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Relationship of the Redox State of Pyridine Nucleotides and Quinone Pool with Spectral Complex Formation in *Rhodobacter sphaeroides* 2.4.1

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The homeostasis of the pyridine nucleotide pool [NAD(P)H and $NAD(P)^+$] is maintained in *Rhodobacter sphaeroides* mutant strains defective in the cytochrome bc_1 complex or the cytochrome c oxidases in terms of its concentration and redox state. Aerobic derepression of the *puf* operon, which is under the control of the PrrBA two-component system, in the CBB3 mutant strain of *R. sphaeroides* was shown to be not the result of changes in the redox state of the pyridine nucleotides and the ubiquinone/ubiquinol pool. Using the bc_1 complex knock-out mutant strain of *R. sphaeroides*, we clearly demonstrated that the inhibitory effect of cbb_3 oxidase on spectral complex formation is not caused indirectly by the redox change of the ubiquinone/ubiquinol pool.

Key words : cbb3 cytochrome c oxidase, photosynthesis, PrrBA two-component system, pyridine nucleotide, redox sensing

Introduction

Rhodobacter sphaeroides is an anoxygenic photosynthetic bacterium which synthesizes the photosynthetic apparatus when oxygen tensions in the environment fall bellow ~3% [17,25]. The three major regulatory systems governing the expression of photosynthesis genes in response to changes in oxygen tensions have been known: PrrBA two-component system, PpsR-AppA repressor-antirepressor system, and FnrL anaerobic regulator [6-8,12,13,36]. The PrrBA two-component system is a global regulatory system which acts as both activator and repressor. The PrrBA two-component system is composed of the membrane-bound PrrB histidine kinase and its cognate response regulator PrrA [6-8]. Most of photosynthesis (PS) genes are regulated positively by the PrrBA two-component system. When oxygen tensions are decreased, the PrrB histidine kinase phosphorylates PrrA by transferring the γ-phosphoryl group from ATP to the conserved aspartate residue (Asp-63) in the N-terminal receiver domain of PrrA [2]. The phosphorylated PrrA serves as a transcriptional activator to stimulate the expression of many PS genes.

Two hypotheses have been suggested regarding how the PrrB histidine kinase recognizes oxygen tensions and controls the state of PrrA phosphorylation. It has been suggested that the PrrBA two-component system constitutes a signal transduction pathway together with the cbb_3 cytochrome c oxidase and that the latter serves as an oxygen sensor [18,22,24,28]. According to this hypothesis, the volume of electron flow through the cbb_3 oxidase serves as the signal that is transduced to the PrrBA two-component system. The greater the volume of electron flow through the cbb_3 oxidase, the stronger the inhibitory signal, which shifts the equilibrium of PrrB activity from the kinase mode to the phosphatase-dominant mode, resulting in the repression of PS gene expression [28]. Since the partial pressure of oxygen in the environment is proportional to the extent of electron flow through the cbb_3 oxidase, the PS genes, which are positively regulated by PrrA, are repressed under aerobic conditions. Recently it was demonstrated that the purified cbb_3 oxidase itself enhances the phosphatase activity of PrrB without any effect on PrrB kinase activity [28]. The other hypothesis was proposed from the study of RegB (a close homolog of PrrB from *Rhodobacter capsulatus*). Ubiquinone of the electron transport chain was demonstrated to inhibit RegB kinase activity, whereas the reduced form of ubiquinone, ubiquinol, did not affect RegB kinase activity [33]. RegB inhibition by ubiquinone was proposed to be mediated through its binding to the transmembrane domain of RegB. The redox state of the ubiquinone/ubiquinol (Q/QH_2) pool of the electron transport chain (ETC) is influenced by the availability of O_2 and the functionality of the terminal oxidases, accounting for repression of PS genes un-

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der aerobic conditions where the relative ratio of ubiquinone to ubiquinol is increased compared to under anaerobic conditions.

In this study, we provide evidence that the redox state of neither $NAD(P)^+$/NAD(P)H nor Q/QH_2 pool did greatly affect spectral complex formation in *R. sphaeroides* and that the cbb_3 oxidase has a negative effect on PS gene expression even in the absence of electron flow through itself.

Materials and Methods

Growth conditions of bacterial strains

R. sphaeroides and *Escherichia coli* strains were grown as described previously [23].

DNA manipulations and conjugation techniques

Standard protocols [23] 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 [4].

