Sidedness of plant plasma membrane vesicles purified by partitioning in aqueous two-phase systems

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Preparations of plant plasma membrane vesicles were obtained by partition in dextran–polyethylene glycol two-phase systems. By this procedure particles are separated according to their surface properties, and an iso-osmotic environment is maintained throughout. The vesicles thus produced are right-side-out and sealed, as measured by enzyme [(K^+ + Mg^{2+})-ATPase and glucan synthetase II] latency on addition of Triton X-100.

Plant plasma membrane vesicle *Sidedness* *Enzyme latency* *K^+-stimulated Mg^{2+}-dependent ATPase* *Glucan synthetase II* *Two-phase system*

1. INTRODUCTION

Isolated plasma membrane vesicles are important material for studies on biological events associated with this membrane. However, for a correct interpretation of results on transport, phytochrome binding, hormone binding etc., it is important to use preparations of plasma membranes with a defined sidedness. The ideal situation, of course, would be to have pure preparations of both right-side-out and inside-out vesicles, respectively. Such preparations can be obtained from erythrocyte and chloroplast thylakoid membranes, and have been of great importance for the analysis of the transverse asymmetry of these membranes (review [1] and [2], respectively). With both erythrocytes and chloroplast thylakoids, membrane preparations of opposite sidedness are obtained by partition in aqueous dextran–polyethylene glycol two-phase systems. In fact, this method is extremely well suited for the preparation of membrane vesicles which are homogeneous with respect to sidedness, since it separates particles with respect to surface properties [3] rather than size and density, as does centrifugation. We have prepared plasma membranes from several plant species using two-phase systems [4,5], and these preparations may therefore be expected to contain vesicles of uniform sidedness. These plasma membrane vesicles may furthermore be expected to be right-side-out, since they partition similarly to intact protoplasts, i.e., they have a high affinity for the polyethylene glycol-rich upper phase [6]. To test this hypothesis, we have studied the latency of K^+-stimulated Mg^{2+}-dependent ATPase and glucan synthetase II on addition of Triton X-100. These two enzymes are known to be localized in the plasma membrane [7] and the ATPase has generally been assumed to have its ATP-binding site on the cytoplasmic side [7], while results on the UDP-glucose-binding site of glucan synthetase II are conflicting [8,9]. Our results, obtained with cauliflower inflorescences and oat roots, suggest that plant plasma membranes collected from the upper phase after purification by phase partition are mainly sealed right-side-out vesicles.

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2. MATERIALS AND METHODS

2.1. *Plant material*

Oats (*Avena sativa*, L. cv. Brighton) were grown for 7 days at 18°C in darkness [5]. Cauliflower inflorescences (*Brassica oleracea* L.) were obtained at the local market.

2.2. *Plasma membranes*

Plasma membranes were purified from microsomal fractions of oat roots (10000–30000 × *g* fraction) and cauliflower inflorescences (10000–50000 × *g* fraction) by partitioning in an aqueous polymer two-phase system [4,5]. To avoid pelleting the material after phase partitioning, to keep the plasma membrane vesicles as intact as possible, we modified the composition of the phase system to allow for measurements of $(K^+ + Mg^{2+})$-ATPase activity on aliquots withdrawn directly from the upper phase. Thus, potassium phosphate was exchanged for Tris–SO_4, and a phase system of the following composition was used: 6.5% (w/w) dextran T 500, 6.5% (w/w) polyethylene glycol 4000, 0.25 M sucrose, 2 mM Cl–Tris, 5 mM SO_4–Tris (pH 7.8).

2.3. *K^+-stimulated Mg^{2+}-dependent ATPase*

K^+-stimulated Mg^{2+}-dependent ATPase was determined essentially as in [10] in an iso-osmotic medium containing 0.25 M sucrose, 1 mM $MgSO_4$, 25 mM KCl, 1 mM ATP, 40 mM Tris–Mes of desired pH, and 5 μg (oats) or 25 μg protein (cauliflower) in a total volume of 0.5 ml. The assay was run for 30 min at 30°C.

