The purified tonoplast $H^+$-ATPase from oat roots (Avena sativa L. var. Lang) consists of at least three different polypeptides with masses 72, 60, and 16 kDa. We have used covalent modifiers (inhibitors) and polyclonal antibodies to identify the catalytic subunit of the $H^+$-pumping ATPase. The inactivation of ATPase activity by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (Nbd-Cl, an adenine analog) was protected by MgATP or MgADP, and showed kinetic properties consistent with active site-directed inhibition. Under similar conditions, $[^{14}C]Nbd-C1$ preferentially labeled the 72-kDa polypeptide of the purified ATPase. This binding was reduced by MgATP or 2'- (3')-O-(2,4,6-trinitrophenyl) ATP. Nbd-Cl probably modified cysteinyl-$SH$ or tyrosyl-$OH$ groups, as dithiothreitol reversed both ATPase inactivation and $[^{14}C]Nbd$-Cl binding to the 72-kDa subunit. The finding that N-ethylmaleimide inhibition of ATPase activity was protectable by nucleotides is consistent with the idea of sulphydryl groups in the ATP-binding site. Polyclonal antibody made to the 72-kDa polypeptide specifically reacted (Western blot) with a 72-kDa polypeptide from both tonoplast-enriched membranes and the purified tonoplast ATPase, but it did not cross-react with the mitochondrial or Escherichia coli F$_1$-ATPase. The antibody inhibited tonoplast ATPase and $H^+$-pumping activities. We conclude from these results that the 72-kDa polypeptide of the tonoplast $H^+$-ATPase contains an ATP- (or nucleotide-) binding site that may constitute the catalytic domain.

In higher plant cells, the vacuole maintains and regulates cell turgor, and consequently controls cell expansion. This organelle transports and stores ions and metabolites (1, 2). Recent studies have shown that an anion-sensitive ATPase on the tonoplast (vacuolar membrane) pumps $H^+$ into the vacuole (3), providing the proton-motive force for transport of various solutes, such as Ca$^{2+}$ (4) and anions (5–7).

The tonoplast ATPase from oat roots has been well characterized. It is resistant to orthovanadate, stimulated by C1-, and inhibited by NO$_3$. Both $H^+$ pumping and ATPase activities are sensitive to inhibition by NEM, DCCD, Nbd-Cl, and DIDS (10, 11). The tonoplast ATPase is insensitive to azide or oligomycin (10, 11). These properties are generally shared by the vacuolar ATPase of fungi (12) and a $H^+$-ATPase associated with clathrin-coated vesicles (13) and the chromaffin granule (14). These $H^+$ pumps may represent a third class of ATPases which differ from the E$_1$E$_2$-type (plasma membrane) and the F$_{1}$F$_{0}$-type ATPases.

The purified tonoplast ATPase from plants and fungi is a multimeric enzyme (holoenzyme is 400–500 kDa) which consists of at least three different subunits of mass 67–72, 57–60, and 16 kDa (11, 15–20). The function of the polypeptides is unclear. Two conflicting reports of potential nucleotide-binding subunits have appeared recently. Manolson et al. (15) found that the ATP analog, Bz-ATP, preferentially labeled the 57-kDa polypeptide of the tonoplast ATPase from red beets. However, Bowman et al. (19) found that an adenine analog, Nbd-Cl, labeled a 70-kDa polypeptide of the vacuolar membrane ATPase from Neurospora crassa. The 16-kDa polypeptide from several different tissues is preferentially labeled by DCCD (11, 12, 15, 16). It has been proposed that the 16-kDa polypeptide constitutes a proton channel (11, 12, 15), analogous to the DCCD-binding protein of the mitochondrial F$_{1}$F$_{0}$-ATPase (21).

To identify the catalytic subunit of the tonoplast $H^+$-ATPase from oat roots, we have examined NEM and Nbd-Cl for their effectiveness as active site-directed inhibitors. Nbd-Cl has been a useful probe for the identification and characterization of the nucleotide-binding sites in F$_{1}$F$_{0}$- and E$_{1}$E$_{2}$-type ATPase (22, 23). Here we show that the Nbd-Cl inhibition of ATPase activity follows inactivation kinetics consistent with a site-specific covalent modification by Nbd-Cl. $[^{14}C]$ Nbd-Cl specifically labeled the 72-kDa polypeptide and this binding is protected by a competitive inhibitor, TNP-ATP.

