Characterization of the *Saccharomyces cerevisiae* Cytosine Transporter Using Energizable Plasma Membrane Vesicles*

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Benoit Pinson‡, Christian Napia‡, Jean Chevallier‡, Peter J. A. Van den Broek§, and Daniel Bréthes‡

*From the ‡Institut de Biochimie et Génétique Cellulaires du Centre National de la Recherche Scientifique, Bordeaux, France and the §Department of Molecular Cell Biology, Syliocrus Laboratory, Leiden University, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands*

The purine-cytosine permease is a carrier localized in the plasma membrane of the yeast *Saccharomyces cerevisiae*. The energetics of cytosine transport catalyzed by this permease has been studied in an artificial system obtained by fusion between proteoliposomes containing beef heart cytochrome c oxidase and plasma membrane-enriched fractions of a *S. cerevisiae* strain overexpressing the permease. Upon addition of an energy donor, a proton-motive force (inside alkaline and negative) is created in this system and promotes cytosine accumulation. By using different phospholipids, it is shown that cytosine uptake is dependent on the phospholipids surrounding the carrier. It was demonstrated that the purine-cytosine permease is able to catalyze a secondary active transport of cytosine. By using nigericin and valinomycin, the ΔpH component of the proton-motive force is shown to be the only force driving nucleobase accumulation. Moreover, transport measurements done at two pH values have shown that alkalization of intravesicular pH leads to a significant increase in cytosine uptake rate. Finally, no specific role of K⁺ ions on cytosine transport could be demonstrated in this system.

Living cells have to take up various ions and metabolites from extracellular medium. Recent reviews have listed many hydrophobic proteins of the yeast *Saccharomyces cerevisiae* plasma membrane involved in specific transport of a large array of molecules, such as hexoses and amino acids, as well as potassium and sulfate ions (1, 2). Many of these proteins have been reported to work as secondary active carriers. Most of them are symports coupling the utilization of the proton gradient, built up by the H⁺-ATPase of the plasma membrane, to the uptake of different kinds of molecules (1).

Purine-cytosine permease from *S. cerevisiae* is one of these plasma membrane carriers (3, 4). In vivo, this permease seems to mediate the co-transport of proton and purine bases (adenine, hypoxanthine, and guanine) or a pyrimidine base (cytosine) (5, 6), the energy source of this active transport being the proton electrochemical gradient built up by the H⁺-ATPase (7, 8). By measuring simultaneously hypoxanthine uptake and H⁺ and K⁺ fluxes, it has been proposed that purine translocation through the *S. cerevisiae* plasma membrane is an electroneutral base/H⁺ symport with a K⁺ antiport (9). In contrast to this, it has been proposed in other experiments carried out on a *S. cerevisiae* strain (lacking cytosine deaminase and overexpressing the purine-cytosine permease) in ATP-depletion conditions, that the pump works as an electrogenic proton symport (10) with a H⁺/base stoichiometry close to 1 (11).

In addition, studies have been carried on *S. cerevisiae* purine-cytosine permease proficient strains carrying plasmid-encoded multiple copies of either wild type or mutated *FCY2* gene, encoding the purine-cytosine permease. We have analyzed the effects of pH on *in vivo* uptake and *in vitro* equilibrium binding of nucleobases, and have shown a key role played by a protonable group of the permease for the solute binding step of the translocation process (12).

Mechanistic studies of solute uptake in whole cells cannot be analyzed in close detail for the following reasons: (a) inside the cell, the solute can be rapidly metabolized obscuring the characteristics of the uptake; (b) since the ionic content of the cytoplasm cannot be controlled, no specific study of the influence of ionic fluxes on solute transport can be performed. However, it is possible to analyze the transport in a well defined medium with an *in vitro* system obtained by membrane fusion between plasma membrane fractions and proteoliposomes containing a so-called “energy-producing system.” Such a system that generates a proton-motive force has been widely used for the study of various transport systems in bacteria (13) and of some carriers of yeast plasma membrane (for a review, see Ref. 1).

In this study, we have used this technique to analyze the energetics of the transport catalyzed by the purine-cytosine permease of the plasma membrane of *S. cerevisiae*. Cytosine uptake has been analyzed in artificial vesicles obtained by fusion between proteoliposomes containing beef heart cytochrome c oxidase and plasma membrane-enriched fractions of a *S. cerevisiae* strain overexpressing the permease. Several properties of the transport system, which were difficult to assess in whole cells, have been characterized with these hybrid vesicles. With this system, it is demonstrated that the purine-cytosine permease is a secondary active transport driven only by the ΔpH component of the proton-motive force. In addition, it is shown that the rate of uptake increases when the internal pH is more alkaline, and that the carrier is able to fully exchange cytosine at the steady state of accumulation. The influence of the phospholipid environment on the uptake is also investigated.

