The Journal of Microbiology, June 2006, p.284-292
Vol. 44, No. 3
Copyright © 2006, The Microbiological Society of Korea

Effect of Mutations of Five Conserved Histidine Residues in the Catalytic Subunit of the *cbb*$_3$ Cytochrome *c* Oxidase on its Function

Jeong-Il Oh*

Department of Microbiology, Pusan National University, Busan 609-735, Republic of Korea

(Received January 26, 2006 / Accepted April 3, 2006)

The *cbb*$_3$ cytochrome *c* oxidase has the dual function as a terminal oxidase and oxygen sensor in the photosynthetic bacterium, *Rhodobacter sphaeroides*. The *cbb*$_3$ oxidase forms a signal transduction pathway together with the PrrBA two-component system that controls photosynthesis gene expression in response to changes in oxygen tension in the environment. Under aerobic conditions the *cbb*$_3$ oxidase generates an inhibitory signal, which shifts the equilibrium of PrrB kinase/phosphatase activities towards the phosphatase mode. Photosynthesis genes are thereby turned off under aerobic conditions. The catalytic subunit (CcoN) of the *R. sphaeroides cbb*$_3$ oxidase contains five histidine residues (H214, H233, H303, H320, and H444) that are conserved in all CcoN subunits of the *cbb*$_3$ oxidase, but not in the catalytic subunits of other members of copper-heme superfamily oxidases. H214A mutation of CcoN affected neither catalytic activity nor sensory (signaling) function of the *cbb*$_3$ oxidase, whereas H320A mutation led to almost complete loss of both catalytic activity and sensory function of the *cbb*$_3$ oxidase. H233V and H444A mutations brought about the partial loss of catalytic activity and sensory function of the *cbb*$_3$ oxidase. Interestingly, the H303A mutant form of the *cbb*$_3$ oxidase retains the catalytic function as a cytochrome *c* oxidase as compared to the wild-type oxidase, while it is defective in signaling function as an oxygen sensor. H303 appears to be implicated in either signal sensing or generation of the inhibitory signal to the PrrBA two-component system.

Keywords: *cbb*$_3$ cytochrome *c* oxidase, gene regulation, photosynthesis, redox sensing, two-component system

The purple nonsulfur photosynthetic bacterium, *Rhodobacter sphaeroides*, contains a branched respiratory electron transport chain that is terminated with at least two cytochrome *c* oxidases and at least one functional quinol oxidase (Oh and Kaplan, 2001). The *aa*$_3$- and *cbb*$_3$-type cytochrome *c* oxidases catalyze the four-electron reduction of dioxygen to water concomitantly with the oxidation of cytochrome *c* (Donohue *et al.*, 1988; Garcia-Horsman *et al.*, 1994; Myllykallio *et al.*, 1998). The cytochrome *c* oxidases also serve as the proton pumps that translocate protons across the cytoplasmic membrane towards the periplamic side (Mills *et al.*, 2000; Zaslavsky and Gennis, 2000). In *R. sphaeroides* the *aa*$_3$ cytochrome *c* oxidase is the major cytochrome *c* oxidase under highly aerobic conditions, while the *cbb*$_3$ oxidase is the predominant and perhaps exclusive cytochrome *c* oxidase under oxygen-limiting and anaerobic conditions (Oh and Kaplan, 1999, 2000). Both the enzymes are assigned to the superfamily of heme-copper oxidases on the basis of the presence of a low-spin heme and a binuclear center composed of a high-spin heme and Cu_B in their catalytic subunits where the reduction of dioxygen to water occurs (Garcia-Horsman *et al.*, 1994).

The *cbb*$_3$ cytochrome *c* oxidase encoded by the *ccoNOQP* (*fixNOQP*) operon consists of four different subunits (Toledo-Cuevas *et al.*, 1998; Zufferey *et al.*, 1998). The CcoN subunit is the catalytic subunit of the oxidase, which is homologous to subunit I of the *aa*$_3$ cytochrome *c* oxidase. Two b-type hemes (high- and low-spin hemes) are non-covalently linked to the CcoN apoprotein. The CcoO and CcoP subunits are membrane-bound monoheme- and diheme cytochromes *c*, respectively. Six canonical histidine residues of CcoN are involved in the coordination of the low-spin heme and the binuclear center (Garcia-Horsman *et al.*, 1994; Toledo-Cuevas *et al.*, 1998; Zufferey *et al.*,

* To whom correspondence should be addressed.
(Tel) 82-51-510-2593; (Fax) 82-51-514-1778
(E-mail) joh@pusan.ac.kr

1998). H118 and H407 provide two axial ligands to the low-spin heme, while H405 provides an axial ligand to the high-spin heme (using the numbering of *R. sphaeroides* CcoN). The Cu_B center is coordinated by H267, H317, and H318. Replacement of any histidine residues to alanine or valine by site-directed mutagenesis was shown to result in the loss of enzyme activity and a defect in enzyme assembly (Zufferey *et al.*, 1998; Oh and Kaplan, 2000). Using in-frame deletion of the individual genes, it was demonstrated that CcoN, CcoO, and CcoP are all required for the catalytic activity of the cbb_3 oxidase, indicating that three subunits comprise the catalytic core complex required for the reduction of dioxygen and the oxidation of a cytochrome *c* (Zufferey *et al.*, 1996; Oh and Kaplan, 2000). The CcoQ subunit is the smallest subunit of the cbb_3 oxidase, consisting of 67 amino acids in the case of the *R. sphaeroides* cbb_3 oxidase. In-frame deletion of the *ccoQ* gene was demonstrated to affect neither the catalytic properties nor the assembly of the enzyme complex in *R. sphaeroides* when examined in cells grown anaerobically (Oh and Kaplan, 1999, 2002). It has been recently revealed that the CcoQ subunit protects the core complex of the cbb_3 oxidase from oxidative destabilization in the presence of oxygen. In the presence of oxygen, the cbb_3 core complex lacking CcoQ lost the CcoP subunit, leading to the inactivation of the enzyme (Oh and Kaplan, 2002).

