The plasma membrane H⁺-ATPase of *Neurospora crassa* was treated with 5,5'-dithiobis(2-nitrobenzoato) to determine its cysteine content and with 2-nitro-5-thiosulfobenzoate to determine its cystine content. Six and seven mol of thiols/mol of H⁺-ATPase were detected in the 5,5'-dithiobis(2-nitrobenzoato) and 2-nitro-5-thiosulfobenzoate reactions, respectively, indicating that 6 of the 8 cysteine residues in the molecule are present as free cysteines and that 2 are present in disulfide linkage. The results of quantitative carboxymethylation experiments using [¹⁴C]iodoacetate under nonreducing and reducing conditions fully support this conclusion. Preparations of the ATPase [¹⁴C]carboxymethylated under the above conditions were treated with trypsin, and the tryptic digests were resolved into hydrophilic and hydrophobic peptide fractions by our recently published procedure (Rao, U. S., Hennessey, J. P., Jr., and Scarborough, G. A. (1988) Anal. Biochem. 173, 251–264). Five of the six labeled free cysteine peptides partitioned into the hydrophilic peptide fraction and were purified and established to contain Cys⁴⁷⁰, Cys⁴⁹⁹, Cys⁷⁴², Cys⁶³³, and Cys⁸⁴⁰. The labeled free cysteine residue in the hydrophobic peptide fraction was identified as either Cys⁴⁰ or Cys⁶⁸⁹ by virtue of its presence in a large ~21-kDa hydrophobic peptide estabishes previously to begin at Ser⁸⁶⁰. This in turn identified either Cys⁴⁰ or Cys⁶⁸⁹ as one of the disulfide bridge cysteines. The other disulfide bridge cysteine was identified as Cys⁴⁰ by purification and NH₂-terminal sequencing of an additional peptide labeled in the reduced enzyme. The disulfide bridge is therefore between Cys⁴⁰ and either Cys⁴⁰ or Cys⁶⁸⁹. Because Cys⁴⁰ is present in a putative membrane-embedded sector near the NH₂ terminus of the ATPase molecule and Cys⁴⁰ and Cys⁶⁸⁹ are present in a similar sector near the COOH terminus, it is possible that the disulfide bridge plays an important structural role in holding the two major membrane-embedded sectors of the molecule, distant in the linear sequence, together.

The primary interest in this laboratory is an understanding of the structure and molecular mechanism of the *Neurospora crassa* plasma membrane H⁺-ATPase, which is a member of a large family of transport enzymes that includes the plasma membrane H⁺-ATPases of fungi, plants, and animals, the plasma membrane K⁺-ATPase of *Escherichia coli*, the plasma membrane H⁺/K⁺-ATPase of gastric mucosa, the plasma membrane Na⁺/K⁺-ATPase of animal cells, and the Ca⁺⁺-ATPases of animal cell plasma membranes, sarcoplasmic reticulum, lysosomes, and Golgi (1). As one approach to this, we are attempting to elucidate the topography of the H⁺-ATPase (2, 3) and to identify active site residues (4–6) using protein chemical techniques. As for all known integral membrane transport proteins, this has been a daunting task because of the presence in these molecules of a large hydrophobic domain that is almost totally refractory to conventional techniques of protein chemistry (7–10). However, in an important first step to surmounting this problem for the *Neurospora* plasma membrane H⁺-ATPase, we have recently reported highly effective methodology for fragmenting this molecule and purifying, with high recoveries, virtually all of the hydrophilic and hydrophobic peptides produced (11). A key feature of the procedure developed is an initial separation of the tryptic hydrolysate into hydrophilic and hydrophobic peptide fractions, presumably representing the cytoplasmic and membrane-embedded domains of the molecule (2), which are then resolved by entirely different techniques. In this paper, this new methodology has been utilized to facilitate the elucidation of the chemical state of the 8 cysteine residues known to exist (12, 13) in the H⁺-ATPase molecule. The results clearly indicate the presence of 5 free cysteine residues in the hydrophilic domain of the ATPase and 1 free cysteine and 1 disulfide bridge in the hydrophobic domain.

**EXPERIMENTAL PROCEDURES**

**H⁺-ATPase Preparation**

The *Neurospora* plasma membrane H⁺-ATPase was purified according to the procedure of Smith and Scarborough (14) with minor modifications (15). Glycerol gradient-purified enzyme obtained from the above procedure was purified further by chromatography on a Sepharose CL-4B-200 column (2.5 × 85 cm) (16) in a solution containing 30% (w/v) glycerol, 1 mM EDTA, and 2 μg/ml chymostatin, pH adjusted to 6.8 with Tris. The fractions that exhibited ATPase activity were pooled and then concentrated to 1–4 mg/ml, using RCF ConFIlk hollow fiber bundles (M, cutoff 6,000; Bio-Molecular Dynamics), centrifuged at 12,000 × g for 25 min at 4 °C, and the supernatant fluid was filtered through a 0.45-μm Nylon-66 filter (Rainin) and stored at −20 °C as described by Hennessey and Scarborough (16). The specific activity of the ATPase was 16–20 μmol of P released/min/mg of protein when assayed as described (14, 15).

**Determination of the Total Free Sulfhydryl Content of the ATPase**

Free sulfhydryl groups in the H⁺-ATPase were estimated by reaction with Ellman’s reagent, DTNB (17). In a typical experiment, 0.8
ml of a buffer solution containing 0.2 M Tris, 3 mM EDTA, and 5 mM recrystallized guanidine HCl (pH 8.0 with HCl), was transferred to a 1-cm path length glass cuvette, and 0.15 ml of the ATPase solution was added with gentle mixing. The cuvette was then placed in a Beckman DU-1 spectrophotometer and the reaction started by adding 0.05 ml of freshly prepared 2 M DTT in 0.2 M Tris containing 3 mM EDTA (pH 8.0 with HCl). The final concentration of enzyme in the reaction mixtures was 1-3.5 μM. The course of the reaction at room temperature was recorded as the increase in absorbance at 412 nm. An extinction coefficient of 13,800 M⁻¹ cm⁻¹ at 412 nm for NTB was used for quantitating the reaction (17).

