The Iron Superoxide Dismutase from the Filamentous Cyanobacterium Nostoc PCC 7120

LOCALIZATION, OVEREXPRESSION, AND BIOCHEMICAL CHARACTERIZATION*

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The nitrogen-fixing filamentous cyanobacterium Nostoc PCC 7120 (formerly named Anabaena PCC 7120) possesses two genes for superoxide dismutase, a unique membrane-associated manganese superoxide dismutase (MnSOD) and a soluble iron superoxide dismutase (FeSOD). A phylogenetic analysis of FeSODs shows that cyanobacterial enzymes form a well separated cluster with filamentous species found in one subcluster and unicellular species in the other. Activity staining, inhibition patterns, and immunogold labeling show that FeSOD is localized in the cytosol of vegetative cells and heterocysts (nitrogenase containing specialized cells formed during nitrogen-limiting conditions). The recombinant Nostoc FeSOD is a homodimeric, acidic enzyme exhibiting the characteristic iron peak at 350 nm in its ferric state, an almost 100% occupancy of iron per subunit, a specific activity using the ferricytochrome in its ferric state, an almost 100% occupancy of iron per enzyme exhibiting the characteristic iron peak at 350 nm.

The filamentous, nitrogen-fixing cyanobacterium Nostoc PCC 7120 (formerly named Anabaena PCC 7120) was recently shown to contain two genes for SODs, namely a sodB gene encoding an iron-containing and a sodA gene encoding a manganese-containing superoxide dismutase (2). Nostoc PCC 7120 forms heterocysts when the filaments are grown under nitrogen-limiting conditions (3). Mature heterocysts are the site of nitrogen fixation and contain nitrogenase, the enzyme that catalyzes the reduction of atmospheric nitrogen to ammonia. Because nitrogenase is irreversibly inhibited upon exposure to molecular oxygen or activated oxygen species (4), several strategies are necessary to protect this ancient enzyme: (i) heterocysts have no oxygen-evolving activity because they lack photosystem II (4), (ii) they are surrounded by a thick cell wall (5), and (iii) they have a high activity of respiration which consumes oxygen (6). In nonheterocyst nitrogen-fixing cyanobacteria, nitrogen fixation and oxygenic photosynthesis are separated temporally or spatially (7).

The protection mechanism in heterocysts is not perfect. The thick cell wall does not totally exclude oxygen penetration into the cells (5), and both the respiratory electron transport and photosystem I contribute to the formation of superoxide radicals (4). Thus, superoxide dismutase activity is necessary in both vegetative cells and heterocysts of cyanobacteria in order to protect against cellular damage by superoxide. We have reported that Nostoc PCC 7120 MnSOD is unique in being membrane-bound and presented a comprehensive biochemical and biophysical characterization of this enzyme (2). MnSOD is the enzyme that catalyzes the reduction of atmospheric nitrogen to ammonia. Because nitrogenase is irreversibly inhibited upon exposure to molecular oxygen or activated oxygen species (4), several strategies are necessary to protect this ancient enzyme: (i) heterocysts have no oxygen-evolving activity because they lack photosystem II (4), (ii) they are surrounded by a thick cell wall (5), and (iii) they have a high activity of respiration which consumes oxygen (6).

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localized in both cytoplasmic and thylakoid membranes (2). Recently, the 2.0-Å structure of the catalytic portion of the MnSOD was reported (8). Membrane attachment of Nostoc MnSOD was confirmed by immunoblotting and activity staining (9).

To complete the characterization of SODs in Nostoc PCC 7120 and understand superoxide detoxification in nitrogen-fixing heterocystous cyanobacteria, we cloned the sodB gene from this organism and performed a comprehensive biochemical and kinetic investigation including the determination of actual bimolecular rate constants at pH 7–10. Its localization in the cytosol of both vegetative cells and heterocysts is presented as well as its phylogenetic relationship with other cyanobacterial FeSODs. Comparing these findings with those from MnSOD obtained under identical experimental conditions, we conclude that FeSOD and MnSOD complement each other in Nostoc PCC 7120 to protect cell components from oxidative stress. The physiological relevance is discussed with respect to the bioenergetic peculiarities of cyanobacteria as well as to nitrogen fixation in heterocysts. The SOD equipment of Nostoc PCC 7120 is compared with that of other cyanobacteria.

