Superoxide Generation by Chlorophyllide a Reductase of Rhodobacter sphaeroides

Received for publication, September 17, 2007 and in revised form, December 12, 2007 Published, JBC Papers in Press, December 12, 2007, DOI 10.1074/jbc.M707774200

Eui-Jin Kim 1, Ju-Sim Kim 1, Il-Han Lee, Hae J. Rhee, and Jeong K. Lee 2

From the Department of Life Science and Interdisciplinary Program of Integrated Biotechnology, Sogang University, Seoul 121-742, Korea

Chlorophyllide a reductase of Rhodobacter sphaeroides, which were reconstituted with the purified subunits of BchX, BchY, and BchZ, reduced ring B of chlorophyllide a using NADH under anaerobic conditions. Interestingly, suppressor mutations rescuing the inability of R. sphaeroides Fe-SOD mutant to grow in succinate-based minimal medium were predominantly mapped to BchZ subunit of chlorophyllide a reductase. The enzyme is labile in the presence of O₂. However, it generates superoxide at low O₂. The enzymes reconstituted with BchX, BchY, and the mutein subunit of BchZ from suppressor mutants showed less activity not only for chlorophyllide a reduction but also for superoxide generation compared with the enzyme reconstituted with the wild-type subunits. BchX, which contains FMN, and BchY are iron-sulfur proteins, whereas BchZ is a hemoprotein containing b-type heme. Neither chlorophyllide a reduction nor superoxide generation was observed with the enzyme reconstituted with the wild-type subunits of BchX and BchY, and the apo-subunit of BchZ that had been refolded without heme, in which FMN of BchX was fully reduced. Thus, superoxide is generated not from FMN of BchX but from heme of BchZ. Consistently, the heme of BchZ mutiens was half-reduced in its redox state compared with that of wild-type BchZ.

Rhodobacter sphaeroides, a facultative photosynthetic bacterium, contains two SODs; CuZn-SOD is detected only under the conditions where photosynthetic complexes are formed (1), whereas Fe-SOD is constitutively expressed, although its activity of the aerobically grown cell nearly doubled as compared with that of the anaerobically grown cell. The role of CuZn-SOD in protecting the photoheterotrophic cells from periplasmic superoxide upon exposure to O₂ was proposed (1). A cam-

bialistic SOD using manganese or iron is the only SOD found in Rhodobacter capsulatus and is essential for cell viability (2, 3). Oxidative stress defense in R. sphaeroides and R. capsulatus is also mediated by catalases as well as by glutathione-glutaredoxin and thioredoxin systems that act as thiol-disulfide redox buffer to reduce the protein thiols that were oxidized (4, 5). Mutations in glutathione-glutaredoxin and thioredoxin systems lowered the formation of the photosynthetic complex (4, 5).

The formation of photosynthetic complexes of R. sphaeroides is redox-dependent. Lowering oxygen tension induces the formation of intracytoplasmic membrane housing the photosynthetic complexes of R. sphaeroides. Light captured by B800–850 and B875 LH complexes is transferred to reaction center complex, where the redox reactions are initiated to convert light energy into ATP and reducing power. The oxygen-regulated expression of apoproteins of LH and reaction center complexes, which are encoded by puc, puf, and puh operons, was elucidated (for reviews see Refs. 6 and 7).

The expression of several enzymes for Bchl a synthesis is also subject to anaerobic induction (8–10). Proporphyrin IX, which is synthesized from 5-aminolevulinic acid, is a common intermediate for heme and Bchl a. It can be easily chelated with magnesium ion, giving Mg-proporphyrin IX. Magnesium-chelatase has much lower Kₘ for proporphyrin IX than ferrochelatase (11, 12). Mg-proporphyrin IX is metabolized to Chlide a, which is either used as a direct precursor of chlorophyll a or further converted to bacteriochlorophyllide a, a precursor of Bchl a (13). The first step of Chlide a metabolism is the reduction of its ring B by a nitrogenase-like enzyme, chlorophyllide a reductase (COR) (14, 15), which is composed of BchX, BchY, and BchZ.

In an effort to understand the role of cytosolic SOD in R. sphaeroides, we disrupted sodB coding for Fe-SOD. sodB mutant SodB1 grows in LB medium but not in Sis minimal medium. Suppressor mutations enabling SodB1 to grow in Sis minimal medium were mainly mapped to BchZ, a hemoprotein subunit of COR, whose other subunits BchX, which contains FMN, and BchY are iron-sulfur proteins. Consistently, COR was found to generate superoxide radical from its reaction at low O₂. Suppressor mutations affected the reduction level of BchZ heme, where superoxide radical is generated.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

R. sphaeroides 2.4.1 was used as a wild-type strain and cultured at 28 °C in Sis minimal medium (16) as described previously (1, 17). Tp and Rif were used at 25 and 50 μg/ml, respectively. Other antibiotics were added as indicated previously (1).
Detection and Determination of SOD Activity
Preparation of cell extracts, electrophoresis of a native polyacrylamide (13.5%) gel, staining of SOD activity, and the quantification of the relative SOD levels between samples were performed as described previously (18, 19). Proteins were determined by a modified Lowry method using bovine serum albumin as a standard (20).

Spectrophotometric Assay of Spectral Complexes and Determination of Bchla
Preparation of membrane fraction of R. sphaeroides grown exponentially under anaerobic dark (with Me₂SO) and photosynthetic growth, with maximum absorbance (Tcr) (29)) to generate pSUPZ100. It was mobilized into R. sphaeroides RbB1 (Kmr and Tps) on Sis minimal agar plate containing sucrose (15%) with Kmr and Tps. The Bchla was mobilized into bchZ mutant (Kms) to obtain bchZ-bchF mutant (Kms and Tps) on Sis minimal agar plate containing sucrose (15%), respectively, contain HindIII site (in boldface). PCR fragments were digested with BamHI and HindIII and cloned into pRK415 downstream (PstI site in boldface) and reverse primer (5'-CGCTAGCTGCGACGTGATATCAGTCGTCGACGTGAC-3'; EcoRV site is in boldface). PCR product was cloned into T-vector, followed by digestion with EcoRV and Sall. The Pstl-EcoRV upstream DNA and EcoRV-Sall downstream DNA were cloned into Pstl-Sall sites of a suicide plasmid pLO-tp (Tp₄), to yield pLO-tp-bchF, in which a 324-bp DNA between 24th and 132nd residues of BchF was deleted. The plasmid was mobilized into bchZ mutant (Kms) to obtain bchZ-bchF mutant (Kms and Tps) on Sis minimal agar plate containing sucrose (15%).

Construction of bchC-lacZ Transcriptional Fusion Plasmids and β-Galactosidase Activity Assay
A 1.1-kb Pstl-EcoRV DNA extending from 1,004 bp upstream from the initiation codon of bchC to its 28th codon was transcriptionally fused to lacZ (23) on IncQ plasmid (24) to generate pCZ1004. The resulting plasmid was mobilized from Escherichia coli S17-1 into R. sphaeroides by conjugation as described previously (25), and the β-galactosidase activity (Miller units) was determined as described previously (26).

Construction of Mutants
Construction of sodB Mutant SodB1—The Stul site (Fig. 1) of sodB within a 714-bp Pstl-Xhol DNA was interrupted with a 2.0-kb transcription and translation stop Ω (Sm'/Sp-resistant (Sm'/Sp') DNA (27), and the resulting DNA fragment was cloned into a suicide plasmid pLO1 (Km-resistant (Km')) (28) to generate pDCS100. It was mobilized into R. sphaeroides, and the single crossover exconjugant (Kmr) was isolated and subjected to segregation to double crossover (mutant SodB1: Km and Sm'/Sp') on LB agar plate containing Sm, Sp, and sucrose (15%).

