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Irreversible inhibition of H^+-ATPase of higher plant tonoplast by chaotropic anions: evidence for peripheral location of nucleotide-binding subunits

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The H^+-translocating ATPase of tonoplast vesicles from storage root of *Beta vulgaris* L. is irreversibly inhibited by a 30 min treatment with the chaotropic anions SCN^-, ClO_4^-, I^- or NO_3^- in the 0.25–1.5 M concentration range. The inhibitory potencies of the anions follow the Hofmeister series ($SCN^- > ClO_4^- > I^- > NO_3^- \gg CH_3COO^-$; $SO_4^{2-} = 0$). The H^+-translocating inorganic pyrophosphatase of the same membrane is, by contrast, unaffected by chaotrope concentrations which completely abolish H^+-ATPase activity. Inhibition of the ATPase is associated with the removal of two polypeptides of 67 and 57 kDa from the membrane, concomitant with their appearance in the supernatant. The chaotrope-dissociated 67 and 57 kDa polypeptides comigrate with the major subunits of the partially purified ATPase upon SDS-polyacrylamide gel electrophoresis and cross-react with antibody raised to the nucleotide-binding subunits of the enzyme. Since the 16 kDa [^{14}C]DCCD-binding proteolipid of the ATPase remains associated with the membrane after treatment with chaotrope, it is concluded that chaotropic anions inhibit the enzyme by specific detachment of the nucleotide-binding subunits. The tonoplast ATPase of *Beta* is therefore deduced to have structure/function partitioning analogous to the F_0F_1 H^+-ATPase of energy-coupling membranes.

Introduction

The vacuolar membrane (tonoplast) of higher plant cells contains two primary H^+-pumps: an ATPase (tonoplast ATPase, EC 3.6.1.3) and an inorganic pyrophosphatase (tonoplast pyrophosphatase; EC 3.6.1.1) [1]. Both enzymes are capable of establishing an inside-acid, inside-positive $\Delta\tilde{\mu}_{H^+}$ across the tonoplast which may be utilized to

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Abbreviations: BTP, 1,3-bis[tris(hydroxymethyl)methylamino] propane (also: bis-Tris propane); BzATP, 3-*O*-(4-benzoyl)benzoyladenosine 5′-triphosphate; DCCD, *N,N′*-dicyclohexylcarbodiimide; EDTA, ethylenediaminetetraacetic acid; IPA, radioiodinated *Staphylococcus* protein A; k_{50}, concentration of inhibitor required for 50% inhibition of activity vs. control; Mes, 4-morpholineethanesulfonic acid; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; PP_i, inorganic pyrophosphate; Tween-20, polyoxyethylene-sorbitan monolaurate; $\Delta\tilde{\mu}_{H^+}$, H^+ electrochemical potential difference.

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energize the H^+-coupled secondary transport of a number of solutes [2].

In common with the endomembrane H^+-ATPases from a wide variety of animal and plant sources, the tonoplast ATPase has an apparent functional mass of 400 kDa [3] and comprises at least three distinct subunits of 70, 60 and 16 kDa, respectively [1]. Affinity-labeling experiments show the 70 and 60 kDa subunits to have the attributes of nucleotide-binding, catalytic/regulatory subunits, whereas the 16 kDa component behaves in a manner consistent with it constituting part or all of a putative H^+-channel [1]. The physical aspects of the association of the subunits with each other and with the membrane, on the other hand, remain unexplored. In view of the ostensibly intermediate properties of the endomembrane ATPases and the E_1E_2 and F_0F_1 categories of H^+-ATPase [1], a paramount question is that of structure/function partitioning. Specifically, whether the high M_r subunits of the tonoplast ATPase, like the nucleotide-binding subunits of the F_1-sector of F_0F_1, are extrinsically associated with the membrane, while the 16 kDa component, by analogy with the H^+-conducting F_0 portion of F_0F_1, is deeply embedded in the phospholipid bilayer [1].

In a preliminary report we have shown that the tonoplast ATPase of *Beta* is subject to irreversible inhibition by high concentrations of chaotropic anions [4]. In agreement with the concept that the apolar groups of macromolecules form hydrophobic bonds because of their thermodynamically unfavorable interaction with water, the relative water structure-breaking properties of chaotropic anions are correlated with their ability to destabilize membranes and multimeric enzymes [5]. Thus, in this communication we investigate the consequences of treatment with high concentrations of chaotropic anions on membrane polypeptide composition with regard to the dissociability of the subunits of the tonoplast ATPase. Nonspecific effects are distinguished from specific effects by comparing the tonoplast ATPase with the tonoplast pyrophosphatase, the latter of which appears to reside on the same membrane [1] but is demonstrated here to be relatively insensitive to inhibition by chaotropic anions. The results show that the irreversible inhibition of ATPase activity by chaotropic anions is accompanied by specific dissociation from the membrane of the nucleotide-binding subunits of the tonoplast ATPase.

Materials and Methods

Isolation of tonoplast vesicles. Tonoplast vesicles were isolated from storage root of fresh red beet (*Beta vulgaris* L.) as detailed elsewhere [6]. For the routine preparation of membranes, the 0.24 M KI-treated pellet from 330 g beet was suspended in suspension medium (1.1 M glycerol, 1 mM BTP-EDTA, 0.5 mM butylated hydroxytoluene and 5 mM dithiothreitol buffered to pH 8.0 with 5 mM BTP-Mes) and layered onto a 10/23% (w/w) sucrose step gradient. After centrifugation at $80\,000 \times g$ for 2 h, the tonoplast-enriched fraction at the 10/23% interface was removed with a Pasteur pipette. A low-speed ($7000–10\,000 \times g$) centrifugation before the sedimentation of microsomes was not employed for most of the work described because the low density of tonoplast vesicles enables their efficient separation from mitochondria by sucrose density gradient centrifugation alone. Pretreatment of the microsomal pellet with 0.24 M KI (above) was omitted for part of the experiment described in Table I.

Treatment with chaotropes. The tonoplast-enriched membrane fraction was treated with chaotrope by the addition of 4 ml chaotrope solution to 1 ml membrane suspension (1–3 mg/ml membrane protein). The mixture was left on ice for 30 min, diluted at least 20-fold with suspension medium and the membranes were pelleted by centrifugation at $100\,000 \times g$ for 30 min or $200\,000 \times g$ for 1 h. The chaotropic anions were added as their K^+ salts dissolved in suspension medium, except for ClO_4^- which was added as its Na^+ salt.