**EXPERIMENTAL PROCEDURES**

**Materials**

Enzymes and chemicals were used from the following companies: alkaline phosphatase from Roche Applied Science; T4 DNA ligase, Neol and XhoI restriction enzymes from MBI Fermentas; ExTaQ DNA polymerase from TaKaRa; Epoxy resin kit from Multilab; bovine serum albumin, lysozyme (from chicken egg white), DNase I (from bovine pancreas), PMSF, leupeptin, pepstatin A, fibrinogen type IV (from bovine plasma), thymon (from bovine plasma), SigmaMarker (wide molecular weight range), electrophoresis kit for isoelectric focusing, cytochrome c (horse heart), xanthine oxidase (from buttermilk grade I), 18-crown-6-ether, 3-Å molecular sieve and potassium permanganate (Sigma-Aldrich, St. Louis, MO); MgCl2, 300 mM mannitol, 0.5% bovine serum albumin, lysozyme (from chicken egg white), DNase I (from bovine pancreas), PMSF, leupeptin, pepstatin A, fibrinogen type IV (from bovine plasma), thymon (from bovine plasma), SigmaMarker (wide molecular weight range), electrophoresis kit for isoelectric focusing, cytochrome c (horse heart), xanthine oxidase (from buttermilk grade I), 18-crown-6-ether, 3-Å molecular sieve and potassium permanganate (Sigma-Aldrich, St. Louis, MO); MgCl2, 300 mM mannitol, 0.5% bovine serum albumin, 0.5% lysozyme, pH 7.2, and incubated at 35 °C for 1 h at a cell density of 50 ml of packed cells per ml. The lysozyme-treated cells were pelleted, washed twice with lysozyme-free mannitol buffer, resuspended in 30 mM Hepes-Pipes-ROH buffer (with 1 mM MgCl2, 0.0075% DNase I, pH 7.2) to a cell density of 1000 ml of packed cells per ml, and extruded through a precooled French pressure cell (Amino) at 34.5 MPa and 4 °C. Unaffected heterocysts were separated from broken vegetative cells by centrifugation (4000 × g, 4 min, 4 °C) and disrupted by French Press extrusion at 138 MPa and 4 °C (6). The membranes and cytosolic components of heterocysts were separated as described for vegetative cells.

**Electron Microscopy Combined with Immunogold Labeling—Sample Preparation of Nostoc PCC 7120 were agglutinated with a thrombin–fibrinogen solution, fixed with 3% glutaraldehyde (in 0.2 M phosphate buffer, pH 7.2) for 2 h, washed twice with 0.2 M phosphate buffer for 5 min, and post-fixed with 1% osmium tetroxide (in 0.2 M phosphate buffer, pH 7.2) for 2 h. Subsequently, samples were dehydrated by incubation in solutions of increasing ethanol and propylene oxide concentrate and emulsiﬁed in epoxy resin using pressurization of increasing resin concentration. After drying at 40 °C for 2 h, the specimens were polymerized at 60 °C for 2 days. Ultrathin sections were produced using the Ultracut E ultramicrotome (Reichert-Jung) and mounted on 200-mesh nickel grids. The grids were treated with 5% H2O2 for 5 min, washed with PBS-T (PBS containing 0.1% Tween-20), and blocked with 5% bovine serum albumin (in PBS-T) for 30 min. The grids were incubated overnight in rabbit anti-FeSOD antibody (1:1000 dilution) or with 1% bovine serum albumin (in PBS-T), and washed three times with PBS-T for 15 min each. Incubation with goat anti-rabbit IgG antibody conjugated with 10-nm colloidal gold particles (diluted 1:20 with PBS-T) was performed for 1 h, the grids were washed twice with PBS-T, and twice with distilled water for 15 min each. Grids were poststained in 2% uranyl acetate for 15 min, washed in distilled water, incubated in lead citrate for 5 min, washed in distilled water, air-dried, and observed with a transmission electron microscope (CEM 902 from Zeiss) operating at 80 kV. Controls were made using preimmune serum instead of anti-FeSOD antibody (13, 14).

**Cloning and Overexpression of FeSOD—DNA and protein sequence of the iron-containing superoxide dismutase was obtained from the Cyanobase, the genome data base for Nostoc PCC 7120, at www.iron.prairiefire.com. The following primers were synthesized from geneXpress (Maria Wörth, Austria): primer 1 (5'-ATA CCA TGG CAT TTG TAC AGG AAC C-3'); 25 bases containing an Nol restriction site), and primer 2 (5'-GTG CTC CAG AGC TTT AGC ATC ATC CGC-3'; 27 bases with a XhoI restriction site). Chromosomal DNA was obtained by treating Nostoc cell pellets with SDS and heat, followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation (15). To amplify the gene for FeSOD, primers 1 and 2, and 2, and chromosomal DNA from Nostoc as the template and the TaKaRa ExTaQ DNA polymerase were used for PCR under the following conditions: 94 °C for 2 min (hot start); 28 cycles at 92 °C for 30 s, 50 °C for 30 s and 72 °C for 1.5 min; finally at 72 °C for 10 min. The PCR product was sequenced by dyeideoxy chain termination method (17). Competent Escherichia coli BL21(DE3) cells were transformed with the expression vector by electroporation (Gene Pulser, Bio-Rad), positive clones carrying the recombinant plasmid were selected and grown overnight on an orbital shaker (180 rpm) at 37 °C in LB medium containing 50 μg/ml kanamycin. M9ZB medium containing 50 μg/ml kanamycin and 100 μM FeSO4 was incubated with an overnight culture, shaken at 37 °C to reach an OD600 of 1.0 and induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside. After 4 h of incubation at 37 °C, cells were harvested by centrifugation (5000 × g, 5 min, 4 °C), frozen, and stored at −80 °C until used. Cells were resuspended in lysis buffer (50 mM Tris/HCl, pH 8.0, containing 2 mM EDTA, 0.1% Triton X-100, 1 mM PMSF, 5 μM leupeptin, and 5 μM pepstatin A) and broken by sonication with short bursts. The cell extract was centrifuged (21,000 × g, 20 min, 4 °C), and from the supernatant the superoxide dismutase was purified.

