Cadimium Transport across Tonoplast of Vesicles from Oat Roots

EVIDENCE FOR A Cd\(^{2+}/H^+\) ANTIPORT ACTIVITY*

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Cadimium accumulates in the vacuole of plant cells, but the mechanism driving its transport across the vacuole membrane is not understood. Here we present evidence for Cd\(^{2+}\) transport via a Cd\(^{2+}/H^+\) antiport activity into tonoplast-enriched vesicles isolated from oat roots. Experimentally, accumulation of Cd\(^{2+}\) into vesicles could be driven by ΔpH generated by either V-type ATPase or artificially using nigericin to exchange K\(^+\) and H\(^+\) in K\(^+\)-loaded vesicles. When tonoplast-enriched vesicles were separated on a linear sucrose gradient, NO\(_2^–\)-sensitive ATPase, total MgATPase, and ΔpH-dependent Cd\(^{2+}\) transport equilibrated at 1.11 g/ml, the density of tonoplast membrane. Cd\(^{2+}\) accumulation in vesicles was accompanied by efflux of protons in a Cd\(^{2+}\) concentration-dependent manner characteristic of an antiport activity. The ΔpH-dependent Cd\(^{2+}\) accumulation process showed saturation kinetics with a K\(_{\text{app}}\) of 5.5 μM. Thus the process is a candidate for transport of Cd\(^{2+}\) from the cytoplasm to the vacuolar sap under conditions of low as well as high Cd\(^{2+}\) exposure.

Cadimium is a nonessential potentially toxic pollutant metal which accumulates in the kidney with a biological half-life exceeding 10 years. Its main pathogenic effects, in humans, are pulmonary emphysema and renal tubular damage (Ryan et al., 1982). Recently, Cd\(^{2+}\) has also been shown to stimulate bone demineralization in ovariectomized mice (Bhattacharyya et al., 1988), which corroborates the role of Cd\(^{2+}\) in the severe osteoporosis/osteomalacia experienced by postmenopausal women during the Itai-Itai episode in Japan. Cadmium principally occurs in the human diet as a result of its uptake and concentration from soil by crop plants. Little is known concerning mechanisms of Cd\(^{2+}\) uptake by plants or animals at the tissue or cell level. Hinkle et al. (1987) suggested that Cd\(^{2+}\) is transported across the plasma membrane of a pituitary cell line via a voltage-gated Ca\(^{2+}\) channel; however, a similar study by Karez et al. (1990) with the alga Crispospora elongata proved inconclusive.

In studies of Cd\(^{2+}\) uptake and distribution in plants exposed to relatively low levels of metal, Cd\(^{2+}\) is found to accumulate both in roots and shoots, the distribution depending on the species (Dabin et al., 1978; Cataldo et al., 1981; Wagner and Yeargan, 1986; and Tukendorf and Rauser, 1990). Since recent evidence suggests that the vacuole is a major compartment for Cd\(^{2+}\) accumulation in plants (Kroetz et al., 1989 and Vogeli-Lange and Wagner, 1990), we have undertaken a study of Cd\(^{2+}\) uptake into tonoplast vesicles isolated from oat roots. Our results demonstrate that Cd\(^{2+}\) transport across the tonoplast in oat seedling roots is coupled to the tonoplast V-type ATPase via ΔpH in a manner similar to the Cd\(^{2+}/H^+\) antiport function shown to occur in this membrane (Schunaker and Sze, 1985, 1986; Bush and Sze, 1986; Blumwald and Poole, 1996; Joyce et al., 1988; DuPont et al., 1990; and Chanson, 1991). We have partially characterized this Cd\(^{2+}/H^+\) antiport function in order to further understand the transport mechanism(s) involved in vacuolar Cd\(^{2+}\) accumulation. This report represents the first direct demonstration, to our knowledge, of a ΔpH-dependent "heavy metal" active transport system in plants or animals.

EXPERIMENTAL PROCEDURES

Plant Material—Oat seeds (Avena sativa) were floated on plastic mesh and germinated in the dark over 0.5 mM CaSO\(_4\). Oats were harvested after 5 days. Oats were purchased from Southern States Cooperative, Lexington, KY.

