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INTERACTION OF NUCLEOSIDE TRIPHOSPHATE DIPHOSPHOHYDROLASE FROM PEA WITH A MEMBRANE PROTEIN FACTOR

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Key Word Index—*Pisum sativum*; pea; Leguminosae; ATP diphosphohydrolase; plant microsome; solubilization.

Abstract—The nucleoside triphosphate diphosphohydrolase (EC 3.6.1.5) purified from pea stem is precipitated at low ionic strength, pH 7, in the presence of Mg^{2+} by a protein released from the microsomal membranes. A Triton X-100 extract from enzyme-depleted microsomes was capable of promoting the sedimentation of the enzyme. The enrichment in this capacity was accompanied by the enrichment in a polypeptide of 34000 Mr.

INTRODUCTION

Diphosphohydrolases or apyrases (EC 3.6.1.5) have been purified from different tissues and species of higher plants [1–5]. The intracellular location and the physiological role of these enzymes are unknown. The ATP diphosphohydrolase which we have previously purified from pea stem is present either in membrane-bound form in the microsomal fraction, or in soluble form in the 220000 *g* supernatant obtained in the absence of Mg^{2+} during homogenization. The enzyme activity of this supernatant binds to the microsomal membranes at low ionic strength in the presence of Mg^{2+}. The same ionic conditions cause a sedimentation of the soluble activity, thus making questionable an evaluation of the microsome-bound activity, based on centrifugation. In the present paper, the factors influencing the physical state of the pure enzyme in the 220000 *g* supernatant and in microsomal extracts have been studied.

RESULTS

Sedimentation of the enzyme from the microsomal supernatant

Previous research showed that the soluble fraction (220000 *g* supernatant), obtained in the absence of Mg^{2+} during homogenization, is endowed with phosphohydrolase activity, part of which (*ca* 40%) is made sedimentable upon addition of 5–10 mM Mg^{2+}. The activity of the Mg-sediment is entirely due to the ATP diphosphohydrolase which has been purified, while the non sedimentable activity is due to other phosphatases [6]. The pure enzyme was not sedimentable when added to the homogenizing buffer, even in the presence of Mg^{2+} (Table 1, A). In the presence of the soluble fraction we found partial sedimentation, strongly stimulated by Mg^{2+}, of both the labelled enzyme, added in trace amounts, and the enzyme activity, coming essentially from the soluble fraction (Table 1, B). The effects of Mg^{2+} on both phenomena were very similar (see the relative values of precipitates in Table 1). The comparison was made possible by the almost complete recovery of the enzyme activity after centrifugation. From these results the lack of sedimentation of the purified enzyme in buffer and the sedimentation of the crude activity from the soluble fraction could not be attributed to different properties of the purified and crude enzyme. The soluble fraction was effective in sedimenting the added enzyme also after desalting by Sephadex G-25 (Table 1, C). No sedimentation of pure enzyme was observed in the presence of 0.1% serum albumin or gelatin (not shown).

Sedimentation of the enzyme in soluble preparations from the microsomes

According to previous findings [5, 6] *ca* 85% of the microsome-bound ATP diphosphohydrolase and 44% of protein were solubilized by resuspending the microsomal pellet in 0.3 M sodium chloride at pH 7. The solubilized activity (220000 *g* supernatant) was made partially sedimentable at 100000 *g* after reducing the ionic strength by dilution and adding Mg^{2+} (Table 2, first sodium chloride washing). After three washes with sodium chloride the microsomes contained *ca* 1% and 33% of the original enzyme activity and protein, respectively. To investigate if some membrane factors, causing the sedimentation of the enzyme, were released from the microsomal membranes, we treated the enzyme-depleted microsomes with Triton X-100 at high ionic strength. The solubilized Triton X-100 preparation (Triton X-100 crude extract) was capable of inducing a partial sedimentation of the added enzyme, while a parallel washing without the detergent (4th sodium chloride washing) was not (Table 2). Starting from the Triton X-100 extract we obtained a preparation strongly enriched in the enzyme sedimenting capacity by using a procedure based essentially on precipitation at low ionic strength, followed by resolubilization with the detergent at high ionic strength (see in Experimental, enzyme-sedimenting, partially purified preparation). The data of Table 2 show 85% sedimentation of the added enzyme in the presence of this partially purified preparation, after lowering the ionic strength by dilution and adding Mg^{2+}. In the presence of the same amount of protein from the Triton X-100 crude extract the sedimentation was only 17%. The enrichment in the enzyme-sedimenting capacity was accompanied by an enrichment in a polypeptide of 34000 Mr (see later). The presence of 1% Triton X-100 in the diluting buffer did not change the results substantially (not shown).

