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# Properties of the Chloride-ATPase from *Limonium* Salt Glands: Activation by, and Binding to, Specific Sugars

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*Summary.* Attempts to separate membrane fractions enriched in $Cl^-$-ATPase activity from *Limonium* leaf microsomes were hampered because, it seemed, the microsomal membranes were aggregated in clumps. We found hemagglutination activity, specific for N-acetylgalactosamine and to a lesser extent galactose, in the soluble phase of the homogenate, and we were able to prevent membrane aggregation by adding galactose to the microsomes. We discovered that the $Cl^-$-ATPase activity of the microsomes was increased by galactose and to an even greater extent by N-acetylgalactosamine. We report that the $Cl^-$-ATPase binds to galactosamine-sepharose, from which it can be eluted in 0.1 M galactose, i.e., the enzyme is associated with a saccharide-binding site similar to that of the hemagglutinins. This procedure results in a 100-fold enrichment of the $Cl^-$-ATPase activity and represents a new way of purifying a membrane-bound enzyme from a heterogeneous membrane preparation in one step. Enzyme isolated by affinity chromatography of Triton-solubilized membranes was essentially free of other ATPase and accounted for a substantial proportion (sometimes all) of the $Cl^-$-ATPase of the microsomes. This purified preparation of the enzyme shows N-acetylgalactosamine-specific hemagglutination activity. However, we can show that the $Cl^-$-ATPase and the hemagglutinins are different entities. Thus, material isolated in the same way from salt-free plants showed hemagglutination but not $Cl^-$-ATPase activity. Also, the hemagglutinins, but not the $Cl^-$-ATPase, will bind to galactosamine-sepharose in the absence of ATP.

This is the first report of enzyme activity associated with a carbohydrate receptor-specific protein. Possible roles for saccharide-binding in the control, assembly, and orientation of the chloride-pump are discussed.

Sea lavendar (*Limonium vulgare* Mill.), a saltmarsh plant, gets rid of the salt which reaches the leaves during transpiration by secreting concentrated brine out of multicellular glands at the leaf surface. Each gland is enclosed in a box of cuticle pierced by plasmodesmata, the points of entry for salt from the cytoplasm of mesophyll cells. The force driving salt through the gland is active pumping of chloride ions

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outwards across the plasma membrane. The result is to draw sodium ions and water out into the extracellular compartment of the gland where this strong salt solution accumulates until it weeps through pores in the cuticle onto the leaf surface (Hill & Hill, 1973*a*).

Hill and Hill (1973*b*) have shown that chloride-pumping is linked to the hydrolysis of ATP and have demonstrated chloride-stimulated ATPase activity in microsomal preparations from *Limonium* leaves. The microsomal $Cl^-$-ATPase correlated strongly with electrophysiologically detected chloride pumping. Thus both activities were low in salt-free plants, but were induced in full over a period of 4 hr by the presence of salt. Again, for both activities this induction was blocked by puromycin, which did not inhibit either activity once it had been induced.

In subsequent experiments $Cl^-$ and ATP isotherms for the enzyme were obtained but activities at lower substrate concentrations were very variable due to the low specific activity of the enzyme in the microsomal preparation. We describe results obtained while attempting to purify the $Cl^-$-ATPase activity from microsomal preparations from *Limonium* leaves.

## Materials and Methods

*Limonium vulgare* Mill. plants from a coastal saltmarsh were grown in the greenhouse. For comparative studies two groups of plants were grown on vermiculite and watered with distilled water (salt-free) or 0.2 M NaCl. $Cl^-$-free Hoagland's solution was added at intervals to replenish essential minerals.

### *Preparation of Microsomes*

Fresh leaves, 20 g, were taken from the appropriate plants, sliced, and incubated overnight in distilled water (salt-free) or 0.15 M NaCl solution. The solutions were aerated and lit continuously by a tungsten filament bulb. All the following steps were carried out at 4 °C. The leaves were homogenized with 20 g polyclar AT in 7 volumes of buffer containing: 0.5 M sucrose, 10 mM $K_2S_2O_5$, 2 mM EDTA, and 0.1 M TES-NaOH, pH 7.0 to 7.1. After being strained through muslin, the homogenate was centrifuged for 20 min at $20{,}000 \times g$ and the pellets were discarded. The supernatant was centrifuged at $100{,}000 \times g$ for 60 min. to obtain microsomal pellets.

