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Surface Properties of Right Side-Out Plasma Membrane Vesicles Isolated from Barley Roots and Leaves[1]

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ABSTRACT

Highly purified plasma membrane vesicles were obtained from roots and leaves of 7-day-old light-grown barley (*Hordeum vulgare* L. cv Kristina) seedlings by partitioning of crude microsomal fractions in a dextran-polyethylene glycol two-phase system. Sodium dodecylsulfate polyacrylamide gel electrophoresis showed the polypeptide composition of plasma membranes from the two organs to be qualitatively similar, but with different relative amounts of some of the polypeptides. Between 80 and 100% of the K^+,Mg^{2+}-ATPase activity was latent indicating that the vesicles were sealed and right side-out. The isoelectric points of the outer surface of root and leaf plasma membranes as determined by cross-partitioning were similar and quite acidic—about pH 3.6. In contrast, the net negative surface charge density at pH 7.0 as measured by 9-aminoacridine fluorescence differed significantly, being -29 mC·m^{-2} for the leaf plasma membrane and only -19 mC·m^{-2} for the root plasma membrane. As isolated, both types of plasma membrane vesicles had Ca^{2+} and Mg^{2+} bound to the outer surface as shown by the combined use of chelators and 9-aminoacridine fluorescence; however, the leaf plasma membrane had a relatively higher proportion of Ca^{2+} bound (0.57) than did the root plasma membrane (0.45). This difference probably reflects differences in the *in vivo* conditions as no chelator was present during the isolation procedure. Also Ni^{2+} could bind to the root vesicles as indicated by the effect of Ni^{2+} on 9-aminoacridine fluorescence, and by the binding of $^{63}Ni^{2+}$ (44 nanomoles bound per milligram protein) at 100 micromolar $NiCl_2$.

Biological membranes carry a net negative surface charge at neutral pH. The isoelectric point of plant (15) and mammalian (13) mitochondrial membranes is at pH 4.7 to 5.4, and of thylakoid membranes at 4.1 to 4.7 (1, 35). The negative surface charge gives rise to a negative surface potential, the size of which depends on the net number of negative charges per unit surface area (the net surface charge density, measured in mC·m^{-2}) as well as on the concentration and type of cations present in the solution with which the membrane surface is in contact. The presence of this surface potential affects the apparent K_m and the apparent pH optimum of membrane-bound enzymes (12, 38) and sometimes even the V_{max} (24). Other processes like protein binding (31), insertion of newly synthesized proteins into membranes (34), and host-pathogen interactions (10) may also be influenced by the surface potential, *i.e.* by electrostatic interactions between the charged membrane surface and charged proteins. Thus, for studies of membrane structure and function it is clearly important to have information on the electrostatic properties of the membrane.

The electrostatic properties of thylakoid membranes have been studied extensively by Barber (2, and references therein). Electrostatic interactions at the outer surface of the inner membrane of plant mitochondria have also been described, particularly the specific and unspecific effects of cations on NADH oxidation (24, 28, and references therein). Although some work has appeared on the electrostatic properties of microsomes (*e.g.* 32) only a few recent reports have dealt with purified plasma membranes (15, 25, 26). This is particularly unfortunate as the plasma membrane forms the outer border of plant cells which is in contact with the variable external medium. In the roots, the plasma membrane is responsible for the discrimination between and the uptake of ions from the soil solution, and one might therefore expect the plasma membrane from roots to show special electrostatic properties when compared with plasma membranes from, *e.g.* leaves. The outer surface of the plasma membrane is also the first site of interaction between invading pathogens and the plant cell.

The plasma membrane preparations isolated by phase partitioning from various plant species and organs are highly purified (36; for review see Larsson [20]) and appear to consist mainly of sealed right side-out vesicles (21). The isolation of purified plasma membrane vesicles from green leaves and the establishment of their polypeptide composition (18) provides the possibility to compare plasma membranes from green and nongreen parts of the same plant. This makes it possible to ascertain how the differentiation into root and shoot affects properties of the plasma membrane.

In the present study, we have isolated plasma membrane vesicles from both roots and leaves of light-grown barley and compared their polypeptide composition, and isoelectric point, as well as other electrostatic properties.

MATERIALS AND METHODS

Chemicals. Dextran T500 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden and PEG 3350 (earlier designated 4000) from Union Carbide. The anionic polymer sodium PEG-sulfonate and the cationic trimethylamino-PEG bromide were obtained from Aqueous Affinity, Lund, Sweden. $^{63}NiCl_2$ was obtained from Amersham Radiochemical Centre, England. 9-Aminoacridine was from Sigma (No. A-1135). All other chemi-

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cals were of analytical grade.

Preparation of Plasma Membrane Vesicles. Barley (*Hordeum vulgare* L. cv Kristina) was grown at 19°C in a low salt medium according to Wignarajah *et al.* (37), except for the use of light (70 $w \cdot m^{-2}$ during a 12-h d). After 7 d, roots and leaves were harvested and plasma membrane vesicles prepared by differential centrifugation and partitioning in an aqueous dextran-PEG two-phase system as described by Kjellbom and Larsson (18). The composition of the phase system: 6.2% (w/w) Dextran T500, 6.2% (w/w) PEG 3350, 0.33 M sucrose, 3 mM KCl, 5 mM K-phosphate (pH 7.8) was the same for the purification of plasma membranes of both roots and leaves. All steps in the preparation procedure were carried out at 4°C. (For a detailed treatise of the method see Larsson [20]).

