Plant Physiol. (1988) 88, 1407–1410
0032-0889/88/88/1407/04/$01.00/0

Regulation of Assimilatory Sulfate Reduction by Cadmium in *Zea mays* L.[1]

Received for publication February 11, 1988 and in revised form June 20, 1988

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ABSTRACT

Plants cultivated with Cd can produce large amounts of phytochelatins. Since these compounds contain much cysteine, these plants should have an increased rate of assimilatory sulfate reduction, the biosynthetic pathway leading to cysteine. To test this prediction, the effect of Cd on growth, sulfate assimilation *in vivo* and extractable activity of two enzymes of sulfate reduction, ATP-sulfurylase (EC 2.7.7.4) and adenosine 5′-phosphosulfate sulfotransferase were measured in maize (*Zea mays* L.) seedlings. For comparison, nitrate reductase activity was determined. In 9-day-old cultures, the increase in fresh and dry weight was significantly inhibited by 50 micromolar and more Cd in the roots and by 100 and 200 micromolar in the shoots. Seedlings cultivated with 50 micromolar Cd for 5 days incorporated more label from $^{35}SO_4^{2-}$ into higher molecular weight compounds than did controls, indicating that the predicted increase in the rate of assimilatory sulfate reduction took place. Consistent with this finding, an increased level of the extractable activity of both ATP-sulfurylase and adenosine 5′-phosphosulfate sulfotransferase was measured in the roots of these plants at 50 micromolar Cd and at higher concentrations. This effect was reversible after removal of Cd from the nutrient solution. In the leaves, a significant positive effect of Cd was detected at 5 micromolar for ATP-sulfurylase and at 5 and 20 micromolar for adenosine 5′-phosphosulfate sulfotransferase. At higher Cd concentrations, both enzyme activities were at levels below the control. Nitrate reductase (EC 1.6.6.1) activity decreased at 50 micromolar or more Cd in the roots and was similarly affected as ATP-sulfurylase activity in the primary leaves.

In 1957 a cysteine-rich, Cd-binding protein was isolated from equine kidney (10) and subsequently called metallothionein. In higher plants the inducible Cd-binding compounds showed properties distinctly different from metallothioneins: in Ouchterlony two-dimensional immunodiffusion tests, no cross-reaction could be observed (7), and attempts to establish the primary structure by Edman degradation failed (5). Similarities were a high cysteine content, $A_{254}:A_{280} > 1$, and comparable circular dichroism spectra (5), indicating the lack of aromatic residues and the possible binding of heavy metals by mercaptide complexes.

The sequence of a Cd-binding peptide from suspension cultures of *Rauwolfia serpentina* was established as (γ-glutamic acid-cysteine)$_n$-glycine (n = 3–7), and the name phytochelatin was proposed for this class of natural products (5). Because of the repetitive γ-glutamic acid bonds, they cannot be regarded as primary gene products. Their primary structure is consistent with what Kondo *et al.* (9) reported as Cd-binding complex from fission yeast, which they called cadystin A (n = 3) and B (n = 2). An amino acid composition of Cd-binding peptides consistent with the structure of phytochelatins was reported for several plant species (1, 6, 12, 14).

The extraordinary high cysteine content of the phytochelatins together with our previous findings about the regulation of assimilatory sulfate reduction (3, 8, 19, 21) led us to predict that plants growing in the presence of Cd should contain increased levels of APSSTase[2] activity. We therefore monitored the activity of this enzyme and of ATP-sulfurylase in Cd treated plants. ATP-sulfurylase is the first enzyme of sulfate reduction, synthesizing APS from ATP and SO_4^{2-}. APSSTase catalyzes the transfer of the sulfate activated in APS to a carrier (Car-SH), thus forming Car-S-SO_3^- (15, 17). Since the regulation of assimilatory sulfate and nitrate reduction is interconnected (8, 13), we also included nitrate reductase into our study.

MATERIALS AND METHODS

Maize kernels (*Zea mays* L. cv LG 11, Limagrain, CHAPPES-63360, Gerzat, France) were soaked for 2 d in aerated water at 4°C. After subsequent germination on damp paper in the dark at 25°C for 36 h, the seedlings were transferred to nutrient solution (21) with or without $CdCl_2$. There were 25 plants in each pot containing 400 mL of medium. They were kept in continuous light (1 Philips TL 40W/33 and 2 Philips TL 40W/32, 30 $\mu Em^{-2} s^{-1}$) at 21 ± 1°C and 80 ± 3% RH.

