Differential Expression and Localization of Mn and Fe Superoxide Dismutases in the Heterocystous Cyanobacterium *Anabaena* sp. Strain PCC 7120

Tao Li,1 Xu Huang,1 Ruanbao Zhou,1 Yingfang Liu,1 Bin Li,1 Chris Nomura,2 and Jindong Zhao1,3*

State Key Lab of Plant Genetic and Protein Engineering and College of Life Sciences, Peking University,1 Beijing 100871, China, and Department of Biochemistry, Pennsylvania State University, University Park, Pennsylvania 168022

Received 12 March 2002/Accepted 14 June 2002

Superoxide dismutases (Sods) play very important roles in preventing oxidative damages in aerobic organisms. The nitrogen-fixing heterocystous cyanobacterium *Anabaena* sp. strain PCC 7120 has two Sod-encoding genes: a sodB, encoding a soluble iron-containing Sod (FeSod), and a sodA, encoding a manganese-containing Sod (MnSod). The FeSod was purified and characterized. A recombinant FeSod was also obtained by over-production in *Escherichia coli*. Immunoblot study of the FeSod during induction of heterocyst differentiation showed that the cells produced six- to eightfold more FeSod 8 h after a shift from a nitrogen-replete condition to a nitrogen-depleted condition. However, the amount of FeSod protein in filaments with mature heterocysts was the same as that in filaments grown with combined nitrogen. Superoxide anion-generating chemicals such as methyl viologen did not induce upregulation of the sodB gene expression. The predicted preprotein of the sodA gene has a leader peptide and a motif for membrane attachment at the N terminus of the mature protein. Activity staining after gel electrophoresis of the purified thylakoid membranes showed that most of the MnSod in *Anabaena* sp. strain PCC 7120 was located on thylakoids toward the lumenal side. Expression of the sodA gene in *E. coli* shows that the leader peptide was required for its activity and the membrane localization of the MnSod. Northern hybridization detected one 0.82-kb transcript of sodA. The sodA gene was upregulated by methyl viologen, whereas its amount was unchanged during heterocyst differentiation. Immunoblotting and activity staining showed that isolated heterocysts contained a lower but still significant amount of FeSod, suggesting that its function is required in heterocysts. No MnSod was observed in isolated heterocysts. These results show that the two different Sod proteins have differentiated roles in *Anabaena* sp. strain PCC 7120.

Active oxygen species (AOS) such as superoxide anion (O$_2^-$), hydroxyl radical (OH), and hydrogen peroxide (H$_2$O$_2$) are inevitably formed in all aerobic organisms and can cause damage to a wide range of biomolecules, such as DNA molecules and proteins (6, 20). They are formed by partial reduction of oxygen in cellular metabolism. Superoxide could disrupt Fe-S centers, and the released irons can react with H$_2$O$_2$, forming highly reactive hydroxyl radical (15). Therefore, both O$_2^-$ and H$_2$O$_2$ must be removed promptly to avoid cellular damage. The defense systems against AOS in prokaryotes and eukaryotes are similar (15, 47). They both use enzymes and nonenzymatic antioxidants to remove AOS. Antioxidant chemicals such as glutathione and ascorbate scavenge AOS directly, whereas enzymatic protection against AOS involves systems of enzymes which remove AOS. The most critical enzymes in removing superoxide anion are superoxide dismutases (Sods), which carry out the following reaction: $2$O$_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. The H$_2$O$_2$ formed is removed by catalases or peroxidases. So far, four kinds of Sods have been found: the copper-zinc type (Cu,Zn-Sod), the manganese type (MnSod), the iron type (FeSod), and the nickel type (NiSod). They all participate in protecting cellular molecules from damage caused by AOS (11, 15).

In plant leaves, algae and cyanobacteria, O$_2^-$ is usually formed by reduction of oxygen at the reducing side of photosystem I (2). It has been demonstrated that Sods play important roles against oxidative damage in cyanobacteria. A mutant strain of *Synechococcus* sp. strain PCC 7942 lacking detectable FeSod activity was shown to be much more sensitive to AOS (19) and chilling stress (40). An FeSod is also shown to be very important against oxidative damage in *Nostoc commune* under prolonged desiccation (37).