### *Preparation of Hemagglutinin Solutions*

The microsomal supernatant was passed through a 0.22 μm pore-size millipore filter and Triton X-100 added to a final concentration of 0.1% vol/vol (supernatant). The material on the millipore filter was extracted in 1% vol/vol Triton X-100 in phosphate buffered saline (filterable membranes) and the microsomal pellets dispersed in a small volume of

the same solution in a glass homogenizer (pelletable membranes). These fractions were passed through 2 × 1-cm columns of Sepharose-N-caproyl galactosamine (Allen & Neuberger, 1975). After washing with phosphate-buffered saline (PBS) to remove the Triton, the columns were eluted with 0.1 M galactose in PBS. The eluates were dialyzed extensively against PBS.

### *Preparation of $Cl^-$-ATPase by Affinity Chromatography*

The microsomal pellets were dispersed in a glass homogenizer in a basic resuspension buffer (a solution of 5 mM dithiothreitol, 2 mM EDTA and 30 mM TES-NaOH, pH 7.0–7.1) with 10 mM ATP and 0.25% vol/vol Triton X-100. A 2 × 1-cm column of Sepharose-N-caproylgalactosamine was equilibrated with resuspension buffer plus ATP at 1 mM before the dispersed microsomes were passed through the column and washed through with care using small aliquots of 5 mM ATP in resuspension buffer; total volume of washing solution was 10 ml. The column was eluted with 4 ml of 0.1 M galactose, 5 mM ATP, in resuspension buffer. The eluate was used for $Cl^-$-ATPase estimation, or dialyzed against PBS for hemagglutination assay, or dialyzed against water for protein assay.

### *$Cl^-$-ATPase Assay*

Microsomal pellets were resuspended in the basic resuspension buffer. When testing the effects of sugars on the $Cl^-$-ATPase activity of microsomes, the resuspension buffer contained the appropriate sugar at 10× the concentration given in Table 2 because aliquots of resuspended microsomes were diluted 10× in the assay.

Enzyme activity was measured (Hill & Hill, 1973*b*) in 3 mM ATP (Na), 0.5–1 mM dithiothreitol, 25 mM TES-NaOH, pH 7.0–7.1, as the activity in 0.1 M NaCl in excess of that in 0.1 M recrystallized sodium benzene sulphonate. Propionate and sulphate have been used previously as "background" anions against which the $Cl^-$-ATPase activity can be measured, but sodium benzene sulphonate was preferred because its properties of ionic strength and osmotic activity are more similar to those of sodium chloride.

In the early assays (Hill & Hill, 1973*b*) $Mg^{++}$ was included. However, experiments showed that the addition of $Mg^{++}$ did not stimulate the $Cl^-$-ATPase activity of microsomal preparations and it was subsequently omitted. (We now need to examine the effect of $Mg^{++}$ and $Ca^{++}$ on the purified $Cl^-$-ATPase.)

### *Hemagglutination Assay*

The activity against trypsinized rabbit erythrocytes was estimated in titer plates (Kauss & Glaser, 1974). Titer numbers were converted to μg equivalents of soybean agglutinin by comparison with a standard curve obtained using solutions of soybean agglutinin, prepared by the method of Allen & Neuberger (1975), freshly dissolved in PBS.

### *Protein Assay*

Resuspended microsomes were dialyzed extensively against water before protein estimation. Bovine serum albumin (BSA) and soybean agglutinin (SBA) were used as standards: Standard curves of optical density *vs.* protein concentration prepared using SBA coincided

exactly with curves prepared at the same time using BSA provided these proteins were trichloroacetic acid-precipitated and only the washed precipitate was used for protein estimation. Concentrations of protein below 10 µg equivalents BSA/1 ml were measured by fluorimetry (Böhlen *et al.*, 1973); above 10 µg/ml by the method of Lowry *et al.* (1951).

