Purification and characterization of a membrane-bound ATP diphosphohydrolase from *Cicer arietinum* (chick-pea) roots

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Microsomal membranes from *Cicer arietinum* (chick-pea) roots contained an ATP phosphohydrolase activity that could be solubilized by high-ionic-strength media. The enzyme has been purified to homogeneity by affinity and ion-exchange chromatography. It has the properties of an ATP diphosphohydrolase (apyrase, EC 3.6.1.5) that hydrolyses different nucleoside di- and tri-phosphates but has no activity towards monophosphoric esters and pyrophosphate. No stimulation by K^+ could be demonstrated for either the membrane-bound or the purified enzyme, and therefore it would seem not to be related to the K^+ -dependent ATPase postulated to mediate K^+ transport in plants.

In spite of its potential physiological significance (Marreé, 1979), the ATPase from plant plasma membrane has neither been purified nor unambiguously identified. A complication with most of the studies in this field is the existence in plant homogenates of several enzymes capable of hydrolysing ATP. In plasma membranes from oat (*Avena sativa*) roots, a phosphohydrolase activity very specific for ATP has been described (Hodges *et al.*, 1972). This activity is stimulated more than 3-fold by K^+ , and this has been taken as evidence for the involvement of the enzyme in K^+ transport (Leonard & Hodges, 1973). Similar plasma-membrane preparations from corn (maize, *Zea mays*) (Leonard & Hotchkiss, 1976), kidney beans (*Phaseolus vulgaris*) (Koehler *et al.*, 1976), soya beans (*Glycine max*) (Hendrix & Kennedy, 1977) and barley (*Hordeum vulgare*) (Nagahashi *et al.*, 1978) contained a phosphohydrolase activity of broad substrate specificity, but only the hydrolysis of ATP was stimulated by K^+ . The small degree of K^+ stimulation in these tissues has been attributed to the presence of a non-specific phosphatase independent of K^+ which masks the specific enzyme activated by K^+ (Leonard & Hotchkiss, 1976; Nagahashi *et al.*, 1978). However, since the stimulation by K^+ is only observed at low pH and high temperatures (Leonard & Hodges, 1973; Leonard & Hotchkiss, 1976), the relationship between the K^+ -dependent and -inde-

pendent activities is unclear. Thus the purification and characterization of the different phosphohydrolase activities present in plant membranes is a prerequisite for further progress in the field.

In the present work we report the purification and characterization of the ATP-hydrolysing activity associated with membranes from *Cicer arietinum* (chick-pea) roots. This plant material was selected for the relative ease of obtaining and manipulating the large quantities required for enzyme isolation.

Experimental

Materials

Seeds from *Cicer arietinum* (chick peas) were obtained from Puebla de Almenara, Spain, and corresponded to a local variety. They were soaked for 5 h in water and planted in trays of Vermiculite moistened with 1 mM- $MgSO_4$ /1 mM- $CaCl_2$ /5 mM- KH_2PO_4 , pH 6.5. After 7 days at 30°C in the dark the roots were excised and washed with ice-cold deionized water.

Sucrose (May and Baker), KCl (Merck), Coomassie Brilliant Blue G-250 (Serva), cytochrome *c* (Sigma type II), ATP (Sigma type I), $NADP^+$ (Sigma grade), NADPH (Sigma type I), hexokinase (Sigma type C-130), pyruvate kinase (Sigma type II) and lactate dehydrogenase (Sigma type II) were obtained as indicated. Cytochrome *c* was reduced with ascorbic acid (Wharton & Tzagoloff, 1967) and the sodium salt of ATP was converted into the Tris salt as described by Hodges & Leonard (1974).

Abbreviations used: Mes, 4-morpholine-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

Methods

Preparation of membrane fractions. All steps were performed at 0–4°C in a buffer containing 0.25 M-sucrose, 10 mM-Tris/HCl, pH 7.5, and 2.8 mM-2-mercaptoethanol. Portions (30–40 g fresh weight) of roots were chopped with a razor blade and ground with mortar and pestle with 3–5 ml of buffer. The brei was filtered through a cheesecloth and the residue extracted with 10 ml of buffer and filtered. The volume of the combined filtrates was adjusted with buffer to 50 ml and the pH adjusted to 7.5 with 1 M-Tris base. Centrifugation at 900 g (r_{av} , 7.6 cm) for 5 min produced the supernatant referred to as the 'homogenate'. Membrane fractions were obtained by centrifuging the homogenate first at 15000 g (r_{av} , 7.6 cm) for 10 min ('15000 g pellet') and then at 110000 g (r_{av} , 6.3 cm) for 30 min ('110000 g pellet').

