In saline environments, plants accumulate \( \text{Na}^+ \) in vacuoles through the activity of tonoplast \( \text{Na}^+/\text{H}^+ \) antiporters. The first gene for a putative plant vacuolar \( \text{Na}^+/\text{H}^+ \) antiporter, \( \text{AtNHX1} \), was isolated from \( \text{Arabidopsis} \) and shown to increase plant tolerance to \( \text{NaCl} \). However, \( \text{AtNHX1} \) mRNA was up-regulated by \( \text{Na}^+ \) or \( \text{K}^+ \) salts in plants and substituted for the homologous protein of yeast to restore tolerance to several toxic cations. To study the ion selectivity of the \( \text{AtNHX1} \) protein, we have purified a histidine-tagged version of the protein from yeast microsomes by \( \text{Ni}^{2+} \) affinity chromatography, reconstituted the protein into lipid vesicles, and measured cation-dependent \( \text{H}^+ \) exchange with the fluorescent \( \text{pH} \) indicator pyranine. The protein catalyzed \( \text{Na}^+ \) and \( \text{K}^+ \) transport with similar affinity in the presence of a \( \text{pH} \) gradient. \( \text{Li}^+ \) and \( \text{Ca}^+ \) ions were also transported with lower affinity. Ion exchange by \( \text{AtNHX1} \) was inhibited 70% by the amiloride analog ethysopropylamiloride. Our data indicate a role for intracellular antiporters in organelle \( \text{pH} \) control and osmoregulation.

In plants, sodium extrusion from the cytosol and compartmentation in vacuoles are key processes for \( \text{Na}^+ \) detoxification and cellular osmotic adjustment (1). Primary proton pumps provide the driving force for the transport of \( \text{Na}^+ \) against its electrochemical gradient by tonoplast and plasma membrane \( \text{Na}^+/\text{H}^+ \) antipoters (2, 3). Existence of plant \( \text{Na}^+/\text{H}^+ \) antipoters was first inferred from biochemical studies of antiport activity in vacuoles or tonoplast vesicles in many plant species (4). The \( \text{Na}^+/\text{H}^+ \) exchange activity could be inhibited by amiloride analogs and showed \( K_m \) values for \( \text{Na}^+ \) in the range of 25–51 mM (4). Recently the first plant \( \text{Na}^+/\text{H}^+ \) antipoter genes, \( \text{NHX1} \) and \( \text{SOS1} \), were identified and shown to be involved in salinity tolerance in \( \text{Arabidopsis} \) (5, 6). The \( \text{Arabidopsis} \) \( \text{AtNHX1} \) protein localizes to the vacuole, and \( \text{Na}^+/\text{H}^+ \) exchange could be detected in tonoplast fractions from transgenic \( \text{Arabidopsis} \) overexpressing the protein (5). \( \text{Na}^+/\text{H}^+ \) exchange activity could also be measured in vacuolar membrane fractions of yeast expressing the \( \text{AtNHX1} \) protein (7). \( \text{AtNHX1} \) shows greatest sequence homology with the \( \text{ScNHX1} \) protein of \( \text{Saccharomyces cerevisiae} \) and the NHE transporters of mammalian cells (8, 9). \( \text{ScNHX1} \) localizes to the prevacuolar compartment (9). The NHE exchangers comprise a family of eukaryotic membrane proteins catalyzing electroneutral \( \text{Na}^+/\text{H}^+ \) countertransport (10–12). At the plasma membrane of animal cells, these antipoters dissipate the \( \text{Na}^+ \) gradient generated by the \( \text{Na}^+/\text{K}^+ \) ATPase to extrude protons from the cell, playing therefore crucial roles in intracellular \( \text{pH} \) regulation, maintenance of cellular volume, transepithelial \( \text{Na}^+ \) reabsorption, and cell proliferation in response to growth factors (13). To date seven isoforms have been described in animal cells. \( \text{NHE1–NHE5} \) localize to the plasma membrane, whereas \( \text{NHE6} \) and \( \text{NHE7} \) reside in mitochondrial and Golgi membranes, respectively (14–16). The \( \text{AtNHX1} \) and \( \text{ScNHX1} \) proteins are most closely related to isoforms \( \text{NHE6} \) and \( \text{NHE7} \), thus confirming a novel family of organellar or endosomal antipoters (8, 9).