## Results

Attempts to separate out $Cl^-$-ATPase-enriched membranes from microsomes by density-gradient centrifugation were unsuccessful because the bulk of the membranes sedimented through conventional sucrose gradients together. It appeared that this was the result of membrane vesicles aggregating in clumps, and we looked to see if the clumping might be lectin-mediated. We found that the supernatant fraction of the homogenate, after millipore-filtering to remove all membrane fragments, showed hemagglutination activity (Table 1) which was inhibited strongly by N-acetylgalactosamine, weakly by galactose, and not at all by galactosamine, N-acetylglucosamine, or glucose. To test whether this activity was responsible for agglutinating the membranes, we attempted to disperse the clumps by including the cheaper hapten, galactose, in the medium for resuspending the microsomes so as to compete with the membranes for sugar-binding site on the lectins. In the absence of galactose, no visible bands were seen in the sucrose gradient (1.06 g $cm^{-3}$ linear to 1.23 g $cm^{-3}$) and the bulk of the material sedimented to the base of the gradient. As the concentration of galactose in the resuspension medium was increased from 10 to 15 mM, this basal pellet

Table 1. Hemagglutination activity and protein content of galactosamine-sepharose-binding material from *Limonium* leaf homogenates

| Preparation | Minimum concentration of protein for full hemagglutination (µg equivalent BSA per ml) | Hemagglutination activity H (µg equivalent SBA per gFW) | Protein[a] bound to galactosamine-sepharose P (µg equivalent SBA per gFW) | Specific activity relative to that of SBA. H/P (SBA = 1.00) |
|---|---|---|---|---|
| Pelletable membranes | 0.3 | 0.03 | 1.4 | 0.02 |
| Filterable membranes | | 0.05 | | |
| Supernatant | 1.5 | 0.20 | 20 | 0.01 |
| Total | | 0.28 | | |

[a] Estimated using a standard curve prepared from known weights of SBA.

was progressively reduced (and at 20 mM galactose eliminated) and increasing amounts of membrane appeared in a broad band halfway down the gradient. This band contained $Cl^-$-ATPase activity. We concluded that sugar binding *was* involved in membrane clumping.

We discovered that when *Limonium* microsomes were resuspended in medium containing 20 mM galactose, the activity of $Cl^-$-ATPase was increased relative to replicate samples from the same leaves resuspended without galactose (Table 2). At first we thought this was because the sugar had dispersed the clumps of membranes. In the absence of galac-

Table 2. Specific activities of $Cl^-$-ATPase preparations from *Limonium* leaves

| Preparation | Specific activity (μmol Pi/h/mg protein) | relative to microsomes (*x*) |
|---|---|---|
| *Experiment 1* | | |
| Microsomes | 0.52 | 1.0 |
| Microsomes with galactose (concentration at assay 2 mM) | 1.9 | 3.7 |
| Microsomes with 0.01% vol/vol Triton X-100 | 1.7 | 3.2 |
| Microsomes with galactose (concentration at assay 2 mM) and 0.01% vol/vol Triton X-100 | 2.9 | 5.6 |
| *Experiment 2* | | |
| Microsomes | 0.26 | 1.0 |
| Microsomes with galactose (concentration at assay 2 mM) | 0.43 | 1.6 |
| Microsomes with N-acetylgalactosamine (concentration at assay 1 mM) | 0.54 | 2.1 |
| *Experiment 3* | | |
| Microsomes | 0.38 | 1.0 |
| Microsomes with galactose (concentration at assay 2 mM) | 0.50 | 1.3 |
| Microsomes with N-acetylgalactosamine (concentration at assay 0.4 mM) | 0.69 | 1.8 |
| *Four Experiments* | | |
| Microsomes | $0.5 \pm 0.2$ | 1 |
| Galactosamine-sepharose-binding material from Triton-solubilized microsomes | $45 \pm 15$ | $10^2$ |

In these experiments the basal rate of nonspecific ATPase activity in different preparations of microsomes varied between 1.4 and 9.1 μmol Pi/h/mg protein. The variability of the basal rate has been discussed before (Hill & Hill, 1973*b*).

tose, a similar increase in activity could be obtained using Triton to disperse the microsomes. Triton might also have increased the ATPase activity by increasing the permeability of the membrane vesicles to ATP and $Cl^-$. However, the increase in $Cl^-$-ATPase activity due to galactose was shown even in the presence of Triton (Table 2) when it is most unlikely that the state of membrane dispersion or its permeability to substrates was limiting the activity of the enzyme.

