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Correlated Induction of Nitrate Uptake and Membrane Polypeptides in Corn Roots[1]

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ABSTRACT

Induction of corn (*Zea mays* L.) seedling root membrane polypeptides was studied by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and two-dimensional gel electrophoresis in relation to induction of nitrate uptake. When nitrate uptake was studied using freshly harvested roots from 4-day old corn seedlings, a steady state rate of uptake was achieved after a lag of 2 to 3 hours. The plasma membrane fraction from freshly harvested roots (uninduced) and roots pretreated in 5 millimolar nitrate for 2.5 or 5 hours (induced) showed no differences in the major polypeptides with Coomassie blue staining. Autoradiography of the ^{35}S-methionine labeled proteins, however, showed four polypeptides with approximate molecular masses of 165, 95, 70, and 40 kilodaltons as being induced by both 2.5 and 5-hour pretreatment in 5 millimolar nitrate. All four polypeptides appeared to be integral membrane proteins as shown by Triton X-114 (octylphenoxypolyethoxyethanol) washing of the membrane vesicles. Autoradiography of the two-dimensional gels revealed that several additional low molecular weight proteins were induced. A 5-hour pretreatment in 5 millimolar chloride also induced several of the low molecular weight polypeptides, although a polypeptide of about 30 kilodaltons and a group of polypeptides around 40 kilodaltons appeared to be specifically induced by nitrate. The results are discussed in relation to the possibility that some of the polypeptides induced by nitrate treatment may be directly involved in nitrate transport through the plasma membrane.

Nitrate transport through the plasma membrane appears to be carrier mediated because the rate of transport shows Michaelis-Menten saturation with increasing nitrate concentration (13). Michaelis-Menten kinetics observed during competitive inhibition of nitrate uptake by chlorate, an analog of nitrate, provides further support for the involvement of carrier proteins in nitrate transport (6, 10). Competition (12) and inhibitor (8) studies suggested that different carrier mechanisms existed for the transport of the major nutrient anions in plant roots. Despite the availability of such information, it has been difficult to identify the plasma membrane polypeptides involved in anion transport. Identification of a nitrate carrier is important for understanding and manipulating the process of nitrate transport at the molecular level.

When nitrate-starved corn roots are incubated in a nitrate solution, an apparent induction in the rate of nitrate uptake occurs after a lag of about 3 h (17, 21, 24). It has been widely postulated that the enhanced rate of nitrate uptake results from induction of more nitrate carriers in the plasma membrane. Recently, a small (mol wt = 31,000) membrane-associated polypeptide was found to be induced by nitrate in a microsomal fraction from corn roots (22). However, it was concluded that the polypeptide might be a regulator of nitrate uptake rather than a component of a nitrate carrier.

Nitrate reductase activity is also induced by nitrate, as well as by environmental factors such as temperature, light, and degree of anaerobiosis (1, 31). Hence, it is difficult to study the induction of a nitrate carrier in intact roots because of the confounding effects of metabolic factors such as nitrate reduction and assimilation (2, 25), cytoplasmic regulation of nitrate influx (5, 7, 30), and shoot regulation of root anion uptake (11, 20). One way to circumvent this problem is to study nitrate uptake in isolated plasma membrane vesicles, but the use of this approach is somewhat limited by the difficulty in preparing sealed right side out vesicles and the lack of a convenient radiotracer for nitrate.

The purpose of this study was to attempt to identify polypeptide(s) of a plasma membrane associated nitrate carrier by comparing membrane polypeptides of plasma membrane fractions from corn roots before and after apparent induction by nitrate.

MATERIALS AND METHODS

Plant Material. Seeds of corn (*Zea mays* L.) hybrid WF9 × M017 were grown in glass baking trays (33 × 23 × 5 cm) on a single layer of blotter paper saturated with 0.1 mM $CaCl_2$ solution in the dark at 28°C (approximately 96 seeds per tray). The trays were covered with a plastic wrap which was perforated to allow air exchange. After 3 d, 30 ml more of 0.1 mM $CaCl_2$ solution were added to keep the blotter paper saturated with water.