R. sphaeroides can synthesize the spectral complexes, which are composed of the reaction center and light harvesting complexes (LHCs) I and II, only under oxygen-limiting (<~3% O_2) or anaerobic conditions (Kiley and Kaplan, 1988). One of the major regulatory systems that are responsible for anaerobic induction of photosynthesis genes encoding the apoproteins of the spectral complexes and enzymes catalyzing photopigment (bacteriochlorophyll and carotenoid) biosynthesis, is the PrrBA two component system (Eraso and Kaplan, 1994; Oh *et al.*, 2000). The PrrBA two-component system is composed of the PrrB histidine kinase and the PrrA response regulator (Eraso and Kaplan, 1994, 1995, 1996). The cbb_3 oxidase has been suggested to play an additional role as a redox sensor that controls the equilibrium between the PrrB kinase/phosphatase activities in response to changes in O_2 availability (Zeilstra-Ryalls and Kaplan, 1996; O'Gara and Kaplan, 1997; O'Gara *et al.*, 1998; Oh and Kaplan, 2000). The cbb_3 oxidase generates a signal, which shifts the relative equilibrium

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	van Neil, 1944
CBB3Δ	2.4.1 derivative, deletion in *ccoNOQP*	Oh and Kaplan, 2002
	E. coli and plasmid	
E. coli DH5α	(Φ80d*lacZ*ΔM15)Δ*lacU169 recA1 endA1 hsdR17 supE44 thi1 gyrA96 relA1*	Jessee, 1986
E. coli S17-1	Pro^- Res^- Mob^+ *recA*; integrated plasmid RP4-Tc::Mu-Km::Tn7	Simon *et al.*, 1983
pUC19	Ap^r; *lacPOZ'*	Yanisch-Perron *et al.*, 1985
pRK415	Tc^r; Mob^+ *lacZα* IncP	Keen *et al.*, 1988
pUI2803	pRK415::4.7-kb *Bam*HI-*Eco*RI fragment containing *ccoNOQP*	O'Gara and Kaplan, 1997
pCCO2	pUC19::4.7-kb *Bam*HI-*Eco*RI fragment containing *ccoNOQP*	Oh and Kaplan, 2000
p19CCON	pUC19::2.6-kb *Bam*HI-*Sac*I fragment from pCCO2	This study
pUI1663	Sp/Sm^r Km^r; IncQ, *puf::lacZYA'*	Eraso and Kaplan, 1996
pUI655	Ap^r; pBS KS+::0.47-kb *StyI* fragment of pufBA	Lee J.K.
pUI2803H214A	pUI2803 in which the codon for H214 is displaced with GCC	This study
pUI2803H233V	pUI2803 in which the codon for H233 is displaced with GTG	This study
pUI2803H303A	pUI2803 in which the codon for H303 is displaced with GCC	This study
pUI2803H320A	pUI2803 in which the codon for H320 is displaced with GCC	This study
pUI2803H444A	pUI2803 in which the codon for H444 is displaced with GCC	This study

of PrrB activity from the kinase mode to phosphatase mode under aerobic conditions. Under oxygen-limiting or anaerobic conditions, the signal emanating from the *cbb*$_3$ oxidase is weakened and the equilibrium of the PrrB activity is thereby in the default state, i.e., the kinase-dominant mode, leading to the induction of photosynthesis (PS) genes (Oh *et al.*, 2001). Using the purified *cbb*$_3$ oxidase, PrrB, and PrrA, it was clearly demonstrated in vitro that the *cbb*$_3$ oxidase inhibits the PrrB activity phosphorylating the PrrA response regulator by increasing the PrrB phosphatase activity without alteration of the PrrB kinase activity (Oh *et al.*, 2004).

The catalytic subunits (CcoN or FixN) of the *cbb*$_3$ cytochrome *c* oxidases contain 12 histidine residues conserved in all *cbb*$_3$ oxidases except for the *Helicobacter pylori cbb*$_3$ oxidase (Toledo-Cuevas *et al.*, 1998; Zufferey *et al.*, 1998). Six histidine residues among them are involved in the coordination of the redox centers as described above. The histidine residue corresponding to H397 of *R. sphaeroides* CcoN was suggested to coordinate a Mg^{2+} or Mn^{2+} ion (Toledo-Cuevas *et al.*, 1998). The roles of the remaining five histidine residues (H214, H233, H303, H320, and H444 of *R. sphaeroides* CcoN) have yet to be elucidated.

In order to investigate the possible roles of the five conserved histidine residues, the histidine residues in CcoN were individually changed to either alanine or valine by site-directed mutagenesis, and catalytic activities and signaling function of the mutant forms of the *cbb*$_3$ oxidase were assessed. We here demonstrate that substitution of H303 to alanine led to the partial defect in signaling function of the *cbb*$_3$ oxidase without alteration of the its catalytic activity.

Materials and Methods

Strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *R. sphaeroides* and *Escherichia coli* strains were grown as described previously (Oh *et al.*, 2000).

DNA manipulations and conjugation techniques

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).

