Initial Steps in the Assembly of the Vacuole-Type H\(^{+}\)-ATPase\(^1\)

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The plant vacuole is acidified by a complex multimeric enzyme, the vacuole-type H\(^{+}\)-ATPase (V-ATPase). The initial association of ATPase subunits on membranes was studied using an in vitro assembly assay. The V-ATPase assembled onto microsomes when V-ATPase subunits were supplied. However, when the A or B subunit or the proteolipid were supplied individually, only the proteolipid associated with membranes. By using poly(A\(^+)\) RNA depleted in the B subunit and proteolipid subunit mRNA, we demonstrated A subunit association with membranes at substoichiometric amounts of the B subunit or the 16-kD proteolipid. These data suggest that poly(A\(^+)\) RNA-encoded proteins are required to catalyze the A subunit membrane assembly. Initial events were further studied in vivo protein labeling. Consistent with a temporal ordering of V-ATPase assembly, membranes contained only the A subunit at early times; at later times both the A and B subunits were found on the membranes. A large-mass ATPase complex was not efficiently formed in the absence of membranes. Together, these data support a model whereby the A subunit is first assembled onto the membrane, followed by the B subunit.

The accumulation of solutes in the vacuole, largely driven by two vacuole H\(^{+}\) pumps, the pyrophosphatase and the V-ATPase, allows the development of an osmotic gradient, which ultimately leads to turgor pressure, a requisite for cell elongation and the development of the mature plant cell. The V-ATPase, although predominantly localized at the vacuole, has also been detected in a variety of organelles that constitute the endomembrane system, including the ER (Herman et al., 1994), Golgi apparatus (Chanson and Taiz, 1985; Ali and Akajuza, 1986), and coated vesicles (Depta et al., 1991). The V-ATPase is a large multimeric enzyme consisting of one or more of 7 to 10 distinct subunits (Parry et al., 1989; Sze et al., 1992). The molecular mass of the holoenzyme has been estimated at 600 to 700 kD (Randall and Sze, 1986; Bremberger and Luttge, 1986; Parry et al., 1989). The 69- to 72-kD (A subunit) and the 57- to 60-kD (B subunit) proteins are major subunits of the V-ATPase, with an apparent stoichiometry of three each per holoenzyme (Sze et al., 1992). There also appear to be six copies of a 16-kD proteolipid (Kaelstner, 1988) and all other subunits are thought to exist in single copies (Mandala and Taiz, 1986; Randall and Sze, 1986; Sze et al., 1992).

The V-ATPase is considered to have two distinct sectors (Rea et al., 1987a). The soluble peripheral sector (V\(_1\)) contains the regulatory (B subunit; Manolson et al., 1985) and catalytic subunits (A subunit; Mandala and Taiz, 1986; Randall and Sze, 1986). The integral membrane sector (V\(_0\)) contains the proteolipid and is thought to form the pore through which protons are passed. Electron micrographs show a “ball-and-stalk” structure with a diameter of 9 to 10 nm resting on a narrower stalk that connects the ball and membrane (Klink and Luttge, 1991; Moore et al., 1991; Taiz and Taiz, 1991; Sze et al., 1992). The V\(_1\) sector (containing the A and B subunits and several other polypeptides) is dissociated from the holoenzyme by chaotropic anions (Lai et al., 1988; Ward et al., 1992) and can be reassOCIated into a functional complex (Ward et al., 1992). This indicates that the reassembly of the peripheral subunits with the membrane sector does not require de novo protein synthesis. Reversible assembly of the ATPase appears to be a regulatory mechanism in yeast (Kane, 1995).

The V\(_0\) sector is dominated by the 16-kD proteolipid, so called because of its solubility in chloroform-methanol (Rea et al., 1987b). The deduced amino acid sequence of the oat 16-kD subunit predicts four membrane-spanning domains (Lai et al., 1991). Along with the 16-kD proteolipid, 32-, 13-, and 12-kD proteins have been identified as V\(_0\) sector proteins in oat (Ward and Sze, 1992). A larger integral membrane protein that often associates with the purified ATPase has been observed in a variety of plant species and yeast (Arai et al., 1988; Parry et al., 1989; DuPont and Morrissey, 1992; Manolson et al., 1992, 1994) and varies in apparent molecular mass from 95 to 115 kD. This subunit appears unnecessary for V-ATPase H\(^{+}\) pumping and ATP hydrolysis activity in mung bean, oat, Kalanchee, and corn vacuoles (Ward et al., 1992). Disruption of this gene in yeast, however, leads to a blockage in ATPase assembly on the tonoplast (Manolson et al., 1992).

