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Isolation and Sequence of Tryptic Peptides from the Proton-Pumping ATPase of the Oat Plasma Membrane[1]

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ABSTRACT

In crude extracts of plant tissue, the M_r = 100,000 proton-pumping ATPase constitutes less than 0.01% of the total cell protein. A large-scale purification procedure is described that has been used to obtain extensive protein sequence information from this enzyme. Plasma membrane vesicles enriched in ATPase activity were obtained from extracts of oat roots by routine differential and density gradient centrifugation. Following a detergent wash, the ATPase was resolved from other integral membrane proteins by size fractionation at 4°C in the presence of lithium dodecyl sulfate. After carboxymethylation of cysteine residues and removal of detergent, the ATPase was digested with trypsin and resultant peptide fragments separated by reverse phase high performance liquid chromatography. Peptides were recovered with high yield and were readily sequenced by automated Edman degradation on a gas-phase sequencer. Of the eight peptides sequenced, six showed strong homology with known amino acid sequences of the fungal proton-pumping and other cation-transporting ATPases.

The plasma membrane of higher plants and fungi contains an electrogenic, proton-pumping ATPase (H^+-ATPase). The H^+-ATPase uses the chemical energy of ATP to extrude protons into the external medium. This generates a proton electrochemical gradient, also termed a protonmotive force, which is used to drive solute uptake systems. In addition to providing a driving force for nutrient uptake, the H^+-ATPase helps to establish the turgor, pH, and ionic composition necessary for growth of the cell (4, 18).

The H^+-ATPase belongs to a class of cation-transporting ATPases with similar enzymatic and structural properties. Other members include the Na^+, K^+-ATPase, the H^+, K^+-ATPase, and the Ca^{2+}-ATPase of animal membranes, and the K^+-ATPase of *Escherichia coli*. All of these enzymes are inhibited by vanadate and have a catalytic subunit of M_r = 100,000 that is phosphorylated during the reaction cycle. The complete amino acid sequences for many of the cation-transporting ATPases (6, 10, 16) and for the H^+-ATPases of the fungi *Neurospora crassa* (5) and *Saccharomyces cerevisiae* (19) have been determined, by direct protein sequencing and by cloning and sequencing the gene encoding the ATPase, and all show a number of highly conserved regions with sequence homology.

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Very little structural information is available on the plasma membrane H^+-ATPase of higher plants. The gene encoding the H^+-ATPase of higher plants has not yet been cloned and sequenced, and only a short stretch of amino acid sequence near the active site of phosphorylation is known (23). Although progress has recently been made in purifying active H^+-ATPase from higher plants, even in the purest preparations the ATPase is recovered in low yield and contains protein contaminants (2, 17). Chemical studies of the ATPase, such as amino acid sequencing, require large amounts of pure polypeptide but the polypeptide need not be enzymatically active. We describe here a method by which large amounts of oat plasma membrane H^+-ATPase can be readily purified. We have isolated and sequenced tryptic peptides derived from this material, providing the first extensive sequence information on a higher plant plasma membrane H^+-ATPase.

MATERIALS AND METHODS

Plasma Membrane Isolation. Oat root plasma membranes were isolated and assayed for ATPase activity as described by Surowy and Sussman (20). Briefly, oat roots were homogenized, strained through cheesecloth, and centrifuged at 8,000*g* for 15 min to remove mitochondria and cellular debris. Microsomes were pelleted from the supernatant by centrifugation at 48,000*g* for 1.5 h, and then applied to a discontinuous sucrose gradient. After centrifugation at 200,000*g* for 1.5 h, plasma membranes were collected at the 33%/46% (w/w) sucrose interface. ATPase activity was assayed colorimetrically by measuring the release of inorganic phosphate from ATP. Plasma membrane protein was assayed by the method of Lowry (9), using BSA as standard. For the experiments described here, 200 mg of oat plasma membrane protein obtained from 3.6 kg of oat roots were used. Specific activity of the H^+-ATPase in these membranes was 1.3 μmol inorganic phosphate released × (mg protein)$^{-1}$ × min^{-1}. Greater than 95% of this activity was resistant to 5 mM NaN_3, 100 mM KNO_3, and 0.5 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, known inhibitors of the mitochondrial, vacuolar, and nonspecific cytoplasmic ATPases, respectively.

