Electrogenicity, pH-Dependence, and Stoichiometry of the Proton-Sucrose Symport

Daniel R. Bush

Photosynthesis Research Unit, U.S. Department of Agriculture, Agricultural Research Service and Department of Plant Biology, University of Illinois, 289 Morrill Hall, Urbana, Illinois 61801

ABSTRACT

The electrogenicity, pH-dependence, and stoichiometry of the proton-sucrose symport were examined in plasma membrane vesicles isolated from sugar beet (Beta vulgaris L. cv Great Western) leaves. Symport mediated sucrose transport was electrogeneric as demonstrated by the effect of membrane potential on ΔΦ-dependent flux. In the absence of significant charge compensation, a low rate of sucrose transport was observed. When membrane potential was clamped at zero with symmetric potassium concentrations and valinomycin, the rate of sucrose flux was stimulated fourfold. In the presence of a negative membrane potential, transport increased six-fold. These results are consistent with electrogenic sucrose transport which results in a net flux of positive charge into the vesicles. The effect of membrane potential on the kinetics of sucrose transport was on V_{max} only with no apparent change in K_m. Sucrose transport rates driven by membrane potential only, i.e. in the absence of ΔΦ, were comparable to ΔΦ-driven flux. Both membrane potential and ΔΦ-driven sucrose transport were used to examine proton binding to the symport and the apparent K_m for H^+ was 0.7 micromolar. The kinetics of sucrose transport as a function of proton concentration exhibited a simple hyperbolic relationship. This observation is consistent with kinetic models of ion-cotransport systems when the stoichiometry of the system, ion:substrate, is 1:1. Quantitative measurements of proton and sucrose fluxes through the symport support a 1:1 stoichiometry. The biochemical details of proton-sucrose-coupled symport transport reported here provide further evidence in support of the chemiosmotic hypothesis of nutrient transport across the plant cell plasma membrane.

Although the higher plant is a photoautotrophic organism, it is composed of several heterotrophic tissue systems that are dependent upon carbon import for normal growth and development. Because these tissues represent a large constituency within the plant system, their impact on the overall carbon economy of the plant dictates that as much as 85% of the carbon assimilated during photosynthesis (11) is exported from the leaf to satisfy the metabolic and biosynthetic needs of the import-dependent cells. Despite the significance of carbon redistribution, however, little is known about the regulation and integration of the systems mediating this essential process. Furthermore, even less is known about the membrane proteins which actively transport metabolically important organic compounds such as sucrose, the predominant form of exported carbon, and amino acids. Therefore, a major research interest in this laboratory is on active transport systems found in the plasma membrane that may be involved in assimilate partitioning.

Sucrose and amino acids are actively transported across the plant cell PM by proton-coupled symports. These transport systems are capable of driving solute accumulation against significant concentration gradients by linking translocation to the free energy available in a Δμ_H^+ (3, 4, 8, 17). It is now well established that the Δμ_H^+ is generated by the primary transport activity of the PM proton-pumping ATPase which has been purified and cloned (9, 19, 21). In contrast, it is just recently that the first biochemical investigations of the proton-coupled symport systems were achieved using isolated PMVs and imposed proton electrochemical gradients (2–4, 13). These initial reports confirmed the existence of ΔΦ-dependent symports and provided new information about the kinetics, electrogenicity, and inhibitor sensitivity for both sucrose and amino acid transport. Although the tissue specific location of these active transport systems has yet to be determined and, consequently, their exact role in long distance transport is unclear, they are nevertheless good candidates as important participants in assimilate partitioning. In the results reported here, a detailed examination of the electrogenicity, pH-dependence, and stoichiometry of the proton-sucrose symport provides specific information about the biochemical characteristics of this critical transport system.

MATERIALS AND METHODS

Plant Material

Sugar beet (Beta vulgaris L. cv Great Western) seeds were germinated in a 1:1 mixture of vermiculite and sand. When the seedlings were 1 inch tall, they were transferred to a hydroponic system, 2 plants per 13 L, containing one-half strength Hoagland solution and 1 mM NaCl (10). Plants were maintained under a 10 h photoperiod (400 μE m^-2 s^-1) at 24°C, 70% RH, and 14 h night period at 18°C, 70% RH. Only fully expanded leaves were harvested for experimental analysis.

