

# A Cyanobacterium Lacking Iron Superoxide Dismutase Is Sensitized to Oxidative Stress Induced with Methyl Viologen but Is Not Sensitized to Oxidative Stress Induced with Norflurazon<sup>1</sup>

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A strain of *Synechococcus* sp. strain PCC 7942 with no functional Fe superoxide dismutase (SOD), designated *sodB*<sup>-</sup>, was characterized by its growth rate, photosynthetic pigments, and cyclic photosynthetic electron transport activity when treated with methyl viologen or norflurazon (NF). In their unstressed conditions, both the *sodB*<sup>-</sup> and wild-type strains had similar chlorophyll and carotenoid contents and catalase activity, but the wild type had a faster growth rate and higher cyclic electron transport activity. The *sodB*<sup>-</sup> was very sensitive to methyl viologen, indicating a specific role for the FeSOD in protection against superoxide generated in the cytosol. In contrast, the *sodB*<sup>-</sup> mutant was less sensitive than the wild type to oxidative stress imposed with NF. This suggests that the FeSOD does not protect the cell from excited singlet-state oxygen generated within the thylakoid membrane. Another up-regulated antioxidant, possibly the MnSOD, may confer protection against NF in the *sodB*<sup>-</sup> strain. These results support the hypothesis that different SODs have specific protective functions within the cell.

Oxygenic PET and aerobic respiration evolved during the Precambrian period, improving the efficiency of C metabolism many-fold. The benefits of these new pathways were partially offset, however, by their tendency to form reactive oxygen species that cause oxidative damage to biological molecules. The most significant reactive oxygen species include excited singlet-state oxygen (<sup>1</sup>O<sub>2</sub><sup>\*</sup>), the superoxide ion (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and the highly destructive hydroxyl radical (·OH). O<sub>2</sub><sup>-</sup> and <sup>1</sup>O<sub>2</sub><sup>\*</sup> mainly occur in the electron-transport chains of PET and respiration (Asada and Takahashi, 1987; Gutteridge and Halliwell, 1990; Shiraishi et al., 1994). They subsequently give rise to H<sub>2</sub>O<sub>2</sub> and ·OH. The reaction of O<sub>2</sub><sup>-</sup> with H<sub>2</sub>O<sub>2</sub> that forms ·OH is often catalyzed by metals, especially Fe<sup>2+</sup> (Halliwell and Gutteridge, 1986). As a consequence, Fe-bearing biomolecules, such as metalloenzymes and electron-transport proteins, may be the first sites of O<sub>2</sub><sup>-</sup> damage in cells (Halliwell and Gutteridge, 1986; Kuo et al., 1987; Fridovich, 1989; Gutteridge and Halliwell, 1990;

Gardner and Fridovich, 1991). Following oxidative damage to Fe-bearing proteins, freed Fe<sup>2+</sup> can adversely react with other cellular components, causing additional damage. O<sub>2</sub><sup>-</sup> may also disrupt Fe-S centers directly in proteins such as Fd, aconitase, succinate dehydrogenase, and PSI (Liochev, 1996). <sup>1</sup>O<sub>2</sub><sup>\*</sup> occurs in the chlorophyll antennae when excited triplet-state chlorophyll transfers excitation energy to molecular oxygen. <sup>1</sup>O<sub>2</sub><sup>\*</sup> can give rise to the O<sub>2</sub><sup>-</sup> anion (Asada and Takahashi, 1987; Symons, 1991). This results in a potential for oxidative damage within the thylakoid membrane. In plants, formation of both <sup>1</sup>O<sub>2</sub><sup>\*</sup> and O<sub>2</sub><sup>-</sup> by PET is favored when normal metabolic pathways are slowed by physiological stress.

Reactive oxygen species are detoxified in cells by a system of antioxidants that co-evolved with PET and respiration. SODs catalyze the conversion of O<sub>2</sub><sup>-</sup> to H<sub>2</sub>O<sub>2</sub>. Catalase subsequently oxidizes the H<sub>2</sub>O<sub>2</sub> to molecular oxygen and water. Plant and algal chloroplasts and some bacteria (e.g. *Streptococcus* spp.) lack catalase and instead use peroxidases that require ascorbate or glutathione as sources of reducing power. In photosynthetic organisms, carotenoid pigments in the light-harvesting antennae quench triplet chlorophyll and <sup>1</sup>O<sub>2</sub><sup>\*</sup> (Siefermann-Harms, 1987; Koyama, 1991), both of which can produce O<sub>2</sub><sup>-</sup> and lipid peroxy radicals. Other nonenzymatic reductants, such as tocopherols and tocotrienols, can quench reactive forms of oxygen as well.

The SODs are metalloenzymes that may be separated into three classes, depending on their metal cofactor. MnSODs are found in the cytosol of eubacteria, in the cytosol and thylakoid membrane of cyanobacteria, and in the mitochondrial lumen of eukaryotes. FeSODs are found in the cytosol of eubacteria and cyanobacteria and in the chloroplast stroma of photosynthetic plant cells. FeSODs are not usually found in eukaryotes other than plants. Cu/ZnSODs are present only in eukaryotes and may be found in the cytosol, chloroplast, and mitochondrial intermembrane spaces (Okada et al., 1979; Campbell and Laudenbach, 1995). FeSODs and MnSODs are proposed to have a common prokaryotic origin, whereas Cu/ZnSODs are proposed to have evolved independently in eukaryotes

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Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; MV, methyl viologen; NF, norflurazon; PET, photosynthetic electron transport; SOD, O<sub>2</sub><sup>-</sup> dismutase.

(Bannister et al., 1987; Campbell and Laudenbach, 1995). Despite differing evolutionary histories, the catalytic activities of the different types of SODs are essentially the same. One class of SODs can complement deletion mutations of other classes of SODs within and between species, families, and even kingdoms (Carlioz and Touati, 1986; Laudenbach et al., 1989; Haas and Goebel, 1992; Purdy and Park, 1994; Takeshima et al., 1994). The existence of multiple SODs may result from the fact that the cells of cyanobacteria and eukaryotes are divided into compartments by internal membranes. Since  $O_2^-$  ions are negatively charged and cannot cross a phospholipid bilayer readily, they are effectively trapped within the compartment where they were generated. This may have selected for the evolution of multiple SODs in compartmentalized cells. Indeed, most organisms with compartmentalized cells have SODs in compartments that are likely to generate  $O_2^-$ , such as mitochondria and chloroplasts (for review, see Fridovich, 1989).

