Biochimica et Biophysica Acta, 1103 (1992) 259–267

BBAMEM 75495

The sucrose carrier of the plant plasmalemma. III. Partial purification and reconstitution of active sucrose transport in liposomes

Ze-Sheng Li, Olivier Gallet, Cécile Gaillard, Rémi Lemoine and Serge Delrot

UA CNRS 574, Laboratoire de Biologie et Physiologie Végétales, Université de Poitiers, Poitiers (France)

(Received 25 July 199)

Key words: Sucrose carrier; Sucrose transport; Plant plasmalemma; Plant plasma membrane; Reconstitution; (Sugar beet)

The proteins from plasma membranes from sugar beet leaves were solubilized by 1% CHAPS and separated by size exclusion chromatography and by ion-exchange chromatography. The fractions enriched in sucrose transporter were monitored in three ways: differential labeling, ELISA, and reconstitution in proteoliposomes. When the plasma membranes were differentially labeled by *N*-ethylmaleimide in the presence of sucrose, a major peak of differential labeling was found at 120 kDa upon gel filtration. When this peak was recovered, denaturated by sodium dodecyl sulfate and reinjected on the gel filtration column, it yielded a peak of differential labeling at 42 kDa. When unlabeled membranes were used, the fractions eluted from the column were monitored by ELISA for their ability to recognize a serum directed against a 42 kDa previously identified as a putative sucrose carrier. The results paralleled those obtained by differential labeling, i.e. a major ELISA-reactive peak was found at 120 kDa upon gel filtration, and this peak yielded a peak most reactive at 40 kDa after denaturation. The 120 kDa peak prepared from unlabeled membranes was further separated on a Mono-Q column. The fractions were monitored by ELISA as described above, and reconstituted into proteoliposomes using asolectin. Active transport of sucrose, but not of valine could be observed with the reconstituted 120 kDa fraction. When the eluates from the Mono-Q column were reconstituted, the fractions exhibiting highest transport activity were enriched with a 42 kDa band. The data provide the first report concerning reconstitution of sucrose transport activity and confirm the involvement of a 42 kDa polypeptide in sucrose transport.

Introduction

According to the mass-flow model, transport of sucrose in the plant affects not only the partitioning of sugars, but also that of other phloem solutes (nitrogenous compounds, ions) [1]. Sucrose transport is therefore very important for plant productivity, not only in terms of sugars, but also more generally in terms of the biomass harvested in the sinks. Long distance transport of sucrose depends directly on the activity and on the distribution of sucrose carriers located at the plasmalemma and possibly at the tonoplast.

While the hexose carrier has now been identified and cloned in *Chlorella* and in *Arabidopsis* [2–4], the data concerning the sucrose carrier are still confuse. In soybean cotyledons, a 42 kDa polypeptide has been identified with a photolyzable derivative of sucrose as a putative sucrose carrier [5]. Although this polypeptide appears in the microsomal fraction of soybean cotyledon cells concomitantly with the onset of actice sucrose influx, no functional evidence concerning the identity of this protein is available, and its sequence does not show any homology with any known bacterial or animal sugar carrier [6]. Differential labeling of broad bean microsomes [7] and purified plasma membrane vesicles from broad bean [8] and sugar beet [9] leaves by NEM allowed the identification of an integral 42 kDa polypeptide which was specifically protected by su-

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; ΔpH, trans-membrane pH gradient; $\Delta\psi$, trans-membrane electrical gradient; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

Correspondence: S. Delrot, Laboratoire de Physiologie et Biochimie Végétales (CNRS UA 574), 25 rue du Faubourg St-Cyprien, 86000 Poitiers, France.

crose. Since polyclonal antibodies raised against the 42 kDa region of the plasma membrane selectively inhibited the uptake of sucrose into broad bean mesophyll protoplasts without affecting the uptake of hexoses or of amino acids, it was concluded that a 42 kDa polypeptide may be a component of the sucrose carrier of the plant plasma membrane [16]. Recently, techniques were designed to study the active uptake of sucrose into purified plasma membrane vesicles artificially energized by a proton motive force [11–15]. The gradients (ΔpH and $\Delta\psi$) obtained after artificial energization of these vesicles have been characterized [16], as well as proton fluxes associated with sucrose uptake in this material [17]. All the data point out to a proton-sucrose cotransport system operating in purified plasma membrane vesicles from plant leaves, confirming earlier physiological evidence [18–20].

Therefore, although several candidates have been proposed as sucrose carriers of the plant plasmalemma, and although it is possible to drive active uptake of sucrose in plasma membrane vesicles by imposing artificial ion gradients, no report of reconstitution of active transport of sucrose in proteoliposomes is available. The present paper makes use of the recent progress summarized above to identify plasma membrane fractions with which transport activity specific for sucrose could be reconstituted. Identification of the sucrose carrier under its native form was based both on the results of differential labeling and on immunological data.

Materials and Methods

Isolation of plasma membranes. Sugar beet plants (*Beta vulgaris* L. var. Aramis) were grown as described in Ref. 9. Mature exporting leaves (4–5 weeks old) were used for the preparation of plasma membrane vesicles. Plasma membranes vesicles (95% pure) were obtained from a microsomal fraction by two-phase-partitioning between Dextran T 500 and poly(ethylene glycol) 3350 [9,16].

