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IMPROVED PREPARATION AND ASSAY AND SOME CHARACTERISTICS OF Cl^--ATPase ACTIVITY FROM *LIMONIUM VULGARE*

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We present a revised method for the preparation, storage and assay of the Cl^--ATPase activity responsible for salt secretion in *Limonium vulgare*. Altering the centrifugation step improved the yield and a linked-enzyme assay for ADP provided a sensitive method for continuous monitoring of Cl^--ATPase activity. The activity of untreated membranes was low but fairly stable. Treatment with detergent gave strong stimulation of Cl^--ATPase activity but caused a rapid decline in activity with time. V_{app} was approx. 6.5 μmoles/h per mg protein and $K_{m,app}$ for ATP between 0.1 and 0.2 mM. Cl^- stimulated the activity up to a maximum at 0.13 M Cl^-, and the pH optimum was around 6.3 to 6.4.

Introduction

Limonium vulgare, a saltmarsh plant, excretes salt through specialized glands on the leaf surface. A chloride-stimulated ATPase has been demonstrated in membrane preparations from leaves of salt-treated plants and appears to be responsible for salt secretion [1].

We present here a modified method for membrane preparation, a more sensitive assay and further data on the Cl^--ATPase activity which these impovements have enabled us to measure.

At present we are not able to purify the enzyme satisfactorily by affinity chromatography [2], and the results of work on the affinity adsorption of membrane components and their haemagglutinating activity will be published elsewhere. We present here some of the characteristics of the enzyme after partial solubilization of the membrane to obtain maximal activity.

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulphonic acid; Tes, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)-ethanesulphonic acid.

Materials and Methods

Most plants were grown in greenhouse conditions, temperature minimum 20°C, with 16 h illumination to supplement daylight. Leaves were harvested regularly to encourage new growth for membrane preparation. Some plants were kept at 25°C under constant illumination (52 W/m^2 between 400 nm and 700 nm wavelength, from 'warm white' tubes manufactured by Thorn). Their leaves were larger and more fragile.

NADH, phospho*enol*pyruvate, pyruvate kinase and lactate dehydrogenase were purchased from Boehringer, 2-([2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino)ethanesulphonic acid (Tes) and detergents from Sigma, 2-(*N*-morpholino)ethanesulphonic acid (Mes) from Ultrol, and polyclar AT from Serva. Other chemicals were from BDH.

Protein concentration was measured by the method of Schaffner and Weissman [3].

Results

Measurement of ATPase activity

We now use a linked enzyme assay, which has

several advantages over the measurement of the release of phosphate more commonly used for plant ATPases [2,4,5]. In the linked-enzyme assay, ADP production is quantitatively related to the oxidation of NADH which can be monitored continuously as absorbance at λ 340 nm, allowing initial rates of ATP hydrolysis to be determined with ease. Provided that ATP hydrolysis is always the rate-limiting step in the reaction sequence, kinetic parameters of the ATPase can be measured in a variety of conditions.

The method is based on an assay [6] for the estimation of ADP, and a similar system has been used by Møller et al. [7] and Warren et al. [8] to measure ATPase activity in sarcoplasmic reticulum from rabbit skeletal muscle.

The following reactions are used:

$$ATP \xrightleftharpoons{ATPase} ADP + P_i$$

ADP + phospho*enol*pyruvate

$$\xrightleftharpoons{\text{pyruvate kinase}} \text{pyruvate} + ATP$$

$$\text{pyruvate} + NADH \xrightleftharpoons{\text{lactate dehydrogenase}} NAD + \text{lactate}$$

ΔA_{340} was monitored continuously by a Pye Unicam 1750 recording spectrophotometer.

Standard conditions. The incubation medium was 30 mM Tes buffer solution, pH 7.9, containing 2 mM $Na_2 \cdot$ EDTA, 330 μM NADH, 660 μM phospho*enol*-pyruvate, 4.4 mM magnesium acetate and 16 mM potassium acetate. Incubation medium was made 0.12 M with respect to sodium benzene sulphonate or NaCl for assay, and the pH readjusted if necessary.

