Biochimica et Biophysica Acta, 771 (1984) 42–52
Elsevier

BBA 72031

LYSOPHOSPHATIDYLCHOLINE-ACTIVATED, VANADATE-INHIBITED, Mg^{2+}-ATPase FROM RADISH MICROSOMES

MARIA CECILIA COCUCCI and ERASMO MARRÈ

Centro di Studio del C.N.R. per la Biologia Cellulare e Molecolare delle Piante, Dipartimento di Biologia dell'Universitá, via Celoria 26, 20133 Milano (Italy)

(Received July 21st, 1983)

Key words: Mg^{2+}-ATPase; Proton pump; Phospholipid activation; Vanadate inhibition; (Radish microsome)

An orthovanadate-inhibited, nitrate-insensitive, phospholipid-requiring Mg^{2+}-ATPase has been partially purified (approx. 40-fold) from microsomal preparations from 24 h germinated radish seedlings. The specific activity obtained was 10–13 μmol $P_i \cdot min^{-1}$ per mg protein, namely by 4- to 10-fold higher than that reported for the known similar enzyme preparations from corn and oat roots, and by 3- to 10-fold lower than that of the extensively purified plasmalemma enzymes from *Neurospora* and yeast. The partially purified activity was fairly specific for ATP, other nucleotide triphosphates being hydrolysed at less than 10% the rate with ATP; no activity was present towards ADP, AMP, *p*-nitrophenyl phosphate and other phosphate esters. The activity was strongly dependent on the presence of phospholipids with a marked preference for lysophosphatidylcholine, and showed an absolute requirement for Mg^{2+} or some other divalent cations (CO^{2+}, Mn^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+} in order of decreasing effectiveness); Ca^{2+} could not substitute for Mg^{2+} and was strongly inhibitory in its presence. K^+, Rb^+ and Na^+ and also to a lesser extent NH_4^+ and Li^+ were significantly stimulatory, while the anions NO_3^-, $H_2PO_4^-$, Cl^- and SO_4^{2-} were ineffective. Orthovanadate, *N,N'*-dicyclohexylcarbodiimide, diethylstilbestrol, *p*-chloromercuribenzensulfonate, tetraiodofluorescein and tetrachlorotetraiodofluorescein were strongly inhibitory. The coincidence of the K_m for ATP with that for Mg^{2+} suggested that ATP-Mg is the true substrate. Accordingly, the enzyme showed a normal Michaelis-Menten kinetics for ATP-Mg with an apparent K_m of approx. 0.5 mM. The similarity of the characteristics of this enzyme with those of the plasmalemma enzymes from lower plants suggests its location at the plasma membrane, while some data 'in vivo' and in native sealed vesicle systems indicate its involvement in active proton transport.

Introduction

In the recent years the importance of active proton transport at the plasmalemma and the tonoplast of higher plants has been widely recognized. Consequently attention has concentrated on the enzymes mediating the conversion of metabolic energy into protonmotive force, and, in particular, on ATP-hydrolyzing enzymes presumably located at these membranes. The recent work on sealed membrane vesicles, on isolated vacuoles and on partially purified enzymes leads to a tenta tive identification of two main classes of microsomal Mg^{2+}-ATPases, with optimum in the 6–7 pH range, most probably involved in electrogenic

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulphonic acid); Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; DCCD, *N,N'*-dicyclohexylcarbodiimide; erythrosine B, tetraiodofluorescein; Rose bengal, tetrachlorotetraiodofluorescein.

proton transport at the plasmalemma or at the tonoplast. One class of these ATPases is characterized by its inhibition by vanadate, some activation by K^+ and other monovalent cations, a high specificity for ATP, a tight binding to lipidic components of the membrane and a marked dependence on phospholipids for maximum activity. The distribution in density gradients and the strict similarity with the plasmalemma ATPases from *Neurospora* and from yeasts [1] strongly suggests that these enzymes are located at the plasmalemma. In contrast, a second group of vanadate-insensitive, anion-sensitive proton transport ATPases, also present in 'microsomal' preparation [2,3] are similar to the ATPases present in latex luteoids [4] and in purified vacuoles [5,6] and are thus considered as belonging to the tonoplast.

Vanadate-sensitive (presumably plasmalemma-bound) ATPases have been reported as present in microsomes from maize roots [7], oat roots [8] and radish seedlings [9]. The enzymes from maize and from oat have been partially purified and characterized and ATP-dependent electrogenic H^+ transport has been demonstrated by experiments of reconstitution of proteoliposomes, in which the purified ATPase from oat roots had been incorporated [8]. Unpublished data obtained in this laboratory indicate that this is also true for the radish enzyme, which is also interesting for its strong dependence for activity on phospholipids and for its similarity to the *Neurospora* and yeast plasmalemma ATPases.

The present paper deals with the partial purification and a preliminary characterization of this enzyme.

Material and Methods

Material. Radish seeds (*Raphanus sativus* L.c.v. Tondo Rosso Quarantino, Ingegnoli, Milano, Italy) were used. Germination conditions were those used by Cocucci and Cocucci [10].

Preparation of the crude membrane fraction. The 24 h germinated seeds (300 g fresh weight) were homogenized at 4°C in a mortar with 1200 ml of 0.1 M Tris-HCl (pH 8)/0.3 M sucrose/0.1 mM $MgCl_2$/1 mM EDTA/5 mM mercaptoethanol. The membrane fraction sedimenting between 13000 and 80000 × *g* was washed twice with 120 ml of 1 mM Tris-HCl (pH 7)/0.25 M sucrose/0.1 mM $MgCl_2$ (centrifugation for 30 min at 105000 × *g*) as described by Cocucci and Ballarin-Denti [9]. The 'washed membrane' fraction (130–150 μg of protein/g fresh weight) was resuspended in 0.5 M Tris-SO_4 (pH 7.5)/0.25 M Na_2SO_4/5 mM mercaptoethanol at a concentration of 5 mg protein/ml and frozen overnight at −70°C.

