# Reconstitution of the *Rhodobacter sphaeroides cbb*$_3$-PrrBA Signal Transduction Pathway in Vitro†

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ABSTRACT: The PrrBA two-component system in *Rhodobacter sphaeroides* 2.4.1, which is composed of the PrrB histidine kinase and the PrrA response regulator, controls the expression of all of the photosynthesis genes, either directly or indirectly, in response to changes in oxygen tension. In vivo under aerobic conditions it is the *cbb*$_3$ cytochrome *c* oxidase which generates an inhibitory signal preventing the accumulation of activated PrrA. Using purified *cbb*$_3$ cytochrome *c* oxidase, PrrB, and PrrA, we demonstrate in vitro that the *cbb*$_3$ oxidase inhibits PrrB activity by apparently increasing the intrinsic PrrB phosphatase activity, which dephosphorylates phosphorylated PrrA without alteration of the PrrB kinase activity. The transmembrane domain of PrrB is required for the enhancement of PrrB phosphatase activity by the *cbb*$_3$ oxidase. Full-length PrrB has a significantly greater ability to phosphorylate PrrA than does truncated PrrB lacking the transmembrane domain. This is at least in part due to the lower autophosphorylation rate of the truncated PrrB relative to the full-length PrrB. This finding provides evidence that the sensing domain (transmembrane domain) of PrrB plays an important role not only in optimally sensing the state of the *cbb*$_3$ oxidase but also in maintaining the correct conformation of PrrB, providing optimal autokinase activity.

*Rhodobacter sphaeroides* is a purple, non-sulfur bacterium that can perform photosynthesis (PS)[1] anaerobically in the presence of light. Oxygen is the primary signal that determines the presence or absence of the spectral complexes required for photosynthesis in *R. sphaeroides*. Expression of the PS genes encoding the apoproteins of the spectral complexes as well as enzymes catalyzing the biosynthesis of the photopigments (bacteriochlorophyll and carotenoid) occurs under $O_2$-limiting ($<\sim$3% $O_2$) or anaerobic conditions (*1*−*3*).

The PrrBA two-component system is one of the major regulatory systems involved in the regulation of PS gene expression in response to changes in $O_2$ tension (*4*−*9*). The PrrBA two-component system consists of the membrane-associated PrrB histidine kinase and its cognate PrrA response regulator. The PrrB histidine kinase is a bifunctional enzyme that possesses both kinase and phosphatase activities (*10*, *11*). PrrB is composed of the conserved C-terminal kinase/phosphatase domain and the N-terminal membrane-spanning domain with six transmembrane helices forming three periplasmic and two cytoplasmic loops (*12*). It was demonstrated through site-directed mutagenesis that the central portion of the PrrB transmembrane domain including the second periplasmic loop plays an important role in the "sensing" function of PrrB (*9*). Either disruption of the structure of the PrrB transmembrane domain through mutagenesis or overexpression of *prrB* leads to constitutive expression of the PrrA regulon in the presence or absence of $O_2$. These results were interpreted to indicate that the intrinsic or default state of PrrB is in the kinase-dominant mode (*9*). In vitro studies conducted on the purified PrrB by Potter et al. (*11*) subsequently confirmed, using the intact protein, that the kinase-positive mode is the default state for PrrB.

On the basis of genetic and biochemical studies performed in vivo, the *cbb*$_3$ cytochrome *c* oxidase possessing a high affinity for $O_2$ was proposed to be the redox sensor that controls the equilibrium between the PrrB kinase/phosphatase activities in response to changes in $O_2$ availability (*9*, *13*−*17*). The *cbb*$_3$ oxidase generates a signal, which results in the inhibition of PrrB and which shifts the relative equilibrium of PrrB from the kinase mode to the phosphatase mode. The strength of the inhibitory signal is proportionally related to the extent of electron flow through the *cbb*$_3$ oxidase (*16*). According to this model, the *cbb*$_3$ oxidase generates a strong inhibitory signal under high $O_2$ conditions where its substrate ($O_2$) is sufficient, resulting in the silencing of PS gene expression. It has also been suggested that the membrane-bound PrrC protein enhances the transmission of the inhibitory signal from the *cbb*$_3$ oxidase to PrrB (*8*).

To gain a fuller understanding of this process, we describe here the reconstruction of this signaling pathway in vitro. Using purified *cbb*$_3$ oxidase, PrrA, and PrrB, we first demonstrate in vitro that the *cbb*$_3$ oxidase itself can inhibit

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[1] Abbreviations: DM, *n*-dodecyl *β*-D-maltoside; PS, photosynthesis; B875, light-harvesting complex I; B800−850, light-harvesting complex II.

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Table 1: Bacterial Strains and Plasmids Used in This Work

| strain or plasmid | relevant phenotype or genotype | source or ref |
|---|---|---|
| | *R. sphaeroides* | |
| 2.4.1 | wild type | *30* |
| PrrB1 | 2.4.1 derivative, Δ*prrB*::ÚSp$^r$/St$^r$ | *6* |
| CBB3Δ | 2.4.1 derivative, deletion of *ccoNOQP* | *20* |
| | *E. coli* | |
| DH5α | Φ80d*lacZ*ΔM15)Δ*lacU169 recA1 endA1 hsdR17 supE44 thi1 gyrA96 relA1* | *31* |
| S17-1 | Pro$^-$ Res$^-$ Mob$^+$ *recA*; integrated plasmid RP4-Tc::Mu-Km::Tn*7* | *32* |
| Bl21(DE3) | F$^-$, *omp*T, *hsdS*$_B$, ($r_{B^-}$, $m_{B^-}$), *dcm*, *gal*, *λ*(DE3) | Promega |
| | Plasmid | |
| pUC19 | Ap$^r$; *lacPOZ′* | *33* |
| pBSIIKS+ | Ap$^r$; *lacPOZ′* | Stratagene |
| pT7-7 | Ap$^r$; T7 promoter, RBS, and translation start codon overlapping with *Nde*I site | *34* |
| pRK415 | Tc$^r$; Mob$^+$ *lacZ*α IncP | *35* |
| pPRRB3 | pBSIIKS+::2.0-kb *Apa*I fragment containing *prrB* with 6 His codons before its stop codon | *9* |
| pUI1643 | pBSIIKS+::4.0-kb *Bam*HI−*Hin*dIII fragment containing *prrB* and *prrA* | *6* |
| pUI2803NHIS | pRK415::4.7-kb *Bam*HI−*Eco*RI fragment containing *ccoNOQP* with 6 His codons before the stop codon of *ccoN* | *20* |
| pHIS9A | pRK415::1.4-kb *Kpn*I−*Xba*I fragment containing *prrB* | this study |
| pHIS9C | pRK415::0.9-kb *Kpn*I−*Xba*I fragment containing the 3′-part of *prrB* | this study |
| pPRRACHIS2 | pT7-7::0.6-kb *Nde*I−*Hin*dIII fragment containing *prrA* with 6 His codons before its stop codon | this study |
| pT7HIS9A | pT7-7::1.4-kb *Nde*I−*Pst*I fragment containing *prrB* with 9 His codons before its stop codon | this study |
| pT7HIS9C | pT7-7::0.9-kb *Nde*I−*Pst*I fragment containing the 3′-part of *prrB* with 9 His codons before its stop codon | this study |