These results provide evidence that the 72-kDa subunit contains the substrate-binding site.

**MATERIALS AND METHODS**

**Plant Material**—Oat seeds (Avena sativa L. var. Lang) were germinated over an aerated solution of 0.5 mM CaSO$_4$. Roots were harvested after 3–5 days of growth.

**Preparation of Tonoplast Vesicles and Purification of the Tonoplast ATPase**—Low density vesicles, enriched in tonoplast ATPase, were obtained by centrifugation of microsomes onto a 6% (w/w) dextran cushion as described previously (11). The ATPase was solubilized with 5% (v/v) Triton X-100 in the presence of 30% (v/v) glycerol, dithiothreitol; DCCD, N,N'-dicyclohexylcarbodiimide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Nbd-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; PBS, physiological buffered saline, pH 7.4; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TNP-ATP, 2'(3')-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate.
and was purified (11) with the following modifications. The solubilized preparation was separated with Sepharose CL-4B, instead of Sepharose 4B, to obtain greater flow rates in the presence of glycerol. As ATP and DTT prevented Nbd-C1 inhibition of the ATPase, they were omitted from the solubilization and elution buffers. MgSO₄ (0.5 mM) was added to stabilize the enzyme activity. Although this modified procedure did not change the apparent mass of the tonoplast ATPase, it did result in the appearance of a few additional polypeptides. Fractions with the highest specific ATPase activities were utilized (11).

**ATPase and Protein Assays**—ATPase activity was measured either by the coupled assay (24) or by P₆₀ release (25). In the former assay, ADP production was monitored spectrophotometrically by measuring NADH oxidation in a coupled lactate dehydrogenase-pyruvate kinase reaction and an ATP-regenerating system (24) in a 1.0-ml reaction mixture (11). Protein concentration was determined according to Lowry (28) after precipitating the proteins twice with 7.5% trichloroacetic acid to remove glycerol (27).

**SDS-Polyacrylamide Gel Electrophoresis**—All samples were precipitated with ice-cold 10% trichloroacetic acid, washed with 100% acetone, and boiled 2-4 min in an electrophoresis sample buffer (11). For Nbd-C1 binding experiments, the reducing agent (DTT) was omitted from the sample buffer. Laemmli gels (28) (10% acrylamide, 0.27% bisacrylamide) were run as described (11). The gels were then washed for 20 min in ice-cold water, equilibrated in Autofluor for 20 min, and dried. Gels were exposed to preflashed (29) Kodak XAR-5 film for 2-3 weeks.

**Proportion of Polyclonal Antibodies to the 72-kDa Subunit**—Antibodies were raised in New Zealand White rabbits. To obtain the purified 72-kDa subunit, the purified holozyme (11) was separated on preparative 10% SDS-polyacrylamide gels. The position of the subunit was determined by staining alignment strips. The 72-kDa polypeptide was cut out and electroeluted from the unstained portion of the gel. The antigen was dialyzed against water and lyophilized. Rabbits were immunized with 20-40 µg of protein by intramuscular injection (30). The first and second immunizations contained Freund’s complete and incomplete adjuvant, respectively, while subsequent injections were in PBS. Booster shots were given at 3-week intervals. After the tier built up (about 5 months), blood was collected and the serum obtained was stored at -70 °C. The antiserum was partially purified with a one-step batch DEAE-cellulose absorption (30) procedure.

**Immunoblotting**—After separating polypeptides by SDSPAGE, the unstained gels were blotted onto diazophenylthioether paper (31, 32). The paper was incubated with antiserum to 72-kDa polypeptide (1:200 dilution) for 3 h at 22 °C; the blots were thoroughly washed (32), and probed with goat anti-rabbit IgG conjugated to alkaline phosphatase. 5-Bromo-4-chloro-3-indolyl phosphate in 0.1 M Tris-Cl, 0.1% NaN₃, and 1.0% agarose at pH 8.3 was utilized for the detection of alkaline phosphatase activity. Reactive bands were visualized as a dark blue color due to the product, indigo.

**Reagents**—[14C]Nbd-C1 (100-120 mCi/mmol) and [3H]NEM (40-50 mCi/mmol) were obtained from Research Products International. Autofluor was obtained from National Diagnostics. TNP-ATP was purchased from Molecular Probes Inc. Diazophenylthioether paper was synthesized (33). Other reagents were obtained from Sigma.