We have chosen cyanobacteria as models for the study of SODs. Like eukaryotes, cyanobacteria have compartmentalized cells (cytosol and thylakoid lumen) and multiple SODs (Okada et al., 1979; Herbert et al., 1992; Campbell and Laudenbach, 1995). The photosynthetic apparatus of cyanobacteria is essentially the same as that of algae and plants, but cyanobacteria are easier to genetically manipulate than plants. For example, genes for the enzymatic antioxidants can be specifically inactivated by insertional mutagenesis (Golden, 1988; Porter, 1988). Also, the antioxidant system of cyanobacteria is simpler than that of plants. Genetic overexpression of SODs and other antioxidant enzymes in plants has been attempted with the goal of increasing their stress tolerance (Bowler et al., 1991; Foyer et al., 1994; Rennenberg and Polle, 1994; Allen, 1995; Van Camp et al., 1996). The antioxidant system in plants is quite complex, however, and attempts to create stress tolerance by overexpression of individual antioxidants have met with mixed success (Bowler et al., 1991; Pitcher et al., 1991; Foyer et al., 1994; Van Camp et al., 1996). Genetic improvements of the antioxidant system in plants can be designed more carefully if the specific intracellular roles of different types of antioxidants are better understood by study in simpler systems.

The cyanobacterium *Synechococcus* sp. strain PCC7942 (hereafter referred to as PCC7942) possesses two SODs. The MnSOD encoded by the *sodA* gene is thylakoid associated, whereas the FeSOD encoded by the *sodB* gene is cytosolic (Herbert et al., 1992). We hypothesize that the two SODs have different protective functions within the cyanobacterial cell. The FeSOD protects against  $O_2^-$  formed within the cytosol, whereas the MnSOD protects against  $O_2^-$  formed in the thylakoid membranes or within the thylakoid lumen. A mutant of PCC7942 lacking detectable FeSOD activity was constructed previously and designated *sodB*<sup>-</sup> (Laudenbach et al., 1989; Herbert et al., 1992). In this study we compared the response of the wild-type and *sodB*<sup>-</sup> strains to damage caused by either  $O_2^-$  formation in the cytosol or by  $^1O_2^*$  formation within the thylakoid membrane. We predicted that, relative to the wild type, the *sodB*<sup>-</sup> mutant would be sensitized to damage by cytosolic

$O_2^-$  but not sensitized to damage by  $^1O_2^*$  formed within the thylakoid membranes. We found that the *sodB*<sup>-</sup> strain was more sensitive to damage by  $O_2^-$  within the cytosol but was actually partially resistant to damage by  $^1O_2^*$  in the thylakoid membranes.

## MATERIALS AND METHODS

### Culture and Experimental Conditions

Stock cultures of wild-type and *sodB*<sup>-</sup> *Synechococcus* sp. PCC7942 were grown in 50-mL tubes of BG-11 broth (Sigma) supplemented with 10 mM NaHCO<sub>3</sub> and adjusted to pH 8.0 with 5 mM KH<sub>2</sub>PO<sub>4</sub>. Cultures were incubated in a water bath at 27°C, sparged with 3% CO<sub>2</sub> in air, and illuminated with cool-white fluorescent tubes with a PAR flux of 15 to 35  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Light intensities were measured using a LI-189 quantum sensor (Li-Cor, Lincoln, NE). Dry cell mass, direct cell counts, and  $A_{750}$  were used to monitor cell concentration. We found a high correlation ( $r = 0.97$ ,  $n = 25$ ) between  $A_{750}$  and cellular dry mass, confirming the use of absorbance at this wavelength as an indicator of biomass (data not shown). Unless otherwise noted, cultures were diluted to  $A_{750} = 0.3$  with fresh BG-11 broth without supplemental NaHCO<sub>3</sub> prior to experiments. Oxidative stress treatments were imposed in water-jacketed beakers maintained at 27°C in air and illuminated with 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  from cool-white fluorescent tubes. Samples were stirred continuously during treatments.

### Growth Measurements

Rapidly growing cells were centrifuged and resuspended in fresh BG-11 broth to an  $A_{750}$  of 0.1 to 0.3 and illuminated at 30  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Aliquots of 3 mL were removed at approximately 24-h intervals and  $A_{750}$  was measured. Periodically, 10-mL samples were removed, vacuum filtered onto 0.45- $\mu\text{m}$  membrane filters, and dried for 24 h at 100°C to determine dry mass.

### Oxidative Stress Induction

$O_2^-$  generation was catalyzed by adding MV (paraquat) to cultures to obtain a final concentration of 0.1 to 5.0  $\mu\text{M}$ . MV catalyzes the formation of  $O_2^-$  at a variety of electron-transport sites, but in photosynthetic organisms in light the vast majority of  $O_2^-$  is generated at the F<sub>A</sub> and F<sub>B</sub> centers of PSI (Fujii et al., 1990; Dodge, 1991). MV is a competitive inhibitor of the PSI cyclic pathway at concentrations of 100  $\mu\text{M}$  (Yu et al., 1993). However, the low concentrations of MV used in our experiments had no effect on the PSI cyclic pathway (Herbert et al., 1995; Martin et al., 1997).  $^1O_2^*$  generation was catalyzed indirectly by adding the carotenoid inhibitor NF to cultures to obtain a final concentration of 0.1 to 5.0  $\mu\text{M}$ . NF inhibits phytoene desaturase and thus blocks synthesis of  $\beta$ -carotene and other carotenoids (Ben-Aziz and Koren, 1974; Kümme and Grimme, 1975). Since carotenoids ( $\beta$ -carotene in particular) normally quench  $^1O_2^*$  in the chlorophyll antenna, the addition of NF pro-

motes the formation of  $^1\text{O}_2^*$  within the thylakoid membrane.

