Inhibition of δ-aminolevulinic acid dehydratase activity by cadmium in excised etiolated maize leaf segments during greening

J. Sarangthem, M. Jain, R. Gadre

School of Biochemistry, Devi Ahilya University, Takshashila Campus, Indore, India

ABSTRACT

Supply of 0.1–0.5 mmol $CdCl_2$ inhibited δ-aminolevulinic acid dehydratase (EC 4.2.1.24, ALAD) activity and total chlorophylls in excised etiolated segments of maize leaves during greening. Due to cadmium supply δ-aminolevulinic acid (ALA) content was reduced significantly at 0.5 mmol Cd only. Also the Cd treatment decreased the protein content and accumulated significantly the Cd in the tissue. Significant correlation between Cd accumulation in the leaves and various parameters measured is observed, with the *R*-squared values being 0.727 with ALAD activity, 0.885 with ALA content, 0.902 with total chlorophylls and 1.00 with proteins. The % inhibition of ALAD activity by Cd was decreased in the presence of nitrogenous compounds, glutamine and NH_4NO_3 and the observed inhibition was 25% and 16%, respectively. More substantial reduction in % inhibition of enzyme activity by Cd was observed during treatment with glutathione, a ubiquitous thiol and levulinic acid, a competitive inhibitor of ALAD, with the inhibition being only 2% and 4%, respectively. Supply of some essential metal ions, such as Mg, Zn, and Mn, also reduced the % inhibition of enzyme activity by Cd. Inclusion of varying concentrations of ALA during assay also affected the % inhibition of enzyme activity by Cd showing an increased inhibition from 17% to 53% with increasing ALA concentration. It is suggested that Cd inhibits ALAD activity by affecting the ALA binding to the enzyme and/or disrupting thiol interaction.

Keywords: pigment biosynthesis; plant tetrapyrrole metabolism; *Zea mays*; toxic elements, metallic stress

Trace metals are important environmental pollutants and their toxicity is a problem of increasing significance for ecological, evolutionary, nutritional and environmental reasons (Benavides et al. 2005). Cadmium is a common environmental contaminant introduced into the soil through anthropogenic activities. It is readily taken up and accumulated by plants despite of being non-essential element, and it increases the potential for contamination of the food chain (Prasad 1995). The toxic effects of Cd on plant growth and metabolism are well documented (Sanita di Toppi and Gabbrielli 1999).

Chlorophylls are often measured to assess the impact of enviromental pollutants, as the pigment content is correlated to visual symptoms and also photosynthetic plant productivity. Decrease in chlorophyll content in response to Cd supply was reported in several systems (Parekh et al. 1990, Al-Hakimi 2007, Jain et al. 2007). Chlorophyll biosynthesis is a vital physiological process of the green plants, which is regulated at several steps (Beale 1999). Earlier steps of the pathway are common with the biosynthesis of other tetrapyrrole derivatives like heme, phytochromes, phycobilins etc. The universal precursor, ALA (δ-aminolevulinic acid), is synthesized by the two pathways, the Shemin pathway involving glycine and succinyl Co-A and the Beale pathway, involving the C-5 compounds, like glutamate and 2-oxoglutarate by an ALA synthesizing system in plants (Beale 1999). The enzyme, ALAD (δ-aminolevulinic acid dehydratase), one of the regulatory enzymes of the pathway, catalyzes the asymmetric condensation of two molecules of ALA leading to the formation of the basic unit of tetrapyrroles, the porphobilinogen. ALADs from different sources are metalloenzymes that utilize a variety of divalent and monovalent cations (Jaffe 2000). The plant enzyme requires Mg^{2+} for enzymatic activity. The present study has been carried out to elucidate the mechanism of inhibition of ALAD activity by cadmium, a metallic toxicant.

