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Purification and Characterization of Tonoplast ATPase from Etiolated Mung Bean Seedlings[1]

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ABSTRACT

The tonoplast ATPase from etiolated seedlings of *Vigna radiata* L. (mung bean) was isolated using a two-step detergent solubilization modified from Mandala and Taiz (S Mandala, L Taiz [1985] Plant Physiol 78: 327–333). After ultracentrifugation on 10 to 28% sucrose gradient, the ATPase showed a 31.6-fold purification over the initial specific activity of the starting tonoplast-enriched membranes. The purified ATPase used Mg^{2+}-ATP as the preferred substrate. The tonoplast ATPase was isolated in a form with characteristics similar to that on its native membrane environment. Analysis by SDS-PAGE revealed two prominent bands with molecular weights of 78,000 (α subunit) and 64,000 (β subunit). The intensity of Coomassie blue staining showed a 1:1 stoichiometry for α and β subunits. The amino acid composition of α and β subunits also confirmed the suggested stoichiometry of the subunit composition of the tonoplast ATPase. Moreover, radiation inactivation analysis yielded a functional size of 414 ± 24 and 405 ± 25 kilodaltons for soluble and membrane bound tonoplast ATPases, respectively. It is possible that the functioning tonoplast ATPase may be in a form of $\alpha\beta$-heteromultimer.

The vacuole of higher plant cells is an acidic organelle that plays a pivotal role in the maintenance and regulation of cell turgor and in the storage and transport of ions and metabolites (3, 29). A body of evidence has revealed the presence of a proton-translocating ATPase associated with the tonoplast membrane (16, 25, 27, 29, 30). The tonoplast ATPase generates the proton motive force for the active transport of ions and solutes across the membrane. This proton pump is insensitive to the plasma membrane ATPase inhibitor, vanadate (6, 7, 10, 11, 29), the mitochondrial ATPase inhibitor, azide (6, 7, 19, 29), or the phosphatase inhibitor, ammonium molybdate (19, 29). However, it is NO_3^--sensitive and can be stimulated by chloride ion (27, 29).

Although tonoplast vesicles from many plant sources have been prepared, little is known of the characteristics of the isolated tonoplast ATPase (25, 29, 31). Tonoplast ATPase from corn coleoptile and *Neurospora crassa* have been partially purified, and they contained two major polypeptides of mol wt of 70,000 and 62,000 along with several minor bands of lower mol wt (5, 20). The functional size of the tonoplast ATPase from corn coleoptile was 400 kD whereas that of *N. crassa* was 520,000 based on radiation inactivation analysis. Although the properties of isolated tonoplast ATPase from both sources were only partially characterized, it is believed that the tonoplast ATPase is structurally different from either the mitochondrial F_0/F_1 ATPase or the plasma membrane E_1/E_2 (20, 21) and represents a novel type of ATPase.

In the present study, we report on the partial purification and characterization of the tonoplast ATPase from etiolated mung bean seedlings.

MATERIALS AND METHODS

Plant Materials

Seeds of *Vigna radiata* L. (mung bean) were soaked for 3 h in tapwater and germinated at room temperature in the dark in a commercial seedling incubator. The hypocotyls of 4-d-old etiolated seedlings were excised and used as starting materials.

Membrane Preparations

Tonoplast vesicles were prepared from etiolated seedlings according to the methods of Yoshida *et al.* (31) and Churchill and Sze (8) with the following modifications. All procedures were carried out at 4°C. The excised hypocotyls from 4-d-old seedlings were washed, and 500 g of chilled hypocotyls were homogenized with a Polytron PT-35 at the medium speed setting for 1 min in a solution (buffer I) containing 75 mM Tris-Cl (pH 7.6), 0.25 M sorbitol, 5 mM EGTA, 0.25% BSA, 2 mM DTT, and 1 mM PMSF. The homogenate was passed through four layers of cheesecloth and centrifuged at 480*g* for 10 min to remove debris. The supernatant was subjected to differential centrifugation at 6,000*g* for 15 min and then 60,000*g* for 30 min. The latter pellets were suspended in a medium (buffer II) containing 10 mM Tris-Mes (pH 7.3), 0.25 M sorbitol, 1 mM EGTA, 1 mM DTT and 0.2 mM PMSF, loaded onto a 4% (w/v) dextran (79,000) cushion made in buffer II and centrifuged at 70,000*g* for 2 h. The turbid band at the 0/4% interface was collected and were referred as resealed tonoplasts.