ATPase assay. The standard incubation mixture contained an appropriate amount of the microsomal suspension or of the membrane fractions (corresponding to different amounts of fresh weight ranging from 0.250 to 2 g), 3 mM $MgCl_2$, 25 mM KCl, 3 mM K-ATP, 10 mM Pipes-Tris (pH 6.4) in 0.5 ml final volume. The assay of ATPase in the presence of phospholipid (usually lysophosphatidylcholine) was performed by adding 50 μg of lipid as liposomes 10 min before the addition of ATP. Liposomes were prepared, as described by Cocucci and Ballarin-Denti [9], in 2 mM Hepes-histidine/0.1 mM EDTA (pH 6.7) to give a final phospholipid concentration of 1 mg/ml. The reaction was started by adding the substrate and allowed to proceed at 30°C for 15 to 20 min. The liberated P_i was assayed by stopping the reaction with 1 ml of a freshly prepared solution containing 0.375 M H_2SO_4/0.75% $(NH_4)_2MoO_4$/3% $FeSO_4 \cdot 7H_2O$/0.75% SDS. The samples were read at 740 nm 10 min after reagent addition; no hydrolysis of the phospholipid was observed during the assay and the presence of the lipid did not interfere with the colorimetric assay of phosphate.

Except where expressly specified, the optimal (or close to optimal) conditions of the ATPase activity assay adopted for its characterization were: Mg^{2+}, 3 mM; ATP, 3 mM; Cl^-, 28 mM; K^+, 25 mM; buffer Pipes-Tris 10 mM (pH 6.4); lysophosphatidylcholine 100 μg/ml; temperature, 30°C. The effects of changes in activity induced by changes in each of these parameters, as well by some other activating or inhibiting factors are described in Results.

In the experiments in which the effect of various concentrations of $H_2PO_4^-$ on ATPase activity was tested, $^{32}P_i$ and [γ-^{32}P]ATP were separated according to Lindberg and Ernster [11]; radioactivity was measured (aliquots 0.5 ml) in 10 ml Instagel by liquid scintillator Packard Tri-Carb.

All data presented are the average of three or

more experiments performed in duplicate or triplicate.

Detection of reversibly phosphorylated proteins. The assay was carried out at 10°C using the partially purified ATPase immediately after the solubilization with lysophosphatidylcholine; the reaction mixture contained in a final volume of 1 ml 500 μM [γ-^{32}P]ATP (350–400 mCi/mmol, about 600 cpm/pmol), 1 mM $MgSO_4$, 5 mg lysophosphatidylcholine, 0.25 M sucrose, 0.5 mM mercaptoethanol, 170–190 μg protein in 10 mM Pipes-Tris (pH 6.4). The reaction was started by the addition of ATP; rapid mixing was provided by a magnetic stir bar. After incubation for 1–2 min, [γ-^{32}P]ATP was isotopically diluted by adding excess of unlabeled ATP and Mg^{2+} (final concentration 5 mM). At appropriate times the reaction was stopped by adding samples of 0.2 to 3 ml of an ice-cold 0.3 M $HClO_4$, 5 mM NaH_2PO_4. After centrifugation for 15 min at 13000 × *g* (4°C), the pellet was resuspended once more in 3 ml of the same solution and centrifuged as before, the resulting pellet was suspended in 3 ml of unlabeled 1 mM ATP and centrifuged again. Aliquots of the $HClO_4$ pellet were transferred to scintillation vials and the radioactivity determined with a Packard Scintillation Counter in 10 ml Instagel. The efficiency of counting was about 70%; controls for unwashed radioactivity were performed by adding the quenching solution before [γ-^{32}P]ATP. Phosphorylated samples for SDS-gel electrophoresis were prepared by successive centrifugations as described above.

SDS-polyacrylamide gel electrophoresis. The pH 2.4, SDS-polyacrylamide gel system of Fairbank and Avruk as modified by Dame and Scarborough [12] was used except that a two-phase gel (4%–5.6% acrylamide) was performed in tubes (gel size: 5 × 125 mm). Samples of the phosphorylated enzyme were prepared by dissolving for 15 min at 30°C the perchloric acid-precipitated proteins in 60 μl of a medium containing 50 mM H_3PO_4 (pH 2.4 with NaOH), 1% SDS, 2% mercaptoethanol, 0.25 M sucrose, 4 M urea and 20 μg/ml pyronin Y; aliquots of 40–50 μl, corresponding to 20–30 μg protein were submitted to electrophoresis. Electrophoresis was performed at 20°C at constant current (8 mA/tube) for 3 h in a Bio-Rad apparatus.

Gel analysis. After electrophoresis, the gels were either fixed overnight at room temperature in trichloroacetic acid/methanol/water (10 : 33 : 57, by vol.) and then stained in acetic acid/methanol/water (10 : 25 : 65, by vol.) containing 0.1% Coomassie blue R 250 or frozen overnight at −70°C. The stained gels were destained in trichloroacetic acid/methanol/water (10 : 25 : 65, by vol.) with a Bio-Rad Diffusion Destainer and scanned at 595 nm in a Gilford spectrophotometer equipped with a linear scanner accessory. Proteins of known molecular weight (LMW protein standards Bio-Rad) were solubilized and stained under the identical conditions used for the phosphorylated samples. A linear relationship was found when the molecular weight logarithm was plotted versus the relative mobility for the standards tested, except at low molecular weights, as shown in Fig. 6. The frozen gels were cut into 2 mm thick sections with Bio-Rad Gel Slicer; the slices were treated overnight at 37°C with 0.5 ml Protosol/water (9 : 1, v/v) and counted in 10 ml of toluene-based scintillant [13].

