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ARTICLE *in* ANALYTICAL CHEMISTRY · AUGUST 2007

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Anal. Chem. **2007,** *79,* 5181−5187

Enhanced Separation of Membranes during Free Flow Zonal Electrophoresis in Plants

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Free flow zonal electrophoresis (FFZE) is a versatile technique that allows for the separation of cells, organelles, membranes, and proteins based on net surface charge during laminar flow through a thin aqueous layer. We have been optimizing the FFZE technique to enhance separation of plant vacuolar membranes (tonoplast) from other endomembranes to pursue a directed proteomics approach to identify novel tonoplast transporters. Addition of ATP to a mixture of endomembranes selectively enhanced electrophoretic mobility of acidic vesicular compartments during FFZE toward the positive electrode. This has been attributed to activation of the V-ATPase generating a more negative membrane potential outside the vesicles, resulting in enhanced migration of acidic vesicles, including tonoplast, to the anode (Morré, D. J.; Lawrence, J.; Safranski, K.; Hammond, T.; Morré, D. M. *J. Chromatogr., A* 1994, *668*, 201−213). We confirm that ATP does induce a redistribution of membranes during FFZE of microsomal membranes isolated from several plant species, including *Arabidopsis thaliana*, *Thellungiella halophila*, *Mesembryanthemum crystallinum*, and *Ananas comosus*. However, we demonstrate, using V-ATPase-specific inhibitors, nonhydrolyzable ATP analogs, and ionophores to dissipate membrane potential, that the ATP-dependent migrational shift of membranes under FFZE is not due to activation of the V-ATPase. Addition of EDTA to chelate Mg^{2+}, leading to the production of the tetravalent anionic form of ATP, resulted in a further enhancement of membrane migration toward the anode, and manipulation of cell surface charge by addition of polycations also influenced the ATP-dependent migration of membranes. We propose that ATP enhances the mobility of endomembranes by screening positive surface charges on the membrane surface.

The tonoplast of plant cells is resident to many membrane transport proteins essential for the functioning of the vacuole, including those for cellular ion homeostasis, carbohydrate metabolism, compartmentalization of toxic ions and xenobiotics, Ca^{2+} signaling, osmotic adjustment, and pH regulation.[1,2] Progress in identifying the proteins responsible for these activities has been slow, and there are many processes for which the transporters involved remain unknown.

The ARAMEMNON database classifies 6475 *Arabidopsis thaliana* proteins, or 25% of the proteome, as putative membrane proteins.[3] Functional annotation can classify many of these as transport proteins, based on homology to characterized gene family members. However, it is not yet possible to detect membrane proteins targeted to the tonoplast from the amino acid sequence, as no decisive sorting signal has been identified. A directed proteomics approach in which the proteome of the tonoplast is deciphered would significantly advance our knowledge of tonoplast transporters, by providing a directory of membrane proteins, including transporters, located to the tonoplast and supply information on proteins whose function is known or unknown.

Fundamental to the analysis of the tonoplast proteome is the purity of the membrane preparation. Contamination from non-tonoplast proteins can subsequently mask the presence of low-abundant proteins such as transporters. Moreover, the erroneous allocation of nonresident proteins to the tonoplast could lead to the incorrect assignment of functions to this membrane. Several groups have attempted to catalog the tonoplast proteome employing traditional fractionation techniques including sucrose density gradients, dextran gradients, and vacuole purification; however, all reported endomembrane cross-contamination and identified known non-tonoplast proteins.[4−8] Free flow zonal electrophoresis (FFZE) allows for the separation of tonoplast from other cellular endomembranes based on net membrane surface charge during matrix free laminar flow through a thin aqueous layer.[9,10] It is a highly versatile and reproducible fractionation technique and has been adapted to effectively separate cells, organelles, membranes, and proteins from a wide variety of organisms and cell types.[11] FFZE run conditions can be easily manipulated to enhance separation of a particular pool of membranes. In one report, inclusion of trypsin to the sample media facilitated separation of

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10.1021/ac070159v CCC: $37.00
Published on Web 06/13/2007

endosomes and lysosomes from other organelles in CHO cells.[12] The mild protease treatment of the sample may function to remove basic peripheral proteins attached to the endosomal membrane, e.g., elements of the cytoskeleton, or the trypsin might facilitate the disaggregation of organelles in the homogenate.[12]

Reports have also suggested it is possible to manipulate the mobility and therefore the degree of separation of acidic membrane vesicles by FFZE with the inclusion of ATP in the sample medium.[13] This was attributed to activation of an H^+−ATPase, and the generation of a more negative membrane potential outside the vesicles through the pumping of H^+ to the interior, resulting in an altered membrane surface charge. This could theoretically enhance the migration of this population of membrane vesicles toward the anode.[13] It is well-known that the plant tonoplast contains a H^+-pumping V-ATPase,[14] suggesting that this method could be used for the enhanced separation of tonoplast vesicles from other cellular membranes during FFZE. In this study, we demonstrate that, while there is an ATP-dependent migrational shift of membranes under FFZE, it is not due to activation of the V-ATPase. We propose that the electrophoretic changes in membrane migration caused by the inclusion of ATP in the separation medium are most likely due to the screening of exposed peripheral positive charges.