Differential labeling. Plasma membrane vesicles were differentially labeled by a procedure using [^{3}H]NEM and [^{14}C]NEM in the presence of either sucrose or palatinose as protecting sugars. According to this procedure, detailed in Ref. 9, the polypeptides protected by the sugar tested (either sucrose or palatinose) bind more [^{3}H]NEM, while [^{14}C]NEM is used to trace background thiols. The [^{3}H]NEM/[^{14}C]NEM ratio which was monitored on the polypeptides separated by SDS-PAGE in the previous work [9], was measured on the different fractions recovered after solubilization and separation of the polypeptides by HPLC in the present work. For each fraction eluted from the column, a differential labeling index was calculated according to the formula: [(dpm ^{3}H of the fraction)/(dpm ^{14}C of the fraction)]/[(total ^{3}H of the sample injected)/(total ^{14}C of the sample injected)]. Differential labeling in the presence of palatinose (6-*O*-α-D-glucopyranosyl-D-fructofuranose, a non-transported analogue of sucrose [21]) was run as a control to identify the peaks of labeling that were specific for sucrose [9].

Solubilization and high performance liquid chromatography. Plasma membrane vesicles were resuspended (4 mg/ml) in a medium containing 20 mM potassium phosphate (pH 6.0), 330 mM sorbitol, 10% glycerol, 0.5 mM $CaCl_2$, and 0.25 mM $MgCl_2$. CHAPS (final concentration, 1%) was added dropwise under constant stirring at 4°C from a 10% stock solution prepared with the same medium. After 45 min solubilization, the insoluble material was pelleted down by centrifugation at 100000 × *g* for 30 min. Under these conditions, CHAPS solubilized about 55% of the total membrane proteins. The supernatant (referred to as 'CHAPS supernatant') was frozen at −70°C until further use. After thawing in a water bath at 25°C, the solubilized proteins (1.5 mg, 500 μl) were applied to a TSK SW-3000 gel filtration column (30 × 0.75 cm, Supelco) equilibrated with 50 mM Tris-acetate (pH 6.7), 100 mM NaCl and 0.1% CHAPS. The proteins were eluted at a flow rate of 0.4 ml/min and collected in 0.4 ml aliquots. The ability of each fraction to react with an antiserum directed against the 42 kDa polypeptide of the plasma membrane assumed to be the sucrose carrier [9,10] was tested by ELISA as described below.

The gel filtration column was calibrated with molecular weight standards, in the presence of 0.1% CHAPS when the 'CHAPS supernatant' was analyzed, or in the presence of 0.1% SDS when proteins denaturated by SDS were analyzed. The molecular weight standards were obtained from Bochringer (Combithek size II) and consisted of cytochrome *c* (12.5 kDa), chymotrypsinogen A (25 kDa), hen egg albumin (45 kDa), bovine serum albumin (68 kDa) and aldolase (158 kDa).

The 120 kDa fraction recovered after gel filtration chromatography was dialyzed and concentrated overnight in a buffer containing 20 mM Tris-HCl (pH 8.3) and 0.15% CHAPS. The '120 kDa' fraction (0.6 mg) was then injected on a mono-Q column (HR5/5; Pharmacia) with a flow rate of 0.75 ml/min. A step gradient was imposed with the two following solvents: A, 20 mM Tris-HCl + 2.5 mM (0.15%) CHAPS; B = A + 1 M NaCl. The solvents were changed as follows: 0–5 min, 100% A; from 5 to 35 min, solvent B was introduced to form a linear gradient up to 50%; 35–40 min, 100% B. The fractions (0.75 ml) were analyzed for UV absorbance and recognition by the anti-42 kDa serum. The polypeptides eluted from the column were separated by SDS-PAGE [9] and visualized after silver staining [22]. In some experiments, 50 mM sucrose or 50 mM lactose were included in buffers A and B, to test the effects of these sugars on the elution and on

the recognition of proteins by the anti-42 kDa serum. While sucrose is obviously a substrate for the sucrose carrier, lactose is not transported by this transporter [21].

Immunological procedures. The 42 kDa antigen was prepared from preparative SDS-PAGE of purified plasma membranes from sugar beet leaves [9]. A mouse ascite fluid directed against this polypeptide was obtained by the immunization procedure described in Ref. 23. This ascite fluid was used to monitor the reactivity of the plasma membranes proteins eluted from the gel filtration column by means of an ELISA test and by Western blot.

200 μl (3–7 μg protein) of each aliquot recovered from HPLC (gel filtration or ion exchange) were used to coat ELISA plates overnight. This amount of protein was higher than that needed to saturate the adsorption sites of the well (2 μg). The wells were washed three times 15 min with phosphate buffer saline containing 3% defatted dry milk and 0.5% (v/v) Tween 20. The ELISA test was run using standard procedures with a 1/500 dilution of primary (anti-42 kDa) ascite fluid and a 1/2000 dilution of horseradish peroxydase-conjugated goat anti-mouse antibody (EIA grade, Bio-Rad). In some experiments, the effect of adding sucrose or lactose in the elution buffer of the column was studied on the ELISA response. In this case, the sugar was present in the coating medium, but all the other steps of the ELISA were run with standard media without sugar.