^{35}S-Methionine Labeling. To prepare the plasma membrane fraction from uninduced roots, 2.5 mCi of ^{35}S-methionine were diluted in 30 ml of 0.1 mM $CaCl_2$ solution and added to one of the trays at the end of 3 d. Roots were excised 8 h later and immediately homogenized for plasma membrane isolation. For other treatments, 20 g of the roots from 3.5-d-old seedlings were incubated at 30°C in a 5 mM nitrate or chloride solution (2.5 mM KNO_3 or KCl, 1.25 mM $Ca(NO_3)_2$ or $CaCl_2$, in 5 mM Mes [pH 6.0]) containing ^{35}S-methionine at a specific activity of 13 to 20 μCi per ml. After 2.5 or 5 h of incubation, radioactive solution was discarded, and roots were washed with ice-cold 1 mM methionine solution twice for 5 min each and used immediately for preparation of the plasma membrane fraction.

Preparation of Plasma Membrane Fraction. The plasma membrane fraction was isolated as described by Gallagher and Leonard (14). In brief, 20 g roots from each treatment were homogenized at ice temperature with a pre-chilled pestle and mortar in 100 ml of 0.25 M sucrose, 3 mM EDTA, 2.5 mM DTT, and 25 mM Tris-Mes (pH 7.7). The filtered homogenate was centrifuged at 13,000*g* for 20 min, and the supernatant so obtained was further centrifuged at 80,000*g* for 30 min. The 80,000*g* pellet was suspended in 2.5 ml of the suspension buffer (0.25 M sucrose,

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1 mM DTT, and 1 mM Tris-Mes [pH 7.2]), which was layered on the top of 34% sucrose in a 36-ml tube containing a 34 to 45% (w/w, 8/28 ml, respectively) discontinuous sucrose gradient. After a 2-h centrifugation at 82,500*g*, the plasma membrane enriched fraction at the interface of 34 to 45% sucrose was collected, diluted with suspension buffer, and centrifuged at 80,000*g* for 1 h. The final pellet was suspended in suspension buffer and stored at −80°C.

Detergent Treatment of the Plasma Membrane Fraction. The plasma membrane fraction was treated with Triton X-114[3] as described by Gallagher and Leonard (15). Briefly, the membrane vesicles were diluted to 2 mg protein/ml in 10 mM imidazole (pH 7.5), 2 mM EDTA, 10% (w/v) glycerol, and 1 mM DTT, on ice. An equal volume of the same buffer containing 2% Triton X-114 was added with continuous stirring. After 20 min on ice, the mixture was centrifuged at 100,000*g* for 30 min. Residual Triton X-114 was removed from the pellet by resuspending it in the imidazole buffer and centrifuging at 100,000*g* for 30 min. The final pellet was suspended in the suspension buffer and stored at −80°C.

Electrophoresis. SDS-PAGE was performed as described by Gallagher and Leonard (15) with some modifications. Discontinuous gels contained 10 and 3.75% acrylamide in the resolving and stacking portions of the gel, respectively. Protein samples for electrophoresis were prepared by mixing 30 μg protein or, alternatively, 65,000 to 125,000 cpm of the ^{35}S-methionine labeled protein in a 10 μl volume of suspension buffer with an equal volume of 2 times sample buffer (0.125 M Tris-HCl [pH 6.8], 8% SDS, 0.2 M DTT, 10% glycerol, and 0.002% bromophenol blue) which was preheated to 75°C. The mixture was heated at the same temperature for another 10 min. Heating the sample at 75°C did not cause any aggregation of high mol wt polypeptides (Fig. 1) but eliminated the need for protease inhibitors used by Gallagher and Leonard (15). Resolution was enhanced by lowering the pH of the resolving gel buffer from 8.80 (15) to 8.45. The gels were electrophoresed at 15 mamp constant current for about 6 h, and then fixed in a 40% methanol and 7% acetic acid solution overnight. The fixed gels were transferred to a 30% methanol solution containing 3% glycerol for 2 h, after which they were dried on Whatman No. 1 MM filter paper. The dried gels were exposed to Kodak X-Omat XAR-5 x-ray film for 3 weeks at −80°C. Nonradioactive gels were stained in a Coomassie blue staining solution (40% methanol, 7% acetic acid, 0.025% Coomassie blue R-250).