Enzyme purification. Unless otherwise indicated, all operations were performed at 0–4°C in a 50 mM-Tris/HCl buffer, pH 7.5. The 110000 g pellet was adjusted to a protein concentration of 2 mg/ml and KCl was added to a final concentration of 0.1 M. After centrifugation at 110000 g (r_{av} , 6.3 cm) for 30 min, the supernatant was discarded. The washed pellet was resuspended at 5 mg/ml and KCl was added to 0.5 M. After centrifugation as just described, the pellet was discarded and the supernatant dialysed against Tris buffer for 3–4 h. A small amount of precipitated material appeared at this step and was removed by low-speed centrifugation. The desalted enzyme (20–30 ml) was applied to an Affi-Gel Blue column (5 ml bed volume, Bio-Rad) at a flow rate of 0.4 ml/min. After washing the column with 20 ml of Tris buffer, the enzyme was eluted with a linear gradient of KCl (50 ml, 0–1 M). Fractions (2 ml) were collected, and those with a specific activity over 200 μ mol of P_i /min per mg were pooled and dialysed against 10 mM-Mes adjusted to pH 6.5 with Tris. After 3–4 h the non-diffusible material (8–10 ml) was applied to a carboxymethylcellulose column (5 ml bed volume; Whatman CM-23) equilibrated with the same buffer. The flow rate was 0.2 ml/min, and fractions (1 ml) were collected. After washing the column with 10 ml of buffer the enzyme was eluted with a linear gradient of KCl (20 ml, from 0 to 1 M) prepared in 10 mM-Tris/HCl, pH 7.5. Fractions with specific activities over 1700 μ mol of P_i /min per mg were pooled and stored frozen.

Electrophoresis. Polyacrylamide-gel electrophoresis in the presence of SDS was performed as described by Fairbanks *et al.* (1971), except that the concentrations of acrylamide and detergent were 7.5 and 0.1% respectively. The samples, containing from 3 μ g of pure-enzyme protein to 60 μ g of crude-fraction protein, were precipitated with 10% (w/v) trichloroacetic acid in order to concentrate the

protein and to remove K^+ . After centrifugation at 110000 g (r_{av} , 6.9 cm) for 30 min, the pellets were resuspended in 50 μ l of medium containing 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.01% Bromophenol Blue, 2 mM-EDTA and 40 mM-Tris/acetic acid, pH 7.4. After neutralization with concentrated NaOH, 25 μ l samples were applied to the gels. Electrophoresis in the absence of SDS was performed in a discontinuous gel system as described by Williams & Reisfeld (1964). A sample containing 300 μ l of dialysed pure enzyme (2–3 μ g) was applied to the gels.

ATP phosphohydrolase activity. ATP phosphohydrolase activity was measured by determination of P_i as previously described (Serrano, 1978). The incubation medium contained 50 mM-Mes (adjusted to pH 6.5 with Tris), 5 mM-MgSO₄ and 2 mM-ATP. Incubation was for 10 min at 30°C, and the enzymic activity is expressed as μ mol of P_i formed/min. To locate the activity after electrophoresis the gels were incubated at 30°C for 10 min in a solution containing 5 mM-ATP/5 mM-MgSO₄/1.35 mM-lead acetate and 0.1 M-Mes, adjusted to pH 6.5 with Tris. The gels were washed for 2 h with several changes of water and stained for 1 min with 0.2% Na₂S. Finally, they were washed with water and stored in 10% (v/v) acetic acid.

Protein determination and staining in the gels. Protein concentration was determined with Coomassie Brilliant Blue G-250 as described by Bradford (1976). For very dilute samples the sensitivity was increased by mixing samples of 0.1 ml (0.3–5 μ g of protein) with 0.4 ml of dye reagent. Bovine serum albumin was employed as standard. The same dye was employed for the rapid staining of protein bands after gel electrophoresis (Reisner *et al.*, 1975). When the gels contained SDS, the detergent was removed before staining by incubation for 3 h with methanol/water/acetic acid (5:4:1, by vol.). After being stained for 2 h the gels were transferred to 10% (v/v) acetic acid for colour intensification (Blakesley & Boezi, 1977). Gels were scanned at 600 nm by using a Gilford densitometer.