2.4. *Glucan synthetase II*

Glucan synthetase II was assayed as in [11] with minor modifications. A total volume of 0.16 ml contained 0.15 M cellobiose, 0.10 M sucrose, 0.2 mM [^{3}H]UDP-glucose (10^4 Bq), 40 mM Tris–Mops of desired pH, and 20 μg protein. The assay was run for 25 min at 25°C.

2.5. *Protein*

Protein was measured as in [12] with 0.01% Triton X-100 present to solubilize membrane proteins; and with bovine serum albumin as standard.

3. RESULTS

3.1. *pH optima of ATPase and glucan synthetase II*

To exclude the possibility that a measured increase in enzyme activity on addition of detergent was merely due to a shift in pH optimum, it was necessary to check the pH dependency of the activities.

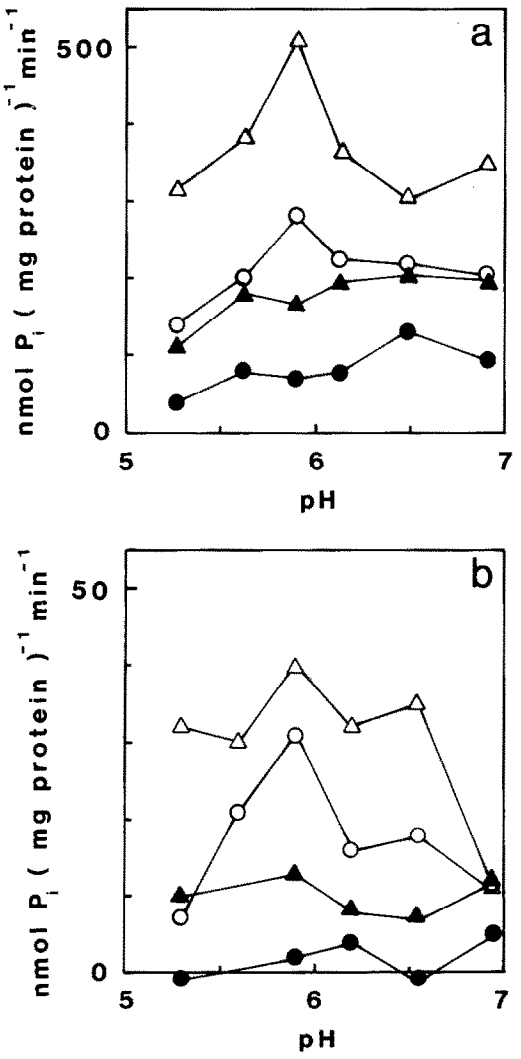

Fig.1. pH dependency of the K^+-stimulated Mg^{2+}-dependent ATPase activity of isolated plasma membrane vesicles from: (a) oat roots and (b) cauliflower inflorescences. The activity was assayed with either Mg^{2+} only (○, ●) or $Mg^{2+} + K^+$ (△, ▲). (○, △) 0.01% Triton X-100 present.

The K^+-stimulated Mg^{2+}-dependent ATPase is usually assayed at pH 6.0 or 6.5 [7,10]. We found the pH optimum to be around 5.9 for both plant materials in the presence of 0.01% Triton X-100 (fig.1a,b). This was the case both when the ATPase was assayed with Mg^{2+} only, and when assayed with Mg^{2+} and K^+. In the absence of detergent the activities were low and no pH optimum was readily found. Thus, the addition of detergent stimulated ATPase activity in the whole pH interval (5.3–6.9), and the stimulation of activity at any fixed pH was not due to a shift in pH optimum.

For glucan synthetase II of cauliflower a pH optimum of 7.0 was found both with and without 0.01% Triton X-100 (fig.2b); and the activity was stimulated by detergent in the whole pH interval (6–8). With oat root, however, glucan synthetase II activity was inhibited by 0.01% Triton X-100; and the pH optimum was shifted from 7.5 to 7 (fig.2a).

3.2. *Enzyme latency*

A broad range of Triton X-100 concentrations was used, since the detergent could be expected to stimulate enzyme activity at low concentrations and inhibit at higher concentrations.