The tonoplast-enriched membranes from corn coleoptiles were isolated according to Mandala and Taiz (20).

Solubilization

Soluble tonoplast ATPase was prepared from resealed tonoplast vesicles using methods modified from those of Man-

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dala and Taiz (20). DOC[2] was added dropwise from a 5% stock solution to a final concentration of 0.1% (w/v) in buffer II containing 1 mg/mL membrane protein and 15% glycerol (w/v) while stirring on ice. After 30 min incubation at 4°C, the DOC-treated tonoplast was centrifuged at 120,000*g* for 1 h. The pellets were suspended in buffer II, containing 15% (w/v) glycerol, to a protein concentration of 2 mg/mL and OG was added from a 100 mM stock solution to a final concentration of 40 mM. The tonoplast solution was incubated for 25 min at 4°C and then centrifuged at 120,000*g* for 1 h. An aliquot (0.5 mL) of supernatant containing ATPase activity as then layered onto a 4 mL 10 to 28% linear sucrose gradient (buffer III) containing 5 mM Tris-Cl (pH 7.3), 0.25 M sorbitol, 1 mM EGTA, 1 mM DTT, 0.2 mM PMSF, and 15% (w/v) glycerol. The gradient was centrifuged at 200,000*g* for 5 h. Aliquots (0.5 mL) were collected from the bottom of gradient and the fraction with highest ATPase activity was used for further characterization.

Enzyme Assay and Protein Determination

Aliquots of tonoplast ATPase or resealed vesicles were assayed in a 0.5 mL volume containing 10 μg protein, 30 mM Tris-Mes (pH 7.5), 3 mM $MgSO_4$, 50 mM KCl, 3 mM ATP, 0.5 mM sodium azide, 0.1 mM sodium vanadate, and 0.1 mM ammonium molybdate. After 30 min incubation at 30°C, the reaction was terminated by adding a solution containing 1.7% (w/v) ammonium molybdate, 2% (w/v) SDS, and 0.02% (w/v) ANSA. The released Pi was measured spectrophotometrically (13).

Protein concentration was determined according to the modified Lowry method (18) using bovine serum albumin as the standard.

SDS-PAGE

SDS-solubilized membrane proteins and soluble ATPase were subjected to electrophoresis according to Laemmli (17) and the polypeptide bands visualized either with Coomassie blue or silver stain.

Amino Acid Analysis

To separate the α and β subunits for amino acid analysis, the polypeptide bands on SDS-PAGE were visualized with 4 M sodium acetate according to the method of Higgins and Dahmus (14). The α and β bands were cut out, chopped into pieces, homogenized, and then eluted overnight in a 20-fold volume of distilled water with stirring. Following exhaustive dialysis against distilled water, the eluants were hydrolyzed anaerobically in 6 N HCl for 24 h at 110°C in the presence of 1% phenol. The hydrolysates were then subjected to analysis with an LKB 4150 Alpha Amino Acid Analyzer at the Taipei Regional Analytical Instrument Center. The number of cysteic acid residues was measured by reaction of cysteines with 2,2′-dithiopyridine (12). The tryptophan content was determined according to the method of Spandee and Witkop (28).

Radiation Inactivation Analysis

Radiation inactivation analysis of the tonoplast ATPase and an enzyme standard was carried out as described elsewhere (24). Samples were frozen at −25°C maintained by a cryothermostat and irradiated using a ^{60}Co irradiator (1000 Ci) at this college. After radiation exposure, the samples were thawed at the same time and assayed for enzyme activity immediately. the functional size of enzyme was calculated according to Beauregard and Potier (1):

$$\log m = 5.89 - \log D_{37,t} - 0.0028t \quad (1)$$

where m is the functional size in daltons, t is the temperature (°C) during irradiation, D_{37} is the dose of radiation in megarads required to reduce the activity to 37% of that found in an unexposed control at temperature t (°C).

Materials

ANSA, deoxycholate, dithiothreitol, glucose-6-phosphate dehydrogenase, octylglucopyranoside, and orthovanadate were purchased from Sigma. Enzyme standards for SDS-PAGE were obtained from Bio-Rad. All chemicals were of reagent grade and used without further purification.