Purification of the ATPase. Plasma membranes were treated with 0.2% sodium deoxycholate, a procedure which removes approximately half of the membrane protein without solubilizing the ATPase (20). Membranes were then recovered by centrifugation at 200,000*g* for 45 min, and the pellet solubilized at 10 mg protein/ml with 1% (w/v) lithium dodecyl sulfate. The M_r = 100,000 ATPase was then resolved from other solubilized oat plasma membrane proteins by size-fractionation on a column of Bio-Gel A-1.5m (2.5 cm × 120 cm) run at 4°C under denaturing conditions with 1% (w/v) lithium dodecyl sulfate. The lithium salt of dodecyl sulfate was used as denaturant for increased solubility at 4°C. Bromophenol blue and blue dextran were included with the sample as visual mol wt markers for the column's included and excluded volumes, respectively. Eluted fractions were

analyzed for protein by SDS-PAGE (8), and those found to contain the M_r = 100,000 ATPase were pooled and concentrated by lyophilization. Excess detergent was then removed by stirring the sample with Biobeads SM-4, with the decrease in detergent concentration monitored using a methylene blue assay (11).

Reduction and carboxymethylation. The conversion of cysteine to carboxymethylcysteine was accomplished by an adaption of the method of Allen (1). Concentrated and lyophilized ATPase (about 500 μg) was suspended at a protein concentration of 2 mg/ml in 1 mM EDTA, 8 M urea, and 100 mM Tris adjusted to pH 8.3 with HCl. Detergent concentration was 1% (w/v) lithium dodecyl sulfate. DTT was added to a concentration of 0.2 mM, and the mixture incubated at 37°C for 1 h under nitrogen. [^{3}H]Iodoacetic acid was then added to a concentration of 0.6 mM and alkylation allowed to proceed in the dark at 37°C for 1 h under N_2. Reagents were added from 20 mM aqueous stock solutions. The alkylation reaction was repeated once more with 0.1 mM DTT and 0.3 mM iodoacetic acid. The ATPase was then dialyzed extensively at room temperature against 0.1 M NH_4HCO_3 (pH 8) to remove unreacted reagents and detergent.

Isolation and Sequencing of Tryptic Peptides. Following dialysis, the volume of the ATPase solution was reduced in a Speed Vac Concentrator to yield a final protein concentration of 2 mg/ml. Diphenylcarbamyl chloride-treated trypsin (1% by weight of protein) was added and the mixture incubated at 37°C for 3 h, followed by a second equivalent addition of trypsin. The ATPase was then digested for an additional 21 h.

Tryptic peptides were purified by reverse phase HPLC using a Vydac C-18 analytical column (25 cm × 0.4 cm, 5 μm particle size, 30 nm pore size). Absorbance of the eluate was monitored on a Hewlett Packard Model 1040A diode array detector. Solvent A was 0.1% TFA in water, and solvent B was 0.083% TFA in 95% acetonitrile. Peptides were separated on a 70 min linear gradient, from 0% to 47% solvent B, with a 1 ml/min constant flow rate. Peptide-containing peaks were collected and, where further purification was necessary, rechromatographed with the same solvents on a shallower gradient.

Automated Edman degradations of the purified peptides were performed on an Applied Biosystems 470A gas-phase protein sequencer equipped with an on-line, microbore HPLC. PTH[2] derivatives of the amino acids were identified by retention time on reverse phase HPLC and quantified by absorbance at 269 nm using PTH-amino acid standards (25 pmol).

Amino acid sequence comparisons were performed on a VAX minicomputer using the PROFILE program of the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package, Version 4.

