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Evidence for a Selective and Electroneutral K^+/H^+-Exchange in *Saccharomyces cerevisiae* using Plasma Membrane Vesicles

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The existence of a K^+/H^+ transport system in plasma membrane vesicles from *Saccharomyces cerevisiae* is demonstrated using fluorimetric monitoring of proton fluxes across vesicles (ACMA fluorescence quenching). Plasma membrane vesicles used for this study were obtained by a purification/reconstitution protocol based on differential and discontinuous sucrose gradient centrifugations followed by an octylglucoside dilution/gel filtration procedure. This method produces a high percentage of tightly-sealed inside-out plasma membrane vesicles. In these vesicles, the K^+/H^+ transport system, which is able to catalyse both K^+ influx and efflux, is mainly driven by the K^+ transmembrane gradient and can function even if the plasma membrane H^+-ATPase is not active. Using the anionic oxonol VI and the cationic $DISC_2(5)$ probes, it was shown that a membrane potential is not created during K^+ fluxes. Such a dye response argues for the presence of a K^+/H^+ exchange system in *S. cerevisiae* plasma membrane and established the non-electrogenic character of the transport. The maximal rate of exchange is obtained at pH 6·8. This reversible transport system presents a high selectivity for K^+ among other monovalent cations and a higher affinity for the K^+ influx into the vesicles (exit from cells). The possible role of this K^+/H^+ exchange system in regulation of internal potassium concentration in *S. cerevisiae* is discussed.

KEY WORDS *Saccharomyces cerevisiae*; plasma membrane purification; vesicles reconstitution; K^+/ID-exchangc

INTRODUCTION

In yeast, recent advances in molecular biology and genome sequencing have led to the identification of many putative solute transport systems. As a result of sequence homology, these genes were proposed to encode solute uptake systems (Bisson *et al.*, 1993; Marger and Saier, 1993; Marini *et al.*, 1994). However, many of these systems are poorly defined mechanistically. This lack of information can be explained by the fact that whole cells were generally used in these studies. In intact cells, metabolism can influence transport in a complex way. One way to avoid these problems is to analyse exchange phenomena using membrane reconstituted vesicles. Such a methodology has been employed for studies of plant-derived transporters (Braun *et al.*, 1988; Amalou *et ai*, 1992; Kasai and Sawada, 1994; Marshall *et al.*, 1994) and in another field for characterization of yeast H^+-ATPase (Serrano, 1988; Monk *et ai*, 1989). After membrane purification and isolation of plasma membrane proteins, plasma membrane vesicles are generally obtained by reconstitution into artificial phospholipid bilayers using freeze-thawing-sonication (FTS; Kasahara and Hinkle, 1977; Ongjoco *et al.*, 1987) or a detergent solubilization/gel filtration protocol (Perlin *et al.*, 1984).

Concerning K^+ transport in *Saccharomyces cerevisiae*, it has been described previously that K^+ can be transported by a membrane potential-driven carrier (Calahorra *et al.*, 1987). Two genes (*TRKI* and *TRK2*) have been cloned and sequenced (Gaber *et al.*, 1988; Ko *et al.*, 1990) and two hypotheses have been proposed related to the

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function of these gene products: (i) *TRK1* and *TRK2* encode plasma membrane independent transporters respectively required for high and low affinity potassium uptake (Gaber *et al*, 1988; Ko *et al.*, 1990; Ko and Gaber, 1991); (ii) Trklp and Trk2p belong to a complex potassium uptake system (Ramos *et al*, 1985) in which Trk2p is not a low-affinity potassium transporter (Ramos *et ai*, 1994). In addition, the existence of an outward-rectifying channel in the plasma membrane of *S. cerevisiae*, implied in regulation of intracellular potassium concentration, has been established by patch-clamp/conductance experiments (Gustin *et al*, 1986; Bertl *et al.*, 1993). However, general mechanisms implied in K^+ efflux from yeast remain unknown.

Due to an improved protocol, which preserves membrane functional properties and produces a high percentage of inside-out tightly sealed plasma membrane vesicles, we have identified a K^+/H^+ exchange system. It has been characterized further, in particular by the determination of its non-electrogenic and reversible character and of the affinity for K^+ efflux and influx. Finally, the possible role of this system in *S. cerevisiae* potassium homeostasis has been discussed.

MATERIALS AND METHODS

Chemicals

9-Amino-6-chloro-2-methoxy-acridine (ACMA), 8-hydroxy-1,3,6-pyrene-trisulfonic acid (pyranine), 3,3'-diethylthiadicarbocyanine iodide ($DISC_2(5)$) and bis-(3-propyl-5-oxoisoxazol-4-yl) pentamethine oxonol (oxonol VI) were purchased from Molecular Probes, Inc. (Eugene, Oregon, U.S.A.). All other chemicals were purchased from Sigma, U.S.A.

Yeast strain and culture medium

The haploid strain of *S. cerevisiae* V5 (*Mata, ura3*) comes from our laboratory collection (Institut National de la Recherche Agronomique, IPV, Montpellier, France). Yeast culture was performed without agitation in a minimal synthetic medium as previously described (Salmon, 1989), purged with argon. This medium contains 12 mMK^+.

Purification of plasma membranes

All the purification steps were performed at 4°C. *Saccharomyces cerevisiae* cells were harvested by centrifugation at the end of the exponential growth phase and washed twice in buffer A (50 mM-bis-tris-propane/2-[N-morpholino]ethanesulfonic acid BTP/MES) pH 7·5, 1 mM-ethylenediaminetetraacetic acid (EDTA), 1 mM-phenylmethylsulfonyl fluoride (PMSF), 1 mM-dithiothreitol (DTT)). About 10 g fresh-weight were mixed with 25 ml of buffer A and 15 g of glass beads (0·5 mm diameter) and the mixture was shaken in an MSK cell homogenizer (Braun Biotec International, Melsungen, Germany) for 3 min under liquid C02 cooling. Glass beads were removed by filtration under vacuum through a nylon mesh and washed with buffer A. The homogenate was centrifuged twice for 5 min at 1000 x g, and once for 10 min at 3000 x g_s. The resulting pellets were discarded, and the supernatant was further centrifuged for 40 min at 15 000 x *g*. The pellet containing the yeast membranes was then suspended in 16 ml of buffer B (10 mM-BTP/MES pH 6·5, 1 mM-DTT, lmM-PMSF, 20% (w/v) glycerol). This membrane suspension (8 ml) was applied to a 43%/53% (w/w) discontinuous sucrose gradient and centrifuged for 2 h at 170 000 x *g*. The membrane fraction located at the sucrose interface was collected, suspended in 4 ml of buffer B and washed by centrifugation (30 min, 150 000 *x g)*. In order to complete the purification, a second 43%/53% (w/w) discontinuous sucrose gradient step was performed under the same conditions. The final plasma membrane pellet was suspended in 4 ml of buffer B without PMSF and stored in liquid nitrogen until use.