Analysis of the protein released by treatment with chaotrope was performed by freeze-drying ($-5\,°C$) and resuspension of the residue in 5 mM BTP-Mes (pH 8.0). Glycerol and KI were removed from the samples by gel filtration on Sephadex G25 before SDS-polyacrylamide gel electrophoresis.

ATPase and pyrophosphatase assays. ATPase and pyrophosphatase activities were determined by measuring the rate of liberation of P_i from ATP and PP_i, respectively. The reaction media

contained 30 mM BTP-Mes (pH 8.0), 50 mM KCl, 5 μM gramicidin-D and 3 mM BTP-ATP or 0.6 mM BTP-PP_i, as appropriate. The reaction was initiated by the addition of membrane protein. The reaction was stopped (and if present, Triton X-100 and added phospholipid were precipitated) by the addition of 1 vol. ice-cold 10% (w/v) trichloroacetic acid, 4% (w/v) $HClO_4$. The samples were left on ice for 2 min, centrifuged for 3 min in an Eppendorf microfuge and the supernatants were assayed for P_i by the method of Ames [7].

The fractions from Sepharose chromatography were assayed in a medium supplemented with 1.33 mg/ml sonicated phospholipid as described previously [8].

Measurement of intravesicular acidification. ATP- and PP_i-dependent intravesicular acidification were assayed with the monoamine dye acridine orange at excitation and emission wavelengths of 495 and 540 nm, respectively, and a slitwidth of 5 nm for both excitation and emission.

Tonoplast vesicles (50 μg membrane protein), 2.5 mM BTP-Mes, 1 mM BTP-ATP or 0.6 mM Tris-PP_i, 50 mM KCl, 0.4 M glycerol and 5 μM acridine orange were added to the sample cell to give a final volume of 2 ml (pH 8.0). H^+-translocation was initiated by the addition of 1 mM or 0.6 mM $MgSO_4$, respectively, and the fluorescence decrease was measured at 25 °C.

The decrease in fluorescence (F) against time (t, measured in minutes) was monitored with a Perkin-Elmer LS-5 luminescence spectrometer and sampled (frequency = 10 Hz) by an IBM-PC/XT microprocessor [9]. The relationship $F = f(t)$ was fitted, for both ATP- and PP_i-dependent H^+-translocation, by a non-linear least squares routine [10] to the function $F = a - b\ e^{-ct}$, with a, b and c all constants. The initial rate ($t = 0$) of fluorescence quenching, which approximates the initial rate of H^+-translocation [11], was taken as the first derivative of this relationship.

Solubilization and partial purification of tonoplast-ATPase. For the preparation of partially purified tonoplast ATPase, tonoplast vesicles were solubilized with 4% (w/v) Triton X-100 in 20% (w/v) glycerol, 5 mM BTP-Mes (pH 8.0), 0.1 mM BTP-EDTA and 5 mM dithiothreitol. The mixture was incubated on ice for 30 min and centrifuged at 200 000 × g for 1 h. Tonoplast ATPase was partially purfied by gel filtration of the 200 000 × g supernatant on Sepharose CL-4B. A 90 × 1 cm ID column packed with Sepharose CL-4B was equilibrated with running buffer (10% glycerol, 0.3% Triton X-100, 0.05 mg/ml Type IV L-α-phosphatidylcholine (Sigma), 5 mM dithiothreitol, 1 mM BTP-EDTA and 5 mM BTP-Mes, pH 8.0) and 2 mg of solubilized protein were applied. The column was operated at a flow-rate of 4 ml/h at 4 °C.

Labeling with [^{14}C]DCCD. Tonoplast vesicles were suspended to a concentration of 250 μg/ml membrane protein in 30 mM Tris-Mes buffer (pH 8.0) containing 1 mM dithiothreitol and an ethanolic solution of [^{14}C]DCCD was added to give a final DCCD concentration of 30 μM. The suspension was incubated for 30 min at 30 °C. The reaction was terminated, and unreacted DCCD removed by at least 40-fold dilution of the membrane suspension into ice-cold 30 mM Tris-Mes (pH 8.0) containing 1 mM dithiothreitol. The diluted sample was centrifuged at 80 000 × g for 35 min and the membrane pellet was resuspended in a small volume of suspension medium for subsequent treatment with chaotrope.

SDS-polyacrylamide gel electrophoresis. One-dimensional SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli [12]. For the routine analysis of membrane pellets and supernatants recovered from the treatments with chaotrope, concave exponential gradient (10–14%) polyacrylamide gels were prepared as described by O'Farrell [13]. For the analysis of [^{14}C]DCCD-labeled polypeptides, the gels were prepared from 14% (w/v) acrylamide and 0.4% (w/v) bisacrylamide, and run at 50 V for 24 h to maximize separation in the low M_r (< 20 000) range. The molecular-weight markers employed were: phosphorylase b (M_r = 97 400), bovine serum albumin (66 000), ovalbumin (45 000), carbonic anhydrase (29 000), lysozyme (14 300) and cytochrome c (12 000).

The protein was detected by Coomassie blue staining. ^{14}C-labeled components were detected fluorographically. After destaining in 7% (v/v) acetic acid, the gels were impregnated with Amplify (Amersham International plc, Amersham, U.K.) before drying and exposure to preflashed

X-ray plates at −80 °C. ^{14}C-label was determined quantitatively by liquid scintillation counting. Radioactive bands on the gels were detected fluorographically, excised and cut into 1 mm strips. The strips were depolymerized in 1 ml volumes of 19 parts 30% (w/v) hydrogen peroxide/1 part 0.88 ammonia for 48 h at 37 °C [14]. The dissolved samples were counted in Optiphase 'MP' (Fisons plc. Loughborough, U.K.).

A modification of the method of Piccioni et al. [15] was employed for the removal of Triton X-100 before denaturation and SDS-polyacrylamide gel electrophoresis of the fractions from Sepharose CL-4B chromatography. Trichloroacetic acid was added to the samples to give a final concentration of 15% (w/v). The samples were left on ice for 30 min and centrifuged for 10 min in an Eppendorf microfuge at 4 °C. The supernatants were aspirated, the pellets were extracted with 1 ml 90% (v/v) ice-cold ethanol, and the centrifugation step was repeated. The pellet from the second centrifugation was extracted again with 0.5 ml 90% ethanol before freeze-drying and denaturation for SDS-polyacrylamide gel electrophoresis.

Densitometry was performed with a Joyce Loebl Chromoscan 3 densitometer at a wavelength setting of 530 nm.