Table 2: Primers Used To Construct the Plasmids Used in This Study

| primer | nucleotide sequence (5′ to 3′) |
|---|---|
| His9A+ | TTGCCCCATATGATACTCGGTCCCGAC |
| His9B+ | GTACGGCATATGTTCGAATTCGGCTTC |
| His9C+ | TTTCGTCATATGTCGGATGCGCTTCTG |
| His9− | ACTCTGCAGTCAATGATGATGGTGGTGGTGATGGTGGTG |
| ACHis+ | GACACATATGGCTGAGGATCTGGTA |
| ACHis− | GTTCAGTGGTGGTGATGGTGGTGGCGCGGGCTGCTCTTCGC |

PrrB-dependent phosphorylation of PrrA. Further, we reveal that the inhibitory effect exerted by PrrB is the apparent result of the increase in the phosphatase activity of PrrB rather than a reduction of its kinase activity.

## EXPERIMENTAL PROCEDURES

*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 (*17*).

*DNA Manipulations and Conjugation Techniques.* Standard protocols (*18*) or manufacturer's instructions were followed for general recombinant DNA manipulations. Introduction of plasmids into *R. sphaeroides* strains was performed by conjugation as described previously (*19*).

*Construction of Plasmids. (A) pT7HIS9A and pT7HIS9C.* A 1424-bp fragment including the *prrB* gene and the nine histidine codons immediately before its stop codon was amplified with primers His9A+ and His9− using the plasmid pPRRB3 as the template and *pfu* DNA polymerase (the nucleotide sequences of the primers are listed in Table 2). The PCR product was restricted with *Nde*I and *Pst*I and cloned into pT7-7 digested with the same restriction enzymes, yielding pT7HIS9A. The DNA sequence of the insert was verified by DNA sequencing. pT7HIS9C was constructed in the same way as pT7HIS9A except for the primers used for PCR. The primer pair His9C+/His9− was employed for the construction of pT7HIS9C (Table 2). pTHIS9C carries the 3′-portion of *prrB* encoding a truncated form of the PrrB protein in which 191 N-terminal amino acids are removed.

*(B) pHIS9A and pHIS9C.* To construct pHIS9A/C, the *Pst*I−*Xba*I fragments from pT7HIS9A/C containing the *prrB* gene were cloned into pBSIIKS+ restricted with *Pst*I and *Xba*I, resulting in pBSHIS9A/C. Subsequently, the *Kpn*I−*Xba*I fragments from pBSHIS9A/C were cloned into pRK415 to yield pHIS9A/C.

*(C) pPRRACHIS2.* A 0.58-kb DNA fragment containing *prrA* with a tail of six histidine codons immediately upstream of its stop codon was generated by PCR using the primers ACHis+ and ACHis− and pUI1643 as the template. The PCR product was cloned into pUC19 digested with *Hin*cII to give the plasmid pPRRACHIS. A 0.6-kb *Nde*I−*Hin*dIII fragment from pPRRACHIS was cloned into pT7-7 restricted with the same enzymes, yielding pPRRACHIS2.

*Protein Purification. (A) Intact PrrB.* The *E. coli* Bl21-(DE3) strain carrying pT7HIS9A was grown in Luria−Bertani (LB) medium supplemented with 100 $\mu$g/mL ampicillin aerobically at 37 °C to an absorbance of 0.4−0.5 at 595 nm. The *prrB* gene was induced by addition of IPTG to a final concentration of 1 mM, and cells were further grown for 4 h at 30 °C. After harvesting of a 4 L culture, cells were washed with buffer A [20 mM Tris-HCl (pH 8.0) containing 20 mM $\beta$-mercaptoethanol]. Cells were resuspended in 30 mL of buffer A and disrupted by two passages through a French pressure cell. Following DNase treatment [150 units of DNase (Promega, Madison, WI)] in the presence of 10 mM $MgCl_2$ for 30 min at room temperature, cell-free crude extracts were obtained by centrifugation two times at 20000*g* for 20 min. Membrane fractions were isolated by ultracentrifugation of crude extracts at 150000*g*

for 90 min at 4 °C. After the membrane fraction (pellet) was washed twice with buffer A, the membranes were solubilized in 30 mL of buffer B [buffer A containing 20% (w/v) glycerol] containing 1% (w/v) *n*-dodecyl $\beta$-D-maltoside (DM) for 2 h at 4 °C and then centrifuged at 150000*g* for 1 h at 4 °C. The supernatant was taken as solubilized membrane proteins and used for affinity chromatography. After addition of imidazole to a final concentration of 5 mM, 1.5 mL of a 50% (v/v) nickel−nitrilotriacetic acid HIS-bind slurry (Novagen, Madison, WI) was added to the solubilized membrane proteins and mixed gently by shaking for 15 h at 4 °C. The protein−resin mixture was loaded into a column, and the column was washed with 20 volumes of buffer C [buffer B containing 0.05% (w/v) DM] containing 5 mM imidazole followed by 50 volumes of buffer C containing 20 mM imidazole. The His$_9$-tagged PrrB protein was eluted with buffer C containing 200 mM imidazole. The fractions containing the PrrB protein were collected and dialyzed against 1 L of buffer C for 5 h at 4 °C. The desalted PrrB was concentrated by means of ultrafiltration (membrane YM30; Millipore Co., Bedford, MA).

*(B) Truncated PrrB.* The truncated form of PrrB was heterologously overexpressed in an *E. coli* Bl21(DE3) strain carrying pT7HIS9C. The strain was grown, and the *prrB* gene was induced as described for overexpression of the intact PrrB. After harvesting of a 1.8 L culture, cells were washed with buffer A. Cells were resuspended in 10 mL of buffer A and disrupted by two passages through a French pressure cell. Following DNase treatment (150 units of DNase) in the presence of 10 mM $MgCl_2$ and 1 mM phenylmethanesulfonyl fluoride for 30 min at room temperature, cell-free crude extracts were obtained by centrifugation twice at 20000*g* for 20 min. The crude extract was filtered through a syringe filter with a pore size of 0.45 $\mu$m, and imidazole was added to the filtered crude extract to a final concentration of 5 mM. Four mL of a 50% (v/v) nickel−nitrilotriacetic acid HIS-bind slurry was added to the crude extract, and the resultant solution was mixed gently by shaking for 1 h at 4 °C. The protein−resin mixture was loaded into a column. Affinity chromatography was performed under the following conditions: 10 volumes of buffer A containing 0.5 M NaCl and 5 mM imidazole in the first wash step, 6 volumes of buffer A containing 0.5 M NaCl and 60 mM imidazole in the second wash step, and elution with buffer A containing 0.5 M NaCl and 500 mM imidazole. The fractions containing the truncated PrrB protein (C-PrrB) were collected and dialyzed against 2 L of buffer C overnight at 4 °C. The desalted PrrB was concentrated by means of ultrafiltration (membrane YM10).