**RESULTS**

**NEM and Nbd-C1 Inhibition of ATPase Activity Is Protectable by ATP and ADP**—We found previously that both NEM and Nbd-C1 inhibition of the tonoplast H⁺-ATPase activity were protected by the substrate, MgATP (10, 11). These preliminary results suggested that both inhibitors were potentially useful probes for identifying the catalytic subunit. The inhibition by NEM and Nbd-C1 were examined in greater detail. Tonoplast membranes were pretreated with NEM or Nbd-C1 at different concentrations, temperature, pH, or time. The pretreatments were terminated by diluting 100-fold and ATPase activity was measured at pH 7.0 and at 25 °C.

Inhibition by NEM was pH dependent with maximum sensitivity at pH 8.0 (Fig. 1). This suggests that NEM may be reacting with the unprotonated sulfhydryl group of a cysteine residue as suggested for NEM inhibition of the plasma membrane ATPase from N. crassa (34). NEM (at pH 8) inhibited ATPase activity with an I₅₀ of 2-3 µM (Fig. 24). At this pH, the protection by MgATP was relatively small compared to pH 7 where the I₅₀ was 20 µM (Fig. 2A).

The reactivity of Nbd-C1 with the tonoplast ATPase was also strongly dependent on both pH and temperature. Fig. 3 shows the loss of ATPase activity as a function of Nbd-C1 concentration at pH 7.0 and 4 °C. Under these conditions, the ATPase was about 50-fold less sensitive to inhibition than at pH 8 (and 23 °C) where the I₅₀ was 0.8 µM (Fig. 2B). It is probable that the increased inhibition is due to the heightened reactivity of Nbd-C1 towards amino groups at alkaline pH (35, 36). Although the inhibition by Nbd-C1 is protected by ATP, inhibition may not be site-directed under these conditions (see below). Therefore all subsequent incubations of Nbd-C1 with the ATPase were conducted at pH 7.0 and 4 °C.

Inhibition by NEM (Fig. 4A) and Nbd-C1 (Fig. 4B) was prevented by the physiological substrate, MgATP, and the competitive inhibitor, MgADP (Kᵢ = 0.35 mM, Ref. 10). MgATP protection of inhibition by NEM and Nbd-C1 was concentration-dependent and saturable. The Kᵢ for MgATP protection against Nbd-C1 inhibition, calculated from several semi-log plots like Fig. 5 (according to Ref. 37), was 0.24 ±
little protection against inhibition by either NEM or Nbd-C1 (Fig. 4B). Adenosine conferred this implied that ATP binding to the catalytic site protects against inhibition by Nbd-C1. MgADP at 0.4 mM protected well protected by MgADP (Fig. 4B).

Kinetics of Nbd-Cl and NEM Inhibition of ATPase Activity—To determine if Nbd-Cl and NEM were valid probes for identifying a catalytic subunit of the ATPase, we conducted a preliminary study to test for site-specific inhibition. In site-specific inhibition, the initial binding of the inhibitor to the enzyme is due to its structural analogy to the physiological substrate (ATP). If the inhibitor is bound at the active site prior to covalent modification, the loss of activity as a function of time should show pseudo first order kinetics, and the rate of inactivation should reach a maximum with increasing concentrations of inhibitor (38, 39). If the presence of substrate retards the rate of inactivation, then a simple interpretation is that the substrate and inactivator compete for binding at the enzyme’s active site.

Tonoplast vesicles were incubated with Nbd-Cl at 4 °C for various times, pH 7.0. ATPase activity was inhibited 33% by 15 μM Nbd-Cl in the absence of ATP. This inhibition was almost completely protected by ATP. The log activity plotted as a function of time showed pseudo first order kinetics, and the rate of inactivation should reach a maximum with increasing concentrations of inhibitor (38, 39). If the presence of substrate retards the rate of inactivation, then a simple interpretation is that the substrate and inactivator compete for binding at the enzyme’s active site.

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polypeptide was consistently reduced when the ATPase was preincubated in the presence of MgATP. In the experiment shown, Nbd-Cl binding was reduced 33% by 3 mM ATP and 86% by 10 \( \mu M \) TNP-ATP (Fig. 9A). The protection by MgATP (33%) was less than might be expected from the near complete protection of activity (Figs. 4B and 5). A large component (67%) of the Nbd-Cl (not protected by ATP) may be bound to reactive site(s) on the 72-kDa polypeptide that are not directly related to catalysis. The near complete protection by TNP-ATP would suggest that some of these reactive sites may bind nucleotides. The results, nevertheless, show that a portion (33%) of the \([^{14}C]Nbd-Cl\) was bound to sites protectable by the substrate.