PAGE. SDS-PAGE of proteins was performed according to the method of Laemmli (8), using a stacking gel of 5% (w/v) acrylamide and a running gel of 8% (w/v) acrylamide. Following electrophoresis, gels were stained in 25% (v/v) isopropanol, 10% (v/v) acetic acid, 0.05% (w/v) Coomassie blue, and destained in 25% (v/v) isopropanol, 10% (v/v) acetic acid. Fluorograms were prepared by soaking gels for 15 min in Amplify (Amersham, Arlington Heights, IL), drying the gel onto a paper backing, and then exposing the gel at −80°C to X-Omat AR film (Kodak, Rochester, NY). To measure the radioactivity contained in protein bands, bands were excised from the gel, dissolved in 30% (v/v) H_2O_2, 1% (v/v) NH_4OH, and counted in liquid scintillation cocktail (fluid type 3a70b, Research Products International, Mount Prospect, IL). Coomassie-stained gel bands were scanned and integrated using a Zeineh Soft Laser Scanning Densitometer, model SL-504-XL (Biomed Instruments, Fullerton, CA).

Materials. Oat seeds (*Avena sativa*, variety Stout) were obtained from Olds Seed Co., Madison, WI. Bio-Gel A-1.5m, Biobeads SM-4, and SDS-PAGE reagents were purchased from BioRad Labs (Richmond, CA). [^{3}H]Iodoacetic acid was purchased from NEN Research Products. HPLC solvents were purchased from either Alltech Associates, Inc. (Deerfield, IL) or Burdick & Jackson Labs (Muskegon, MI). All other reagents were purchased from Sigma.

[2] Abbreviation: PTH, phenylthiohydantoin.

RESULTS AND DISCUSSION

Purification of the ATPase. The M_r = 100,000 H⁺-ATPase of the oat plasma membrane was purified from other integral plasma membrane proteins by size-fractionation under denaturing conditions. Oat plasma membranes were first isolated using routine differential and density gradient centrifugation procedures and then treated with 0.2% sodium deoxycholate. This detergent wash procedure removes approximately half the membrane protein without extracting the ATPase (20). Most important, the deoxycholate wash removes a few other polypeptides with a mol wt near that of the M_r = 100,000 ATPase. Experiments using glycerol density gradient centrifugation to purify the H⁺-ATPase indicate that, in these preparations of detergent-washed oat plasma membranes, the H⁺-ATPase is the predominant M_r = 100,000 polypeptide (14). Washed membranes were then solubilized in 1% (w/v) lithium dodecyl sulfate, and the ATPase purified from the other solubilized proteins by size-fractionation on a column of Bio-Gel A-1.5m at 4°C (to minimize proteolysis). When column fractions were analyzed by SDS-PAGE and stained with Coomassie blue (Fig. 1A), a prominant band at M_r = 100,000, corresponding to the oat plasma membrane H⁺-ATPase (7), was observed in several fractions. The H⁺-ATPase was recovered from the column in high yield with very little contamination from other proteins. Based on densitometer scans of Coomassie-stained gels, the M_r = 100,000 polypeptide constituted at least 83% of the protein when the peak ATPase-containing fractions were pooled.

ATPase recovered from the column was concentrated by lyophilization and resuspended in a 1/10 smaller volume. The excess lithium dodecyl sulfate was removed by stirring samples with

