% CO₂ to an OD₆₀₀ of 0.5–0.6. (A) Western blot analysis using a polyclonal antibody against CcoO and CcoP proteins. Solubilized membrane proteins (50 μg) were loaded in each lane. H405A: pUI2803-H405A, H407A: pUI2803-H407A, H267A: pUI2803-H267A, *cbb₃*: ~2 μg of the purified *cbb₃* oxidase. The relative amounts of the CcoO and CcoP subunits are given below the western blots. (B) Dithionite-reduced minus air-oxidized difference spectra between 500 and 600 nm recorded at RT. Membrane proteins (0.2 mg/ml) solubilized with dodecyl maltoside were used for the assay. The 551 and 561 nm peaks represent *c*-type and *b*-type cytochromes, respectively. The ratio of heme *c* to heme *b* (*c/b*) was estimated by using their respective extinction coefficients (see Materials and methods).

western analysis, which were not present in the purified *cbb₃* oxidase, is unknown.

Figure 5B shows the dithionite-reduced minus air-oxidized visible absorption spectra of membranes isolated

from the three histidine replacement mutants as well as from the control strains. Because the stoichiometries of heme *b* and heme *c* in the *cbh₃* oxidase are not equal (two heme *b* and three heme *c* per enzyme molecule), an increase in *cbh₃* oxidase results in a higher ratio of heme *c* to heme *b* (*c/b*) in the visible spectrum profile. As expected, *CCONΔ* (pRK415) exhibited significantly reduced levels of both heme *c* and heme *b* as well as a decreased *c/b*. In the mutants H405A and H267A the levels of both heme *b* and *c* were significantly reduced as compared with the positive control strain *CCONΔ* (pUI2803). However, mutant H407A displayed only slightly (13–14%) decreased levels of *b*- and *c*-type hemes compared with *CCONΔ* (pUI2803). Furthermore, this mutant showed the same *c/b* as the positive control strain *CCONΔ* (pUI2803).

The results presented in Table II and Figure 5 reveal that with but one exception, all histidine to alanine substitutions result in the loss of *cbh₃* activity, a change in the *c/b* ratio and the derepression of PS gene expression. Only mutant H407A is relatively normal with respect to *cbh₃* structure and catalytic function, but still leads to the derepression of PS gene expression under aerobic conditions.

We further investigated the effects of mutations leading to replacements of the histidine residues in three heme-binding motifs (C-X-X-C-H-X_n-M-P) present in the CcoO and CcoP subunits. All three mutants showed the same phenotype as the H405A mutant, i.e. some defect in the assembly or stability of the *cbh₃* oxidase, the lack of *cbh₃* activity and oxygen-insensitive spectral complex formation under aerobic conditions (data not shown).

Redox state of the cyclic photosynthetic ETC serves as a signal that is in part mediated by PpsR

Collectively, these results strongly suggest that the *cbh₃* oxidase itself is the signal generator that controls the PrrBA two-component system and thereby controls both *puc* and *puf* operon expression. Yet, as observed above (Figure 2), *puc* operon expression is highly variable as compared with *puf* operon expression. One possible explanation is that another regulatory element, in addition to the *cbh₃*-PrrBA signal transduction pathway, is operative in regulating *puc* expression. We do know that the AppA-PpsR antirepressor-repressor system and FnrL are active in regulating *puc* expression and it is possible that this(these) system(s) derive(s) a signal elsewhere in the ETC.

To address this question, we examined the effect of DMSO and light, which affect the redox state of the cyclic photosynthetic ETC, upon spectral complex formation in the CCOP1, PPS1 and CCOP1FNR mutant strains as well as in the wild type as a control. The ability of the CCOP1FNR mutant (Oh *et al.*, 2000), in which both *ccoP* and *fnrL* genes are disrupted, to grow photosynthetically enabled us to examine the involvement of FnrL in the light response. As shown in Figure 6, when grown photosynthetically at high light (100 W/m²) or at medium light in the presence of DMSO (10 W/m² + DMSO), the CCOP1 mutant and the wild-type strain produced very substantially reduced levels of the B800–850 and B875 complexes, when compared with the same strains grown at medium light (10 W/m²). In the case of the CCOP1FNR