PM Vesicle Isolation

PMVs were prepared at 4°C as described previously (3), with the exception that 10 mM KCl was included in both the

1 Abbreviations: PM, plasma membrane; PMV, plasma membrane vesicle; Δμ_H^+, proton electrochemical potential difference; Btp, 1,3-bis[tris(hydroxymethyl)methylamino]propane; CCCP, carbonyl cyanide m-chlorophenylhydrazone; LCRB, low buffer capacity resuspension buffer; PCMBs, p-chloromercuribenzenesulfonic acid; ΔΨ, membrane potential.
homogenization and resuspension buffers. Briefly, 40 g of leaves were homogenized in 400 mL of 240 mM sorbitol, 50 mM Hepes, 10 mM KCl, 3 mM EGTA, 3 mM DTT, and 0.5% (w:v) BSA (final pH was adjusted to 8.0 with solid Btp). After filtration and a 10 min, 10,000 g centrifugation to eliminate large organelles, the supernatant was centrifuged at 50,000 g for 45 min. The resulting microsomal pellets were resuspended in 3 mL 340 mM sorbitol, 5 mM K$_2$HPO$_4$ (pH 7.8), and 0.1 mM DTT. PMVs were purified from the resuspended microsomes with the aqueous phase partitioning method which yields predominantly right-side-out vesicles (12). After the final phase separation, the PMVs were washed in 30 mL resuspension buffer containing 330 mM sorbitol, 2 mM Hepes, (pH 7.8 with Btp)10 mM KCl, and 0.1 mM DTT and pelleted with a 45 min centrifugation at 50,000 g. The final PMV pellet was resuspended in 1 mL resuspension buffer (≈5 mg protein/mL).

Freeze-Thaw Equilibration and Loading

Five cycles of freeze-thawing were used to equilibrate the intravesicular volume of isolated PMVs with solutions of desired composition. Diluted PMVs (1–5 mg membrane protein per 2 mL treatment solution) were frozen in liquid nitrogen and subsequently thawed at 35°C. Multiple cycles of freeze-thawing were necessary to efficiently equilibrate PMVs with the treatment solution. This was shown by measuring the number of cycles required to saturate the accumulation of an impermeable solute, $^4$C-inulin, or by demonstrating that vesicles equilibrated at pH values less than 8.0 required multiple cycles before CCCP-sensitive sucrose accumulation, used as an indirect measure of ΔpH, was eliminated (data not shown). The composition of specific treatment solutions are given in the legends of their respective experiments. It should be noted that the total concentration of osmotically active solutes was always maintained at 350 ± 10 mmol/L to minimize changes in osmotic potential.

Sucrose Transport and Tetraphenyl Phosphoium Accumulation

Sucrose transport experiments were conducted at 10°C using the pH-jump approach previously described (3). Briefly, transport was initiated by diluting a small aliquot (20–50 µL) of PMVs into an acidic transport solution (400–600 µL) containing resuspension buffer (pH adjusted from 7.8 to 6.0 with solid Mes, about 10 mM), 0.8 to 2.5 µCi [4$^4$C]sucrose, and unlabeled sucrose to the desired final concentration. In some experiments, 5 µM valinomycin was also present in the transport solutions. At predetermined time points membrane vesicles were collected with the Millipore$^2$ filtration technique as described (3). Accumulated radioactivity was measured with scintillation spectrometry. ΔpH-dependent sucrose transport was defined as the difference between that accumulated in the absence and in the presence of 5 µM CCCP. Each experiment was repeated at least three times and multiple time points were sampled per treatment, with the exception of the Δp-driven experiments where only one time point was taken per duplicate. When appropriate, rates were taken from best fit lines generated with linear regression analysis. Standard errors within any given experiment never exceeded 8% of the experimental average. Results from single representative experiment are given.

Tetraphenyl phosphoium (TPP$^+$) accumulation was measured using the same protocol as used in the sucrose transport experiments except glass fiber filters were used to collect and wash the PMVs. This was necessary because TPP$^+$ binds to the cellulose based Millipore filter.