Preparation of Sealed Tonoplast-enriched Vesicles—The procedure was after that of Randall and Sze (1987). All steps were conducted at 4 °C, and where used, DTT\(^{\text{IV}}\) was added just before use. Oat roots (30–50 g) were homogenized in a mortar and pestle in a medium containing 250 mM mannitol, 60 mM EGTA, 0.1% BSA, 0.1 mM PMSF, 50 mM Hepes adjusted with BTP to pH 7.4 and 1 mM DTT, using a medium-to-tissue ratio of 1 to 1.5 ml g\(^{-1}\). The brei was strained through four layers of cheesecloth and reextracted as before. The resulting suspension was centrifuged for 5 min at 1000 × g and then 10 min at 12,000 × g. Membranes in the supernatant were pelleted by centrifugation at 60,000 × g for 30 min. The pellets (total microsomal membranes) were gently resuspended in 1 ml of medium (resuspension buffer) containing 250 mM mannitol, 0.1% BSA, 0.1 mM PMSF, 2.5 mM Hepes–BTP at pH 7.2, and 0.1 mM DTT, pooled, and diluted to 6 ml with resuspension buffer. The resuspended pellet was layered onto 10 ml of a 6% (w/v) dextran cushion containing 6% dextran (w/v) (Sigma D-4751, average M, 74,200), 2.5 mM Hepes–BTP at pH 7.2, and 250 mM mannitol. Gradients were centrifuged for 2 h at 70,000 × g. The visible band at the 0/6% dextran interface was collected, diluted 10-fold with resuspension buffer, and centrifuged at 90,000 × g for 30 min. Pelleted membranes were resuspended in resuspension buffer (approximately 200–300 μl) to give a concentration of 10–20 mg protein/ml to yield a tonoplast enriched vesicle preparation.

Protein Concentration Determination—Except for sucrose density gradient samples protein concentration was estimated using a modification of the method of Bensadoun and Weinstein (1976) for mem-

1 The abbreviations used are: DTT, dl-dithiothreitol; BTP, bis-[tris(hydroxymethyl)methylamino]propane; BSA, bovine serum albumin (fraction V); PMSF, phenylmethylsulfonyl fluoride; FCCP, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone.
brane proteins. To 10–50 μl of vesicles (containing 10–20 μg of protein) 30 μl of 1% sodium deoxycholate was added, followed by incubation at 65 °C for 5 min. Solubilized protein was precipitated with 1 ml of cold 12% trichloroacetic acid (10 min on ice), and the suspension was centrifuged at 1000 x g for 20 min. The supernatant was removed, 1 ml of Pierce BCA protein assay reagent was added and, the sample was incubated at 37 °C and allowed to cool for 15 min and A562 nm was measured (BSA as a standard). For sucrose gradient samples, protein content was measured using the dye-binding procedure of Bradford (1976).

Assay of ATP Hydrolysis—Tonoplast-enriched vesicles were added to an assay mixture containing 25 mM Hepes-BTP at pH 7.0, 175 mM mannitol, 1.5 mM Tris-ATP, 0.2 mM NaN3, 40 mM KCl, and 40 μM FCCP (in ethanol, giving a final concentration of ethanol in the assay mixture of 1%), to give a final protein concentration of 0.1–0.2 mg/ml. Generally, the reaction was initiated in a 2 ml sample by addition of 20 μl of 300 mM MgSO4. After incubation for 30 min at 35 °C, samples were divided into 0.45 ml aliquots and the reaction stopped by addition of 0.65 ml of cold 10% trichloroacetic acid. NO3-sensitive ATPase activity in fractions from sucrose gradients was assayed after adding 0.25 ml of fraction to 0.25 ml of 50 mM Hepes-BTP at pH 7.0, 0.4 mM NaN3, and 100 mM KCl. Tris-ATP was added to give a concentration of 1.5 mM, and the addition of 5 μl of 300 mM MgSO4. Samples were incubated as above and the reaction stopped by the addition of 0.5 ml of cold 10% trichloroacetic acid. Reactions contained either 20 mM KNO3 or 20 mM K-glucanate (as indicated in the figure legends). Inorganic phosphate in the above samples was measured using a slight modification of the method of Martin and Doty as described by Wagner (1983), described below. MgATP-dependent ApH generation in vesi-
cles was measured by addition of K+-loaded vesicles to a reaction mixture containing Buffer A with 1.5 mM Tris-ATP (to give desired concentration) were added. The reaction was then initiated by addition of 1 μl of 300 mM MgSO4. The rate of H+ translocation generated by the V-type ATPase was taken as the initial linear rate of decrease in A490 (lag, 0.18 pmol of Pi/mg of protein/h), a rate comparable with that reported by Churchill et al., 1983, 1984; Wang and Sze, 1985) using