Site directed mutagenesis

To mutate H214, H233, H303, H320, and H444 of CcoN to alanine or valine 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, USA). Synthetic oligonucleotides 33-34 bases long containing an alanine (GCC) or valine (GTG) codon 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 DNA sequencing, a 2.6 kb *Bam*HI-*Sac*I fragment containing the mutated sequence was cloned into pUI2803 in which the 2.6 kb *Bam*HI-*Sac*I fragment was removed. The resulting plasmids, pUI2803H214A, pUI2803H233V, pUI2803H303A, pUI2803H320A, and pUI2803H444A, were introduced into *R. sphaeroides* CBB3Δ, a strain in which the *ccoNOQP* operon has been deleted by gene replacement.

RNA isolation and analysis

Total RNA was isolated from *R. sphaeroides* strains as described by Oelmuller *et al.* (1990). Northern hybridization experiments were performed using AlkPhos DIRECT System (American Pharmacia Biotech, USA) as instructed by the manufacturer. The signal levels were normalized by those of processed 23S rRNA (14S).

Quantitative analysis of spectral complexes

The B800-850 and B875 complexes levels were determined spectrophotometrically as described previously (Oh and Kaplan, 1999).

Enzyme assays, 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 spectrophotometrically with reduced horse heart cytochrome *c* (Oh and Kaplan, 1999). Protein concentration was determined by the bicinchoninic acid protein assay (Pierce, USA) using bovine serum albumin as the standard protein.

Immunoblotting analysis

Preparation of solubilized membrane proteins, SDS-PAGE, and Western blotting were performed as described previously (Oh and Kaplan, 2000), except that samples were denatured for 40 min at RT in SDS loading buffer prior to electrophoresis.

Results and Discussion

12 histidine residues are conserved in the catalytic subunits of most cbb$_3$ *cytochrome* c *oxidases*

As shown in Fig. 1, the catalytic subunits (CcoN or

```
CcoN-R.s.   -------------------------------MWDYVKLVALGVIALCAAIAANYAR--DLA  28
CcoN-P.d.   -------------------------------MLDTIKLIALGTIAVLAAIAANYARPDDLA
CcoN-A.t.   MLQPTQRRVCALPGAYLPFVGRLARWGKPTMNYTLETAIVALGAFLALLGAAFAHDSLFA
FixN-B.j.   ------------------MSQPSISKSMTIGESGLAVVFAATAFLCVIAAAKALDAPFAF

CcoN-R.s.   YMVNAVSVMLVAGGLFLWQVRRVG-DEVRPKPALQTEYMDGVIRYGVVATAFWGVVGFLV  87
CcoN-P.d.   YLVNALIIMLAAGIMFLRVLRQMGNEQPALEPHPETQYMDDVVRAGVIATAFWGVVGFLV
CcoN-A.t.   AHMWVLFFTLVVSTVLLLRRVSFAPVDPAARARRNSEYFDEVVKYGVIATVFWGVVGFLV
FixN-B.j.   HAALSAAASVAAVFCIVNRYFERPAALPPAEINGRPNYNMGPIKFSSFMAMFWGIAGFLV
                                                   O
CcoN-R.s.   AVIIAFQLAFPQLNFEWAHG-YLNFGRLRPLHTSAVIFAFGGNALIATSFYVVQRTSAAR  146
CcoN-P.d.   GVVIAFQLAFPALNLSDITMGYTNFGKLRPLHTSAVIFAFGGNGLIATSFYVVQRTSAAR
CcoN-A.t.   GVVVALQLAFPDLNIAPYFN----FGRMRPLHTSAVIFAFGGNALIATSFYVVQRTCRAR
FixN-B.j.   GLIIASQLAWPALNFDLPWIS---FGRLRPLHTSAVIFAFGGNVLIATSFYVVQKSCRVR

CcoN-R.s.   LWGGNLGWFVFWGYNLFIVLVAQSYLLGATQSKEYAEPEWYLDLWLTIVWVCYLAAFLGT  206
CcoN-P.d.   LWGGNAAWFVFWGYQLFIVLAATGYILGATQSKEYAEPEWYVDWWLTVVWVVYLAVFLGT
CcoN-A.t.   LFGGNLGWFVFWGYNLFIIMAATGYLLGITQGREYAEPEWYVDIWLTIVWVAYLATFLGT
FixN-B.j.   LAGDLAPWFVVVGYNFFILVAGTGYLLGVTQSKEYAEPEWYADLWLTIVWVVYLLVFLAT
                    214                 233
CcoN-R.s.   IIKRKEPHIYVANWFYLAFIVTVAMLHIFNNLSIPVSFFGSKSVQVFSGVQDAMVQWWYG  266
CcoN-P.d.   ILKRKEPHIYVANWFYLSFIVTIAMLHIVNNLAIPVSLFGSKSVQLFSGVQDAMTQWWYG
CcoN-A.t.   ILTRKEPHIYVANWFYLSFIVTIAMLHIVNNLAVPVSFLGVKSYSAFSGVQDALTQWWYG
FixN-B.j.   IIKRKEPHIFVANWFYLAFIVTIAVLHLGNNPALPVSAFGSKSYVAWGGIQDAMFQWWYG
            O                                  303            OO320
CcoN-R.s.   HNAVGFFLTAGFLGMMYYFVPKQAERPVYSYKLSIVHFWALIFLYIWAGPHHLHYTALPT  326
CcoN-P.d.   HNAVGFFLTAGFLGMMYYFIPKQAERPVYSYKLSIIHFWALIFLYIWAGPHHLHYTALPD
CcoN-A.t.   HNAVGFFLTAGFLGMMYYFIPKQVNRPVYSYRLSIIHFWALIFMYIWAGPHHLHYTALPD
FixN-B.j.   HNAVGFFLTAGFLAIMYYFIPKRAERPIYSYRLSIIHFWALIFLYIWAGPHHLHYTALPD

CcoN-R.s.   WTSTLGMVFSIMLWMPSWGGMINGLMTLSGAWDKLRTDPIIRMMVVSIGFYGMSTFEGPM  386
CcoN-P.d.   WASTLGMVFSIILWMPSWGGMINGLMTLSGAWDKLRTDPIIRMMVVAVGFYGMATFEGPM
CcoN-A.t.   WAQTLGMVFSIMLWMPSWGGMINGLMTLSGAWDKIRTDPIVRMMVMAVAFYGMATFEGPM
FixN-B.j.   WTQTLGMTFSIMLWMPSWGGMINGLMTLSGAWDKLRTDPVLRMLVVSVAFYGMSTFEGPM
                      O       O O                                444
CcoN-R.s.   MSIKAVNSLSHYTDWTIGHVHSGALGWNGMITFGALYFLTPKLWNKERLYSLSLVSWHFW  446
CcoN-P.d.   MSIKAVNFVSHYTDWTIGHVHSGALGWNGMITFGALYYLVPRLWGRERLYSTGLVSWHFW
CcoN-A.t.   MSIKAVNSLSHYTDWTIGHVHSGALGWNGMITFGAIYYLTPKLWGRDRLYSLQLVNWHFW
FixN-B.j.   MSIKVVNSLSHYTDWTIGHVHSGALGWVGFVSFGALYCLVPWAWNRKGLYSLKLVNWHFW

CcoN-R.s.   LATIGIVLYASSMWVSGIMEGLMWREVDANGFLVNAFADTVAAKFPMNVVRGLGGVLYLT  506
CcoN-P.d.   LATIGLVLYAASMWVSGIMEGLMWREVDAQGFLVNAFADTVAAKFPMNVVRALGGVLYLG
CcoN-A.t.   LATLGIVVYAAVMWVAGIQQALMWREYDSQGFLVYSFAESVAALFPYYVMRALGGLMFLS
FixN-B.j.   VATLGIVLYISAMWVSGILQGLMWRAYTSLGFLEYSFIETVEAMHPFYIIRAAGGGLFLI

CcoN-R.s.   GALIMCYNLWKTVTSAPSRVVRAAAVPAE-----  535
CcoN-P.d.   GALIMCYNLWATVAKQPKTQSTAAAVPAE-----
CcoN-A.t.   GALIMAYNVTMTILGHQREEGASKGAAPSLQPAE
FixN-B.j.   GALIMAYNLWMTVRVGEAEVQMPVALQPAE----
```