Reconstitution of plasma membrane vesicles

In order to obtain a high percentage of inside-out sealed plasma membrane vesicles, a solubilization/gel filtration reconstitution procedure was used. Soybean phospholipids (L-a-phosphatidylcholine type II-S, 60 mg) were dispersed under argon by vigorous mixing on a vortex mixer in the presence of glass beads (2 mm diameter) in 1 ml of reconstitution buffer (10 mM-BTP/MES pH 7·5, 10% (w/v) glycerol). In experiments using cation pre-loaded vesicles, 50 mM-Li, Cs, K or Rb (sulphate or nitrate salts) were added to the reconstitution buffer. In order to obtain small unilamellar vesicles, the suspension of phospholipid vesicles was sonicated for 15 min to clarity in a bath sonicator. 50 mM-octylglucoside and 0T mg of proteins were added to 2 mg of phospholipids in a final volume of 246 pi containing 10 mM-BTP/MES pH 7·5, 10% (w/v) glycerol (lipid/protein weight ratio: 20). The

mixture was applied to the top of a Sephadex G 50 column (3·5 cm x 0·5 cm disposable syringes fitted with porous glass filters), pre-equilibrated with reconstitution buffer. The column was centrifuged at 150 x *g* for 5 min. Octylglucoside was removed during this chromatographic step, and sealed plasma membrane vesicles were directly eluted. Vesicles were used immediately or frozen and stored in liquid nitrogen.

When pyranine dye was used as encapsulated fluorescent probe, it was added (0·5 mM final) to the phospholipid/plasma membrane/detergent solution just before gel filtration. Non-encapsulated pyranine was removed during the chromatographic step.

Determination of enzymatic activities

The purity of the plasma membrane preparation was checked by the following marker enzymes.

Vanadate-sensitive ATPase activity at pH 6·5 was measured. Plasma membrane marker (Alhers, 1984) was measured in a basal medium (0·5 ml final volume) containing 10 mM-BTP/MES pH 6·5. 1 mM-EDTA, 5 mM-ATP, 10 mM-MgS04 and 40 pg protein, in the presence or absence of 50 pM-orthovanadate. The inorganic phosphate released between 5 and 15 min of reaction at 30°C was determined according to Ames (1966).

a-Mannosidase (vacuolar marker) was determined by measuring 4-nitrophenol-a-mannopyranoside cleavage at 37°C. The incubation mixture contained 100 mM-sodium acetate buffer pH 4·5, 50 pg ml^{-1} bovine serum albumin, 0T mM-ZnS04, and 4 mM-4-nitrophenol-a-mannopyranoside. The reaction was stopped by addition of one volume of 133 mM-glycine/NaOH buffer pH 10·7; the 4-nitrophenol liberated was measured spectrophotometrically at 400 nm.

Cytochrome *c* oxidase (mitochondrial marker) was evaluated by reduced cytochrome *c* oxidation determined at 30°C. Oxidation of 25 pM-cytochrome *c* (reduced by addition of sodium dithionite and stored under argon) was measured spectrophotometrically at 550 nm in 50 mM-potassium phosphate buffer pH 7. It was verified that the obtained activity was always inhibited by 10 mM-sodium azide.

All the specific activities are expressed in milliunits (mU) per mg of protein. One mU corresponds to one nmol of substrate transformed per minute.

Fluorescence measurements

Fluorescence quenching of ACMA (Dufour *et al.*, 1982) or pyranine (Kano and Fendler, 1978) was used to monitor the formation and dissipation of pH gradients (acid inside) across plasma membrane vesicles. Transmembrane potential gradients (AVP) were measured using the fluorescent probes oxonol VI (Apell and Bersch, 1987; Freedman and Novak, 1989) and $DISC_2(5)$ (Herrero *et al*, 1991). Fluorescence intensities of these probes were recorded with an SLM Aminco 8000 C photon-counting spectrofluorimeter. In all experiments where the dye quenching was induced by vesicle dilution, the fluorescence signal was previously stabilized by addition of 30 pi K^+-free liposomes.

In ACMA experiments, unless otherwise stated, plasma membrane vesicles (12 pg of protein) were added to a cuvette containing 2 ml of 10 mM-BTP/MES pH 7·5, 10% (w/v) glycerol and 2 pM-ACMA. The cuvette was stirred and maintained at 30°C. Emission signals were recorded at 480 nm after excitation at 412 nm.

For pyranine experiments, vesicles pre-loaded with this dye were added to a cuvette containing 10 mM-BTP/MES pH 7·5 and 10% (w/v) glycerol. Pyranine fluorescence was acquired at an emission wavelength of 511 nm, after excitation at 456 nm and 419 nm corresponding to the non-protonated species and the isobest point of the dye. The obtained values corresponded to the ratio of 456/419 nm signal.

Measurement of membrane potential was performed using the same procedure as above except that 50 nM-oxonol VI or 400 nM-$DISC_2(5)$ was added instead of ACMA. In this case, excitation and emission wavelengths were 646 and 614 nm for oxonol VI and 643 nm and 680 nm for $DISC_2(5)$, respectively.

Determination of accessibility of mannose residues in plasma membrane vesicles

The accessibility of mannose residues in sealed plasma membrane vesicles was determined using the specific binding of concanavalin A (Con A) as described by Monk *et al.* (1989). Incubations were performed at 28°C. All the enzymatic reactions and washing steps were completely automated using a Beckman Biomek 1000 workstation.

Protein determination

Protein concentrations were determined, after precipitation of the different fractions with 5% trichoro-acetic acid, by the BCA Proteic Reagent

Table 1. Evolution of vacuolar (a-mannosidase), mitochondrial (cytochrome *c* oxidase) and plasma membrane (H^+-ATPase) markers during the plasma membrane purification procedure.

	Purification step		
	Cell homogenate (1)	Differential centrifugations (2)	Discontinuous sucrose gradient centrifugations (3)
a-Mannosidase	2 mU mg^{-1}	4·3 mU mg^{-1}	0·62 mil mg^{-i}
Cytochrome *c* oxidase	89 mU mg^{-1}	60 mU mg^{-1}	28 mU mg^{-1}
H+-ATPase (pH 6·5)	53 mU mg^{-1}	167 mU mg^{-1}	555 mU mg^{-1}

The enzymatic activities were determined as described in Materials and Methods at the following steps of the purification procedure: (1) after cell breakage; (2) after differential centrifugation steps; (3) after discontinuous sucrose gradient centrifugation steps (end of the purification procedure).

method (Pierce). Bovine serum albumin was used as standard.

RESULTS

Properties of reconstituted vesicles

The separation procedure previously described by Serrano (1988), applied to the cell homogenate, resulted in plasma membrane purification. Indeed, the ratio between vanadate-sensitive H^+-ATPase (plasma membrane marker) and cytochrome *c* oxidase (mitochondrial marker) activities on the one hand, or between vanadate sensitive H^+-ATPase and a-mannosidase (vacuolar marker) activities on the other hand, increased around 34 times during plasma membrane preparation (Table 1). Fermentation conditions used for yeast growth (no agitation, argon purged medium, catabolite repression by glucose used as only carbon source) are unfavourable for the formation of active mitochondria. In this way, the ATPase hydrolytic activity contained in our preparation presented a high specificity for ATP (around 0·6 nmol of Pi/min per mg) and we did not find any hydrolytic activity in the presence of GTP (data not shown). It has been demonstrated that only ATP, but not other nucleotides, can be hydrolysed by plasma membrane ATPases (Serrano, 1983). Mitochondrial and vacuolar ATPases, as well as apirases and phosphatases, can use ATP but also GTP as substrate. All these experimental data are in agreement with the removal of a large fraction of mitochondrial and vacuolar membrane contaminants during the preparation steps, which leads to a purified plasma membrane suspension.