Determination of the Cystine Content of the H⁺-ATPase

The cysteine content of the ATPase was determined by reaction with NTBS according to the procedure of Thanhauser et al. (18, 19). The NTBS assay solution (0.85 ml) containing 0.5 mM NTBS, 3 mM guanidine thiocyanate, 0.2 M Tris, 0.1 mM sodium sulfate, and 3 mM EDTA (pH 9.5 with NaOH), was transferred to a 1-cm path length cuvette, after which 0.15 ml of the ATPase solution was added with mixing, and the change in absorbance at 412 nm was recorded in a Beckman DU-1 spectrophotometer. The final concentration of enzyme in the reaction mixtures was 1-3.5 μM. The NTB extinction coefficient indicated above was used for quantitating the reaction (18, 19).

Carboxymethylation of the Free Cysteine Residues in the H⁺-ATPase

Carboxymethylation of the H⁺-ATPase was carried out using iodoacetate essentially according to the procedure of Hirn (20). In a typical experiment, 0.9 ml of the H⁺-ATPase solution (3 mg of protein) was transferred to a 15-ml siliconized (21) Corex test tube, degassed for 20 min using a water aspirator, and 0.1 ml of nitrogen-flushed 2 M Tris containing 20 mM EDTA (pH 8.3 with HCl) was added. The tube containing the reaction mixture was then placed in a room temperature water bath and the solution flushed with nitrogen for 5 min. Following this, 0.24 ml of 3 M iodo[2-¹⁴C]acetic acid (pH 7.0 with 1 N NaOH) was added, and the resulting solution was incubated at room temperature in the dark for 25 min with a continuous gentle flow of nitrogen over the surface of the reaction mixture. After this, 1 ml of β-mercaptoethanol was added, and the reaction mixture was dialyzed against 1% (w/v) ammonium bicarbonate (three changes, 2 liters each) for 2 days at 4 °C. The protein and the radioactivity contents were then quantitated as described below.

Carboxymethylation of the Disulfide Cysteine Residues in the ATPase

The H⁺-ATPase was first carboxymethylated with nonradioactive iodoacetate by the above procedure to block the free cysteine sulphydryl groups. No β-mercaptoethanol was added at the end of the reaction, and the reaction mixture was dialyzed against 0.2 M Tris containing 2 mM EDTA (pH 8.3 with HCl) (three changes, 2 liters each) for 2 days at 4 °C in the dark. The ATPase in the dialysis bag was recovered and the solution degassed using a water aspirator for 25 min. Freshly prepared 0.25 M DTT in water was then added to the dialyzed ATPase solution to a final concentration of 5.2 M. The solution was then adjusted to 5.2 M guanidine HCl using solid recrystallized guanidine HCl and the resulting solution incubated under a stream of nitrogen for 30 min. The tube was then sealed with Parafilm and for 3 h at room temperature, an additional 0.05 ml of 3 M iodo[2-¹⁴C]acetic acid (pH 7.0 with 1 N NaOH) was added, and the resulting solution was dialyzed against 0.2 M Tris containing 2 mM EDTA and 2 mM DTT (pH 8.3 with HCl) (three changes, 2 liters each) for 2 days at 4 °C to adjust the DTT concentration to 2 mM. After dialysis, the somewhat hazy protein solution was again adjusted to 5.2 M guanidine HCl as above. The resulting solution was then degassed for 20 min using a water aspirator after which nitrogen was passed over the surface of the solution for 25 min. The reduced ATPase was then carboxymethylated with iodo[2-¹⁴C]acetic acid as follows. The ATPase solution in a 15-ml Corex test tube was placed in a room temperature water bath and flushed gently with nitrogen. While flushing with nitrogen, 1.4 ml of 3 M iodo[2-¹⁴C]acetic acid (pH 7.0 with NaOH) was added/ml of ATPase solution (based on the volume before the addition of guanidine HCl), and the carboxymethylation reaction was allowed to proceed in the dark for 25 min. The reaction mixture was then dialyzed against 0.2 M Tris containing 2 mM EDTA and 2 mM DTT (pH 8.3 with HCl) for 2 days (three changes, 2 liters each) at 4 °C to adjust the DTT concentration to 2 mM. After dialysis, the protein solution was collected and then adjusted to 5.2 M guanidine HCl as above. The resulting solution was then degassed for 20 min using a water aspirator after which nitrogen was passed over the solution for 25 min. The tube was then placed in a room temperature water bath and the solution flushed gently with nitrogen over the surface. 1.4 ml of 3 M iodo[2-¹⁴C]acetic acid (pH 7.0 with NaOH) was then added/ml of the ATPase solution (based on the volume before addition of guanidine HCl) and the reaction allowed to proceed for 25 min in the dark. The reaction mixture was then dialyzed against 1% (w/v) ammonium bicarbonate (three changes, 2 liters each) at 4 °C for 2 days. The protein solution in the dialysis tubing was then collected and used for further analysis.

Tryptic Digestion of the Carboxymethylated ATPase and Separation of the Hydrophilic and Hydrophobic Tryptic Peptides

The carboxymethylated protein samples obtained after dialysis against 1% (w/v) ammonium bicarbonate were digested with freshly prepared trypsin and the hydrophilic and hydrophobic peptide fractions separated by extraction with 0.75 M ammonium bicarbonate as described previously (11).

