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PURIFICATION AND CHARACTERIZATION OF A DIVALENT CATION-ACTIVATED ATP-ADPase FROM PEA STEM MICROSOMES *

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Summary

The microsomal fraction (13 000—60 000 × *g* pellet) from pea stem shows a very high divalent cation-dependent, diethylstilbestrol- and orthovanadate-inhibited ATPase and ADPase activity. No detectable inorganic or organic pyrophosphatase and adenylate kinase and almost negligible activities of mitochondrial ATPase and of other phosphomonoesterases are present in this preparation.

The ATPase and ADPase activities of microsomes have been solubilized with $NaClO_4$ and then purified by gel filtration and DEAE-Sephadex fractionation to a final specific activity of 71.5 and 102 μmol P_i/min per mg for ATP and ADP, respectively. The purified enzyme hydrolyzes triphosphonucleosides (ATP, CTP, GTP, UTP) and diphosphonucleosides (ADP and to a lesser extent CDP, UDP, IDP) and presents pH optima of 6 for ATP and 7 for ADP. It requires Mg^{2+}, Mn^{2+} or Ca^{2+} and is inhibited by diethylstilbestrol and orthovanadate.

The conclusion that the ATPase and ADPase activities belong to the same enzyme is based on the following results: (1) parallel effects of diethylstilbestrol and orthovanadate on both ATPase and ADPase; (2) parallel behavior of ATPase and ADPase throughout all the purification steps; (3) non-additivity of ATPase and ADPase and (4) lack of dilution of β-^{32}P formed from [β-^{32}P]-ATP by unlabelled ADP.

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* A preliminary report of this work has been recently published [9].

Abbreviations: Mes,4-morpholineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

Introduction

In recent years, considerable interest has been centered on Mg^{2+}-dependent, *N,N'*-dicyclohexylcarbodiimide- and diethylstilbestrol-sensitive ATPase, present in membrane preparations from tissues of higher plants. Several lines of evidence suggest that at least some of these enzymes are involved in ion transport across the cell membrane [1,2] and possibly represent the main constituent of an electrogenic proton pump, similar to that described for bacteria and fungi [3—8]. The purification, biochemical characterization and localization at the cell level of membrane ATPases are necessary for the understanding of their functions. However, work on plant ATPases other than those of mitochondria and chloroplasts is still in an initial stage.

In the present paper, we describe the purification and a preliminary characterization of a divalent cation-dependent ATPase present in microsomal preparations from pea stem tissue *.

A high activity of an Mg^{2+}-dependent ATPase present in pea stem microsomal preparations and probably located at the plasmalemma has been previously reported by Pierce and Hendrix [10].

Materials and Methods

Preparation of the microsomal fraction. Segments of the second internode of etiolated 'Alaska' pea seedlings grown for 5 days at 26°C in the dark were weighed, washed, then ground (with a mortar and pestle) at 4°C, in 1/2 fresh wt./vol. of a medium consisting of 0.3 M sucrose, 40 mM Hepes and 3 mM $MgCl_2$, the pH being adjusted to 7 with KOH. The homogenate was strained through cheese-cloth and centrifuged at 1000 × *g* for 10 min. This centrifugation was omitted in the purification of the enzyme. The supernatant was spun at 13 000 × *g* for 20 min. The resulting pellet is referred to as the mitochondrial fraction. The supernatant was centrifuged at an average 60 000 × *g*. The resulting microsomal pellet was resuspended by a Teflon pestle in 1 mM $MgCl_2$, 1 mM Tris-HCl (pH 7), in the proportions of 1 ml buffer to 1.5—2 g of fresh tissue weight. The ATPase activity of the suspension did not change after storage at 0°C for 4 days.

Assay of phosphohydrolase activities. To determine the enzyme activities, the microsomal suspension or the enzyme solution was diluted into the incubation medium without the substrate and aliquots of the dilution were added to the test tubes. The incubation mixture contained 30 mM KCl, 1 or 2 mM $MgCl_2$, 1 mM substrate and 10 mM Tris-Mes (pH 6), in 1 ml final volume. The liberated orthophosphate was assayed by stopping the reaction with 2 ml of a freshly prepared solution containing 0.75 N H_2SO, 0.75% $(NH_4)_2MoO_4$, 3% $FeSO_4 \cdot 7H_2O$ and 0.75% SDS. The samples were read at 740 nm, 10 min after

* In this paper, the term 'microsomal preparation' or 'microsomes' indicates the fraction of homogenate in 0.3 M sucrose, sedimenting between 13 000 and 60 000 × *g*. This fraction contains, besides a large amount of plasmalemma vesicles (approx. 30%) identified by the phosphotungstate reaction [11], also other cell structures, such as endoplasmic reticulum, Golgi vesicles and plastid fragments. Contamination by mitochondria is very low and the contribution of mitochondrial ATPase to the microsomal ATPase activity is negligible (see text).

reagent addition. Substrate blanks were subtracted, the enzyme blank being null under the described conditions. To prevent the acid hydrolysis of substrate during the readings, the ATPase assays were run sequentially. Alternatively, the samples were kept cold (10—15°C) during the readings. When ^{32}P-labelled ATP or ADP was used, the reaction was stopped by adding 0.2 ml of 6% SDS; the samples were then frozen till $^{32}P_i$ was determined according to the method of Seals et al. [12]. Enzyme activity was determined at 26°C, except where indicated. All the experiments were performed in triplicate and repeated at least three times.