Mg^{2+}-dependent ATPase activity, as well as the K^+-stimulated Mg^{2+}-ATPase activity, was stimulated by increasing concentrations of Triton X-100 up to 0.05%; at higher concentrations an inhibitory effect became evident (fig.3a,b). The latency curves obtained with cauliflower and oat root were similar, but the activities obtained with oat root material were higher, particularly the rates without detergent. Assuming that the ATP-binding site of the ATPase is at the cytoplasmic side of the plasma membrane, the latencies observed at 0.05% Triton X-100 suggest that the plasma membrane preparation obtained from cauliflower contained about 90% sealed right-side-out vesicles, while that from oat root contained about 70% sealed right-side-out vesicles, sealed in this case being defined as not leaky to ATP.

It was recently shown [13] that relatively high concentrations (0.5 mM) of vanadate may be used to inhibit completely plasma membrane-bound K^+-stimulated Mg^{2+}-ATPase in oat root microsomes, without affecting the activities of other K^+- or Cl^--stimulated Mg^{2+}-ATPases. Under these conditions, the K^+-stimulated

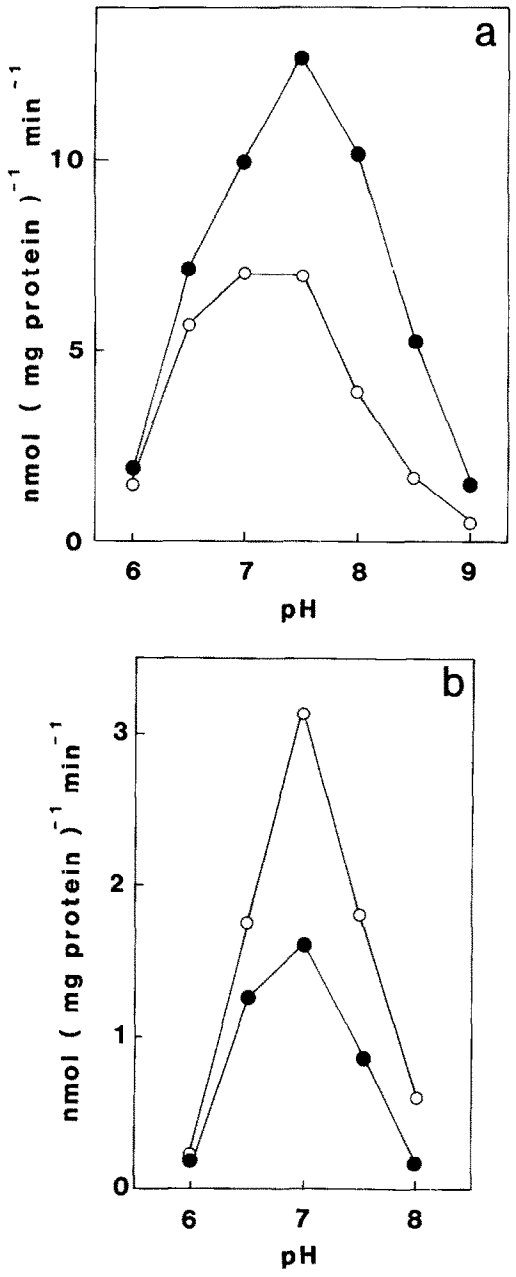

Fig.2. pH dependency of glucan synthetase II activity of isolated plasma membrane vesicles from: (a) oat roots and (b) cauliflower inflorescences. (○) 0.01% Triton X-100 present.

Mg^{2+}-ATPase activity present in oat root plasma membrane vesicles purified by phase partition was completely inhibited (M. Sommarin, personal communication); and the activity in cauliflower