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‡ To whom correspondence should be addressed: Institut de Biochimie et Génétique Cellulaires, UPR 9026, 1, rue Camille Saint-Saëns, F-33077 Bordeaux Cedex, France. Fax: 33-5-56-99-90-59; E-mail: daniel.br ethes@ibgc.u-bordeaux2.fr.

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Materials—(2-14)C(Cytosine (1.85 GBq·mol⁻¹) was purchased from Moravek. Other chemicals were the following: Escherichia coli l-a-phosphatidylethanolamine (type IX), soybean l-a-phosphatidylcholine (type IV-S), fresh frozen egg yolk l-a-phosphatidylcholine (type XV-E), nigericin, valinomycin, and cytochrome c were from Sigma; tetraphenylphosphonium (TPP⁺) bromide, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), and ascorbic acid from Fluka; pyruvate from Eastman; N,N,N',N'-tetramethyl-p-phenylene diamine (TMPD) from Merck; octadecyl rhodamine from Molecular Probes Inc.; m-nigericin, valinomycin, and cytochrome c oxidase liposome-plasma membrane vesicles; l-glucose (20 gliter⁻¹), and 25 mM sodium phosphate at pH 5.

Purification and Reconstitution of Cytochrome c Oxidase into Proteoliposomes—Commercially obtained phospholipids and phospholipids extracted from yeast or bacteria plasma membrane were purified with acetone/ethyl ether (3:1) and then dissolved in chloroform. The purified phospholipids were stored at −20 °C under nitrogen.

Plasmatic Membrane Isolation—Plasmatic membrane-enriched fractions were isolated as already described (14). Cells were grown at 28 °C under agitation in a liquid medium containing yeast nitrogen base without amino acid and ammonium sulfate was from Difco; scintillation mixture was Ready Value from Beckman. All other reagents were of analytical grade from Aldrich.

Characterization of Hybrid Vesicles—The vesicles used in this work have already been described (3). NC233-10[pJpDB] is a permease-proficient strain, carrying plasmid-encoded multiple copies of the purine-cytosine permease. The strains used in this work have been previously described by Hoekstra et al. (19) using a TPP⁺-inhibitable cation distribution technique as already described (26) in K⁺ buffer, pH 5.7, containing NaCl (100 mM) and [2-14]Cytosine (620 MBq·mol⁻¹) at concentrations ranging from 0.1 to 30 μM in the presence (nonspecific binding) or absence (total binding) of 4 mM adenine. B_max (maximal amount of specifically bound cytosine) and K_d (affinity constant) of cytosine binding were calculated by nonlinear regression analysis of the saturation curve.

Cytosine uptake measurements were done with CL-PMV (0.8 mg of plasma membrane protein·ml⁻¹) in K⁺ buffer containing MgCl₂ (1 mM) at pH 5.7 or 6.2 in a magnetically stirred vessel thermostated at 30 °C. Energization and additions of nucleobase and ionophores were done as indicated in the figures. Ionophores and uncoupler stock solutions were made in ethanol and, when added to the incubation medium, were diluted 100-fold. At given intervals, aliquots (10 μl) were withdrawn and added to 2 ml of ice-cold LiCl (0.1 M), filtered on cellulose nitrate filters (0.45 μm, Schleicher & Schuell), and washed once with 2 ml of ice-cold LiCl (0.1 M). For K₃ (Michaels constant of transport) and V_max (maximal rate of uptake) determinations of cytosine uptake, plasma membrane protein concentration was 0.2 mg·ml⁻¹. Aliquots (30 μl) were withdrawn at 10-s intervals during the first minute of the uptake.

Miscellaneous—Protein concentration was determined by the Lowry procedure (27) for plasma membrane and by the biuret method (28) for cytochrome c oxidase with bovine serum albumin as standard.

