Oxygen adaptation: the role of the CcoQ subunit of the \textit{cbb}_3 cytochrome \textit{c} oxidase of \textit{Rhodobacter sphaeroides} 2.4.1

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Running title: The functional role of subunit IV of the \textit{cbb}_3 oxidase
Summary

The cbb$_3$ cytochrome c oxidase of Rhodobacter sphaeroides consists of four nonidentical subunits. Three subunits (CcoN, CcoO, and CcoP) comprise the catalytic “core” complex required for the reduction of O$_2$ and the oxidation of a c-type cytochrome. On the other hand, the functional role of subunit IV (CcoQ) of the cbb$_3$ oxidase was not obvious, although we previously suggested that it is involved in the signal transduction pathway controlling photosynthesis gene expression (J.–I. Oh and S. Kaplan, Biochemistry 38. 2688-2696, 1999). Here we go on to demonstrate that subunit IV protects the core complex, in the presence of O$_2$, from proteolytic degradation by a serine metalloprotease. In the absence of CcoQ, we suggest that the presence of O$_2$ leads to the loss of heme from the core complex, which destabilizes the cbb$_3$ oxidase into a “degradable” form, perhaps by altering its conformation. Under aerobic conditions the absence of CcoQ appears to most severely affect the CcoP subunit. It was further demonstrated, using a series of C-terminal deletion derivatives of CcoQ, that the minimum length of CcoQ required for stabilization of the core complex under aerobic conditions is the amino-terminal ~48 to 50 amino acids.
Introduction

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 (Qxt) (1). The *aa*$_3$- and *cbb*$_3$-type cytochrome *c* oxidases both belonging to the heme-copper oxidase superfamily catalyze the reduction of molecular oxygen to water using electrons derived from the oxidation of cytochromes *c*$_2$ and *c*$_y$ (2-4). In *R. sphaeroides* the *aa*$_3$ oxidase is the major cytochrome *c* oxidase under highly aerobic conditions, whereas the *cbb*$_3$ oxidase is the predominant and perhaps exclusive cytochrome *c* oxidase under oxygen-limiting and anaerobic conditions, respectively (5).

The bacterial *aa*$_3$ cytochrome *c* oxidase has been demonstrated both genetically and from x-ray crystallographic analyses to consist of four subunits (6,7). Subunits I and II form the functional unit containing all of the redox centers. These consist of a low spin heme and a binuclear center composed of a high spin heme and Cu$_B$ in subunit I, and Cu$_A$ in subunit II as well as amino acid residues required for enzyme activity and proton translocation. Subunit III appears not to be essential for catalytic function of the enzyme since a functional two-subunit enzyme consisting of subunits I and II has been demonstrated (8). It was suggested that the removal of subunit III leads to “suicide inactivation” of the enzyme during turnover (9). The function of the smallest subunit IV is unknown. Deletion of the gene encoding subunit IV (CtaH in *Paracoccus denitrificans*) was shown to have no effect on either the integrity of the enzyme complex nor its spectral and enzymatic properties (7).
The $cbb_3$ cytochrome $c$ oxidase encoded by the $ccoNOQP$ ($fixNOQP$) operon is also composed of four subunits (10,11). The $ccoN$ ($fixN$) gene encodes the catalytic subunit of the oxidase which is homologous to subunit I of the $aa_3$ oxidase. The $ccoO$ ($fixO$) and $ccoP$ ($fixP$) genes encode membrane-bound mono- and diheme cytochromes $c$. On the basis of determined redox potentials, it was suggested that electrons are transferred from cytochrome $c$ to CcoN via CcoP and CcoO in that order (12). CcoN, CcoO, and CcoP subunits are all required for catalytic activity of the $cbb_3$ oxidase (13,14). Elimination of any redox prosthetic center by site-directed mutagenesis was shown to lead to the loss of enzyme activity and a defect in enzyme assembly (14). The CcoQ (FixQ) is the smallest subunit of the $cbb_3$ oxidase, consisting of 48 – 73 amino acids depending upon its source. Its primary structure shows a basal level of homology to subunit IV (CtaH) of the $aa_3$ oxidase in the membrane-spanning helix region. 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 (5). This is also true for $Bradyrhizobium japonicum$ (13).

We have shown the $cbb_3$ oxidase to play an additional role as an redox sensor in a signal transduction pathway. Electron flow through the $cbb_3$ oxidase is postulated to generate a signal under aerobic conditions which is inhibitory to and mediated by the PrrBA two-component activation system and which leads to the repression of photosynthesis (PS) gene expression (14,15). Although a $ccoQ$ in-frame deletion mutant of $R. sphaeroides$ grown under anaerobic conditions retains a catalytically intact $cbb_3$ oxidase, under highly aerobic conditions it produces spectral complexes accompanied by the aerobic derepression of photosynthesis (PS) genes as observed for Cco null mutants (5). Based
upon these observations, we initially suggested that CcoQ was involved in the process of actually transducing the inhibitory signal from the \textit{cbb}_3 oxidase to the PrrBA two-component system (5). However, further study of the role of CcoQ reveals a more subtle mode of action, leading us to suggest that the CcoQ subunit stabilizes the \textit{cbb}_3 oxidase complex under aerobic conditions and in its absence, the core complex is destabilized, thereby removing the inhibitory signal. We report these findings here.
Experimental Procedures

Strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table I. *R. sphaeroides* and *Escherichia coli* strains were grown as described previously (5).

DNA manipulations and conjugation techniques

Standard protocols or manufacturer’s instructions were followed for recombinant DNA manipulations. Mobilization of plasmids from *E. coli* strains into *R. sphaeroides* strains was carried out as described elsewhere (16).

