Cysteine 254 of the 73-kDa A Subunit Is Responsible for Inhibition of the Coated Vesicle (H\(^+\))-ATPase Upon Modification by Sulphydryl Reagents*

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The vacuolar class of (H\(^+\))-ATPases are highly sensitive to sulphydryl reagents, such as N-ethylmaleimide. The cysteine residue which is responsible for inhibition of the coated vesicle (H\(^+\))-ATPase upon modification by N-ethylmaleimide is located in subunit A and is able to form a disulfide bond with the cysteine moiety of cystine through an exchange reaction. This unique property distinguishes this cysteine residue from the remaining cysteine residues of the (H\(^+\))-ATPase. Using this reaction, we selectively labeled the cysteine-reactive cysteine residue of subunit A with fluorescein-maleimide. After complete digestion of the labeled subunit A by V8 protease, a single labeled fragment of molecular mass 3.9 kDa was isolated and the amino-terminal sequence was determined. This fragment contains 2 cysteine residues, Cys\(^{254}\) and Cys\(^{264}\). Since Cys\(^{254}\) is conserved among all vacuolar (H\(^+\))-ATPases whereas Cys\(^{264}\) is not, it is likely that Cys\(^{254}\) is the residue which is responsible for the sensitivity of the vacuolar (H\(^+\))-ATPase to sulphydryl reagents.

The vacuolar class of (H\(^+\))-ATPases\(^*\) carry out acidification of endosomal and other intracellular compartments in eucaryotic cells (for review, see Ref. 1). Members of this class of ATPases have been isolated from clathrin-coated vesicles (Z), lytic cells (for review, see Ref. 1). Members of this class of ATPases, resembling F-type ATPases, are composed of a peripheral membrane sector containing ATP-hydrolytic sites and a membrane-embedded sector involved in proton translocation (10, 11). In the clathrin-coated vesicle (H\(^+\))-ATPase, the peripheral sector consists of subunits of molecular mass 73 (A), 58 (B), 40, 34, and 33 kDa and the intrinsic membrane sector contains subunits of molecular mass 100, 38, 19, and 17 (c) kDa (10, 11).

All vacuolar ATPases share the property of being inhibited by low concentrations of N-ethylmaleimide (NEM) (1–2 \(\mu\)M) (1). This distinguishes them from the F-type ATPases, which are inhibited by higher concentrations of NEM (0.1–1 mM), and the F-type ATPases, which are virtually resistant to inhibition by NEM. The cysteine residue(s) which are responsible for NEM sensitivity are present in subunit A (2, 12–14).

Binding of ATP to subunit A prevents reaction of the cysteine residue(s) of subunit A with NEM or 7-chloro-4-nitrobenz-2-oxa-1,3-diazole and thus protects the enzyme from inhibition (2, 12–14). On the other hand, modification of subunit A by NEM also prevents binding of nucleotides to this subunit (12). Identification of the cysteine residues which are responsible for NEM inhibition will provide information concerning the role of particular cysteine residues in catalysis.

A cDNA encoding subunit A of the bovine vacuolar ATPase has been cloned and sequenced recently in this laboratory (18). According to the deduced amino acid sequence, we have identified Cys\(^{254}\) as the cysteine residue responsible for NEM sensitivity of the coated vesicle (H\(^+\))-ATPase.

EXPERIMENTAL PROCEDURES

Materials—Calf brains were obtained from a local slaughterhouse. Phosphatidylcholine and phosphatidylethanolamine were obtained as chloroform solutions from Avanti Polar Lipids, Inc., and stored at −20 °C. 9-Amino-6-chloro-2-methylacridine and fluorescein maleimide were purchased from Molecular Probes, Inc. [3H]NEM was obtained from Du Pont-New England Nuclear. Most of the other chemicals were purchased from Sigma.