RESULTS

Characterization of Hybrid Vesicles—Plasma membrane preparations containing the purine-cytosine permease were fused with cytochrome c oxidase proteoliposomes using a freeze-thaw-extrusion procedure. Fusion efficiency was estimated to be more than 95% as measured by the octadecyl rhodamine fluorescence method. The internal volume of the fused vesicles was close to 0.9 μl·mg⁻¹ of phospholipids for CL-PMV obtained with a protein/phospholipid ratio of 1:20 (w/w). Upon addition of the energization mixture, the rate of oxygen consumption measured with these vesicles was 5.4 ± 0.4 μmol of oxygen atom⁻¹·mg⁻¹ of cytochrome c oxidase⁻¹. It could be stimulated by FCCP, 10 μM, up to 8.9 ± 1.3 μmol of oxygen atom⁻¹·mg⁻¹·min⁻¹ of cytochrome c oxidase⁻¹ and was totally inhibited by KCN (160 μM).

Addition of the energization mixture to CL-PMV induced the generation (in about 5 min) of a steady state transmembrane electric potential difference (Δψ) of about 95 mV, which was nearly stable for at least 30 min. It was increased by nigericin and collapsed by valinomycin addition (Fig. 1A). In these conditions, a ΔpH of 1.1–1.2 units was simultaneously created. It was also stable for at least 30 min, slightly increased by valinomycin, and abolished by nigericin (Fig. 1B) or KCN (not shown) addition. Thus, energized CL-PMV showed a proton-motive force of about 160 mV stable for at least 30 min.

To establish whether the membrane fusion process affected the ligand binding properties of the purine-cytosine permease, cytosine equilibrium binding experiments were done on CL-PMV. Binding parameters obtained at pH 5.7 (K_d, μM) of 8.4 ± 0.8 μM and B_max of 1700 ± 70 pmol·mg⁻¹ of plasma membrane protein⁻¹ were very similar to those obtained for the plasma membrane preparations used to prepare the CL-PMV (K_d, μM) of 13.6 ± 0.4 μM and B_max of 1750 ± 96 pmol·mg⁻¹ of plasma membrane protein⁻¹.

Cytosine Transport in CL-PMV—As shown in Fig. 2, CL-PMV were able to accumulate cytosine upon energization. The cytosine uptake was carrier-dependent since CL-PMV prepared from plasma membrane fractions isolated from the pericellullar mutant strain NC233-10[pJpDB] were unable to promote the uptake. Moreover, solute accumulation was dependent on the same energization conditions as those used for Δψ determinations. Pyranine (300 μM) was added to the fusion buffer and, after fusion, the dye remaining in the external medium was eliminated by elution through a Sephadex G-25 column.

Activity Measurements on CL-PMV—Ligand equilibrium binding measurements were performed at 4 °C on CL-PMV by a centrifugation technique as already described (26) in K⁺ buffer, pH 5.7, containing NaCl (100 mM) and [2-14]Cytosine (620 MBq·mol⁻¹) at concentrations ranging from 0.1 to 30 μM in the presence (nonspecific binding) or absence (total binding) of 4 mM adenine. B_max (maximal amount of specifically bound cytosine) and K_d (affinity constant) of cytosine binding were calculated by nonlinear regression analysis of the saturation curve.
the proton-motive force since: (a) it did not occur in the absence of energy supply; (b) it was completely abolished by addition of FCCP at the steady state of accumulation. Therefore, cytosine translocation is a secondary active transport system. The low amount of radioactivity taken up by CL-PMV of permease null strain in the presence of energy or by CL-PMV made with the purine-cytosine permease proficient strain in the absence of energy was mainly due to diffusion.

Adenine and hypoxanthine were also actively taken up by energized CL-PMV, but the levels of accumulation were lower than that observed for cytosine. The accumulation ratios obtained at the plateaus were 6.8 for cytosine (Fig. 2) and 3.4 for purine bases (not shown).

The curve showing initial velocities of the carrier-mediated transport versus external cytosine concentrations (Fig. 3) could be fitted as a simple Michaelian hyperbola. Under these conditions, $K_T$ and $V_{\text{max}}$ were $13.0 \pm 3.3 \mu M$ and $3.3 \pm 0.3 \text{ nmol of cytosine-min}^{-1}\text{mg of plasma membrane protein}^{-1}$, respectively. Corresponding values of $K_T$ and $V_{\text{max}}$ for NC233-10B[pAB4] cells, were $1.9 \pm 0.1 \mu M$ and $8.8 \pm 0.1 \text{ nmol-min}^{-1}\text{10}^7\text{ cells}^{-1}$, respectively.