Immunodetection of 67 and 57 kDa subunits of tonoplast-ATPase. Polypeptides were transferred from gels to 0.45 μm nitrocellulose paper (Schleicher and Schuell, D-3354 Dassel, F.R.G.) at 0.4 mA for 5 h at 4 °C in a transfer cell (LKB 2005 Transphor Electroblotter, S-161 26 Bromma, Sweden) containing buffer consisting of 25 mM Tris, 150 mM glycine, 20% (v/v) methanol and 0.1% (w/v) SDS [16]. The nitrocellulose blots were blocked by incubation in 3% (w/v) bovine serum albumin in phosphate-buffered saline (157 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 and 3 mM KH_2PO_4) for 1 h followed by incubation in 5 changes of 0.1% (v/v) Tween-20 in phosphate-buffered saline. The blots were then incubated for 1.5 h in rabbit antiserum raised against the 67 and 57 kDa subunits of the tonoplast ATPase from *Beta* [17] in 3% (w/v) bovine serum albumin in phosphate-buffered saline. The blots were washed in five changes of phosphate-buffered saline containing 0.1% (v/v) Tween-20 and probed with $5 \cdot 10^5$ dpm/ml [^{125}I]IPA suspended in 1% (w/v) bovine serum albumin in phosphate-buffered saline for 1 h. The blots were finally washed in 5 changes of 0.1% (v/v) Tween-20 in phosphate-buffered saline and one change of phosphate-buffered saline alone before being dried overnight at room temperature. Bound [^{125}I]IPA was detected autoradiographically.

Protein. Protein was estimated routinely by a modification of the dye-binding method of Bradford [18]. Protein in the column fractions was estimated by the BCA method [19] which is not subject to interference by detergents. To overcome interference from dithiothreitol, the samples were made 15% (w/v) with trichloroacetic acid and protein was estimated on the pellet.

Chemicals. All of the general laboratory reagents were obtained from Sigma Chemical Co Ltd (Poole, Dorset, U.K.) or BDH Ltd (Poole, Dorset, U.K.). Sephadex G25 and Sepharose CL-4B were purchased from Pharmacia (Milton Keynes, U.K.) and acridine orange (3,6-bis[dimethylamino]acridine) from Fluka (Glossop, U.K.). [^{14}C]DCCD (57 mCi/mmol) and [^{125}I]IPA (30 mCi/mg total protein A) were from Amersham International PLC, Amersham, U.K. BTP-ATP and BTP-PP_i were prepared from Na_2ATP and Na_4PP_i, respectively by cation-exchange chromatography.

Results

Influence of standard KI treatment

Potassium iodide has been employed to diminish the contamination of some membrane preparations from plant tissues by acid phosphatase [20]. Since I^- is itself chaotropic, it was therefore important to assess the effect of this standard treatment on the tonoplast H^+-pumps before investigating their response to chaotropes in general. The results are summarized in Table I.

Pretreatment of the microsomes from fresh red beet with 0.24 M KI had no effect on the specific activity of NO_3^--sensitive, N_3^--insensitive ATPase but caused a small (approx. 20%) increase in the specific activity of K^+-stimulated, Na^+-inhibited pyrophosphatase. Contamination by MoO_4^{2-}-sensitive (nonspecific phosphatase), orthovanadate-sensitive (E_1E_2-ATPase) and N_3^--sensitive (F_1-

TABLE I

EFFECT OF PRETREATMENT WITH 0.24 M KI ON ATP AND PP_i HYDROLYSIS BY TONOPLAST-ENRICHED MEMBRANE FRACTION

The microsomal pellets were resuspended in suspension medium in the presence (+KI) or absence (−KI) of 0.24 M KI, sedimented at 80000 × *g* and then subjected to sucrose gradient density centrifugation. The tonoplast-enriched fraction was collected from the 10/23% (w/w) sucrose interface. 50 mM KCl was included in all of the assay media, except when substituted by 50 mM NaCl for the pyrophosphatase assays. Potassium nitrate (100 mM), Na_2MoO_4 (200 μM), sodium orthovanadate (100 μM) or NaN_3 (2 mM) were added as indicated. The % activities relative to the control are shown in parenthesis.

Effector	Activity (μmol/mg per h)			
	ATPase		Pyrophosphatase	
	−KI	+KI	−KI	+KI
None	71.4 (100.0)	69.6 (100.0)	17.5 (100.0)	21.6 (100.0)
KNO_3	17.4 (24.3)	17.4 (25.0)	–	–
Na_2MoO_4	70.9 (99.3)	66.8 (96.0)	–	–
Orthovanadate	68.5 (95.9)	72.6 (104.3)	–	–
NaN_3	75.9 (106.2)	71.4 (102.6)	–	–
$KNO_3 + NaN_3$	15.4 (21.5)	17.1 (24.6)	–	–
NaCl	–	–	4.7 (27.0)	5.1 (23.6)

ATPase) activities was negligible in both the KI-treated and non-treated tonoplast-enriched fraction. The standard KI treatment consequently appears to have no inhibitory effect on the activities of either the tonoplast ATPase or tonoplast pyrophosphatase. It should, however, be noted that in some membrane preparations from *Beta*, sugar beet especially, pretreatment of the microsomal pellet with KI is necessary to minimize contamination of the final tonoplast-enriched fraction with F_1-ATPase and mitochondrial membrane fragments (cytochrome *c* oxidase; Rea, P.A., Griffith, C.J. and Sanders, D., unpublished data). Treatment with 0.24 M KI during the preparation of membranes was therefore retained throughout as a precaution against significant contamination by F_1-ATPase.

Differential susceptibilities of tonoplast-ATPase and tonoplast pyrophosphatase to irreversible inhibition by chaotropic anions

The tonoplast ATPase was markedly more susceptible to irreversible inhibition by chaotropic anions than the tonoplast pyrophosphatase and this was manifest both at the level of H^+-translocation and of substrate hydrolysis. Thus, 0.5 and 0.6 M KI decreased the rate and extent of ATP-dependent fluorescence quenching by 80% or more but left PP_i-dependent H^+-translocation unaffected (Fig. 1). The k_{50} values for irreversible