Preparation of Clathrin-coated Vesicle ATPase Modified by Cystine—Clathrin-coated vesicles were prepared from calf brain as previously described (2). Vesicles were stripped of their clathrin coat according to Adachi et al. (19). Stripped vesicles were suspended in 1 mM EDTA, 10% glycerol, and 20 mM HEPES (pH 7.0) at a protein concentration of 2 mg/ml. The stripped vesicles were then dialyzed against two changes of the above suspension buffer (300 volumes) for 30 h at 4 °C to remove trace dithiothreitol which is present in the coated vesicle preparation. The vesicles were then dialyzed against 1 mM cystine, 1 mM EDTA, 10% glycerol, and 20 mM HEPES (pH 7.0) for 24–30 h at 4 °C. The cystine-treated vesicles were then placed on ice, and an aliquot was assayed for the ATP-dependent proton transport described below. The vesicles were kept on ice until proton transport was completely inhibited (usually less than 24 h). The (H\(^+\))-ATPase was then solubilized from the cystine-treated vesicles and purified by glycerol gradient centrifugation as previously described (2) with the exception that mercaptoethanol was omitted from solutions for solubilization and purification.

Labeling of the Cystine-modified Coated Vesicle (H\(^+\))-ATPase with [\(^{3}\)H]NEM—The (H\(^+\))-ATPase was labeled with [\(^{3}\)H]NEM as previously described (2). Briefly, 2.5 \(\mu\)M [\(^{3}\)H]NEM (40 Ci/mmol) was added to various coated vesicle (H\(^+\))-ATPase preparations (10 \(\mu\)g of protein) in 0.6 ml of 0.06% C\(_{12}\)E\(_{4}\), 8 \(\mu\)g of phosphatidylcholine, 0.2 mM EGTA, 10% glycerol, and 20 mM HEPES (pH 7.0). The mixtures were incubated for 10 min at 23 °C. The reaction was stopped by addition of 1 mM unlabeled NEM followed by 6% trichloroacetic acid and 20 \(\mu\)g of cytochrome c (a carrier protein). The precipitates were
centrifuged and washed. The pellets were dissolved in 50 μl of Laemmli sample buffer and run on a 12.5% acrylamide Laemmli gel as described below.

**Labeling of the Coated Vesicle (H')-ATPase with Fluorescein Mal- eimide** - Stripped vesicles were incubated with fluorescein maleimide-labeled cysteine moiety of cystine. The mixture was kept at 23 °C for 50 min before being dialyzed against 1 mM EDTA, 10% glycerol, and 20 mM HEPES (pH 7.0) to remove dithiothreitol. The dialysis was allowed to proceed for 2 days with four changes of dialysis buffer.

Fluorescein maleimide (1 mM) was then added to the dialyzed vesicles, and the reaction was allowed to proceed at 23 °C for 1 h. Dithiothreitol (20 mM) was then added to the vesicles to neutralize excess fluorescein maleimide and the mixture was incubated at 23 °C for 30 min. The fluorescein maleimide-labeled stripped vesicles were then centrifuged for 1 h at 100,000 × g in a Beckman SW-50.1 rotor. The pellet was suspended in 50 mM NaCl, 30 mM KCl, 0.2 mM EDTA, 10% glycerol, and 20 mM HEPES (pH 7.0). The fluorescein maleimide-labeled (H')-ATPase was solubilized from the stripped vesicles and purified by glycerol gradient centrifugation as previously described (2).

