The Effect of Activated Oxygen Species on Nitrogenase of *Anabaena variabilis*

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The heterocyst-forming cyanobacterium *Anabaena variabilis* (ATCC 29413) is deficient in catalase activity, but especially heterocysts are rich in superoxide dismutase and peroxidases. It exhibits a light-dependent methylviologen-catalyzed oxygen uptake which is accompanied by a massive release of hydrogen peroxide. Methylviologen caused a transient concentration-dependent inhibition of nitrogenase ending in a complete loss of activity. Suppression of hydrogen peroxide accumulation by the photosynthesis inhibitor DCMU (diuron, 3-[3,4-dichlorophenyl]-1,1-dimethylurea), or by addition of catalase prevented nitrogenase inhibition indicating that methylviologen affects nitrogenase activity *via* hydrogen peroxide produced by photosynthetic electron transport. Immunospecific Western blotting of cell extracts separated on SDS polyacrylamide gel electrophoresis showed that the transient inhibition of nitrogenase is accompanied by rapid modification of its Fe-protein followed by enzyme destruction. In a cell-free system the enzyme exhibited only moderate sensitivity towards hydrogen peroxide but was found susceptible against activated oxygen species generated by the xanthine/xanthine oxidase system and free iron. The results indicate that H_2O_2 itself has little direct effect on nitrogenase, but endogenous peroxide produced by methylviologen in the light may initiate oxygen radical formation leading to nitrogenase modification and subsequently its destruction.

Introduction

Several mechanisms of oxygen toxicity are due to biological activation of molecular oxygen. In photosynthetic organisms activated oxygen species arise from reactions between oxygen and components of the photosynthetic electron transport chain leading to singlet oxygen, superoxide anions (O_2^-), hydrogen peroxide and hydroxyl radicals (OH) [1, 2]. Partially reduced oxygen species have been implicated in the inhibition of nitrogen fixation catalyzed by nitrogenase although *in vivo* interaction with nitrogenase has not been documented [3, 4]. This notation is supported by the presence of high superoxide dismutase activity found in all nitrogen-fixing organisms ([5, 6]; see [7] for an actual survey on protective mechanisms of nitrogenase). However, many cyanobacteria, including some diazotrophs, have low or negligible catalase activity [8] and some of them release high amounts of hydrogen peroxide under autotrophic growth conditions [9]. This peroxide not only results from pseudocyclic electron transport at photosystem I (Mehler reaction) but, at least in some strains, from a photosystem II-linked reaction [10], possibly produced by an arginine oxidase [11]. The question arises whether activated oxygen species interfere with nitrogenase activity and stability.

With *Anabaena variabilis* a rapid switch-off of nitrogenase following incubation under pure oxygen was demonstrated [12, 13]. An oxygen-dependent inhibition of nitrogenase was also found in carbon-limited, glycogen-depleted cells, or by addition of ammonia under alkaline conditions [14]. In all cases, inactivation of nitrogenase was accompanied by a modification of its subunit, the Fe-protein, causing decrease of mobility of this subunit in SDS polyacrylamide gel electrophoresis. Evidence was presented that Fe-protein modification is involved in oxygen protection of nitrogenase [15]. However, the signals leading to nitrogenase modification are unknown.

This study contributes data to the role of oxygen radicals and hydrogen peroxide with respect to nitrogenase activity and modification. Because radicals and peroxide are difficult to detect directly in intact cells the herbicide methylviologen (paraquat 1,1′-dimethyl-4,4′-bipyridylium chloride) was

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used to increase radical formation inside the cell. In the light, methylviologen is reduced by photosystem I and subsequently autoxidized producing the free radical O_2^- [16]. With metal ions present the superoxide anion may give rise to the powerful oxidant $OH^{\cdot}$ (Fenton reaction). Hydrogen peroxide is formed during dismutation of superoxide catalyzed either by superoxide dismutase or, as in chloroplasts, by oxidation of ascorbate [1]. In our experiments, production of H_2O_2, the stable intermediate of partial oxygen reduction, was followed by a fluorimetric method [17] and the levels of enzymes which apparently control radical formation and possibly protect nitrogenase were determined in both heterocysts and vegetative cells of *Anabaena*.