RESULTS AND DISCUSSION

Partial Purification of Tonoplast ATPase

The preparation of microsomal membrane from etiolated mung bean seedlings was described in "Materials and Methods." Several detergents were tested to solubilize the tonoplast ATPase from vacuolar membranes, including Triton X-100, LDAO (lauryl dimethylamine *N*-oxide), cholate, deoxycholate, and OG. We concurred with Mandala and Taiz (20) that DOC and OG were the most effective detergents. Therefore, two successive detergent extractions, modified from methods described by Bowman *et al.* (5), were used to solubilize the ATPase from tonoplast membrane. Table I gives a summary of the recovery of tonoplast ATPase activity during each step. At a concentration of 0.1% DOC (detergent/protein ratio of 1.0), the ATPase specific activity increased by about 30%, while 50% of the protein was removed from membrane. In the higher concentrations of DOC, the ATPase activity declined. For this reason, 0.1% DOC was chosen as a first step in purification to solubilize unwanted proteins from the tonoplast membrane.

In a second detergent (OG) treatment, the ATPase was solubilized into the supernatant (Fig. 1, A and B) at concentrations of OG up to 40 mM. Therefore, 40 mM OG was employed routinely to release ATPase from DOC-treated membranes (at protein concentration of 2 mg/mL). The OG supernatant was then centrifuged on a 10 to 28% sucrose gradient (buffer III). In many cases, detergent was included in sucrose gradient buffer to prevent aggregation and allow better

[2] Abbreviations: DOC, sodium deoxycholate; ANSA, 1-amino-2-naphthol-4-sulfonic acid; DCCD, *N,N′*-dicyclohexyl-carbodiimide; DIDS, 4,4′-dithiocyanostilbene-2,2′-disulfonate; EDAC, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NEM, *N*-ethylmaleimide; OG, octyl glucopyranoside.

Table I. *Tonoplast ATPase Purification*

Tonoplast-enriched membranes were solubilized and ATPase was partially purified as described in the text. Reaction conditions were as described under "Materials and Methods." Specific activity (SA) is expressed as μmol Pi released/mg protein·h. Percentage yield is the ratio of ATPase activity in each step to that of the tonoplast-enriched membrane (×100).

Fraction	Activity		Protein	
	SA	Purification	Amount	Yield
		-fold	*mg*	%
Tonoplast-enriched membrane	1.23	1.00	39.6	100.0
0.1% DOC pellet	1.78	1.44	28.6	72.2
40 mM OG supernatant	3.30	2.67	24.7	62.5
Peak sucrose gradient fraction No. 6	39.00	31.60	8.8	22.3

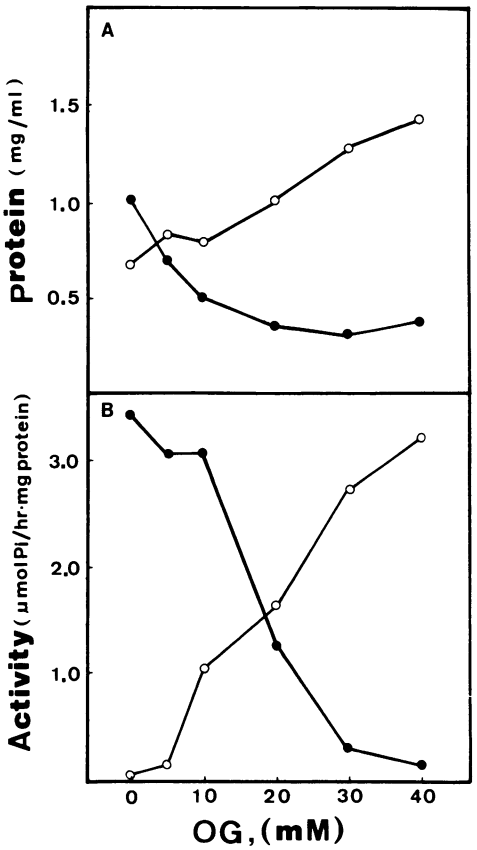

Figure 1. Solubilization of ATPase and proteins by OG. The ATPase activity was assayed in supernatant (○) and pellet (●) after centrifugation at 120,000*g* for 1 h. Reaction conditions were as described under "Materials and Methods." The protein concentration was determined by a modified Lowry method (18).

resolution. However, in our preparation, extra OG was not needed in the sucrose gradient for the better performance. Most ATPase activity was found on the fraction 5–7 from the bottom of gradient (Fig. 2). The ATPase activity was strongly inhibited by KNO_3 but was insensitive to the mitochondria inhibitor, azide, the plasma membrane inhibitor, vanadate,