EXPERIMENTAL SECTION

Chemicals. All chemicals were of standard analytical grade and purchased from either Sigma Aldrich or MP Biomedicals. Na_2-ATP was converted to BTP/ATP pH 8.0 by cation exchange with Dowex 50W (Bio-Rad, Mexico).

Plant Materials. *Mesembryanthemum crystallinum*, *A. thaliana* and *Thellungiella halophila* plants were grown from seed in soil (Metro Mix 500; Sun Gro Horticulture, WA), in propagation trays. Three weeks following germination, individual seedlings were transplanted; plants of *M. crystallinum* or *T. halophila* were placed in pots containing soil mixture, with two plants per 15 cm diameter pot; *A. thaliana* plants were transplanted to hydroponics trays with roots immersed in half-strength Hoagland's media. *Ananas comosus* was propagated vegetatively from the crowns of commercially obtained fruits. All plants were grown to maturity in a glasshouse under natural irradiation and photoperiod. Minimum temperatures ranged from 12 to 18 °C, and the maximum temperature was maintained below 28 °C.

Microsomal Membrane Isolation. Plant material (30 g) was harvested and sliced into small pieces (following the removal of major veins from leaf tissue). Tissue was placed directly into 300 mL of ice-cold homogenization medium, and all subsequent operations were carried out at 4 °C. The homogenization medium consisted of 400 mM mannitol, 10% (w/v) glycerol, 5% (w/v) PVP-10, 0.5% (w/v) BSA, 1 mM PMSF, 30 mM Tris, 2 mM DTT, 5 mM EDTA, 5 mM $MgSO_4$, 0.5 mM butylated hydroxytoluene, 0.25 mM dibucaine, 1 mM benzamidine, and 26 mM K^+−metabisulfite, adjusted to pH 8.0 with H_2SO_4. Microsomal membranes were isolated as previously described.[15] Protein in microsomal preparations was measured by a modification of the Bradford method,[16] in which membrane protein was partially solubilized with 0.5% (v/v) Triton X-100 for 5 min before the addition of the dye reagent concentrate, the final concentration of Triton X-100 in the assay being 0.015%.[17] BSA was employed as the protein standard. Membranes were frozen directly in liquid N_2 and stored at −80 °C.

Free Flow Zonal Electrophoresis. Microsomal membranes were fractionated by FFZE using the BD FFE system (BD Proteomics, Germany). Prior to fractionation, the microsomal sample was diluted 1:1 (v/v) in separation medium and centrifuged at 14 000g_{av} for 20 min at 4 °C. The sample (3 mg/mL) was injected continuously via a peristaltic pump at a rate of 1.2 mL/h using the anodic sample inlet. The media inlets of the chamber had the following buffer compositions: inlets 2−6, separation medium (10 mM TEA, 10 mM acetic acid, 2 mM KCl, and 250 mM sucrose); inlets 1 and 7, stabilization medium (40 mM TEA, 40 mM acetic acid, 8 mM KCl, and 180 mM sucrose). The cathodic and anodic circuit electrolyte solutions consisted of 100 mM TEA, 100 mM acetic acid, and 20 mM KCl adjusted to pH 7.4 with NaOH, with 0.4% formaldehyde added to the anodic solution to prevent loss of chloride by anodic oxidation. The counter flow medium for inlets C1, C2, and C3 was the same as the separation medium.

FFZE was performed in horizontal mode at a constant voltage of 750 V (118 mA), with a media and counter flow rate of 250 mL/h. The temperature during the run was maintained at 5 °C by the continual flow of coolant, below the glass separation plate, from a circulating water bath.

Following separation in the chamber, membrane fractions were collected continually in 96 deep well microtiter plates (4 mL/well; Sunergia Medical, VA). Fractions from sequential runs were pooled, and membranes were concentrated by centrifugation in a Beckman 55.2 Ti rotor in an L8-M ultracentrifuge at 100 000g_{av} for 50 min at 4 °C. Membrane pellets were resuspended in 50−100 μL of suspension buffer containing 250 mM mannitol, 10% glycerol (w/v), 10 mM Tris/MES pH 8, and 2 mM DTT and frozen in liquid N_2 for storage at −80 °C. Separation by FFZE was monitored by collecting microtiter plates (250 μL/well) at several time points during the run and measuring protein (OD_{280}) using a microplate scanning spectrophotometer (Power Wave$_X$, Bio-Tek Instruments, VT).