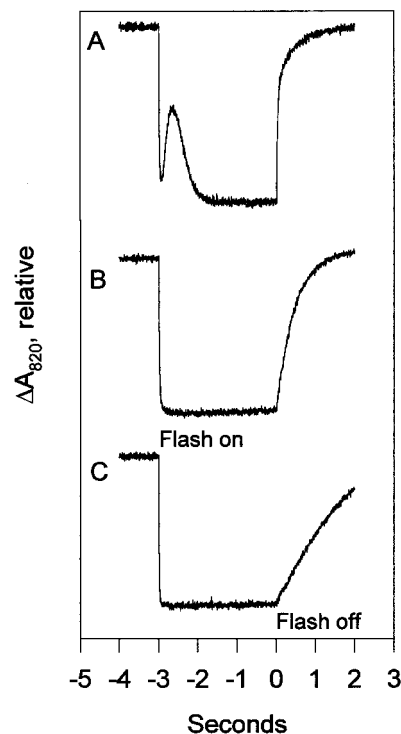
### $P_{700}$ Oxidation Reduction

The photooxidation and dark-reduction kinetics of  $P_{700}$  were measured in intact cells using the broadband  $A_{820}$  change ( $\Delta A_{820}$ ), as described elsewhere (Herbert et al., 1995). The  $\Delta A_{820}$  was monitored by reflectance using a modulated detection system (Walz, Effeltrich, Germany) consisting of a PAM 101 control unit and an ED 800T emitter-detector unit. A branched fiber optic cable was used to deliver modulated 820 nm and white actinic light to the sample and to collect reflected 820 nm light. Actinic light ( $1000 \mu\text{M photons m}^{-2} \text{s}^{-1}$ ) was provided by a tungsten projector lamp (model EJV, General Electric) fitted with three Calflex C heat filters (Balzers, Liechtenstein) and a mechanical shutter (Uniblitz VS25, Vincent Associates, Rochester, NY). Output from the PAM 101 control unit was collected and analyzed with a MacLab/2e data acquisition system using Scope v3.3 software (AD Instruments, Milford, MA) on a Macintosh computer. Samples for  $\Delta A_{820}$  measurements were prepared at room temperature by vacuum filtering 10 mL of culture onto 0.45- $\mu\text{m}$  membrane filters (type HA, Millipore). The filter and sample were then placed under an acrylic light guide at the end of the trifurcated fiber optic cable. Electron transport inhibitors (DCMU and/or DBMIB) were added to the samples prior to filtration.

$A_{820}$  transients were analyzed and interpreted as described by Yu et al. (1993), with the exception that we measured the initial slopes instead of using half-times to determine rates of oxidation and re-reduction of  $P_{700}$ . Typical traces of raw data are shown in Figure 1. Inhibitors of electron transport were used to block different inputs of electrons to PSI, as has been done previously (Maxwell and Biggins, 1976; Herbert et al., 1992; Yu et al., 1993). Input from PSII was abolished with 25  $\mu\text{M}$  DCMU. Input from the plastoquinone pool was blocked with 25  $\mu\text{M}$  DBMIB, leaving only "nonspecific reductants" to slowly re-reduce  $P_{700}$ . The nature of these nonspecific reductants is uncertain (Maxwell and Biggins, 1976). Recent findings indicate that DBMIB itself may act as a slow electron donor to PSI (Martin et al., 1997).

### Pigment Measurements

Measurements of photosynthetic pigments were made using a DW-2000 scanning spectrophotometer (SLM/Aminco, Urbana, IL). Estimates of chlorophyll *a* and phycocyanin concentrations were made by measuring  $A_{625}$ ,  $A_{678}$ , and  $A_{700}$  in whole-cell suspensions and by applying the calculations of Myers et al. (1980). Chlorophyll and carotenoids were then extracted in 100% acetone. Chlorophyll *a* was quantified from  $A_{663}$  using an absorption coefficient of 11.3. The ratio of total carotenoids to chlorophyll *a* in the extract was estimated by integrating the absorbance at the intervals of 400 to 520 nm and 640 to 690 nm. Relative amounts of specific carotenoids and chlorophyll *a* were measured by reverse-phase HPLC. A liquid



**Figure 1.**  $P_{700}$  oxidation-reduction trace. These are typical traces from  $P_{700}$  measurements with and without added inhibitors. Each trace is an average of four measurements. Oxidation occurs when the sample is flashed with actinic light ("flash on"). Re-reduction occurs in the dark when the light is turned off ("flash off"). The difference in absorbance between the oxidized and reduced states is proportional to the amount of  $P_{700}$  present. The initial oxidation rate ("on rate") is proportional to the efficiency with which the photosynthetic antennae deliver excitation energy to  $P_{700}$ . The initial re-reduction rate ("off rate") is proportional to the rate of electron transfer to  $P_{700}$  from the cyclic and noncyclic electron transport pathways. The transient seen just after the actinic flash in untreated samples (A) is thought to be due to electron input from PSII. In the presence of DCMU (B), the input of electrons from PSII to PSI is blocked, resulting in a lower rate of re-reduction. In the presence of DBMIB (C), electrons from both the cyclic and noncyclic pathways are blocked, resulting in very slow re-reduction. For comparison, in an unstressed wild-type sample, the relative re-reduction rates are: A = 116, B = 79, and C = 14.

chromatograph (series 4, Perkin-Elmer) was used in conjunction with an LC-95 UV/visible spectrophotometric detector (Perkin-Elmer) and a Supelcosil LC-18-DB 15-cm  $\times$  4.6-mm, 3- $\mu\text{m}$  pore-size column (Supelco, Bellefonte, PA). The carrier protocol was described previously (Millie et al., 1990), but we made one minor alteration. The carrier that we used consisted of 90% methanol, 6% acetonitrile, and 4% water for 7 min, followed by 32% methanol, 3% acetonitrile, and 65% acetone for 10 min. Fifteen milliliters of cell suspension ( $A_{750} = 0.3$ ) was centrifuged at 1800g for 10 min. The supernatant was discarded and the pellet extracted in 1 mL of methanol for 1 h at  $-20^\circ\text{C}$  in the dark. Fifty microliters of the extract was injected into a 20- $\mu\text{L}$  sample loop. Carotenoid and chlorophyll peaks were identified at 420 nm using known standards, absorbance spectra, and/or previously published chromatograms. The

presence or absence of tocopherols was investigated using the same HPLC system at 290 nm in a 100% methanol carrier with known standards (Lang et al., 1992).