The CHAPS supernatant and some of the fractions recovered after gel filtration and/or ion exchange chromatography were analyzed by Western blot according to Ref. 10, after separation of the proteins contained by SDS-PAGE. About 60 μg protein were deposited in each well. The proteins transferred to the nitrocellulose sheet were stained for 10 min in a solution containing 3% (w/w) trichloracetic acid and 0.2% (w/w) Ponceau red. Proteins were assayed according to Ref. 24.

Reconstitution experiments. Reconstitution was done following a procedure modified from Ref. 25. Soybean asolectin (Sigma IV-S) was dissolved in chloroform at a concentration of 200 mg/ml and stored at −20°C under N_2. To prepare lipids for use during reconstitution, 4.4 mg asolectin were dried under N_2, rotating the tube to form a thin film. To remove residual chloroform, the phospholipids were washed with 1 ml of cold (−20°C) diethyl ether and evaporated to dryness. The lipids were dried for an additional 30 min under vacuum to remove all traces of solvent. The lipids were resuspended in 400 μl of solution containing 50 mM potassium phosphate (pH 7.5) and 1 mM DTT. The mixture was bath-sonicated (Bransonic-5 sonicator) to clarity (about 30 min) in a tube immersed in ice. CHAPS (1%, final concentration) was added from a 10% stock solution prepared in 50 mM potassium (pH 7.5), and the tube was vortexed for 15 s. Glycerol (20%, final concentration, v/v) was added and the tube was vortexed again for 15 s. 1 mg (1.7 ml) of membrane protein 'CHAPS supernatant', 120 kDa fraction (as referred to in the results) or Mono-Q eluate were added to the tube, and incubated 30 min at 4°C. The insoluble material was then removed by pelleting at 100000 × g for 45 min. Reconstitution was done by adding 2 ml of the supernatant in a tube containing 5 mg dried asolectin and 178 μl of 50 mM potassium phosphate buffer (pH 7.5). The tube was sonicated for 30 min, and 44 μl of a 10% CHAPS solution were added before vortexing, and incubation for 30 min at 4°C. Proteoliposomes were formed by rapid injection (Pasteur pipet) of the ice-cold solubilized protein/phospholipid mixture into 25 ml of 50 mM potassium phosphate (pH 7.5). The tube was incubated 20 min at room temperature and centrifuged for 1 h at 100000 × g. The final pellet was resuspended into 100 μl potassium phosphate buffer (pH 7.5).

The CHAPS supernatant contained enough protein to be reconstituted 'directly'; yet, to reconstitute the 120 kDa fraction, it was necessary to pool the 120 kDa eluates of three runs on the gel filtration column. The 120 kDa fraction pooled in this way was concentrated under pressure using an Amicon system equipped with a 10 kDa cut-off size filter, and used for reconstitution as described above. To reconstitute the fractions eluted from the Mono-Q column, it was necessary to pool the corresponding eluates of 30 injections on the column. The fractions were concentrated as described above for the 120 kDa fraction before being reconstituted.

Uptake experiments. Uptake experiments with reconstituted proteoliposomes containing either the 'CHAPS supernatant', the '120 kDa fraction' or fractions eluted from the Mono-Q column were run according to the technique previously designed to study active uptake by native plasma membrane vesicles [13], after imposition of ΔpH and $\Delta\psi$ across the membrane. For comparison, uptake experiments were also run with native plasma membrane vesicles. Briefly, the membranes were first equilibrated with a medium (medium K) containing 0.3 M sorbitol, 50 mM potassium phosphate (pH 7.5), 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$ and 0.5 mM DTT, at a concentration of 15 mg protein per ml. At the beginning of incubation, 2 μl of this suspension were suspended in 400 ml of a medium containing 0.3 M sorbitol, 50 mM sodium phosphate (pH 5.5), 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, 0.5 mM DTT, 5 μM valinomycin and 1 mM [6,6′(n)-^{3}H]sucrose (26 kBq) (medium Na). The pH buffers used and the combination of K^+/valinomycin energized the vesicles by the creation of ΔpH (interior alkaline) + $\Delta\psi$ (interior negative). At the end of incubation, uptake was terminated by addition of 1.75 ml of chilled rinsing medium (medium

Na + 5 mM $HgCl_2$), and filtration on a Millipore HAWP filter, pore size 0.42 μm for the plasma membrane vesicles, or on a Millipore GS filter, pore size 0.22 μm, for the proteoliposomes. Sucrose uptake was measured as the difference between uptake at the time selected and time zero (i.e. the samples were immediately diluted with ice-cold blocking medium containing 1 mM $HgCl_2$ and filtrated). Uptake under non-energized conditions was also studied by equilibrating the vesicles in medium K, and resuspending them in the same medium, in the presence of 5 μM nigericin to collapse any gradient.

In some experiments, the same procedure was used to study the uptake of valine, except that the incubation medium contained 1 mM L-[3,4(n)-^{3}H]valine (18.5 kBq) instead of labeled sucrose.

All experiments (HPLC and uptake experiments) were repeated at least three times with similar results. In each uptake experiment, data points were the mean of four independent samples.