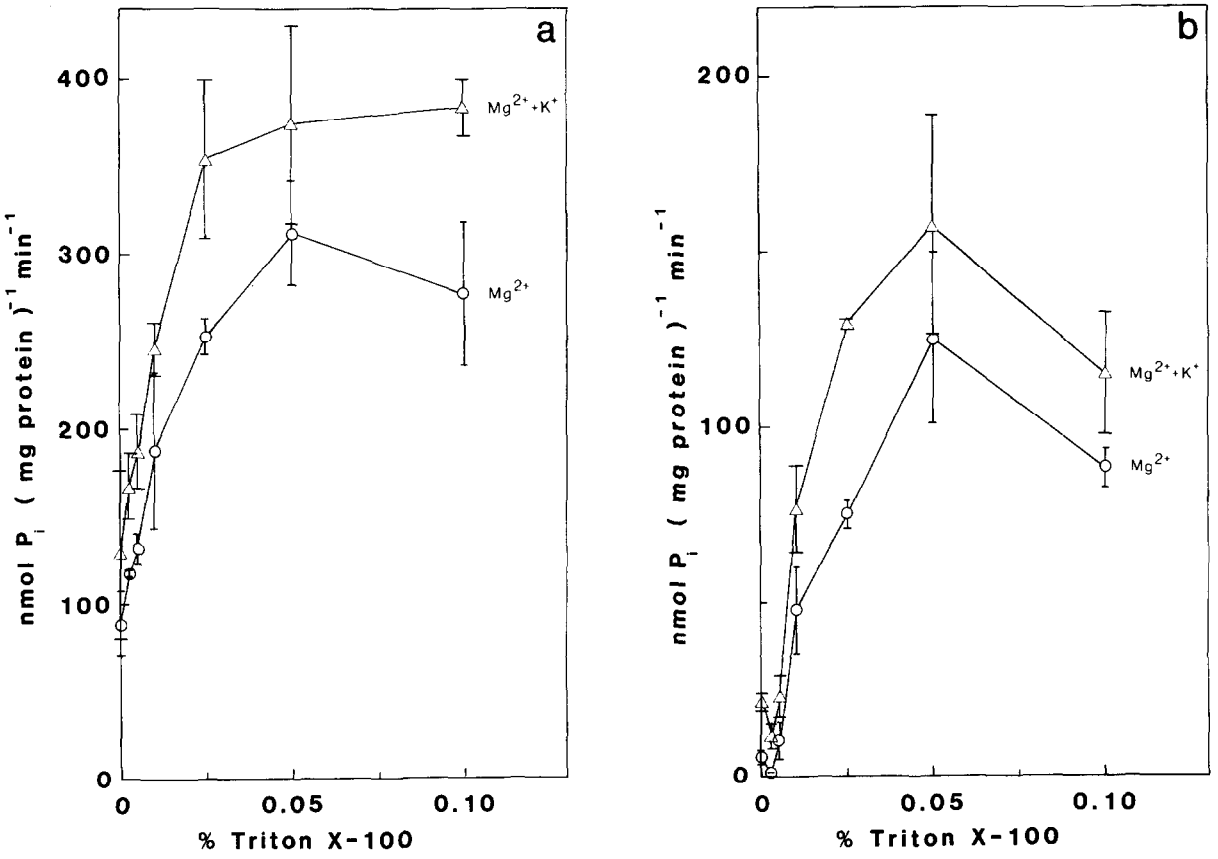

Fig.3. Latency of K^+-stimulated Mg^{2+}-dependent ATPase activity of isolated plasma membrane vesicles from: (a) oat roots and (b) cauliflower inflorescences. The activity was assayed ith either Mg^{2+} only (○), or Mg^{2+} + K^+ (△). Each value represents the mean and maximum variation of two independent experiments.