\( \text{AtNHX1} \) can functionally substitute for the \( \text{ScNHX1} \) protein of yeast (8, 17). Both \( \text{NHX1} \) proteins impart tolerance to \( \text{Na}^+ \) and \( \text{Li}^+ \) through ion sequestration inside vacuoles, but they also mediate resistance to other toxic cations such as \( \text{TMA}^+ \) or hygromycin B (8, 17, 18). Moreover, \( \text{ScNHX1} \) was required for yeast tolerance to acute hypertonic shock, and \( \text{AtNHX1} \) mRNA accumulated in plants treated with KCl (8, 19). These findings have raised questions on the specificity of cation transport by NIX exchangers that need to be addressed. In contrast to their animal counterparts, structural or functional studies on the only recently cloned plant and fungal antipoters are just starting. Biochemical approaches have to be adopted to progress in the understanding of the structure-function relationships and regulations of the NHX-like transporters. Such studies are most conveniently done using highly purified protein. In this paper we describe a method for the purification of the \( \text{Arabidopsis} \) \( \text{Na}^+/\text{H}^+ \) antipoter \( \text{AtNHX1} \) by \( \text{Ni}^{2+} \) affinity chromatography. The purified protein could be functionally reconstituted into liposomes, and its transport characteristics could be determined. Contrary to most NHE transporters, high transport activity could be measured in the absence of \( \text{K}^+ \). The monovalent cations \( \text{Li}^+ \) and \( \text{Ca}^+ \) were also transported with lower affinity, whereas \( \text{TMA}^+ \) was not a substrate. This procedure will permit a detailed description of structure-function relationships of the protein as well as identification of regulatory mechanisms and protein-protein interactions.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—A RGS1 histidine tag was inserted into the yeast multicyclic vector pMP658, a derivative of YeEp51 containing the yeast \( \text{PMA1} \) promoter and transcriptional terminator (20). For this, oligonucleotides with sequence 5′-TCGAGGATCGCATCACCATCAC-3′ were annealed into the yeast yeast multicopy vector pMP658, a derivative of YeEp51 containing the yeast \( \text{PMA1} \) promoter and transcriptional terminator (20). For this, oligonucleotides with sequence 5′-TCGAGGATCGCATCACCATCAC-3′ were annealed 1. The abbreviations used are: TMA, tetramethylammonium; BTP, Bis-Tris propane; MES, 4-morpholineethanesulfonic acid; EIPA, ethylisopropylamiloride; MIA, 5-(N-ethyl-N-isopropyl)amiloride.
CATCAGTGAA-3 (forward) and 5'-CTAGTTGATGATGATGATGGTG-GATGCGGATCC-3' (reverse), encoding a RGS12 tag followed by a stop codon after the last histidine and flanked by XhoI and SpeI restriction sites, were ligated between the PM1A promoter and terminator of pMM65, resulting in plasmid pKV61. The AnNHX1 gene was amplified from pAtNHX1-1 (17), using a forward primer 5'-GCTATCTC-GGCAAATGATTGATC-3' and a reverse primer 5'-GGCCGTG-GAGCCTTACGATGATCT-3', resulting in a sequence with two terminal XhoI sites and without stop codon at the C-terminal end. This AnNHX1 fragment was ligated into plasmid pKV61, giving rise to plasmid pKV62. The construct was verified by restriction enzyme digestion and sequencing. For yeast complementation tests, the pKV62 construct was verified by restriction enzyme digestion and fragment was ligated into plasmid pKV61, giving rise to plasmid pAtNHX1-1 and pAtNHX1-H6 were transferred into S. cerevisiae W303-1B derivative AXT3K (Mata leu2-13, 112 ura3-1 trpl-1 his3-11, 15 ade2-1 can1-100 Δnhx1::KanMX ana1::LEU2 Δena1-4::HIS3) lacking the endogenous NHX1 protein, as well as the plasma membrane efflux transporters NHA1 and ENA1-4. Yeast transformants were grown in selective YDA-glucose medium (22). A saturated 3-ml preculture in SDA-galactose and grown at 30 °C in YPD (1% yeast extract, 2% peptone, 2% glucose). For reconstitution of AtNHX1:RGSH6, plasmid pAtNHX1-1 and pAtNHX1-H6 were transformed into S. cerevisiae W303-1B strain RS72 (23) with a 50-kDa cut-off. The sample was frozen in liquid nitrogen and stored at −80 °C.

