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Rapid purification and reconstitution of a plant vacuolar ATPase using Triton X-114 fractionation: subunit composition and substrate kinetics of the H^+-ATPase from the tonoplast of *Kalanchoë daigremontiana*

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A rapid procedure for the purification and reconstitution into proteoliposomes of the H^+-translocating ATPase of plant vacuolar membranes is reported. It involves fractionation of the tonoplast with Triton X-114, resolubilization of the ATPase with octyl glucoside in the presence of a mixture of phosphatidylcholine, phosphatidylserine and cholesterol (27:53:20, by weight), and removal of the detergent by gel-filtration. Starting with partially purified vacuolar membranes, the procedure can be accomplished in about 2 hours. It has been applied to the H^+-ATPase from the crassulacean plant *Kalanchoë daigremontiana*, from which it yields vesicles with a specific ATPase activity of about 3 μmol/min per mg protein. The purified enzyme contains polypeptides of apparent molecular mass 72, 57, 48, 42, 39, 33 and 16 kDa; these polypeptides also co-sediment on centrifugation of the solubilized ATPase through glycerol gradients. The 16-kDa subunit is labelled with [^{14}C]dicyclohexylcarbodiimide. There is no evidence for a larger ATPase subunit in this preparation. The reconstituted ATPase proteoliposomes undergo ATP-dependent acidification, which can be measured by quenching of the fluorescence of 9-aminoacridine. The initial rate of fluorescence quenching is a measure of the rate of H^+ translocation, and is directly proportional to the vesicle protein concentration, so the preparation is suitable for studying the kinetics of the tonoplast H^+-ATPase. The dependence of the rate of fluorescence quenching on the concentration of MgATP is well fitted by the Michaelis equation, with a K_m value about 30 μM. ATP can be replaced by dATP, ITP, GTP, UTP or CTP, and Mg^{2+} by Mn^{2+} or Ca^{2+}; kinetic parameters for these substrates are reported. In contrast, hydrolysis of MgATP shows complex kinetics, suggestive either of negative cooperativity between nucleotide-binding sites, or of two non-interacting catalytic sites. Both the hydrolytic and the H^+-translocating activities of the proteoliposomes are inhibited by nitrate, though not in parallel, the latter activity being the more sensitive. Both activities are inhibited in parallel by bafilomycin A_1, which does not produce complete inhibition; the bafilomycin-insensitive component has complex ATPase kinetics similar to those of the uninhibited enzyme.

Introduction

Many succulent plants assimilate CO_2 photosynthetically by a process known as crassulacean acid metabolism (CAM) [1,2]. This is characterized by the nocturnal fixation of atmospheric CO_2 into malic acid, which accumulates to high concentrations in the large central vacuoles of photosynthetic cells [3]. The transport of malate into the vacuole is believed to be energized by two distinct H^+-translocating enzymes located at the tonoplast, an adenosinetriphosphatase and an inorganic pyrophosphatase [4,5].

The plant tonoplast ATPase is a member of the 'vacuolar' (V-type) class of H^+-ATPases. These multisubunit enzymes are associated with the endomembrane systems of eukaryotic cells and include proton pumps present in lysosomes, endosomes, Golgi, secretory vesicles and clathrin-coated vesicles of animal cells, as well as the vacuolar membranes of plants and fungi [6–8]. They hydrolyse cytosolic ATP and translocate protons into the interior of the organelles, thus generating an inside-acid transmembrane pH difference and an inside-positive membrane potential which may be used for secondary processes such as ion transport or solute uptake. Exceptionally, V-type ATPases are found in the plasma membrane [9,10].

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; CAM, crassulacean acid metabolism.

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V-type ATPases are composed of two distinct domains, a transmembrane proton channel and an attached catalytic complex [6,11]. The exact subunit composition of the holoenzymes is not known. All contain at least three subunit types, of molecular masses approximately 70, 57 and 16 kDa, but other subunits may be present. For example, mammalian V-type ATPases have been found to contain additional subunits of 33, 40 and 115–120 kDa [12–15], and comparable compositions have been reported for the V-type ATPases of yeast [16] and higher plants [17–19].