Yeast genomic deletion mutants have been useful tools in determining requirements for assembly and targeting of the V-ATPase. Mutants lacking the 69-, 60-, or 27-kD V\(_1\) subunits failed to assemble into soluble complexes (Doherty and Kane, 1993), and mutants lacking the 69-, 60-, 42-, 32-, or 27-kD V\(_1\) subunits also failed to assemble the V\(_1\) subunits onto the vacuole membrane (Kane et al., 1992; Ho et al., 1993b; Graham et al., 1994). Mutants lacking the

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Abbreviations: DEPC, diethyl pyrocarbamate; V-ATPase, vacuole-type H\(^{+}\)-ATPase.
54-kD subunit could assemble and target the peripheral complex to the tonoplast but could not translocate protons (Ho et al., 1993a). Although complete assembly of the holoenzyme requires all but the 54-kD subunit, a number of “intermediate” complexes have been identified using a combination of native and denaturing gel electrophoresis (Tomashek et al., 1996). The Vₐ sector appears to be assembled as a separate entity and is not affected by the presence or absence of the peripheral sector subunits (Kane et al., 1992; Manolson et al., 1992; Bauerle et al., 1993; Doherty and Kane 1993; Ho et al., 1993a, 1993b; Graham et al., 1994). The 95-, 36-, and 16-kD subunits are all required for the assembly of a stable integral membrane sector (Kane et al., 1992; Doherty and Kane, 1993) and for tonoplast targeting of the peripheral complex (Kane et al., 1992; Manolson et al., 1992; Bauerle et al., 1993; Doherty and Kane, 1993). In addition to the ATPase subunits, a 25.5-kD protein (Hirata et al., 1993) and a 21-kD (ER-associated) protein (Hill and Stevens, 1995) are necessary for V-ATPase assembly. These studies have thus demonstrated the requirements for various subunits for final localization to the vacuole and for the functionality of the proton pump. It is important to note that all of these analyses address the steady-state situation in cells, and thus may not address the mechanisms or requirements for the initial steps of assembly.

Because little is known about the early events in biosynthesis and assembly of the ATPase, we have developed an in vitro assay with which we can observe the initial events of V-ATPase assembly in plants. This assay exploited a heterologous translation system and ER membranes (non-plant) so that the role of plant-specific factors could be determined. Using this assay we show that although the V₁ subunit to the membrane. These data are consistent with a model of assembly in which the A subunit is first assembled on the membrane (perhaps in conjunction with V₀ₐ sector proteins) followed by the B subunit. This suggests a potential regulatory mechanism by which the proton pump would be inactive until it reaches an appropriate intracellular destination (possibly a subdomain of the ER or perhaps the medial or trans Golgi), at which time it would be activated by assembly of the appropriate remaining subunits.

RNA Extraction

Oat roots were frozen in liquid nitrogen and pulverized to a fine powder. The powder was then extracted with 4 M guanidinium thiocyanate and RNA was sedimented through a 5.66 M cesium chloride cushion (Chirgwin et al., 1979). Poly(A⁺) RNA was purified with oligo(dT)-cellulose in a batchwise fashion (Maniatis et al., 1982).

In Vitro Transcription of Cloned V-ATPase Subunit Genes

In vitro transcription of cDNAs encoding V-ATPase subunits was performed using a commercially obtained transcription kit (Promega) according to the manufacturer’s instructions. A cDNA (accession no. J03769; Zimniak et al., 1988) encoding the A subunit from carrot (Daucus carota) was graciously provided by Dr. Lincoln Taiz; a cDNA (accession no. J04185; Manolson et al., 1988) encoding the B subunit from Arabidopsis was graciously provided by Dr. Morris Manolson; and a cDNA (accession no. M73232; Lai et al., 1991) encoding the 16-kD subunit from oat was graciously provided by Dr. Heven Sze.

Fractionation of Poly(A⁺) RNA on Suc Gradients

Poly(A⁺) RNA purified as described above was fractionated on linear 30% to 60% (w/w) Suc gradients. Suc solutions were prepared in 10 mM Heps, pH 7.5, 1 mM EDTA (pH 7.5), 0.25% Sarcosyl, and treated overnight with 0.1% DEPC. After 20 min of autoclaving, the volumes were adjusted to the original volume with DEPC-treated water. To reduce possible RNase contamination, the gradient maker was cleaned with 5 M hydrochloric acid, rinsed extensively with DEPC-treated water, and then rinsed with the 30% and 60% Suc stocks. Ultracentrifuge tubes were treated with 3% (v/v) hydrogen peroxide for 1 h and rinsed three times with DEPC-treated water. Poly(A⁺) RNA (100 μL, approximately 40 μg) was loaded on top of a 5.3-mL Suc gradient. Gradients were centrifuged for 6 h at 70,000 rpm in an NVT90 rotor (Beckman). Fractions (200 μL) were collected dropwise from the bottom of the tube. After dilution to 400 μL with DEPC-treated water, 2.5 μg of yeast tRNA was added as a carrier, and the RNA was precipitated with 0.3 M ammonium acetate and 2.5 volumes of ethanol. The final precipitate was resuspended in 10 μL of DEPC-treated water. Generally, 0.5 μL was used in a 12-μL translation assay.