**Functional Testing of Recombinant AnNHX Proteins—** Yeast cells were grown at 30 °C in YPD (1% yeast extract, 2% peptone, 2% glucose) or YDA-glucose medium. This culture was then inoculated into 6 liters of YPD medium and grown for 20 h. The cells were harvested, and the microsomes were isolated as described (23). The microsomal membrane fraction (4 ml, 5 mg protein/ml) was mixed with 20 ml of solubilization buffer (50 mM KH2PO4, pH 7.4, 500 mM NaCl, 10 mM imidazole, 20% glycerol, 0.5% n-dodecyl-β-D-maltoside, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml chymostatin, 2 μg/ml pepstatin) and incubated for 30 min at 4 °C under gentle shaking. Unsolubilized material was removed by centrifugation for 30 min at 30,000 g and the supernatant was mixed with 1 ml of Pentadentate Chelator resin charged with Ni2+ (AffiLynx) and incubated overnight at 4 °C with gentle stirring. The resin was then poured into a polypropylene column and washed with 3 × 4 ml of buffer A, 3 × 4 ml of buffer B, and 1 × 4 ml of buffer A (buffers supplemented with protease inhibitors as above and 0.15% n-dodecyl-β-D-maltoside), according to the manufacturer’s instructions. Thereafter the protein was eluted with an imidazole step gradient containing 0, 100, 200, 300, and 1000 mM imidazole, pH 7.4, in 50 mM KH2PO4, pH 7.4, 500 mM NaCl, 20% glycerol, 0.075% n-dodecyl-β-D-maltoside, 2 μg/ml pepstatin, and 0.2 mM phenylmethylsulfonyl fluoride. The purified protein eluted in the 100–200 mM imidazole fractions. Finally, the 200 mM fraction was concentrated to 500 μl using a centrifugal concentrator (Pall Microsep) with a 50-kDa cut-off. The sample was frozen in liquid nitrogen and stored at −80 °C.

**Gel Electrophoresis and Western Blotting—** Membrane fractions or purified proteins were separated by SDS-PAGE on 10% acrylamide using the system of Laemmli (24). Western blotting was performed as described (25). After protein electrotransfer to a polyvinylidene difluoride membrane (Pall Gelman), the blot was incubated with a monoclonal antibody raised against the RGS12 epitope (Quagen, Chatsworth, CA).

**Reconstitution of AnNHX1:RGSH6 into Vesicles—** For reconstitution of purified AnNHX1:RGSH6 into artificial membranes, essentially the same protocol was used as previously developed for reconstitution of the purified His-tagged plasma membrane proton ATPase AHA2 (23). Protein (2 μg) was mixed with soybean phospholipids type H-S (Sigma) at a lipid to protein ratio of 5:1 in a total volume of 280 μl of reconstitution buffer containing 20 mM BTP-MES, pH 7.5, 10% glycerol, 25 mM (NH4)2SO4, and 2.5 mM pyrinate (converted to BTP salt using Dowex 50W×8 ion exchange resin). The sample was solubilized by the addition of 12 μl of 1 M octylglucoside and loaded onto a 2.5 ml spin column filled with Sephadex G-50 (fine; Amersham Biosciences, Inc.) preloaded with 200 μl of 2.5 mM pyrinate in reconstitution buffer. After centrifugation for 5 min at 180 × g, the eluate was incubated for 30 min at room temperature with 100 mg of wet BioBeads (SM-2; Bio-Rad) and passed again over a 25-50 spin column.

**Measurement of Cation/H+ Exchange in Vitro—** Pyranine fluorescence was recorded in 463-nm excitation wavelength and 510-nm emission wavelength. Fluorescence of the sample was adjusted to adequate level of fluorescence using the sample with reconstitution buffer. Next, 50 μl of liposomes containing AnNHX1:RGSH6 were diluted in reconstitution buffer without (NH4)2SO4 in a 1 ml stirred reaction cuvette thermostated at 20 °C. The 20-fold NH4+ dilution resulted in acid loading of the vesicles because of outward diffusion of NH3 (26). The resulting pH inside the vesicles determined from the fluorescence of pyranine was 6.6. Thereafter, infinite inward cation gradients were imposed by the addition of chloride salts at the outside of the vesicles. Proton efflux coupled to cation influx was monitored from the increase of pyranine fluorescence.