**Protein Purification**—The supernatant was adjusted to 1 M NaCl and 20 mM Pipes-ROH buffer (pH 6.6) charged with 30 μmol of Zn2+ /ml gel and equilibrated with 67 mM phosphate buffer, pH 7.0, containing 1 M NaCl and 20 mM imidazole at 4 °C. The column was washed with 200 ml of the equilibration buffer, and bound proteins were eluted with 100 ml of a gradient of 20–500 mM imidazole in 67 mM phosphate buffer, pH 7.0, containing 1 M NaCl. The eluted proteins were concentrated with Centriprep concentrators. Proteins were loaded onto a Superdex 200 column equilibrated with 67 mM...
Iron Superoxide Dismutase of Nostoc PCC 7120

RESULTS

Detection of Superoxide Dismutase Activity in Nostoc PCC 7120—To search for enzymes that are able to destroy the superoxide radical anion, cultures of the filamentous cyanobacterium Nostoc PCC 7120 were grown under photautotrophic conditions. Cells were broken, and one part was separated into a cytosolic and a membrane fraction. Because cyanobacteria possess two types of membrane, the membrane component was further separated into cytoplasmic and thylakoid membranes by density gradient centrifugation. The four cell extracts (whole cell extract, cytosol, cytoplasmic membranes, and thylakoids) were used for SOD activity assays and native gel electrophoresis combined with SOD-specific staining. Membrane-containing extracts were solubilized in 2% dodecyl maltoside before application to the native gel. After the electrophoretic run SOD staining of whole cell extracts showed two bands, which were interpreted as an indication of the presence of two different types of SOD (Fig. 1, lane 1). Separation of cytosol and membranes showed that the upper band is only

Fig. 1. Gel electrophoresis of cell lysates and purified enzyme. Lanes 1–4, non-denaturing PAGE of cell lysates from Nostoc PCC 7120 stained for SOD activity; lane 1, crude membranes; lane 2, cytosolic extract; lane 3, whole cell extract; lane 4, whole cell extract incubated with 10 mM hydrogen peroxide. The upper band represents the MnSOD and the lower band the FeSOD. Lanes 5 and 6, SDS-PAGE and Coomassie Blue staining; lane 5, molecular mass markers (molecular masses are given on the left; lane 6, purified FeSOD from Nostoc PCC 7120 overexpressed in E. coli.
The next step was to show the presence and distribution of SODs in heterocysts. Therefore *Nostoc* PCC 7120 was grown in nitrogen-depleted medium and heterocysts were selectively separated from vegetative cells and broken. Whole heterocyst extracts also gave two bands at the same positions as vegetative cells in the native gel after SOD staining. Quantitative measurements using the ferrocyanochrome c assay showed the following values (specific activities in units per mg protein in parenthesis): cytosolic fraction (15.5 ± 2), cytoplasmic membrane (3.1 ± 0.5), and thylakoids (2.0 ± 0.7).

**Localization of FeSOD in *Nostoc PCC 7120* Cells by Immunogold Labeling**—To determine the distribution of FeSOD in vegetative cells and heterocysts, an antibody raised against the *Anabaena cylindrica* FeSOD was used (13). SDS-PAGE and Western blotting had shown that the antibody did not cross-react with MnSOD and is specific for FeSOD. Ultrathin sections of filaments from *Nostoc* PCC 7120 grown under nitrogen-depleted conditions were incubated with this anti-FeSOD antibody and decorated with gold particles. FeSOD was present in both cell types and distributed throughout the cells. Fig. 2 depicts a gold-labeled and post-stained *Nostoc* heterocyst with the typical thick envelope. Quantitative analysis resulted in 5.3 gold particles per μm² within vegetative cells and 4.0 gold particles per μm² in heterocysts. Control experiments without antiseraum gave no labeling. Also a significantly reduced amount of gold particles was seen when the preimmune serum was used. This confirmed that the gold particles bound to the antibody do reflect the presence of FeSOD. The even distribution of particles throughout the cell indicate a cytosolic occurrence of FeSOD in both cell types, heterocysts, and vegetative cells.

