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Inactivation of Cyanobacterial Nitrogenase After Exposure to Ultraviolet-B Radiation

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Abstract. Exposure of the N_2-fixing cyanobacterium *Anabaena* BT2 to ultraviolet-B radiation (2.5 W m^{-2}) for 30 min resulted in complete loss of nitrogenase activity but 100% cell killing occurred only after a 90-min exposure. Inactivation of nitrogenase activity was not specific to *Anabaena* BT2; other species also showed a similar effect. The time required for 100% killing and inactivation of nitrogenase activity differed in various species, and this difference may be ascribed to the presence of different levels of UV-B protection mechanisms in individual species. Inhibition of nitrogenase activity was immediate, since exposure of cultures to UV-B for as little as 5 min elicited some inhibition of activity. The activity of UV-B-inhibited nitrogenase did not recover upon transfer of exposed cells to fluorescent light, suggesting that the inhibition may be due to specific inactivation of the enzyme. By employment of inhibitors of protein synthesis and PS-II activity, it was demonstrated that restoration of nitrogenase activity in a UV-B-treated culture occurred by fresh synthesis of nitrogenase polypeptide. Our findings suggest that estimation of nitrogenase activity in diazotrophic species may be used as a marker enzyme for assessing the impact of UV-B radiation.

The damaging effects of solar ultraviolet (UV-B, 280–320 nm) radiation have been studied in a variety of biological systems [2, 8, 17, 24]. Depletion of stratospheric ozone leads to an increased incidence of UV-B on the earth's surface, causing worldwide environmental effects, leading to imbalances in natural ecosystems [4, 21]. Most studies on UV-B have been confined to only a few species of higher plants, algae, and animals, and hence no general conclusions pertaining to the deleterious effects of UV-B have been put forward [2, 17]. Furthermore, the intensity of UV-B reaching the earth surface varies in different ecosystems, and in certain ecosystems only particular types of organisms are exposed to UV-B radiation [11, 21]. There is a great need for studying the effects of UV-B on a much wider spectrum of organisms from a diversity of habitats than hitherto explored.

Cyanobacteria, the oldest oxygenic-photosynthetic prokaryotes [1], occur widely in nature, in soil and water and in association with a variety of plant and marine life [12, 14]. Many species can tolerate various climatic conditions and are found in diverse habitats including hot springs, arctic regions, desert soils, and rocky surfaces [1, 3, 22]. Members show considerable metabolic plasticity and, in addition to CO_2 and O_2 assimilation, some also assimilate N_2 and H_2 [22]. Because of efficient N_2 fixation potential, certain members of cyanobacteria are known to play an important role in the fertility of soil, especially rice fields in the tropics. In spite of these added features in cyanobacteria, no systematic investigations related to UV-B effects on a particular metabolic event have been made [17]. Studies so far conducted reveal that UV-B affects the growth, survival, pigmentation, orientation, development, general metabolism, photosynthesis, nitrogen fixation, and assimilation of nitrogen in diverse types of cyanobacterial species [5, 10, 13, 16, 18]. It has been suggested that these effects are in part due to direct effects of UV-B radiation on membrane proteins, photosystem-II, DNA, enzymes, pig-

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ments, growth regulators, or to its indirect effect through the formation of reactive oxygen species [17, 18, 24]. Information gathered so far does not reveal the exact mechanism(s) of UV-B action on growth and survival or the damaging effects on various metabolic processes [17]. In our previous study dealing with the effects of UV-B radiation on certain enzymes of nitrogen metabolism in *Nostoc* species, we demonstrated that the enzyme nitrogenase is more sensitive to UV-B radiation than nitrate reductase, whereas glutamine synthetase activity remains more or less unaffected [10, 24].

In the present study, we have made an attempt to identify a metabolic process of cyanobacteria for possible use as an indicator for assessing the deleterious effect of UV-B. The enzyme nitrogenase, which reduces N_2 to ammonia, was selected to study the above hypothesis and seems to be a suitable indicator enzyme for assessing the effect of UV-B.

Materials and Methods

Test organism and growth conditions. The test organisms *Anabaena* BT2, *Anabaena doliolum*, *Calothrix* sp., *Nostoc spongiaeforme*, *Nostoc commune*, and *Scytonema* sp. were isolated from a rice field of Banaras Hindu University. *Nostoc muscorum Ag ex Born et Flah* (ATCC 27893) was generously supplied to us by R. Haselkorn, U.S.A. The symbiotic cyanobacterium *Anabaena cycadeae* was isolated from the coralloid root of *Cycas revoluta.* All these strains have been maintained in our laboratory for the last 5–6 years. Axenic cultures were routinely grown in modified Chu-10 medium [15] in a culture room at 27° ± 2°C and illuminated with Sylvania 40 W T12 fluorescent lamp at an intensity of 14.4 ± 1 W m^{-2} for a 14/10 h light/dark cycle. Unless otherwise stated, all the experiments were performed with the log phase cultures having an initial dry wt. of approximately 0.15 mg mL^{-1}.