1 ml of incubation medium was warmed to 25°C in semi-micro cuvettes. 20 μl of membranes and 2 μl each of pyruvate kinase and lactate dehydrogenase were added to both reference and sample cuvettes. After ensuring a level baseline, a small, measured volume (μl) of 0.1 M ATP, 0.1 M magnesium acetate solution was added to the sample cuvette, and the rate of hydrolysis measured. More ATP was added to provide a series of initial rates at different ATP concentrations. Since pyruvate kinase regenerates ATP, initial concentrations of ATP are maintained. Chloride-dependent ATPase activity was measured as the difference between the rate in NaCl and the rate in sodium benzene sulphonate at the same concentration of ATP.

Mg^{2+} was included as a constant excess of Mg over ATP as recommended by Cornish-Bowden [9]. This gave more linear recorder-traces than before which suggests that Mg ATP is a substrate for the reaction. Mg^{2+}-excess up to 10 mM did not affect Cl^--specific activity but there was some indication that it reduced ATPase activity in the benzene sulphonate blank.

For chloride-dependency studies up to 0.12 M, NaCl and sodium benzene sulphonate incubation media were mixed in varying proportions. Some higher concentrations of chloride were used but not of benzene sulphonate which was found to inhibit the linking enzymes at higher concentrations.

NaCl did not affect the activity of the linking enzymes at any of the concentrations used.

For pH-dependency studies, a mixture of 30 mM Tes and 30 mM Mes was used over the whole pH range investigated to eliminate any chemical effect of the buffers.

Preparation of membranes

In the previous method [2], leaf slices were incubated with aeration in 0.15 M NaCl solution overnight, homogenised in the presence of Polyclar AT, centrifuged for 20 min at 12 000 × *g* and then for 60 min at 100 000 × *g*. This second, 'microsomal' pellet was used initially for activity studies but Cl^--ATPase activity was not always reproducible. The edges of leaf slices showed severe tissue damage and browning characteristic of polyphenols.

During attempts to improve the yield of Cl^--ATPase we took the youngest fully expanded leaves, split the midrib with a razor and floated them on 0.15 M NaCl solution overnight under tungsten filament illumination. Such leaves do not show any damage or browning and vigorously secrete salt solution. After rinsing, the leaves were sliced into homogenization buffer [2] containing Polyclar AT and approx. 3 mg/ml bovine serum albumin to try to protect Cl^--ATPase activity.

The unique protection afforded by bovine serum albumin during enzyme preparation has been attributed to its ability to react in all the principal ways in which proteins can combine with phenols. It is also an effective quinone scavenger and has a high capacity to bind to lipids [10].

The low speed supernatant was much greener and more viscous and the high speed pellet larger than

TABLE I

CONSEQUENCES OF VARYING SOME OF THE PARAMETERS OF MICROSOME PREPARATION

Homogenization regime		Initial centrifugation (pellet discarded)		Subsequent centrifugations (pellet assayed)				Cl-ATPase activity
				Time (min)	Fraction taken		Size of the pellet	
Bovine serum albumin: present + absent 0	Time (s)	Time (min)	x (1000 × g)		from x (1000 × g)	to y (1000 × g)		high: +++ low: +
0	35	20	20	75	20	100	small (brown)	0/+
+	35	20	20	75	20	100	large (green)	+++
0	35	10	5	15	5	10	small	+
				15	10	20	small	+
+	35	10	5	15	5	10	medium	+
				15	10	20	medium	++
0	60	10	5	15	5	12	large	++
				15	12	20	medium	+
0	60	10	2	75	2	100	large	+++

when bovine serum albumin was not included. Cl^--stimulated ATPase activity was detectable in all preparations using bovine serum albumin. It seemed that one effect of bovine serum albumin might be to bring into the high speed pellet membranes which before had sedimented at low speed. SDS-polyacrylamide gel electrophoresis showed this protein to be in the high speed pellet and, even, in membrane fractions distributed through a sucrose linear density gradient. Specific activities, therefore, could not be estimated.

We investigated different homogenization and centrifugation regimes to try to obtain, in the absence of bovine serum albumin, the membranes which appeared in the 12000 to 100000 × g pellet in the presence of this protein. Results are shown in Table I. The yields of membranes and Cl^--ATPase activity varied with the age and growth conditions of leaves, and the homogenization procedure, in addition to the presence or absence of bovine serum albumin. The results confirm that in previous methods Cl^--ATPase activity had been discarded in the low speed pellet, sometimes all of it perhaps.