*(C) PrrA.* To overproduce the His$_6$-tagged PrrA protein, *E. coli* Bl21(DE3) carrying pPRRACHIS2 was employed. The same procedures were applied to overexpression and purification of PrrA as truncated PrrB except that the buffer used in PrrA purification did not contain $\beta$-mercaptoethanol and DM.

*(D) cbb$_3$ Cytochrome c Oxidase.* The His$_6$-tagged *cbb*$_3$ cytochrome *c* oxidase was purified as described elsewhere (*20*).

*In Vitro Phosphorylation Assay.* Protein phosphorylation was performed at room temperature in assay buffer containing the appropriate protein components, 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 10−15% (w/v) glycerol, 10 mM $\beta$-mercaptoethanol, and 0.025% (w/v) DM. For autophosphorylation assays of PrrB, an appropriate amount of PrrB was incubated in assay buffer in the presence or absence of the *cbb*$_3$ oxidase for 5 min at room temperature, and the reaction was initiated by the addition of a mixture of [$\gamma$-$^{32}$P]ATP and unlabeled ATP to final concentrations of 100 (1000 Ci/mol) or 200 $\mu$M (500 Ci/mol). In the case of time course experiments, samples (10 $\mu$L) were removed at various time intervals, and reactions were stopped by the addition of 3 $\mu$L of 4× loading buffer [40 mM Tris-HCl (pH 6.8), 4% SDS, 16% (w/v) glycerol, 16 mM DTT, 1% $\beta$-mercaptoethanol, 0.1% (w/v) bromophenol blue, and 100 mM EDTA]. PrrB-dependent phosphorylation of PrrA was performed in assay buffer for the autophosphorylation assay containing purified PrrA as described above.

*In Vitro Assay for Phosphotransferase Activity of PrrB.* PrrB (7.5 $\mu$g, 143.3 pmol) was maximally phosphorylated in assay buffer containing 100 $\mu$M [$\gamma$-$^{32}$P]ATP (1000 Ci/mol) in the presence or absence of 7.6 $\mu$g (60 pmol) of the *cbb*$_3$ oxidase for 20 min at room temperature. Following the addition of 5 $\mu$g (233.5 pmol) of PrrA to the reaction (the total reaction volume is 50 $\mu$L), 10 $\mu$L of aliquots was removed at various time intervals and added to 3 $\mu$L of 4× sample buffer to stop the reaction. Given the fact that the presence of the *cbb*$_3$ oxidase does not affect the autophosphorylation rate of PrrB, the phosphotransferase activity of PrrB for PrrA was measured by monitoring the dephosphorylation rate of phosphorylated PrrB in the presence of PrrA.

*In Vitro Assay for Phosphatase Activity of PrrB.* PrrA (8 $\mu$g, 373.6 pmol) was phosphorylated in 72 $\mu$L of assay buffer containing 2.4 $\mu$g (45.8 pmol) of PrrB and 100 $\mu$M ATP (1000 Ci/mol) for 20 min at room temperature. The *cbb*$_3$ oxidase (12.2 $\mu$g, 96.4 pmol) and 8 $\mu$L of 33 mM unlabeled ATP were added to the reaction. Samples (10 $\mu$L) were removed at various time intervals and added to 3 $\mu$L of sample buffer to stop the reaction. As a negative control, the same assay was performed in the absence of the *cbb*$_3$ oxidase.

Samples were denatured for 40 min at room temperature in sample buffer, and $^{32}$P-labeled PrrB and PrrA were resolved by SDS−PAGE using 12.5% (w/v) polyacrylamide gels. Gels were dried at 80 °C under vacuum, and the labeled proteins were visualized by autoradiography. Quantitation of the labeled proteins was performed using the densitometer program BIO-PROFIL Bio-1D (v97.04, Vilber-Lourmat).

*Protein Determination.* Protein concentration was determined by the bicinchoninic acid protein assay (Pierce) using bovine serum albumin as the standard protein.

*Quantitative Analysis of Spectral Complexes.* The levels of the B800−850 and B875 complexes were determined spectrophotometrically as described previously (*17*).

## RESULTS

*Overexpression of the ccoNOQP Operon Leads to a Reduction in Spectral Complex Formation under Anaerobic Conditions.* Previously, it was reported from this laboratory that Cco-minus mutants of *R. sphaeroides* exhibited increased levels of PS gene expression accompanying an increase in spectral complex formation under anaerobic growth conditions when compared with the wild-type strain grown under the same conditions (*13*, *14*). By contrast, overexpression

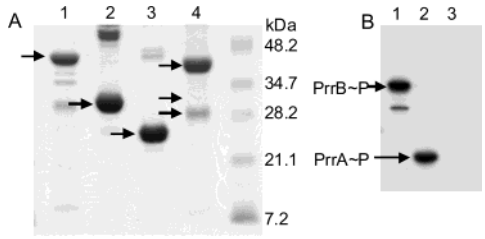


FIGURE 1: Purification of PrrB, C-PrrB, PrrA, and $cbb_3$ oxidase (A) and in vitro phosphotransfer assay (B). The purified proteins were resolved by SDS−PAGE (12.5% polyacrylamide gel) and visualized by staining with Coomassie brilliant blue. Lanes: 1, purified PrrB (10 $\mu$g); 2, purified C-PrrB (10 $\mu$g); 3, purified PrrA (9 $\mu$g); 4, purified $cbb_3$ oxidase (12 $\mu$g). The positions of each protein are indicated by arrows. The $cbb_3$ oxidase is composed of four different subunits (CcoN, CcoP, CcoO, and CcoQ in decreasing order of the molecular mass). The CcoN, CcoP, and CcoO subunits are indicated by the arrows. The CcoP subunit was previously demonstrated not to stain well with Coomassie brilliant blue. In vitro phosphotransfer assays were performed in assay buffer [20 mM Tris-HCl (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 10−15% (w/v) glycerol, 10 mM $\beta$-mercaptoethanol, and 0.025% (w/v) DM] containing the following protein components and 100 $\mu$M ATP for 10 min at room temperature. Lanes: 1, 2 $\mu$g of PrrB; 2, 1 $\mu$g of PrrA and 2 $\mu$g of PrrB; 3, 3 $\mu$g of $cbb_3$ oxidase and 1 $\mu$g of PrrA.

of the *ccoNOQP* operon by virtue of a 5−7-fold increase in copy number in the wild-type strain 2.4.1 resulted in a decrease in the levels of light-harvesting complexes (B800−850 + B875) to ~55% of those detected in the control strain (2.4.1 containing one copy of the *ccoNOQP* operon) when both strains were grown under anaerobic photosynthetic conditions at a light intensity of 10 W/m$^2$ (*17*). These results imply that the $cbb_3$ oxidase can generate an inhibitory signal sufficient to shift the equilibrium of the PrrB kinase/phosphatase activity in the direction of phosphatase activity even in the absence of $O_2$.