**Quantification of Proton Fluxes and Antiporter Turnover Rate—** The fluorescence signal was calibrated with pH as described (27) and used to calculate the initial rate of pH variation after the addition of cations. An internal vesicle volume of 1.92 liters/mol corresponding to a radius of 30.5 nm was calculated from the fluorescence of entrapped pyranine using a surface area of 75 Å2 for one phospholipid molecule (28) and an approximate molecular mass of 1000 KDa for crude soybean phospholipids (27, 29). The net initial rate of proton efflux was calculated from the initial rate of pH variation as described (27, 30) correcting for the buffer capacity of 20 mM BTP-MES at the starting pH inside vesicles. The number of antiporter molecules/vesicle was estimated from the lipid to protein ratio used during the reconstitution, assuming a molecular mass of 50 KDa for the antiporter. The turnover rate of the antiporter (cycles−1) was calculated from the maximum proton flux and density of the antiporters, assuming the transport of 1 proton/cycle.

**Protein Determination—** Protein was determined by the method of Bradford (31) with the Bio-Rad protein assay reagent and bovine serum albumin as a standard. Concentration of purified protein was estimated from quantification of the intensity of the band on Coomassie-stained SDS gels using bovine serum albumin as a standard and Scion Image software (version 3.62, www.scioncorp.com).

**RESULTS**

**Functionality of the Tagged Protein—** The AtNHX1 protein was tagged with a C-terminal RGS12 tag. To test whether the tagged protein was still functional, yeast strain AXT3K, in which the endogenous Na+/H+ vacular antiporter gene NHX1 is disrupted, was transformed with a yeast multicopy vector containing the gene for the tagged protein or the wild type, nontagged protein. Both constructs restored tolerance to NaCl when expressed in the nhx1 deletion strain (Fig. 1). No phenotypic differences could be detected between both AtNHX1 recombinant strains.

**Expression and Purification of the AtNHX1:RGSH6 Protein—** Microsomal membranes were isolated from yeast cells expressing the tagged AtNHX1 protein or not. Although no extra protein bands could be observed on a Coomassie-stained gel of microsomes of the strain expressing the AtNHX1:RGSH6 protein, indicative of relatively low levels of expression, the

![Fig. 1. Complementation of the cation sensitivity of nhx1 yeast mutants by AtNHX1:RGSH6.](http://www.jbc.org/Downloaded from by guest on July 25, 2018)
tagged polypeptide was detected by Western blotting using a monoclonal antibody raised against the RGSH₄ epitope (Fig. 2) and with a polyclonal antibody raised against the C-terminal domain of AtNHX1 (data not shown and Ref. 17). Two cross-reactive bands were observed in microsomes, one of about 100 kDa and another broader band at ~45–50 kDa (Fig. 2). The absence of a signal in control microsomes proved that the AtNHX1:RGSH₄ protein was responsible for both bands. After solubilization of the microsomal membrane fraction, the 47-kDa band could be purified by immobilized metal affinity chromatography using the pentadentate metal-binding resin Pentadentate Chelator. From Western blotting experiments with the RGSH₄ antibody, we determined that the highest amount of tagged polypeptide was bound to the resin using Ni²⁺ or Zn²⁺ ions (data not shown). Bound proteins were eluted from the resin using an imidazole step gradient. The AtNHX1: RGSH₄ protein was recovered in the 100–200 mM imidazole fractions, as determined with the RGSH₄ antibody (Fig. 3). Because purity of AtNHX1:RGSH₄ was maximal in the 200 mM imidazole fraction, this was the source of recombinant protein for subsequent studies.

**Measurement of Cation/H⁺ Exchange in Vitro**—The 200 mM imidazole fraction was concentrated by ultrafiltration, and the purified AtNHX1:RGSH₄ protein was reconstituted in soybean phospholipid vesicles containing the pH indicator pyranine and in the presence of (NH₄)₂SO₄. Dilution of the proteoliposomes in reconstitution buffer without (NH₄)₂SO₄ resulted in an instantaneous fluorescence diminution of trapped pyranine, reflective of the internal acidification of the vesicles (Fig. 4). The internal pH estimated from the ratio of fluorescence at 404 and 463 nm was 6.60 (Fig. 4, trace a). When vesicles were diluted in the presence of (NH₄)₂SO₄ (no pH gradient), the internal pH as indicated from pyranine fluorescence was 7.55 (Fig. 4, trace b). The variation of pyranine fluorescence (pK₇.2) is approximately linear between pH 6.6 and 7.5 and therefore directly related to intravesicular pH changes (27).