Construction of bchZ Mutant—The BamHI site of bchZ within a 3.9-kb Ball-Pstl DNA spanning bchXYZ was interrupted with a 2.2-kb Ω (Km') DNA, and the resulting DNA fragment was cloned into a suicide plasmid pSUP202 (Tc-resistant (Tc') (29)) to generate pSUPZ100. It was mobilized into wild type to obtain bchZ mutant (Km' and Tc') on Sis minimal agar plate containing Km.

Construction of bchZ-sodB Mutant—sodB of bchZ mutant was disrupted. The 2.7-kb DNA of sodB::Ω (Sm'/Sp') from pDCS100 was cloned into pSUP202 to generate pSUPB100. It was mobilized into bchZ mutant (Km') to obtain bchZ-sodB mutant (Km', Sm'/Sp', and Tc') on LB agar plate containing Km, Sm, and Sp.

Construction of bchZ-bchF Mutant—A 520-bp DNA upstream from the 24th residue of BchF was PCR-amplified with forward primer (5'-CACCCGGATCCGTCGACGGTATATCAGTCGTCGACGTGAC-3'; the Pstl site is in boldface and the mutated sequence is underlined unless noted otherwise) and reverse primer (5'-GGCTAGCTGCGACGTGATATCAGTCGTCGACGTGAC-3'; EcoRV site is in boldface). PCR product was cloned into T-vector, followed by digestion with Pstl and EcoRV. Likewise, a 455-bp DNA downstream from the 132nd residue of BchF was amplified with forward primer (5'-GATATCAGTCGACGTGATATCAGTCGTCGACGTGAC-3'; EcoRV site is in boldface) and reverse primer (5'-GTGAGATCCGTCGACGTGATATCAGTCGTCGACGTGAC-3'; Sall site is in boldface). PCR product was cloned into T-vector, followed by digestion with EcoRV and Sall. The Pstl-EcoRV upstream DNA and EcoRV-Sall downstream DNA were cloned into Pstl-Sall sites of a suicide plasmid pLO-tp (Tp₄), to yield pLO-tp-bchF, in which a 324-bp DNA between 24th and 132nd residues of BchF was deleted. The plasmid was mobilized into bchZ mutant (Km') to obtain bchZ-bchF mutant (Km' and Tp₄) on Sis minimal agar plate containing sucrose (15%).

Overexpression and Purification of BchX, BchY, and BchZ
Purification with His Tag—A 308-bp insert DNA, including R. sphaeroides rrnB promoter (31), was cloned into pRK415 to yield pRKrrnB. Then a 142-bp Xbal-BamHI DNA encompassing a coding region of His₆ tag of pRSET-C (Invitrogen) was cloned at the cloned downstream of R. sphaeroides rrnB promoter to generate pRKrrnB-His. The DNAs of bchX, bchY, and bchZ were PCR-amplified from plasmid pSKXYZ, a recombinant pBluescript SK(-) having a 4.2-kb Ball-HincII insert DNA spanning bchXYZ. Forward primers 5'—CCGCGAAGAGGAGATCCGATCTGCA-GAG-3' (BamHI site is in boldface), 5'-GGCCGAGGAGATCCGATCTGCA-GAG-3' (BamHI site is in boldface), and 5'-GATTCTAGATCTGCA-GAG-3' (BamHI site in boldface) contain BamHI/BglII sites that were changed from the initiation codons of bchX, bchY, and bchZ, respectively. Reverse primers 5'-CGCGAAGAGGAGATCCGATCTGCA-GAG-3', 5'-TGCGAGGAGATCCGATCTGCA-GAG-3', and 5'-CGCGAAGAGGAGATCCGATCTGCA-GAG-3' for the amplification of bchX, bchY, and bchZ, respectively, contain HindIII site (in boldface). PCR fragments were digested with BamHI (or BglII) and HindIII and cloned into BamHI-HindIII site of pRKrrnB-His to yield prrnB-hX, prrnB-hY, and prrnB-hZ (for His₆-tagged proteins of BchX, BchY, and BchZ, respectively). Primers prrnB-hZ V90E, and prrnB-hZ C310Y (for His₆-tagged BchZ S74L, 5% CO₂, and 90% N₂. They were removed for centrifugation and returned to the chamber before being opened. All the following procedures for the purification of proteins were performed in an anaerobic chamber. Cells were broken by sonica-
S

Superoxide from Chlorophyllide a Reductase Reaction

tion, followed by purification of His6-tagged proteins by nickel-nitrilotriacetic acid-agarose (Qiagen, Hilden, Germany) column chromatography. If necessary, His6 of the expressed proteins was cleaved off with enterokinase (New England Biolabs).

Purification with Strep Tag II—The DNAs of bchX and bchYZ were PCR-amplified from plasmid pSKXYZ. Forward primers Fx1 (5'-GAG GCC GAGGAC ATG ACG GAT GC-3'; SacI site is in boldface) and Fy1 (5'-GGC AGG TAC CAT GAG CCA GAC-3'; KpnI site is in boldface) and Fz1 (5'-GAG CCA TGT TGA CG-3') correspond to the sites downstream from bchX and bchZ, respectively. Primer sets Fx1-Rx1 and Fz1-Rz1 were used to amplify bchX and bchYZ respectively. Reverse primers Rx1 (5'-GAG GCC GAGGAC ATG GCC TC-3') and Rz1 (5'-GGA CCG TCG TGT TGA CG-3') are the amplification codons of bchX and bchY, respectively. Primer sets Fx1-Rx1 and Fy1-Rz1 were used to amplify bchX and bchYZ respectively. bchX DNA was digested with ScaI and NcoI, whereas bchYZ DNA was digested with KpnI and HinClI (NcoI and HinClI are the sites between the amplified genes and reverse primers), and cloned into pASK-IBA7plus (IBA, Göttingen, Germany) to direct them between XbaI and HindIII. The resulting XbaI-HindIII DNAs were cloned into XbaI-HindIII sites of pRKrrnB to generate prnB-sX and prnB-sYZ (for Strep-tagged BchX and Strep-tagged BchY co-expressed with BchZ, respectively). Plasmids prnB-sYZ574L, prnB-sYZ906E, and prnB-sYZ310Y (for Strep-tagged BchY co-expressed with BchZ574L, BchZ906E, and BchZ310Y, respectively) were also constructed in the same way as prnB-sYZ. Strep-tagged proteins were purified in anaerobic chamber by affinity chromatography on Strep-Tactin-Sepharose columns (IBA, Göttingen, Germany) after the expression of recombinant plasmids in bchCXYZ mutant.

Purification and Detection of Chlide a

bchZ-bchF mutant was cultured in Sis minimal medium under anaerobic dark conditions (with Me2SO) until culture turbidity reached ~100 Klett units. The pigment in culture supernatant was extracted and dried as described previously (15). The dried pigment was dissolved in Me2SO and used as a substrate for COR. The concentration of Chlide a was determined after dissolution of the dried pigment in 80% acetone based on the extinction coefficient of 77.1 mm$^{-1}$ cm$^{-1}$ as described previously (15).