The purified plasma membrane vesicles were resuspended in 0.33 M sucrose and kept on ice or frozen in liquid N_2. Freshly prepared vesicles were used for most measurements. Exceptions were (a) SDS-PAGE and protein determinations (both frozen and unfrozen material have been used, without detectable differences), and (b) binding studies with $^{63}Ni^{2+}$ and IDPase assays (only frozen material in both cases). All measurements where the sidedness of the vesicles was of importance were performed in iso-osmotic media to avoid rupturing of the vesicles. Average yields of plasma membranes were 4.4 and 2.8 mg of protein from 125 g of roots and leaves, respectively.

SDS-PAGE was run on gradient gels (total amount of monomers, 12 to 20%; degree of cross-linking, 2.7%; gel dimensions 140·80·2.7 mm) in the buffer system of Laemmli (19) using a circulating running buffer at 12°C. During the passage through the stacking gel, a current of 5 mamp/gel was applied, and during the rest of the electrophoresis 7.5 mamp/gel. Total electrophoresis time was 20 h. Silver staining was essentially as described by Guevara *et al.* (16). Staining with Coomassie brillant blue R-250 was according to standard procedures.

ATPase Activity. K^+-Stimulated Mg^{2+}-dependent ATPase activity was determined essentially as described by Hodges and Leonard (17). The assay medium was iso-osmotic and contained 0.25 M sucrose, 1 mM ATP, 40 mM Tris-Mes (pH 5.9), and 5 μg protein in a total volume of 0.5 ml, as well as 1 mM $MgSO_4$ and 25 mM KCl where indicated. The assay was run at 30°C for 30 min. Enzyme latency on addition of Triton X-100 was calculated according to Larsson *et al.* (21) (Fig. 3).

IDPase Activity. Latent IDPase was assayed at pH 7.5 according to Green (14), except that latency was tested by addition of detergent (0.05% [w/v] Triton X-100) rather than by letting the samples stand for 3 d at 4°C.

Determination of the Isoelectric Point. The isoelectric point of the plasma membrane vesicles was determined by cross-partitioning (1, 35). The vesicles were partitioned in two series of phase systems where the pH was varied using a citrate buffer. A relatively large electrostatic potential difference between the bulk phases was produced by including either a cationic or an anionic PEG derivative in the phase systems: In one series of phase systems trimethylamino-PEG bromide was used, in the other sodium PEG-sulfonate.

A final two-phase system weighed 2.00 g and contained: 6.8% (w/w) Dextran T500, 4.1% (w/w) PEG 3350, 2.7% (w/w) trimethylamino-PEG or PEG-sulfonate, 0.33 M sucrose, 2 mM citrate buffer at different pH, and sample. Fresh solutions of the PEG derivatives were always used to avoid any problems due to possible instability of these polymers in solution.

Fluorescence of 9-Aminoacridine. The fluorescence of 9-aminoacridine was measured as described by Møller *et al.* (26) in a medium containing 0.3 M sucrose, 5 mM MOPS[2]-KOH (pH 7.0), 20 μM 9-aminoacridine and plasma membrane vesicles from roots or leaves as indicated. The relative effect of chelators, EDTA and EGTA, as well as the effect of titrations with cations, especially Ni^{2+}, was measured as specified in the legends to the figures and tables. The surface charge density at neutral pH was determined as described by Møller *et al.* (26) and the calculations performed as in Chow and Barber (9).

Binding of $^{63}Ni^{2+}$. Binding of Ni^{2+} to root plasma membrane vesicles was also determined using the radioactive tracer $^{63}Ni^{2+}$. The binding solution contained 0.3 M sucrose, 5 mM MOPS-KOH (pH 7.0), and ^{63}Ni-labeled $NiCl_2$ (68.4 GBq/mol) in the concentration range 0 to 100 μM. Incubation of vesicles in 0.5 ml binding solution was stopped by addition of 3 ml ice-cold desorption solution (0.3 M sucrose, 5 mM MOPS-KOH [pH 7.0], and 100 μM unlabeled $NiCl_2$) after 10 min at room temperature. The vesicles were immediately collected on a 0.2 μm Millipore filter (EGWP 025). For each Ni^{2+} concentration, a filter background was run without sample. The filters were dried and the radioactivity measured by liquid scintillation counting.

Protein Determination. Protein was determined with a modified Bearden (3) procedure, using Triton X-100 to solubilize the membrane proteins. The fractions were first incubated with 0.1% (w/v) Triton, and then water and reagents were added to bring the Triton concentration to 0.01%. This final concentration of Triton X-100 did not disturb the assay. BSA was used as the standard.

RESULTS

Polypeptide Composition. The crude microsomal fraction from barley leaves contained several dominant polypeptides which were completely absent in the final plasma membrane fraction (Figs. 1 and 2) in agreement with earlier results (18). By comparison with the polypeptide pattern of chloroplast thylakoids isolated from barley leaves (Fig. 1) the more prominent bands in the microsomal fraction may be identified as thylakoid polypeptides. The absence of these major thylakoid polypeptides in the plasma membrane fraction underlines the efficiency of the phase partitioning procedure in separating thylakoid fragments from plasma membranes. Similarly, several bands were more pronounced in the plasma membrane fraction from roots as compared with the root microsomal fraction (Fig. 2), whereas other bands were only found in the microsomal fraction.