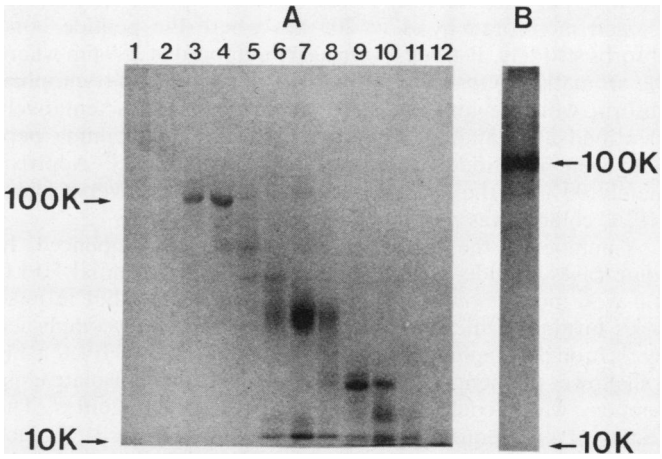

FIG. 1. SDS-PAGE analysis during purification of H⁺-ATPase from oat plasma membranes. (A) Coomassie-stained polypeptide profile of fractions obtained off a Bio-Gel A-1.5m column. Deoxycholate-stripped oat plasma membranes were solubilized, denatured with 1% (w/v) lithium dodecyl sulfate, and applied to the column. Flow rate was 20 ml/h and the fraction volume was 6 ml. An aliquot from each of the even-numbered fractions, 6 to 28, was analyzed by SDS-PAGE (lanes 1 through 12 in the figure). Arrows and mol wt indicate the positions of the M_r = 100,000 ATPase (derived from position of mol wt standards) and the dye front. (B) Fluorogram from SDS-PAGE analysis of the purified ATPase, following reduction and carboxymethylation of the cysteine residues with [^{3}H]iodoacetic acid and dialysis against 0.1 M NH_4HCO_3.

Biobeads SM-4. Previous experiments established that this procedure absorbs 90% of the detergent without loss of protein. Because not all of the detergent is removed, the hydrophobic ATPase stays in solution. By sequentially lyophilizing and resuspending the sample at 1/10 the former volume, it was possible to reduce the sample volume 100-fold, while maintaining a concentration of 1% (w/v) lithium dodecyl sulfate.

Cysteine residues of the ATPase were reduced and carboxymethylated with [^{3}H]iodoacetic acid. This was done for several reasons. First, the introduction of charged groups makes the largely hydrophobic ATPase more soluble, allowing subsequent removal of all detergent and facilitating enzymatic digestion (1). Second, by radioactively labeling one can detect protein with enhanced sensitivity and accuracy. Finally, the radiolabel can be used to identify cysteine-containing peptides, some of which have been implicated in the H^+-ATPase reaction mechanism (7). Following reduction and carboxymethylation, the ATPase was dialyzed extensively to remove unincorporated radiolabel, reagents, and remaining detergent, and then analyzed by SDS-PAGE. A fluorogram of a dried gel is shown in Figure 1B. The M_r = 100,000 ATPase band is strongly labeled, indicating the conversion of cysteine residues to [^{3}H]carboxymethylcysteine. The M_r = 100,000 band was excised from the gel, dissolved with 30% (v/v) H_2O_2, 1% (v/v) NH_4OH, and the amount of incorporated radiolabel determined by liquid scintillation counting. Protein content in the excised band was estimated by intensity of Coomassie-stain relative to standard mol wt markers (phosphorylase *a* and β-galactosidase). From these measurements we calculated an estimated stoichiometry of 330 Bq/μg ATPase, suggesting that 5 mol of cysteine were modified per mol of ATPase.

Isolation and Sequencing of Tryptic Peptides. Preliminary experiments indicated that the amino terminus of the ATPase is blocked, preventing use of the Edman degradation with the intact protein. In order to obtain amino acid sequence information on the H^+-ATPase, we digested the M_r = 100,000 polypeptide with 2% (w/w) trypsin in 0.1 M NH_4HCO_3 pH 8 at 37°C for 24 h. The resulting peptides were separated on reverse phase HPLC (Fig. 2), and monitored by *A* at 205 nm where the peptide bond absorbs strongly. Peptides were also monitored at 280 nm where the aromatic groups of tyrosine, phenylalanine, and tryptophan absorb, with the most strongly absorbing peptides tentatively identified as containing tryptophan. Cysteine-containing peptides were identified by the presence of radiolabel. Approximately 90% of the radioactivity applied to the reverse phase HPLC column was recovered.

A number of the peptides were isolated and sequenced. In some cases peptide-containing fractions from the initial HPLC run were pure enough for immediate sequencing, but in most cases further purification was required. This was accomplished by rechromatographing the peptide by reverse phase HPLC using a shallower gradient. By this means we were able to isolate eight peptides which could be sequenced without ambiguities. The results of one sequencing run are shown in Figure 3. In each cycle only one amino acid, present as the PTH-derivative, is observed in appreciable quantity. In the first few cycles some peaks cochromatographing with standard amino acids are present, but these are present at low levels and do not obscure the sequence.