Preparation of Extracts. Extracts were prepared either from 5 cm long root tips of 20 plants or from five primary leaves randomly chosen from these plants. The root tips were extensively washed with deionized water. One part of plant material was ground in a glass homogenizer with 5 or 10 parts (w/v) buffer (0.1 M Tris-HCl [pH 8] containing 0.1 M KCl, 2 mM $MgCl_2$, 10 mM DTE) for roots and leaves, respectively. After filtration through four layers of 100% viscose fleece (Milette, Migros, Switzerland), the filtrate was immediately used for enzyme assays.

Enzyme Assays. Nitrate reductase (EC 1.6.6.1) activity was measured following the method of Neyra and Hageman (11) but omitting EDTA. The assay mixture contained 25 mM potassium phosphate (pH 7.5), 3.5 mM KNO_3, 0.15 mM NADH, and extract in a final volume of 1 mL. The reaction was stopped by the addition of 0.125 M Zn-acetate. ATP-sulfurylase (EC 2.7.7.4) activity was measured in the back reaction, determining the ATP produced from APS and PPi with a luciferin-luciferase system (18) on a Lumac/3M Biocounter M 2010 (Lumac, Basel, Switzerland). Adenosine 5′-phosphosulfate sulfotransferase activity was measured as the production of ^{35}S-sulfite, assayed as acid volatile radioactivity from AP^{35}S (specific activity: 45–70 Ci/

[1] Supported in part by the Swiss National Science Foundation.

[2] Abbreviations: APSSTase, adenosine 5′-phosphosulfate sulfotransferase; APS, adenosine 5′-phosphosulfate; DW, dry weight; FW, fresh weight; NR, nitrate reductase.

mol) in the presence of DTE as previously described (3, 16). The incubation mixture was 100 mM Tris-HCl (pH 9) containing 4 mM DTE, 0.8 M $MgSO_4$, 75 μM $AP^{35}S$, 200 mM Na_2SO_3, and plant extract in a total volume of 500 μL.

Cd was routinely measured by atomic absorption spectrometry. Nutrient solutions were pretreated with 10 μL per mL 36% (w/w) HCl, plant extracts with 40 μL 30% (w/w) H_2O_2, and 40 μL 97% H_2SO_4 (w/w) per ml.

Determination of Assimilation of $^{35}SO_4^{2-}$ *in Vivo*. Maize seedlings were cultivated for 5 d on nutrient solution (21) with or without 50 μM $CdCl_2$ and with carrier-free $^{35}SO_4^{2-}$ (0.25 μCi·mL^{-1}) in addition to SO_4^{2-} in the medium. The plant material was frozen in liquid nitrogen and ground to a fine powder with mortar and pestle under a stream of argon. The powder was suspended in 100 mM Tris-HCl (pH 8.0), saturated with argon at room temperature and containing 10 mM Na-ascorbate. Two mL of buffer solution were added per g FW. The suspension was filtered as described above and centrifuged under argon for 10 min at 20,000 *g* and 4°C. Three mL of the supernatant fluid were applied to a Sephadex G-25 column (1.6 × 60 cm), equilibrated at 4°C with 10 mM Tris-HCl (pH 8.6) saturated with argon, and containing 10 mM Na-ascorbate. The equilibration buffer was used for elution at 4°C and a flow rate of 9 mL h^{-1} in an atmosphere of argon. Fractions of 3 mL were collected and the radioactivity of each fraction was determined. Cd was measured with an ion specific electrode (Metrohm, Herisau, Switzerland). The protein content of the extracts was measured according to Bradford (2) with BSA as a standard.

Chemicals. Na_2SO_3, $Na_4P_2O_7 \cdot 10\ H_2O$, and $MgCl_2 \cdot 6\ H_2O$ were from Merck, Darmstadt (Germany), ATP and APS from Sigma, St. Louis, MO (U.S.A.) Luciferin-luciferase was the Lumit kit from 3M, St. Paul, MN (U.S.A.). All other chemicals were purchased from Fluka, Buchs (Switzerland). $AP^{35}S$ was prepared according to Tsang *et al.* (20) with $^{35}SO_4^{2-}$ from the Radiochemical Centre, Amersham (U.K.).

