The Photoreduction of H_2O_2 by *Synechococcus* sp. PCC 7942 and UTEX 625[1]

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It has been claimed that the sole H_2O_2-scavenging system in the cyanobacterium *Synechococcus* sp. PCC 7942 is a cytosolic catalase-peroxidase. We have measured in vivo activity of a light-dependent peroxidase in *Synechococcus* sp. PCC 7942 and UTEX 625. The addition of small amounts of H_2O_2 (2.5 μM) to illuminated cells caused photochemical quenching (qP) of chlorophyll fluorescence that was relieved as the H_2O_2 was consumed. The qP was maximal at about 50 μM H_2O_2 with a Michaelis constant of about 7 μM. The H_2O_2-dependent qP strongly indicates that photoreduction can be involved in H_2O_2 decomposition. Catalase-peroxidase activity was found to be almost completely inhibited by 10 μM NH_2OH with no inhibition of the H_2O_2-dependent qP, which actually increased, presumably due to the light-dependent reaction now being the only route for H_2O_2-decomposition. When ^{18}O-labeled H_2O_2 was presented to cells in the light there was an evolution of $^{16}O_2$, indicative of $H_2{}^{16}O$ oxidation by PS 2 and formation of photoreductant. In the dark $^{18}O_2$ was evolved from added $H_2{}^{18}O_2$ as expected for decomposition by the catalase-peroxidase. This evolution was completely blocked by NH_2OH, whereas the light-dependent evolution of $^{16}O_2$ during $H_2{}^{18}O_2$ decomposition was unaffected.

Light-dependent excretion of H_2O_2 by various strains of *Anacystis nidulans* (*Synechococcus* sp. PCC 6301, PCC 7942, UTEX 625, R2) has been well documented (Van Baalen, 1965; Patterson and Myers, 1973; Stevens et al., 1973; Roncel et al., 1989, Morales et al., 1992). Production of H_2O_2 by *A. nidulans* is not surprising, as it photoreduces O_2 at high rates (Hoch et al., 1963, Miller et al., 1988a; Badger and Schreiber, 1993; Mir et al., 1995; Li and Canvin, 1997a, 1997b). The rate of O_2 photoreduction can be as much as 40% the rate of concomitant photosynthetic CO_2 fixation with rates of about 100 μmol O_2 mg^{-1} chlorophyll (Chl) h^{-1} (Miller et al., 1988a; Mir et al., 1995; Li and Canvin, 1997a, 1997b). The photoreduction of two molecules of O_2 is required to produce the two superoxide radicals that are required to form one molecule of H_2O_2 in the reaction catalyzed by superoxide dismutase (Badger, 1985) so if none of the H_2O_2 were decomposed within the cells one would expect sustained excretion rates of about 50 μmol H_2O_2 mg^{-1} Chl h^{-1} based upon the observed rates of O_2 photoreduction. Upon illumination of cells, Patterson and Myers (1973) observed a rate of about 24 μmol H_2O_2 mg^{-1} Chl h^{-1} that lasted no longer than 5 min and was followed by a rate of no more than about 0.5 μmol H_2O_2 mg^{-1} Chl h^{-1}. Roncel et al. (1989) reported a rate of excretion of 32.2 μmol H_2O_2 mg^{-1} Chl h^{-1}, but also mentioned that this rate was not long sustained. Morales et al. (1992) found that azide, an inhibitor of the H_2O_2-decomposing enzyme catalase, substantially increased the sustained portion of the light-dependent excretion of H_2O_2. Overall, the low rates of sustained H_2O_2 excretion and the involvement of catalase indicate that much of the H_2O_2 produced as a result of O_2 photoreduction in *A. nidulans* is decomposed within the cells and that excretion is only one mode of H_2O_2 detoxification.

Recently, a catalase-peroxidase has been purified and characterized from *A. nidulans* and the relevant gene has been cloned and sequenced (Mutsuda et al., 1996; Obinger et al., 1997). The gene showed a very high similarity to other members of the bacterial catalase-peroxidase family (Mutsuda et al., 1996). These enzymes are bifunctional enzymes that can catalyze the reduction of H_2O_2 to H_2O and O_2 by using either another H_2O_2 molecule as a reductant (catalase activity) or by using a reduced organic molecule, such as pyrogallol (peroxidase activity). The natural reductant for this peroxidase activity in *A. nidulans* is unknown but the enzyme did not readily accept electrons from ascorbate, reduced glutathione, or NADH (Obinger et al., 1997). With the best reductant available, *o*-dianisidine, the relative peroxidase activity was still much lower than the catalase activity. The catalase-peroxidase was the only H_2O_2-decomposing enzyme found in the cytosol of this strain of *A. nidulans*, but the thylakoid fraction was not investigated (Obinger et al., 1997). Work by Miyake and Asada (1991) indicated that *A. nidulans* decomposed H_2O_2 only via catalase and that there was no involvement of a peroxidase, such as ascorbate peroxidase, coupled indirectly to photochemically produced reductant, as occurs in chloroplasts

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(Asada, 1984, 1992). It was found that illuminated cells provided with 18-labeled H_2O_2 released only $^{18}O_2$, whereas cyanobacteria, such as *Synechocystis* sp. PCC 6803, thought to have a peroxidase linked to the use of a photoreduced compound such as ascorbate, also released $^{16}O_2$ as a manifestation of the required electron flow through PS 2 (Miyake and Asada, 1991). The addition of H_2O_2 to illuminated *A. nidulans* also did not cause photochemical quenching (qP) of Chl fluorescence (Miyake and Asada, 1991). The addition of H_2O_2 to *Synechocystis* sp. PCC 6803 (Miyake and Asada, 1991) or to chloroplasts from higher plants (Neubauer and Schreiber, 1988) did cause quenching, indicating the use of photoreductant for H_2O_2 decomposition. Badger and Schreiber (1993) found, unlike Miyake and Asada (1991), that H_2O_2 did cause quenching in *A. nidulans* that was relieved as the H_2O_2 was consumed, and they suggested that a peroxidase, possibly ascorbate peroxidase, was involved. The presence of such an enzyme would agree with the work of Mittler and Tel-Or (1991), who not only measured appreciable levels of ascorbate peroxidase in the same strain, *Synechococcus* sp. PCC 7942, studied by Badger and Schreiber (1993) but also found the peroxidase activity to be higher than the catalase activity. They came to the conclusion that in this strain of *A. nidulans* catalase plays only a minor role in the decomposition of H_2O_2.

Given the conflicting results as to the presence of peroxidase activity linked to the use of photoreductant in *A. nidulans*, we have re-investigated the decomposition of H_2O_2 in *Synechococcus* sp. PCC 7942 (formerly R2) and UTEX 625. We have found that addition of H_2O_2 does cause qP and that when ^{18}O-labeled H_2O_2 is added to cells there is evolution of $^{16}O_2$ in the light and only $^{18}O_2$ in the dark. We have also found that the catalase activity can be selectively inhibited with 10 μM NH_2OH without inhibition of the light-dependent decomposition pathway. The results clearly demonstrate the presence of a light-dependent peroxidase activity in *A. nidulans*, a species widely used in the study of O_2 metabolism in cyanobacteria.