COR Reaction

An equimolar (8.0 nm) mixture of BchX and co-purified BchY-BchZ was put in a 10-ml glass vial containing 2-ml reaction mixture (0.1 M potassium phosphate (pH 7.4), 300 μM NADH, 5 mM MgCl2, and an ATP-generating system (2 mM Mg$^{2+}$-ATP, 5 mM creatine phosphate, and 40 units/ml creatine phosphokinase)). It was tightly sealed with rubber cap and flushed with N2 for 10 min, followed by incubation at 30 °C in the dark for 30 min to reconstitute the enzyme. Alternatively, an equimolar (8.0 nm) mixture of BchX, BchY, and BchZ was put in 0.1 M potassium phosphate (pH 7.4) buffer containing sodium cholate (2 mM) and incubated at 30 °C in the dark for 30 min to reconstitute the enzyme. After removal of detergent by dialysis against the same buffer for 1–2 h, the enzyme was mixed in reaction mixture, and reaction in glass vial was initiated by adding Chlide a (1–2 μM in Me2SO). An aliquot (200 μl) of assay mixture was taken out after reaction in the dark and mixed with 800 μl of acetone to stop the reaction. The pigment was extracted with hexane as described (15) and analyzed for absorption spectrum from 550 to 800 nm with UV 2550-PC spectrophotometer (Shimadzu, Japan). The reaction product 3-vinyl bacteriochlorophyllide a was determined based on the extinction coefficient 44.7 mm$^{-1}$ cm$^{-1}$ of its Qy peak (15).

Determination of Half-lives of COR and Its Subunits during Exposure to O2

BchXYZ complex (80 nm) or each subunit (80 nm) was put in 2 ml of 0.1 M potassium phosphate (pH 7.4) buffer and sparged at 20 ml/min with a gas mixture of 20% O2 and 80% N2 (DO, 230 ± 8 μM) or the mixture of 2% O2 and 98% N2 (DO, 42 ± 6 μM). An aliquot (8.0 nm) of BchXYZ complex was taken out intermittently and measured for enzyme activity, whereas an aliquot of a subunit was taken out and mixed in equimolar ratio with the other two subunits, which had not been exposed to O2, to reconstitute BchXYZ in the presence of cholate prior to enzyme reaction. The proteins sparged with N2 (20 ml/min) were included as positive controls. Stability was expressed as percentage of activity by the enzyme that had not been exposed to O2. The experiments were repeated three times, yielding similar results; data shown are the representatives of triplicate experiments.

Superoxide Determination

EPR Measurement—EPR was measured on a Bruker model EMX spectrometer. COR reaction was performed under the gas phase of 2% O2 and 98% N2 (DO, 29 ± 3 μM) in the presence of 150 mM DMPO (Sigma). Desferoxamine (100 μM) was included to chelate iron impurities. After COR reaction at 30 °C in the dark for 10 min, the mixture was placed in quartz EPR flat cell, and the spectrum was recorded at ambient temperature. The EPR-settings were as follows: modulation amplitude 0.32 G, time constant 0.16 s, scan time 60 s, modulation frequency 100 kHz, microwave power 20 milliwatts, and microwave frequency 9.76 GHz (32). A standard superoxide-generating mixture of xanthine (100 μM) and xanthine oxidase (0.004 units) in 0.1 M potassium phosphate (pH 7.4) buffer (33) under the gas phase of 20% O2 and 80% N2 (DO, 205 ± 11 μM) was included as a positive control.

Cyt c Reduction Assay—COR reaction was performed with 20 μM Cyt c under the gas phase of 2% O2 and 98% N2, and aliquots (200 μl) were taken out intermittently to measure the reduced Cyt c (550 nm) in a 1-ml anaerobic cuvette based on the net extinction coefficient (reduced-oxidized) of 21.0 mm$^{-1}$ cm$^{-1}$ as described previously (34, 35). Superoxide production was examined from the difference in the rates of Cyt c reduction in the absence and presence of E. coli MnSOD (10 units).

Detection, Identification, and Determination of Flavin

Protein was digested with trypsin and chemotrypsin, heat-denatured to coagulate undigested protein, and centrifuged to obtain supernatant of cofactor extract and analyzed for absorption spectrum from 550 to 800 nm with UV 2550-PC spectrophotometer (Shimadzu, Japan). The reaction product 3-vinyl bacteriochlorophyllide a was determined based on the extinction coefficient 44.7 mm$^{-1}$ cm$^{-1}$ of its Qy peak (15).
Shimadzu 10AVP series HPLC system equipped with a Phenomenex Gemini C-18 reversed-phase column was used to distinguish FMN from FAD as described previously (37). FMN was also distinguished from FAD by measuring the ratio of fluorescence at pH 2.6 over pH 7.7 (emission at 535 nm; excitation at 450 nm) with a spectrofluorometer (Quanta masterQM-4/2005, PTI, Lawrenceville, NJ) as described previously (38). The concentration of free FMN was calculated with molar extinction coefficient of 12.2 mM$^{-1}$ cm$^{-1}$ at 450 nm (39).

**Heme Staining and Heme Binding Assay**

Proteins for heme staining were prepared in 62.5 mM Tris-HCl buffer (pH 8.0) containing 2% SDS and 10% glycerol and incubated at 37 °C for 15 min. Heme staining was done after low temperature (4 °C) SDS-PAGE as described previously (40, 41).

Heme binding to BchZ was assayed by absorption spectrophotometry following equilibrium dialysis. One-mL solution of BchZapo or BchZmapo (10 µM), which had been refolded in the absence of heme, was dialyzed at 4 °C for 12 h against 1.0 liter of 0.1 M potassium phosphate (pH 7.4) buffer containing the varying amounts of hemin. Unbound heme was removed by dialysis against the same buffer under the same conditions. The heme content of BchZ was measured in an anaerobic cuvette based on 20.9 ± 0.5 mm$^{-1}$ cm$^{-1}$ at 558 nm (α-band) in 0.1 M potassium phosphate (pH 7.4) buffer, which was experimentally determined from the extinction coefficient of 34.4 mm$^{-1}$ cm$^{-1}$ for hemin in pyridine hemochrome (42).

**Refolding of Denatured BchZ**

BchZ in 50 mM Tris-HCl (pH 8.0) buffer containing 150 mM NaCl and 1 mM dithiothreitol was denatured under anaerobic conditions at 4 °C in the presence of 6 M GdnHCl. Renaturation was carried out under the same conditions through stepwise dialysis (3 h per step) against the same buffer containing decreasing concentrations of GdnHCl. The renaturation of polypeptide was followed by scanning the emitted fluorescence between 300 and 400 nm after excitation at 280 nm (43). Fraction unfolded ($F_U$) was calculated by an equation of $F_U = (Y - Y_N)/(Y_U - Y_N)$, where $Y$ is the observed fluorescence emission at 354 nm, and $Y_N$ and $Y_U$ are the fluorescence emission at 354 nm for the native and fully unfolded conformations, respectively (44).

**Determination of Iron-Sulfur Clusters**

Metal contents of purified BchX, BchY, and BchZ were measured with an ICP mass spectrometer (Elan 6100, PerkinElmer Life Sciences) and ICP atomic emission spectrometer (138 Ultrace, Jobin Yvon, Edison, NJ). The contents of Sulfide ($S^{2-}$) and sulfane sulfur ($S^0$) in each protein were analyzed as described previously (45).