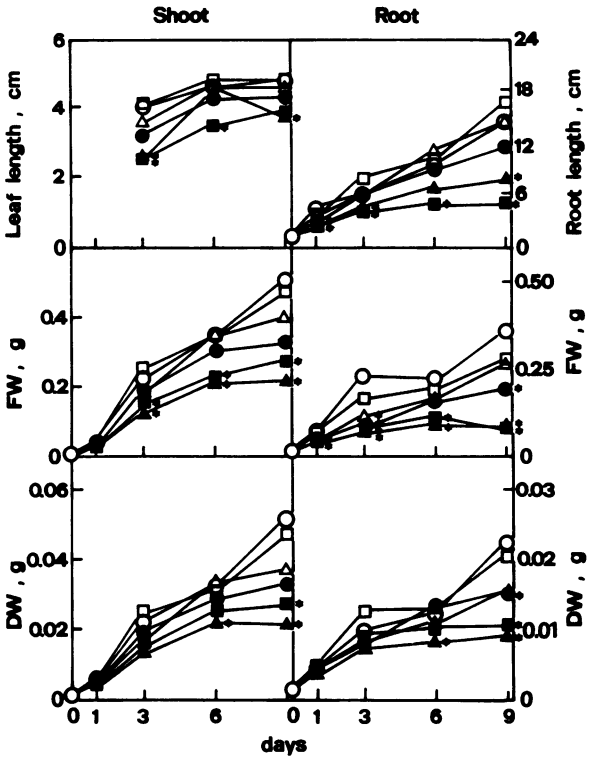

FIG. 1. Changes in FW and DW of roots and shoots and in length of roots and primary leaves of maize seedlings cultivated with 0 (○), 0.5 (△), 5 (□), 50 (●), 100 (▲), and 200 (■) μM $CdCl_2$. Mean values from six plants are presented. Values followed by asterisk are different from the control at $P \leq 0.01$.

RESULTS

Plants exposed to 50 μM and more Cd for 9 d showed significant growth reduction of the roots on a FW as well as a DW basis (Fig. 1). These roots turned brownish within 1 d after exposure to Cd, were less flexible, and broke off more easily than those cultivated with less Cd. Growth of the shoot was inhibited with 100 and 200 μM Cd. The primary leaves unfolded between d 5 and 6 instead of d 2 and 3. Shortly after unfolding, these leaves showed chlorosis progressing from the tip backward, followed by necrosis at the tip on d 8 or 9.

Since the nutrient solutions were not changed during the course of the experiments, their Cd content was monitored. The culture with 0.5 μM Cd was at half the initial concentration after 3 d, and Cd was no longer detectable after 9 d. In the culture with 5 μM Cd, the content dropped almost linearly to 0.21 μM within 6 d and then to 0.09 μM within the next 3 d. The loss of Cd in the nutrient solution due to plant uptake was less than 10% at 50, 100, and 200 μM (data not shown). These findings were consistent with the Cd content of the plant material: For the cultures with more than 5 μM the accumulation of Cd in roots followed a linear pattern. After 9 d, it reached a concentration of 1.3 μmol g FW^{-1} at 200 μM and of 0.75 μmol Cd g FW^{-1} at 50 μM. In roots exposed to an initial Cd concentration of 0.5 and 5 μM, a maximal cadmium content was reached after 6 d (0.03 and 0.16 μmol g FW^{-1}, respectively) followed by a decline up to d 9 to 0.01 and 0.13 μmol per g FW^{-1}, respectively. This decrease was probably due to the fact that Cd was almost completely depleted from the medium after 6 d, so that the plants could no longer take up appreciable amounts. The Cd content in the roots was thus reduced by transport to the shoots and by

