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Inhibition and Labeling of the Plant Plasma Membrane H^+-ATPase with *N*-Ethylmaleimide[1]

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ABSTRACT

H^+-ATPase activity in plasma membranes isolated from *Avena sativa* root cells is inhibited by *N*-ethylmaleimide, a covalent modifier of protein sulfhydryl groups. The rate of inhibition is reduced by ADP, MgADP, and MgATP, but even at 40 millimolar ADP the enzyme is only partially protected against inactivation. When plasma membranes are treated wth *N*-[2-^{3}H]ethylmaleimide and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, prominent radioactive bands appear at M_r=100,000 and several other positions. However, only radioactivity in the M_r=100,000 protein is reduced by the presence of MgADP. These results provide independent evidence that the M_r=100,000 polypeptide which is observed in purified preparations of the enzyme is the catalytic subunit of the H^+-ATPase. When tryptic peptides are produced from *N*-[2-^{3}H]ethylmaleimide labeled M_r=100,000 protein and separated by reverse phase high performance liquid chromatography, two radioactive peaks are observed for which *N*-[2-^{3}H]ethylmaleimide incorporation is reduced in the presence of MgADP.

An electrogenic proton pump (H^+-ATPase) has been identified in the plasma membrane of fungi and higher plants (6, 15). The enzyme converts chemical energy derived from hydrolysis of ATP into a protonmotive force. This protonmotive force in turn drives the transport of solutes across the membrane and in plants may also be involved in regulating growth (15). The structural and enzymological properties of the H^+-ATPase relegate this protein to a group of cation-translocating ATPases which includes the (Na^+, K^+)-ATPase of animal cell plasma membranes, the Ca^{2+}-ATPase of sarcoplasmic reticulum, the (H^+, K^+)-ATPase of gastric mucosa and the K^+-ATPase of *Escherichia coli*. All of these enzymes have a catalytic subunit of M_r=100,000 which forms a phosphorylated intermediate during the reaction cycle and all are sensitive to inhibition by vanadate. At present, the fungal H^+-ATPase is much better characterized than the plant H^+-ATPase and, in fact, procedures to prepare nearly pure plant H^+-ATPase have been reported only recently (16, 21). For unknown reasons, the plant enzyme is partially denatured during purification, and relatively low specific activities are obtained compared with purified preparations of related enzymes.

Recently, complete amino acid sequences for the M_r=100,000 catalytic subunit of certain of these enzymes have been deduced from nucleotide sequences (8, 12, 17, 18) and the polypeptides have conserved regions showing sequence homology (17). Several of these enzymes have been reported to be inhibited by covalent sulfhydryl reagents, such as NEM[3] and 5,5′-dithiobis-(2-nitrobenzoic acid) and are partially or completely protected from inhibition in the presence of nucleotides (4, 24, 26). This may indicate a conserved functional domain containing cysteine whose accessibility to these inhibitors is decreased sterically or allosterically by nucleotides. Neither the location of the involved cysteine(s) nor the surrounding sequence has been determined in any of the proteins.

We have investigated the nucleotide-protectable inhibition of the H^+-ATPase of oat roots by NEM. This information was used to determine conditions in which [^{3}H]NEM could be targeted to the essential cysteine(s) which are protected by MgADP. As a first step to determining the location of the essential cysteine(s) in the primary sequence, we have tentatively identified two tryptic peptides whose reaction with NEM is decreased by MgADP.

MATERIALS AND METHODS

Plasma Membrane Preparation. Plasma membranes were isolated from the roots of 6-d-old etiolated oat seedlings (*Avena sativa* cv Stout). Membranes were isolated as described by Surowy and Sussman (20). Briefly, a root homogenate was strained through cheesecloth, and then was centrifuged for 10 min at 8,000*g* to remove debris and intact mitochondria. Microsomes were collected by centrifugation at 48,000*g* for 1.5 h and sedimented through a sucrose density gradient. Plasma membranes were collected at the interface between 33% (w/w) sucrose and 46% (w/w) sucrose and stored at −80°C. Immediately before use, plasma membranes were washed with deoxycholate (20) to remove extraneous protein without loss of ATPase.