Construction of the CBB3Δ mutant and plasmids

CBB3Δ mutant: In order to construct the CBB3Δ mutant, a 1400-bp *Stu*I fragment containing a 3’-portion of *ccoN*, all of *ccoO* and *ccoQ*, and a 5’-portion of *ccoP* was deleted by the restriction of pCCO1 with *Stu*I and subsequent recircularization, yielding the plasmid pCBB3Δ1. A 3.2-kb *Eco*RV-*Sac*I fragment from pCBB3Δ1 was cloned into the suicide vector pLO1 digested with *Pme*I and *Sac*I. The resulting plasmid pCBB3Δ2 was transferred from *E. coli* strain S17-1 to *R. sphaeroides* 2.4.1 by conjugation. Heterogenotes of *R. sphaeroides*, generated by a single recombination event, were selected for kanamycin resistance on Sistrom’s medium A (SIS) agar plates incubated under aerobic conditions. Isogenic homogenotes were obtained from the heterogenotes after a second recombination selecting for sucrose resistance on SIS agar plates containing 15% (w/v) sucrose, 1% (w/v) Bactotryptsin, 0.5% (w/v) yeast extract, 0.5%
(w/v) NaCl, and 0.5% (v/v) DMSO under dark anaerobic conditions. The allelic exchange was verified by PCR.

pUI2803NHIS and pUI2803NHIS-CCOQ: A 0.8-kb fragment containing the 3′ portion of *ccoN* with a tail of six histidine codons immediately upstream of its stop codon was generated by a two-round recombination PCR using *pfu* Turbo polymerase (Stratagene, La Jolla, CA). With pCCO2 as the template, two primary PCR reactions were performed with the primers HIS+ (5′-GTTCCCGCCACCACCATCACCATCACTGAGTGAAGATAAGGGGACA-3′) and OUT+ (5′-CGAATAGCGCTCGCCGACGCGGGC-3′), and HIS- (5′-TTCACTCAGTGATGGTGATGGTGGTGCGGCGGGAACGGGCCGCGGC-3′) and OUT- (CTACGGCATGTCGACCTTCGAGGG-3′) to generate two DNA fragments containing a 34-bp overlapping region. The two primary PCR products were used as templates for the secondary PCR which was performed using the primers OUT+ and OUT-. A 0.8-kb PCR product was restricted with *Apa*I and cloned into pCCO2 digested by *Apa*I, replacing its 0.8-kb wild-type *Apa*I fragment. The resulting plasmid pCCO2NHIS with the correct orientation of the 0.8-kb *Apa*I fragment was digested with *Bam*HI and *Eco*RI and a 4.7-kb *Bam*HI-*Eco*RI fragment was ultimately cloned into pRK415 restricted with the same enzymes, giving the plasmid pUI2803NHIS.

For the construction of pUI2803NHIS-CCOQ, a 0.83-kb *Sac*I-*Stu*I fragment from pCCOQΔ1 was cloned into the vector portion of p19CCOQP, in which the 0.85-kb wild-type *Sac*I-*Stu*I fragment was removed, to give the plasmid pCCOOP. A 2-kb *Eco*RI-*Sac*I fragment was cloned into the vector portion of pUI2803NHIS restricted with the same enzymes, yielding the plasmid pUI2803NHIS-CCOQ.
Site-directed mutagenesis

In order to obtain a set of truncated forms of CcoQ, a series of nonsense mutations was introduced into the plasmid-borne ccoQ gene using the Quick Change Site-Directed Mutagenesis kit (Stratagene) with the template plasmid pOYQ. Synthetic deoxyoligonucleotides 34 bases long containing the TGA stop codon in place of S60, D56, T51, or R48 codons in the middle of their sequences were employed to mutagenize the corresponding codons. Following verification of the mutations by DNA sequencing, a 490-bp SalI-StuI fragment containing the mutated ccoQ gene was cloned into pBBR1MCS2 restricted with EcoRV and SalI. The resulting plasmids pBBRQTGA1-pBBRQTGA4 (see Table I) were introduced into R. sphaeroides CCOQΔ in which 171 bp of the ccoQ gene was deleted in frame by gene replacement.

Purification of the His\textsubscript{6}-tagged cbb\textsubscript{3} cytochrome c oxidase

The R. sphaeroides CBB3Δ strain carrying either pUI2803NHIS or pUI2803NHIS-CCOQ was grown semiaerobically or under anaerobic dark-DMSO conditions to the late exponential phase in SIS medium supplemented with 2 \(\mu\)g of tetracycline per ml. For semiaerobic growth, where the expression of the ccoNOQP operon is highest, 1-liter flasks were filled with 700 ml of SIS medium and shaken at 150 rpm on a rotary shaker. Two-liter cultures were harvested, resuspended in 50 ml of buffer A (20 mM Tris-HCl, pH8.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and disrupted by two passages through a French pressure cell at 120 Mpa. Following DNase treatment (150 unit DNase [Promega, Madison, WI] in the presence of 10 mM MgCl\textsubscript{2} for 30 min at room
temperature [RT]), cell-free crude extracts were obtained by centrifugation at 20,000 x g for 15 min at 4°C two times. Membrane fractions were isolated by ultracentrifugation of crude extracts at 150,000 x g for 1 h at 4°C. After the membrane fraction (pellet) was washed twice with buffer A, the membranes were solubilized in 15 ml of buffer A containing 1% (w/v) n-dodecyl β-D-maltoside (DM), 1 mM PMSF, and 100 mM NaCl, and then centrifuged at 150,000 x g for 1 h at 4°C. The supernatant was taken as solubilized membrane proteins and used for affinity chromatography. In order to reduce the concentration of DM, 12 ml of buffer A containing 100 mM NaCl was added to the solubilized membrane protein and then imidazole was added to a final concentration of 5 mM. 2 ml of the 50% (v/v) Ni-NTA HIS-bind slurry (Novagen, Madison, WI) was added to 30 ml of solubilized membrane protein and mixed gently by shaking at 4°C for 2 h. The protein-resin mixture was loaded into a column and the column was washed with 10 volumes of buffer B (buffer A with 100 mM NaCl and 0.01% (w/v) DM) containing 5 mM imidazole followed by 6 volumes of buffer B containing 60 mM imidazole. The red-colored cbb₃ oxidase was finally eluted with 250 mM imidazole in buffer B. The fractions containing the cbb₃ oxidase were dialysed overnight against 2 liter of bufferA containing 0.01% (w/v) DM to remove imidazol and NaCl. The desalted cbb₃ oxidase was concentrated by means of ultrafiltration (membrane YM10, Millipore Co., Bedford, MA).
Spectroscopic and immunoblotting analyses

Both heme $b$ and heme $c$ contents were determined by using the pyridine hemochrome difference spectra and the cognate calculation matrix as described by Berry and Trumpower (17).