Fig. 1. Amino acid sequence alignments of CcoN and CcoN homologues. The canonical histidine residues involved in coordination of the redox centers and Mg^{2+} (Mn^{2+}) are shaded in gray and indicated by the symbols "O" above the shadings. The remaining five conserved histidine residues, which were subjected to site-directed mutagenesis in this study, are shaded in gray and indicated by the amino acid numbers (using numbering of *R. sphaeroides* CcoN). Abbreviations: R.s., *R. sphaeroides*; P.d., *Paracoccus denitrificans*; A.t., *Agrobacterium tumerfaciens*; B.j., *Bradyrhizobium japonicum*.

FixN) of the *cbb*3 cytochrome *c* oxidases contain 12 conserved histidine residues, of which 6 histidines are predicted to serve as the cofactor ligands based on sequence similarities with the *Paracoccus denitrificans* *aa*3 cytochrome *c* oxidase whose structure is known (Iwata *et al.*, 1995). H118 and H407 provide two axial ligands for the low-spin heme within the *R. sphaeroides* CcoN, while H405 coordinates the high-spin heme. H267, H317, and H318 bind the Cu_B center in CcoN. The replacement each of H267 and H405 to alanine led to assembly defect and loss of the *cbb*3 oxidase activity in *R. sphaeroides* (Oh and