Reaction with NEM. Deoxycholate-washed membranes were resuspended in 50 mM Tris-HCl (pH 8.0), 100 mM KCl, containing 10% (w/w) sucrose, hereafter called NEM reaction medium. The buffer also contained 1 mM EDTA, except where indicated. In labeling experiments, the Tris-HCl was replaced by Tricine-KOH. In early experiments, the membranes were pelleted through a sucrose cushion to ensure removal of residual DTT from the original membrane isolation, but this proved to be unnecessary and was omitted from later experiments. The stock solution of NEM was freshly prepared in 1 mM Mes-NaOH (pH 5.5). Where concentrations of free Mg^{2+} and MgADP are indicated, these were calculated using the methods of Wolf and Adolph (25). After membranes were incubated with NEM, the reaction was stopped by a dilution of at least 20-fold into ATPase reaction mix containing 0.005% 2-mercaptoethanol, or by dilu-

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[3] Abbreviations: NEM, *N*-ethylmaleimide; [^{3}H]NEM, *N*-[2-^{3}H]ethylmaleimide; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; DPCC, diphenylcarbamyl chloride.

tion into at least an equal volume of either 50 mM PIPES-Tris (pH 6.7) or 50 mM imidazole (pH 6.7), both containing excess 2-mercaptoethanol.

Labeling of Plasma Membranes with [^{3}H]NEM. Deoxycholate-washed membranes at a protein concentration of 1 mg/ml were first reacted with 50 μM unlabeled NEM for 45 min at room temperature as described above. The reaction was stopped by mixing with an equal volume of cold 50 mM imidazole (pH 6.7), containing 1 mM 2-mercaptoethanol. The membranes were then pelleted at 200,000g for 1 h through 1 ml of NEM reaction medium to remove excess reductant. After resuspension in fresh reaction medium, plasma membranes were reacted with 50 μM [^{3}H]NEM (N-[2-^{3}H]ethylmaleimide (New England Nuclear)) at 1 mg/ml protein concentration at room temperature for 45 min. The radioactive NEM reaction was stopped by mixing with an equal volume of cold 50 mM imidazole (pH 6.7), 10 mM 2-mercaptoethanol and the subsequent pellet was washed twice by resuspension and centrifugation in the same buffer. The final pellets were resuspended in electrophoresis stop buffer containing 125 mM Tris-HCl (pH 6.8), 5% (w/v) SDS, 25% (v/v) glycerol, 3% (v/v) 2-mercaptoethanol, and a trace of bromphenol blue. The samples were then frozen at −80°C prior to electrophoresis.

Polyacrylamide Gel Electrophoresis. Discontinuous SDS-PAGE was carried out according to Laemmli (10). The labeled membranes were run on 7.5% (w/v) acrylamide gels and stained with Coomassie blue. Fluorograms were made of stained and destained gels which had been soaked in Amplify (Amersham) for 15 min, and dried onto filter paper. Bands containing [^{3}H] NEM were visualized by exposure of X-Omat AR film (Kodak, Rochester, NY) to the gel at −80°C. Some of these bands were cut out, digested in 30% H_2O_2 containing 1% NH_4OH, and counted in scintillation fluid (3a70b, Research Products International, Mount Prospect, IL).

Trypsinization of Gel Slices and Separation of Peptides by Reverse-Phase HPLC. The stained band at M_r=100,000 was cut out of the gel and trypsinized as described (1), with modifications. The gel slice was homogenized in 50 or 100 mM NH_4HCO_3 (pH 8.0), 0.1 mM $CaCl_2$, containing 50 to 100 μg/ml DPCC-treated trypsin (type XI, Sigma Chemical Co.) and incubated at 37°C. The homogenate was then briefly centrifuged to remove gel particles, and the supernatant was filtered (Millex-GV, Millipore, Bedford, MA). Filtered supernatant was lyophilized and stored at −80°C until use. Peptides were separated by HPLC using a Vydac 218TP54 reverse-phase C-18 column (The Separations Group, Hesperia, CA). Prior to injection, the dry peptides were redissolved in 100 μl 50% (v/v) acetic acid. The chromatogram was developed with a 60 min linear gradient from 0 to 57% acetonitrile, with 0.1% HCOOH throughout, at a flow rate of 1 ml/min. Fractions were collected every 0.5 min and counted in scintillation fluid.