Published procedures for the isolation of plant V-type ATPases involve several steps and typically yield a product of specific activity 2.1–6.0 μmol/min per mg protein [19–22], although a specific activity of 20–25 μmol/min per mg protein has been reported for the purified tonoplast ATPase of red beet [17]. Here we describe a rapid method for the partial purification of the tonoplast ATPase from the CAM plant *Kalanchoë daigremontiana* and its reconstitution into proteoliposomes. The method involves the fractionation of tonoplast membranes with the detergent Triton X-114 and resolubilization of the ATPase with *n*-octyl glucoside in the presence of purified lipids. The detergent is then removed by gel filtration, allowing the formation of proteoliposomes. An efficient reconstitution procedure of this kind is an essential prerequisite for detailed study of the kinetics and mechanism of regulation of the enzyme.

Materials and Methods

Materials

Triton X-114 was obtained from Fluka AG, Buchs, Switzerland and purified by the method of Bordier [23], and its concentration determined by the method of Garewal [24]. *n*-Octyl β-glucoside and 9-aminoacridine were purchased from Sigma. Bio-Gel P6-DG was from Bio-Rad. Cholesterol was supplied by Boehringer, Lewes, Sussex, UK. Bovine spinal-cord phosphatidylcholine and phosphatidylserine and egg phosphatidylcholine were obtained from Lipid Products, Redhill, Surrey, UK. Bafilomycin A_1 was a gift from Dr. K. Altendorf, University of Osnabrück, Germany. Antiserum to phospho*enol*pyruvate carboxylase from *Kalanchoë daigremontiana* was a gift from Dr. P. Maier and Dr. M. Kluge, Institut für Botanik, Technische Hochschule Darmstadt, Germany. Antiserum directed against the 120-kDa subunit of chromaffin granule H^+-ATPase was prepared by Ms. J.M. Percy in this Department.

Plant material

Plants of *Kalanchoë daigremontiana* Hamet et Perrier de la Bathie were propagated vegetatively and grown in John Innes No. 3 potting compost in a heated glasshouse. Natural solar radiation was supplemented by mercury-vapour lamps (400 W MBF; Thorn EMI, London UK) for 12 h daily. When 6–9-month old, plants were transferred to a reverse-phase controlled-environment room, where they were illuminated by a combination of metal-halide fluorescent lamps (400 W MBIF/BU; Thorn) and tungsten lamps (PAR 38 150 W Flood; General Electric Co., Wembley, Middlesex, UK) for 12 h daily at a photosynthetic photon flux density (400–700 nm) of 300 μmol/m^2 per s at mid-plant height. Air temperature was maintained at 25°C (light)/14°C (dark), with a relative humidity of approx. 35% (light)/70% (dark). Plants were maintained in the controlled-environment room for at least 2 days before use.

Tonoplast isolation

Tonoplast fractions were prepared from the mesophyll tissue of *Kalanchoë daigremontiana* according to published methods [5,18] with minor modifications. The leaf midrib and margins were removed and the mesophyll tissue was homogenized at 4°C in a blender in 450 mM mannitol, 10 mM EGTA, 2 mM dithiothreitol, 0.5% (w/v) polyvinylpyrrolidone (PVP-40), 100 mM Tricine, adjusted to pH 8.0 with Tris base; 100 ml medium were used for every 60 g tissue. The homogenate was filtered through two layers of cheesecloth and the filtrate was centrifuged (10000 rev./min, Beckman JA-14 rotor, $g_{av} = 9820$) for 15 min. The resulting supernatant was layered over a 0.78 M sucrose cushion containing 5 mM Tricine-Tris (pH 8.0) and 2 mM dithiothreitol. The gradients were centrifuged (36000 rev./min, Beckman Ti45 rotor, $g_{av} = 100006$) for 1 h. Membrane vesicles were removed from the interface using a Pasteur pipette and diluted 1:1 (v/v) with 150 mM mannitol, 25 mM Tricine-Tris (pH 8.0), 2 mM dithiothreitol. The vesicles were then pelleted by centrifugation (36000 rev./min, Beckman Ti45 rotor) for 30 min, and finally resuspended in 150 mM sucrose, 10 mM Tricine-Tris (pH 8.0), 2 mM dithiothreitol. All fractionation steps were performed at 4°C and the tonoplast preparation was stored at −20°C.