Materials and Methods

Organism and growth conditions

Anabaena variabilis (ATCC 29413) was cultivated in mineral medium without combined nitrogen at 30 °C in a water bath. Cultures were illuminated with white light (130 μE m^{-2} sec^{-1}) and supplied with CO_2-enriched air (1.5%, v/v) [14]. Cells from 1 day-old cultures were used in whole-cell assays and for preparation of cell-free extracts. Some cultures were flushed with 50% (v/v) O_2 added to air/CO_2 2.5 h prior to harvest.

Preparation of cell extracts

Cells were harvested (10 min, 6000 × *g*, 21 °C) and resuspended in buffer containing (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes)), 30 mM; piperazine-N′N-bis(ethanesulfonic) acid (Pipes), 30 mM; $MgCl_2$ 1 mM, which was used also for the preparation of extracts. Extracts of vegetative cells were prepared from filaments after mild lysozyme incubation (1 mg lysozyme per mg chlorophyll (Chl), 30 min at 30 °C) followed by removal of lysozyme by centrifugation (3000 × *g*, 5 min), and subsequent destruction of vegetative cells in an ultrasonic cleaning bath (Bandelin Electronics KG, Berlin, Germany). Heterocysts were separated from vegetative cell fragments by centrifugation (3000 × *g*, 5 min). Heterocysts were further purified by several washing steps resulting in preparations with less than 2% vegetative cells. Cell-free extracts of heterocysts were prepared by French-press treatment (1.38×10^5 kPa), and non-broken cells were removed by centrifugation (3000 × *g*, 5 min). For nitrogenase measurement, cell-free extracts were prepared by French-press treatment of whole filaments in the presence of 8 mM Na-dithionite under a hydrogen atmosphere. Cell fragments were removed by centrifugation (100,000 × *g*, 2 h). All extracts were frozen in liquid nitrogen immediately after preparation.

Immunospecific Western blotting

5 ml of intact filaments were rapidly filtered (Millipore, RA, 1.2 μm) and quickly frozen in liquid nitrogen. Nitrogenase was extracted by boiling the cells in 0.4 ml of sodium dodecylsulfate (SDS) buffer (4 min), and extracts equivalent to 1–2 μg of chlorophyll per slot were separated by SDS polyacrylamide gel electrophoresis [14]. The Fe-protein of nitrogenase was identified by immunospecific Western blotting using an antibody prepared against the SDS-denatured Fe-protein from *A. variabilis* [13]. The antibody complex was visualized with a peroxidase-conjugated *anti*-rabbit antibody (Dianova, Hamburg, Germany) as described [13].

Hydrogen peroxide production

One day-old cultures were harvested and resuspended in a medium defined by Patterson and Myers [18] at a culture density of 18 to 20 μg ml^{-1} chlorophyll. Hydrogen peroxide production was quantified in the supernatant using the H_2O_2-dependent decay of scopoletine fluorescence catalyzed by horse radish peroxidase [17]. Scopoletin fluorescence was measured with a Hitachi F 2000 fluorescence spectrophotometer at excitation and emission wavelengths of 342 nm and 456 nm, respectively. Additions of scopoletin (6-methoxy-7-hydroxy-1,2-benzopyrone, 10 μM) and horse radish peroxidase (EC 1.11.1.7, 10 units) were made immediately before start of the assay by illumination (650 μE m^{-2} s^{-1}). At times indicated an aliquot was removed and the reaction stopped by rapid centrifugation (2 min, 13,000 × *g*). Replicate analyses of the same sample were found within ± 5%.

Enzyme assays

For determination of cellular nitrogenase activity one day-old cultures were harvested (1500 × *g*) and resuspended in the same medium at a chlorophyll density of 20 μg ml^{-1}. Activity was measured by acetylene reduction with 5 ml cell suspension in 25 ml glass vessels closed with serum stoppers at 30 °C under white light (650 μE m^{-2} s^{-1}). The assay was started by addition of 13% (v/v) acetylene to a gas phase consisting of air or H_2 as indicated (further details in ref. [19]).

Cell-free nitrogenase activity was estimated in the presence of Na-dithionite and an ATP-regenerating system under argon [20]. 30 mM glucose and 15 units of glucose oxidase were included in the assay mixture before an anaerobically prepared cell-free extract of *A. variabilis* was added. The reaction was initiated by addition of acetylene.