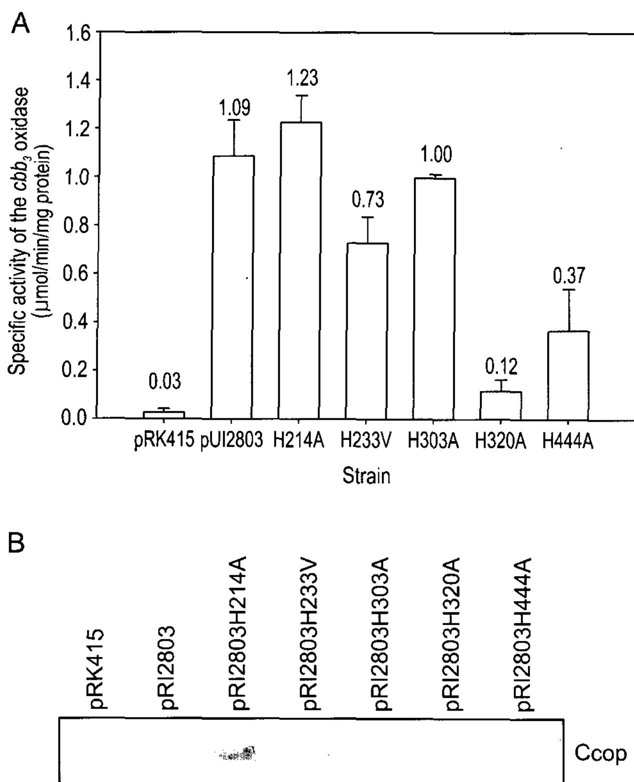

Fig. 2. Activity and immunological analyses of the cbb_3 cytochrome *c* oxidase from a set of histidine replacement mutants of CcoN. (A) cbb_3 oxidase activity was measured spectrophotometrically by monitoring the oxidation of prereduced horse heart cytochrome *c* using cells grown anaerobically with DMSO as a terminal electron acceptor in the dark. All values provided are the average of two independent determinations. (B) Western blot analysis using a polyclonal antibody against the CcoP protein. Membrane fractions were isolated from the strains haboring the corresponding plasmid (in parentheses) grown anaerobically with DMSO. Solubilized membrane proteins (50 µg) were loaded in each lane.

Kaplan, 2000). The mutation of H407 to alanine resulted in a partial loss of the oxidase activity (Oh and Kaplan, 2000). It was demonstrated in *B. japonicum* that mutations of aforememtioned 6 histidines to alanine or valine brought about the inactivation of the cbb_3 oxidase, indicating that the histidine residues involved in coordination of the redox cofactors within CcoN (FixN) are essential for the oxidase activity (Zufferey *et al.*, 1998). H397 of the remaining histidine residues within the *R. sphaeroides* CcoN is assumed to be implicated in binding of Mg^{2+} or Mn^{2+} (Lauraeus *et al.*, 1991; Puustinen *et al.*, 1991). The corresponding histidine residue of the *B. japonicum* FixN (H410) was shown to be required for assembly and activity of the cbb_3 oxidase (Zufferey *et al.*, 1998).

The role of the remaining five histidine residues (H214, H233, H303, H320, and H444) and the reason why they were conserved during evolution of almost all of the cbb_3 oxidases remain to be elucidated.

Relevant phenotypes of a set of histidine replacement mutants of catalytic subunit of* R. sphaeroides cbb₃ *oxidase

In order to test the importance of the five histidine residues (H214, H233, H303, H320, and H444) in oxidase activity and assembly in the membrane, the individual histidine residue was substituted by a small and neutral amino acid such as alanine and valine by site-directed mutagenesis. The plasmid pUI2803 contains the *ccoNOQP* operon cloned into a broad host-range vector pRK415 as well as its own promoter region of the *ccoNOQP* operon. The plasmids pUI2803H214A, pUI2803H233V, pUI2803H303A, pUI2803H320A, and pUI2803H444A have the same construction as pUI2803 except that each histidine in the plasmid name is mutated to either alanine or valine. The pUI2803 and pUI2803 variants were introduced into a *ccoNOQP*-deletion strain of *R. sphaeroides* (CBB3Δ) by conjugation and the functionality of the cbb_3 oxidase was assessed by complementation tests.

We first examined the effect of the histidine mutations on cbb_3 oxidase activity. Cytochrome *c* oxidase activity was determined in the mutant strains as well as the control strains grown under anaerobic, dark-DMSO conditions where the cbb_3 oxidase is the sole cytochrome *c* oxidase (Oh and Kaplan, 1999). As shown in Fig. 2A the negative control strain CBB3Δ (pRK415) showed virtually no cbb_3 oxidase activity. On the other hand, the positive control strain CBB3Δ (pUI2803) as well as mutant strains CBB3Δ (pUI2803 H303A) and CBB3Δ (pUI2803H214A) displayed similar levels of the cbb_3 oxidase activity. The cbb_3 oxidase activities detected in the strains CBB3Δ (pUI2803H233V) and CBB3Δ (pUI2803H444A) were reduced by approximately 30% and 65%, respectively, as compared with that in the positive control strain CBB3Δ (pUI2803). The H320A mutant showed 10% of cbb_3 oxidase activity observed for the positive control strain. Taken together, the results presented in Fig. 2A suggest that H214A and H303A mutations of CcoN did not affect the catalytic activity of the cbb_3 oxidase, while H320A mutation led to the almost complete loss of the cbb_3 oxidase activity. H233V and H444A mutations led to the partial loss of the oxidase activity with more negative effect of H444A mutation on the oxidase activity than H233V mutation.

To determine whether the assembly of the cbb_3 oxidase in the membrane was affected by the histidine replacements, we performed Western blot analyses with a polyclonal antibody against CcoP of *R.*

Table 2. Relevant phenotypes of a set of histidine replacement mutants of catalytic subunit of *R. sphaeroides* cbb_3 oxidase

Strain	Level of spectral complexes (nmole/mg protein)	
	B800-850	B875
CBB3Δ (pRK415)	0.33 ± 0.02	3.22 ± 0.20
CBB3Δ (pUI2803)	< 0.1	< 0.1
CBB3Δ (pUI2803H214A)	< 0.1	< 0.1
CBB3Δ (pUI2803H233V)	0.13 ± 0.01	1.04 ± 0.03
CBB3Δ (pUI2803H303A)	0.20 ± 0.01	1.69 ± 0.08
CBB3Δ (pUI2803H320A)	0.24 ± 0.01	1.95 ± 0.09
CBB3Δ (pUI2803H444A)	0.19 ± 0.00	1.40 ± 0.08

For determination of spectral complex levels, strains carrying the corresponding plasmids were grown aerobically by sparging with 30% O_2, 69% N_2, 1% CO_2 to an A_{600} of 0.45 to 0.5.