Kylin and Gee [11] working on *Avicennia* describe similar problems in using mature leaves, and for maize coleoptiles Hendricks [12] comments on variation in apparent activities and distribution between pellet and supernatant of the plasma membrane markers glucan synthetase and K^+-ATPase after different homogenization and centrifugation procedures.

The method described below yielded reproducible, high levels of Cl^--stimulated ATPase activity:

About 20 g leaves were slit along the midrib and floated on 0.15 M NaCl solution overnight at room temperature under illumination. All subsequent steps were carried out between 0 and 4°C. The leaves were rinsed and sliced into 270 ml homogenization buffer (0.1 M Tes, 2 mM Na_2 · EDTA, 0.5 M sucrose, 20 mM $K_2S_2O_5$ solution, pH 7.0) containing 20 g Polyclar AT. The slices were homogenised for 1 min in a Kenwood blender and the brei squeezed through four layers of muslin. The supernatant from a 10-min centrifugation at 2000 × g (Beckman J2-21) was then centrifuged for 75 min at 100000 × g (Beckman L5-50). The 100000 × g pellets were resuspended in 4 ml of 30 mM Tes buffer solution, pH 7 containing 2 mM Na_2 · EDTA and 5 mM dithiothreitol, and assayed.

Properties of the Cl^--dependent ATPase

Usually Cl^--ATPase activity was undetectable until detergent was added. Triton X-100, poly(oxyethanol-9-lauryl ether) and poly(oxyethylene ether) W-1, all

at 0.25% v/v in resuspended membranes gave comparable, strong stimulation up to the same level of activity. Brij 56 and 96 stimulated the activity to a lesser degree. Because in most cases the specific activity of untreated membranes was zero, it is difficult to quantify the stimulation. In a few preparations Cl^--ATPase activity was detectable in the absence of detergent, but never at more than 8% of the fully stimulated level.

However, detergent treatment also caused a rapid decline in activity with time. If the medium was made 20% v/v in glycerol this decline was slower, as described by Dean and Tanford [13] and Møller et al. [7] for a Ca^{2+}-dependent ATPase. Membranes prepared in the presence of bovine serum albumin were less susceptible to detergent denaturation of the activity. The presence of Cl^- or ATP in the detergent-treated preparation did not help to stabilize the activity.

Cl^--ATPase activity in membrane preparations was stable on ice for several hours. During this time the addition of detergent immediately before assay increased the activity to the same level. Preparations were stored as resuspended membranes in small proportions under liquid N_2. Consistent activity was measured at intervals during several months storage, although after the single freeze-thaw process the activity was slightly lower than that of freshly prepared membranes.

Identical preparations from plants grown without salt treatment showed no activity in the presence of detergent using the new, linked-enzyme assay, indicating that we are still measuring only salt-inducible activity associated with chloride-pumping.

For the experiments reported below, two large preparations were stored in liquid N_2. Both gave similar results although one preparation gave a K_m that was apparently lower under the conditions used (discussed later).

Determination of kinetic parameters

Estimates of $K_{m,app}$ and V_{app} were made under standard assay conditions. For each experiment, parameters were estimated by the method of Eisenthal and Cornish-Bowden [14]. The data are also represented in Fig. 1, combined for each of the two preparations.

For the first preparation, *a*, ATP was used at 0.2 to 1.2 mM. At high ATP concentrations, recorder-traces were occasionally curved, possibly an effect on the linking enzymes, and so rates could not be measured accurately. Therefore for the second preparation, *b*, a lower range of ATP concentration, 0.05 to 0.5 mM, was used. V_{app} for both preparations was similar: *a*, 6.6 ± 0.32; *b*, 6.4 ± 0.21 μmol/h per mg protein. The estimates for $K_{m,app}$ are: *a*, 0.197 ± 0.03, and *b*, 0.09 ± 0.014.