Other analytical procedures. Cytochrome *c* oxidase (EC 1.9.3.1; Wharton & Tzagoloff, 1967), NADPH:cytochrome *c* oxidoreductase (EC 1.6.2.4; Schatz & Klima, 1964), adenylate kinase (EC 2.7.4.3; Oliver, 1955), ATP and ADP (Lowry & Passonneau, 1972) were determined as described in the references cited.

Results

The homogenate from *Cicer arietinum* roots was separated into three main fractions by differential centrifugation. ATP phosphohydrolytic activity and the marker enzymes (Quail, 1979) cytochrome *c* oxidase (for mitochondria) and NADPH:cyto-

Table 1. *Distribution of ATP phosphohydrolase and marker enzymes among the fractions separated by differential centrifugation*

Conditions of the assays for enzyme activity are described in the Experimental section. Results are the mean values ($\pm$ S.D.) for four experiments. The amount of protein corresponds to the fractions derived from 1 g (fresh weight) of root tissue.

Fraction	Protein (mg)	ATP phosphohydrolase (μ mol of P_i /min per mg)	Activity (μ mol of cytochrome <i>c</i> /min per mg)	
			Cytochrome <i>c</i> oxidase	NADPH:cytochrome <i>c</i> oxidoreductase
Homogenate	3.3 $\pm$ 0.6	1.0 $\pm$ 0.1	0.06 $\pm$ 0.02	0.04 $\pm$ 0.01
15000 g Pellet	1.2 $\pm$ 0.3	0.5 $\pm$ 0.1	0.14 $\pm$ 0.04	0.03 $\pm$ 0.01
110000 g Pellet	0.6 $\pm$ 0.2	1.7 $\pm$ 0.5	0.02 $\pm$ 0.01	0.07 $\pm$ 0.02
110000 g Supernatant	1.1 $\pm$ 0.2	1.1 $\pm$ 0.3	<0.01	0.02 $\pm$ 0.01

chrome *c* oxidoreductase (for endoplasmic reticulum) were measured in these fractions. As shown in Table 1, the 15000 g pellet was enriched in mitochondria and the 110000 g pellet in endoplasmic reticulum. Most of the ATP phosphohydrolytic activity distributed between the 110000 g pellet and the supernatant, and the highest specific activity corresponded to the 110000 g pellet. This microsomal fraction is probably a mixture of different types of organelle membranes, and in other plant tissues it contained the plasma membranes (Hodges & Leonard, 1974). Therefore this fraction was selected for our subsequent studies.

Solubilization

More than 80% of the ATP phosphohydrolytic activity associated with the 110000 g pellet could be solubilized with concentrations of different salts in excess of 0.5 M. EDTA at low concentrations (2 mM) was also effective, suggesting that bivalent cations are involved in the electrostatic binding of the enzyme to the membranes.

The solubilization could be made more specific by first treating the membranes with 0.1 M-KCl and then solubilizing the ATP phosphohydrolytic activity with 0.5 M-KCl. This two-step procedure resulted in an increase of specific activity of more than 14-fold.

Purification of the solubilized enzyme

The solubilized enzyme could be purified by affinity chromatography on an Affi-Gel Blue column. Most of the solubilized protein was not retained by the column, whereas the ATP phosphohydrolytic activity was absorbed and eluted by a linear gradient of KCl (Fig. 1). The specific activity increased more than 15-fold with this step. Equally useful were a carboxymethylcellulose column, which specifically retained the ATP-hydrolysing activity and not the contaminating proteins eluted from the Affi-Gel Blue column (Fig. 2). At this stage the enzyme was homogeneous as judged by polyacryl-

Fig. 1. *Affinity chromatography of solubilized ATP phosphohydrolase*

Enzyme solubilized by KCl (30 ml, 8 mg of protein) was dialysed and passed through a 5 ml Affi-Gel blue column at a flow rate of 0.4 ml/min. After washing the column with 20 ml of buffer, the enzyme was eluted with a linear gradient of KCl.

amide-gel electrophoresis in the absence and in the presence of SDS (results not shown). The protein and activity in the course of the purification appear in Table 2. A 1000-fold purification was achieved with only two chromatographic columns. The loss of activity is due to the fact that only the column fractions with high specific activity were selected during the purification. As indicated in Figs. 1 and 2, no evidence was obtained for more than one enzyme hydrolysing ATP in the material solubilized from the microsomal fractions. After the Affi-Gel Blue step, the protein concentration was very low (less than 50 μ g/ml), and the protein tended to stick to the dialysis bags when K^+ was removed. Therefore some loss of activity (20–40%) was always found in the dialysis before the carboxymethylcellulose column. Desalting by gel filtration resulted in even higher losses of activity.