capsulatus. It was previously shown that the deletion of the *ccoN* gene in *R. sphaeroides* led to a defect in the assembly of both CcoP and CcoO in the membrane (Oh and Kaplan, 2000). Fig. 2B shows that the H233V, H303A, and H444A mutant strains have similar levels of CcoP in the membrane as the positive control strain CBB3Δ (pUI2803). The amount of CcoP in the H320A mutant strain was decreased as compared with that in the positive control strain, whereas the CcoP level in the H214A strain was rather slightly increased relative to that of the positive control strain. Taken together, the data in Fig. 2 suggest that H320A mutation of CcoN leads to either some assembly defect or instability in the cbb_3 oxidase in the membrane, which results in almost complete loss of the oxidase activity and that although the mutant forms (H233V and H444A) of the cbb_3 oxidase are normally assembled in the membrane and retain their stability, their catalytic activities are significantly reduced by mutations when compared with the wild-type oxidase. H214A mutation of CcoN appears to increase the stability of the cbb_3 oxidase.

It was previously reported that the mutation of H316 to valine in *B. japonicum* FixN did not affect the assembly of the oxidase in the membrane, but led to the complete loss of the oxidase activity (Zufferey *et al.*, 1998). By contrast, the corresponding mutation (H303A) in *R. sphaeroides* affected neither the assembly nor the activity of the oxidase. The H333V mutant form of the *B. japonicum* cbb_3 oxidase was demonstrated to retain the activity and stability of the oxidase (Zufferey *et al.*, 1998), whereas the corresponding mutant form (H320A) of the *R. sphaeroides* cbb_3 oxidase had defects in assembly and activity of the oxidase. The differences observed between *B. japonicum* and *R. sphaeroides* might result from the fact that the histidine residues were mutated to valine in *B. japonicum*, but to alanine in *R. sphaeroides*.

The H303A mutant form of the cbb$_3$ *oxidase has a defect in signaling function without alteration of its catalytic activity*

The cbb_3 cytochrome *c* oxidase constitutes a signal transduction pathway together with the PrrBA two-component system, which controls PS gene expression in response to changes in oxygen tension in the environment (Oh and Kaplan, 2001). In this pathway the cbb_3 oxidase serves as an oxygen sensor. It was suggested that under aerobic conditions the cbb_3 oxidase generates a signal that turns off PS gene expression via the PrrBA two-component system (Oh *et al.*, 2004). To assess the functionality of the mutant forms of the cbb_3 oxidase as an oxygen sensor, we examined the effect of the histidine replacements on spectral complex formation in the mutants grown under 30% O_2 conditions where the wild-type strain of *R. sphaeroides* cannot synthesize the spectral complexes (Table 2). The positive control strain CBB3Δ (pUI2803) produced virtually no light harvesting complexes (LHCs) under 30% oxygen conditions whereas the CBB3Δ mutant containing the empty vector pRK415 synthesized the LHCs under the same conditions, indicating that the deletion of the *ccoNOQP* operon in the CBB3Δ mutant is fully complemented by pUI2803.

The mutant strain CBB3Δ (pUI2803H214A), which retained similar levels of the cbb_3 oxidase activity as the positive control strain, did not synthesize the LHCs under 30% O_2 conditions like the positive control strain. The mutant strains CBB3Δ (pUI2803 H233V), CBB3Δ (pUI2803H320A), and CBB3Δ

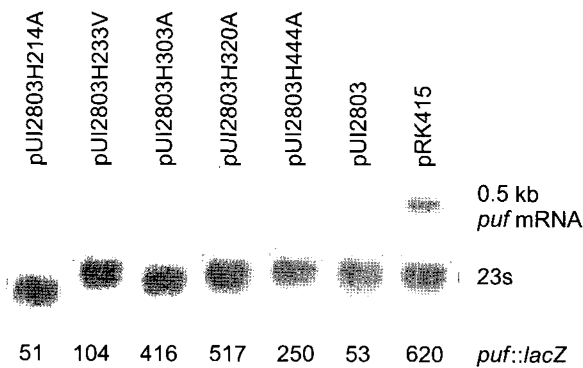

Fig. 3. Expression of the *puf* operon in a set of histidine replacement mutants of CcoN. The expression levels of *puf* were measured by Northern blot analysis as well as by promoter activity assay using a *puf::lacZ* transcriptional fusion. *R. sphaeroides* CBB3Δ strains harboring the corresponding plasmids were grown under 30% O_2 conditions as described in Table 2. Total RNA was isolated from *R. sphaeroides* strains and approximately 20 μg of total RNA was loaded in each lane. The Northern blot was probed with a labeled 0.47 kb *Sty*I fragment from pUI655 which is specific for *puf* mRNA. The same levels of processed 23S rRNA (14S) in each lane indicate that the same amounts of total RNA were used in Northern blot analysis. The levels of *puf* expression were quantitatively determined in *R. sphaeroides* strains harboring the transcriptional fusion plasmid pUI1663 by measuring the β-galactosidase activity. The activity is expressed as nmole/min mg protein.

(pUI2803H444A) produced intermediate levels of LHCs relative to the positive and negative control strains. The levels of LHCs in the mutants are inversely proportional to the activities of the cbb_3 oxidase detected in the mutant strains. Interestingly, significant amounts of LHCs were synthesized in the mutant strain CBB3Δ (pUI2803H303A), although this mutant strain showed nearly the same levels of the cbb_3 activity as the positive control strain. When grown under anaerobic, dark-DMSO conditions, all the mutant strains synthesized similar levels of the LHCs as the positive control strain CBB3Δ (pUI2803) (data not shown). This observation implies that the H303A mutant form of the cbb_3 oxidase retains the catalytic function as a cytochrome *c* oxidase, while it is defective in signaling function as an oxygen sensor. H303 appears to be implicated in either signal sensing or generation of the inhibitory signal to the PrrBA two-component system.