EPR measurements to determine iron-sulfur clusters of purified proteins were performed on an X-band ESR spectrometer (JES-PX2300, JEOL, Japan) equipped with a liquid helium flow cryostat (ESR900A, Oxford, UK). The EPR settings were as follows: modulation amplitude 0.5 millitesla, time constant 0.03 s, modulation frequency 100 kHz, microwave power 2.0 milliwatt, and microwave frequency 9.05 GHz.

**RESULTS**

*R. sphaeroides* sodB Mutant Grew in LB Medium but Failed to Grow in Sis Minimal Medium—We were never successful in obtaining sodB mutant of *R. sphaeroides* on Sis minimal agar plate even under anaerobic dark (with Me$_2$SO) and photoheterotrophic conditions. The sodB mutant SodB1 (Fig. 1A), however, was observed on LB agar plate with a frequency (number of double-crossover colonies per CFU of the wild-type cells spread on the medium) comparable with that routinely observed for the disruption of other genes such as *pucB* and *pufB* (data not shown). The growth of SodB1 in Sis minimal medium was restored with sodB in trans. SodB1 was cultured and maintained in LB unless stated otherwise. CuZn-SOD expression was not changed by the lack of Fe-SOD (Fig. 1B). SodB1 grew slowly under aerobic and photoheterotrophic conditions to show doubling times of 5 and 4 h, respectively, compared with 3-h doubling of wild-type cells. The amount of LH...
complexes of SodB1, however, was not different from that of wild-type cells (Table 1).

SodB1 grew to form colonies on Sis minimal agar plate supplemented with lysine (7 mM). Each or all of the other 19 amino acids were not effective in supporting the growth of SodB1 in minimal medium. SodB1 may be considered an auxotroph like the cytosolic SOD mutant of *E. coli* (46, 47). However, SodB1 grew on Sis minimal medium supplemented with cadaverine (7 mM), which is a lysine decarboxylase reaction product, and also known to scavenge superoxide (48, 49). Consistently, we failed to disrupt *sodB* of lysine decarboxylase (RSP1991) mutant of *R. sphaeroides* even on LB medium.

Spontaneous mutation conferring resistance to rifampin (Rif) on SodB1 was determined in LB medium and compared with that of wild-type cells to examine whether SodB1 is genetically stable in LB medium. The mutation frequency under aerobic conditions was expressed in the number of Rifr colonies as a proportion of total viable count from triplicate experiments, each with duplicate colony counts. The frequency (0.8 ± 0.3 × 10⁻⁶) observed with SodB1 was not higher than that (1.8 ± 0.3 × 10⁻⁶) determined with wild-type cell, indicating that SodB1 is genetically stable during growth in LB medium.

Suppressor Mutations Rescuing the Inability of SodB1 to Grow in Sis Minimal Medium Were Predominantly Mapped to *bchZ* of COR—SodB1 was grown aerobically in LB medium, and a 0.1-ml aliquot (10⁶–0⁷ CFU) was spread on Sis minimal agar plate after a brief wash with the minimal medium. Two kinds of suppressor mutants showed up after a 7-day incubation under aerobic conditions. Pale pigmented colonies (collectively grouped as BsupA) were obtained with a frequency of 1.4 ± 0.8 × 10⁻⁴ from the total viable counts on LB medium, whereas the other wild-type pigmented colonies (collectively grouped as BsupB) showed up with a frequency of 9.2 ± 0.3 × 10⁻⁶. The representative isolates of BsupA mutant BsupA1, BsupA2, and BsupA3 were chosen for further analyses. The B800–850 and B875 LH complexes of BsupA mutants reached 40–65% of the wild-type level (Table 1). However, the -galactosidase activities from *puc::lacZ* (24) and *puf::lacZ* (8) transcriptional fusion constructs in trans in BsupA were not different from those measured in wild-type cell under aerobic and anaerobic dark conditions (with Me₂SO) (data not shown). Thus, any transcription regulator(s) governing *puc* and *puf* expression did not appear to be associated with the suppressor mutation of BsupA.

Pale pigmentation of BsupA mutants was complemented with a cosmid clone pUI8487 (31) of *R. sphaeroides*. The DNA was further narrowed down to *bchZ* coding for a subunit of COR composed of BchX, BchY, and BchZ (Fig. 2). When *bchZ* was mobilized into BsupA, the exconjugant stably grew on LB but failed to grow in Sis minimal medium. They were easily

---

4 E.-J. Kim and J. K. Lee, unpublished observations.

---

**FIGURE 2.** *In vitro* COR reaction and Bchl *a* biosynthetic pathway (13). A, formation of 3-vinyl bacteriochlorophyllide *a* was examined with the enzyme complex that had been reconstituted with equimolar (8.0 nM) mixture of three subunits without cholate (X + Y + Z) or with cholate (X + Y + Z + cholate). The reaction by the enzyme reconstituted with BchX and co-purified BchY-BchZ was also examined (X + co-purified Y-Z). B, protoporphyrin IX, which is synthesized from 5-aminolevulinic acid (ALA), is used by either ferrochelatase (*hemH*) or magnesium chelatase (*bchH* and *bchlD*). The B-ring of Chlorophyllide *a* or 3-hydroxyethyl chlorophyllide *a* is reduced by COR (BchXYZ) to form 3-vinyl bacteriochlorophyllide *a* or 3-hydroxyethyl bacteriochlorophyllide *a*. The absorption maxima of Qy bands of the intermediates in 80% acetone are shown in parentheses. Only genes mentioned in the text are illustrated.
mutated therein to generate BsupA-type and BsupB-type mutants again with frequencies of 2.4 (±0.7) × 10⁻⁴ and 1.2 (±0.2) × 10⁻⁵, respectively. The same was true with 10 other BsupA mutants, which had been randomly isolated in several independent trials. Thus, the complementation of BsupA pigmentation with bchZ was also accompanied with SodB1-like growth phenotype.

It was determined whether the suppressor mutation of BsupA was caused by any change in the level of bchZ expression. Because the genes coding for BchX, BchY, and BchZ are transcribed in an operon of bchCXYZ (14), in which bchC codes for 3-hydroxyethyl bacteriochlorophyllide a dehydrogenase (13), bchC::lacZ transcriptional fusion construct was used to examine bchZ expression. The β-galactosidase activity from the fusion construct in trans in BsupA was not different from those measured in SodB1 and wild-type cell under aerobic and anaerobic dark conditions (with Me₃SO) (data not shown). Thus, neither sodB mutation nor suppressor mutation of BsupA affected bchZ expression.

DNA sequence analysis of bchZ cloned from the chromosome of BsupA1 revealed a substitution mutation of leucine for serine at its 74th residue (S74L), whereas bchZ of BsupA2 and BsupA3 contained mutations of V90E and C310Y, respectively. Identification of BchZ as a major suppressor for sodB mutation further suggests the generation of oxidative stress by COR.

We have interrupted sodB of another strain of R. sphaeroides KCTC 12085 that had been isolated for its higher ability to produce molecular hydrogen under phototrophic conditions (50). Its sodB mutant was obtained in LB medium as described for 2.4.1. Like SodB1, sodB-disrupted mutant of KCTC 12085 did not grow in Sis minimal medium. Moreover, BsupA- and BsupB-type suppressor mutants were also obtained with frequencies similar to those observed with 2.4.1. BsupA-type mutants from KCTC 12085 were also complemented with bchZ, and the analyses of their genomic DNA sequence revealed missense mutations at bchZ.⁵ Thus, the missense mutation at BchZ of COR to suppress the superoxide stress of R. sphaeroides is not a phenotype unique to 2.4.1.