Analytical methods. ATP was assayed by incubation with glucose and hexokinase (EC 2.7.1.1) and measuring the glucose 6-phosphate formation with glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and $NADP^+$.

ADP was assayed with pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (EC 1.1.1.27), phospho*enol*pyruvate and NADH.

AMP was phosphorylated with ATP and adenylate kinase (EC 2.7.4.3) and the resulting 2 equiv. ADP were determined as above.

Adenylate kinase activity was assayed by a spectrophotometric method, measuring the formation of ATP, as above. The reaction mixture (3 ml) contained 14 mM triethanolamine hydrochloride, pH 7.6, 3 mM $MgCl_2$, 1.5 mM ADP, 5 mM glucose, 0.15 mM $NADP^+$, hexokinase (1 I.U.) and glucose-6-phosphate dehydrogenase (1 I.U.). The reaction was started by adding an aliquot of the microsomal or enzyme preparation with ATPase activity of 10—20 nmol P_i/min. The assay system was checked by adding a trace amount of pure adenylate kinase. The adenylate kinase activity was also determined with the ATPase medium, at pH 6.

The cytochrome *c* oxidase activity was measured according to the method of Hodges and Leonard [13].

Proteins were determined by using the method of Lowry et al. [14].

Solubilization and purification of ATP-ADPase. A microsomal suspension from 68 g fresh tissue weight was centrifuged at 60 000 × *g* for 60 min. The supernatant was discarded and the pellet suspended with gentle homogenization in 15 ml of 0.3 M $NaClO_4$, 20 mM Tris-HCl (pH 8). The suspension was incubated at 0°C for 30 min with occasional shaking, then centrifuged at 100 000 × *g* for 30 min. 13 ml of the supernatant were loaded on an LKB AcA34 Ultrogel column (200 ml bed volume, 40 cm gel, 26 ml/h flux) and 7-ml fractions were collected. Fractions containing most of the activity were pooled, dialysed overnight against 50 mM NaCl, 12.5 mM Tris-HCl, pH 7.8, and passed through a DEAE-Sephadex A 50 column (40 ml bed), eluting with a continuous gradient of 50—500 mM NaCl in 12.5 mM Tris-HCl, pH 7.8 (flux 34 ml/h). A good resolution was obtained despite the shrinking of the bed. In the experiment described in Results and Discussion, two fractions containing most of the activity were pooled and, after dialysis as above, passed through a second DEAE-Sephadex column. Aliquots of the purified preparations were stored at —80°C for 5 months, with 20—25% loss of activity.

Electrophoresis. SDS-acrylamide gel electrophoresis was performed according to the method of Laemmli [15], with 12% acrylamide and 6-cm separating gels. An aliquot of the enzyme solution was lyophilized, dissolved in the running buffer containing 1% SDS and 0.1% mercaptoethanol and the sample

immersed in boiling water for 2 min. After dialysis against the running buffer containing 2% sucrose and 0.001% bromophenol, 10—12 μg protein were loaded on each gel and electrophoresis was carried out at 1.5 mA per tube. The staining and destaining were performed as described by Weber and Osborn [16].

Reagents. Nucleosides and assay enzymes were all obtained from Boehringer Mannheim GmbH. The potassium and magnesium salts of ATP were prepared by passing Na_3ATP through Dowex 50 cationic exchanger resin then neutralizing with KOH or $Mg(OH)_2$. [γ-^{32}P]ATP and [β-^{32}P]ATP were bought from The Radiochemical Centre, Amersham. [β-^{32}P]ADP was prepared by incubating [γ-^{32}P]ATP with AMP and myokinase. The reaction was stopped with $HClO_4$. After centrifugation, K_2CO_3 was added to neutrality, thus precipitating $KClO_4$. [β-^{32}P]ADP was purified by passage through a DEAE-Sephadex A 25 column and the pure fraction was checked for the complete absence of myokinase. Sigma 'low calcium' ATP was used in studying the effect of divalent cations.