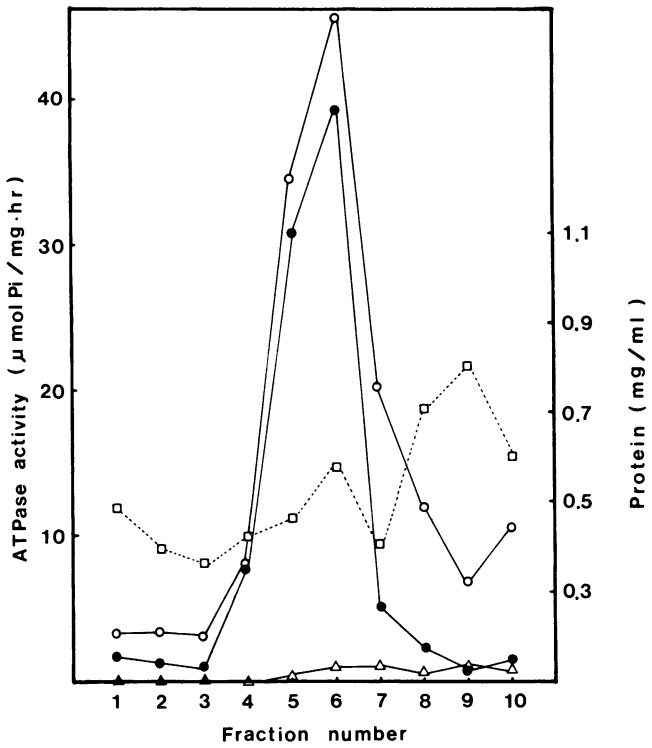

Figure 2. Distribution of tonoplast ATPase on a linear sucrose gradient. A 10 to 28% sucrose gradient was centrifuged at 200,000*g* for 5 h. Fractions of 0.5 mL were collected from the bottom of the gradient. The measurements of ATPase activity (○), tonoplast ATPase activity in the presence of 0.5 mM azide, 0.1 mM vanadate, and 0.1 mM ammonium molybdate (●), and protein concentrations (□) were as described in Figure 1, except 50 mM KNO_3 was added (△) if present.

or the phosphatase inhibitor, ammonium molybdate (6, 7, 10, 11, 19, 29). The presence of exogenous lipids was not the prerequisite for the ATPase activity (data not shown). In the peak fraction, the protein yield was 22.3% while its specific activity was 31.6-fold higher than the starting tonoplast-enriched membrane (Table I). The protein contamination of the purified fraction from sucrose gradient was less than 10% determined from SDS-PAGE.

Analysis of the sucrose gradient fraction on SDS-PAGE showed two prominent bands, at 78 kD (α subunit) and 64 kD (β subunit), concomitantly with ATPase activity (Fig. 3). These values are slightly larger than that obtained from the vacuolar ATPase of other higher plants, fungi, and beef chromaffin granules (*cf.* Table I of ref. 21) even though their subunit compositions are very similar. The smaller polypeptide of M_r = 16,000, which was reported to be involved in proton translocation by other workers (5, 21, 26), was not visualized in our sucrose gradient fraction (Fig. 3). It is probably that this smaller protein was released during the purification. Despite the absence of other smaller polypeptides in our preparation, the ATPase consisting of αβ subunits retained an appreciable activity. Although the active site was believed to locate at larger subunit of most vacuolar ATPases (5, 21), the actual site of ATP hydrolysis in this preparation remains to be determined. Nevertheless, the intensities of

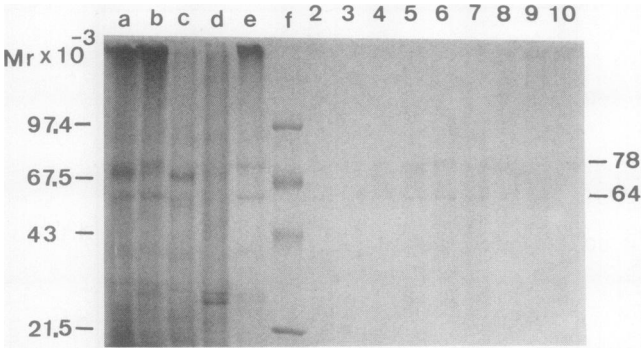

Figure 3. SDS-PAGE (12.5%) of the tonoplast ATPase at each step. Approximately 5 μg protein of tonoplast-enriched membrane (a), DOC pellet (b), DOC supernatant (c), and OG pellet (d), OG supernatant (e), mol wt standards (f), and 10 μL of fraction No. 2 to 10 from the bottom of the sucrose gradient (2–10). Numbers on both sides of the gel indicate mol wt in kD.