The polypeptide composition of the plasma membranes from roots and leaves appears to be nearly identical (Fig. 2; compare tracks 2 and 3) although the relative abundance of some of the polypeptides vary. Thus, the bands at 16, 27, 44, and 85 kD are stronger in the leaf plasma membrane, whereas the bands at 22, 37, and 110 kD are stronger in the root plasma membrane (indicated with arrowheads in Fig. 2). Prominent polypeptides in both root and leaf plasma membranes are found at 26, 28, 31, 40, 56, and 73 kD. Note the difference in staining obtained with silver and Coomassie brilliant blue (compare Figs. 1 and 2). For instance, the 26, 27, and 28 kD polypeptides of the leaf plasma membrane appear as major bands with silver staining (Fig. 2) but are barely detected with Coomassie brilliant blue (Fig. 1).

IDPase Activity. Latent IDPase, a suggested marker for the Golgi apparatus (14, 27), could not be detected in the plasma membrane fractions nor in the microsomal fractions, possibly due to a low content of Golgi in the plant material used. Furthermore, most of the Golgi should have been removed already in the first centrifugation step (14, 27). Nonlatent IDPase, however, was detected in both root and leaf microsomal fractions (960 and 250 nmol (mg protein)$^{-1} \cdot min^{-1}$, respectively) as well as in the corresponding plasma membrane fractions (180 and 80 nmol (mg protein)$^{-1} \cdot min^{-1}$, respectively). Nonlatent IDPase activity of the latter order has earlier been reported in plasma membrane-enriched fractions free from Golgi (27).

[2] Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; $(DM)Br_2$, decamethylene-1,10-bistrimethylammonium bromide.

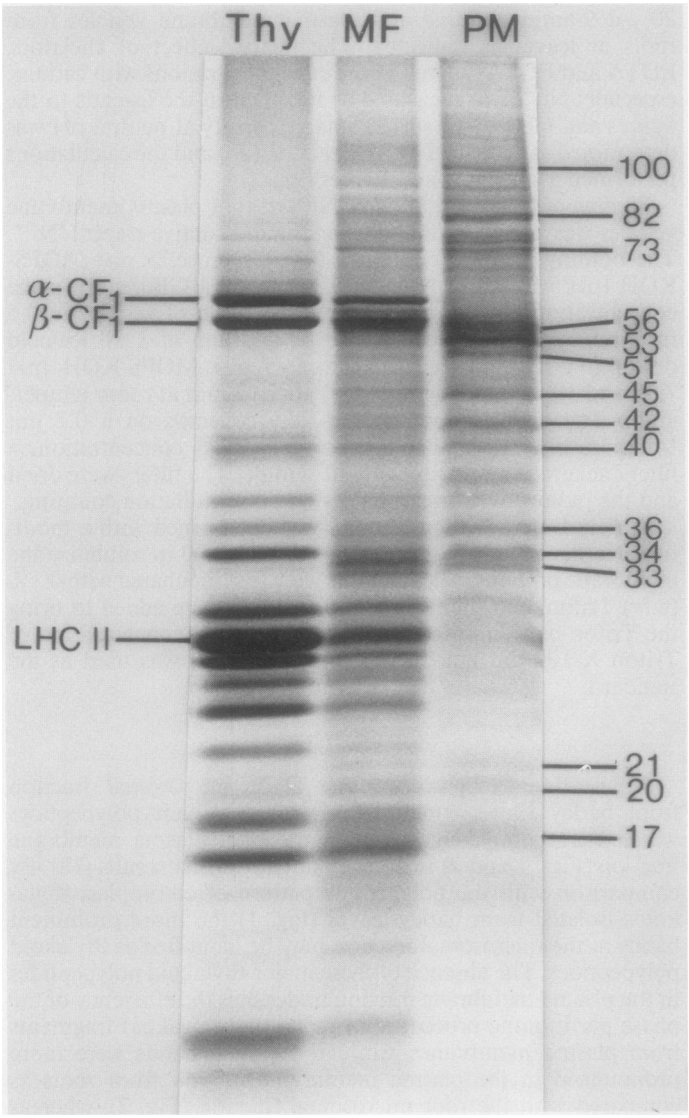

FIG. 1. SDS-PAGE of plasma membranes (PM) obtained from barley leaves. The polypeptide patterns of the microsomal fraction (MF) used as starting material, and chloroplast thylakoids (Thy) are shown for comparison. Mol wt (kD) of prominent plasma membrane polypeptides are indicated to the right. To the left, the positions of some major thylakoid polypeptides, namely the α- and β-subunits of the ATP synthase (α-CF_1, β-CF_1), and the apopolypeptide of the Chl a/b-protein complex of PSII (LHC II), are indicated. Note the absence of thylakoid polypeptides in the plasma membrane fraction. Tracks 2 and 3 were loaded with 100 μg of protein from the plasma membrane and microsomal fractions, respectively. The gel was stained with Coomassie brillant blue R-250.