**FIG. 1.** Energization of CL-PMV. Vesicles were prepared by fusion between proteoliposomes (composed of phosphatidylethanolamine from E. coli and cytochrome c oxidase from beef heart mitochondria) and plasma membranes isolated from S. cerevisiae NC233-10B[pAB4] strain. The protein/phospholipid ratio was 1:20 (w/w). CL-PMV were diluted in K+ buffer, pH 5.7, containing 1 mM MgCl2 (final concentration: 30 μg of plasma membrane protein ml−1). Energization was done by adding potassium ascorbate, TMPD, and cytochrome c (final concentrations 30 mM, 150 μM, and 15 μM respectively). A, Δψ measurements: TPP+ uptake was monitored with a TPP+-selective electrode. Calibration was done with 1 and 2 μM TPP+ in the incubation medium. After 30 min of energization, valinomycin or nigericin were added (arrow) to final concentrations of 100 and 37 nM, respectively. B, ΔpH measurements: CL-PMV were prepared in the presence of pyranine (300 μM). pH variations were monitored by measuring the fluorescence of the dye trapped inside the vesicles. After 30 min of energization, valinomycin or nigericin were added (arrow) to final concentrations of 100 and 37 nM, respectively. Fluorescence-pH calibration was done by addition of NaOH (0.1 M) aliquots after nigericin addition.

**FIG. 2.** Cytosine uptake in CL-PMV. Vesicles were prepared in K+ buffer at pH 5.7 containing MgCl2 (1 mM), either from plasma membrane fractions isolated from NC233-10B[pAB4] strain overexpressing the permease or from NC233-10B[pDB] null strain and proteoliposomes containing cytochrome oxidase. Cytosine uptake was measured by filtration at 0.8 mg of plasma membrane protein ml−1. [2-14C]Cytosine (1.85 GBq mmol−1, 25 μM final concentration) and energization mixture were added at 0 and 10 min, respectively. When added, FCCP was at a final concentration of 10 μM. NC233-10B[pAB4]: ○, no FCCP addition; ■, FCCP addition 20 min after energization (arrow); □, FCCP addition 1 min before energization: +, NC233-10B[pDB].

**FIG. 3.** Initial rates of uptake as a function of cytosine concentration. The experiments were performed with CL-PMV prepared with plasma membrane fractions isolated from the NC233-10B[pAB4] strain. CL-PMV (0.2 mg of plasma membrane protein ml−1) were incubated 5 min in the energization mixture in the presence of FCCP (10 μM, ■) or in its absence (□). Then, [2-14C]cytosine (620 MBq mmol−1) was added to the desired final concentration (from 5 to 65 μM). Initial rates of uptake were determined on 30-μl aliquots withdrawn from the incubation medium every 10 s during the first minute of the kinetic after cytosine addition. At each concentration tested, carrier-mediated initial rate of uptake was obtained from the difference between the initial rate measured in the absence (active transport and diffusion) and in the presence (diffusion) of uncoupler.
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Cytosine uptake experiments were done at two external pH values of 5.7 and 6.2; in each case, the ΔpH and Δψ components of Δ were tested as described and were not significantly different from those described in Fig. 1 (not shown). Among the various phospholipids used for the preparation of CL-PMV, phosphatidylethanolamine from E. coli was found to be the most efficient phospholipid for active transport (Fig. 4). For this reason, all subsequent experiments described in this work were done with CL-PMV prepared with this phospholipid.

Cytosine Exchange—An important point was to know whether the solute accumulation plateau corresponded to cessation of the influx or to the same influx and efflux rates. When the accumulation plateau was reached, addition of an excess of non-labeled cytosine led to a rapid and complete efflux of the internal radioactive solute (Fig. 5A). On the other hand, when radioactive cytosine was added to energized CL-PMV pre-loaded with non-labeled solute, accumulation of the radioactive cytosine inside the vesicles was observed (Fig. 5B). These data showed that the accumulation plateau was the result of equal influx and efflux rates. Moreover, influx and efflux apparent rates of radioactive cytosine observed at the steady state of accumulation (exchange rates) were very similar and significantly higher than the corresponding apparent uptake rate.

Roles of the ΔpH and Δψ Components of Δp on Cytosine Uptake—Uptake experiments were done at two external pH values of 5.7 and 6.2; in each case, the ΔpH and Δψ component of the Δp were collapsed by use of the appropriate ionophores added to the CL-PMV suspension 1 min before energization (Fig. 6).