The *puf* operon encodes the apoproteins of the LHC I (B875) and reaction center and its expression is under the control of PrrBA two-component system (Kiley and Kaplan, 1988; Eraso and Kaplan, 1994). The expression of the *puf* operon was investigated at the transcriptional level by means of Northern hybridization analyses using total RNA isolated from the mutant strains and control strains grown under 30% O_2 conditions. We also confirmed the Northern blotting results by measuring the promoter activity of the *puf* operon using the *puf::lacZ* transcriptional fusion plasmid pUI1663. As shown in Fig. 3, *puf* transcripts were hardly detectable in the positive control strain CBB3Δ (pUI2803) and the mutant strain CBB3Δ (pUI2803H214A). By contrast, the levels of the *puf* transcripts were increased in the negative control strain CBB3Δ (pRK415) and the other mutant strains. The β-galactosidase activities detected in the control and mutant strains were in good agreement with the Northern blot results. The expression levels of *puf* in all the strains but the mutant strain CBB3Δ (pUI2803H303A) were inversely proportional to the cbb_3 oxidase activities detected in the strains, indicating the catalytic function of the cbb_3 oxidase is coupled with the sensory and signaling function of the oxidase. The *puf* operon was derepressed in the mutant strain CBB3Δ (pUI2803H303A) grown under 30% O_2 conditions, although the similar level of the cbb_3 oxidase activity was measured for this mutant strain as compared with the positive control strain in which the *puf* operon was not expressed under 30% O_2 conditions. This result implies that the H303A mutation of CcoN uncouples the sensory (signaling) function of the oxidase from the catalytic function and that H303 appears to be important in sensory and signaling function of the cbb_3 oxidase.

It was recently suggested that the autokinase activity of the RegB (PrrA homologue in *R. capsulatus*) histidine kinase is controlled by the ubiquinone/ubiquinol pool of the electron transport chain (Swem *et al.*, 2006). Under aerobic conditions the ubiquinone/ubiquinol pool is more oxidized relative that under anaerobic conditions. Oxidized ubiquinone was demonstrated to decrease the autophosphorylation rate of RegB. If this result were true, PS genes would be more derepressed in the aa_3 oxidase mutant grown aerobically than in the cbb_3 oxidase mutant grown under the same conditions since the aa_3 oxidase is the major cytochrome *c* oxidase in *R. sphaeroides* grown under aerobic conditions. However, the aa_3 oxidase mutant grown under aerobic conditions did not synthesize spectral complexes in contrast to the cbb_3 oxidase mutant (Oh and Kaplan, 2000). The H303A mutant of *R. sphaeroides* retains the same cbb_3 activity as the wild type, suggesting that the redox state of the ubiquinone/ubiquinol pool could not be changed in this mutant. Aerobic derepression of PS genes in this mutant strongly suggests that the PrrB activity is controlled by the cbb_3 oxidase rather than by the redox state of the ubiquinone/ubiquinol pool

This is the first report regarding identification of an amino acid residue within the cbb_3 oxidase which is

involved in O_2 sensing and signaling. We are now in the process of purification of the H303A mutant form of the *cbb*$_3$ oxidase and the comparative study on this mutant form and wild-type of the *cbb*$_3$ oxidase with regard to the catalytic activity, cofactor composition, and in vitro kinase/phosphatase assay with the purified PrrB and PrrA.

Acknowledgment

This work was supported by Korea Research Foundation Grant (KRF-2004-015-C00488) to Jeong-Il Oh.

References

Davis, J., T.J. Donohue, and S. Kaplan. 1988. Construction, characterization, and complementation of a Puf- mutant of *Rhodobacter sphaeroides*. *J. Bacteriol.*. 170, 320-329.

Donohue, T.J., A.G. McEwan, S. Van Doren, A.R. Crofts, and S. Kaplan. 1988. Phenotypic and genetic characterization of *cytochrome* c_2 deficient mutants of *Rhodobacter sphaeroides*. *Biochemistry*. 27, 1918-1925.

Eraso, J.M., and S. Kaplan. 1994. *prrA*, a putative response regulator involved in oxygen regulation of photosynthesis gene expression in *Rhodobacter sphaeroides*. *J. Bacteriol.* 176, 32-43.

Eraso, J.M., and S. Kaplan. 1995. Oxygen-insensitive synthesis of the photosynthetic membranes of *Rhodobacter sphaeroides*: a mutant histidine kinase. *J. Bacteriol.* 177, 2695-2706.

Eraso, J.M., and S. Kaplan. 1996. Complex regulatory activities associated with the histidine kinase PrrB in expression of photosynthesis genes in *Rhodobacter sphaeroides* 2.4.1. *J. Bacteriol.* 178, 7037-7046.

Garcia-Horsman, J.A., B. Barquera, J. Rumbley, J. Ma, and R.B. Gennis. 1994. The superfamily of heme-copper respiratory oxidases. *J. Bacteriol.* 176, 5587-5600.

Iwata, S., C. Ostermeier, B. Ludwig, and H. Michel. 1995. Structure at 2.8 Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans*. *Nature* 376, 660-669.

Jessee, J. 1986. New subcloning efficiency competent cells: >1 × 10^6 transformants/μg. *Focus* 8, 9.

Keen, N.T., S. Tamaki, D. Kobayashi, and D. Trollinger. 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene* 70, 191-197.