In heterocystous cyanobacteria, special cells called heterocysts are formed when the filaments are grown under a nitrogen-limiting condition (16, 46). Mature heterocysts contain a nitrogenase system and are the site for nitrogen fixation. Heterocysts have no oxygen evolving activity, and the thick wall of heterocysts limits but does not totally exclude oxygen penetration into the cells (46). Heterocysts also have a high activity of respiration, which consumes oxygen. Because photosystem I is present in heterocysts, oxygen can still intercept electrons of the electron transport chain to form O$_2^-$. Respiratory electron transport could also lead to the formation of O$_2^-$. Sod is thus probably required in heterocysts to protect against cellular damage by O$_2^-$. However, little information is available on what kind of Sod is present in heterocysts and how the sod gene expression is regulated. We have recently cloned the

---

*Corresponding author. Mailing address: College of Life Sciences, Peking University, Beijing 100871, People’s Republic of China. Phone: 86-10-6275-6421. Fax: 86-10-6275-1526. E-mail: jzhao@pku.edu.cn.
sodB gene from Anabaena sp. strain PCC 7120 and showed that its expression was upregulated upon shifting from a nitrogen-replete condition to a nitrogen-depleted condition (26). Here we report the results of isolation, purification, and characterization of the FeSod and MnSod from Anabaena sp. strain PCC 7120. Their localization and gene expression are also reported.

MATERIALS AND METHODS

Strains and culture conditions. Axenic cultures of Anabaena sp. strain PCC 7120 were grown on agar plates (1.5% Difco Bacto Agar) or in liquid medium of BG11 (34) with or without combined nitrogen. Liquid cultures were grown at 28°C and bubbled with 0.5% CO₂ in filtered air. The cultures were illuminated with cool white fluorescent light at an intensity of ca. 80 μmol m⁻² s⁻¹. Escherichia coli strain DH5α was used for all routine cloning. The E. coli BL21(DE3) was used for overproduction of the sodI gene, and the E. coli strain CC774 (10) was used to express the sodI gene from Anabaena sp. strain PCC 7120.

Purification of FeSod from Anabaena sp. strain PCC 7120. Anabaena cells from 10 liters of late exponential culture in BG11 medium were collected by centrifugation. The pellet was resuspended in buffer A (20 mM Tris-HCl, pH 8.0; 10 mM NaCl) and centrifuged. The cells were resuspended in buffer A containing 100 mM phenylmethylsulfonyl fluoride and broken by passage through a French press twice at a pressure of 160 MPa. The cell extracts were centrifuged at a speed of 20,000 rpm in an A252 rotor (Beckman) for 1 h to remove membranes and unbroken cells. Solid ammonium sulfate was added to the supernatant to a saturation of 60%. The solution was stirred for 30 min at 4°C before centrifugation at 25,000 × g for 1 h at 4°C. The pellet was discarded, and the supernatant was dialyzed against buffer A containing 100 mM sodium sulfate to a saturation of 70%. The solution was stirred and centrifuged as described above. The pellet containing Sod activity was resuspended in buffer A and diazylated against buffer A overnight at 4°C. The precipitate formed during the dialysis was removed by centrifugation, and the supernatant was loaded to a DEAE-Sephadex column (40 by 2.5 cm) equilibrated with buffer A. The column was eluted with a linear NaCl gradient from 10 to 500 mM in buffer A. The fractions containing FeSod activity were pooled and dialyzed against buffer A before being concentrated by ultratitration and loaded onto a Sephacryl-100 HR (Sigma) column (50 by 4 cm). The column was eluted with buffer A at a flow rate of 25 ml h⁻¹. The fractions containing Sod activity were pooled and concentrated as described above, applied to a Mono-Q HR column, and eluted with a linear NaCl gradient. The fractions containing Sod activity were collected and concentrated as described above. The molecular mass of the purified Sod protein was determined with a G2025A MS instrument as described by Zhou et al. (49). The isoelectric point of the purified Sod was estimated by isoelectrofocusing electrophoresis (48), followed by activity staining.