Protein assay. Protein concentration during the preparation of ATPase was measured by the Coomassie blue dye-binding procedure [14]. The protein determination for the lysophosphatidylcholine extract and the more purified fractions were performed in the 1 to 50 μg range according to Bensadoun and Weinstein [15] after removal of lipid from the proteins precipitated with sodium deoxycholate-trichloroacetic acid by successive washing with 0.1 M potassium acetate in ethanol, ethanol, ether as suggested by Lowry et al. [16]; the recovery (i.e. the sum of supernatant and pellet) varied from 92% to 105%. Bovine serum albumin was used as standard.

Chemicals. Nucleotides were obtained from Boehringer Biochemia Robin; ATP was converted to its Tris salt by passing Na-ATP through Dowex 50 cationic exchanger resin than neutralizing with Tris; *p*-nitrophenyl phosphate and DCCD were purchased from BDH, diethylstilbestrol from Merck, erythrosin B, Rose bengal and egg lysophosphatidylcholine from Sigma and other phospholipids from Supelco; [γ-^{32}P]ATP was bought from The Radiochemical Centre, Amersham.

Results

Partial purification of the DCCD- and vanadate-sensitive ATPase activity

Just as with the plasmamembrane ATPases from *Neurospora* and yeasts the main obstacle met in the purification of the DCCD-, vanadate-sensitive ATPase of radish 'microsomes' is the difficulty of obtaining it in a soluble state. Washing the microsomes with 1% cholate had been reported to efficiently remove the contaminating phosphatases, leaving in the sediment a phospholipid-dependent, vanadate- and DCCD-inhibited ATPase [9]. Our attempts to solubilize this 'insoluble' activity, using different detergents, ionic strength, pH values, were unsuccessful. Instead, we obtained a partial (approx. 50%) solubilization of the phospholipid-dependent, DCCD-sensitive ATPase (still contaminated by *p*-nitrophenylphosphatase) by extracting the microsomes ('washed membrane preparation', see Methods) with 0.35% cholate under strictly controlled conditions. The soluble preparation thus obtained could then be further purified by $(NH_4)_2SO_4$ fractionation, followed by treatment with EDTA and with cholate, which finally led to a highly active enzyme preparation sedimentable by high speed centrifugation. This could be re-solubilized by treatment with lysophosphatidylcholine; the 'soluble' preparation thus obtained (referred to in this paper as 'partially purified enzyme') is that utilized for characterization. The purification procedure adopted after a number of trials is described in detail hereafter.

The frozen 'washed membrane' preparation (see Methods) was thawed at room temperature and a solution of 10% potassium cholate, pH 7.5, was slowly added with stirring at 4°C to give a final concentration of 0.35% cholate (0.7 : 1, cholate/protein ratio). After 15 min the suspension was centrifuged at $105\,000 \times g$ for 45 min and the pellet was discarded. A saturated ammonium sulphate solution was added to the supernatant (4°C, with stirring) to reach 26% saturation. After 20 min the mixture was centrifuged at $105\,000 \times g$ for 30 min. The ammonium sulphate pellet was washed with 50 mM Tris-SO_4, 1 mM Na-EDTA, 0.5 mM mercaptoethanol (pH 7.5) at approx. 3 mg protein/ml. After centrifugation for 30 min at $105\,000 \times g$, the supernatant was discarded and the washing was repeated once. The resulting pellet was resuspended at 2.5 mg protein/ml in 0.375 M Tris-SO_4/ 0.187 M Na_2SO_4/ 0.5 mM

TABLE I

PARTIAL PURIFICATION OF A LIPID-ACTIVATED, DCCD-SENSITIVE, MICROSOMAL Mg^{2+}-ATPase

Data from a typical experiment starting with 100 g fresh weight. The assay mixture contained in 0.5 ml: 25 mM KCl, 3 mM $MgCl_2$, 10 mM Pipes-Tris (pH 6.4). The P_i-hydrolizing activities present in each fraction were assayed using as substrate: (a) 3 mM *p*-nitrophenylphosphate in absence of lysophosphatidylcholine (which does not influence this activity); (b) 3 mM ATP either in absence or in presence of lysophosphatidylcholine (lysoPC) (100 μg/ml) with or without 0.3 mM DCCD. DCCD was added as ethanolic solution (1% final concentration). Incubation was carried out for 20 min at 30°C. pNPPase, *p*-nitrophenylphosphatase.

Fraction	Protein (mg)	pNPPase activity (μmol $P_i \cdot min^{-1}$) (a)	ATPase activity			
			Total (μmol $P_i \cdot min^{-1}$) (b)			Specific (μmol $P_i \cdot min^{-1} \cdot (mg\ protein)^{-1}$) +lysoPC
			−lysoPC	+lysoPC	+lysoPC +DCCD	
A: crude microsomes	14.7	2.16	3.84	5.04	2.04	0.34
B: cholate extract	12.1	2.40	3.48	4.20	2.04	0.34
C: 0–26% satn. $(NH_4)_2SO_4$ precipitate	3.6	0.21	0.72	0.96	0.36	0.26
D: residue from C after washing with EDTA	1.8	0	0.40	1.32	0.48	0.83
E: residue from D after washing with cholate	0.63	0	0.09	1.55	0.08	2.46
F: lyso-PC extract from E	0.16	0	–	1.92	0.07	12