The cation/H⁺ exchange reaction was initiated upon the addition of monovalent cations (80 mM, Cl⁻ salts), and the rate of pH variation inside the vesicles was estimated from the change in pyranine fluorescence (Fig. 4). The most effective cations for dissipating the pH gradient were Na⁺ and K⁺. Li⁺ and Cs⁺ were much less competent, and no recovery of the signal was observed using the organic cation TMA⁺. In control liposomes no significant recovery of pyranine fluorescence was observed upon the addition of these monovalent cations (data not shown). The addition of (NH₄)₂SO₄ fully collapsed the pH gradient in both liposomes and proteoliposomes (Fig. 4). When transport measurements were made at equilibrium pH inside and outside the vesicles and in the presence of the permeant anion SCN⁻, the addition of KCl to the outside still caused intravesicular alkalinization in response to the inward K⁺ gradient (Fig. 5). Vesicle alkalinization was abolished by addition of 10 μM benzamid, a potent inhibitor of Na⁺/H⁺ antiporters (Fig. 5).

The ion exchange reaction showed a saturable kinetics with increasing cation concentrations (Fig. 6). In control liposomes, no significant ion exchange was observed even at the highest salt concentration used (Fig. 7B and data not shown). The affinity of AtNHX1 for Na⁺ and K⁺ was similar, with apparent Km values of 42 and 45 mM respectively. The affinity for Li⁺ was much lower, with a Km of about 226 mM. The maximum initial rate of vesicle alkalinization (Vₘₐₓ) obtained from fitting the data in Fig. 6 was 5.90 × 10⁻³ ΔpH s⁻¹ for Na⁺, which corresponds to 1.34 nmol H⁺·m⁻²·s⁻¹ after calibrating the pyranine fluorescence signal with pH and correcting for buffer

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**Fig. 2. Detection of the tagged protein in yeast microsomes.** Microsomal membranes (25 μg of protein) of control cells (lanes 1) and cells expressing the tagged AtNHX1 protein (lanes 2) were subjected to SDS-PAGE electrophoresis using a 10% acrylamide gel. The gel was stained with Coomassie Brilliant Blue (A) or used for Western blot analysis (B) using a monoclonal antibody raised against the RGSH₄ epitope.

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**Fig. 3. Purification of the AtNHX1-RGSH₆ protein by Ni⁰⁰²⁺ affinity chromatography.** Microsomal membranes were solubilized, and the proteins were bound to Pentadentate Chelator resin as described under "Experimental Procedures." The fractions were eluted with indicated buffers, and the proteins were precipitated with trichloroacetic acid from aliquots before SDS-PAGE electrophoresis. Lane FT, flow through fraction (100 μl); lane A, buffer A wash fraction (1 ml); lane B, buffer B wash fraction (1 ml); lanes 0, 50, 100, 200, 300, and 1000 indicate the millimolar concentration of the imidazole step gradient used for elution. Left panel, Coomassie-stained gel. Right panel, Western blot of the indicated fractions using the monoclonal antibody raised against the RGSH₄ epitope.

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**Fig. 4. Na⁺/H⁺ exchange activity in reconstituted liposomes and ion selectivity of the cation/H⁺ exchange reaction.** Purified protein was reconstituted in liposomes containing ammonium. An acid inside pH gradient was created by 20-fold dilution of proteoliposomes in ammonium free medium at pH 7.5. After dilution the inside pH was 6.60, as estimated from the fluorescence of encapsulated pyranine (left panel, trace a). When proteoliposomes were diluted at equilibrium conditions for ammonium in and outside, no acid inside pH was generated (left panel, trace b). The cation/H⁺ exchange reaction was started by the addition of 80 mM of chloride salts of the indicated cations (right panel, arrow 1). A fast fluorescence recovery, i.e. vesicle alkalinization, was observed upon addition of Na⁺ or K⁺ to the vesicles, indicative of cation/H⁺ exchange. Li⁺ and Cs⁺ were 3–10-fold less efficient. TMA⁺ did not induce vesicle alkalinization. The addition of 25 mM (NH₄)₂SO₄ (right panel, arrow 2) resulted in full recovery of fluorescence.
Inhibition of the cation/H\(^+\) exchange reaction by EIPA.

- The exchange reaction was initiated by adding 80 mM KCl (A, arrow 1) or 500 mM KCl (B, arrow 1). Maximal inhibition was observed by 30 \(\mu\)M EIPA (A, inset). The addition of the K\(^+\)/H\(^+\) exchanger ionophore nigericin (arrow 2) resulted in a rapid augmentation of fluorescence that was not affected by EIPA. In control liposomes (B), the addition of 30 \(\mu\)M EIPA (dissolved in dimethyl sulfoxide (DMSO)) in the presence of 500 mM KCl induced dissipation of the pH gradient as estimated from pyranine fluorescence, indicative of perturbation of the membrane permeability by EIPA. DMSO addition alone did not induce pH gradient dissipation.