Construction of the plasmids

A 4.7-kb *Eco*RI/*Bam*HI fragment containing the *ccoNOQP* operon was excised from pUI2803 and cloned into pBBR1MCS2, yielding the plasmid pCCO3.

A 0.8-kb DNA fragment containing *prrC* and its ribosome-binding site was amplified by polymerase chain reaction (PCR) using the primers, PrrC-EcoRI (5′-TAAAGAATTCAATCGGCAACCGCAAGC-3′) and PrrC-Hind3 (5′-CAAGAAGCTTCAGGAAGGGCTCGT-3′), and pUI1640 as the template. The resulting fragment was restricted with *Eco*RI and *Hind*III and cloned into pCCO3 restricted with the same enzymes, resulting pCCO3C which contains both *ccoNOQP* and *prrC* in the collinear direction.

Quantitative analysis of spectral complexes

The levels of B800-850 and B875 complexes were determined spectrophotometrically as described previously [22].

β-galactosidase assay and protein determination

Preparation of crude cell extracts and determination of β-galactosidase activity were performed as described previously [19]. Protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL) using bovine serum albumin as the standard protein.

Quantitative analysis of pyridine nucleotides

50 ml of cultures of the *R. sphaeroides* strains were harvested and resuspended in 2 ml of 100 mM Tris-HCl buffer (pH 8.0). Cells were disrupted by two passages through a French pressure cell, and cell-free crude extracts were obtained by centrifugation at 20,000× *g* for 15 min. Determination of $NADP^+$/NADPH and NAD^+/NADH ratios and concentrations in the crude extracts were carried out as described elsewhere [3].

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	[35]
BC1	2.4.1 derivative, Δ*fbcBC*::ΩTp^r	[20]
AA3	2.4.1 derivative, Δ*ctaD*::ΩSp/St^r	[24]
CBB3	2.4.1 derivative, deletion in *ccoNOQP*	[27]
PrrB1	2.4.1 derivative, Δ*prrB*::ΩSp/St^r	[7]
	E. coli	
DH5α	(Φ80d*lacZ*ΔM15)Δ*lacU169 recA1 endA1 hsdR17 supE44 thi1 gyrA96 relA1*	[15]
S17-1	Pro$^-$ Res$^-$ Mob$^+$ *recA*; integrated plasmid RP4-Tc::Mu-Km::Tn*7*	[32]
	plasmid	
pUI1643	pBSIIKS+::4.0 kb *Bam*HI-*Hind*III fragment containing *prrA*, *prrC*, and *prrB*	[7]
pCCO3	pBBR1MCS2::4.7 kb *Bam*HI-*Eco*RI fragment containing *ccoNOQP*	This study
pCCO3C	pBBR1MCS2::5.5 kb DNA fragment containing *ccoNOQP* and *prrC*	This study
pUI1663	Sp^r St^r Km^r; IncQ, *puf*::*lacZYA*′	[8]
pUI2803	pRK415::4.7 kb *Bam*HI-*Eco*RI fragment containing *ccoNOQP*	[21]
pA-9	pRK415::1.45 kb *Xba*I-*Kpn*I fragment containing *prrB*	[26]
pBBR1MCS2	Km^r; Mob$^+$, *lacZ*α, IncP	[19]
pRK415	Tc^r; Mob$^+$, *lacZ*α	[16]

Determination of the redox state of Q/QH_2 pool in the membrane

10 ml of *R. sphaeroides* cells were harvested and re-suspended with 2 ml of 0.2 M HClO in methanol. The Q/QH_2 pool in the membrane was subsequently extracted by mixing the mixture with 2 ml of petroleum ether and thorough vortexing for 30 sec. After centrifugation at 3,000× *g* for 5 min, the upper petroleum ether phase was taken and evaporated to dryness with a stream of nitrogen. Quantitative analysis of ubiquinone and ubiquinol was performed using HPLC equipped with the reverse phase column as described previously [34].