Overproduction of Anabaena sod genes in E. coli. The sodB gene from Anabaena sp. strain PCC 7120 was amplified by PCR in the presence of the high fidelity enzyme PfuTurbo (Stratagene). The primers used for the amplification were 5'-TCAACATGATCCCATCTGAGCAGAAGC-3' and 5'-TTGATATCCGAGTCATTTGAGCTTTAGCATATG-3'. The restriction sites of NcoI and BamHI in the primers are underlined. The amplified fragment was first cloned into the pGEM-T vector (Promega, Beijing, People’s Republic of China). The fragment from the resultant plasmid obtained by digestion with NcoI and BamHI was cloned into pET3d (38) and transformed into E. coli strain BL21(DE3) for overexpression. The overproduced Sod from Anabaena sp. strain PCC 7120 encodes a protein with a leader peptide. The DNA fragment encoding full-length protein was amplified by PCR with the primers 5'-TTCAGATGGGAAATCCTGCTGTTGGCGAAAAGC-3' (NcoI) and 5'-TTGATATCCGCCGAGTCCAGAGC-3' (BamHI). The DNA fragment encoding only the mature protein was amplified by PCR with the primer 5'-TTGATGTCGACGCTCATGACCGAGAGACG-3' (AflII) and 5'-TTGATATCCGCCGAGTCCAGAGC-3' (BamHI). The amplified fragments were first cloned into the pGEM-T vector. The resultant plasmids were digested with NcoI and BamHI or with AflII and BamHI. The generated DNA fragments containing the sodB genes were cloned into pSE380 (5), generating the expression plasmids pSE380sodA-A and pSE380sodA-A, respectively. These plasmids were transformed into the E. coli strain Q774 for expression of the sodB genes.

Isolation of heterocysts and purification of membranes. Heterocysts were isolated according to the method of Zhou et al. (35). The plasma membranes and thylakoid membranes were purified from 4 liters of culture by using the two-phase partition system (25, 30). The purity of the isolated membranes was determined according to the method of Norling et al. (30). No P700 activity or PSI electron transport activity was detected in the isolated plasma membranes.

Detection of Sod. The measurement of Sod activity was based on its inhibition of the reduction of nitroblue tetrazolium by superoxide according to the method of Lutterbourn et al. (45). The FeSod activity staining procedure was performed according to the method of Beauchamp and Fridovich (4) as follows. The proteins were separated with nondenaturing polyacrylamide gel (8%) electrophoresis. The gel was then soaked in 2.5 mM nitroblue tetrazolium in buffer F (20 mM NaH₂PO₄ and 20 mM K₂HPO₄, pH 7.8) for 30 min before being placed in buffer P containing 28 μM TEMED (N,N,N',N'-tetramethylethylenediamine) and 28 μM flavin and soaked for another 20 min. The gel was then rinsed twice with buffer P before being illuminated with a tungsten light bulb at an intensity of 450 μmol m⁻² s⁻¹.

The cells of Anabaena sp. strain PCC 7120 grown in BG-11 medium in late exponential phase were collected and broken either with a French press as described above or by osmotic shock after lysosome treatment. The cell extracts obtained with the French press were centrifuged (5,000 × g, 5 min) to remove unbroken cells. The supernatant was centrifuged with high-speed centrifugation at 20,000 rpm (27,200 × g) with an A252 rotor for 1 h. The supernatant was the soluble fraction and used directly for in-gel Sod assay. The pellet was the membrane fraction. The membranes were washed three times with buffer A, and they were named total membranes. Heterocysts were broken with a French press according to the method of Zhou et al. (48). The total membranes, the purified membranes, and the total cell extracts from isolated heterocysts were precipitated with 80% acetone to remove lipids. The precipitants were solubilized in buffer A before they were assayed for Sod activity by in-gel activity staining. The procedure for breaking the cells by osmotic shock was as follows. The Anabaena sp. strain PCC 7120 cells in buffer A containing 0.5 M sucrose. To the cell suspension, lysosome was added to a final concentration of 0.5 mg ml⁻¹ and incubated at 30°C for 30 min before it was centrifuged at 5,000 × g for 5 min. The cell pellet was gently resuspended in an equal volume of buffer A without sucrose and centrifuged at 15,000 rpm for 30 min. The supernatant was used directly for in-gel assay for Sod activity.

Immunoblotting for detection of FeSod was performed as follows. The purified FeSod was used to raise antibodies in rabbits (38). The proteins of the total cellular extracts from Anabaena sp. strain PCC 7120 filaments or from isolated heterocysts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22) before being blotted on a polyvinylidene difluoride membrane. Proteins cross-reacted with the anti-FeSod antibodies were detected by using secondary antibodies conjugated with horseradish peroxidase (Promega) as described previously (48). Quantification of proteins after immunoblotting or in-gel staining was done by using ImageMaster VDS software (Pharmacia, Hong Kong).

