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# SOLUBILIZATION AND RECONSTITUTION OF THE GLUCOSE TRANSPORT SYSTEM FROM *SACCHAROMYCES CEREVISIAE*

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**The glucose transport system from *Saccharomyces cerevisiae* was solubilized from isolated plasma membranes by the nonionic detergent, octylglucoside. The transport system was reconstituted into proteoliposomes with removal of detergent from the extract by dialysis, followed by the addition of asolectin liposomes to the dialyzed proteins with a freeze-thaw and brief bath-sonication step. The reconstituted proteoliposomes exhibit specific carrier-mediated facilitated diffusion of D-glucose, including stimulated equilibrium exchange and influx counterflow. Furthermore, the reconstituted facilitated diffusion system shows substrate specificities similar to those of the intact cell D-glucose transport system.**

## Introduction

The facilitated diffusion of glucose is characterized by a stereospecific, carrier-mediated transport mechanism. The study of sugar transport in the yeast, *Saccharomyces cerevisiae*, has been confined principally to measurements on intact cells by the use of kinetic analyses with substrate analogs, inhibitors and transport-associated mutations [1–3]. Despite these efforts, the identity and mechanism of the protein(s) involved in the D-glucose facilitated diffusion process remain unresolved by studies with whole yeast cells.

In recent work, we demonstrated D-glucose-specific, carrier-mediated facilitated diffusion in hybrid liposome-plasma membrane vesicles ('vesicles' [3]) prepared by an adaptation of the procedure of Kasahara and Hinkle [4]. We reported that the addition of soybean phospholipid liposomes to the isolated membranes followed by freezing, thawing and brief sonication in a bath sonicator produces vesicles which exhibit specific carrier-mediated facilitated diffusion of D-glucose, including stimulated equilibrium exchange and influx counterflow. These vesicle preparations were an important step in our work towards the solubilization and reconstitution of yeast glucose transport into artificial liposomes, as described below.

The glucose transport system from *S. cerevisiae* was reconstituted by a procedure adapted from Kasahara and Hinkle [4], and Newman and Wilson [5]. The nonionic detergent, octylglucoside, was used to solubilize the yeast glucose transport system from the isolated plasma membranes. After dialyzing the solubilized extract, the proteins were incorporated into bath-sonicated phospholipid liposomes by a freeze-thaw-sonication step, forming the reconstituted proteoliposomes.

In this communication, we demonstrate that the reconstituted carrier-mediated facilitated diffusion system for D-glucose shows kinetic properties and substrate specificities similar to those of the intact yeast cell.

## Materials and Methods

*Yeast.* Red Star commercial 1 lb yeast cakes were purchased from a local bakery. They were washed two or three times with glass distilled deionized water just before use.

*Isolation of plasma membranes.* This procedure has been described in detail elsewhere [3], and remained unmodified for this study.

*Preparation of liposomes.* This procedure has been described in detail previously [3]. Liposomes were used within one week of their formation. Asolectin was obtained from Associated Concentrates, Woodside, NY; other phospholipids were from Sigma Chemical Co., St. Louis, MO.

*Solubilization of plasma membranes.* The procedure for vesicle preparation, which involves the mixing of plasma membrane protein with exogenous liposomes by freezing-thawing followed by a brief bath-sonication step, has been described in detail previously [3]. Solubilization of the plasma membranes, and reconstitution of the D-glucose transport system into artificial liposomes was performed as follows. The solubilization mixture consisted of 0.6 ml buffer A (50% glycerol/10 mM 2-mercaptoethanol/5 mM EDTA/0.02% sodium azide/250 mM potassium phosphate (pH 7.5) at 4°C), 3.75 mg yeast plasma membrane protein, 37.5 mg octylglucoside (Calbiochem, La Jolla, CA), 11.25 mg bath-sonicated asolectin liposomes (from a 48 mg/ml stock solution), and glass-distilled water brought to a final volume of 3 ml. The detergent was added to the mixture on ice in two 0.125 ml portions (from a 15% stock solution) approx. 1 min apart, with gentle vortexing in between. The liposomes (in 0.236 ml) were added after the detergent, and the tube was vortexed gently and incubated for 20 min at 4°C. The suspension was centrifuged at $140\,000 \times g_{av}$ in a Beckman 50 Ti rotor for 60 min at 4°C. The supernatant, containing the solubilized extract, was transferred to dialysis bags (Spectropor 6, molecular weight cut-off $12\,000 \times 14\,000$, Spectrum Medical, Los Angeles, CA) and dialyzed overnight against 1 liter buffer B (1 mM EDTA/2 mM 2-mercaptoethanol/0.02% sodium azide/0.5 mM phenylmethylsulfonylfluoride/10 mM potassium phosphate (pH 7.5)). After dialysis, in earlier experiments, the dialysis bag was buried for 2 h in Sephadex G-200 (Pharmacia, Uppsala, Sweden) at 4°C to concentrate the suspension approx. 2-fold. This procedure has since been found to be unnecessary. The material was kept on ice until use, or stored at −80°C.