The reduced plus CO minus reduced difference spectra and the oxidized minus oxidized plus CN$^-$-spectra were obtained as described previously (7,18). The CO- and CN$^-$-reactivity of the $cbb_3$ oxidase reflecting the content of the high-spin heme $b$ within the purified enzyme was estimated for the following wavelength pairs: 415.5 and 436 nm (CO binding); 404 and 420 nm (CN$^-$ binding) (19). All spectra were recorded using the purified 3-subunit and 4-subunit $cbb_3$ oxidase equilibrated in buffer A containing 0.01% (w/v) DM. The B800-850 and B875 spectral complex levels were determined spectrophotometrically as described elsewhere (5). Preparation of solubilized membrane proteins, SDS-PAGE, and Western blotting were performed as described previously (14), except that samples were denatured for 40 min at RT in SDS loading buffer prior to electrophoresis.

Enzyme assays, protein determination, and heme staining

Preparation of crude cell extracts and determination of β-galactosidase activities were performed as described previously (5). Cytochrome $c$ oxidase activities were measured spectrophotometrically with reduced horse heart cytochrome $c$ (5). Protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL) using bovine serum albumin as the standard protein.
After SDS-PAGE the \( c \)-type cytochromes were visualized via their intrinsic peroxidase activity, using \( 3,3',5,5' \)-tetramethyl benzidine (TMBZ) and \( \text{H}_2\text{O}_2 \) (20).
Results

_CcoQ and its effect on the activity and integrity of the core oxidase_

In good agreement with our previous report (5), the levels of the CcoO and CcoP subunits detected in the CCOQΔ in-frame deletion mutant were similar to those found in the wild-type strain of _R. sphaeroides_ 2.4.1 when both were grown anaerobically in the dark with DMSO as an external electron acceptor (Fig. 1A). Surprisingly, when grown under either aerobic (30% O₂) or semiaerobic (2% O₂) conditions, the CCOQΔ mutant showed significant decreases in both the CcoO and especially the CcoP polypeptides in comparison with the wild type (Fig. 1A). In the case of CcoP, the polypeptide was undetectable by means of immunoblotting. When assayed using reduced cytochrome _c_, the cytochrome _c_ oxidase activity of the CCOQΔ mutant was only marginally lower than that detected in the wild type 2.4.1 when both strains were grown under anaerobic dark-DMSO conditions (Fig. 1B). The CCONΔ mutant when used as a negative control grown under the same conditions showed no cytochrome _c_ oxidase activity, which is consistent with previous results revealing that the _cbb₃_ oxidase appears to be the exclusive cytochrome _c_ oxidase activity present in _R. sphaeroides_ grown under anaerobic conditions (5). In contrast, the CCOQΔ mutant grown under semiaerobic conditions possessed only 37% of the cytochrome _c_ oxidase activity found in the wild type grown under the same conditions. The negative control strain CCONΔ contained only marginal cytochrome _c_ oxidase activity which is most likely attributable to some residual _aa₃_ cytochrome _c_ oxidase. When normalized to the cytochrome _c_ oxidase activity present in the CCONΔ mutant, the _cbb₃_ oxidase activity in the CCOQΔ mutant amounted to ~30% of that detected in the wild type, when both strains were grown under semiaerobic...
conditions and reduced cytochrome c was employed in the assay of cytochrome c oxidase.

When the cco operon with an in-frame deletion in ccoQ was overexpressed in the CBB3Δ mutant under 2% O2 conditions using the plasmid pUI2803NHIS-CCOQ (see Table I for strain and plasmid definition), the negative effect of the ccoQ deletion on the accumulation of the CcoP and CcoO polypeptides in membrane fractions was partially overcome (Fig. 1C). This was anticipated based upon the known increase in cbb3 oxidase when the intact ccoNOQP operon is expressed in trans (14). However, the levels of CcoO and CcoP in membranes were still less than those observed in the control strain CBB3Δ with pUI2803NHIS carrying the entire ccoNOQP operon. In this experiment we were able to probe the level of CcoN in the membrane by means of immunoblotting using anti-His4 antibody. The removal of CcoQ affected to a lesser extent the accumulation of CcoN in the membrane as compared with the accumulation of CcoO and CcoP. Taken together, the results presented here demonstrate that removal of the CcoQ subunit affects both the activity and integrity of the cbb3 oxidase under aerobic growth conditions, but not under anaerobic conditions. The magnitude of the effect, due to the removal of CcoQ, on the cbb3 subunits under aerobic conditions appears to be in the order of CcoP > CcoO > CcoN as judged by the immunoblotting results (Fig 1A and C).

**Characterization of the purified 3-subunit cbb3 oxidase lacking CcoQ**

The intact 4-subunit cbb3 oxidase (holoenzyme) and 3-subunit cbb3 oxidase (core enzyme) lacking CcoQ were purified from semiaerobically grown CBB3Δ carrying pUI2803NHIS and pUI2803NHIS-CCOQ, respectively, as described in Experimental
Procedures. As shown in Fig. 2A, two bands of apparent molecular masses of 45 and 31 kDa, which correspond to the CcoN and CcoO subunits of the \( cbb_3 \) oxidase, respectively, were clearly visible in the stained SDS-PAGE gel. The CcoP band was more diffuse and less well stained than the other bands. SDS-PAGE using a Tris-tricine gel revealed a protein band with a molecular mass of \(~11\) kDa in the purified holoenzyme which was missing from the purified core enzyme, suggesting that it is the CcoQ polypeptide with a theoretical molecular mass of 7778 Da (Fig. 2A). Due to the difference in purity and heme content of the different oxidase preparations, the amounts of the core- and holoenzymes used for comparative biochemical analyses were adjusted so that the band intensity of the catalytic CcoN subunit of each preparation was visually the same following Coomassie-blue staining of the SDS-PAGE gel. This is unavoidable due to the instability of the core enzyme. When approximately the same amount of CcoN was applied to an SDS-PAGE gel, the band intensity of CcoO from the core enzyme was similar to that of the holoenzyme (Fig. 2A). However, immunoblotting showed that the purified core enzyme contained a lower amount of CcoP than did the holoenzyme.