RESULTS

H_2O_2-Dependent Quenching of Chl Fluorescence

The addition of low concentrations of H_2O_2 to illuminated cells of *Synechococcus* sp. PCC 7942 resulted in quenching of Chl fluorescence (Fig. 1). Most of this quenching was transiently relieved during a saturating flash (SF) (Fig. 1) indicating it was qP. Before the addition of H_2O_2 the cells were allowed to deplete the medium of contaminant inorganic carbon (C_i) by photosynthetic CO_2 fixation, which then allows measurement of F_M during a SF (Fig. 1; Miller et al., 1991). The addition of 25 μM C_i then caused quenching of Chl fluorescence that was predominantly qP; until this C_i was consumed by photosynthetic CO_2 fixation (Fig. 1). The subsequent addition of H_2O_2 also caused fluorescence quenching, mainly qP, that was relieved as the H_2O_2 was consumed; this was evident by the O_2 evolution (Fig. 1). As expected for any mechanism

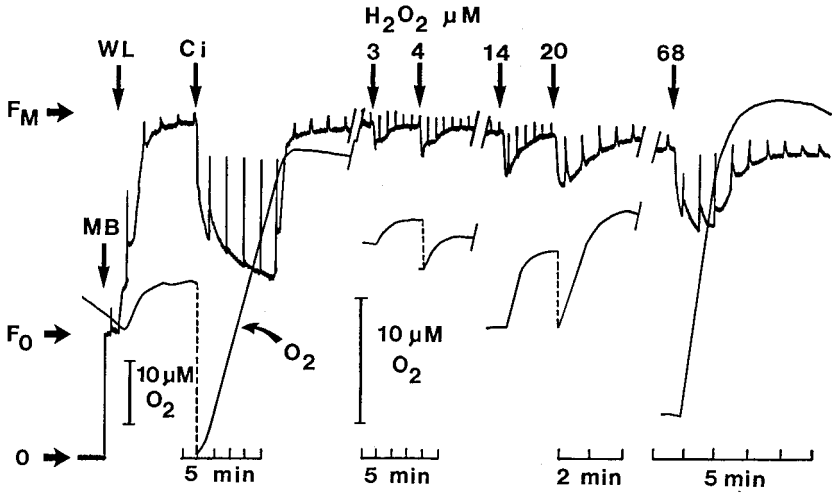

Figure 1. H_2O_2-dependent quenching of Chl fluorescence (*F*) in *Synecococcus* sp. PCC 7942. The cells were dark-adapted for 10 min and the fluorescence signal was measured in the absence (O) and presence (F_o) of the weak, pulse-modulated measuring beam (MB). A single SF was given during this time, indicated by the transient increase in the *F* signal. The non-modulated WL was then turned on (120 μmol photon m^{-2} s^{-1}, PAR). The cells were then allowed to deplete the medium of C_i, manifest as attainment of maximal fluorescence yield (F_M) during an SF, as described by Miller et al. (1991). The addition 50 μM C_i then resulted in reappearance of *F* quenching until this C_i was consumed. The additions of H_2O_2 then also resulted in *F* quenching, which was relieved as the H_2O_2 was decomposed, manifest as cessation of the H_2O_2-dependent O_2 evolution indicated by the traces below the *F* trace. SFs were periodically given so that qP could be estimated.

of H_2O_2 decomposition (Asada, 1984) there was evolution of one O_2 molecule for every two H_2O_2 molecules decomposed. For 17 separate cell suspensions to which 30 to 50 μM H_2O_2 was added in the light at the CO_2-compensation point, the ratio of O_2 evolved to H_2O_2 added was 0.48 ± 0.05 ($\bar{x}$ ± SE). Concentrations of H_2O_2 as low as 4 μM gave easily measurable quenching (Fig. 1).

It was necessary to rule out the possibility that contaminant C_i in the H_2O_2 solutions was the cause of the fluorescence quenching, even though H_2O_2 solutions were prepared to avoid this (see "Materials and Methods"). C_i in the very low micromolar range causes significant quenching (Miller and Canvin, 1987; Crotty et al., 1994; Li and Canvin, 1997b). We, therefore, determined whether H_2O_2 would still cause quenching under conditions that would prevent any contaminant C_i from doing so. When C_i is added to cells that have depleted the medium of C_i the quenching of Chl fluorescence that occurs (Fig. 1) is due both to the photoreduction of the added C_i and to a C_i stimulation of O_2 photoreduction (Miller et al., 1988a, 1991; Badger and Schreiber, 1993). The photoreduction of C_i can be prevented by the addition of glycolaldehyde (Miller and Canvin, 1989) and the photoreduction of O_2 can be prevented by the removal of O_2 by addition of Glc oxidase and Glc (Miller et al., 1991). As the intention was to observe the effect of H_2O_2, it was necessary to use a Glc oxidase preparation with very low levels of contaminant catalase (catalog no. G9010, Sigma-Aldrich, St. Louis). The H_2O_2 produced during the consumption of the O_2 in the medium was undoubtedly consumed by these illuminated cells themselves. This was indicated by a temporary qP, predicted from the results described in Figure 1, following initiation of the reaction by Glc (data not shown). The experiments were begun when the O_2 in the medium had been completely consumed. In Figure 2, the magnitude of the fluorescence increase during the flash is a measure of the qP that was obtained before the flash. The presence of both C_i and O_2 as electron acceptors resulted in the expected large amount of qP, that was transiently relieved during the SF and resumed very rapidly after the flash terminated (Fig. 2A). The addition of C_i in the presence of both glycolaldehyde and the Glc oxidase O_2 trap did not result in any qP, so there was no increase in fluorescence intensity during the SF (Fig. 2B). The addition of H_2O_2 under the same conditions did cause qP, which was relieved during a SF, resulting in an increase in fluorescence intensity (Fig. 2C). The resumption of qP following the flash was slow and complex (Fig. 2C), quite unlike the recovery observed when qP is due to CO_2 and O_2 photoreduction (Fig. 2A). The reoxidation kinetics following a SF are very similar for H_2O_2 photoreduction (Fig. 2C) and CO_2 photoreduction in the absence of O_2 (Miller et al., 1991). Electron flow to either H_2O_2 or CO_2 thus is compromised by the absence of O_2, perhaps by over-reduction of electron carriers during the SF. The results in Figures 1 and 2 clearly show that H_2O_2 can serve as an acceptor of electrons form PS 2.