Two-dimensional gel electrophoresis was performed mainly as described by Jones (18), with some modifications. The concentration of SDS in 2 times sample buffer was raised to 8% from the recommended 4.6%. The membrane vesicles were initially solubilized as described for SDS-PAGE above. Next, samples were prepared for IEF by mixing solubilized membrane vesicles, IEF sample buffer (9.5 M urea, 2% [w/v] Nonidet P-40 [NP40], 1.6% [v/v] pH 5–7 ampholine, 0.4% pH 3–10 ampholine, and 5% [v/v] 2-mercaptoethanol) and NP40 in a 2:2:1 ratio. Gel mixture consisting of 9.2 M urea, 4% acrylamide, 2% NP40, 1.6% pH 5 to 7 ampholine, 0.4% pH 3 to 10 ampholine, 1% ammonium persulfate, and 0.07% TEMED was poured into 15-cm-long glass tubes with an internal diameter of 2.5 mm up to a 12.5 cm mark and overlaid with glass-distilled water. The overlay was removed after 1 h and replaced with 20 μl of IEF buffer which was overlaid with water. After an additional hour, the overlays were removed and replaced with 20 μl of IEF sample buffer which was overlaid with cathode solution (0.02 M sodium hydroxide). The anode solution was 0.01 M phosphoric acid.

Tube gels were prefocused for 15 min at 200 V, 30 min at 300 V, and 30 min at 400 V each at constant voltage. The overlay was removed and either 200 μg protein or 600,000 cpm of the ^{35}S-methionine labeled protein in a total volume of 80 μl were loaded on the cathode side and overlaid with 20 μl of sample overlay solution (9 M urea, 0.8% pH 5–7 ampholine, and 0.2% pH 3–10 ampholine). Focusing was done for 4800 V/h at a constant voltage of 300, at the end of which period the voltage was raised to 800 V for 1 h. Tube gels were then equilibrated in 1 times sample buffer carrying 4% SDS for 30 min, frozen in a dry ice-ethanol bath, and stored at −80°C. Before running the second dimension, the frozen tube gels were thawed at room temperature and equilibrated in fresh 1 times sample buffer for another 10 min. The second dimension was run on the SDS-PAGE discontinuous system as described above. The gels were fixed, dried, and autoradiographed as described above for one-dimensional gels.

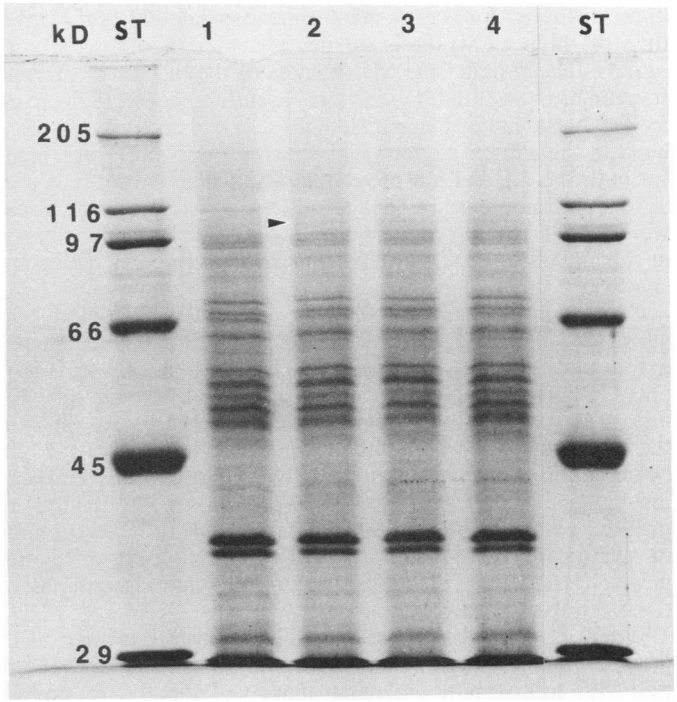

FIG. 1. SDS-PAGE comparison of polypeptide pattern of the plasma membrane fraction from uninduced and nitrate induced corn seedling roots. Roots were induced in an aerated 5 mM nitrate solution for 2.5 or 5 h. Lanes 1 to 4 each contained 30 μg total protein from the plasma membrane fraction of uninduced roots (lane 1), roots induced for 2.5 h (lane 2), roots induced for 5 h (lane 3), and roots treated in 5 mM chloride solution for 5 h (lane 4). Lanes marked ST contain high mol wt standards from Sigma Chemical Company which were: myosin (205 kD), β-galactosidase (116 kD), phosphorylase B (97.4 kD), BSA (66 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD).