Results

Differential labeling and fractionation of membrane proteins

First, the labeling pattern of plasma membrane vesicles differentially labeled by [^{3}H]NEM/[^{14}C]NEM in the presence of sucrose was studied after solubilization of the integral proteins by CHAPS, and size exclusion chromatography of these proteins under non-denaturating conditions. These data were compared with the labeling pattern of plasma membrane vesicles differentially labeled in the presence of a non-protecting sugar, i.e. palatinose. Fig. 1A shows that the patterns obtained were quite similar, except for a peak differentially labeled only in the presence of sucrose, located at 120–125 kDa. This peak was collected and pooled as shown by the dotted area in Fig. 1A. From the UV absorbance data, the proteins pooled in this way accounted for about 23% of the total plasma membrane proteins. In the following, this fraction will be referred to as the '120 kDa fraction'.

The 120 kDa fraction from membranes differentially labeled by [^{3}H]NEM/[^{14}C]NEM either in the presence of sucrose or palatinose was denaturated by SDS (1%, 60°C for 15 min) and resubmitted to gel filtration. Under these conditions, the 120 kDa fraction from plasma membranes differentially labeled in the presence of sucrose yielded a rather large peak centered around 42 kDa (Fig. 1B). The differential labeling was much more apparent after denaturation by SDS, since the labeling index reached 4 for the denaturated proteins (Fig. 1B), compared to 1.4 for the native proteins (Fig. 1A).

Fractionation of membrane proteins as monitored with the anti-42 kDa ascite fluid

Various polyclonal anti-42 kDa sera, including the ascite fluid used for the present study, inhibit active uptake of sucrose into protoplasts [8] and into purified plasma membrane vesicles [18,26]. The ascite fluid reacted with a rather wide region of the plasmalemma around 42 kDa, but no other region of the plasma membrane cross-reacted with this serum [27].

After gel filtration under non-denaturating conditions, a broad region around 120 kDa was the most reactive in ELISA with the anti-42 kDa serum (Fig. 2A). This peak accounted for about 23% of total plasma membrane proteins. When the 120 kDa peak was recovered, denaturated by SDS and resubmitted to gel filtration, the major ELISA-reactive peak was observed around 40 kDa, while the 120 kDa peak was strongly reduced (Fig. 2B) (two experiments) or completely disappeared (three experiments), depending on the experiments.

When the 120 kDa peak was subjected to ion-exchange chromatography under non-denaturating conditions, a broad peak recognized by the anti-42 kDa ascite fluid was eluted between 0.27 and 0.31 M NaCl

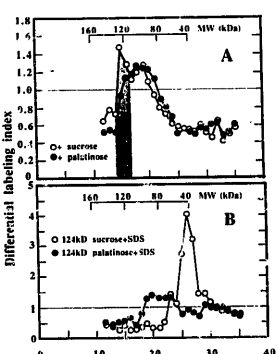

Fig. 1. Labeling profile of plasma membranes proteins differentially labeled by NEM in the presence of either sucrose (○) or palatinose (●), after size exclusion chromatography on a TSK-SW 3000 column. (A) The plasma membrane proteins were differentially labeled, solubilized by 1% CHAPS, and injected on the gel filtration column. (B) The 120 kDa fraction (dotted area in Fig. 1A) of membranes differentially labeled in the presence of either sucrose or palatinose was collected, denaturated by SDS, and analyzed again by gel filtration chromatography. Typical UV absorbance profiles are shown in Fig. 2. Dextran blue 2000, used as a marker of void volume, was eluted in fraction 10.

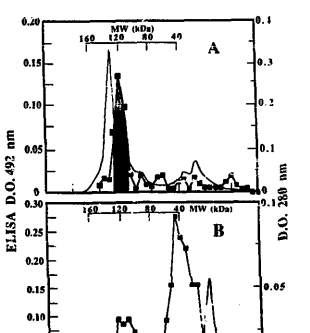

Fig. 2. ELISA monitoring (left ordinate, ■) and UV absorbance at 280 nm (right ordinate) of unlabeled membranes solubilized by 1% CHAPS and separated by size exclusion chromatography. Aliquots of the fractions eluted from the column were coated on ELISA plates, and their ability to be recognized with an anti-42 kDa polyclonal ascite fluid was measured by standard ELISA. (A) Chromatography of the proteins solubilized by CHAPS. (B) The 120 kDa area (dotted area in Fig. 2A) was pooled and denaturated by SDS before being separated again on the same column.

(Fig. 3A). The binding of antibodies directed towards transport proteins may be affected by conformational changes induced in the presence of the substrate [29]. Therefore, in an attempt to identify more precisely which fractions would contain the highest amounts of proteins specifically recognizing sucrose, either 50 mM (final concentration) sucrose or 50 mM lactose were included in the elution buffer. These inclusions did not affect the elution pattern from the ion-exchange column, as estimated from the UV absorbance. However, upon inclusion of sucrose in the elution medium, and hence in the medium used to coat the eluted proteins on the ELISA plates, fraction No. 27 exhibited a strong increase in reactivity with the anti-42 kDa ascite fluid (Fig. 3B), compared to an elution medium without added sugar (Fig. 3A) or with addition of lactose (Fig. 3C). In order to obtain enough protein for reconstitution, and to avoid extensive contamination by the large protein peak occurring in fractions 28–29, fractions 24–27 were pooled together. These fractions represented about 2 to 3% of the 120 kDa fraction. Starting from 100 mg purified plasma membranes, only 0.35 mg of fractions 24–27 were obtained after all the steps involved in preparation, i.e. solubilization, gel filtration, ion-exchange chromatography, and several steps of concentration dialysis.