mercaptoethanol (pH 7.5) and concentrated potassium cholate was added to a final concentration of 7 mg/ml (2.8 : 1, cholate/protein ratio). After centrifugation for 30 min at 105 000 × *g*, the supernatant was discarded and the pellet was resuspended in the same buffer without potassium cholate (approx. 1 mg protein/ml). After centrifugation for 30 min at 160 000 × *g*, the pellet was suspended in 10 mM Pipes-Tris, 0.5 mM mercaptoethanol (pH 6.4) at a concentration of about 2 mg protein/ml and stored at −70°C. In this condition the preparation was stable for at least two months. The next step of purification involved the solubilization with lysophosphatidylcholine: the thawed suspension was treated with a solution of sonicated egg lysophosphatidylcholine (8 mg/ml), supplemented after sonication with sucrose and mercaptoethanol; the resulting mixture contained 500 μg protein/ml with lysophosphatidylcholine at a ratio of 10 mg/mg of protein in 10 mM Pipes-Tris/0.25 M sucrose/0.5 mM mercaptoethanol (pH 6.4); incubation was carried out for 15 min at 20°C, then the mixture was centrifuged at 105 000 × *g* for 30 min at 15°C. The supernatant is referred to hereafter as the partially purified ATPase.

Some observations can be made concerning this procedure. When the microsomes were treated with cholate, a progressive activation of DCCD-sensitive ATPases (measured as the sum of the soluble and pelletable activity) took place, while higher concentrations of the detergent (above 1.7%) led to progressive inactivation of the ATPase (data not shown). The cholate concentration adopted is that giving the highest ATPase solubilization together with the highest activation of the total DCCD-sensitive ATPase. In this condition, the DCCD-sensitive ATPase detectable in the cholate-soluble extract accounted for about 50% of the total DCCD-sensitive ATPase. In the following steps, the fractionation of the cholate extract with ammonium sulphate and the washing with Tris-EDTA completely removed the *p*-nitrophenylphosphatase activity, together with a large amount of inactive protein. The treatment with cholate of the $(NH_4)_2SO_4$ pellet was useful in removing most of residual lipids and in increasing the specific activity. The concentration of cholate in this step was again critical, due to the inactivating effect of high concentrations of the detergent on the ATPase activity. The solubilization of ATPase with lysophosphatidylcholine was concentration-dependent, as found by Addison and Scarborough [17] for *Neurospora crassa* and by Dufour and Goffeau [18] for *Schizosaccharomyces pombe*; in our conditions 80% of the ATPase activity is solubilized at a lysophosphatidylcholine/protein ratio of 10 : 1, whereas at detergent/protein ratio 2.5 : 1 only 40% of the ATPase is solubilized. The ATPase thus solubilized was unstable and lost about 70–80% of its activity within 5–6 h; however, when ATP was added at a final concentration of 1 mM, the ATPase activity decreased only by 15%, and was stable for at least a week at −70°C.

The progress in purification of the enzyme by means of the adopted procedure is summarized in Table I, showing that: (a) the *p*-nitrophenylphosphate activity initially present is completely eliminated during purification together with the activity towards various other phosphate esters (including ADP, AMP, glucose 6-phosphate, glycerophosphate, data not shown); (b) the specific activity of the purified preparation is 40-fold higher than that of the initial microsomal pellet; (c) the sensitivity to DCCD as an inhibitor increases during purification from 59% to 96% (with 0.3 mM DCCD) (a parallel behaviour is shown by the sensitivity to vanadate, data not shown); (d) the phospholipid-dependence rises from a relatively modest to a very high value.

Characterization of the partially purified enzyme activity

(1) Polar lipid requirement. Previous work by Cocucci and Ballarin-Denti [9] had shown that in radish microsomal preparations the removal of phospholipids by cholate almost completely inactivates the DCCD-sensitive Mg^{2+}-ATPase activity, this effect being efficiently reversed by the addition of polar lipids extracted from germinating seeds. A marked stimulation of the ATPase activity by phospholipids was observed at all steps of the purification procedure here adopted. As shown by Table II the very low activity of the partially purified enzyme (corresponding to step 'E' of Table I) preparation was strongly increased by the addition of a mixture of soybean phospholipids, as well as by some, although not all, individual phospholipids.

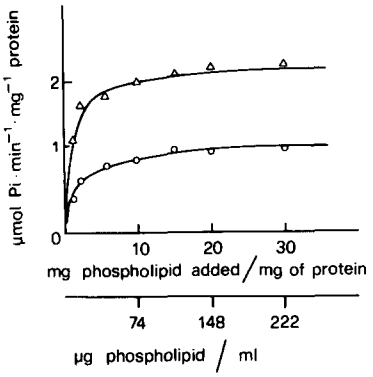

Fig. 1. Effects of soybean phospholipids and of lysophosphatidylcholine. Experimental conditions as in Table II. △, lysophosphatidylcholine; ○, soybean phospholipids.

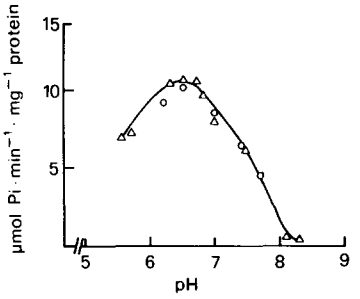

Fig. 2. Effects of pH. ATPase activity was tested in the standard assay conditions with 10 mM either Pipes (△) or Hepes (○), final pH having been adjusted with Tris or KOH at the indicated values.

Interesting differences appeared in the activating effect of the varius phospholipids tested: lysophosphatidylcholine was by far the most active, followed by phosphatidylinositol. Phosphatidylglycerol and phosphatidylserine had little effect, while phosphatidylethanolamine and phosphatidylcholine had no effect.

The dose-effect curves of Fig.1 show that for both lysophosphatidylcholine and the soybean phospholipids the activation effect was saturated by a phospholipid/protein ratio of approx. 30 : 1 (w/w), half-maximum activation being observed at about 1 : 1 (lysophosphatidylcholine) and about 2 : 1 (soybean phospholipids) ratio.