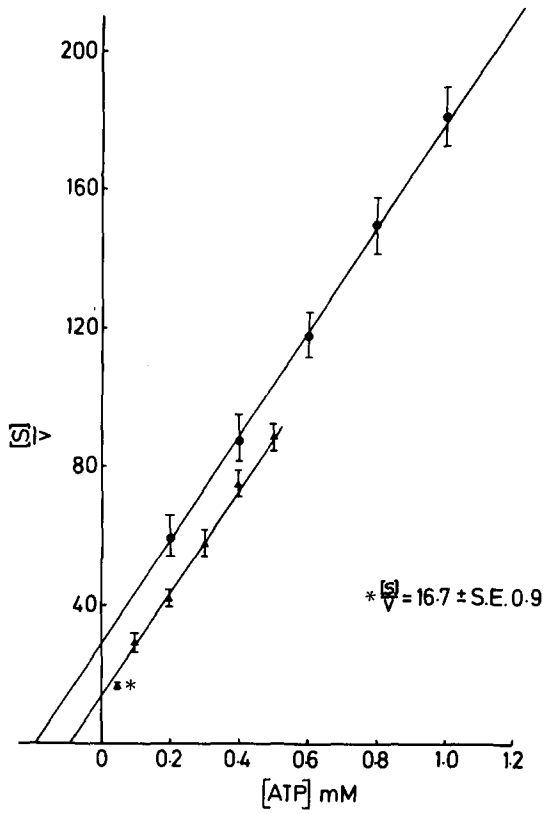

Fig. 1. A plot of $[S]/V$ against [S] for preparation *a* (•) and preparation *b* (▲). The initial velocity, *v*, was measured, as described in the text, in a chloride concentration of 0.12 M, pH 7.0 and at 25°C. The error bars represent the standard error from 11 determinations for preparation *a* and six determinations for preparation *b*.

Note in Fig. 1 that at 0.05 mM ATP the point is off the precise line dictated by the other points and the standard error is so small we believe this deviation is real. We suggest there may be a site with higher affinity for ATP detected at low substrate concentration. Two values for $K_{m,app}$ have been reported for Ca^{2+}-ATPase using concentration ranges of ATP of

0.45 to 50 μM and 0.01 to 10 mM on detergent-solubilized vesicles of sarcoplasmic reticulum [7].

Since rates of ATP hydrolysis by Cl^--stimulated ATPase were low, they were especially difficult to estimate at ATP concentrations lower than those presented here.

The relatively large standard errors for $K_{m,app}$ could be because slight differences in the degree of solubilization by detergent may have a greater effect on $K_{m,app}$ than on V_{app}. It is also possible that slight denaturation of the enzyme during extraction affects $K_{m,app}$ which could explain the difference in $K_{m,app}$ between the two preparations.

Chloride dependency

Rates of Cl^--stimulated ATP-hydrolysis were measured at chloride concentrations between 0.015 M and 0.24 M. The results are plotted as points in Fig. 2. We have drawn the best-fitting Michaelis-Menten curve through the points since, though they do not establish it, these data strongly suggest that Cl^--stimulation obeys Michaelis-Menten kinetics. The data are not sufficient to determine the characteristics of Cl^--stimulation quantitatively but they show that any concentration of Cl^- will stimulate Cl^--pumping, and that ATP-hydrolysis increases with chloride concentration up to a maximum at approx. 0.13 M Cl^-.

The lower-than-expected values of V at higher Cl^- concentrations may be due to inhibition by high ionic strength. Also, because benzene sulphonate could not be used at 0.24 M, Cl^--stimulation may have been underestimated.

At the other extreme, measurement of activity at very low Cl^- concentrations is difficult because Cl^--specific activity is so low.

Note in Fig. 2 that values obtained from the two preparations, *a* and *b*, are distributed on both sides of the line indicating that, despite the different $K_{m,app}$ of the two preparations, there is no difference in the characteristics of Cl^--dependency. $K_{m,app}$ for ATP did not vary much over this range of Cl^--concentration for either preparation, indicating that Cl^--binding by the ATPase does not alter its affinity for its substrate, ATP, i.e. there are no obvious co-operative effects.

pH dependency

The effect of pH on chloride-stimulated ATP-hydrolysis is shown in Fig. 3. Results from prepara-