Polarity of Vesicles. From previous studies we know that plasma membrane preparations consist of vesicles (*e.g.* 18, 36). It is important to determine the sidedness and degree of leakiness of such vesicles before characterizing their surface properties. We used the latency of the K^+,Mg^{2+}-ATPase to do this (21). In an iso-osmotic medium, the K^+,Mg^{2+}-ATPase activity was very low for both root and leaf plasma membrane preparations (Fig. 3). When Triton X-100 was added to rupture the vesicles the K^+,Mg^{2+}-ATPase activity increased several-fold. This indicates that: (a) the active site of the K^+,Mg^{2+}-ATPase is located on the inside of the vesicles and that these therefore are right side-out, since the active site of this ATPase is assumed to be located on the cytoplasmic side of the plasma membrane; (b) the vesicles are tightly sealed so that no Mg-ATP can approach the active site of the enzyme. The degree of latency was found to be between 80 and 100% for the plasma membrane vesicles from both roots and leaves (Table I; Fig. 3).

The plasma membrane vesicles used in this work thus have the apoplastic side exposed to the medium and all of the following data pertain to this surface.

The Isoelectric Point of the Plasma Membrane. The isoelectric point of the outer surface of the vesicles was determined by cross-partitioning and found to be at about pH 3.6 for both the root and leaf plasma membrane (Fig. 4; Table I). At the isoelectric point, the partition of the vesicles between the upper and lower phase is independent of the electrostatic potential difference between the phases created by the charged polymers; *i.e.* the isoelectric point is identical to the cross-point of the two curves in Figure 4. At the cross-point, root plasma membrane vesicles were mainly in the upper phase whereas leaf plasma membrane vesicles were more evenly distributed (Fig. 4). This cross-point represents the net-charge-independent partitioning of the vesicles and the difference indicates that the outer surface of root and leaf plasma membranes differ in hydrophobic/hydrophilic properties (35).

The data obtained for the partitioning at the lowest pH values tested (around pH 3) repeatedly deviated from the curve given by the other values (Fig. 4). This deviation might be due to rearrangements in the membrane at this unphysiological pH, or alternatively be due to release of divalent cations bound by carboxyl groups (which are titrated at this pH), thus changing the net charge of the membrane surface.

The Surface Charge Density of the Plasma Membrane. The surface charge density was about 50% more negative for the outer surface of leaf than for root plasma membrane vesicles as determined by 9-aminoacridine fluorescence at pH 7.0 (Table I). Thus, although the type and relative amounts of charged groups are similar for root and leaf vesicles, as indicated by the very similar isoelectric point (Fig. 4; Table I), the number of charged groups per unit area differ.

Bound Divalent Cations. The fluorescence of 9-aminoacridine can be used to monitor changes in the surface potential of membranes as originally shown by Searle *et al.* (30). An increase in the size of the surface potential is followed by a decreased fluorescence and vice versa. Thus, when bound divalent cations are removed from the surface of membranes by chelators, this is observed as a decrease in fluorescence as the membranes become more negative (23, 25). Since EGTA is specific for Ca^{2+}, whereas EDTA removes both Ca^{2+} and Mg^{2+}, the two dominant divalent cations on the membrane surface, the relative effects of EGTA and EDTA gives information on the relative amounts of bound Ca^{2+} and Mg^{2+}.

Titrations of barley plasma membrane vesicles with chelators in the presence of 9-aminoacridine showed that both root and leaf vesicles are isolated with bound Ca^{2+} and Mg^{2+} (Fig. 5; Table II). Since the vesicles were prepared in the absence of any chelator (even in the homogenization medium; the 5 mM Pi used in the phase system is a weak complexor and allows the presence of 100 μM free Ca^{2+}), the amounts of bound divalent cations are probably similar to those found *in vivo*. It is therefore interesting that the relative effects of EGTA and EDTA are different for root and leaf vesicles. Plasma membrane vesicles from barley leaves appear to have relatively less Mg^{2+} bound to their outer surface than do plasma membrane vesicles from roots as suggested by the smaller relative additional effect of EDTA (Fig. 5; Table II).

The Binding of Added Cations. When cations are added to membranes suspended in a low-cation medium they cause a decrease in the size of the surface potential due to electrostatic