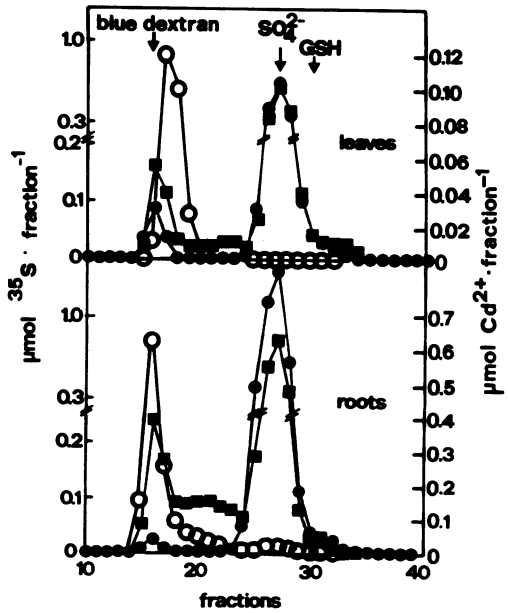

FIG. 2. Radioactive sulfur (^{35}S) in fractions obtained after Sephadex G-25 filtration of crude extracts from roots and primary leaves of maize seedlings cultivated with 50 μM $CdCl_2$ (■) or without Cd (●) and with $^{35}SO_4^{2-}$. Cd^{2+} (○) was measured in the fractions from extracts of seedlings cultivated with this heavy metal. Mean values from two experiments are presented.

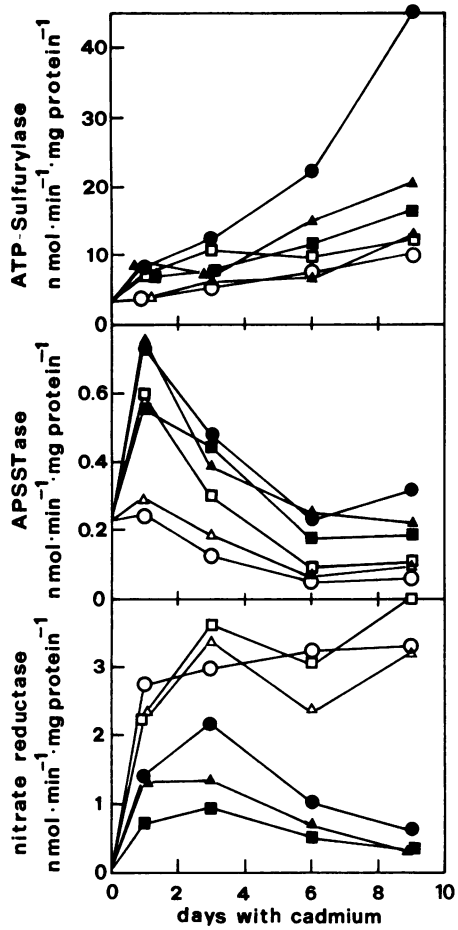

FIG. 3. Effect of 0 (○), 0.5 (△), 5 (□), 50 (●), 100 (▲), and 200 (■) μM $CdCl_2$ on the extractable activity of ATP-sulfurylase, APSSTase, and nitrate reductase from roots of maize seedlings.

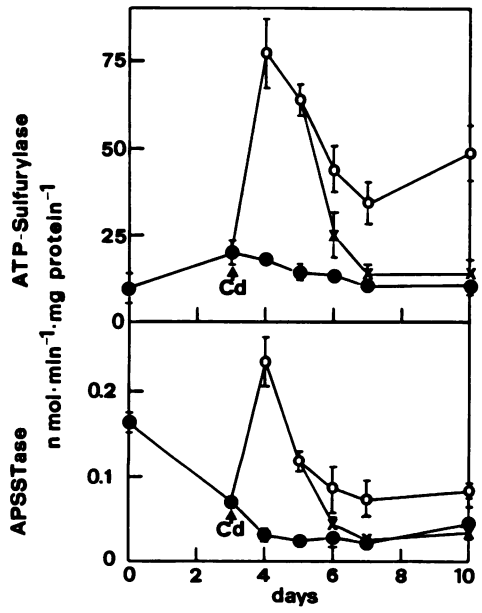

FIG. 4. Effect of 50 μM $CdCl_2$ on the extractable activity of ATP-sulfurylase and APSSTase from roots of maize seedlings (○). Control plants only had 50 μM Cd from d 3 to 5 (×) or were cultivated without Cd (●). Mean values from five experiments ± SD are presented.

dilution by growth. On a FW basis, shoots contained 3 to 4 times less Cd than roots.