Results and Discussion

General characteristics of the microsomal ATPase activity

The 13 000—60 000 × *g* fraction from the second internode of the pea stem microsomal fraction (see footnote, p. 2), has been chosen as a favorable source of Mg^{2+}-activated ATPase, among similar preparations from tissues of different species, because of its high specific activity (about 1 μM P_i/min per mg protein) and low contamination by the mitochondrial ATPase. A relevant contamination by mitochondria or mitochondrial fragments seemed in fact to be excluded on the basis of the following observations: (1) the ATPase/cytochrome *c* oxidase activity ratio (in μmol P_i/μmol oxidized cytochrome *c*) was 9.6 in the microsomal and 0.37 in the mitochondrial fraction (1000—13 000 × *g*), thus indicating a mitochondrial contamination of less than 5%; (2) the ATPase activity was about 5-times higher at pH 6 than at pH 9 (a ratio higher than has been observed in other materials), thus indicating the prevalence of an activity with a much lower pH optimum than that of the mitochondrial ATPase; (3) the microsomal ATPase was insensitive to oligomycin (20 μg/ml), which strongly inhibited the ATPase activity of the mitochondrial fraction (data not shown).

The ATPase and ADPase activities of the microsomal preparation were strongly stimulated by Mg^{2+}, but much less so by K^+ (Table I). The Mg^{2+} effect may have been reduced by the presence of vesicle-bound Mg^{2+} and the K^+ effect refers to the difference in stimulation from 4 to 34 mM K^+. Diethylstilbestrol (0.3 mM) and orthovanadate (0.1 mM) inhibited by 52—53% and by 44—45%, respectively, both the ATPase and ADPase activities of our microsomal preparations. Diethylstilbestrol and Na_3VO_4 are known to inhibit transport ATPases in fungi and plant cells [17—20].

Pea stem microsomes actively hydrolyzed ATP, ADP and other nucleoside triphosphates, while very little phosphatase activity was found with AMP, phospho*enol*pyruvate, *p*-nitrophenyl phosphate, glucose-6-phosphate and none with inorganic or organic pyrophosphates (Table II). The release of P_i from ADP was not changed by the addition of a large excess of the ATP-trapping

TABLE I

CHARACTERISTICS OF THE MICROSOMAL ATPase AND ADPase ACTIVITIES

The complete medium contained 30 mM KCl, 10 mM Tris-Mes (pH 6), 1 mM ATP or ADP and microsomes (2.8 μg protein). The reaction was started by adding ATP or ADP and the incubation was carried out at 26° C for 1 h. Where KCl was absent, the final concentration of K^+ from ATP-K was 4 mM. Diethylstilbestrol was dissolved in ethanol to yield 1% final ethanol concentration, present in each sample. This ethanol concentration did not influence the microsomal activity.

Medium	ATPase activity		ADPase activity	
	nmol P_i	Relative	nmol P_i	Relative
Complete	156	100	220	100
Minus $MgCl_2$	46	29	63	29
Minus KCl	132	84	200	91
Plus 0.3 mM diethylstilbestrol	73	47	105	48
Plus 0.1 mM Na_3VO_4	85	54	121	55

system, hexokinase-glucose, showing that ADP hydrolysis depended on 'true' ADPase activity and not on the formation of ATP by adenylate kinase. Moreover, adenylate kinase activity, very carefully determined as NADP reduction in the presence of hexokinase and glucose at either pH 6 or 7.6, was completely absent. In the presence of ATP plus ADP, both at 1 mM, the amount of released P_i was intermediate between those observed with each substrate, thus excluding additivity.

The ATPase activity of the microsomal preparation was determined, besides being measured colorimetrically as P_i formed, also by means of an enzymatic method, which allows the determination of the ADP formed as NADH oxidized in the presence of an ADP-trapping, ATP-regenerating system, including phospho*enol*pyruvate, pyruvate kinase and lactate dehydrogenase. Under our conditions, NADH oxidation by the microsomes in the presence of ATP reached a constant rate in about 5 min. Doubling the concentrations of pyruvate kinase and lactate dehydrogenase did not influence the reaction rate, once this was linear (Table III), showing that the ADP-trapping system was in large excess,

TABLE II

PHOSPHOHYDROLASE ACTIVITIES OF THE MICROSOMAL PREPARATION

Experimental conditions as in Table I, except for the substrates, each at 1 mM PEP, phospho*enol*pyruvate; G6P, glucose 6-phosphate; pNPP, *p*-nitrophenyl phosphate.

Substrate(s)	Relative activity	Substrate	Relative activity
ATP	100	CTP	128
ADP	138	GTP	112
ADP + hexokinase (1 I.U.) + 0.3 M glucose	140	UTP	101
ATP + ADP	124	PEP	6
		AMP, G6P, pNPP	⩽1
		PP_i or NAD^+ *	0

* No formation of AMP was detected.