Purification of the Hydrophilic [¹⁴C]Carboxymethylated Free Cysteine-containing Peptides by HPLC

HPLC of the hydrophilic trypsin peptide fraction obtained from ATPase treated to [¹⁴C]carboxymethylate the free cysteine residues as described above was carried out according to the procedure of Rao et al. (11) with minor modifications. The hydrophilic peptide fraction was filtered through a 0.45-μm Nylon-66 filter, degassed for 10 min using a water aspirator, and then directly applied to a Bio-Rad HPLC instrument equipped with model 440 and 441 UV detectors and interfaced with an NEC APC IV computer and Waters base line 810 chromatography work station software. After the initial separation of the peptides, the individual radioactive peptides were purified further as follows.

Peptide I—The peptide that eluted at 15.0-17.5 min in the initial HPLC run was dried by lyophilization and dissolved in 0.1 ml of water. The solution was then filtered through a 0.45-μm Nylon-66 filter, degassed, and injected onto the above mentioned C18 column equilibrated with 0.1% (v/v) trifluoroacetic acid in water. The column was then washed with 0.1% (v/v) trifluoroacetic acid in water for 30 min at a flow rate of 0.5 ml/min after which a linear gradient of 0-5% acetonitrile in 0.1% (v/v) trifluoroacetic acid was developed over a period of 30 min at a flow rate of 0.5 ml/min. The radioactivity peak eluted at 32 min with complete recovery.

Peptide II—The radioactive peptide eluting at 67.5-72.5 min in the initial HPLC separation was collected and dried by lyophilization. The peptide was then dissolved in 0.1 ml of 10% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid, and the solution was filtered through a 0.45-μm Nylon-66 filter, degassed, and injected onto the above mentioned C18 column. The chromatogram was then developed using a 60-min linear gradient of 10-14% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml/min. The radioactive peptide eluted at 20 min and was then rechromatographed under the same conditions. The recovery of this peptide was also complete.

Peptide III—The radioactive peptide eluting at 162-167.5 min in the initial HPLC separation was lyophilized to dryness, dissolved in 0.1 ml of 30% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid, and further purified on the C18 column, using a linear gradient of 30-33% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.5 ml/min for 60 min. A single radioactive peptide peak with a retention time of 11.0 min was collected and
purified further by chromatography under the same conditions. The recovery was greater than 94%.

Peptide IV—The radioactive peptide mixture eluting at 162.5-175 min, designated peptide IV, was pooled, dried by lyophilization, dissolved in 1% (w/v) ammonium bicarbonate, and then redigested with trypsin (0.3 μg of trypsin/nmol of radioactive peptide) at 37 °C for 7 h. The digest was then applied directly to the C₈ column and eluted with a linear gradient of 30-35% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml/min for 30 min. Radioactivity was found in one major peak (65% of the total radioactivity, retention time 8.5 min) and two minor peaks (20 and 15% of the total radioactivity, retention times 5 and 7 min, respectively).

Peptide V—the radioactive peptide eluting at 175-182.5 min was collected, dried by lyophilization, dissolved in 0.1 ml of 35% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid, applied to the C₁₈ column, and eluted with a linear gradient of 33-38% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.5 ml/min for 60 min. A single radioactive peptide eluted at 23 min with complete recovery.

Purification of the Hydrophobic ¹⁴C-Carboxymethylated Free Cysteine Peptide

The hydrophobic ¹⁴C-carboxymethylated free cysteine peptide was purified by Sephadex LH-60 column chromatography in chloroform-methanol (1:1, v/v) containing 0.1% (v/v) trifluoroacetic acid after neat trifluoroacetic acid treatment of the hydrophobic peptide fraction, as described by Rao et al. (11). Aliquots of the fractions were dried in scintillation vials and the radioactivity determined as described below. The recovery of radioactivity from the column was complete.

Isolation of the Hydrophilic Disulfide Bridge Cysteine-containing Peptide

ATPase treated with ¹⁴C-carboxymethyl the disulfide bridge cysteine residues was digested as described above except that an additional 7-h digestion with freshly added trypsin was included, and the hydrophilic and hydrophobic peptide fractions were then prepared as described (11). The hydrophilic peptide fraction was dialyzed against distilled water in 2,000-Da cutoff dialysis tubing for 1.5 days (three additional 7-h digestion with freshly added trypsin was included, and the resulting moderately turbid solution in the dialysis tubing was collected, centrifuged at 12,000 x g for 25 min, and the supernatant fluid filtered through a 0.45-Mm Nylon-66 filter. The solution remaining after these steps retained 75% of the original radioactivity. The solution was then adjusted to 1 M guanidine HCl by adding an equal volume of 2 M guanidine HCl and was then subjected to chromatography on three tandem columns (86 x 1.5 cm each) of Sephacyr S-100 HR equilibrated with 1 M guanidine HCl at a flow rate of 55 ml/h. Fractions (4.3 ml) were analyzed and the radioactivity in the fractions analyzed as described below. The recovery of radioactivity from the column was 80%.

Amino Acid Analysis and Sequence Determination

Peptide amino acid analysis and sequencing were carried out at the UCLA Microsequencing Center under the supervision of Dr. Audree V. Fowler. For the amino acid analyses, the peptide samples were digested with 6 N HCl for 18 h at 110 °C in vacuo and analyzed using a Waters Partisil system. Peptide samples for sequencing were dried down on polyvinylidene difluoride membrane and sequenced using an Applied Biosystems 470A sequencing system.

Analysis of Radioactivity

Radioactivity in the test samples was determined by scintillation counting in the mixture of Patterson and Greene (22) after removal of organic solvents by evaporation, if necessary.