Nitrate Uptake Assay. Nitrate uptake was studied as previously described (8). Briefly, 3-g root segments from 3.5-d-old seedlings were incubated in 30 ml of 1 mM nitrate solution (0.5 mM KNO_3, 0.25 mM $Ca(NO_3)_2$, 2 mM Mes [pH 6.0]) at 30°C. Nitrate uptake was studied by monitoring the loss of nitrate from the uptake solution (8). To see if induction was nitrate specific or not, root segments were incubated in 5 mM chloride (2.5 mM KCl, 1.25 mM $CaCl_2$, 5 mM Mes [pH 6.0]) or 5 mM Mes (pH 6.0) for 3 h, at the end of which period they were transferred to 1 mM nitrate solution. Nitrate uptake was studied as described above.

RESULTS

The apparent induction of nitrate uptake in nitrate-starved roots has been reported to occur in about 2 to 3 h (17, 24). If, as originally suggested, this apparent induction occurs due to *de*

[3] Abbreviations: Triton X-114, octylphenoxypolyethoxyethanol; IEF, isoelectric focusing; TEMED, *N,N,N*^{1},*N*^{1}-tetramethylethylenediamine.

novo synthesis of a plasma membrane associated nitrate carrier, then the induced synthesis of such a carrier should occur very rapidly. The results from SDS-PAGE of the plasma membrane fraction are presented in Figure 1. No differences were detected for any of the major Coomassie blue-stained bands. A very light band in the 100 kD region (Fig. 1, arrow) was more prominent for both the 2.5 and 5 h pretreatments in 5 mM nitrate, as well as for 5-h treatment in 5 mM chloride. Likewise, no differences were detected for any of the major Coomassie blue-stained bands in the 13,000 to 80,000*g* pellet (microsomal fraction) (data not shown).

Coomassie blue-stained gels may not be sensitive enough to allow the detection of newly synthesized proteins that are not major plasma membrane polypeptides. Moreover, the differences in polypeptide pattern shown by Coomassie blue may also arise from posttranslational modification of the previously synthesized proteins. On the other hand, the inclusion of radiolabeled amino acid(s) in the uptake solution during the period of incubation should result in the incorporation of label into the proteins being synthesized during that period. Induced proteins in such a case may constitute a high enough proportion of total newly synthesized proteins for detection by autoradiography of the electrophoresed gels.

When proteins of excised corn roots were labeled with [^{35}S]methionine, four polypeptides of the plasma membrane fraction with approximate molecular masses of 165, 95, 70, and 40 kD were apparently induced by both 2.5- and 5-h treatments in a nitrate solution (Fig. 2B). None of these four appeared to be detectably induced in the microsomal fraction as shown in Figure 2A. However, two other polypeptides, which did not appear in the plasma membrane fraction, appeared to be induced in the microsomal fraction. While a 47 kD (approximate) polypeptide appeared to be induced in the microsomal fraction at both 2.5 and 5 h of pretreatment with nitrate, a low mol wt band of about 30 kD was enhanced after 5 h of nitrate treatment (Fig. 2A).

The proteins that act as ion carriers are expected to be integral proteins or protein complexes (15, 19, 33). Triton X-114, a nonpolar detergent, has been successfully used to remove peripheral proteins from corn root plasma membrane vesicles (15). To further resolve the induced integral membrane proteins, the plasma membrane fraction was washed with Triton X-114 as described by Gallagher and Leonard (15). Both the Triton X-114 solubilized polypeptides and those not solubilized were separated by SDS-PAGE. All four induced polypeptides appeared to be integral in nature, although some proportion of the 70 and 40 kD polypeptides also partitioned into the supernatant (Fig. 3). Another diffuse radioactive band of around 60 kD in the Triton X-114 pellet and two bands of around 50 kD in the supernatant, which were not detected in the nondetergent treated plasma membrane fraction, also appeared to be induced, both at 2.5 and 5 h in the aerated nitrate solution.