Data concerning SDS-PAGE of the different fractions obtained after solubilization, size exclusion chromatography, and ion-exchange chromatography are presented in Fig. 4. CHAPS (Fig. 4, lane C) solubilized most of the proteins of the plasmalemma. The '120 kDa' fraction yielded numerous polypeptides after denaturating SDS-PAGE (Fig. 4, lane D), but surprisingly bands at 42 kDa were hardly visible. Although some bands of the CHAPS supernatant were missing in the 120 kDa fraction (for example, at 70, 37, 33 and 24 kDa), silver staining suggested that solubilization by CHAPS and size exclusion chromatography allowed poor separation of the membrane polypeptides. The pattern was quite different after ion exchange, since the different fractions collected (Fig. 4, lanes E–J) showed marked differences in the polypeptide pattern. Given the previous identification of a 42 kDa polypeptide as component of a sucrose transport system, and

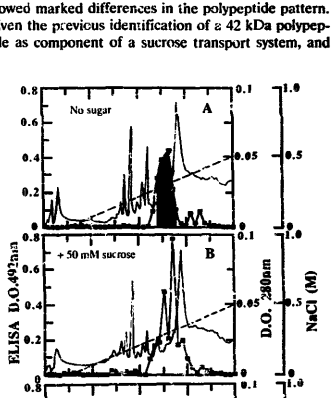

Fig. 3. Separation of the 120 kDa fraction by ion-exchange chromatography on a Mono-Q column. Eluates were monitored by ELISA and for UV absorbance as explained in the legend of Fig. 2. The NaCl gradient used for elution is shown by the dotted line, and by the ordinate at the extreme right. (A) The elution medium contained no sugar. (B and C) The elution medium contained 50 mM sucrose and 50 mM lactose, respectively. Appropriate blanks were run to check for the effects of sugars on ELISA detection.

given the data presented below, it was interesting to note the presence of two close bands at 42 kDa in fractions 24–27 collected from the mono-Q column (Fig. 4, lane E). Compared to fractions 28–30, fractions 24–27 also contained additional bands at 29 and possibly 73 kDa. Immunoblots show that this fraction was clearly recognized the anti-42 kDa serum (Fig. 4, lane E'), while no reaction was found for fractions 28–30 (Fig. 4, lane F'), and only a poor reaction could be seen with the 120 kDa fraction (Fig. 4, lane D').

Active uptake by reconstituted proteoliposomes

When the 120 kDa fraction was used for reconstitution experiments, measurements of protein recovered into the proteoliposomes showed that about 67% of the fraction was actually reincorporated. Since the 120 kDa fraction accounts for 23% of total plasma membrane proteins, about 15% of the total membrane proteins were incorporated in the liposomes. In the absence of energization, little sucrose was retained in the reconstituted proteoliposomes (Fig. 5A, lower curve). A proton-motive force-driven sucrose uptake was obtained after imposition of $\Delta pH + \Delta\psi$ (Fig. 5A, upper curve). The difference between the energized conditions and the non-energized conditions is representative of an active, proton-driven uptake of sucrose. After 2 min incubation, active uptake of sucrose was completely abolished in the presence of 0.1 mM $HgCl_2$, and was inhibited to 20% of the control value in the presence of 0.5 mM NEM. Using the same proteoliposomes that were competent for active transport of sucrose, no active uptake of valine could be observed (Fig. 5B). The amounts of valine retained passively on the vesicles were higher than the amounts of sucrose retained passively, possibly due to adsorption of charged valine to the membrane proteins. Comparison of Figs. 5A and 5B shows that the active uptake system reconstituted was specific for sucrose.

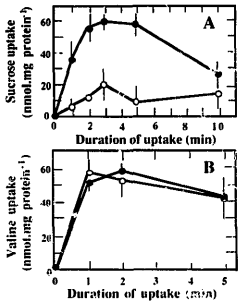

Fig. 5. Time course of uptake of 1 mM sucrose (A) or 1 mM valine (B) by proteoliposomes reconstituted with the 120 kDa fraction. Experiments were run in the presence (●) or in the absence (○) of imposed $\Delta pH + \Delta\psi$. Means of 12 measurements (three independent experiments) ± S.E.

The time course of active uptake of sucrose into native plasma membrane vesicles, into proteoliposomes made from the CHAPS supernatant and into proteoliposomes made from the 120 kDa fraction was com-

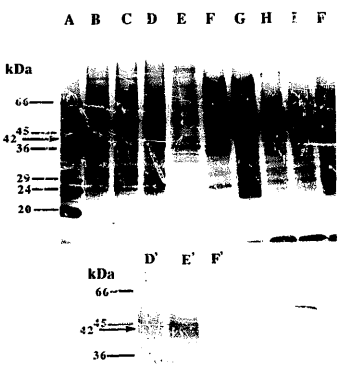

Fig. 4. Silver-staining (top) and immunoblot (bottom) of various plasma membrane fractions. (A) Molecular weight standards; (B) total plasma membranes; (C) CHAPS supernatant; (D) 120 kDa fraction; (E–J) fractions 24–27, 28–30, 31–33, 34–37, 37–40 and 40–45 of the Mono-Q eluate (Fig. 3). The same amount of protein (about 7 mg) was deposited in each lane. (D', E', F') Lanes D (120 kDa fraction), E (fractions 24–27) and F (fractions 27–30) were probed by immunoblotting with the anti-42 kDa polyclonal ascite fluid.