MATERIAL AND METHODS

Plant material. Seeds of *Zea mays* L., cv. Ganga safed-2 were surface sterilized with 0.1% $HgCl_2$ for 1–2 min and then washed thoroughly with distilled water. Seedlings were raised in small plastic pots containing acid washed sand in continuous dark for 7–8 days at 25 ± 3°C. They were watered on alternate days with half strength Hoagland's solution, which was modified to exclude nitrogen. For various treatments, primary leaves from uniformly grown seedlings were cut into about 0.5 × 0.5 cm^2 pieces and floated on 1/4th strength Hoagland's solution (pH 6.0) containing the desired compounds for 24 h in continuous light of intensity about 40 W/m at 25 ± 2°C.

Assay procedure. ALAD activity was assayed colorimetrically by estimating the amount of PBG (porphobilinogen) formed by using modified Ehrlich reagent. Extraction and assay of ALAD was carried out according to the procedure described in Jain and Gadre 2004. One unit of enzyme activity is defined as 1 nmol of PBG formed per hour. The ALA content was determined according to the method of Tewari and Tripathy 1998. For total chlorophyll estimation, the absorbance of the chlorophylls extracted in 80% acetone was measured at 663 and 645 nm and the content was calculated by using extinction coefficients according to the method mentioned in Jain et al. (2007). Protein content was estimated by the method of Lowry et al. 1951. Cadmium level of the treated material was estimated by using an atomic absorption spectrophotometer (Perkin Elmer 5100 PC, Waltham, USA) after digestion of the dried material using a mixture of conc. HNO_3 and 60% perchloric acid in a ratio of 3:1 (Pandey and Sharma 2002).

Data analysis. Data presented in the paper are average of at least four independent experiments with ± S.E. Significance of difference obtained for various treatments was tested by the Student's *t*-test.

RESULTS AND DISCUSSION

Supply of 0.1–0.5 mmol $CdCl_2$ to excised maize leaf segments during greening inhibited total chlorophylls and ALAD activity in a concentration-dependent manner showing increased inhibition with increasing concentration (Table 1A). Cadmium supply decreased the ALA content prominantly at 0.5 mmol and slightly at 0.2 mmol, but remained unaffected at 0.1 mmol (Table 1A). Cadmium treatment reduced the protein content gradually with increasing concentration, however, substantial Cd accumulation in the range of 750–2238 fold resulted with the supply of 0.1 to 0.5 mmol $CdCl_2$ (Table 1B). Correlation analysis between Cd accumulation and ALAD activity (Figure 1a), Cd accumulation and ALA content (Figure 1b), Cd accumulation and total chlorophylls (Figure 1c) and Cd accumulation and proteins (Figure 1d) was performed by using Microsoft Excel chart type XY scatter. The *R*-squared values for Cd accumulation with these parameters were highly significant being 0.727 with ALAD activity, 0.885 with ALA content, 0.902 with total chlorophylls and 1.00 with proteins.

Incubation of greening leaf segments with 5 mmol glutamine, 5 mmol NH_4NO_3 and 5 mmol GSH (glutathione) increased the ALAD activity substantially in the presence of Cd, while in the absence of Cd it was marginally reduced (Table 2). Thus, the % inhibition of enzyme activity by Cd is significantly reduced in all the treatments compared to control with the effect being the most prominent in case of GSH supply. With the supply of 2 mmol LA (levulinic acid), % inhibition of ALAD activity by Cd was substantially reduced due to significant inhibition of activity in the absence of Cd only (Table 2).