SDS−PAGE and Western Blotting. FFZE fractions were precipitated by dilution of the samples 50-fold in 1:1 (v/v) ethanol/acetone and incubated overnight at −30 °C. Samples were then centrifuged at 13 000g_{av} for 20 min at 4 °C using an F2402 rotor in a GS-15R table-top centrifuge (Beckman, Mexico). Pellets were air-dried, resuspended with sample buffer (2.5% SDS), and heated at 60 °C for 2 min before loading (15 μg of protein per lane) onto 10% (w/v) linear acrylamide extrawide minigels (Scie-Plas Ltd., Warwickshire, U.K). After electrophoresis, SDS−PAGE-separated proteins were electrophoretically transferred onto nitrocellulose membranes (ECL, Amersham, U.K.), and western blot analysis

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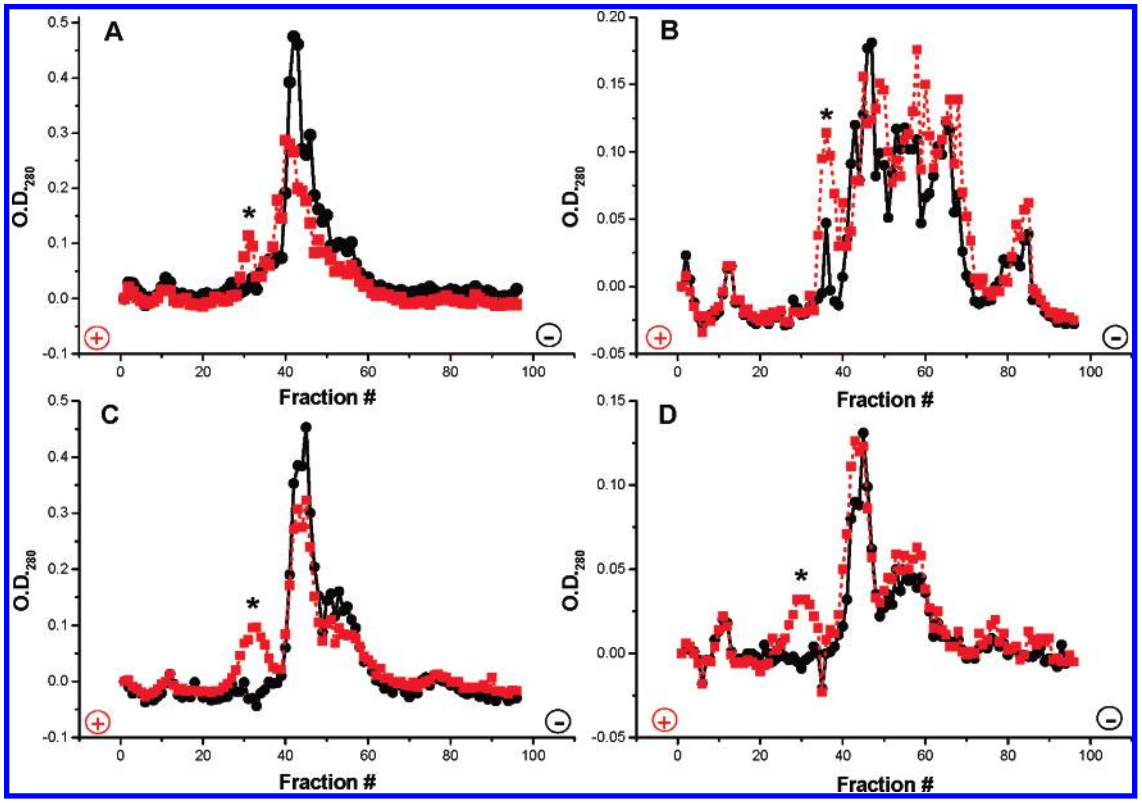

Figure 1. FFZE of plant microsomal membranes from *M. crystallinum* (A), *A. comosus* (B), *T. halophila* (C), and *A. thaliana* (D) in the presence (■) or absence (●) of 3 mM ATP. Increased electrophoretic mobility of a population of membranes toward the anode can be observed in the fractions in the presence of ATP (∗).

was carried out as previously described.[18] Images were captured using a Hewlett-Packard flatbed scanner (Scan Jet 8250, Hewlett-Packard, Mexico).