**The Gene for the Iron-containing Superoxide Dismutase of *Nostoc PCC 7120*—**Searching the entire genome of the cyanobacterium *Nostoc PCC 7120* revealed two genes with high homology to superoxide dimutases. One of them (sodA) codes for a MnSOD. Analysis of the protein sequence showed that it contains a hydrophobic N terminus, which is responsible for membrane-anchoring (2). The second gene (sodB) encodes an iron-containing superoxide dismutase (FeSOD). Inspection of the amino acid sequence does not reveal any hydrophobic membrane anchor or signal sequence. Therefore, it should be a soluble cytosolic protein. The nucleotide sequence contains an open reading frame including 600 bp from the start site at ATG to the termination codon TAG, coding for a polypeptide chain of 7942 (58/72), 72/81), 6803 (60/72), 7942 (68/72), 7942 (57/71), E. coli (51/64). These homologies reflect the phylogenetic distance between the listed species; the phylogenetic tree constructed with ClustalX groups the cyanobacterial FeSODs within a separated cluster (Fig. 4). Filamentous cyanobacterial enzymes are combined in one sub-
Iron Superoxide Dismutase of Nostoc PCC 7120

Absorption Spectroscopy of Nostoc 7120 FeSOD—Iron content was determined from the absorption spectrum using the peaks at 280 and 350 nm (see Fig. 5A). The peak at 280 nm corresponds to the content of aromatic amino acids, and from the sequence a molar absorbance coefficient ε280 of 46,410 M⁻¹ cm⁻¹ was calculated. The second characteristic peak of FeSOD is that of the iron at 350 nm with ε350 = 1850 M⁻¹ cm⁻¹ (27). Using the absorbances at these wavelengths from the spectrum, the iron occupancy of the recombinant enzyme was determined to be 0.98 Fe per subunit. Addition of 1 mM sodium dithionite to 200 μM recombinant enzyme led to the complete loss of the peak at 350 nm, showing the reduction of iron(III) to iron(II). After removal of the dithionite by gel filtration the catalytic activity was fully present.

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Molecular Mass and Isoelectric Point of the FeSOD from Nostoc PCC 7120—All FeSODs characterized until now are homodimeric or homotetrameric proteins. To determine the oligomeric state of the Nostoc FeSOD, three methods were used. The first one was gel filtration chromatography on a Superdex 200 column, which showed that the purified recombinant protein has a mobility similar to that of chicken ovalbumin with 43 kDa. Nondenaturing polyacrylamide gel electrophoresis at several acrylamide concentrations combined with a Ferguson plot provided a native molecular mass of 42 kDa. Cross-linking with dimethyl suberimidate in Tris buffer at pH 9 and subsequent SDS-PAGE gave a band at 45 kDa. With these very similar results obtained by three different methods and the calculated molecular mass of the monomer of 23 kDa, the homodimeric nature of this FeSOD was confirmed.

Fig. 3. Amino acid sequence alignment of iron-containing superoxide dismutases from various cyanobacterial species and E. coli. The protein sequences are aligned using the program ClustalX, version 1.8. The four amino acid residues coordinating the iron center are highlighted in black, residues involved in substrate entrance and hydrogen bond network are boxed. The last line depicts secondary structural elements (α-helices and β-strands) deduced from the three-dimensional structure of FeSOD from E. coli. Abbreviations: Fe, FeSOD; A.7120, Nostoc (Anaeebana) PCC 7120; N. comm., N. commune; N. linc., N. linckia; N. puncticorne; P. bory., P. boryanum; S.031, Synechocystis PCC 6301; S.803, Synechococcus PCC 7942; T. elon., T. elongatus BP-1.

The classification of FeSOD is based on secondary structure, metal binding, and/or the catalytic mechanism. Most important are four residues that coordinate the metal ion. In FeSOD these are His-28, His-80, Asp-162, and His-166 (see Fig. 3), which normally form together with a hydroxide ion a trigonal bipyramidal coordination sphere. Also conserved are amino acids forming the substrate entrance (Trp-84, Tyr-36, His-32) and a hydrogen bond network (Gln-76, Tyr-36, Trp-129, Asn-79, Asp-185). The peak at 280 nm corresponds to the content of aromatic amino acids, and from the sequence a molar absorbance coefficient ε280 of 46,410 M⁻¹ cm⁻¹ was calculated. The second characteristic peak of FeSOD is that of the iron at 350 nm with ε350 = 1850 M⁻¹ cm⁻¹ (27). Using the absorbances at these wavelengths from the spectrum, the iron occupancy of the recombinant enzyme was determined to be 0.98 Fe per subunit. Addition of 1 mM sodium dithionite to 200 μM recombinant enzyme led to the complete loss of the peak at 350 nm, showing the reduction of iron(III) to iron(II). After removal of the dithionite by gel filtration the catalytic activity was fully present.