*Reconstitution of glucose transport into proteoliposomes.* Bath-sonicated asolectin liposomes were mixed with 20–50 μg of octylglucoside-extracted, dialyzed proteins at a weight ratio of 50–80 to 1 in a final volume of 0.15–0.2 ml in a 16 × 122 mm screw-cap tube. The tube was gently vortexed, then frozen in a solid $CO_2$/acetone bath and thawed at room temperature (approx. 10 min). The turbid mixture was then bath-sonicated at the maximum setting (see Ref. 3 for explanation) for 7 s, which clears the suspension slightly, to yield the reconstituted proteoliposome preparation.

*Assays of sugar transport.* Zero-trans influx, influx equilibrium exchange and influx countertransport assays have been described previously [3]. Radioactive D-[$^{14}$C]glucose was obtained from Research Products International, Mount Prospect, IL; D-[$^{3}$H]glucose, L-[$^{3}$H]glucose and 2-deoxy D-[$^{14}$C]glucose were obtained from New England Nuclear, Boston, MA. Millipore GSTF (0.22 μm pore size) filters were wetted on both sides in a 1 mg/ml solution of poly-L-lysine) (90 000 average molecular weight, Sigma) and used immediately without drying. This treatment has been shown to increase the retention of liposomes and vesicles on the membrane filters, even after the multiple washings of the filters for the assays of transport [3]. Transport activity is expressed as nmol D-glucose minus L-glucose transported per unit time of uptake and as the ratio of D-glucose to L-glucose transported. The difference between D-glucose and L-glucose uptake is a measure of D-glucose-specific transport activity, whereas the D/L-glucose ratio is a measure of nonspecific glucose leakiness. Ideally, the D- minus L-glucose uptake and D/L ratio should both be significant. Liquiscint (National Diagnostics, NJ) was used as the scintillation fluid for counting in a Beckman Model 3300 scintillation spectrometer.

*Protein assays.* Protein was determined by the procedure of Schaffner and Weismann [6]. The final concentration of sodium dodecyl sulfate in the assay was increased to 2%, and protein precipitated with trichloroacetic acid was filtered on a

47 mm diameter (0.45 μm; Amicon) microporous filter. For the determination of protein with a high concentration of lipid present, the filters were rinsed with 5 ml of chloroform, and thoroughly air-dried before staining with 0.15% Naphthol blue-black in methanol/acetic acid/water (45 : 10 : 45, v/v) for 5 min.

*Fluorescence assay.* Quantitative assay of the amount of phospholipid vesicles was carried out by the method of London and Feigenson [3,7].

## Results and Discussion

*Solubilization of plasma membranes*

A variety of approaches to solubilization of the plasma membranes from *S. cerevisiae* were attempted to reconstitute the yeast D-glucose-specific transport activity. The addition of exogenous liposomes during membrane solubilization was found to be essential, and recovery of transport activity was slightly improved if the liposomes were introduced just after rather than just before the addition of the detergent.