Properties of vesicles obtained from the last plasma membrane fraction using the detergent dilution/gel filtration reconstitution procedure described by Kasamo (1987) were then determined. The sidedness of reconstituted vesicles was approached by measuring specific binding of Con A to mannose residues, located on the external face of yeast plasma membrane in whole cells, and specific H^+-ATPase hydrolytic activity, normally located on the internal face of yeast plasma membranes. Only 20% of the vesicles exhibit accessible mannose residues (data not shown). Specific H^+-ATPase hydrolytic activity in the vesicles suspension (486 mU) represents 87% of the activity obtained on the crude membrane preparation (555 mU). These results suggest that at least 80% of the vesicles are sealed with an inside-out orientation.

The generation of a pH gradient, measured by ACMA quenching, was used to determine the integrity of the vesicles. As a result of plasma membrane H^+-ATPase activation, vesicle acidification was observed (Figure IB). Addition of $(HN_4)_2S04$, which equilibrates H^+ across the membrane (by neutral diffusion of NH_3), reversed the fluorescence quenching, indicating that this signal is effectively due to proton transport. These results confirm the tightly-sealed nature of plasma membrane vesicles since they accumulate protons without leakage. It should be noticed that GTP did not induce proton pumping (Figure 1A). This observation provides further evidence that the H^+-ATPase activity contained in our membrane preparation comes from the plasma membrane.

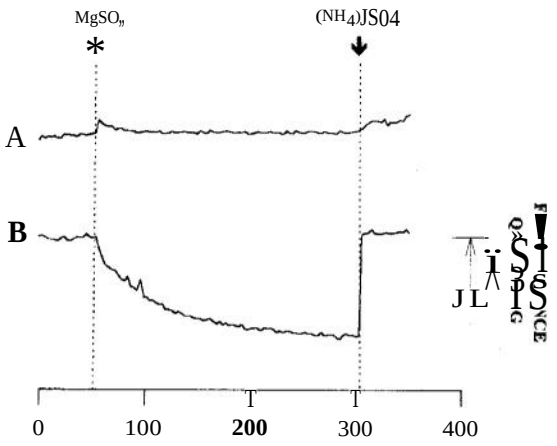

Figure 1. ACMA fluorescence quenching produced by activation of the H^+-ATPase contained in the plasma membrane preparation. Vesicles (30 pi, which corresponds to 12 pg of membrane proteins) were incubated in 2 ml of reaction mixture (10 mM-BTP-MES pH 7·5; 10% (w/v) glycerol; 25 mM-K_2S04; 25 mM-KN03; 250 nM-valinomycin) in the presence of 5 mM-GPT (trace A) or 5 mM-ATP (trace B) as substrate, until a stable fluorescence signal was observed. Proton-pumping was induced by addition of 2 mM-MgS04. 10 mM-$(NH_4)_2S04$ was added after stabilization of the signal.

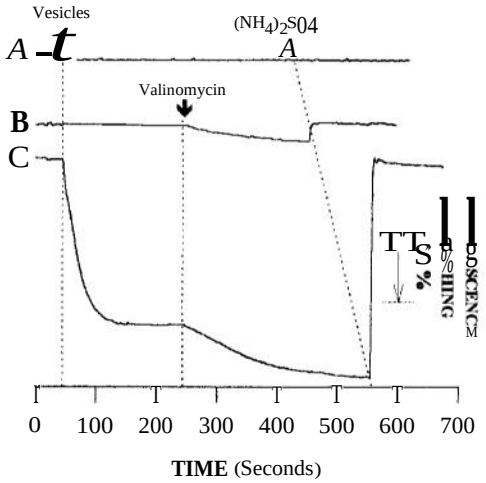

Figure 2. Difference between K^+ pre-loaded and K^+-free plasma membrane vesicles in proton permeability as measured by ACMA fluorescence quenching. 30 pi of pre-boiled plasma membrane vesicles reconstituted in the presence of 25 mM-K_2S04 (trace A), 30 pi of plasma membrane vesicles reconstituted in the absence (trace B) or the presence of 25 mM-K_2S04 (trace C) were added to 2 ml of 10 mM-BTP/MES pH 7·5, containing 10% (w/v) glycerol, 30 pi K^+-free liposomes and 2 pM-ACMA. 250 nM-valinomycin and 10 mM-$(NH_4)_2S04$ were added after stabilization of the signal as indicated.

Evidence for a K^+IH^+-exchange system

The evidence for potassium permeability of the plasma membrane vesicles is summarized in Figure 2. When 50 mM-K^+ pre-loaded plasma membrane vesicles were diluted in a K^+-free buffer, a high pH gradient (acid inside), monitored by ACMA fluorescence quenching, was formed (Figure 2C). On the contrary, plasma membrane vesicles reconstituted in the absence of K^+ did not quench the fluorescence of ACMA (Figure 2B), indicating that the acidification response was due to the presence of K^+ inside the vesicles. The quenching of ACMA fluorescence was rapidly abolished by addition of 10 mM-$(NH_4)_2S04$. This result confirms the formation of an acid-inside pH gradient. In these assays, H^+ fluxes linked to anion transmembrane exchanges were avoided by the use of sulphate salts. Indeed, $S042^-$ exhibits a very weak transmembrane diffusion. In addition, such a pH gradient formation could not be observed with control vesicles (Figure 2A), prepared from a boiled membrane suspension in order to denature proteins. Consequently, the strong internal acidification of the plasma membrane vesicles pointed to the existence of a proteic K^+ transport system. These results were confirmed by another set of experiments using the impermeable pH-sensitive probe pyranine (data not shown).

In order to verify that the observed ACMA signals were not due to a proton leakage of the vesicles, the experiments shown in Figure 3 were performed. Proton entrance into the vesicles and liposomes, after external acidification of the media with MES, was studied before and after addition of the proton ionophore carbonyl cyanide para-(trifluoromethoxy) phenylhydrazone (FCCP). The assays were performed in the absence or presence of octylglucoside in order to obtain closed or opened vesicles (or liposomes), respectively. To follow this study, K^+ pre-loaded vesicles and liposomes prepared at pH 7·5 were diluted in BTP/MES buffer pH 7·5 containing 25 mM-K_2S04 (identical K^+ concentration and pH inside and outside vesicles). External acidification with MES to pH 6·9 always led to an immediate increase of ACMA fluorescence signal, attributed to a modification of the balance between protonated and unprotonated forms of the dye. ACMA fluorescence signal before FCCP addition remained constant in all experiments where potassium exchange is not possible (absence of transporter; 3A, 3C and absence of gradient in the presence of

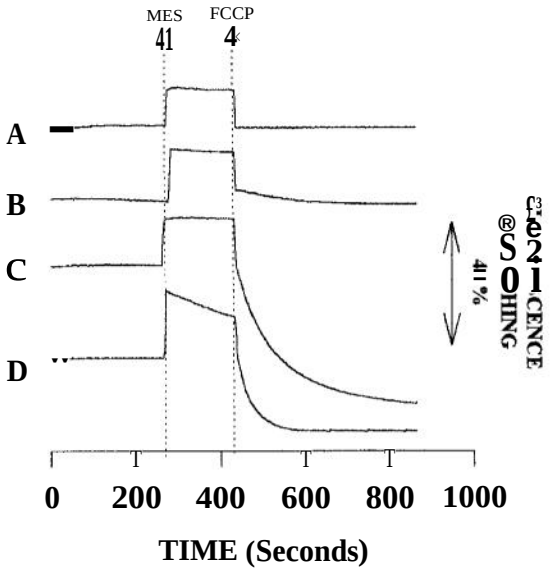

Figure 3. Proton permeability of vesicles and liposomes prepared using detergent solubilization/gel filtration procedure. 30 pi of 25 mM-K_2SO_4 pre-loaded liposomes (traces A, C) or 25 mM-K_2SO_4 pre-loaded vesicles (traces B, D) were diluted in 2 ml of 10 mM-BTP/MES pH 7·5, 10% (w/v) glycerol, 25 mM-K_2SO_4 buffer in the absence (traces C, D) or in the presence of 750 pM-octylglucoside (traces A, B). In order to decrease external pH from 7·5 to 6·9, 5 mM-MES was added (as indicated in the figure). Addition of 10 pM carbonyl cyanide para-(trifluoromethoxy)phenylhydrazone (FCCP) was used to collapse the pH gradient.