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Whereas for the wild-type SOD an isoelectric point (pI) of 5.14 was calculated using the amino acid sequence, the recom-
binant enzyme with a C-terminal hexahistidine tag gave a pi of 5.77. Isoelectric focusing of the purified recombinant FeSOD confirmed the calculated pi with a value of pi 5.9 showing that it is an acidic protein.

Activity Measurements of Recombinant FeSOD—Catalytic activity was determined by the ferriytochrome c assay using xantine and xantine oxidase as source of superoxide. The specific activity was (2040 ± 90) units mg⁻¹ at pH 7.8. Incubation of 100 μm recombinant enzyme with 10 μm H₂O₂, NaCN, or Na₃N₃ for 30 min and thereafter performing the activity assay led to distinct results. No inhibition was detectable with cyanide, whereas azide reduced the activity by 30%. Hydrogen peroxide led to the complete loss of activity. Removing the H₂O₂ by gel filtration does not restore the activity, which shows irreversible inactivation of FeSOD (28). Similar results were obtained on a nondenaturing PAGE. 5 μg of FeSOD were loaded per lane and electrophoresed. All spectra were recorded at room temperature in 100 mM phosphate buffer, pH 7.0.

Several anions act as competitive inhibitors of FeSODs, which can be grouped into two classes according to their ability to change the iron peak at 350 nm (24). Spectral changes were analyzed by mixing 1 ml of 28 μM recombinant Nostoc FeSOD with 25 μl 2 mM Na₂SO₄, NaCl, KSCN, HCOONa, NaF, or NaN₃ (50 mM final concentration of anion) after recording the spectrum of free FeSOD. Sulfate, chloride, thiocyanate, and formate inhibit FeSOD activity but do not change the spectral

FIG. 4. Phylogenetic relationships of iron-containing superoxide dismutases. The phylogenetic tree was constructed using ClustalX, version 1.81, and the Bootstrap Neighbor Joining Tree option. Branch lengths represent calculated phylogenetic distances. The branch containing all cyanobacterial FeSODs is highlighted. Abbreviations: Cm, cambialistic SOD; F1-3, FeSODs; A.7120, Nostoc (Anabaenopsis) PCC 7120; A. eso., Aquifex aeolicus; A. pyro., Aquifex pyrophilus; A. thal., Arabodopsis italiana; A. tume., Agrobacterium tumefaciens; B. bovi., Babesia bovis; B. pseu., Burkholderia pseudomallei; C. coli., Campylobacter coli; C. cres., Caulobacter crescentus; D. vulg., D. vulgaris; E. coli., Escherichia coli; G. poly., Gonyaulax polyedra; H. pylo., Helicobacter pylori; L. chag., Leishmania chagasi; L. pneum., Legionella pneumophila; N. comm., N. commune; N. gono., Neisseria gonorrhoeae; N. linc., N. lineia; N. punc., N. punctiforme; P. aeur., Pseudomonas aeruginosa; P. bory., P. boryanum; P. freu., Propionibacterium freudenreichii; P. ginge., Porphyromonas gingivalis; P. oeal., Pseudomonas ovalis; P. pyro., Aquifex pyrophilus; P. syri., Psychrobacter syriacus; P. vag., Pseudomonas vaginilis; P. vulgaris, Pseudomonas vulgaris; R. caps., Rhodobacter capsulatus; R. prow., Rickettsia prowazekii; S.6301, Synechococcus PCC 6301; S.6803, Synechocystis PCC 6803; S.7942, Synechococcus PCC 7942; S. acid., Sulfolobus acidocaldarius; S. meli., Sinorhizobium meliloti; S. so., Sulfolobus solfataricus; S. thyl., Salmonella typhimurium; T. acid., Thermoplasma acidophilum; T. cruz., Trypanosoma cruzi; T. elon., T. elongatus; T. foet., Trichomonas fetus; T. vagi., Trichomonas vaginalis; T. chol., Vibrio cholerae; Y. pest., Yersinia pestis; Z. aeth., Zantedeschia aethiopica.
properties of FeSOD. This is interpreted as an indication that the anion does not directly bind to the metal ion but blocks the access of superoxide to the active site. However fluoride led to disappearance of the iron peak, whereas azide gave rise to two new bands in the visible region (see Fig. 5B), which showed that these two anions directly bind to the iron of the enzyme. To measure the dissociation constant for the complex between the *Nostoc* FeSOD and azide, 100 μM enzyme was titrated by increasing the NaNO₃ concentration from 1 to 68 mM in 100 phosphate buffer at pH 7.0, and absorption spectra were recorded (Fig. 5, B and C). The dissociation constant ($K_d$) was calculated from the difference spectra of cyanide complex minus oxidized FeSOD and showed a value of $K_d = 2.1$ mM. This value is very similar to the $K_d$ determined for the FeSOD of *E. coli*, which gave $K_d = 2.3$ mM (24).