**Discussion**

The Tagged AtNHX1 Protein Is Active in Yeast—The histidine tag was added to the C-terminal end of AtNHX1 because the existence of N-terminal signal peptides in NHE type transporters has been suggested (14, 15). As judged from phenotypic comparisons of yeast expressing the tagged and nontagged AtNHX1 proteins, addition of the histidine tag did not alter significantly the activity of the exchanger. Tolerance to Na\(^+\) ions, which relies on ion compartmentation in the vacuole of AYP7K cells (17), was identical for cells expressing either recombinant form of AtNHX1 (Fig. 1). For unknown reasons, the function of the yeast ScNHX1 protein confers greater resistance to the cationic antibiotic hygromycin B (8). Again, both tagged and nontagged AtNHX1 proteins conveyed equal resistance to hygromycin B when expressed in yeast.

Purification of the His-tagged AtNHX1 Protein from Yeast Microsomes—Using a monoclonal antibody raised against the RGSH4 epitope, two cross-reactive bands of 100 and 45–50 kDa were observed in microsomes (Fig. 2). Although the 45–50-kDa band is slightly below the predicted molecular mass of AtNHX1, amiloride indicated that this drug strongly interfered with pyranine fluorescence at the concentration needed to inhibit the process (data not shown). However, the amiloride analogs ethylisopropyl-amiloride (EIPA), 5-(N-isopropyl)amiloride (MIA), and benzamil could be used successfully together with pyranine to inhibit the exchange reaction at low inhibitor concentrations (Table I). The cation exchange reaction, using KCl salts, was inhibited by EIPA in a dose-dependent manner, with a maximal 70% inhibition at 30 \(\mu\)M EIPA and a \(K_{0.5}\) of about 10 \(\mu\)M (Fig. 7). The addition of the K\(^+\)/H\(^+\) ionophore nigericin collapsed the pH gradient, independently of the EIPA concentration. A greater inhibition of AtNHX1-driven cation exchange by EIPA could not be attained in proteoliposomes because higher concentrations of the drug induced significant vesicle alkalinization of control liposomes, probably because of nonspecific perturbation of the lipidic permeability barrier (Fig. 7). Both MIA and benzamil were somewhat more efficient than EIPA, with \(K_{0.5}\) below 10 \(\mu\)M (Table I). Quinine, a compound not related to amiloride and known to inhibit cation/H\(^+\) exchange in mitochondria, could also inhibit the exchange reaction at much higher concentrations (Table I).
The addition of Na\(^{+}\) capacity of the buffers used, this is close to the expected value. The Na\(^{+}\)K\(^{+}\) band found in microsomes likely corresponds to dimers of ScNHX1, which has been suggested to be part of the network that transports Na\(^{+}\) and K\(^{+}\) during protein trafficking and maturation (34). The 100-kDa ScNHX1 is a glycoprotein, AtNHX1 could also become glycosylated during protein trafficking and maturation (34). Because NHX1 sequence, and glycosylation and phosphorylation are well demonstrated for mammalian NHE isoforms (14). Because NHX1 is a glycoprotein, AtNHX1 could also become glycosylated during protein trafficking and maturation (34). The 100-kDa band found in microsomes likely corresponds to dimers of the Na\(^{+}/H\(^{+}\) antiporter. Oligomerization seems to be a common feature of many transmembrane proteins, and dimerization of NHE1 and NHE3 isoforms has been reported (35).