In order to compare the heme content of each subunit of each form of the oxidase, heme-staining analysis was performed. Protein samples were denatured under mild heating conditions prior to subjecting them to SDS-PAGE to visualize the CcoN subunit which contains noncovalently bound \( b \)-type hemes. Heme staining showed that the CcoP and CcoN subunits of the core enzyme contained significantly decreased amounts of heme compared to the intact holoenzyme. A substantial decrease in the heme content of the CcoP subunit is undoubtedly due, in part, to the reduced level of the CcoP apoprotein in the core enzyme. The heme content of CcoP was reduced to a greater extent than the
CcoP apoprotein as judged by comparison of the heme-stained gel and immunoblot. The heme content of CcoO in the core enzyme was also decreased when compared to the holoenzyme, but appeared to be less susceptible to loss than observed for the heme of CcoN and CcoP (Fig. 2A). In addition to the three heme-containing subunits of the \( cbb_3 \) oxidase, two bands of molecular masses 26 and 24 kDa were also observed after heme staining. The 24-kDa band is cytochrome \( c_y \) since heme staining showed this band to be missing in membranes derived from a \( cycY \) null mutant (data not shown). The 26-kDa band is either a degradation product of CcoP or a CcoP conformer since this band could not be detected by heme staining of membrane fractions from the CBB3\( \Delta \) mutant (data not shown) and it also cross-reacted with CcoP-specific antibody (Fig. 2A).

The core enzyme exhibited \(~37\%\) of the cytochrome \( c \) oxidase activity of the holoenzyme (Fig. 2B). In 20 mM potassium phosphate buffer (pH 7.5) the core- and holoenzymes showed virtually the same \( Km \) value for reduced horse heart cytochrome \( c \) (3.4 and 3.3 \( \mu \)M, respectively), indicating that both oxidase preparations have the same binding affinity for reduced cytochrome \( c \) regardless of the presence or absence of CcoQ. This observation rules out the possibility that reduced cytochrome \( c \) can donate electrons to both CcoO and CcoP. Both the heme \( b \) and heme \( c \) contents of the core- and holoenzymes were determined by pyridine hemochrome analysis. The heme \( b \) content in the core enzyme was 71\% of that observed for the holoenzyme. Similarly, the core enzyme contained 87\% of the heme \( c \) detected in the holoenzyme. The heme \( c \) content of the core enzyme appears to be overestimated since the heme \( c \) content determined by spectroscopic analysis represents not only the heme \( c \) present in the CcoO and CcoP subunits, but also that of the copurified cytochrome \( c_y \). Due to a lower purification yield
of the core oxidase complex resulting from a lower cellular level of the core enzyme over
the holoenzyme, contamination of the core oxidase with cytochrome $c_y$ was always
greater than that of the holoenzyme complex (Fig. 2A). The difference in the heme $b$
content of the purified core- and holooxidases estimated by heme-staining analysis
appears greater than that determined by spectroscopic analysis. Because heme $b$ is
noncovalently bound to the CcoN subunit, there is a possibility that CcoN of the core
enzyme may lose heme more readily during SDS-PAGE than does the holoenzyme. We
should also point out that heme staining is at best semiquantitative, especially in the case
of noncovalently bound heme. The content of the functional high-spin heme $b_3$ was
estimated by spectroscopic changes concomitant with CO binding to the reduced
oxidases and CN$^-$ binding to the oxidized enzymes. Reduced heme $b_3$ of the core enzyme
bound 78% of the amount of CO observed for the holoenzyme. Similarly, the oxidized
core enzyme showed 76% of the CN$^-$ binding as compared with the holoenzyme. Both
ligand-binding experiments indicated that the CcoN subunit of the core enzyme retained
76 – 78% of the functional high-spin heme $b$ when compared to the holoenzyme. The fact
that the total heme $b$ content of the core enzyme is 71% that in the holoenzyme, also
suggests that both high- and low-spin hemes in CcoN of the core enzyme are
compromised.

As shown in Fig. 2C, when the core enzyme was purified from the CBB3$\Delta$ with
pUI2803NHIS-CCOQ grown anaerobically with DMSO, it contained significantly more
heme $b$ and heme $c$ than the same core enzyme purified from semiaerobically grown
cells, again indicating that a likely role for CcoQ is to protect the heme moieties of the
$cbb_3$ oxidase under aerobic conditions. Even in anaerobically grown cells the heme

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content of the core enzyme was still lower than that in the holoenzyme. Since the purification of the oxidase was carried out in the presence of air, there is a likely possibility that some heme moieties were lost during purification. This possibility is addressed below.

**Removal of CcoQ and the stability of the cbb\textsubscript{3} oxidase under aerobic conditions**

The induction of the *ccoNOQP* operon encoding the *cbb\textsubscript{3}* oxidase under semiaerobic and anaerobic conditions is mediated by the global anaerobic activator FnrL (21). The fact that the CCOQ\textsubscript{Δ} mutant showed similar levels of *cbb\textsubscript{3}* oxidase activity as the wild type under anaerobic dark-DMSO conditions, makes it highly unlikely that a decrease in *cbb\textsubscript{3}* oxidase activity in the CCOQ\textsubscript{Δ} mutant grown under semiaerobic conditions resulted from a decrease in the transcriptional activity of the *ccoNOQP* operon in the mutant. The decline in *cbb\textsubscript{3}* activity in the mutant appears to come from either the instability of- or an assembly defect in the *cbb\textsubscript{3}* oxidase in the presence of O\textsubscript{2}. To examine the former possibility, cell-free crude extracts of the wild-type and CCOQ\textsubscript{Δ} mutant strains grown under anaerobic dark-DMSO conditions were prepared, and the stability of the *cbb\textsubscript{3}* oxidase in crude extracts exposed to air was determined by measuring *cbb\textsubscript{3}* oxidase activity over time. As shown in Fig. 3, *cbb\textsubscript{3}* oxidase activity in cell-free crude extracts of the wild type remained unaltered after a 3-day incubation. In contrast, a relatively steep decline in *cbb\textsubscript{3}* activity was observed for the CCOQ\textsubscript{Δ} mutant and after 3 days the activity was reduced to 39% of the initial activity and was apparently still declining. This result strongly suggested that the presence of O\textsubscript{2} directly leads to instability of the *cbb\textsubscript{3}* oxidase in the absence of the CcoQ subunit.
To further investigate a possible role for CcoQ in protecting the *cbb*<sub>3</sub> oxidase from “oxidative” damage, the following analyses were performed. The core enzyme was purified from cells grown anaerobically with DMSO and as a control the intact holoenzyme was isolated from semiaerobically grown cells. As shown in Fig. 4A, the holoenzyme remained relatively stable as judged by both heme- and Coomassie-blue staining of CcoN, CcoP, and CcoO after a 3-day incubation. The CcoN subunit of the core enzyme appeared relatively stable. In contrast, the heme content of CcoP and CcoO and the amount of the CcoO polypeptide of the core enzyme were decreased in a time-dependent manner to a significantly greater extent than observed for the holoenzyme. In particular, the heme content of CcoP was most severely affected. These results suggest that the loss of heme from the purified core enzyme is most likely associated with degradation of the CcoP and CcoO apoproteins, which is consistent with *in vivo* results (see Fig. 1A and C).