**Stopped Flow Measurements**—Stopped flow technology was used to quantitate steady-state SOD activity at 15 or 20 °C and pH values between pH 7 and 10. Two different final superoxide concentrations (220 μM and 4.6 mM) were employed to follow uncatalyzed and SOD-catalyzed superoxide decay. The solubility of the potassium superoxide (KO₂) in the Me₂SO:DMF mixture was increased by utilizing crown ethers. The amount of organic solvent in the final mixture was reduced to 5% (v/v) by first mixing superoxide solution in Me₂SO:DMF with a weak buffer at pH 10 in a 1:10 ratio. The resulting solution was combined with a strong buffer of the desired pH in a second mixer. High quality chemicals guaranteed second-order self-dismutation in the absence of enzyme at pH 7–10 as could be seen from a linear fit of reciprocal superoxide concentration versus time. To monitor enzyme kinetics the concentrations of recombinant *Nostoc* FeSOD were varied between 12.5 nM (lowest concentration at pH 7) and 20 μM (highest concentration at pH 10). Measurements at low and high superoxide concentrations with FeSOD showed no indication of saturation. From these curves it was not possible to calculate turnover numbers and Michaelis constants ($K_m$) using the model described by Bull and Fee (24, 29). Our equipment did not reach temperatures as low as 3 °C or 5.5 °C which were used to measure superoxide decay with *E. coli* FeSOD or bovine CuZnSOD to demonstrate a rate-limiting first-order process and calculate turnover numbers and $K_m$ (24, 29). But the curves fit very well by a first-order superoxide decay and a plot of the logarithm of absorbance versus time is linear. This is similar to the behavior of bovine CuZnSOD at 20 °C (29, 30). At constant superoxide concentrations, the enzyme was varied from nanomolar to micromolar concentrations (Fig. 6A) and the obtained pseudo-first-order rate constants ($k_{obs}$) were plotted against concentration. The plots were linear over the enzyme concentration range confirming that the reaction is first-order in enzyme concentration (Fig. 6B). The contribution to the observed rate by the second-order self-dismutation is small (see intercept in Fig. 5B) under the conditions of the experiment over the pH 7–10. The slope of the $k_{obs}$ versus enzyme concentration plot allowed the calculation of the second-order rate constant ($k$). These second-order rate constants decrease with increasing pH value by a factor of about ten per pH unit (Fig. 6C), $k = 5.3 \times 10^9$ M⁻¹ s⁻¹ (pH 7), 1.7 $\times 10^8$ M⁻¹ s⁻¹ (pH 8), 3.9 $\times 10^7$ M⁻¹ s⁻¹ (pH 9), and 4.8 $\times 10^6$ M⁻¹ s⁻¹ (pH 10). This is in accordance with other FeSODs and MnSODs, which also show reciprocal pH dependence and is in contrast to the pH independence of CuZnSOD over the range 7–9 (30).

**DISCUSSION**

Cyanobacteria form a diverse group of bacteria that use oxygenic photosynthesis for energy production from sunlight. Many cyanobacteria are able to reduce atmospheric dinitrogen to ammonia by an enzyme called nitrogenase. In some filamentous cyanobacteria, nitrogen-fixing heterocysts are formed. These heterocysts are terminally differentiated cells with thick cell walls whose interior becomes nearly anaerobic, mainly as a consequence of respiration, allowing the oxygen-sensitive process of nitrogen fixation to continue (31). Nitrogenase is highly sensitive to dioxygen and is irreversibly inactivated by it. Organisms able to perform N₂ fixation in aerobic conditions have evolved mechanisms to protect this nitrogenase from the deleterious effects of O₂. Reactive oxygen species produced from O₂ may be involved in these toxic reactions (32). Superoxide directly reacts with iron-sulfur clusters in enzymes, such as aconitate, containing 4Fe-4S clusters. Thereby, the enzyme is inactivated by releasing iron from the cluster (33). It is tempting to speculate that a similar mechanism could be responsible for irreversible inactivation of nitrogenase reductase and/or nitrogenase through the destruction of their iron-sulfur clusters. Reactive oxygen species may also change the redox state of the 4Fe-4S clusters, thus inhibiting the acceptance or donation of electrons (4). Therefore, in a nitrogen-fixing cyanobacterium it is extremely important to control the level of oxygen and reactive oxygen molecules (4). This is underscored by the recent investigations of the proteome of *N. punctiforme* (35), where the cells were grown in nitrogen-deficient media, and a cytosolic fraction was analyzed by two-dimensional electrophoresis and mass spectrometry. Based on optical density from the two-dimensional gel, FeSOD was shown to be the most abundant non-photobacterin protein found in the cytosolic extract. In addition three putative peroxidases with homology to peroxiredoxins were shown to also belong to the most highly expressed proteins of this filamentous nitrogen-fixing cyanobacterium (35). These findings clearly emphasize the importance of free radical management in cyanobacteria.