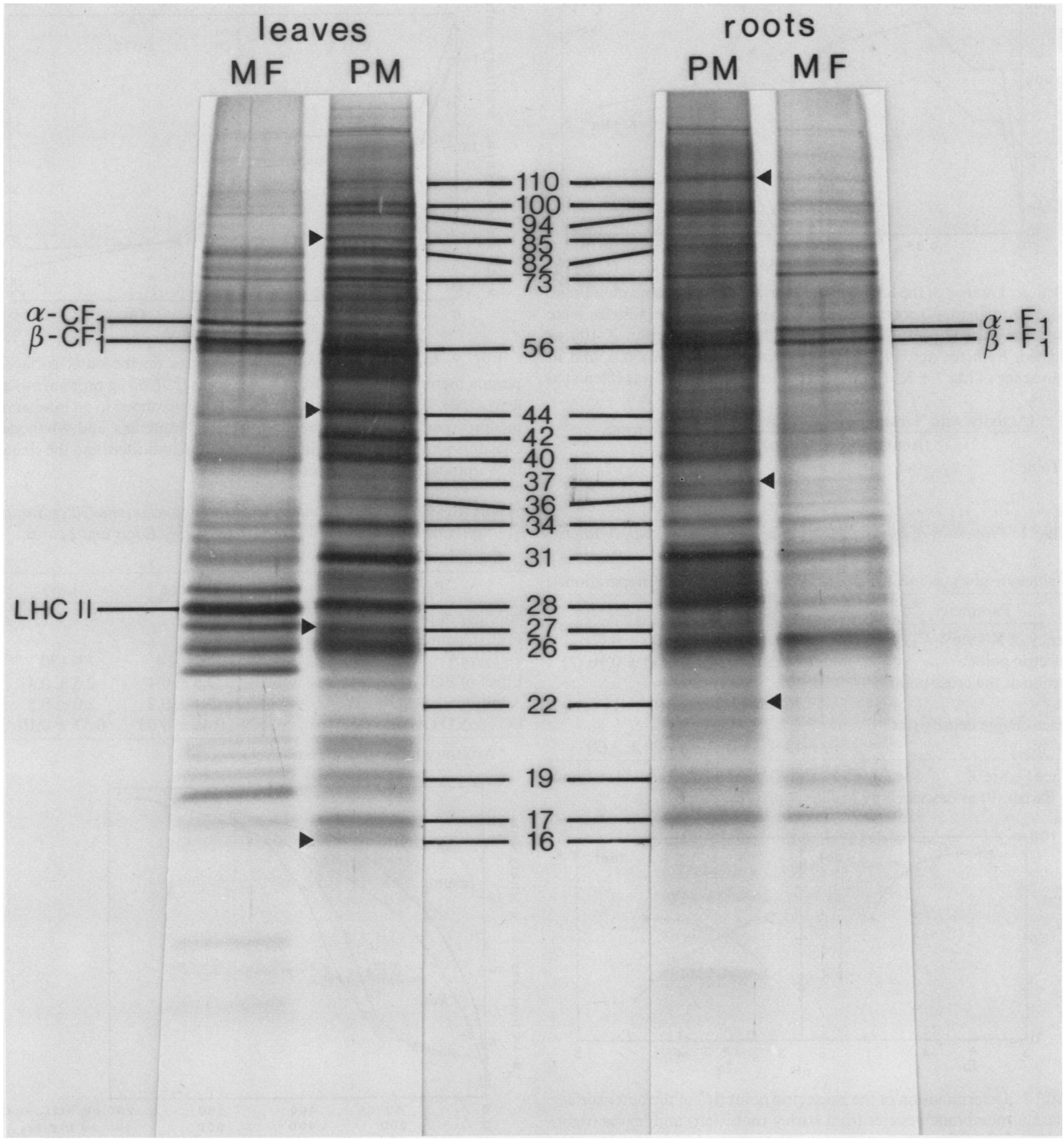

FIG. 2. SDS-PAGE of plasma membranes (PM) and microsomal fractions (MF) from barley leaves and roots. The mol wt (kD) of prominent plasma membrane polypeptides are indicated in the middle. Note that leaf and root plasma membranes were run on neighboring tracks; the photograph has been cut to facilitate the indication of mol wt. Major differences between leaf and root polypeptides are indicated by arrowheads. The 56 and 58 kD polypeptides of the root microsomal fraction are tentatively identified as the α- and β-subunits, respectively, of the mitochondrial ATP synthase (α-F_1, β-F_1). Other abbreviations as in Figure 1. Ten μg protein of each fraction was loaded on the gel, which was silver stained (16).

screening. This can be observed as an increase in 9-aminoacridine fluorescence. In the presence of 100 μM EDTA to remove all bound divalent cations (Fig. 5), the addition of 0 to 20 mM KCl or 0 to 600 μM $(DM)Br_2$ gave hyperbolic increases in 9-aminoacridine fluorescence (Fig. 6) consistent with the low binding constants for K^+ and $(DM)^{2+}$. The addition of low amounts (0–50 μM) of $CaCl_2$ or $NiCl_2$, on the other hand, had very little effect probably due to complexing with residual EDTA in the solution (23). Above 50 μM, the effect of Ca^{2+} and Ni^{2+} was hyperbolic and both ions were much more efficient at affecting the surface potential than $(DM)^{2+}$ (at least 4-fold, [Fig. 6]) probably due to binding of Ni^{2+} and Ca^{2+} to the membrane surface. The concentration of Ca^{2+} and Ni^{2+} at which the half maximal effect was observed was 90 ± 14 (mean ± SD, $n = 4$) and 73 ± 13 ($n = 5$) μM, respectively.

Ni^{2+} was chosen as a representative of the heavy metal ions

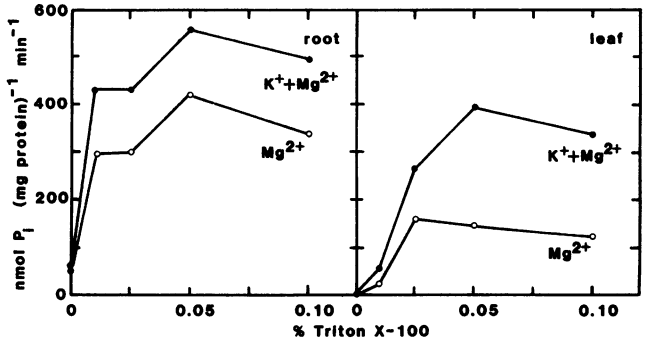

FIG. 3. Latency of the K^+,Mg^{2+}-ATPase activity in plasma membrane vesicles from barley roots (left) and leaves (right). The vesicles were assayed in an iso-osmotic medium with addition of Triton X-100 as indicated. Both the activities in the presence of Mg^{2+} only (O), and in the presence of Mg^{2+} + K^+ (●) were recorded. The latency was calculated as:

$$\frac{\text{(Activity with Triton)} - \text{(activity without Triton)}}{\text{(Activity with Triton)}} \cdot 100\%$$

see Table I.