Fig. 2. Carboxymethylcellulose chromatography of the Affi-Gel Blue-purified enzyme

The active fractions from an Affi-Gel Blue column (Fig. 1, 15 ml and 0.4 mg of protein) were dialysed and passed through a 5 ml carboxymethylcellulose column at a flow rate of 0.2 ml/min. After washing the column with 10 ml of buffer, the enzyme was eluted with a linear gradient of KCl.

Molecular properties of the purified enzyme

The purified preparation contained a single polypeptide of mol.wt. 50 000 as determined by polyacrylamide-gel electrophoresis in the presence of SDS (results not shown). This polypeptide was a very minor component of the 110 000 g pellet and its enrichment in the course of the purification correlated with the specific ATP phosphohydrolytic activity. The molecular weight was also determined by gel filtration on a Sephadex G-200 column equilibrated with 0.2 M-KCl to prevent inactivation (see above) and calibrated as described by Andrews (1964). The activity eluted slightly behind bovine serum albumin and a mol.wt. of 55 000 could be calculated (results not shown). These results indicate that the enzyme is a monomer, because similar molecular weights can be determined under native conditions and after dissociation with SDS. Electrophoresis in the absence of detergent in order to preserve the enzymic activity demonstrated that the major protein component of the preparation contained the ATP phosphohydrolytic activity (results not shown).

Table 2. Purification of the ATP phosphohydrolase activity of the 110 000 g pellet

Step	Protein (mg)	Total activity (μmol of P _i /min)	Yield (%)	Specific activity (μmol of P _i /min per mg)	Purification (fold)
110 000 g Pellet	240	440	100	1.8	1
Pellet washed with 0.1 M-KCl	170	400	90	2.4	1.3
Solubilized enzyme	12	310	70	26	14.5
Affi-Gel Blue	0.4	160	36	400	220
Carboxymethylcellulose	0.02	36	8	1800	1000

Table 3. Substrate specificity of the phosphohydrolase activity in the 110 000 g pellet and in the purified enzyme

The incubation medium contained 50 mM-Mes adjusted to pH 6.5 with Tris, 5 mM-MgSO₄, 2 mM-substrate and enzyme. Protein concentration was 50 μg/ml for the 110 000 g pellet and 40 ng/ml for the purified enzyme. The hydrolysis rate of the different substrates has been compared with that for ATP, which was 1.6 μmol of P_i/min per mg for the 110 000 g pellet and 1900 μmol of P_i/min per mg for the purified enzyme. Incubation was for 10 min at 30°C.

Substrates	Relative phosphohydrolase activity	
	110 000 g pellet	Purified enzyme
ADP, GDP or UDP	1.8	2.0
GTP	1.2	1.3
ATP, IDP, ITP or UTP	1.0	1.0
CTP or CDP	0.3	0.5
AMP	0.2	0.0
PP _i or triphosphate	0.1	0.0
p-Nitrophenyl phosphate	0.2	0.0
β-Glycerophosphate	0.0	0.0
Adenosine 5'-tetraphosphate	—	0.0
Adenosine 5'-[α,β-methylene]triphosphate	—	0.0

Substrate specificity

The purified enzyme hydrolysed several nucleoside di- and tri-phosphates at similar rates. The fact that both ADP and ATP are substrates (Table 3) indicates that the enzyme must be classified as ATP diphosphohydrolase (apyrase, EC 3.6.1.5) and not as ATP phosphohydrolase (ATPase, EC 3.6.1.3). The possibility that ADP hydrolysis occurs by prior conversion into ATP catalysed by contaminating adenylate kinase (EC 2.7.4.3) and hydrolysis afterwards by an ATP-specific enzyme can be discarded by the following evidence. The hydrolysis of ADP is faster than that of ATP, and this is not compatible with the indirect mechanism suggested above. Little or no adenylate kinase activity could be detected in the purified enzyme (less than 0.1% of the ATP phosphohydrolytic activity) and the specific adenylate kinase inhibitor P^1,P^5 -di(adenosine-5')penta-phosphate (Lienhard & Secemski, 1973) at a concentration of 1 mM was without effect on ADP hydrolysis. In addition, the presence of hexokinase (EC 2.7.1.1; 10 μ g/ml) and glucose (10 mM) did not affect ADP hydrolysis, and this combination should trap any intermediate ATP formed by adenylate kinase. Thermal inactivation of the enzyme by exposure for 10 min to temperatures between 40 and 70°C did not alter the relative rate of hydrolysis of ATP and ADP. Another indication that ATP and ADP hydrolysis are catalysed by the same enzyme is that the phosphohydrolytic activity with the mixture of both nucleotides is the mean value of the activities obtained with each nucleotide alone and not the addition of these activities.