Purification and reconstitution of the H^+-ATPase

Tonoplast membranes were thawed and sedimented (100000 rev./min, Beckman TL-100.3 rotor, $g_{av} = 340000$, 10 min). All subsequent operations were performed at 0°C. The membrane pellet was resuspended to a final protein concentration of 2.0 mg/ml, using gentle homogenization in a glass homogenizer. The resuspending buffer was 10 mM Hepes/KOH (pH.7.6), 0.15 M KCl, 1 mM EDTA, 2 mM dithiothreitol, containing 6.2 mg/ml Triton X-114. Triton-insoluble material was collected by centrifugation as described above, washed by homogenisation in the same buffer

containing Triton X-114 (6.2 mg/ml), and sedimented again. It was then resuspended in half the original volume of buffer, containing *n*-octyl β-glucoside (68 mM), phosphatidylcholine (2.7 mg/ml), phosphatidylserine (5.3 mg/ml) and cholesterol (2.0 mg/ml); the solution was centrifuged and the clear supernatant carefully removed from the pellet. This supernatant was the solubilized, partially purified ATPase.

Aliquots of 0.2 ml of this solution were loaded onto 1-ml columns of Bio-Gel P6-DG that had been equilibrated with 10 mM Hepes-KOH (pH 7.6), 0.15 M KCl, 2 mM dithiothreitol, 1 mM EDTA, 10% (v/v) methanol, and packed by centrifugation in a bench centrifuge (1400 rev./min; $g_{av} = 180$; 1 min). The column eluates were collected by centrifugation under the same conditions and stored on ice. These proteoliposomes were used for studying ATP-hydrolytic and proton-pumping activities.

Further purification of the tonoplast ATPase resolubilized after Triton X-114 fractionation was performed by centrifugation on glycerol gradients. Gradients (5 ml) were of 5–15% (w/v) glycerol, in 0.15 M KCl, 10 mM Hepes-KOH (pH 7.6), 0.1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) methanol, 35 mM *n*-octyl β-glucoside, 2.7 mg/ml phosphatidylcholine, 5.3 mg/ml phosphatidylserine and 2 mg/ml cholesterol. Aliquots of 0.4 ml of the solubilized ATPase were layered onto these gradients, which were centrifuged for 5 h (Beckman SW50.1 rotor; 45000 rev./min; $g_{av} = 190000$). After centrifugation, the gradients were fractionated into 0.5-ml samples which were assayed for ATPase activity and analysed by SDS-polyacrylamide gel electrophoresis.

[^{14}C]DCCD labelling of tonoplast ATPase

Tonoplast membranes (2 mg/ml), reconstituted ATPase vesicles (0.5 mg/ml) and the fractions from the peak of ATPase activity on glycerol gradients were labelled with [^{14}C]DCCD (1.85 TBq/μmol) by incubation of 100 μl aliquots of the samples with 11 kBq of [^{14}C]DCCD for 3 h at room temperature. After separation by electrophoresis, fluorography of the gels was carried out using 1 M sodium salicylate.

Analytical methods

ATP hydrolysis was measured at 37°C by a coupled spectrophotometric assay described previously [25]. H^+ translocation by reconstituted ATPase proteoliposomes was measured at 30°C by recording the quenching of 9-amino-acridine fluorescence in a Perkin-Elmer 3000 fluorimeter, with excitation and emission wavelengths of 420 and 480 nm, respectively. The standard assay medium (0.5 ml) contained 0.3 M sucrose, 10 mM Hepes/NaOH buffer (pH 7.4), 1 mM ATP, 1 mM $MgSO_4$, 9-aminoacridine (1.0 μM), valinomycin (0.36 μM) and reconstituted ATPase (2 μg protein). The initial rate of fluorescence quenching was shown to be directly proportional to the amount of ATPase protein in the assay (data not shown) and was taken to be directly proportional to the rate of H^+ translocation (an assumption that has also been justified on theoretical grounds [26]). Initial rate data were fitted to the appropriate rate equations by non-linear regression analysis using a computer program written in C programming language and run on the Sequent computer of Edinburgh University Computing Service. The variances of initial-rate estimates were found to be proportional to the square of the measured rate (data not shown), so these were appropriately weighted in the data-fitting procedure.