Protein Estimation

Protein was determined by the procedure of Lowry et al. (23) as modified by Rennaudou and Weinstein (24), using bovine serum albumin as a standard. The H⁺-ATPase preparation used in these studies was approximately 90% pure (16). Therefore, all ATPase protein estimations were corrected for a 10% non-ATPase protein content.

Materials

The sources of most of the materials used have been described previously (11, 14, 16). DTNB was obtained from Aldrich. Iodo[2-³⁵S]acetic acid (specific activity of 50-60 mCi/mmol) was obtained from Amersham Corp. Acetyl-CoA, palmitoyl-CoA, cytochrome c, aprotinin, ricin, and nonradioactive iodoacetic acid were from Sigma. Nonradioactive iodoacetic acid was recrystallized from carbon tetra chloride and stored in the dark at -20°C. Guanidine HCl was purchased from Mallinckrodt and recrystallized as described (25). Guanidine thiocyanate was obtained from Kodak. Septamyl chloride 1-500 HR was from Pharmacia LKB Biotechnology Inc. The trypsin used was L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin from Worthington. Dialysis tubing was from Spectrum; unless otherwise indicated, the molecular mass cutoff of the tubing used was 12-14 KD.}

RESULTS

Reaction of the ATPase with DTNB and NTSB—Fig. 1 shows the results obtained when the purified ATPase is treated with the widely used sulfhydryl reagent, DTNB (17), in 4 M guanidine HCl. The reaction of DTNB with the enzyme is rapid, and within a few min a maximum of 6.15 mol of NTB is released/mol of ATPase, indicating the presence of 6 free cysteine residues in the molecule. There is no further release of NTB over a period of 30 min. This experiment has been carried out 12 times using several different ATPase preparations, with essentially identical results. Similar assays of the H⁺-ATPase denatured in 2.4 M guanidine thiocyanate, 6.4 M guanidine HCl, or 0.8% (w/v) SDS also indicate the presence of six free sulfhydryl groups in the molecule.

Since there are 8 cysteine residues in the H⁺-ATPase molecule as deduced from the gene sequence (12, 13), these findings suggest that the remaining 2 cysteines in the molecule may be present in disulfide linkage and thereby not reactive with DTNB. This possibility was investigated by reacting the ATPase with NTSB under conditions described by Thannhauser et al. (18, 19) for quantitating the disulfide bridge content of proteins. Fig. 1 also illustrates the results of the NTSB reaction with the H⁺-ATPase. About 6 mol of NTB/mol of ATPase is released in the first 5 min followed by a slower rise to a final value of about 7.1. This experiment has been carried out 10 times using several different ATPase preparations, with essentially the same results. As explained by Thannhauser et al., the additional 1 mol of NTR released in the NTSB reaction compared with the DTNB reaction is the expected result of the sulfonolysis of a single disulfide bond in the enzyme. The above data thus account for all 8 of the cysteine residues in the ATPase molecule.

The presence of fatty acylated cysteine residues has been demonstrated for several proteins (26-30), and it was therefore considered possible that the 2 cysteine residues in the H⁺-ATPase molecule which are unreactive with DTNB might
be present as fatty acyl derivatives. To test this possibility, the behavior of two acyl thioesters, acetyl-CoA and palmitoyl-CoA, in the NTSB reaction was investigated. The results (not shown) showed clearly that acyl thioesters liberate NTB only slowly under the conditions of the NTSB assay, much more slowly than any of the seven NTBs liberated by the ATPase (Fig. 1), presumably reflecting a slow rate of alkaline hydrolysis of the acyl thioesters followed by facile reaction of the liberated CoA with NTB. In addition, when allowed to proceed to completion, the stoichiometry of such reactions was 1 mol of NTB produced/mol of acyl-CoA, not 1/2 as would be required to explain the observed extra 1 mol of NTB liberated/mol of ATPase in the NTSB reaction. Thus, it is extremely unlikely that the cysteine residues in the ATPase molecule which are unreactive with DTNB are present as fatty acyl thioesters.

Carboxymethylation of the H+-ATPase—To corroborate the results of the DTNB and NTSB assays, the stoichiometry of carboxymethylation of the ATPase with the well established thiol reagent iodo[2-14C]acetic acid was investigated under nonreducing and reducing conditions. Table I summarizes the results obtained. ATPase treated with iodo[2-14C]acetic acid as 3-fold excess over the 8 cysteine residues in the H+-ATPase molecule in the presence of 5.2 mM guanidine HCl as described under “Experimental Procedures” incorporates almost exactly 6 mol of [14C]-carboxymethyl group/mol of enzyme. In these experiments, excess reagent was avoided in order to minimize the unwanted side reactions that take place with methionine, histidine, lysine, and tyrosine residues (20). However, the use of a 23-fold excess of [14C]iodoacetate did not result in any increased labeling of the enzyme (not shown). Moreover, the specific radioactivity of the iodo[2-14C]acetic acid used for these experiments varied anywhere from 1,000 to 21,000 cpm/nmol without any difference in the stoichiometry of labeling of the ATPase. These results are thus in complete agreement with the results of the DTNB assays of the free cysteine content of the H+-ATPase.