Quantitation of the 16-kD Proteolipid RNA

RNA fractions separated on Suc gradients (1 μL each) were blotted onto Nytran Plus membranes (Schleicher & Schuell) using a Minifold II slot-blot apparatus (Schleicher & Schuell). Blots were probed with a 32P-labeled antisense riboprobe generated from the cloned 16-kD proteolipid sequence (Lai et al., 1991) under conditions that revealed a single-sized message on northern blots (not shown). The autoradiograms were quantitated by densitometry using the National Institutes of Health Image 1.44 image...
In Vivo Labeling of Oat Roots

Labeling and extraction of [35S]Met-labeled oat roots was performed as described previously (Randall and Sze, 1989).

In Vitro Translation and Immunoprecipitation of ATPase Subunits

In vitro translations of poly(A+) RNA and of in vitro-synthesized mRNAs were performed using a commercially obtained rabbit reticulocyte lysate (Promega) and [35S]Met (Amersham) according to the manufacturers’ instructions. Where cDNA-encoded mRNAs were used as the template, 9 μL of reticulocyte lysate, 2 μL of [35S]Met (10 μCi/μL), 0.5 μL of cold amino acids (1 mm each), and 0.5 μL of RNA were used in the translations. In general, the amount of RNA in a translation mixture was not quantified, but the amount of RNA added to translations was optimized for the most efficient translation in the presence of microsomes for each batch. When mixtures were treated with proteinase K, 11.5 μL of translation mixture was diluted to 100 μL with KMH (10 mm Hepes, 80 mm KCl, and 2 mm magnesium acetate, pH 7.5) and treated with 0.5 mg/mL proteinase K for 30 min on ice. The reaction was stopped by the addition of 5 mg/mL PMSF (in DMSO, final concentration 1 mm) for 10 min on ice. Sample mixtures were then centrifuged in a tabletop microcentrifuge at the maximum speed for 2 min to remove nonspecifically binding proteins. Immune or preimmune sera (anti-ATPase; Randall and Sze, 1989) were generally used at a 1:1000 dilution for immunoprecipitation. The mixtures were rotated end-over-end overnight at 4°C. The antigen-antibody complex was incubated with 20 μL of 10% protein A-Sepharose for 30 min at 4°C. Samples were centrifuged in a tabletop microcentrifuge at maximum speed for 2 min and the resultant pellets were washed five times with 0.75 mL of 50 mm Tris, 5 mm EDTA, and 150 mm NaCl (adjusted to pH 7.4 with hydrochloric acid) containing 0.1% Triton X-100, and two times with the same wash buffer minus the detergent. The immunoprecipitated ATPase subunits were resuspended in 2× electrophoresis sample buffer minus DTT (Laemmli, 1970). SDS-PAGE (generally 10% acrylamide) was performed according to the method of Laemmli (1970).

In Vitro Assembly Assay

Translations were performed as described previously in the presence or absence of commercially obtained canine microsomes (Promega). One microliter (20 equivalents) of microsomes per 12 μL of translation mixture was added either at the time of translation (cotranslational) or after the translation process was terminated by the addition to a final concentration of 5 μg/mL cyclohexamide (posttranslational). After a 1-h incubation at 37°C, translation mixtures were diluted to 100 μL with 0.25 M Suc in KMH. The diluted translations were loaded onto a cushion consisting of 500 μL of 0.5 M Suc in KMH. After centrifugation in a TLA 100.3 rotor (Beckman) for 35 min at 75,000 rpm (245,000g at maximal radius), the membranes were recovered in the pellet. The top 200 μL of the overlay was retained and considered nonmembrane associated (supernatant). The pellet was resuspended in 200 μL of 0.25 M Suc in KMH. If necessary, immunoprecipitations were conducted as described above.

Suc-Gradient Fractionation of in Vitro-Synthesized ATPase Subunits

Translation mixtures were loaded onto a 20% to 60% (w/w) continuous gradient of Suc in KMH. The gradients were centrifuged in an NVT90 rotor for 2.25 h at 70,000 rpm (390,000g at maximal radius). Fractions of approximately 300 μL were collected by piercing the bottom of the tubes with a syringe needle and collecting drops manually. Proteins ([35S]Met labeled) derived from translations using poly(A+) mRNAs were immunoprecipitated as described previously. Proteins derived from in vitro-synthesized mRNAs were analyzed directly. BSA (66 kD), amylase (200 kD), catalase (250 kD), and apoferritin (443 kD), run in parallel gradients, were used as standards to estimate the size of the protein complexes present at various Suc densities.