Effect of varying concentrations of ALA on the ALAD activity of enzyme preparations obtained from leaf segments treated without cadmium (–Cd enzyme) and with 0.5 mmol cadmium (+Cd enzyme) was analysed. Increasing concentration of

Table 1A. Effect of Cd on ALAD activity, ALA content and total chlorophylls in excised etiolated maize leaf segments during greening. Leaf segments from dark-grown maize seedlings were treated with 1/4th strength Hoagland's solution containing the desired concentration of $CdCl_2$ in continuous light for 24 h at 25 ± 2°C

$CdCl_2$, Conc. (mmol)	ALAD activity (units/g FW)	ALA content (nmol ALA/g FW)	Total chlorophylls (µg/g FW)
0.0	177 ± 6 (100)	175 ± 30 (100)	355 ± 23 (100)
0.1	121 ± 5*** (68)	178 ± 11 (102)	294 ± 35 (83)
0.2	97 ± 5 *** (55)	161 ± 38 (92)	222 ± 19*** (63)
0.5	83 ± 5*** (47)	66 ± 3** (38)	148 ± 10*** (42)

Values relative to control are given in parentheses. $P < 0.05$*; $P < 0.01$**; $P < 0.001$***

Table 1B. Effect of Cd on protein content and Cd accumulation in excised etiolated maize leaf segments during greening. Leaf segments from dark-grown maize seedlings were treated with 1/4th strength Hoagland's solution containing the desired concentration of $CdCl_2$ in continuous light for 24 h at 25 ± 2°C

$CdCl_2$ Conc. (mmol)	Protein content (mg/g FW)	Cd accumulation (mg/g DW)
0.0	8.1 ± 1.0 (100)	0.08 ± 0.002 (100)
0.1	7.5 ± 1.2 (93)	0.60 ± 0.02*** (750)
0.2	7.4 ± 1.1 (91)	0.72 ± 0.11** (900)
0.5	6.2 ± 1.0 (77)	1.79 ± 0.08*** (2238)

Values relative to control are given in parentheses. $P < 0.05$*; $P < 0.01$**; $P < 0.001$***

ALA increased the enzyme activity with both –Cd as well as +Cd enzymes, but to a lesser extent in the latter (Table 3). Hence, observed inhibition of enzyme activity by Cd increased with increasing concentration of ALA.

Treatment with 1 mmol $MgCl_2$ and 1 mmol $ZnCl_2$ increased the enzyme activity in the presence of Cd only (Table 4). However, supply of 1 mmol $MnCl_2$ caused an increase in enzyme activity in the absence as well as presence of Cd with the increase being more substantial for the latter (Table 4). Thus, the % inhibition of enzyme activity by Cd is reduced in presence of all the metals used.

The results demonstrate a concentration-dependent inhibition of ALAD activity by Cd accumulation at 0.6 to 1.79 mg/g DW which corresponds to the supply of 0.1 to 0.5 mmol $CdCl_2$ in excised etiolated maize leaf segments during greening; however, ALA content decreased significantly at 1.79 mg Cd/g dry leaves (0.5 mmol $CdCl_2$) only (Table 1A). Thus, ALAD activity seems to be more sensitive than ALA accumulation up to 0.72 mg Cd/g DW (0.2 mmol $CdCl_2$), while at 1.79 mg Cd/g DW (0.5 mmol $CdCl_2$) it is less sensitive than ALA accumulation. Severe inhibition of ALAD activity by Cd along with accumulation of ALA was reported in leaves, roots and nodules of soybean plants and the ALA accumulation was suggested to be due to Cd induced oxidative stress (Noriega et al. 2007). In our study, a decrease in ALAD