*Purification and Characterization of PrrB and PrrA.* To examine whether the $cbb_3$ oxidase directly affects PrrB activity which determines the phosphorylation state of PrrA, the full-length PrrB (PrrB), PrrA, and $cbb_3$ oxidase as well as the truncated form of PrrB lacking the entire transmembrane domain (C-PrrB) were overexpressed and purified as described in Experimental Procedures. As shown in Figure 1, SDS−PAGE analysis showed that intact PrrB, C-PrrB, PrrA, and $cbb_3$ oxidase were purified to greater than 90% homogeneity. The purified $cbb_3$ oxidase was able to oxidize reduced cytochrome *c*, indicating that the purified enzyme is catalytically active [the specific activity of the purified enzyme was 0.393 $\mu$mol min$^{-1}$ (mg of protein)$^{-1}$]. The apparent molecular mass of intact PrrB was estimated to be approximately 45 kDa as judged by SDS−PAGE, which is consistent with the value reported previously (*9*, *11*). The determined molecular masses of purified C-PrrB and PrrA were in relatively good agreement with the theoretical values (31.4 and 21.4 kDa, respectively). To examine the functionality of purified PrrB and PrrA, the ability of intact PrrB to autophosphorylate using ATP and to transfer the phosphoryl group to PrrA was probed in the presence of a detergent, DM, in the reaction mixture. When 2 $\mu$g (38.2 pmol) of purified PrrB was incubated with [$\gamma$-$^{32}$P]ATP for 10 min at room temperature, PrrB was autophosphorylated (Figure 1B, lane 1). When the same reaction was performed in the presence of 1 $\mu$g (46.7 pmol) of purified PrrA, only a phosphorylated PrrA band was observed (Figure 1B, lane 2), indicating that both purified PrrA and PrrB are active and that the phosphotransfer reaction from phosphorylated PrrB to PrrA is much faster than the autophosphorylation reaction of PrrB. An additional phosphorylated protein with a molecular mass of 34 kDa was observed below the PrrB band. This band might be a fragmented form of the PrrB protein, which was produced during purification, since this protein appears to have the ability to transfer the phosphoryl group to PrrA (Figure 1B, lane 2). Incubation of 3 $\mu$g (23.7 pmol) of the purified $cbb_3$ oxidase and 1 $\mu$g (46.7 pmol) of PrrA with [$\gamma$-$^{32}$P]ATP did not lead to any band of phosphorylated protein, which indicates that PrrA does not possess the capability of autophosphorylation and that the purified $cbb_3$ oxidase does not contain any contaminating histidine kinases, which can phosphorylate PrrA (Figure 1B, lane 3).

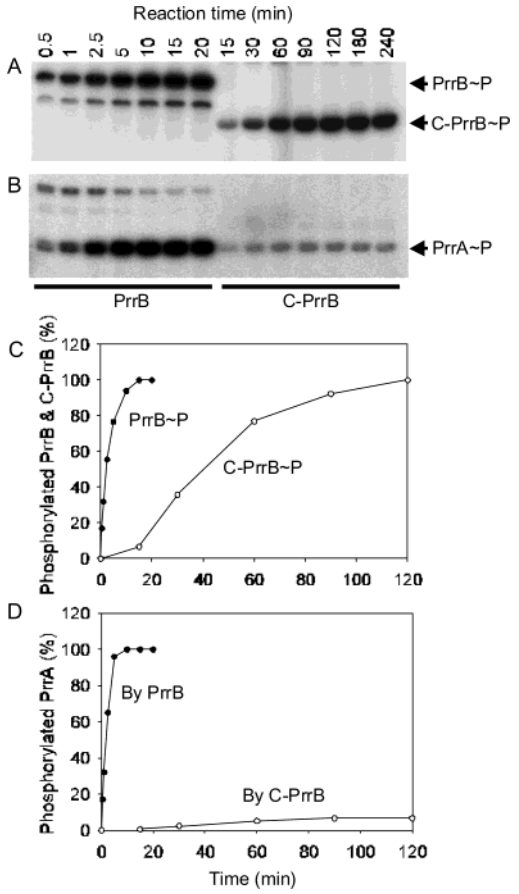


FIGURE 2: Autophosphorylation of PrrB and C-PrrB (A and C) and PrrB- (C-PrrB-) dependent phosphorylation of PrrA (B and D). The autophosphorylation reactions were performed by using either 4 $\mu$M PrrB or 4 $\mu$M C-PrrB and 100 $\mu$M ATP at room temperature. For PrrB-dependent phosphorylation of PrrA, the reactions were initiated by the addition of 100 $\mu$M ATP to the reaction mixture containing 4 $\mu$M PrrB (or C-PrrB) and 7 $\mu$M PrrA. At the time points indicated, samples (10 $\mu$L) were removed and added to 3 $\mu$L of loading buffer to stop the reaction. The amount of protein phosphorylation was quantified by SDS−PAGE and subsequent densitometer analysis.

Although the full-length PrrB and truncated PrrB (C-PrrB) have been purified and their autophosphorylation rates have been determined separately by two different research groups (*10*, *11*), no comparative assays using both full-length PrrB and truncated PrrB have been performed to date. As shown in Figure 2A,C, when the autophosphorylation rate of PrrB was measured in the presence of 100 $\mu$M ATP, the extent

Table 3: Levels of Spectral Complexes in *R. sphaeroides* Strains Grown under Aerobic (30% $O_2$) and Anaerobic (Dark DMSO) Conditions[a]

| | 30% $O_2$ | | dark DMSO | |
|---|---|---|---|---|
| strain | B800−850 | B875 | B800−850 | B875 |
| 2.4.1 (pRK415) | <0.1 | <0.1 | 37.0 ± 0.0 | 9.3 ± 0.2 |
| PrrB1 (pRK415) | <0.1 | <0.1 | 2.0 ± 0.1 | 6.3 ± 0.2 |
| PrrB1 (pHIS9A) | 0.2 ± 0.0 | 2.0 ± 0.1 | 68.4 ± 0.9 | 19.7 ± 0.2 |
| PrrB1 (pHIS9C) | <0.1 | <0.1 | 1.9 ± 0.1 | 5.8 ± 0.2 |

[a] Strains were grown aerobically by sparging with 30% $O_2$, 69% $N_2$, and 1% $CO_2$ to an $A_{600}$ of 0.4−0.5. Anaerobic growth with DMSO as a terminal electron acceptor was performed in completely filled screw-cap tubes in the dark. All values provided are an average of two independent determinations. The levels of spectral complexes are expressed as nmol/mg of protein.

of PrrB phosphorylation increased over time with a half-maximal phosphorylation ($t_{1/2}$) of ~2.5 min. Under the same reaction conditions C-PrrB displayed a significantly slower autophosphorylation rate with a $t_{1/2}$ of ~40 min, indicating that although the C-PrrB protein lacking the transmembrane domain retains autokinase activity, its activity is significantly reduced when compared with the intact PrrB. Furthermore, we measured the PrrB- and C-PrrB-dependent phosphorylation rate of PrrA by including purified PrrA in the reaction mixture (Figure 2B). PrrA was observed to be phosphorylated by PrrB at essentially the same rate as PrrB was autophosphorylated. This is also true for PrrA phosphorylation by C-PrrB. However, a conspicuous difference between PrrA phosphorylation by PrrB and C-PrrB is that the amount of PrrA phosphorylated by PrrB is much greater at steady state (where the kinase and phosphatase activities of PrrB are at presumed equilibrium) than the amount of PrrA phosphorylated by C-PrrB, despite the considerable autokinase activity of C-PrrB. This result implies that the equilibrium of C-PrrB kinase/phosphatase activities lies in favor of the relative phosphatase activity when compared with that of PrrB, which can be explained by the impaired autokinase activity of C-PrrB.