Reconstitution was carried out as described in Materials and Methods. The data presented in Fig. 1A show a representative time-course for zero-trans uptake from a 5 mM glucose solution into reconstituted proteoliposomes compared with uptake into plasma membrane vesicles ('vesicles'). The results show an initial velocity of D-glucose specific uptake of 22 nmol/mg protein per min for the reconstituted proteoliposomes and 58.5 nmol/mg protein per min for the vesicles. The specific activity of transport in reconstituted proteoliposomes relative to that of vesicles has varied from 60 to 120%.

Fig. 1B shows a representative time-course of zero-trans uptake, equilibrium exchange, and influx countertransport with the reconstituted proteoliposomes. For equilibrium exchange, the proteoliposomes were preincubated with 5 mM D-glucose at room temperature for 60–90 minutes. The assays were initiated by the addition of tracer amounts of L-[$^3$H]glucose plus D-[$^{14}$C]glucose. The characteristics of uptake for equilibrium exchange at 5 mM are similar to those for zero-trans, but the initial 'fast' phase is stimulated (apparent initial velocity is 44 vs. 22 nmol D-glucose/mg protein per min).

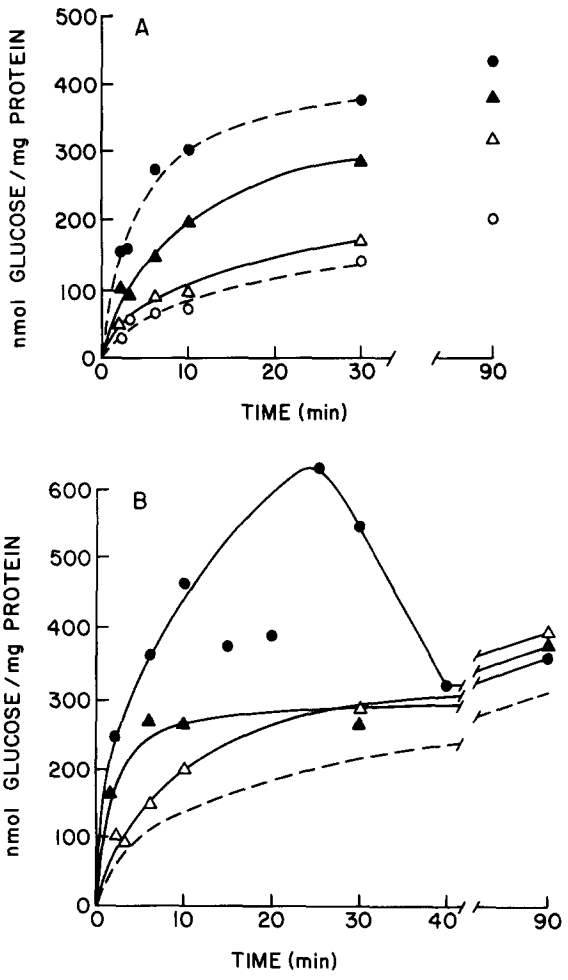


Fig. 1. Time-course assays of D-glucose transport. Plasma membrane protein was extracted with 1.25% octylglucoside and reconstitution was carried out as described in Materials and Methods. (A) A representative experiment of zero-trans uptake into vesicles (●, D-glucose; ○, L-glucose) and into reconstituted proteoliposomes (▲, D-glucose; △, L-glucose). (B) A representative experiment of 5 mM D-glucose zero-trans uptake (△, D-glucose), equilibrium exchange uptake (▲, D-glucose) into reconstituted proteoliposomes, and influx countertransport into proteoliposomes preloaded with 125 mM D-glucose and diluted to 5 mM D-glucose (●, D-glucose). For the first 10 min, each point represents the average of triplicate determinations. After 10 min, each point represents the average of duplicate determinations. The average values of L-glucose uptake in all three modes of transport are described by the dotted line.

The definitive diagnosis for a carrier-mediated facilitated diffusion system is the overshoot phenomenon observed with influx countertransport. To demonstrate this phenomenon, the recon-

stituted proteoliposomes were preloaded with a high concentration (125 mM) of unlabeled sugar followed by a dilution to a lower concentration (5 mM) of labeled sugar (Fig. 1B). The overshoot is a consequence of the rapid equilibration of the label with intraliposomal sugar before the intraliposomal concentration falls to that of the external medium.