Other methods. Induction of heterocyst differentiation was as described previously (48), and the oxidative stress condition was introduced by addition of 5 μM methyl viologen (MV) to the culture. Multiple sequence alignment was performed with CLUSTALW (41). Northern analysis was performed as described above or by osmotic shock after lysozyme treatment. The cell extracts were centrifuged at a speed of 20,000 rpm in an AJ25 rotor (Beckman) for 1 h. The supernatant was centrifuged with high-speed centrifugation at 20,000 rpm (27,200 × g) with an A252 rotor for 1 h. The supernatant was the soluble fraction and used directly for in-gel Sod assay. The pellet was the membrane fraction.

RESULTS

Characterization of FeSod. The gene encoding the FeSod from Anabaena sp. strain PCC 7120 has been cloned and sequenced (26). The gene was named sodB based on the homology of its amino acid sequence with other FeSods at several key positions. To confirm that its product is indeed an FeSod, the soluble Sod from Anabaena sp. strain PCC 7120 was purified. Table 1 summarizes the purification process. The Sod in the supernatant was first concentrated with ammonium sulfate precipitation since it was found that ammonium sulfate precipitation was effective in removing most of phycobiliproteins.

Downloaded from http://jb.asm.org/ on March 26, 2021 by guest
as well as other proteins. The Sod was precipitated with 70% ammonium sulfate. The pellet was resuspended in buffer A, and the Sod was purified with a DEAE anion-exchange column, followed by purification on a gel filtration column. The DEAE ion-exchange step would separate the FeSod from the MnSod of *Anabaena* sp. strain PCC 7120 because the FeSod has a pI of 4.8 (Fig. 2), whereas the MnSod has a predicted pI of 6. The Sod was further purified with a MonoQ column. A total of 262-fold purification was achieved. Table 1 also shows that each protein contains approximately one molecule of Fe. One FeSod contained <0.01 Mn atom (data not shown). The results of the FeSod purification were analyzed by SDS-PAGE, as shown in Fig. 1A. After the MonoQ column step, only one band was present, as shown by Coomassie blue staining (lane 5). The purified Sod had a molecular mass of ca. 23 kDa as determined by SDS-PAGE. N-terminal sequencing revealed that it had a sequence of (A)FVQEPLPYDFNALEQY. The N-terminal sequence is identical to the predicted sequence of the *sodB* gene (26), confirming that the gene is indeed encoding an FeSod. The initial Met residue of the FeSod was apparently posttranslationally removed. We found that the first residue of ca. 80% of the native FeSod was an Ala residue (in parenthesis), whereas 20% started with a Phe residue, indicating some protease cleavage at its amino terminus during purification. No other sequences were found with N-terminal sequencing. The purified native Sod has a molecular mass of 22.3 kDa as determined with mass spectroscopy (data not shown). The isoelectric point of the purified native FeSod was estimated to be 4.8 with isoelectrofocusing gel electrophoresis, followed by activity staining (Fig. 2B). The purified Sods maintained 80% activity after treatment with high temperature at 55°C for 30 min (data not shown). We also found that the FeSod activity was inhibited by H_2O_2 but not by KCN (not shown), a finding typical for FeSod.

The *sodB* gene product was also overproduced in *E. coli*, and the results are shown in Fig. 1B. More than 50% of the total cellular proteins were the overproduced FeSod (lane 3). Most of the overproduced Sod protein was in soluble form (lane 4). The purification of the recombinant FeSod was similar to that for the native FeSod, and the purified recombinant Sod is shown in lane 5 of Fig. 1B.