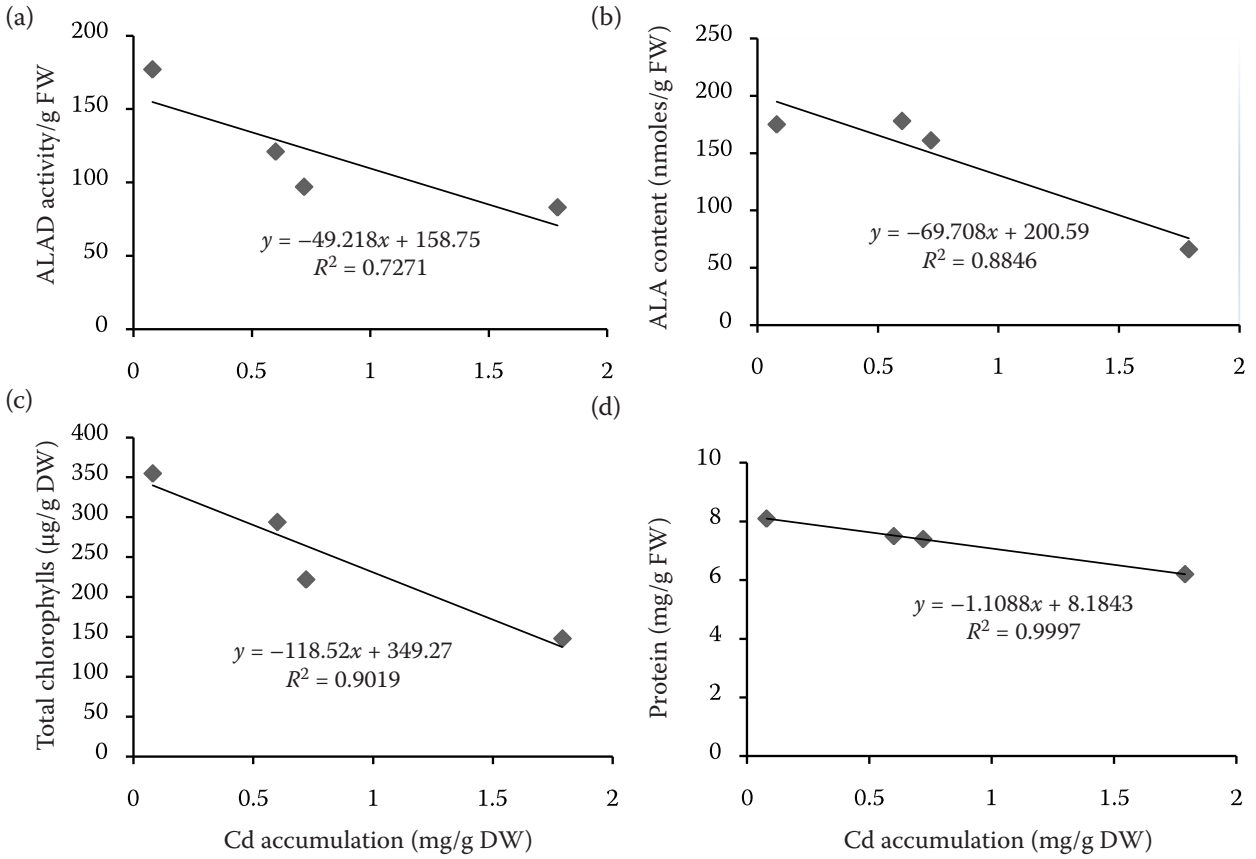

Figure 1. Microsoft Excel chart type XY (scatter) for Cd accumulation with (a) ALAD activity, (b) ALA content, (c) total chlorophylls and (d) protein

Table 2. Effect of metabolites/inhibitor on inhibition of ALAD activity by Cd in greening maize leaf segments. Leaf segments from dark-grown maize seedlings were treated with 1/4th strength Hoagland's solution containing the desired compounds either in the absence or presence of 0.5 mmol $CdCl_2$ in continuous light for 24 h at 25 ± 2°C

Metabolites/inhibitor	ALAD activity (units/g FW)		% Inhibition
	–Cd	+Cd	
None	177 ± 6 (100)	83 ± 5*** (100)	53
Glutamine (5 mmol)	162 ± 6 (92)	121 ± 4*** (146)	25
NH_4NO_3 (5 mmol)	161 ± 5 (91)	135 ± 7** (163)	16
GSH (5 mmol)	162 ± 19 (92)	159 ± 19 (191)	2
LA (2 mmol)	93 ± 2 (53)	86 ± 2* (104)	4