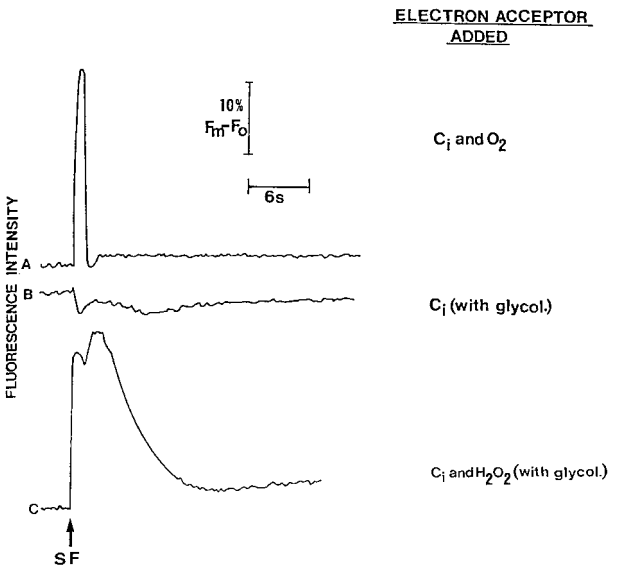

Figure 2. Quenching of Chl *F* due to addition of H_2O_2 solutions is not due to contaminant C_i. *Synechococcus* sp. PCC 7942 cells were allowed to deplete the medium of C_i described in Figure 1, then the response to an SF was monitored in the presence of: 500 μM C_i and about 280 μM O_2 (A); 500 μM C_i, 15 mM glycolaldehyde, and the Glc oxidase O_2 trap (B); or the latter adddition plus 50 μM H_2O_2 (C). F_v is defined as the difference between the F_M signal measured during an SF given while the cells were in WL after depleting the medium of C_i and the F_o signal measured with dark-adapted cells illuminated only by the weak MB (see Fig. 1).

NH_2OH as a Selective Inhibitor of Catalase

Kono and Fridovich (1983) found that a low concentration, 8 μM, of hydroxylamine (NH_2OH) completely inhibited the pseudocatalase of *Lactobacillus plantarum.* The pseudocatalse is so named because, unlike most catalase, it lacks a heme group. Takeda et al. (1995) have subsequently used a much higher concentration, 1 mM, to inhibit the catalase activity in cyanobacteria. We have found that the catalase activity in *Synechococcus* sp. PCC 7942 can be almost fully inhibited by as little as 10 μM NH_2OH (Fig. 3A). The decomposition of 50 μM H_2O_2 by cells in the dark was rapidly stopped by the addition of the 10 μM NH_2OH (Fig. 3A). At H_2O_2 concentrations around 100 μM and higher, some catalase activity was observed at low NH_2OH concentrations (data not shown), perhaps indicating a competitive relationship between H_2O_2 and NH_2OH. Also, there was some slow decay of the ability of the NH_2OH to inhibit the catalase in cells after it has been added, especially in the light, probably as a result of its metabolism. An assay for dark catalase activity was always performed, by the addition of 30 to 50 μM

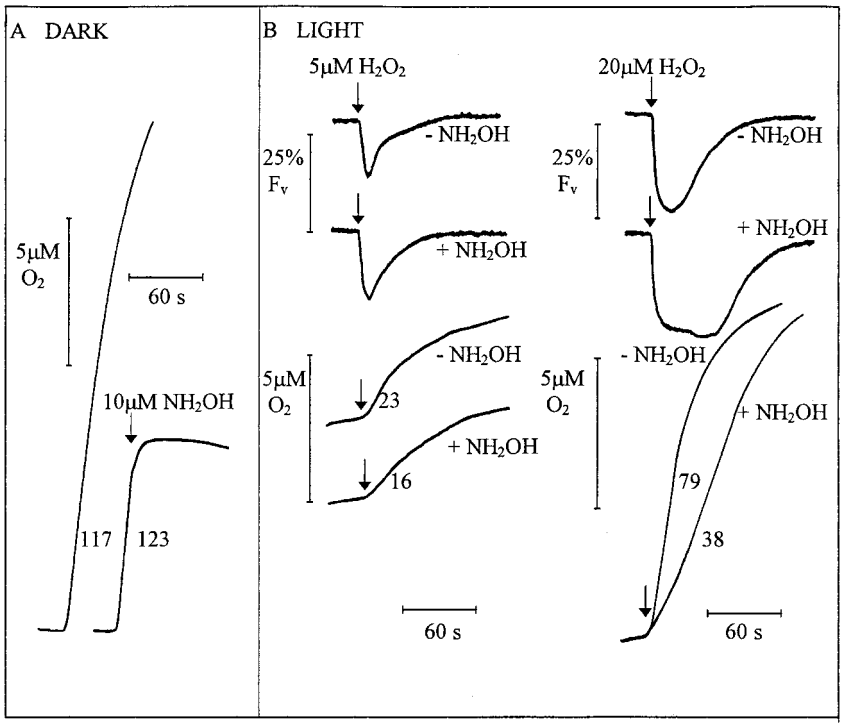

Figure 3. Selective inhibition of in vivo catalase activity of 10 μM NH_2OH. A, 50 μM H_2O_2 was added to dark-adapted cells. O_2 evolution was monitored with or without subsequent addition of 10 μM NH_2OH. B, Cells were allowed to deplete the medium of C_i during illumination with WL (120 μmol photons m^{-2} s^{-1}, PAR) and then *F* quenching and O_2 evolution were monitored after the addition of either 5 or 20 μM H_2O_2 in the absence or presence of 10 μM H_2O_2. For both A and B the maximum O_2 evolution rates, in terms of μmol O_2 mg^{-1} Chl h^{-1}, are given beside the O_2 traces. F_v is as defined for Figure 2.

H_2O_2 and monitoring O_2 evolution, at the end of each run to ensure that the catalase was still inhibited. High concentrations of NH_2OH, up to at least 50 μM can be used without inhibiting photosynthetic CO_2 fixation (data not shown). The NH_2OH did not inhibit the light-dependent decomposition of H_2O_2, as monitored by Chl fluorescence quenching (Fig. 3B). The actual rate of H_2O_2 decomposition, monitored as O_2 evolution was reduced by NH_2OH, whereas the extent of fluorescence quenching was actually increased (Fig. 3B). With inhibition of the catalase pathway by NH_2OH the light-dependent route becomes the sole route of H_2O_2 decomposition, resulting in the reduced rate of H_2O_2 decomposition but also in increased ability of the H_2O_2 to cause quenching.

NH_2OH (10 μM) completely inhibited the catalase activity in cell-free extracts of *Synechococcus* sp. PCC 7942 but did not inhibit the ascorbate peroxidase activity (Table I). In fact, the rate of H_2O_2-dependent ascorbate oxidation was faster in the presence of the 10 μM NH_2OH (Table I), perhaps due to a lack of competition for the substrate H_2O_2 by an active catalase. The presence of measurable ascorbate peroxidase activity in *Synechococcus* sp. PCC 7942 confirms the findings of Mittler and Tel-Or (1991). The peroxidase activity of the cyanobacterial catalase-peroxidase can be observed in the presence of artificial reductants such as pyrogallol (Mutsuda et al., 1996). We found in the several assays we have performed that the ability of pyrogallol to reduce H_2O_2 in cell-free extracts of *Synechococcus* sp. PCC 7942 was inhibited by about 80% in the presence of 10 μM NH_2OH (data not shown).