RESULTS

Characterization of Tonoplast-enriched Vesicles—To confirm that vesicles isolated from the 0/6% dextran interface were enriched in tonoplast, a number of characteristics of the V-type ATPase were investigated. ATPase activities reported here were performed in iso-osmotic medium to maintain the side of the vesicle population so that ATPase and transport properties could be compared directly. In the absence of osmoticum, the ATPase activity increased 4-fold to 18 μmol Pi of protein/h, a rate comparable with that reported for the V-type ATPase of oats by others (Churchill and Sze, 1983; Churchill et al., 1983, 1984; Wang and Sze, 1985) using similar assay conditions. ATPase activity was relatively insensitive to 50 μM sodium orthovanadate (4.55 ± 0.37 ng 4 ± 0.18 μmol of Pi of protein/h) and 0.2 mM NaN3 (6.25 ± 0.4 ng 4 ± 0.18 μmol of Pi of protein/h), stimulated by 20 mM KCl (2.66 ± 0.14 ng 3.4 ± 0.19 μmol of Pi of protein/h) and inhibited by 20 mM NO3 (3.83 ± 0.04 ng 2.21 μmol of Pi of protein/h).
± 0.1 μmol of P/mg of protein/h and 57 nmol/mg protein N,N'-dicyclohexylcarbodiimide (6.25 ± 0.4 vis 3.47 ± 0.29 μmol of P/mg of protein/h) as reported by others for the V-type ATPase (Churchill et al., 1983; Churchill and Sze, 1983 and 1984; Wang and Sze, 1985; Randle and Sze, 1986; Kae- 

nister et al., 1988). ATPase activity measured as ATP hydrolysis had a Ka, for ATP of 0.35 ± 0.02 mM and a v, of 14.3 ± 0.3 μmol/mg of protein/h (Eadie-Hofstee), values which are comparable with those reported by others for the V-type ATPase of oats (Wang and Sze, 1985). The presence of molybdate had no effect on the hydrolysis of ATP, indicating an absence of nonspecific phosphatase activity (Leigh and Walker, 1980). Taken together these data indicate that the activities of the vesicle preparations used in this study are similar to the oat root vesicles described previously and identi- 

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fied as tonoplast vesicles by others. Cadmium was found to be associated with washed vesicles occurred only in the presence of MgATP or nigericin. The remaining associated Cd²⁺ may result from passive diffusion into the vesicles, as this result was not observed on other like gradients. Results of Fig. 2 confirm that the bulk of Cd²⁺ transport activity and MgATPase activity of 0/6% dextran-prepared membranes is associated with tonoplast. These results are supported by the sodium orthovanadate sensitivity of MgATPase activity of 0/6% dextran-prepared membranes. Protein occurring at the top of the gradient presumably is the result of lysis of vesicles.
during resuspension prior to loading onto the gradient.