We have observed variable patterns of the time-course and height of the overshoot phenomenon of influx countertransport with different preparations and different assay conditions. It should be noted that the final quantity of sugar taken up per mg protein (i.e., the sugar space) approaches a value between 250–350 nmol/mg protein for D-glucose as for L-glucose in the three modes of facilitated diffusion studied (Fig. 1B).

The most important criterion of reconstitution is that the solubilized and reconstituted protein(s) demonstrate transport with the characteristics of the intact cell, with respect to kinetic parameters, as demonstrated above, and to substrate specificities. The stereospecificity of the intact-cell D-glucose transporter, as well as the ability to transport the D-glucose analog, e.g., 2-deoxy D-glucose, is retained by our reconstituted preparation. The data of Fig. 2 show a representative time-course of equilibrium exchange of 2-deoxy D-glucose for D-glucose. In this assay, reconstituted proteoliposomes were preincubated with unlabeled D-glucose (5 mM) followed by dilution into a mixture of labeled 2-deoxy D-[$^{14}$C]glucose and L-[$^{3}$H]glucose (5 mM). For comparison, the preloaded proteoliposomes were diluted into 5 mM D-[$^{3}$H]glucose alone, or 5 mM L-[$^{3}$H]glucose alone. There is no apparent difference between the equilibrium exchange of D-glucose with itself or with 2-deoxy D-glucose. This shows that 2-deoxy D-glucose is as effective a substrate analog for the reconstituted D-glucose-specific transport system as it is for the intact cell system [11].

The determination of an efficient octylglucoside concentration [8] for extraction of the yeast glucose transport system from plasma membranes is shown in a representative experiment in Table 1A. Quadruplicates of 3-min equilibrium exchange uptakes were used to compare the specific activity of the solubilized reconstituted sugar transport protein(s) relative to the specific activity of the vesicles

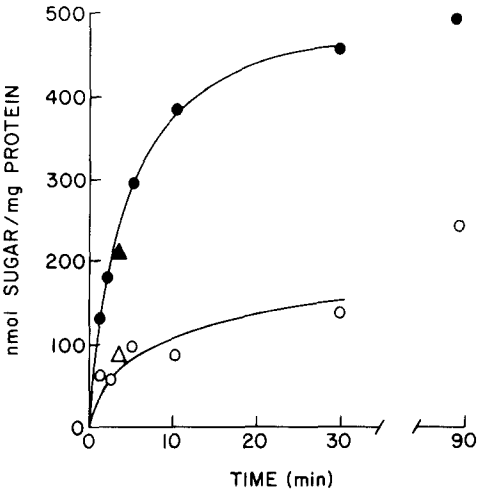


Fig. 2. Time-course of 2-deoxy D-glucose uptake into reconstituted proteoliposomes. Solubilized, dialyzed proteins were reconstituted into proteoliposomes as described in Materials and Methods. The proteoliposomes were equilibrated with 5 mM D-glucose for 60–90 min at room temperature. The equilibrium exchange assay was initiated by the addition of 75 $\mu$l of proteoliposomes to 1.9 ml of 5 mM (final concentration) 2-deoxy D-[$^{14}$C]glucose and L-[$^{3}$H]glucose and agitated by a small magnetic stirrer at room temperature. Aliquots (0.2 ml, 1.9 $\mu$g protein) were removed at various times and processed as described in Materials and Methods. ● and ○ represent 2-deoxy D-[$^{14}$C]glucose and L-[$^{3}$H]glucose equilibrium exchange uptake, respectively. ▲ and △ represent the triplicate mean of single time-point equilibrium exchange uptakes of D-[$^{14}$C]glucose and L-[$^{3}$H]glucose, respectively, as described in Table I.

[3]. The highest relative specific activity (57%) was observed with protein solubilized by both 1% and 1.25% octylglucoside; however, the concentration of protein solubilized by 1.25% octylglucoside was nearly 20% greater.