Mode and source of UV-B irradiation. The source of artificial UV-B irradiation was a UV-B lamp (Cat. No. 3-4408, Fotodyne Inc., USA) giving its main output at 312.67 nm. The desired intensity of UV-B reaching the culture suspension was obtained by adjusting the distance between the UV-B source and the sample. UV-B intensity was measured by Black-Ray J-221, Longwave Ultraviolet Intensity Meter (UVP, Inc., San Gabriel, CA, USA). Treatment of the culture suspension with UV-B was given in complete darkness in a 75-mm sterile petri dish (Corning) with an open lid, each containing 25 ml of homogeneous algal suspension. The culture suspension was gently stirred magnetically during UV-B irradiation to facilitate uniform exposure.

Determination of percentage survival. For determining percentage survival, 0.5-ml aliquots were withdrawn at known time intervals after UV-B treatment and were spread on agar plates. After a 48-h dark incubation, plates were transferred to light in the culture room. Percentage survival was scored by colony counts in a colony counter and was plotted semilogarithmically.

Estimation of nitrogenase activity. Nitrogenase activity was determined by the acetylene reduction technique per the method of Stewart et al. [23].

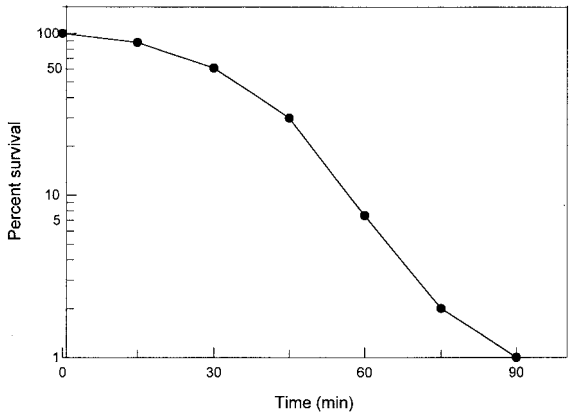

Fig. 1. UV-B survival of *Anabaena* BT2. An exponentially grown culture was exposed to a 2.5-W m^{-2} dose of UV-B radiation and thereafter incubated under fluorescent light. Results are based on the average of three experiments performed independently under identical conditions.

Results and Discussion

Before assessing the effects of UV-B radiation (2.5 W m^{-2}) on nitrogenase activity, we tested its effect on percentage survival in different N_2-fixing cyanobacteria. Exposure of cultures to 2.5 W m^{-2} of UV-B radiation was based on the fact that during exposure the intensity of UV-B in solar radiation averaged about 2.3 W m^{-2}. Figure 1 shows the percentage survival of *Anabaena* BT2 after UV-B radiation. As expected, cells exposed to UV-B for 30 min showed about 62% survival, and complete killing occurred after continuous exposure to UV-B for 90 min. The pattern of percentage survival in other species was similar, but there were differences in the time required for 100% killing. Complete killing of *A. doliolum*, *N. spongiaeforme*, and *N. commune* occurred after 100 min; *A. cycadeae* and *N. muscorum* required 80 and 90 min respectively; whereas *Calothrix* sp. and *Scytonema* sp. showed total killing only after continuous exposure to UV-B for 180 min. The complete killing of cells after exposure to UV-B radiation might be due to inactivation of a number of processes [17–19, 24]. Disintegration of phycobilisomes, followed by release of phycobiliproteins in the medium by exposure of UV-B (2.5 W m^{-2} or above) for 2–3 h to cultures of cyanobacteria, has been reported earlier [18, 24]. Such cultures fail to revive upon transfer to fluorescent light and fresh medium. Differential effects on percentage survival appear to be due to the presence of various types of UV-B protecting mechanisms in different species [3, 14, 20]. *Scytonema* sp. contains a yellow-brown, lipid-soluble dimeric pigment, scytonemin, located in the extracellular sheath, which has been proposed to serve as a UV-B sun-screen compound [3, 14]. Similarly, many species

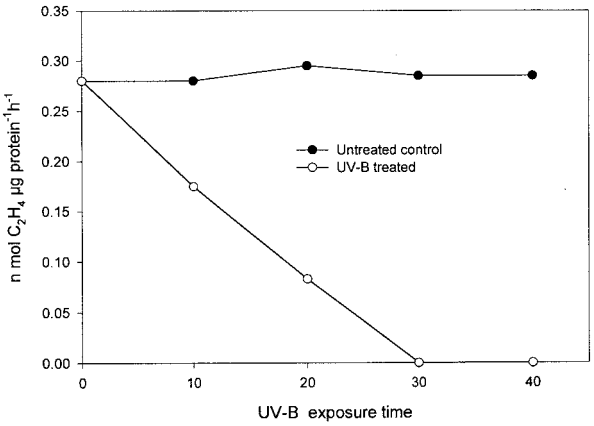

Fig. 2. Effect of UV-B treatment on nitrogenase activity. An actively N_2-fixing culture was exposed to UV-B (2.5 W m^{-2}) for different time periods, and thereafter the C_2H_2 reduction assay was made under fluorescent light. Other conditions as in Fig. 1.