*Nostoc* PCC 7120 possesses two types of superoxide dismutase, a soluble FeSOD in the cytosol and one and the same membrane-bound MnSOD in both membrane types. The solu-
FeSOD has a 5–8-fold higher specific activity than that of cytoplasmic or thylakoid membranes, respectively. Topology analysis of the MnSOD predicts that the catalytic domain is pointing outwards, in the case of a cyanobacterium meaning to the periplasm or the thylakoid lumen (2). Fig. 7 schematically depicts the different compartments of a cyanobacterial cell including the electron transport components of photosynthesis and respiration, which are the main source of superoxide production. The chlorophyll-free cytoplasmic membrane contains only the respiratory machinery whereas thylakoid membranes possess both systems. Additionally the localization of the two SODs is also illustrated. Because most of the superoxide radicals are produced at the iron-sulfur clusters of photosystem I on the cytosolic site, FeSOD seems to be more important to keep the superoxide level low. But also the thylakoid lumen may contain superoxide, and an additional SOD is necessary to remove it in this compartment. In particular, cyanobacterial membranes have a high content of polynsaturated fatty acids (36), so the concentration of reactive oxygen species should be low to prevent oxidative damage. The membrane-bound MnSOD could play this important role in thylakoid lumen and periplasm.

From plant chloroplasts it is known that the stromal superoxide dismutase is an important component of a system that scavenges reactive oxygen species and dissipates excess photons, the water-water cycle (37, 38). At high light intensities, low CO₂ concentrations, or other stress conditions, electrons released in photosystem II from water are used for photoreduction of dioxygen in photosystem I. The superoxide radical formed is rapidly converted to hydrogen peroxide, which can be detoxified by ascorbate peroxidase providing water. The resulting oxidized forms of ascorbate are re-reduced by ferredoxin, monodehydroascorbate reductase, or dehydroascorbate reductase (37, 38).

Analogous to plant chloroplasts, the water-water cycle also functions in cyanobacteria (39). The FeSOD at the cytosolic site of the thylakoid membranes of Nostoc PCC 7120 and many other cyanobacteria removes superoxide at the production site at photosystem I. But the scavenging of hydrogen peroxide is different in cyanobacteria compared with chloroplasts. Cyanobacteria do not contain an ascorbate peroxidase, but they use catalase and/or thioredoxin peroxidase (2-cysteine peroxiredoxin) activities to remove H₂O₂ (40). In the catalase reaction, catalase peroxidase (or classical, monofunctional catalase) is utilized to disproportionate H₂O₂ without the need of any electron donor. In the second type thioredoxin peroxidase reduces H₂O₂ to water using reduced thioredoxin, which is regenerated via ferredoxin-thioredoxin reductase by electrons from photosystem I (39). In the genome of Nostoc PCC 7120 one gene for manganese-containing catalase (61% sequence identity to that of Bacillus halodurans), one gene for thioredoxin peroxidase and two genes for 1-cysteine peroxiredoxin can be found (40). Therefore, both types of H₂O₂ removal systems could be used in the water-water cycle of Nostoc PCC 7120 (Fig. 7).

The water-water cycle (including the FeSOD) fulfills several physiological functions. It keeps the concentration of photoproduced reactive oxygen species low and prevents the generation of hydroxyl radicals. At high light intensities this cycle can also prevent the over-reduction of electron carriers (which leads to photoinhibition) by working as an alternative photon and electron sink (38). The electron flux produced through the water-water cycle delivers ATP for the formation of an inorganic carbon pool within the cell and for the CO₂ fixation in the Calvin-Benson cycle (39).

What is known about the distribution and function of both SODs in heterocysts of Nostoc PCC 7120? Separation of heterocystous cytosol from total membranes clearly depicts that both fractions contain SOD activity to a similar extent (see Table I) and that the FeSOD is again in the cytosol and MnSOD membrane-bound. This is in contrast to a previous article (9) where the authors agree with our group that the FeSOD is soluble and the MnSOD is membrane-bound and both SODs are present in vegetative cells; although in heterocysts they could only detect the FeSOD. The reason for this difference may perhaps lie in deviations of the exact growth conditions. They described that FeSOD increased within the first few hours of induction of heterocyst formation, remained at a high level until heterocyst differentiation was complete, and thereafter declined to normal levels. The increased expression of FeSOD during the phase of heterocyst differentiation corresponds to the high energy need during this phase, which is always correlated with an increased oxygen metabolism. The MnSOD was only elevated during periods of high oxidative stress (9). Therefore, the FeSOD could be more important for Nostoc PCC 7120 under nitrogen-depleted conditions where growth is dependent on heterocyst formation and nitrogenase activity.