Table I. *Catalase and ascorbate peroxidase activity in cell-free extracts of Synechococcus sp. PCC 7942*

Values are means ± SE for assays from five separate cell-free extract preparations in each case.

Assay	Rate
	μmol H_2O_2 mg^{-1} protein h^{-1}
Catalase (−NH_2OH)	17.2 ± 2.1
Catalase (+NH_2OH)	1.3 ± 0.4
Ascorbate peroxidase (−NH_2OH)	42.6 ± 10.5
Ascorbate peroxidase (+NH_2OH)	64.8 ± 9.3

Kinetics of Light-Dependent H_2O_2 Decomposition

With the ability to selectively inhibit catalase activity with NH_2OH, the kinetics of the light-dependent decomposition with respect to H_2O_2 concentration were studied in terms of O_2 evolution (Fig. 4) and the extent of Chl fluorescence quenching (Fig. 5). In the dark there was a linear relationship between the rate of H_2O_2 decomposition, and H_2O_2 concentration (Fig. 4). There was very little decomposition in the presence of 10 μM NH_2OH below 50 μM H_2O_2, but as mentioned previously, there was a small amount at higher concentrations (Fig. 4A). In the light there was still substantial H_2O_2 decomposition in the presence of NH_2OH, as monitored by O_2 evolution (Fig. 4B). The light-dependent reaction, measured as O_2 evolution in the presence of NH_2OH, was saturated by an H_2O_2 concentration of about 50 μM and had a K_m of about 16 μM H_2O_2 (Fig. 4B). This response to H_2O_2

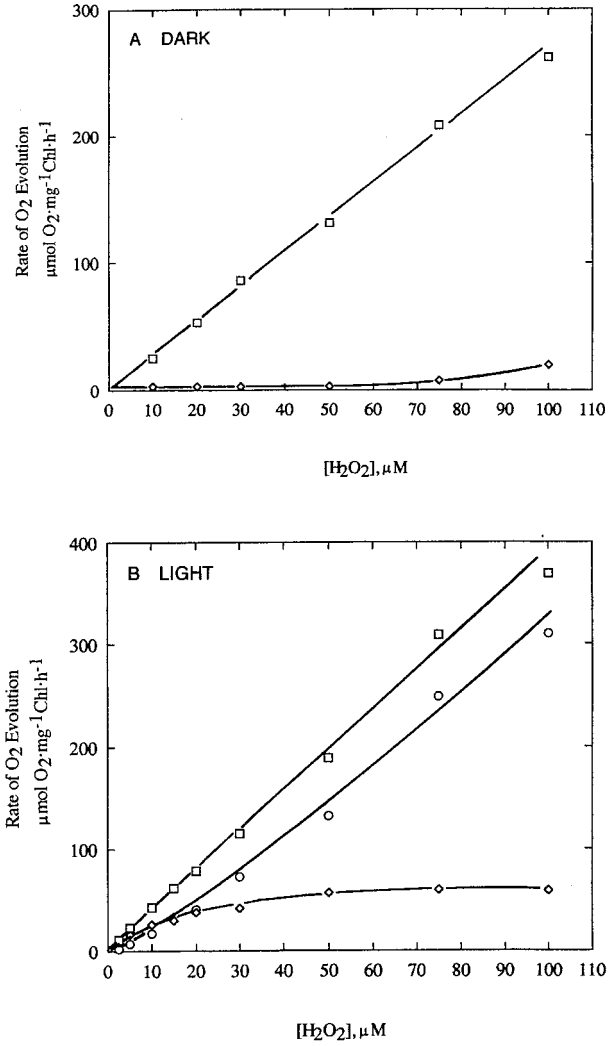

Figure 4. Decomposition of H_2O_2 in dark (A) and light (B) in response to H_2O_2 concentration. The maximum rate of O_2 evolution was used as the measure of H_2O_2 decomposition in the absence (□) or presence (◇) of 10 μM NH_2OH. The rates for cells in the dark (A) were corrected for rates of O_2 uptake due to respiration measured just prior to H_2O_2 addition. For measurement of H_2O_2 decomposition in the light the cells were allowed to first deplete the medium of C_i. The difference between the rates of H_2O_2 decomposition in the light in the absence and presence of 10 μM NH_2OH is also given (○). The WL was 120 μmol photon m^{-2} s^{-1} (PAR).

concentration is very different from the non-saturating response of the catalase reaction, measured in the dark (Fig. 4A). At H_2O_2 concentrations below 10 μM the light-dependent reaction could be the major route for H_2O_2 decomposition in the light (Fig. 4B), although it will be difficult to estimate the fraction that actually goes through each pathway when the catalase is not inhibited.

When the light-dependent reaction was monitored as the extent of fluorescence quenching caused by increasing H_2O_2 concentrations, a rate saturating relationship was observed (Fig. 5). In this case, the reaction rates were similar in the absence and presence of the 10 μM NH_2OH (Fig. 5) because the decomposition of H_2O_2 by the catalase route obviously does not cause fluorescence quenching. The presence of the NH_2OH did, however, have two noticeable effects. First, as previously observed (Fig. 3B), the extent of florescence quenching at concentrations below 10 μM H_2O_2 was greater in the presence of NH_2OH, presumably due to a longer duration of light-dependent peroxidase activity when catalase activity is not participating in the decline of the H_2O_2 concentration. Second, at high H_2O_2 concentrations there was some inhibition of the light-dependent reaction (Fig. 5). This inhibition can be explained as a result of the cells being exposed to a high H_2O_2 for a longer period of time when the catalase route is inhibited (Fig. 3B). In some experiments the inhibition was not as great as that described in Figure 5. If the extent of fluorescence quenching is taken as a measure of the rate of H_2O_2 decomposition (Neubauer and Schreiber, 1988) then a K_m (H_2O_2) of about 7 μM can be estimated (Fig. 5). The same K_m was calculated when the rate, rather than the extent, of fluorescence quenching was considered (data not shown).