Two-dimensional gel electrophoresis of the radiolabeled plasma membrane fraction revealed an additional set of low mol wt polypeptides (marked by arrows numbered 1 through 5 in Figs. 4, 5, and 6) as being induced. While several of the induced polypeptides appeared to be common (numbers 2, 3, and 4) between the plasma membrane fractions prepared from chloride or nitrate pretreated roots (Figs. 5 and 6), some were different. Besides a polypeptide of about 30 kD (Fig. 5, number 5), a series of polypeptides in the 40 kD range (Fig. 5, number 1), appeared to be specifically induced by nitrate. Likewise, chloride induced two polypeptides in the 28 kD region which were not induced

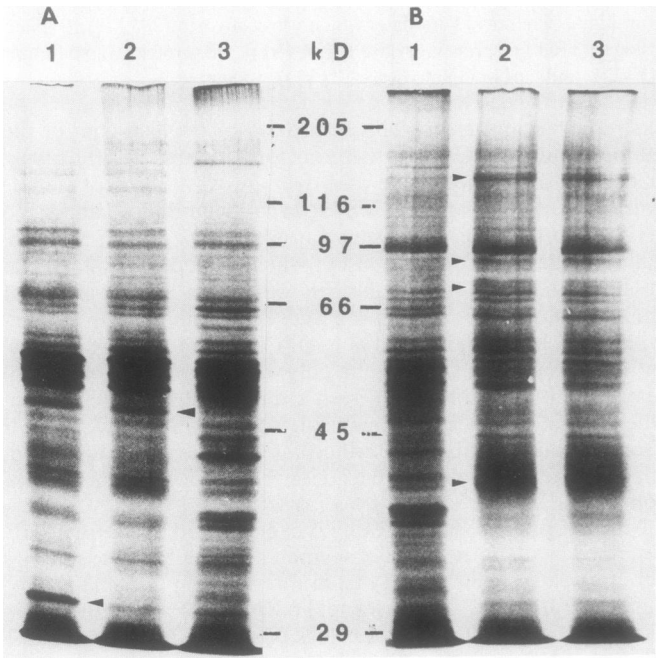

FIG. 2. Autoradiographic comparison of [^{35}S]methionine labeled polypeptides of the microsomal fraction (A) and the plasma membrane fraction (B) from uninduced and nitrate induced corn seedling roots. The induction was done by pretreating the roots in an aerated 5 mM nitrate solution for 2.5 or 5 h in the presence of [^{35}S]methionine. A, Lanes contained microsomal fraction of roots induced for 5 h (lane 1), roots induced for 2.5 h (lane 2), and uninduced roots (lane 3). B, Lanes contained plasma membrane fraction of uninduced roots (lane 1), roots induced for 2.5 h (lane 2), and roots induced for 5 h (lane 3.)

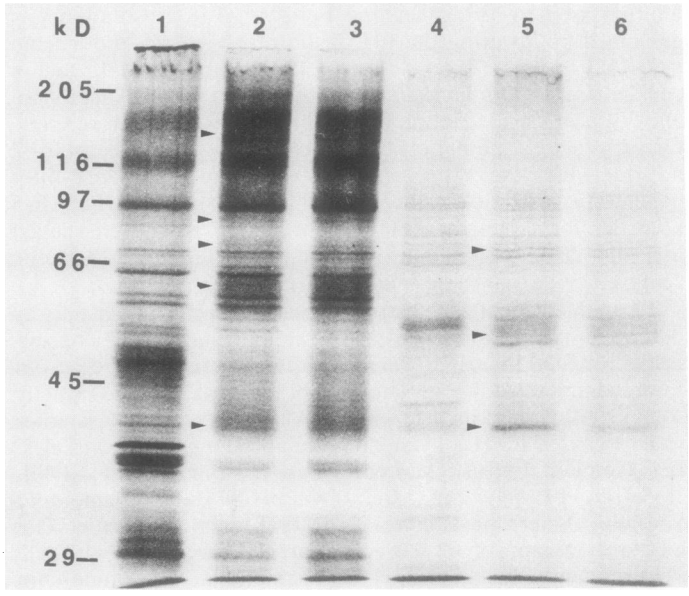

FIG. 3. Autoradiographic comparison of Triton X-114 fractionated polypeptides of the plasma membrane fraction from uninduced and nitrate-induced corn seedling roots. The [^{35}S]methionine labeled plasma membrane fraction was extracted with 1% Triton X-114. Both the Triton X-114 insoluble (lanes 1–3) and soluble polypeptides (lanes 4–6) were separated by SDS-PAGE and autoradiographed. Lanes 1 to 3 each contained 65,000 cpm of the Triton X-114 pelleted plasma membrane from uninduced roots (lane 1), roots induced for 2.5 h (lane 2), and roots induced for 5 h (lane 3). Lanes 4 to 6 each contained 50,000 cpm of the supernatant from Triton X-114 washing of the plasma membrane fraction from uninduced roots (lane 4), roots induced for 2.5 h (lane 5), and roots induced for 5 h (lane 6).