The net recovery of transport activity from the reconstituted supernatant of plasma membrane protein solubilized with 1.25% octylglucoside and the resuspended pellet are shown in a representative experiment in Table IB. Both the D-glucose specific activity of transport and the ratio of D-glucose over L-glucose are significantly higher in proteoliposomes from the supernatant extract compared to membrane vesicles prepared from the pellet of the solubilization mixture. The observation that only approx. 50% of the initial vesicle transport activity is recovered from the supernatant plus the pellet may be due to (i) the inactivation of the transport system by exposure to

TABLE I

EFFICIENCY OF OCTYLGLUCOSIDE SOLUBILIZATION

(A) Plasma membranes were solubilized in the presence of liposomes with varied concentrations of octylglucoside and reconstituted as described in Materials and Methods. The preparations (2–4 μg protein) were assayed for equilibrium exchange uptake of 5 mM D-glucose (in quadruplicate), and these results were compared to those from the vesicles. Percentage of protein solubilized was determined from the supernatant of the solubilization mixture, before dialysis. Percentage of vesicle transport activity reconstituted is calculated from the percentage of vesicle specific activity and the fraction of the plasma membrane protein that was solubilized. (B) The net recovery of transport activity was examined after solubilization of the plasma membranes with 1.25% octylglucoside, and equilibrium exchange uptake of 5 mM D-glucose was assayed in quadruplicate.

| Membrane preparation | Protein solubilization | | Specific activity (nmol glucose/mg protein per 3 min) | | % Vesicle activity | % Vesicle activity reconstituted |
|---|---|---|---|---|---|---|
| | % octyl-glucoside | % solubilized | D−L | D/L | | |
| A. Vesicles | – | – | 281 | 6.9 | 100 | – |
| Proteoliposomes | 0.75 | 28 | 121 | 2.5 | 43 | 12 |
| | 1.0 | 38 | 161 | 2.8 | 57 | 21 |
| | 1.25 | 43 | 161 | 3.0 | 57 | 25 |
| | 1.5 | 42 | 116 | 2.3 | 41 | 17 |
| B. Vesicles | – | – | 239 | 5.3 | – | 100 |
| Octylglucoside fractions | | | | | | |
| supernatant | 1.25 | 54 | 164 | 2.7 | 69 | 37 |
| pellet | – | – | 61 | 1.9 | 25 | 12 |

TABLE II

THE EFFECTS OF FREEZE-THAW-SONICATION AND LIPID ADDITION

The procedure is identical to that described in Materials and Methods for treatment of plasma membranes with 1% octylglucoside, except that in a, the dialyzed supernatant was not supplemented with liposomes, nor frozen-thawed-sonicated. For c–f, the solubilization mixture was bath sonicated at room temperature for 1 min before centrifugation, then treated as described in Materials and Methods. The weight ratio of asolectin liposomes added to a constant protein concentration before freeze-thaw-sonication is indicated for b–f. Equilibrium exchange uptake of 5 mM D-glucose was assayed in quadruplicate as described in Materials and Methods.

| Membrane preparation | Protein solubilization | | Specific activity (nmol glucose/mg protein per 3 min) | | % Vesicle activity | % Vesicle activity reconstituted |
|---|---|---|---|---|---|---|
| | Sonication | % solubilized | D−L | D/L | | |
| Vesicles | – | | 150 | 3.0 | 100 | – |
| Proteoliposomes | | | | | | |
| (a) (no additional liposomes) | – | 36 | 91 | 3.1 | 61 | 22 |
| (additional liposomes : protein) | | | | | | |
| (b) 75 : 1 | – | 36 | 180 | 3.3 | 120 | 43 |
| (c) 75 : 1 | + | 38 | 112 | 2.4 | 75 | 28 |
| (d) 65 : 1 | + | 38 | 103 | 2.4 | 69 | 26 |
| (e) 40 : 1 | + | 38 | 79 | 2.0 | 53 | 20 |
| (f) 15 : 1 | + | 38 | 41 | 1.8 | 27 | 10 |