Table 1. Sedimentation of the added enzyme and of the enzyme activity present in the soluble fraction

Labelled enzyme, added to:	$MgCl_2$ (mM)	ATP diphosphohydrolase activity (units)			Radioactivity (cpm)		
		sn + ppt	ppt	ppt, relative*	sn + ppt	ppt	ppt, relative*
(A) Homogenizing buffer	0	1.437	0.007	—	17200	370	—
	5	1.350	0.010	—	17900	350	—
(B) Soluble fraction	0	0.808	0.167	100	9800	4000	100
	1	0.785	0.216	133	9300	5100	136
	3	0.792	0.267	163	9500	6600	173
	5	0.759	0.275	175	9500	6700	175
(C) Desalted soluble fraction	0	0.928	0.083	100	7300	740	100
	5	0.743	0.230	244	6000	2100	235

^{125}I-ATP diphosphohydrolase (0.1 μg) was alternatively added to 20 ml of: A, homogenizing buffer, supplemented with cold enzyme; B, soluble fraction; C, the same, after desalting. The soluble fraction contained *ca* 8 units of enzyme activity in 20 ml, thus, the contribution of the added labelled enzyme (0.03 units) was negligible. Samples of 2 ml of the enzyme mixture were incubated at 0° for 1 hr, in the presence or absence of $MgCl_2$, as indicated, then centrifuged at 100 000 *g* for 20 min. The enzyme activity was determined in the supernatants (sn) and in the precipitates (ppt), as described in Experimental. The radioactivity was assayed on parallel aliquots of the supernatants and the resuspended precipitates. The data refer to typical experiments, run in duplicate or triplicate and repeated at least three times each, with similar results.

*The relative values of sedimentation were calculated from the ratios of activity or radioactivity: ppt/sn + ppt, made equal to 100 in the absence of Mg^{2+}.

Table 2. Sedimentation of the enzyme added to microsomal extracts

Preparation	Protein (μg)	Activity (units)			Sedimentation $\left(\frac{ppt}{sn+ppt} \times 100\right)$
		from preparation	added	recovered (sn + ppt)	
First NaCl washing	108	0.125	none	0.121	28%
		0.125	0.250	0.343	29%
4th NaCl washing	5.2	0.002	0.250	0.230	2.6%
Triton X-100 crude extract	80	0.020	1.620	1.540	17%
Partially purified preparation	80	0.041	0.405	0.372	90%
		0.041	1.625	1.550	85%
		0.041	13.000	12.700	80%
Triton X-100 buffer	—	—	0.405	0.355	2%
	—	—	1.625	1.580	1.3%
	—	—	13.000	12.800	1.8%

In each case the indicated preparations or the buffer were mixed with pure enzyme (sp. act.: 740 units/mg in 50 μl volume, in which the NaCl concentration was 0.3 M. After 10 min at 0°, 450 μl of 1.5 mM $MgCl_2$, 20 mM Hepes (pH 7) were added to each sample, bringing the NaCl concentration to 30 mM. The final concentration of EDTA coming from the enzyme preparation was negligible (less than 10 μM). After 60 min incubation at 0°, the samples were centrifuged at 100 000 *g*, and the enzyme activity was determined in the supernatants (sn) and in the precipitates (ppt), as described in Experimental. The table summarizes data of typical experiments, run in duplicate and each repeated at least three times with comparable results.