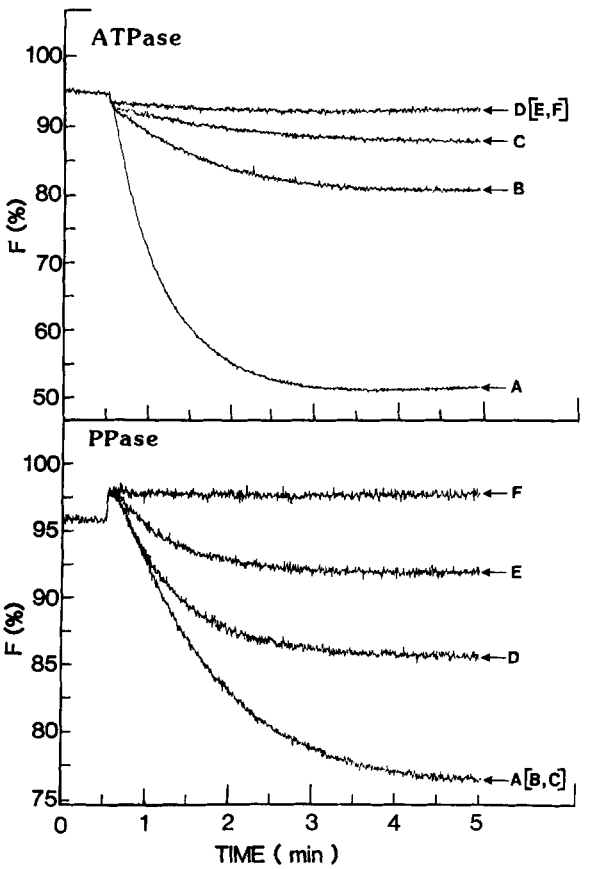

Fig. 1. Differential sensitivities of ATP- and PP_i-dependent H^+-translocation to irreversible inhibition by KI. Potassium iodide concentrations of 0.25 M (A), 0.5 M (B), 0.6 M (C), 0.8 M (D), 1.0 M (E) and 1.5 M (F) were employed. The letters in square brackets indicate that these concentrations of KI gave identical results to the concentration corresponding to the letter outside the square brackets. The membranes were washed free of KI before measuring the decrease in fluorescence against time with 50 μg membrane protein per assay. PPase, pyrophosphatase.

inhibition of ATP hydrolysis by SCN^-, ClO_4^-, I^- and NO_3^- were 0.2–0.3, 0.3–0.4, 0.4–0.5 and over 1.0 M, respectively (Fig. 2A). Pyrophosphate hydrolysis by the same preparations was, on the other hand, not inhibited by ClO_4^-, I^- or NO_3^- and only appreciably inhibited by SCN^- concentrations in excess of 0.5 M (Fig. 2A).

Although broadly correspondent results were obtained for H^+-translocation (Fig. 2B), chaotrope concentrations in excess of the k_{50} for ATP hydrolysis did appear to have non-specific effects on H^+-translocation. For instance, 0.75 M ClO_4^- largely abolished both ATP- and PP_i-dependent intravesicular acidification (Fig. 2B), but had little or no effect on pyrophosphatase-mediated substrate hydrolysis (Fig. 2A). The non-specific effects of high chaotrope concentrations are attributed to an increase in the passive permeability of the membranes to H^+, thereby limiting the rate and extent of intravesicular acidification while leaving substrate hydrolysis unaffected when assayed in the presence of uncoupler (5 μM gramicidin-D, see Materials and Methods).

The non-chaotropic anions, SO_4^{2-} (data not shown) and CH_3COO^-, neither inhibited the tonoplast ATPase nor tonoplast pyrophosphatase whether activity was measured as substrate hydrolysis (Fig. 2A) or as H^+-translocation (Fig. 2B). Since both of these anions were added as their K^+-salts, as were most of the chaotropes, it