The protein concentration of the tonoplast suspension was determined by the method of Bradford [27]. All other protein concentrations were measured by an adaptation of the Folin-Lowry method [28].

Results

Purification and reconstitution of the tonoplast H^+-ATPase

The method used in the purification and reconstitution of the tonoplast H^+-ATPase was adapted from a procedure developed for the chromaffin granule H^+-

TABLE I

Reconstitution of tonoplast ATPase

The data shown are for a typical preparation; values in parentheses are the means ± S.D. of specific activities and recoveries obtained in four separate preparations.

Stage	Activity (nmol/min per ml)	[Protein] (mg/ml)	Specific activity (nmol/min per mg)	Recovery (%)
Membranes	1722	2.0	861 (1006 ± 21)	(100)
Precipitate	1672	1.55	1079 (1449 ± 494)	72.9 (86.7 ± 13.0)
Washed precipitate	1371	1.28	1075 (1265 ± 357)	59.7 (56.8 ± 7.2)
Solubilised ATPase	568	0.59	963 (1131 ± 517)	16.5 (17.2 ± 8.0)
Reconstituted ATPase	1597	0.59	2707 (2897 ± 542)	46.4 (42.9 ± 8.9)

ATPase [29]. Starting with native tonoplast membranes, we were able to produce H^+-pumping vesicles of high specific ATPase activity within 2 h. The lower concentration of Triton X-114 (0.62%, rather than 2.0%) and the conditions used for solubilization of the ATPase in *n*-octyl glucoside were critically important for successful reconstitution of the plant enzyme. When reconstituting the chromaffin granule V-type ATPase [29], the enzyme was solubilized in *n*-octyl glucoside before adding purified lipids. However, this method proved to be unsuitable when applied to the tonoplast ATPase, as this enzyme was rapidly inactivated when solubilized in *n*-octyl glucoside alone. In order to reconstitute the plant enzyme in an active state, it was essential that the solubilization with *n*-octyl glucoside was performed in the presence of lipids already dissolved in the detergent.

The purification and reconstitution of the tonoplast H^+-ATPase is summarized in Table I, which shows data for a typical preparation and the average specific activities from four preparations. The final specific activity reported in Table I is that obtained on reconstitution with a mixture of phosphatidylcholine, phosphatidylserine and cholesterol, in the ratio 27:53:20 by weight (see Materials and Methods). This mixture was found to be close to the optimum for both the ATP-hydrolytic and proton-pumping activities of the enzyme, although as the two activities had different lipid dependencies (Table II) the standard conditions adopted were a compromise, reflecting both the intrinsic lipid-dependence of the ATPase and the requirement for sealed vesicles in which H^+ translocation could be measured. There was a three-fold stimulation of ATPase activity on reconstitution of the solubilized enzyme into proteoliposomes. Similar stimulation by lipids was observed with the chromaffin-granule H^+-ATPase [29], although in this case the optimal lipid composition was quite different.

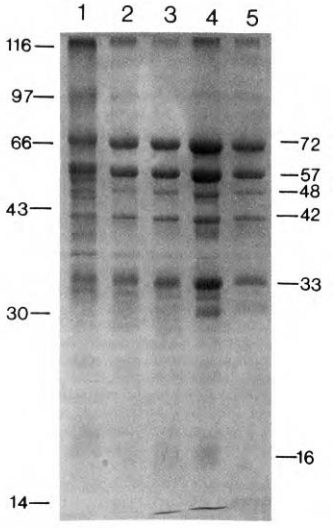

Fig. 1. 12%-polyacrylamide/SDS gel of ATPase fractions at different stages of purification (see Table I), stained with Coomassie blue. Track 1, tonoplast membranes; track 2, protein precipitated with Triton X-114; track 3, washed precipitate; track 4, resolubilised ATPase; track 5, reconstituted ATPase. Each track contained 20 μg of protein. Figures on the left indicate the positions of molecular mass standards; figures on the right show the apparent molecular masses of major polypeptides (kDa).