Fig. 6. Effect of light intensity and DMSO on spectral complex formation in *R. sphaeroides* strains under photosynthetic conditions. Strains were grown photosynthetically at a light intensity of either 10 W/m² (10W) or 100 W/m² (100W). To examine the DMSO effect, strains were grown photosynthetically with supplementation of DMSO to a final concentration of 0.5% (v/v) at 10 W/m² (10W+DMSO). Cells were harvested at an OD₆₀₀ of 0.5–0.6 except for the CCOP1FNR mutant grown at 100 W/m² which was harvested at an OD₆₀₀ of 0.2 because of its slow growth under these conditions. All values provided are the average of two independent determinations. Vertical bars represent the standard deviation from the mean.

mutant only the light effect was examined, since the synthesis of DMSO reductase is severely affected in this mutant. The CCOP1FNR mutant showed a similar response to changes in light intensity to that of the wild-type and CCOP1 mutant strains with regard to the B800–850 and B875 complex levels. In contrast, the PPS1 mutant, in which the *ppsR* gene is disrupted, was much less responsive to either changes in light intensity or addition of DMSO to photosynthetic cultures.

Taken together, the data presented here imply that the redox state of the cyclic photosynthetic ETC, probably the quinone pool (see Discussion), is at least in part sensed by the AppA-PpsR system to control PS gene expression and that the *cbh₃*-PrrBA pathway and FnrL appear not to be involved in sensing the redox state of the photosynthetic ETC in any major way.

Discussion

Previously it was reported from this laboratory that inactivation of the *cbh₃* oxidase leads to derepression of PS genes (PS^D) under aerobic conditions, which is accompanied by the oxygen-insensitive formation of the spectral complexes (Zeilstra-Ryalls and Kaplan, 1996; O’Gara and Kaplan, 1997). Inactivation of the PrrBA two-component activation system in a Cco-minus background overrides the Cco-minus phenotype (O’Gara *et al.*, 1998). The spectrum of PS genes that are derepressed under aerobic conditions by the inactivation of the *cbh₃* oxidase (*puc*, *puf*, *hemA*, *bchE*, *hemN* and *hemZ*) is coincident with those genes that are shown to be regulated by the PrrBA two-component system (Eraso and Kaplan, 1994; Zeilstra-Ryalls and Kaplan, 1996; O’Gara and Kaplan, 1997; Oh *et al.*, 2000). Based on these and other findings,

we proposed that the *cbb₃* cytochrome *c* oxidase functions as an O₂/redox sensor, which transduces an inhibitory signal to the membrane-bound sensor histidine kinase, PrrB, under aerobic conditions to prevent PS gene expression. The unique role of the *cbb₃* oxidase in this sensory transduction pathway came from studies of a *cbb₃* lacking the CcoQ subunit. This mutant contains a fully functional *cbb₃* oxidase but it produces spectral complexes aerobically like other Cco mutant strains, further suggesting that the *cbb₃* oxidase itself is an O₂/redox sensor (Oh and Kaplan, 1999). This assumption is strengthened by the finding that inactivation of the major cytochrome *c* oxidase *aa₃* revealed only a marginal effect upon PS gene expression under aerobic conditions. Thus, the available evidence directly implicates the *cbb₃* oxidase in regulating the PrrBA system and makes it highly unlikely that any other electron carriers of the ETC such as the *bc₁* complex and quinone pool are involved in this pathway.

The finding that strong PS^D occurred in both the BC1 and C2Cy mutants grown under high oxygen conditions allowed us to speculate that it is the interruption of electron flow into the *cbb₃* oxidase that leads to aerobic PS^D. Employing myxothiazol, it was clearly demonstrated that it is the extent of the electron flux through the *cbb₃* oxidase, rather than the binding *per se* of an oxygen molecule to the *cbb₃* oxidase that determines the degree of PS^D. The greater the electron flow through the *cbb₃* oxidase, the stronger the inhibitory signal generated by the *cbb₃* oxidase which in turn represses the PrrBA two-component system, resulting in repression of PS gene expression.