Table 1. Test for the inactivation of nitrogenase activity in different cyanobacteria following exposure of UV-B radiation

Organisms[a]	Time (min) required for the inactivation of nitrogenase activity[b]
Anabaena BT2	30
A. doliolum	30
A. cycadeae	25
Calothrix sp.	40
N. spongiaeforme	30
N. muscorum	25
N. commune	30
Scytonema sp.	40

[a] Equal volumes of cultures having an optical density of 0.25 (at 663 nm) from all the species were exposed to UV-B (2.5 W m^{-2}) as described in Materials and Methods. The acetylene reduction assay was made at intervals of 10 min after UV-B exposure.
[b] Results represent the average of three independent experiments.

are known to protect themselves against UV-B damages by synthesizing UV-B-absorbing substances such as mycosporine-like amino acids (MAAs) [20]. It is pertinent to mention here that marine bacterial isolates also display diverse responses to UV-B radiation [8].

Once it became evident that UV-B treatment does have drastic effect on survival of various diazotrophic cyanobacteria, we became interested in testing its effect on nitrogenase activity. Figure 2 shows that exposure of actively N_2-fixing cultures of *Anabaena* BT2 to UV-B for as little as 5 min elicited some inhibition of nitrogenase activity, which was completely inhibited after 30 min. The inhibition, followed by complete loss of nitrogenase by UV-B treatment, was not at all specific to *Anabaena* BT2; virtually all the strains employed in this study elicited loss of nitrogenase activity (Table 1). Nitrogenase activity in the majority of the strains was completely abolished within 30 min of UV-B treatment; however, the same effect was attained in 40 min in *Calothrix* sp. and *Scytonema* sp. Complete loss of nitrogenase activity seems surprising, because survival of all the test organisms was not affected significantly by 30 min of UV-B treatment.

The virtually instant inhibition induced by UV-B, followed by complete loss of nitrogenase activity, prompted us to seek answers to two questions: a) is the loss of activity due to complete inactivation of nitrogenase polypeptide and/or some conformational changes?; b) do the reductant and ATP limit the activity? To answer the above hypotheses, the cyanobacterium *Anabaena* BT2 was employed, and UV-B-mediated effects were tested in the presence of inhibitors of protein synthesis and photosystem-II.

That nitrogenase is indeed inactivated/denatured is

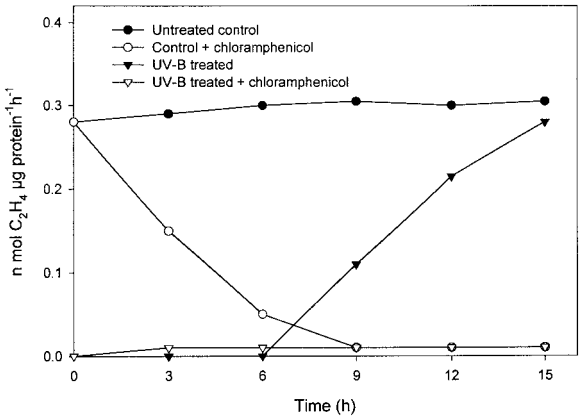

Fig. 3. Restoration of nitrogenase activity in a UV-B-treated culture. An actively N_2-fixing culture was exposed to UV-B (2.5 W m^{-2}) continuously for 30 min so as to attain complete loss of nitrogenase activity. In a UV-B-treated culture, chloramphenicol (25 µg mL^{-1}) was added and then transferred to fluorescent light, and C_2H_2 reduction was measured at different time intervals.

evident from the fact that restoration of C_2H_2 reduction activity upon transfer of *Anabaena* BT2 to fluorescent light does not occur instantly, but after about 6 h (Fig. 3). This suggests that cells either do not contain active nitrogenase or, if some enzyme is present, it does not react with its substrate. The activity detectable after 6 h appears to be due solely to de novo synthesis of the nitrogenase complex (Fig. 3). This is further supported by the data on the effects of chloramphenicol on protein synthesis. Addition of chloramphenicol to UV-B-exposed cells did not restore nitrogenase activity. (Some nitrogenase activity also declined in UV-B unexposed controls treated with chloramphenicol, but complete loss occurred only after 8–9 h.) Thus, if active nitrogenase