Table II. *Substrate Specificity of Tonoplast ATPase*

Reaction conditions were as described under "Materials and Methods" with a concentration of 3 mM for all substrates.

Substrates	Membrane-Bound ATPase		Soluble ATPase	
	μmol Pi / mg protein·h	%	μmol Pi / mg protein·h	%
ATP	1.02	100	26.60	100
ADP	0.61	60	2.50	9
AMP	0.45	44	1.20	5
UTP	1.33	130	8.15	31
GTP	1.49	146	6.09	23
PPi	2.57	235	2.03	8

Coomassie blue staining of α and β subunits was 1:1, suggesting that the tonoplast ATPase might be heteromultimer of these two polypeptides. This speculation was further supported by amino acid analysis and the radiation inactivation technique in this study (see below).

Characterization of Purified Tonoplast ATPase

We characterized the purified ATPase obtained from the sucrose gradient to determine whether the soluble tonoplast ATPase was purified in a form similar to that in its native membrane environment. Substrate specificity of tonoplast ATPase was examined, and Table II indicates that ATP was the preferred substrate for the soluble ATPase although the hydrolysis of UTP, GTP, and pyrophosphate were even all greater in the tonoplast-enriched membranes. Furthermore, the soluble ATPase had a higher affinity for Mg^{2+}-ATP than membrane-bound tonoplast ATPase (data not shown). Lineweaver-Burk analysis yielded an apparent K_m for ATP of 1.0 mM and a V_{max} of 2.1 μmol Pi/mg protein·h for membrane-bound ATPase while an apparent K_m for ATP of 0.5 mM and a V_{max} of 30.8 μmol Pi/mg protein·h were obtained for the soluble ATPase.

Soluble and membrane-bound ATPase activity had distinct pH profiles though optimum pH values were both around 7.0 (Fig. 4). The membrane-bound ATPase displayed a broad pH optimum from 6.5 to 8.0 as observed by Yoshida *et al.* (31). On the other hand, the activity of the soluble ATPase showed a dramatic pH dependence either below or above pH 7.0.

Table III depicts the effects of monovalent ions on activity of the soluble and membrane-bound ATPases. Monovalent cations had little effect on membrane-bound activity, while the activities of soluble ATPase were more or less stimulated. Among anions, only NO_3^- was inhibitory to membrane-bound ATPase activity. However, the soluble ATPase was inhibited by I^- as well as NO_3^-. The effects of divalent cations on tonoplast ATPase were also studied (Table IV). The ATPase activity was stimulated by Mg^{2+} and Mn^{2+}. On the contrary, divalent cations such as Zn^{2+} and Cu^{2+} and Cd^{2+} inhibited activity.

The inhibitor sensitivity of soluble and membrane-bound

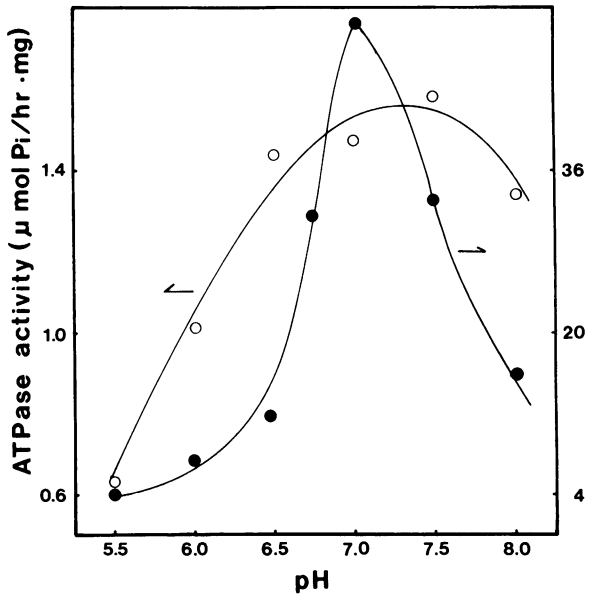

Figure 4. pH Profile of tonoplast ATPase. Reaction conditions were as described under "Materials and Methods." (●), soluble ATPase; (○), membrane bound ATPase.

Table III. *Effects of Monovalent Ions on Tonoplast ATPase*

Reaction conditions were as described under "Materials and Methods" with ion concentrations of 50 mM.