To ascertain whether exposure to air (most probably O<sub>2</sub>) led to the instability of CcoP and CcoO of the core enzyme, we performed the same experiment in the absence of air. In order to remove air, the 1.5-ml incubation tube was stoppered with a rubber septum, and gently flushed with argon gas for 2 min. Sodium ascorbate (pH8.0) was also used to a final concentration of 10 mM to remove O<sub>2</sub> dissolved in the purified oxidase preparations since ascorbate serves as an electron donor for the *cbb*<sub>3</sub> oxidase during its turnover. After a 3 day incubation in the presence of argon and ascorbate, heme staining of the core enzyme revealed a strong CcoO band as well as a strong 26-kDa band. The 26-kDa band appeared to be derived from CcoP because this band cross-reacted with the CcoP antibody (see Fig. 2A) and it also appeared concomitantly with the disappearance of the
CcoP band. The intact holoenzyme showed the same band pattern as the core enzyme when incubated in the presence of argon and ascorbate for 3 days. Although we do not know what caused the change in the migration behavior of CcoP on SDS-PAGE, the reducing conditions resulting from both the removal of air and the presence of ascorbate stabilized both CcoP and CcoO of the core enzyme. Neither argon-flushing nor the addition of ascorbate alone was sufficient to stabilize the core enzyme as judged from the intensity and location of the heme-stainable bands on the gel. As shown in Fig. 4B, a mixture of protease inhibitors protected the core enzyme even in the presence of air, indicating that proteolysis is responsible for the instability of the core enzyme under oxidizing conditions. In order to more accurately assess which type of protease was involved in degradation of the core enzyme under oxidizing conditions, we employed several individual protease inhibitors for the stability test (Fig. 4B). After a 4-day incubation heme staining showed that Bestatin and Pefabolic SC, which are inhibitors of metallo- and serine proteases, respectively, stabilized the core enzyme. In contrast, neither Pepstatin (aspartate protease inhibitor) nor antipain-dihydrochloride (cysteine protease inhibitor) showed any protection of the core enzyme under oxidizing conditions. The difference in stability between the holo- and core oxidases could be the result of a greater contamination by protease(s) of the purified core oxidase preparation compared to the purified holooxidase preparation. If this was the case, mixing the two preparations might be expected to affect the stability of the holooxidase. As shown in Fig. 4C, a mixture of the holo- and core oxidases remained as stable as the holooxidase alone, after a 4-day incubation. The core enzyme complex in the mixture appeared to be protected by the presence of the holoenzyme. In contrast, CcoP of the core oxidase was undetectable
and the CcoO subunit was nearly absent after the same period of incubation of the core oxidase. This result suggests that it is not simply the difference in the amount of protease contamination in each of the purified preparations which results in the different stability of the holo- and core oxidases under oxidizing conditions. The protection effect of the holooxidase on the core oxidase leads us to speculate that the holo- and core oxidases might associate to form a supramolecular complex and the presence of the CcoQ subunit even in a stoichiometry of less than 1 relative to the core complex, affords protection of the core complex. Taken together, these results clearly indicate that the CcoQ subunit protects the core complex of the \( cbb_3 \) oxidase from degradation under oxidizing conditions and that a serine metalloprotease is most likely involved in the proteolytic degradation of the \( cbb_3 \) core complex in the absence of CcoQ. We can exclude the possibility that the core enzyme is destabilized in the process of catalytic turnover since the purified oxidase contains no electron donor.

**Delineation of the functional portion of CcoQ**

When the amino acid sequence of *R. sphaeroides* CcoQ was multiply aligned with those CcoQ homologues from various organisms, only the central portions of the CcoQ homologues were found to be well conserved (Fig. 5A). This central region also shows some homology to the smallest subunit (CtaH) of the \( aa_3 \) cytochrome \( c \) oxidase of *P. denitrificans* (7). According to the resolved crystal structure of the *P. denitrificans aa_3* oxidase, this region comprises the membrane-spanning region of CtaH and the C-terminus of CtaH faces the periplasm, implying that CcoQ might have a similar topology in the cytoplasmic membrane as CtaH (6).
Among the CcoQ homologues, the CcoQ polypeptide of *R. sphaeroides* deduced from its nucleotide sequence is longer than the others (Fig. 5A). In order to define the C-terminal boundary of the functional portion of CcoQ as judged by *cbb*$_3$ stabilization, a series of C-terminal deletion derivatives of CcoQ were constructed by replacing the codons for S60, D56, T51, or R48 of CcoQ by the TGA stop codon. The *ccoQ* gene carrying each nonsense mutation was introduced into the CCOQ$\Delta$ mutant using the vector pBBR1MCS2 and its functionality was assessed by complementation.

Cytochrome *c* oxidase activity was determined in the mutant strains as well as the control strains grown under 2% O$_2$ conditions where the *cbb*$_3$ oxidase is the predominant cytochrome *c* oxidase and where the removal of CcoQ severely affects *cbb*$_3$ oxidase activity. As shown in Fig. 5B, the negative control strain CCOQ$\Delta$ (pBBR1MCS2) showed approximately half the cytochrome *c* oxidase activity present in the control strain CCOQ$\Delta$ (pBBRQ). The deletion of 8 and 12 amino acids from the C-terminus of CcoQ did not affect *cbb*$_3$ oxidase activity in CCOQ$\Delta$ (pBBRQ-TGA1) and CCOQ$\Delta$ (pBBRQ-TGA2), respectively. In contrast, the *cbb*$_3$ oxidase activity in the CCOQ$\Delta$ (pBBRQ-TGA4) strain showed oxidase levels similar to those of the negative control strain CCOQ$\Delta$ (pBBR1MCS2), indicating that the removal of 20 amino acids from the C-terminus of CcoQ makes CcoQ nonfunctional. The CCOQ$\Delta$ (pBBRQ-TGA3) strain, in which 17 amino acids were removed from the C-terminus of CcoQ, retained 81% of the cytochrome *c* oxidase activity detected in the positive control strain CCOQ$\Delta$ (pBBRQ) and is therefore considered to be partially functional.