TABLE II

Effect of lipid composition (expressed as the fraction by weight of total lipid) on ATP hydrolysis and H^+ translocation by the reconstituted ATPase

All rates are expressed relative to the activity of proteoliposomes made with the standard lipid composition (underlined).

Phosphatidyl-choline	Phosphatidyl-serine	Cholesterol	ATPase activity	H^+ translocation activity
0.50	0.50	0.0	74	0
0.475	0.475	0.05	77	0
0.45	0.45	0.10	88	0
0.425	0.425	0.15	93	105
0.40	0.40	0.20	85	154
0.375	0.375	0.25	83	59
0.80	0.0	0.20	100	0
0.53	0.27	0.20	126	50
0.40	0.40	0.20	91	89
0.27	0.53	0.20	100	100
0.0	0.80	0.20	62	152

Subunit composition

Fig. 1 shows a Coomassie blue stained SDS-polyacrylamide gel of the ATPase at various stages of its purification. The reconstituted enzyme contains major polypeptides of apparent molecular mass 72, 57, 48, 42 and 33 kDa; a 16 kDa subunit stains poorly, but is revealed by radioactive labelling (see below). Small amounts of a 100 kDa protein co-purify with the ATPase, but immunoblotting of the fractions shown in Fig. 1 identified this protein as the cytosolic enzyme phospho*enol*pyruvate carboxylase (result not shown). Furthermore, antibodies raised against the 120 kDa subunit of bovine chromaffin granule H^+-ATPase [30]

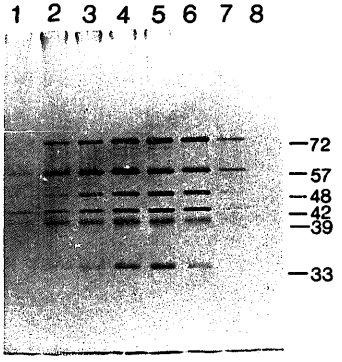

Fig. 2. Subunit composition of the purified ATPase. 10%-polyacrylamide/SDS gel of fractions with ATPase activity on glycerol density-gradient centrifugation, stained with silver. Fraction 4 had the highest activity. Figures on the right of the gel indicate apparent molecular masses (kDa) of subunits.

failed to recognize any antigen in the tonoplast ATPase.

The solubilized ATPase was further purified by centrifugation through glycerol gradients. This procedure removed some minor contaminants from the solubilized ATPase, but the protein concentrations were too low for accurate measurement, so the specific activity could not be determined. A silver-stained polyacrylamide gel of the gradient fractions of highest ATPase activity is shown in Fig. 2. These fractions contain the same polypeptides as the reconstituted enzyme vesicles (Fig. 1); silver-staining also reveals another polypeptide, of apparent molecular mass 39 kDa. The 16 kDa subunit (not seen in Fig. 2, which is a 10%-polyacrylamide gel) was strongly labelled by [^{14}C]DCCD (not shown); some labelling of the 72 and 57 kDa subunits also occurred, although this was not seen when unfractionated tonoplasts were treated with [^{14}C]DCCD.

Kinetic studies of the reconstituted tonoplast ATPase

The reconstituted ATPase vesicles undergo rapid, ATP-dependent acidification, as revealed by the quenching of 9-aminoacridine fluorescence (Fig. 3). This quenching was absolutely dependent on the presence of valinomycin, and was reversed by nigericin or FCCP. The initial rate of quenching of 9-aminoacridine fluorescence was directly proportional to the amount of vesicle protein in the assay, up to the maximum rate that could be measured. The dependence of this rate on the concentration of $MgATP^{2-}$ (in the presence of a fixed concentration of 1 mM free Mg^{2+}) fits a simple Michaelis function, with K_m 33 ± 1 μM (Fig. 4a). However, the kinetics of ATP hydrolysis were more complex, and showed deviation from Michaelis-Menten kinetics (Fig. 4b). The best fit of these data was obtained by assuming two independent catalytic sites, with K_m values of 776 ± 63 and 1.9 ± 1.2 μM, and V_{max} values of 3.65 and 0.27 μmol/mg per min, respectively. The large S.D. in the lower K_m value is due to the difficulty of measuring reaction rates at very low concentrations of MgATP. Previous studies of ATPase activity in fractions of the native tonoplast from *Kalanchoë daigremontiana* yielded apparent K_m values of 310 μM [31] to 810 μM [32], depending on the ionic composition of the assay medium.