### Catalase Assay

Activity of the catalase in wild-type and *sodB*<sup>-</sup> strains of PCC 7942 was determined in intact cells by oxygen evolution in response to exogenous H<sub>2</sub>O<sub>2</sub>. Cultures were washed and resuspended to an  $A_{750}$  of 0.3 in fresh medium. Aliquots (1.5 mL) of these samples were placed in a DW1 liquid-phase oxygen electrode cuvette (Hansatech, Norfolk, UK) and stirred vigorously at 27°C. Oxygen in the cuvette was decreased to 30% of air-saturated values with a stream of N<sub>2</sub> gas, and the cuvette was sealed and allowed to stabilize. A rate-saturating amount of H<sub>2</sub>O<sub>2</sub> was then injected (100  $\mu$ L of a 30% solution), and the rate of oxygen evolution was measured 10 to 30 s after injection within the linear range of the reaction. The background rate of spontaneous oxygen formation was measured by injecting the same amount of H<sub>2</sub>O<sub>2</sub> into a sample of fresh medium containing no cells. This background rate was subtracted from the rates obtained with cell samples and was typically less than 20% of the rate obtained with cells. The assays were performed in darkness to avoid contributions from photosynthetic oxygen evolution. Since PCC7942 cells are small and a rate-saturating amount of H<sub>2</sub>O<sub>2</sub> was added for the assay, breakage of the cells was not considered necessary to obtain comparable catalase activities of the two strains. Both H<sub>2</sub>O<sub>2</sub> and oxygen diffuse rapidly in and out of cells, and cell breakage introduces the risk of catalase degradation during the assay.

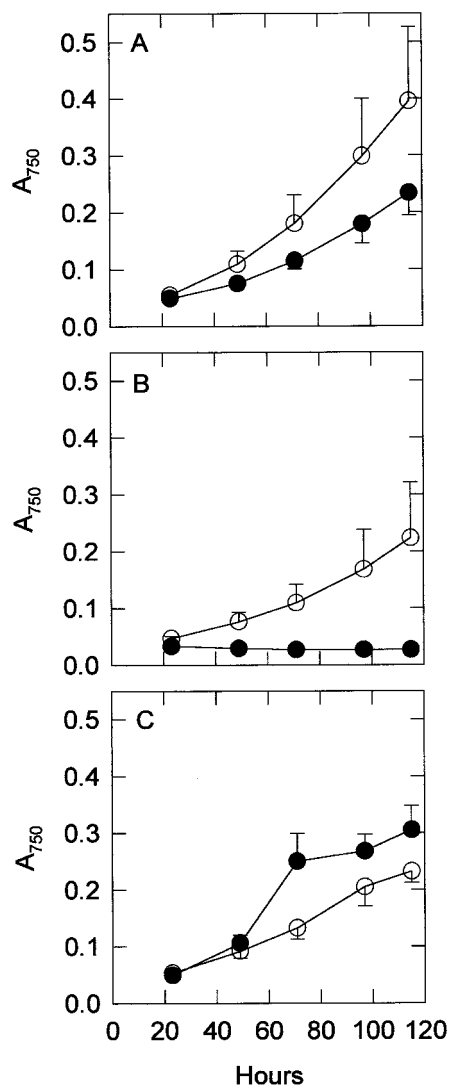
## RESULTS

### Growth Data

Figure 2 shows the growth rates of both strains with and without oxidative stresses. Without oxidative stress, the wild-type strain grew somewhat faster than the *sodB*<sup>-</sup> strain. In the presence of 0.5  $\mu$ M MV, the growth rate of the wild type was markedly slower, but the *sodB*<sup>-</sup> strain did not grow at all. In the presence of 5  $\mu$ M NF, both strains had lower growth rates, but initially the *sodB*<sup>-</sup> strain had a higher growth rate than the wild type. Eventually, both strains died in NF, but the wild type was the first to succumb.

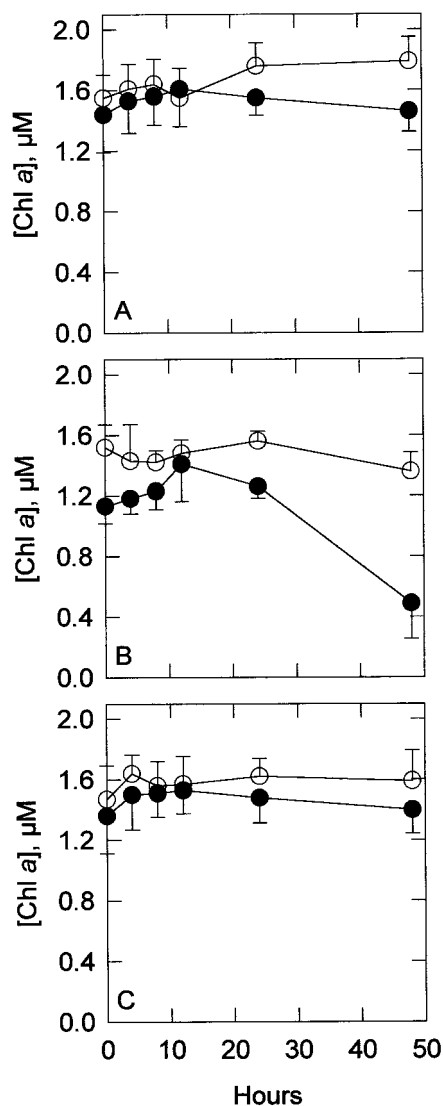
### Photosynthetic Pigments

The amount of phycocyanin changed little during all treatments (data not shown). Measurements of chlorophyll *a* in acetone extracts were consistently lower in the *sodB*<sup>-</sup> strain than in the wild type during all treatments, as shown in Figure 3. Measurements of chlorophyll *a* in whole cells were very similar to the acetone extracts (data not shown). The most marked difference between the two strains was seen during the MV treatments, when the wild type had approximately 3 times more chlorophyll *a* than the *sodB*<sup>-</sup> strain at the end of 48 h. The ratio of total carotenoids to