The purified enzyme exhibited hardly any activity (less than 3% of the rate of ATP hydrolysis) with PP_i , tripolyphosphate and several monophosphoric esters as AMP, *p*-nitrophenyl phosphate and β -glycerophosphate (Table 3). These results clearly differentiate the enzyme from inorganic pyrophosphatases (EC 3.6.1.1) and from the group of phosphoric monoester hydrolases (EC 3.1.3). The observation that ATP and ADP are substrates, but AMP, adenosine 5'-tetrphosphate, adenosine 5'-[α,β -methylene]triphosphate, PP_i and tripolyphosphate are not hydrolysed may provide clues for the understanding of the catalytic mechanism of the enzyme.

The substrate specificity of the phosphohydrolase activity in the membranes from which the ATP diphosphohydrolase was purified is also presented in Table 3. In accordance with the observation that most of the ATP phosphohydrolase activity was solubilized and purified as a single component (see above), the specificity of the membranes and of the purified enzyme is very similar. The only difference is the presence in the membranes of some activity with phosphoric monoesters and pyrophosphate. Most of this phosphatase activity remained asso-

ciated with the membranes after solubilization of the ATP diphosphohydrolase and it hydrolysed ATP, AMP and *p*-nitrophenyl phosphate at similar rates.

Kinetic properties of the purified enzyme

The pH optimum for both ATP and ADP hydrolysis is 6.5 and the activity with 2 mM-substrate was decreased to 50% at pH 5.2 and 7.5. At pH values below 4 and above 9 the activity decreased to less than 10%. When Mg^{2+} was omitted in the assay, the rate of ATP and ADP hydrolysis was decreased to 10–20%, and EDTA in excess of 0.1 mM completely abolished the activity. Apparently traces of contaminant bivalent cations in the assay buffer partially satisfy the requirements of the enzyme. With 2 mM-ATP and 1 mM-bivalent cation, Mg^{2+} , Ca^{2+} , Mn^{2+} and Co^{2+} gave similar activity whereas Cu^{2+} and Zn^{2+} produced no increase in activity over that observed in the absence of added bivalent cation. In the presence of an excess of $MgSO_4$ over nucleotide of 1–10 mM, the enzyme exhibited hyperbolic kinetics with ATP and ADP concentrations in the range of 0.2 to 5 mM. The K_m values estimated from double-reciprocal plots were 0.2 mM for ATP and 0.5 mM for ADP.

K^+ , in the concentration range of 5–200 mM, was without effect on the activity of both the purified enzyme and the 110 000 g pellet. For these experiments the sodium salt of ATP was substituted for the Tris salt and the enzyme was dialysed to remove the KCl employed to elute the enzyme from the column. In addition, changing the pH from 4 to 9 and the temperature from 20 to 40°C failed to reveal any significant effect of K^+ .

The time course of ATP hydrolysis by the purified enzyme is depicted in Fig. 3. The stoichiometry of the reaction is two P_i molecules liberated from every ATP molecule, in accordance with the classification of the enzyme as an ATP diphosphohydrolase. ADP is formed transiently in the course of the reaction. In the initial phase the production of ADP and P_i is similar, but as the ADP is also hydrolysed by the enzyme, P_i production maintains its initial velocity, whereas the amount of ADP reaches a transient steady state and finally decreases to zero.