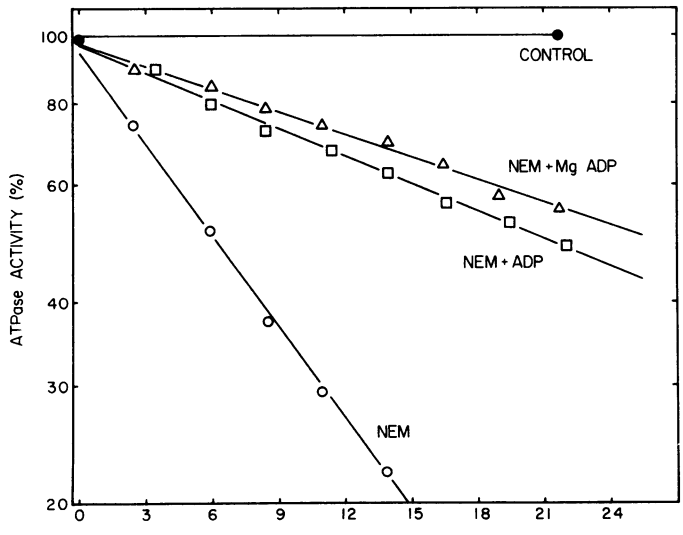

FIG. 1. Time course of NEM inhibition in the presence and absence of ADP or MgADP. Plasma membranes (1.2 mg protein/ml) were incubated on ice with 1 mM NEM. The NEM reaction medium contained 1 mM EDTA and either no $MgCl_2$ or ADP (○), or 5 mM ADP (□), or 5 mM MgADP and 5 mM free Mg^{2+} (△). At various times after NEM was added, aliquots were diluted into pH 6.7 ATPase reaction medium containing 0.005% (v/v) 2-mercaptoethanol and assayed for residual activity. Controls (●) were incubated on ice in the absence of NEM. ATPase activity at 100% was 1.2 μmol phosphate released/mg protein·min.

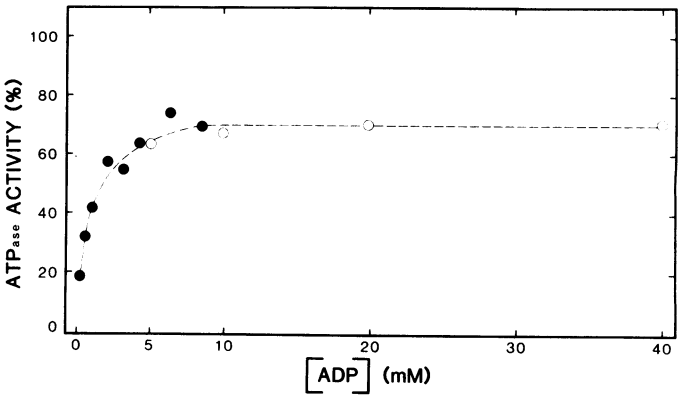

FIG. 2. NEM inhibition as a function of ADP concentration. Plasma membranes (1.4 mg protein/ml) were incubated on ice with 1 mM NEM as described. The NEM reaction medium contained 0.7 mM EDTA and 0 to 40 mM ADP. In the first experiment (●), the incubation was stopped after 20 min by dilution into ATPase reaction medium containing 2-mercaptoethanol, and there was one aliquot per ADP concentration. For this experiment, 100% activity was 1.2 μmol phosphate released/mg protein·min. In the second experiment (○), 9 aliquots were taken from each of the 4 ADP concentrations at various times between 0 and 60 min after addition of NEM and the reaction stopped as before. The data points shown represent the percent activity remaining at 20 min estimated from a logarithmic regression of activity against time for these 9 aliquots. The activity at 0 time (100% activity) was 0.9 μmol phosphate released/mg protein·min. The pseudo first-order decay constants show no significant difference in the rate of decay between 10 and 40 mM ADP.

ATPase Assay. ATPase activity was assayed colorimetrically by release of Pi from ATP, as described (20). To stop any further NEM reaction, 2-mercaptoethanol was sometimes included in the reaction mixture, but had no effect on the assay.

Protein Determination. Plasma membrane protein was determined by the method of Lowry *et al.* (11), using BSA as standard (fraction V, Sigma Chemical Co.).

Materials. Oat seeds were obtained from Olds Seed Co., Madison, WI. ATP was purchased from Boerhinger Mannheim, Indianapolis, IN. Gel reagents were from BioRad Labs, Richmond, CA. All other reagents were of the best available quality, and were purchased from Sigma Chemical Co.