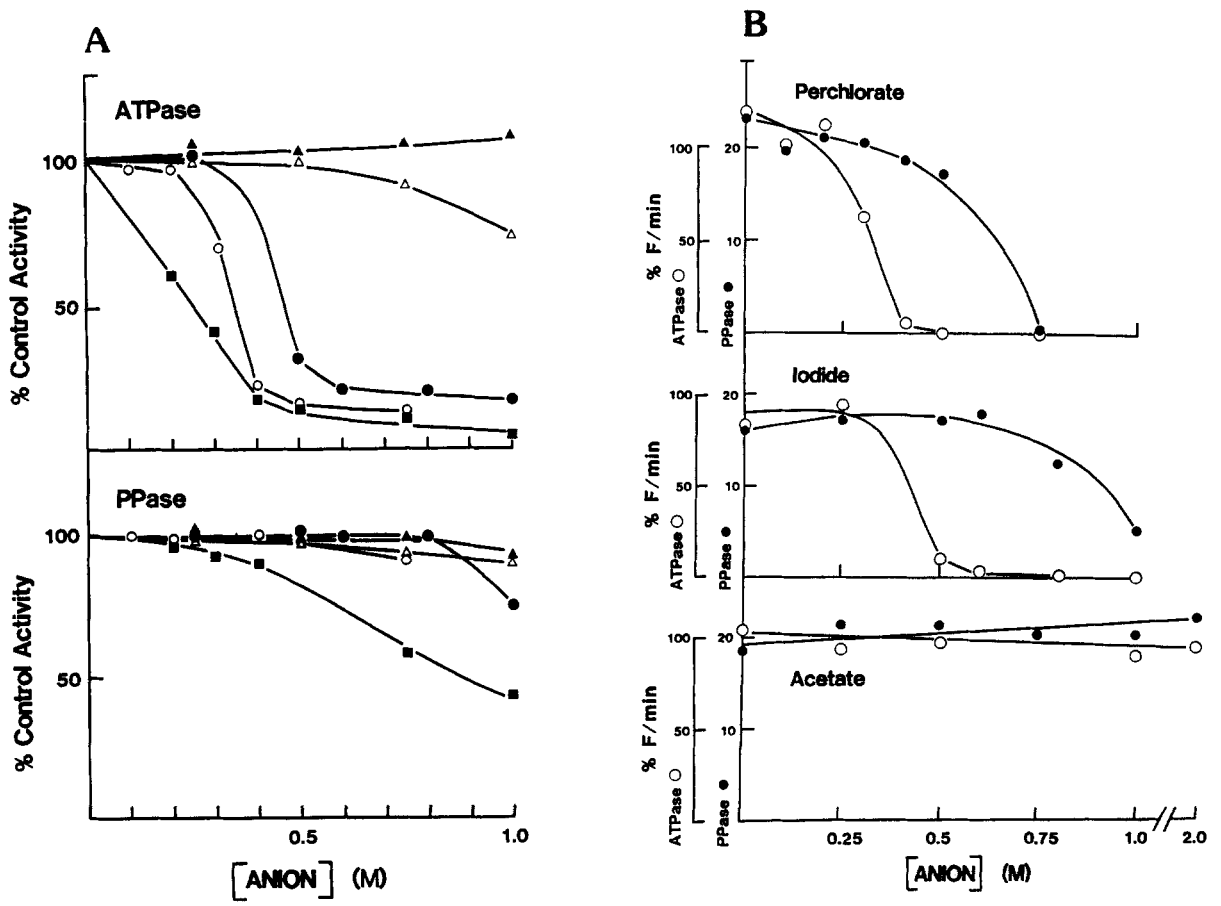

Fig. 2. Differential sensitivities of tonoplast ATPase and tonoplast pyrophosphatase to irreversible inhibition by chaotropic anions. (A) Substrate hydrolysis after treatment with SCN^- (■), ClO_4^- (○), I^- (●), NO_3^- (△) or CH_3COO^- (▲). The control activities varied between 45 and 75 μmol/mg per h (tonoplast ATPase) and 10 and 25 μmol/mg per h (tonoplast pyrophosphatase). (B) Initial rates of ATP- (○) and PP_i-dependent (●) H^+-translocation (% F/min) after treatment with ClO_4^-, I^- or CH_3COO^-. PPase, pyrophosphatase.

appears that the counter-cation does not participate in inhibition.

Potassium iodide and $NaClO_4$ were the chaotropes chosen for all subsequent manipulations because they yielded the widest separations between the tonoplast ATPase and tonoplast pyrophosphatase for the onset of inhibition and appeared to have only minor non-specific effects. The potential for the control and imposition of specific alterations of membrane function and composition without, for example, oxidative damage [5] was considered to be greater for a chaotrope of intermediate rather than high potency.

Differential dissociation of membrane proteins

SDS-polyacrylamide gel electrophoresis analysis of the membrane pellets recovered after treatment of the vesicles with chaotrope revealed a significant diminution of three bands of 67, 57 and 33 kDa (Fig. 3). Quantitative densitometry over a range of ClO_4^- or I^- concentrations further demonstrated that the attenuation of the 67 and 57 kDa bands in the membrane pellet parallels the irreversible abolition of tonoplast ATPase activity (Fig. 4).

Complementary analysis of the supernatants from the treatments with 0.6 M KI (Fig. 5) and 0.3 M $NaClO_4$ (data not shown) revealed the presence of two major 67 and 57 kDa polypeptides and several minor components (lane 4, Fig. 5A). Since vesicles subjected to the same treatment without KI or $NaClO_4$ in the suspension medium yielded essentially no detectable polypeptides in the supernatant (lane 3, Fig. 5A),

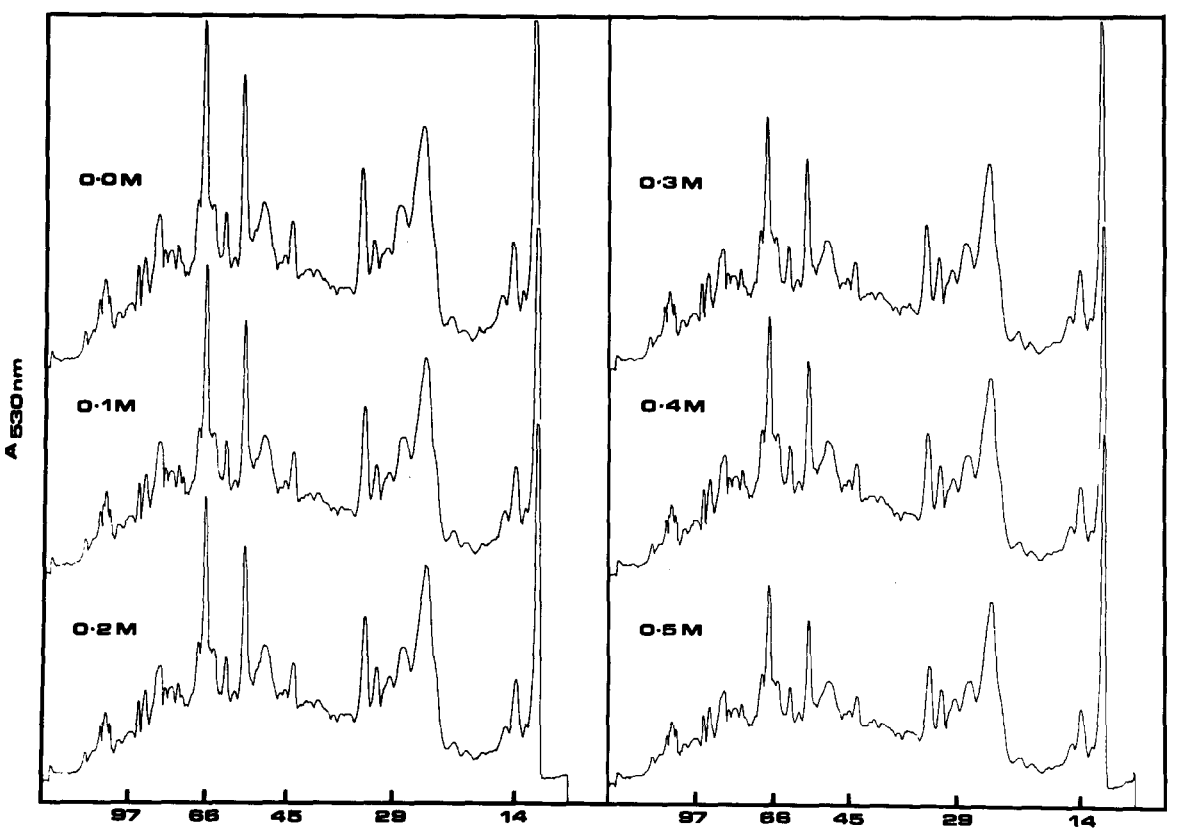

Fig. 3. SDS-polyacrylamide gel electrophoresis analysis of pelletable protein after treatment of tonoplast vesicles with $NaClO_4$. All of the pellets were resuspended to the same volume and the same proportions of the total resuspension volume for each treatment were electrophoresed on a 10–14% concave exponential polyacrylamide gel. The amounts of protein loaded ($NaClO_4$ concentrations in parenthesis) were 30 μg (0.0 M), 30 μg (0.1 M), 26.8 μg (0.2 M), 24.6 μg (0.3 M), 24.2 μg (0.4 M) and 24.0 μg (0.5 M), respectively. The numbers at the bottom of the figure show the location of the molecular-weight markers (kDa).