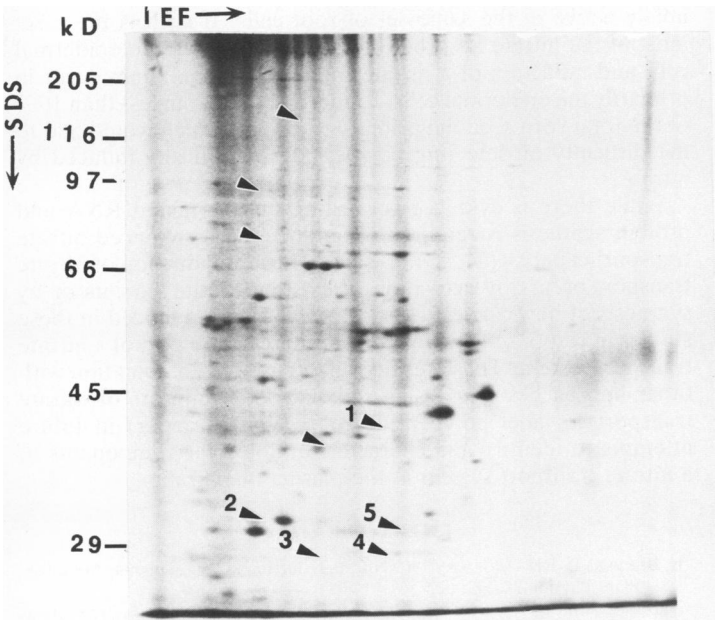

FIG. 4. Two-dimensional autoradiogram of plasma membrane fraction from uninduced corn seedling roots. The gel contained 600,000 cpm of [^{35}S]methionine labeled proteins. Arrows indicate the positions where polypeptides were induced by nitrate or chloride washing (see Figs. 5 and 6).

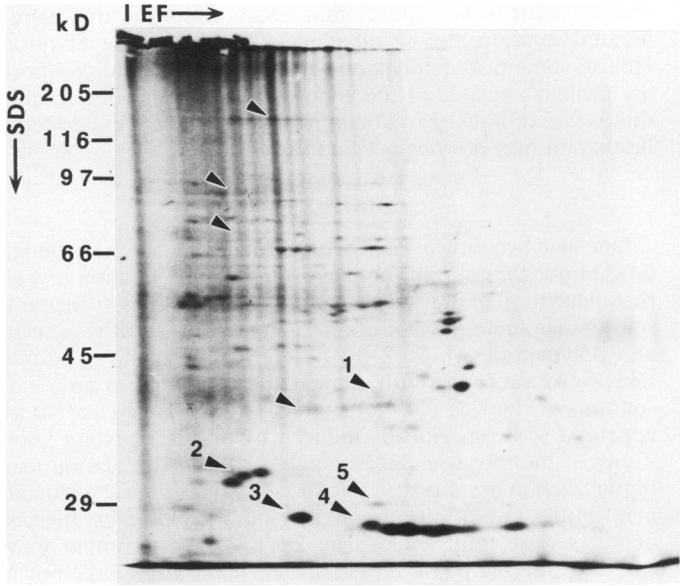

FIG. 6. Two-dimensional autoradiogram of plasma membrane fraction from corn seedling roots washed in a 5 mM chloride solution containing [^{35}S]methionine for 5 h. The gel contained 600,000 cpm of [^{35}S]methionine labeled proteins. Arrows with numbers indicate the positions where polypeptides were induced by nitrate washing (see Fig. 5). Arrows without numbers indicate the location of induced polypeptides as revealed by SDS-PAGE in Figure 2.

