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Action of Heavy Metals on Hill Activity and O_2 Evolution in *Anacystis nidulans*[1]

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ABSTRACT

Addition of 5 micromolar Cu^{2+}, Cd^{2+}, and Zn^{2+} was inhibitory to 10 micromolar H_2O_2-supported Hill activity (dichlorophenolindophenol reduction) and O_2 evolution in membrane preparation from *Anacystis nidulans*. The reversal of Cd^{2+} and Zn^{2+} inhibition, in contrast to Cu^{2+}, by exogenously added catalase (EC 1.11.1.6) suggested that the former cations were inhibitory to H_2O_2 degradation. Ascorbic acid (20 micromolar) supported 27% of the Hill activity which was insensitive to DCMU (10 micromolar) and the remaining activity, attributable to the DCMU sensitive process, was sensitive to inhibition by Cu^{2+} only. It is suggestive that the action site of Cd^{2+} and Zn^{2+} is located between the electron donation sites of H_2O_2 and ascorbic acid, while that of Cu^{2+} is located beyond it. Electron donation by reduced glutathione was insensitive to DCMU and Cu^{2+}, indicating that the action site of Cu^{2+} is prior to its electron donation site. Further, the phenanthroline (10 micromolar) reversal of Cu^{2+} inhibition of Hill activity suggested a tentative action site of Cu^{2+} at the level of cytochrome.

Photosynthetic microorganisms including cyanobacteria are highly sensitive to heavy metal ions (17, 21, 27). The prokaryotic cyanobacteria serve as an excellent tool for studying the effect of heavy metals on photosynthetic activity owing to their close similarity with the chloroplast (16). Heavy metal toxicity is attributable to binding of heavy metals to enzymes, resulting in alteration of their catalytic function (9).

Metals like Cu^{2+}, Cd^{2+}, and Zn^{2+} have similar electronic characteristics, but different affinities for biological ligands. They inhibit photosynthetic electron transport in PSII (2, 8, 12). The possibility has been raised that Cd^{2+} and Zn^{2+} may share the same site of action on the oxidizing side of PSII (25, 26)—a site different from the Cu^{2+} inhibition site (19). Studies on heavy metal toxicity to photosynthesis have not precisely elucidated the nature of the photochemical reactions being affected by metals, and various reports on the site and mode of action for Cu^{2+}, Cd^{2+}, and Zn^{2+} remain controversial. The present investigation on the unicellular cyanobacterium *Anacystis nidulans* is an attempt to elucidate the possible sites of action of Cu^{2+}, Cd^{2+}, and Zn^{2+} on the electron transfer process in PSII.

MATERIALS AND METHODS

Organism and Growth Conditions. *Anacystis nidulans* IU 625 (ATCC 27144) was obtained through the courtesy of Dr. R. S. Safferman, U.S. Environmental Protection Agency, Cincinnati, OH. The alga was routinely grown in Allen's (1) medium supplemented with 16.5 mM $NaNO_3$. The cultures were maintained in a culture room at a temperature of 24 ± 1°C and illuminated for 14 h per d with cool daylight fluorescent tubes with an approximate intensity of 8 W m^{-2} on the culture vessel surface.

Membrane Preparation. The cells of *A. nidulans* were disrupted by osmotic shock treatment (20). Exponential phase (5 d old) cells were harvested by centrifugation and the pellet resuspended in Na-phosphate (40 mM, pH 7.0) containing 0.1% (w/v) lysozyme (Sigma) and 0.4 M sucrose. The cell suspension was incubated in light for 2 h (37°C) and then centrifuged (3000*g*, 5 min). The pellet was again suspended in Na-phosphate of low osmotic concentration containing 0.2 M sucrose and 0.2 M NaCl. The unbroken cells and heavier cell wall fragments were removed by centrifugation at low speed (1000*g*, 10 min). The remaining homogenous preparation was used in the present investigation.

Measurement of O_2 Evolution and Hill Acitivity. O_2 evolution was measured with a Clark type O_2 electrode (Century Instruments, India) fitted with a circulating water jacket. The temperature was adjusted to 20°C. The light intensity on the vessel surface was 10 W m^{-2}.

Hill activity was measured as dye reduction by the addition of 100 μM DCPIP[2] as described by Holt and French (14). Light from a projector lamp (500 W) was filtered through a 2.5 cm thick water column at 20°C and focused on a cuvette of 5 ml capacity.