Addition	Membrane-Bound ATPase		Soluble ATPase	
	μmol Pi / mg protein·h	%	μmol Pi / mg protein·h	%
None	0.82	100	13.95	100
Cations				
KCl	0.98	121	28.61	205
NH_4Cl	0.90	115	33.42	240
NaCl	0.86	106	25.98	186
LiCl	0.93	115	26.74	192
Anions				
KCl	0.98	121	28.61	205
KNO_3	0.02	3	1.95	14
KI	0.91	112	6.56	47
K_2SO_4	0.85	105	13.74	98

Table IV. *Effects of Divalent Cations on Tonoplast ATPase*

Reaction conditions were as described under "Materials and Methods" with cation concentration of 3 and 50 mM Cl^-.

Cations	Membrane-Bound ATPase		Soluble ATPase	
	$\frac{\mu mol\ Pi}{mg\ protein \cdot h}$	%	$\frac{\mu mol\ Pi}{mg\ protein \cdot h}$	%
None	1.0	100	9.7	100
Mg^{2+}	1.4	140	27.4	282
Mn^{2+}	1.9	190	24.0	247
Ca^{2+}	0.1	10	10.0	103
Zn^{2+}	0.6	60	5.2	54
Cu^{2+}	0.2	20	0.0	0
Cd^{2+}	0.0	0	8.1	84

Table V. *Effects of Inhibitors on Membrane-Bound and Soluble Tonoplast ATPase*

Reaction mixture contained 30 mM Tris-Mes (pH 7.5), 3 mM $MgSO_4$, 50 mM KCl, 3 mM ATP, 0.5 mM sodium azide, 0.1 mM sodium vanadate, 0.1 mM ammonium molybdate, 20 μg protein/mL, and various concentrations of inhibitors. Reactions were performed at 30°C for 30 min. NBD-Cl and DCCD were made up in 1:1 ethylene glycol/ethanol. The carried-over concentrations of ethylene glycol and ethanol were less than 1%. The I_{50} values were interpolated from their concentration curves at 50% inhibition.

Inhibitors	Membrane-Bound ATPase ($I_{(50,MB)}$, μM)[a]	Soluble ATPase ($I_{(50,S)}$, μM)	$\frac{I_{(50,MB)}}{I_{(50,S)}}$ Ratio
KNO_3[b]	12.0	4.5	2.67
NEM	2.6	1.6	1.60
NBD-Cl	12.0	4.0	3.00
DCCD	11.5	3.0	3.83
EDAC[b]	33.3	6.0	5.55
DIDS	9.0	0.2	52.94

[a] $I_{(50,MB)}$ and $I_{(50,S)}$ represent I_{50} values for membrane-bound and soluble ATPases, respectively. [b] The units of I_{50} values are mM.

tonoplast ATPases was investigated. The I_{50} of inhibitors is shown in Table V. The I_{50} of KNO_3 for soluble ATPase is 4.50 mM while that for the membrane-bound ATPase was 12.0 mM. Lineweaver-Burk analysis revealed K_i values for NO_3^- of 4.75 and 0.25 mM for membrane-bound and soluble ATPases, respectively, and the mode of NO_3^- inhibition appeared to be noncompetitive with respect to ATP (data not shown). The ATPase was highly sensitive to DIDS. The I_{50} values of DIDS were 9.0 and 0.17 μM for membrane-bound and soluble ATPases, respectively. DCCD and its hydrophilic analog EDAC were inhibitors of most ATPases by reacting with carboxylate residue(s) at active site. The I_{50} values of DCCD were 11.5 and 3.00 μM and of EDAC, 33.3 and 6.0 mM for membrane-bound and soluble ATPases, respectively. Hydrophilic inhibitors such as DIDS and EDAC had lower apparent I_{50}s for the soluble ATPase activity than for the membrane-bound one. These properties imply that the tonoplast ATPase is deeply embedded in the membrane which makes it less accessible in its native state. Nevertheless, the ATPase purified was in a form having characteristics similar to those found in its native membrane environment.

Amino Acid Analysis

Direct amino acid analysis of the subunits of the soluble tonoplast ATPase represents the first such report on this topic (Table VI). The molar composition on a percentage basis is similar for most amino acid residues of both subunits. The α subunit contains 714 amino acid residues/mol while the β subunit contains 584 amino acid residues/mol resulting in calculated mol wt of 77,948 and 64,011, respectively. The number of amino acid residues in the α subunit and thus its calculated mol wt are higher than that of the larger subunit of vacuolar ATPase from carrot and *Neurospora crassa* determined by gene sequence (4, 32). The content of hydrophilic residues are 56.4 and 57.9% for α and β subunits, respectively. There are slightly more negative than positive charges: 19.0 *versus* 13.8% and 20.5 *versus* 12.5% for α and β subunits, respectively. The most frequent amino acids are glutamic (including glutamine), aspartic, leucine, alanine, and glycine. The content of tryptophan was determined by the method of Spandee and Witkop (28). Table VI shows that α and β subunits contained 3 and 6 tryptophan residues per mol of polypeptide resulting in the percentage of aromatic amino acid residues of 8.35 and 8.64%, respectively.