Catalase (EC 1.11.1.6) activity was determined in broken vegetative cells and heterocysts by the production of O_2 from H_2O_2 after addition of catalase using a Clark-type oxygen electrode (Yellow Springs Instruments, OH, U.S.A.) in a thermostated chamber at 30 °C [8], or spectrophotometrically by following the disappearance of H_2O_2 in the supernatant of the cell suspension at 240 nm [21]. Superoxide dismutase (EC 1.15.1.1) was assayed by its effect to decrease the rate of cytochrome *c* (horse heart) reduction by a xanthine/xanthine oxidase (EC 1.2.3.2) system as described [22]. One unit of superoxide dismutase activity causes 50% decrease of the rate of cytochrome *c* reduction under these conditions. Peroxidase (EC 1.11.1.7) was assayed spectrophotometrically by following *p*-phenylenediamine oxidation at 485 nm [23]. Glutathione reductase, NADPH-dependent (EC 1.6.4.2) was assayed by NADPH oxidation at 340 nm [24]. Glutathione peroxidase (EC 1.11.1.9) was measured by coupling the oxidation of glutathione to NADPH oxidation in the presence of *t*-butylhydroperoxide using excess glutathione reductase, NADPH-dependent, according to [25] and adapted by Tözüm and Gallon [4]. Activity of glutathione dehydrogenase, ascorbate-dependent (EC 1.8.5.1), was estimated by coupling the oxidation of glutathione to NADPH oxidation in the presence of dehydroascorbate using excess glutathione dehydrogenase, NADPH-dependent [4]. Ascorbate peroxidase was determined by measuring the disappearance of H_2O_2 essentially as described [4]. Glutathione and ascorbate content of the *Anabaena* extracts were determined after Tiebe [26] and Rose [27], respectively. Protein was determined after [28] using bovine serum albumin as standard.

Chemicals

Enzymes were purchased from Boehringer, Mannheim, xanthine and *p*-phenylenediamine from Merck, Darmstadt, and scopoletin from Sigma, München, all in Germany. Other chemicals were of the highest analytical grade available.

Reliability

Data of typical experiments are shown. All experiments were repeated three and more times. The deviation from the mean did not exceed ±10%.

Results

Methylviologen was rapidly taken up by vegetative cells of *Anabaena variabilis* as it caused an immediate light-stimulated oxygen uptake (Mehler reaction, Fig. 1). In one day-grown filaments with

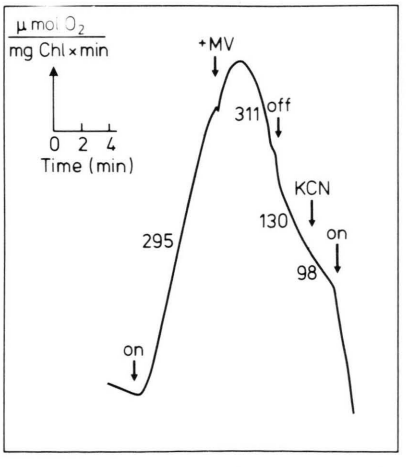

Fig. 1. Oxygen gas exchange of *Anabaena variabilis* before and after addition of methylviologen. Filaments of one day-grown cultures were concentrated (10.8 μg Chl ml^{-1}) and placed into an oxygen electrode chamber. Arrows indicate start and end of illumination and addition of methylviologen (10 μM) and KCN (1 mM), respectively. Figures indicate rates in μmol O_2 mg^{-1} Chl h^{-1}. On, off denotes +, − illumination.

a high glycogen pool (compare ref. [19]) and strong ferricyanide-inducible respiration [29] methylviologen stimulated dark oxygen uptake over endogenous respiratory rates, and KCN (1 mM) was without marked effect on this dark activity (Fig. 1). These results indicate catalase deficiency of the cells (see ref. [8] and Table III).