**Separation and Sequencing of the Fluorescein Maleimide-labeled Fragment**—SDS was added to the fluorescein maleimide-labeled (H')-ATPase as described above to a final concentration of 0.2%. The protein sample was transferred to a 25,000 molecular weight cut-off dialysis tubing and dialyzed against 150 mM NaCl, 0.02% SDS, and 50 mM Tris-HCl (pH 8.0) for 2 days and then against 0.02% SDS for 1 day at 23 °C. The (H')-ATPase sample containing 1 mg of protein was then lyophilized to reduce the volume to 500 μl and run on a 10% acrylamide gel according to Fling and Gregerson (20). The M subunit A (subunit A) labeled by fluorescein maleimide was cut out when illuminated by UV light. Subunit A was then electroeluted from the gel as previously described (21). The eluted subunit A was dialyzed in 25,000 molecular weight cut-off dialysis tubing against 0.02% SDS and 50 mM Tris-HCl (pH 8.0) for 2 days and then against 0.02% SDS for 1 day at 23 °C. The (H')-ATPase sample containing 1 mg of protein was then lyophilized to reduce the volume to 500 μl and run on a 10% acrylamide gel according to Fling and Gregerson (20). The M, 73,000 band (subunit A) labeled by fluorescein maleimide was cut out when illuminated by UV light. Subunit A was then electroeluted from the gel as previously described (21). The eluted subunit A was dialyzed in 25,000 molecular weight cut-off dialysis tubing against 0.02% SDS and 50 mM Tris-HCl (pH 8.0) for 2 days and then against 0.02% SDS for 1 day at 23 °C to remove excess SDS. The protein concentration of the subunit A solution was estimated from the absorbance at 210 nm with bovine serum albumin as a standard. 50 mM sodium phosphate (pH 7.8) and 2 mM EDTA were added to the dialyzed subunit A followed by V8 protease. The mixture was incubated at 37 °C for 2-3 days until subunit A was completely digested. V8 protease was added to the solution by three additions over the course of incubation so that the final ratio of protein/V8 protease was 20:1. The V8 fragments were dialyzed in a 1,000 molecular weight cut-off tubing against 0.02% SDS at 23 °C for 5 days to reduce the protease activity to less than 10%. The dialyzed sample was then concentrated to 100 μl and run on a 20% acrylamide gel according to Fling and Gregerson (20). The fluorescent band of 3.9 kDa was cut out and washed in 10% methanol and 10% acrylamide gel according to Fling and Gregerson (20). The fluorescent band of 3.9 kDa was then electrotransferred to Immobilon for 30 min as previous described (10). The fluorescent band was then cut out from the Immobilon and sequenced using an Applied Biosystems 477A protein Sequencer.

**Other Methods**—ATPase activity was determined by spectroscopic assay as previously described (22). The proton transport activity was measured by 9-amino-6-chloro-2-methoxy- acetylfluorescein fluorescence quenching using a Perkin-Elmer LS-5 spectrofluorometer as previously described (2) with minor modifications. Briefly, less than 200 μl of the ATPase samples were diluted with 50 mM NaCl, 30 mM KCl, and 20 mM HEPES (pH 7.0) to a final volume of 500 μl. 9-Amino-6-chloro-2-methoxyacridine (1 μM) and valinomycin (1 μM) were then added to the solution. The ATP hydrolysis was started by addition of 20 μl of 10 mM ATP and 20 mM MgSO4. When fluorescence quenching reached the maximum, 1 μM of carbonyl cyanide m-chlorophenylhydrazone was added to the reaction mixture. The proton transport activity was determined from the rate of carbonyl cyanide m-chlorophenylhydrazone-sensitive fluorescence quenching. Reconstitution of the purified (H')-ATPase was carried out as previously described (2). Protein concentration was determined by the method of Lowry et al. (23) or, for samples containing C3, by the method of Schaffner and Weissman (24). SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (25) or Fling and Gregerson (20). Following electrophoresis, gels were fixed in 30% methanol and 7.5% acetic acid for 2 h before silver staining as described by Oakley et al. (26). For radiolabeled samples, the fixed gel was soaked in En'Hance (Du Pont-New England Nuclear), dried under vacuum and exposed to Kodak XAR-5 film using an intensifier screen.