TABLE III

MICROSOMAL ATPase ACTIVITY AS ADP AND P_i FORMATION

The salt composition of the medium (3 ml final volume) was as in Table I, with 1 mM ATP. The ATP-regenerating system, added in b, consisted of 0.15 mM NADH, 0.5 mM phospho*enol*pyruvate and 15 I.U. of both pyruvate kinase and lactate dehydrogenase. Before starting the reaction by adding the microsomal suspension (3 μg protein), the complete system was incubated for 20 min in order to deplete the trace of ADP present in ATP. The NADH oxidation proceeded linearly from 8 min to at least 1 h. Recordings of ΔA_{340} (t = 8—58 min) have been compared with ΔP_i in the same period, determined colorimetrically in parallel samples. The linearity of P_i formation was checked by stopping the reaction at different times (t = 23, 58 min). The ratio, ADP/P_i, determined in four experiments was in the range of 0.4—0.45.

Incubation medium	Enzymatic assay for ADP		Colorimetric P_i assay (nmol/ml)
	$-\Delta A_{340}$	nmol/ml	
(a) Control	0.000	0.0	120
(b) Plus ATP-regenerating system	0.310	50.0	112
(c) As b, with doubled pyruvate kinase and lactate dehydrogenase concentrations	0.303	48.9	—
(d) As b, ATP omitted (NADH oxidase test)	0.007	1.1	0

and consequently, that the free ADP concentration was kept exceedingly low. Parallel determinations of P_i and ADP formed in 50 min, in which both NADH oxidation and P_i formation proceeded linearly, showed that the oxidation of NADH, namely ADP formation from ATP, accounted for only 45% of total P_i, determined colorimetrically (Table IIIb). The amount of P_i released was approximately equal in the presence and in the absence of the ADP-trapping system, in spite of the very large difference in the possibility of accumulating ADP under the two conditions. This is in contrast to what would be expected if part of the P_i released were a product of the hydrolysis of ADP arising from ATP. On the other hand, this result together with the unbalanced ADP/P_i formation could be explained if part of the ADP formed from ATP were directly split to AMP + P_i without becoming available to the ADP-trapping system. This, together with the effects of Mg^{2+}, diethylstilbestrol and Na_3VO_4 on the ATPase and ADPase activities and with the non-additivity of the two activities, suggested that a single enzyme was carrying out the sequential hydrolysis of ATP to ADP and then to AMP. This important point will be taken up again, when discussing the characteristics of the purified enzyme.

Solubilization and purification

The ATPase and ADPase activities were solubilized by incubating the microsomal suspension with the chaotropic salt, $NaClO_4$, as described in Materials and Methods. After solubilization, 82% of both the microsomal ATPase and ADPase activity was found in the 150 000 × *g* supernatant and 15% of both was recovered in the precipitate. The soluble activity was first fractionated by gel filtration on an Ultrogel AcA34 column (Fig. 1A). This gave parallel profiles for both the ATPase and ADPase activities, with a quite constant ATPase/ADPase ratio of close to 0.75. Both activities presented a minor peak just after the protein front and a major peak with K_{av} = 0.4, corresponding to an approximate molecular weight of 70 000—80 000. The fractions included in the

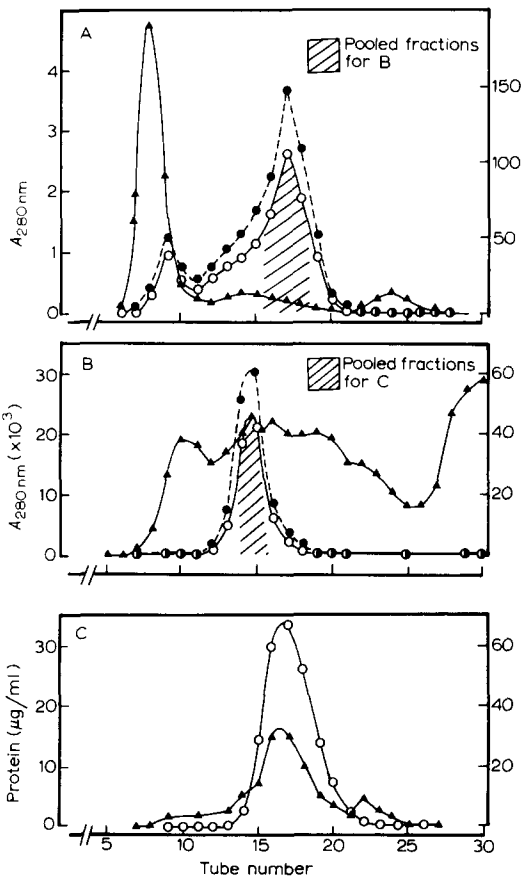


Fig. 1. Purification of the solubilized ATPase and ADPase activities. A, gel filtration (Ultrogel AcA34 column); B, DEAE-Sephadex fractionation; C, repeated DEAE-Sephadex fractionation. ▲, A_{280nm} in A and B, protein concentration in C; ○, ATPase activity; ●, ADPase activity. The enzyme was eluted in C with an NaCl gradient, steeper than in B, in order to obtain an increased enzyme concentration.