Because the hemagglutination activity from *Limonium* showed a higher specificity for N-acetylgalactosamine than for galactose, we investigated whether this sugar might also increase $Cl^-$-ATPase activity. Comparison of the specific activity of $Cl^-$-ATPase in replicate samples of microsomes resuspended in the presence of N-acetylgalactosamine or galactose showed that N-acetylgalactosamine was a more potent activator (Table 2).

We considered that galactose and N-acetylgalactosamine were stimulating activity probably via direct interaction with the $Cl^-$-ATPase, which ought therefore to possess a galactose/N-acetylgalactosamine-binding site.

We isolated the proteins which bound to galactosamine-sepharose from Triton-solubilized microsomes. Provided the microsomes were solubilized in the presence of ATP, a substantial proportion (between 20 and 100% in different experiments) of the $Cl^-$-ATPase activity of the microsomes was recovered in material which bound to galactosamine-sepharose, whilst almost all of the $Cl^-$-independent ATPase passed through the column without binding. In different experiments, between 88 and 100% of the nonspecific ATPase present in the microsomes was separated from the $Cl^-$-ATPase in this step. The specific activity of the $Cl^-$-ATPase was increased *ca.* 15-fold over the value obtained for microsomes in the presence of galactose and Triton (Table 2).

After dialysis, this galactosamine-sepharose-binding material showed hemagglutination activity which was inhibited by N-acetylgalactosamine, very weakly by galactose, and not at all by galactosamine, N-acetylglucosamine, or glucose, We have compared the hemagglutination activity of the galactosamine-sepharose-binding proteins from *Limonium* leaves with that of soybean agglutinin, a potent seed lectin with the same sugar specificity (Table 1). The *Limonium* leaf preparations had much lower lectin activity per mg protein.

To confirm that the $Cl^-$-ATPase which binds to galactosamine-sepharose was the enzyme associated with salt stress and not some other cell component, galactosamine-spharose-binding proteins were prepared

in the same way using microsomes from leaves of *Limonium* plants grown in salt-free conditions. No $Cl^-$-ATPase activity could be detected in these preparations, although the hemagglutination activity was comparable to that of equivalent preparations from salt-treated plants.

## Discussion

Lectin-mediated agglutination of membrane-vesicles so that they cannot be resolved into component membrane types may well be a problem, though as yet unreported, with tissue homogenates of other plant species. The phenomenon leads us to suspect that receptors for the *Limonium* leaf lectins must be present in the tissue.

Although the hemagglutinins and the $Cl^-$-ATPase co-purify on affinity chromatography, we consider that they are probably different proteins for the following reasons. First, the amount of hemagglutination activity obtained from solubilized microsomes of salt-free and salt-treated leaves is comparable, whereas $Cl^-$-ATPase activity is absent from the preparation from salt-free leaves. (Preliminary results from SDS-polyacrylamide gel electrophoresis of these preparations shows that they only differ from each other in one band. This polypeptide, mol wt 58,000, is very much the major component of the preparation from salt-stressed leaves, but a minor component of that from salt-free leaves. The low specific lectin activity relative to SBA of galactosamine-sepharose-binding material from salt-stressed leaves will therefore be due, at least partly, to the apparently nonhemagglutinating $Cl^-$-ATPase protein). Second, we have found recently that while the hemagglutination activity binding to galactosamine-sepharose is the same whether ATP is present or not, $Cl^-$-ATPase activity does not bind to galactosamine-sepharose unless ATP is added to the solubilized microsomes. We are currently using this property to purify the $Cl^-$-ATPase further, but as yet we are not able to measure the concentration of protein in the solutions of the enzyme produced.

As far as we know, this is the first report of enzyme activity in a carbohydrate-binding protein where the carbohydrate is not a substrate but, as our results indicate, an activator. Not only does it seem that enzymic activity is stimulated by receptor binding, but we have found that receptor binding is only possible in the presence of an enzymic substrate (ATP). Because the function of the $Cl^-$-ATPase is known and

the physiology of chloride pumping at least partly understood, a number of points can be made about the biological significance of our results.