**RESULTS**

The clathrin-coated vesicle (H')-ATPase is highly sensitive to sulphydryl reagents. When treated with NEM, the ATPase activity of this enzyme is irreversibly abolished. This enzyme is also sensitive to cysteine. Before treatment with cysteine, coated vesicles stripped of clathrin were dialyzed over 30 h to remove sulphydryl-reducing reagents present during isolation of the coated vesicles. The dialyzed vesicles were incubated with 1 mM cysteine. This resulted in complete inhibition of the proton transport (Table I). However, unlike NEM, the cysteine inhibition can be reversed by dithiothreitol. Moreover, when the ATPase is inactivated by cysteine, it becomes resistant to NEM. The proton transport of the cysteine-treated enzyme can be restored by dithiothreitol after NEM treatment (Table I). This result indicates that the cysteine residue of the coated vesicle ATPase which is responsible for NEM inhibition is able to form a disulfide bond with the cysteine moiety of cystine through an exchange reaction. Formation of the disulfide bond thus prevents this cysteine residue from reacting with NEM. Stripped vesicles which were not treated with cysteine also showed dithiothreitol-enhanced proton transport activity after incubation in a solution depleted of sulphydryl reagents over 30 h. Presumably the coated vesicle ATPase becomes less active due to formation of internal disulfide bonds between the cysteine residues of the ATPase under moderate oxidation and can be fully reactivated by reducing these disulfide bonds. However, these disulfide bonds did not protect the ATPase from NEM inhibition. After NEM treatment, no proton transport could be restored by subsequent dithiothreitol reduction. Therefore, these cysteine residues can be distinguished from that which forms a disulfide bond with the cysteine moiety of cystine.

Formation of the disulfide bond between the cysteine residue of subunit A and the cysteine moiety of cystine is a slow process. Typically, 24-30 h are required to reach complete inhibition of the proton-pumping activity (Fig. 1). ATP (2.5 mM) protects the cysteine residue of the ATPase from reaction with cysteine as it protects the enzyme from NEM inhibition (Fig. 1). In addition to ATP, increasing the ionic strength of the medium also protects the ATPase from cysteine inhibition but is less effective than ATP. This suggests that the conformation of the ATPase is affected by ionic strength and that either as a result of some conformational change or through direct steric hindrance by ATP, certain cysteine residues are hindered from reacting with sulphydryl reagents.

The cystine modified coated vesicle ATPase can be solubilized from the native membranes and reconstituted into artificial membranes as with the normal vacuolar ATPase except that sulphydryl reagents must be avoided. The reconstituted ATPase-containing liposomes did not show any proton transport nor ATPase activity (Table II). However, both activities can be restored by dithiothreitol treatment. Like the cystine-treated vacuolar ATPase on the native membrane, the enzyme purified from the cysteine-treated vesicles is also resistant to NEM. About 70% of the activity can be recovered by dithiothreitol following NEM treatment. Although reaction of NEM with cysteine residues other than the cysteine-reactive one causes partial inhibition of the enzyme (about 30% in our experiments, Table II), modification of the cysteine-reactive cysteine residue results in complete inhibition of the ATPase regardless of whether other cysteine residues are modified by sulphydryl reagents. Thus only the cysteine-reactive cysteine residue is responsible for NEM sensitivity.
Cystine-reactive Cysteine Residue of Vacuolar ATPase

TABLE I
Inactivation and protection of ATP-dependent proton transport of stripped vesicles against NEM inhibition by cystine

Stripped vesicles (1 mg protein/ml) were dialyzed against 10% glycerol, 1 mM EDTA, and 20 mM HEPES (pH 7.0) for 30 h. The dialyzed stripped vesicles were then divided into two parts. One part continued to be dialyzed against the same buffer. The other part was dialyzed against 1 mM cysteine, 10% glycerol, 1 mM EDTA, and 20 mM HEPES (pH 7.0). The dialysis proceeded for 24 h. Aliquots were then assayed for ATP-dependent proton transport as described under "Experimental Procedures." Where indicated, the vesicles (1 mg of protein/ml) were incubated with 5 mM NEM or 50 mM dithiothreitol for 1 h at 23 °C in the order indicated.

| Preparations                 | Initial Incubation conditions | Secondary Incubation conditions | Relative ATP-dependent proton transport |
|------------------------------|-------------------------------|---------------------------------|----------------------------------------|
| Stripped vesicles            | DTT                           | NEM                             | 100 ± 4                                 |
| Stripped vesicles            | NEM                           | DTT                             | 156 ± 10                                |
| Cystine-treated stripped vesicles | DTT               | NEM                             | 0                                      |
| Cystine-treated stripped vesicles | NEM               | DTT                             | 0                                      |
| Cystine-treated stripped vesicles | NEM               | DTT                             | 72 ± 2                                  |

* DTT, dithiothreitol.