RESULTS

Inactivation of ATPase by NEM. The H^+-ATPase of oat root plasma membranes is inhibited by NEM. At 2°C (pH 8), 1 mM NEM totally eliminates all activity in 20 min (Fig. 1). This inactivation shows pseudo first-order kinetics, with no deviation from linearity on a semi-log plot of activity *versus* time down to 5% of the initial activity (data not shown).

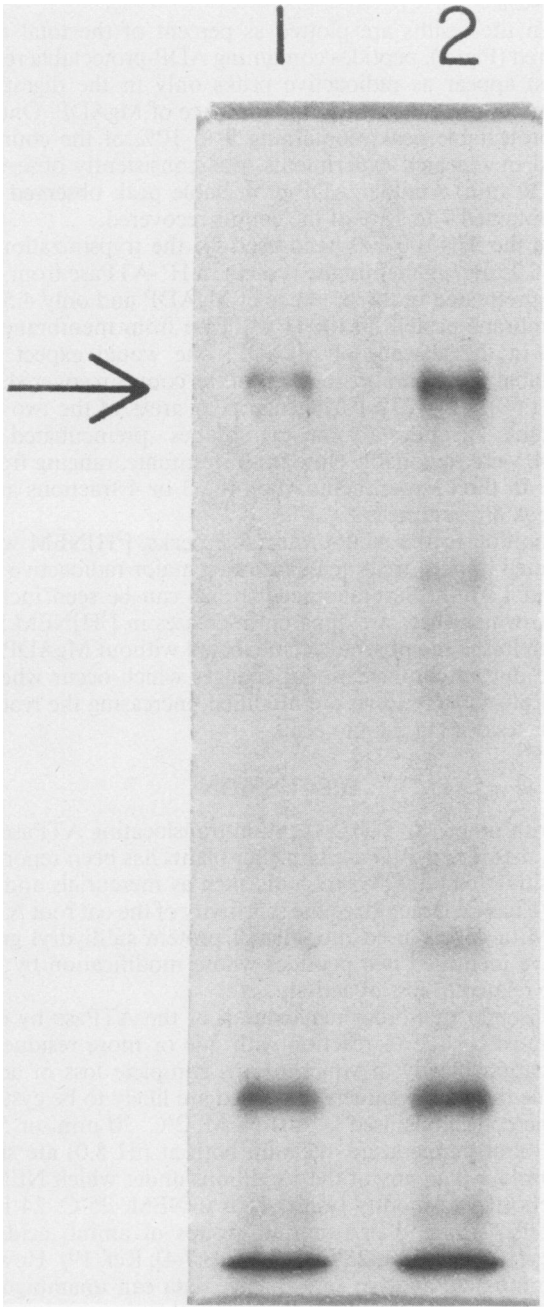

FIG. 3. [^{3}H]NEM-labeled membranes analyzed by SDS-PAGE and fluorography. Plasma membranes (1 mg protein/ml) were prereacted with 50 μM NEM at room temperature in the absence (lane 1) or presence (lane 2) of 10 mM ADP and 15 mM $MgCl_2$. After the reaction was stopped, the membranes were labeled with 50 μM [^{3}H]NEM (2.3×10^{13} Bq/mol) in the presence (lane 1) or the absence (lane 2) of 10 mM ADP and 15 mM $MgCl_2$. After labeling, the membranes were pelleted, washed, resuspended in electrophoresis stop buffer, and frozen at −80°C. Aliquots of 5 μl (4.2 mg protein containing 22,000 dpm) were run on 7.5% acrylamide SDS gels and fluorographed as described in experimental procedures. The arrow denotes position of a M_r=100,000 band which comigrates with protein that is immunologically reactive with antibody to purified H+-ATPase. When cut out, digested with 30% H_2O_2, and counted, this band contained 19.5 Bq in lane 1 and 26.7 Bq in lane 2.

Protection Against Inactivation by Nucleotides. Either ADP or MgADP greatly reduces the rate of inactivation by NEM (Fig. 1). MgADP protects ATPase activity slightly better than does ADP alone, but in either case, the rate of inactivation is pseudo first-order. MgATP also protects against inactivation of the ATPase by NEM, though it does not protect as well, on a molar basis, as MgADP (data not shown). For further studies we used ADP rather than ATP to avoid problems with nucleotide hydrolysis. The protection against NEM inactivation by ADP was incomplete, even at an ADP concentration of 40 mM (Fig. 2).