Figure 2A shows activity staining of the native Sods from the whole-cell extracts, the total membranes, the supernatant obtained with the French press method, the supernatant obtained with osmotic shock, and the purified FeSod after a nondenaturing gel electrophoresis. Only one band can be observed in the purified native Sod (lane 5). There are several bands

### Table 1. Purification of FeSod from *Anabaena* sp. strain PCC 7120 grown under nitrogen-replete conditions

<table>
<thead>
<tr>
<th>Fraction obtained with</th>
<th>Sod activity (U/mg of protein)</th>
<th>Amt of FeSod (Sod/total protein [%])</th>
<th>Purification (fold)</th>
<th>Fe/protein ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH_4)_2SO_4 precipitation</td>
<td>13.8</td>
<td>0.4</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>Fraction obtained by 70% (NH_4)_2SO_4 precipitation</td>
<td>76.5</td>
<td>2.0</td>
<td>5.5</td>
<td>ND</td>
</tr>
<tr>
<td>Fraction obtained with DEAE column</td>
<td>2,678</td>
<td>70.2</td>
<td>194</td>
<td>ND</td>
</tr>
<tr>
<td>Fraction obtained by gel filtration</td>
<td>3,293</td>
<td>86.7</td>
<td>239</td>
<td>ND</td>
</tr>
<tr>
<td>Fraction obtained with MonoQ column</td>
<td>3,611</td>
<td>95.1</td>
<td>262</td>
<td>0.87 ± 0.07</td>
</tr>
</tbody>
</table>

* a Protein concentration determination, enzyme activity assay, and purification steps are all described in Materials and Methods. The amount of iron in purified protein was determined by atomic absorption.

* b ND, not determined.

**A** SDS-PAGE analysis of purification of the native FeSod. Soluble fraction (lane 2) was first treated with ammonium sulfate (60%) to remove most of the phycobiliproteins and other proteins. The Sod was precipitated with 70% ammonium sulfate followed by activity staining (Fig. 2A). The purified native Sod has a molecular mass of 22.3 kDa as determined with mass spectroscopy (data not shown). The isoelectric point of the purified native FeSod was estimated to be 4.8 with isoelectrofocusing gel electrophoresis, followed by activity staining (Fig. 2B). The purified Sods maintained 80% activity after treatment with high temperature at 55°C for 30 min (data not shown). We also found that the FeSod activity was inhibited by H_2O_2 but not by KCN (not shown), a finding typical for FeSod.

The *sodB* gene product was also overproduced in *E. coli*, and the results are shown in Fig. 1B. More than 50% of the total cellular proteins were the overproduced FeSod (lane 3). Most of the overproduced Sod protein was in soluble form (lane 4). The purification of the recombinant FeSod was similar to that for the native FeSod, and the purified recombinant Sod is shown in lane 5 of Fig. 1B.

Figure 2A shows activity staining of the native Sods from the whole-cell extracts, the total membranes, the supernatant obtained with the French press method, the supernatant obtained with osmotic shock, and the purified FeSod after a nondenaturing gel electrophoresis. Only one band can be observed in the purified native Sod (lane 5). There are several bands

**B** SDS-PAGE analysis of purification of the recombinant FeSod. The *sodB* gene was overexpressed in *E. coli* BL21(DE3) by using the expression vector pET3d. Lane 2, total cellular extracts from *E. coli* BL21(DE3) without pET3dSodB; lanes 3 and 4, total cellular extracts and soluble fraction from *E. coli* BL21(DE3) with pET3dSodB induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside), respectively; and lane 5, purified recombinant FeSod. The purification procedure was the same as that described for panel A. In both panels A and B, the gels were stained with Coomassie blue, and the first lanes are the standards with molecular masses (indicated on the left side in kilodaltons).
present in whole-cell lysate (lane 1). The major band observed in whole-cell extract corresponded to the purified FeSod, which moved fastest in the nondenaturing gel. The top band was from MnSod (see below) and the middle bands could be heterodimer and hetero-oligomers of MnSod and FeSod (10, 12). All bands except the top band showed strong reaction with anti-FeSod by immunoblotting after nondenaturing electrophoresis (data not shown), suggesting that most of these bands in lane 1 (Fig. 2A) contained FeSod. The total membranes (lane 2) contained a major band corresponding to the band with lowest mobility (MnSod) in the total cell extracts and a faint band at the position of FeSod. The supernatant obtained with the French press (lane 3) contained two bands: a strong band at the position of FeSod and a weak band at the MnSod position. The supernatant obtained with osmotic shock contained only one band at the position of FeSod.

Characterization of the MnSod from Anabaena sp. strain PCC 7120. The activity staining result (Fig. 2) suggested that there would be at least another Sod protein present in Anabaena sp. strain PCC 7120. The sequence of the genome of Anabaena sp. strain PCC 7120 has recently been determined (www.cyanosite.bio.purdue.edu). A BLAST search based on its sodB gene revealed the presence of another sod gene, which showed a high homology to the sodA gene from Plectonema boryanum (8). No Cu,Zn-type sod gene was found in the Anabaena sp. strain PCC 7120 genome with the BLAST search. Multiple sequence alignment of the sodA gene product with other MnSod proteins shows that it has a sequence typical of bacterial MnSod (Fig. 3).