Peroxide release due to catalase deficiency is reflected by oxygen evolution in the dark of methylviologen-treated cells after addition of exogenous catalase. Unpoisoned filaments liberated little H_2O_2 under growth conditions (data not shown) and under conditions used for acetylene reduction assays (4-fold higher light intensity than used under growth conditions, and a gas phase without additional CO_2, control in Fig. 2). Under the latter conditions methylviologen induced a massive H_2O_2 formation, whose net accumulation after 15 min, but not the initial kinetics, was concentration-dependent. Addition of the photosystem II inhibitor DCMU efficiently suppressed H_2O_2 production in the presence and absence of methylviologen. Catalase added together with methylviologen prevented H_2O_2 accumulation. Noteworthy, the autoxidizable electron acceptor DBMIB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone), which generates H_2O_2 with isolated chloroplasts [30], did not significantly increase H_2O_2 formation in *A. variabilis* at a concentration used to inhibit nitrogenase activity (Fig. 2 and Table I).

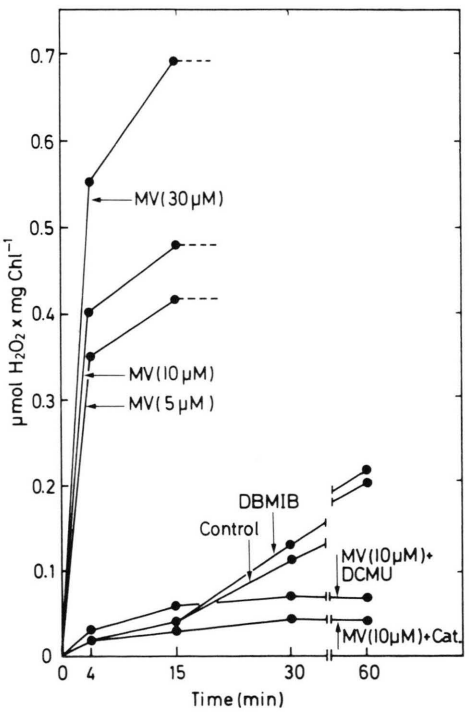

Fig. 2. Effect of methylviologen (MV), MV + DCMU (5 μM), MV + catalase (7 units) and DBMIB (0.2 mM) alone on peroxide formation in *Anabaena variabilis* in the light. Cells were incubated at 30 °C and 650 μE m^{-2} s^{-1} in the presence of scopoletin and peroxidase. At the times indicated 1 ml samples were withdrawn and the reaction was stopped as described in Materials and Methods. The supernatant was analyzed for loss of scopoletin in fluorescence.

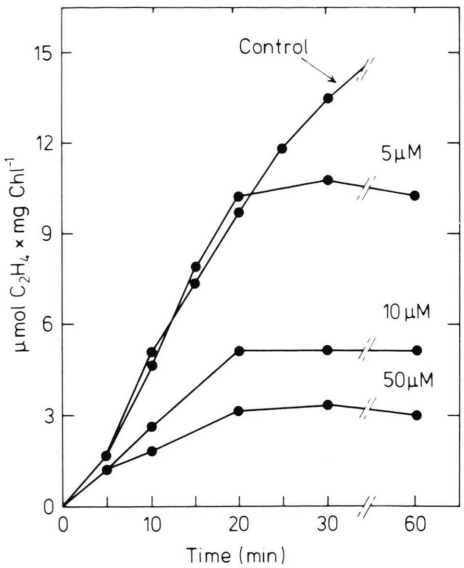

Fig. 3. Inhibition of nitrogenase activity by 5, 10, and 50 μM methylviologen. Nitrogenase activity was followed by acetylene reduction (10% C_2H_2 in air, v/v, 30 °C; cells illuminated with 650 μE m^{-2} s^{-1}). At the times indicated the gas phase was analyzed for ethylene formation.

Methylviologen caused a concentration-dependent inhibition of nitrogenase activity within initial 20 min, which eventually led to complete loss of activity. Even a low concentration (5 μM) hardly affecting the initial activity, but later caused complete inactivation (Fig. 3). To explain this biphasic effect we analyzed the Fe-protein of nitrogenase from above samples by immunospecific Western blotting. The Fe-protein migrated as a single band in SDS polyacrylamide gel electropho-

Table I. Effect of methylviologen and photosynthesis inhibitors on acetylene reduction in the light using intact filaments of *Anabaena variabilis*.