**Fig. 1.** Time course for inhibition of proton transport of stripped vesicles by cystine. Stripped vesicles were sedimented at 100,000 × g for 1 h in a Beckman SW-50.1 rotor and resuspended in 10% glycerol, 1 mM EDTA, and 20 mM HEPES (pH 7.0) (C), or with addition of 1 mM cystine (C), or 1 mM cysteine and 2.5 mM ATP (O), or 1 mM cysteine and 100 mM NaCl (O). The proton transport activity was assayed after various lengths of time as described under "Experimental Procedures."

To determine which cysteine residues in the vacuolar (H+)ATPase form disulfide bonds with the cysteine moiety of cystine, the cysteine-reactive residues were labeled by [3H]NEM. The cystine-treated vacuolar ATPase was purified from coated vesicles and treated with NEM to block cysteine residues in the reduced form. The cysteine moiety which forms disulfide bonds with the cysteine residues of the vacuolar ATPase was then removed by dithiothreitol. The reduced cysteine residues were subsequently labeled by [3H]NEM. The autoradiograph in Fig. 2 shows that [3H]NEM predominately reacts with cysteine residues in the A subunit and reacts slightly with the B subunit as well. Nevertheless, only the cysteine residues in subunit A are protected from [3H]NEM labeling by forming disulfide bonds with the cysteine moiety of cystine (Fig. 2). To evaluate the total NEM-accessible cysteine residues of the ATPase, the cysteine-modified ATPase was first treated with dithiothreitol to remove the cysteine moiety and then labeled with [3H]NEM after removal of dithiothreitol. It is shown that the radioactivity incorporated into the cysteine-reactive cysteine residues is much less than the total NEM-accessible cysteine residues in subunit A (Fig. 2). Therefore, it appears that the cysteine-reactive cysteine residues are only a small fraction of the NEM-reactive cysteine residues in subunit A.

In order to identify which cysteine residues in subunit A form disulfide bonds with the cysteine moiety of cystine, the cysteine-reactive cysteine residues were labeled by fluorescein maleimide, which provides a visual marker to follow the labeled cysteine residues during isolation. Stripped vesicles were first dialyzed to remove trace sulphydryl reagents and to let putative disulfide bonds form. The cysteine-reactive cysteine residues will still be in the reduced form under these conditions (Table I, Fig. 1). Subsequently, the stripped vesicles were incubated with cystine until proton transport was completely inhibited as described in Table I. The cystine-modified coated vesicle (H+)ATPase was then solubilized, purified, and reconstituted into liposomes. Proton transport and ATPase activity were assayed as described under "Experimental Procedures." Where indicated, the reconstituted vesicles were incubated with 1 mM NEM or 50 mM dithiothreitol in the order shown. The specific ATPase activity of the reconstituted vesicles which had been incubated with dithiothreitol was 9.8 μmol of ATP/min (mg of protein)−1 at 37 °C.

**Table II**
ATP-dependent proton transport and ATPase activity of reconstituted vesicles containing the cystine-modified coated vesicle (H+)ATPase

Stripped vesicles were incubated with cystine until proton transport was completely inhibited as described in Table I. The cystine-modified coated vesicle (H+)ATPase was then solubilized, purified, and reconstituted into liposomes. Proton transport and ATPase activity were assayed as described under "Experimental Procedures." Where indicated, the reconstituted vesicles were incubated with 1 mM NEM or 50 mM dithiothreitol in the order indicated. The specific ATPase activity of the reconstituted vesicles which had been incubated with dithiothreitol was 9.8 μmol of ATP/min (mg of protein)−1 at 37 °C.