RESULTS

Assembly of V-ATPase Subunits onto Microsomes

To investigate the initial interactions of the V-ATPase with membranes, we developed an in vitro assembly assay. Using total cellular poly(A+) mRNA from oat roots as a template, radiolabeled proteins were synthesized in vitro in the presence or absence of microsomes. The translation mixtures were centrifuged through a Suc cushion, yielding a particulate fraction containing membranes and membrane-associated proteins in the pellet and soluble proteins (nonmembrane associated) on top of the cushion. To determine the location of the V-ATPase subunits, the overlay and the particulate fraction were subjected to immunoprecipitation using anti-V-ATPase antisera followed by SDS-PAGE and fluorography. In immunoprecipitation reactions this antibody is reactive primarily to the A and B subunits (Randall and Sze, 1989). When the mRNA was...

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translated in the absence of microsomes, the A (69 kD) and the B (60 kD) V-ATPase subunits were found only in the overlay (Fig. 1A), indicating that they were soluble (i.e. not aggregated). When translations were performed in the presence of microsomes, the A and B ATPase subunits were localized primarily in the membrane pellet, consistent with a membrane association of these V-ATPase subunits (Fig. 1A). Thus, poly(A\(^+\)) RNA from oat root homogenates contained sufficient information to direct in vitro membrane association of the A and B V-ATPase subunits. It should be noted that although we cannot visualize all of the ATPase subunits under these conditions, it is likely that all are represented in the poly(A\(^+\)) RNA.

Previously, it was observed that mRNA encoding the A subunit was associated with membrane-bound ribosomes, whereas the mRNA encoding the B subunit was not membrane associated (Randall and Sze, 1989). This suggested that the A subunit might become membrane associated at the time of synthesis (cotranslational association). To determine if membrane association of the subunits could occur cotranslationally or posttranslationally (after protein synthesis), translations of poly(A\(^+\)) mRNAs were performed in the presence and absence of microsomes. In the posttranslational association experiments, protein synthesis was terminated by the addition of 5 \(\mu\)g mL\(^{-1}\) cyclohexamide before the addition of membranes. Both the A and B subunits became membrane associated whether membranes were present during or after translation (Fig. 1B). This raises the possibility that in vivo, assembly of these ATPase subunits onto the membrane could occur posttranslationally.

The A subunit is not an integral membrane protein, yet it is apparently synthesized by membrane-associated ribosomes (Randall and Sze, 1989). Proteins synthesized in this manner typically are translocated into the lumen of the ER or are inserted into the membrane (Singer et al., 1987). To determine whether the A and B subunits, when synthesized in vitro, were located on the extraluminal or the lumenal side of the microsome, their susceptibility to proteinase K was tested. When pre-\(\beta\)-lactamase (a protein known to be translocated to the lumen of the ER) was translated in the presence of microsomes, it became proteinase K resistant (Fig. 1C). On the other hand, both ATPase subunits associated with the microsomes were degraded by proteinase K (Fig. 1C), consistent with an extraluminal location. These results indicate a topology consistent with a cytosolic exposure of the \(V_1\) sector, as is found on the vacuole membrane.

When the 16-kD V-ATPase proteolipid subunit (the product of an mRNA transcript derived from a cDNA; see "Materials and Methods") was translated in the presence of microsomes, it associated with the membrane pellet (Fig. 2). The proteolipid was soluble in chloroform:methanol (2:1, v/v), which is consistent with the hydrophobic nature of this protein (Kaestner et al. [1988]; Fig. 2). The majority of the higher-molecular-mass protein products observed in some translations (Fig. 2, Total Translation) do not appear in chloroform-methanol extracts. This suggests that they likely represent aggregations of the proteolipid that are mostly disrupted by chloroform-methanol treatment (Kaestner et al., 1988). Because the translation of the proteolipid was programmed by a unique mRNA (derived from its cDNA), this membrane association does not require the presence of any additional plant proteins.

The proteolipid also associated with the microsomes whether membranes were present during or after translation (Fig. 3). Membrane association of the proteolipid appeared to be significantly more efficient, however, when synthesis occurred in the presence of microsomes. This suggests that this integral membrane protein is cotranslationally assembled in vivo. It also appears that the nonmembrane-associated protein was sensitive to proteolysis, since the total amount of proteolipid recovered was consistently much less when the translation was conducted in the absence of membranes. An alternative explanation
may be a decreased rate of synthesis caused by translational arrest occurring in the absence of membranes.

To test the competency of the individual A and B subunits to associate with membranes, unique mRNAs transcribed in vitro from cDNAs encoding these subunits were translated in the presence of microsomes. These subunits did not associate with microsomes, whether translated alone or together (Fig. 4). Note that the largest molecular mass, B subunit protein likely represents the full-length B subunit, whereas the smaller protein may be the result of internal initiation of translation (Manolson et al., 1988).