Evolution of $^{18}O_2$ or $^{16}O_2$ during $H_2{}^{18}O_2$ Decomposition

When cyanobacteria decompose $H_2{}^{18}O_2$ by the catalase route, there is an evolution of one $^{18}O_2$ molecule for every $H_2{}^{18}O_2$ coming from the $H_2{}^{18}O_2$ molecule that serves as reductant (Miyake and Asada, 1991). When $H_2{}^{18}O_2$ is decomposed by a peroxidase pathway that uses photoreductant, then $^{16}O_2$ is evolved instead. The $^{16}O_2$ results from the oxidation of $H_2{}^{16}O$ at PS2, as photoreductant is produced, and the ^{18}O remains in the water molecules formed from peroxidase catalyzed reduction of the $H_2{}^{18}O_2$ (Asada and Badger, 1984; Miyake and Asada, 1991). The addition of $H_2{}^{18}O_2$ to *Synechococcus* sp. PCC 7942 in the dark resulted predominantly in $^{18}O_2$ evolution (Fig. 6A), as expected for decomposition by catalase. The same was found with *Synechococcus* UTEX 625 (Fig. 7A). When non-labeled O_2 was used to measure the rate of H_2O_2 decomposition in the dark it was necessary to make substantial corrections for the concomitant uptake of O_2 by respiration, as was done for the calculations presented in Figure 4. The lower than expected amount of $^{16}O_2$ observed for the catalase-dependent decomposition of $H_2{}^{18}O_2$ in the dark (Figs. 6 and 7) is presumably also due mainly to concomitant uptake of $^{16}O_2$ by respiration. In the light the addition of $H_2{}^{18}O_2$ resulted in $^{16}O_2$ evolution as well as $^{18}O_2$ evolution in both strains (Figs. 6B and 7B). The amount of $^{16}O_2$ evolved, indicative of photoreduction, was always greater than the amount of $^{18}O_2$ evolved, indicative of catalase activity. The amount of $^{16}O_2$ evolved by *Synechococcus* sp. PCC 7942 was sometimes greater than that shown. In all

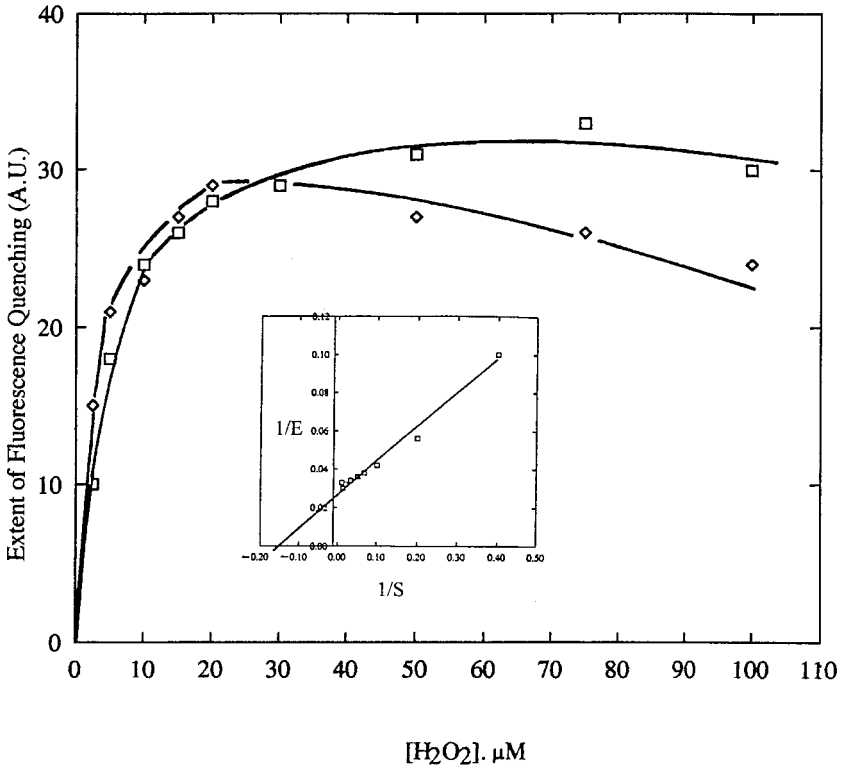

Figure 5. Light-dependent H_2O_2 decomposition monitored as *F* quenching in *Synechococcus* sp. PCC 7942.Cells were allowed to deplete the medium of C_i in WL of 100 μmol photons m^{-2} s^{-1} (PAR) and then the degree of total *F* quenching was monitored after the addition of various concentrations of H_2O_2 in the absence (□) and presence (◇) of 10 μM NH_2OH.

cases with this strain the initial rates of $^{16}O_2$ and $^{18}O_2$ evolution were similar but were followed by a relatively slower rate of $^{18}O_2$ evolution, as seen in Figure 6B. It seems that, as expected, the peroxidase reaction with its relatively low K_m (H_2O_2) (Fig. 5) was less affected by the declining H_2O_2 concentration than the catalase reaction (Fig. 4A). It needs to be noted that in these experiments the light intensity (210 μmol photons m^{-2} s^{-1}) was higher than in the previous experiments (100 or 120 μmol photons m^{-2} s^{-1}) in which a subsaturating light intensity was needed so that qP could be easily measured. The light-dependent H_2O_2 decomposition would be expected to be about 30% higher than in the previous experiments (data not shown) and this may account for the higher relative rate of light-dependent ($^{16}O_2$) versus light-independent ($^{18}O_2$) evolution in these experiments than in those described in Figures 1, 3, and 4. In the presence of 50 μM NH_2OH, in the light, there was only $^{16}O_2$ evolution during H_2 $^{18}O_2$ decomposition by *Synechococcus* sp. PCC 7942 (Fig. 6C) and predominantly $^{16}O_2$ evolution by *Synechococcus* UTEX 625 (Fig. 7C). The rate of $^{16}O_2$ evolution was not increased in the presence of these NH_2OH in these (Figs. 6C and 7C) or other similar experiments (data not shown). This is constant with a low K_m peroxidase that would be saturated at the initial rather high H_2O_2 concentration, 50 μM, used in these experiments. The evolution of $^{16}O_2$, indicative of photoreductant generation by PS 2, was completely inhibited by 20 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (data not shown).

DISCUSSION

This study provides firm evidence for a photoreductant based pathway of H_2O_2 decomposition in two strains, *Synechococcus* sp. PCC 7942 and UTEX 625, of the former taxon *A. nidulans.* Three distinct methods were used to distinguish a light-dependent pathway from the well-described catalase-peroxidase pathway of decomposition of H_2O_2. First, addition of H_2O_2 resulted in the quenching, mainly qP, of Chl fluorescence that was relieved as the H_2O_2 was decomposed (Figs. 1–4). This development of qP was a manifestation of electron flow through PS 2 and of H_2O_2 serving as the terminal electron acceptor. Second, at a concentration of NH_2OH that completely inhibited H_2O_2 decomposition by catalase (Figs. 3 and 4B) there was still H_2O_2-dependent evolution of O_2 in the light (Fig. 4B). Third, when illuminated cells were exposed to H_2 $^{18}O_2$ there was evolution of $^{16}O_2$, indicative of PS 2 activity (Fig. 6B and 7B). In the presence of NH_2OH the evolution of $^{18}O_2$ from the catalase activity was greatly inhibited but the evolution of $^{16}O_2$ from PS 2 activity was not inhibited (Fig. 6C and 7C). There is some indication that the in vivo catalase activity, monitored as $^{18}O_2$ release from H_2 $^{18}O_2$ (Figs. 6 and 7), may be inhibited by light. Unfortunately, our limited access to the mass spectrometer prevented us from acquiring enough data to statistically test this possibility. The duration of fluorescence quenching, qP, was greater when NH_2OH was present so that H_2O_2 could only be decomposed