Kiley, P.J., and S. Kaplan. 1988. Molecular genetics of photosynthetic membrane biosynthesis in *Rhodobacter sphaeroides*. *Microbiol. Rev.* 52, 50-69.

Lauraeus, M., T. Haltia, M. Saraste, and M. Wikstrom. 1991. *Bacillus subtilis* expresses two kinds of haem-A-containing terminal oxidases. *Eur. J. Biochem.* 197, 699-705.

Mills, D.A., L. Florens, C. Hiser, J. Qian, and S. Ferguson-Miller. 2000. Where is 'outside' in cytochrome *c* oxidase and how and when do protons get there? *Biochim. Biophys. Acta.* 1458, 180-187.

Myllykallio, H., F. Drepper, P. Mathis, and F. Daldal. 1998. Membrane-anchored cytochrome c_y mediated microsecond time range electron transfer from the cytochrome bc_1 complex to the reaction center in *Rhodobacter capsulatus*. *Biochemistry*. 37, 5501-5510.

O'Gara, J.P., J.M. Eraso, and S. Kaplan. 1998. A redox-responsive pathway for aerobic regulation of photosynthesis gene expression in *Rhodobacter sphaeroides* 2.4.1. *J. Bacteriol.* 180, 4044-4050.

O'Gara, J.P., and S. Kaplan. 1997. Evidence for the role of redox carriers in photosynthesis gene expression and carotenoid biosynthesis in *Rhodobacter sphaeroides* 2.4.1. *J. Bacteriol.* 179, 1951-1961.

Oelmuller, U., N. Kruger, A. Steinbuchel, and C.G. Friedrich. 1990. Isolation of procaryotic RNA and detection of specific mRNA with biotinylated probes. *J. Microbiol. Methods.* 11, 73-84.

Oh, J.I., J.M. Eraso, and S. Kaplan. 2000. Interacting regulatory circuits involved in orderly control of photosynthesis gene expression in *Rhodobacter sphaeroides* 2.4.1. *J. Bacteriol.* 182, 3081-3087.

Oh, J.I., and S. Kaplan. 1999. The cbb$_3$ terminal oxidase of *Rhodobacter sphaeroides* 2.4.1: structural and functional implications for the regulation of spectral complex formation. *Biochemistry* 38, 2688-2696.

Oh, J.I., and S. Kaplan. 2000. Redox signaling: globalization of gene expression. *EMBO J.* 19, 4237-4247.

Oh, J.I., and S. Kaplan. 2001. Generalized approach to the regulation and integration of gene expression. *Mol. Microbiol.* 39, 1116-1123.

Oh, J.I., and S. Kaplan. 2002. Oxygen adaptation: The role of the CcoQ subunit of the cbb$_3$ cytochrome *c* oxidase of *Rhodobacter sphaeroides* 2.4.1. *J. Biol. Chem.* 277, 16220- 16228.

Oh, J.I., I.J. Ko, and S. Kaplan. 2001. The default state of the membrane-localized histidine kinase PrrB of *Rhodobacter sphaeroides* 2.4.1 is in the kinase-positive mode. *J. Bacteriol.* 183, 6807-6814.

Oh, J.I., I.J. Ko, and S. Kaplan. 2004. Reconstitution of the *Rhodobacter sphaeroides* cbb$_3$-PrrBA signal transduction pathway *in vitro*. *Biochemistry* 43, 7915-7923.

Puustinen, A., M. Finel, T. Haltia, R.B. Gennis, and M. Wikstrom. 1991. Properties of the two terminal oxidases of *Escherichia coli*. *Biochemistry*. 30, 3936-3942.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Simon, R., U. Priefer, and A. Puhler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio/ Technol.* 1, 784-791.

Swem, L.R., X. Gong, C.A. Yu, and C.E. Bauer. 2006. Identification of a ubiquinone binding site that affects autophosphorylation of the sensor kinase RegB. *J. Biol. Chem.* In press

Toledo-Cuevas, M., B. Barquera, R.B. Gennis, M. Wikstrom, and J.A. Garcia-Horsman. 1998. The cbb$_3$-type cytochrome *c* oxidase from *Rhodobacter sphaeroides*, a proton-pumping heme-copper oxidase. *Biochim. Biophys. Acta* 1365, 421-434.

van Neil, C.B. 1944. The culture, general physiology, morphology, and classification of the non-sulfur purple and brown bacteria. *Bacterial Rev.* 8, 1-118.

Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved

M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33, 103-119.

Zaslavsky, D., and R.B. Gennis. 2000. Proton pumping by cytochrome oxidase: progress, problems and postulates. *Biochim. Biophys. Acta.* 1458, 164-179.

Zeilstra-Ryalls, J.H., and S. Kaplan. 1996. Control of *hemA* expression in *Rhodobacter sphaeroides* 2.4.1: regulation through alterations in the cellular redox state. *J. Bacteriol.* 178, 985-993.

Zufferey, R., E. Arslan, L. Thony-Meyer, and H. Hennecke. 1998. How replacements of the 12 conserved histidines of subunit I affect assembly, cofactor binding, and enzymatic activity of the *Bradyrhizobium japonicum* cbb_3-type oxidase. *J. Biol. Chem.* 273, 6452-6459.

Zufferey, R., O. Preisig, H. Hennecke, and L. Thony-Meyer. 1996. Assembly and function of the cytochrome cbb_3 oxidase subunits in *Bradyrhizobium japonicum*. *J. Biol. Chem.* 271, 9114-9119.