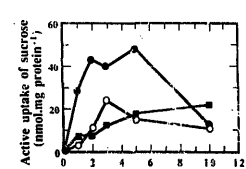

Fig. 6. Active transport of sucrose into native plasma membrane vesicles (■) and into proteoliposomes reconstituted with the CHAPS supernatant (○) or the 120 kDa fraction (●). Active uptake was obtained from the difference of uptake under energized and non-energized conditions.

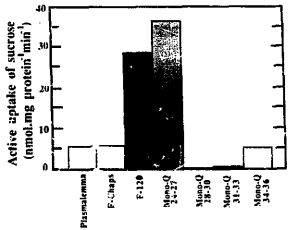

Fig. 7. Ability of different plasma membrane fractions (see abscissa) to take up sucrose actively. The data are expressed as active uptake per min per mg protein actually reincorporated in the proteoliposomes, and compared with native plasma membrane vesicles. Active uptake was measured after 1 min incubation (means of 6–8 measurements, one experiment).

pared (Fig. 6). Proteoliposomes made from the CHAPS supernatant did not show a stronger accumulation than the native plasma membranes, at least for short incubation times. Yet, proteoliposomes made from the 120 kDa fraction accumulate more sucrose than native plasma membranes. Also, the initial rate of influx was much higher in these proteoliposomes than in the native plasma membranes (Fig. 6).

Fig. 7 shows the specific activity of active transport of sucrose (expressed as proton-driven accumulation of sucrose per protein actually reincorporated in the proteoliposomes) of the various fractions obtained after solubilization, gel filtration, and ion-exchange chromatography. The largest increase of specific activity of transport was obtained by size exclusion chromatography. Rather surprisingly, only a marginal increase was noted for fractions 24–27 of the Mono-Q column. The other fractions obtained after ion-exchange chromatography exhibited virtually no transport activity.

Discussion

Diffferential labeling of plasma membranes by NEM led us to identify, after denaturating SDS-PAGE, a 42 kDa polypeptide that was differentially labeled in the presence of sucrose, but not of palatinose [7–9]. These data as well as selective inhibition of sucrose uptake in protoplasts by a polyclonal serum directed against the anti-42 kDa region of the plasma membrane [10] led us to conclude that this polypeptide was a component of the sucrose uptake system. The present work was conducted to try to identify the sucrose carrier under a functional state, in the light of these former results, and as a further test for our hypothesis.

The only information we had on the putative sucrose carrier was that it was differentially labeled, and behaved as a 42 kDa polypeptide after denaturation. Although antibodies were available, the preparation of the antigen needed to raise the antibodies is very time-consuming, and the amount of serum was limited. This precluded the use of immunoaffinity chromatography to prepare fractions enriched with the sucrose carrier. Attempts to identify the native sucrose carrier were therefore based on size exclusion chromatography of plasma membrane proteins differentiallly labeled by NEM, and monitoring of native proteins by ELISA with an anti-42 kDa polyclonal ascite fluid.

After differential labeling, solubilization and gel filtration under non-denaturating conditions, we found no peak of differential labeling at 42 kDa, but a single, rather broad, peak specific for sucrose at 120 kDa (Fig. 1A). The breadth of the peak is not unexpected in view of the poor separating power of the gel filtration columns, particularly with detergent having a high critical micellar concentration (0.5% for CHAPS). This poor resolution was found also, for example, after photoaffinity labeling of the calcium channel of the plant plasma membrane [29]. After denaturation, the 120 kDa peak yielded a peak at 42 kDa (Fig. 1B). These data, which were confirmed by that obtained after monitoring of the eluate in ELISA with the anti-42 kDa ascite fluid (Fig. 2), suggest that under these experimental conditions, the 42 kDa polypeptide is part of a bigger polymer. Although trimerization of transport proteins has already been reported for bacteria [50], more work is needed to conclude on the nature of the macromolecular structure in which the 42 kDa polypeptide is associated. The fact that the differential labeling index (Fig. 1) as well as the ELISA response (Fig. 2) of the 42 kDa peak are higher than that of the 120 kDa peak would support rather an association of the 42 kDa polypeptide with other polypeptides, rather than a trimerization process. However, the relevance of this oligomerization observed *in vitro*, after solubilization by a detergent, with the state of the 42 kDa polypeptide in the membrane *in vivo* needs further work to be tested.

The good agreement found between the differential labeling and the ELISA showed that the ascitic fluid could be used to monitor the purification of the 42 kDa polypeptide. This was important since the further purification step by ion-exchange chromatography was made in order to reconstitute the transport activity. It was impossible to use proteins differentially labeled by NEM, which blocks transport activity. Parallel runs on the Mono-Q could have been made, first with differentially labeled samples to localize the protein, then with unlabeled samples to recover the peak and to reconstitute it, but we have no evidence that blocking the protein with NEM would not alter its properties in ion-exchange chromatography. Therefore, the purification of the polypeptides on the Mono-Q was monitored

with the ELISA test. A single, rather broad peak was also found after ion-exchange. Interestingly, the ELISA response of this peak (or at least part of this peak) was modified by sucrose, but not by lactose (Fig. 3). This strengthens the hypothesis that the proteins present in this peak interact specifically with sucrose since the ELISA response of proteins may be affected by the presence of their substrate [28].