detergent; 3A, 3B). On the contrary, it should be noted that only in the experiment done with K^+-pre-loaded vesicles in the absence of detergent, a slow acidification inside was observed after MES addition, even if a transmembrane K^+ gradient does not exist in this case (Figure 3D). This fact can be explained if we consider that, due to the presence of the K^+/H^+ exchange system in the vesicles, an established transmembrane pH gradient can also drive K^+-coupled efflux. The slow flux of protons cannot be attributed to proton leak, because it was not observed in the control experiment performed with closed liposomes. Finally, addition of proton ionophore FCCP resulted in significant quenching of ACMA fluorescence in the assays performed with closed vesicles and liposomes (Figure 3C,D). This signal is the consequence of pH gradient abolishment (previously created by MES addition). Therefore, these data show that before ionophore addition, liposomes and vesicles are able to maintain a pH gradient without leakage. Such an evolution did not occur with opened liposomes and vesicles (Figure 3A,B). The decrease in dye fluorescence observed after FCCP addition, in experiments done in the presence of detergent, is due to an interference between ACMA and FCCP. This effect was also observed in control experiments performed without external acidification (data not shown).

The presence of K^+ in the external medium greatly affects K^+ efflux from pre-loaded vesicles. The ACMA fluorescence quenching obtained after dilution of 50 mM-K^+ pre-loaded vesicles in a buffer containing 10 mM-K_2SO_4 (Figure 4B), represents only 42% of the signal obtained after dilution of the same vesicles in a K^+-free medium (Figure 4C). Moreover, the use of the same K^+ concentration on both sides of the vesicles completely prevented the establishment of K^+ and H^+ fluxes (Figure 4A). Successive addition of K^+ (below 5 mM) to the medium, during K^+ efflux from pre-loaded vesicles, induced a decrease in the rate of intravesicular acidification (Figure 4D). The supply of 5 mM-K^+ completely inhibited the generation of a pH gradient, indicating that a K^+ concentration balance is reached. Further external K^+ additions (up to 50 mM) reversed ACMA fluorescence quenching, revealing a K^+ influx into plasma membrane vesicles (Figure 4C). Identical phenomena were observed with pyranine dye (data not shown). We can conclude that the K^+ transmembrane gradient drives the transport system, and that this system is capable of either influx or efflux.

The intravesicular H^+ accumulation observed during dilution of K^+ pre-loaded vesicles using pH-sensitive fluorescent dyes can be explained by the existence of a true K^+/H^+ exchange system or by the presence of a K^+ uniporter (creating a negative inside membrane potential) which should promote a passive H^+ influx. To determine which system is operating, different sets of experiments were performed. The possible generation of a membrane potential during K^+ fluxes was investigated using an anionic fluorescent probe (oxonol VI) and a cationic fluorescent probe ($DISC_2(5)$). In both cases, valinomycin (K^+ uniporter) and nigericin (electroneutral K^+/H^+ antiporter) were used as controls.

When 50 mM-K^+ was added to K^+-free liposomes in the presence of valinomycin (Figure 5A), the oxonol VI response (rapid fluorescence increase) showed the formation of a positive inside membrane potential corresponding to K^+ entry into vesicles via the ionophore. In order to reach a thermodynamic equilibrium, a proton efflux is then

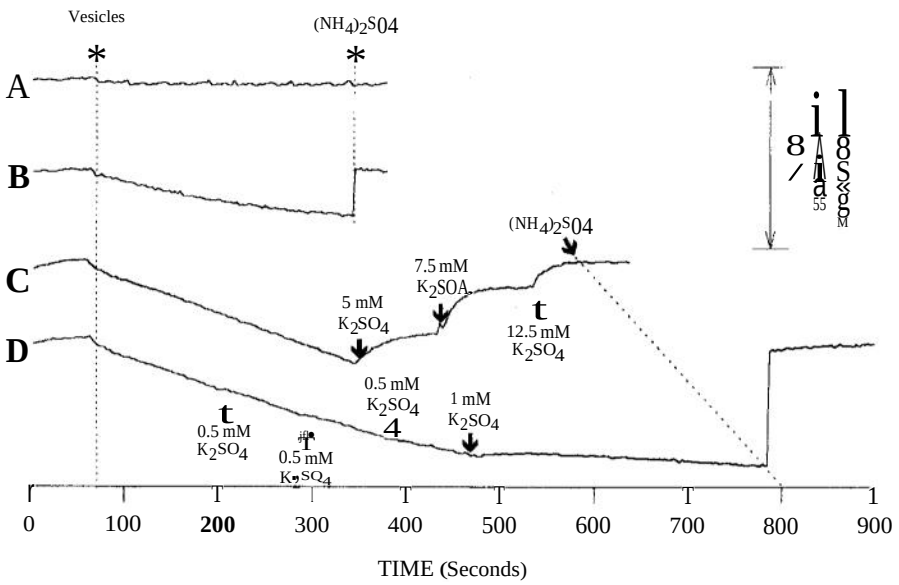

Figure 4. Decrease of the intravesicular acidification rate by external K^+ pulses monitored by ACMA fluorescence quenching. 10 pi of plasma membrane vesicles reconstituted in the presence of 25 mM-K_2SO_4 were added to 2 ml of 10 mM-BTP/MES pH 7·5, 10% (w/v) glycerol, 30 pi K^+-free liposomes, 2 pM-ACMA plus 25 mM-K_2SO_4 (trace A), 10 mM-K_2SO_4 (trace B) or without K_2SO_4 (traces C and D). Successive external K^+ pulses (K_2SO_4) and addition of 10 mM-$(NH_4)_2SO_4$ were made after stabilization of the signal.

induced, resulting in the clamp of the created potential. As expected, oxonol VI fluorescence signal was not modified during the assay achieved in the presence of nigericin (Figure 5B). In Figure 5C we observed that oxonol VI fluorescence signal did not increase but remained constant after addition of 50 mM-K^+ to K^+-free vesicles. Later addition of valinomycin shows that we are also able to detect the creation of a positive inside potential on these vesicles (Figure 5C). This result refutes the hypothesis of a K^+ uniport present in the plasma membrane vesicles, because K^+ entry into the vesicles through such a transporter would necessarily imply the formation of a positive inside membrane potential. Figure 5D shows no generation of a positive inside membrane potential, thus indicating that the system is not an electrogenic K^+/nH^+ antiport-like system. The initial increase in oxonol signal, concomitant to addition of vesicles (Figure 5C,D), is due to an interaction between the probe and the lipids present in the vesicles and not to the formation of a true potential (the same effect is observed with K^+-free vesicles and K^+ pre-loaded vesicles). These observations led us to consider that the K^+ transporter present in *S. cerevisiae* plasma membrane probably functions as an electroneutral K^+/H^+ exchange.