Protein was measured by the method of Lowry *et al.* (18), modified by Herbert *et al.* (13) using lysozyme as standard.

Chemicals. DCPIP, GSH, Phen, DTT, Asc, DCMU, and catalase (EC 1.11.1.6) were obtained from Sigma. $CuCl_2 \cdot 2H_2O$,

Table I. *Effect of H_2O_2, Catalase, Phen, and Fe^{3+} on DCPIP Reduction in the Presence of Cu^{2+}, Cd^{2+}, and Zn^{2+} in a Membrane Preparation of A. nidulans*

Substrate	DCPIP Reduced			
	Control	5 μM Cu^{2+}	5 μM Cd^{2+}	5 μM Zn^{2+}
	nmol/mg protein·min			
None	1.83 (100)[a]	0 (0)	0.34 (18.5)	0.45 (24.5)
H_2O_2, 10 μM	2.37 (100)	0 (0)	1.18 (49.7)	0.94 (39.6)
Catalase, 1 mg/ml	2.10 (100)	0 (0)	1.68 (80)	1.52 (72.3)
Phen, 10 μM[b]	0 (0)	0.76 (42)	0 (0)	0 (0)
Fe^{3+}, 5 μM	2.17 (114)	1.32 (65.2)	1.53 (80.5)	1.70 (89.4)

[a] Percentage Hill activity in comparison to control for each substrate is given in parentheses. [b] In case of Phen and Fe^{3+}, a control (100%) value obtained without addition of Phen and Fe^{3+} is 1.90 nmol DCPIP reduced/mg protein·min.

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[2] Abbreviations: Asc, ascorbic acid; Phen, 1,10-bis-phenanthroline; DCPIP, 2,6-dichlorophenolindophenol.

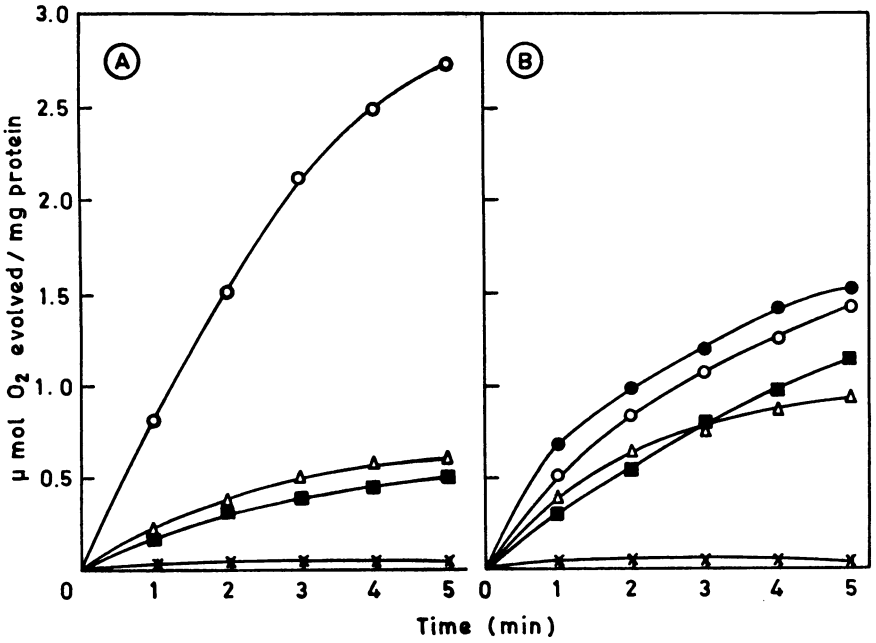

FIG. 1. A, Oxygen evolution with 10 μM H_2O_2 added to membrane preparations of *A. nidulans* as control (○) and in the presence of 5 μM of Cu^{2+} (×), Cd^{2+} (■) and Zn^{2+} (△). B, O_2 evolution without H_2O_2 with membrane preparations of *A. nidulans* in the absence (○) and presence (●) of catalase (1 mg/ml), catalase + Cu^{2+} (×), catalase + Cd^{2+} (■) and catalse + Zn^{2+} (△).