Simultaneous translation of the 16-kD proteolipid with the A and B subunits did not catalyze assembly of the latter two peripheral subunits to the membrane (Fig. 5). Because the A and B subunits associate with the membrane when they are supplied as total cellular poly(A\(^+\)) RNA (Fig. 1), but not when they are supplied to the assembly assay individually (Fig. 5), it is likely that components other than the A and B subunits and the 16-kD proteolipid are required to achieve assembly (see “Discussion”).

**The A Subunit Can Become Membrane Associated in the Presence of Substoichiometric Amounts of Several of the ATPase Subunits**

It was clear that the in vitro assembly assay could sensitively measure membrane association of ATPase subunits. When poly(A\(^+\)) mRNA served as the template, very efficient assembly was detected (Fig. 1); however, when only the A, B, or 16-kD proteolipid subunit was supplied, no assembly of the A or B subunit occurred. Because previous results suggested that the A subunit might be assembled on the membranes in the absence of the B subunit

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**Figure 2.** The 16-kD proteolipid alone is sufficient for membrane association. Assembly assays were conducted as described in Figure 1, except that only the RNA (transcribed from the cDNA; Lai et al. [1991]) encoding the 16-kD proteolipid was used to program the translation. Total Translation shows the entire translation mixture before extraction in chloroform-methanol. Additional bands greater than 16 kD are likely aggregates of the extremely hydrophobic 16-kD proteolipid. The remaining lanes were all extracted in chloroform-methanol (2:1, v/v) before loading on the gel. S, Soluble; P, pellet.

**Figure 3.** The 16-kD subunit associates with microsomal membranes cotranslationally but inefficiently posttranslationally. Assembly assays were conducted as described in Figure 1. RNA for the 16-kD proteolipid (as in Fig. 2) programmed translations. All samples were extracted in chloroform-methanol before separation on the gel. S, Soluble; P, pellet.

**Figure 4.** The A and B subunits, either singly or together, do not associate with the membrane. Assays were conducted as described in Figure 1 except that translations were programmed with RNAs (derived from cDNAs) for the A or B subunit. S, Soluble; P, pellet.

**Figure 5.** The A and B subunits translated in concert will not assemble on membranes even in the presence of the membrane-associated 16-kD subunit. Assays were conducted as described in Figures 1 and 2. Note that radioactivity at the gel front in lane 5 is caused by unincorporated \(^{[35]S}\)Met in that fraction. S, Soluble; P, pellet.
(Randall and Sze, 1989), and because the A subunit itself did not appear to be sufficient for self-association, we were interested in whether a distinct entity was required for catalysis of membrane association of the A subunit. To address this question, total mRNAs were first fractionated. The mRNA encoding the A, B, and proteolipid subunits were largely separated from each other by Suc-gradient centrifugation (Fig. 6). The fractionated mRNA was translated in vitro (Fig. 6A). Fraction 5 was substantially depleted in both translatable mRNA for the B subunit (approximately 3.1-fold) and mRNA for the 16-kD proteolipid (approximately 5-fold). Fraction 6 was slightly enriched in the B subunit over the A subunit, but was depleted in 16-kD proteolipid mRNA. We have assumed that a depletion in proteolipid mRNA resulted in a commensurate decrease in protein synthesis in vitro, which is a reasonable assumption in that translations were linearly dependent on mRNA concentrations. The partially purified mRNAs were then translated in vitro and assayed for in vitro assembly (Fig. 7). In the fraction most highly enriched in the A-subunit RNA (fraction 5 in Fig. 6A), the A subunit associated efficiently with membranes (Fig. 7). In fraction 6, both the A and B subunits were efficiently associated. Because the A-subunit protein derived from a unique RNA alone did not associate with the membrane in the presence of the B subunit or the 16-kD proteolipid (Fig. 4), yet the poly(A\(^+\)) RNA enriched in the A subunit did (Fig. 7), we must conclude that an additional, as-yet-unknown plant mRNA-encoded component(s) catalyzes the association of the A subunit with the membrane. The establishment of the identity of this component (or components) will be essential to obtain an understanding of the assembly of the V\(_{1}\) sector.

V\(_{1}\) Subunits Inefficiently Form a High-Molecular-Mass Complex

The peripheral subunits of the plant V-ATPase have been solubilized from vacuole membranes by chaotropic anions, and after removal of the chaotrope in the presence of membranes, they reassembled into a functional enzyme (Ward et al., 1992). Thus, it is possible to reassemble the V\(_{1}\)-sector proteins with the membrane components in vitro.

![Figure 6](image)

**Figure 6.** Suc-gradient centrifugation of total poly(A\(^+\)) RNA results in a fraction enriched in A-subunit RNA and depleted in B-subunit RNA and 16-kD proteolipid RNA. A, An aliquot from each fraction was translated in the presence of \(^{35}\)S-Met and the incorporation (protein synthesis) into a portion was determined (Randall and Sze, 1989). The remaining translation mixture was immunoprecipitated with anti-ATPase, separated on a 12% gel, and fluorographed (inset). B, The individual immunoprecipitated subunits were quantitated as described in “Materials and Methods” and plotted as the percentage of maximal peak. The 16-kD message was determined by hybridization analysis of each fraction on a slot-blot apparatus using a \(^{32}\)P-labeled antisense transcript from the oat 16-kD proteolipid clone (Lai et al., 1991) as the probe.