Additions[1]	Gas phase	Acetylene reduction[2] [μmol mg^{-1} Chl h^{-1}]	% of control
None	air	35.7	100
DCMU, 5 μM	air	30.3	85
MV, 10 μM	air	14.3	40
DCMU; MV, 10 μM	air	27.1	76
None	H_2	52.9	148
DCMU, 5 μM	H_2	47.6	133
MV, 10 μM	H_2	34.4	96
DCMU; MV, 10 μM	H_2	47.0	132
MV, 10 μM; SOD, 14 U	air	15.7	44
MV, 10 μM; catalase, 7 U	air	34.8	97
DBMIB, 0.2 mM	air	5.7	16
DBMIB, 0.2 mM	H_2	6.8	19
DBMIB, catalase, 7 U	air	7.2	20

[1] All additions were made at zero time. Aliquots from ethanolic stock solutions were added directly. Controls contained equal volumes of ethanol; MV, methylviologen; SOD, superoxide dismutase; U, units (other abbreviations in the text).
[2] Calculated from the production of ethylene during 30 min of incubation.

resis in the control (at 30 min) and at start of the methylviologen treatment. After a short time, dependent on the methylviologen concentration used, the antibody detected a second form which migrated slower. A double band (both forms present) was fully expressed after 20 min with 10 μM methylviologen or less present. After prolonged incubation, however, the polyclonal antibody detected a smear formed below the double band apparently indicative of disintegrated Fe-protein (Fig. 4). We could not resolve the double band with the higher viologen concentrations and the smear appeared much in advance.

The immunoblotting showed that the methylviologen-dependent loss of nitrogenase activity of the second phase of inhibition (Fig. 3) is due to enzyme destruction. The initial inhibition phase, accompanied by the formation of a double band of the Fe-protein, could be due to a competition for reductants between nitrogenase and methylviologen at the acceptor side of photosystem I or due to H_2O_2 produced as consequence of the Mehler reaction in the presence of oxygen. A hydrogen atmosphere (flushing of assay vessels prior to addition of acetylene) stimulated nitrogenase activity but only

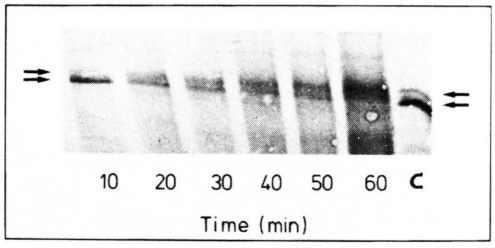

Fig. 4. Modification and destruction of the Fe-protein of *Anabaena* nitrogenase following methylviologen addition. Cell extracts from illuminated filaments, treated with 10 μM methylviologen (according to Fig. 3), for the periods indicated were separated on SDS polyacrylamide gel electrophoresis and transferred to a blotting membrane (the lanes became distorted due to this procedure). The Fe-protein was detected with an antibody as described in Materials and Methods. C, control. See text for further explanation.

partly prevented methylviologen-induced nitrogenase inhibition (Table I). DCMU which prevents H_2O_2 formation (Fig. 2), virtually reversed the methylviologen-induced inhibition of nitrogenase under H_2/C_2H_2, and to a lesser extent under air/C_2H_2. This finding implies that the reduction of

methylviologen initiates inactivation of nitrogenase. Addition of superoxide dismutase (to control the release of superoxide anions) had no effect in intact filaments but catalase brought the enzyme activity near to control level (Table I). DBMIB (200 μM), which was shown not to induce H_2O_2 formation (Fig. 2), inhibited nitrogenase activity irrespective of the gas phase or the presence of catalase. DBMIB treatment alone did not cause Fe-protein modification or Fe-protein degradation (data not shown).