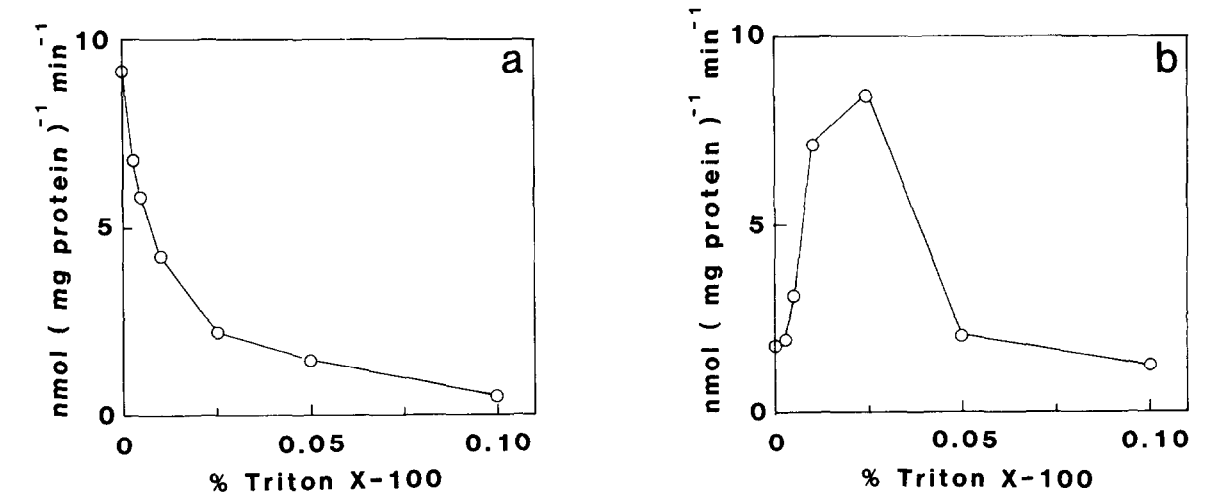

Fig.4. Latency of glucan synthetase II activity of isolated plasma membrane vesicles from: (a) oat roots and (b) cauliflower inflorescences.

plasma membrane was inhibited to more than 90%, whereas azide (1 mM), a known inhibitor of mitochondrial ATPase, had no effect on the activities. Moreover, ammonium molybdate (0.1 mM), an inhibitor of non-specific acid phosphatases, had no effect on the activity in oat plasma membrane (M. Sommarin, personal communication). Thus, there are strong indications that the measured ATPase activities are associated with the plasma membrane, and not due to enclosure of mitochondrial or tonoplast membranes, or of soluble ATPase within plasma membrane vesicles.

Glucan synthetase II of oat root was strongly inhibited by Triton X-100 (fig.4a); and not even at the lowest concentration (0.0025%) of detergent was any latency observed. With cauliflower, however, the glucan synthetase II activity was stimulated several-fold up to 0.025% Triton X-100 (fig.4b); only at higher concentrations was inhibition observed. These results suggest that the UDP-glucose-binding site of glucan synthetase II is located on the same side of the plasma membrane as the ATP-binding site of the ATPase, i.e., most probably on the cytoplasmic side. In fact, the latency curves for glucan synthetase II and $(K^+ + Mg^{2+})$-ATPase of cauliflower are almost identical below 0.025% Triton X-100, where inhibition of the former enzyme probably starts to become severe.

4. DISCUSSION

Results from recent studies on the sidedness of plasma membrane vesicles purified by density gradient centrifugation are conflicting. Authors in [14], using plasma membrane vesicles prepared from carrot protoplasts, obtained evidence from latency of concanavalin A binding, that their preparations contained a mixture of right-side-out and inside-out vesicles. Authors in [15], on the other hand, using the same assay for sidedness, found only right-side-out vesicles in their preparations, and suggested that all plasma membrane vesicles formed after homogenization of corn coleoptiles are right-side-out. This is questioned by the author in [16], using the same material and an assay based on *N*-1-naphthylphthalamic acid (NPA) binding. He provides evidence for a partial separation of right-side-out and inside-out plasma membrane vesicles by polyethylene glycol-facilitated sedimentation. The presence of plasma membrane vesicles with different orientation in preparations obtained by density gradient centrifugation would not be unexpected, since this method separates particles according to density and not according to surface properties, as does phase partitioning [3].

About 50% of the plasma membrane vesicles prepared by density gradient centrifugation were not tightly sealed, as measured by latency of ATPase [14], and UDPase [15] activity. This may be due to the low and changing osmotic potential experienced by the vesicles in the gradient. By contrast, the use of phase partitioning allows the preparation of plasma membrane vesicles in an iso-osmotic environment throughout the procedure.

Thus, preparations with a large proportion of sealed vesicles, which are homogeneous with respect to sidedness, may be obtained by phase partitioning. With the present procedure right-side-out vesicles are obtained. However, if inside-out vesicles of plasma membrane are present in the homogenates, it should also be possible to purify these by a modification of the procedure.

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