Figure 2 shows that maize seedlings cultivated with 50 μM Cd incorporated more label from $^{35}SO_4^{2-}$ into compounds with higher mol wt than did the controls. The fractions eluted in the excluded volume or directly afterward contained Cd^{2+} when extracts from plants cultivated with this heavy metal were analyzed. This indicates that Cd-binding peptides were present in these fractions and that the increased level of radioactivity resulted partly from incorporation of ^{35}S-cysteine into these compounds. The increased level of radioactivity in these fractions may be taken as a sign of an increased sulfate reduction. Consistent with this, higher levels of the extractable activity of the first two enzymes of assimilatory sulfate reduction were detected in the roots of maize seedlings cultivated with 50 μM Cd (Fig. 3) as compared to controls. ATP-sulfurylase activity constantly increased during the experimental period and was at 240 and 440% of the controls after 1 and 9 d, respectively. APSSTase activity was at 400% of the control after 1 d with Cd, then decreased relatively less pronounced than the controls. Increased levels of both enzyme activities were also measured at 100 and 200 μM Cd. Cadmium at 0.5 μM did not appreciably affect ATP-sulfurylase and APSSTase activity, whereas 5 μM initially induced a higher level in both enzymes. This effect was no longer detectable after 6 and 9 d, probably because the Cd concentrations were too low at this time to affect the enzyme activities.

NR activity in roots of maize seedlings cultivated with 50 μM or more Cd was below the level of control plants (Fig. 3).

The changes in ATP-sulfurylase and APSSTase activity at 5 μM Cd (Fig. 3) indicate that the Cd-induced increase is reversible, because both enzyme activities decreased to the level of the controls when the Cd concentration in the nutrient solution became low. The reversibility of the Cd effect was further tested by transferring plants after 2 d on 50 μM Cd to Cd-free medium (Fig. 4). In order to eliminate growth effects, only the 5 cm of the roots next to the kernel were extracted for these measurements. After 1 d with Cd, ATP-sulfurylase and APSSTase activity had increased to levels which were at 343 and 634% of the controls, respectively. Two d later, both enzyme activities were still about 3 times higher than in the controls. Seedlings put back to Cd-free medium after this time had ATP-sulfurylase and APSSTase activities comparable to the controls within 2 d. In roots of seedlings which remained on Cd during this period, ATP-sulfurylase and APSSTase activity was still at 230 and 360% of the controls, respectively. Mixing of extracts from seedlings cultivated with Cd and containing high activities of ATP-sulfurylase and APSSTase with extracts from control cultures resulted in additive activities, indicating that no activating or inactivating substance was involved. The effect of Cd concentrations from 0.5 to 200 μM on the activity of ATP-sulfurylase, APSSTase and NR in primary leaves of 9-d-old plants is shown in Figure 5. At 5 μM Cd, all enzyme activities were at higher levels than in controls. Higher Cd concentrations induced enzyme activities below the control values: only APSSTase activity was still significantly higher at 50 μM.

DISCUSSION

We have previously shown that APSSTase plays an important role in the regulation of assimilatory sulfate reduction (3, 8, 19, 21). The extractable activity of this enzyme was at very low levels in plant material cultivated with H_2S or cysteine as a sulfur source. It was at high levels during sulfur starvation or during an increased demand for sulfur amino acids due to an increased rate of protein synthesis. These results can be best explained by assuming that APSSTase is low when sufficient amounts of the end-products of assimilatory sulfate reduction are available from the nutrient solution or the atmosphere, and is high when there is an increased demand for or a lack of these compounds. On this basis we can predict that the reported (1, 5, 6, 12, 14) Cd-induced synthesis of large amounts of cysteine-rich Cd-binding