![Figure 7](image)

**Figure 7.** The A subunit in the presence of substoichiometric amounts of the B subunit and 16-kD proteolipid associates with membranes. Fractionated poly(A\(^+\)) RNA fractions 5 (F5) and 6 (F6) were obtained as shown in Figure 6. Although the RNAs were obtained from a single experiment, this assembly experiment using those fractionated RNAs is representative of two experiments.
Furthermore, studies of yeast V-ATPases have shown that the A, B, and 27-kD peripheral subunits can be found in a soluble complex, the V₁ sector (Kane et al., 1992). The yeast V₁ complex has an apparent molecular mass of 400 to 500 kD, as determined by glycerol gradients (Doherty and Kane, 1993). To test whether the plant enzyme could be assembled into a soluble V₁ complex, we translated the individual subunits, a combination of the A and B subunits, or total poly(A₁) mRNA, all in the absence of microsomes (but otherwise under conditions similar to those in the translation and assembly assays). The sizes of the complexes were estimated by ultracentrifugation in Suc gradients. When the A and B subunits were translated together (Fig. 8) and fractionated on a Suc gradient, the estimated molecular masses for the A and B subunits in both cases were less than 100 kD. This indicated that these subunits did not aggregate and could not form partial complexes. The failure of the A and B subunits alone to form a stable complex is perhaps not surprising, given the requirement for the additional 27-kD subunit in yeast (Kane et al., 1992). This is also consistent with the observation (by two-hybrid analysis) that the A and B subunits can only weakly interact (Tomashek et al., 1996).

The in vitro-synthesized subunits (poly[A₁] programmed) have been shown in this study to assemble efficiently on membranes (Fig. 1), but do not appear to efficiently form a high-molecular-mass complex in the absence of membranes (Fig. 9). The majority of the A and B subunits migrated in the gradient at a molecular mass of less than 100 kD. However, a small proportion of the A and B subunits migrated at a molecular mass of greater than 443 kD, suggesting the inefficient formation of the V₁ sector (not discernible in Fig. 9). It is possible that this complex could also contain chaperone proteins. Note that these same subunits are very efficiently associated when membranes are present under virtually identical conditions (i.e. the same buffers and centrifugation through a Suc cushion; Fig. 1).

**Discontinuity of A- and B-Subunit Membrane Assembly in Vivo**

The data described above suggest that the A subunit is capable of associating with the membrane in the absence of stoichiometric amounts of the B subunit and the 16-kD proteolipid. However, the Suc-gradient analysis indicated that in the presence of all subunits (poly[A₁] programmed translation), a portion of the A subunit was found in a higher-molecular-mass complex containing the B subunit. The former observation suggested that an initial step in V-ATPase assembly could be A-subunit association with the membrane, whereas the latter observation suggested that the formation of a soluble V₁ sector containing the A subunit might be an initial step.

**Figure 8.** The A and B subunits alone do not assemble into a high-molecular-mass complex. After translation, the synthesized proteins were fractionated on 30% to 60% (w/w) Suc gradients. Aliquots were directly loaded onto SDS-PAGE gels and analyzed by fluorography. Note that the B subunit typically runs as a doublet after translation of cDNA-derived message. The migration positions of molecular-mass markers, run in a parallel gradient, are indicated at the top of the panels. Data are representative of two experiments.

**Figure 9.** The poly(A₁)-encoded A and B subunits do not assemble into a high-molecular-mass complex. After translation of total poly(A⁺) RNA, the synthesized proteins were fractionated on 30% to 60% (w/w) Suc gradients and immunoprecipitated with anti-ATPase antibody. Aliquots were loaded onto SDS-PAGE gels and analyzed by fluorography. A and B designate the positions of the A and B subunits. Band 1 represents a 91-kD polypeptide that reacts with the polyclonal antibody, and band 2 represents a 64-kD polypeptide that is likely to be calnexin. The migration positions of molecular-mass markers, run in a parallel gradient, are indicated at the top of the panels. Data are representative of two experiments.
To determine which mechanism would be the most likely in vivo, we tested whether the A and B subunits were simultaneously associated with the membrane immediately after synthesis. Oat roots were briefly (1 h) labeled with \([^{35}\text{S}]\text{Met}\). After this labeling period roots were immediately homogenized using conditions previously shown to maintain ATPase activity (Randall and Sze, 1986), and membrane and soluble fractions were obtained. Under these isolation conditions, no A or B subunits in the soluble fraction have ever been observed (data not shown), indicating the stability of the assembled complex. ATPase subunits were immunoprecipitated from both membrane and soluble fractions and analyzed by SDS-PAGE. After this short labeling period a significant portion of the A subunit, but not the B subunit, was found to be associated with the membrane fraction (Fig. 10). In the soluble fraction all of the B subunit and a portion of the A subunit were present. Based on longer-time-course labeling experiments, by 2 h approximately similar amounts of the A and B subunits were present on the membranes (data not shown).