The findings indicate that methylviologen-dependent inhibition of nitrogenase results from photosynthetic H_2O_2 formation. Attempts were made to check this with a cell-free nitrogenase assay (Table II). Small amounts of air-saturated buffer caused inhibition of nitrogenase. To avoid errors caused by molecular oxygen introduced together with other components we included glucose plus glucose oxidase in the assay mixture. Under these conditions nitrogenase was not inhibited by 10 μl of air-saturated buffer, the volume used to add the radical forming xanthine/xanthine oxidase mixture or H_2O_2. With glucose/glucose oxidase present nitrogenase exhibited only moderate sensitivity towards H_2O_2 vanishing in the presence of catalase. Addition of xanthine/air plus xanthine oxidase which produced superoxide assayed by cytochrome *c* reduction [22], caused stronger inhibition which was not completely relieved by catalase. Glucose/glucose oxidase or any other component of nitrogenase reaction mixture did not quench superoxide, because the cytochrome reduction ability was not influenced. Addition of extract caused some minor decrease of superoxide due to some endogenous superoxide dismutase. High toxicity, however, was observed in the xanthine/xanthine oxidase system with ferric ions present. Fe^{3+} by itself did hardly influence nitrogenase activity. By oxygen-electrode measurement an anoxygenic atmosphere of nitrogenase assays was confirmed even after addition of aerated components.

These findings give evidence that *in vitro* oxygen radicals are much more effective than H_2O_2 itself to inactivate nitrogenase. Obviously, without methylviologen present the cellular concentration of all partially reduced oxygen species will be markedly influenced by enzymes responsible for removal of radicals. Therefore, broken cell preparations of vegetative cells and heterocysts were examined for activities of enzymes possibly involved in oxygen radical control. The results are summarized in Table III. Like in *Gloeocapsa* [4] catalase is missing in *A. variabilis* when assayed spectrophotometrically or polarographically. Superoxide dis-

Table II. Effect of hydrogen peroxide and oxygen radicals on a cell-free preparation of nitrogenase from *Anabaena*.

Additions	Acetylene reduction[1] [nmol mg^{-1} protein min^{-1}]	% of control
None	5.7	100
Air-saturated buffer, 10 μl	4.0	69
Air-saturated buffer, 10 μl*	5.3	93
H_2O_2, 50 μM	4.6	81
100 μM	3.7	58
1000 μM	1.4	21
H_2O_2, 50 μM; catalase, 7 U	6.8	119
Xanthine; 0.2 mM/ xanthine oxidase, 0.04 U	2.0	35
Xanthine/xanthine oxidase, catalase	4.7	82
$FeCl_3$, 50 μM	5.4	95
$FeCl_3$, xanthine/xanthine oxidase	0.3	6

All assays contained 30 mM glucose and 15 units (U) glucose oxidase except for that of the second line. All experiments were conducted under Ar/C_2H_2 and additions were made with previously flushed solutions. Xanthine/xanthine oxidase was mixed in air and added directly in a volume of 10 μl.

Table III. Distribution of oxygen-radical scavenging enzymes in homogenates of vegetative cells and heterocysts of air-grown and oxygen-enriched cultures of *Anabaena variabilis*[a].

Enzymes and metabolites	Air-grown culture		O_2-enriched culture	
	Vegetative cells	Heterocysts	Vegetative cells	Heterocysts
Catalase	n.d.	n.d.	n.d.	n.d.
Superoxide dismutase[1]	n.d.	46	2.3	33
Peroxidase[2]	0.18	1.3	0.10	2.4
Glutathione reductase[3]	18	10	40	18
Glutathione peroxidase[3]	14	16	29	18
Ascorbate-glutathione dehydrogenase[3]	49	170	14	86
Ascorbate peroxidase[4]	n.d.	n.d.	1.6	n.d.
Ascorbic acid[5]	11.4	1.2	6.4	0.4
Glutathione[5]	5.3	4.8	4.5	1.8

[a] Homogenates of vegetative cells and heterocysts were prepared under air as described in Materials and Methods from one day-grown cultures. O_2-enriched cultures were incubated for 2.5 h with 50% oxygen (v/v) in the normal air/CO_2 cultivation atmosphere; n.d., not detectable.
[1] One unit (U) caused 50% inhibition of superoxide formation per mg protein produced by the xanthine/xanthine oxidase system *vs.* control.
[2] μmol *p*-phenylenediamine oxidized mg^{-1} protein min^{-1}.
[3] nmol NADPH oxidized mg^{-1} protein min^{-1}.
[4] μmol H_2O_2 consumed mg^{-1} protein min^{-1}.
[5] μg mg^{-1} protein.