We determined the amino acid sequence for one peptide containing radiolabel and observed a peak of radioactivity in cycle 12 (Fig. 4). On the basis of *A* at 269 nm, this cycle was identified as cysteine by the presence of PTH-carboxymethylcysteine, the PTH derivative resulting from the modification of cysteine with iodoacetic acid. Although only 11% of the radioactivity initially present was recovered in cycle 12, this amount is in accordance with the yield of PTH-amino acid (as the carboxymethylcysteine derivative), which was also 11% of that observed in the first cycles. The remaining 89% of the radioactivity and PTH-amino acid was lost due to peptide washout at each cycle of the Edman degradation, an unavoidable problem with gas- and liquid-phase protein sequencing. Repetitive yields for all peptides varied from 82% to 92%.

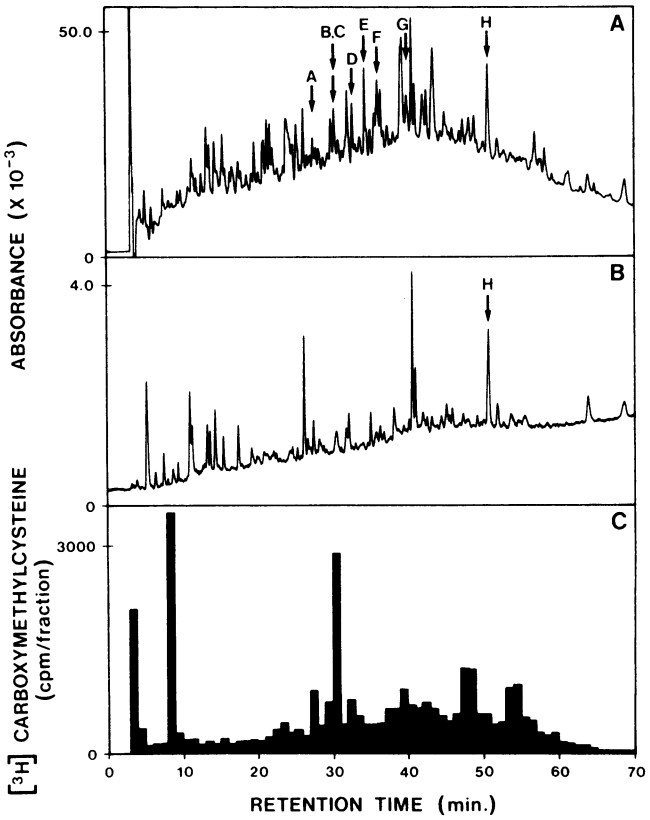

FIG. 2. Reverse phase HPLC separation of tryptic peptides of the oat root plasma membrane H^+-ATPase. Peptides were separated on a 70 min gradient, from 0% to 44% acetonitrile in 0.1% TFA, with a constant flow rate of 1 ml/min. Absorbance at 205 nm (A) and at 280 nm (B) and radioactivity (C) were monitored. Letters and arrows indicate peptide-containing peaks that were purified and sequenced.

Peptide H, which has strong peak of absorption at 280 nm, was shown to contain tryptophan in cycle 1 of this 18 amino acid-long peptide (Fig. 5).

Homology with Other Cation-Transporting ATPases. We isolated and sequenced 8 peptides covering a total of 110 amino acids, or about 11% of the entire amino acid sequence of the oat M_r = 100,000 H^+-ATPase. Six of the oat peptides (peptides A, C, D, E, F, and H) show a high degree of homology with regions that have been implicated in the reaction mechanism of the other cation-transporting ATPases (Fig. 5). Two of the oat peptides correspond to the two most highly conserved regions of the ATPases: peptide A corresponds to a region adjacent to the phosphorylation site at aspartyl residue 378 in the fungal enzyme (5), and peptide F corresponds to a region that in the animal M_r = 100,000 ATPases has been shown to bind the ATP analog 5′-(*p*-fluorosulfonyl)benzoyladenosine in an ATP-protectable manner, suggesting that it is part of the active site (12). Peptides C, D, E, and H were found to be homologous to sequences between the postulated second and third transmembrane segments, a region that has been implicated in the energy transduction mechanism (15). The homology between peptides isolated from the M_r = 100,000 polypeptide of the oat plasma membrane and the known cation-transporting ATPases confirms