Table 2. Reappearance of nitrogenase activity in UV-B-, DCMU-, and dark-pretreated cultures of *Anabaena* BT2

Treatment[a]	Nitrogenase activity (nmol C_2H_4 μg protein^{-1} h^{-1})[b]			
	Time (h)			
	0	1	2	3
Untreated control[c]	0.28	–	–	–
UV-B-treated (30 min)	0.00	0.00	0.00	0.0
UV-B (30 min) + 0.5% glucose	0.00	0.00	0.00	0.04
DCMU pretreatment (6 h)	0.02	0.10	0.22	0.25
Dark preincubation (8 h)	0.00	0.06	0.14	0.24

[a] UV-B exposure for 30 min, DCMU (2×10^{-5} M) pretreatment for 6 h, and dark preincubation for 8 h of *Anabeana* BT2 showed complete loss of nitrogenase activity. Such cultures were used for testing reactivation of nitrogenase activity. Other conditions as in Table 1.

[b] Nitrogenase activity was measured after incubating cultures under fluorescent light at different time intervals as indicated above.

[c] The activity was almost constant up to 3 h in untreated control culture.

was present in UV-B-exposed material, there should have been perceptible but constant activity at least during the initial period of chloramphenicol treatment. Chloramphenicol-treated cultures revived on transfer to inhibitor-free medium, indicating that the concentration of inhibitor used was non-toxic. Delayed appearance of nitrogenase activity also appears to rule out the alternative possibility of conformational change in the enzyme complex.

Nitrogenase requires a continued and abundant supply of suitable reductant and ATP [7, 25] for conversion of N_2 to NH_3. We presumed that UV-B might impair the synthesis and/or supply of the reductant. To test this hypothesis, we treated the cells with DCMU (specific inhibitor of photosystem-II) and a readily utilizable reductant source. Cells treated with DCMU (3-(3, 4-dichlorophenyl)-1, 1-dimethylurea), or incubated in the dark, showed complete loss of nitrogenase activity after 6 and 8 h respectively (Table 2). In neither case was there an instantaneous loss; rather, the activity declined gradually. When such cultures were transferred to light in DCMU-free medium, the activity started to reappear and by 3 h was almost comparable to that in control cultures. In contrast, the UV-B-exposed material failed to restore activity, even with glucose treatment, up to at least 3 h. Failure of activity restoration with glucose may be due to the fact that nitrogenase enzyme is probably not present in the cells. The data suggest that DCMU treatment or dark preincubation does not inactivate a presynthesized nitrogenase complex. Furthermore, loss of reductant and ATP supply does not occur immediately under the above conditions, and the time required for the above loss may differ among different species [9, 12]. These observations suggest that loss of nitrogenase activity after UV-B treatment is not related to the instant impairment of reductant/ATP supply. It is pertinent to mention here that a number of cyanobacteria have been reported to drive nitrogenase activity in the dark or, in the absence of functional PS-II, at the expense of endogenous reductant/ATP for short durations [12]. Furthermore, the activity of other molybdo-enzyme such as nitrate reductase is not lost completely even after 2–3 h of continuous exposure to UV-B [5, 10].

Both transcriptional and post-translational modes of regulation of nitrogenase are known [7]. Inhibition of nitrogenase activity by several factors, especially the utilizable inorganic nitrogen sources, has been reported in a number of N_2-fixing microorganisms [7, 22]. However, these inhibitors do not cause immediate loss of in vivo nitrogenase activity, and inhibition does not occur with the purified enzyme in vitro. Instant and complete inactivation of nitrogenase both under in vivo and in vitro conditions has been demonstrated after exposure to O_2, but only at a concentration higher than the ambient level [6, 22]. Inactivation of nitrogenase by ADP-ribosylation of Fe-protein involving the roles of the NAD^+-dependent enzymes, dinitrogenase reductase ADP-ribosyltransferase (DRAT) and its partner, dinitrogenase reductase-activating glycohydrolase (DRAG) after UV-B treatment does not seem possible, since the inactivation of nitrogenase is not reversible [7]. Thus, inactivation of nitrogenase by UV-B appears to be a novel phenomenon. Our findings allow us to conclude that nitrogenase is very sensitive to UV-B exposure, and inactivation of activity is possibly due to complete damage to the nitrogenase polypeptide. Most probably the amino acids present or the general structure of nitrogenase makes it more susceptible to UV-B radiation. The inhibition of nitrogenase activity by UV-B is by no means specific to the members of cyanobacteria; we have observed a similar effect on two other N_2-fixing bacteria. Thus, our findings suggest that estimation of nitrogenase activity both under laboratory and field conditions may be used as a marker enzyme for assessing the impact of UV-B radiation.

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