Measuring Proton and Sucrose Uptake for Stoichiometric Determinations

For these experiments PMVs were loaded with a low buffer capacity resuspension buffer (LCRB; 260 mM sorbitol, 30 mM K$_2$SO$_4$, 0.15 mM Hepes pH adjusted to 6.0 with Btp) and 1 mg/mL acid invertase using the freeze-thaw procedure outlined above. After five freeze-thaw cycles, the vesicles were washed in 33 mL LCRB, pelleted with a 45 min centrifugation at 50,000 g, and resuspended in 1.5 mL LCRB. To measure sucrose-driven proton uptake, PMVs (1–1.5 mg protein) were diluted into 2.0 mL LCRB + 5 µM valinomycin in a thermostatically controlled chamber at 10°C. Changes in the proton concentration of the transport solution were measured by recording changes in potential across a proton-sensitive glass electrode with a Keithley Instruments electrometer (model 610C) and strip chart recorder. After a 5-min equilibration period, a concentrated aliquot of sucrose was diluted into the treatment solution, final concentration 18 mM, to initiate sucrose-dependent proton transport. The electrode system was calibrated by adding 10.0 nmol of HCl to the treatment solution at the end of each experiment.

Symport-dependent sucrose transport was measured under the same conditions except 50 µL PMVs were diluted into 250 µL LCRB + 5 µM valinomycin in the presence or absence of 0.4 mM PCMBS. After the 5 min equilibration period, an aliquot of concentrated [4$^4$C]sucrose (final concentration 18 mM) was added to each treatment solution to initiate transport. Membrane vesicles were collected and washed with the Millipore filtration technique. Symport-mediated sucrose transport was defined as the difference between that accumulated in the absence and presence of 0.4 mM PCMBS. A 5-min pretreatment in 0.4 mM PCMBS completely inactivates the symport system (DR Bush, unpublished data).

Protein Determinations

Proteins were determined by the method of Markwell (15) after solubilization in 0.02% Na deoxycholate and precipitation in 6.2% TCA (1).

RESULTS AND DISCUSSION

Electrogenicity

Because sucrose is an uncharged molecule, proton-coupled sucrose transport should result in a net influx of positive...
charge into isolated PMVs. However, it is possible that compensating charge is obligatorily translocated by the carrier resulting in an electroneutral exchange. Therefore, it was of interest to determine if the sucrose transport system is electrogenic. The electrogenicity of the proton-sucrose symport was explored by examining the effect of $\Delta \phi$ on $\Delta p\phi$-dependent sucrose transport. A trans-membrane proton concentration difference (basic interior) was generated with a pH-jump as previously described (3) and potassium gradients and valinomycin were used to alter the $\Delta \phi$ of the isolated PMVs. In the presence of 10 mM KCl on both sides of the vesicle membrane, linear rates of $\Delta p\phi$-dependent sucrose transport were observed (Fig. 1). When valinomycin, a potassium ionophore, was included in the transport solution the rate of sucrose transport increased. Under these conditions, valinomycin clamps the $\Delta \phi$ at zero because potassium ions can rapidly exit the vesicle as protons enter. Stimulation of sucrose transport in this experiment suggests the native conductance of potassium and chloride ions was too slow to compensate for the influx of positive charge. Therefore, in the absence of valinomycin, proton-sucrose cotransport (as well as unspecific proton conductances) resulted in the generation of a positive $\Delta \phi$ that began to thermodynamically oppose proton-coupled sucrose uptake. In the presence of valinomycin, $\Delta \phi$ remained at zero and, consequently, the proton electrochemical potential difference driving sucrose accumulation was not significantly reduced and the rate of sucrose transport was not affected. These data are consistent with electrogenic sucrose transport and support recent reports from other laboratories (2, 13).

In a more detailed study of the electrogenicity of the proton-sucrose symport, the concentration dependence of $\Delta p\phi$-dependent sucrose transport was examined as a function of $\Delta \phi$ (Fig. 2). Under conditions of minimum charge compensation, as seen in Figure 1, low rates of sucrose transport were recorded. When the membrane potential was clamped at zero, however, transport activity was stimulated fourfold at the apparent $K_m$ (1 mM) for sucrose. When the KCl loaded vesicles were diluted into a potassium free transport solution in the presence of valinomycin a negative membrane potential developed, as a result of the steep potassium diffusion potential, and a sixfold increase in the rate of sucrose transport was observed (Fig. 2). The generation of a stable, negative membrane potential under these conditions was confirmed using $\Delta \phi$-dependent accumulation of the lipophilic cation tetraphenyl phosphonium (Fig. 3) (18). The effect of membrane potential on sucrose transport kinetics provides further evidence in support of the electrogenicity of the proton-sucrose symport. These data also indicate that both components of the $\Delta \mu^+$, $\Delta p\phi$, and $\Delta \phi$, can contribute to active sucrose transport.