In the same way, the cationic probe $DISC_2(5)$ was used to measure the evolution of negative inside membrane potential during K^+ efflux experiments (Figure 6). An extinction of dye fluorescence revealed the formation of a negative inside potential when K^+ pre-loaded control liposomes were added to K^+-free medium in the presence of valinomycin (Figure 6A). The same experiment was done in the presence of FCCP to prove that the signal obtained with valinomycin in Figure 6A is not an artefact. In this case, valinomycin addition does not produce any decrease in $DISC_2(5)$ fluorescence (Figure 6B). However, there was no decrease in $DISC_2(5)$ fluorescence, neither in control K^+-pre-loaded liposomes in the presence of nigericin (Figure 6C), nor with K^+-pre-loaded vesicles (Figure 6D). This last experiment excluded the possibility of an electrogenic nK^+/H^+ exchange and pointed again to the electroneutral nature of the system. The initial increase of $DISC_2(5)$ fluorescence after addition of liposomes or reconstituted vesicles into K^+-free medium was not linked to the creation of a membrane potential, due to K^+ fluxes, but to an interaction between

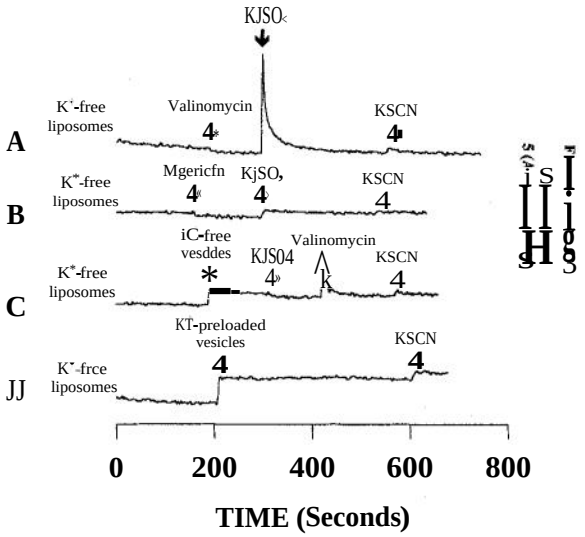

Figure 5. Determination of membrane potential (AT) during K^+ fluxes across plasma membrane vesicles using the anionic probe oxonol VI. Before 25 mM-K_2SO_4 addition, 250 nM-valinomycin (trace A) or 250 nM-nigericin (trace B) were provided to 30 pi of K^+-free liposomes diluted into 2 ml of 10 mM-BTP/MES pH 7·5 buffer, containing 10% (w/v) glycerol and 50 nM-oxonol VI. Trace C corresponds to the dilution of 30 pi of plasma membrane vesicles reconstituted in the absence of potassium, into the same buffer. 250 nM-valinomycin was added after 25 mM-K_2SO_4. In trace D, 30 pi of 25 mM-K_2SO_4 pre-loaded plasma membrane vesicles were diluted in 2 ml of 10 mM-BTP/MES pH 7·5, containing 10% (w/v) glycerol and 50 nM-oxonol VI. After stabilization of the signal, 10 mM-KSCN was added to the reaction medium in order to collapse the membrane potential.

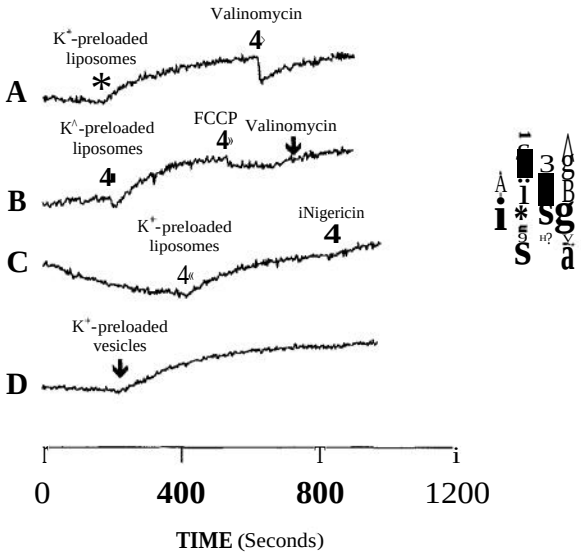

Figure 6. Determination of membrane potential (AT) during K^+ fluxes across plasma membrane vesicles using the cationic probe $DISC_2(5)$. 250 nM-valinomycin were added to 30 pi of 25 mM-K_2SO_4 pre-loaded liposomes diluted into 2 ml of 10 mM-BTP/MES pFI 7·5, containing 10% (w/v) glycerol, 30 pi K^+-free liposomes and 400 nM-$DISC_2(5)$ in the absence (trace A) or in the presence (trace B) of 10 pM-FCCP. The experiment shown in trace C was performed in the same conditions as A, but valinomycin was replaced by 250 nM-nigericin. Trace D corresponds to an activation of the K^+ transporter by dilution of 30 pi of plasma membrane vesicles reconstituted in the presence of 25 mM-K_2SO_4 in 2 ml of K^+-free BTP/MES/10% (w/v) glycerol buffer.

the dye and the lipids. All these results are in agreement with the presence of an electroneutral K^+/H^+ exchange system.

Finally, these data were confirmed by the comparison of H^+ fluxes in the absence (Figure 7A) or in the presence (Figure 7B) of a permeant anion NO_3^-. The free diffusion of this ion through the vesicles abolishes the formation of membrane potential. In this case, H^+ fluxes promoted by the creation of membrane potential linked to K^+ efflux (via a uniport system) will never occur. When 50 mM-KNO_3 pre-loaded vesicles were diluted in 50 mM-BTP-NO_3 buffer, however, an internal acidification of vesicles arose (Figure 7B), which was quicker than in the control experiment (Figure 7A). Therefore, the hypothesis of an antiport-like exchange system working in the vesicles was supported and the existence of two electrically coupled uniporters was again discarded. Moreover, it clearly appears that a collapse of electrical potential greatly activates K^+/H^+ exchange. This probably reflects some modulation of activity of the electroneutral antiport system by membrane potential.

Taken together, these results demonstrate that K^+ is transported using a reversible electroneutral K^+/H^+ exchange system which can be composed of one or more proteins. In order to assess the cation selectivity of this K^+/H^+ antiport-like system, pH gradients obtained by dilution of vesicles pre-loaded with different monovalent cations (K^+, Cs^+, Rb^+, Li^+, 50 mM ion intravesicular concentration, reconstitution buffer pH 7·5) were compared (Figure 8). Rb^+ and Cs^+ appeared to be transported to a lesser extent than K^+ and only a very weak Li^+ efflux was observed. The initial rates of ion transport were measured from the initial quenching of ACMA fluorescence (curves slope of the initial linear part). Cs^+, Rb^+ and Li^+ transport rates represented 61, 54 and 8% respectively of the initial rate for K^+ efflux (deter-