Table I. *Properties of Plasma Membrane Vesicles from Barley Roots and Leaves*

Values are given as mean ± SD (number of independent preparations).

Parameter	Root	Leaf
Latency of K^+,Mg^{2+}-ATPase[a]	87 ± 3 (2)	100 ± 0 (2)
Isoelectric point[b]	3.55 ± 0.10 (4)	3.65 ± 0.10 (2)
Partition at the cross-point (%)[b]	76 ± 20 (4)	41 ± 11 (2)
Surface charge density (mC·m^{-2})[c]	−19 ± 1 (4)	−29 ± 3 (3)

[a] See Figure 3. [b] See Figure 4. [c] Determined according to Chow and Barber (9) as described by Møller *et al.* (26).

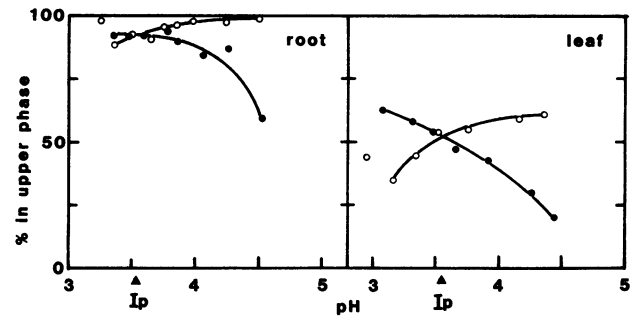

FIG. 4. Determination of the isoelectric point (Ip) of the outer surface of plasma membrane vesicles from barley roots (left) and leaves (right) by cross-partitioning. Right side-out plasma membrane vesicles were partitioned in phase systems of different pH containing either trimethylamino-PEG (positive upper phase [O]) or PEG-sulfonate (negative upper phase [●]). At the isoelectric point of the membrane surface the partition is independent of the electrostatic potential difference between the phases created by the charged polymers *i.e.* the isoelectric point is identical to the cross-point. The unexpected increase in partition at very low pH (unconnected points) is treated in "Discussion".

which contaminate our environment to an increasing extent and whose uptake into plants might cause structural and functional changes. The binding of Ni^{2+} to the outer surface of the plasma membrane of the root is clearly an important step in this uptake process and we therefore attempted to determine at what concentration binding of Ni^{2+} to the plasma membrane occurred.

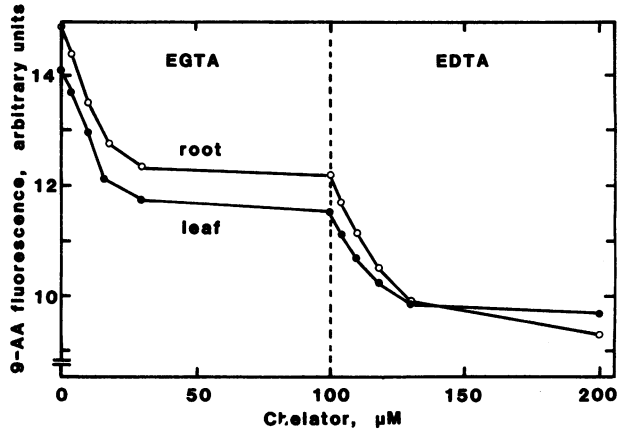

FIG. 5. Monitoring bound divalent cations on the outer surface of plasma membrane vesicles from barley roots ([O], 62 μg protein/ml) and leaves ([●], 77 μg protein/ml). Samples were suspended in an iso-osmotic medium containing 9-aminoacridine (see "Materials and Methods"), chelators were added to the final concentration indicated, and the changes in 9-aminoacridine fluorescence recorded.

Table II. *Monitoring of Bound Divalent Cations on the Outer Surface of Plasma Membrane Vesicles from Barley Roots and Leaves*

Experimental details as in Figure 5.

Parameter	Root	Leaf
Number of preparations	3	4
Protein concentration in assay (μg/ml)	62–114	77–130
Effect of EGTA	2.5 ± 0.4[a]	2.5 ± 0.4
Additional effect of EDTA	3.1 ± 0.2	2.0 ± 0.5
EGTA/EDTA ratio	0.45 ± 0.03	0.57 ± 0.10

[a] Arbitrary units, mean ± SD.

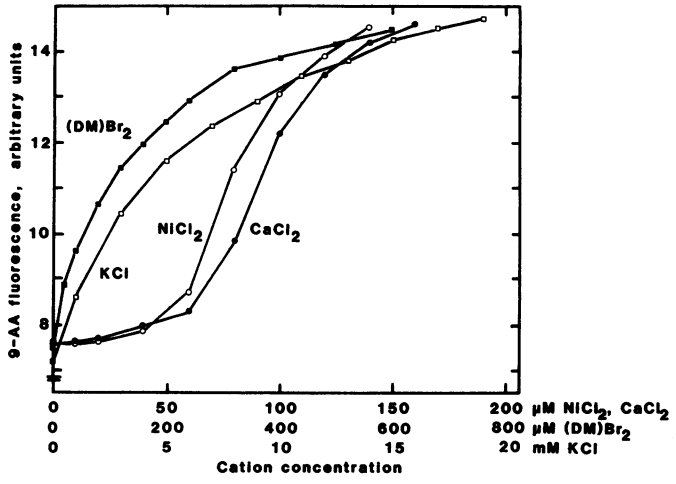

FIG. 6. Effect of cations on the surface potential of the outer surface of plasma membrane vesicles from barley roots. The vesicles (92 μg protein/ml) were suspended in the medium described in "Materials and Methods" plus 100 μM EDTA, salts were added to the final concentrations indicated, and the changes in 9-aminoacridine fluorescence recorded.