As further proof that the signal transduction pathway originates within the structure of the *cbb₃* oxidase, we demonstrated that altering the structure of the *cbb₃* oxidase by changing a series of histidine residues involved in ligand coordination to alanine all led to the aerobic PS^D. However, only in the case of H407A were both the catalytic function and structure of the *cbb₃* oxidase maintained. The H407A mutant contains near normal levels of apparently properly assembled *cbb₃* oxidase containing normal amounts of heme *b*, which is consistent with the data obtained from the corresponding mutant of *Bradyrhizobium japonicum* (Zufferey *et al.*, 1998). The partial decrease in *cbb₃* activity in H407A despite the normal levels of heme *b* indicates that the loss of His407 might alter the conformation or placement of the low spin heme in CcoN. The absence of one axial ligand (His407) might result in displacement of the heme iron within the porphyrin plane toward the other axial ligand His118, which in turn causes a partial reduction of *cbb₃* oxidase activity. Considering that the H407A mutant contains more *cbb₃* oxidase activity than the wild type with pRK415, which contains a single copy of the *ccoNOQP* operon (see Table II), oxygen-insensitive formation of spectral complexes in this mutant implies that either the His407 itself or precise placement of the low spin heme might be required in order for the *cbb₃* oxidase to generate the inhibitory signal to repress PS gene expression in the presence of O₂. The removal of His407, as well as the non-polar deletion of the *ccoQ* gene, unambiguously dissociates the sensory function of the *cbb₃* oxidase from its catalytic function, reinforcing the idea that the CcoQ subunit of the *cbb₃* oxidase and the His407 residue or the

low spin heme are intimately involved in signal generation and transduction. These results together with the lack of any phenotypic effect associated with the AA3 mutant indicate that no other component of the aerobic ETC is involved in the signal transduction pathway from *cbb₃* to the PrrBA two-component system.

The *cbb₃* oxidase has excellent properties as an oxygen sensor in order to provide for the orderly control of PS gene expression in *R.sphaeroides*: (i) it has a high affinity for O₂ (*K_m* of *B. japonicum cbb₃* oxidase is 7 nM which is 6- to 8-fold lower than that for the *aa₃* oxidase: Preisig *et al.*, 1996) and (ii) it is present in cells grown under anaerobic conditions (Shapleigh *et al.*, 1992; Oh and Kaplan, 1999). The high affinity of the *cbb₃* oxidase for O₂ enables *R.sphaeroides* not to activate the PrrBA two-component system until O₂ tensions in the environment fall to sufficiently low levels. The anaerobic presence of the *cbb₃* oxidase allows *R.sphaeroides* both to maintain transcriptional control of the PS genes under anaerobic conditions and to turn off the PrrBA system quickly as soon as it is exposed to O₂.

How then does the inhibitory signal move from the *cbb₃* oxidase to the membrane-bound PrrB histidine kinase? We suggest that the CcoQ subunit, which is a part of the *cbb₃* complex, can monitor the 'volume' of electron flow through the *cbb₃* oxidase (Oh and Kaplan, 1999). Furthermore, recent genetic studies performed by Eraso and Kaplan proposed that the membrane-bound PrrC protein, whose gene is located immediately upstream of *prrA*, is located between the *cbb₃* oxidase and the PrrBA two-component system in the signal transduction pathway (Eraso and Kaplan, 2000).

Rhodobacter sphaeroides contains two functional cytochromes *c* under normal physiological conditions, which participate in electron transfer between the membrane-associated *bc₁* complex and the two terminal cytochrome *c* oxidases (Donohue *et al.*, 1988; Myllykallio *et al.*, 1999). The soluble periplasmic cytochrome *c₂* supports both respiratory and photosynthetic ETC, while the membrane-anchored cytochrome *c_y* participates only in respiratory electron transfer. Based upon our assumption that the 'extent' of electron flow through the *cbb₃* oxidase ultimately determines the level of PS gene expression under aerobic conditions, then the differential expression of both the *puc* and *puf* operons observed in the C2 and Cy mutants grown under 30% O₂ conditions permits us to assign, albeit indirectly, the relative contribution of cytochromes *c₂* and *c_y* to channel electrons from the *bc₁* complex to the *cbb₃* oxidase (see Figure 2). From these results, we suggest that under 30% O₂ conditions the membrane-bound cytochrome *c_y* is approximately two to three times more effective in its ability to channel electrons from the *bc₁* complex to the *cbb₃* oxidase than is cytochrome *c₂*.