We previously demonstrated that the extent of electron flow through the *cbb*$_3$ oxidase is inversely related to the level of spectral complexes as well as to the expression of PS
genes which are under control of the PrrBA two-component system (14). The positive control strain CCOQΔ (pBBRQ) as well as the mutant strains CCOQΔ (pBBRQ-TGA1) and CCOQΔ (pBBRQ-TGA2) containing the fully active \( cbb_3 \) oxidase, produced only basal levels of the light harvesting complexes under 30% \( O_2 \) conditions like the wild type 2.4.1 (pBBR1MCS2). In contrast, substantial levels of the light harvesting complexes were synthesized in CCOQΔ (pBBRQ-TGA4) and the negative control strain CCOQΔ (pBBR1MCS2). Only marginal increases in spectral complex levels were observed in CCOQΔ (pBBRQ-TGA3).

As anticipated, the \( puf \) operon which is regulated by the PrrBA two-component system, was derepressed 4.3-fold in the negative control strain CCOQΔ (pBBR1MCS2) grown under 30% \( O_2 \) conditions when compared with the CCOQΔ (pBBRQ) strain grown under the same conditions. Similarly, the CCOQΔ (pBBRQ-TGA4) strain showed a 3.8-fold increase in \( puf \) expression. The CCOQΔ (pBBRQ-TGA3) exhibited only marginal derepression in \( puf \) expression under the same growth conditions compared to the control strain CCOQΔ (pBBRQ). The introduction of the \( B. \) japonicum \( fixQ \) gene into the CCOQΔ mutant led to complementation of the CcoQ-minus phenotype as judged by \( puf \) operon expression under aerobic conditions (data not shown).

Taken together, the results clearly suggest that the minimum length of CcoQ required for protection of the \( cbb_3 \) terminal oxidase is 48 – 50 amino acids, which is consistent with the fact that the CcoQ homologues of \( P. \) denitrificans and \( S. \) meliloti consist of 48 and 50 amino acid residues, respectively. This observation also suggests that the CcoQ subunit may play a similar role in these strains. These results also confirmed that the “functional
state” of the \( cbb_3 \) oxidase is inversely proportional to the extent of aerobic derepression of those PS genes which are regulated by the PrrBA two-component system.
Discussion

Bacterial quinol and cytochrome c oxidases belonging to the heme-copper superfamily are generally composed of four different subunits. The smallest subunit IV differs in size and membrane topology depending on the type of oxidase. The $aa_3$ cytochrome c oxidase from *P. denitrificans* contains subunit IV (CtaH) with a single membrane-spanning helix (6). This is also the case for the $cbb_3$ cytochrome c oxidase (11). To the contrary, subunit IV of the well-studied $bo_3$ quinol oxidase of *E. coli* has three membrane-spanning helices and is larger than those corresponding to the $aa_3$- and $cbb_3$ cytochrome c oxidases (22,23). According to the three-dimensional crystal structures of the *P. denitrificans* $aa_3$ cytochrome c oxidase (6) and the *E. coli* $bo_3$ quinol oxidase (24), subunit IV is positioned in the cleft between subunits I and III. However, the crystal structure of the mitochondrial $aa_3$ cytochrome c oxidase consisting of 13 different subunits reveals no obvious counterpart to the bacterial subunit IV (25).

The removal of subunit IV from the $bo_3$ quinol oxidase leads to inactivation of the enzyme and defects in the heme-copper binuclear center of subunit I (26). Likewise, a deletion of the *Bacillus subtilis* *qoxD* encoding subunit IV of the $aa_3$ menaquinol oxidase was shown to significantly reduce respiration and proton pumping (27). In contrast, deletion of *ctaH* in *P. denitrificans* was reported to have no effect on the spectral and enzymatic properties of the $aa_3$ cytochrome c oxidase (7).

In this report, we first demonstrated that subunit IV (CcoQ) of the $cbb_3$ cytochrome c oxidase plays a role in protecting the $cbb_3$ oxidase under aerobic conditions. The rationale for this argument is: i) Subunit abundance and $cbb_3$ oxidase activity in the CCOQΔ mutant were significantly reduced under aerobic growth conditions (2% and 30% O$_2$), but
not under anaerobic dark-DMSO or photosynthetic conditions (5) when compared with measurements of the wild-type strain grown under the same conditions. ii) The activity of the $cbb_3$ oxidase in cell-free crude extracts of the CCOQΔ mutant was unstable when exposed to air, whereas the $cbb_3$ activity in cell-free crude extracts of the wild type was stable under the same experimental conditions. iii) When the purified core- and holoenzymes were compared regarding their stability in the presence of air, the absence of CcoQ was directly related to the stability of both the CcoP and CcoO subunits as well as the heme content of these subunits. Reducing conditions resulting from the removal of air and the addition of ascorbate, stabilized the hemes of the core enzyme.

The absence of CcoQ appears to most severely affect the CcoP subunit of the $cbb_3$ oxidase under oxidizing conditions as judged by the following observations: i) The most significant reduction in the heme content was observed for CcoP of the purified core enzyme (see Fig. 2A and 2C). The stoichiometry of CcoP to CcoN and CcoO in the purified core enzyme is altered compared to that of the holoenzyme (see Fig. 2A). ii) The level of CcoP in the membranes of the CCOQΔ mutant grown under aerobic conditions was most severely affected (see Fig. 1A). iii) In vitro stability tests showed that the loss of heme from CcoP of the purified core enzyme occurred to the greatest extent compared to the other subunits (see Fig. 4A).