Results and Discussion

Relationship of the redox state of pyridine nucleotides with the expression rate of *puf*

The respiratory ETC of *R. sphaeroides* is terminated by two active cytochrome c oxidases (aa_3- and cbb_3-type) which belong to the heme-copper superfamily [11,14,31]. The aa_3 cytochrome c oxidase is a major cytochrome c oxidase in *R. sphaeroides* grown under high oxygen conditions (30% O_2), while the cbb_3 oxidase is dominantly synthesized under semiaerobic and anaerobic conditions [24]. It was previously reported that inactivation of the cbb_3 oxidase or cytochrome bc_1 complex led to oxygen-insensitive formation of the photosynthetic apparatus accompanying with aerobic derepression of PS genes in *R. sphaeroides* [24]. In good agreement with the previous result, the *puf* operon encoding the apoproteins of the photosynthetic reaction center and light harvesting complex I was derepressed in the BC1 and CBB3 knock-out mutant strains of *R, sphaeroides* grown under 30% O_2 conditions (Table 2). As depicted in Fig. 1, the inactivation of the bc_1 complex and cbb_3 oxidase must severely affect the electron flow through the ETC, which might result in changes in the cellular redox state. We first examined the

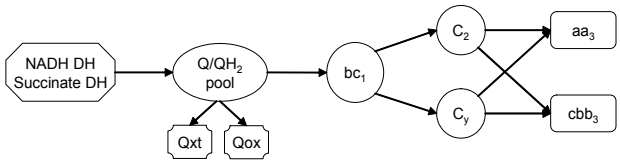

Fig. 1. Respiratory electron transport chain of *R. sphaeroides*. Abbreviations: DH, dehydrogenase; Q/QH_2, ubiquinone/ubiquinol pool; bc_1, cytochrome bc_1 complex; c_2 and c_y, cytochromes c_2 and c_y aa_3, aa_3 cytochrome c oxidase; cbb_3, cbb_3 cytochrome c oxidase; Qxt and Qox, Qxt- and Qox-quinol oxidases.

redox state of the pyridine nucleotide pool in several ETC mutants as well as the wild-type strain 2.4.1 grown under 30% O_2 conditions. The total concentration of NADH and NAD^+ in *R. sphaeroides* strains ranged from 17 to 22 pmol/μg protein, whereas the concentration of NADPH and $NADP^+$ was much lower (1-3 pmol/μg protein) than $NADH/NAD^+$ (data not shown). Since NADH serves as an electron donor for the ETC, we expected that inactivation of the bc_1 complex or the cytochrome c oxidases results in the more reduced state of the $NADH/NAD^+$ pool. However, as shown in Table 2, the redox state of the $NADH/NAD^+$ pool was maintained relatively constantly despite the inactivation of the bc_1 complex and the cytochrome c oxidases. This result indicates that aerobic derepression of *puf* observed in the BC1 and CBB3 mutant strains grown under high oxygen conditions is not due to changes in the redox state of the cellular $NADH/NAD^+$ pool. The relative portion of the reduced NADPH in the $NADPH/NADP^+$ pool is small (~1-3.4%) relative to NADH in the $NADH/NAD^+$ pool. With the exception of the BC1 mutant, the redox state of the cellular $NADPH/NADP^+$ pool in the wild-type and mutant (AA3 and CBB3) strains was approximately the same, implying that aerobic derepression of *puf* in the CBB3 mutant grown aerobically is not caused by changes in the redox state of the $NADPH/NADP^+$ pool, either. It is also noteworthy

Table 2. Expression of *puf* and redox state of $NAD(P)H/NAD(P)^+$ in *R. sphaeroides* strains grown under aerobic conditions

Strain	*puf*::*lacZ* (nmol/min·mg protein)	$NADH/(NADH+NAD^+)$ (×100)	$NADPH/(NADPH+NADP^+)$ (×100)
2.4.1	65.0±9.2	18.8±1.2	3.0±0.2
BC1	307.5±25.3	21.3±2.0	0.9±0.2
AA3	105.2±1.7	23.3±0.7	3.4±0.5
CBB3	291.3±15.6	19.6±0.6	3.1±0.3

The strains were grown aerobically by sparging with 30% O_2-69% N_2-1% CO_2 to an OD_{600} of 0.4 to 0.5. β-galactosidase activity was measured for the *R. sphaeroides* strains carrying pUI1663 to determine the expression rate of *puf*. All values are the averages of two independent determinations.

that the redox state of the pyridine nucleotides in *R. sphaeroides* is first reported here. The decline of the relative amount of NADPH in the NADPH/$NADP^+$ pool in the BC1 mutant might result from high levels of the photosynthetic apparatus synthesized in the mutant grown aerobically since NADPH is required for biosynthesis of macromolecules.