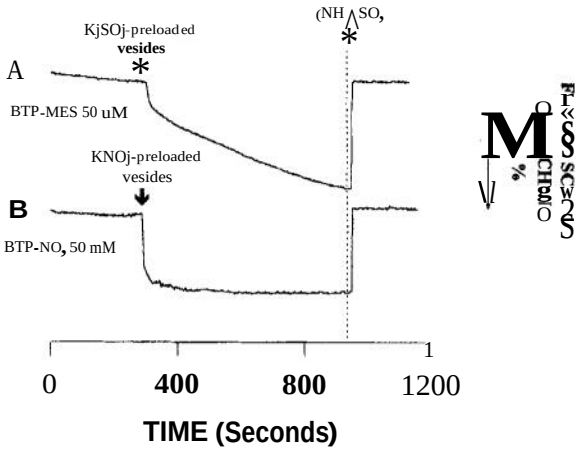

Figure 7. Effect of the permeant anion NO_3^- on the K^+ transporter present in *Saccharomyces cerevisiae* plasma membranes. Measurement of ACMA fluorescence quenching linked to K^+ fluxes obtained after dilution of: (A) 25 mM-K_2SO_4 pre-loaded vesicles in 50 mM-BTP/MES, 10% (w/w) glycerol buffer (pH 7·5); (B) 50 mM-KNO, pre-loaded vesicles in 50 mM-BTP/NO_3, 10% glycerol buffer (pH 7·5). In all experiments, the signal was stabilized prior to reaction by addition of 30 pi K^+-free liposomes. At the end of the experiments, 10 mM-$(NH_4)_2SO_4$ were added to collapse the pH gradient.

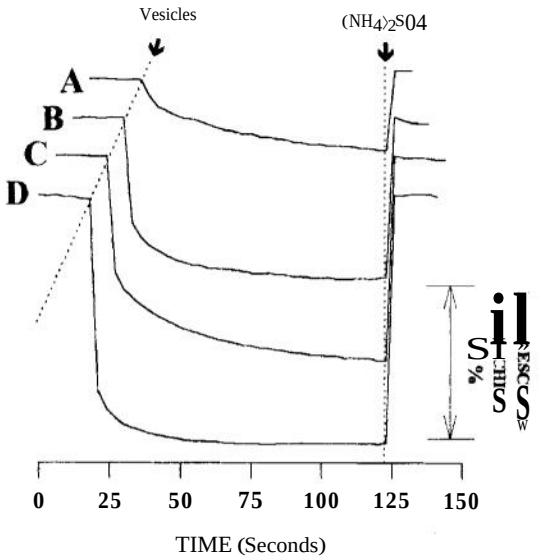

Figure 8. Determination of the cation selectivity of the exchange system using ACMA fluorescence quenching. 20 pi of plasma membrane vesicles reconstituted in the presence of 25 mM-Li_2SO_4 (trace A), 25 mM-Rb_2SO_4 (trace B), 25 mMCs_2SO_4 (trace C) and 25 mM-K_2SO_4 (trace D) were added to 2 ml of 10 mM-BTP/MES 7·5, containing 10% (w/v) glycerol, 30 pi K^+-free liposomes and 2 pM-ACMA. 10 mM-$(NH_4)SO_4$ was added after stabilization of the signal to collapse the pH gradient.

mined under the same experimental conditions). The order of effectiveness $K^+ > Cs^+ \geq Rb^+ > Li^+$ demonstrates the selectivity of the K^+/H^+ exchange system present in the reconstituted plasma membrane vesicles.

The optimum pH for the exchange was determined by varying the external pH, even if the concomitant pH gradients created were different in each experiment. Proton accumulation measured by ACMA fluorescence quenching was initiated by dilution of K^+ pre-loaded vesicles (reconstitution buffer pH 7·5) in K^+-free buffers at different pH values (Figure 9). The maximum rate of acidification was obtained at pH 6·8. This value, which did not correspond to the highest pH gradient used in this characterization, can be considered as the K^+/H^+-exchange optimum pH.

The K^+ affinity of the antiport was determined by measuring initial rates of proton influx induced by K^+ efflux from pre-loaded vesicles at different K^+ concentrations (Figure 10) or measuring the kinetics of dissipation of pre-formed pH gradient by the addition of various K^+ concentrations to the external medium (Figure 11). The saturation curves observed in both cases exhibit Michaelis-Menten kinetics but were quite different: a Km of 22 mM was calculated by the least-square fitting method in the case of K^+ efflux and a Km of 6 mM for K^+ influx. The differences in measured Km values indicate that the plasma membrane K^+/H^+ antiport-like system functions with a lower affinity and a higher efficiency in the direction of K^+ efflux than in the direction of K^+ influx.

DISCUSSION

In this work, fully functional reconstituted vesicles were obtained from a purified plasma membrane preparation in order to study *S. cerevisiae* K^+ transport systems. The use of the octylglucoside dilution/gel filtration procedure described by Kasamo (1987) applied to *S. cerevisiae* plasma membrane extract allows us to obtain more than 80% of inside-out sealed vesicles, with the rest of the preparation consisting of open vesicles (which do not interfere with measurements using fluorescent dyes) and a small proportion of right-side-out sealed vesicles. Such homogeneity was not achieved by the FTS method, generally employed for reconstitution of vesicles (Kasahara and Hinkle, 1977; Ongjoco *et al.*, 1987; Serrano, 1988). Indeed, Monk *et al.* (1989) obtained equal amounts of right-side-out and inside-out vesicles in

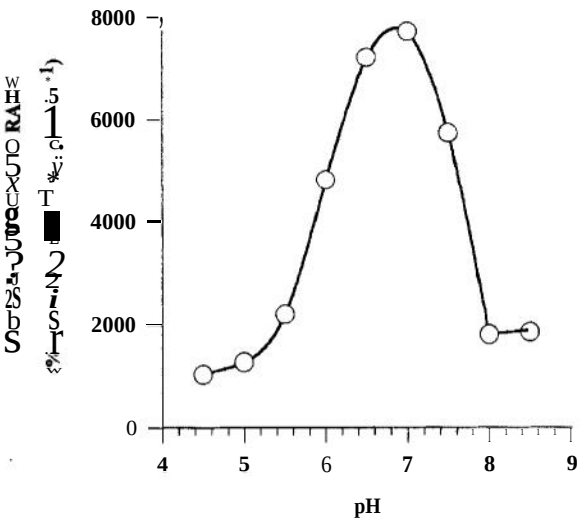

Figure 9. Estimation of the K^+/H^+ exchange pH optimum measured by ACMA fluorescence quenching. 30 pi of plasma membrane vesicles reconstituted in the presence of 25 mM-K_2SO4 were added to 2 ml of 10 mM-BTP buffered at different pH values by MES (pH 4·5 to pH 8·5), and containing 10% (w/v) glycerol, 30 pi K^+-free liposomes and 2 pM-ACMA. Initial fluorescence quenching rates were plotted as a function of external pH.

a plasma membrane vesicle suspension prepared by the FTS method. The high proportion of inside-out tightly sealed vesicles obtained in this study is a characteristic of the reconstitution method and makes them particularly useful for the study of transmembrane transport systems.

This ability of reconstituted vesicles to exhibit transmembrane exchange phenomena was used to characterize K^+ transport. Concerning potassium uptake in *S. cerevisiae*, the existence of two parallel uptake systems which differ in their substrate affinity (Km values of 24 \XM and 2 mM respectively) has been proposed (Rodriguez-Navarro and Ramos, 1984; Ramos *et al.*, 1985). Moreover, two genes implied in potassium uptake (*TRK1* and *TRK2*) have been cloned and sequenced (Gaber *et al.*, 1988; Ko and Gaber, 1991), but the function of these gene products (regulation proteins or transporters) has not been clearly defined yet. The existence of a membrane potential-driven carrier for monovalent cations (K^+ and Rb^+) has also been described (Calahorra *et al.*, 1987). In addition, evidence of an outward-rectifying K^+ channel in *S. cerevisiae* plasma membrane must also be noted (Gustin *et al.*, 1986; Bertl *et al.*, 1993).