Lineweaver-Burk plots of the data reporting concentration dependent sucrose transport show that the apparent $K_m$ for sucrose was unaltered by membrane potential (Fig. 4). This suggests the binding affinity of the porter for sucrose is unaffected by $\Delta \phi$. Since sucrose is an unchanged molecule, direct interaction with $\Delta \phi$ would not be expected. Membrane potential appears to exert its influence over a charge translocation step that, according to the observed changes in $V_{\text{max}}$, may be rate limiting in the translocation process. It is not possible, based on the available data, to determine what the binding status of the carrier is when it was most sensitive to $\Delta \phi$.

The increased rate of sucrose transport in the presence of a negative membrane potential (Fig. 2) suggests the symport may be driven by $\Delta \phi$ as well as $\Delta p\phi$. Buckhout (2) previously provided data in support of this hypothesis by demonstrating $\Delta \phi$-dependent transport in the absence of $\Delta p\phi$, although the rate of flux reported was only 10% of that seen for $\Delta p\phi$. In the experiments reported here, much higher concentrations of potassium, 60 mM versus 2 mM (2), were loaded into the PMVs to ensure a stable potassium diffusion potential when
equal AAH+ solutions whose
of (3).

The dependence lowered. Interestingly, transport pH of histidine, an amino acid residue whose possible participation in the translocation mechanism has been previously suggested based on sensitivity to diethyl pyrocarbonate (3).

Proton binding to the symport

The ability to drive the symport with Δψ provided a simple experimental approach to examine the pH dependence of the symport, as opposed to the ΔpH dependence. Thus, Δψ-dependent transport in Figure 5 was reported as a function of the pH of the transport solution. The acidic pH optimum observed here was previously reported for intact tissues (5, 6, 16). However, in contrast to whole cell experiments, the pH dependence of transport activity reported here reflects proton binding to the symport only, and is not complicated by an indirect increase in the proton motive force as the pH is lowered. Interestingly, the curve in Figure 5 is typical of curves generated with pH titrations. This suggests an important pK may occur around a pH of 6.5. Significantly, this is close to the pK of histidine, an amino acid residue whose possible participation in the translocation mechanism has been previously suggested based on sensitivity to diethyl pyrocarbonate (3).

Proton binding to the symport was also examined using ΔpH to drive sucrose accumulation. In these experiments, equal ΔμH+ potentials were used to drive sucrose flux as a function of the transport solution pH. To accomplish this, PMVs were equilibrated at desired pH values using a series of freeze-thaws. The vesicles were then diluted into transport solutions whose pH was buffered at exactly 1 pH unit less than the equilibrated pH of the PMVs. Vesicles were equilibrated at pHs of 8.0, 7.5, 7.0, and 6.0 and they were jumped into pH 7.0, 6.5, 6.0, and 5.0 transport solutions, respectively. Since the Δψ of the PMVs was clamped at zero, these one-unit pH jumps should generate equivalent proton potential differences. This expectation was supported in experiments where the accumulation of [14C]acetate, which is a reporter of ΔpH (18), was the same for each one unit pH jump (not shown).

The rate of ΔpH-driven sucrose transport as a function of the transport solution pH exhibited an acidic pH optimum. When these data were plotted as a function of proton concentration, rather than pH, a simple hyperbolic curve was observed (Fig. 6) and the Lineweaver-Burk plot was linear, yielding an apparent Km of 0.7 μM protons (Fig. 6, inset). It is noteworthy that Δψ (Fig. 5, pK ≈ 6.5 (0.3 μM) and ΔpH (Fig. 6, Km = 0.7 μM) driven sucrose transport yielded similar results regarding proton binding.

The pH optimum for proton binding has important physiological consequences. Since the apoplast pH of the leaf is approximately 5.5 (21), the results presented here suggest the symport is probably not limited by proton binding. It would appear that the proton binding site of the symport is always occupied (protonated) and, therefore, only sucrose binding and the transmembrane ΔμH+ limit optimum symport activity. Of course, specific regulation such as phosphorylation or allosteric binding could significantly modify symport activity.