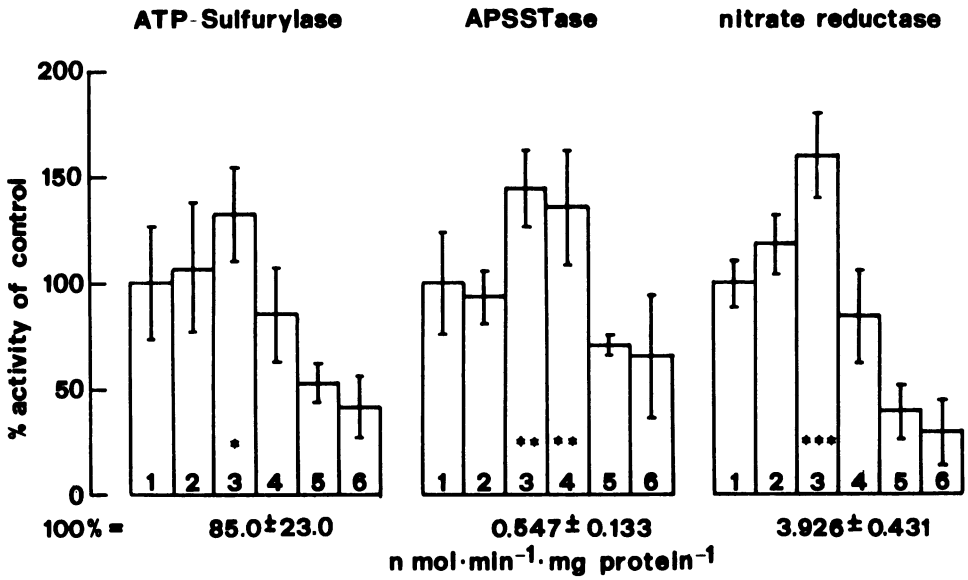

FIG. 5. Effect of 0 (1), 0.5 (2), 5 (3), 50 (4), 100 (5), and 200 (6) μM $CdCl_2$ on the extractable activity of ATP-sulfurylase, APSSTase, and nitrate reductase from leaves of 9-d-old maize seedlings. Mean values from five experiments ± SD are presented. Values with asterisks are significantly higher than the control at $P \leq 0.10$ (*), $P \leq 0.02$ (**), and $P \leq 0.01$ (***).

proteins should result in an increased level of APSSTase activity. The results with maize roots presented here confirm this prediction. Therefore, two conclusions seem appropriate: (a) the increase in APSSTase activity in a situation of increased demand for cysteine presents further evidence for a function of this enzyme in the pathway of assimilatory sulfate reduction of plants; (b) the increase in both activity of APSSTase *in vitro* and sulfate assimilation *in vivo* in the roots of Cd-treated roots indicate that these organs contribute to the production of cysteine needed for phytochelatin synthesis.

In other systems, APT-sulfurylase activity turned out to be much less susceptible to regulatory effectors than APSSTase activity (8). As shown in this paper for roots of maize, ATP-sulfurylase activity was affected similarly to APSSTase by Cd. This may be related to the low level of ATP-sulfurylase activity present in the roots of control seedlings as compared to other heterotrophic (8) and autotrophic (18) systems. An increased demand for cysteine could thus only be met by increasing the extractable activity of ATP-sulfurylase parallel to that of APSSTase.

In the primary leaves, the relative increase in ATP-sulfurylase at 5 μM and in APSSTase activity at 5 and 50 μM Cd is small compared to that in the roots, but the absolute increase is similar in both types of tissue. The decrease at higher Cd concentrations may be due to the fact that growth is almost completely inhibited. Furthermore, premature senescence, a Cd effect reviewed by Fuhrer (4), may be involved.

The molar ratio in which nitrogen and sulfur occur is 25:1 in proteins (13) and approximately 2.5:1 in phytochelatins (12). If the extractable NR activity is regulated according to the need for amino acids for petide synthesis as is APSSTase, we would therefore expect the following changes to be induced by Cd: plants producing substantial amounts of phytochelatins instead of proteins should have a decrease in NR and an increase in APSSTase activity. These changes were detected at Cd concentrations of 50 and more μM Cd in the roots and at 50 μM in the leaves. When the production of phytochelatins takes place in addition to protein synthesis, increased levels of both enzyme activities should be detected. This situation was found in leaves at 5 μM Cd. Plants with inhibited peptide synthesis or subjected to premature senescence should contain reduced levels of both NR and APSSTase activity. A corresponding effect was observed at 100 and 200 μM Cd in the leaves.

Acknowledgments—We thank M. Suter for performing preliminary experiments and Dr. U. Feller for measuring Cd.

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