The purification and the identification of transport proteins strongly depend on the possibility to reconstitute the transport system. The reconstitution procedure was first designed with the 120 kDa fraction (Fig. 5). Upon imposition of an artificial protonmotive force, the reconstituted 120 kDa fraction was able to take up sucrose, but not valine, against a concentration gradient. The study of the specific activity of active sucrose transport (nmol sucrose taken up per min per mg reconstituted protein, Fig. 7) shows that the transport activity of the 120 kDa fraction was about 5.6-fold higher than that of native plasma membrane vesicles. Given that the reconstituted proteins represents about 15% of the total membrane proteins initially present in the vesicles, a 6.6-fold increase in the specific activity of transport should be expected if no activity was lost. The difference between the expected value and the measured value indicates that about 15% of activity was lost during the solubilisation/reconstitution procedure. This small loss suggests that CHAPS is a suitable detergent to solubilize the sucrose carrier and to reconstitute it. This high activity also implies either that most of the carrier was reconstituted in the right orientation, and/or that the functioning of the carrier is symmetrical. Preliminary experiments with octyl glucoside or Triton X-100 did not give results better than CHAPS. Yet, after 1 min incubation, no increase of specific activity was observed for the CHAPS supernatant (Fig. 7), and a decrease was even sometimes observed, compared to native plasma membrane vesicles (Fig. 6). Some increase was expected since the CHAPS supernatant contains only 55% of the initial protein content. The reason for this may be due to the fact that the data of Fig. 7 were obtained after 1 minute of incubation, a time assumed to give the best account of the initial velocity of transport. Although this assumption seems valid for native plasma membrane vesicles and for the 120 kDa fraction, the time course study shows that this is not necessarily true in all cases, and at least for the CHAPS supernatant (Fig. 6). This illustrates the limit of comparing initial velocities of transport in proteoliposomes, where the proton motive force may take some time to build up, and is rapidly dissipated due to the lack of any system compensating for the dissipation of ΔpH and $\Delta\psi$ due to the operation of the proton-cotransport system. Another limitation to the comparison of the specific activities of transport is the assumption that the size and the sealingness of the vesicles is the same for the different fractions reconstituted, which is not necessarily true.

Since the fractions 24–27 of the Mono-Q eluate represent about 2 to 3% of the 120 kDa fraction, a large increase of specific transport activity was expected, unlike the marginal increase actually observed (Fig. 7). A considerable loss of activity seems therefore to occur during ion-exchange chromatography. Numerous reasons might explain this observation. First, this loss may be only apparent if the activity was very high and the initial velocity conditions were maintained just during a few seconds. In this case, measurements made after 1 min incubation would concern the declining phase of the overshoot usually observed in membrane systems artificially energized. One way to check this hypothesis would be to run time course studies, which is hardly feasible with the Mono-Q eluates, due to the very low amount of proteins recovered. Another possibility is to run the uptake assay with lower concentrations of sucrose, to dissipate less rapidly the proton motive force. Another reason which this loss of activity could be only apparent may be due to kinetic limitations, for example, if the maximum number of carriers that can be incorporated in the vesicles is already attained with the 120 kDa fraction.

Of course, the loss of transport activity observed during after ion-exchange chromatography may be real, and again, several reasons may explain it, e.g. sensitivity of the carrier to the conditions used for chromatography, dialysis, reconstitution, loss of an active component (polypeptide, ion) during ion-exchange chromatography, wrong orientation of the carrier, etc... Loss of activity is unfortunately commonly found when membrane proteins are removed from their natural lipidic environment, even for proteins that do not exhibit transport activity, but simply chemical activity. For example, solubilization and HPLC purification of 1,3-β-glucan synthase from plasma membranes of *Brassica oleracea* was not accompanied by any increase in specific activity compared to the plasma membranes [31].

Although the data suggest that a large part of activity was lost during ion-exchange chromatography, fractions 24–27 possess a much higher transport activity than the other fractions recovered from the Mono-Q column. Comparisons of the polypeptide pattern of this fraction with that of fractions 28–30 (Fig. 4), which exhibit very little transport activity (Fig. 7) shows that only a few bands differ between both samples: two very close bands at 42 kDa, one minor band at 29 kDa, and possibly another band at 75 kDa are present in fractions 24–27 but not in fractions 28–30. The two bands at 42 kDa could hardly been distinguished in the 120 kDa fraction after silver staining, but a positive reaction was seen at 42 kDa in this fraction after immuno-

blotting (Fig. 4). The presence of two well-defined at 42 kDa in the Mono-Q eluate showing the highest activity of proton-driven sucrose transport after reconstitution gives strong support to the previous hypothesis that a 42 kD band is involved in sucrose transport.