To account for the 2 remaining cysteines in the ATPase molecule, the ATPase was first carboxymethylated with nonradioactive iodoacetate under nonreducing conditions as described under “Experimental Procedures” to derivatize the 6 free cysteines and was then reduced with a high concentration of DTT under denaturing conditions, dialyzed to lower the DTT concentration to 2 mM, and then treated with a slight excess of iodo[2-14C]acetic acid in the presence of 5.2 mM guanidine HCl. The results of this experiment are also presented in Table I. It can be seen that blocking of the free cysteines with nonradioactive iodoacetate followed by reduction and carboxymethylation with iodo[2-14C]acetic acid results in the radiolabeling of 2 additional cysteine residues, arising from the reduction of the disulfide bridge in the H+-ATPase molecule. To ensure that the labeling of the 2 additional cysteine residues in this experiment was indeed due to the reduction of a disulfide bridge and not due to any of the other manipulations required by this experiment, the same experiment was repeated without DTT and with a 400-fold m excess of iodo[2-14C]acetic acid. The results are also shown in Table I. In this case, almost no cysteine labeling occurred, demonstrating that reduction by DTT is required for labeling of the additional 2 cysteine residues.

Finally, the ATPase was fully carboxymethylated under reducing conditions. The enzyme was first reduced with a high concentration of DTT in the presence of 5.2 mM guanidine HCl for 3 h and then dialyzed as described under “Experimental Procedures.” The enzyme was then carboxymethylated in the presence of guanidine HCl with iodo[2-14C]acetic acid at a concentration slightly in excess of the thiol content present in the reaction mixture. The results of this experiment (Table I) showed that all 8 cysteine residues present in the enzyme are modified with iodoacetate under reducing conditions. These combined results of the carboxymethylation experiments thus fully corroborate the results of the DTNB and NTSB assays and indicate the presence of 6 free cysteines and a single disulfide bridge in the ATPase molecule. Importantly, they also pave the way for identifying both the free and disulfide bridge cysteine residues.

The enzyme preparation used for all of the above studies was isolated in a 30% (w/v) glycerol solution that did not contain DTT (see “Experimental Procedures”). It is thus possible that isolation of the ATPase in the absence of a reducing agent could allow the formation of an artificial disulfide bond in the enzyme during purification. To check this possibility, the ATPase was purified as described under “Experimental Procedures” but in the presence of 1 mM DTT. The enzyme was then carboxymethylated with a slight excess of nonradioactive iodoacetate as above to block the free cysteines, dialyzed, and then assayed for free thiols and disulfide cysteines using the DTNB and NTSB assays described above. No reaction occurred with DTNB, indicating that all of the free cysteines had been derivatized with iodoacetate. However, in the NTSB reaction, 1.19 mol of NTB was released/mol of ATPase, indicating that the disulfide bridge is

| Condition | mol [14C]-carboxymethyl group/mol ATP | Distribution of [14C]-carboxymethyl groups |
|-----------|------------------------------------------|------------------------------------------|
| Nonreduced | 5.90 ± 0.27<sup>a</sup> | 4.87 ± 0.27<sup>a</sup> | 1.00 ± 0.11<sup>a</sup> |
| [14C] Carboxymethylated, reduced, and then [14C] carboxymethylated | 2.05 ± 0.14<sup>b</sup> | 1.04 ± 0.05<sup>b</sup> | 1.02 ± 0.15<sup>b</sup> |
| [14C] carboxymethylated and then [14C] carboxymethylated without reduction | 0.24 | ND<sup>c</sup> | ND<sup>c</sup> |
| Reduced | 8.05<sup>d</sup> | 6.04<sup>d</sup> | 2.01<sup>d</sup> |

<sup>a</sup> Mean of six independent experiments.
<sup>b</sup> Mean of five independent experiments.
<sup>c</sup> ND, not determined.
<sup>d</sup> Average of two experiments.

Table 1
Stoichiometry of labeling of the H+-ATPase by iodo[2-14C]acetic acid under various conditions
The ATPase was labeled under the various conditions indicated, and the extent of [14C] carboxymethylation of the ATPase and hydrophilic and hydrophobic peptide subfractions of the ATPase were determined as described under “Experimental Procedures.”
present even when the ATPase is isolated in the presence of DTT. These results strongly argue that the disulfide bridge in the H*-ATPase is a natural and possibly important structural feature. As isolated, the purified ATPase used for these experiments is in the form of hexamers (31), and it is possible that the disulfide bridge could be present between monomers in the hexamers. This possibility was ruled out by the absence of any higher molecular mass species above the 100,000-Da ATPase band in SDS-polyacrylamide gel electrophoresis analyses of the enzyme run under nonreducing versus reducing conditions (not shown). The behavior of a ricin standard that contains a known intersubunit disulfide bridge (32) in these gels indicated that this procedure would have detected an intermonomer disulfide bridge if it were present.

Tryptic Digestion of the Carboxymethylated H*-ATPase and Separation of the Products into Hydrophilic and Hydrophobic Peptide Fractions—We have recently reported a highly effective salt extraction procedure for quantitatively fractionating tryptic digests of the H*-ATPase into hydrophilic and hydrophobic peptide fractions (11). As a preliminary step toward identifying the free and disulfide bridge cysteines, the H*-ATPase was carboxymethylated by several of the procedures described above, digested with trypsin, and the distribution of the labeled peptides in the hydrophilic and hydrophobic peptide fractions was determined. Table I shows the results obtained. When ATPase carboxymethylated to label the 6 free cysteine residues is used in such an experiment, 5 of the labeled free cysteines are found in the hydrophilic peptide fraction, and 1 is found in the hydrophobic peptide fraction. These peptides will be referred to below as the five hydrophilic 14C-carboxymethylated free cysteine peptides and the hydrophobic 14C-carboxymethylated free cysteine peptide, respectively.

When ATPase specifically labeled at the disulfide bridge cysteines is used, 1 labeled cysteine is found in the hydrophilic peptide fraction, and 1 is found in the hydrophobic peptide fraction. The hydrophilic cysteine-containing peptide labeled in this way will be referred to below as the hydrophilic 14C-carboxymethylated disulfide bridge cysteine peptide.