The number of cysteic acid residues was measured by the reaction of cysteines with 2,2′-dithiopyridine (12). The α subunit contains 4.0 cysteine residues/mol polypeptide while the β, 2.1 cysteine residues/mol protein. The cysteine content of α subunit of mung bean tonoplast ATPase is close to that from carrot and *Neurospora crassa* (5 and 4 cysteine residues/mol protein, respectively, refs. 4 and 32), but slightly higher

Table VI. *Amino Acid Analysis of the Subunits of Tonoplast ATPase*

The subunits of tonoplast ATPase were isolated by SDS-PAGE. The procedures of amino acid analysis were described under "Materials and Methods." Apparent molecular weights of α and β subunits were measured by SDS-PAGE as shown in Fig. 3. The calculated molecular weights of both subunits were determined from amino acid analysis.

Amino Acid	Number and Percentage of Amino Acids			
	α	%	β	%
Lys	45.5	6.38	31.9	5.46
His	13.7	1.92	10.2	1.75
Arg	40.1	5.62	30.9	5.30
Asp	66.4	9.30	62.3	10.66
Thr	40.1	5.62	40.2	6.88
Ser	39.3	5.52	37.9	6.49
Glu	69.7	9.77	57.7	9.88
Pro	35.7	5.00	42.0	7.20
Gly	61.0	8.55	47.6	8.15
Ala	72.2	10.12	46.2	7.91
Val	48.7	6.82	36.9	6.32
Met	11.2	1.57	3.2	0.55
Ile	38.6	5.41	34.2	5.85
Leu	68.6	9.61	51.3	8.78
Tyr	22.7	3.18	18.0	3.08
Phe	33.2	4.65	25.9	4.43
Trp	2.9	0.41	5.8	0.99
Cys	4.0	0.56	2.1	0.36
Total	713.6	100.00	584.3	100.00
Calculated M_r	77,948		64,011	

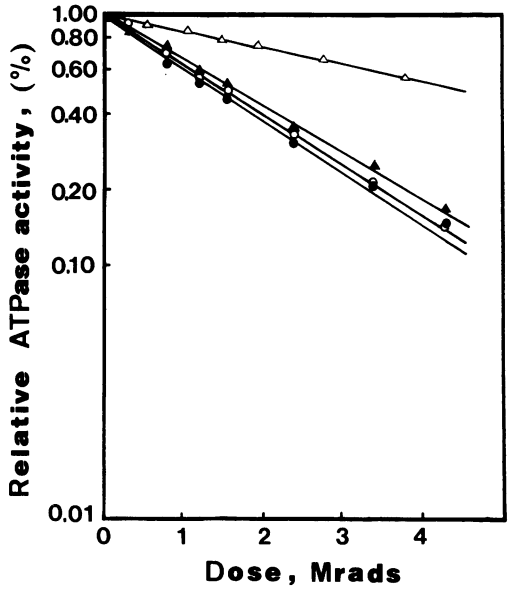

Figure 5. Radiation inactivation of tonoplast ATPase. Reaction conditions were described as under "Materials and Methods." (○), membrane-bound tonoplast ATPase from mung bean; (●), soluble tonoplast ATPase from mung bean; (▲), membrane-bound tonoplast ATPase from corn coleoptiles. All data points are means of 12 assays with lines fitted by regression analysis ($r > 0.98$). The control activities were 38.5, 1.6, and 4.1 μmol Pi released · mg protein · h^{-}, respectively. The intersections of regression lines at 37% control activity give the D_{37} dose values. The functional size was calculated using Beauregard and Potier equation (1). The survival curve of glucose-6-phosphate dehydrogenase (M_r = 104,000) is shown (Δ) to demonstrate the feasibility of radiation inactivation technique (for details, see ref. 24). D_{37} value for glucose-6-phosphate dehydrogenase is 7.7 ± 0.4 Mrads which yields a functional size of 116 ± 6 kD.

than that from F_1-F_0 ATPase of spinach chloroplast (2 cysteine residues/mol of β subunit, ref 2). The relative abundance of cysteine residues may partially account for the higher sensitivity of vacuolar ATPase than F_0-F_1 ATPase to sulfhydryl group inhibitors such as NEM. Nevertheless, if the cysteines for each subunit are rounded off to the nearest whole number and then compared to that determined from the whole tonoplast ATPase (data not shown), we come to a conclusion that the stoichiometry of α and β subunits of the tonoplast ATPase is 1:1.