Conclusions

The results presented here, based on three different approaches (differential labeling, ELISA, reconstitution) provide the first data concerning the partial purification and reconstitution of a carrier system exhibiting a proton-driven transport specific for sucrose. Altogether, the data confirm the previous conclusion that a 42 kDa polypeptide is part of the sucrose uptake system of the plant plasma membrane [7–10]. Other evidence supporting this conclusion has been recently obtained in our lab. Antisera from rabbits or from mice directed against the 42 kDa region of the plasma membrane inhibited the uptake of sucrose, but not the uptake of valine into plasma membrane vesicles artificially energized by a proton motive force [26]. Although improvements may be still needed to increase the transport activity measured in proteoliposomes, and to purify and to characterize further the carrier (role of the 73 kDa and 29 kDa bands, kinetics of the reconstituted system), severe limitations are imposed to these biochemical approaches by the large amounts of plasma membranes to prepare and to handle, due to large losses of proteins during the various steps of purification, particularly during dialysis and concentration. These limitations might be avoided by the cloning of the corresponding genes and their expression in appropriate systems.

In spite of the difficulty of the biochemical approach, significant progress is presently being made in the reconstitution of carriers possessing a key role in carbon compartmentation, since partial purification and reconstitution of the malate carrier of the tonoplast was also recently reported [32].

Acknowledgements

We are grateful to Janine Bonmort and Catherine Charlot for help in the preparation of the plasma membranes and of the ascite fluid. This work was supported in part by the EEC under the Bridge Programme (Contract BIOT-0175-C).

References

1 Munch, E (1930) Die Stoffbewegungen in der Pflanze, Jena, Gustav Fisher.
2 Sauer, N. and Tanner, W. (1989) FEBS Lett. 259, 43–46.
3 Sauer, N., Caspari, T., Klebl, F. and Tanner, W. (1990) Proc. Natl. Acad. Sci USA 87, 7949–7952.
4 Sauer, N., Friedländer, K. and Gram-Wicke, U. (1990) EMBO J. 9, 3045–3050.
5 Ripp, K.G., Viitanen, P.V., Hitz, W.D. and Franceschi, V.R. (1988) Plant Physiol. 88, 1435–1445.
6 Hitz, W.D., Ripp, K.G. and Warmbrodt, R.D. (1990) Abst. Int. Conf. on Phloem Transport and Assimilate Compartmentation, Cognac, August 19–24, 1990.
7 Pichelin-Poitevin, D., Delrot, S., M'Batchi, B. and Everat-Bourbouloux, A. (1987) Plant Physiol. Biochem. 25, 597–607.
8 Pichelin-Poitevin, D. and Delrot, S. (1987) CR Acad. Sci. Paris 304, 371–374.
9 Gallet, O., Lemoine, R., Larsson, C. and Delrot, S. (1989) Biochim. Biophys. Acta 978, 56–64.
10 Lemoine, R., Delrot, S., Gallet, O. and Larsson, C. (1989) Biochim. Biophys. Acta 78, 65–71.
11 Bush, D.R. (1989) Plant Physiol. 89, 1318–1323.
12 Buckhout, T.J. (1989) Planta 178, 393–399.
13 Lemoine, R. and Delrot, S. (1989) FEBS Lett. 249, 129–133.
14 Bush, D.R. (1990) Plant Physiol. 93, 1590–1596.
15 Williams, L.E., Nelson, S.J. and Hall, J.L. (1990) Planta 182, 540–545.
16 Lemoine, R., Bourquin, S. and Delrot, S. (1991) Physiol. Plant. 82, 377–384.
17 Slone, J.H. and Buckhout, T.J. (1991) Planta 183, 584–589.
18 Giaquinta, R.T. (1977) Plant Physiol. 59, 750–755.
19 Komor, E. (1977) Planta 137, 119–131.
20 Delrot, S. (1981) Plant Physiol. 68, 706–711.
21 M'Batchi, B. and Delrot, S. (1988) Planta 174, 340–348.
22 Guevara, J., Jr., Johnston, D.A., Ramagali, L.S., Martin, B.A., Capetillo, S. and Rodriguez, L.V. (1982) Electrophoresis 3, 197–205.
23 Turano, F.J., Jordan, R.L. and Matthews, B.F. (1990) Plant Physiol. 92, 395–400.
24 Bearden, J.C., Jr. (1978) Biochim. Biophys. Acta 533, 525–529.
25 Schumaker, K.S. and Sze, H.(1990) Plant Physiol. 92, 340–345.
26 Gallet, O., Lemoine, R., Gaillard, C., Larsson, C. and Delrot, S. (1991) Plant Physiol, 97, in press.
27 Li, Z.S., Gallet, O., Gaillard, C., Lemoine, R. and Delrot, S. (1991) FEBS Lett. 286, 117–120.
28 Bernotat-Danielowski, S. and Koepsell, H. (1988) J. Immunol. Methods 115, 275–287.
29 Thuleau, P., Graziana, A., Canut, H. and Ranjeva, R. (1990) Proc. Natl. Acad. Sci. USA 87, 10000–10004.
30 De Cock, H., Hendriks, R., De Vrije, T. and Tommassen, J. (1990) J. Biol. Chem. 265, 4646–4651.
31 Fredrikson, K., Kjellbom, P. and Larsson, C. (1991) Physiol. Plant. 81, 289–294.
32 Martinoia, E., Vogt, E. Rentsch, D. and Amrhein, N. (1991) Biochim. Biophys. Acta 1062, 271–278.