Analysis of the enzyme precipitates by SDS gel electrophoresis

The enzyme, purified as previously described [5] showed a single band of 47 000 Mr (Fig. 1, A). The enzyme band was present in the precipitate obtained by adding Mg^{2+} to the soluble fraction (Fig. 1, B). A soluble preparation obtained by treating the intact microsomes with Triton X-100 at high ionic strength gave, upon dilution and Mg^{2+} addition, a precipitate which contained the enzyme band and a major polypeptide band of 34 000 Mr (Fig. 1, C). The Triton X-100 crude extract from the enzyme depleted microsomes also showed the 34 000 Mr band (Fig. 1, D). The enzyme-sedimenting, partially purified preparation was strongly enriched in this band (Fig. 1, E). As expected, the precipitate obtained from a mixture of this preparation and the pure enzyme contained both the enzyme and the 34 000 Mr polypeptide (Fig. 1, F). This polypeptide was also present in the precipitate from the soluble fraction, though at relatively lower amount (Fig. 1, B).

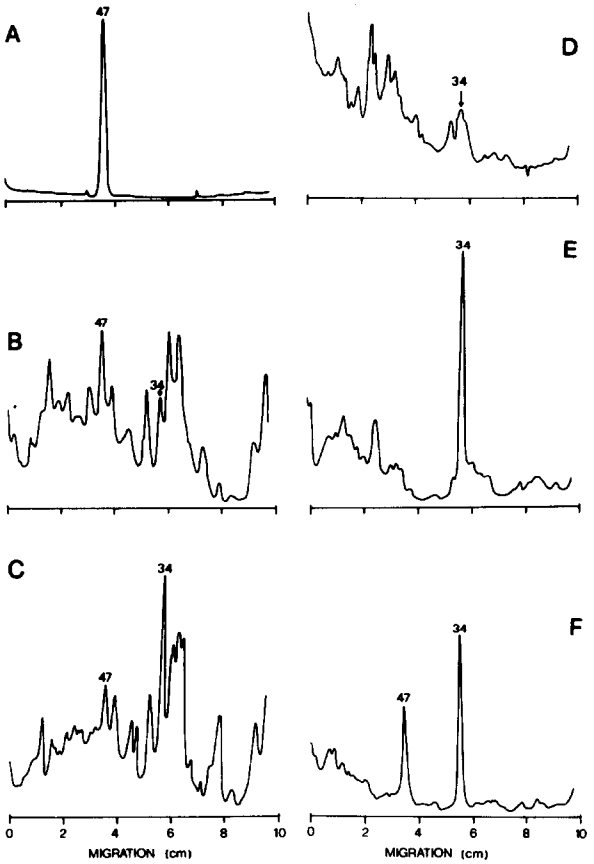

Fig. 1. SDS polyacrylamide gel electrophoresis of the enzyme precipitates. Densitometric tracings of gels stained with Coomassie brilliant blue R-250. The numbers indicate the values of Mr $\times 10^{-3}$. For reference, the Biorad low MW standard was used. Gels were loaded in A, with 2.5 μg of enzyme; in B–F, with 30–50 μg protein, including 1 μg of enzyme in F. The samples (usually 10% TCA-precipitates, washed once with water) were treated with 2% SDS, 1% mercaptoethanol and left to stand at 30° for 15 hr prior to electrophoresis.

DISCUSSION

The ATP diphosphohydrolase from pea stem is associated with the microsomal particles, from which it can be solubilized by salt washings. In the absence of Mg^{2+} in the homogenizing medium part of the microsome-bound enzyme is recovered in the soluble fraction (220 000 *g* supernatant). This soluble activity can either re-associate to the microsomes or sedimentate in their absence at low ionic strength and in the presence of Mg^{2+} [6]. The present data show that at pH 7 the pure enzyme is soluble at low ionic strength in the presence of Mg^{2+} and that the sedimentation of this enzyme, added to the soluble fraction, as well as the sedimentation of the enzyme activity of this fraction, depends on the presence of at least one factor of high MW.

When sodium chloride-washed, enzyme-depleted microsomes are treated with Triton X-100 at high ionic strength they also release a factor capable of promoting the sedimentation of the enzyme at low ionic strength and in the presence of Mg^{2+}. This factor can be tentatively identified with a polypeptide of 34 000 Mr, as its enrichment in a partially purified preparation from the Triton X-100 extract is accompanied by increased enzyme-sedimenting capacity. The presence of this polypeptide in the soluble fraction further supports this idea, suggesting that the 34 000 Mr polypeptide is involved in the enzyme sedimentation also in this case. The interaction of the enzyme with this polypeptide, independently of their co-precipitation, could represent a step of a reconstitution process.