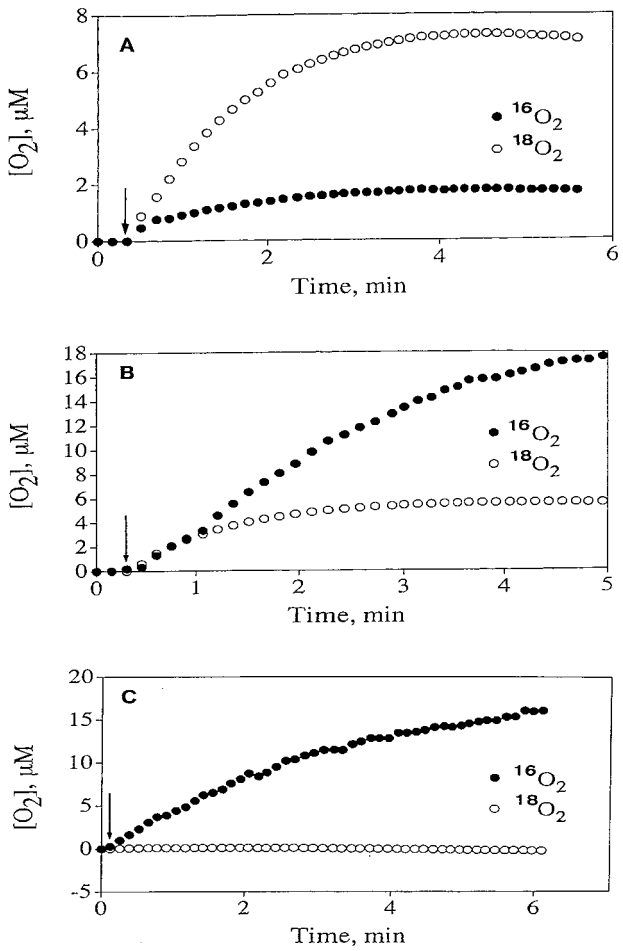

Figure 6. Decomposition of $H_2{}^{18}O_2$ by *Synechococcus* sp. PCC 7942. Cells (12 μg Chl mL^{-1}) were incubated in the dark (A) or in WL (206 μmol photons m^{-2} s^{-1}; B and C) in the absence (A and B) or presence of (C) of 50 μM NH_2OH. At the times indicated by the arrows, 50 μM $H_2{}^{18}O_2$ was added and $^{16}O_2$ and $^{18}O_2$ evolution were monitored by MS. Cells incubated in the light were allowed to deplete the medium of C_i before addition of the $H_2{}^{18}O_2$. A correction was made for $^{18}O_2$ contamination of the $H_2{}^{18}O_2$ solution (see "Materials and Methods").

by the light-dependent peroxidative pathway (Fig. 3). As expected, the rate of O_2 evolution was lower and, the duration of O_2 evolution was higher, when NH_2OH was present (Fig. 3). It would be expected that the release of $^{16}O_2$, during $H_2{}^{18}O_2$ decomposition, would also be of longer duration in the presence of NH_2OH_2, with the $^{18}O_2$ releasing catalase pathway inhibited. This was true in one case (Fig. 7C) but not evident in the other (Fig. 6C). More mass spectrometry (MS) studies are required to obtain more quantitative data on this point. The MS data does in all cases, provide firm evidence for a H_2O_2 decomposition pathway that involves $^{16}O_2$ evolution from PS2.

The nature of the light-dependent pathway in these strains for H_2O_2 decomposition remains unknown. The complete inhibition of the catalase activity of the catalase-peroxidase, and the almost complete inhibition of its pyrogallol peroxidase activity, in cell-free extracts by 10 μM NH_2OH (data not shown) show that this enzyme is not part of the light-dependent pathway. Thus, although the catalase-peroxidase may be the only H_2O_2 decomposing enzyme in the cytosol of *Synechococcus* sp. PCC 6301 (equals UTEX 625), as reported by Obinger et al. (1997), it cannot represent the only peroxidase activity in the cell. The situation seems to be the same in *Synechocystis* sp. PCC 6803, where elimination of the katG gene, which codes for the catalase-peroxidase, did not eliminate the light-dependent peroxidase activity (Tichy and Vermaas, 1999). In the latter case the peroxidase is thought to be thioredoxin peroxidase. In chloroplasts, of both higher plants and various green algae, the light-dependent peroxidase is usually ascorbate peroxidase (Asada, 1984, 1992; Miyake and Asada, 1991; Noctor and Foyer, 1998). In the reaction catalyzed by this enzyme, ascorbate reduces H_2O_2 and is oxidized to monodehydroascorbate (MDHA), which can be reduced back to ascorbate by reduced ferre-

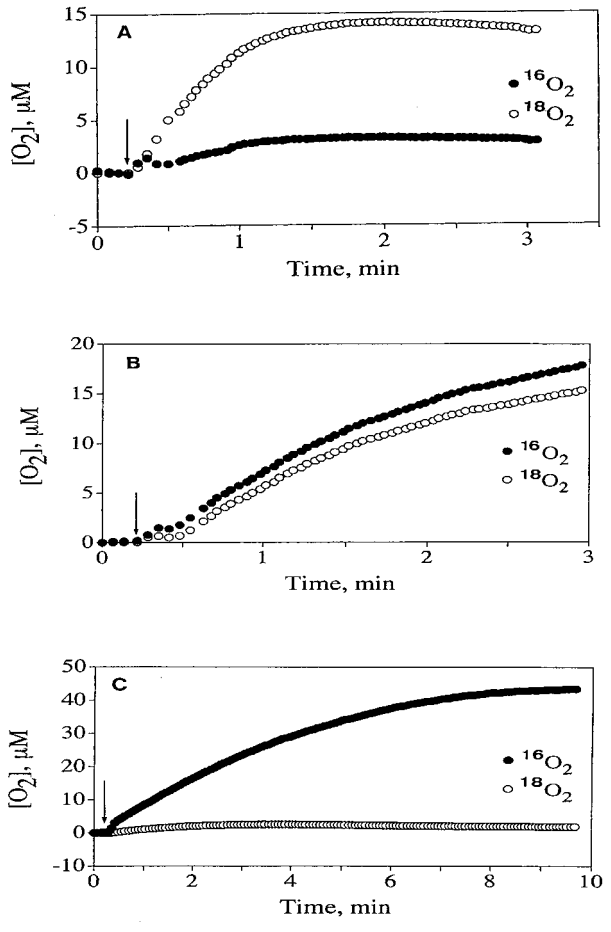

Figure 7. Decomposition of $H_2{}^{18}O_2$ by *Synechococcus* UTEX 625. Cells (11 μg Chl mL^{-1}) were incubated in the dark (A) or in WL (206 μmol photons m^{-2} s^{-1}; B and C) in the absence (A and B) presence (C) of 50 μM NH_2OH. Conditions as described in Figure 6 except that 80 μM $H_2{}^{18}O_2$ was added.