Anabaena sp. strain PCC 7120 sodA has conserved amino acid residues specific to MnSod in boldface. Gaps are indicated by dashes.

FIG. 3. Partial sequence comparison of Anabaena sp. strain PCC 7120 SodA (residues 1 to 178) with other MnSods by multiple sequence alignment by using CLUSTALW. SodAs: 7120, Anabaena sp. strain PCC 7120 (www.cyanosite.bio.purdue.edu); Bsub, Bacillus subtilis (P54375); Nostoc, N. punctiforme; Pbo, P. boryanum (U17609, U17610, and U17611 for SodA1, SodA2 and SodA3, respectively); Taq, Thermus aquaticus (E04306); Valg, Vibrio alginolyticus (AF085191). The leader peptides are underlined, and the predicted lipid attachment motifs (COPO and CASA) are italic. The characteristic amino acid residues specific to MnSod are in boldface. Gaps are indicated by dashes.

To study the roles of the leader peptide in the correct folding of the MnSod, the recombinant SodA from Anabaena sp. strain PCC 7120 was overproduced. Figure 4 shows the activity staining of the recombinant SodA produced in E. coli strain QC774 which lacks both sodB and sodA (10). When the full-length sodA gene encoding both leader peptide and mature protein was used to produce recombinant SodA, Sod activity could be detected and it was found in total cell extracts (lane 3) and associated with membranes (lane 4). When the sodA gene lacking the sequence encoding the leader peptide was expressed in E. coli, little Sod activity was detected in the E. coli cell extract (lane 2), whereas SDS-PAGE analysis showed that the gene product was present. However, it formed inclusion bodies lacking Sod activity (not shown).

Anabaena sp. strain PCC 7120, like most of the cyanobacteria, has two membrane systems: plasma membranes and thylakoid membranes. To study the location of SodA in Anabaena sp. strain PCC 7120, plasma membranes and thylakoid membranes were separated by using a two-phase system (25, 30). The results show that plasma membranes and thylakoid membranes were separated by using a two-phase system (25, 30). The differences show that plasma membranes and thylakoid membranes were separated by using a two-phase system (25, 30).
staining band of the thylakoid membrane was at the same position in the gel as the recombinant MnSod produced in E. coli (lane 3). Thus, the detected band on thylakoid membranes was the MnSod. The Sod activity associated with both the thylakoid membranes and the E. coli plasma membranes was inhibited neither by H₂O₂ nor by KCN, a finding typical of bacterial MnSod (data not shown). It is worthwhile to point out that only one band of MnSod was detected with the isolated membranes.

Expression of sodA and sodB in Anabaena sp. strain PCC 7120. It has been shown that the sodB gene was upregulated by the shifting Anabaena sp. strain PCC 7120 culture from a nitrogen-replete condition to a nitrogen-depleted condition (26). To carry the present study further, we raised antibodies against FeSod to study the expression of sodB in Anabaena sp. strain PCC 7120 during the entire period of heterocyst differentiation and under oxidative stress by superoxide anions. When Anabaena sp. strain PCC 7120 culture was shifted from a nitrogen-replete condition to a nitrogen-depleted condition, the amount of FeSod increased about six to eight times within the first few hours of induction (Fig. 6A). It remained at a high level until heterocyst differentiation was complete (24 h) and gradually declined to the original low level in 48 h. The polyclonal antibodies used in the present study seemed to be specific to FeSod since we found that they showed no cross-reaction with MnSod even at the concentration 10 times higher than that used under our experimental conditions.

The expression of sodB genes in some bacteria can be induced by oxidants (12). The expression of sodB of Anabaena sp. strain PCC 7120 subjected to a superoxide anion stress by addition of MV to the culture was studied (Fig. 6B). No increase of FeSod was observed by immunoblotting up to 12 h after the addition of MV. The cells died after longer incubation with MV (data not shown).