In addition, our data suggest that a serine metalloprotease activity is related to the instability of the $cbb_3$ core complex lacking the CcoQ subunit. The proteolytic degradation of the core enzyme occurred only in the presence of $O_2$, indicating that the presence of $O_2$ triggers the degradation process of the core enzyme lacking the CcoQ subunit. At this point we are faced with the question of why the core enzyme is degraded.
only under oxidizing conditions. A plausible answer can be drawn from the observation that although the purified core oxidase consists of the same three subunits having the same molecular mass as those of the holoenzyme, it shows decreased levels of heme \textit{b} as well as heme \textit{c} when compared to the same amount of the holoenzyme (see Fig. 2A and C). This finding leads us to hypothesize that in the presence of O$_2$, the loss of the heme moieties in the core oxidase occurred prior to degradation of the apoproteins. That is, the loss of heme would be expected to convert the $cbb_3$ oxidase into a conformationally altered, degradable protein which presumably becomes susceptible to proteolysis.

The complementation experiments using a series of C-terminal deletion derivatives of CcoQ demonstrated that the minimum length of CcoQ required for stabilizing the $cbb_3$ oxidase under aerobic conditions is ~48 to 50 amino acids. All CcoQ homologues, which have been so far identified, consist of 48 to 73 amino acids, implying that the minimum size of the functional CcoQ homologues may correspond to the 48 amino-acid CcoQ homologues when these are multiply-aligned (see Fig 5A). As shown in Fig. 5A, there are a number of conserved amino acid residues in the first 48 amino acids. It is possible that an analysis of these may shed light on the mechanism of CcoQ protection of the core complex, perhaps through an alteration of its binding to the core enzyme.

The $cbb_3$ cytochrome $c$ oxidase of \textit{R. sphaeroides} has multiple functions, i.e., as a terminal oxidase in the presence of O$_2$ (19), as an O$_2$/redox sensor (1,14) and finally as a conduit for reducing power under anaerobic conditions (5). Under aerobic conditions the $cbb_3$ oxidase generates the signal which is transmitted to the PrrBA two-component system most likely via PrrC, to repress PS gene expression (1,15). We have previously demonstrated that the $cbb_3$ oxidase does not sense O$_2$ itself, but it is the extent of electron
flow through the oxidase which serves to generate the inhibitory signal (14). The greater the volume of electron flow through the \textit{cbb}_3 oxidase, the stronger the inhibitory signal generated by the oxidase to shift the equilibrium of PrrB activity toward the phosphatase mode and away from kinase mode, resulting in the absence of activation of PS genes. Therefore, under aerobic conditions where the substrate for the \textit{cbb}_3 oxidase (O\textsubscript{2}) is sufficient, PS genes are not induced. The complementation experiment using the truncated forms of CcoQ reconfirms that the functional state (activity) of the \textit{cbb}_3 oxidase is inversely related to the extent of aerobic derepression of those PS genes which are under the control of the PrrBA two-component system.

Although the CCOQ\textsubscript{\Delta} mutant has near normal \textit{cbb}_3 oxidase activity under anaerobic photosynthetic and dark-DMSO conditions, it produces significant levels of the spectral complexes under highly aerobic conditions like the various Cco null mutant strains (5). This phenotype originally suggested to us that the CcoQ subunit was part of the signaling pathway from the \textit{cbb}_3 oxidase to the PrrBA two-component system. However, noting the stabilizing effect of CcoQ on the \textit{cbb}_3 oxidase in the presence of O\textsubscript{2} as described here, provides an alternative explanation. The \textit{cbb}_3 oxidase, as we have shown, is severely affected in both its cellular levels and activity in the CCOQ\textsubscript{\Delta} mutant grown under aerobic conditions. This destabilization of the altered \textit{cbb}_3 oxidase attenuates the inhibitory signal to the PrrBA two-component system, resulting in the aerobic formation of the spectral complexes.

The \textit{cbb}_3 cytochrome \textit{c} oxidase is considered to be a primitive form of terminal oxidase. It is suggested that the \textit{cbb}_3 oxidase evolved from the anaerobic enzyme, NO-reductase on the basis of the similarity in the catalytic subunit and heme composition (3). The high
affinity of the \textit{cbb}$_3$ oxidase for O$_2$ leads us to hypothesize that this form of oxidase evolved during the transition period from an anoxic to an oxygenic atmosphere to enable the more efficient aerobic respiration. But such an “anaerobic” enzyme system might have been sensitive to oxygen even at low levels. Therefore, subunit IV might have been “recruited” to protect the enzyme complex from oxidative damage. If the \textit{aa}$_3$ cytochrome \textit{c} oxidase evolved from a \textit{cbb}$_3$-type oxidase, subunit IV of the bacterial \textit{aa}$_3$ oxidase may be an “evolutionary remnant”.

**Acknowledgements**

We thank Dr. Hans-Martin Fischer for providing the plasmid pRJ4504 carrying the \textit{fixQ} gene of \textit{B. japonicum}. This work was supported by grant GM15590 to S.K.

**References**


Footnotes

The abbreviations used in the text are: DM, n-dodecyl β-D-maltoside; DMSO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; PS, photosynthesis; RT, room temperature; SIS, Sistrom’s medium A; TMBZ, 3,3’,5,5’-tetramethyl benzidine.
**Figure legends**

**Fig. 1.** Effect of *ccoQ* disruption on the integrity and activity of the *cbb*3 oxidase under various growth conditions. 

(A) Western blot analysis using a polyclonal antibody against CcoO and CcoP. 50 µg of solubilized membrane protein isolated from *R. sphaeroides* strains grown under aerobic (30% O2), semiaerobic (2% O2), or anaerobic dark-DMSO conditions (DMSO) was loaded onto each lane. 

(B) Cytochrome c oxidase activity was determined using cell-free crude extracts of *R. sphaeroides* strains grown semiaerobically (2% O2) or anaerobically in the dark with DMSO (Dark DMSO). The specific activity is expressed as micromoles per minute per milligram protein. 

(C) The CBB3∆ strains containing either pUI2803NHIS (lane 1) or pUI2803NHIS-CCOQ (lane 2) were grown under semiaerobic conditions and 50 µg of each solubilized membrane protein was employed for Western blotting. The CcoN polypeptide was detected using anti-His4 monoclonal antibody (Quiagen) and the CcoO and CcoP proteins using the polyclonal antibody of CcoO and CcoP. Cells were grown aerobically by sparging with 30% O2-69% N2-1% CO2 to an OD600 of 0.9-1.0 or semiaerobically by sparging 2% O2-97% N2-1% CO2 to an OD600 of 0.3-0.4.