Labeling of Membranes with [^{3}H]NEM. To identify the essential residue(s) whose rate of modification by NEM is reduced by ADP, we wished to specifically label these residues with [^{3}H]NEM. To ensure adequate label incorporation into the ATPase of intact membranes at reasonable cost, it was important to maximize the specific radioactivity of [^{3}H]NEM. Thus, labeling had to be performed at low NEM concentrations. Preliminary experiments indicated that inactivation with low concentrations of NEM had to be performed at room temperature in order for complete inhibition of the ATPase to take place in a reasonable time. At 1 mg/ml protein, 50 μM NEM was the lowest concentration for which the ATPase was 100% inhibited and the kinetics of inactivation were still pseudo first-order.

To reduce the background of label incorporated into residues whose modification is not affected by MgADP, we first pretreated the membranes with 50 μM unlabeled NEM for 45 min at room temperature in the presence and absence of 10 mM ADP and 15 mM $MgCl_2$. During this preincubation, 90% of the ATPase activity was inactivated in the absence of MgADP, while only 35% of the activity was lost when MgADP was present. After stopping the reaction with 2-mercaptoethanol and pelleting the membranes to remove excess reductant, we then labeled with 50

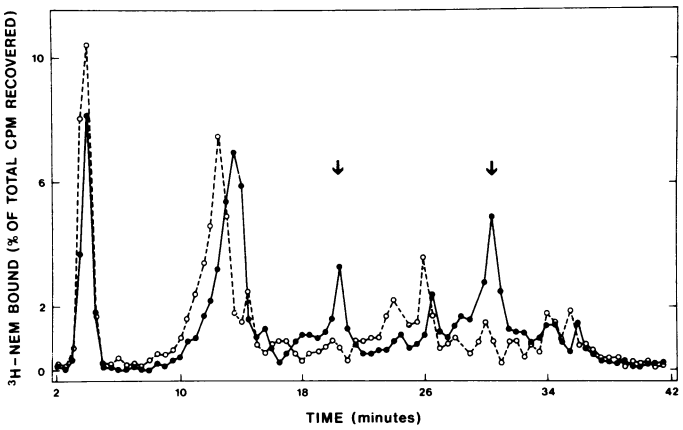

FIG. 4. Reverse-phase HPLC separation of tryptic fragments of [^{3}H]NEM labeled M_r=100,000 protein. The Coomassie-stained, radioactive M_r=100,000 protein obtained by SDS-PAGE of labeled membranes was cut out and trypsinized as described in experimental procedures. Peptide fragments were separated by reverse-phase HPLC using a 60 min gradient from 0 to 57% acetonitrile in 0.1% trifluoroacetic acid. Fractions (0.5 ml) were collected and counted in a scintillation counter. Data are presented as percent of the total cpms recovered which is observed in each individual fraction. The Figure shows a chromatogram of one replicate of each treatment trypsinized for 8 h. (○) and (---) represent membranes pretreated in the absence and labeled in the presence of MgADP and corresponds to lane 1 in Figure 3. (●) and (——) represent membranes pretreated in the presence and labeled in the absence of MgADP and corresponds to lane 2 in Figure 3. Arrows point to peaks which show ADP-protectable labeling. The two ADP-protectable peaks have been observed in chromatograms of five independently trypsinized M_r=100,000 gel bands from two batches of [^{3}H]NEM-labeled membranes.

μM [^{3}H]NEM for 45 min at room temperature. Membranes which had been pretreated with MgADP to protect the residues of interest were now labeled in its absence, while membranes which had been pretreated in the absence of nucleotide were labeled in its presence. In this way we maximized the difference between the two treatments with respect to the amount of label incorporated in MgADP-protected residues. In membranes which were pretreated with NEM in the presence of MgADP and then labeled with [^{3}H]NEM in the absence of MgADP, residues whose reaction with NEM is reduced by MgADP should be specifically labeled. In the converse treatment (pretreatment in the absence of MgADP and labeling in its presence) nucleotide-protectable residues should incorporate little or no label. If loss of activity corresponds to 1 mol NEM/mol essential residue, then one would expect 0.65 mol [^{3}H]NEM/mol essential residue in the former treatment (based on 65% residual activity after the pretreatment and 100% loss of that residual activity during labeling), but only 0.035 mol [^{3}H]NEM/mol essential residue in the latter (based on 10% residual activity after pretreatment, and a 35% loss of that residual activity during labeling). Other residues can show up to 0.25 mol [^{3}H]NEM/mol residue, if the reaction conditions allow 50% reaction with NEM in each incubation.