(2) pH optimum. The curve of ATPase activity as a function of pH with either Hepes or Pipes as buffers showed an optimum at 6.5 ± 0.15, with a rather sharp decrease for pH values higher than 6.8 (Fig. 2).

(3) Temperature. In the assay conditions the enzyme appeared fairly stable (hours) for temperatures up to 40°C. A 10 degree C increase in

TABLE II

EFFICIENCY OF VARIOUS PHOSPHOLIPIDS IN RESTORING THE ATPase ACTIVITY

The incubation mixture contained in 0.5 ml: 25 mM KCl, 3 mM $MgCl_2$, 10 mM Pipes-Tris (pH 6.4), 50 µg of the indicated lipid and ATPase preparation (3.7 µg protein) corresponding to step E of Table 1. Lipids were added as dispersions (see Material and Methods) 10 min before the reaction was started with 3 mM Tris-ATP. Incubation was carried out for 30 min at 30°C.

Addition	ATPase activity (μmol $P_i \cdot min^{-1} \cdot (mg\ protein)^{-1}$)	Efficiency (%)
None	0.25	–
Soybean phospholipids	1.24	396
Lysophosphatidylcholine	2.08	732
Phosphatidylserine	0.39	56
Phosphatidylinositol	0.68	172
Phosphatidylethanolamine	0.28	12
Phosphatidylcholine	0.22	−12
Phosphatidylglycerol	0.43	72

TABLE III

SUBSTRATE SPECIFICITY

The ATPase activity of the partially purified enzyme (1.6 µg) was assayed in the standard conditions described under Material and Methods: the assay mixture contained 25 mM KCl, 3 mM $MgCl_2$, 10 mM Pipes-Tris (pH 6.4), 100 µg/ml lysophosphatidylcholine and 3 mM substrates (potassium salts). Activity on CTP, GTP, UTP, ITP was tested using 5.1 µg of protein.

Substrate	ATPase activity (μmol $P_i \cdot min^{-1} \cdot (mg\ protein)^{-1}$)	Relative activity (%)
ATP	12.3	100
ADP	0	0
AMP	0	0
CTP	0.87	7
GTP	0.94	7
UTP	0.68	5
ITP	1.21	9
Glucose 6-phosphate	0	0
p-Nitrophenyl phosphate	0	0

TABLE IV

EFFECT OF MONOVALENT CATIONS

The incubation mixture contained in 0.5 ml: 3 mM Tris-ATP, 3 mM $MgCl_2$, 10 mM Pipes-Tris (pH 6.4), and 100 μg/ml lysophosphatidylcholine, 1,6 mg of purified enzyme and chloride salts of the indicate monovalent cations.

Addition	ATPase activity (μmol $P_i \cdot min^{-1} \cdot (mg\ protein)^{-1}$)	Stimulation (%)
None	8.7	–
K 3 mM	11.2	28
K 25 mM	13.3	52
K 50 mM	13.6	56
Rb 50 mM	13.6	56
Na 50 mM	13.5	55
NH_4 50 mM	12.4	42
Li 50 mM	10.8	24

temperature enhanced the activity by a factor of 1.97 in the 17–27°C and of 1.77 in the 27–37°C intervals.

(4) Substrate specificity. The data of Table III show that the purified preparation was highly specific for ATP. Among other nucleotides tested, ITP, CTP, GTP and UTP were attacked at rates lower than one tenth that for ATP. No activity was observed towards ADP, AMP, glucose-6-phosphate and *p*-nitrophenyl phosphate.

(5) Monovalent cations. K^+, Rb^+ and Na^+ and also, in decreasing order of effectiveness, NH_4^+ and Li^+ significantly stimulated the partially purified ATPase activity (Table IV). For K^+, maximum stimulation and half-maximum stimulation corresponded to approximately the 25 mM and the 3 mM cation concentrations. It has to be

TABLE V

DIVALENT CATION REQUIREMENT

The incubation mixture in 0.5 ml: 3 mM Tris-ATP, 3 mM of indicated cations as chloride salt, 100 μg/ml lysophosphatidylcholine, 10 mM Pipes-Tris (pH 6.4) and 1.6 μg of purified enzyme.

Addition	ATPase activity (μmol $P_i \cdot min^{-1} \cdot (mg\ protein)^{-1}$)	Relative activity (%)
None	0	0
Mg	12.7	100
Mg + 0.5 mM EGTA	14.2	112
Mn	14.1	111
Co	17.9	140
Zn	10.6	83
Ni	4.4	34
Ca	0	0

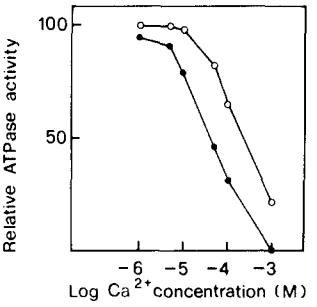

Fig. 3. Effect of Ca^{2+} in the presence of Mg^{2+}. The ATPase activity of the purified enzyme (1.6 μg) was assayed in 0.5 ml of incubation mixture containing: 25 mM KCl, 100 μg/ml lysophosphatidylcholine, 10 mM Pipes-Tris (pH 6.4) and $CaCl_2$ at the indicated concentration. The reaction was started with either 1 or 3 mM equimolar ATP-Mg. Results are expressed as per cent of the controls without Ca^{2+}: 12,4 μmol $P_i \cdot min^{-1} \cdot (mg\ protein)^{-1}$ with 3 mM ATP, 8.2 μmol $P_i \cdot min^{-1} \cdot (mg\ protein)^{-1}$ with 1 mM ATP. ●, 1 mM ATP-Mg; ○, 3 mM ATP-Mg.