The *puf* operon, which appears to be under the exclusive control of the PrrBA system (Zeilstra-Ryalls *et al.*, 1998), was shown to be derepressed to similar levels in the BC1, C2Cy, CCONΔ and CCOQΔ mutant strains (see Figure 2B). However, in the case of the *puc* operon its degree of derepression in the aforementioned mutants was highly variable, i.e. derepression of the *puc* operon in the BC1 and C2Cy mutants was substantially higher than that observed for the CCONΔ and CCOQΔ mutant strains (see

Fig. 7. Respiratory and photosynthetic electron transport pathways in *R. sphaeroides* and model for redox sensing and signal transduction. The solid and dotted arrows demonstrate the electron and signal flows, respectively. The red arrows indicate the photosynthetic cyclic electron transport pathway. The thickness of the arrow indicates the presumptive relative contribution of cytochromes c_2 and c_y to channel electrons from the bc_1 complex to the cbb_3 oxidase. The '+' sign indicates the activation of genes. Abbreviations: Q-pool, ubiquinone pool; bc_1 , cytochrome bc_1 complex; C_2 , soluble cytochrome c_2 ; C_y , membrane-bound cytochrome c_y ; aa_3 , aa_3 -type cytochrome c oxidase; RC, photochemical reaction center; Qox and Qxt, two quinol oxidases; PS, photosynthesis; fix, fixation; $h\nu$, light; red, reduced form.

Figure 2A). These differences in *puc* and *puf* operon expression imply that 'factors', in addition to the PrrBA activation system, are operative together with the PrrBA system in regulating the *puc* operon. These factors, in a cbb_3 -PrrBA-independent manner, sense another redox signal associated with the interruption of electron flow through the bc_1 complex to enhance *puc* operon expression. Other factors known to affect *puc* operon expression are FnrL, IHF and the AppA-PpsR antirepressor-repressor system (Zeilstra-Ryalls *et al.*, 1998). We postulate that the redox state of the quinone pool, which is positioned upstream of the bc_1 complex in the ETC, might be the source of another signal that is transduced to enhance *puc* operon expression independently of the cbb_3 -PrrBA signal transduction pathway. The redox state of the quinone pool can be affected by the rate at which electrons are removed to the terminal oxidases under aerobic conditions or to the terminal reductases such as DMSO reductase under anaerobic conditions, when a steady flow of electrons from a given organic electron donor is entering the pool. In addition, changes in the light intensity also affect the redox state of the quinone pool. Therefore, under 30% O₂, inactivation of the bc_1 complex or both cytochromes c_2 and c_y is likely to shift the quinone pool toward a more reduced state, which might preferentially enhance the derepression of the *puc* operon relative to the *puf* operon.

What then might sense the redox state of the quinone pool? The best candidate to our knowledge is the AppA antirepressor-PpsR repressor system (Gomelsky and Kaplan, 1995a,b, 1997). The reasons are that: (i) it is involved in the regulation of the *puc* operon and not the *puf* operon (Gomelsky and Kaplan, 1995a); (ii) the PpsR mutant PPS1 was shown to be much less responsive to the transition from medium light (10 W/m²) to high light (100 W/m²) as well as to the addition of DMSO to photosynthetic cultures when compared with the wild type

with regard to spectral complex formation; (iii) the CCOP1 and CCOP1FNRL mutant strains showed a similar response to changes in light intensity under photosynthetic conditions to the wild type, eliminating the involvement of both the cbb_3 -PrrBA signal transduction pathway and FnrL in sensing and transduction of the redox state of the quinone pool; (iv) the AppA protein contains at least FAD as a possible redox prosthetic group (Gomelsky and Kaplan, 1998), which further raises the possibility that it might sense the redox state of the quinone pool; and finally (v) there is no indication that the IHF protein is involved in redox control, although it has been shown to be involved in *puc* operon regulation (Lee *et al.*, 1993).