When ATPase labeled at all 8 residues is used, 6 labeled cysteines are found in the hydrophilic peptide fraction, and 2 labeled cysteines are found in the hydrophobic peptide fraction. The combined results are thus highly quantitative and internally consistent and indicate that 5 of the free cysteine residues and 1 of the disulfide bridge cysteine residues in the H*-ATPase molecule are present in hydrophilic tryptic peptides and that 1 free and 1 disulfide bridge cysteine residue are present in hydrophobic tryptic peptides.

Identification of the Hydrophilic 14C Carboxymethylated Free Cysteine-containing Peptides—Fig. 2 shows the results obtained when the hydrophilic tryptic peptide mixture derived from ATPase 14C carboxymethylated to label the free cysteine residues is resolved by HPLC as described under "Experimental Procedures." Two of the five 14C-carboxymethylated cysteine peptides, designated peptide I and II, respectively, are well resolved with retention times around 15 and 65 min. The other labeled peptides elute as a partially resolved group between 155 and 180 min. Further purification of peptides I, II, III, and V by two or three additional HPLC runs as described under "Experimental Procedures" results in relatively pure peptide preparations as judged by the constant A_{214} to A_{380} ratios in the purified peptide peaks (not shown). Each of these peaks could therefore be identified on the basis of its amino acid composition. Identification of these peptides on the basis of their amino acid compositions was straightforward since they each contain cysteine, and their amino acid sequences and NH$_2$ and COOH termini are predictable from the gene sequence (12, 13) and expected tryptic cleavage sites (11). Table II shows the amino acid compositions of peptides I, II, III, and V and their identities based on this information. Peptide I contained the 6 predominant residues shown and was therefore unambiguously identified as a hexapeptide containing Cys$_{545}$ with the sequence Thr-Val-Cys-Glu-Ala-Lys beginning at Thr$_{413}$ and ending at Lys$_{548}$. Peptide II was similarly identified as a pentapeptide containing Cys$_{572}$ beginning at Ile$_{490}$ and ending at Lys$_{548}$. Peptides III and V could also be identified in this way as Cys$_{586}$ and Cys$_{590}$ containing peptides comprising residues 363-379 and 388-414, with the sequences Leu-Ser-Ala-Ile-Glu-Ser-Leu-Ala-Gly-Val-Glu-Ile-Leu-Cys-Ser-Asp-Lys and Leu-Ser-Leu-His-Asp-Pro-Tyr-Thr-Val-Ala-Gly-Val-Asp-Pro-Glu-Asp-Leu-Met-Thr-Ala-Cys-Leu-Ala-Ala Sor Arg, respectively.

A second HPLC run of the pooled fractions from 162.5 to 175 min designated as peptide IV in the initial HPLC separation of the hydrophilic peptides (Fig. 2) indicated that there are several radioactive peptides in this region. Because the total amount of radioactivity in this region was equivalent to about 1 mol of 14C-carboxymethylated cysteine/mol of the ATPase, it was suspected that this region contained incomplete tryptic cleavage products. The pooled peptide fractions from a similar preparation were therefore dried, redissolved, redigested with trypsin, and separated by IIPLC as described under "Experimental Procedures." After redigestion, one major peak representing 65% of the radioactivity applied, and...
### TABLE II

Amino acid compositions and identities of the hydrophilic $^{14}$C-carboxymethylated free cysteine-containing peptides

ATPase $^{14}$C carboxymethylated under nonreducing conditions to label the 6 free cysteine residues was digested with trypsin, and the hydrophilic free cysteine-containing peptides were purified and subjected to amino acid analysis as described under "Experimental Procedures."

| Amino acid | Peptide I | Peptide II | Peptide III | Peptide IV | Peptide V |
|------------|-----------|------------|-------------|------------|-----------|
|            | pmol$^a$  | pmol$^a$   | pmol$^a$    | pmol$^a$   | pmol$^a$  |
| Cys (cm)$^d$ | 100       | 1          | 230         | 1          | 83        |
| Ala        | 11        | 0.1        | 42          | 0.2        | 76        |
| Glx        | 78        | 0.8        | 56          | 0.2        | 140       |
| Ser        | 11        | 0.1        | 106         | 0.4        | 196       |
| Gly        | 31        | 0.3        | 113         | 0.5        | 147       |
| His        | 1         | 0          | 20          | 0.1        | 7         |
| Arg        | 5         | 0          | 81          | 0.3        | 10        |
| Thr        | 69        | 0.7        | 175         | 0.8        | 115       |
| Ala        | 78        | 0.8        | 35          | 0.1        | 141       |
| Pro        | 10        | 0.1        | 68          | 0.3        | 18        |
| Tyr        | 5         | 0          | 85          | 0.4        | 0         |
| Val        | 68        | 0.7        | 205         | 0.9        | 57        |
| Met        | 6         | 0.1        | 4           | 0          | 0         |
| Ile        | 5         | 0          | 195         | 0.8        | 98        |
| Leu        | 3         | 0          | 49          | 0.2        | 196       |
| Phe        | 3         | 0          | 10          | 0          | 7         |
| Lys        | 61        | 0.6        | 219         | 0.9        | 80        |

| Position in deduced amino acid sequence |
|----------------------------------------|
| Peptide I: 543-548                      |
| Peptide II: 470-474                     |
| Peptide III: 363-379                    |
| Peptide IV: 520-537?                    |
| Peptide V: 388-414                      |

$^a$ Actual pmol of amino acids obtained from the amino acid analysis except for carboxymethylcysteine, which was obtained from the radioactivity content.

$^b$ Calculated on the basis of the [$^{14}$C]carboxymethylcysteine content of the sample analyzed.