In the present paper, fluorimetric measurements of proton fluxes in reconstituted plasma membrane vesicles demonstrate the existence of a K^+ transporter in *S. cerevisiae*. This system is driven mainly

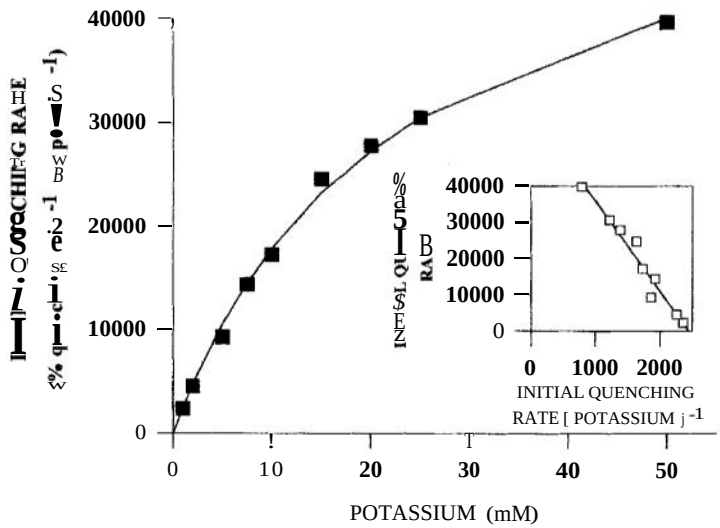

Figure 10. Measurement of H^+ influx (coupled to K^+ efflux) kinetic parameters using ACMA fluorescence quenching. 30 pi of plasma membrane vesicles reconstituted in the presence of variable concentrations of K_2SO4 (0·5 to 25 mM) were added to 2 ml of 10 mM-BTP/MES pH 7·5, containing 10% (w/v) glycerol, 30 pi K^+-free liposomes and 2 pM-ACMA. Initial quenching rates (% quenching min^{-1} per mg protein) were determined during the first 20 s after dilution of the vesicles and were plotted as a function of the extravesicular potassium concentrations. Inset: Eadie-Hofstee plot. A Km of 22 mM and a maximum velocity of 58 300% quenching min^{-1} per mg protein were determined.

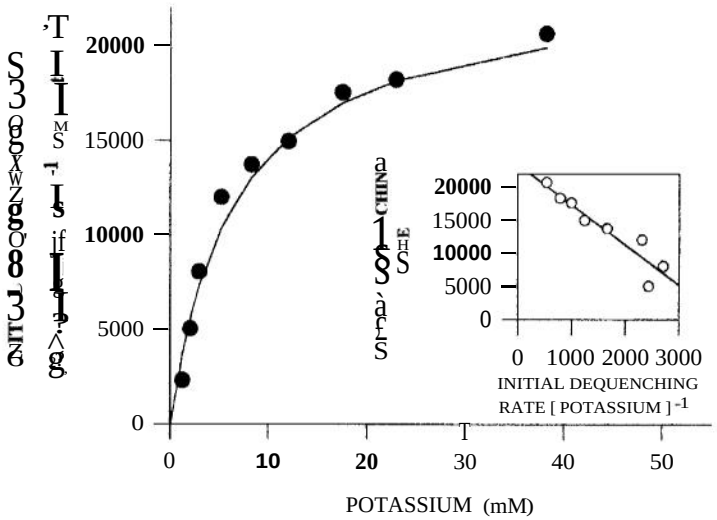

Figure 11. Measurement of H^+ efflux (coupled to K^+ influx) kinetic parameters using ACMA fluorescence quenching. 30 pi of plasma membrane vesicles reconstituted in the presence of 25 mM-K_2SO_4 were added to 2 ml of 10 mM-BTP/MES pH 7·5, containing 10% (w/v) glycerol, 30 pi K^+-free liposomes and 2 pM-ACMA. After stabilization of the signal, various concentrations of K_2SO_4 (0·5 to 20 mM) were added to the reaction medium, initiating a dequenching of ACMA fluorescence. Initial quenching reversion rate (% dequenching min^{-1} per mg protein) was plotted as a function of the intravesicular potassium concentration. Inset: Eadie-Hofstee plot. A *Km* of 6mM and a maximum velocity of 23 500% dequenching min^{-1} per mg protein were determined.

by the transmembrane K^+ concentration gradient, and it catalyses both K^+ influx and efflux. The non-generation of a membrane potential (characteristic of an electrical coupling between K^+ and H^+ uniporters) during K^+ exchange allows us to consider this system as an electroneutral one. However, membrane electrical potential (even if it is not generated during exchange) modulates activity of the electroneutral K^+/H^+ antiport-like system. The presence of such an operating system in yeast plasma membrane is not surprising since a K^+/H^+ exchange, involved either in control of intracellular pH or in regulation of internal K^+ concentration, has been described previously from oil-seed rape by Cooper *et al*. (1991). The K^+/H^+ transport system presented here has a specificity for potassium over other monovalent cations. The observed specificity ($K^+ > (Cs^+ \simeq Rb^+) \gg Li^+$) is in good agreement with recent observations on plant K^+ transporter selectivity (Cooper *et al*, 1991; Schatchtman *et al.*, 1992; Schatchtman and Schroeder, 1994). Finally, the kinetic parameters of K^+ fluxes across *S. cerevisiae* plasma membranes were determined using this reconstituted system. It should be noted that, as a result of the inside-out nature of the reconstituted compared with the native vesicles, a K^+ influx corresponds to a K^+ transport out of the cell. Significant differences between inward and outward K^+ fluxes were observed: a higher affinity for potassium and a lower rate of transport were measured for K^+ efflux out of yeast cells than for the influx. In addition, potassium is transported into the cells by this K^+/H^+ antiport-like system with an apparent *Km* of 22 mM, which is considerably higher than the affinity constant values of the K^+ uptake transport systems previously described (Ramos *et al.*, 1990, 1994).

The potassium transport system reported in this paper differs from K^+ channels previously described by Gustin *et al.* (1986), Calahorra *et al.* (1987) and Bertl *et al*. (1993) in its way of working (K^+/H^+ electroneutral exchange). It also differs from the K^+ uptake system described (Rodriguez-Navarro and Ramos, 1984) in its ability to function in both ways: potassium influx and efflux. Because of the polarity of this system, it seems that its main function is K^+ excretion from cells and

so, it must be implied in *S. cerevisiae* potassium homeostasis. These data are in agreement with the observations of Gaber *et al*. (1988), who proposed the existence of a K^+ transporter in order to explain K^+ efflux from *trkl* mutant cells. The existence of such a system was supported by the fact that internal K^+ concentration did not exceed a threshold value (around 70 mmol K^+ per g fresh weight) when external K^+ concentration was increased (up to 50 mM), supposing the induction of a specific K^+ efflux from the cells.

REFERENCES

Ahlers, J. (1984). Effects of different salts on the plasma membrane ATPase and on proton transport in yeast. *Can. J. Biochem. Cell. Biol.* 62, 998–1005.