The substrate specificity of the reconstituted ATPase was also investigated by measuring initial rates of

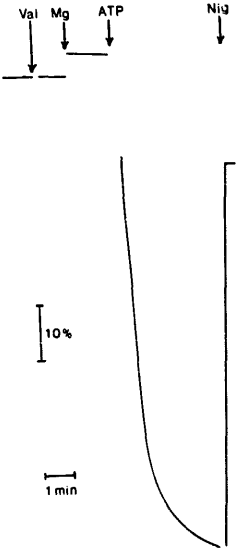

Fig. 3. Quenching of fluorescence of 9-aminoacridine by reconstituted ATPase proteoliposomes. The assay mix (0.5 ml) contained 2 μg of reconstituted ATPase protein. Additions were: Val, valinomycin (0.36 μM); Mg, $MgSO_4$ (2 mM); ATP, adenosine triphosphate (1 mM); Nig, nigericin (0.67 μM).

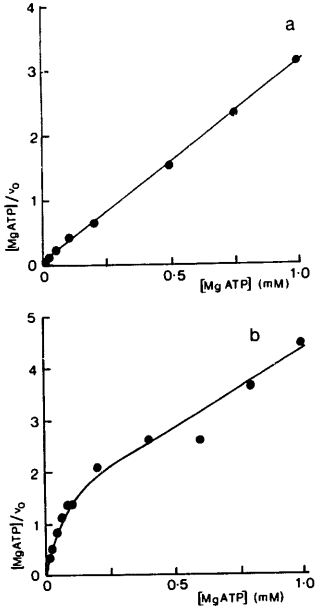

Fig. 4. Kinetics of the reconstituted ATPase. (a) Hanes plot of the dependence of the rate of H^+ translocation on the concentration of MgATP. The data points are experimental (each a mean of four observations). The line is the best fit, calculated for $K_m = 33\ \mu M$. Initial rates (v_0) are expressed as fractional fluorescence quench/min per μg protein. (b) Hanes plot of the dependence of the rate of ATP hydrolysis on the concentration of MgATP. The data points are experimental (each a mean of two observations). The line is the best fit, calculated for K_m values of 776 and 1.9 μM. Initial rates (v_0) are expressed as μmol ATP hydrolysed/min per mg protein.

H^+ translocation produced by a range of concentrations of ATP and of other nucleoside triphosphates. The kinetic parameters are shown in Table III. In terms of the relative V_{max} values obtained with the different nucleoside triphosphates (as their magnesium complexes), the relative substrate effectiveness is

dATP > ATP > ITP > GTP = UTP ≫ CTP

With CTP as substrate the activity of the ATPase was so low that the K_m value could not be measured. This result demonstrates that hydrolysis of alternative nucleoside triphosphates, which was observed previously in preparations of isolated vacuoles [31], is a genuine property of the ATPase.

Table III also shows the effect of different divalent cations on the H^+-translocating ability of the reconstituted ATPase. No H^+ translocation was measurable with Zn^{2+} as the activatory cation; investigation of the effects of Co^{2+} or Ni^{2+} was precluded by their quenching of the fluorescence of 9-aminoacridine.

Inhibition of the reconstituted ATPase by nitrate and bafilomycin

Nitrate is a characteristic inhibitor of V-type ATPases [6]. The inhibition by nitrate of ATP hydrolysis and H^+ translocation is shown in Fig. 5a. Nitrate is a noncompetitive inhibitor of H^+ translocation, the K_m for $MgATP^{2-}$ being unaltered by concentrations of NO_3 up to 50 mM (data not shown). As with native membranes from *Kalanchoë* [32], H^+ translocation is more sensitive to nitrate inhibition than is ATP hydrolysis, the inhibitor concentrations producing half-maximal inhibition of the reconstituted enzyme being 4 and 9 mM, respectively.