$^c$ Theoretical amino acid content of the peptides identified at the bottom of the column deduced from the gene sequence (12, 13).

$^d$ Cys(cm). carboxymethylcysteine.
two minor radioactive peaks were obtained. Although the amino acid composition of the major peptide (Table II) suggested that it comprises residues 520-537 with the sequence Gly-Glu-Gly-Ser-Trp-Glu-Ile-Leu-Gly-Ala-Met-Pro-X-Met-Asp-Pro-Pro, the identification was not as certain as for peptides I, II, III, and V. This peptide was therefore subjected to NH₂-terminal amino acid sequencing and yielded the sequence Gly-Glu-Gly-Ser-Trp-Glu-Ile-Leu-Gly-Ala-Met-Pro-X-Met-Asp-Pro-Pro, confirming the identity of this peptide.

The five hydrophilic [14C]-carboxymethylated free cysteine peptides designated I-V in Fig. 2 thus comprise residues 520-537 with the sequence Gly-Glu-Gly-Ser-Trp-Glu-Ile-Leu-Gly-Ala-Met-Pro-X-Met-Asp-Pro-Pro, the identification was not as certain as for peptides I, II, III, and V. This peptide was therefore subjected to NH₂-terminal amino acid sequencing and yielded the sequence Gly-Glu-Gly-Ser-Trp-Glu-Ile-Leu-Gly-Ala-Met-Pro-X-Met-Asp-Pro-Pro, confirming the identity of this peptide.

Identification of the Hydrophobic [14C]-Carboxymethylated Free Cysteine Peptide—Fig. 3 shows the results obtained when the H⁺-ATPase hydrophobic peptide mixture containing the free [14C]-carboxymethylated cysteine (Table I) is resolved by Sephadex LH-60 column chromatography by our recently described procedure (11). About half of the radioactivity emerges near the void volume in the fractions we have established previously (11, 34) to contain only a ~21-kDa hydrophobic H⁺-ATPase peptide beginning at Ser⁶⁶⁰ and ending near Lys⁶⁶⁵ and a small amount of a concanavalin A contaminant (11) that contains no cysteine residues (35). This indicates that the free cysteine residue in the hydrophobic portion of the ATPase molecule is either Cys⁶⁶⁰ or Cys⁶⁶⁵ because there are no other cysteine residues in the ~21-kDa hydrophobic peptide as deduced from the gene sequence (12, 13). Since our original analysis (11), we have established by NH₂-terminal sequencing (not shown) that a previously unidentified, minor ~14-kDa hydrophobic ATPase peptide eluting just to the right of the ~21-kDa peptide in the Sephadex LH-60 profile also begins at Ser⁶⁶⁰ and because of its size, presumably ends at Arg⁶⁸⁴. SDS-polyacrylamide gel electrophoresis analysis (34) of the column fractions collected in the experiment of Fig. 3 (not shown) showed the presence of roughly equal quantities of the ~21- and ~14-kDa bands, indicating an increased efficiency of tryptic digestion of the ~21-kDa peptide compared with our earlier studies (11), presumably as a result of denaturation and carboxymethylation of the ATPase. Conversion of the ~21-kDa hydrophobic peptide to the ~14-kDa peptide would release one or more smaller peptides containing both Cys⁶⁶⁰ and Cys⁶⁶⁵, which explains the presence of radioactivity in the smaller peptides eluting after the ~21-kDa peptide in the experiment of Fig. 3.

The establishment of either Cys⁶⁶⁰ or Cys⁶⁶⁵ as the 6th free cysteine residue in the ATPase molecule also establishes one or the other of these residues as the hydrophobic disulfide bridge cysteine (Table I) because the other 5 free cysteines have already been accounted for.

Identification of the Hydrophobic [14C]-Carboxymethylated Disulfide Bridge Cysteine Peptide—Identification of the hydrophilic [14C]-carboxymethylated disulfide bridge cysteine peptide defined as described above was by no means straightforward because of its extremely peculiar properties. Attempts to purify the peptide by HPLC using C₁₈ and C₄ reversed phase columns, DEAE-ion exchange column chromatography, Sephadex LH-60 column chromatography in chloroform/methanol/trifluoroacetic acid (11), gel filtration on Sephadex G-50 in anionic and cationic detergents, two-dimensional peptide mapping on thin layer cellulose sheets, SDS-polyacrylamide gel electrophoresis, and electroblotting onto polyvinylidene difluoride membranes following SDS-polyacrylamide gel electrophoresis were all unsuccessful for a variety of reasons. However, molecular exclusion chromatography on a long column of Sephacryl S-100 HR equilibrated with 1 M guanidine HCl as described under "Experimental Procedures" eventually proved to be a satisfactory means of purifying this peptide. Fig. 4 shows the results of a typical column run. The eluted radioactivity profile is characterized by a small peak at around 310 ml with a high A₂₅₀ followed by a major radioactive peak representing more than half of the radioactivity applied at about 330 ml with a much smaller A₂₅₀.

The major labeled peptide in this experiment was identified by NH₂-terminal amino acid sequencing. Although the repetitive yields for this peptide dropped rapidly, again presumably reflecting its peculiar properties, the sequence obtained clearly establishes that this peptide begins at Val⁹⁹ with the sequence Val-Val-Pro-Glu-Asp-Met-Leu-Gln-Thr. . . Because of its apparent size and because it must contain a cysteine residue, the hydrophobic peptides generated from the H⁺-ATPase [14C] carboxymethylated under nonreducing conditions. The ATPase was labeled to [14C] carboxymethylate the free cysteine residues and then digested with trypsin. The tryptic digest obtained was then separated into hydrophilic and hydrophobic peptide fractions, and the hydrophobic peptide fraction representing 2.2 mg of ATPase was dried by lyophilization and subjected to Sephadex LH-60 column chromatography and the fractions analyzed for radioactivity as described under "Experimental Procedures." The void volume of the column was about 115 ml (11).
this peptide probably ends at Lys175, indicating that the hydrophilic disulfide bridge cysteine is Cys148. The recalcitrant properties of this peptide presumably result from its large size and the presence of roughly equal amounts of hydrophilic and hydrophobic residues in extended linear stretches (11).