Are the investigations in Nostoc PCC 7120 relevant for superoxide detoxification in other cyanobacteria? Which superoxide dismutases are present in other cyanobacteria? To answer these questions we looked into data bases of completely or partially sequenced cyanobacteria of different habitats to determine if the superoxide dismutases are present in other cyanobacteria? To answer these questions we looked into data bases of completely or partially sequenced cyanobacteria of different habitats to determine if the superoxide dismutases are present in other cyanobacteria? To answer these questions we looked into data bases of completely or partially sequenced cyanobacteria of different habitats to determine if the superoxide dismutases are present in other cyanobacteria?
and the MnSOD of crystallography was performed for the FeSOD of marine cyanobacterium Synechococcus (43). Four genes of SOD, one coding for a cytosolic SOD, which contain iron (freshwater cyanobacteria) or nickel (marine cyanobacteria) at the active site and could be membrane-bound MnSODs to effectively lower superoxide concentration. Other species with additional SODs, in particular filamentous species with heterocysts having nitrogenase activity appear to need one or more supplementary MnSOD (gene 257.5591) Membrane-bound FeSOD (gene 461.70) Cytosol

<table>
<thead>
<tr>
<th>Cyanobacterium</th>
<th>Superoxide dismutase (gene number)</th>
<th>Putative localization</th>
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</thead>
<tbody>
<tr>
<td>Anabaena (Nostoc) PCC 7120</td>
<td>FeSOD (alr2938)</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Anabaena variabilis ATCC 29413</td>
<td>MnSOD (aln0070)</td>
<td>Membrane-bound</td>
</tr>
<tr>
<td>N. punctiforme ATCC 29133</td>
<td>FeSOD (gene 218.395)</td>
<td>Cytosol</td>
</tr>
<tr>
<td>P. boryanum UTEX 485</td>
<td>MnSOD (gene 257.3591)</td>
<td>Membrane-bound</td>
</tr>
<tr>
<td>P. marinus MIT9313</td>
<td>FeSOD (461.70)</td>
<td>Cytosol</td>
</tr>
<tr>
<td>P. marina SS120</td>
<td>MnSOD (gene 458.34)</td>
<td>Membrane-bound</td>
</tr>
<tr>
<td>Trichodesmium erythraeum</td>
<td>MnSOD (gene 507.217)</td>
<td>Membrane-bound</td>
</tr>
<tr>
<td>G. violaceus PCC 7421</td>
<td>MnSOD (gene 7063)</td>
<td>Membrane-bound</td>
</tr>
<tr>
<td>Synechocystis PCC 6803</td>
<td>MnSOD (gfg1327)</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Synechococcus PCC 7942</td>
<td>MnSOD (gb0692)</td>
<td>Membrane-bound</td>
</tr>
<tr>
<td>T. elongatus BP-1</td>
<td>CuZnSOD (gb1981)</td>
<td>Membrane-bound</td>
</tr>
<tr>
<td>Synechococcus WH8102</td>
<td>CuZnSOD (gbr2170)</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Prochlorococcus marinus MED4</td>
<td>CuZnSOD (gbr1516)</td>
<td>Cytosol</td>
</tr>
<tr>
<td>P. marinus PCC 70027</td>
<td>NfSOD (gene 6285)</td>
<td>Cytosol</td>
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<tr>
<td>P. marinus PCC 70027</td>
<td>NfSOD (gene 7063)</td>
<td>Membrane-bound</td>
</tr>
<tr>
<td>P. marinus PCC 70027</td>
<td>ThfSOD (thf0036)</td>
<td>Membrane-bound</td>
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<tr>
<td>P. marina PCC 70027</td>
<td>NfSOD (SYN1626)</td>
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<td>P. marina PCC 70027</td>
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<td>NfSOD (PM10340)</td>
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<tr>
<td>P. marina PCC 70027</td>
<td>NfSOD (Pro1368)</td>
<td>Cytosol</td>
</tr>
</tbody>
</table>

Table II lists twelve cyanobacterial species or strains where sequences of the whole or nearly complete genome are available. Included are also data from P. boryanum (44). All filamentous cyanobacteria seem to contain a membrane-bound MnSOD, whereas unicellular cyanobacteria only have a cytosolic superoxide dismutase with the exception of Gloeobacter violaceus and T. elongatus, which also have a membrane-bound MnSOD. What is especially interesting is the fact that all freshwater cyanobacteria contain a cytosolic FeSOD, but marine cyanobacteria do not comprise a gene for FeSOD; however, they encode a protein with 30–40% identity and 50–60% similarity to NiSOD of Streptomyces species (12). This may be related to the very low content of dissolved iron in upper layers of the open ocean. To summarize, all cyanobacteria contain a cytosolic SOD, which contain iron (freshwater cyanobacteria) or nickel (marine cyanobacteria) at the active site and could be the housekeeping superoxide dismutase. Some cyanobacteria have additional SODs, in particular filamentous species with nitrogenase activity appear to need one or more supplementary membrane-bound MnSODs to effectively lower superoxide concentration.

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The Iron Superoxide Dismutase from the Filamentous Cyanobacterium *Nostoc* PCC 7120: LOCALIZATION, OVEREXPRESSION, AND BIOCHEMICAL CHARACTERIZATION

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