Addition of nigericin (Δp = Δψ) totally prevented active cytosine transport. Addition of valinomycin (Δp = −0.06 ΔpH) and no ionophore addition (Δp = Δψ − 0.06 ΔpH) led to active transport of cytosine for which the observed accumulation ratios were related to the existing ΔpH component of the Δp (Table I). This suggests that the ΔpH component was the only driving force of the active solute transport process. In these experiments, showing that the ΔpH was totally collapsed and transformed at least partly in Δψ, nigericin was added at a ratio of 6.6 pmol/mg of phospholipids−1, a value which is much lower than the value of 115 pmol/mg of phospholipids−1 used for Δψ and ΔpH measurements displayed in Fig. 1. Therefore, at the ratio used for cytosine uptake, nigericin had negligible or no uncoupling effect.

Moreover, at each of the external pH values tested, the apparent rate of cytosine uptake was higher in the presence of valinomycin than in its absence (Fig. 6, A and B). Valinomycin addition to energized CL-PMV transforms, at least partially (see Fig. 1), the Δψ into ΔpH leading to alkalinization of the internal pH. In each of the four experiments shown in Fig. 6, the cytosine accumulation ratio was related to the ΔpH (Table I). The stimulation of the apparent rate of cytosine uptake observed in the presence of valinomycin could have to deal with modification of ΔpH value or alkalinization of vesicle interior or both.

In this respect, one has to compare the cytosine uptake experiments shown in Fig. 6A in the presence of valinomycin and in Fig. 6B in the absence of valinomycin. These two experiments, done at different external pH (5.7 and 6.2, respectively), showed almost the same internal pH value and thus, different ΔpH values. One can see that the two curves display very similar apparent rates of uptake despite the fact that ΔpH were different in both experiments. Thus, if the CL-PMV ΔpH controls the cytosine accumulation ratio, the apparent rate of cytosine uptake is related to the internal pH value.

Cytosine uptake experiments in buffers containing Na+ instead of K+ ions were also carried out and the effects of an Na+ ionophore (nonactin) and of an Na+/H+ exchanger (monensin) were tested. The results shown in Fig. 6C were analogous to those described for K+ ions (Fig. 6A), confirming that the ΔpH was the only driving force for cytosine uptake, and that the active transport did not display any strict specificity for K+ ions.

DISCUSSION

In this report, cytosine transport was analyzed in artificial energizable vesicles obtained by fusion of plasma membrane-enriched fractions of S. cerevisiae containing the purine-cytosine permease and proteoliposomes containing cytochrome c oxidase. CL-PMV displayed an internal volume value very similar to that already obtained by other methods (19, 29, 30). The cytochrome c oxidase embedded in CL-PMV displayed specific activity, total inhibition by cyanide, and activation upon FCCP addition similar to that observed with isolated beef heart mitochondria (17). FCCP stimulation of oxygen consumption indicated that CL-PMV were not very leaky for protons. The equilibrium binding parameters values (Kd(app) and Bmax) measured for cytosine were almost the same for CL-PMV as those measured with plasma membrane-enriched fractions. However, because of a dramatic decrease in cytochrome c oxidase activity at pH values below 5.5, the experiments presented here were done at pH 5.7 or 6.2. These pH conditions, which were not the optimum for purine-cytosine permease activity, were acceptable since nucleobase uptake on intact cells and equilibrium binding on plasma membrane fractions were still accurately measured at these pH (12).

Upon energy supply, CL-PMV containing purine-cytosine permease were able to actively transport nucleobases in a solute/H+ symport process (Fig. 2 and Table I). The apparent Michaelis constant of cytosine uptake measured for CL-PMV (Fig. 3) was not dramatically different to that of intact cells: they were 13.0 ± 3.3 and 1.9 ± 0.1 µM, respectively. The uptake catalytic constant (calculated from Bmax and Vmax values given

FIG. 4. Influence of CL-PMV phospholipid composition on the cytosine uptake. CL-PMV were made by fusion between plasma membrane fractions isolated from NC233-10[pBAB4] and proteoliposomes containing cytochrome c oxidase made with different kinds of phospholipids. ●, phosphatidylethanolamine from E. coli; ○, purified phospholipids from E. coli plasma membrane; ×, purified phospholipids from S. cerevisiae plasma membrane; □, soybean phosphatidylcholine; and ■, egg yolk phosphatidylcholine. The plasma membrane protein/phospholipid ratio was 1:20 (w/w). For all CL-PMV, cytosine uptake experiments were performed as described.
FIG. 5. Cytosine exchange at the steady state of accumulation. CL-PMV were prepared from the NC233-10B[pAB4] strain. A, cytosine uptake was initiated by addition of [2-14C]cytosine (1.85 GBq mmol$^{-1}$) at a final concentration of 25 μM. After 15 min of uptake, either a large excess of non-labeled cytosine (5 μl, final concentration 1 mM) or the same volume of buffer (5 μl) was added in the incubation medium. B, CL-PMV were preincubated for 5 min with the following energization mixture: K$^+$ ascorbate (80 mM), TMPD (400 μM), and cytochrome c (40 μM). Cytosine uptake was initiated by addition of either [2-14C]cytosine (120 μM, 482 MBq mmol$^{-1}$) or, non-labeled cytosine (90 μM) followed, after 15 min, by a subsequent addition of [2-14C]cytosine (30 μM, 1.85 GBq mmol$^{-1}$). The arrow indicates FCCP addition (10 μM).