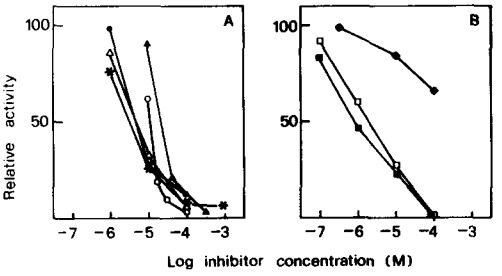

Fig. 4. Effects of inhibitors. The incubation mixture contained in 0.5 ml: 3 mM $MgCl_2$, 25 mM KCl, 3 mM K-ATP, 100 μg/ml lysophosphatidylcholine and the partially purified enzyme (1.6 μg of protein). DCCD, diethylstilbestrol, erythrosin B, Rose bengal, fluorescein, were added as ethanolic solution to yield 1% final ethanolic concentration; no significative inhibition was observed by 1% ethanol alone. All the inhibitors were added at the indicated concentration before the addition of enzyme and substrate. Incubations were carried out for 15 min at 30°C. Results are expressed as percent of the controls without the inhibitors (12.7 μmol $P_i \cdot min^{-1} \cdot (mg\ protein)^{-1}$. (A) ●, *p*-Chloromercuribenzensulfonate; *, $CuSO_4$; △, DCCD; ○, orthovanadate; ▲, diethylstilbestrol. (B) ■, erythrosine; □, Rose bengal; ◆, fluorescein.

observed that these effects of the monovalent cations were measured in the presence of 13 mM Tris, reported to somewhat stimulate the ATPase activity of other plant membrane preparations [19].

(6) Divalent cations. The activity of the partially purified preparation showed an absolute requirement for Mg^{2+} or some other divalent cations: in decreasing order of effectiveness $Co^{2+} > Mn^{2+} \geqslant Mg^{2+} > Zn^{2+} > Ni^{2+}$ (Table V). (More information on the effects of Mg^{2+} is presented below in (9) kinetics).

Ca^{2+} was completely inactive, when supplied in the absence of other divalent cations, and strongly inhibitory, when present at concentrations higher than 10 μM together with Mg^{2+}-ATP either 1 or 3 mM (Fig. 3). Moreover in the presence of 3 mM Mg^{2+} the addition of the Ca^{2+} chelating agent, EGTA, either left unchanged or slightly stimulated the ATPase activity (Table V). These results rule out the possibility of a contamination of our preparation by a (Ca^{2+} + Mg^{2+})-ATPase.

(7) Anions. Effects of anions on the ATPase activities of plant membrane preparations have been reported (in particular, activation by Cl^- and inhibition by NO_3^- [20,21]). In our partially purified preparation the substitution of Cl^- in the standard ATPase assay with SO_4^{2-}, NO_3^- (up to 50 mM) or H_2PO_{-4} (up to 10 mM) did not induce significant changes in activity (Table VI).

TABLE VI

EFFECT OF ANIONS

The standard medium contained 25 mM KCl, 3 mM Mg-ATP, 100 μg lysophosphatidylcholine/ml, 10 mM Pipes-Tris (pH 6.4 and 1.6 μg enzyme. In (b) KCl was increased to 50 mM, in (c) KCl was replaced by 50 mM KNO_3, in (d) by 25 mM K_2SO_4, in (e) the standard medium was supplemented with 10 mM KH_2PO_4. [γ-^{32}P]ATP (15 μmol/μCi) was added and the radioactivity in P_i and ATP was measured as indicated in Methods (appropriate control not shown).

Medium	ATPase activity (μmol $P_i \cdot min^{-1}$ $\cdot$(mg protein)$^{-1}$)	Relative activity (%)
(a) standard	11.2	100
(b) Cl^- (×2)	11.7	105
(c) NO_3^-	12.7	113
(d) SO_4^{2-}	13.4	119
(e) $Cl^- + H_2PO_4^-$	10.3	92

(8) Inhibitors. The data of Fig. 4A show that the ATPase activity of the partially purified preparation was strongly inhibited by DCCD, diethylstilbestrol, orthovanadate, Cu^{2+} and *p*-chloromercuribenzensulfonate.