The requirement for both salt and detergent for the removal of the 34 000 Mr polypeptide from the microsomes indicates the involvement of both electrostatic and hydrophobic forces in its binding to the membrane. The former seem to prevail in the sedimentation, which only depends on the ionic environment and not on the removal of the detergent.

The presence in homogenates of a protein which co-precipitates with the enzyme may explain the difficulty in assessing the location of the enzyme by using centrifugal methods and, consequently, the high number of locations which have been proposed, including cell wall [7], plasmalemma [3], nucleus [8] and multiple locations and soluble [9]. The presence of ATP diphosphohydrolase in the soluble fraction clearly depends on low Mg^{2+} concentration during cell disruption, although a certain solubilization might also result from the action of phospholipases and proteases coming from the cells.

EXPERIMENTAL

The microsomal fraction (13 000–80 000 *g* pellet) was prepared from etiolated pea epicotyls as described in ref. [6], using the Mg-less homogenization buffer. The 80 000 *g* supernatant was further centrifuged at 220 000 *g* for 60 min to obtain the 220 000 *g* supernatant, referred to as the soluble fraction. To deplete the microsomes of the enzyme, the microsomal pellet from 1 kg of tissue was washed × 3, each by resuspending the ppt in 100 ml of 0.3 M NaCl, 20 mM Hepes, pH 7 with KOH, and centrifuging the suspension at 220 000 *g* for 20 min. All the preparative procedures were performed at 0–4°.

The Triton X-100 crude extract from the enzyme-depleted microsomes was obtained by resuspending the salt-washed microsomal pellet (315 mg protein) in 100 ml of 1% Triton X-100, 0.3 M NaCl, 20 mM Hepes, pH 7. After 10 min at 0° the suspension was centrifuged at 220 000 *g* for 20 min. The super-

natant, containing 220 mg protein, was used immediately or after storing at −80°.

The enzyme-sedimenting, partially purified preparation was obtained from the Triton X-100 crude extract (100 ml) by dialysis for 14 hr against 2 l. of 2 mM $MgCl_2$, 20 mM Hepes, pH 7, followed by centrifugation at 100000 *g* for 20 min. The ppt (5.3 mg protein) was resuspended in 1.5 ml of 2% Triton X-100, 0.3 M NaCl, 20 mM Hepes, pH 7, then clarified by sonication (20 kHz, three pulses of 30 sec, each) and centrifuged at 220000 *g* for 20 min. The supernatant, containing most of the protein, was used in the experiments.

The enzyme was purified as previously described [5]. By keeping the central part of the activity peak, eluted from the DE-52 column, we eliminated the residual 5% of impurity and obtained a single polypeptide band on SDS electrophoresis (see Results). The enzyme was iodinated at specific radioactivity of 15–25 μCi of $^{125}I/\mu g$ by using the method described in ref. [10]. The sp. act. of the unlabelled enzyme was 740 units/mg. After labelling it decreased to 350 units/mg.

The incubation of the enzyme with the soluble fraction or the preparations from microsomes in sedimentation experiments (0° for 60 min) was run in glass tubes, as some adsorbtion of the enzyme on plastic tubes was detected. The samples were transferred to centrifuge polycarbonate tubes immediately prior to centrifugation (100000 *g* for 20 min). After centrifugation, 0.3 M NaCl (final concn) was added to the supernatants, and the ppts were resuspended with a teflon pestle in the respective supernatant buffer supplemented with 0.3 M NaCl, at a vol. corresponding to the vol. of the supernatant. The salt was added to have the enzyme in the soluble form, as this allowed good reproducibility of data and high recovery of enzyme activity.

The ATP diphosphohydrolase activity was assayed at 26°, in the presence of 2 mM ATP-Mg, 30 mM KCl, 20 mM Tris–Mes (pH 6), in one ml vol. The reaction was started by addition of the enzyme mixture and stopped by adding the Pi reagent, as described in ref. [6]. One unit is defined as the amount of enzyme catalysing the liberation of 1 μmol of orthophosphate/min at 26°. The SDS polyacrylamide gel electrophoresis was performed as described previously [4], except for the gel length (100 mm) and the acrylamide concentration (12.5%). The concn of protein was determined by the method of ref. [11], with bovine serum albumin as a standard.

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