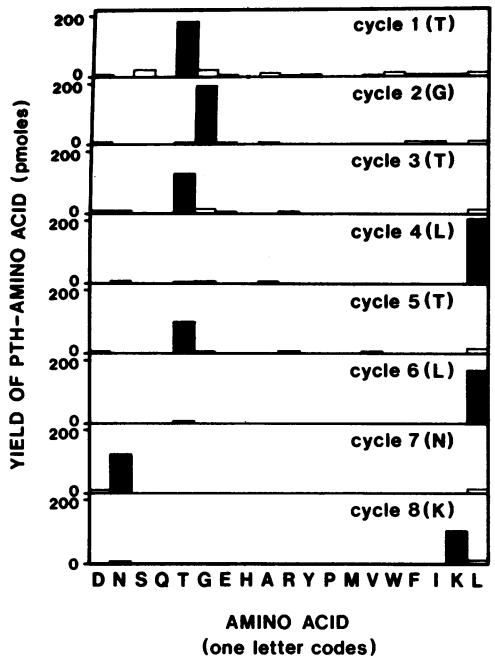

FIG. 3. Amino acid sequence analysis of peptide A. Peptide A (40% of the sample) was sequenced by automated Edman degradation on an Applied Biosystems gas-phase protein sequencer. The yield of all the PTH-amino acids are plotted from each cycle of the sequence. Darkened blocks indicate the primary PTH-amino acid observed in each cycle. Clear blocks indicate contaminating PTH-amino acids also present. Amino acids are identified by their single-letter code.

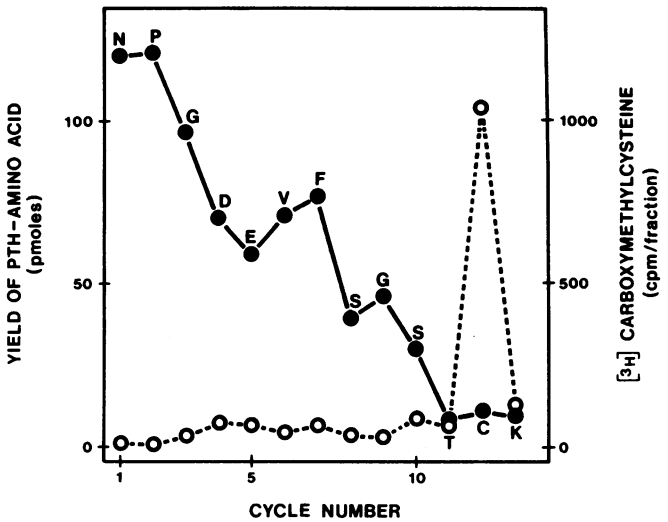

FIG. 4. Amino acid sequence analysis of peptide C. Peptide C was purified from a reverse phase HPLC fraction that contained radioactivity, and 40% of the sample sequenced via the Edman degradation. The primary PTH-amino acid observed (single-letter codes) and its recovery are shown for each cycle of the degradation (closed circles). The radioactivity released at each cycle is also shown (open circles).

that we have isolated the H⁺-ATPase of higher plants, and indicates that the oat H⁺-ATPase has a mechanism for ATP binding and hydrolysis similar to that of the other cation-transporting ATPases.

Two peptides (peptide B, Glu-Leu-Ser-Glu-Ile-Ala-Glu-Gln-Ala-Lys; and peptide G, Thr-Leu-His-Gly-Leu-Gln-Ala-Pro-Glu-Ser-Thr-Ser/Ala-Leu-Asn-Leu-Phe-Asn-Asp-Lys) do not show