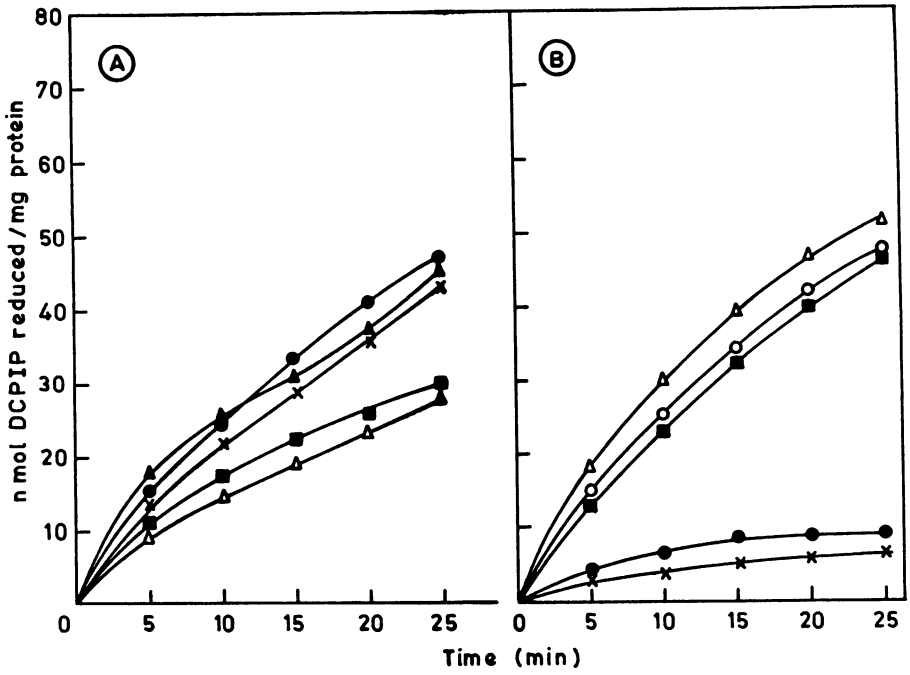

FIG. 2. A, Rate of 20 μM GSH supported DCPIP reduction in the presence of GSH alone (●), GSH + 10 μM DCMU (▲), GSH + Cu^{2+} (×), GSH + Cd^{2+} (■) and GSH + Zn^{2+} (△). B, Rate of DCPIP reduction in the presence of 20 μM Asc alone (○), Asc + 10 μM DCMU (●), Asc + Cu^{2+} (×), Asc + Cd^{2+} (■), and Asc + Zn^{2+} (△).

$CdCl_2 \cdot 2H_2O$, $ZnCl_2$, $FeCl_3 \cdot 6H_2O$ and H_2O_2 were obtained from British Drug House, India. DCMU was dissolved in ethanol in such a way, so that the final concentration of ethanol in the assay mixture did not exceed 0.1%.

Equimolar concentrations (5 μM) of Cu^{2+}, Cd^{2+}, and Zn^{2+} were used throughout the present investigation.

RESULTS

Effect of Cu^{2+}, Cd^{2+}, and Zn^{2+} on Hill Activity and O_2 Evolution. O_2 evolution was completely inhibited by Cd^{2+} and Zn^{2+} (data not shown), while Hill activity was inhibited to a level of 81.5 and 75.5%, respectively (Table I). The same level of Cu^{2+} completely abolished Hill activity as well as O_2 evolution. The apparent difference in the data on Hill activity and O_2 evolution in the presence of Cd^{2+} and Zn^{2+} may be due to the enhanced rate of O_2 consumption favored by such cations (4), and not to a complete supression of O_2 evolution.

Effect of H_2O_2 and Catalase. Partial reactions of the Hill activity were used to delineate the sites of action of Cu^{2+}, Cd^{2+}, and Zn^{2+}. H_2O_2 is a DCMU sensitive electron donor to PSII (22). The addition of 10 μM H_2O_2 to the membrane preparation resulted in a high rate of dye reduction and O_2 evolution (Table I and Fig. 1A). In the presence of Cd^{2+} and Zn^{2+}, the H_2O_2-mediated Hill activity was inhibited by 50.3 and 60.4%, respectively, in comparison to control. However, the addition of Cu^{2+} was lethal to the H_2O_2-supported Hill activity as well as to the O_2 evolution process. Thus, the results indicated that all the cations are inhibitory to the H_2O_2-supported Hill activity and to O_2 evolution; but the degree of inhibition differs for each cation.