main activity peak were then pooled and run through a DEAE-Sephadex column. The ATPase and ADPase activities were eluted from the DEAE-Sephadex column at about 250 mM NaCl; both were recovered in a single well defined peak (Fig. 1B). A typical purification experiment, in which the DEAE-Sephadex step was repeated (Fig. 1C), is represented in Table IV. The final specific activity of ATPase was 34 μmol P_i/min per mg, determined at 26°C and 71.5 μmol P_i/min per mg, determined at 37°C. This is a much higher value than that reported by Benson and Tipton [21] (14.9 μmol/min per mg), for the purified K^+-ATPase of maize microsomes, with 90% purity as estimated using SDS-acrylamide gel electrophoresis.

The high recovery of solubilization (82%) and purification (79% for gel filtration, 95% in DEAE-Sephadex first run) suggests that the purified enzyme is the main component of the microsomal activity.

SDS-acrylamide electrophoresis of the DEAE-Sephadex enzyme showed two main bands accounting for most of the protein and corresponding to molecular

TABLE IV

PURIFICATION OF ATP-ADPase

The specific activity refers to standard conditions (Table I), at 26°C. The specific activity of the fractions 16 + 17 from the DEAE-Sephadex II column was 71.5 μmol/min per mg at 37°C. The DEAE-Sephadex II step was omitted in other preparations.

Material	Volume (ml)	Total ATPase activity (μmol P_i/min)	Total protein (mg)	Specific activity (μmol P_i/min per mg)
Microsomes	39.0	49.40	48.50	1.02
$NaClO_4$ supernatant	14.2	40.00	9.94	4.02
Ultrogel, pooled fractions	21.0	14.30	1.03	13.90
DEAE-Sephadex I, pooled fractions	14.6	10.10	0.33	30.60
DEAE-Sephadex II, fractions 16 + 17	4.0	2.06	0.06	34.30

weights of 30 000 and 48 000, respectively (Fig. 2). Both may represent subunits, as the sum of molecular weights (78 000) is in the range of 70 000—80 000 estimated for the main peak obtained by gel filtration. In this case the purity of the enzyme would be about 90%.

Characteristics of the purified enzyme

The activity with various phosphorylated substrates and the effects of some inhibitors are shown in Table V. Triphosphonucleosides were attacjed rather unspecifically, while ADP was preferred among the diphosphonucleosides. Diethylstilbestrol and orthovanadate (0.1 mM) inhibited the purified ATPase activity by 79 and 63%, respectively, namely somewhat more than in the microsomal preparation. The sulfhydryl reagents, *p*-chloromercuribenzenesulfonate and mersalyl, were scarcely inhibitory. AMP inhibited the ATP activity competitively, with an estimated K_i value of 295 μM (data not shown). This value is much higher than the apparent K_m value for ATP (see later), thus indicating a low effectiveness of the inhibitor.

Both the ATPase and ADPase activities of the purified enzyme were divalent cation-dependent. With 2 mM ATP or ADP and 0.5 mM, Mg^{2+} induced the

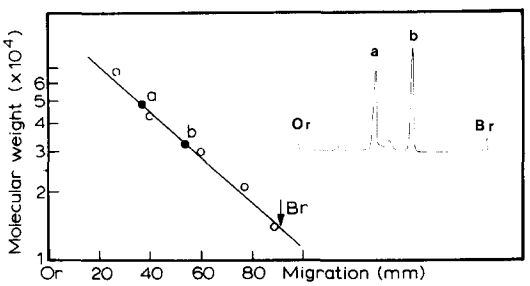


Fig. 2. SDS-polyacrylamide electrophoresis of the purified enzyme. Insert: densitometric tracings of gel loaded with 10—12 μg of protein and stained with Coomassie blue; Or, origin; Br, bromophenol dye. Migrations of the bands a and b (in mm) are plotted together with those of the standard proteins (○), from left to right: bovine serum albumin, ovalbumin, carbonic anydrase, soybean trypsin inhibitor, lysozyme.