Regarding the minor 13C-carboxymethylated peptide peak in the column eluate shown in Fig. 4, from the molar absorbance of standard peptides at 220 nm and the radioactivity content of this peak, it could be estimated that the peak contained extensive amounts of contaminating peptide material. This was confirmed by NH2-terminal sequence analysis of the material in this peak, which indicated the presence of at least four recognizable H+-ATPase peptides. Judging from the molecular size of this material (~10 kDa as estimated from its behavior on the Sephacryl column) and the presence of recognizable H+-ATPase sequences, it must represent a collection of minor incomplete tryptic cleavage products. However, one of the sequences obtained in pmol amounts approximately equal to the radioactivity content was Tyr-Gly-Leu from residues 100-102, which then dropped off rapidly as did the major 13C-carboxymethylated peptide. The radioactivity present in the minor peak therefore probably also represents 13C-carboxymethylated Cys148 in a different peptide originating at Tyr190 and ending at an as yet unidentified residue. Thus, the great majority of the 13C-carboxymethylated material in the column eluate of Fig. 4 contains Cys148, identifying Cys148 as the hydrophilic disulfide bridge cysteine. The identification of this cysteine as the other disulfide bridge cysteine is of course fortified by the fact that it is the only cysteine of the 8 cysteine residues in the ATPase molecule left to be accounted for.

**DISCUSSION**

The results presented above indicate that 6 of the 8 cysteine residues in the N. crassa plasma membrane H+-ATPase are present in the reduced state and that the other 2 are present in disulfide linkage. The evidence for this comes from three independent sets of experiments. First, spectrophotometric titrations of the H+-ATPase with DTNB indicate that there are six free sulfhydryl groups in the enzyme. Second, similar titrations of the DTNB and NTSB titrations are fully corroborated by the results of carboxymethylation experiments using iodoacetate. Carboxymethylation of the ATPase under nondenaturing conditions derivatizes 6 cysteine residues, and carboxymethylation after reduction derivatizes all 8. It is thus reasonably certain that the native ATPase molecule contains 6 free cysteine residues and 1 disulfide bridge.

We have recently introduced the use of an ammonium bicarbonate extraction procedure for efficiently separating the hydrophobic and hydrophilic peptides generated by limited digestion of the H+-ATPase with trypsin, and additional methodology for further fractionating the numerous peptides present in the resulting hydrophilic and hydrophobic peptide mixtures, with essentially complete recoveries (11). In the studies presented here, this methodology proved invaluable for the identification of the free and disulfide bridge cysteine residues. Thus, utilizing the ammonium bicarbonate extraction procedure, it could be shown that 5 of the carboxymethylated free cysteine peptides and 1 of the disulfide bridge cysteine peptides partition into the hydrophilic peptide fraction, and the peptides containing the remaining 2 cysteines partition into the hydrophobic peptide fraction. Purification of the 5 carboxymethylated free cysteine peptides present in the hydrophilic peptide fraction by HPLC was reasonably straightforward, allowing the identification of these cysteines as Cys175, Cys396, Cys472, Cys532, and Cys545 and leaving only 3 cysteine residues to be identified. Fractionation of the hydrophobic peptide mixture allowed the identification of the remaining free cysteine as either Cys462 or Cys547 in the extremely hydrophobic ~21-kDa ATPase peptide beginning at Ser565, which automatically defined one or the other of these cysteine residues as a disulfide bridge cysteine because the 6 free cysteines were already identified. This was corroborated by the demonstration that 1 of the disulfide bridge cysteines partitions into the hydrophobic peptide fraction. Finally, the remaining disulfide bridge cysteine present in the hydrophilic peptide fraction was identified as Cys578 by purification and direct amino acid sequence analysis and by default as the only remaining cysteine in the molecule. In addition to delineating the chemical status of the 8 cysteine residues in the H+-ATPase molecule, these results attest to the effectiveness of our previously reported procedures (11).

The demonstration of a disulfide bridge between Cys148 and either Cys462 or Cys547 has interesting implications as to the three-dimensional structure of the H+-ATPase molecule. Cys148 is present in an extremely hydrophobic stretch of residues in the ATPase molecule, which, from hydropathy analyses (11-13) and from the preliminary results of our direct protein chemical analyses of the membrane-embedded sectors of the H+-ATPase (2), appears to contain two of the four to six membrane-spanning segments at the NH2-terminal end of the molecule. On the other hand, both Cys462 and Cys547 are present in the extremely hydrophobic ~21-kDa ATPase peptide, which, by the same criteria, appears to contain four to six membrane-spanning segments at the COOH-terminal end of the molecule. It is thus possible that the disulfide bridge between Cys148 and either Cys462 or Cys547 may serve a critical structural role in holding the two major membrane-embedded sectors of the H+-ATPase molecule, distant in the linear sequence, together. It will therefore be of interest in the future to see if this structural feature is conserved in other ATPases in the aspartyl-phosphoryl-enzyme intermediate family and other transporters as well. When a suitable expression system for the H+-ATPase is developed, it will also be of considerable interest to assess the importance of the H+-ATPase disulfide bridge by site-directed mutagenesis.

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