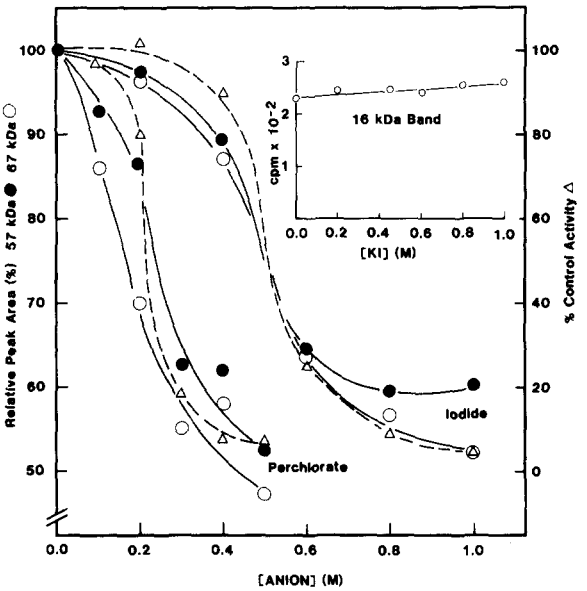

Fig. 4. Quantitative analysis of attenuation of 67 and 57 kDa bands in the membrane pellets and abolition of tonoplast ATPase activity after treatment with the indicated concentration of chaotrope. The samples were electrophoresed as in Fig. 3 and the relative peak areas (% of 0.0 M KI control) were measured. Inset, cpm ($\times 10^{-2}$) [^{14}C]DCCD retained in the 16 kDa proteolipid of tonoplast vesicles treated with 0.0–1.0 M KI. See legend to Fig. 6 for details.

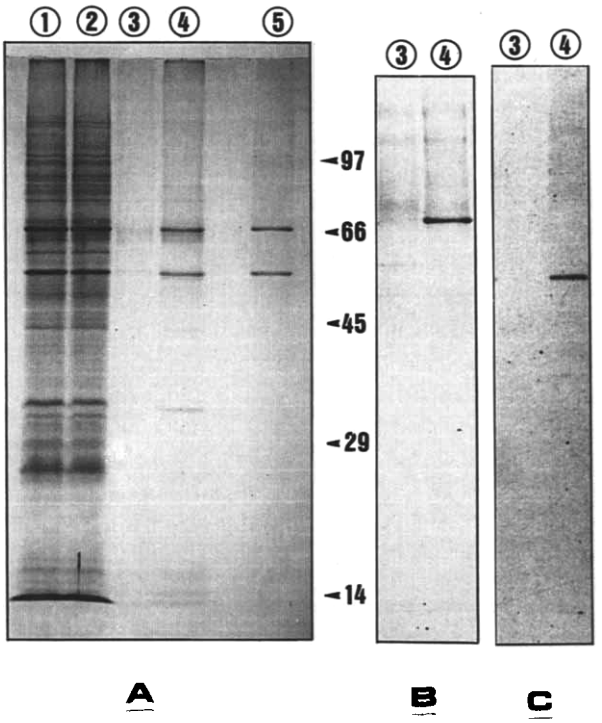

Fig. 5. SDS-gel and immunoblot of pelletable and non-pelletable protein after treatment of tonoplast vesicles with KI. (A) Coomassie blue-stained gel. (B) Immunoblot of lanes 3 and 4 of gel in A probed with antibody raised to the 67 kDa subunit of the partially purified tonoplast ATPase. (C) Immunoblot of lanes 3 and 4 of gel in A probed with antibody raised to the 57 kDa subunit of the partially purified tonoplast ATPase. Lanes 1 and 2 correspond to the membrane pellets recovered after treatment of the tonoplast vesicles with 0.0 and 0.6 M KI, respectively. The pellets were resuspended to the same volume and the same proportions of the total volume for each treatment were electrophoresed. The amounts of protein loaded were 45 μg (1) and 38 μg (2). In these and other experiments, there was a 15–18% loss of protein from the membranes after treatment with 0.6 M KI. Lanes 3 and 4 correspond to the protein recovered from the 200000 × *g* supernatant after treatment with 0.0 M and 0.6 M KI, respectively. 3% of the total protein recovered from the supernatants was electrophoresed in each case: less than 1 μg (lane 3) and 5 μg (lane 4). 30–40% of the protein lost from the membranes upon treatment with KI was recovered from the supernatant after freeze-drying and Sephadex G25 chromatography. Partially purified tonoplast ATPase (lane 5, A: 5 μg protein) is shown for comparative purposes. Cross-reacted antibody was detected autoradiographically using [^{125}I]IPA (Materials and Methods).

it is concluded that the chaotropic anion is the factor responsible for the preferential detachment of these components from the membrane.

The major subunits of the partially purified tonoplast ATPase (lane 5, Fig. 5A) and the principal components in the 0.6 M KI supernatant (lane 4, Fig. 5A) and the 0.3 M $NaClO_4$ supernatant (data not shown) comigrate when electrophoresed under identical conditions. This finding, together with the similar I^- and ClO_4^- concentrations required for the onset of irreversible inhibition of the tonoplast ATPase and attenuation of the 67 and 57 kDa bands in the membrane pellet, indicates that the 67 and 57 kDa subunits of the tonoplast ATPase and their comigrants in the I^- and ClO_4^- supernatants are identical.

Although all attempts to demonstrate catalytic (ATPase) activity in the 0.6 M KI supernatant before and after removal of chaotrope failed, identification of the 67 and 57 kDa polypeptides of the 0.6 M KI supernatant as the major subunits of the tonoplast ATPase is confirmed by the fact that antibodies raised to the 67 and 57 kDa subunits of the partially purified tonoplast ATPase cross-react with the 67 and 57 kDa polypeptides, respectively, of the KI supernatant (Fig. 5B and C).

The other identified component of the tonoplast ATPase, the 16 kDa DCCD-binding proteolipid [21], by contrast, shows no detachment from the membrane after treatment with chaotrope. Incubation of tonoplast vesicles with 30 μM [^{14}C]DCCD for 30 min yields three major low M_r components upon SDS-polyacrylamide gel electrophoresis and fluorography: two bands of 16 and 8 kDa, respectively, and an intensely radioactive fraction at the front of the gel (Fig. 6). The latter represents [^{14}C]DCCD-labeled phospholipid, since it can be quantitatively extracted with acetone/ethanol (1 : 1), whereas the 8 kDa band corresponds to contamination by the *c* peptide of mitochondrial F_0F_1-ATPase [21]. Only the 16 kDa band copurifies with the tonoplast ATPase [22] and shows the appropriate kinetics of labeling with [^{14}C]DCCD [21].