Data were obtained from experiments similar to those displayed in Figs. 2 and 6. Valinomycin and nigericin were added 1 min before energization. ΔpH and Δψ values (internal − external) were measured as described in the legend to Fig 1. Cytosine accumulation ratio is obtained from the calculated values of internal and external cytosine concentrations at the steady state of accumulation. Data presented in this table are the average of 2–12 independent experiments.

| External pH | Addition   | ΔpH     | Δψ     | Cytosine accumulation ratio |
|------------|------------|---------|--------|-----------------------------|
| 5.7        | None       | -95 ± 7 | 6.8 ± 0.2 |
| 5.7        | Valinomycin| 1.35 ± 0.06 | 0°      | 7.2 ± 0.1 |
| 5.7        | Nigericin  | 0°      | -112 ± 9 | 1° |
| 6.2        | None       | 1.0 ± 0.05 | ND$^a$ | 5.8 ± 0.2 |
| 6.2        | Valinomycin| 1.45 ± 0.05 | ND | 8.8 ± 0.1 |
| 6.2        | Nigericin  | 0°      | ND | 1° |

$^a$ Nondetectable by the experimental method used. 

$^b$ No cytosine accumulation detectable.

$^c$ ND, nondetermined.

in this text) was around 2 min$^{-1}$, a value 30 times lower than that already published for intact cells which is close to 60 min$^{-1}$ (14). Such reduced efficiency of a carrier is rather common in reconstituted systems and mainly attributable to effects of physical and chemical constraints imposed during plasma membrane preparation and vesicle fusion, and to the nature of the phospholipid environment of the carrier in the artificial system.

Data in Fig. 2 demonstrate that cytosine uptake is a secondary active transport process depending on the presence of a proton-motive force or, at least, one of its components. The observed cytosine accumulation ratio was only 6.8 when the Δp was 160 mV. Various attempts have been done to improve this low accumulation ratio by modifying the preparation of CL-PMV. Instead of the extrusion procedure, sonication methods described for other carriers were tried (19, 29). In addition, in the fusion procedure, the protein/phospholipid ratio, the pore size of the extruder filters, and the number of passes through the filter were also varied. The vesicles obtained from these numerous attempts always displayed the same or lower accumulation ratio (not shown). Modifications of the phospholipid environment of the carrier have also been tried by preparing CL-PMV with purified phospholipids from various sources (Fig. 4). Phosphatidylethanolamine from E. coli was more efficient than all the other phospholipids, and particularly more so than the natural purified phospholipids extracted from plasma membrane fractions of S. cerevisiae. Such behavior has already been observed for some secondary active carriers (19, 31). Moreover, phosphatidylethanolamine from E. coli was also shown to confer the best carrier environment for the well-investigated branched amino acid permease of Streptococcus cerevisiae, located in vivo in a membrane totally lacking this phospholipid (31). Clearly, more work is needed to understand why natural yeast phospholipids did not allow good uptake and accumulation of cytosine in CL-PMV.

Use of ionophores collapsing either the ΔpH or the Δψ component of the Δp (Fig. 6) showed that the cytosine active transport was solely driven by the ΔpH component and that the solute accumulation ratios observed were related with the ΔpH (Table 1). In all cases, the accumulation plateaus were stable for about 10–15 min except for the experiment shown in Fig. 6B in the presence of valinomycin, where the curve showed a maximum instead of a plateau followed by a decrease which was related to the energy source exhaustion (as observed by the appearance of a blue color of the incubation medium due to the stable free radical form of TMPD).