Values relative to control are given in parentheses. $P < 0.05$*; $P < 0.01$**; $P < 0.001$***

activity due to Cd supply correlates more with the decrease in chlorophyll content (Table 1A). Hence, it is likely that during Cd supply ALAD activity controls the overall effect on chlorophyll formation. Moreover, no significant effect of Cd on protein content in spite of the accumulation of Cd in the tissue suggests that the effect of Cd on ALAD activity does not appear to be related to general metabolic activities of the system. In radish leaves inhibition of ALAD activity, reduced growth, DNA content and protein content by Cd was also observed with marked accumulation of Cd (Morsch et al. 2002). Strong correlation between Cd accumulation in the leaves and various parameters measured with the *R*-squared values being 0.727 with ALAD activity, 0.885 with ALA content, 0.902 with total chlorophylls and 1.00 with proteins (Figures 1a, b, c and d, respectively) suggests that Cd stress affecting the chlorophyll metabolism is caused by accumulation of Cd in the leaves. In spinach also the contents of toxic elements, such as Cd affecting plant metabolism upon exposure to sewage sludge were reported (Pavlíková et al. 2002).

Effects of various metabolites, such as glutamine, a key organic nitrogenous compound regulating overall nitrogen metabolism, NH_4NO_3, an inorganic nitrogenous source and GSH, a ubiquitous thiol, were observed in relation to inhibition of ALAD activity by Cd. With all these metabolites, the % inhibition of enzyme activity by Cd was reduced (Table 2) showing counteracting effects with Cd. Glutamine may overcome Cd inhibition of ALAD by complexation with it. Glutamine content in the tomato leaves was found to be increased upon treatment with Cd (Chaffei et al. 2004). Moreover, glutamate and 2-oxoglutarate being the precursors of ALA in higher plants, it is likely that glutamine supply even under the condition of Cd toxicity maintains the supply of C_5 compounds for ALA formation. ALA synthesis from glutamate involves a three-step reaction including the ligation of glutamate to $tRNA^{Glu}$ catalysed by glutamyl tRNA synthetase, the reduction of glutamate to glutamate-1-semialdehyde by glutamyl tRNA reductase and a final transamination step mediated by glutamate-1-semialdehyde aminotransferase (Kannangara et al. 1988). Alternatively, the glu-

Table 3. Effect of varying concentrations of ALA on ALAD activity of the enzyme preparations obtained from greening maize leaf segments treated without cadmium (–Cd enzyme) or with 0.5 mmol cadmium (+ Cd enzyme). Leaf segments from dark-grown maize seedlings were treated with 1/4th strength Hoagland's solution either in the absence or presence of 0.5 mmol $CdCl_2$ in continuous light for 24 h at 25 ± 2°C. ALAD activity of the enzyme preparations (–Cd enzyme and +Cd enzyme) were assayed using desired concentrations of ALA

ALA conc. (µmol)	ALAD activity (units/g FW)		% Inhibition
	–Cd	+Cd	
66	23 ± 2 (100)	19 ± 2 (100)	17
133	54 ± 4 (235)	45 ± 3 (237)	17
200	82 ± 5 (356)	54 ± 3*** (284)	34
400	100 ± 8 (435)	66 ± 5** (347)	34
600	177 ± 6 (769)	83 ± 5*** (437)	53

Values relative to control are given in parentheses. $P < 0.05$*; < 0.01**; < 0.001***

Table 4. Effect of some metal ions on inhibition of ALAD activity by Cd in greening maize leaf segments. Leaf segments from dark-grown maize seedlings were treated with 1/4th strength Hogland's solution containing the desired metal either in the absence or presence of 0.5 mmol $CdCl_2$ in continuous light for 24 h at 25 ± 2°C