Radiation Inactivation Analysis of Tonoplast ATPase

Radiation inactivation analysis has been employed to estimate the functional size of membrane enzymes (15). We utilized this technique to determine the functional mass of the tonoplast ATPase either on the membrane or in soluble form (Fig. 5). The decrease of ATPase activity displayed a simple exponential function of irradiation exposure. D_{37} values of 2.20 ± 0.11 and 2.15 ± 0.11 Mrads were obtained for membrane-bound and soluble tonoplast ATPases, yielding the functional size of 405 ± 25 and 414 ± 25 kD, respectively. The functional mass of the tonoplast ATPase from corn coleoptiles was also determined as 387 ± 19 kD compared to 400 kD measured by Mandala and Taiz (20). Radiation inactivation of an internal standard, glucose-6-phosphate dehydrogenase, is shown in Figure 5, verifying the feasibility of this technique in determining the functional size of the ATPase in this study (*cf.* ref. 24). The mol wt of glucose-6-phosphate dehydrogenase was 116,000 ± 6,000 compared to 104,000 measured by conventional biochemical methods. The functional size of soluble ATPase is similar to that of membrane-bound ATPase. It is suggested that soluble ATPase was purified in a form similar to that at its native state on membrane under present conditions. Furthermore, the functional mass for tonoplast ATPase was larger than the sum of the mol wt (142,000) of its α and β subunits on SDS-PAGE indicating the possible functional unit of tonoplast ATPase is an αβ heteromultimer. The arrangement of α and β subunits in the functional unit requires further elucidations.

Studies on partially purified vacuolar membrane ATPases from various sources indicate its difference from either the mitochondrial F_0/F_1 or plasma membrane E_1/E_2 ATPases (25, 29). The soluble F_1 portion of the mitochondrial ATPase is composed of five subunits ranging between 8 and 62 kD in size. It is obvious that the mol wt of the tonoplast ATPase subunits are apparently larger in mass than the two largest (58 and 62 kD) subunits of the mitochondria ATPase. Furthermore, the sensitivity of mitochondrial and tonoplast ATPases to inhibitors is quite different. The tonoplast ATPase can be inhibited by NO_3^- but not azide. However, mitochondrial ATPase is inhibited by azide but unaffected by NO_3^- (6, 19, 29). Nevertheless, more evidence suggests that the vacuolar ATPase may be structurally similar to the F_0-F_1 ATPase. For instance, F_0F_1 ATPase of spinach is a large enzyme with molecular mass of approximately 420 kD (23), while the vacuolar ATPases form maize (20), mung bean (this study), and *Neurospora crass* (5) are in the range of 400 to 500 kD. Furthermore, a homology between the large subunit of the vacuolar ATPase and the β subunit of F_0-F_1 ATPase was recently demonstrated (4, 32).

The characteristics of plasma membrane ATPase are also different from the tonoplast ATPase (29). Most E_1/E_2-type H^+-ATPases consist of a single polypeptide with a mol wt of about 100,000. The insensitivity of the tonoplast ATPase to vanadate suggests that it does not form phosphorylated intermediates as the plasma membrane ATPase does. However, Mito *et al.* (22) recently isolated from mung bean hypocotyl a new type of higher plant plasma membrane ATPase which contained two major polypeptides with molecular masses of 67 and 55 kD as well as a 15 to 20 kD [^{14}C]DCCD-binding protein. Their ATPase superficially resembles that from the tonoplast membrane, except in its sensitivity to vanadate and nitrate. Further studies on both ATPases from different membrane sources of mung bean hypocotyls may provide more insights into the nature of these ATPases.

On the other hand, the tonoplast ATPase strikingly resembles the ATPase from the chromaffin granule membrane of the adrenal cortex (9). Increasing lines of evidence suggest that H^+-transducing ATPases from storage or hydrolytic organelles, including tonoplasts and chromaffin granule, may represent a novel type of ATPase differing both kinetically and in subunit composition from the F_0/F_1-type and E_1/E_2-type ATPases (29). The purified mung bean tonoplast ATPase

offers a means to study its function and structure and to make comparisons among various types of ATPase mentioned above. Further investigations are currently in progress in this laboratory.

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