COR Activity Was Illustrated by the Enzyme Reconstituted with BchX, BchY, and BchZ—The amino acid sequence of BchX reveals ~50% similarity to that of NiFH, a dinitrogenase reductase. The conserved regions include an ATPase motif and three cysteine residues, two of which are thought to be involved in chelating a 4Fe-4S cluster (51). Like BchY and BchZ of R. capsulatus (15), cysteine residues of BchY (55th, 80th, and 138th) and BchZ (35th) of R. sphaeroides also appear to be conserved at similar positions compared with those possibly responsible for the chelation of two 4Fe-4S clusters of NifE-NifN complex, another NiFDK-like protein, which is involved in MoFe cofactor biosynthesis (52, 53). Moreover, the co-purification of BchY-BchZ of R. capsulatus supported a nitrogenase model of COR (15). BchY-BchZ of R. sphaeroides was also co-purified when Strep-tagged BchY was purified after co-expression with BchZ (Fig. 3).

Considering the nitrogenase-like structural features, COR may be inactivated by any exposure to oxygen. Accordingly, all the procedures from cell harvest to protein purification were manipulated in an anaerobic chamber. His₆ tag was removed from the purified subunits by enterokinase, and an equimolar mixture (8.0 nm) of three subunits was incubated to reconstitute the enzyme. However, three subunits failed to reduce Chlde a unless they were preincubated in the presence of sodium chloride (2 mm) (Fig. 2A). The preincubation with cholate was not necessary if co-purified Strep-tagged BchY-BchZ was included to reconstitute the enzyme (Fig. 2A). Consistently, the detergent was required for the pretreatment of BchY and BchZ only (data not shown), implying the necessity for the correct interaction between two subunits. There was no difference in the level of 730-nm pigment between reactions by the enzymes reconstituted in two ways (Fig. 2A).

COR Is Labile in the Presence of Oxygen—Because the primary sequence of BchX and the co-purification of BchY-BchZ support a nitrogenase model of the enzyme, its oxygen sensitivity was examined. After a subunit was sparged with 20% O₂ (DO, 230 ± 8 μM) (Fig. 4), aliquots were taken out intermittently and preincubated in equimolar ratio with the other two subunits in the presence of cholate, followed by enzyme assay. BchX was inactivated with a half-life of ~60 min. BchY, also a putative iron-sulfur protein, was more sensitive to show a half-life of ~20 min, whereas BchZ was relatively stable under the conditions (Fig. 4), suggestive of the relative rate of oxygen (54, 55). Moreover, the half-life of BchXYZ complex, which had been sparged with 20% O₂ following the reconstitution of complex, was ~40 min. Thus, COR is labile in the presence of oxygen. Consistently, no COR activity was found under the gas phase of O₂ higher than 5% (DO higher than 65 ± 6 μM). However, ~33% enzyme activity (15.9 ± 2.6 nmol/min/mg protein) was reproducibly observed under the gas phase of 2% O₂ (DO, 29 ± 3 μM), suggestive of the relatively longer half-life of COR

⁵ J.-S. Kim and J. K. Lee, unpublished observations.
Superoxide from Chlorophyllide a Reductase Reaction

A

B

FIGURE 4. Inactivation of COR by O2. A subunit or the reconstituted enzyme was sparged with a gas mixture of 20% O2 and 80% N2, or the mixture of 2% O2 and 98% N2. Aliquots were taken out intermittently. A subunit was mixed with the other two subunits to reconstitute enzyme prior to reaction. The half-life of each subunit and the reconstituted enzyme is shown as mean ± S.D.

at low O2. Indeed, the half-life of BchXYZ complex, which had been sparged with 2% O2 (DO, 42 ± 6 μM), was ~210 min (Fig. 4). All the subunits and enzyme complex were quite stable under anaerobic conditions (100% N2) (Fig. 4).

COR Generates Superoxide at Low O2—It was examined whether superoxide was generated from COR reaction at low O2. Enzyme reaction was performed under the gas phase of 2% O2 (Fig. 5A). Remarkably, EPR spectroscopy revealed signals characteristics of the DMPO-OH spin adduct with quartet showing intensity ratios of 1:2:2:1 and hyperfine coupling constants of $\alpha^N = \alpha^H = 14.89$ G (Fig. 5A). DMPO-OH is formed through spontaneous decay of the superoxide spin adduct DMPO-OOH (54). Xanthine reaction by xanthine oxidase also produced the same DMPO-OH spin adduct (Fig. 5A). Reaction without oxygen resulted in the total disappearance of the EPR signal, and no EPR signal was detected when Chlide a, NADH, ATP-generating system, or one of BchX, BchY, and BchZ was omitted from the reaction mixture (data not shown), implying that superoxide is not chemically yielded through one-electron reduction of O2 from NADH by metallic impurities such as iron (55). The signal was abolished by E. coli MnSOD (Fig. 5A), thus establishing superoxide as the source of DMPO-OH. Therefore, COR indeed generates superoxide at low O2.

Superoxide from COR reaction at varying levels of O2 was determined by SOD-sensitive Cyt c reduction assay (Fig. 5B). Cyt c was reduced by superoxide generated from the enzyme reaction under the gas phase of 2% O2 (Fig. 5B), but no such reduction was observed at higher O2, possibly reflecting the oxygen sensitivity of COR. Hydrogen peroxide, determined using titanium sulfate (56), was not detected (data not shown). Thus, COR specifically generates superoxide radical at low O2 (DO, 29 ± 3 μM DO).

BchX Contains FMN—Chlide a was not reduced when NADH was substituted for NADH in reaction, indicating that NADH is a reductant specific to COR. Dithionite (0.7 mM) was also effective as a reductant (data not shown). Assuming the presence of iron-sulfur cluster in BchX, the electron transfer from NADH to its Fe-S may require flavin. The cofactor extract was prepared from BchX in anaerobic chamber, but its UV-visible spectrum did not show any spectral profile (Fig. 6A). However, a spectrum typical of flavin with the absorbance maxima at 371 and 444 nm was exhibited after sparging of the cofactor extract with a gas mixture of 20% O2 and 80% N2 for 1 min (Fig. 6A). The spectrum disappeared upon reduction with sodium dithionite (data not shown but the same as “reduced” spectrum of Fig. 6A). The HPLC purification of the BchX extract revealed a peak showing the same retention time as that of standard FMN (data not shown). Moreover, the pH-dependent ratio of fluorescence (535 nm) by flavin extract at pH 2.6 over pH 7.7 was 0.74, which was similar to 0.69 of standard FMN but different from 4.44 of standard FAD. Determination of FMN from the absorption (450 nm) of BchX extract under aerobic conditions revealed a molar ratio of ~0.83 (± 0.03), indicative of one FMN per BchX. Thus, BchX contains FMN and may act as an NADH:FMN oxidoreductase subunit.