**Figure 2.** Growth rates of wild-type (○) and *sodB*<sup>-</sup> (●) strains. Growth curves measured at  $A_{750}$  are shown here for control (A), 0.5  $\mu$ M MV-treated (B), and 5  $\mu$ M NF-treated (C) groups. Each point is the mean of three samples. Error bars are SDs.

chlorophyll, shown in Figure 4, followed a pattern similar to that seen with total chlorophyll. In the control and NF groups, the ratio remained similar in both strains, increasing slowly in the control and decreasing slowly in the NF treatment. The MV treatment resulted in a larger difference between the two strains at the end of 48 h, with the *sodB*<sup>-</sup> strain losing carotenoids. HPLC separation of the pigments, shown in Figure 5, coincides with the spectrophotometry results. Before any treatments, the two strains had approximately the same amount of total carotenoids per unit chlorophyll. Four carotenoids were identified from the HPLC data by absorbance spectra and comparison with previous chromatograms (Masamoto and Furukawa, 1998): nostoxanthin, caloxanthin, zeaxanthin, and  $\beta$ -carotene. As has been shown previously, zeaxanthin and  $\beta$ -carotene were the most abundant carotenoids in this cyanobacterium (Omata and Murata, 1983; Guillard et al., 1985). No



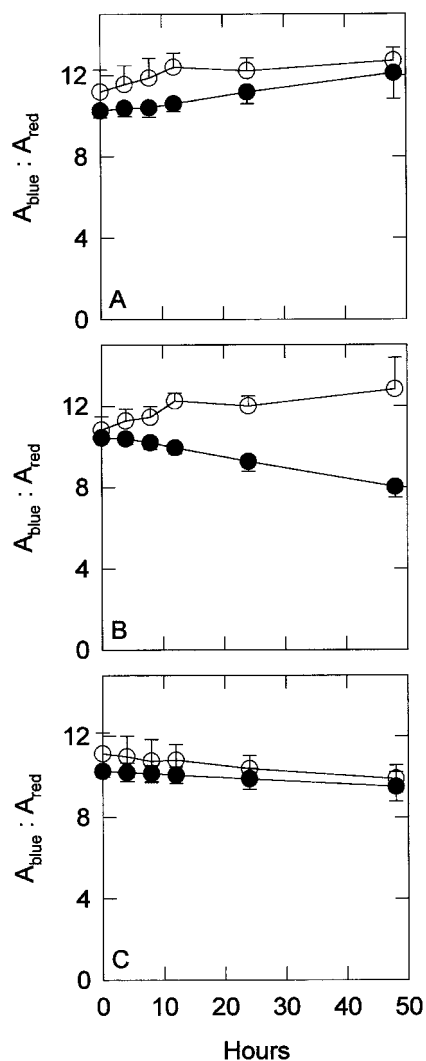
**Figure 3.** Spectrophotometric measurements of acetone-extractable chlorophyll (Chl) *a*. Chlorophyll was extracted into 100% acetone. Extractable chlorophyll concentrations are shown for wild type (○) and *sodB*<sup>-</sup> (●) over 48-h treatment periods for control (A), 0.5  $\mu\text{M}$  MV-treated (B), and 5  $\mu\text{M}$  NF-treated (C) groups. Each point is the mean of five or six samples. Error bars are SDs.

evidence of tocopherols was found (data not shown), confirming previous investigations (Powls and Redfeam, 1967).

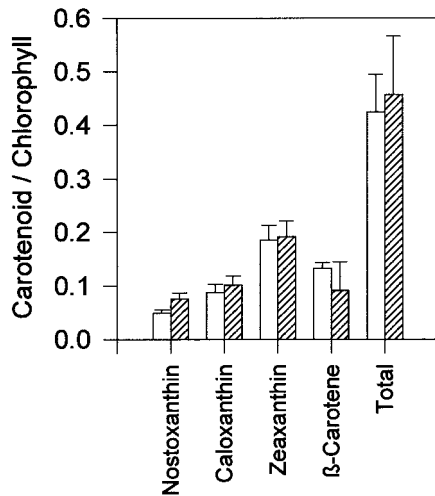
#### $P_{700}$ Oxidation Rate

The rates of  $P_{700}$  oxidation, and thus the efficiency of excitation energy transfer from the photosynthetic antennae to  $P_{700}$ , are shown in Figure 6. DBMIB (25  $\mu\text{M}$ ) was added to slow competing re-reduction of  $P_{700}$  and to ensure the maximum oxidation rate. In the wild-type control, the oxidation rate initially increased during the first 4 h and then decreased to a steady rate that was higher than the initial rate. The *sodB*<sup>-</sup> control oxidation rate decreased

slightly during the 24-h period. During treatments with MV, the wild-type oxidation rate remained constant throughout most of the treatment, with a slight decrease by the end of 24 h. The *sodB*<sup>-</sup> oxidation rate with MV showed only a slight decrease as well but was lower than the rate in the wild type. A much more dramatic difference was seen in the NF treatments. The wild-type oxidation rate decreased almost linearly to less than 20% of the original rate after 48 h. During the same duration, the *sodB*<sup>-</sup> rate decreased to only about 70% of the original rate.



**Figure 4.** Carotenoid-to-chlorophyll ratios of wild-type (○) and *sodB*<sup>-</sup> (●) strains. Chlorophyll and carotenoids were extracted into 100% acetone. The resulting values are the ratios of blue peak absorbance ( $A_{\text{blue}}$ ) to red peak absorbance ( $A_{\text{red}}$ ) over 48-h treatment periods for control (A), 0.5  $\mu\text{M}$  MV-treated (B), and 5  $\mu\text{M}$  NF-treated (C) groups (see "Materials and Methods"). Each point is the mean of five or six samples. Error bars are SDs. For comparison, purified chlorophyll *a* from spinach yielded a blue:red ratio of 4.53. Symbols are as in Figure 3.