**Transport Assays**—The purified protein could be efficiently reconstituted in pyranine containing liposomes. The pH indicator pyranine has been shown to accurately report intravesicular pH (27) and is extremely sensitive in the range used. An inside acid pH gradient of 0.9 units could be created by 20-fold dilution of ammonium-loaded vesicles in ammonium-free medium (Fig. 4, left panel). Taking into account the buffering capacity of the buffers used, this is close to the expected value. The addition of Na\(^{+}\) at the outside of the vesicles resulted in a rapid initial recovery of fluorescence indicative of Na\(^{+}/H\(^{+}\) exchange. Maximal ion exchange mounted to almost half of the total pH gradient (Fig. 4, right panel). This indicates that only half of the vesicles exhibit Na\(^{+}/H\(^{+}\) exchange and thus contain active antiporter molecules. The density of the antiporters calculated from the lipid to protein ratio used during reconstitution was 8.55 × 10\(^{11}\) m\(^{-2}\). Using the estimated vesicle radius of 30.5 nm, this predicts an average of 1.06 antiporters/vesicle. At this density, random distribution of antiporter monomers into proteoliposomes predicts the presence of inactive vesicles that do not contain an antiporter and vesicles with inside-out proteins that could be inhibited by their cytosolic side facing the acidic intravesicular pH. The observed maximal rate of proton efflux of 1.34 nmol H\(^{+}\) m\(^{-2}\) s\(^{-1}\) together with the density of the antiporters indicates the transport of 9.4 protons s\(^{-1}\) antiporter molecule, without correcting for the presence of inactive vesicles. Thus, assuming a stoichiometry of 1 proton/rotation cycle, the turnover rate of the enzyme would be at least 9.4 protons s\(^{-1}\). This is at the lower end of the range reported for exchangers (36), but even much lower turnover rates have been suggested for the NHE isoforms (37).

The most efficient Na\(^{+}/H\(^{+}\) exchange reaction was observed when an inward gradient of alkali cations was applied in the presence of an outward pH gradient (compare Figs. 4 and 5). These conditions combine a strong driving force for cation/H\(^{+}\) exchange with favorable pH conditions for antiporter activity. However, proton efflux could be electrically coupled to cation influx in these conditions rather than by enzymatic catalysis of ion fluxes. Notwithstanding this possibility, vesicle alkalization was also observed when an alkali cation gradient was applied without a pH gradient and in the presence of the lipophilic anion SCN\(^{−}\), likely to short circuit any indirect electrical coupling of cation/H\(^{+}\) exchange (Fig. 5). Moreover, vesicle alkalization was abrogated by benzamil, an inhibitor of cation exchangers. Thus, cation/H\(^{+}\) exchange was directly catalyzed by the AtNHX1 antiporter. Our procedure for transport assays using pyranine and purified enzyme thus permits us to describe transport kinetics on a molecular and quantitative basis, representing an improvement compared with classical indirect assays on tonoplast vesicles using relief of quenching of permeant amine dyes after pH gradient creation by tonoplast V-ATPase.

**Functional Implications of ΔpH-dependent Low Affinity Na\(^{+}\) and K\(^{+}\) Transport**—A rapid recovery of fluorescence (vesicle alkalization) was observed when using Na\(^{+}\) or K\(^{+}\) salts, whereas Li\(^{+}\) and Ca\(^{2+}\) were much less efficient. The organic cation TMA\(^{+}\) did not induce fluorescence recovery (Fig. 4). In animal NHE-like antiporters, the cation-binding site is not totally selective for Na\(^{+}\), because it can also accommodate H\(^{+}\) and Li\(^{+}\). By contrast, larger K\(^{+}\) ions have either no effect (NHE2 and NHE3) or inhibit Na\(^{+}\) transport (NHE1) (14). Only recently, the novel isoform NHE7 localized in the trans-Golgi network has been found to transport Na\(^{+}\) and K\(^{+}\) (16). It has been suggested that the concerted interplay of the V-ATPase and cation/H\(^{+}\) antiporters to set the luminal pH of vesicles is important for protein sorting through the exocytic and endocytic pathways. The yeast homolog ScNHX1, localized in the prevacuolar compartment, is required for endosomal protein trafficking (38), adaptation to acute hyposmotic shock (19), and resistance to various toxic organic cations (8). Significantly, a mutation in a gene of *Ipomoea nil* most similar to AtNHX1 abrogated capacity to increase vacuolar pH, a requirement for corolla color transition (39), clearly indicating that plant NHX1-like proteins are instrumental to vacuolar pH regulation. Moreover, the AtNHX1 gene was not only induced in *Arabidopsis* by NaCl but also by treatment with KCl or the dehydration-related hormone ABA (8, 17), thus suggesting a function of AtNHX1 in plant responses to changes in external osmolarity. Our finding that AtNHX1 couples H\(^{+}\) transport in exchange for K\(^{+}\) and Na\(^{+}\) with similar affinity indicates that a primary role of AtNHX1 is pH control and osmotic regulation of organelles and endosomes. The utilization of the more physiological K\(^{+}\) cation may be shared by other endomembrane antiporters, as is NHE7 of mammalian cells (16). We suggest that the emerging family of intracellular cation/H\(^{+}\) antiporters with broad specificity are important for organelle volume regulation and control of luminal pH.