Stoichiometry of the Proton-Sucrose Symport

The stoichiometry of the proton-sucrose symport is an important biophysical parameter because it defines, thermodynamically, the maximum level of sucrose accumulation attributable to symport activity and because this ratio provides physical information about the symport mechanism. Based on the kinetic models describing cotransport systems developed by Turner (22), the hyperbolic dependence of sucrose flux on proton concentration shown in Figure 6 is consistent
with a stoichiometry of 1:1. If two or more protons were required per sucrose translocated, the data in Figure 6 would exhibit a sigmoidal relationship. Although the data in Figure 6 are consistent with predictions based on Turner’s kinetic model (22), it is not valid to use this fit as the only criterion in support of a specific molecular mechanism. Therefore, a direct measurement of both proton and sucrose flux was also used to determine the stoichiometry of the proton-sucrose symport.

The rationale behind these experiments was to use a steep sucrose diffusion gradient to drive the symport and to measure independently the flux of both protons and sucrose. By using a sucrose concentration difference to drive the system, it was possible to use a sensitive proton selective-electrode and electrometer to quantify the sucrose-dependent depletion of protons from a weakly buffered transport solution. In parallel experiments, symport-dependent sucrose flux was measured as PCMB-sensitive $[^{14}C]$sucrose accumulation. This was necessary because a ΔpH-independent transport system, which is insensitive to PCMB, is also present in these vesicle preparations (3). Initially, both ΔpH and Δψ were clamped at zero. However, although a sucrose-dependent increase in the transport solution pH was observed, it saturated too quickly to get meaningful rates of initial proton flow. One possible explanation for this observation is that the sucrose concentration within the vesicles rose quickly and, thereby, dissipated the diffusion gradient driving the symport. To overcome this limitation, the PMVs were preloaded with invertase using a series of freeze-thaws. With invertase inside the vesicles, accumulated sucrose would by hydrolyzed into glucose and

![Figure 5. Δψ-Driven sucrose transport as a function of the transport solution pH. The freeze-thaw procedure (see "Materials and Methods") was used to equilibrate the intravesicular volume of the PMVs in treatment solutions which included 260 mM sorbitol, 30 mM K$_2$SO$_4$, 1 mM Mes, and 1 mM Hepes. The pH of these solutions was adjusted to 8.0, 7.0, 6.0, or 5.0 with Btp. The PMVs were diluted 20-fold into transport solutions of equal pH which included 350 mM sorbitol, 1 mM Mes, 1 mM Hepes, 0.5 mM sucrose, 5 μM valinomycin, and Btp, whose concentration varied depending on the final pH. In control experiments (not shown), sucrose transport was not altered by the changing concentrations of Btp. This experimental treatment resulted in a negative Δψ in the absence of a trans-membrane proton concentration difference.]

![Figure 6. ΔpH-dependent sucrose transport as a function of the transport solution proton concentration. The freeze-thaw procedure was used to equilibrate the intravesicular volume of the PMVs in treatment solutions which included 260 mM sorbitol, 30 mM K$_2$SO$_4$, 1 mM Mes, and 1 mM Hepes. The final pH was adjusted with Btp to 8.0, 7.0, 6.7, or 6.0. The PMVs were diluted 14-fold into transport solutions whose composition was exactly the same as the intravesicular volume of the diluted vesicles, except it also included 0.5 mM sucrose, 5 μM valinomycin, and the pH was adjusted at one pH unit less than the vesicles pH. This treatment resulted in a 10-fold proton concentration difference across the plasma membrane and clamped Δψ at zero mV.]

![Figure 7. Sucrose-dependent proton uptake into PMVs. The PMVs were equilibrated in a low buffer capacity treatment solution and subsequently diluted in the same solution to measure the depletion of protons with a proton sensitive electrode as described in "Materials and Methods." After a 5-min equilibration period, a concentrated aliquot of sucrose or raffinose was added to the treatment solution (−) to a final concentration of 18 mM. Sucrose increased the rate of proton flux into the vesicles whereas raffinose had no effect. Controls included sucrose and raffinose additions to the treatment solutions in the absence of PMVs to show that changes in solution proton concentration were not vesicle independent events.]