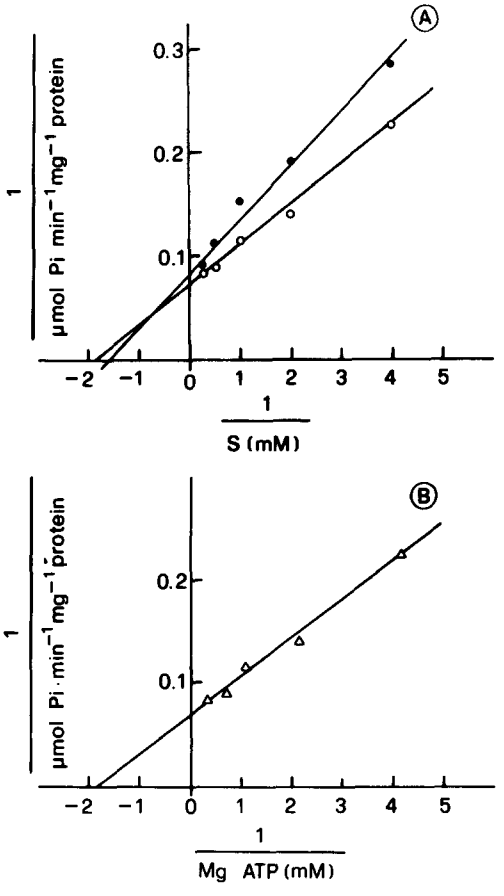

Fig. 5. Kinetic parameters of ATPase activity estimated by Lineweaver-Burk plot; influence of ATP and $MgCl_2$ concentrations. The activity of the partially purified enzyme (1.6 μg of protein) was assayed in: 10 mM Pipes-Tris (pH 6.4), 25 mM KCl, 100 μg/ml lysophosphatidylcholine with 4 mM $MgCl_2$ and concentrations of ATP varying from 0.250 to 4 mM (O——O), or in the same medium with 4 mM ATP and concentrations of $MgCl_2$ varying from 0.250 to 4 mM (●——●). Incubation was carried out for 7 min to keep ATP hydrolysis below 20% (A) Lineweaver-Burk plot of ATPase activity as a function of ATP or Mg^{2+} varying concentrations. (B) Lineweaver-Burk plot of ATPase activity as a function of Mg-ATP concentration. Mg-ATP was calculated using the equation of Wolf and Adolph [24] with $K_{Mg\text{-}ATP} = 0.215$ mM. Kinetic constants, calculated by linear regression analysis and expressed as millimolar concentrations were for ATP: K_m 0.52, V_{max} 13.8; for Mg: K_m 0.56, V_{max} 11.23; for Mg-ATP: K_m 0.53, V_{max} 14.5.

Fig. 4B shows the effects of a class of interesting inhibitors of some animal transport ATPases, erythrosin B and Rose bengal. The two compounds appeared markedly active (I_{50} at approx. 1 μM) in inhibiting our ATPase; while a much smaller effect was observed for fluorescein (which is also ineffective on rabbit muscle sarcoplasmic ATPase) [22].

No effects were observed for the mitochondrial ATPase inhibitors oligomycin and azide (up to 1 mM), and for the sulfhydryl reagent ethylmaleimide. Also AMP and ADP (at concentrations up to 2 mM) did not significantly influence the ATPase activity, when added in the presence of Mg-ATP concentrations ranging from 0.4 to 3 mM (data now shown).

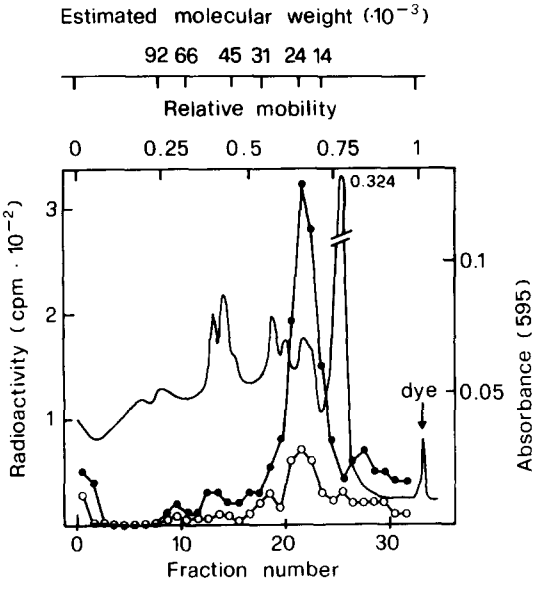

Fig. 6. Electrophoretic profile of the ^{32}P labeling of the partially purified ATPase preparation. 180 μg of the purified enzyme were incubated for 2 min at 10°C with 0.5 mM [γ-^{32}P]ATP in Pipes-Tris 10 mM, 0.25 M sucrose, 1 mM $MgCl_2$, 0.5 mM mercaptoethanol in presence of lysophosphatidylcholine (5 mg/ml). Isotopic dilution of [γ-^{32}P]ATP chase was performed by adding ATP and Mg at 5 mM final concentration and the reaction was stopped 2 min later. Samples were precipitated with perchloric acid and 30 μg of protein were processed as described in Methods. ————, protein as stained with Coomassie blue and scanned at 595 nm. ●————●, ^{32}P radioactivity after 2 min of incubation of the enzyme with [γ-^{32}P]ATP. ○————○, ^{32}P radioactivity after chase with unlabeled 5 mM ATP and 5 mM Mg^{2+} (see Methods for detection of radioactivity). Molecular weights were estimated from a plot of log mol.wt. versus relative mobility using as protein standards (from Bio-Rad): phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme.

(9) Kinetic properties and mechanism. The results of experiments in which the concentrations of either Mg^{2+} or ATP were changed between 0.25 and 4 mM are summarized by the Lineweaver-Burk plots of Fig. 5A, indicating very close values of the apparent K_m for ATP and for Mg^{2+}. This may be taken as an indication that Mg-ATP is the true substrate [23]. In fact the data of Fig. 5 B show Michaelis-Menten kinetics for Mg-ATP, with a K_m of approx. 0.53 mM.

A mechanism of action involving the formation of a phosphorylated intermediate was suggested by the strong inhibition by vanadate [1,25]. In agreement with this view are the results of some experiments, in which the enzyme was incubated for a short time with [γ-^{32}P]ATP, followed or not by a chase with an excess of unlabeled ATP, and peptides in the preparation were then analyzed by SDS-electrophoresis. In fact, the protein-radioactivity profiles of Fig. 6 show the formation of a rapidly turning over phosphoprotein. More detailed kinetic studies are required, however, to decide whether it really corresponds to a phosphorylated enzyme intermediate.

Discussion and Conclusions

Purity and characteristics

The finding that our final preparation still shows several protein bands when analyzed by SDS electrophoresis indicates that it is still rather impure. On the other hand its specific activity is the highest yet described for the similar vanadate-sensitive plant enzymes (13 μmoles · min^{-1} per mg protein against 2.5 μmoles · min^{-1} of the enzyme from oat roots and 1.5 μmol · min^{-1} for that from maize; to be compared with the 97 μmol · min^{-1} for the plasmalemma enzyme from *Neurospora*, the 35 μmol · min^{-1} for that from *S. pombe*, and the 21 μmol · min^{-1} for that from *S. cerevisiae*). This, together with the high specificity for ATP, and the general pattern of the responses to activators and inhibitors suggests that the ATPase activity present in our preparation belongs to a single enzyme.