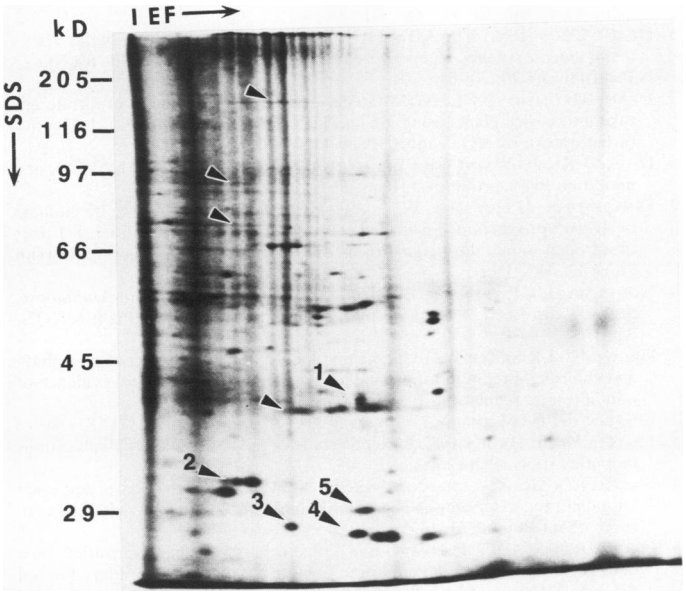

FIG. 5. Two-dimensional autoradiogram of plasma membrane fraction from induced corn seedling roots. Induction was done by washing the roots in a 5 mM nitrate solution containing [^{35}S]methionine for 5 h. The gel contained 600,000 cpm of [^{35}S]methionine labeled proteins. Arrows with numbers point to the polypeptides or groups of polypeptides that are induced as compared to uninduced roots (see Fig. 4). Arrows without numbers indicate location of induced polypeptides as revealed by SDS-PAGE in Figure 2.

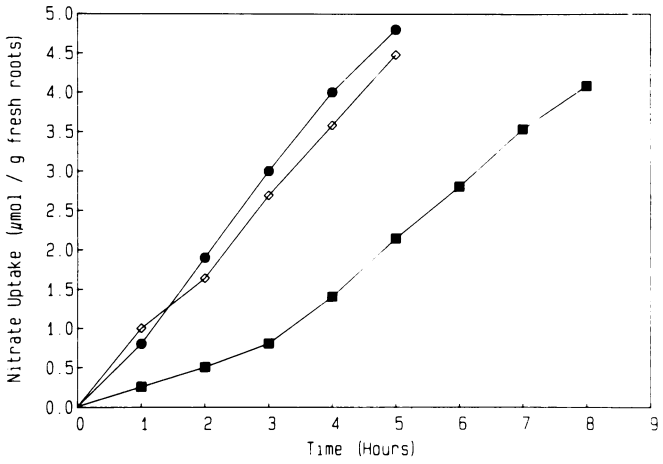

FIG. 7. Effect of washing treatment on nitrate uptake in corn seedling roots. Control (■) roots were kept in 1 mM nitrate solution throughout the period of uptake. Other treatments were washing the roots for 3 h in 2 mM Mes (□) or 5 mM chloride (●) prior to transfer to 1 mM nitrate. The rates of uptake were 0.64, 0.86, and 0.97 μmol/(g fresh roots h) for the control between 4 and 8 h, roots prewashed in Mes, and roots prewashed in chloride, respectively.

by nitrate. This set of low mol wt polypeptides was not readily apparent on one-dimensional SDS-PAGE. The high mol wt polypeptides that were detected with SDS-PAGE were not as clearly resolved on the two-dimensional gels (Figs. 4–6, arrows without numbers) but could be detected.

Since nitrate and chloride pretreatment induced several similar polypeptides, it was of interest to determine if enhanced nitrate uptake occurs with incubation of excised root segments in an aerated chloride solution. A general enhancement of ion uptake is well documented to occur following incubation of excised root segments in aerated solution. Excised corn root segments were pretreated for 3 h in either a chloride or a Mes buffer solution at pH 6.0 and then nitrate uptake was measured (Fig. 7). The initial rate of nitrate uptake for chloride or Mes-pretreated roots was comparable to the enhanced rate of nitrate uptake observed after 3 h of incubation in nitrate (7). It appears that the general

enhancement in ion uptake that occurs following treatment in aerated solution also resulted in enhanced uptake of nitrate. Hence, some of the polypeptides induced by nitrate or chloride are probably related to the general enhancement in ion uptake that occurs following treatment of root segments in aerated solution and may not be specific to either ion.