**Fig. 2.** Properties of the purified core- and holooxidases. 

(A) 12 µg of the holoenzyme (lane 1) and 17 µg of the core enzyme (lane 2) purified from semiaerobically grown cells were used for SDS-PAGE giving the same band intensity for the CcoN polypeptide. Following SDS-PAGE the gel was vertically cut into three slices containing the same samples. One gel slice was stained with Coomassie brilliant blue, a second heme-stained with TMBZ and H2O2, and the third used for Western blotting using a polyclonal
antibody against CcoP. To resolve the small CcoQ polypeptide by SDS-PAGE, Tris-tricine SDS-PAGE was performed (Tris-tricine). Quantitation of band intensity was performed using the program NIH Imager 1.62. The background-corrected values are placed below the photos. The value of each subunit of the holoenzyme is set at 100 and the relative values are expressed for the subunits of the core enzyme. (B) Cytochrome c oxidase activity and $K_m$ for reduced horse heart cytochrome c were measured spectrophotometrically. Both heme $b$ and heme $c$ contents were determined by using the pyridine hemochrome difference spectra. CO- and CN$^{-}$-reactivities (the ability to bind CO and CN$^{-}$) of the holo- and core enzymes were obtained employing the reduced plus CO minus reduced difference spectra and the oxidized minus oxidized plus CN$^{-}$ difference spectra, respectively. All spectra were recorded in buffer A containing 0.01% (w/v) DM at RT using 100 $\mu$g/ml of the holoenzyme and 141 $\mu$g/ml of the core enzyme which contained the same amount of CcoN. Except for the $K_m$ values, the values of the holoenzyme are set at 100 and the relative values are expressed for the core enzyme. Cbb3: purified holoenzyme, Cbb3-CCOQ: purified core enzyme. (C) Heme staining of the core oxidase purified from cells grown under anaerobic dark-DMSO conditions (lane 3): holo- (lane 1) and core (lane 2) enzymes purified from semiaerobically grown cells were subjected to heme staining as the controls. Protein concentration of each enzyme preparation was adjusted to give the same band intensity of CcoN after Coomassie-blue staining. Coomassie blue: Coomassie-blue-stained gel, Heme: heme-stained gel.

Fig. 3. Effect of the absence of CcoQ on the stability of the $cbb_3$ oxidase. Cell-free crude extracts of the wild-type (2.4.1) and CCOQ$^{\Delta}$ mutant strains were prepared from the cells
grown anaerobically in the dark with DMSO. 50 µl of crude extracts containing chloramphenicol and tetracycline to a final concentration of 0.005% (w/v) were placed in the 1.5-ml Eppendorf tubes and incubated at RT. Crude extracts were assayed for cytochrome c oxidase activity at the times indicated. The enzyme activity is expressed as micromoles per minute per milligram protein. All values provided are the average of two independent determinations.

**Fig. 4.** *In vitro* stability analyses of the holo- and core enzymes (A) 10 µl of each aliquot of the purified core- (Cbb3-CCOQ, 17 µg) and holo- (Cbb3, 12 µg) oxidases was placed in the 1.5-ml Eppendorf tube and incubated over a period of 3 days at RT. The incubation times are indicated at the top of the gel figures. Samples were frozen rapidly at the indicated time points by placing them in ethanol-dry-ice slurry and stored at –20°C until SDS-PAGE analysis. The samples were subjected to SDS-PAGE followed by heme and Coomassie-blue staining. (B) 10 µl of each aliquot of the purified core- (Cbb3-CCOQ, 17 µg) and holo- (Cbb3, 12 µg) oxidases was placed in the 1.5-ml eppendorf tube and incubated for 3 days at RT. C: control samples before incubation, none: control samples incubated for 3 days without treatment with argon and ascorbate, Ar: argon was flushed, ascorbate: sodium ascorbate was added to the samples to a final concentration of 10 mM, P inhibitors: the mixture of protease inhibitors was added to the samples (antipain-dihydrochloride, 50 µg/ml; Bestatin, 40 µg/ml; Pefabolic SC, 500 µg/ml; Pepstatin, 0.7 µg/ml). When the core enzyme was treated with each protease inhibitor, the samples were incubated for 4 days. Cysteine, Metal, Serine, Aspartate: the corresponding protease inhibitor was added to the samples to a final concentration as described above. (C) the
purified holo- (12 µg), core- (17 µg) oxidases, and a mixture of both purified oxidases (Holo+Core) were incubated for 4 days at RT. Samples were subjected to SDS-PAGE followed by heme staining.

**Fig. 5.** Multiple alignment of the CcoQ homologues and CtaH of *P. denitrificans* (A) and determination of the minimal length of CcoQ capable of complementing the CcoQ mutant of *R. sphaeroides* (B). Identical or conservatively substituted residues are highlighted by a gray background. The numbers on the right of the aligned sequences indicate the length of the polypeptides deduced from the corresponding nucleotide sequences. pBBRQ is the pBBR1MCS2-based plasmid harboring the wild-type *ccoQ* gene. pBBRQ-TGA1 - pBBRQ-TGA4 are derivatives of pBBRQ and each contains the *ccoQ* gene with a nonsense mutation encoding the truncated form of CcoQ. aLevels of spectral complex in *R. sphaeroides* strains grown under aerobic conditions. bβ-Galactosidase activities in aerobically grown *R. sphaeroides* strains bearing the *puf::lacZ* transcriptional fusion plasmid (pUI1830). cCytochrome *c* oxidase activities in *R. sphaeroides* strains grown semiaerobically.

Strains were grown aerobically (30% O₂) to an OD₆₀₀ of 0.3-0.4 or semiaerobically (2% O₂) to an OD₆₀₀ of 0.5-0.6. Abbreviations: P.d., *P. denitrificans*; R.c., *Rhodobacter capsulatus*; V.c., *Vibrio cholerae*; B.j., *B. japonicum*; S.m., *Sinorhizobium meliloti*; R.s., *R. sphaeroides*. 
### Table I. Bacterial strains and plasmids used in this work

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### Table: Strain Plasmid Levels of Spectral Complexes

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Oxygen adaptation: The role of the CcoQ subunit of the cbb3 cytochrome c oxidase of Rhodobacter sphaeroides 2.4.1
Jeong-Il Oh and Samuel Kaplan

J. Biol. Chem. published online February 25, 2002

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