doxin or NADP (Miyake and Asada, 1994; Noctor and Foyer, 1998). MDHA is a rather unstable radical and can disproportionate nonenzymatically to form ascorbate and dehydroascorbate. The dehydroascorbate can be reduced to ascorbate by the ascorbate-glutathione cycle, which is driven by NADPH produced at the thylakoids (Noctor and Foyer, 1998). Ascorbate peroxidate activity, as well as activity of all the enzymes needed for its regeneration, have been found in cell-free extracts of *Synechococcus* sp. PCC 7942 (Mittler and Tel-Or, 1991). Ascorbate peroxidase activity was also detected in this strain in the present study and it was not inhibited by 10 μM NH_2OH (Table I). Mittler and Tel-Or (1991) found that following a challenge of the cells with added exogenous H_2O_2 the ascorbate peroxidase activity increased 2-fold and MDHA-reductase activity increased 4-fold.

Although on the one hand there appears to be good evidence for an ascorbate peroxidase pathway *Synechococcus* sp. PCC 7942, on the other there is evidence against it. Levels of ascorbate that have been measured in cyanobacteria are much lower, at 30 to 100 μM (Tel-Or et al., 1986), than the 15 to 25 mM characteristic of chloroplasts (Foyer et al., 1983). Even so the level of ascorbate, although low, did vary as expected for an involvement in H_2O_2 decomposition (Mittler and Tel-Or, 1991). The ascorbate level was almost 3-fold lower in the dark than in the light and was lowered by the presence of H_2O_2, dropping 10% in the light and to zero in the dark. It is interesting, however, that the genome, now completely sequenced, of *Synechocystis* sp. PCC 6803 contains no gene for ascorbate peroxidase (Tichy and Vermaas, 1999). Based on H_2O_2 quenching of Chl fluorescence and $^{16}O_2$ release during H_2 $^{18}O_2$ decomposition in the light it had been thought that this species was one of the cyanobacteria that would posses ascorbate peroxidase (Miyake and Asada, 1991). Tichy and Vermaas (1999) have found that the peroxidase can use dithiothreitol as reductant instead of the normal, unknown photoreductant.

Two gene sequences that have significant similarity to the thiol-specific peroxidase of yeast (Chae et al., 1994) are in the *Synechocystis* sp. PCC 6803 genome (Tichy and Vermaas, 1999). In yeast the normal reductant for this peroxidase is thioredoxin but dithiothreitol can also be used (Chae et al., 1994). *Synechocystis* sp. PCC 6803 contains two gene sequences closely similar to the gene for glutathione peroxidase but no activity of this peroxidase could be measured (Tichy and Vermaas, 1999). In *Synechococcus lividus*, however, glutathione peroxidase is thought to be the main route for H_2O_2 detoxification, with catalase actually being completely absent (Dupouy et al., 1985). In their definitive study of the catalase-peroxidase of *A. nidulans*, Obinger et al. (1997) found only this one enzyme to have peroxidase activity. However, the thylakoid fraction was not assayed. At present we are searching for a thylakoid-bound peroxidase in *Synechococcus* sp. PCC 7942 that could be a thioredoxin, ascorbate, or a glutathione peroxidase.

It is worth noting that light-dependent H_2O_2 decomposition occurred in the absence of added C_i; in fact, cells were always allowed to deplete the medium C_i before tests of H_2O_2 photoreduction were performed (Fig. 1). This was done to avoid the complication of C_i-induced Chl fluorescence quenching (Fig. 1). The photoreduction of O_2, NO_2^-, and the artificial PS 1 electron acceptor *N*′,*N*′-dimethyl-*p*-nitrosoalinine are all stimulated by the active accumulation of C_i within the cells (Miller et al., 1988a, 1991; Badger and Schreiber, 1993; Mir et al., 1995; Li and Canvin, 1997b). The light-dependent H_2O_2 decomposition observed in this study presumably requires PS l, as does operation of the ascorbate-glutathione pathway of chloroplasts (Asada, 1984; Noctor and Foyer, 1998). So far we have been unable to demonstrate any stimulation of H_2O_2 photoreduction by C_i in *Synechococcus* sp. PCC 7942 (data not shown). It is not at all clear why O_2 photoreduction, for example, should be stimulated by intracellular C_i accumulation whereas it seems unnecessary for H_2O_2 photoreduction. More information on the exact mechanisms of O_2 and H_2O_2 photoreduction and on the exact site of C_i-stimulation of photosynthetic electron transport (Miller et al., 1991; Badger and Schreiber, 1993; Mir et al., 1995; Li and Canvin, 1997a, 1997b) is required to answer this question.

Tichy and Vermaas (1999) argued that in the katG mutant the rate of H_2O_2 production was less than 1% the rate of total photosynthetic electron transport. The rate of O_2 photoreduction, and thus presumably of H_2O_2 production, can certainly be greater in wild-type *Synechocystis* sp. PCC 6803 (Goosney and Miller, 1997). High rates of O_2 photoreduction appear to be necessary to prevent photoinhibition in *Synechococcus* sp. PCC 7942 and UTEX 625 (Li and Canvin, 1997a; Campbell et al., 1999). An assessment of the total rate of H_2O_2 production, not only excretion, in various cyanobacteria under various conditions is necessary. It can already be seen, however, that these cyanobacteria have a battery of defenses against H_2O_2 that can include the unusual resistance of some enzymes (Takeda et al., 1995; Tamoi et al., 1996), excretion (Patterson and Myers, 1973), the catalase-peroxidase (Mutsuda et al., 1996; Obinger et al., 1997), and at least one light-dependent peroxidase.

MATERIALS AND METHODS

Strains and Culture Conditions

Synechococcus sp. PCC 7942, also known as *Anacystis nidulans* R2 (Rippka et al., 1979; Golden et al., 1989) was obtained from the University of Toronto Culture Collection as UTCC #100. *Synechococcus* UTEX 625, also known as *Synechococcus* sp. PCC 6301 and *A. nidulans* (Rippka et al., 1979; Golden et al., 1989), was obtained from Dr.

George Espie at the University of Toronto. In this paper we use the taxon *A. nidulans* when it is unclear which strain was being used by other workers or when the discussion refers to both strains. Cells were grown in the medium of Allen (1968), lacking the sodium silicate, and buffered at pH 8.0 with 50 mM HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid)-NaOH. Cultures (50 mL) were grown in glass culture tubes (25 × 200 mm) and were sparged with humidified air (approximately 70 mL min^{-1}) at 31°C. Illumination was provided by an equal number of wide spectrum Gro and Sho and Cool White (General Electric, Fairfield, CT) fluorescent tubes providing and incident photon flux density of photosynthetically active radiation of 100 μmol photons m^{-2} s^{-1}. Cells were kept in rapid growth phase by daily sterile dilution with fresh medium and were harvested at Chl concentrations of 3 to 5 μg mL^{-1}. Chl was determined after the extraction of cells with methanol (MacKinney, 1941).