**Figure 5.** Initial relative amounts of chlorophyll and carotenoids as measured by reverse-phase HPLC. All values are ratios of carotenoid to chlorophyll a peak areas (milliabsorbance units  $s^{-1}$ ) measured by  $A_{420}$ . Values are means  $\pm$  SD;  $n = 4$ . Open bars, Wild type; hatched bars, *sodB*<sup>-</sup>.

#### $P_{700}$ Activity

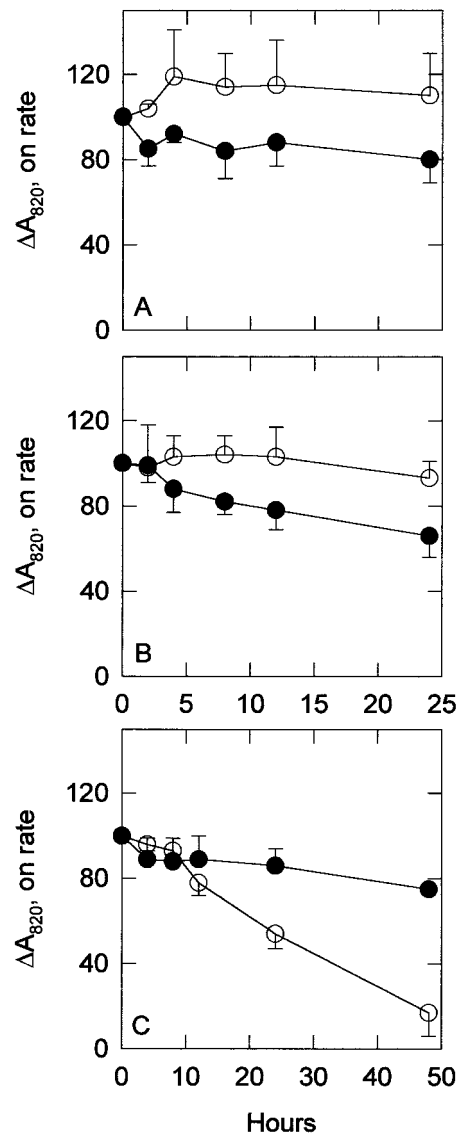
The data of Figure 7 show that the extent of  $P_{700}$  oxidation remained approximately the same in both strains during the control and the  $0.5 \mu M$  MV treatments. However, the *sodB*<sup>-</sup> strain had slightly lower values and was more inhibited by MV than the wild type. When treated with  $5 \mu M$  NF, the wild-type strain showed a marked decrease in  $P_{700}$  oxidation extent after 12 h, whereas the *sodB*<sup>-</sup> strain showed a steadier and much slower decrease. To ensure that the  $P_{700}$  was fully oxidized when measuring the extent of  $P_{700}$  oxidation, saturating actinic light was used and the samples were treated with  $25 \mu M$  each of DCMU and DBMIB.

#### PSI Electron Transport

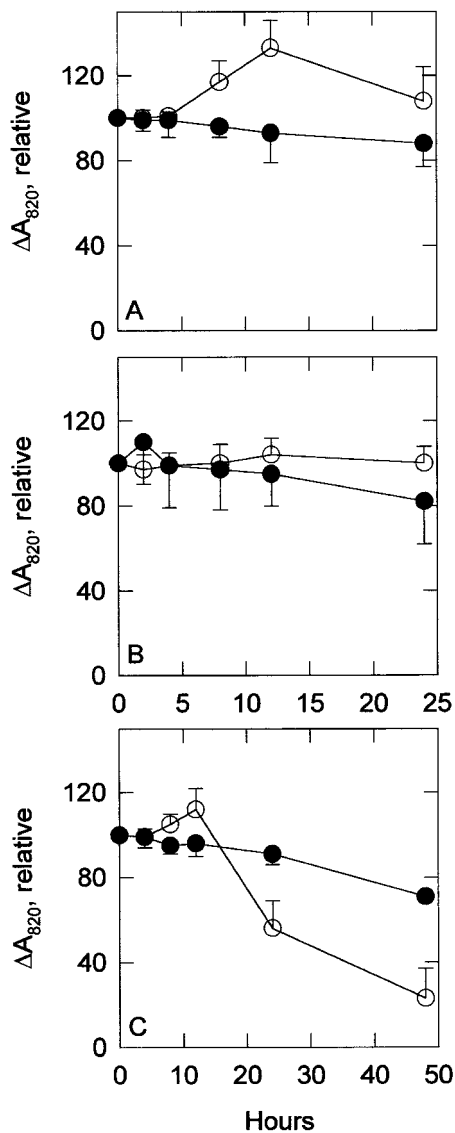
The rate of  $P_{700}$  re-reduction, shown in Figure 8, was measured in both strains in the presence of  $25 \mu M$  DCMU. This rate is an indicator of PSI-driven cyclic electron transport (Herbert et al., 1995). The wild-type control group initially showed an increase in cyclic electron transport, as indicated by the increased rate of re-reduction. After 12 h, cyclic electron transport activity stabilized at a rate of almost 160% of the initial rate. The *sodB*<sup>-</sup> control group had relatively constant cyclic electron transport activity throughout the time course. Both strains exhibited decreased cyclic activity when treated with MV, but the *sodB*<sup>-</sup> strain lost activity twice as fast as the wild type. In the NF treatment the re-reduction rate in the wild-type strain increased during the first 4 h and then decreased drastically after 12 h. The re-reduction rate in the *sodB*<sup>-</sup> strain increased to a lesser degree during the first 8 h and then remained fairly constant at a rate of approximately 130% of the starting rate.