The main characteristics of the enzyme investigated here are: (a) a high specificity for Mg-ATP

(Mg^{2+} can be substituted by Co^{2+} and Mn^{2+} but not by Ca^{2+}, which results in strong inhibition; (b) a marked dependence of the reactivation of the detergent de-lipidated enzyme on phospholipid, and in particular on lysophosphatidylcholine; (c) a pH optimum at 6.6; (d) some activation by monovalent cations, particularly K^+ and Na^+, in contrast with the lack of sensitivity to nitrate or other anions; (e) a very high sensitivity to DCCD and vanadate and also to the iodinated derivatives of fluorescein such as erythrosin B and Rose bengal. These characteristics are so similar to those of the plasmamembrane enzymes of *Neurospora crassa, Saccaromyces cerevisiae* and *Saccaromyces pombe* to legitimate its attribution to the same class of enzymes. On the other hand, most of the above mentioned characteristics also correspond to those of the two other vanadate-sensitive membrane ATPases as yet partially purified from non-mitochondrial, non-plastidial membrane preparations from higher plants, namely the oat and the corn ATPases described by Vara and Serrano [8] and by Dupont et al. [7], while they are clearly different from those of the vanadate-insensitive plant membrane ATPases. It thus seems legitimate to propose that the vanadate-sensitive radish enzyme dealt with here, as well as those described for oats and for maize, belongs to the same membrane ATPase type or class as the more thoroughly investigated *Neurospora* and yeast vanadate-sensitive ATPases.

Intracellular localization and function

At least three considerations strongly suggest that the vanadate-sensitive radish ATPase is located at the plasmalemma and catalyses the utilization of ATP hydrolysis energy for active proton transport: (a) the similarity of characteristics with the vanadate-sensitive fungal and yeast enzymes, which almost certainly represent the ATPase component of the plasmalemma proton pump; (b) the results of 'in vivo' experiments, showing that in various materials, among which the intact germinating radish seeds, vanadate markedly inhibits electrogenic proton extrusion [26]; (c) the recent findings of an ATP-dependent, vanadate-sensitive activity of electrogenic proton transport in the sealed vesicles of membrane preparations from radish seedlings recovered in a high-density region ($d = 1.16–1.17$) of the sucrose gradient [3].

In conclusion, the results of this investigation integrate and extend the previous reports concerning the enzymes from the corn and oat root membrane preparation, inasmuch as they confirm the presence in higher plants of a class of vanadate-sensitive, low pH-optimum membrane ATPases similar to those extensively studied in *Neurospora* and yeasts, and bring a further support to the hypothesis of a rôle of these enzymes in the plasmalemma proton pump.

Acknowledgements

We are indebted to Mr. Sergio Dalla Rosa for precious and clever technical collaboration.

References

1 Goffeau, A. and Slayman, C.W. (1981) Biochim. Biophys. Acta 639, 197–233
2 Churchill, K.A. and Sze, H. (1983) Plant Physiol. 71, 610–617
3 De Michelis, M.I., Pugliarello, M.C. and Rasi-Caldogno, F. (1983) FEBS Lett. 162, 85–90
4 D'Auzac, J. (1977) Phytochemistry 16, 1881–1885
5 Wagner, G.J. and Lin, W. (1982) Biochim. Biophys. Acta 689, 261–266
6 Bowman, E.J. and Bowman, B.J. (1982) J. Bacteriol. 151, 1326–1337
7 Du Pont, F.M., Burke, L.I. and Spanswick, R.M. (1981) Plant Physiol. 67, 59–63
8 Vara, F. and Serrano, R. (1982) J. Biol. Chem. 257, 12826–12830
9 Cocucci, M. and Ballarin-Denti, A. (1981) Plant Physiol. 68, 377–381
10 Cocucci, S. and Cocucci, M. (1977) Plant Sci. Lett. 10, 85–95
11 Lindberg, O. and Ernster, L. (1956) in Methods of Biochemical Analysis (Glick D., ed.), Vol. 3, pp. 1–22, Interscience, New York
12 Dame, J.B. and Scarborough, G.A. (1980) Biochemistry 19, 2931–2937
13 Floyd, R.W., Stone, M.P. and Joklik, W.K. (1974) Anal. Biochem. 59, 599–609
14 Bradford, M. (1976) Anal. Biochem. 72, 248–254
15 Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Chem. 193, 265–275
17 Addison, R. and Scarborough, G.A. (1981) J. Biol. Chem. 256, 13165–13171
18 Dufour, J.P. and Goffeau, A. (1978) 253, 7026–7032
19 Beffagna, N., Marré, E. and Cocucci, S. (1979) Planta 146, 387–391

20 Du Pont, F., Giorgi, D.L. and Spanswick, R.M. (1982) Plant Physiol. 70, 1694–1699
21 Stout, R.G. and Cleland, R.E. (1982) Plant Physiol. 69, 798–803
22 Morris, S.J., Silbergeld, E.K., Brownd, R.S. and Haynes, D.H. (1982) Biochem. Biophys. Res. Commun. 104, 136–1311
23 Dixon, M. and Webb, E.C. (eds.) (1964) Enzymes, pp. 421–443, Longmans, Green, London
24 Wolf, H.R. and Adolph, L. (1969) Eur. J. Biochem. 8, 68–74
25 Briskin, D.P. and Poole, R.J. (1983) Plant Physiol. 71, 507–512
26 Cocucci, M., Ballarin-Denti, A. and Marré, M.T. (1980) Plant Sci. Lett. 17, 391–400