RESULTS AND DISCUSSION

In searching for an improved method to better separate tonoplast vesicles from a plant microsomal mixture, we followed the work by Morré et al.,[13] who reported that FFZE in the presence of ATP enhanced separation of acidic membrane vesicles including plant tonoplast. Preincubation of microsomal membranes with 3 mM Mg−ATP, assumed to activate the tonoplast H^+−ATPase (V-ATPase), resulted in a change in membrane separation profile of the FFZE fractions and, specifically, the appearance or increase of a peak of membranes migrating toward the anode (positive electrode) that was absent in the fractions from membranes separated without ATP (Figure 1). This ATP-dependent migration was observed in all four plant species separated by FFZE; however, the position and shape of the peak appeared to be species specific, as was the general separation profile. In microsomal membranes separated from *M. crystallinum*, *A. comosus*, *T. halophila*, and *A. thaliana*, the peak of membranes showing increased electrophoretic mobility in the presence of ATP was detected in FFZE fractions 31, 36, 33, and 29, respectively (Figure 1, asterisks). In contrast to other species tested, ATP preincubation of *A. comosus* membranes did not induce the appearance of a novel peak of membranes but, rather, increased the amplitude of an existing peak (Figure 1B). When ATP was injected with sample buffer, in the absence of membranes, no peaks were observed and only a baseline trace was measured in all FFZE fractions at OD_{280} (data not shown).

To identify the origin of this population of membranes, FFZE fractions of *M. crystallinum* microsomal membranes were collected and subjected to western blot analysis (Figure 2). On the basis of protein marker analysis for different membrane compartments, including the plasma membrane Na^+/K^+ cotransporter, HKT1,[19] the tonoplast H^+-transporting V-PPase, AVP1,[20] and the endoplasmic reticulum Ca^{2+}-binding protein, calreticulin, CRT1,[21] we could confirm that the ATP-dependent peak of membranes at fraction 31 corresponded to tonoplast (Figure 2). Due to the complexity of the vacuolar biogenesis pathways in plant cells and the lack of confirmed markers,[22] we cannot rule out the presence of other prevacuolar endomembrane compartments in this peak. Only a subpopulation of tonoplast showed increased mobility as tonoplast was also detected in fractions 34−48. This is most likely due to the presence of both inside out and right-side out tonoplast vesicles with different surface properties, which, as demonstrated by Morré et al.,[13] show different mobility, with only right-side out vesicles showing ATP-dependent migration.

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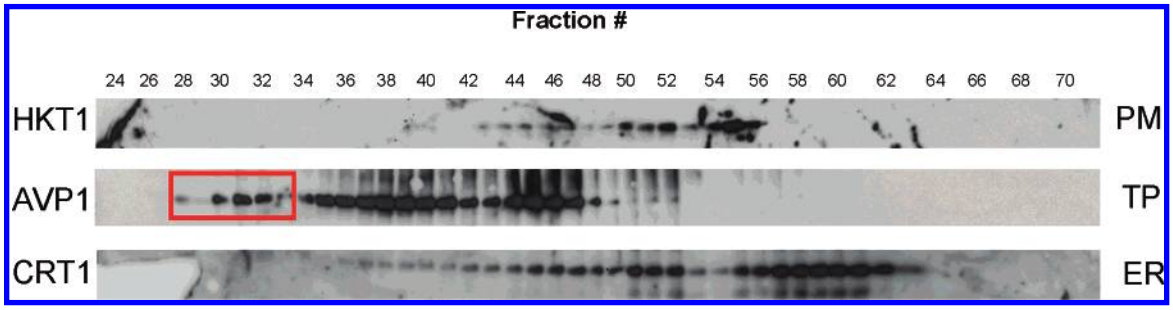

Figure 2. Western blot analysis of FFZE fractions collected from ATP-treated *M. crystallinum* microsomal membranes. Immunological detection in the respective fractions of (from top to bottom) the plasma membrane (PM) marker HKT1 (55 kDa), the tonoplast (TP) marker AVP1 (70 kDa), and the endoplasmic reticulum marker CRT1 (57 kDa). The fractions corresponding to the ATP-dependent peak in Figure 1A are enclosed in the red box.

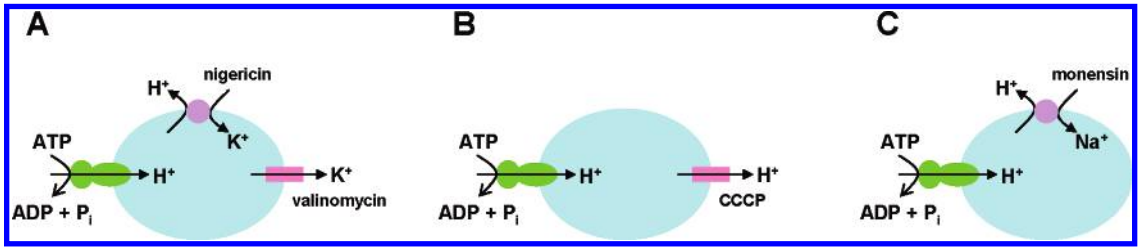

Figure 3. Schematic representation of the mode of action of ionophores on the tonoplast membrane potential and pH gradient generated by the V-ATPase.