Although initial experiments suggested that NEM could be useful in probing the functional subunits of the ATPase, \( [^{14}C] \) NEM labeled several polypeptides (including a 72-kDa polypeptide) in the purified ATPase preparation, and little specific MgATP protection could be discerned from these experiments (not shown). This may be due to NEM modification of several sites on the subunits of the ATPase.

Reversal of Nbd-Cl Inhibition and Binding by DTT — The carbonion ion, formed as chloride leaves Nbd-Cl, is highly reactive with nucleophiles (such as —SH, —OH, and —NH_2) and can form 4-substituted 7-nitrobenzofurazan derivatives (42). Nbd-Cl could potentially react with cysteine, tyrosine, or lysine residues (42). To distinguish between these alternatives, we tested the effect of DTT on Nbd-Cl inhibition and binding. Sulphydryl reducing agents can reverse N-Nbd or tyrosyl O-Nbd adducts but not N-Nbd adducts (22, 43). Dithiothreitol partially reversed Nbd-Cl inhibition (75%) (Table I) suggesting that the site of inhibition on the tonoplast ATPase contains sulphydryl (cysteinyl) or hydroxyl (tyrosyl) groups. Consistent with these results, dithiothreitol reduced \( [^{14}C] \) Nbd-Cl binding to the 72-kDa polypeptide of the purified ATPase. It is clear that a substantial amount (at least 65%) of the Nbd-Cl binding was reversed (compare lane 1 in Fig. 9A to lane 2 in Fig. 9B). These results suggest that the nucleotide-binding site on the 72-kDa subunit contains either a cysteine or a tyrosine, or both residues. It is possible the remaining (25–35%) \( [^{14}C] \) Nbd-Cl is bound as N-Nbd (44).

Inhibition of Tonoplast ATPase Activity and H^+ Pumping with Antiseras to the 72-kDa Polypeptide — Polyclonal antibodies were made to the 72-kDa polypeptide. The antibody made to the 72-kDa polypeptide was tested for specificity. In Western (immuno) blots, the antibody reacted with a single polypeptide (mass = 72 kDa) in tonoplast-enriched membranes, or the purified tonoplast ATPase (Fig. 10). It did not cross-react with either the mitochondrial or Escherichia coli F_1-

### Table I

| Pre-treatment | ATPase activity | Immunoblot | (Anti Tp-72) |
|---------------|-----------------|------------|-------------|
|               | control         | control    | control     |
|               | Nbd-Cl          | Nbd-Cl     | Nbd-Cl      |
|               | DTT             | DTT        | DTT         |

**DISCUSSION**

In this paper we have used Nbd-Cl as an active site-directed probe to identify a putative catalytic subunit of the tonoplast H^+·ATPase complex. We first showed that Nbd-Cl inhibition of ATPase activity is consistent with a modification of the catalytic site based on the following results. (i) Loss of activity followed pseudo first-order kinetics and the inactivation rate constant saturated with respect to Nbd-Cl concentration (Figs. 5 and 6); (ii) the physiological substrate, MgATP, protected against inactivation by Nbd-Cl with an average dissociation constant of 0.24 mM, similar to the \( K_m \) for MgATP hydrolysis of 0.25 mM (10, 11); and (iii) MgADP, the product and a competitive inhibitor, protected against inactivation by Nbd-Cl (Fig. 4B). These results support the idea that Nbd-Cl, an adenine analog, binds and modifies the cat-
The catalytic site may contain a cysteine, a tyrosine, or both residues. The ATPase is inhibited by NEM (Fig. 2A; Refs. 10 and 11) suggesting an essential role for —SH groups. The reversal of Nbd-Cl inhibition of activity by DTT (Table I), suggests that a cysteinyl —SH or tyrosyl —OH group had been modified (22, 43). We hope to identify the chemical nature of the Nbd derivatives from their UV spectra, fluorescent properties, and by amino acid analyses. Such studies will demonstrate whether a tyrosine residue is in the catalytic site of the tonoplast ATPase as was found for the β subunit of the F₅-ATPase (22, 43) and the Na/K-ATPase of eel electroplax (23).