Thus, if we assume a H$^+$/solute stoichiometry of 1 (11) and if the system was to reach its thermodynamic equilibrium, one
\( \Delta \text{pH} \) unit should lead to a solute accumulation ratio of 10. In the various uptake experiments described, we observed values ranging from 32 to 58% of that value (Table I). Two main reasons could account for these results. First, in the fusion procedure, there was a large excess of cytochrome c oxidase proteoliposomes as compared with the plasma membrane fraction. Thus, in the CL-PMV population not all the energizable vesicles might contain purine-cytosine permease. Unfortunately, it was not possible to determine the amount of functional vesicles as already done for reconstituted vesicles containing other permeases, where the reconstituted material contained from 10 to 50% of functional vesicles (19, 30, 32). Second, because of the low affinity of the carrier in CL-PMV for the nucleobases, and because of the low specific radioactivity of the commercially available \( [^{2,14}\text{C}] \)-cytosine, a rather high external solute concentration had to be used to measure the uptake accurately. In such conditions, as solute accumulation proceeds, it causes the internal solute concentration to reach values for which the efflux via passive diffusion becomes large for such a hydrophobic compound and, consequently, limits its accumulation ratio. This is shown by uptake analyses done at external cytosine concentrations of 25 \( \mu \text{M} \) (Fig. 5A) and 120 \( \mu \text{M} \) (Fig. 5B) which led to accumulation ratios of 6.8 and 3.4, respectively. This has been observed and discussed for other carriers (33). Therefore, this made it very difficult to determine accurately the \( \text{H}^+/-\text{cytosine stoichiometry} \). However, our data (Table I) fit with the postulated stoichiometry of 1 (11). Moreover, replacement of \( \text{K}^+ \) by \( \text{Na}^+ \) ions in the uptake buffer did not change the behavior of the transport (Fig. 6). Clearly, this is consistent with the results of Hopkins et al. (10). Therefore, if the nucleobase transport is electroneutral, \( \text{K}^+ \) or \( \text{Na}^+ \) counterflow should not occur via the permease itself, but rather through some unidentified monocation/\( \text{H}^+ \) exchange system located in the plasma membrane. But, an alternative to this would be that the cytosine transport is actually electroneutral by itself. This means that the carrier would take up, instead of the cytosine neutral form, its anionic specie (very scarce at the experimental \( \text{pK}_a \) of cytosine is 12.2) with a tremendous affinity: this mechanism would be in good agreement with the fact that the cytosine accumulation ratio depends only on the \( \Delta \text{pH} \) component of the proton-motive force.

On the other hand, another important finding was the role played by the internal \( \text{pH} \) value on the uptake kinetics, alkaline values leading to stimulation of the apparent rate of cytosine uptake (Fig. 6). In this respect, it is to be noted that there was a clear dependence of the apparent rate of cytosine uptake (Fig. 6). In this respect, it is to be noted that there was a clear dependence of the apparent rate of cytosine uptake (Fig. 5). Such behavior has already been observed in other transport system (35). In contrast, it has been shown that yeast arginine and maltose permeases are totally irreversible (29, 35). An interesting case is the histidine permease of \( \text{Salmonella typhimurium} \). By using energized right-side-out plasma membrane vesicles, it was first shown that the transport was fully reversible (36). Recently, by using a totally reconstituted system, the same group showed it to be fully irreversible and regulated by transinhibition (37). As, in our present work, CL-PMV were prepared from plasma membrane fractions, caution is needed about the reversible aspect of cytosine accumulation and the answer to that question would be obtained by total reconstitution.

In conclusion, the CL-PMV system used in this work is a very useful tool in the study of the purine-cytosine permease. It has allowed us to make the following statements: (a) the carrier catalyzes a nucleobase-active transport process; (b) the \( \Delta \text{pH} \) component of the \( \Delta \text{p} \) is the sole driving force for solute accumulation; (c) the vesicle internal \( \text{pH} \) plays a crucial role in the uptake kinetics; and (d) the cytosine permease does not show any strict specificity for \( \text{K}^+ \) ions.

However, our observations indicate the limitations of the CL-PMV system for complete characterization of the translocation process and show that further elucidation of the transport mechanism will require a totally reconstituted system.