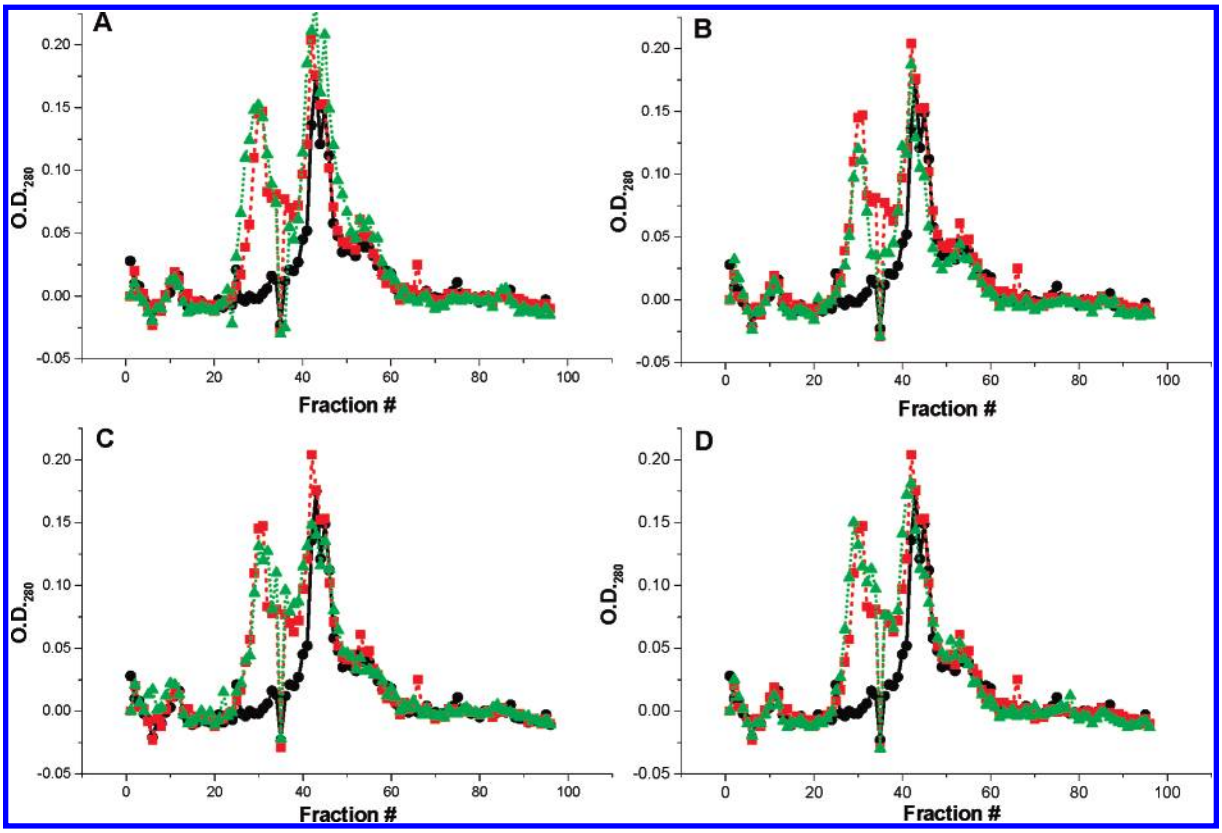

Figure 4. Effect of V-ATPase inhibitors and ionophores on the ATP-induced shift in membranes during FFZE of *M. crystallinum* microsomal fractions; membranes were incubated in the presence (■) or absence (●) of 3 mM ATP or in the presence of ATP with the indicated ionophore or inhibitor (▲): (A) 5 μM nigericin + 1 μM valinomycin, (B) 5 μM CCCP, (C) 100 nM bafilomycin, and (D) 50 mM nitrate.