Our findings with the tonoplast ATPase from a higher plant tissue confirm and extend a recent report of the vacuolar ATPase from the fungus, N. crassa. Although Bowman et al. (19) did not demonstrate whether Nbd-Cl modified the active site specifically, they showed that Nbd-Cl binding to a 70-kDa polypeptide of the vacuolar membrane ATPase was protected by ATP. In a recent study with tonoplast ATPase from corn coleoptiles, it was also concluded that the 72-kDa polypeptide is the catalytic subunit (47).

The role of the 60-kDa polypeptide is less clear. We have observed ATP-protectable, [¹⁴C]NEM binding to both the 60-kDa as well as the 72-kDa subunit.³ Manolson et al. (15) showed that Bz-ATP (an ATP analog) preferentially bound to the 57-kDa polypeptide of the tonoplast ATPase from red beet, but it did not inhibit with simple competitive kinetics. Taken together, the results show that the 57–60 kDa subunit also has a nucleotide-binding site, possibly a regulatory site as in the mitochondrial F₁-ATPase (48). Alternatively, the catalytic site may be composed of both the 72-kDa and the 60-kDa polypeptides, and different probes may react with different portions of the same nucleotide-binding pocket, as postulated for the α and the β subunits of the mitochondrial F₁-ATPase (49).

Although the H⁺-ATPases from clathrin-coated vesicles (13) and chromaffin granules (14) are inhibited by Nbd-Cl, the catalytic subunits of these enzymes have not as yet been identified. The polypeptide profiles of the partially purified ATPases from clathrin-coated vesicles (13) or chromaffin granules (14) show sufficient similarities (major polypeptides at about 70, 60, and 16 kDa) with the plant tonoplast H⁺-ATPase to suggest these enzymes may belong to one class of H⁺-ATPases. Furthermore, the remarkable similarity of these ATPases to that of the primitive anaerobic bacteria, Clostridium pasteurianum (50), suggests that these ATPases may have had an evolutionary antecedent in the prokaryotic kingdom.

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Supplement to:
PROBING THE CATALYTIC SUBUNIT OF THE TONOPLAST H+-ATPase FROM OAT ROOTS

FIG. 1. NEM inhibition of tonoplast ATPase activity at a function of pH. Tonoplast membranes were treated with 2 mM NEM at the pH indicated for 12 min at 0°C. The pretreatment was terminated by a 100-fold dilution into the reaction mixture. The optima of ATPase activity at each pH was calculated from the difference in activity in the presence and absence of NEM.

FIG. 2. ATPase activity as a function of pH. ATPase activity was measured in the presence of 1 mM ATP and 1 mM MgATP at pH 7.5 and 25°C. The ATPase activity was determined by a 100-fold dilution into the reaction mixture. Pi released after 35 min at 35°C was measured.

Nbd-Cl (M)
Nbd-C1 Binding to the 72-kDa Subunit of the Tonoplast H+·ATPase

**FIG. 4.** Protection against NEM (A) and Nbd-C1 (B) inhibition by ATP, ADP, or adenosine. Tonoplast vesicles were preincubated with 100 µM NEM or 20 µM Nbd-C1 in the presence of the indicated concentrations of MgATP, MgADP, or Mgadenosine for 30 min at 4°C (pH 7.0). After dilution, ATPase activity was measured with a coupled assay as in Fig. 3. ATPase activities of membranes preincubated in the presence of ATP, ADP, or adenosine alone were set to 100%.

**FIG. 5.** Effect of Nbd-C1 concentration on the rate constant of ATPase inactivation. ATPase activities were measured after membranes were incubated with Nbd-C1 for 0 to 12 min under the conditions described in Fig. 3 (in the absence of ATP). The results were plotted as in Fig. 3 and the inactivation rate constants were calculated from the slopes. The double reciprocal plot (inset) yielded a limiting inactivation rate constant of 52 min⁻¹ and a kᵦ of 26 µM.

**FIG. 6.** Inhibition of tonoplast ATPase activity by NEM. Membranes were preincubated at pH 7.0 and 4°C with NEM alone (at 1, 10, or 30 µM), 30 µM ATP, or 0.5 mM NEM and 30 µM MgATP (C). Membranes were then diluted and assayed for ATPase activity as in Fig. 3. Percent activity is plotted on a log scale.