Determination of Iron-Sulfur Clusters of BchX, BchY, and BchZ—Labile sulfur was barely detected from BchZ, suggestive of no possible iron-sulfur cluster in the protein (Fig. 7C). However, 1.62 and 3.04 mol of labile sulfur were observed per BchX and BchY respectively (Fig. 7C). Labile sulfur content from BchY was not different from that of the BchY-BchZ complex, suggestive of no bridged iron-sulfur cluster between the subunits. Proteins were further examined for seven kinds of metal (vanadium, manganese, iron, cobalt, nickel, copper, and molybdenum). Cobalt and molybdenum were barely detected, whereas vanadium, manganese, nickel, and copper were detected at the level close to detection limit (Fig. 7C). Consistently, BchX and BchY upon oxidation with ferricyanide resulted in absorption increase with a pronounced shoulder at ~410 nm, a characteristic of iron-sulfur cluster (Fig. 6B).
Superoxide from Chlorophyllide a Reductase Reaction

FIGURE 5. Superoxide generation by COR at low O2. A, EPR spectrum resulting from the COR reaction at 2% O2 was examined in the presence of the spin trap DMPO (150 mM). The same reaction by COR in the presence of E. coli MnSOD (10 units) is also shown. A standard superoxide-generating reaction by xanthine oxidase at 20% O2 was included as a control. B, superoxide from COR reaction at varying O2 was measured by SOD-sensitive Cyt c reduction method (upper panel). The quantitative value of Cyt c reduced by superoxide (lower panel) is shown as mean ± S.D.

FIGURE 6. Cofactors of BchX and BchZ. A, UV-visible absorption spectra of the reduced and oxidized cofactor extract of BchX are shown. B, spectral profiles of each subunit of BchXYZ are shown after treatment with 2 mM sodium dithionite (solid line) or 1 mM ferricyanide (dotted line). The bands typical of b-type heme of BchZ are illustrated with α at 558 nm, β at 529 nm, and Soret (γ) at 425 nm. C, SDS-PAGE of the three subunits of BchXYZ (left panel) and the heme-stained SDS-PAGE of the same sample (right panel) are shown. Each lane contained 1 µg of protein. Lane m contains molecular marker.

The iron-sulfur clusters present in BchX and BchY were characterized by measuring EPR spectra of the proteins after reduction with dithionite (10 mM) or oxidation with ferricyanide (1 mM). BchX upon reduction with dithionite exhibits EPR signals with g1 = 2.02, g2 = 1.93, and g3 = 1.92 (Fig. 7A). The value of g2 is characteristic of [2Fe-2S]1+ or [4Fe-4S]1+ centers (57–59), and the EPR signals were significantly decreased upon oxidation with ferricyanide. Thus, the contents of iron and labile sulfur and EPR spectra of BchX indicate the presence of [2Fe-2S]1+ per protein molecule or [4Fe-4S]1+ per two protein molecules. It remains to be determined whether BchX forms a homodimeric structure or heteromultimeric complexes with other Bchl a biosynthetic enzymes. An EPR signal at g = 2.00 (Fig. 7A), which may reflect flavo-semiquinone radicals (59, 60), was intensified when BchX was exposed for 3–5 min to air after reduction with dithionite (data not shown), indicative of the presence of FMN.

BchY upon oxidation with ferricyanide revealed EPR signals with g1 = 2.04, g2 = 2.00, and g3 = 1.97 (Fig. 7B), which were significantly attenuated upon reduction with dithionite. The appearance of EPR signal upon oxidation and its characteristic g2 value suggest the presence of [3Fe-4S]1+ or [4Fe-4S]3+ cluster in BchY (57, 61), which is further corroborated by its higher contents of iron and labile sulfur compared with BchX. The co-purified BchY-BchZ also showed EPR signals similar to those of BchY. Consistently, no EPR signals reflecting iron-sulfur cluster were revealed with BchZ (data not shown).

BchZ, Which Appears to Provide a Substrate-binding Site, Contains b-type Heme That Is Essential Not Only for Chlide a Reduction but Also for Superoxide Generation—Top-added Chlide a was significantly retained in Strep-Tactin-Sepharose column that held Strep-tagged BchY-BchZ or in nickel-nitrilotriacetic acid agarose column that held His6-tagged BchZ, whereas no such retention was observed with the columns that held Strep-tagged BchX or His6-tagged BchY (data not shown). Thus, BchZ appears to provide a binding site for Chlide a.

Interestingly, the purified subunit of BchZ, when reduced with sodium dithionite, revealed a spectrum characteristic of the noncovalently bound b-type heme (typical bands of α at 558 nm, β at 529 nm, and Soret (γ) at 425 nm) (Fig. 6B). The Soret band was shifted from 425 to 415 nm upon oxidation with ferricyanide (Fig. 6B), indicative of redox-active heme. Neither BchX nor BchY revealed heme spectra. The ability of BchZ to bind heme was also assessed by heme-dependent peroxidase activity, as shown by heme staining in SDS gel (Fig. 6C).

BchZ was denatured in the presence of 6 M GdnHCl, followed by renaturation through stepwise dialysis against the decreasing concentration of GdnHCl in the presence or absence of an equimolar concentration of hemin. BchZref, which had been refolded in the presence of hemin, revealed a spectrum typical of BchZ upon reduction with dithionite (Fig. 8B). No such spec-
The incubation of BchZapo with equimolar concentration irrespectively of the presence of heme (data not shown). Consistently, the blue shift of fluorescence emission maxima (from 354 to 336 nm) during renaturation was also observed after excitation at 280 nm, was not affected by heme (Fig. 8A). The renaturation of polypeptide, which was followed by measuring the emitted fluorescence of exogenous hemin at ambient temperature resulted in a spectrum of hemoprotein (data not shown but the same as that of BchZref of Fig. 8A). Thus, heme of BchZ is essential not only for Chlide a reduction but also for superoxide generation. Because flavin of BchX but heme of BchZ.

COR Reconstituted with BchX, BchY, and BchZ Mutant (BchZm) of BsupA Showed Less Activity Not Only for Chlide a Reduction but Also for Superoxide Generation Compared with Wild-type Enzyme—His6 tags of purified subunits were removed, and the equimolar (8.0 nm) mixtures of BchX, BchY, and BchZm (either BchZS74L, BchZV90E, or BchZC310Y) were preincubated in the presence of cholate to reconstitute the enzyme. COR activities were measured from the spectral profiles of acetone extracts of the reaction mixtures taken out intermittently throughout the reaction and compared with that of wild-type enzyme BchXYZ (Fig. 9A). The BchXYZm showed ~50% COR activity of BchXYZ (Fig. 9A), and this could account for the lower levels of LH complexes of BsupA compared with wild type (Table 1). The SOD-sensitive Cyt c reduction assay also revealed that superoxide generation of BchXYZm is approximately half that of BchXYZ (Fig. 9B), accounting for the nature of BsupA mutations that suppress the lack of Fe-SOD. Given that COR is a major superoxide generator, mutation in bchXYZ should rescue the inability of SodB1 to grow in Sis minimal medium. Consistently, bchZ-sodB double mutant formed the same CFU on Sis minimal agar plate as that appeared on LB agar plate.

Reduction Level of Heme of BchZm from BsupA Is Half That of Wild-type BchZ—It was examined whether the binding between BchY and BchZ was altered by suppressor mutations. SDS-PAGE of Streptagged BchY that was purified after coexpression with BchZ or BchZm revealed the presence of two proteins of 55-kDa (Strep-tagged BchY) and 53-kDa (BchZ) apparent molecular mass that were at similar intensity as proteins of 55-kDa (Strep-tagged BchY) and 53-kDa (BchZ) expression with BchZ or BchZm revealed the presence of two subunits, retained fully reduced flavin (data not shown but the same as the reduced spectrum of Fig. 6A). Thus, it appears that the site for superoxide formation is not flavin of BchX but heme of BchZ.