Copyright © 1990 American Society of Plant Biologists. All rights reserved.
Sucrose with the data as flux (22), in observed resultant proton ± sucrose developed and, porter the although physiological considerations was an an and, thus, a steep sucrose concentration gradient would be maintained. By accident, a small ΔpH (basic interior) was also introduced into the system when this protocol was developed and, as it turns out, both the small ΔpH and the presence of invertase contributed to the maintenance of linear rates of proton flux in the presence of the sucrose concentration gradient.

Typical results of sucrose-dependent proton flux are shown in Figure 7. In the absence of sucrose, a linear rate of proton uptake was measured as protons entered the vesicle by sucrose-independent pathways. This flux was driven by the ΔpH introduced as a consequence of preloading with invertase in a weakly buffered solution. When 18 mM sucrose was added, an increased proton conductance was recorded. This proton flux was specific for sucrose since a similar increase was not observed when 18 mM raffinose, a carbohydrate which shows no affinity for the proton-sucrose symport, was added (3). In parallel experiments, symport mediated sucrose flux was measured as the difference between [%C]Sucrose accumulation in the absence and presence of 1 mM PCMBs. Based on these quantitative measurements, symport-dependent proton and sucrose uptake were 2.4 ± 0.3 nmol H+/mg min⁻¹ and 2.95 ± 0.56 nmol sucrose/mg min⁻¹, respectively, and the resultant proton to sucrose stoichiometry was 0.81. Taken together with the data in Figure 6 and Turner’s kinetic model (22), it is concluded that the stoichiometry of the proton-sucrose symport is 1:1. The apparent underestimation of proton flux may be the result of buffering by the membrane vesicles or the result of proton-glucose efflux from the PMVs as glucose builds up due to invertase activity. Proton-glucose cotransport has been observed in these vesicles (DR Bush, unpublished data). A 1:1 stoichiometry is consistent with the measured stoichiometries of other ion-sugar cotransport systems (23) and Lin’s estimate for proton-sucrose cotransport into protoplasts isolated from soybean cotyledons (14). These results are also significant because they provide unequivocal evidence of coupled sucrose and proton transport.

Since the proton:sucrose stoichiometry sets a thermodynamic limit on the maximum sucrose accumulation ratio which the symport can achieve (i.e., ΔμSuc ≤ ΔμH+), it is important to ask whether the 1:1 stoichiometry reported here can account for physiologically relevant sucrose concentration gradients. Although a direct role for the proton-sucrose symport in phloem transport has yet to be demonstrated (3), the sucrose concentration gradient between the leaf mesophyll cells and vascular tissue is one of the largest found in the plant and, therefore, the following calculations are based on these concentration differences. If we use standard estimates of cytoplasmic pH (7.5), apoplastic pH (5.5), and the electrogenic component of Δψ (—80 mV), then the proton motive force available to drive sucrose transport is —198 mV at 25°C. This represents enough free energy to drive a reaction, assuming 100% efficiency, at least three orders of magnitude away from equilibrium. Based on osmotic pressure differences and estimates of apoplastic sucrose concentrations, the sucrose concentration of sieve elements and mesophyll cells in sugar beet leaves is approximately 0.8 and 0.02 M, respectively (7, 20). The free energy needed to maintain this concentration difference is —94 mV. Even if the concentration differences were 0.8 and 0.001 M, representing a ΔG of 172 mV, the free energy available in the proton electrochemical gradient is theoretically large enough to maintain this difference. Thus, thermodynamic considerations suggest a 1:1 stoichiometry can account for the sucrose concentration differences found in photosynthetically active leaf tissue.

The results presented here provide insight into the biochemical characteristics of the proton-sucrose symport. Symport mediated sucrose transport was electrogenic (Fig. 2), it was driven by both ΔpH and Δψ (Figs. 1 and 5), the binding affinity of the symport for protons (Km ≈ 0.7 μM) was much higher than that for sucrose (Km = 1 mM), and the stoichiometry of the symport was 1:1. These observations are summarized in a simple model of the symport system (Fig. 8). This model also underscores several important biochemical questions that must be answered before the molecular details of proton-sucrose cotransport can be understood.

LITERATURE CITED

substrate for the sucrose carrier during phloem loading in *Vicia faba* leaves. Plant Physiol 67: 560–564