detergent in either the supernatant or the pellet, or both; (ii) the use of soybean lipids as opposed to more compatible phospholipids which might improve the efficiency of transport activity (see below); and (iii) the inactivation of transport activity by proteolysis during the time elapsed between plasma membrane solubilization and the assays for sugar transport in proteoliposomes. The first possibility was studied by the use of other detergents. Triton X-100, sodium dodecyl sulfate, sodium deoxycholate and sodium cholate were not effective detergents in our hands for reconstituting the yeast glucose transport system. We have had variable success with a zwitterionic detergent, Zwittergent TM3-14, used by Malpartida and Serrano [9] in the solubilization and reconstitution of the yeast plasma membrane ATPase (data not shown). We also tested the effect of bath sonication during the solubilization step, which was the procedure used to extract the ATPase with Zwittergent [9]. Bath sonication during solubilization (1 min at room temperature) with 1% octylglucoside has a small effect on the percentage of protein solubilized (36% (a,b) vs. 38% (c–f), Table II), but the specific activity of the reconstituted 'sonication-solubilized' proteins (Table II, c, 112 nmol D-glucose/mg of protein per 3 min) is only 63% of the specific activity of the extracts prepared without sonication during solubilization (Table II, b, 180 nmol D-glucose/mg of protein per 3 min).

In the procedure described above, liposomes are added at two steps: (i) during solubilization, at a phospholipid-to-protein ratio of 3 : 1, and (ii) after dialysis, but before the final freeze-thaw-sonication, at a phospholipid-to-protein ratio of 75 : 1. The lipid-to-protein ratio after dialysis was 10–14 : 1, measured by the fluorescence method of London and Feigenson [7]. If the dialyzed extract were not supplemented with additional liposomes, and the mixture was, furthermore, not freeze-thaw-sonicated, the specific activity of transport (Table II, a) was up to one-half that of proteoliposomes made from dialyzed extracts mixed with additional liposomes followed by freeze-thaw-sonication (Table II, b). The improved activity of reconstituted proteoliposomes was dependent on the concentration of liposomes added before the freeze-thaw-sonication step. This is shown in Table II (c–f), where the protein concentration for freeze-thaw-sonication was kept constant, and the phospholipid liposome concentration added was varied to increase the phospholipid-to-protein ratio from 15 : 1 to 75 : 1.

Preliminary observations of the electrophoretic patterns in 7.5% sodium dodecyl sulfate polyacrylamide gels (Laemmli [10]) of the proteins solubilized with octylglucoside show significant qualitative and quantitative differences compared to the plasma membrane protein patterns before extraction (data not shown).

Preliminary experiments have also shown that liposomes prepared from a mixture of egg phosphatidylcholine and bovine phosphatidylethanolamine (1 : 1) were more effective for the reconstitution of yeast D-glucose transport than liposomes prepared from other phospholipid mixtures, including asolectin, but the transport activity observed using asolectin liposomes was sufficient to warrant its use for these preliminary studies.

By the criteria used, we have reconstituted 25–50% of the vesicle D-glucose transport activity into artificial liposomes by a 1.25% octylglucoside solubilization of plasma membrane protein to which is added, after overnight dialysis, a weight ratio of asolectin liposomes to protein (75 : 1) followed by freeze-thaw-sonication. We are currently involved in the optimization of the reconstituted D-glucose transport activity by several means. Negative purification of the starting plasma membrane proteins and positive purification of the solubilized extract should lead to an increase in the specific activity of transport. As mentioned earlier, alteration of the phospholipid composition of the liposomes is expected to increase further the relative specific activity of transport. The kinetic parameters of facilitated diffusion will then be studied in such an improved reconstituted yeast D-glucose transport system.

It has been reported that while phosphorylation is not a requisite step in the facilitated diffusion of D-glucose, the affinity of glucose and its metabolizable analogues for transport is greatly enhanced under phosphorylating conditions [2,12], which suggests that carrier-kinase associations might occur. A close association between the sugar kinases and transporters is also indicated by the results of pool labeling experiments [13–16]. Such carrier-

kinase associations can be studied in the solubilized reconstituted transport system. Furthermore, methods for studying the mechanism and regulation of the yeast facilitated diffusion of glucose can be extended by the use of a purified, reconstituted system.

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