The effect of MV on the membrane-associated MnSod was also studied by using activity staining, and the results are shown in Fig. 6C. The addition of MV at a concentration of 5 μM...
induced a four- to sixfold increase of MnSod activity on membranes. The activity increased to its maximum level at 8 h after the addition of MV. The activity started to decline at 12 h, probably due to the cell death induced by MV. On the other hand, heterocysts contained only the FeSod band with activity staining (lane 3). No MnSod was detected in the other hand, heterocysts contained only the FeSod band with activity staining (lane 3). No MnSod was detected in whole-cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that heterocysts contained less active SodB protein. Immunoblotting with the cell extracts from isolated heterocysts also showed that hetero-
double bonds (42). The *Anabaena* sp. strain PCC 7120 used in this study has at least four different fatty acid desaturases (www-cyanosite.bio.purdue.edu). The complex membrane system in an environment of high oxygen concentration may create a special situation that requires a membrane-associated Sod to protect membrane molecules from oxidative damage. Some MnSod in other cyanobacteria are also found to be associated with membranes. *N. punctiforme* has two *sodA* genes, and their products are predicted to be membrane associated (Fig. 3). *P. boryanum* has three *sodA* genes, and at least one of them (*sodA1*) is predicted to be membrane associated (8). *Synechococcus* sp. strain PCC 7942 probably has one membrane-associated MnSod based on assays of enzymatic activity (19, 23). With isolated thylakoid membranes and plasma membranes, we demonstrated that the MnSod from *Anabaena* sp. strain PCC 7120 was associated with thylakoid membranes (Fig. 5), with little activity on plasma membranes. This result implies that membrane-associated MnSod is possibly related to the function of protecting thylakoid membranes from superoxide anion damage. A membrane-associated MnSod was also found in rat liver mitochondria, and it was suggested to protect the membranes from superoxide damage (31).

Similar ways have been adapted for the association of MnSods with the membranes in different cyanobacteria. Both the MnSod from *Anabaena* sp. strain PCC 7120 and the SodA1 from *N. punctiforme* have a leader peptide and a fatty acid attachment site (Fig. 3). The mature forms of the two MnSods are predicted to be lipoproteins. The leader peptides have also been found in other cyanobacterial MnSods such as the *sodA1* gene product *P. boryanum* (8). We demonstrated that the leader peptide of the MnSod from *Anabaena* sp. strain PCC 7120 is not only critical to the MnSod association with membranes but also important in the correct folding of the MnSod and its activity in *E. coli* (Fig. 4). Since the signal sequences in prokaryotes are similar and the membrane topology is similar in both cyanobacteria and *E. coli*, the leader peptide of the MnSod is very likely also important in the *Anabaena* sp. strain PCC 7120 cells.

The presence of the leader peptide and the association of MnSod with thylakoid membranes suggest that the MnSod of *Anabaena* sp. strain PCC 7120 is located within the lumen, whereas the FeSod is in cytoplasm, although it cannot be completely ruled out that the membrane-anchored MnSod is facing the cytoplasmic side of the membranes. The suggestion that the MnSod is within the lumen is supported by the results shown in Fig. 2A. Only FeSod, which did not form aggregates by itself (Fig. 2A, lane 5), was released outside the cells with osmotic shock, whereas some MnSod and FeSod were present in the supernatant obtained by the French press method. The MnSod and FeSod obtained by the French press method formed no aggregates of heterodimer or hetero-oligomers. The aggregation could occur if MnSod and FeSod are located within the same compartment of the cells (10, 12) or after the concentration process in the case of the Sods from *Anabaena* sp. strain PCC 7120 (Fig. 2A, lane 1). Thus, the two Sods from *Anabaena* sp. strain PCC 7120 are likely separated spatially. The differential localization of MnSod and FeSod in *Anabaena* sp. strain PCC 7120 suggests that they have different roles in protecting cellular molecules from superoxide damage. By using different superoxide-generating agents, Thomas et al. (39) demonstrated that different Sods of *Synechococcus* sp. strain PCC 7942 might have different roles in protection against superoxide damages generated at different locations within the cells. The expression of the *sodA* and *sodB* genes in *Anabaena* sp. strain PCC 7120 also suggests that they have differentiated roles in protection against superoxide. While both *sodA* and *sodB* of *Anabaena* sp. strain PCC 7120 are expressed under normal growth conditions, suggesting that they are required for prevention of superoxide damage under these conditions, the upregulation of the two genes in *Anabaena* sp. strain PCC 7120 is induced under different conditions. Northern blotting analysis revealed that shifting from a nitrogen-replete condition to a nitrogen-depleted condition induced a severalfold increase of the *sodB* transcripts in *Anabaena* sp. strain PCC 7120 (26). The immunoblotting result shown in Fig. 6 confirmed the observation. The increase of the FeSod level during heterocyst differentiation suggests that the cells are under a more stressful condition during heterocyst differentiation, probably due to the limitation of nitrogen supply. This stress condition is relieved once nitrogen fixation takes place and the amount of FeSod returns to the same level as that under nitrogen-replete conditions. On the other hand, no upregulation of *sodA* expression is observed under nitrogen step-down conditions (Fig. 6 and 7). Northern analysis showed that the *sodA* gene from *Anabaena* sp. strain PCC 7120 was transcribed as a monocistron with an mRNA size of 0.82 kb (Fig. 7). The expression of *sodA* is upregulated by MV, which generates superoxide anions in both photosynthetic and respiratory electron transfers (Fig. 6). A previous study reported that the *sodA2* gene transcript increased rapidly upon addition of MV in *P. boryanum* (8). We did not observe any FeSod increase induced by MV, as was the case with *E. coli* whose *sodB* gene expression remained unchanged after addition of MV (7, 10).