To examine the in vivo activity of C-PrrB, the plasmid pHIS9C containing the gene encoding C-PrrB was introduced into the *R. sphaeroides* mutant PrrB1, a PrrB null mutant strain, and complementation of the PrrB-minus phenotype was determined. As a control, plasmid pHIS9A containing the intact *prrB* gene was placed into the PrrB1 mutant. Both pHIS9A and pHIS9C are based on the broad host range vector pRK415, and the *prrB* genes in each construct are transcribed from the Tc-resistance gene promoter. As anticipated, spectral complex formation was shown to be severely impaired in the PrrB1 mutant containing pRK415 grown under anaerobic conditions when compared with the wild type containing pRK415 grown under the same conditions (Table 3). Introduction of pHIS9A into the PrrB1 mutant led to complementation of the PrrB-minus phenotype, revealing the induction of spectral complex formation under anaerobic conditions. Furthermore, the PrrB1 mutant with pHIS9A synthesized substantially increased levels of spectral complexes under both aerobic and anaerobic conditions in comparison with the wild type containing pRK415 grown under the same conditions. This effect of the overexpression of *prrB* was discussed previously (*9*). On the other hand, introduction of pHIS9C into the PrrB1 mutant did not restore the PrrB-minus phenotype with regard to spectral complex

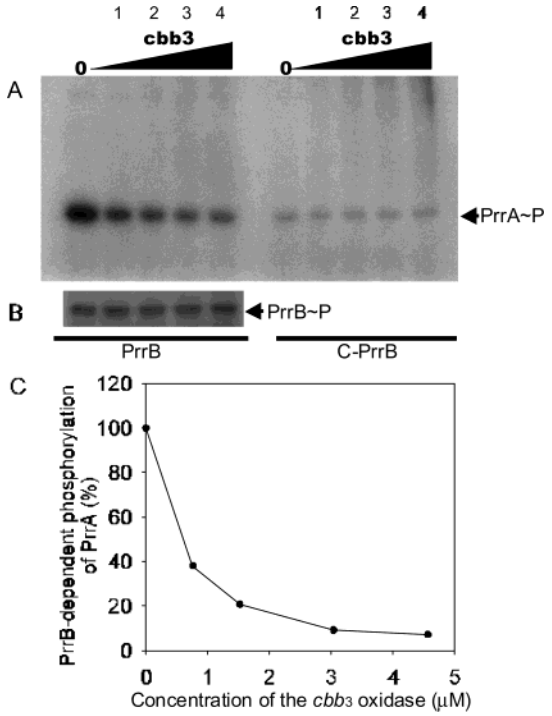


FIGURE 3: Effect of the *cbb*$_3$ oxidase upon PrrB-dependent phosphorylation of PrrA. (A and C) PrrA phosphorylation by PrrB was performed in a reaction mixture containing 4.7 $\mu$M PrrA and 2.8 $\mu$M PrrB with various concentrations of the *cbb*$_3$ oxidase (lane 0, no *cbb*$_3$ oxidase; lane 1, 0.6 $\mu$M *cbb*$_3$ oxidase; lane 2, 1.2 $\mu$M; lane 3, 2.4 $\mu$M; lane 4, 3.6 $\mu$M). The reaction was initiated by the addition of ATP to a final concentration of 200 $\mu$M and performed for 20 min at room temperature. When 2.6 $\mu$M C-PrrB in place of PrrB was used in the control experiment, the reaction was incubated for 60 min at room temperature due to the weak kinase activity of C-PrrB. (B) The effect of the *cbb*$_3$ oxidase upon PrrB autophosphorylation was examined under the same reaction conditions as above except for the absence of PrrA in the reaction.

formation under anaerobic conditions. By means of Western blot analysis using an anti-$His_6$ antibody, the presence of C-PrrB in the PrrB1 mutant containing pHIS9C was probed. PrrB was detected in the PrrB1 mutant containing pHIS9A, whereas C-PrrB was not detected in the PrrB1 mutant containing pHIS9C (data not shown). These results suggest that the PrrB protein lacking the transmembrane domain is subject to proteolysis in *R. sphaeroides*, probably due to its incorrect conformation.

*Effect of the cbb*$_3$ *Oxidase on PrrB Activity.* To ascertain whether the purified *cbb*$_3$ oxidase inhibits PrrB activity that in turn phosphorylates PrrA in vitro, the extent of PrrA phosphorylation by PrrB was determined in a reaction mixture containing fixed amounts of PrrA and PrrB with the addition of various concentrations of the *cbb*$_3$ oxidase. We measured the extent of PrrA phosphorylation at steady state by incubating the reactions for 20 min after the addition of ATP. Panels A and C of Figure 3 show that the amounts of phosphorylated PrrA at steady state decreased with increasing concentrations of the *cbb*$_3$ oxidase added to the reaction. Treatment of ~28 pmol of the PrrB monomer with ~24 pmol of the *cbb*$_3$ oxidase gave maximum inhibition of PrrB activity (lane 3), suggesting that the presence of the *cbb*$_3$ oxidase in a stoichiometry of 1 relative to the PrrB monomer is sufficient to afford maximum inhibition of PrrB activity. The same experiment was performed in the absence of PrrA in the reaction to assess the effect of the *cbb*$_3$ oxidase upon

the autophosphorylation rate of PrrB (Figure 3B). The extent of PrrB autophosphorylation was shown to remain constant with increasing amounts of the *cbb*$_3$ oxidase added to the reaction. When C-PrrB lacking the transmembrane domain was used in place of PrrB, increasing concentrations of the *cbb*$_3$ oxidase did not appear to influence the activity of C-PrrB which determines the phosphorylation state of PrrA (Figure 3A). From these results the following conclusions can be drawn: (i) *cbb*$_3$ oxidase inhibits the PrrB activity which phosphorylates PrrA, and this inhibitory effect upon PrrB activity is not exerted through a decrease in the PrrB autophosphorylation rate. (ii) The transmembrane domain of PrrB is required for PrrB to "communicate" with the *cbb*$_3$ oxidase. The most likely result of this "communication" is the relative increase in the intrinsic phosphatase activity of PrrB.