mutase is low, if detectable in vegetative cells, but is significantly confined to heterocysts. Using phenylenediamine and reduced glutathione as substrates peroxidases were found in both cell types, but heterocysts had a higher peroxidase activity than vegetative cells as suggested from microscopic staining studies [31]. In addition, glutathione reductase, the enzyme responsible to recycle reduced glutathione from the oxidized form, was present in both cell types. Ascorbic acid may non-enzymatically interact with O_2^- and hydrogen peroxide thus generating dehydroascorbic acid, which is reduced by glutathione dehydrogenase, located preferentially in the heterocysts. Exposing the cells to excess oxygen did not dramatically alter the enzyme pattern but caused a decrease in cellular ascorbate levels and in ascorbate-dependent glutathione dehydrogenase, while glutathione reductase activity increased and superoxide dismutase activity became detectable in vegetative cells.

Discussion

Anabaena variabilis belongs to a group of cyanobacteria which exhibit low H_2O_2 production by the untreated control (Fig. 2, comp. also ref. [9]). The low peroxide release of unpoisoned filaments reflects an effective control of peroxide levels by the activity of peroxidases and by non-enzymatic oxidation of ascorbic acid (Table III). As catalase activity is low or absent (Fig. 1 and Table III) addition of methylviologen induced rapid O_2-uptake in the light without oxygen release after darkening (Fig. 1, comp. ref. [8]), leading to a high H_2O_2 production (Fig. 2). This shows that the capacity of the ascorbate-regenerating enzymes glutathione reductase and glutathione reductase and that of peroxidases, all depending on the supply of reduced substrates, is insufficient in the presence of methylviologen.

DCMU strongly inhibits methylviologen-catalyzed light-induced H_2O_2 formation (Fig. 2) and protects nitrogenase activity against methylviologen action (Table I). In heterocysts, photosystem II is absent [32]. Reduced pyridine nucleotides derive from dissimilation of photosynthates directly imported from photosynthetically active vegetative cells or transiently stored as glycogen (see ref. [33] for review). In one day-grown cultures high glycogen reserves make nitrogenase independent

from direct supply of photosynthates and therefore activity remains high in the presence of DCMU [19, 34]. The protective effect of DCMU under oxygen-free conditions demonstrates that methylviologen itself does not affect electron transport to nitrogenase in heterocysts. In the presence of exogenous oxygen (air) inhibition by methylviologen-catalyzed peroxide formation is increased and DCMU is less protective. Catalase added to intact filaments quantitatively removes hydrogen peroxide from the medium generated through the action of methylviologen (Fig. 2), thereby increasing the H_2O_2 efflux. The protective effect by catalase strengthens the hypothesis that nitrogenase inhibition is primarily due to endogenous light-induced H_2O_2 accumulation in vegetative cells and heterocysts.

Peroxide toxicity, however, was found to be low in the cell-free nitrogenase assay (Table II). On the other hand, oxygen radicals like O_2^- formed by xanthine/xanthine oxidase and of hydroxyl ions formed in the presence of iron (Fenton reaction) caused drastic inhibition of nitrogenase. Air-induced inhibition (assay without glucose/glucose oxidase) apparently is a cumulative effect of O_2 and O_2^- produced from dithionite oxidation by molecular oxygen. From these results we conclude, that the methylviologen-dependent inactivation of nitrogenase is due to H_2O_2-supported radical formation rather than to peroxide itself.

Inactivation of nitrogenase is followed by disintegration as indicated by the immunoreactive smear (Fig. 4). Excess of activated oxygen species as produced in our experiments apparently leads to oxidation of the proteins. Oxidized proteins may be more prone to proteolytic attack. Protein oxidation is a general mechanism to regulate the enzyme turnover [35], and nitrogenase was shown to exhibit an increased turnover under aerobic conditions [36]. It should be mentioned, however, that an accumulation of degradation products of the Fe-protein as observed after methylviologen treatment is not normally seen in the intact cell even after prolonged maintenance of the nitrogenase in the inactive, enzymatically modified form [37].