These experiments were performed in the absence of EDTA to leave the membranes with their natural complement of bound divalent cations (see above) and to avoid interference from EDTA (as observed in Fig. 6).

Two methods were used to follow the binding of Ni^{2+}: (a) titration of 9-aminoacridine fluorescence, and (b) binding of the

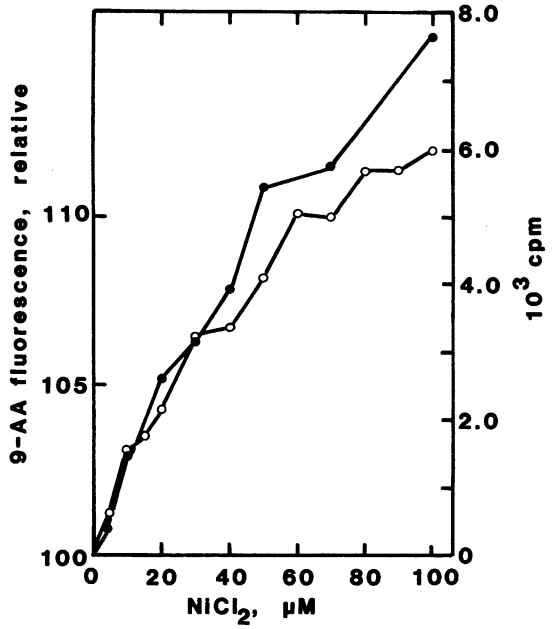

FIG. 7. Binding of Ni^{2+} to the outer surface of plasma membrane vesicles from barley roots. Binding was monitored using 9-aminoacridine fluorescence or $^{63}NiCl_2$ as described in "Materials and Methods". The curve showing the change in 9-aminoacridine fluorescence (O) is the average of four individual experiments (identical titrations with four separate samples containing 170 μg protein/ml). These are normalized at zero $NiCl_2$ to cancel out small differences in the initial fluorescence and the values given are relative. Each point for the $^{63}NiCl_2$ curve (●) is the average of two separate samples (containing 149 μg protein/ml). Similar curves were observed with other plasma membrane preparations.

radioactive $^{63}Ni^{2+}$. The effect of Ni^{2+} on 9-aminoacridine fluorescence (Fig. 7) never gave rise to a smooth hyperbolic curve, however, as observed with K^+ and $(DM)^{2+}$ (compare Figs. 6 and 7). The experiments with $^{63}Ni^{2+}$ confirmed that binding took place (order of magnitude of 44 nmol Ni^{2+}/mg protein at 100 μM $NiCl_2$) and again the curves were not smooth (Fig. 7).

DISCUSSION

Plasma membranes prepared by partitioning in aqueous two-phase systems are in general very pure, and have been shown to be practically free from contamination by intracellular membranes (for review see Larsson [20]). Thus, even Chl-free preparations of plasma membrane are readily obtained from light-grown barley leaves (18; Fig. 1), and the close similarity in the polypeptide composition between root and leaf plasma membranes (Fig. 2) indicates that also the root plasma membrane preparation was very pure. Although the polypeptide composition appears to be qualitatively the same for root and leaf plasma membranes, the relative amounts of some of the polypeptides differed (Fig. 2). However, the overall similarity in polypeptide composition indicates that the function of the plasma membrane is similar in roots and leaves and that the two membranes differ mainly in the balance between the different processes taking place at their surfaces. The extensive similarity between plasma membranes from different organs of the same plant is consistent with recent observations that differentiation in general only causes the appearance/disappearance of relatively few polypeptides (11).

Plasma membranes from both roots and green leaves show bands in the 100 kD region where a subunit of the K^+,Mg^{2+}-ATPase is found (33). However, none of these polypeptides belong to the main bands which are all found at lower mol wt in both root and leaf plasma membranes. It is not surprising that the ATPase constitutes only a small percentage of the plasma membrane protein since an ATPase may well have a high turnover number, making each molecule highly efficient. Assuming that the barley ATPase has the same specific activity as that of the *Neurospora* plasma membrane (140 μmol [mg protein]$^{-1}$ min^{-1}, calculated from the purification scheme of Bowman *et al.* [7]) the barley ATPase should constitute less than 0.5% of the plasma membrane protein.

Almost all of the K^+,Mg^{2+}-ATPase activity was latent in both types of vesicles (Fig. 3; Table I). This is consistent with results on plasma membrane preparations from cauliflower influorescences and oat roots (21), and shows that the vesicles are sealed (the substrate cannot penetrate to the active site of the ATPase) and right side-out. The latter conclusion is based on the assumption that the ATPase is located on the inner, cytoplasmic side of the plasma membrane. Unfortunately, the active site of the other established plasma membrane marker enzyme, glucan synthetase II (EC 2.4.1.34), also appears to be located on the cytoplasmic side (21). Thus, there is presently no known enzyme marker activity for the outer surface of the plasma membrane.