In membranes labeled as described, the M_r=100,000 band on SDS polyacrylamide gels was the only major radioactive band which showed differences between the treatments in the amount of label incorporated as visualized on a fluorogram of a dried gel (Fig. 3). Furthermore, when labeled membranes were mixed with membranes containing active ATPase and solubilized and subjected to glycerol density gradient centrifugation (16), radioactive M_r=100,000 polypeptide, Coomassie-stained M_r=100,000 polypeptide, and ATPase activity all co-purified (data not shown). These results provide independent evidence that a M_r=100,000 polypeptide which is observed in purified preparations of the H$^+$-ATPase is the catalytic subunit.

When [^{3}H]NEM radioactivity in the M_r=100,000 band was measured by liquid scintillation counting, we observed 6.25 Bq/μg membrane protein in H$^+$-ATPase from membranes pretreated in the presence of MgADP and only 4.58 Bq/μg membrane protein in the H$^+$-ATPase from membranes pretreated in the absence of MgADP. The difference in radioactivity at M_r=100,000 between the lanes in Figure 3 appears more striking than the counts would indicate due to the lack of linearity of response of x-ray film to radioactivity in the absence of preflashing.

Trypsinization of Labeled H$^+$-ATPase and Resolution of Peptides on Reverse-phase HPLC. Deoxycholate-washed membranes, labeled as described above, were run on SDS gels and the M_r=100,000 band (identified by Coomassie blue staining) was cut out, homogenized in 50 or 100 mM NH_4HCO_3, and exposed to 50 to 100 μg/ml trypsin at 37°C. The peptides recovered in the digestion supernatant were separated by HPLC on a Vydac C-18 column using a linear acetonitrile gradient in the presence of 0.1% TFA.

In preliminary experiments we observed that the HPLC peaks due to NEM-modified cysteine broaden and increase in number as the time of incubation at pH 8 is increased (data not shown). Thus, for treatment of labeled enzyme, we minimized trypsinization times. Trypsinization for 4 to 8 h at 50 to 100 μg/ml trypsin was sufficient to release 81 to 96% of the radioactivity from the homogenized gel slice into the supernatant in five experiments. An 8 h incubation time was used in the experiment shown in Figure 4.

On the average, in 17 injections, 97% of the radioactivity injected was recovered in the fractions collected. Therefore, it is unlikely that there are any ADP-protectable peptides that were not extracted from the gel slice or were irreversibly adsorbed to the reverse-phase column. All the radioactivity was recovered in the first 40 min of the gradient, at acetonitrile concentrations below 40%.

When the results are plotted as percent of the total counts recovered (Fig. 4), peptides containing ADP-protectable residues (arrows) appear as radioactive peaks only in the digest from membranes preincubated in the presence of MgADP. One such ADP-protectable peak, containing 9 to 10% of the counts recovered in repeated experiments, was consistently observed at about 30 min. Another ADP-protectable peak observed at 20 min contained 7 to 12% of the counts recovered.

Since the M_r=100,000 band used for the trypsinization contained 6.25 Bq/μg membrane protein in H$^+$-ATPase from membranes pretreated in the presence of MgADP and only 4.58 Bq/μg membrane protein in the H$^+$-ATPase from membranes pretreated in the absence of MgADP, one would expect ADP-protectable peaks comprising 27% of the counts recovered (since [6.25−4.58]/6.25=27%). The total peak areas of the two ADP-protectable peptides from membranes preincubated with MgADP were reasonably close to this estimate, ranging from 16 to 22% in three experiments when the 3 or 4 fractions around each peak are summed.

In addition to the ADP-protectable peaks, [^{3}H]NEM was incorporated in both treatments into two major radioactive peaks at 5 and 13 min. Several smaller peaks can be seen including some in which there are apparent increases in [^{3}H]NEM incorporation in the membranes preincubated without MgADP. This may be due to conformational changes which occur when the ADP-protectable residues are modified, increasing the reactivity of other residues in the molecule.