Spectrophotometry of BchZm following equilibrium dialysis against increasing concentration of hemin showed the heme-binding capacity of the subunit that saturates at ~0.9 heme per BchZ molecule, with an apparent $K_d$ of 5.91 $\times$ 10$^{-7}$ M drawn by Scatchard analysis (Fig. 10). The heme content of BchZ was further corroborated by its iron content (0.88 mol of iron per BchZ) (Fig. 7C).

The same heme-binding capacity was true for BchZS74Lapo and BchZV90Eapo with similar $K_d$. However, ~2.0 heme molecules are required to saturate BchZC310Yapo with 20-fold higher...
K_d values (Fig. 10). Thus, the relative molar ratio of heme bound per BchZ\textsubscript{C310Y} is half that of heme bound to wild-type BchZ when the molar ratio of hemin to protein is less than 1.

The redox state of heme of BchZ\textsubscript{m} from BsupA was examined with co-purified BchY-BchZ and BchY-BchZ\textsubscript{m}. The spectra of co-purified complexes revealed that the relative level of the \(\alpha\)-band (558 nm) shown by BchZ\textsubscript{m} was approximately half that of BchZ (Fig. 11A). Thus, the heme of BchZ\textsubscript{m} is half-reduced compared with that of wild-type BchZ, which may account for the lower superoxide generation by BchXYZ\textsubscript{m}. Heme \(\alpha\)-bands at 558 nm of BchZ\textsubscript{S74L} and BchZ\textsubscript{V90E} were intensified 2-fold to reach the wild-type level when the samples were fully reduced with sodium dithionite (Fig. 11B). However, no further increase of \(\alpha\)-band was observed with BchZ\textsubscript{C310Y}, as shown by % reduced heme of the subunit. This corroborated the result that heme content of BchZ\textsubscript{C310Y} was half that of the wild-type BchZ (Fig. 10). Therefore, the reduction level of heme of BchZ\textsubscript{m} is approximately half that of wild-type BchZ either by altering its redox state as observed with BchZ\textsubscript{S74L} and BchZ\textsubscript{V90E} or by decreasing its heme affinity as observed with BchZ\textsubscript{C310Y}.

Taken together, the major suppressor mutation for sodB mutation of \textit{R. sphaeroides} was mapped to COR, which reduces ring B of Chlide \(a\). Consistently, COR was found to generate superoxide through heme of its BchZ subunit at low O_2.

**DISCUSSION**

The results of this work clearly indicate that COR is the main superoxide generator of \textit{R. sphaeroides}. Because bchCXYZ expression is induced upon lowering O_2 tension (62), and superoxide is generated by COR at low O_2, the formation of photosynthetic complexes under the conditions is accompanied with the generation of intracellular superoxide stress. Thus, the cytosolic Fe-SOD of \textit{R. sphaeroides} is required to decrease oxidative stress caused by COR when the photosynthetic complexes are induced.

As shown by the wild-type-like growth phenotype of BsupA and bchZ-sodB double mutant, the partial or total loss of COR activity enabled SodB\textsubscript{1} to grow in Sis minimal medium. The results, however, do not necessarily mean the lack of other superoxide-generating component(s) in this bacterium, because SodB\textsubscript{1} was unable to grow even in LB medium unless lysine decarboxylase-forming cadaverine is intact. Consistently, SodB\textsubscript{1} grew in Sis minimal medium supplemented with cadaverine, which can scavenge superoxide radicals (48, 49).

Protochlorophyllide oxidoreductase (DPOR) of \textit{R. capsulatus}, which reduces ring D of protochlorophyllide \(a\).
lide to form Chlide a, was characterized in vitro with the purified proteins of BchL, BchN, and BchB, supporting a nitrogenase model of the enzyme complex (63–65). Comparing the primary structures of BchXYZ of COR with BchLN& of DPOR and NifHDK of nitrogenase, significant (~50%) similarities were found to exist between the deduced amino acid sequences of BchX, BchL, and NifH. Cysteine residues (Cys-71, Cys-130, and Cys-165) for iron-sulfur clusters and an ATP-binding motif between Gly-42 and Ser-49 (66) are well conserved. Thus, the proteins in common appear to act as ATP-dependent reductase, transferring electrons from suitable sources, including NADH or ferredoxin, to the catalytic complexes composed of the other two subunits. The comparison of the primary structures between BchY-BchZ, BchN-BchB, NifD-NifK, and NifE-NifN reveals less similarity (15, 63–65), which may reflect their own differing catalytic activities. Our results clearly indicate that BchX and BchY harbor iron-sulfur clusters, but BchZ does not even though it is co-purified with BchY. This result further corroborates a much longer half-life of BchZ in the presence of O2 compared with BchX and BchY.

Because Chlide a specifically binds to BchY-BchZ and BchZ, BchZ appears to acts as a substrate-binding site. Moreover, it was found that the b-type heme of BchZ was essential for Chlide a reduction. However, it remains to be determined how this heme is involved in the path of electron transfer to Chlide a. As far as we know, this is the first example of a Bchl biosynthetic enzyme containing a hemoprotein subunit. The interaction of two tetrapyrroles, one as a cofactor (heme) and the other as a substrate (Chlide a), in BchZ awaits more detailed information about the protein structure. The formation of superoxide through the heme of BchZ may further explain why BsupA suppressor mutations are predominantly mapped to BchZ.

It remains to be determined whether superoxide is generated by DPOR, as no suppressor mutations were mapped to DPOR. DPOR may not generate superoxide, and if it does, it may only be a very low level. The oxygen sensitivity of DPOR, especially at low O2, also remains to be determined.

Chlide a is either used as a direct precursor for the attachment of C20 phytol group to form chlorophyll a or subjected to ring B reduction by COR, followed by conversion of its C-3-vinyl group of ring A into an acetyl group through hydration (BchF) and dehydrogenation (BchC). The resulting bacteriochlorophyllide a is a substrate for the attachment of the same C20 phytol chain to form Bchl a. Thus, COR is mostly found in anoxygenic photosynthetic bacteria synthesizing Bchl a. On the evolutionary path leading to oxygenic photosynthesis, a new shortcut of Bchl a biosynthetic pathway to chlorophyll a might have evolved in response to the rise in atmospheric oxygen levels perhaps before or during the Proterozoic era (~2,500 million years ago to 543 million years ago) (67). Concomitant evolutionary emergence of light-dependent protochlorophyllide oxidoreductase compensated for the sensitivity of DPOR to oxygen (65). The superoxide generation by COR in the presence of oxygen could result in strong oxidative stress in primitive anoxygenic photosynthetic organisms. As a consequence, a new shortcut metabolic pathway, which employs direct attachment of C20

**FIGURE 10.** Heme binding to BchZ and BchZm. Proteins were used at 10 μM. Heme binding to the apoproteins of BchZapo, BchZapo S74L, BchZapo V90E, and BchZapo C310Y was determined by equilibrium dialysis against increasing concentration of hemin, followed by absorption spectrophotometry. Kd is shown for each protein.

**FIGURE 11.** The redox state of heme of BchZ and BchZm. A, BchZapo, BchZapo S74L, BchZapo V90E, and BchZapo C310Y were co-purified with Strep-tagged BchY after expression from prrnB-sYZapo, prrnB-sYZ S74L, prrnB-sYZ V90E, and prrnB-sYZ C310Y, respectively. Wild-type BchZ was also co-purified in the same way using the recombinant plasmid prrnB-sYZ. All the spectra, which were determined with the co-purified proteins, reflect the difference profile of no treatment minus ferricyanide (1 mM) treatment, B, same sample shown in A was reduced with sodium dithionite (2 mM), and the difference spectra of dithionite treatment minus ferricyanide treatment are shown. The % heme reduced before and after sodium dithionite treatment was calculated from a-band of each sample and is shown to the right of the panel.
phytol group to Chlide a, might emerge to bypass COR reaction.