It has been proposed that ATP activates the V-ATPase on the tonoplast vesicles resulting in the generation of a more negative membrane potential on the outer vesicular surface that enhances the migration of these vesicles toward the positive electrode.[13] To test this hypothesis, prior to FFZE, membranes were incubated in the presence of ATP with the ionophores nigericin and valinomycin, which function as K^+/H^+ exchangers and K^+ channels, respectively. The combined presence of the ionophores would act to dissipate the negative outside membrane potential generated by the pumping of H^+ into the vesicle by activation of the V-ATPase by ATP (Figure 3A). As observed in Figure 4A, the presence of the ionophores had no effect on the ATP-induced shift

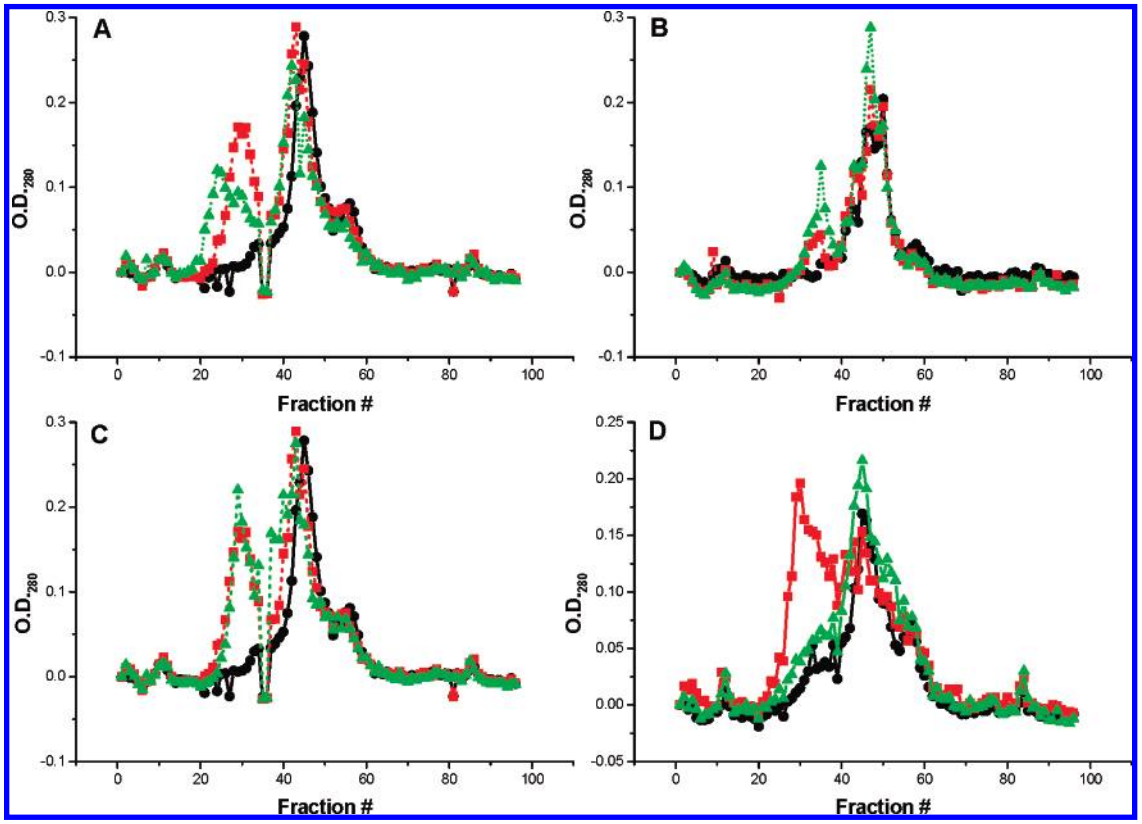

Figure 5. Effects of nonhydrolyzable ATP analogs, alternative nucleotides, and V-ATPase-independent generation of membrane potential on the migration of membranes during FFZE of *M. crystallinum* microsomal fractions. FFZE was carried out in the presence (■) or absence (●) of 3 mM ATP or in the presence (▲) of ATPγS (A), or ADP−PNP (B), or GTP (C), or PP_i (D).

in membrane fractionation during FFZE, indicating that the establishment of membrane potential is not responsible for the observed changes. This conclusion was further supported by incubation of membranes in the presence of ATP and the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) that also functions to dissipate the membrane potential generated by the H^+ pumping of the V-ATPase, as well as the pH gradient (Figure 3B). Again, the presence of CCCP did not inhibit the ATP-induced membrane shift (Figure 4B).

These findings contrast those reported by Morré et al.;[13] however, in that study the Na^+ ionophore monensin was incorrectly employed to eliminate the charge difference across the membrane. Monensin is an electroneutral monovalent cation/proton exchanger, and its addition would lead to neutralization of the vesicular pH without resulting in a change in the vesicular membrane potential, as there would remain a net accumulation of positive charges from the uptake of Na^+ (Figure 3C).