On the basis of the results presented here, as well as those reported previously (Zeilstra-Ryalls *et al.*, 1998; Oh and Kaplan, 1999; Oh *et al.*, 2000), we present a model describing the interrelationship between the regulation of the PS genes and the activity of the ETC in *R. sphaeroides* (Figure 7). This model together with other recent studies of the anaerobic regulator FnrL, which participates in the selective control of both *puc* and the tetrapyrrole biosynthesis genes such as *hemaA*, *hemN*, *hemZ* and *bchE* as well as the *ccoNOQP* operon as conditions become increasingly less aerobic (Zeilstra-Ryalls and Kaplan, 1995, 1998; Zeilstra-Ryalls *et al.*, 1997; Mouncey and Kaplan, 1998a; Oh *et al.*, 2000), allows us to describe fully the regulation of PS gene expression in *R. sphaeroides*.

In summary, we here demonstrated that the regulation of PS gene expression in *R. sphaeroides* is closely coupled with the activity of the ETC, i.e. the functional state of the cbb_3 oxidase and the redox state of the quinone pool. The fact that the cbb_3 oxidase has dual function as both a terminal oxidase and an O₂ sensor and that it, together with the PrrBA two-component system, constitutes a signal transduction pathway, provides a new paradigm for O₂ sensing and gene regulation. The advantage of redox

Table III. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant phenotype or genotype	Source or reference
2.4.1	<i>R.sphaeroides</i>	van Neil (1944)
BC1	wild type	N.J.Mouncey, E.Gak, M.Choudhary, J.Oh and S.Kaplan, in preparation
	2.4.1 derivative, $\Delta fbcBC::Tp$ cassette	Donohue <i>et al.</i> (1988)
C2	2.4.1 derivative, formerly CYCA1, $\Delta cycA::Km$ cassette	this study
Cy	2.4.1 derivative, $cycY::Tp$ cassette	this study
C2Cy	2.4.1 derivative, $\Delta cycA::Km$ cassette $cycY::Tp$ cassette	Oh and Kaplan (1999)
CCON Δ	2.4.1 derivative, in-frame deletion in <i>ccoN</i>	this study
AA3	2.4.1 derivative, $\Delta actA::Sp/St$ cassette	O'Gara and Kaplan (1997)
CCOP1	2.4.1 derivative, $ccoP::Tp$ cassette	Oh <i>et al.</i> (2000)
CCOP1FNRL	2.4.1 derivative, $ccoP::Tp$ cassette, $\Delta fnrL::Km$ cassette	Gomelsky and Kaplan (1997)
PPS1	2.4.1 derivative, $ppsR::Km$ cassette	Oh and Kaplan (1999)
CCOQ Δ	2.4.1 derivative, in-frame deletion in <i>ccoQ</i>	
	<i>E.coli</i>	
S17-1	Pro ⁻ Res ⁻ Mod ⁺ <i>recA</i> ; integrated plasmid RP4-Tc::Mu-Km::Tn7	Simon <i>et al.</i> (1983)
	Plasmids	
pNHG1	Km ^r Tc ^r ; SacB RP4-oriT ColE1-oriT	Jeffke <i>et al.</i> (1999)
pRK415	Tc ^r ; Mob ⁺ <i>lacZ</i> α IncP	Keen <i>et al.</i> (1988)
pUI624	Ap ^r ; pBS::0.53 kb <i>Xma</i> III fragment of <i>puc</i>	Lee <i>et al.</i> (1989)
pUI655	Ap ^r ; pBS::0.47 kb <i>Sty</i> I fragment of <i>pufBA</i>	J.K.Lee
pUI2803	pRK415::4.7 kb <i>Bam</i> HI- <i>Eco</i> RI fragment containing <i>ccoNOQP</i>	O'Gara and Kaplan (1997)
pUI2803-H405A	pUI2803 in which His405 of CcoN is changed to Ala	this study
pUI2803-H407A	pUI2803 in which His407 of CcoN is changed to Ala	this study
pUI2803-H267A	pUI2803 in which His267 of CcoN is changed to Ala	this study
pUI8180	Tc ^r ; pLA2917 derivative, cosmid containing <i>cycY</i> , <i>ccoNOQP</i> and <i>rdxBHIS</i>	Zeilstra-Ryalls and Kaplan (1995)
pTP	Ap ^r Tp ^r ; pBSISK+::1110 bp <i>Apa</i> I fragment containing the Tp cassette	this study
pCTS1	pJS1 derivative, $\Delta actA::Sp/St$	Shapleigh and Gennis (1992)
pCY Δ 3	Km ^r Tc ^r Tp ^r ; pNHG1 derivative, $cycY::Tp$	this study
pCCO2	pUC19::4.7 kb <i>Bam</i> HI- <i>Eco</i> RI fragment from pUI2803	this study
p19CCON	pUC19::2.6 kb <i>Bam</i> HI- <i>Sac</i> I fragment from pCCO2	this study
pCF200Km	Sp ^r St ^r Km ^r ; IncQ, <i>puc::lacZYA'</i>	Lee and Kaplan (1992)
pUI1663	Sp ^r St ^r Km ^r ; IncQ, <i>puf::lacZYA'</i>	Eraso and Kaplan (1996)