Dissipation of a ΔpH by Cd²⁺—Results observed thus far are consistent with the occurrence of a Cd²⁺/H⁺ antiport activity. If an antiport were responsible for the observed uptake, one would expect that methylamine accumulation in the presence of Cd²⁺ would be decreased. In MgATP-energized vesicles the accumulation of methylamine was reduced by 25% after 20 min in the presence of 10 μM Cd²⁺. The direct inhibitory effect of Cd²⁺ on the ATPase could account only for approximately 4% of this decrease. Thus most H⁺ depletion is due to the utilization of the ΔpH for Cd²⁺ transport into the vesicles. To establish the effect of Cd²⁺ on an existing ΔpH, vesicles were loaded with H⁺ using K⁺/nigericin. Mg-ATPase-energized vesicles were not used in these experiments because results would have been complicated by the steady state inward flux of H⁺ caused by MgATPase activity and the small, but perhaps significant, inhibitory effect of Cd²⁺ on this enzyme. It is clear from Fig. 3 that addition of Cd²⁺ to vesicles preloaded with H⁺ causes a Cd²⁺ concentration-dependent outward flux of H⁺. However it appears to be saturable (see inset to Fig. 3) over the range of [Cd²⁺] with 10, 25, 50, and 100 μM Cd²⁺ causing a H⁺ efflux of 40, 80, 90, and 100 pmol/mg protein/min, respectively (measured as loss of methylamine). High cadmium concentration (1000 μM) appeared to make vesicles nonspecifically leaky to H⁺. Data for 100 and 1000 μM [Cd²⁺] were from a separate experiment and were adjusted for comparison. This outward flux of H⁺ is consistent with the occurrence of a Cd²⁺/H⁺ antiport activity. It is also possible that this H⁺ efflux could be due in part to the dissipation of a membrane potential (negative inside) by the formation of a Cd²⁺ diffusion potential, allowing for an increased H⁺ conductance. However there is no evidence that a membrane potential of this type is significant in K⁺/nigericin-energized vesicles. The effect of Cd²⁺ on a pre-existing pH gradient was also confirmed using acridine orange to monitor H⁺ efflux in MgATP-energized vesicles (Fig. 4). Here hexokinase was added in the presence of glucose to remove any residual ATP. Calcium, Cd²⁺, and Mg²⁺ were added (as chloride salt) to a final concentration of 100 μM.

![Figure 2: Linear sucrose density gradient separation of oat root tonoplast-enriched vesicles.](image)

![Figure 3: Effect of Cd²⁺ on a pH gradient in tonoplast-enriched vesicles.](image)

![Figure 4: Effect of Ca²⁺, Cd²⁺, and Mg²⁺ on a pH gradient in tonoplast-enriched vesicles.](image)
these experiments. Thus initial rates could only be observed after this lag.

Kinetics of ΔpH-dependent Cd²⁺ Transport—The kinetic properties of Cd²⁺/H⁺ transport activity were measured as a function of Cd²⁺ concentration (Fig. 5). As Cd²⁺ was found to inhibit the ATPase activity, ΔpH was generated in these experiments using K⁺/nigericin to eliminate any effect of Cd²⁺ on H⁺ loading. K⁺/nigericin-dependent Cd²⁺ transport represents uptake dependent on the ΔpH, generated by the exchange of K⁺ and H⁺ mediated by nigericin, and was calculated by subtracting the rate of Cd²⁺ accumulation in the presence and absence of nigericin. Initial rates (60 s) of nigericin-dependent Cd²⁺ transport showed saturation kinetics (Fig. 5), and a $K_{m}$ of 5.5 ± 1.1 µM and $V_{max}$ of 13.9 ± 0.7 nmol of Cd²⁺/mg of protein/min, were predicted from an Eadie-Hofstee plot. Data analysis using a nonlinear least squares method (Ligor program) produced values for $K_{m}$ of 7.7 µM and $V_{max}$ of 15.2 nmol of Cd²⁺/mg of protein/min. Initial rates (60 s) of Cd²⁺ accumulation in the absence of nigericin did not show saturation kinetics over the Cd²⁺ concentration range tested (Fig. 5). This uptake could represent passive movement of Cd²⁺ and/or its nonspecific binding to membrane lipids or membrane proteins. This type of nonsaturable component has been reported for Ca²⁺ transport in barley root tonoplast vesicles (DuPont et al., 1990).