Growth of glycophytes like *Arabidopsis* is severely inhibited in saline conditions, but AtNHX1 overexpression enhanced the salt tolerance of transgenic *Arabidopsis* (5). It is plausible that in land plants that grow in nonsaline conditions the specificity of vacuolar NHX-like Na\(^{+}/H\(^{+}\) transporters has shifted from Na\(^{+}/Li\(^{+}\) toward K\(^{+}/Na\(^{+}\) and that these proteins contribute to salinity tolerance not only through Na\(^{+}\) compartmentation but also by facilitating osmotic adjustment using the readily available K\(^{+}\) in the cytoplasm. However, biochemical studies using tonoplast vesicles of various plant species showed that the antiporter activity was specific for Na\(^{+}\) (40–42). This would indicate that NHX1-like isoforms are not related to the activity seen in these assays. Because the *Arabidopsis* genome contains

| Inhibitors | Concentration | Activity |
|-----------|---------------|----------|
|           | μM | %  |
| Amiloride  | 10 | 101 |
| EIPA      | 1  | 85  |
| MIA       | 10 | 58  |
| Benzamil  | 1  | 43  |
| Quinine   | 10 | 70  |
|           | 100| 57  |
six recognizable NHX isogenes, it is reasonable to expect that variant forms of NHX protein may have distinct substrate specificity and cellular localization.

The affinity of the transport reaction for K⁺ or Na⁺ measured in reconstituted vesicles is quite low, with a $K_m$ of about 45 mM, respectively. AtNHX1 suppressed the NaCl sensitivity of a yeast nhx1 mutant under reduced K⁺ availability, which also indicates that AtNHX1 is a low affinity Na⁺/H⁺ antiporter (8, 17). A $K_m$ of about 17 mM was determined in vacuolar membrane vesicles from yeast expressing AtNHX1 (7), whereas a $K_m$ of about 7 mM for Na⁺ was reported for tonoplast vesicles isolated from transgenic Arabidopsis expressing the AtNHX1 protein (5). Absence of regulatory molecules to efficiently activate the plant enzyme expressed in an heterologous host could be responsible for these discrepancies. Furthermore it is likely that removal of loosely bound yeast regulatory proteins during the purification procedure would result in lower affinities.

Changes in affinity by binding of regulatory molecules or phosphorylation is well documented for NHE-like transporters (14). Therefore, our procedure would be very well suited for the study of regulatory properties of these proteins and the characterization of protein–protein interactions.

Inhibitor Studies—NHE-like Na⁺/H⁺ transporters are inhibited by the diuretic drug amiloride. More specific and potent inhibition is observed by amiloride analogs like EIPA or MIA (43). Pyramine fluorescence at 463 nm is relatively insensitive to low concentrations of these compounds. In the range used, EIPA did not significantly affect fluorescence (Fig. 7). The signal did not vary upon addition of EIPA to the vesicles, nor was the final level after the addition of nigericin altered. The K⁺/H⁺ transport reaction was inhibited up to 70% by EIPA with a $K_{0.5}$ of about 10 μM. This is in accordance with data published for the mammalian NHE1 isoform in reconstituted membrane vesicles from yeast cells expressing the protein (44). In control liposomes the addition of EIPA had the opposite effect, which was most clearly seen using a very strong inward K⁺ gradient (Fig. 7B). This indicated that the hypotonic drug also interacts nonspecifically with the lipid bilayer, increasing passive H⁺ and K⁺ permeability and causing dissipation of the pH gradient. Therefore, we could probably not obtain 100% inhibition of the exchange reaction. Like NHE7 of mammalian cells (16), AtNHX1 activity could also be inhibited by quinine (Table I). However, although NHE7 was relatively insensitive to benzamil, this compound was the most effective in inhibiting AtNHX1.

Concluding Remarks—In this paper we have shown that the AtNHX1 protein, when purified and reconstituted in liposomes, can transport Na⁺ and K⁺ with equal affinity. This would imply a function of this protein in K⁺ transport for endosomal osmoregulation and/or pH control under physiological growth conditions and in Na⁺ detoxification upon saline stress. The purification and reconstitution assay presented in this paper will open an avenue for performance of fine biochemical studies on the catalytic and regulatory properties of this class of plant enzymes.

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*J. Biol. Chem.* 2002, 277:2413-2418.  
doi: 10.1074/jbc.M105043200 originally published online November 13, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105043200

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