Biophysical studies indicate that chloride pumping takes place across the plasma membrane, because the salt is extruded by the glands from the leaf symplasm and the characteristics of this process make transport in vesicles unlikely (Hill & Hill, 1973*a*). There is evidence that the plasma membrane differs from the other membranes of the cell in its high content of glycoproteins and glycolipids (Hanke, 1977). We propose that stimulation of the $Cl^-$-ATPase by N-acetylgalactosamine, a glycoprotein component, is involved in the activation of chloride pumping specifically at the plasma membrane, if, e.g., the native receptors are restricted to this membrane.

Hill and Hill (1970) distinguish two phases of the induction process. An early phase of synthesis is not inhibited by temperatures down to 3 °C but is prevented by dactinomycin and puromycin. A subsequent "assembly" phase is not affected by the inhibitors but *is* arrested at 6 °C or below. We propose that activation of the $Cl^-$-ATPase is part of the "assembly" phase, e.g., the newly synthesized enzyme *in transit* will remain inactive until it is inserted in the plasma membrane where it will bind an N-acetylgalactosaminoylated component and begin to drive the vectorial pumping of ions. That the addition of N-acetylgalactosamine increases the $Cl^-$-ATPase activity of microsomes (Table 2) shows that the activity of some of the enzyme in a preparation of membranes from the whole cell is limited by the availability of receptors. We think that a portion of the enzyme in the homogenate is inactive because it had not yet bound receptor when the cells were broken. Alternatively, $Cl^-$-ATPase-stimulation by sugars could involve an increase in the activity of all the enzyme. Measurements of the activity of the purified enzyme at different concentrations of N-acetylgalactosamine should enable us to check this. We shall test whether the purified enzyme is stimulated by N-acetylgalactosamine and investigate whether control sugars such as N-acetylglucosamine activate it as soon as we can prepare galactose-free enzyme, the objective of current work.

In yeast and animal cells, receptors for plant lectins are found on the external surface of the plasma membrane, but not on its internal surface (Boller, Dürr & Wiemken, 1976, and references therein). If the same is true in plant cells, receptor binding could be involved in maintaining the correct orientation of the ion pump across the plasma membrane. In that the membranes undergo lectin-mediated agglutination when *Limonium* leaf cells are homogenized, at least some of the lectin receptors

must be kept separate from lectins *in vivo,* perhaps on the outer surface of the plasma membrane. We are currently preparing protoplasts from *Limonium* leaves to study the receptors and sugar-binding proteins at the outer surface of the plasma membrane.

We feel that it cannot be a coincidence that the soluble and membrane lectins share the same sugar specificity as the $Cl^-$-ATPase. It may be that the hemagglutinins are part of the mechanism controlling the activity of the $Cl^-$-ATPase, e.g., through competition for the same receptor molecules. In the absence of enzyme activity, the functions currently proposed for lectins in plant cells are suggested by their ability to attach erythrocytes to each other, e.g., joining together P-protein filaments (Sabnis & Hart, 1978), wall components (Kauss & Glaser, 1974), endomembranes (Bowles, Schnarrenberger & Kauss, 1976), or *Rhizobia* to legume roots (Bohlool & Schmidt, 1974). The idea that intracellular binding to specific receptors is involved in the regulation of cellular activities has been mooted (Bowles & Hanke, 1977) and our evidence provides support for this view.

Proteins that bind specific carbohydrate residues are not unique to the *Limonium* salt-gland, and it is not likely that this mechanism of activation for plasma membrane enzymes is either. We were given a purified preparation of the $Na^+ - K^+$ pump from porcine kidney (Jørgensen, 1974) by Professor I.M. Glynn, which we tested for lectin activity. The enzyme showed hemagglutination activity (minimum concentration for full hemagglutination 20 μg/ml) which appeared to be specific for mannose. It is interesting to note that the purified $Na^+ - K^+$-ATPase consists of two polypeptides: a large one containing the phosphorylation site, connected to a smaller glycopeptide (Glynn & Karlish, 1975) and that while the large subunit is confined to the baso-lateral membranes, the smaller glycoprotein can be detected over the whole plasma membrane surface (Dibona & Mills, 1979).

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