| Initial Additions | Secondary Additions | Proton transport | ATPase activity |
|-------------------|---------------------|-----------------|-----------------|
| None              | 0                   | 0               | 0               |
| NEM               | 0                   | 0               | 0               |
| DTT*              | 100 ± 4             | 100 ± 2         | 0               |
| NEM               | DTT                 | 77 ± 3          | 59 ± 3          |

* DTT, dithiothreitol.
moved to allow those putative disulfide bonds to form again so that those cysteine residues involved would not be labeled. The cysteine residues which are responsible for the NEM sensitivity will not be able to form disulfide bonds with other cysteine residues under these conditions (Table I). The resulting vesicles were then treated with fluorescein maleimide to label the remaining reduced cysteine residues. The labeled ATPase was then solubilized from the vesicle membranes by C12E9 and purified by glycerol gradient centrifugation. The coated vesicle ATPase thus prepared was predominantly labeled by fluorescein maleimide in subunit A (Fig. 3A). The fluorescein maleimide-labeled subunit A was isolated and subjected to complete digestion by V8 protease which specifically hydrolyzes peptide bonds on the carboxylic side of glutamic acid and aspartic acid in a phosphate buffer (pH 7.8)(27). The proteolytic fragments were separated by SDS-polyacrylamide gel electrophoresis. Fig. 3B shows that among the fragments separated on the SDS gel, only one peptide with an apparent molecular mass of 3.9 kDa is labeled by fluorescein maleimide. The labeled peptide was then transferred to Immobilon and sequenced. The first 11 amino acids from the amino terminus of this peptide are listed in Table III. It matches residues 220–230 of the amino acid sequence deduced from the cDNA encoding subunit A of the bovine vacuolar ATPase (18). The molecular mass of the labeled peptide is 3.9 ± 0.1 kDa (n = 2). According to the cDNA sequence, a peptide of molecular mass 3.9 kDa beginning at residue 220 would have the following sequence: KLPAHIPNL7GQRVLDAIFPCVQGGTTAIPGAFGC. This peptide, after labeling by fluorescein maleimide, has a molecular mass of 3.987 kDa. A peptide which continued through the next acidic residue (Asp227) would have a molecular mass of 5.681 kDa, while one which continued through Glu229 would have a mass of 5.614 kDa, both of which are much larger than the observed molecular mass. It thus appears that the labeled fragment was generated by cleavage at Glu228 and Cys229. It is

| Residues | Yield (pmol) |
|----------|-------------|
| Lys      | 2.01        |
| Leu      | 1.53        |
| Pro      | 1.09        |
| Ala      | 1.64        |
| Asn      | 1.05        |
| His      | 0.50        |
| Pro      | 0.59        |
| Leu      | 1.24        |
| Leu      | 2.22        |
| Thr      | 0.96        |
| Gly      | 3.34        |
Cystine-reactive Cysteine Residue of Vacuolar ATPase

likely that addition of the negatively charged fluorescein group to Cys254 has generated a cleavage site for V8 protease. To test this possibility, we modified the cysteine residue of a peptide, CVRWKQPRPTYQKL, with fluorescein maleimide. This peptide contains no glutamic and aspartic acid residues. However, digestion of the modified peptide with V8 protease resulted in cleavage of the peptide at the carboxyl side of the fluorescein maleimide-modified cysteine residue. Therefore, it is evident that it is Cys254 of subunit A which reacts with fluorescein maleimide and becomes hydrolyzable by V8 protease. Moreover, Cys246, which is one of the 2 cysteine residues in the fragment from Lys270 to Cys254, is unlikely to be modified by fluorescein maleimide because such modification would result in cleavage at the carboxyl side of Cys254 by V8 digestion to generate a fragment of 2.728 kDa which is much smaller than the observed molecular mass.

**Discussion**

Vacuolar (H+)-ATPases are characterized by their sensitivity to inhibition by sulfhydryl reagents, such as NEM (1). The cysteine residue which is responsible for NEM inhibition is located on the A subunit and is able to form a disulfide bond with the cysteine moiety of cysteine through an exchange reaction which results in complete inactivation of the (H+)-ATPase. After removal of the blocking cysteine group, this residue can be selectively labeled by fluorescein maleimide. Isolation and cleavage of the labeled A subunit with V8 protease generated a single-labeled fragment of molecular mass 3.9 ± 0.1 kDa which contains 2 cysteine residues, Cys246 and Cys254. Cys254 is likely the only cysteine residue to form a disulfide bond with the cysteine moiety of cysteine.