A more specific and potent inhibitor of V-type ATPases is bafilomycin A_1 [33], which inhibits ATP hydrolysis and H^+ translocation in parallel (Fig. 5b). The apparent I_{50} value in this experiment (0.18 ng bafilomycin/μg protein) extrapolates to saturation at approximately 0.3 mol inhibitor/mol ATPase (assuming that the enzyme is pure and has a molecular mass of about 500 kDa [18]). This suggests that bafilomycin binds with very high affinity, and effectively titrates out the enzyme, so that the I_{50} value is dependent on the protein concentration. Even at high concentrations of bafilomycin, there is a residual activity (approx. 10% of the uninhibited rate) which is resistant to this inhibitor.

TABLE III

Kinetic parameters of the reconstituted ATPase

Initial rates of H^+ translocation were determined by the quenching of 9-aminoacridine fluorescence, and fitted to the Michaelis equation. Because the rates were measured in arbitrary units, the quoted values of V_{max} are relative to those with MgATP. Each data set contained 28 rate measurements, over a 100-fold range of substrate concentration (cf. Fig. 3a). n.d., not determined.

Substrate (μM)	K_m (?)	Relative V_{max}
MgATP	33 ± 1	100
MgdATP	17 ± 1	204
MgCTP	n.d.	4
MgGTP	86 ± 4	56
MgITP	162 ± 5	70
MgUTP	263 ± 9	46
CaATP	24 ± 1	44
MnATP	10 ± 0.3	111

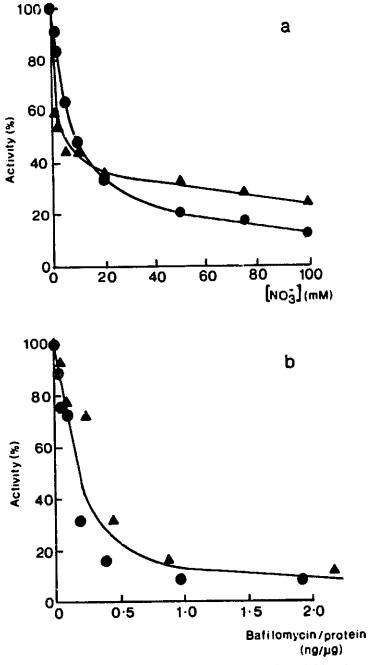

Fig. 5. Inhibition of the reconstituted ATPase. (a) Inhibition by nitrate. (b) Inhibition by bafilomycin A_1. ▲, H^+ translocation; ●, ATP hydrolysis.

However, this activity was found to be nitrate-sensitive and to display the complex kinetics of ATP hydrolysis described above.

Discussion

Although considerable progress has been made in defining the structures of V-type H^+-ATPases, rather few kinetic studies have so far been reported. The procedure for ATPase purification and reconstitution, involving the fractionation of membranes with Triton X-114, resolubilization with octyl glucoside and exchange of detergent for lipid by gel-exclusion chromatography, was originally developed for the enzyme from bovine chromaffin- granule membranes [29]. It was found to be applicable to plant tonoplast with some modifications, the most important of which was the inclusion of lipids throughout the process of resolubilization. It is likely that this procedure will be of general applicability in purifying V-type ATPases; it can be completed within 2 hours, and yields ATPase proteoliposomes of high specific activity and good stability, suitable for kinetic studies. A procedure for reconstitution of the tonoplast H^+-ATPase from mung bean has recently been published [34]. Although the specific activity of this preparation was not reported, the rate of H^+ translocation was apparently much less than in the present work (compare Fig. 3 with Ref. 34, Fig. 2); in terms of fractional fluorescence quenching, the rates are 0.37 and 0.0005 min^{-1} per μg protein, respectively.