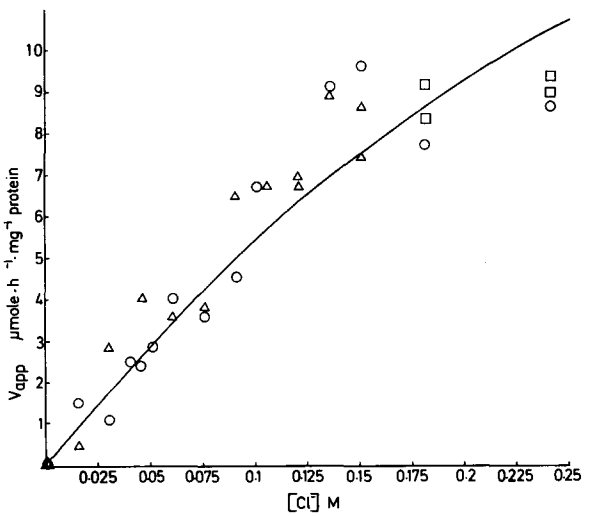

Fig. 2. A graph showing the relationship of V_{app} and $[Cl^-]$ for Cl^--specific ATPase activity. Data from preparations *a* (△) and *b* (○) are included. (□, individual points at saturating ATP concentration.)

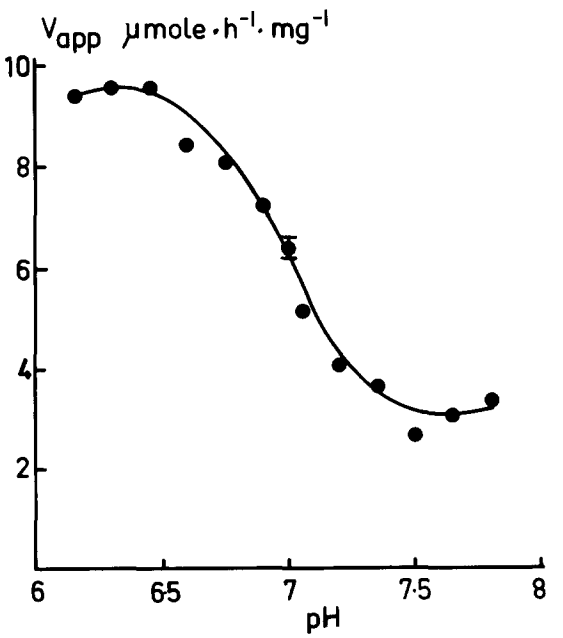

Fig. 3. The effect of pH on Cl^--specific ATPase activity measured at 25°C and a chloride concentration of 0.12 M.

tion *a* are presented. Similar results have been obtained with other preparations.

One limitation of this assay method is that above pH 8, where other ATPases have an optimum, and below pH 6.2 are outside the effective range of the linking enzymes. We have checked and confirmed the effectiveness of the linking enzymes in our system at each pH at which Cl^--ATPase was measured.

Within the range measured, the pH optimum of the Cl^--ATPase appeared to be around 6.3 to 6.4 which is quite common for ATPases [4,15]. We cannot exclude the possibility of other pH optima outside this range. The maximum effect of pH was around neutrality.

$K_{m,app}$ showed a tendency to increase with increasing pH, probably reflecting ionization of ATP unfavourable for enzyme-binding.

Discussion

Up till now progress in the investigation of the control of salt excretion by the salt-glands of *Limonium* leaves has been hampered by wide variations in the activity of Cl^--ATPase prepared from the tissue [1,2].

We found that at least part of this variation was due to low and variable recoveries of the total activity of the homogenate during centrifugation. In the improved method of preparation reported here, the recovery of activity is increased and the variability of recovery greatly decreased.

The original assay method [1] also contributed to the variation in activity. The method of Fiske and SubbaRow for the determination of phosphate was found to be unsuitable for Cl^--ATPase assay because it is subject to interference by detergents, and because the substrate, ATP, released more phosphate during phosphate determination. Also the original assay involved measuring the accumulated product, phosphate, after one hour and so the initial rate of reaction could not be measured or monitored. The linked-enzyme assay we present here is not subject to these limitations. It has provided consistent values for the specific activity of Cl^--ATPase in *Limonium* leaf microsomes which are much higher than those obtained using the previous assay.

Our results support the suggestion that the Cl^--specific ATPase is the enzymatic activity responsible for salt excretion [1]. The marked response to detergent suggests that the activity is associated with an integral membrane protein. The response to different concentrations of chloride is consistent with a chloride-pumping mechanism capable of operating over a wide range of concentrations. Finally, the $K_{m,app}$ for ATP is appropriately low, indicating that this activity could operate in physiological concentrations of ATP.

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