TABLE V

RELATIVE ACTIVITY OF THE PURIFIED ENZYME WITH VARIOUS SUBSTRATES, AND EFFECTS OF SOME INHIBITORS

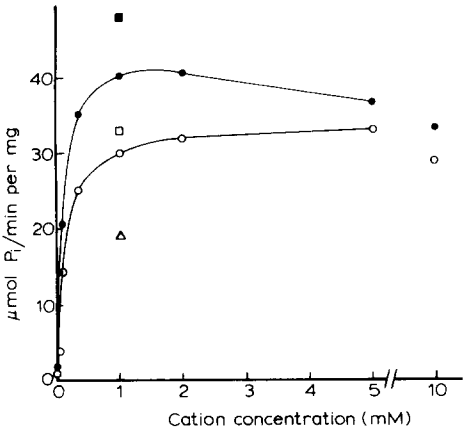
The reaction was started by adding 0.1 μg of enzyme. Samples contained 30 mM KCl, 2 mM $MgCl_2$, 1 mM substrate, 10 mM Tris-Mes (pH 6), in 1 ml final volume. Activity was nil with AMP, glucose 6-phosphate, phospho*enol*pyruvate, *p*-nitrophenyl phosphate, PP_i, thiamine pyrophosphate, 3′,5′-cyclic AMP. The rate of hydrolysis of ATP was in the range of 135—155 nmol P_i formed in 1 h of incubation at 26°C. The effects of the inhibitors were determined with 1 mM ATP. Diethylstilbestrol was dissolved in ethanol to yield 0.5% final ethanol concentration, and the effect of the inhibitor was referred to a separate control with 0.5% ethanol, which inhibited 7.7% by itself. CMBS, *p*-chloromercuribenzenesulfonate.

Substrate	Relative activity	Inhibitor	Inhibition (%)
ATP	100	10^{-5} M diethylstilbestrol	19
CTP	116	10^{-4} M diethylstilbestrol	79
GTP	100	10^{-5} M Na_3VO_4	31
UTP	91	10^{-4} M Na_3VO_4	63
ADP	150	10^{-5} M CMBS	22
CDP	64	10^{-4} M CMBS	40
UDP	43	10^{-5} M mersalyl	10
IDP	57	10^{-4} M mersalyl	13

maximal activation at concentrations between 1 and 5 mM, 10 mM being slightly inhibitory. Half-maximal activation was at approx. 120 and 60 μM $MgCl_2$ for ATPase and ADPase, respectively (Fig. 3). If EGTA was omitted, 13% of the maximal activity was found in the absence of Mg^{2+}, due probably to the calcium impurities of the reagents. At 1 mM concentration, Mn^{2+} and Ca^{2+} were more effective and Co^{2+} less effective than Mg^{2+}. Ni^{2+} did not active the enzyme. Various monovalent ions (K^+, Na^+, Li^+, NH_4^+, $Tris^+$, $choline^+$), tested at a mM in the presence of 1 mM MgATP (no buffer present), stimulated slightly the ATPase activity, the maximal stimulation (24%) being exibited by KCl (not shown). The pH curves of ATPase and ADPase are presented in Fig. 4. At pH 6 the ratio ATPase/ADPase was 0.75. Double-reciprocal plots of ATPase and ADPase velocities as a function of substrate concentration in the range of 30 μM—1 mM and in the presence of 2 mM $MgCl_2$ were linear, indicating an apparent K_m value of 70—80 μM for ATP and 21—35 μM for ADP (not shown). These values refer to total nucleotides, not to the free or metal-complexed substrates.

Table VI reports the stoichiometric relationship between the degradation of the substrate and the formation of the products under the conditions of: (a) 14% degradation of ATP, and (b) complete degradation of ATP or ADP. In the first case, both ADP and AMP were formed in amounts closely accounting for the disappearance of ATP, while the amount of P_i was very close to the sum of P_i equivalents corresponding to the ADP and AMP formed. Neither adenylate kinase nor pyrophosphate even in trace amounts could be detected. Therefore, these data exclude the formation of reaction products other than ADP, AMP and P_i. Also, in the experiments in which the hydrolysis was allowed to proceed to complete disappearance of either ATP or ADP, the values of P_i found closely accounted for ATP/P_i and ADP/P_i ratios of 2 and 1, respectively.

A peculiarity of this enzyme is the activity on both ATP and ADP. This



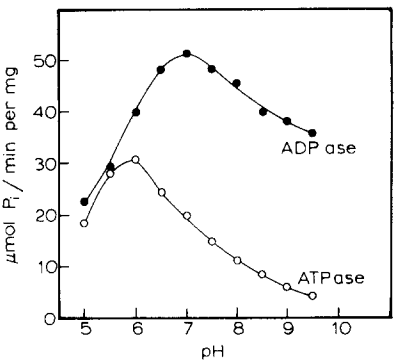


Fig. 3. Effects of the divalent cations on the enzyme activity. Experimental conditions as in Table V. The curves represent the ATPase (○) and the ADPase (●) activities as a function of $MgCl_2$ concentration. If EGTA was omitted, the ATPase activity, determined in the absence of $MgCl_2$, was 13% of the maximal. The single symbols represent the values of ATPase activity obtained in the presence of: ■, $MnCl_2$; □, $CaCl_2$; △, $CoCl_2$ (1 mM each, EGTA omitted).