Tonoplast preparations from the CAM plant *Kalanchoë daigremontiana*, from which purification of the vacuolar (V-type) ATPase is reported in this paper, are a particularly good source of the enzyme, as judged by their high specific ATPase activity and the relative abundance of ATPase subunits in the unfractionated membranes (Fig. 1). The purified enzyme contains polypeptides of apparent molecular mass 72, 57, 48, 42, 39, 33 and 16 kDa, and these co-sediment during glycerol-gradient centrifugation. This subunit composition is very similar to that determined by Bremberger et al. [18] for *Kalanchoë daigremontiana*, except that the present work revealed an additional polypeptide of 39 kDa. Although a large (approx. 100 kDa) glycosylated subunit is a component of most, if not all animal V-type ATPases [11–15,30] the function of such a subunit remains unknown. We found no evidence for a subunit of this size in the *Kalanchoë* enzyme, either by gel staining or by immune blotting, although small amounts of phospho*enol*pyruvate carboxylase (one of the major leaf proteins in CAM plants) co-purified with the ATPase. However, the presence of a 100 kDa subunit in vacuolar H^+-ATPase of red beet has been reported [17], so it is still unclear whether a polypeptide of 100–120 kDa is a necessary component of all V-type ATPases, or is a species- or tissue-specific subunit.

The kinetics of ATP-dependent H^+ translocation were studied by measuring the initial rates of quenching of 9-aminoacridine fluorescence, which is proportional to the net H^+-flux into the vesicles [26]. In studying the reconstituted ATPase from chromaffin granules [29], 9-amino-6-chloro-2-methoxyacridine (ACMA) was used as a probe, but in the present work 9-aminoacridine was found to be more suitable, as it was not subject to instantaneous fluorescence changes on the addition of valinomycin, Mg^{2+} or ATP (Fig. 3). The difference between the two systems is presumably due to the different lipid compositions, which were

dictated by the requirements of the enzymes (compare the data in Table II with those reported in Ref. 29).

In the absence of ADP and the presence of 1 mM free Mg^{2+}, the dependence of the rate of H^+ translocation on the concentration of $MgATP^{2-}$ was adequately described by the Michaelis equation, with K_m = 33 μM (Fig. 4a). Similar kinetics were found with other purine nucleotide triphosphates, or when Mg^{2+} was replaced with other divalent cations (Table II). The rate of ATP hydrolysis, however, showed complex kinetics, with nonlinear Hanes plots (Fig. 4b). What is the basis of this unusual substrate dependence, and why does it not apply to H^+ translocation too? In principle, plots of this type would be expected if two or more catalytic centres with different K_m values contribute to the measured rate; or alternatively if there is negative cooperativity between nucleotide-binding sites. Hanada et al. [36] reported similar kinetic behaviour in a study on the chromaffin granule membrane ATPase, and determined three separate K_m values by graphical procedures. The data shown in Fig. 4b could be satisfactorily fitted by summing two Michaelis functions, but were not better fitted by the sum of three Michaelis functions. Alternatively, the data could be fitted to the empirical Hill function

$$v_o = V_{max}/(1 + K/[S]^{n_h})$$

with $n_h = 0.55$. This low value of the Hill coefficient would suggest negative cooperativity between ATP-binding sites, as occurs in F-type ATPases [37], but the fit was significantly worse than with the two-K_m model. It seems unlikely that the reconstituted preparation is contaminated with other ATPases, as it is insensitive to efrapeptin and vanadate, inhibitors of F-type and P-type ATPases, respectively. It might contain small amounts of a modified or dissociated form of the enzyme, formed perhaps during solubilization with octyl glucoside, that is incompetent in H^+ translocation. However, it is noteworthy that kinetics of this type, as well as being a property of the reconstituted chromaffin granule enzyme [36,38], also apply to ATP hydrolysis by resealed chromaffin granule ghosts (Pérez-Castiñeira, J.R. and Apps, D.K., unpublished data), although a further complication in this system is the presence of a second membrane ATPase [39].

It is not clear why the kinetics of ATP hydrolysis and ATP-dependent H^+ translocation are different, but it is noteworthy that the two activities are differentially inhibited by nitrate, and such differential inhibition has been observed elsewhere [40].

Fractionation of the tonoplast membrane with Triton X-114 has proved to be an effective method for rapid purification and reconstitution of the V-type H^+-ATPase. Leaf mesophyll cells of *Kalanchoë daigremontiana* are a rich source of this enzyme, since the H^+-ATPase constitutes over 30% of the tonoplast protein in this CAM plant [18,41]. Further studies on the reconstituted enzyme should therefore provide an improved understanding of the molecular structure and function of this V-type ATPase.

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