Although our results demonstrated that generation of an outside negative membrane potential was not required for the shift in membranes during FFZE, it was still possible that proton pumping by the V-ATPase was involved. To investigate this possibility proton flow through the H^+ channel of the V-ATPase was inhibited by the presence of either bafilomycin A1 or nitrate.[23,24] Both inhibitors failed to affect the ATP-induced migration of membranes (Figure 4, parts C and D), strongly indicating that proton pumping did not play a role and suggesting that the V-ATPase was not contributing to this effect. This was further confirmed by the use of nonhydrolyzable or slowly hydrolyzable ATP analogs, including ATP−PNP and ATPγS, which would bind to the ATP-binding site on the V-ATPase but prevent its activation.[25] The presence of these analogs was also unable to prevent the ATP-induced membrane migration (Figure 5, parts A and B), and interestingly, ATPγS slightly enhanced the migration of the membranes toward the anode over that which was observed in the presence of ATP (Figure 5A). These results not only ruled out a role for the V-ATPase, but because the hydrolysis of the γ-phosphate is not involved, we can also exclude mediation by protein and/or lipid kinases or other ATPases such as aminophospholipid translocases, which when activated by ATP could increase negatively charged phospholipids at the cytoplasmic side of the membrane.[26]

To examine if the shift in vesicle separation showed specificity for the nucleotide, membranes were incubated in the presence of GTP. As observed in Figure 5C, GTP was also able to induce membrane migration to the same extent as ATP, suggesting that the effect was not nucleotide specific but likely due to the anionic nature of these molecules ($Mg-ATP^{2-}$ or $Mg-GTP^{2-}$ at pH 7.4), indicating that they may act by screening positive surface charges on the membranes.

In plants, a second, distinct H^+-transporting pump, the vacuolar pyrophosphatase (V-PPase), is localized on the tonoplast, and in

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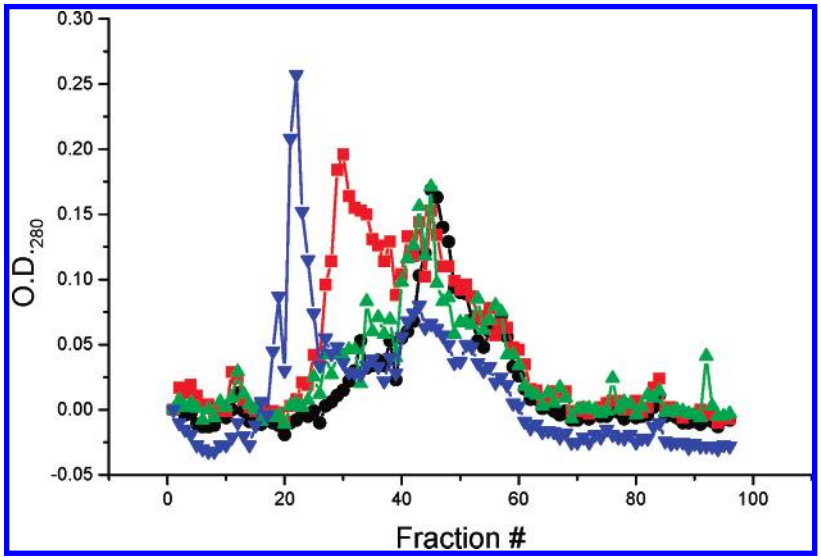

Figure 6. Effect of the chelation of divalent cations on the ATP-induced shift in membranes during FFZE of *M. crystallinum* microsomal fractions. FFZE was carried out in the presence (■) or absence (●) of 3 mM ATP or in the presence of 3 mM ATP + 1 mM EDTA (▼) or 1 mM EDTA alone (▲).