#### Catalase Activity

Activity of the single catalase known in PCC7942 (Mutsuda et al., 1996) was measured by oxygen evolution in response to  $H_2O_2$  in whole cells. The rates obtained from wild-type and *sodB*<sup>-</sup> cells were similar. Wild-type cultures diluted to an  $A_{750}$  of 0.3 produced oxygen at a maximum rate of  $0.28 \pm 0.09 \mu mol \min^{-1} mL^{-1}$  of sample in response to exogenous  $H_2O_2$ . *sodB*<sup>-</sup> cultures measured in the same way produced oxygen at a maximum rate of  $0.30 \pm 0.10 \mu mol \min^{-1} mL^{-1}$  of sample (values are means  $\pm$  sample SD). The sample size for both numbers was seven. Each of



**Figure 6.**  $P_{700}$  oxidation rate. The  $\Delta A_{820}$  over the initial 10 to 30 ms after the onset of actinic light was used to calculate the initial rate of  $P_{700}$  oxidation in control (A),  $0.5 \mu M$  MV-treated (B), and  $5 \mu M$  NF-treated (C) cultures.  $\circ$ , Wild-type strain;  $\bullet$ , *sodB*<sup>-</sup> strain. Each point represents the mean of three to eight samples and all data are percent of initial values. Error bars are SDs. DBMIB ( $25 \mu M$ ) was added to all samples to prevent re-reduction by the cyclic and noncyclic pathways.



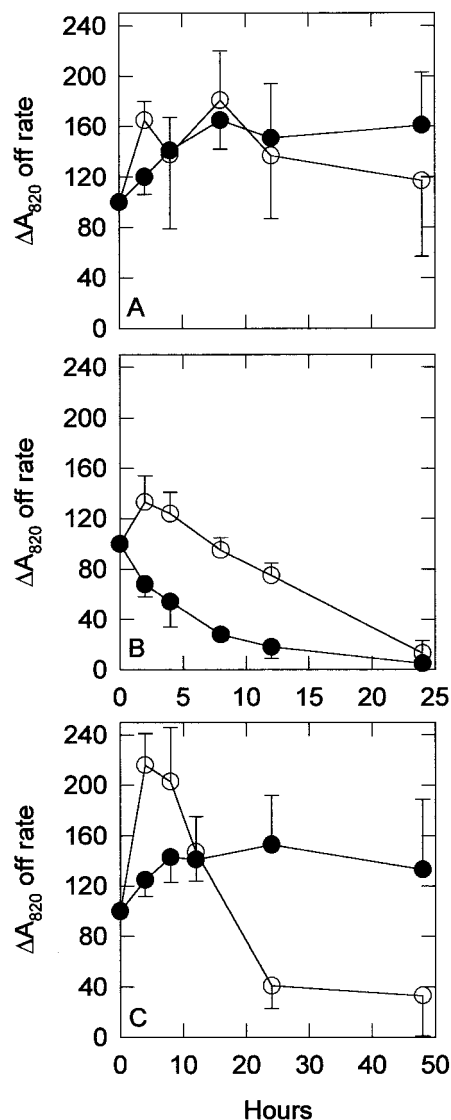
**Figure 7.** P<sub>700</sub> oxidation extent. ΔA<sub>820</sub> was used to measure the relative amount of photooxidizable P<sub>700</sub> in control (A), 0.5 μM MV-treated (B), and 5 μM NF-treated (C) cultures. ○, Wild-type strain; ●, *sodB*<sup>-</sup> strain. All data are percent of starting values. Values are means of three to eight samples. Error bars are SDs. DCMU and DBMIB (25 μM each) were added to ensure complete oxidation of P<sub>700</sub>.

these seven samples was a separate culture that was assayed two or three times to get an average value. Values from any one culture were typically very repeatable.

## DISCUSSION

Loss of cytosolic FeSOD activity in the *sodB*<sup>-</sup> mutant sensitized it to oxidative stress imposed with MV and light. Inhibition of growth, loss of photosynthetic pigments, and inhibition of electron transport caused by MV were all much more severe in the *sodB*<sup>-</sup> strain than in the wild type. MV catalyzes O<sub>2</sub><sup>-</sup> formation from electron transport at the F<sub>A</sub>/F<sub>B</sub> centers of PSI, close to the cytosolic compartment. The sensitivity of the *sodB*<sup>-</sup> strain to MV suggests that the

FeSOD has a specific role in protecting cellular components from O<sub>2</sub><sup>-</sup> formed in the cytosol. Surprisingly, the *sodB*<sup>-</sup> mutant was not sensitized to oxidative stress imposed with the carotenoid synthesis inhibitor NF. In fact, the *sodB*<sup>-</sup> strain was partially resistant to NF in comparison with the wild type, exhibiting less inhibition of growth, a similar loss of photosynthetic pigments, and less inhibition of electron transport. NF inhibits the synthesis of colored carotenoid pigments at the phytoene desaturase step and thus promotes formation of <sup>1</sup>O<sub>2</sub>\* in the chlorophyll antennae. The resistance of the *sodB*<sup>-</sup> strain to NF indicates that the cytosolic FeSOD plays no role in protecting cell compo-



**Figure 8.** P<sub>700</sub> re-reduction rate with DCMU. The ΔA<sub>820</sub> over the initial 50 to 300 ms after the removal of actinic light was used to calculate the initial rate of P<sub>700</sub> re-reduction in control (A), 0.5 μM MV-treated (B), and 5 μM NF-treated (C) cultures. ○, Wild-type strain; ●, *sodB*<sup>-</sup> strain. Each point represents the mean of three to eight samples and all data are percent of initial values. Error bars are SDs. The addition of 25 μM DCMU blocks input from PSII. The data shown represent cyclic electron transport.

nents from damage by  $^1\text{O}_2^*$  formed within the thylakoid membranes. A different antioxidant that is up-regulated to compensate for the lack of FeSOD may account for NF resistance in the *sodB*<sup>-</sup> strain.