sensing through the ETC, as demonstrated here, appears to be the ability to respond rapidly and precisely to environmental stimuli as well as to provide for a mechanism to integrate all cellular metabolic activities.

Materials and methods

Strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table III. *Rhodobacter sphaeroides* and *Escherichia coli* strains were grown as described previously (Oh and Kaplan, 1999).

DNA manipulations and conjugation techniques

Genomic DNA from *R.sphaeroides* was isolated according to Ausubel *et al.* (1988). Standard protocols (Sambrook *et al.*, 1989) or manufacturer's instructions were followed for recombinant DNA manipulations. Mobilization of plasmids from *E.coli* strains into *R.sphaeroides* strains was performed as described elsewhere (Davis *et al.*, 1988).

Construction of mutants

Cy and C2Cy mutants. In order to construct the Cy mutant, a 1232 bp fragment containing the *cycY* gene was amplified from pUI8180 by PCR using pfu turbo polymerase (Stratagene, La Jolla, CA) using primers CyEcoRI (5'-GCAAAAGAATTCTCGACCGGAGCATC-3') and CyHindIII (5'-CTCGCAAGCTTGGGTGCGTGGGATTG-3'). The PCR product was restricted with *Eco*RI and *Hind*III, and cloned into pUC19 to give plasmid pCY Δ 1. A 1.1 kb *Apa*I fragment containing the Tp cassette from pTP was cloned into pCY Δ 1 digested with *Apa*I, resulting in the plasmid pCY Δ 2. Finally, a 2.3 kb *Eco*RI-*Hind*III fragment from pCY Δ 2 was filled in by treatment of Klenow fragment of *E.coli* polymerase I and subsequently cloned into the suicide vector pNHG1, which was restricted with *Sac*I and blunt-ended, yielding the plasmid pCY Δ 3. The resulting plasmid pCY Δ 3 was transferred from *E.coli* strain S17-1 to *R.sphaeroides* 2.4.1 and C2 by conjugation to

generate Cy and C2Cy mutants, respectively. Heterogenotes of *R.sphaeroides*, generated by a single recombination event, were selected for their Km and Tc resistance on SIS agar plates incubated under aerobic conditions. Isogenic homogenotes were obtained from the heterogenotes after a second recombination selecting for sucrose and Tp resistance on SIS agar plates containing 15% (w/v) sucrose, 1% (w/v) Bactotrypton, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl and 0.5% (v/v) DMSO under dark anaerobic conditions.

AA3 mutant. The AA3 mutant was constructed by crossing pCTS1 into *R.sphaeroides* 2.4.1 and selecting for the double cross-over recombinant.