The isoelectric point for both root and leaf plasma membrane vesicles was at about pH 3.6 (Fig. 4; Table I) which is very close to the value of pH 3.4 obtained for the outer surface of the spinach leaf plasma membrane (35) also using cross-partitioning, but significantly lower than the pH 4.4 observed on plasma membranes from pea by Griffing and Quatrano (15) using isoelectric focusing. This latter technqiue may not, however, give the correct isoelectric point (29). It should be noted that the isoelectric point only gives information on the net surface charge at that particular pH (where it is zero per definition) and not at other pH values, in contrast to what has been implied by, *e.g.* Griffing and Quatrano (15). Therefore the net surface charge density of a membrane surface must be determined by a separate technique as done here using the fluorescence of 9-aminoacridine (Table I). The outer surface of plasma membrane vesicles from barley leaves was 50% more negative at pH 7.0 than that of root plasma membrane vesicles in spite of their identical isoelectric point (Table I; Fig. 4) and similar polypeptide pattern (Fig. 2). Root and leaf plasma membrane vesicles also differed markedly in their partitioning at the isoelectric point (Fig. 4; Table I) indicating a difference in hydrophobic/hydrophilic properties (35). Since the polypeptide patterns were relatively similar, the large differences in surface net charge density and hydrophobic/hydrophilic properties may be due to differences in the lipid composition of the membranes, a property not investigated so far. The barley leaf plasma membrane is a very lipid-rich membrane (70% w/w, [18]) why the contribution to the surface properties from the lipid fraction should be important.

The values of net surface charge density for the outer surface of barley plasma membranes (Table I) are compared to other values from the literature (Table III). All values presented were estimated by use of 9-aminoacridine fluorescence (2, 4, 9, 22, 26) since it is difficult to compare absolute values determined using different techniques, *e.g.* particle electrophoresis consistently gives lower values for the surface charge density probably due to the presence of the shearing layer (2, 9). Barley root plasma membrane vesicles have a similar surface charge density as plasma membrane vesicles and mitochondria from wheat roots which appear to belong to the membranes of intermediate charge (Table III). In contrast, the surface charge density of barley leaf plasma membrane vesicles resembles that of oat root plasma membrane and Jerusalem artichoke mitochondria. The surface charge density of different regions of the thylakoid membrane has been reported to span the entire range found for plasma membranes and mitochondria (Table III). The inside surface of plasma membrane vesicles (*i.e.* the cytoplasmic surface) is more

Table III. *Surface Charge Density of Plant Membranes*

Data were all measured by the use of 9-aminoacridine fluorescence.

Membrane	Source	Surface	
		Outer	Inner
		$mC \cdot m^{-2}$	
Plasma membranes	Wheat roots[a]		
	Low K^+	−21	−32
	High K^+	−19	−24
	Oat roots[a]	−29	−38
	Barley roots[b]	−19	
	Barley leaves[b]	−29	
Mitochondria	Jerusalem artichoke tubers[a]	−33	
	Wheat roots[c]	−19 to −21	
Thylakoid membranes	Pea leaves[d]	−21 (randomized thylakoids)	−37 (appressed region)

[a] Møller *et al.* (26). [b] This paper. [c] Bérczi *et al.* (4). [d] Mansfield *et al.* (22).

negative than the outer surface (Table III).

Both types of vesicles investigated in the present study had divalent cations bound to their outer surface as revealed by the reduction in 9-aminoacridine fluorescence (= increase in the size of the surface potential) upon addition of chelators (Fig. 5). The difference in the EGTA/EDTA ratio between root and leaf vesicles (Fig. 5; Table II) indicates that leaf vesicles have a relatively lower proportion of bound Mg^{2+} on their outer surface. Wheat leaves contain at least twice as much Ca^{2+} as wheat roots (6) and since the concentration of free Ca^{2+} is thought to be in the micromolar range more than 99% of this total Ca^{2+} must be bound. This would point to the existence of relatively more Ca^{2+}-binding sites in the leaves than in the roots of cereals consistent with our observation (Table II). These bound divalent cations would reduce the net surface charge density *in vivo* (the values in Table II were determined in the presence of enough EDTA to strip the vesicles' outer surface of divalent cations). The electrostatic interaction between cations and charged cell membranes could be important for the regulation of the ion content in plant cells (4, 5). Of course, specific effects of membrane-bound divalent cations are also important; one could here mention the external NADH dehydrogenase in plant mitochondria which is dependent on bound Ca^{2+} (23).

The binding of Ni^{2+} to the outer surface of the root plasma membrane would be the first step in the uptake of Ni^{2+} and this uptake has been reported to have two phases in the concentration range 0 to 100 μM (8). It was therefore of interest to see if the binding showed several phases. The effect of Ni^{2+} on 9-aminoacridine fluorescence was never smoothly hyperbolic as would be expected for pure electrostatic screening (see, *e.g.* Fig. 6, curves with KCl and $[DM]Br_2$) and it appeared as if several phases could indeed be observed in the absence of EDTA (Fig. 7) which interfered with the assay (Fig. 6). The use of $^{63}Ni^{2+}$ also did not give rise to smooth binding curves (Fig. 7) and we therefore think that there may be several classes of binding sites with different affinities for Ni^{2+} on the outer surface of plasma membrane vesicles from barley roots.

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