The transcription of bchCXYZ is positively regulated by PrrA (68) and AppA, an anti-repressor for PpsR (69). Interestingly, AppA was found to contain noncovalently bound heme (70, 71), and the recognition of redox signal by heme at low O₂ was proposed to strengthen its interaction with PpsR. Upon lowering O₂ tension, heme coordination by AppA (71) would be a prerequisite for COR induction. It would be interesting to examine whether the heme binding of AppA is affected by the rapid rise of the newly synthesized hemoprotein BchZ under the conditions.

The photosynthesis in the presence of oxygen is detrimental because singlet oxygen (¹O₂) is formed from an excited triplet state photosensitizer such as Bchl. Unless quenched by carotenoid and resolved by a number of oxygen (O₂) channels, ¹O₂ causes various damage to proteins, lipids, and DNA (72–74). Superoxide generation from COR reaction, which is readily dismutated by cytosolic Fe-SOD, might be considered electron leakage through heme. This in turn rapidly diminishes COR activity at low O₂, which would result in keeping the newly synthesized Bchl a at low levels. Consequently, less ¹O₂ could be expected in the presence of oxygen under irradiated conditions.

In summary, we identified and characterized COR with respect to the oxidative stress in R. sphaeroides, demonstrating that COR is responsible for the generation of superoxide, particularly when oxygen tension is lowered. Moreover, the results of this work provide a molecular basis for the COR activity of R. sphaeroides.

REFERENCES

1. Kho, D. H., Yoo, S. B., Kim, J. S., Kim, E. J., and Lee, J. K. (2004) FEMS Microbiol. Lett. 234, 261–267
2. Tabares, L. C., Bittel, C., Carrillo, N., Bortolotti, A., and Cortez, N. (2003) J. Bacteriol. 185, 3223–3227
3. Cortez, N., Carrillo, N., Pasternak, C., Balzer, A., and Klug, G. (1998) J. Bacteriol. 180, 5413–5420
4. Li, K., Hein, S., Zou, W., and Klug, G. (2004) Biochemistry 43, 10420–10428
5. McKord, J. M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049–6055
6. Kearney, E. B. (1960) J. Biol. Chem. 235, 865–877
7. Latimer, M. T., Painter, M. H., and Ferry, J. G. (1996) J. Biol. Chem. 271, 24023–24028
8. Faeder, E. J., and Siegel, L. M. (1973) Anal. Biochem. 53, 332–336
9. Kimmich, N., Das, A., Sevrioukova, I., Meherennya, Y., Silar, S. G., and Poulos, T. L. (2007) J. Biol. Chem. 282, 27006–27011
10. Klatt, P., Pfeiffer, S., List, B. M., Lehner, D., Glatter, O., Bachinger, H. P., Werner, E. R., Schmidt, K., and Mayer, B. (1996) J. Biol. Chem. 271, 7336–7342
11. Krolickiewski, J., Hombek-Urban, K., and Szczepaniak, A. (2005) Biochemistry 44, 7570–7576
12. Fulhkop, J. H., and Smith, K. M. (1975) in Laboratory Methods in Porphyrogenesis and Metalloporphyrogenesis Research (Smith, K. M., ed) pp. 804–807, Elsevier Science Publishing Co., Inc., New York
13. Holmgren, A. (1972) J. Biol. Chem. 247, 1992–1998
14. Calsudinelli, L., Iamettti, S., Barbirioli, A., Bonomi, F., Fessas, D., Molla, G., Pinone, M. S., and Pollegioni, L. (2005) J. Biol. Chem. 280, 22572–22581
15. Beintn, H. (1983) Analyst Biochem. 131, 373–378
16. Benov, L., Khedik, N. M., and Fridovich, I. (1996) J. Biol. Chem. 271, 21037–21040
17. Benov, L., and Fridovich, I. (1999) J. Biol. Chem. 274, 4202–4206
18. Kim, J. S., Choi, S. H., and Lee, J. K. (2006) J. Bacteriol. 188, 8586–8592
19. Rhee, H. J., Kim, E. I., and Lee, J. K. (2007) J. Cell. Mol. Med. 11, 685–703
20. Lee, I. H., Park, J. Y., Kho, D. H., Kim, M. S., and Lee, J. K. (2002) Appl. Microbiol. Biotechnol. 60, 147–153
21. Burke, D. H., Heast, E. J., and Sidow, A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7134–7138
22. Brigit, K. E., Weiss, M. C., Newton, W. E., and Dean, D. R. (1987) J. Bacteriol. 169, 1547–1553
23. Goodwin, P. J., Agar, J. N., Roll, J. T., Roberts, G. P., Johnson, M. K., and Dean, D. R. (1998) Biochemistry 37, 10420–10428
24. Finkelstein, E., Rosen, G. M., and Rauckman, E. J. (1980) Arch. Biochem. Biophys. 206, 283–307
25. Barquera, B., Ramirez-Silva, L., Morgan, J. E., and Nilges, M. J. (2006) J. Biol. Chem. 281, 36482–36491
26. Ohnishi, T., King, T. E., Slarno, J. C., Blum, H., Bowyer, J. R., and Maida, T. (1981) J. Biol. Chem. 256, 5577–5582
Superoxide from Chlorophyllide a Reductase Reaction

61. Johnson, M. K., Kowal, A. T., Morningstar, J. E., Oliver, M. E., Whittaker, K., Gunsalus, R. P., Ackrell, B. A., and Cecchini, G. (1988) J. Biol. Chem. 263, 14732–14738
62. Armstrong, G. A., Cook, D. N., Ma, D., Alberti, M., Burke, D. H., and Hearst, J. E. (1993) J. Gen. Microbiol. 139, 897–906
63. Fujita, Y., and Bauer, C. E. (2000) J. Biol. Chem. 275, 23583–23588
64. Nomata, J., Swem, L. R., Bauer, C. E., and Fujita, Y. (2005) Biochim. Biophys. Acta 1708, 229–237
65. Nomata, J., Kitashima, M., Inoue, K., and Fujita, Y. (2006) FEBS Lett. 580, 6151–6154
66. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) EMBO J. 8, 945–951

67. Des Marais, D. J. (2000) Science 289, 1703–1705
68. Abada, E. M., Balzer, A., Jager, A., and Klug, G. (2002) Mol. Genet. Genomics 267, 202–209
69. Gomelsky, M., and Kaplan, S. (1997) J. Bacteriol. 179, 128–134
70. Han, Y., Meyer, M. H., Keusgen, M., and Klug, G. (2007) Mol. Microbiol. 64, 1090–1104
71. Moskvin, O. V., Kaplan, S., Gilles-Gonzalez, M. A., and Gomelsky, M. (2007) J. Biol. Chem. 282, 28740–28748
72. Frank, H. A., and Brudvig, G. W. (2004) Biochemistry 43, 8607–8615
73. Anthony, J. R., Warczak, K. L., and Donohue, T. J. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 6502–6507
74. Glaeser, J., and Klug, G. (2005) Microbiology 151, 1927–1938