Metal ions	ALAD activity (units/g FW)		% Inhibition
	–Cd	+Cd	
None	177 ± 6 (100)	83 ± 5*** (100)	53
$MgCl_2$ (1 mmol)	173 ± 13 (98)	119 ± 8** (143)	31
$ZnCl_2$ (1 mmol)	169 ± 9 (95)	112 ± 8*** (135)	34
$MnCl_2$ (1 mmol)	246 ± 12 (143)	173 ± 11*** (208)	30

Values relative to control are given in parentheses. $P < 0.05$*; $P < 0.01$**; $P < 0.001$***

tamate may also be required for proline synthesis and the activity of the regulatory enzyme, glutamate kinase was reported to be inhibited by Cd (Pavlíková et al. 2007). Proline accumulation during Cd toxicity was observed in Sharma et al. 1998. Another key metabolite, GSH, exerted a strong protective effect against inhibition of ALAD activity by Cd (Table 2). Thus, it is likely that GSH protects reactive – SH groups of enzyme blocked by Cd. Alternately, GSH being the precursor to metal binding peptides, phytochelatins, involved in metal detoxification prevents Cd to cause enzyme inhibition. Involvement of phytochelatins in metal detoxification was reported in some plant systems (Scheller et al. 1987, Cobbett 2000, Thangavel et al. 2007). Further, in spinach after sludge treatment leading to Cd accumulation, majority of Cd was contained in the fraction with oligopeptides, mainly phytochelatins (Pavlíková et al. 2002). Inorganic nitrogenous source, particularly NO_3^- may protect the enzyme against Cd inhibition by increasing vacuolar mobilization of Cd. Vacuoles were suggested to be the sites for accumulation of heavy metals (Rauser and Ackerly 1987, Heuillet et al. 1989). Moreover, in wheat seedlings the uptake of nitrate was found to be inhibited by adding Cd to the nutrient medium (Veselov et al. 2003).

Analysis involving metal ions to mediate Cd effect on ALAD activity revealed that the divalent ions, such as Mn, Mg, and Zn, increased the ALAD activity in the presence of Cd (Table 4) and thereby reducing the sensitivity of the enzyme towards Cd. Hence, it is likely that these ions reduce Cd uptake to overcome the inhibition. Decreased Cd uptake and accumulation in plants by Zn application were reported (Oliver et al. 1997, Koleli et al. 2004). Further, competitive interaction between Zn and Cd for uptake to show existence of a common system on plasma membrane was observed (Hart et al. 2002).

ALAD is a substrate-modulated enzyme (Tchuinmogne et al. 1989). In the present study, the % inhibition of ALAD activity by Cd is strongly reduced by LA, a competitive inhibitor of the enzyme, due to significant inhibition of activity in the absence of Cd only (Table 2). This suggests that Cd affects LA binding to the enzyme and hence also its substrate ALA. Further, observed increase in % inhibition of ALAD activity by Cd with increasing concentration of ALA in the present study (Table 3) demonstrates that Cd affects ALA binding to the enzyme. ALADs from different sources are metalloenzymes that utilize a variety of divalent and monovalent cations (Jaffe 2000). Metal chelators, such as EDTA, or divalent heavy metal ions inhibit the enzyme activity (Gibbs et al. 1985). In the present study, reduced sensitivity of the enzyme towards Cd in the presence of the divalent ions, such as Mn, Mg, and Zn (Table 4) suggested that Cd binding to enzyme causing inhibition is checked by these metal ions. ALAD was found to contain binding determinants for allosteric Mg (Jaffe 2003). Maximal binding of three Mg (II) per subunit was shown, two being functionally distinct type and the third being non-physiological and inhibitory (Kervinen et al. 2000).

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Corresponding author:

Prof. Rekha Gadre, Ph.D., Devi Ahilya University, School of Biochemistry, Takshashila Campus, Khandwa Road, Indore – 452017, India
phone: + 91 731 2460 276, e-mail: gdr_rkh@yahoo.co.in