Addition of catalase (1 mg/ml) to the membrane preparation alleviated the Cd^{2+} and Zn^{2+} induced inhibition of Hill activity

(80 and 72.3%, respectively) and O_2 evolution (50 and 60%, respectively) with respect to the control (Table I and Fig. 1B). In contrast, the addition of catalase did not alter the Cu^{2+} induced inhibition. It may be suggested that Cd^{2+} and Zn^{2+} are inhibitory to the H_2O_2 degradation process, while the inhibition by Cu^{2+} occurred at a different site.

Effect of GSH and Asc. GSH is an electron donor (10). Its biological importance is presumed to be due to its ability to bind with metals (24). The DCPIP reduction (Fig. 2A) by 20 μM GSH was insensitive to the addition of 10 μM DCMU. Addition of Cd^{2+} and Zn^{2+} was not very inhibitory (26.7 and 40%, respectively) to the GSH-mediated dye reduction, while Cu^{2+} showed no inhibition. Similar results have been obtained with DTT (data not shown). Since electron donation by GSH was insensitive to the addition of DCMU and Cu^{2+}, it may be suggested that GSH donates electrons beyond both the DCMU (6) and Cu^{2+} sites of inhibition.

Asc is an electron donor to PSII (3). When DCMU was used to inhibit PSII, Asc donated electron around the plastocyanin level (11). Our results indicated that 27% of the 20 μM Asc supported DCPIP reduction was insensitive to DCMU inhibition and that the remaining 73% of the activity may be attributed to a DCMU sensitive process (Fig. 2B). The Asc + DCPIP reduction (without DCMU) was least affected by Cd^{2+} and Zn^{2+}, while Cu^{2+} showed 80% inhibition compared to the control. Thus, the results suggested that the site of Cd^{2+} and Zn^{2+} action may be located prior to the Asc electron donation site.

Effect of Phen and Fe^{3+}. Phen is an iron chelator and inhibitor of H^+ exchange in Cyt (5). Phen (10 μM) + Cd^{2+} and Phen + Zn^{2+} were fully inhibitory to Hill activity (Table I), while Phen + Cu^{2+} exhibited 42% activity in comparison to control. Thus, the reversal of Cu^{2+} induced inhibition in presence of Phen indicated a tentative site of Cu^{2+} action at the level of Cyt.

Interaction of 5 μM Fe^{3+} with Cu^{2+}, Cd^{2+}, and Zn^{2+} resulted in the reduced toxicity of Cu^{2+}, Cd^{2+}, and Zn^{2+} (65.2, 80.5, and 89.4%, respectively) in comparison to control. These results point toward a competition between iron and other metals at the level of their cellular binding sites.

DISCUSSION

The results on H_2O_2 and Asc-supported Hill activity in presence of Cu^{2+}, Cd^{2+}, and Zn^{2+}, and a reversal of Cd^{2+} and Zn^{2+} induced inhibition by catalase (7) suggested that the latter two cations are inhibitory to H_2O_2 degradation process and their site of action lies between the electron donation sites of H_2O_2 and Asc (DCMU sensitive), while the Cu^{2+} action site seems to be located beyond the Asc donation site. Thus, our results agree with the suggestions of De Filippis *et al.* (8) that Cd^{2+} and Zn^{2+} may share the same site of action on the oxidizing side of PSII.

Since the GSH + DCPIP reduction was insensitive to DCMU and Cu^{2+}, it may be suggested that GSH donates electrons beyond the Cu^{2+} and DCMU sites of inhibition. However, the inhibitory action of Cd^{2+} and Zn^{2+} with respect to GSH and H_2O_2 supported DCPIP reduction calls for a close relation between H_2O_2 degradation and GSH electron donation processes as suggested by Kalt-Torres *et al.* (15) and Tel-or *et al.* (23). The specific action of Phen (an inhibitor of Cyt) with Cu^{2+} tentatively suggested that copper acts at the level of Cyt. Our results show some similarity with those of Shioi *et al.* (19) showing that Cu^{2+} acts beyond the DCMU sensitive site, but it may be invariably prior to the electron donation site of GSH. However, at present, we do not rule out other effects of these cations on photosynthetic activity.

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