## MV

The earliest target of MV inhibition in both strains was electron transport into  $P_{700}$  (Fig. 8). Without DCMU, PSII is the major source of these electrons. With DCMU added, the major sources of these electrons are the two cyclic paths of photosynthetic electron transport present in cyanobacteria (Herbert et al., 1992, 1995; Yu et al., 1993). The rate of this cyclic input to  $P_{700}$  declines rapidly in the wild type when treated with MV and much more rapidly in the *sodB*<sup>-</sup> strain. The *sodB*<sup>-</sup> strain of PCC7942 was shown previously to be sensitive to MV, and the primary site of damage to PET was observed to be in the cyclic electron transport path between the  $F_A/F_B$  centers of PSI and Cyt *f* (Herbert et al., 1992; Samson et al., 1994). The present study confirms and extends those previous results. Inhibition of the two cyclic paths in cyanobacteria is consistent with oxidative damage to the  $F_A/F_B$  centers of PSI or to Fd. The  $F_A/F_B$  centers of PSI are known to be an early site of oxidative damage to this photosystem (Fujii et al., 1990; Dodge, 1991). They and Fd also contain Fe-S centers, which are known to be disrupted by  $\text{O}_2^-$  (Liochev, 1996).

In contrast to  $P_{700}$  re-reduction, chlorophyll concentration, the rate of  $P_{700}$  oxidation by the light-harvesting antenna of PSI, and the quantity of photooxidizable  $P_{700}$  decreased only slightly in both the *sodB*<sup>-</sup> strain and the wild type during the first 24 h of MV treatment (Figs. 3, 6, 7, and 8). The  $P_{700}$  reaction center and its chlorophyll antenna are both within the thylakoid membrane and may not be fully exposed to  $\text{O}_2^-$  and subsequent  $\cdot\text{OH}$  formed by MV. This inaccessibility may account for the fact that the rate and extent of  $P_{700}$  oxidation are not sensitized to MV in the *sodB*<sup>-</sup> strain. Alternatively, these targets may not be strongly sensitized to MV because the membrane-associated MnSOD or an unknown lipophilic antioxidant protects them to an equal extent as in the wild type.

It should be noted that MV is a competitive inhibitor of PSI cyclic electron transport at 100  $\mu\text{M}$  (Yu et al., 1993), and such inhibition of the cyclic path could account for inhibition of  $P_{700}$  re-reduction. However, the 0.5  $\mu\text{M}$  MV used in our experiments did not inhibit re-reduction of  $P_{700}$  (data not shown).

## CO<sub>2</sub> and Irradiance Side Effects

When the cells were transferred from growth conditions (30  $\mu\text{M}$  photons  $\text{m}^{-2} \text{s}^{-1}$ , 3% CO<sub>2</sub>) to the stress chambers (100  $\mu\text{M}$  photons  $\text{m}^{-2} \text{s}^{-1}$ , ambient CO<sub>2</sub>), the wild type exhibited an increase in both the amount of photooxidizable  $P_{700}$  and in the  $P_{700}$  oxidation rate. Under the same conditions, the *sodB*<sup>-</sup> strain was less variable for both parameters. Previous research has shown that cyclic electron transport increases in response to increased light intensity (Herbert et al., 1995). In addition, the transfer from high to low concentrations of CO<sub>2</sub> would activate

inorganic carbon-concentrating mechanisms (for review, see Kaplan et al., 1991) that require the cyclic pathway (Ogawa, 1991).

## NF

The most interesting result from this study was that the *sodB*<sup>-</sup> strain is at least partially resistant to the effects of NF. Unlike MV, NF caused a decrease in the rate and extent of  $P_{700}$  oxidation. Both effects would result from oxidation of chlorophyll in PSI by the formation of  $^1\text{O}_2^*$  within the thylakoid membrane. When treated with NF, the wild type shows a decreased rate of  $P_{700}$  photooxidation (Fig. 6C), a loss of photooxidizable  $P_{700}$  (Fig. 7C), decreased cyclic PET (Fig. 8C), and a low growth rate (Fig. 2C). The effects of NF on PSI activity in the wild type are consistent with oxidative damage at or near the reaction center. Loss of photooxidizable  $P_{700}$  represents damage to the reaction center itself. Decreased oxidation rate suggests an interruption of the delivery of excitation to the reaction center. Figure 3C shows that oxidative loss of total chlorophyll did not coincide with the decrease of the oxidation rate, so the interruption must occur close to  $P_{700}$ .

The NF effects observed in wild-type PCC7942 were much less marked in the *sodB*<sup>-</sup> strain. One explanation of this would be that the phytoene desaturase of the *sodB*<sup>-</sup> strain is resistant to inhibition by NF, as has been observed in other organisms (Windhövel et al., 1994). The data in Figure 4A do not support this hypothesis, however. With NF treatment, the ratio of total carotenoids to chlorophyll *a* declined in both the wild-type and *sodB*<sup>-</sup> strains, as would be expected if carotenoid synthesis was inhibited. This ratio declines equally in both strains and is a little lower to begin with in the *sodB*<sup>-</sup> strain (Figs. 4C and 5).

## The Cause of NF Resistance in the *sodB*<sup>-</sup> Strain

Since NF-resistant phytoene desaturase does not appear to be the cause of the NF resistance, what is? PCC7942 has a relatively simple antioxidant system consisting of FeSOD and MnSOD (Laudenbach et al., 1989), catalase (Mutsuda et al., 1996), and  $\beta$ -carotene. The ascorbate peroxidase and glutathione systems found in plants have not been found in PCC7942 (Takeda et al., 1994). Also, unlike other cyanobacteria and higher organisms, PCC7942 lacks the lipid-soluble antioxidant  $\alpha$ -tocopherol (Powls and Redfearn, 1967). Both the wild-type and *sodB*<sup>-</sup> strains have similar catalase activities and carotenoid complements. Increased expression of the MnSOD in the *sodB*<sup>-</sup> strain has been reported, however (Herbert et al., 1992). Presumably, the MnSOD activity is increased to compensate for the lack of FeSOD. We propose that the *sodB*<sup>-</sup> strain resists the effects of NF because of overexpression of MnSOD, which detoxifies the  $\text{O}_2^-$  formed within the thylakoid as a product of  $^1\text{O}_2^*$ . Work in progress to obtain a *sodA*<sup>-</sup> deletion mutant will confirm this putative role of MnSOD in PCC4972 and further define the different intracellular roles of antioxidants in this organism.

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