To gain more detailed insight into the mechanism by which the *cbb*$_3$ oxidase affects the activity of PrrB, the rate of PrrA phosphorylation by PrrB was determined as a function of time in the presence or absence of the *cbb*$_3$ oxidase in the reaction mixture (Figure 4A). The amounts of phosphorylated PrrA were shown to increase for the first 2.5−5 min after the addition of [$\gamma$-$^{32}$P]ATP to the reaction and then to decrease slightly thereafter when the *cbb*$_3$ oxidase was present. Without the addition of the *cbb*$_3$ oxidase the amounts of phosphorylated PrrA increased throughout the reaction. Between 2.5 and 10 min after the reaction was initiated by the addition of ATP when the level of PrrA phosphorylation was maximal, the level of phosphorylated PrrA in the presence of the *cbb*$_3$ oxidase was significantly reduced when compared with that in the absence of the *cbb*$_3$ oxidase. This observation confirms that the presence of the *cbb*$_3$ oxidase shifts the equilibrium of the PrrB kinase/phosphatase activities toward the phosphatase mode.

Three possible mechanisms by which the *cbb*$_3$ oxidase can regulate the PrrA phosphorylation/dephosphorylation activity of PrrB are possible. (i) The regulation of PrrB occurs exclusively at the level of PrrB kinase activity. (ii) It is the PrrB phosphatase activity rather than the kinase activity that is regulated. (iii) PrrB kinase/phosphatase activities are regulated reciprocally.

The PrrB reaction that transfers a phosphoryl group from ATP to PrrA consists of two half-reactions: (i) a PrrB autophosphorylation reaction in which the conserved histidine residue within the dimerization domain of PrrB becomes phosphorylated by the phosphoryl group derived from ATP; (ii) a phosphotransfer reaction from phosphorylated PrrB to PrrA. To ascertain whether the *cbb*$_3$ oxidase affects the rate of PrrB autophosphorylation, the rate of PrrB autophosphorylation in the presence or absence of the *cbb*$_3$ oxidase was measured as a function of time. As shown in Figure 4B, the rate of PrrB autophosphorylation in the presence of the *cbb*$_3$ oxidase in the reaction was shown to be the same as that in the absence of the *cbb*$_3$ oxidase. This result indicates that the *cbb*$_3$ oxidase does not regulate the rate of PrrB autophosphorylation, which is in keeping with the result demonstrated above (see Figure 3B). We next examined whether the *cbb*$_3$ oxidase affects the rate of phosphotransfer from phosphorylated PrrB to PrrA. For the phosphotransfer assay, PrrB was first maximally autophosphorylated with 100 $\mu$M ATP for 20 min in the presence or absence of the *cbb*$_3$ oxidase, and the rate of PrrB dephosphorylation, which reflects the phosphotransfer activity, was determined over time following the addition of PrrA (Figure 4D). Since the rate of PrrB autophosphorylation is identical in the presence or absence

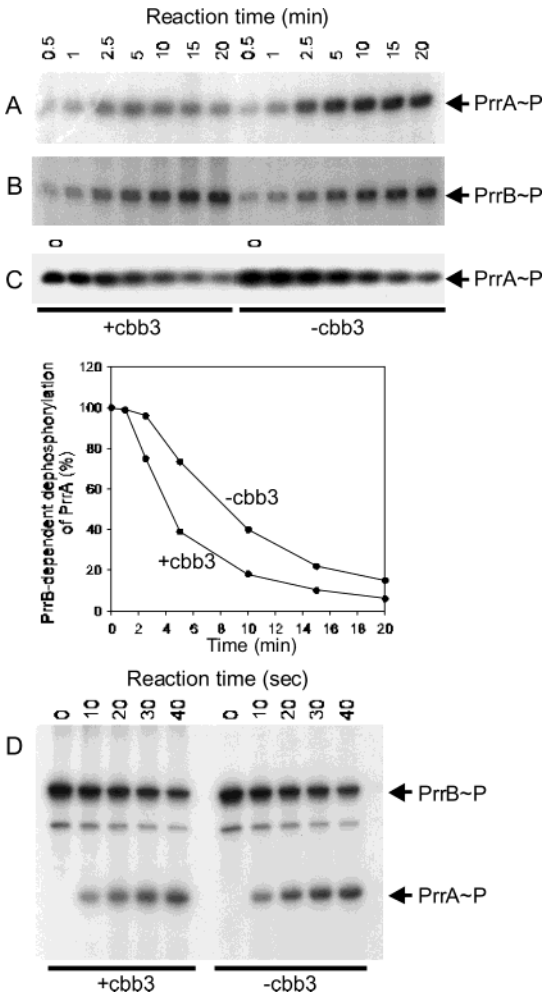


FIGURE 4: Effect of the *cbb*$_3$ oxidase upon PrrB kinase/phosphatase activities. (A) PrrB-dependent phosphorylation of PrrA in the presence (+*cbb*$_3$) or absence (−*cbb*$_3$) of the *cbb*$_3$ oxidase. The reaction was started by the addition of ATP (500 Ci/mol) to the reaction mixture containing 0.6 $\mu$M PrrB and 4.7 $\mu$M PrrA to a final concentration of 200 $\mu$M. The reaction was performed in the presence or absence of 1.2 $\mu$M *cbb*$_3$ oxidase. (B) PrrB autophosphorylation in the presence or absence of the *cbb*$_3$ oxidase. The phosphorylation reaction of PrrB was initiated by the addition of ATP (500 Ci/mol) to the reaction mixture containing 0.6 $\mu$M PrrB to a final concentration of 200 $\mu$M. (C) PrrA dephosphorylation by PrrB in the presence or absence of the *cbb*$_3$ oxidase. 4.7 $\mu$M PrrA was phosphorylated in the reaction mixture containing 0.6 $\mu$M PrrB and 100 $\mu$M ATP (1000 Ci/mol) for 20 min at room temperature. The *cbb*$_3$ oxidase and nonlabeled ATP were added to the reaction at final concentrations of 1.2 $\mu$M and 3.3 mM, respectively, to monitor the rate of PrrA dephosphorylation. For all of the time course assays (A−C), aliquots (10 $\mu$L) were removed at the indicated time points, and the reactions were stopped by the addition of 4× loading buffer. (D) Phosphotransfer reaction from phosphorylated PrrB to PrrA in the presence or absence of the *cbb*$_3$ oxidase. 2.8 $\mu$M PrrB was maximally phosphorylated in assay buffer containing 100 $\mu$M ATP (1000 Ci/mol) in the presence or absence of 1.2 $\mu$M *cbb*$_3$ oxidase for 20 min at room temperature. Following the addition of PrrA to the reaction to a final concentration of 4.7 $\mu$M (the total reaction volume is 50 $\mu$L), 10 $\mu$L aliquots were removed at the indicated time points, and the reaction was stopped by the addition of 4× loading buffer. The phosphotransfer rate from phosphorylated PrrB to PrrA was measured by monitoring the rate of PrrB dephosphorylation.

of the *cbb*$_3$ oxidase, it was not necessary to remove ATP from the reaction prior to the addition of PrrA. Irrespective of the presence or absence of the *cbb*$_3$ oxidase in the reaction, phosphorylated PrrB lost its phosphate group over time at the same rate, indicating no effect of the *cbb*$_3$ oxidase upon the phosphotransfer reaction from phosphorylated PrrB to PrrA. Interestingly, the rate of PrrA phosphorylation in the phosphotransfer reaction from phosphorylated PrrB to PrrA appeared to be lower in the presence of the *cbb*$_3$ oxidase than in the absence of the *cbb*$_3$ oxidase (Figure 4D), although the rate of PrrB dephosphorylation was the same in the presence or absence of the *cbb*$_3$ oxidase. This result implies that the presence of the *cbb*$_3$ oxidase in the reaction might result in an increase in PrrB phosphatase activity acting on phosphorylated PrrA. Taken together, the results presented in Figure 4 suggest that the *cbb*$_3$ oxidase does not alter the actual PrrB kinase activity that catalyzes the phosphotransfer reaction from ATP to PrrA.