ATP phosphohydrolase activities in other fractions of the homogenate

The soluble fraction of the homogenate (110 000 g supernatant) contained a considerable amount of ATP phosphohydrolytic activity (Table 1). About half of this activity was not retained by a DEAE-cellulose column and corresponded to a non-specific enzyme(s) that hydrolysed *p*-nitrophenyl phosphate, β -glycerophosphate, AMP and PP_i in addition to ATP and ADP. The activity retained by the column could be purified by the same chromatographic methods employed for the enzyme solubilized from

Fig. 3. Time course of ATP hydrolysis by the purified enzyme

The purified enzyme (30 ng/ml) was incubated at 30°C with 0.75 mM-ATP, and at the indicated times, samples were boiled for 2 min to stop the reaction and were analysed for ATP (Δ), ADP (●) and P_i (○) as described under 'Methods'.

the 110000g pellet, and the final preparations of both enzymes exhibited identical kinetic and molecular properties (substrate specificity, K_m for ATP and ADP, pH-dependence and electrophoretic mobility in the presence or absence of SDS; results not shown). Most of the ATP phosphohydrolase sedimented at 15000g could be solubilized by 0.5 M-KCl, and although it could not be purified to homogeneity, it exhibited the same kinetic properties as the enzyme purified from the other fractions of the homogenate.

Discussion

In homogenates from *Cicer arietinum* roots, over 90% of the ATP phosphohydrolase activity associated with the membranes corresponds to an apyrase or ATP diphosphohydrolase (EC 3.6.1.5) that is non-stimulated by K⁺ and that can be solubilized by high-ionic-strength media. This enzyme has been purified to homogeneity, and its molecular and kinetic properties have been determined. The only ATP phosphohydrolytic activity previously purified from plant membranes is, to our knowledge, a non-specific phosphohydrolase solubilized from corn root microsomal fractions by NaClO₄ (Benson & Tipton, 1978). This enzyme hydrolyses several nucleotide triphosphates, ADP,

PP_i and *p*-nitrophenyl phosphate, and its activity was stimulated by salts such as KCl and NaCl. Clearly, neither this enzyme nor the apyrase we have purified from *Cicer arietinum* correspond to the ATP-specific enzyme stimulated by K⁺ described by Hodges & Leonard (1974) in oat root plasma membranes. Only preliminary attempts to purify this enzyme have been described (Du Pont & Leonard, 1980), and given the difficulties in characterizing enzymic activities in crude fractions, the nature of this hypothetical cation-transport ATPase remains obscure.

The apyrases were first described by Meyerhof (1945) in potato tubers, and further characterized in soluble form from the same source. Potato apyrase has a mol. wt. of 40000 (Traverso Cori *et al.*, 1970), requires Ca²⁺ or Mg²⁺ for activity (Molnar & Lorand, 1961), and hydrolyses nucleoside di- and tri-phosphate, but not monophosphoric esters or PP_i (Molnar & Lorand, 1961; Del Campo *et al.*, 1977). Two isoenzymes differing in their relative activity towards ATP and ADP have been described. Type A hydrolyses ATP more than 10-fold faster than ADP, whereas type B exhibits similar reaction rates with both nucleotides (Molnar & Lorand, 1961; Traverso-Cori *et al.*, 1970). The apyrase we have purified from *Cicer arietinum* resembles isoenzyme B of potato apyrase. Both enzymes can be differentiated by the observation that potato apyrase exhibits optimal activity at very alkaline pH (Valenzuela *et al.*, 1973), whereas the enzyme purified in the present work has no activity at pH above 9.

The subcellular distribution of potato apyrase is not known, but an insoluble form of the enzyme has been described (Krishnan, 1949), and as the purification of the soluble form involves treatment of crude homogenates with (NH₄)₂SO₄, it is possible that this enzyme is also bound to membranes by electrostatic interactions (Traverso-Cori *et al.*, 1965). In *Cicer arietinum* roots, the same apyrase purified from the microsomal membranes is also found in the soluble fraction of the homogenate. Solubilization of membrane-bound enzyme only occurs at ionic strengths greater than 0.3 M, and therefore under physiological conditions the enzyme is probably associated with membranes. The presence of the same apyrase in the soluble fraction of the homogenate may indicate that part of the activity is soluble *in vivo*. However, the homogenization of the tissue could cause some solubilization, for example by dilution of cellular bivalent cations.

The physiological function of apyrases is not known, but their association with cellular membranes and the fact that they hydrolyse energy-rich phosphate bonds in nucleotides is compatible with an energy-transducing activity. Recently, an apyrase associated with pig pancreas membranes has

been suggested to operate as a Ca^{2+} pump (Le Bel *et al.*, 1980). Further investigation is required to clarify the subcellular distribution and physiological function of apyrases. The identification of this type of enzyme in plant membranes may be useful in the search for the biochemical basis of H^+ and K^+ transport in plants. Even if apyrases are not involved in these functions, their presence in plant membranes may mask the ATP-hydrolysing activity of any other enzyme responsible for cation transport.

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