Site-directed mutagenesis

To mutate His267, His405 and His407 of CcoN to alanine, a 2.6 kb *Bam*HI-*Sac*I fragment carrying the *ccoN* gene was cloned from pCCO2 into pUC19. The resulting plasmid, p19CCON, was used as a template plasmid for site-directed mutagenesis. Mutagenesis was carried out using the Quick Change Site-Directed Mutagenesis Kit (Stratagene). Synthetic oligonucleotides 33–34 bases long containing an alanine codon (GCC) in place of the histidine codon in the middle of their sequences were used to mutagenize the histidine codons. Following the verification of mutations by sequencing, a 2.6 kb *Bam*HI-*Sac*I fragment containing the mutated sequence was cloned back into pCCO2, in which the 2.6 kb fragment was removed, to give pCCO2 with the mutated sequence. The entire insert of the mutated pCCO2 (the 4.7 kb *Bam*HI-*Eco*RI fragment containing the entire *ccoNOQP* operon) was cloned in pRK415. The resulting plasmids, pUI2803-H267A, pUI2803-H405A and pUI2803-H407A, were introduced into *R.sphaeroides* CCON Δ , a strain in which a part of *ccoN* has been deleted in frame by gene replacement.

RNA isolation and analysis

Total RNA was isolated from *R.sphaeroides* strains as described by Oelmüller *et al.* (1990). Northern hybridization experiments were performed using the AlkPhos DIRECT System (Amersham Pharmacia Biotech, Piscataway, NJ) as instructed by the manufacturer. Quantitation of signals was performed with NIH Imager 1.62. The signal levels were normalized by those of processed 23S and 16S rRNA.

Preparation of solubilized membrane proteins

The harvested cells were resuspended in buffer A (20 mM Tris-HCl pH 7.5) and disrupted by passage through a French pressure cell. Cell-free crude extracts were obtained following centrifugation at 27 000 g for 20 min at 4°C to remove unbroken cells and cell debris. Membrane fractions were isolated by ultracentrifugation of crude extracts at 150 000 g for 1 h at 4°C. After membrane fractions (pellet) were washed twice with buffer A, the membranes were solubilized in buffer A containing 0.5% (w/v) sodium dodecyl maltoside by pipetting and then centrifuged at 13 000 r.p.m. for 10 min in a benchtop minicentrifuge. The supernatant was taken as solubilized membrane proteins.

Spectroscopic and immunoblotting analyses

Solubilized membrane proteins (0.2 mg/ml) in buffer A containing 0.5% (w/v) sodium dodecyl maltoside was used to record an air-oxidized spectrum on an UV 1601PC spectrophotometer (Shimadzu Corp., Columbia, MD). The same sample was reduced with 30 µl of 15% (w/v) sodium dithionite for 2 min at room temperature (RT) and then a reduced spectrum was recorded. The dithionite-reduced minus air-oxidized spectrum was obtained by subtraction of these two spectra. The amount of the b- and c-type hemes was estimated by using the extinction coefficients $\epsilon_{561-575}$ of 22 mM⁻¹ cm⁻¹ and $\epsilon_{551-540}$ of 19 mM⁻¹ cm⁻¹, respectively.

The B800–850 and B875 complex levels were determined spectrophotometrically as described previously (Oh and Kaplan, 1999). SDS-PAGE and western blotting were performed as described elsewhere (Laemmli, 1970; Mouncey and Kaplan, 1998b).

Enzyme assays and protein determination

Preparation of crude cell extracts and determination of β -galactosidase activities were performed as described previously (Oh and Kaplan, 1999). Cytochrome *c* oxidase activities were measured either spectrophotometrically with reduced horse heart cytochrome *c* (Sigma, St Louis, MO) as described previously (Oh and Kaplan, 1999) or polarographically with a Clark-type oxygen electrode (YSI Inc., Yellow Springs, OH). Prepared membrane fractions washed twice with 50 mM potassium phosphate buffer pH 7.0 were resuspended in the same buffer. The rate of oxygen uptake at 30°C was determined with 0.2 mg resuspended membrane fractions in 5 ml 30% O₂ saturated buffer after addition of 20 µM horse heart cytochrome *c* and 12.5 mM ascorbate. Protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL) using bovine serum albumin as the standard protein.

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