The predominant role of the cbb_3 oxidase, rather than the redox state of ubiquinone/ubiquinol, in the regulation of spectral complex formation

It was previously demonstrated that the binding of ubiquinone, the oxidized form of ubiquinol, to the second periplasmic loop of the transmembrane domain of RegB (PrrB) led to the decrease in kinase activity of RegB [33]. This result allowed the authors to suggest that the redox state of the Q/QH_2 pool of the respiratory ETC is shifted to the oxidized state under aerobic conditions, leading to the inhibition of RegB kinase activity. If the RegB histidine kinase senses the oxygen tension in the environment in such a way, more photosynthetic apparatus must be synthesized in the AA3 mutant strain than the CBB3 mutant strain when both the strains are grown under aerobic conditions because the aa_3 cytochrome c oxidase is the major cytochrome c oxidase in *R. sphaeroides* grown under aerobic conditions (approximately 70% of cytochrome c oxidase activity detected in *R. sphaeroides* grown aerobically is attributable to the aa_3 cytochrome c oxidase) [24]. However, as reported elsewhere [24] and shown in Table 2, the *puf* operon was not derepressed in the AA3 mutant grown under aerobic conditions as much as in the CBB3 mutant grown under the same conditions, indicating that the direct signal controlling the RegB kinase activity is not the redox state of Q/QH_2 pool. To confirm this assumption, we determined the redox state of Q/QH_2 pool of the AA3 and CBB3 mutant strains grown under 30% O_2 conditions. The HPLC profile in Fig. 2 shows that the Q/QH_2 pool in the AA3 mutant is more reduced than that of the CBB3 mutant as expected. The higher proportion of ubiquinone relative to ubiqunol was observed in the CBB3 mutant than the AA3 mutant, excluding the possibility that the redox state of Q/QH_2 pool is a major determinant for the regulation of PrrB (RegB) kinase activity.

Based on the observation that the inactivation of the cbb_3 oxidase led to aerobic derepression of PS genes, which are under the control of the PrrBA two-component system, as well as the results derived from the ETC mutant strains of

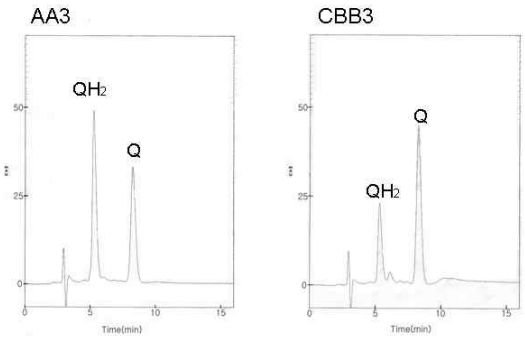

Fig. 2. The redox state of the ubiquinone/ubiquinol pool in the membranes of *R. sphaeroides* mutant strains (AA3 and CBB3) grown under aerobic conditions (30% O_2). QH_2 and Q denote ubiquinol and ubiquinone, respectively. The unit of the y axis is the arbitrary unit (AU) set by the HPLC program.

R. sphaeroides [24], we previously suggested that the cbb_3 cytochrome c oxidase generates an inhibitory signal to decrease the PrrB kinase activity [18,24,28]. The inhibitory signal was suggested to is stronger when the cbb_3 oxidase is functional in the presence of O_2 than in the absence of O_2. The purified cbb_3 oxidase was shown to inhibit the PrrB activity phosphorylating the PrrA response regulator by increasing PrrB phosphatase activity [28]. As shown in Table 3, the overexpression of *prrB* resulted in increased synthesis of the photosynthetic apparatus in *R. sphaeroides* strain [PrrB1 (pA-9+pBBR1MCS2)] grown anaerobically, which is consistent with the previous result that the default state of PrrB is in the kinase dominant mode [26]. When the *ccoNOQP* operon encoding the cbb_3 oxidase was overexpressed together with *prrB* [PrrB1 (pA-9+pCCO3)],

Table 3. Levels of the spectral complexes synthesized in *R. sphaeroides* strains grown under anaerobic conditions

Strain	B800-850	B875
2.4.1 (pRK415)	37.1±0.7	8.5±0.1
PrrB1 (pRK415)	2.3±0.2	6.5±0.0
PrrB1 (pA-9 + pBBR1MCS2)	43.6±0.5	12.0±0.1
PrrB1 (pA-9 + pCCO3)	22.6±0.1	6.6±0.2
PrrB1 (pA-9 + pCCO3C)	36.4±1.4	9.1±0.1

The strains were grown anaerobically in SIS medium supplemented with 0.5% (v/v) dimethyl sulfoxide (DMSO) and 0.1% (w/v) yeast extract in screw-cap tubes in the dark. B875 and B800-850 indicate the light harvesting complexes I and II, respectively. The levels of spectral complexes are expressed as nmol/mg protein. All values are the averages of two independent determinations. pA-9 is the pRK415 derivative containing *prrB*. pCCO3 and pCCO3C are the pBBR1MCS2 derivatives containing *ccoNOQP* and *ccoNOQPprrC*, respectively.