**DISCUSSION**

This study reports the results of the development of an in vitro membrane-assembly system, using either poly(A\(^{+}\)) RNAs or unique RNAs derived from cDNAs as the source of ATPase subunits. The role of canine microsomes and reticulocyte lysate in this study was to supply generalized functions necessary for protein synthesis and assembly so that plant-specific requirements could be demonstrated. The microsomes were competent to import and process pre-\(\beta\)-lactamase, indicating a functional translocation apparatus. In addition to the components necessary for the translocation and cleavage of precursors, the canine microsomes may also possess membrane-associated proteins, which might have an effect on V-ATPase assembly. However, through the addition of the plant subunits we could examine the unique requirements of the plant V-ATPase assembly.

We have demonstrated that activities contributed by canine ER, rabbit reticulocyte lysate, and oat mRNA-encoded proteins can together catalyze peripheral \(V\) assembly onto the membrane. Although we chose the heterologous system deliberately, there are several aspects of the in vitro-assembly assay over which we had no control. When this study was begun, none of the plant ATPase subunits was available from the same organism, so we used cDNAs derived from different plant species. Not all of the putative ATPase subunits from plants have yet been cloned and sequenced. The ATPase subunits, however, are highly conserved in amino acid sequences in both the animal and plant kingdoms. For example, the bovine- and carrot-derived full-length A subunit sequences are 67% identical and 84% similar. Specifically relevant to this study is the fact that if one compares sequences of two Arabidopsis expressed sequence tags (accession nos. H76218 and T14078) encoding the A subunit with the full-length carrot cDNA (Zimniak et al., 1988), a deduced sequence (overlap of approximately 181 amino acids, corresponding to amino acids 401–582 of the carrot protein) shows an identity of 98% with the carrot sequence, where the differences are extremely conservative changes (e.g. Gly for Ala, Ser for Thr, etc.). In addition, the phenotypes of yeast mutants defective in the A subunit (T. Wilkins, personal communication) and a newly identified 54-kD subunit, VMA13, in yeast (Lu et al., 1997) have been suppressed by the plant protein cognates. We believe that heterology (between the A subunit from carrot and the B subunit from Arabidopsis) is thus an unlikely explanation for the failure of the A and B subunits to form a complex or to associate with the membrane when supplied from cDNAs, although the possibility of a confounding effect of multiple isoforms of these subunits cannot be discounted.

ATPase subunits were observed to be assembled onto membranes when all subunit RNAs were present; however, when supplied individually the A and B ATPase peripheral subunits did not assemble on the membrane. This is not a surprising result because current models suggest a stalk-like arrangement of subunits that would connect the \(V\) sector to the membrane-associated \(V\) subunit. However, when mRNAs were fractionated to remove mRNA encoding the 16-kD proteolipid and the B subunit (such that substoichiometric amounts of the B subunit and 16-kD proteolipid were synthesized), the A subunit was still able to assemble on the membrane. These assembly assays using fractionated plant RNA imply a requirement for a distinct plant-encoded protein, since the cDNA-encoded A subunit did not associate with the membrane in vitro. The ability to assemble the A subunit in the absence of stoichiometric amounts of the B subunit and proteolipid (Fig. 7) implies that this subunit could be assembled in the absence of these subunits in vivo, and that this assembly in turn could result in a temporal discontinuity in membrane association between the A and B subunits. In support of this, in vivo labeling (Fig. 10) of oat roots indicated a temporary discontinuity in assembly of the A and B subunits, with the A subunit becoming membrane associated first.

In contrast to the peripheral subunits, the 16-kD proteolipid (\(V\) subunit) subunit can be synthesized and assembled on the membrane in the absence of any other ATPase subunit. The 16-kD proteolipid associates with membranes

![Figure 10](image-url)
in a posttranslational manner very inefficiently (Fig. 3). This suggests that cotranslational assembly and integration of the proteolipid is more likely.