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[1] K. Asada, Methods Enzymol. **105,** 422–429 (1984).
[2] I. Fridovich, Arch. Biochem. Biophys. **247,** 1–11 (1986).
[3] A. G. Buchanan, Can. J. Microbiol. **23,** 1548–1553 (1977).
[4] D. S. R. Tözüm and J. R. Gallon, J. Gen. Microbiol. **111,** 313–326 (1979).
[5] L. E. A. Henry, I. N. Gogotov, and D. O. Hall, Biochem. J. **174,** 373–377 (1978).
[6] A. Puppo and J. Rigaud, FEBS Lett. **201,** 187–189 (1986).
[7] M. Becana and C. Rodriguez-Barrueco, Physiol. Plant. **75,** 429–438 (1989).
[8] N.-H. Chua, Biochim. Biophys. Acta **245,** 277–287 (1971).
[9] S. E. Stevens, C. O. P. Patterson, and J. Myers, J. Phycol. **9,** 427–430 (1975).
[10] J. Bednarz, S. Höper, M. Bockstetter, K. Bader, and G. H. Schmid, Z. Naturforsch. **44c,** 946–954 (1989).
[11] E. K. Pistorius and H. Voss, Eur. J. Biochem. **126,** 203–209 (1982).
[12] R. L. Smith, C. van Baalen, and F. R. Tabita, J. Bacteriol. **169,** 2537–2542 (1987).
[13] S. Reich and P. Böger, FEMS Microbiol. Lett. **58,** 81–86 (1989).
[14] A. Ernst, S. Reich, and P. Böger, J. Bacteriol. **172,** 748–755 (1990).
[15] F. R. Tabita, C. Van Baalen, R. L. Smith, and D. Kumar, in: Nitrogen Fixation: Hundred Years After (H. Bothe, F. J. de Bruijn, and W. E. Newton, eds.), pp. 189–194, G. Fischer, Stuttgart, New York 1988.
[16] J. A. Farrington, M. Ebert, E. J. Land, and K. Fletcher, Biochim. Biophys. Acta **314,** 372–381 (1973).
[17] H. Perschke and E. Broda, Nature **190,** 257–258 (1961).
[18] C. O. P. Patterson and J. Myers, Plant Physiol. **51,** 104–109 (1973).
[19] A. Ernst, H. Kirschenlohr, J. Diez, and P. Böger, Arch. Microbiol. **140,** 120–125 (1984).
[20] B. Schrautemeier, H. Böhme, and P. Böger, Arch. Microbiol. **137,** 14–20 (1984).
[21] B. Chance and A. C. Maehly, Methods Enzymol. **2,** 764–775 (1955).
[22] T. W. Kirby and I. Fridovich, Anal. Biochem. **127,** 435–440 (1982).
[23] H. Lück, in: Methoden der enzymatischen Analyse (H. V. Bergmeyer, ed.), pp. 894–897, Verlag Chemie, Weinheim 1962.
[24] E. M. Scott, I. W. Duncan, and V. Ekstrand, J. Biol. Chem. **238,** 3928–3933 (1963).
[25] C. Little and P. J. O'Brian, Biochem. Biophys. Res. Commun. **31,** 145–150 (1968).
[26] F. Tiebe, Anal. Biochem. **27,** 502–522 (1969).

[27] J. H. Rose, in: Methods of Biochemical Analysis (D. Glick, ed.), **Vol. 1,** pp. 115–139, Wiley Interscience, New York 1954.
[28] B. O. Fanger, Anal. Biochem. **162,** 11–17 (1987).
[29] S. Scherer and P. Böger, Z. Naturforsch. **40c,** 138–141 (1985).
[30] E. F. Elstner and D. Frommeyer, FEBS Lett. **86,** 143–145 (1978).
[31] M. A. Murry, A. G. Olafson, and J. R. Benemann, Curr. Microbiol. **6,** 201–206 (1981).
[32] H. Almon and H. Böhme, Biochim. Biophys. Acta **952,** 113–120 (1980).
[33] S. Scherer, H. Almon, and P. Böger, Photosynth. Res. **15,** 95–114 (1988).
[34] A. Ernst and P. Böger, J. Gen. Microbiol. **131,** 3147–3153 (1985).
[35] E. R. Stadtman, Trends Biol. Sci. **11,** 11–12 (1986).
[36] M. A. Murry, A. J. Horne, and J. R. Benemann, Appl. Environ. Microbiol. **47,** 449–454 (1984).
[37] A. Ernst, Y.-D. Liu, S. Reich, and P. Böger, Botan. Acta **103,** 183–189 (1990).