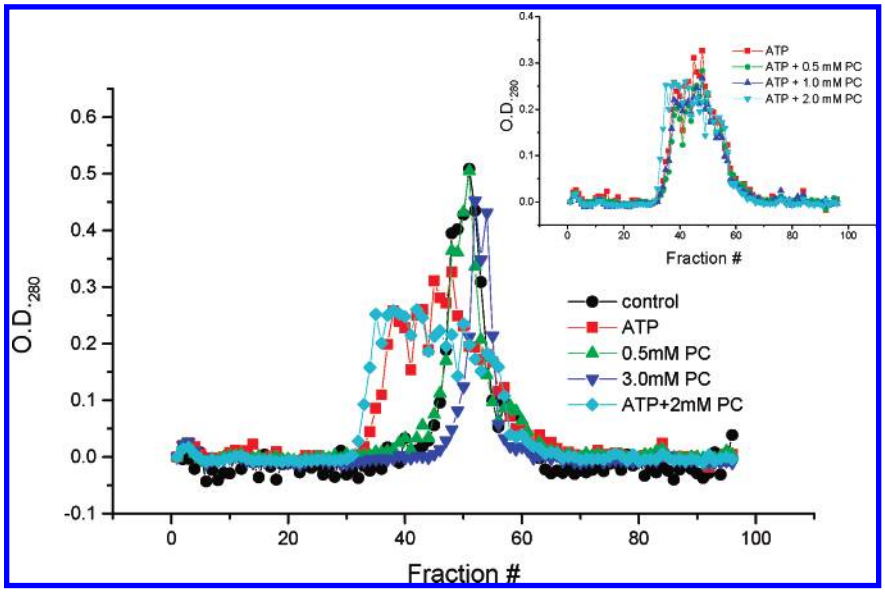

Figure 7. Effect of the polycation poly-L-lysine (PC) on the ATP-induced shift in membranes during FFZE of *M. crystallinum* microsomal fractions. FFZE was carried out in the presence (■) or absence (●) of 3 mM ATP, or in the presence of 0.5 mM (▲) or 3.0 mM (▼) poly-L-lysine alone, or in the presence of 3 mM ATP + 2 mM poly-L-lysine (◆). Inset: increasing concentrations of poly-L-lysine results in an increase in the ATP-dependent migration of endomembranes. FFZE was carried out in the presence of 3 mM ATP with 0 (■), 0.5 (▲), 1.0 (▼), and 2 mM (◆) poly-L-lysine.

parallel with the V-ATPase, establishes the inside-acid, inside-positive H^+ electrochemical potential difference responsible for energizing the H^+-coupled transport of solutes into the vacuole.[27] However, unlike the V-ATPase, this enzyme uses inorganic pyrophosphate as its energy source. This allowed us to test explicitly the requirement for the generation of an outside negative membrane potential without requiring the presence of ATP or the activity of the V-ATPase. Figure 5D further confirms that membrane potential is not required for the ATP-induced separation of membranes; as in the presence of 300 μM PP_i no membrane migration was observed, proving that it is solely the presence of the negatively charged nucleotide that results in the membrane shift during FFZE.

In an attempt to provide more insight into the role of ATP in this study, FFZE was carried out in the presence of EDTA to chelate divalent cations. This would allow us to investigate whether nucleotide charge could play a role in the process. The addition of EDTA, in the absence of ATP, had no effect on the membrane mobility (Figure 6). However, interestingly, EDTA in the presence of ATP resulted in greater membrane mobility than that observed in the presence of ATP alone (Figure 6). This result suggests that by chelating Mg^{2+}, the proposed effect caused by ATP on surface charges would be increased, as the ATP should be in its tetravalent anionic form (ATP^{4-}) and not in the form $Mg{-}ATP^{2-}$. Binding of the more negatively charged ATP^{4-} to the membrane would result in an enhancement of the overall net negative surface charges responsible for the observed shift in membrane separation.

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Additionally, these results also substantiate that neither ATP hydrolysis nor the establishment of a membrane potential are factors responsible for the shift in membrane migration as the substrate for the V-ATPase is $Mg-ATP^{2-}$, and ATP^{4-} alone does not function as a substrate; in the presence of EDTA, no $Mg-ATP^{2-}$ would be available as substrate for the V-ATPase.

Decisive evidence in support of a role for membrane surface charge on the ATP-induced shift in migration was obtained when membranes were preincubated in the presence of the polycation poly-L-lysine.[28] Binding of poly-L-lysine to the membrane would result in an increase in the net positive charge of the membrane and allow for a greater binding of ATP. Figure 7 shows that in the presence of poly-L-lysine there is an increase in the ATP-dependent membrane shift toward the anode compared to that observed in the presence of ATP alone.

This is dependent upon the concentration of polycation, with the greatest shift observed in the presence of 2 mM poly-L-lysine (Figure 7, inset). Addition of poly-L-lysine alone (in the absence of ATP) resulted in a concentration-dependent shift toward the cathode, confirming the increase in positive surface charge of the membranes by the polycation (Figure 7).

CONCLUSIONS

This study demonstrates that preincubation of microsomal membranes from different plant species in a medium containing millimolar concentrations of ATP^{4-} modifies the separation of membranes, increasing the migration of a specific population toward the anode during FFZE. However, neither changes in membrane potential nor V-ATPase activity can explain this occurrence. Additionally, the lack of requirement for the high-energy γ-phosphate suggests there is no involvement of other ATPases or lipid and protein kinases, which all require the hydrolysis of ATP. Evidence suggests that the shift in membrane migration toward the anode is solely the result of screening of positive surface charges caused by the anionic nature of the nucleotide on a particular population of membranes. The incubation of plant microsomal fractions with ATP prior to FFZE can be employed to enhance the resolution of endomembrane fractions, which will allow for more precise proteomic studies.

ACKNOWLEDGMENT

The authors acknowledge financial support from CONACyT (Grant 49735) to B.J.B. and DGAPA Grants IN-205505 to O.P. and IN-2002205 to R.V-E.

Received for review January 26, 2007. Accepted May 10, 2007.

AC070159V

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