Comparison of the available A subunit sequences from bovine (18), Neurospora (16), carrot (15), and yeast (17), indicates that Cys254 is conserved in all four species while Cys246 is cysteine in bovine and yeast but serine in Neurospora and carrot. Because all of the vacuolar (H+)-ATPases identified have been shown to be sensitive to micromolar concentrations of NEM (1), the reactive cysteine residue would be expected to be common to all of them. In fact, only 3 cysteine residues are conserved in all of the V-ATPase A subunit sequences obtained thus far: Cys254, Cys277, and Cys330 (using the bovine A subunit residue numbers). Our data indicate that it is Cys254 which is responsible for the NEM sensitivity of the vacuolar (H+)-ATPases.

Further support for this comes from sequence comparisons with the archaeobacterial and F-type ATPases. The A subunit of the V-ATPases has been shown to be approximately 50% identical to the A subunit of the archaeobacterial ATPases (15–18, 28, 29) and approximately 25% identical to the β subunit of F1 (15–18, 30). The conservation is even higher in regions which are believed, from chemical labeling and mutagenesis studies of the β subunit of F1, to be involved in nucleotide binding or hydrolysis (for review, see Ref. 31). Consistent with the insensitivity of both the archaeobacterial and F-type ATPases to NEM, the residue corresponding to Cys254 is serine in the archaeobacterial ATPases (28, 29) and valine in the F-type ATPases (30).

Residues 250–258 of the bovine A subunit correspond to the consensus sequence GXXGXGKTV, termed the Walker “A” sequence (32). This region forms a glycine-rich loop which is perfectly conserved in all of the V, F, and archaeobacterial ATPases and has been shown from x-ray crystallographic studies to form part of the nucleotide binding site of adenylate kinase (33). Chemical labeling and mutational analysis of the β subunit of F1 have strongly implicated this region in nucleotide binding or hydrolysis (34–37). In addition, a 50-amino acid peptide containing this sequence has been shown to bind nucleotides (38). Thus, modification of Cys254, which is located in the middle of this consensus sequence, would be expected to disrupt binding of ATP. Similarly, ATP might be expected to block reaction of Cys254 with sulfhydryl reagents. These predictions are both born out for the V-ATPases since NEM labeling of the A subunit is ATP-protectable (2, 12–14) and inhibition of ATP binding to the A subunit occurs on reaction with NEM (12).

The coated vesicle (H+)-ATPase contains nine subunits. Six of these subunits (100, 73, 58, 40, 38, and 33 kDa) have cysteine residues which are able to react with NEM (Fig. 2) or 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (2). Cysteine reversibly reacts with a single cysteine residue (Cys254) in subunit A (73 kDa). This property makes cysteine a useful sulfhydryl reagent to study the various cysteine residues of the vacuolar ATPases. Cys254 of subunit A is responsible for NEM sensitivity. As long as Cys254 is modified by sulfhydryl reagents, the ATPase is completely inhibited, in spite of whether other cysteine residues are modified. On the other hand, NEM modification of cysteine residues of the coated vesicle ATPase other than Cys254 results in partial loss of activity. In addition, the coated vesicle ATPase in the native membrane has at least 2 cysteine residues which are able to form a disulfide bond under moderate oxidation. Reducing this disulfide bond enhances the proton pumping activity up to 50% (Table 1). However, oxidative formation of disulfide bonds between these cysteine residues does not protect the vacuolar ATPase from NEM inhibition. Therefore, these 2 cysteine residues can be distinguished from Cys254 which renders NEM resistance to the ATPase once protected by formation of a disulfide bond.

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Cystine-reactive Cysteine Residue of Vacuolar ATPase

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