Amalou, Z., Gibrat, R., Brudigou, C., Trouslot, P. and d'Auzac, J. (1992). Evidence for an amiloride-inhibited $Mg^{2+}/2H^+$ antiporter in luto-vfd (vacuolar) vesicles from latex *Hevea brasiliensis. Plant Physiol.* **100,** 255–260.

Ames, B. N. (1966). Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol.* 8, 115–118.

Apell, H. J. and Bersch, B. (1987). Oxonol VI as an optical indicator for membrane potentials in lipid vesicles. *Biochim. Biophys. Acta* **903,** 480–194.

Bertl, A., Slayman, C. L. and Gradmann, D. (1993). Gating and conductance in outward-rectifying K^+ channel from the plasma membrane of *Saccharomyces cerevisiae. J. Memb. Biol.* **132,** 183–199.

Bisson, L. F., Coons, D. M., Kruckeberg, A. L. and Lewis, D. A. (1993). Yeast sugar transporters. *CRC Crit. Rev. Biochem. Mol Biol.* **28,** 259–308.

Braun, Y., Hassidim, M., Lerner, H. and Reinhold, L. (1988). Evidence for a Na^+/H^+ antiporter in membrane vesicles isolated from roots of the halophyte *Atriplex nummularia. Plant Physiol.* **87,** 104–108.

Calahorra, M., Ramirez, J., Clemente, S. M. and Pena. (1987). Electrochemical potential and ion transport in vesicles of yeast plasma membrane. *Biochem. Biophys. Acta* **899,** 229–238.

Cooper, S., Lemer, H. R. and Reinhold, L. (1991). Evidence for a highly specific K^+/H^+-antiporter in membrane vesicles from oil-seed rape hypocotyls. *Plant Physiol.* **97,** 1212–1220.

Dufour, J. P., Goffeau, A. and Tsong, T. Y. (1982). Active proton uptake in lipid vesicles reconstituted with the purified yeast plasma membrane ATPase. Fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine. *J. Biol. Chem.* **257,** 9365–9371.

Freedman, J. C. and Novak, T. S. (1989). Optical measurement of membrane potential in cells, organelles, and vesicles. *Meth. Enzymol.* **172,** 102–122.

Gaber, R., Styles, C. and Fink, G. (1988). *TRK1* encodes a plasma membrane protein required for high affinity potassium transport in *Saccharomyces cerevisiae. Mol. Cell. Biol.* 8, 2848–2859.

Gustin, M. C., Martinac, B., Saimi, Y., Culbertson, M. R. and Kung, C. (1986). Ion channels in yeast. *Science* 233, 1195–1197.

Herrero, I., Miras-Portugal, M. T. and Sanchez-Prieto, J. (1991). Inhibition of glutamate release by arachidonic acid in rat cerebrocortical synaptosomes. *J. Neurochem.* **57,** 718–721.

Kano, K. and Fendler, J. H. (1978). Pyranine as a sensitive pH probe for liposome interiors and surfaces. pH gradients across phospholipids vesicles. *Biochim. Biophys. Acta* **509,** 289–299.

Kasahara, M. and Hinkle, P. (1977). Reconstitution and purification of the D-glucose transporter from human erythrocytes. *J. Biol. Chem.* 252, 7384–7390.

Kasai, M. and Sawada, S. (1994). Evidence for a decrease in vanadate-sensitive Mg^{2+}-ATPase activity of higher plant membrane preparations in sucrose solutions. *Plant Cell. Physiol.* 35, 697–700.

Kasamo, K. (1987). Reconstitution and characterisation of H^+-translocating ATPase from the plasma membrane of *Phaseolus mungo L.* roots. *Plant Cell. Physiol.* **28,** 19–28.

Ko, C. H. and Gaber, R. F. (1991). *TRK1* and *TRK2* encode structurally related K^+ transporters in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **11,** 4266–1273.

Ko, C. H., Buckley, A. M. and Gaber, R. F. (1990). *TRK2* is required for low affinity K^+ transport in *Saccharomyces cerevisiae. Genetics* **125,** 305–312.

Krasne, S., Eisenman, G. and Szabo, G. (1971). Feeding and melting of lipids bilayers and the mode of action of nonactin, valinomycin, and gramicidin. *Science* **174,** 412–415.

Marger, M. D. and Saier, M. H. (1993). A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. *T.I.B.S.* **18,** 13–20.

Marini, A. M., Vissers, S., Urrestarazu, A. and Andre, B. (1994). Cloning and expression of the MEP1 gene encoding an ammonium transporter in *Saccharomyces cerevisiae. EMBO J.* **13, 345623463**.

Marshall, J., Corzo, A., Leigh, R. and Sanders, D. (1994). Membrane potential-dependent calcium transport in right-side-out plasma membrane vesicles from *Zea mays L* roots. *Plant J.* 5, 683–694.

Monk, B., Montesinos, C, Leonard, K. and Serrano, R. (1989). Sidedness of yeast plasma membrane vesicles and mechanisms of activation of the ATPase by detergents. *Biochim. Biophys. Acta* **981,** 226–234.

Ongjoco, R., Szkutnicka, K. and Cirillo, V. (1987). Glucose transport in vesicles reconstituted from *Saccharomyces cerevisiae* membranes and liposomes. *J. Bacterial.* **169,** 2926–2931.

Ovchinnikov, Y. A. (1979). Physico-chemical basis of ion transport through biological membranes: ionophores and ions channels. *Eur. J. Biochem.* **94,** 321–336.

Perlin, D., Kasamo, L., Broocker, R. and Slayman, C. (1984). Electrogenic H^+ translocation by the plasma membrane ATPase of *Neurospora*. Studies on plasma membrane vesicles and reconstituted enzyme. *J. Biol Chem.* **259,** 7884–7892.

Ramos, J., Alijo, R., Haro, R. and Rodriguez-Navarro, A. (1994). TRK2 is not a low-affinity potassium transporter in *Saccharomyces cerevisiae. J. Bacteriol.* **176,** 249–252.

Ramos, J., Contreras, P. and Rodriguez-Navarro, A. (1985). A potassium transport mutant of *Saccharomyces cerevisiae. Arch. Microbiol.* **143,** 88–93.

Ramos, J., Haro, R. and Rodriguez-Navarro, A. (1990). Regulation of potassium fluxes in *Saccharomyces cerevisiae. Biochim. Biophys Acta* **1029,** 211–217.

Rodriguez-Navarro, A. and Ramos, J. (1984). Dual system for potassium transport in *Saccharomyces cerevisiae. J. Bacteriol.* **159,** 940–945.

Salmon, J. M. (1989). Effect of sugar transport inactivation in *Saccharomyces cerevisiae* on sluggish and stuck enological fermentations. *Appl. Environ. Microbiol.* **55,** 951–958.

Schachtman, D. P., Schroeder, J. L, Lucas, W. J., Anderson, J. A. and Gaber, R. F. (1992). Expression of an inward-rectifying potassium channel by the *Arabidopsis KAT1* cDNA. *Science* **258,** 1654–1657.

Schachtman, D. P. and Schroeder, J. I. (1994). Structure and transport mechanism of high-affinity potassium uptake transporter from higher plants. *Nature* 370, 655–658.

Serrano, R. (1983). Purification and reconstitution of the proton-pumping ATPase of fungal and plant plasma membranes. *Arch Biochem.* **227,** 1–8.

Serrano, R. (1988). H^+-ATPase from plasma membrane of *Saccharomyces cerevisiae* and *Avena sativa* roots: purification and reconstitution. *Methods Enzymol.* **157,** 533–578.