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REFERENCES

1. Van der Rest, M. E., Kamminga, A. H., Nakano, A., Anraku, Y., Poolman, B., and Konings, W. N. (1990) Microbiol. Rev. 59, 304–322
2. André, B. (1995) Yeast 11, 1575–1611
3. Weber, E., Rodriguez, C., Chevallier, M. R., and Jund, R. (1990) Mol. Microbiol. 4, 585–596
4. Schmidt, R., Manolson, M. F., and Chevallier, M. R. (1994) Proc. Natl. Acad. Sci. U. S. A. 81, 6276–6280
5. Paluck, A., and Grenson, M. (1973) Eur. J. Biochem. 32, 276–282
6. Chevallier, M. R., Jund, R., and Lacroute F. (1975) J. Bacteriol. 122, 629–641
7. Geofeau, A., and Slayman, C. W. (1981) Biochim. Biophys. Acta 639, 197–223
8. Serrano, R. (1988) Biochim. Biophys. Acta 947, 1–28
9. Reichert, U., and Forêt, M. (1977) FEBS Lett. 83, 325–328
10. Hopkins, P., Chevallier, M. R., Jund, R., and Eddy, A. A. (1988) FEMS Microbiol. Lett. 49, 173–177
11. Hopkins, P., Shaw, R., Aris, L., Oliver, S., and Eddy, A. A. (1992) Yeast 8, 1053–1064
12. Bréthès, D., Napais, C., Torchut, E., and Chevallier, J. (1992) Eur. J. Biochem. 210, 785–791
13. Dreesen, A. J. M., De Vrij, W., and Konings, W. N. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7555–7559
14. Chirio, M. C., Bre`thes, D., Grandier-Vazeille, X., Rakotomanana, F., and Chevallier, J. (1990) Eur. J. Biochem. 194, 293–299
15. Folch-Pi, J., Lees, M., and Sloane-Stanley, G. H. (1957) J. Biol. Chem. 226, 497–509
16. Viitanen, P., Newman, M. J., Foster, D. L., Wilson, T. H., and Kaback, H. R. (1986) Methods Enzymol. 125, 429–452
17. Yu, C., Yu, L., and King, T. E. (1975) J. Biol. Chem. 250, 1383–1392
18. Guérin, M., Camougrand, N., Velours, G., and Guérin, B. (1982) Eur. J. Biochem. 124, 457–467
19. Van Leeuwen, C. C. M., Postma, E., Van den Broek, P. J. A., and Van Steveninck, J. (1991) J. Biol. Chem. 266, 12146–12151
20. Mac Donald, R. C., Mac Donald, R. I., Menco, B. Ph. M., Takeshita, K., Subbarao, N. K., and Hu, L. (1991) Biochim. Biophys. Acta 1061, 297–303
21. Hoekstra, D., De Boer, T., Klappe, K., and Wilschut, J. (1984) Biochemistry 23, 5675–5681
22. Shinbo, T., Kami, N., Kurihara, K., and Kohatake, Y. (1978) Arch. Biochem. Biophys. 187, 414–422
23. Lolkema, J. S., Hellingwerf, K. J., and Konings, W. N. (1982) Biochim. Biophys. Acta 681, 85–94

\(^2\) M. C. Chirio and D. Bréthès, unpublished results.
Energy Coupling of the Purine-cytosine Permease

24. De Vrij, W., Driessen, A. J. M., Hellingwerf, K. J., and Konings, W. N. (1986) *Eur. J. Biochem.* **156**, 431–440
25. Clément N. R., and Gould, J. M. (1981) *Biochemistry* **20**, 1534–1538
26. Bréthes, D., Chirio, M. C., Napias, C., Chevallier, M. R., Lavié, J. L., and Chevallier, J. (1992) *Eur. J. Biochem.* **204**, 699–704
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
28. Gornall, A. G., Bardawill, C. J., and David, M. M. (1948) *J. Biol. Chem.* **177**, 751–766
29. Opekárková, M., Caspari, T., and Tanner, W. (1993) *Eur. J. Biochem.* **211**, 683–688
30. Geros, H., Cassio, F., and Leao, C. (1996) *Yeast* **12**, 1263–1272
31. Driessen, A. J. M., Zheng, T., Veld, G. I., Op den Kamp, J. A. F., and Konings, W. N. (1988) *Biochemistry* **27**, 865–872
32. Ongjoko, R., Szkutnicka, K., and Cirillo, V. P. (1987) *J. Bacteriol.* **169**, 2926–2931
33. Driessen, A. J. M., Hellingwerf, K. J., and Konings, W. N. (1987) *J. Biol. Chem.* **262**, 12438–12443
34. Driessen, A. J. M., De Jong, S., and Konings, W. N. (1987) *J. Bacteriol.* **169**, 5193–5200
35. Van den Broek, P. J. A., Van Gompel, A. E., Luttik, M. A. H., Pronk, J. T., and Van Leeuwen, C. C. M. (1997) *Biochem. J.* **321**, 487–495
36. Prossnitz, E., Gee, A., and Ames, G. F.-L. (1989) *J. Biol. Chem.* **264**, 5006–5014
37. Liu, C. E., and Ames, G. F.-L. (1997) *J. Biol. Chem.* **272**, 859–866