Tonoplast vesicles labeled with [^{14}C]DCCD (so as to visualize the hydrophobic 16 kDa polypeptide which stains poorly with Coomassie blue [1]) and subsequently treated with 0.0–1.0 M KI show no attenuation of this band as determined

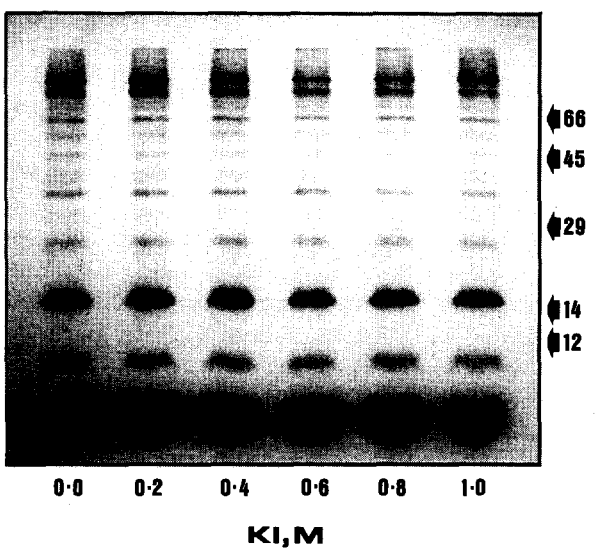

Fig. 6. Fluorogram of KI-treated [^{14}C]DCCD-labeled membranes. Membranes (250 μg/ml) were labeled with 30 μM [^{14}C]DCCD for 30 min, excess reagent was removed and the resuspended samples were treated with KI as described in Materials and Methods. The membrane pellets from the treatments with KI were electrophoresed on 14% slab gels, fluorographed and the 16 kDa bands excised for counting (Fig. 4, inset).

both radiometrically (Fig. 4, inset) and fluorographically (Fig. 6). It therefore appears that of the three known subunits of the tonoplast ATPase, chaotropic anions specifically detach the 67 and 57 kDa polypeptides, while the 16 kDa component remains associated with the membrane.

Retention of latent ATPase activity

A possible objection to the above deductions is that although essentially all overt tonoplast ATPase activity is abolished by treatment of the tonoplast vesicles with 0.5 M $NaClO_4$ or 0.8 M KI (Figs. 2 and 4), densitometry shows approx. 50% of the 67 and 57 kDa components to remain associated with the membrane after treatment (Fig. 4). Detergent-activation experiments, however, provide a possible reconciliation of these two apparently conflicting findings in demonstrating quantitative retention of the latent component of the tonoplast ATPase activity after treatment with chaotrope.

The inclusion of increasing concentrations of Triton X-100 in the ATPase reaction medium causes maximal activation at a concentration of 0.025–0.030% (w/v), whether or not the vesicles have been treated with 1.0 M KI (Fig. 7A). Moreover, the detergent activations seen with non-treated and KI-treated membranes yield latent components of similar magnitude (51–59 μmol/mg per h) to each other and to the control activity in the absence of KI (i.e., 45 μmol/mg per h). It therefore appears that these tonoplast preparations consist of a mixed (approx. 1 : 1) population of inside-out and rightside-out vesicles and that the ATPase of inside-out vesicles is rendered accessible to substrate by Triton X-100 concentrations in the vicinity of its critical micelle concentration (0.03%). Since the latent components are of similar absolute magnitude whether or not the membranes have been treated with KI, chaotropic anions seem to abolish largely the outwardly oriented (overt), but not the inwardly oriented (latent) components of activity.

The lack of inhibition of latent activity is unlikely to result from poor transmembrane permeation of the anion concerned, since chaotropic anions, by virtue of their low charge/mass ratios, are also highly lipophilic. Furthermore, since irreversible inhibition of the tonoplast ATPase is

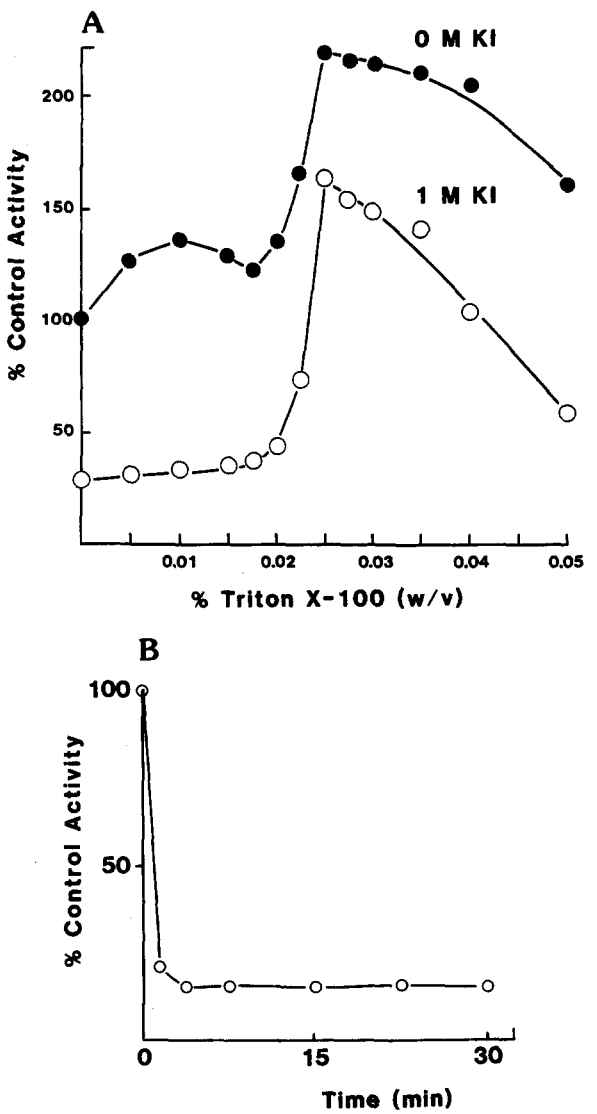

Fig. 7. (A) Latency of tonoplast ATPase activity before (●) and after (○) treatment of tonoplast vesicles with 1 M KI. Control activity = 100% = activity of tonoplast ATPase measured in the absence of Triton X-100 without pretreatment with KI. Triton X-100 was added to the ATPase assay media at the concentrations indicated. (B) Time-dependence of abolition of tonoplast ATPase activity upon treatment of tonoplast vesicles with 1 M KI. The vesicles were treated with KI for the times indicated and washed free of KI by dilution and centrifugation. ATPase activity was measured on the pellet.

complete within 5 min of the addition of KI to the medium (Fig. 7B), inadequate equilibration is unlikely to explain the maintenance of the latent component. Consequently, we propose that the ATPase of inside-out vesicles is accessible to chaotrope, but because of their large size the subunits dissociated cannot exit the intravesicular compartment and so reassociate with the membrane to restore activity upon removal of the chaotrope. However, other explanations, such as differential detergent-activation of the chaotrope-treated and control ATPases, cannot be eliminated.