The dephosphorylation rate of phosphorylated PrrA by PrrB was determined in the presence or absence of the *cbb*$_3$ oxidase as described in Experimental Procedures. As shown in Figure 4C, the absence of the *cbb*$_3$ oxidase in the reaction resulted in an ~2-fold increase, i.e., 100%, in the half-life of the phosphorylated PrrA as compared to the presence of the *cbb*$_3$ oxidase ($t_{1/2}$ values of phosphorylated PrrA in the presence or absence of the *cbb*$_3$ oxidase are ~4.2 or ~8.4 min, respectively), indicating that the *cbb*$_3$ oxidase increases the phosphatase activity of PrrB toward phosphorylated PrrA. This must be considered a minimal estimate since PrrA dephosphorylation begins immediately.

## DISCUSSION

Histidine kinases of the bacterial two-component system normally have two enzymatic activities, an autophosphorylation (kinase) activity and a phosphatase activity, that together determine the phosphorylation state of their cognate response regulators (*21*). When a signal is sensed by the sensing domain of a histidine kinase through either direct binding of a signal molecule (ligand) to the kinase or protein−protein interactions between the kinase and other sensory proteins, the equilibrium of the kinase and phosphatase activities of the histidine kinase is changed to elicit an appropriate response under a given condition.

Here, we demonstrated that the presence of the *cbb*$_3$ oxidase in vitro alters the equilibrium of the PrrB kinase/phosphatase activities toward the phosphatase mode. This result is consistent with our previous observations: (i) Overexpression of the *ccoNOQP* operon leads to a decrease in PS gene expression under anaerobic conditions (*17*). (ii) Inactivation of the *cbb*$_3$ oxidase in *R. sphaeroides* through mutations of the *ccoNOQP* operon brings about increased expression of the PrrBA regulon under both aerobic and anaerobic conditions (*13*, *14*, *16*, *17*). The membrane-spanning domain of PrrB appears to be essential for the acquisition of the inhibitory signal from the *cbb*$_3$ oxidase since the activity of the truncated PrrB lacking the transmembrane domain remains constant irrespective of the presence or absence of the *cbb*$_3$ oxidase. This finding is in keeping with previous results showing that transmembrane helices 3 and 4 as well as the second periplasmic loop of PrrB play a significant role in the sensing function of PrrB (*9*) and that a Leu-78 to Pro mutation of PrrB leads to the constitutive expression of the PrrBA regulon (*6*). Recently, we found that site-directed mutations affecting Asp-90, Gln-93, and Asn-106 within the second periplasmic loop of PrrB resulted in the loss of the redox-sensing function of PrrB in vivo (Oh and Kaplan, unpublished experiments). The precise mechanism through which the *cbb*$_3$ oxidase controls PrrB activity remains elusive at the present time. However, the fact that, in the absence of any other protein components, purified *cbb*$_3$ oxidase is sufficient to decrease PrrB activity which phosphorylates PrrA implies that protein−protein interactions between the *cbb*$_3$ oxidase and PrrB might be the mechanism by which PrrB activity is controlled.

At least three mechanisms by which the enzymatic activities of a histidine kinase are regulated to ultimately result in an altered ratio of kinase to phosphatase activity, are conceivable: First, only the kinase activity of the histidine kinase is regulated in response to a signal. The activity of the CheA histidine kinase of *E. coli* was demonstrated to be regulated at the level of autophosphorylation by a chemoreceptor (*22*). Second, it is the phosphatase activity of the histidine kinase that is regulated in response to a signal. Using the Taz1 protein, a hybrid protein in which the periplasmic receptor domain of Tar is fused to the cytoplasmic kinase/phosphatase domain of EnvZ, it was demonstrated that the equilibrium between the kinase and phosphatase activities of EnvZ is controlled by the regulation of its phosphatase activity, while its autokinase activity remains constant (*23*). Third, both kinase and phosphatase activities of the histidine kinase are regulated reciprocally in response to a signal. In the NtrBC two-component system of *E. coli* which is involved in the regulation of nitrogen metabolism, both autokinase and phosphatase activities of NtrB are regulated by an auxiliary protein, $P_{II}$, which senses the cellular level of 2-ketoglutarate (*24*). It was also reported that the binding of $O_2$ to the heme of the FixL histidine kinase reduces the autophosphorylation rate of FixL and simultaneously increases its phosphatase activity to the FixJ response regulator (*25*).

The *cbb*$_3$ oxidase appears to control PrrB activity exclusively at the level of the phosphatase activity on the basis of the following observations: (i) The *cbb*$_3$ oxidase affected neither the PrrB autophosphorylation rate nor the phosphotransfer rate from phosphorylated PrrB to PrrA. (ii) The PrrB phosphatase activity acting on phosphorylated PrrA was shown to be increased by the *cbb*$_3$ oxidase.

The transfer of the phosphoryl group from ATP to PrrA by PrrB consists of two steps, autophosphorylation of PrrB and phosphotransfer from phosphorylated PrrB to PrrA (*10*, *11*). Relatively little phosphorylated PrrB accumulates when both PrrB and PrrA are incubated with ATP (see Figure 2B), indicating that the phosphotransfer reaction is more rapid than PrrB autophosphorylation. Since the autophosphorylation reaction of PrrB is the rate-limiting step in PrrA phosphorylation and phosphorylated PrrA is very stable with regard to decay kinetics ($t_{1/2}$ of autodephosphorylation of phosphorylated PrrA is ~330 min) (*10*), the phosphorylation state of PrrA is ultimately controlled by the balance of the autophosphorylation rate of PrrB and the phosphatase activity of PrrB acting on phosphorylated PrrA.