ENERGY TRANSDUCTION REGION

OAT PEPTIDE H	- W G E Q E A S I L V P G D I V S I K -	
H^+-ATPase (N)	188 L K E I E A P E V V P G D I L Q V E	205
H^+-ATPase (S)	188 L V E I P A N E V V P G D I L Q L E	205
Ca^{2+}-ATPase	137 V Q R I K A K D I V P G D I V E I A	154
Na^+,K^+-ATPase	177 . . S I N A E Q V V V G D I V E V K	192
K^+-ATPase	118 A D K V P A D Q L R K G D I V L V E	135
OAT PEPTIDE D	- L G D I V P A D A R -	
H^+-ATPase (N)	206 E G T I I P A D G R	215
H^+-ATPase (S)	206 D G T V I P T D G R	215
Ca^{2+}-ATPase	155 V G D K V P A D I R	264
Na^+,K^+-ATPase	193 G G D R I P A D L R	202
K^+-ATPase	136 A G D I I P C D G E	145
OAT PEPTIDE E	- I D Q S G L T G E S L P V T K -	
H^+-ATPase (N)	225 V D Q S A L T G E S L A V D K	239
H^+-ATPase (S)	225 I D Q S A I T G E S L A V D K	239
Ca^{2+}-ATPase	175 V D Q S I L T G E S V S V I K	189
Na^+,K^+-ATPase	211 V D N S S L T G E S . . . E P	222
K^+-ATPase	153 V D E S A I T G E S A P V I R	167
OAT PEPTIDE C	- N P G D E V F S G S T C K -	
H^+-ATPase (N)	240 H K G D Q V F A S S A V K	252
H^+-ATPase (S)	240 H Y G D Q T F S S S T V K	252
Ca^{2+}-ATPase	203 D K K N M L F S G T N I A	215
Na^+,K^+-ATPase	236 E T R N I A F F S T N G V	248
K^+-ATPase	174 . . . A S V T G G T R I L	183

PHOSPHORYLATION REGION

OAT PEPTIDE A	- T G T L T L N K -	
H^+-ATPase (N)	380 T G T L T K N K	387
H^+-ATPase (S)	380 T G T L T K N K	387
Ca^{2+}-ATPase	353 T G T L T T N Q	360
Na^+,K^+-ATPase	376 T G T L T Q N R	383
K^+-ATPase	309 T G T I T L V N	316

ATP-BINDING REGION

OAT PEPTIDE F	- G I V G M T G D G V N D A P A L K -	
H^+-ATPase (N)	627 Y L V A M T G D G V N D A P S L K	643
H^+-ATPase (S)	627 Y L V A M T G D G V N D A P S L K	643
Ca^{2+}-ATPase	696 E I T A M T G D G V N D A P A L K	712
Na^+,K^+-ATPase	708 A I V A V T G D G V N D S P A L K	724
K^+-ATPase	511 R L V A M T G D G T N D A P A L A	527

FIG. 5. Amino acid homology between tryptic peptides isolated from the oat plasma membrane H⁺-ATPase and known sequences of cation-transporting ATPases. Homologies were identified using the PROFILE program of the University of Wisconsin Genetics Computer Group Sequence Analysis Software. Peptides from the oat plasma membrane H⁺-ATPase are designated according to Figure 2. H⁺-ATPase (N) refers to the *N. crassa* enzyme, H⁺-ATPase (S) refers to the yeast *S. cerevisiae* enzyme, Ca^{2+}-ATPase refers to the enzyme isolated from the rabbit sarcoplasmic reticulum, Na^+, K^+-ATPase refers to the enzyme isolated from the sheep kidney, and K^+-ATPase refers to the product of the *KdpB* gene of *E. coli*. Amino acid residues (single-letter codes) identical to those in the oat ATPase peptides are boxed.

significant homology to the cation-transporting ATPases. These may represent portions of the oat H⁺-ATPase that share little homology with the other ATPases, or could represent contamination from other polypeptides present in our ATPase preparation. Several lines of evidence suggest that one would expect to find regions of the oat H⁺-ATPase which do not show significant homology with the other ATPases. Polyclonal antibodies have been generated against the H⁺-ATPase of oat roots and of the fungus *N. crassa* and only weak cross-reactivity between the two enzymes was observed, indicating that there are substantial differences in structure even between these two closely related ATPases (20). In addition, a comparison of the complete sequences of the different cation-transporting ATPases reveals that although certain specific regions of the enzymes are highly conserved there are other regions in which no obvious homology in primary sequence is detected.