Discussion

A number of significant conclusions emerge from the results of this investigation.

(1) Caution is required when using chaotropic anions for the removal of loosely associated proteins from tonoplast during the routine preparation of membranes [6,20,23] because the tonoplast ATPase is itself sensitive to irreversible chaotropic effects. Although KI at a concentration of 0.24 M, as employed by Poole et al. [20], Bennett et al. [23] and Rea and Poole [6], has no effect on the specific activity of the tonoplast ATPase, higher concentrations are inhibitory.

(2) The differential sensitivities of the tonoplast ATPase and tonoplast pyrophosphatase to irreversible inhibition by chaotropic anions demonstrate that the two activities are physically and functionally distinct both with respect to H^+-translocation and substrate hydrolysis, without resort to kinetic [6] or chromatographic analyses [8].

(3) Chaotrope concentrations maximally inhibiting the tonoplast ATPase but leaving the tonoplast pyrophosphatase unaffected detach two major polypeptides of 67 and 57 kDa from the membrane which cross-react with antibodies raised to the 67 and 57 kDa subunits of the partially purified tonoplast ATPase.

The tonoplast ATPase from higher plant tissues consists of at least three subunits: a 67–70 kDa polypeptide which is labeled by [^{14}C] *N*-ethylmaleimide and [^{14}C] NBD-Cl in the absence, but not in the presence of Mg-ATP [24]; a 57–60 kDa polypeptide which labels with [α-^{32}P] BzATP [22]; and a 16 kDa component which is labeled by [^{14}C]DCCD [21,22,24,25]. Preferential detachment of two major bands of 67 and 57 kDa from the tonoplast, as indicated by their attenuation in the membrane pellet and their appearance in the supernatant at chaotrope concentrations maximally

inhibiting the tonoplast ATPase, but not appreciably inhibiting the tonoplast pyrophosphatase, therefore indicates relatively specific dissociation of the 67 and 57 kDa subunits of the tonoplast ATPase from the membrane. Although it is not possible to establish an unambiguous correlation between loss of enzymic activity and dissociation of subunits (because of the large latent component and the possibility of non-dissociative denaturation), these data indicate a relatively peripheral disposition for the 67 and 57 kDa components. This notion is consistent with the observation that the high M_r subunits are readily labeled by hydrophiles and are accessible to antibody [24]. The resistance of the DCCD binding 16 kDa subunit to chaotrope-elicited detachment, on the other hand, implies an intrinsic association with the membrane.

The behavior of the tonoplast ATPase is reminiscent of the structure/function partitioning of the F_0F_1-category of H^+-ATPases. Thus, the soluble nucleotide-binding F_1 sector of F_0F_1 is removed from the DCCD-binding sector by washing with EDTA at low ionic strength [26], while the 67 and 57 kDa components of the tonoplast ATPase are dissociated from the 16 kDa subunit by chaotropic anions. Likewise, both the DCCD-binding F_0 sector of F_0F_1 and the DCCD-binding 16 kDa component of the tonoplast ATPase, but not the nucleotide-binding subunits of either complex, freely partition into chloroform/methanol mixtures (F_0F_1, [27]; tonoplast ATPase [21]). There is, however, one difference: unlike the EDTA-dissociated F_1-component of F_0F_1, the chaotrope-detached nucleotide-binding subunits of the tonoplast ATPase are catalytically inactive.

An important characteristic of the tonoplast ATPase is its anion sensitivity: Cl^- is stimulatory and NO_3^- is inhibitory in the millimolar range. While the mechanism of stimulation by Cl^- has been investigated by several laboratories [11,28], information concerning the inhibitory action of NO_3^- is scant. Recent studies, however, show that NO_3^- is a pseudo-competitive inhibitor of the tonoplast ATPase and potently chaotropic anions can simulate this effect [29]. It has therefore been proposed that NO_3^- exerts its inhibitory effect in the millimolar range chaotropically [29].

Such a proposal now appears to be incorrect on the basis of the results presented above insofar as the reversible, competitive inhibitions reported previously for low concentrations of chaotropic anions [29] and the irreversible chaotropic effects in the high concentration range reported here are unrelated. The reversible, competitive inhibitions imposed by the anions tested follow the sequence $NO_3^- > SCN^- > ClO_4^- \gg CH_3COO^- = 0$, whereas their irreversible inhibitory efficacies exactly coincide with the Hofmeister series [30] for chaotropic potency, i.e., $SCN^- > ClO_4^- \gg I^- > NO_3^- \gg CH_3COO^-$, $SO_4^{2-} = 0$ (Table II). Hence, NO_3^- is an anomalously potent competitive inhibitor if reversible chaotropism is the principal cause of the inhibitions seen in the low concentration range. There are numerous examples of weak chaotropes, such as NO_3^-, being more potent destabilizing agents then potent ones [5], since electrostatic as well as hydrophobic interactions may participate in the maintenance of enzyme functional integrity (see Ref. 29 for further discussion). However, the fact that NO_3^- is not also an anomalously potent irreversible inhibitor in the high concentration range argues against the idea of simple destabilization as its primary mechanism of inhibition at low concentrations. Since we have already shown that NO_3^- does not exert its competitive effect in the low-concentration range by stereochemically simulating the trigonal planar

TABLE II

COMPARISON OF SENSITIVITY OF TONOPLAST ATPase TO REVERSIBLE AND IRREVERSIBLE INHIBITION BY ANIONS

The numbers shown are the K_I values for competitive inhibition with respect to Mg-ATP (data from Ref. 29) and the k_{50} values for the irreversible inhibition of hydrolytic activity (data from Fig. 2B), respectively. n.d. = not determined.

Anion	K_I (mM)	k_{50} (mM)
SCN^-	38	200–300
ClO_4^-	54	300–400
ClO_3^-	120	n.d.
I^-	stimulatory [a]	400–500
NO_3^-	22	>1250
CH_3COO^-	not inhibitory	not inhibitory
SO_4^{2-}	non-competitive	not inhibitory

[a] Data of Briskin et al. [31].

geometry of the terminal phosphoryl-group of ATP to form a dead-end complex with Mg-ADP [29], the mode of action of this inhibitory anion in the lower concentration range remains unresolved.

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