The amount of Sods in heterocysts has been reported in several studies, and the results varied by species. While the heterocysts of *Anabaena variabilis* contained more Sod protein than the vegetative cells (3), the heterocysts of *Anabaena cylindrica* contained less Sod than the vegetative cells (9). In this study, we show that the heterocysts of *Anabaena* sp. strain PCC 7120 contain a smaller amount of Sod (Fig. 8) by both activity staining and immunoblotting. We also demonstrate that FeSod is the only significant Sod present in the heterocysts by activity staining (Fig. 8). Because a major site of production of superoxide anions in heterocysts is at excited P700 of photosystem I, a soluble Sod is likely required to remove superoxide in cytosol. On the other hand, a heterocyst is an environment with a much reduced oxygen concentration, and it resembles some anaerobic bacteria. It is thus likely that much less Sod would be required and that the expression of *sodB* gene is low in heterocysts. The heterocyst thylakoids would also be under much less oxidative stress, and much less MnSod would be required in heterocysts to protect the thylakoid membranes.

A mutant lacking *sodB* was constructed in *Synechococcus* sp. strain PCC 7942 (19), and the mutant strain helped in the understanding of the roles that FeSod plays in that organism. A mutant strain of *Corynebacterium melasecola* lacking *sodA* was also useful in elucidating the roles of MnSod in that organism. A mutant strain of *Sinorhizobium melloti* lacking *sodA* was severely impaired (35). Conversely, the increase in activ-
ity of antioxidant enzymes through genetic engineering extends the average life span in *Drosophila melanogaster* (32) and *Caen-
norhabditis elegans* (28), and it improves plant tolerance to stress conditions (1, 36). *Anabaena* sp. strain PCC 7120 pre-
sents an ideal case for studying the separate roles of MnSod and FeSod in heterocystous cyanobacteria because it has only one *sodA* and one *sodB*. We are currently trying to overexpress the *sodA* and *sodB* genes, as well as to construct mutant strains lacking either *sodA* or *sodB* or both, by the conjugal transfer method (13). These mutants, if constructed, will be very help-
ful in our understanding of different Sods in both vegetative cells and heterocysts under various conditions.

**ACKNOWLEDGMENTS**

The skillful technical assistance of C. Dong and Z. Wang is appre-
ciated.

This research was supported by the National Natural Science Foun-
dation of China (grant 39535002) and by the Department of Science and Technology of China (G1998010100, J99-A-032, and 00CB1089).

**REFERENCES**

10. Norling, B., E. Zak, B. Andersson, and H. Pakrasi. 1998. 2D-isolation of pure plasma and thylakoid membranes from the cyanobacterium *Synecho-
zymol. 185:56–69.
24. von Heijne, G. 1989. The structure of signal peptides from bacterial lipopro-
25. Winterbourn, S., R. Hawkins, M. Brain, and R. Carrell. 1975. The estima-

*VOL. 184, 2002* EXPRESSION AND LOCALIZATION OF MnSOD AND FeSOD 5103