DISCUSSION

One- and two-dimensional gel electrophoresis of [^{35}S]methionine labeled polypeptides of the plasma membrane fraction revealed that incubation of excised roots from corn seedlings in an aerated solution containing 5 mM nitrate enhanced the synthesis of several polypeptides (Figs. 2–6). While several of these polypeptides were also apparently induced by treatment in an aerated solution of chloride (Fig. 6), others (*e.g.* about 30 and 40 kD) appeared to be specifically induced by nitrate. It is not known if any of the nitrate-induced polypeptides of the plasma membrane fraction are directly or indirectly involved in the enhancement in the rate of nitrate uptake (Fig. 7). However, the polypeptides were fully induced by 2.5 h, which is similar to the length of the lag phase observed before nitrate uptake reaches a steady state induced level (9, 16, 17, 24).

McClure *et al.* (22) found that nitrate treatment enhanced the synthesis of a 31 kD polypeptide in a microsomal fraction from corn roots but found no consistent differences in [^{35}S]methionine labeled polypeptides in the plasma membrane fraction. We observed enhanced synthesis of 30 and 47 kD polypeptides in a microsomal fraction (Fig. 2), as well as several polypeptides in the plasma membrane fraction following treatment in an aerated nitrate solution. In addition to the technical difficulties of comparing polypeptides of membrane preparations isolated from control and treated root samples, there is a difference in the procedure used for nitrate treatment that may contribute some uncertainty when comparing the results from the two laboratories. McClure *et al.* grew the corn seedlings in an aerated nutrient solution for several days before treating with nitrate in a similarly aerated solution. In our experiments, roots were excised from seedlings germinated on moist absorbent paper and transferred to aerated treatment solutions. This procedure is well documented to produce a general enhancement of ion uptake in addition to an apparent induction in nitrate transport in response to the presence of nitrate (24, 32). We used treatment with chloride as a control for a general enhancement in ion transport induced by excision and transfer to an aerated solution, but we cannot be certain about the adequacy of this control.

Roots treated for several hours in aerated solution may show symptoms in common with flooded roots because of lower oxygen concentration in water than in air. When roots are subjected to flooding, cytoplasmic acidosis occurs leading to root death (27). A nitrate pretreatment of corn root tips greatly reduced cytoplasmic acidosis, presumably by inducing the capacity to use excess protons (26). It is possible that, in the present study, the nitrate uptake system is induced in response to partial hypoxia, which could result when the roots of seedlings grown on absorbent paper are transferred to the nitrate (or chloride) pretreatment solution. Several polypeptides with molecular masses of about 33 kD are known to be induced under anaerobic conditions in maize seedlings (29), and these may account for some of the polypeptides induced by treatment in the aerated nitrate or chloride solutions. However, other polypeptides were specific to the nitrate (or chloride) treatment and may be involved in induction of increased capacity for nitrate transport.

Most of the nitrate reductase activity in corn roots has been localized in the epidermal cells, even after treatment with 20 mM nitrate for 20 h (28). Similar kinetics of induction of nitrate uptake and nitrate reductase activities (3, 4, 24) are consistent with the idea that nitrate transport and reduction may be maximally active in the same set of root cells. If such is the case, most of the nitrate absorbed by the root may be into epidermal cells and induction of a nitrate transport system might occur in primarily the epidermal cells. Epidermal cells from less than 10% of the total corn seedling primary root (28), which would add to the difficulty of detecting polypeptides specifically induced by nitrate.

While there is evidence suggesting that sustained RNA and protein synthesis is required for induction of enhanced nitrate transport capacity (21, 23), it is not known if induction of nitrate transport occurs by activation of existing nitrate carriers or by synthesis of new carriers. Hence, polypeptides induced in these studies in response to nitrate may or may not be part of a nitrate transport system. These results will be used in combination with other approaches (*e.g.* using radiolabeled inhibitors of nitrate transport to label polypeptides of a nitrate carrier) in future attempts to identify and characterize molecular components of a nitrate transport system at the plasma membrane.

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