It is unlikely that the 16-kD proteolipid is necessary for the initial A subunit assembly, because poly(A⁺) depleted in 16-kD proteolipid still supports the A subunit membrane assembly (Figs. 6 and 7). It is remotely possible that canine microsomes contain unassembled proteolipid, which might allow assembly of the A subunit. However, the proteolipid is clearly not sufficient to catalyze the A subunit assembly (Fig. 5). These observations suggest that a specific component or components may be necessary to attach the A subunit to the membrane. One could speculate that this component is a final component of the functional ATPase holoenzyme, or alternatively, that it may be a temporary scaffolding structure that might be dismantled after ATPase assembly. It may be that membrane association of the A subunit occurs without any interaction with the V₁₁ or V₀ sector components. If a membrane-bound scaffolding apparatus is involved in the assembly of the V₁ sector onto the V₀ sector, then the A subunit may interact with a bound scaffolding protein and therefore partition as a membrane-bound protein, as demonstrated here during the initial assembly phase. This latter hypothesis would explain the observations we have made, in which under both in vitro and in vivo conditions the A subunit is membrane associated (but the B subunit is not). It remains to be determined whether this putative receptor/scaffold is a protein homologous to the yeast 95-kD protein necessary for membrane localization of the ATPase, or to the 21- and 25.5-kD yeast proteins necessary for V-ATPase targeting and assembly, or whether it is a distinct protein. Recent evidence suggests calnexin, a membrane-associated chaperone, as a putative scaffold, since it can be found associated with the assembled ATPase (Li et al., 1998). Consistent with this idea is the observation that small amounts of calnexin are present in isolated vacuoles (Seals and Randall, 1997).

The localization of the A subunit on the membrane in the absence of the B subunit (demonstrated both in vitro [Fig. 7] and in vivo [Fig. 10]) and the localization of mRNA encoding the A subunit but not the B subunit on the membrane (Randall and Sze, 1989) suggests the following model. Although it may be possible to assemble the V₁ sector as a soluble complex that is later attached to the V₀ sector through stalk proteins, as suggested for the yeast ATPase, it appears more likely that in vivo the plant A subunit is first assembled on the membrane. This could be accomplished through the use of a membrane-localized receptor (perhaps a “scaffolding” protein). At a later time the B subunit (and perhaps other subunits) is integrated into the nascent complex. One important ramification of this model may be in the regulation of V-ATPase activity. If the ATPase were assembled in an active form on the ER, then it might have proton-pumping activity. An active V-ATPase, however, has not been observed on the ER, which is consistent with our results here. Because the Golgi apparatus is acidified and an active V-ATPase pump has been demonstrated on this organelle (Chanson and Taiz, 1985), the ATPase must be assembled into a functional enzyme by the time it reaches that location. In this manner temporal control of assembly could result in the spatial regulation of V-ATPase activity.

An alternative model, differing only in the site of V-ATPase assembly, is suggested by the results of Herman et al. (1994), in which immunological observations in oat roots indicated the presence of the B subunit at specific subdomains of the ER. This model would involve the soluble B subunit (and perhaps other subunits) joining with the A subunit, which is already membrane associated, but in this case at a specialized region of the ER destined to be targeted to the vacuole. In support of this model, it was also noted in this immunological study that no B subunit was observed on the Golgi apparatus and little to no B subunit was observed at all in cells that were already vacuolated (cells at a distance greater than 1 cm from the root tip). Unfortunately, the A subunit could not be visualized in this study. Both models should be viewed as viable possibilities in light of a number of recent reports of alternative pathways to the vacuole (Hoh et al., 1995; Matsuo et al., 1995; Robinson and Hinz, 1996). The work by Herman et al. (1994) focused on very “young cells” that lacked large central vacuoles and were likely undergoing de novo vacuolation, whereas our work more likely describes the replenishment of existing vacuoles. It is tempting to speculate that the pathway suggested by Herman et al. (1994) (a more direct route from ER to vacuole) might occur during de novo vacuole biogenesis, whereas the pathway suggested by this paper (ER to vacuole via the Golgi complex) might be the route for the continued maintenance of existing vacuoles.

The in vitro assembly assay described here will be a useful tool in the study of V-ATPase assembly as cDNAs encoding other V-ATPase subunits become available for study. Further identification of the components requisite for individual assembly events could be achieved using the poly(A⁺) mRNA pool and antisense mRNAs, which would eliminate a single mRNA species from the poly(A⁺) pool. This would allow assembly studies of initial steps lacking one or more subunits in an otherwise functional assembly assay. When cDNAs corresponding to all subunits of the V-ATPase have been cloned and can be transcribed together in vitro, the minimum subunit assembly requirements and the sequence of events leading to functional assembly can then be characterized. The role of plant proteins corresponding to the yeast 21- and 25.5-kD ER-associated proteins in V-ATPase assembly could also be examined. In addition, site-directed mutagenesis of the subunits would further our understanding of the amino acid sequence requirements for the initial stages of assembly of the V-ATPase holoenzyme. Finally, by generating a cDNA library (derived from fractionated poly[A⁻] RNA, as in Fig. 6, which is enriched in the A subunit but depleted in the B subunit and the 16-kD proteolipid), one may obtain a functional screen for cDNAs encoding assembly factor(s) necessary for the initial steps in A-subunit membrane association.
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