Fig. 4. pH curve of ATPase and ADPase. Medium composition as in Table V, with 10 mM Tris-Mes buffer (various pH values were obtained with different concentrations of Mes). ○, 1 mM ATP; ●, 1 mM ADP.

feature, rather unusual amongst ATPase (see, however, Refs. 21—23), deserved a critical experimental study. Table VII reports an experiment among several carried out reproducibly with the purified enzyme, showing that the activities on ATP and ADP were not additive, as already observed for the microsomes, but rather an intermediate amount of P_i was liberated when the two substrates were added together (Table VII, first column). This may suggest either a cross-

TABLE VI

STOICHIOMETRY OF THE PARTIAL AND COMPLETE ATP HYDROLYSIS AND OF COMPLETE ADP HYDROLYSIS

Experimental conditions as in Table V, except for the indicated concentrations of substrates and enzyme. A, 14% of ATP (initially 0.9 μmol) was hydrolyzed during the incubation time in the presence of 0.1 μg of enzyme. B, the hydrolysis of either ATP or ADP (0.18 μmol initially) was complete with 1 μg of enzyme.

Conditions	Substrate and products determined	$\Delta(t_0 - t_{60min})$ (nmol/ml)
A		
Partial ATP hydrolysis (0.9 μmol initial ATP)	ATP	−130
	ADP	+ 53
	AMP	+ 68 total 121
	P_i	+175 (calculated from ΔADP + ΔAMP : 189)
B		
Complete hydrolysis		
0.18 μmol initial ATP	P_i	+345
0.18 μmol initial ADP	P_i	+157

TABLE VII

MUTUAL INHIBITORY EFFECTS OF ADP AND ATP ON ATPase AND ADPase

Experimental conditions as in Table V. The experiment was repeated four times, with substantially identical results (maximal deviations for the effects of the cold substrate are in brackets).

Substrates (1 mM each)	P_i released (nmol/ml)			Effects of the cold substrate
	Total *	γ-$^{32}P_i$	β-$^{32}P_i$	
[γ-^{32}P]ATP	135	101	—	—
[γ-^{32}P]ATP + ADP	187	59	—	—42% (±3)
[β-^{32}P]ATP	135	—	36	—
[β-^{32}P]ATP + ADP	187	—	22	—39% (±4)
[β-^{32}P]ADP	212	—	208	—
[β-^{32}P]ADP + ATP	187	—	103	—50% (±6)

* Colorimetrically assayed P_i.

inhibition between two substrates at the level of two different enzymes, one specific for ATP and the other for ADP, or a mutual competition between ATP and ADP for the same enzyme. To discriminate between these hypotheses we took advantage of the possibility to follow the fate of the γ- and β-P of ATP and β-P of ADP, by using substrates in which the phosphate group was specifically labelled with ^{32}P either in the γ- or β-position.

The data of Table VII show that with ATP and ADP (1 mM each) present simultaneously, and when the reaction is still in its initial phase (namely only 6% of ATP has disappeared), the presence of (unlabelled) ADP inhibits by approx. 40% the release of both the γ- and β-P of ATP, while (unlabelled) ATP inhibits by 50% the release of the β-P of ADP. Consequently, 32% of the total P_i formed is accounted for by the γ-P (ATP), 12% by the β-P (ATP) and 56% by the β-P (ADP) released. According to preliminary observations, this cross-inhibition between ATP and ADP appears competitive (data not shown).

The analysis of the contribution of the β-$^{32}P_i$ from ATP to the total β-P_i released in the presence of ATP and ADP allows discrimination between the action of a single ATP-ADPase or an ATPase plus an ADPase. In the experiment presented in Table VII, the maximal concentration reached by the ADP produced from ATP in the presence of ADP can be estimated by the difference: 59 nmol/ml γ-P_i (corresponding to the ATP degraded) — 22 nmol/ml β-P_i (corresponding to the AMP formed) = 37 nmol/ml ADP. This calculation is justified by the finding that ADP, AMP and P_i are the only products of ATP hydrolysis, and that no pyrophosphatase is present. Due to the presence of added unlabelled ADP, the amount of 37 nmol of [β-^{32}P]ADP formed from [β-^{32}P]ATP is diluted by 1000 nmol of the latter (896 nmol at the end of incubation). Thus, in the case of the action of a distinct ADPase, the contribution of the β-$^{32}P_i$ from ATP to the total β-P_i released from ATP and ADP should not exceed the approximated ratio of 37/934 = 4%. The amount of β-P_i released in the presence of 1 mM ADP and 1 mM ATP is given by the 103 nmol from ADP plus a practically negligible contribution (less than 4%) of ADP arising from ATP. Hence, the contrinution of β-$^{32}P_i$ from ATP to total β-P_i should not exceed $37/934 \times 103 = 4$ nmol. This is in strong contrast with the