DISCUSSION

As with other M_r=100,000 cation-translocating ATPases, the plasma membrane ATPase of higher plants has been reported to be sensitive to sulfhydryl reagents such as mercurials and Cu^{2+} (21). We have characterized the sensitivity of the oat root ATPase to NEM, a widely used modifier of protein sulfhydryl groups, and have identified two peptides whose modification by NEM may be related to loss of activity.

The pseudo first-order inactivation of the ATPase by excess NEM must be due to reaction with one or more residues, the modification of any of which causes complete loss of activity (14). The essential residue(s) modified are likely to be cysteines, since the conditions used (1 mM NEM, 2°C, 20 min, or 50 μM NEM, room temperature, 45 min, both at pH 8.0) are significantly milder than any of the conditions under which NEM has been reported to modify lysines (1 mM NEM, 25°C, 24 h [pH 8.0]; Refs. 2 and 9) or α-amino groups of amino acids and peptides (10 mM NEM, 25°C, 1–3 h [pH 7.4]; Ref. 19). However, only amino acid analysis or sequence data can unambiguously identify the residue modified.

At least one of the essential residues is partially protected from reaction with NEM in the presence of MgADP, ADP, or MgATP. Protection by ADP and/or ATP against inactivation by NEM is also observed in other related ATPases (4, 24, 26). In those enzymes (fungal H$^+$-ATPase, [Na$^+$+K$^+$]-ATPase, and Ca^{2+}-ATPase from sarcoplasmic reticulum) nucleotides are reported to provide nearly complete protection from inactivation (4, 7, 13), while we observed only partial protection (Fig. 3). This may be due to an essential residue which is not protected by nucleotides, or to one which is only partially protected. However, it is also possible that addition of another effector or optimization of conditions might give more complete protection. For the [Na$^+$+K$^+$]-ATPase (24) the rate of inactivation by NEM proved to be very sensitive to the specific combination of effectors used.

Since the rate of inactivation is greatly reduced by the presence of either substrates or products, at least one essential residue is

less accessible to NEM when nucleotides are bound to the protein. This reduction in reaction rate at the protected residue could be due to either steric blocking of the reactive residue by the bound nucleotide or to some change in the accessibility of the residue due to a conformational change caused by nucleotide binding.

Sequence information for several members of this family of enzymes, including the H+-ATPase from yeast (12, 17, 18) indicates that there is a cysteine two residues from the phosphorylation site which is conserved in all three eukaryotic vanadate-sensitive ATPases sequenced. This cysteine is known to be conserved in higher plants as well (23), and could be the residue whose modification by NEM causes inactivation.

We chose to further investigate the nucleotide-protectable essential residue(s) by labeling and trypsinization because identification and purification of nucleotide-protectable labeled fragment(s) will enable the unambiguous identification of the residue(s) modified and their location in the primary sequence. In addition, labeling of membranes with [^{3}H]NEM after first pretreating with unlabeled NEM in the presence of nucleotides provides a means of identifying the catalytic polypeptide of the H+-ATPase. Such an approach has been used previously on the related animal ATPases (7, 13) and in this case confirms that the M_r=100,000 polypeptide, which copurifies with ATPase activity on glycerol gradients after solubilization (16, 21) and is reversibly phosphorylated by ATP (3, 22), is the catalytic subunit.

Reverse-phase HPLC provided a means to compare the profile of the radioactivity of the tryptic fragments between the two treatments in a highly reproducible manner. Trypsinization of labeled membranes produced two ADP-protectable peaks that were separable on HPLC. However, the same modified residue could appear in two different peaks due to incomplete cleavage, or due to modifications affecting their chromatographic retention. NEM-modified cysteines are particularly prone to such modifications. In *Neurospora* (5), only one tryptic peptide containing an ADP-protectable, NEM-labeled residue was detectable when tryptic peptides of the [H+]-ATPase were separated by two dimensional thin layer electrophoresis. Peptide sequencing will be required to resolve whether this discrepancy between the *Neurospora* and oat enzymes reflect a true difference in the number of nucleotide-protectable residues. The identification of the essential residues in these tryptic peptides and their location in the primary sequence will help to determine if the nucleotide-protected residues are part of a functional domain conserved throughout this family of proteins.

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