Distribution of ΔpH-dependent Cd²⁺ Transport Activity in Microsomal Membranes—Cadmium must pass the plasma membrane prior to transport into the vacuole and could also be sequestered in organelles other than the vacuole. Therefore, we made a preliminary examination of the distribution of NO₃⁻-sensitive and -insensitive ΔpH-dependent Cd²⁺ transport in total microsomal membranes separated on a sucrose step gradient. A total microsomal membrane preparation was separated on a sucrose step density gradient of 6/29% sucrose (Fig. 1A), confirming that vesicle preparations used here were enriched in tonoplast, like those described previously by others (Sze, 1982; Churchill and Sze, 1983, 1984; Randall and Sze, 1986; Schumaker and Sze, 1985, 1986). ΔpH, generated by V-type ATPase, was capable of driving Cd²⁺ accumulation into tonoplast vesicles (Fig. 1A). Cadmium transport under these conditions was sensitive to NO₃⁻, relatively insensitive to sodium orthovanadate, and inhibited in the presence of H⁺ ionophores (gramicidin and FCCP), confirming that the ΔpH formed by the MgATPase was responsible for driving this transport. Cadmium accumulation was also observed when the ΔpH was artificially generated using K⁺/nigericin-energized vesicles (Fig. 1B), supporting the conclusion that Cd²⁺ transport is driven by the ΔpH. Cadmium transport activity co-equilibrated with NO₃⁻-sensitive MgATPase activity at a density of 1.11 g/ml in linear sucrose density gradients, confirming the tonoplast origin of the membranes (Fig. 2). Cadmium was able to dissipate a pre-existing ΔpH in a concentration-dependent manner as would be expected for a Cd²⁺/H⁺ antiport activity (Fig. 3). We note that the kinetics of methylamine efflux do not match those for Cd²⁺ transport. Cd²⁺ transport reaches saturation after 4 min at 10 µM Cd²⁺ and remains stable for up to 20 min (Fig. 1A), whereas methylamine efflux shows a linear decline over 10 min (Fig. 3). There are a number of factors which could explain this difference. Residual K⁺ inside the vesicles will help to sustain ΔpH (Schumaker and Sze, 1986) and hence reduce depletion of ΔpH by Cd²⁺ influx. At steady state the [Cd²⁺]ᵢ is presumably determined by the magnitude of influx and efflux due to antiport and leak processes. If steady state [Cd²⁺]ᵢ is not limited by ΔpH, then the H⁺ efflux needed to balance Cd²⁺

![Figure 5](image_url)  
**Figure 5.** Cadmium concentration dependence of Cd²⁺ uptake into oat root tonoplast-enriched vesicles. K⁺/nigericin-dependent Cd²⁺ uptake. Cadmium, containing tracer ³⁶⁹Cd, was added to give the desired final concentration. Cadmium uptake was monitored in the presence (●) and the absence of nigericin (▲). Uptake in the absence of nigericin was subtracted from uptake in its presence and plotted as nigericin-dependent uptake (●).
leak will continue without affecting \([\text{Cd}^{2+}]_{\text{m}}\). Only when the \(\Delta p\)H becomes limiting would one expect to see a decrease in \([\text{Cd}^{2+}]_{\text{m}}\). This phenomenon has been observed for Ca\(^{2+}\) by others (Schumaker and Sze, 1985; DuPont et al., 1990).

Taken together these observations are consistent with a \(\text{Cd}^{2+}/\text{H}^{+}\) antiport activity associated with the tonoplast. The majority of microsomal membrane \(\Delta p\)H-dependent \(\text{Cd}^{2+}\) transport activity appears to be associated with the tonoplast (Table I); however, significant NO\(_3\)-insensitive \(\Delta p\)H-dependent \(\text{Cd}^{2+}\) transport activity was observed to be enriched in a heavier membrane fraction, perhaps representing plasma membrane. Further study is under way to confirm this.

For \(\text{Cd}^{2+}/\text{H}^{+}\) antiport activity to transport \(\text{Cd}^{2+}\) across the tonoplast in \textit{vivo}, and therefore be involved in cellular detoxification of \(\text{Cd}^{2+}\) via compartmentation/sequestration in the vacuole (Vogeli-Lange and Wagner, 1990), its \(K_m\) for \(\text{Cd}^{2+}\) would be expected to be within the predicted range of physiological cellular \(\text{Cd}^{2+}\) concentration. It has been estimated that plants growing in a typical uncontaminated soil are exposed to \(\text{Cd}^{2+}\) levels of 0.005 to 0.4 pM in uncontaminated soils and to 0.4 to 10 pM in Cd\(^{2+}\) polluted soils. It is also possible that the \(\text{Cd}^{2+}/\text{H}^{+}\) antiport mechanism described here is a nonspecific cation transporter capable of transporting a number of different cations, including Ca\(^{2+}\). Both the \(\text{Cd}^{2+}\) transport activity described here and the \(\text{Ca}^{2+}\) transport activity described by others in oat roots (Schumaker and Sze, 1985, 1986) may be based on the same transporter. Further work is needed to clarify this aspect.

REFERENCES


Blumwald E., and Poole, R. J. (1986) \textit{Plant Physiol.} 80, 727-731