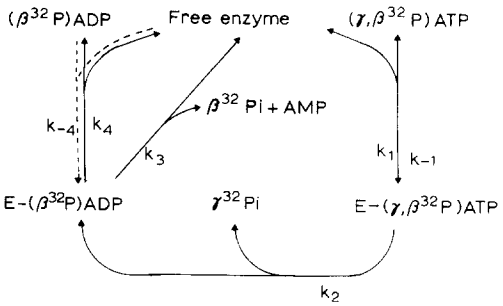


Fig. 5. Possible scheme of the ATP-ADPase catalytic sequence, as suggested by the results presented. The reaction, E-[β-^{32}P]ADP → E + [β-^{32}P]ADP, is considered irreversible, the back reaction remaining negligible during the initial phase, due to the very low concentration of the product [β-^{32}P]ADP.

experimental data, showing that β-$^{32}P_i$ from ATP contributes 22 nmol. This seems to rule out the possibility of the coexistence of an ADPase distinct from an ATPase in our purified preparation. On the contrary, the data of Table VII are fully consistent with the hypothesis of a single enzyme sequentially hydrolysing ATP to ADP and then to AMP. In this case, the addition of unlabelled ADP should inhibit (by competing with ATP for the free enzyme) to the same extent the release of γ- and β-P of ATP, which indeed is the case. Fig. 5 represents a possible scheme of the ATP-ADPase catalytic sequence which could explain the results discussed above.

Conclusions

An enzyme able to hydrolyze triphosphonucleosides (ATP, CTP, GTP, UTP) and diphosphonucleosides (ADP and to a lesser extent CDP, UDP, IDP) has been purified from the microsomal fraction of pea stem. This enzyme does not attack AMP, *p*-nitrophenyl phosphate, PP_i, NAD^+, thiamine pyrophosphate, phospho*enol*pyruvate and glucose 6-phosphate, is activated by Mn^{2+}, Ca^{2+} or Mg^{2+}, and is inhibited by the plasmalemma ATPase inhibitors diethylstilbestrol and Na_3VO_4. The partial hydrolysis of ATP by the purified enzyme gives as only products ADP, AMP and P_i, while AMP and P_i are the only products of the complete hydrolysis of either ATP or ADP.

The conclusion that the ATPase and ADPase activities belong to the same enzyme is based on the following results: (1) in the microsomal preparation both activities are stimulated by Mg^{2+} and inhibited by diethylstilbestrol and Na_3VO_4 to the same extent; (2) the ATPase and ADPase activities have parallel behavior and the ATPase/ADPase ratio remains constant (approx. 0.75) throughout all the purification steps; (3) ATPase and ADPase activities are not additive, with the microsomes as well as with the purified enzyme; (4) experiments with γ-^{32}P- or β-^{32}P-labelled ATP or ADP show that the ATPase and ADPase activities are reciprocally inhibited by ADP and ATP, respectively, while the β-^{32}P of ATP is not diluted by unlabelled ADP, as would be expected if two different enzymes were present (see Results and Discussion). The features of this enzyme are thus quite different from those of other known

diphosphonucleoside phosphatases from plant tissues, such as a soybean nucleoside diphosphatase (ineffective with ATP [24]) and a latent pea diphosphatase of the Golgi fraction, supposed to represent an inactivated form of polysaccharide synthetase [25]. An ADPase activity of maize microsomal ATPase has been previously suggested, although not unequivocally demonstrated, by Benson and Tipton [21]. In animal tissues, a Ca^{2+}-sensitive ATP diphosphohydrolase also attacking diphosphonucleosides and producing only AMP and P_i without any accumulation of ADP or PP_i has been purified from pig pancreas microsomes [22]. An enzyme similar to that described in this paper with respect to its activity on nucleoside triphosphates and ADP, as well as to its dependency on divalent cations, has been purified from kidney cells, where it is thought to be involved in Ca^{2+} and Mg^{2+} transport [23].

The very high activity of the pea microsome enzyme (approx. 1 μmol P_i/min per mg protein, corresponding to 1.3 μmol P_i/min per g fresh weight of tissue) would account for the complete consumption of ATP produced by respiration, on the basis of the Q_{O_2} value of 6.7 μl O_2/min per g fresh weight, observed in the same tissue (Rasi-Caldogno, F., Cerana, R. and Pugliarello, M.C., personal communication) and assuming an average P/O ratio of 2. This suggests that the enzyme is involved in some important process and, at the same time, that some conditions or factors inhibit its full operation in the intact cell.

The subcellular localization of the enzyme remains open to investigation. A mitochondrial origin can be excluded on the basis of several indications. The sensitivity to the plasmalemma inhibitors diethylstilbestrol and Na_3VO_4 may suggest a plasmalemma origin, even if other ATPases can be affected by these compounds. Further work is in progress in this laboratory to elucidate this important point.

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