Table 4. Effect of expression of the *ccoNOQP* operon on spectral complex formation in *R. sphaeroides* BC1 strain

Strain	+O_2 (30% O_2)		- O_2 (dark DMSO)	
	B800-850	B875	B800-850	B875
BC1 (pRK415)	0.4±0.1	2.5±0.1	24.8±0.4	4.8±0.1
BC1 (pUI2803)	0.2±0.0	1.7±0.3	18.2±0.0	2.4±0.1

The strains were grown aerobically (+O_2) or anaerobically (-O_2) as described in Tables 2 and 3. The levels of spectral complexes are expressed as nmol/mg protein. All values are the averages of two independent determinations. pUI2803 is the pRK415 derivative containing the *ccoNOQP* operon.

the formation of the photosynthetic apparatus was significantly reduced as compared with PrrB1 (pA-9+ pBBR1MCS2), indicating that the cbb_3 oxidase itself inhibits the PrrB kinase activity. Intriguingly, when *prrC* is coexpressed with *ccoNOQP*, the inhibitory effect of *ccoNOQP* expression on spectral complex formation was partially mitigated, implying that PrrC is somehow involved in the cbb_3-PrrBA signal transduction pathway. The *prrC* gene forms an operon with *prrA* and was suggested to be involved in the PrrBA signal transduction pathway in a unknown way [9]. The PrrC protein is a copper-binding membrane protein and its periplasmic domain possesses the thiol-disulfide oxidoreductase activity [1]. The PrrC homolog called Sco proteins were found to be localized in mitochondria and to be required for synthesis of the active cytochrome c oxidase [5]. The presence of *prrC* within the *prrCA* operon implicates that the cbb_3 cytochrome c oxidase might be related with the PrrBA two-component system.

To rule out the possibility that aerobic derepression of *puf* in the CBB_3 mutant strain grown aerobically was the result of changes in the redox state of the Q/QH_2 pool, we overexpressed the *ccoNOQP* operon in the BC1 mutant strain in which the electron transfer through the cytochrome c oxidases is completely blocked due to the inactivation of the cytochrome bc_1 complex (Fig. 1) and determined the levels of spectral complexes. The redox state of the Q/QH_2 cannot be altered by the overexpression of the *ccoNOQP* operon in the BC1 mutant. As shown in Table 4, when the *ccoNOQP* operon is overexpressed in the BC1 mutant strain [BC1 (pUI2803)] grown under aerobic or anaerobic conditions, the levels of the synthesized spectral complexes were decreased in comparison with the control strain BC1 (pRK415). This result strongly indicates that the inhibitory effect of *ccoNOQP* overexpression on spectral complex formation is not caused by changes in the redox state of the Q/QH_2 pool, implying that the lack of the cbb_3 oxidase itself, rather than the redox change of the Q/QH_2 pool, brings about aerobic derepression in the CBB3 mutant.

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초록 : *Rhodobacter sphaeroides* 2.4.1 내의 pyridine nucleotide와 quinone pool의 redox 상태와 광합성기구의 합성과의 상관관계

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호흡전자전달계의 cytochrome bc_1 complex 또는 cytochrome c oxidase가 기능을 하지 않는 *Rhodobacter sphaeroides* mutant 내에서 pyridine nucleotide[NAD(P)H와 $NAD(P)^+$]의 농도와 redox 상태는 wild type과 비교할 때 큰 변화가 없었다. 높은 산소분압 조건에서 키운 *Rhodobacter sphaeroides* cbb_3 oxidase mutant 내에서 PrrBA two-component system에 의해서 조절되는 *puf* 오페론의 발현은 pyridine nucleotide나 전자전달계의 ubiquinone/ubiquinol pool의 redox 상태의 변화에 의해 유도된 것이 아니다. *R. sphaeroides* cytochrome bc_1 complex mutant를 이용하여 광합성기구 합성에 대한 cbb_3 cytochrome c oxidase의 억제 효과는 ubiquinone/ubiquinol pool의 redox 변화에 의해 간접적으로 일어나는 것이 아님을 증명하였다.