It is noteworthy that the full-length PrrB has a significantly higher activity toward the phosphorylation of PrrA than does

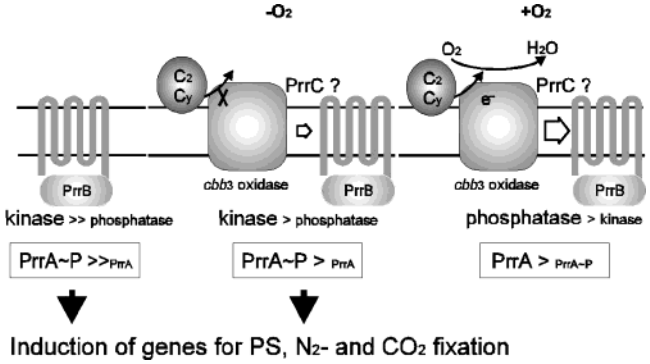


FIGURE 5: Model for $O_2$ signaling through the *cbb*$_3$-PrrBA signal transduction pathway. The default state of the PrrB activity is in the kinase-dominant mode. In the presence of $O_2$ ($+O_2$) electron flow through the *cbb*$_3$ oxidase generates an inhibitory signal which shifts the equilibrium of PrrB activity from the kinase-dominant mode to the phosphatase-dominant mode, leading to dephosphorylation of PrrA. The transmembrane domain of PrrB is required for the acquisition of the inhibitory signal from the *cbb*$_3$ oxidase. In the absence of $O_2$, electron flow through the *cbb*$_3$ oxidase is minimized, which attenuates the signal, thereby returning PrrB in the kinase-dominant mode. This leads to phosphorylation of PrrA, resulting in the induction of PS genes. The nature of the inhibitory signal emanating from the *cbb*$_3$ oxidase is that which increases the phosphatase activity of PrrB while keeping the kinase activity unchanged.

C-PrrB lacking the transmembrane domain. This is at least in part due to the lower autophosphorylation rate of C-PrrB relative to the full-length PrrB. In vivo studies using a yeast two-hybrid system recently demonstrated that the truncated form of NtrB lacking the N-terminal sensing domain interacts with its cognate NtrC response regulator. However, protein−protein interactions between the truncated NtrB and NtrC were shown to be much weaker than that between the full-length NtrB and NtrC (*26*), suggesting that phosphotransfer activity from phosphorylated C-PrrB to PrrA might be compromised due to the absence of the N-terminal sensing domain. In light of these observations it is reasonable to assume that the N-terminal sensing domain of the histidine kinase is important not only for its sensing function but also for the maintenance of a correct conformation which provides both optimal autokinase activity and protein−protein interactions between the histidine kinase and its cognate response regulator. Finally, it is evident that electron flow through the *cbb*$_3$ oxidase in vitro is not taking place. This suggests that the *cbb*$_3$ oxidase can generate the inhibitory signal, although weak, even in the absence of oxygen, which is in keeping with the in vivo result that overexpression of the *ccoNOQP* operon leads to a decrease in PS gene expression under anaerobic conditions.

On the basis of our previous in vivo results (*9*, *14*, *16*, *17*) as well as those described here, we present a model for $O_2$ sensing through the *cbb*$_3$-PrrBA signal transduction pathway (Figure 5). The intrinsic (default) state of the PrrB histidine kinase is in the kinase-dominant mode. The *cbb*$_3$ oxidase generates an inhibitory signal which is transferred either directly or indirectly and which shifts the equilibrium between the relative kinase/phosphatase activities of PrrB toward the phosphatase activity. The inhibitory signal emanates from the *cbb*$_3$ oxidase under both aerobic and anaerobic conditions. However, the strength of the inhibitory signal is proportional to the extent of electron flow through the *cbb*$_3$ oxidase, which is dependent on $O_2$ availability: the greater the electron flow through the *cbb*$_3$ oxidase (the higher the $O_2$ tensions), the stronger the inhibitory signal generated by the *cbb*$_3$ oxidase. Under high $O_2$ conditions the magnitude of electron flow through the *cbb*$_3$ oxidase results in the generation of the strong inhibitory signal which alters the PrrB kinase/phosphatase activity toward the phosphatase-dominant mode. Therefore, under these conditions, PS genes are not induced. When $O_2$ tensions are reduced or under anaerobic conditions, the reduced or weakened electron flow through the *cbb*$_3$ oxidase dampens the inhibitory signal and PrrB returns to its default state, i.e., the kinase-dominant mode, to induce PS gene expression. Here we have demonstrated for the first time that the nature of the inhibitory signal emanating from the *cbb*$_3$ oxidase is that which increases the phosphatase activity of PrrB without alteration of the potential PrrB kinase activity.

Recently, it was suggested that Cys-265 of RegB (PrrB homologue in *Rhodobacter capsulatus*) has a role in controlling the RegB autokinase activity through the formation of an intermolecular disulfide bond in response to redox changes (*27*). Since the Cys-265 residue is located in the cytoplasmic domain, this result alone cannot explain the constitutive expression of the PrrB regulon in PrrB mutant strains having changes in the transmembrane domain of PrrB nor the effect of the overexpression of the *ccoNOQP* operon upon PS gene expression. Similarly, the effect of the overexpression of *prrB* under aerobic conditions upon PS gene expression is also difficult to reconcile with these observations.

The *prrC* gene is located immediately upstream of *prrA* and forms the *prrCA* operon together with *prrA* (*6*, *8*). The PrrC protein is a membrane-associated protein with a single membrane-spanning helix (*8*). It contains two conserved cysteine residues in its periplasmic domain, which are involved in binding of a copper ion (*28*). PrrC was suggested to serve as a chaperone protein to deliver a copper ion to the cytoplasmic domain of RegB (PrrB) (*27*). However, this would not seem to be the case for *R. sphaeroides* since the copper-binding domain of PrrC is in the periplasm. At the present time we find it so difficult to explain these disparate observations that much more needs to be done. Inactivation of *prrC* leads to aerobic derepression of PS genes, which is similar to the Cco-minus phenotype (*8*). However, the extent of PS gene expression in PrrC-minus mutants grown under aerobic conditions is not as strong as that observed for Cco-minus mutants and is intermediate between those of the wild type and Cco-minus mutants grown under the same conditions. From these observations we have suggested that the membrane-bound PrrC protein could transmit the inhibitory signal from the *cbb*$_3$ oxidase to PrrB (*8*), but other possibilities exist. In these experiments, we have not included the PrrC protein, yet the direct role of the *cbb*$_3$ oxidase on PrrB is clear. There are several possible explanations: (i) PrrC has a role other than a direct link between the *cbb*$_3$ oxidase and PrrB, perhaps to either stablize this connection or facilitate this interaction in the membrane, thereby enhancing the signal transduction between the *cbb*$_3$ oxidase and PrrB. (ii) Another possibility is that the absence of PrrC in the membrane alters the conformation of the PrrB protein such that the phosphatase activity is decreased. (iii) PrrC is required for PrrB to sense the extent of electron flow through the *cbb*$_3$ oxidase.

Using this established in vitro system opens the door for numerous additional studies which will be aimed at reconstructing the complete system including the $cbb_3$ oxidase and PrrB, as well as the PrrC protein and the Rdx system (*29*), which have been suggested to be involved in the $cbb_3$-PrrBA signal transduction pathway. Further, through the use of mutant forms of these proteins already in hand, we should be able to assess the mechanism involved in the $cbb_3$-PrrBA signal transduction.

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