One might expect the higher plant H^+-ATPase to show more homology to the fungal H^+-ATPases than to the Na^+, K^+- and Ca^{2+}-ATPases of animal membranes. The H^+-ATPases of *S. cerevisiae* and *N. crassa* are 74% homologous overall, but share only 14% homology with the other cation-transporting ATPases (5). The high degree of conservation between fungal enzymes is caused in part by evolutionary closeness and in part by similarities in reaction mechanism, since both differ from the other ATPases in being exclusively proton pumps. Although the plant plasma membrane ATPase shares cation specificity with the fungal enzymes, only one of the peptides from the oat enzyme, peptide C, shows significantly more homology to the fungal H^+-ATPases than to the animal ATPases. This suggests that the higher plant H^+-ATPase has evolutionarily diverged from the fungal H^+-ATPases. In addition, the proton transport function could reside in a section of the enzyme not revealed in the peptides we sequenced (21). Based upon these results, further amino acid sequence information should be particularly revealing as to the H^+-ATPase reaction mechanism. Sequences common to all the cation-transporting ATPases are probably involved in either ATP hydrolysis or cation-translocation. Homologous regions particular to the H^+-ATPases of higher plants and fungi are probably related directly to the proton-specificity of the enzyme.

CONCLUSION

We have described a method to obtain sequencable amounts of the M_r = 100,000 polypeptide of the oat root plasma membrane proton pump (H^+-ATPase). This procedure relies on the observation that in detergent-washed plasma membrane preparations obtained from oat roots and fungal hyphae the ATPase is the predominant polypeptide of $M_r > 70{,}000$. It is unlikely that the enzyme obtained with our method is completely pure. Estimates based on the Coomassie-stained SDS-PAGE profile indicate that at least 83% of the protein is accounted for by this M_r = 100,000 polypeptide. Previous experiments indicate that this M_r = 100,000 polypeptide is predominantly the H^+-ATPase (14). Thus, when detergent-washed oat plasma membranes were solubilized and the various proteins resolved using glycerol density gradient centrifugation, a single polypeptide at M_r = 100,000 was observed and this polypeptide co-purified with ATPase activity. Two-dimensional gel electrophoresis cannot be used to assess the homogeneity of this preparation because hydrophobic integral membrane proteins such as the H^+-ATPase aggregate in the absence of SDS and do not enter the isoelectric focusing gel with good yield (22).

From the intensity of the Coomassie-stained M_r = 100,000 ATPase polypeptide, we estimated that about 5 nanomol (500 μg) of protein were purified, starting from a crude extract of 3.6 kg oat roots. Based on the level of PTH-amino acid observed in the first cycle of Edman degradation, the yield of tryptic peptides varied from 300 pmol to about 3 nmol. The differences in yield could be due to incomplete digestion of the ATPase with trypsin or be the result of aggregation and loss due to the hydrophobic nature of peptides derived from transmembrane fragments.

By sequencing tryptic peptides we have obtained the first extensive amino acid sequence information on a plasma membrane H^+-ATPase from a higher plant. The results indicate that the higher plant enzyme has significant homology with the other known M_r = 100,000 cation-transporting ATPases, particularly in regions thought to be involved in the ATPase reaction mechanism. Slightly more homology was observed with the fungal H^+-ATPases than with the other cation-transporting ATPases. Chemical modification studies indicate that the plant H^+-ATPase contains an essential *N*-ethylmaleimide-reactive cysteine residue in the nucleotide-binding site (7) and a dicyclohexylcarbodiimide-reactive residue located in a hypothetical proton 'channel' (13, 21). The procedures described in this report will be useful for isolating and sequencing peptide fragments containing these modified amino acids as well as phosphorylated serine and threonine residues that may be involved in kinase-mediated regulation of this enzyme (3, 14). In addition, the protein sequence data we have generated will prove useful in studies aimed at cloning and sequencing the structural gene(s) encoding the oat plasma membrane H^+-ATPase.

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