Cells were harvested and washed (four times) by centrifugation (10,000*g* for 45 s) in a microfuge at room temperature. Washed cells were resuspended, under N_2, to reduce contamination with CO_2, in 50 mM BTP (1,3-bis[tris (hydroxymethyl)-methylamino] propane)/HCl buffer (pH 8.0), containing only about 25 μM dissolved C_i (Miller et al., 1988b). NaCl (50 mM) was added to the cell suspension to ensure optimal rates of active CO_2 and HCO_3^- transport (Espie et al., 1988). The Chl concentration was 8 to 12 μg mL^{-1}.

Chl Fluorescence and O_2 Evolution

Simultaneous measurements of Chl fluorescence and O_2 evolution were made in a DW2/2 O_2-electrode chamber from Hansatech Ltd. (King's Lynn, Norfolk, UK). The convergent arm of a four-armed fiber optic bundle was inserted into one port of the chamber assembly. The white light (WL) was transmitted through one arm of this bundle at 100 or 120 μmol photons m^{-2} s^{-1} obtained from a 300-W tungsten-halogen bulb and passed through a Calflex C heat filter (Balzers, Marlborough, MA). This beam could be interrupted with an electronic shutter and its intensity was varied with neutral density filters. The fiber optic bundle was also used to carry the modulated (100-kHz fluorescence measuring beam (MB, approximately 1.0 μmol photons m^{-2} s^{-1}, peak 650 nm) of a pulse amplitude modulation fluorometer (Walz, Effeltrich, Germany) to the cell suspension and to return the fluorescence emission to the pulse amplitude modulation fluorometer detector. The fourth arm of the fiber optic bundle was used to deliver a SF of WL (approximately 12,000 μmol photons m^{-2} s^{-1} from the FL103 saturation pulse lamp (Walz) for the determination of the magnitude of the qP (Schreiber et al., 1986). The duration of the SF was 600 ms. Changes in the O_2 concentration of the cell suspension were measured with the Hansatech electrode calibrated with N_2 and air. All measurements were performed at 30°C.

Catalase and Ascorbate Peroxidase Activity

The activity of catalase and ascorbate peroxidase was assayed in cell-free extracts of *Synechococcus* sp. PCC 7942. To prepare cell-free-extracts, cells were washed, frozen, and then thawed and resuspended to a density of about 200 μg Chl mL^{-1} in 1.0 mL of 100 mM potassium-phosphate buffer (pH 7.5) containing 5 mM EDTA. The cells were then disrupted with 0.5-mm glass beads in a Mini BeadBeater (Biospec Products, Bartlesville, OK). The glass beads were removed by passage of the homogenate through a small column of glass wool and the eluate was centrifuged (15 min at 14,000*g*) to remove unbroken cells. The cell-free extracts were stored at −70° until assayed.

Catalase activity was monitored as the decrease in A_{240} as H_2O_2 was decomposed (Aebi, 1984). The assay solution contained 100 μL of cell-free extract in 2.8 mL of 100 mM potassium-phosphate buffer (pH 7.5) and the reduction was initiated by the addition of 100 μL of H_2O_2 solution to yield a final concentration of 12.7 mM. Rates were corrected for the low rate of H_2O_2 decomposition observed in the absence of cell-free extract. Ascorbate peroxidase activity was monitored as the decrease A_{290} as ascorbate was oxidized in the presence of H_2O_2 (Tel-Or et al., 1986). The same assay solution was used as for the catalase assay but with the addition of 300 μM sodium ascorbate. Rates were corrected for the rate of ascorbate disappearance observed in the absence of H_2O_2. Protein concentrations of the cell-free extracts were determined with bicinochonic acid reagent (Pierce Chemical, Rockford, IL) using bovine serum albumin as a standard.

Synthesis and Use of $H_2{}^{18}O_2$

$H_2{}^{18}O_2$ was prepared essentially as described by Miyake and Asada (1991) by the reaction of $^{18}O_2$ with Glc catalyzed by Glc oxidase. The reaction solution (1.5 mL containing 10 mM potassium-phosphate at pH 6.0, 0.5 mM EDTA, 20 mM β-D-Glc, and 100 units of Glc oxidase) was placed in the O_2-electrode chamber at 30°C and the $^{16}O_2$ was removed with N_2 bubbling. The chamber was then stoppered and bubbles of $^{18}O_2$ (97.4 atom % ^{18}O [v/v], MSD Isotopes, Montreal, Canada) were introduced through the capillary port. The reaction was allowed to proceed for 3 h, with more $^{18}O_2$ being added periodically. The reaction was terminated by the addition of 30 μL of 1 M HCl and the unreacted $^{18}O_2$ was removed by N_2 bubbling. Unlike Miyake and Asada (1991), no KCN was added to the solution, as a Glc oxidase preparation (no. G9010, Sigma) containing very low catalase contamination was used. Thus the ion-exchange step to remove CN^- was unnecessary. The $H_2{}^{18}O_2$ solution was neutralized to pH 7.0 with KOH. Solutions prepared in this way were 8.3 to 10.8 mM with respect to H_2O_2 (measured as O_2 evolution after catalase addition) and had about 90 atom % ^{18}O. Solutions were kept on ice and used within several hours of preparation. During this time some nonenzymatic decomposition did occur and a correction for the resulting contaminant ^{18}O was obtained by adding samples to BTP buffer without cells in the MS cuvette. The signal (m/e = 36) due to this

$^{18}O_2$ was subtracted from the signal obtained with cells present. The leak from the MS cuvette for $^{16}O_2$ was 0.4% per minute and for $^{18}O_2$ was 0.8% per minute; because runs lasted only about 6 min, no corrections for leakage were made.

The changes in the concentrations of $^{16}O_2$ and $^{18}O_2$ in cell suspensions due to H_2 $^{18}O_2$ metabolism were monitored using a magnetic sector mass spectrometer (model no. MM 14–80 SC, VG Gas Analysis, Middlewich, UK) equipped with a membrane inlet system (Miller et al., 1988b). The system was calibrated using $^{16}O_2$ and N_2. The same calibration factor was used for $^{18}O_2$ measurements. Cells were illuminated with WL at 210 μmol photons m^{-2} s^{-1} (PAR).

Unlabeled stock H_2O_2 solutions were prepared by a 100-fold dilution of a commercial (Stanley Pharmaceuticals Ltd., Vancouver) 3% (v/v) solution with distilled water from which CO_2 had been removed by bubbling with N_2. The solutions were kept on ice and the H_2O_2 content was determined periodically by measuring the O_2 evolution from samples in the presence of catalase (Sigma-Aldrich).

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